

Histones facilitate α -synuclein aggregation during neuronal apoptosis

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Abstract Ample in vitro and in vivo experimental evidence supports the hypothesis that intercellular transmission of α -synuclein (α S) is a mechanism underlying the spread of α S pathology in Parkinson's disease and related disorders. What remains unexplained is where and how initial transmissible α S aggregates form. In a previous study, we demonstrated that α S aggregates rapidly form in neurons with impaired nuclear membrane integrity due to the interaction between nuclear proaggregant factor(s) and α S and that such aggregates may serve as a source for α S seeding. In the present study, we identify histones as a potential nuclear proaggregant factor for α S aggregation in both apoptotic neurons and brains with α S pathology. We further demonstrate that histone-induced aggregates contain a range of α S oligomers, including protofibrils and mature fibrils, and that these α S aggregates can seed additional aggregation. Importantly, we demonstrate transmissibility in mouse brains from stereotaxic injection. This study provides new clues to the mechanism underlying initial pathological aggregation of α S in PD and related disorders, and could lead to novel diagnostic and therapeutic approaches.

Keywords Aggregation · Apoptosis · Nuclear membrane integrity · Parkinson's disease · Proaggregant nuclear factors · α -synuclein · Histone

Introduction

Abnormal filamentous human α -synuclein (α S) aggregates are a pathological feature of Parkinson's disease (PD) and related disorders [32, 33]. Increasing evidence suggests that cell-to-cell transmission of α S aggregates contributes to progression of PD [5, 6, 24, 27]. It remains an enigma, however, as to where and how the initial transmissible α S aggregates form. In a previous study, we demonstrated that filamentous α S aggregates rapidly form in neurons undergoing apoptosis, facilitated by a proaggregant factor or factors in nuclear material released upon disruption of nuclear envelope integrity [16]. Breakdown of apoptotic bodies results in release of α S aggregates, spread to neighboring healthy neurons, and seeding of additional aggregation. These findings suggest that a nuclear proaggregant factor exists that is responsible for facilitating rapid aggregation of cytoplasmic α S due to the loss of nuclear membrane integrity.

We hypothesize that a candidate for the proaggregant nuclear factor released upon the breakdown of the nuclear membrane during apoptosis is histone. Histones are a major class of nuclear proteins that have been shown to promote α S fibril formation in vitro, possibly due to their net positive charge [11]. Previous studies have also shown that histones are released into the cytoplasm during apoptosis [36]. To test this hypothesis, we used cellular models, animal models, and immunohistochemistry of brains with Lewy body pathology to examine the role of histones in α S aggregation. Our data show that histones associate with α S

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aggregates in apoptotic neurons and are a component of a subset of Lewy bodies in humans. More importantly, histone-induced α S aggregates are transmissible to neurons in both in vitro and in vivo conditions. Therefore, our study strongly suggests that histones may be a nuclear proaggregant factor that triggers α S aggregation.

Materials and methods

Cell culture and maintenance

Three neuronal cell models were used in the present study. The first two were 3D5 and α S-Flag derived from human dopaminergic neuroblastoma BE2-M17D cell line, which displays a neuronal phenotype under retinoic acid-induced differentiation. 3D5 cells express wild-type human α S upon TetOff induction and have been described previously [18]. α S-Flag is derived from BE2-M17D cell line with lentivirus overexpression of Flag-tagged α S. All neuronal cell models were maintained in DMEM/10% fetal bovine serum at 37 °C and 5% CO₂, but 3D5 also required 2 μ g/ml tetracycline. Neurons were seeded at a density of 3×10^6 cells/plate (100 \times 20 mm, BD Biosciences) for biochemical analysis and 2×10^4 cells/well on coverslips in 24-well plates for immunocytochemistry. For differentiation, media were replaced with Neurobasal medium (Invitrogen), 2% B-27 supplement (Invitrogen), 2 mM L-glutamine (Sigma) and 10 μ M RA (Sigma). To induce α S expression in 3D5 cells, tetracycline was deprived from the medium.

The third cell line used is the Lund human mesencephalic (LUHMES) cell line (LUHMES ATCC[®] CR-2927), which was differentiated into morphologically and biochemically mature dopamine-like neurons in advanced DMEM/F-12/Glutamax/N2 medium (Invitrogen) containing 2 ng/ml human recombinant glial cell-derived neurotrophic factor (R&D Systems), 1 mM cAMP (Sigma-Aldrich) and 1 μ g/ml tetracycline as described previously [16, 23]. Cells with at least 4 days of differentiation were used for experiment.

Isolation of cytoplasmic proteins and subsequent western blot analysis

Neuronal cultures were harvested and centrifuged at 200 \times g for 15 min to obtain cell pellets. Cytoplasmic fractions were isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific) following the manufacturer's instructions, but with minor modifications. Briefly, cells were resuspended in cytoplasmic extraction reagent-I and incubated for 10 min, followed by the addition of cytoplasmic extraction reagent-II

and centrifugation at 16,000 \times g for 5 min. Supernatants were considered the cytoplasmic fraction. The whole process was done on ice or at 4 °C. The bicinchoninic acid (BCA) assay was used for protein quantitation. The same amount of proteins (30 μ g) from each sample was mixed with Tricine-SDS sample buffer (Invitrogen) and 2% β -mercaptoethanol, boiled for 5 min and resolved by SDS-PAGE using 10–20% Tris/Tricine gel (Bio-Rad). Precision-plus protein standards (Bio-Rad) were included as references. After gel electrophoresis, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes for further probing with antibodies against α S (Syn1, 610787, BD Biosciences) or α S (NACP98, Mayo Clinic [7]), H1.2 (ab17677, Abcam), H3 (ab1791, Abcam). Western Lightning Plus ECL (PerkinElmer) or ECL[™] Prime Western Blotting Detection Reagent (Fisher Scientific) was used for visualization of protein immunoreactivities. Data from at least 3 sets of independent experiments were analyzed by one-way ANOVA with Dunnett's post hoc test for statistical significance.

Immunoprecipitation

Cytoplasmic fractions were isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents as described above, and then incubated overnight with Dynabeads[®] (Invitrogen) coated with rabbit IgG, antibodies to α S (NACP98, Mayo Clinic [7]), H1.2 (ab17677, Abcam), H3 (ab1791, Abcam), following the manufacturer's instruction. Proteins bound to the beads were released by boiling in sample loading buffer and probed with antibodies of interest by western blotting.

Lentiviral plasmids and virus preparation

Lentiviral plasmids carrying α S-Flag [EX-G0543-Lv121] were purchased from GeneCopoeia. The preparation of lentivirus carrying genes of interest was the same as described previously [15].

Immunocytochemistry and co-localization measurement for cell cultures

Cells grown on cover slips were rinsed with PBS, fixed in 4% paraformaldehyde, and permeabilized with 0.1 M Tris-buffered saline (TBS; pH 7.6) containing 0.5% Triton X-100 for 5 min. They were subsequently blocked with 3% skim milk in TBS, incubated with antibodies to proteins of interest in TBS containing 3% skim milk overnight at 4 °C and then incubated for 1 h with secondary antibodies. Immunolabeled cells were stained with nuclei stain DAPI (Invitrogen) for 10 min and observed by confocal fluorescence microscopy (Zeiss LSM 510, Carl Zeiss MicroImaging).

Confocal images (60 × objective magnification) of at least 20 cells randomly chosen from 5 fields (upper left, upper right, center, lower left and lower right) per group were captured. The same exposure time was used throughout the course of image acquisition. Zen 2009 Light Edition software (Zeiss) was used to evaluate co-localization between interesting proteins for each single cell. An Overlap Coefficient (Carl Zeiss Advanced Imaging Microscopy, Jena, Germany) was measured from each cell in each group. The data were analyzed for statistical difference between the two groups with Student *t* test.

Recombinant proteins

Recombinant human α S was freshly prepared as previously described [17]. Recombinant histone H1.0 (M2501S) was purchased from New England Biolabs (NEB).

Electron microscopy (EM)

Samples were processed for EM and immuno-EM using protocols described previously [17]. Briefly, samples were adsorbed onto carbon-coated copper grids, fixed with 2% formaldehyde in phosphate-buffered saline (PBS), negatively stained with 5% uranyl acetate, and examined with a Philips EM 2008 electron microscope (Phillips). For immunogold labeling, samples were adsorbed onto carbon-coated copper grids, fixed in 2% formaldehyde in PBS, blocked with PBS plus 5% normal goat serum, 0.1% bovine serum albumin, and 0.1% gelatin, and incubated for 1 h with a mixture of anti-H1.0 (ab125027, 1:10, Abcam) and anti- α S (610787, 1:10, BD Biosciences) antibodies. The grids were then incubated with a mixture of secondary antibodies containing anti-rabbit IgG (1:25) and anti-mouse IgG (1:25), respectively, conjugated with 5- and 10-nm colloidal gold. The immunogold-labeled samples were negatively stained with 2% uranyl acetate and examined by EM.

Stereotaxic surgery

All animal procedures were approved by the Mayo Clinic Institutional Animal Care and Use Committee (IACUC) and were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996.

Recombinant protein solutions of α S and H1.0 were subjected to 100,000×*g* at 4 °C for 60 min to pellet any aggregated material as previously described [34]; the resultant supernatants were then measured by bicinchoninic acid assay (BCA) for determining protein concentration followed by 4-h incubation of α S/H1.0 mixtures, α S and H1.0

alone for preparation of injection materials. C57BL/6 mice (12 month of age) were anesthetized with 3% isoflurane and stereotaxically injected with 10 μ g α S/H1.0 complexes (α S/H1.0 = 6/4), 6 μ g α S or 4 μ g H1.0 (NEB) per brain following methods of Luk et al. [25]. In addition, sham-operated mice with PBS injection were included as controls to exclude any effects due to surgery and injection. At least 7 mice were used for each group. A single needle insertion (coordinates: X = 2.0 mm; Y = 0.2 mm; Z = 0.8 and 2.6 mm, respectively) was used to deliver the inoculum to somatosensory cortex and dorsal neostriatum. Material was injected via a Hamilton syringe at a rate of 0.5 μ l per min (5 μ l total per site). After recovery from surgery, animals were monitored regularly. A week later, animals were anesthetized and subjected to transcardial perfusion with PBS and brain fixation with 10% formalin followed by paraffin embedding of brains. Coronal sections were cut at 5 microns thickness for immunohistochemical and immunofluorescent staining.

Immunohistochemical and immunofluorescent staining

Sections of paraffin-embedded tissue were sequentially subjected to deparaffinization, rehydration, pretreatment with 98% formic acid for 30 min (only for staining with NACP98 antibody), steaming in DAKO target retrieval solution (pH 6.1) for 30 min, digestion with Protease 24 for 8 min at room temperature (only for staining with LB509), and blocking with Protein Block (X0909, DAKO) for 1 h at room temperature. Following blocking of endogenous peroxidase with 3% hydrogen peroxide, the sections were incubated with 5% normal goat serum for 20 min to reduce non-specific labeling. Tissue sections were then incubated with antibodies to H1 (sc-8030, 1:50, Santa Cruz), H3 (ab1791, 1:1500, Abcam) and α S (LB509, 1:100, Invitrogen) for 45 min and Envision-Plus labeled polymer HRP, rabbit or mouse (DAKO) for 30 min. Peroxidase labeling was visualized with a solution containing 3, 3'-diaminobenzidine (DAB-Plus). The sections were subsequently counterstained with Lerner 1 hematoxylin (14-930-11, Fisher Scientific) and coverslipped with cytoaseal mounting medium (8310-16, Richard-Allan Scientific).

For immunofluorescence, sections were incubated with Protein Block (X0909, DAKO) for 1 h at room temperature, followed by incubation with antibodies to MAP2 (Chicken, 1:3000, EnCor Biotechnology Inc.) plus H1 (sc-8030, 1:50, Ms, Santa Cruz) and α S (NACP98, 1:500, Rb), or H3 (ab1791, 1:500, Rb, Abcam) and α S (LB509, 1:100, Ms, Invitrogen) at 4 °C overnight, followed by incubation with three individual secondary antibodies (1:500, 1.5 h per antibody). For mouse brain sections, antibodies to H1.0 (ab125027, 1:100, Abcam) and α S (LB509, 1:100,

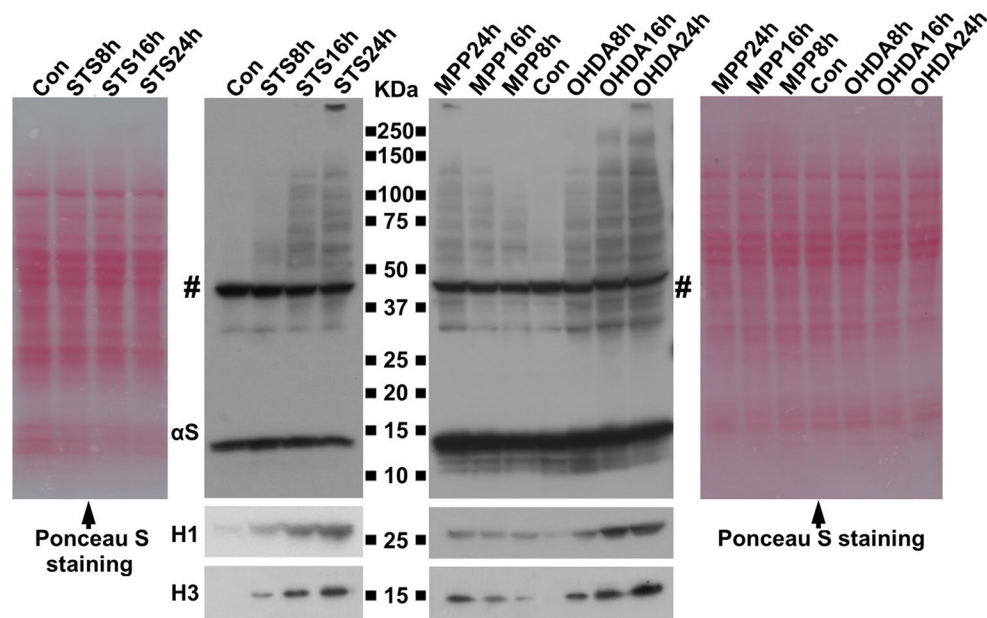


Fig. 1 Increase of histones in cytoplasm is concurrent with α S aggregation during neuronal apoptosis. Differentiated 3D5 cells with 5 days of α S induction were exposed to staurosporine (STS), 1-methyl-4-phenylpyridinium (MPP) or 6-hydroxy-dopamine (6-OHDA) for 0, 8, 16, 24 h. Cytoplasmic proteins of each sample were extracted and subjected to SDS-PAGE and western blotting with antibodies to histone H1 and H3, as well as α S. Ponceau S staining

was performed before immunostaining to demonstrate that all samples had comparable levels of total proteins. Statistical analysis of immunoreactivity of various proteins is shown in Fig. A1 in Supplemental Materials. Results demonstrate that H1 and H3 gradually increase in the cytoplasm during neuronal apoptosis, concurrent with gradual increase in α S aggregates. Number sign (#) denotes a non-specific Syn1-immunoreactive band on western blots of cell lysates

Invitrogen) were used. Non-specific fluorescence signals were blocked by staining with Sudan Black stain for 2 min. Sections were coverslipped with Vectashield mounting media (H-1200, Vector Laboratories) and evaluated by confocal fluorescence microscopy.

Co-localization measurement for human brain sections

One section per human brain sample was used for immunofluorescence staining and subsequent confocal imaging. A total of 10 cases of diffuse Lewy body disease (DLBD) and 10 age- and sex-matched neurologically and pathologically normal controls with minimal or no Alzheimer or PD type pathology (Thal phase and Braak stage) were included. Images ($20 \times$ objective magnification) of multiple fields (at least 3 randomly chosen fields) per section from the basal forebrain of postmortem brain sections were captured. The same setting of exposure time was used throughout the course of image acquisition. We used Zen 2009 software to measure the Overlap Coefficient (Carl Zeiss Advanced Imaging Microscopy, Jena, Germany) between H1 and α S, or H3 and α S. The average of overlap coefficient measured from each section was collected. Differences between DLBD and control were assessed by Student's *t* test.

Results

Histones H1 and H3 increase in cytoplasm in a time-dependent manner during neuronal apoptosis

We previously employed multiple neuronal cell models to show that during neuronal apoptosis, insoluble α S aggregates increase over time in cytoplasm and α S filaments can be found in apoptotic bodies. We also found that the formation of aggregates is associated with reciprocal diffusion and interaction between cytoplasmic α S and nuclear proaggregant factors due to the disruption of nuclear envelope integrity induced by apoptosis [16]. To determine if histones represent a nuclear proaggregant factor, we followed previous methods to induce apoptosis of differentiated 3D5 cells by exposing them to 25 nM staurosporine (STS) for 8, 16 and 24 h [16]. Cell lysates were subjected to SDS-PAGE and subsequent western blotting with antibodies to α S and two representative histone proteins—H1 and H3. As we expected, both H1 and H3 increase in cytoplasm in a time-dependent manner, which is concurrent with a gradual increase of cytoplasmic α S aggregates (Fig. 1 and Supplementary Fig. A1). Similar results were observed when apoptosis was induced by the PD-associated neurotoxins 1-methyl-4-phenylpyridinium (MPP) and

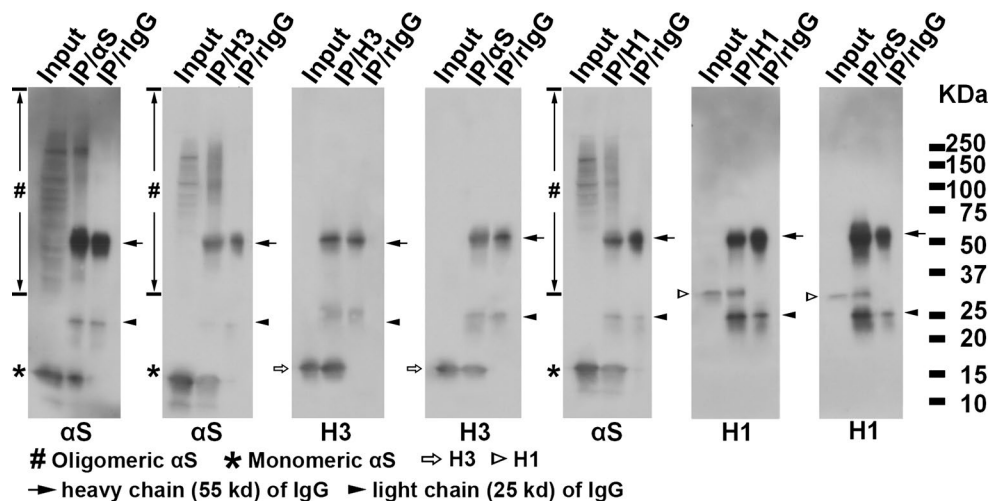


Fig. 2 Co-immunoprecipitation experiments show that histone and α S interact in apoptotic neuronal cultures. Differentiated 3D5 cells with 5 days of α S induction were exposed to STS for 24 h. Cytoplasmic fractions were aliquoted and, respectively, incubated with antibodies to α S, histone H1, H3 or rabbit IgG to generate immunoprecipitates (IP), referred to as IP/ α S, IP/H1, IP/H3 and IP/rIgG. Cell lysates lacking the aforementioned incubation were included as Input. A portion of each sample was probed with α S, histone H1, H3 to con-

firm enrichment of these proteins in IP products, and evaluate their interactions. IP/H1 and IP/H3 were shown to contain different sizes of α S-positive proteins corresponding to monomer (denoted by *asterisk*) and oligomers (denoted by *number sign*); and IP/ α S was also shown to contain H1 (denoted by *empty arrowhead*) and H3 (denoted by *empty arrow*) proteins. Solid arrow and *arrow head*, respectively, denote the heavy chain (55 kDa) and light chain (25 kDa) of IgG in IP

6-hydroxy-dopamine (6-OHDA) (Fig. 1 and Supplementary Fig. A1).

Histones H1 and H3 interact with α S in apoptotic neurons

To determine if histones interact with α S during neuronal apoptosis, differentiated 3D5 cells were exposed to STS for 24 h and then harvested. Cell lysates were prepared for immunoprecipitation with antibodies to α S, H1, H3 and rabbit IgG (rIgG), respectively. As expected, both H1 and H3 were detected in the immunoprecipitate of α S (IP/ α S), and α S was detected in the reverse IP/H1 and IP/H3 (Fig. 2). In contrast, neither α S, H1 nor H3 was detected in the immunoprecipitate of IP/rIgG (Fig. 2). These results suggest that histone and α S interact in neurons undergoing apoptosis.

Histones are associated with the formation of cytoplasmic α S aggregates in apoptotic neurons

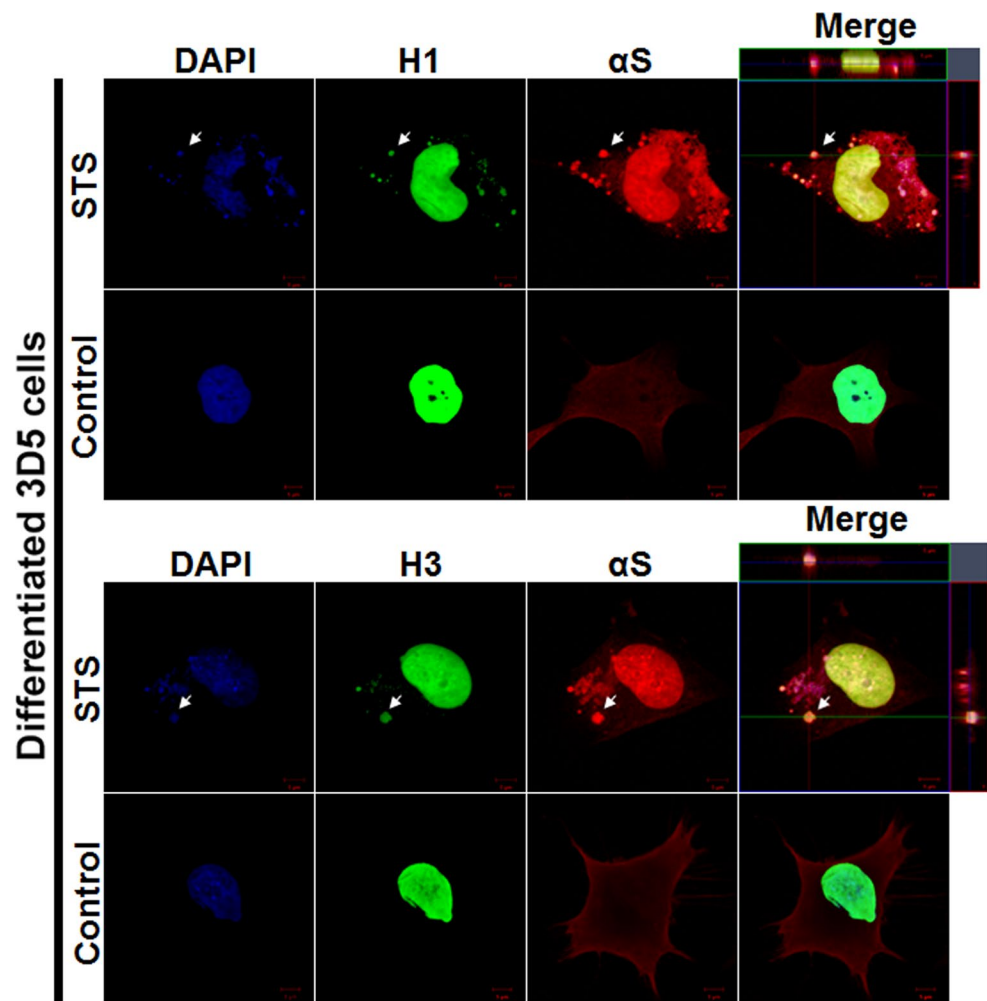
To further confirm the association between histones and cytoplasmic α S aggregates in apoptotic neurons, differentiated 3D5 cells were subjected to treatment with STS or vehicle, followed by fixation and dual immunocytochemical staining with antibodies to H1 (sc-34464, Santa Cruz) and α S, or H3 (ab1791, Abcam) and α S. A well-known α S antibody (LB509 [19, 22, 31]) that is sensitive to aggregated forms of α S was used to demonstrate the distribution

of α S aggregates. LB509 was generated against purified Lewy bodies as immunogen [13]. Using confocal microscopy, we observed that after STS treatment some α S translocate into the nucleus, which is consistent with our previous findings [16], leading to the co-localization between α S and H1 or H3 in nucleus. Simultaneously, H1 and H3 also diffuse into the cytoplasm, concurrent with the appearance of H1 and H3 immunopositive α S aggregates (arrow denoted structures in Fig. 3). In non-apoptotic cells treated with vehicle, α S and H1 or H3 remain in the cytoplasm and nucleus, respectively, and no α S aggregates can be observed (Fig. 3). Furthermore, the ratio of cells containing H1 or H3 co-immunopositive α S aggregates to total cells is, respectively, about 70 ± 10 and $17 \pm 8\%$. Co-localization between histones and α S can also be detected in differentiated LUHMES dopamine cells after exposure to STS (Supplementary Fig. A2). These data suggest that H1 and H3 are associated with cytoplasmic α S aggregates in apoptotic neurons.

Histones are associated with α S pathology in human brain

To determine if histones are associated with α S pathology in humans with Lewy-related pathology, tissue sections of basal forebrain from 10 patients with diffuse Lewy body disease (DLBD) and 10 age- and sex-matched normal controls were immunostained with antibodies to α S, histones H1 and H3. A subset of neurons in the basal nucleus

Fig. 3 Immunocytochemical staining demonstrates that histones co-localize with α S aggregates in apoptotic neurons. Differentiated 3D5 cells with 5 days of α S induction were subjected to treatment with staurosporine (STS) or vehicle (Control), followed by dual immunocytochemical staining with antibodies to histone H1/ α S or H3/ α S. Co-localization of α S and H1 or α S and H3 can be observed in both nucleus and cytoplasm in cells with STS treatment, but not in control cells. Z-stack confocal imaging is used to confirm the intracellular location of α S aggregates. Arrows denote a cluster of α S aggregates immunopositive to H1 or H3. Scale bar 5 μ m



of Meynert of DLBD had both nuclear and cytoplasmic immunoreactivity to H1 and H3 (Fig. 4a–c). Normal controls had only nuclear immunostaining. In addition, both H1 and H3 were associated with a subset of Lewy bodies and Lewy neurites (Fig. 4b, c). Co-localization analysis (Fig. 4d) demonstrated that overlap coefficients of both H1/ α S and H3/ α S in DLBD were significantly higher than those in controls. The overlap coefficient of H1/ α S was much higher than that of H3/ α S in DLBD cases, suggesting that of the two histone proteins, H1 may have a more significant role than H3 as a proaggregant factor for α S aggregation in DLBD. Therefore, we focused further investigations on histone H1.

Histone H1 interacts with α S to form complexes with a filamentous ultrastructure

To determine if histone H1 can induce α S aggregation, recombinant α S, histone H1, or a mixture of both proteins were incubated at 37 °C for 10 min and immediately subjected to native gel electrophoresis and western blotting,

as well as EM and immuno-EM. Duplicate samples were incubated at 37 °C for 4 h and then subjected to the same analysis. As a positive control, α S aggregates were prepared by constant shaking at 37 °C for one week. Native gel analysis demonstrates that α S and histone H1 can form complexes within 10 min of incubation, characterized as a single band near the cathode that is immunopositive for both H1 and α S (Fig. 5a). In contrast, H1 and α S each remained at the cathode or migrate close to the anode and are immunopositive only for H1 or α S (Fig. 5a). EM showed that after only 10 min incubation with H1, α S formed annular structures (Fig. 5b) similar to protofibrils as described previously [37], as well as short filamentous structures. After 4 h of incubation with H1, α S formed mature fibrils (Fig. 5b, panel i2) that were morphologically similar to fibrils formed from α S subjected to a week of constant shaking (Fig. 5b, panel i3). In contrast, samples of α S or H1 alone did not form distinctive structures after 4 h of incubation (Fig. 5b, panel i4 & i5). The filamentous structures observed in α S and H1 mixtures were decorated by both 5 nm gold particles for H1 and 10 nm gold particles

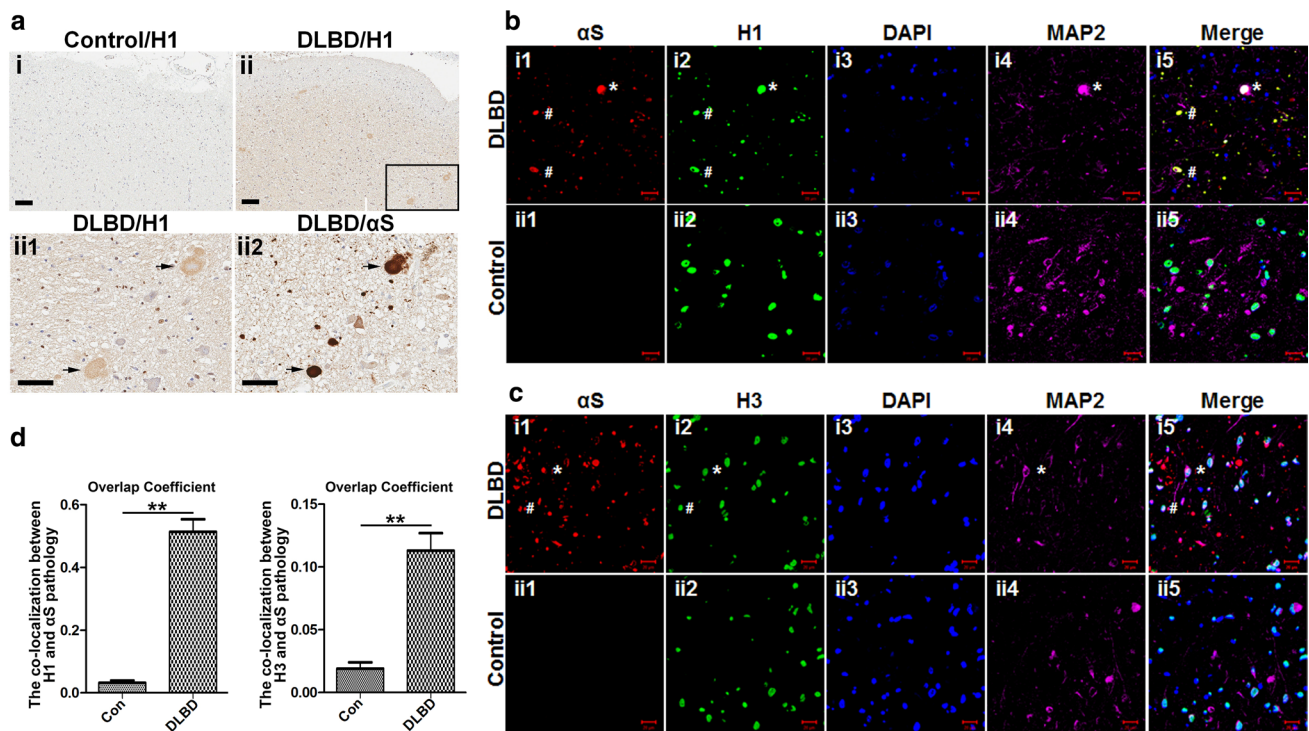


Fig. 4 Histone is associated with α S pathology in human brain tissues. Human specimens containing basal nucleus of Meynert from different cases with diffuse Lewy body disease (DLBD) and controls were probed with antibodies to α S and histone H1, or H3 by **a** immunohistochemistry and **b, c** immunofluorescence. *i, ii* in (**a**), respectively, show the representative pattern and intensity of H1 staining in brain tissue with α S pathology from DLBD patients and the same area from controls. *Scale bar* 100 μ m. *ii1* Enlarges the framed area in (*ii*) showing two H1 positive non-nuclear structures (*arrow denoted*). *ii2* Shows two α S positive structures (*arrows denoted*) which are detected at the same location in the same area of an adjacent section

for α S (Fig. 5b, panels *ii1* & *ii2*). Dual-labeled filaments are not detected in controls with only α S or H1 (Fig. 5b panel *ii3, ii4* & *ii5*). The fibrils formed by incubation of α S and H1 for 4 h (α S/H1 complexes) were sonicated for subsequent *in vitro* and *in vivo* experiments.

Histone-induced α S aggregates can spread to neurons and seed α S aggregation

To determine if histone-induced α S aggregates can be spread in the brain, fresh recombinant H1 and α S were labeled with Lightning-Link[®] Rapid DyLight[®] 550 and DyLight[®] 650 from Innova Biosciences, respectively (referred to as “H1-550” and “ α S-650”). The two samples were briefly mixed and incubated for 4 h at 37 °C to form complexes/aggregates of α S-650/H1-550. The samples of α S-650 and H1-550 alone, and α S/H1 mixtures (without DyLight fluorescent dye labeling) were incubated in parallel to serve as negative and positive controls, respectively.

of (*ii1*). *Scale bar* 60 μ m. **b, c** Respectively, show the representative triple immunofluorescence staining results of α S/H1/MAP2 and α S/H3/MAP2 in brain tissue with α S pathology from DLBD patients (*i1* to *i5*) and the same area from controls (*ii1* to *ii5*). Both *asterisk* * and *number sign* “#” randomly denote some co-localized staining of α S/H1 and α S/H3, but *asterisk* also denotes a third co-localization with MAP2. *Scale bar* 20 μ m. **d** *Bar graph* summarizes the co-localization analysis with Zen 2009 Light Edition software for H1/ α S and H3/ α S in aforementioned immunofluorescence staining studies of DLBD and controls

All samples were then subjected to negative staining followed by EM observation. Both α S-650/H1-550 and α S/H1 formed comparable filamentous structures, whereas no visible structures were found in the samples of α S-650 and H1-550 alone (Fig. 6a). Therefore, DyLight fluorescent dye-labeled α S and H1 were considered suitable to follow uptake, seeding and spreading.

Differentiated M17D neuronal cells overexpressing α S fused with Flag tag (referred to as “ α S-Flag”) were incubated with the α S-650/H1-550 complexes for 3 days and then subjected to immunocytochemistry with antibodies to Flag. As a control, neurons were treated only with α S-650 and H1-550 alone. Co-localization of α S-650 and H1-550 was observed in $92 \pm 6\%$ cells of the group treated with α S-650/H1-550 complexes/aggregates, indicative of highly efficient internalization (Fig. 6b). Moreover, these cells had abundant intracellular α S-Flag aggregates, whose distribution was similar to that of intracellular α S-650/H1-550 complexes. Quantitative analysis with Zen 2009 from at

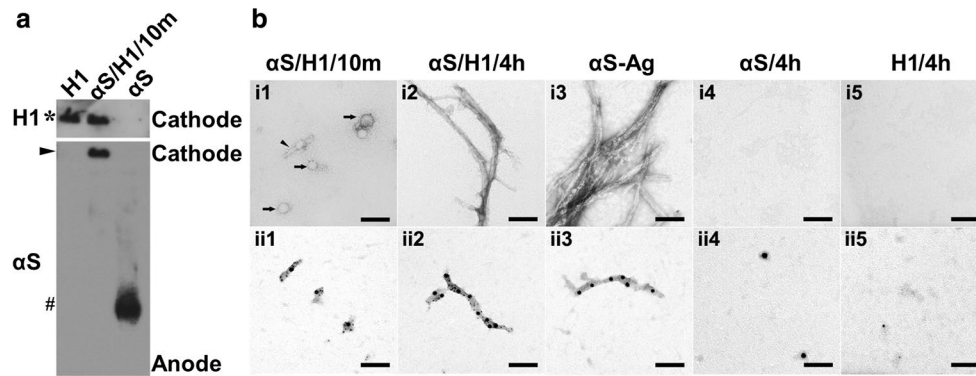


Fig. 5 α S rapidly forms aggregates upon interaction with histone H1. Recombinant α S with or without histone H1 was mixed and incubated at 37 °C for 10 min and 4 h, respectively. **a** Native gel electrophoresis and Western blotting analysis were performed to assess histone H1-induced α S aggregates. Asterisk * denotes two different bands staying closest to the cathode, one is the complexes formed by mixing α S with histone H1, which are immunostained by both H1 and α S (also see arrow head denoted band); the other is the histone H1, which is negative with α S immunostaining. Number sign # denotes the band of fresh recombinant α S, which migrates towards

anode. **b** Negative (*i1* to *i5*) and immunonegative (*ii1* to *ii5*) stained transmission electron micrographs of histone H1 induced α S aggregates and different controls. The α S aggregates prepared by a week of constant shaking at 37 °C were included as a positive control and named as “ α S–Ag.” Fresh H1 and α S protein solutions incubated at 37 °C for 4 h, referred to as α S/4 h and H1/4 h, were used as negative controls. Arrows denote annular structures, resembling protofibrils; arrowhead denotes short filamentous structure. All samples labeled with dual immunogold (5- and 10-nm gold particles for H1 and α S, respectively) are shown in *ii1* to *ii5*. Scale bar 100 nm

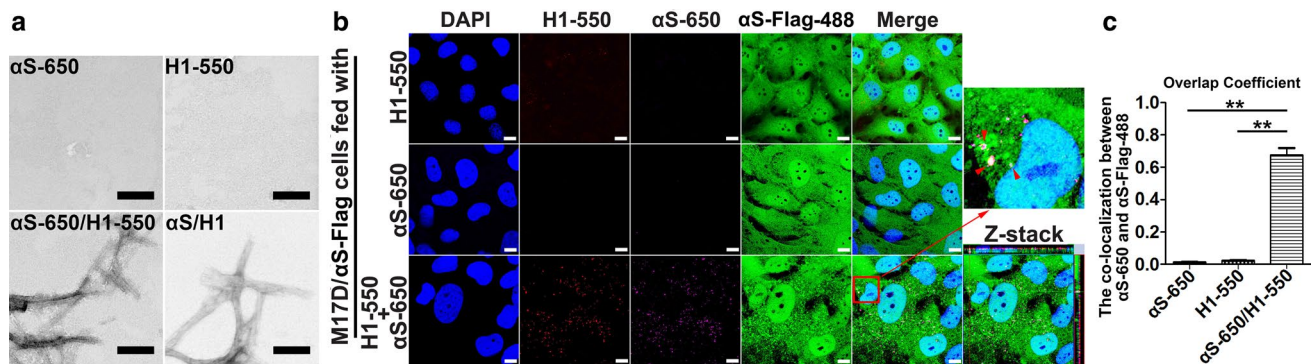


Fig. 6 Histone-induced α S aggregates can spread into neurons and seed α S aggregation in vitro. **a** Freshly prepared histone H1 and α S were, respectively, labeled with Lightning-Link[®] Rapid DyLight[®] 550 and DyLight[®] 650 (Innova Biosciences), referred to as “H1-550” and “ α S-650”. The two samples were briefly mixed and incubated for 4 h at 37 °C to form complexes/aggregates of α S-650/H1-550; the latter were then subjected to negative staining followed by EM observation. The samples of α S-650 and H1-550 alone, and α S/H1 mixtures (without DyLight fluorescent dye) were prepared in parallel to serve as negative and positive controls. Scale bar 100 nm. **b** The filamentous α S-650/H1-550 complexes were added to differentiated M17D cells overexpressing α S-Flag. Cells fed with only α S-650 or H1-550 were used as controls. After 3 days, cells were fixed and immunostained with primary antibody against Flag (F3165, Sigma),

followed by secondary antibody conjugated with AlexaFluor 488 and DAPI counterstaining. Results show that both α S-650 and H1-550 co-localize in cells fed with α S-650/H1-550 complexes, indicative of their internalization. Moreover, these cells have abundant intracellular α S-Flag aggregates, whose distribution is similar to that of intracellular α S/H1 complexes. Scale bar: 10 μ m. Z-stack confirms the intracellular location of seeded aggregates, which is further enlarged above the Z-stack picture. Arrows denote co-localization of α S-Flag aggregates and α S/H1 complexes. **c** Bar graph summarized the co-localization analysis with Zen 2009 software from at least 20 randomly chosen cells per group which shows that the overlap coefficient of α S-Flag aggregates and α S-650 is significantly higher in α S-650/H1-550 group compared with another two control groups

least 20 randomly chosen cells per group showed that the overlap coefficient of α S-Flag aggregates and α S-650 was significantly higher in α S-650/H1-550 compared to the two controls (Fig. 6c). These results suggest that H1-induced α S aggregates can be internalized in neurons and seed α S aggregation.

Histone-induced α S aggregates can be internalized by neurons in vivo

Wild-type C57BL/6 mouse brains were stereotactically injected into somatosensory cortex and dorsal neostriatum with sonicated α S/H1 complexes or with fresh recombinant

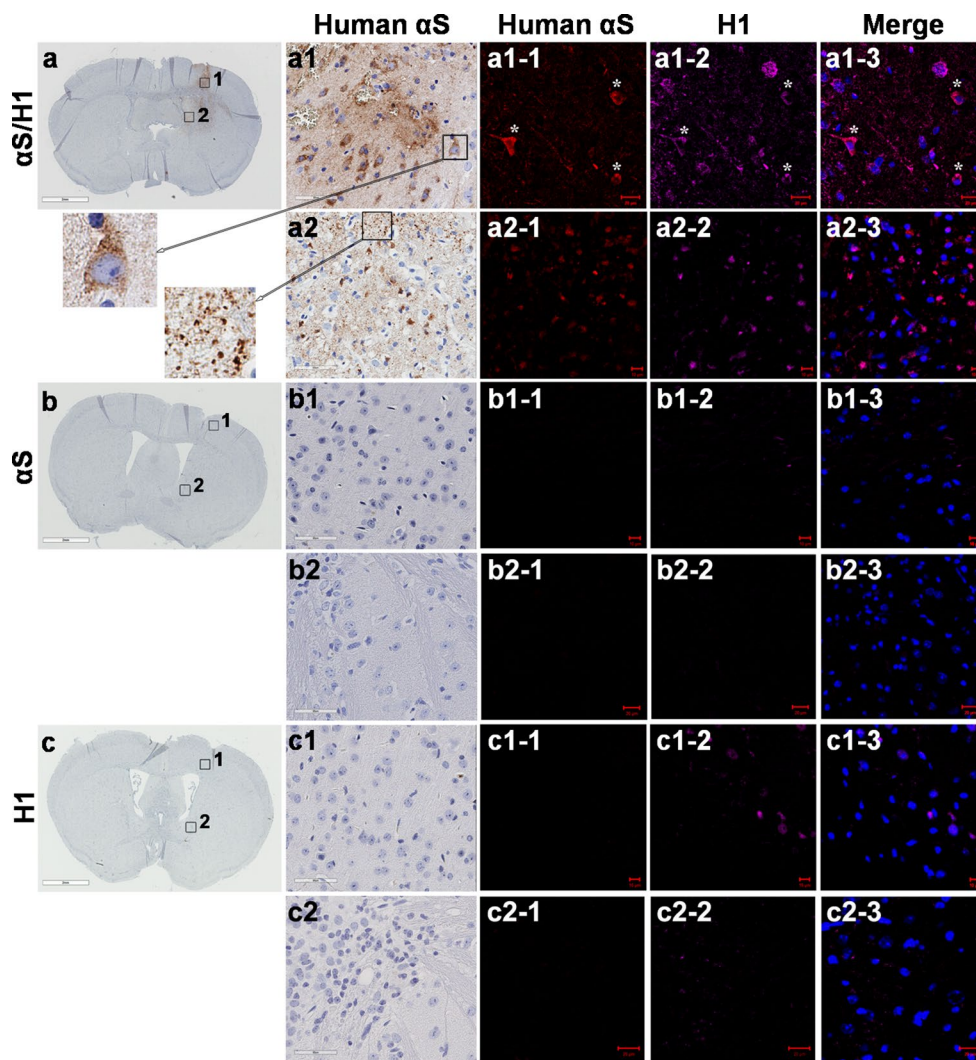


Fig. 7 Histone-induced α S aggregates can spread to neurons upon inoculation into mouse brain. Wild-type C57BL/6 mouse brains were inoculated with α S/H1 aggregates, fresh recombinant α S or histone H1 via stereotaxic brain injection and harvested a week later. **a–c** Paraffin sections of brains injected with **(a)** α S/H1 aggregates, **b** fresh recombinant α S or **c** fresh recombinant H1 were, respectively, subjected to immunohistologic staining with antibody to human α S (LB509) to demonstrate human α S pathology. Scale bar 2 mm. Panels *a1*, *a2*, *b1*, *b2*, *c1* and *c2* enlarge framed areas labeled 1 and 2

a–c. Scale bar 60 μ m. The framed areas in (*a1*) and (*a2*) are further enlarged to show intra-neuronal α S deposits and the thread- or dot-like α S deposits. Panels (*a1-1* to *a1-3*), (*a2-1* to *a2-3*), (*b1-1* to *b1-3*), (*b2-1* to *b2-3*), (*c1-1* to *c1-3*) and (*c2-1* to *c2-3*) are brain regions corresponding to *a1*, *a2*, *b1*, *b2*, *c1* and *c2* in adjacent sections, which were subjected to immunofluorescence staining with primary antibodies to human α S (mouse monoclonal) and H1 (rabbit polyclonal), and goat secondary antibodies to mouse (Alexa Fluor 568) and rabbit (Alexa Fluor 647). Scale bar 10 μ m

α S and H1 alone. After one week, the animals were killed and brains harvested, fixed and embedded in paraffin for immunohistochemical studies. Coronal sections were processed for double labeling immunofluorescence microscopy. Human α S pathology was observed at the site of the injection of sonicated α S/H1 complexes using immunohistochemistry. Perinuclear α S deposits were detected in neurons in the neocortex (Fig. 7a, panel a1), while thread-like or dot-like α S deposits were detected in the white matter (Fig. 7a, panel a2). Dual immunofluorescence demonstrated that α S pathology was immunopositive for H1

(Fig. 7a, asterisk-marked cells), indicating that the α S/H1 complexes were stable to sonication. In contrast, sections from mice injected with recombinant α S or H1, alone, did not show neuronal or neuritic α S pathology.

Discussion

A decade ago, Goers et al. reported that histones could accelerate α S aggregation in vitro [11], but whether histones might play a role in inducing pathological α S in PD

and related disorders has never been addressed. This is partly because histones and α S rarely interact in healthy neurons due to their distinct subcellular locations—histones in the nucleus and α S in the cytoplasm. Our previous study provided a theoretical basis for a role for nuclear proaggregant factor(s) in the formation of cytoplasmic α S aggregates upon loss of nuclear membrane integrity [16]. The present study identifies histones as a potential proaggregant nuclear factor involved in α S aggregation in neurons undergoing apoptosis and possibly in neurons with sub-lethal nuclear membrane abnormalities that lead to leakiness.

We found that in apoptotic neurons, histones H1 and H3 diffuse into the cytoplasm in a time-dependent manner and interact with α S to form cytoplasmic aggregates. More importantly, histones (especially H1) co-localized with a subset of Lewy bodies and Lewy neurites in DLBD. Because H1/ α S had a comparatively higher overlap coefficient compared with H3/ α S, we speculate that H1 may play a more important role in the formation of pathological α S. This might be due to different roles of H1 and H3 in nucleosomes [30]. It is known that H3 is a core protein of the nucleosome that is tightly wrapped around by DNA. It can be unmasked to interact with other proteins only after DNA is removed. In contrast, H1 serves as a linker histone that is less tightly associated with DNA. Therefore, it is possible that H1 is more labile and more likely to interact with α S in the nucleus and more likely to diffuse to cytoplasm upon disruption of nuclear membrane integrity, as occurs during apoptosis.

We showed that histone accelerated α S aggregation into filamentous structures as reported previously by Goers et al. [11], and further demonstrated that histone-induced aggregates contain a range of α S oligomers, including protofibrils and mature fibrils. More interestingly, we found that histone-induced α S aggregates are transmissible to neurons both in vitro and in vivo. In cultured neurons, histone-induced α S aggregates triggered aggregation of endogenous α S, demonstrating its seeding activity. We were unable to determine if histone-induced α S aggregates (α S/H1) have seeding activity after injection into mouse brain because no antibody is currently available that can distinguish endogenous mouse α S from injected human α S. To address this issue, an animal model needs to be developed in which mouse and human α S can be differentiated using tagged α S. Alternatively, considering that human α S is not likely to cause seeding in mouse brain, sonicated mouse α S/H1 fibrils might be used to promote in vivo seeding followed by immunostaining for S129-phosphorylated α S, a strategy successfully employed in previous studies [24, 26]. It will also be important to determine if mouse α S and H1 can form fibrils comparable to those observed after exposing human α S to human H1.

It is worth noting that histones might play roles beyond their proaggregant effect. It has been shown that the positive charge of histones renders them capable of crossing cellular membranes [12]. It has also been shown that the positively charged transfection reagent Lipofectamine can mediate uptake of α S aggregates into neuronal cells [29]. Thus, it is intriguing to speculate that endogenous histones, like the experimental agent Lipofectamine, might facilitate internalization of α S aggregates and, thus, favor cell-to-cell propagation.

Evidence suggests that histones can be secreted into extracellular fluids from a range of cell types, including neurons [1], glia [28] and some activated immune cells [2, 9, 35] in response to cell stress. Moreover, extracellular histones are significantly elevated in response to brain injury [4, 20], which is a risk factor for PD [3, 10]. There is increasing evidence that α S can be secreted into extracellular fluids by non-classical, endoplasmic reticulum- and Golgi-independent protein export pathways [21] and that the level of extracellular α S is increased with cellular stress and brain injury [8, 14]. Thus, it is reasonable to hypothesize that under conditions of cell stress or brain injury, histones may interact with α S in extracellular fluids to facilitate the formation of transmissible aggregates that could facilitate more widespread cell-to-cell propagation.

Although transmission of α S oligomers or aggregates has been previously reported, most studies used aggregates from pathologic brain samples or α S prepared in vitro by prolonged (several days to a week) shaking of recombinant α S [6, 24, 27]. In contrast, α S aggregates used in present study form quickly after incubating α S with histone. Therefore, a secondary outcome of our study is the identification of a faster method for preparing experimental α S aggregates to study transmission in animal models.

While previous work [11] and the present study provide compelling evidence that histones have proaggregant properties with respect to α S aggregation, there may be other nuclear factors that promote α S aggregation during neuronal apoptosis. In-depth studies are warranted to define the nature of other potential nuclear proaggregant factors.

Overall, our study suggests histones as prime candidates for one of the nuclear proaggregant factors involved in the formation of transmissible α S aggregates during neuron apoptosis. This study provides new insights into the pathogenesis of initial pathological α S seeds in PD and related disorders, and could lead to novel diagnostic and therapeutic approaches. Specifically, detection of excessive histone release from cells into body fluids (e.g., cerebrospinal fluid) might be a new strategy for early detection of disease-associated processes that favor α S pathology. It remains to be determined if limiting or preventing histone release could be an upstream target to decrease α S aggregation and subsequent propagation.

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