Low Dose DMSO Treatment Induces Oligomerization and Accelerates Aggregation of α -Synuclein

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Low dose DMSO treatment induces oligomerization and accelerates aggregation of α -synuclein

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Abstract

Dimethyl sulfoxide (DMSO) is a highly utilized small molecule that serves many purposes in scientific research. DMSO offers unique polar, aprotic and amphiphilic features, which makes it an ideal solvent for a wide variety of both polar and nonpolar molecules. Furthermore, DMSO is often used as a cryoprotectant in cell-based research. However, recent reports suggest that DMSO, even at low concentration, might interfere with important cellular processes, and cause macromolecular changes to proteins where a shift from α -helical to β -sheet structure can be observed. To investigate how DMSO might influence current research, we assessed biochemical and cellular impacts of DMSO treatment on the structure of the aggregation-prone protein α -synuclein, which plays a central role in the etiology of Parkinson's disease, and other brain-related disorders, collectively termed the synucleinopathies. Here, we found that addition of DMSO increased the particle-size of α -synuclein, and accelerated the formation of seeding-potent fibrils in a dose-dependent manner. Moreover, as evident through aggregation specific antibody recognition and a bimolecular fluorescence complementation assay, cells exposed to DMSO experienced increased aggregation of α -synuclein. However, no observable α -synuclein abnormalities nor neuronal survival was affected by oral DMSO-treatment in either C57BL/6- or α -syn transgenic F28 mice. In summary, we demonstrate that low concentrations of DMSO makes α -synuclein susceptible to undergo aggregation both in vitro and in cells. This may affect experimental outcomes when studying α -synuclein in the presence of DMSO, and should call for careful consideration when such experiments are planned.

Introduction

α -synuclein (α -syn) is central in the pathogenicity of the subgroup of neurodegenerative disorders collectively designated the synucleinopathies; a subgroup comprised by Parkinson's disease (PD), Dementia with Lewy Bodies (DLB) and Multiple System Atrophy (MSA). α -syn itself presents as a 140-amino acid protein that is widely expressed in the brain, where it primarily localizes to the presynaptic terminals in neurons. The native structure of α -syn is still debated, but a majority of reports suggest, that under physiological conditions, α -syn exists mainly as an intrinsically disordered monomer [1, 2]. However, tetramerization of α -syn under physiological conditions has been proposed [3, 4]. Besides α -syn, two other synuclein family members exist, namely β - and γ -synuclein. While γ -synuclein primarily resides in the peripheral nervous system, α - and β -synuclein (β -syn) exhibit comparable brain-specific expression patterns [5]. Yet, structurally, β -syn diverges from α -syn within the non-amyloid- β component (NAC) domain indispensable for α -syn aggregation [5].

Under pathological conditions α -syn aggregates into larger insoluble species, but α -syn is also hypothesized to exist as intermediate structures, referred to as oligomers, between native- and insoluble states. Such soluble α -syn oligomer species have been detected in post-mortem PD, DLB and MSA patient brain extracts [6-8], and accumulating evidence suggest direct participation of oligomers in disease progression in the brain of patients suffering from synucleinopathies [9-11]. Based on the hypothesis that aggregation of α -syn constitutes the major decisive event in development of pathology, several intervention strategies to prevent oligomerization have been developed, including immunization- and small molecule based strategies. Furthermore, a number of tools have been developed to visualize and assess the size and structure of α -syn, e.g. conformation specific antibodies.

In the current study, we investigate how dimethyl sulfoxide (DMSO), a widely utilized polar aprotic solvent, affects α -syn structure and aggregation. DMSO is often used to dissolve water-insoluble organic and inorganic compounds, including compounds associated with modulatory effects on α -syn aggregation as well as chemical cross-linkers used to stabilize tetrameric α -syn [3, 4, 12]. Although a few reports mention an effect of DMSO treatment on α -syn structure [13, 14] the effect of DMSO in experiments are often neglected.

Here, we demonstrate that low concentrations of DMSO induces a shift in size-distribution of α -syn into larger species. Furthermore, incubation of monomeric α -syn with DMSO gives rise to enhanced aggregation-specific antibody binding, and accelerates the rate of fibrillation without affecting the overall structure and seeding properties of the fibrils. Simultaneously, cells exposed to DMSO exhibit higher levels of α -syn multimerization as well as inclusion-like structures positive for aggregate-specific staining. By contrast, no clear pathological changes were observed in α -syn structures in wt and α -syn transgenic mice orally treated with DMSO. Together, our findings suggest that when interpreting α -syn aggregation-specific data in experiments where DMSO is included caution should be taken: especially when working with α -syn *in vitro* and *in cellulo*.

Results

DMSO treatment increases α -syn particle-size and stimulates *in vitro* aggregation

Due to the widespread usage of DMSO in biomedical research, and limited but concerning information regarding the influence of DMSO treatment on α -syn behavior [13, 14], we decided to perform a comprehensive study, to elucidate the role of DMSO treatment on α -syn assembly. First, to assess the size distribution of recombinant α -syn in the absence and presence of low doses of DMSO, we performed a series of dynamic light scattering (DLS) analyses in the presence or absence of DMSO. Here, short exposure of α -syn to DMSO increased the particle size of α -syn in a concentration-dependent manner (Fig. 1A). Contrarily, DMSO did not induce a size shift in the globular control protein carbonic anhydrase (Fig. 1B).

Based on this finding, we sought to examine if the increased particle size observed was caused by α -syn aggregation. Utilizing the aggregation-specific antibody MJFR-14-6-4-2 that preferentially recognizes oligomeric and fibrillar forms of α -syn [15], we performed dot-blots on recombinant α -syn treated with different concentrations of DMSO. To control for equal loading between samples we used the SYN-1 antibody, which measures total α -syn levels (Fig. 1C). Contrary to SYN-1 detection, the MJFR-14-6-4-2 signal was significantly enhanced by DMSO treatment, suggesting that DMSO facilitates a structural change of α -syn which shares aggregation-specific antibody recognition with that of aggregated α -syn present in neuronal inclusions in brain tissue (Fig. 1C and 1D) [16].

Next, to assess the effect of the DMSO treatment on fibrillation, we used Thioflavin T (ThT) to measure β -sheet content in recombinant α -syn incubated at low levels with increasing concentrations of DMSO under agitating conditions. We observed that the ThT signal of α -syn, incubated in the presence of 10%, 5% and 1% DMSO, increased and reached plateau intensity after two, 10 and 17 days of incubation respectively (Fig. 1E). In contrast, α -syn incubated in the absence of DMSO did not yet plateau after 17 days of incubation (Fig. 1E). Sedimentation of samples after 7 days of incubation, confirmed the presence of insoluble aggregates only in samples exposed to 5% and 10 % of DMSO (Fig. 1F). By contrast, we did not observe any increase in ThT signal of β -syn, when exposed to DMSO (Fig. 1G). Together, these experiments suggest that DMSO treatment specifically increases the aggregation-rate of α -syn *in vitro*.

Pre-formed fibrils made in the presence of DMSO are seed competent and resemble naïve fibrils in structure

To evaluate the structure of the ThT positive and insoluble α -syn we examined *in vitro* aggregated α -syn by transmission electron microscopy (TEM) (Fig. 2A), which revealed a well-ordered fibrillary structure of both α -syn incubated in PBS alone (α -syn wt) and α -syn incubated with 2% DMSO (α -syn DMSO) (Fig. 2A).

To evaluate if the addition of DMSO to the aggregation process could create a distinguishable fibrillar polymorph of α -syn we performed a proteolytic peptide mapping of α -syn wt- and α -syn DMSO pre-formed fibrils (PFFs) and compared their proteolytic fingerprints: a technique that has been extensively used to determine structural differences between strains of α -syn [17-19]. Based on digests performed on purified PFFs with increasing concentrations of the serine protease, Proteinase K (PK), we evaluated the cleavage pattern (Fig. 2B). Here we observed that increasing amounts of PK produced gradually more and more degraded α -syn products as expected (Fig. 2B). Yet, between the two groups of fibrils, an identical cleavage patterns was observed (Fig. 2B), suggesting that DMSO stimulates α -syn aggregation without affecting the overall fibrillary structure.

To test the seeding potential of DMSO induced fibrils, we treated human α -syn-overexpressing OLN-AS7 cells with α -syn PFFs to induce aggregation of cellularly expressed α -syn. These PFFs were formed in PBS alone or induced by 2% DMSO. Yet, to distinguish between externally added PFF seeds, and the cellular expressed α -syn, the PFFs were made from S129A-substituted α -syn (S129A PFF). First, we confirmed the ability of both 2% DMSO S129A PFFs and regular S129A PFFs to induce aggregated MJF-14-6-4-2-positive α -syn structures (Fig. 2C). Further, using a phospho-Ser129 specific antibody, we observed the formation of phospho-Ser129 inclusion in cells treated with both types of fibrils (Fig. 2D). Monomeric S129A α -syn (control) did not induce inclusion formation (Fig. 2C-D). These data demonstrate that α -syn aggregates stimulated by DMSO, possess seeding-potential, and exhibit functional characteristics resembling those of naïve PFFs.

DMSO stimulates α -syn multimerization in cells

Small molecules used to modulate cellular pathways are often dissolved in DMSO prior to experimental application. Although these compounds are most frequently diluted manifold upon the addition to cells the final molar concentration of DMSO is still relatively high. Based on our *in vitro* findings we therefore investigated whether DMSO in its often used concentration of 0.1%-1% [20, 21] causes any significant changes in cellular α -syn structure.

First, we utilized the human neuroblastoma SH-SY5Y cell-line, which by the addition of retinoic acid, is readily differentiated into a non-mitotic phenotype where early developmental features of neurons are mimicked [22]. Here we used a Tet-Off inducible variant (SH-SY5Y ASYN) [23], where α -syn overexpression is suppressed by doxycycline (dox). We observed that α -syn overexpression, as a result of dox removal, gave a detectable baseline MJFR-14-6-4-2-specific antibody staining after 7 days of differentiation relative to dox-treated cells (Fig. 3A-C). Additionally, this aggregation-specific MJFR-14-6-4-2 staining was significantly enhanced in cells continually treated with doses of DMSO that exceeded 0.25% during differentiation (Fig. 3A-C), but already at 0.1% a trend was observed. Interestingly, the MJFR-14-6-4-2 staining morphologically appeared as small inclusions present in both the perinuclear region and in the processes within these cells (Fig. 3B). Contrary to the aggregate-specific MJFR-14-6-4-2-staining, we did not observe elevated levels of phospho-Ser129 α -syn in cells treated with DMSO at any of the applied concentrations (Fig. 3D).

As an orthogonal approach to the aggregate detection using MJFR-14-6-4-2 antibody, we assessed α -syn oligomerization in cells using a bimolecular fluorescence complementation (BiFC) assay with a split fluorescent venus YFP construct (Fig. 3E-F) [24, 25]. This construct consists of two non-fluorescent pairs, namely V1S (N-terminal half of Venus YFP fused to N-terminus of full-length α -syn) and SV2 (C-terminal half of Venus YFP fused to C-terminus of full-length α -syn) that upon approximation reconstitute the intact fluorescent Venus structure. C to N-terminal dimerization of α -syn associated with V1S- and SV2 will bring, the N- and C-terminal fluorescent fragments of Venus YFP within proximity, thereby allowing emission of its yellow fluorescent signal (Fig. 3E). Expression of these fluorescent complementation pairs in HEK-293T cells, allowed evaluation of live α -syn multimerization within cells (Fig. 3F). To assess the role of low dose DMSO treatment we incubated the transfected cells in the presence or absence of DMSO continuously from 20 h prior to fluorescent analysis. Here we observed that even at low doses of 0.1% and 0.25% DMSO, equivalent to 1:1000 and 1:400 dilution respectively, induced an increase in α -syn multimerization based on YFP fluorescence (Fig. 3F). As expected, expression of individual V1S and SV2 construct did not induce YFP fluorescence in the presence or absence of DMSO (Fig. 3F).

Together, these results suggest that DMSO treatment not only induce detectable α -syn aggregation-like species under *in vitro* experimental conditions, but also in a more complex cellular environment.

Oral DMSO treatment does not induce gastrointestinal- or brain α -syn abnormalities in mice

Misfolding of α -syn in the enteric nervous system has been proposed to precede visible α -syn brain abnormalities, suggesting a model where PD pathology originating from the gut can spread to the central nervous system in a prion-like manner [26, 27]. The initial site of aggregation in the gut is unknown, but α -syn-expressing endocrine cells that line the intestinal epithelium and connects to enteric neurons could be the site of initiation [28, 29]. These cells are only separated by their plasma membrane from the gut lumen that may contains environmental toxins, pathogens and metabolites that might affect and initiate α -syn aggregation.

Based on this hypothesis we designed an experiment wherein wt C57BL/6 or transgenic F28 mice overexpressing human α -syn under the control of the endogenous mouse α -syn promoter [30] were orally treated once a day with water, 10% DMSO (1 g/kg bodyweight) or 30% DMSO (3 g/kg bodyweight) for 14 days. Mice were subsequently sacrificed, and α -syn structures in the gastrointestinal tract and brain analyzed by immunohistochemistry (IHC) using both aggregation-specific MJF-14-6-4-2- and phospho-Ser129-specific α -syn antibodies (Fig. 4).

In the duodenum and ileum of both C57bl/6 and F28 mice in all experimental groups specific MJF-14-6-4-2 α -syn immunopositive profiles could be seen (Fig. 4 A and B). The structures localized both to the muscular layer, in typical enteric plexi formations and in the mucosal cells, where enteroendocrine cells could be present (Fig. 4A and B). Although the number and intensity of positive structures were higher in the F28 mice compared to the C57bl/6 mice, no visible differences could be observed among the different treatments (Fig. 4A and B). In the brain, phospho-Ser129-specific staining of α -syn appeared as typical punctae in the striatum for all animals (Fig. 4A and B). Very little staining was observed in the substantia nigra (not shown). Additionally, in the striatum and substantia nigra no visible differences between animals were detected (Fig. 4A and B). Taken together, these data indicate that oral DMSO treatment did not have an observable effect on α -syn aggregation or dopaminergic neuron survival in young wildtype or transgenic mice expressing human α -syn.

Discussion

Due to DMSO's versatility as a polar, aprotic and amphiphilic molecule, it continues to serve as one of the most popular solvents in scientific research used to dissolve a variety of polar and nonpolar constituents. Yet, in line with our observations, multiple reports suggest that DMSO can induce gross changes even at low concentrations, when applied in scientific research experiments. Researchers have found that low DMSO concentrations induce compaction of the native protein structure in hen egg-white lysozyme and myoglobin [31], while similar low DMSO concentrations (0.5-3%) treatment of human growth hormone receptor and the phosphatase domain of PFKFB1 led to protein structure destabilization and aggregation [32]. On the other hand, opposing stabilizing and destabilizing effects of avidin and CYP142A1 was induced by DMSO, suggesting that the effect of DMSO is highly protein-dependent [33].

DMSO treatment evidently also affects cell signalling and macromolecule structure in multiple ways. For instance more than 2000 genes were differentially expressed upon 0.1% DMSO treatment of microtissues compared to non-treated conditions [21]. Similarly, low dose DMSO treatment (0.1-1.5%) of cells led to a comprehensive alteration of especially protein- and nucleic acid organization where a shift towards β -sheet structure was observed in a dose-dependent manner [20]. Based on our *in vitro*- (Fig. 1) and cellular experiments (Fig. 3) which suggest that α -syn multimerization occurs in the presence of DMSO, as well as previous reports showing that α -syn adopt β -sheet structure upon oligomerization and fibrillation [15, 34], we cannot exclude that α -syn is one of these cellular proteins adopting β -sheet structure upon DMSO treatment.

The underlying mechanism of α -syn multimerization during DMSO exposure is unknown, but DMSO can influence the solubility of specific amino acids. Glycine solubility in aqueous DMSO is low compared to water, while this decrease in solubility is less pronounced in hydrophobic amino acids such as alanine and leucine [35]. On the other hand the solubility of tryptophan increases in aqueous DMSO, suggesting that DMSO does still interact with hydrophobic amino acid residues [35]. In agreement with this, a different study described non-covalent interactions between the sulfoxide group of DMSO and aromatic side chains [36]. A possible mechanism of DMSO induced aggregation, could therefore involve disassembly of intramolecular interactions by disruption of hydrophobic clusters via its sulfoxide group, potentiating intermolecular interactions leading to nucleation and aggregation. The extent of oligomerization is uncertain from the presented results, however enhanced MJF14-6-4-2 antigenicity on dot blot is evident after just 30 minutes of DMSO treatment even at low α -syn concentrations (10 μ g/ml, 0.69 μ M) (Fig. 1), and in cells at longer DMSO exposure times (Fig. 3), and furthermore we do observe proposed α -syn dimerization by BiFC in cells (Fig. 3). Our *in vivo* data on the other hand do not suggest that oral DMSO treatment in mice can induce gross changes to enteric α -syn or α -syn in the brain. However, we do not know how much of the orally administered DMSO actually reaches the gut, how long the cells are exposed, and at what concentration. Yet, given the high concentrations of DMSO used in our experimental setup *in vivo*, we do not expect the low DMSO concentrations usually applied in animal research to interfere with, or perturb, the structure of α -syn *in vivo*, and therefore see no reason to substitute DMSO with another solvent when working with animals.

The DMSO concentrations we applied in our cell culture experiments on the other hand (0.1-0.75%), are easily within the range normally used to treat cells, e.g. when DMSO is used as a co-solvent for drug testing. Although a final concentration of 0.1-0.75% does not appear particularly high, the molar concentration of 0.75% DMSO, as calculated as a volume percentage in this work, translates into 106 mM. This means that DMSO at 0.75%, is the third most prevalent molecule in the culture media, but already at 0.1% (14 mM) DMSO is relatively abundant. Concentrations that high of any molecule, will undoubtedly affect the cells. A number of cellular effects induced by DMSO could induce aggregation, however combined with the direct

induction of aggregation *in vitro*, direct interaction of DMSO and α -syn appears a likely causative effect. Interestingly, we did not observe increased phospho-Ser129 levels of α -syn upon DMSO treatment. This could be due to the nature of the aggregation-like cellular α -syn structure that might serve as poor kinase substrates compared to physiological α -syn aggregates.

Our data clearly suggest that precautions should be taken when addressing questions regarding the native structure of α -syn in the presence of DMSO. One experimental setting where DMSO hypothetically could play an unfortunate and misleading role, is within the field of α -syn tetramerization [4, 37]. DMSO has routinely been used to solubilize cross-linkers, and in experiments used to verify the existence of α -syn tetramers in cells, DMSO was present in a 0.2% final concentration [4, 37]. Our experiments suggest that DMSO in this concentration is sufficient to induce vast multimerization of α -syn in cells (Fig. 3). Based on these findings we propose that the existence of α -syn tetramers ought to be verified using water-soluble cross-linkers.

DMSO is also frequently used as a co-solvent in drug effect screens on α -syn aggregation [38, 39], and testing of various drugs effect on α -syn aggregation is often accompanied by a buffer-control with DMSO. However, our results on DMSO induced aggregation, suggest that the assayed effects would still be on a perturbed conformational state of α -syn. Careful consideration should therefore be made when looking at α -syn aggregation and this work indicates that biochemical experiments on α -syn might be better suited, using a different solvent or water soluble reagents. At the very least, one should consider preparing high concentration stock solutions of the used compounds, in order to dilute DMSO to the highest possible extent when conducting α -syn-related research *in vitro* and in cells.

Materials and methods

Antibodies

Primary antibodies: α -syn monoclonal mouse-anti- α -syn (SYN-1, BD Biosciences, catalog no. 610787), polyclonal rabbit-anti- α -syn (ASY1 [40]), rabbit-anti- α -syn (phospho S129) (abcam, catalog no. ab51253), MJFR-14-6-4-2 (MJF14) (abcam, catalog no. 209538, rabbit monoclonal antibody, generated against full length α -syn protein filament), anti- α -tubulin (T9026 Sigma-Aldrich Co.) and anti-Tyrosine Hydroxylase (GeneTex, GTX113016). Secondary antibodies: horseradish peroxidase (HRP)-conjugated polyclonal rabbit-anti-mouse (Dako, P0260), HRP-conjugated polyclonal swine-anti-rabbit (Dako, P0217).

Production and purification of α -syn

Human recombinant α -syn and β -syn were purified from transformed BL21(DE3) competent cells as previously described [41, 42]. Briefly, protein purification involved dialysis of proteins against 20 mM Tris pH 6.5 overnight, followed by ion-exchange chromatography on a Poros HQ50 column (Thermo Fisher Scientific) with a 0–2 M NaCl gradient. Next, a reverse phase-high pressure liquid chromatography purification step was performed on a Jupiter C18 column (Phenomenex, Torrance, CA) in 0.1% trifluoroacetic acid with an 0–90% acetonitrile gradient. Isolated proteins were dialysed in PBS pH 7.4 overnight followed by an additional dialysis step in 20 mM ammonium bicarbonate overnight. Protein concentration was determined by bicinchoninic acid (BCA) protein concentration assay (Pierce). The proteins were subsequently aliquoted, lyophilized, and stored at -20°C until use.

Dynamic light scattering (DLS)

For size determination using DLS, recombinant α -syn (0.5 mg/mL, 35 μM) or carbonic anhydrase (0.5 mg/mL, 17 μM) were incubated for 1 h at a concentration in phosphate-buffered saline (PBS, pH 7.4) alone or in combination with 2%, 5% or 10% DMSO at room temperature (RT) and placed in a disposable plastic cuvette. Particle size within the samples was analyzed as previously described [15] using a Wyatt Technology DynaPro NanoStar. Laser strength was set to 100% and scatter angle fixed at 90° . Total sample measurement was assembled via ten consecutive 5 second measurements. Data was analyzed with Dynamics V7.5.0.17 with solvent signal subtracted from each sample.

Immunoblotting

Dot blotting was performed on 100 ng of protein blotted on nitrocellulose (Hydrobond-C Extra, GE Healthcare). Prior to loading, α -syn was incubated for 30 min at a concentration of 10 $\mu\text{g/mL}$ (0.69 μM) in PBS alone or in combination with 0.1%, 0.5%, 1%, 2%, 5% and 10% of DMSO at room temperature (RT). All membranes were blocked in non-fat milk (Tris-buffered saline (TBS) with 5% non-fat milk, 0.05% Tween 20 and 0.02% sodium azide) for 30 minutes at RT followed by incubation with primary antibodies overnight. Membranes were washed in TBS-T (TBS with 0.1% Tween) and incubated with secondary antibodies for 1.5 hour. Membranes were washed, developed using ECL reagent (Pierce™, Thermo Scientific), and subsequently imaged on a LAS-3000 imaging system (Fuji). Blot quantification was done using ImageJ

Fibrillation

Lyophilized recombinant α -syn or β -syn were re-suspended in sterile PBS (Gibco) to a final concentration of 0.5 mg/mL (35 μM) and placed in Eppendorf Thermotop shaker at 37°C , 1050 rpm for 17 days. For ThT measurements 10 μL of samples was added to 100 μL of 40 μM ThT (final concentration) dissolved in measure buffer (90 mM glycine, pH 8.6) and incubated for 5 min at RT before measuring ThT signal on an EnSpire 2300

Multilabel Plate Reader (PerkinElmer Life Sciences) (excitation at 450 nm and emission at 486 nm). For sedimentation assay, the sample was centrifuged at 25.000 x g for 30 min, at 20°C. Supernatant was collected and considered as soluble fraction, while insoluble fibrils in the pellet fraction were washed and re-suspended in PBS, in the same volume as the isolated supernatant. Similar volumes of supernatant and pellet fractions were resolved on SDS-PAGE and stained at RT, using Coomassie Brilliant Blue. The gel was subsequently destained ON at RT and photographed on Fuji LAS-3000 Intelligent Dark Box (Fujifilm, Japan).

Negative staining transmission electron microscopy

Negative staining transmission electron microscopy (TEM) was performed as described previously [15, 43]. In brief, samples were loaded onto a glow discharged carbon coated copper grid and three times stained with 2% uranyl formate. Images were acquired using a Tecnai Spirit 12G TEM (ThermoFisher – former FEI) in combination with a GATAN 4096 x 4096 CCD detector at a magnification of 42000x.

Proteinase K digestion

To assay the conformational differences between the PFFs generated, the fibrils were digested with proteinase K (PK). Prior to digestions, PFFs were centrifuged at 25.000 x g, at 20°C re-suspended in PBS to remove DMSO. 20 µL of 2 mg/mL (138 µM) PFF (40 µg) was diluted 1:1 in digest buffer in an Eppendorf tube. PK (Sigma- Aldrich) was diluted in digest buffer and added to the PFF samples at final concentrations of 50, 10 and 2 µg/mL. A control PFF sample was run as control without PK. The samples were incubated at 37°C with slow agitation for 30 min. Afterwards the reaction was stopped using 5 µL of Pefabloc® (Sigma-Aldrich) and left at RT for 10 min. Samples were then diluted 1:1 in Special SDS-loading buffer (100 mM Tris pH 6.8, 8% SDS, 24% glycerol, 0.02% bromophenol blue, 25µM 1,4-Dithioerythritol) and then boiled for 5 min, thoroughly vortexed and left at RT for 30 min. The samples were then boiled another 5 min to ensure total dissociation of fibrils. Samples were applied on a 10 well Novex™ 16% Tricine Gel (Invitrogen™) and stained using Coomassie Brilliant Blue.

Cell cultures

All cell-lines were maintained as previously described [44]. In brief, OLN-AS7 cells [45] expressing human AS were made from an immortalized oligodendroglia cell line (OLN-93 [46]) by stable transfection with a pcDNA3.1 zeo(-) AS vector, and maintained with 100 µg/mL zeocin (Invitrogen). These cells were cultured at 37 °C under 5% CO₂ and grown in DMEM (Lonza) supplemented with 10% fetal calf serum, 50 U/mL penicillin, and 50 µg/mL streptomycin. For experiments, cells were seeded without zeocin 24 h before treatment.

HEK293T cells were cultured as described for OLN-93, but without zeocin treatment.

The SH-SY5Y cell-line, is a sub-cloned variant of the human neuroblastoma SK-N-SH cell line. We used a variant (SH-SY5Y ASYN) that stably overexpressed wt human α -syn via a Tet-Off-system [23], kindly provided by Professor, Dr. Leonidas Stefanis, Division of Basic Neurosciences, Biomedical Research Foundation of the Academy of Athens, Athens, Greece. Here, α -syn expression is suppressed by doxycycline (dox). Mitosis was inhibited by administration of 10 µM retinoic acid (RA) final concentration. SH-SY5Y ASYN cells were cultured at 37 °C under 5% CO₂ in RPMI 1640 medium with l-Glutamine (Lonza) supplemented with 15% FCS, 50 U/mL penicillin and 50 µg/mL streptomycin. Cells were maintained in 50 µg/mL hygromycin B (Invitrogen, Life Technologies), 200 µg/mL geneticin (VWR), 1 µg/mL dox (VWR), and 5 µg/mL Plasmocin. For experiments, cells were seeded without hygromycin B, geneticin, plasmocin.

Seeding of cellular aggregation

For immunocytochemistry OLN-AS7 cells were seeded onto poly-L-lysine-coated coverslips and 24 h post-seeding exposed to 28 µg/mL (1.9 µM) S129A α -syn PFFs made in the presence or absence of 2% DMSO or left untreated. Before addition to cells the PFFs were sonicated (20min, using a Branson Ultrasonics™ Sonifier S-250A, duty cycle 30 and output control 3, in a water bath continuously renewed by cold running water to prevent heating.) to produce a uniform population of elongation-prone seeds. The S129A PFFs are made from monomeric substituted S129A α -syn and cannot be phosphorylated on the S129 residue. After 24h PFF treatment, the cells were washed in PBS and fixed in 4% PFA for 10 minutes, washed in PBS and incubated briefly in 15 mg/mL NH₄Cl in PBS. Coverslips were blocked and incubated with primary and secondary antibodies in the blocking buffer (PBS, 3% bovine serum albumin (BSA), 0.1% saponin), with washings in between in PBS with 0.1% saponin and finally double-distilled H₂O, before mounting with Mounting Medium (DAKO, S3023) on glass slides and left in dark O/N at 4 °C.

DMSO induced α -synuclein aggregation in neuron-like cell model

Neuroblastoma SH-SY5Y cells with inducible α -syn expression controlled by the Tet-Off system were seeded on poly-L-lysine coated coverslips and differentiated with 10 µM retinoic acid. Retinoic acid was included in the media for the duration of the experiment. Induction of α -syn expression were started 24 h after seeding, and the cells were simultaneously treated with DMSO for 7 days, with media renewal every second day. The cells were then fixed, blocked and then probed with anti-tubulin (1:1000) and MJF14-6-4-2 (1:25000). Fluorescently labeled secondary antibodies anti-mouse (1:1000) and anti-rabbit (1:1000) and DAPI were subsequently added. Images were taken with X20 and X40 objectives on a Zeiss ObserverZ1 microscope, and pictures were quantified with ImageJ. MJF14-6-4-2 and anti-phospho-Ser129 α -syn signal were quantified by taking the area above a set threshold based on the negative control, and normalizing it to the area of signal from α -Tubulin. For MJF14-6-4-2, four independent experiments were conducted with ~10 images quantified for each experiment. For anti-phospho-S129 α -syn, three independent experiments were conducted with ~8 images quantified for each experiment.

Bimolecular fluorescence complementation visualization of α -syn oligomers

Venus-YFP vectors described in [24, 25] for BiFC visualization of α -syn oligomers were developed and kindly provided by Professor Pamela J McLean (Mayo Clinic, Jacksonville, USA). In short, fluorescent venus YFP was used generating the fluorescent complementation pairs V1S (N-terminal half of Venus YFP fused to the N-terminus of α -syn) and SV2 (C-terminal half of Venus YFP fused to the C-terminus of α -syn). For experiments, 500.000 HEK cells were seeded into 6-well plates and transfected with V1S or SV2 alone or in combination using Lipofectamine 3000 (Thermo Fisher Scientific) transfection reagent according to the manufacturer's protocol. One day post transfection 25.000 cells were re-seeded into Poly-L-lysine-coated 96-well plates. DMSO was added to the media in described concentrations four hours post re-seeding. Cells were incubated for an additional 20h before analysis on an EnSpire 2300 Multilabel Plate Reader (PerkinElmer Life Sciences) (excitation at 513 nm and emission at 528 nm).

Animal experiments

All animal experiments were conducted in accordance with ethical permits approved by the local Animal Welfare and Ethics Committee Malmö/Lund (ethical permit numbers M73/16 and the amendment 5.8.18-03524/2020). Furthermore, all animal experiments were performed in accordance with relevant guidelines and regulations and reporting in the manuscript follows the recommendations in the ARRIVE guidelines.

Twelve wildtype C57bl/6 mice and 12 transgenic F28 mice expressing human full-length wildtype α -syn under the mouse's endogenous α -syn promoter were kept in a 12-hour light/12-hour dark cycle with access to food and water ad-libitum. At 4 months of age, the mice were given a single dose of PBS, 10% DMSO (1 g/kg) or 30% DMSO (3 g/kg) by oral gavage daily for 2 weeks. The animals were weighed daily and closely monitored. At the end of the 2 weeks, the mice were injected intraperitoneally with 0.6 mg/g sodium pentobarbital. After loss of righting reflexes, the mice were transcardially perfused with 0.9% saline. Brains and gastrointestinal tracts were collected and either snap frozen in liquid nitrogen or post-fixed in 4% PFA pH 7.4 for 48 hours. Tissues were then either cryopreserved in 30% sucrose or prepared for paraffin embedding in 70% ethanol and stored at 4°C.

Immunohistochemistry

Brain samples were cut in 30 μ m coronal sections on a freezing microtome and stored in Walter's antifreeze solution. Sections were washed in PBS and endogenous peroxidase quenched with 3% hydrogen peroxide. Sections were pre-incubated in a solution with normal serum, BSA and Triton-X100. Primary antibody solution (anti-phospho-Ser129 (1:5000) or (1:300000) GTX113016) was added overnight at room temperature. Sections were rinsed, incubated with secondary antibody solution (1:500 BA-1000), amplified with ABC solution (VEPK-6100), and developed with DAB (SK-4100). Sections were mounted onto gelatin-coated glass-slides, dehydrated, cleared and coverslipped with pertex.

Intestinal segments from the duodenum and jejunum were paraffin embedded and sectioned at 6 μ m thickness. Samples were deparaffinized before antigen retrieval for 10 min in 80% formic acid at room temperature and 40 min in 1 M citrate buffer pH 8.5 at 95 °C. Immunohistochemistry was then performed as described with aggregation-specific MJF14-6-4-2 antibody (1:10000) at 4 °C overnight. After developing with DAB, samples were counterstained with HTX before dehydration, clearing and coverslipping. All stained tissues were imaged with Olympus BX53.

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Figures

Figure 1. DMSO increases α -syn particle-size, aggregation-dependent antibody recognition and stimulates fibrillation **A and B)** Dynamic light scattering analysis of α -syn (0.5 mg/mL, 35 μ M) and carbonic anhydrase (0.5 mg/mL, 17 μ M) incubated for 1 h with increasing concentrations of DMSO (0%, 2%, 5% and 10%). The figure demonstrates a mass distribution based on the scattering intensity, with log scaled hydrodynamic radius depicted on the X-axis. Representative figure of three independent replicates. **C)** Dot blot of 100 ng of α -syn protein. Prior to loading, recombinant α -syn was incubated for 30 minutes at room temperature (RT) under non-agitating conditions in a concentration of 10 μ g/mL (0.69 μ M) in PBS alone or in combination with indicated concentrations of DMSO. α -syn was visualized using total α -syn (SYN-1) and aggregation-specific (MJF-14-6-4-2) antibodies. **D)** Quantification of MJF-14-6-4-2/SYN-1 α -syn signal ratio normalized to 0% DMSO ($n = 3$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, student t-test). **E)** Lyophilized recombinant α -syn was re-suspended in sterile PBS alone (0% DMSO) or in combination with increasing amounts of DMSO (1%, 5% or 10%). A sample with 10% DMSO alone was included as negative control. The samples were incubated at 37°C and 1050 rpm in a final α -syn concentration of 0.5 mg/mL (35 μ M). ThT measurements were performed on 10 μ L samples added to 100 μ L of 40 μ M ThT (final concentration) and signal measured (excitation at 450 nm and emission at 486 nm). Values were normalized to day 0 measurements for each sample. Representative figure of three independent replicates. **F)** Sedimentation assay of samples depicted in E) after 7 days of incubation. Equal volumes of each sample (0%, 1%, 5% and 10% DMSO) were pelleted by 25,000 x g centrifugation for 30 min at 20°C. The pellets were re-suspended and resolved on SDS-PAGE and stained using Coomassie Brilliant Blue. Depicted supernatant and pellet gel stainings were developed on two individual SDS-PAGE gels. **G)** Lyophilized recombinant β -syn was re-suspended in sterile PBS alone (0% DMSO) or in combination with 10% DMSO. The samples were incubated at 37°C and 1050 rpm in a final β -syn concentration of 0.5 mg/mL (35 μ M). ThT measurements were performed on 10 μ L samples (excitation at 450 nm and emission at 486 nm), and normalized to day 0 measurements. Representative figure of three independent replicates.

Figure 2. DMSO stimulated fibrils resemble naïve α-syn fibrils and maintain seeding capabilities **A)** Representative image of negative stain EM shows structure of α-syn wt- and α-syn DMSO PFFs made in the presence of 2% DMSO. Scalebar = 100 nm. **B)** Digest samples of 25.000 x g pelleted α-syn wt- and α-syn DMSO PFFs. Samples were digested with depicted concentrations of Proteinase K (PK). Digested samples were resolved on SDS-PAGE and stained at RT, using Coomassie Brilliant Blue. Representative gel of three independent replicates. **C)** OLN-AS7 cells were exposed to 28 μg/mL (1.9 μM) S129A α-syn monomer or sonicated α-syn wt- and S129A α-syn DMSO PFFs. After 24h PFF treatment, the cells were fixed and visualized using DAPI (blue), MJF-14-6-4-2 (green), Asy-1 (red) or α-tubulin (purple). **D)** Human α-syn expressing OLN-AS7 cells were exposed to 28 μg/mL (1.9 μM) S129A α-syn monomer or sonicated S129A α-syn wt- and α-syn DMSO PFFs. After 24h PFF treatment, the cells were fixed and visualized using DAPI (blue), anti-phospho-S129 α-syn (green), Asy-1 (red) or α-tubulin (purple). Representative images of three replicates.

Figure 3. DMSO treatment induces α -syn multimerization in cells **A)** SH-SY5Y ASYN cells were treated with dox to suppress α -syn expression (- α -syn) or relieved from dox treatment to induce α -syn overexpression. Cells relieved from dox were treated with increasing amounts of DMSO (0%, 0.1%, 0.25%, 0.5% or 0.75%) for 7 days prior to fixation, and visualized using DAPI (blue), MJF-14-6-4-2 (green) or α -tubulin (red). To stop mitosis, the SH-SY5Y ASYN cells were treated with retinoic acid (10 μ M final concentration) during the experiment. **B)** High magnification image of α -syn expressing SH-SY5Y ASYN cells treated with 0%- (left) or 0.75% (right) of DMSO for 7 days prior to fixation, and visualization using DAPI (blue), MJF-14-6-4-2 (green) and α -tubulin (red). **C)** Quantification of area of MJF-14-6-4-2 signal from individual coverslips relative to area of α -Tubulin signal in cells without α -syn expression (- α -syn) or in α -syn expressing cells treated with increasing amounts of DMSO (0%, 0.1%, 0.25%, 0.5% or 0.75%) for 7 days ($n = 4$, with ~ 10 images for each condition in each experiment, $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$, student t-test). **D)** Quantification of area of anti-phospho-S129 α -syn signal from individual coverslips relative to area of α -Tubulin in cells without α -syn expression (- α -syn) or in α -syn expressing cells treated with increasing amounts of DMSO (0%, 0.1%, 0.25%, 0.5% or 0.75%) for 7 days ($n = 3$ with ~ 8 images for each condition in each experiment, $**p < 0.01$, student t-test). **E)** Explanatory figure of principle behind bimolecular fluorescence complementation (BiFC) assay using the fluorescent venus YFP construct. Upon correct dimerization of α -syn within the two fluorescent complementation pairs, V1S and SV2 will bring, the N- and C-terminus fluorescent fragments of Venus YFP within proximity, thereby allowing emission of its yellow fluorescent signal. **F)** Quantification of YFP signal (excitation at 513 nm and emission at 528 nm) from Venus- α -syn oligomerization in non-treated and 0.1%- and 0.25% DMSO treated HEK-293 cells (20 h. treatment). Signal was normalized to non-treated V1S/SV2-expressing cells. ($n = 3$ with 10 measurements in each experiment, $*p < 0.05$, student t-test)

Figure 4. Oral treatment with DMSO does not induce α -syn aggregation in vivo. Free floating 30 μ m sections of brain tissues from striatum or substantia nigra or paraffin embedded sections of duodenum and ileum from **A)** wt C57BL/6- or **B)** transgenic F28 mice, overexpressing human α -syn under the control of the endogenous mouse α -syn promoter. Mice were treated with water (0% DMSO), 10% DMSO (1 g/kg bodyweight) or 30% DMSO (3 g/kg bodyweight) for 14 days prior to sacrificing and tissue collection. Striatum or substantia nigra sections were stained using a anti-tyrosine hydroxylase antibody and striatum additionally using phospho-Ser129-specific α -syn antibody. The duodenum and ileum samples were stained with the α -syn aggregation-specific MJF14-6-4-2 antibody. Scalebar = 20 μ m for striatum, duodenum and ileum and 50 μ m for substantia nigra.

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Figures

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DMSO stimulated fibrils resemble naïve α -syn fibrils and maintain seeding capabilities A) Representative image of negative stain EM shows structure of α -syn wt- and α -syn DMSO PFFs made in the presence of 2% DMSO. Scalebar = 100 nm. B) Digest samples of 25.000 x g pelleted α -syn wt- and α -syn DMSO PFFs. Samples were digested with depicted concentrations of Proteinase K (PK). Digested samples were resolved on SDS-PAGE and stained at RT, using Coomassie Brilliant Blue. Representative gel of three independent replicates. C) OLN-AS7 cells were exposed to 28 μ g/mL (1.9 μ M) S129A α -syn monomer or sonicated α -syn wt- and S129A α -syn DMSO PFFs. After 24h PFF treatment, the cells were fixed and visualized using DAPI (blue), MJF-14-6-4-2 (green), Asy-1 (red) or α -tubulin (purple). D) Human α -syn expressing OLN-AS7 cells were exposed to 28 μ g/mL (1.9 μ M) S129A α -syn monomer or sonicated S129A α -syn wt- and α -syn DMSO PFFs. After 24h PFF treatment, the cells were fixed and visualized

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Figure 4

Oral treatment with DMSO does not induce α -syn aggregation in vivo. Free floating 30 μ m sections of brain tissues from striatum or substantia nigra or paraffin embedded sections of duodenum and ileum from A) wt C57BL/6- or B) transgenic F28 mice, overexpressing human α -syn under the control of the endogenous mouse α -syn promoter. Mice were treated with water (0% DMSO), 10% DMSO (1 g/kg bodyweight) or 30% DMSO (3 g/kg bodyweight) for 14 days prior to sacrificing and tissue collection. Striatum or substantia nigra sections were stained using a anti-tyrosine hydroxylase antibody and striatum additionally using phospho-Ser129-specific α -syn antibody. The duodenum and ileum samples were stained with the α -syn aggregation-specific MJF14-6-4-2 antibody. Scalebar = 20 μ m for striatum, duodenum and ileum and 50 μ m for substantia nigra.

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