

Cationic carbosilane dendrimers prevent abnormal α -synuclein accumulation in Parkinson's disease patient-specific dopamine neurons

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ABSTRACT

Accumulation of misfolded α -synuclein (α -syn) is a hallmark of Parkinson's disease (PD) thought to play important roles in the pathophysiology of the disease. Dendritic systems, able to modulate the folding of proteins, have emerged as promising new therapeutic strategies for PD treatment. Dendrimers have been shown effective at inhibiting α -syn aggregation in cell-free systems and in cell lines. Here, we set out to investigate the effects of dendrimers on endogenous α -syn accumulation in disease-relevant cell types from PD patients. For this purpose, we chose cationic carbosilane dendrimers of bow tie topology based on their performance at inhibiting α -syn aggregation *in vitro*. Dopamine neurons were differentiated from induced pluripotent stem cell (iPSC) lines generated from PD patients carrying the *LRRK2*^{G2019S} mutation, which reportedly display abnormal accumulation of α -syn, as well as from healthy individuals as controls. Treatment of PD dopamine neurons with non-cytotoxic concentrations of dendrimers was effective at preventing abnormal accumulation and aggregation of α -syn. Our results in a genuinely human experimental model of PD highlight the therapeutic potential of dendritic systems and open the way to developing safe and efficient therapies for delaying or even halting PD progression

1. Introduction

Alpha-synuclein (α -syn) is a small presynaptic protein widely and abundantly expressed by neurons throughout the nervous system¹. Even though the normal functions of α -syn are not completely understood, abnormal accumulation in neurons has been found in a variety of neurological diseases collectively known as synucleinopathies^{2,3}, including Parkinson's disease (PD) in both sporadic and familial cases⁴. PD is an age-related, chronic and progressive neurodegenerative disorder, characterized by a loss of dopamine neurons (DAn) within the substantia nigra pars compacta, which contributes to the cardinal motor symptoms of the disease^{5,6}. A pathological hallmark of PD is the presence of α -syn-containing intraneuronal inclusions, known as Lewy bodies². Both the presence and intensity of Lewy bodies closely correlate with disease progression⁷, and are thus thought to play a pivotal role in PD pathophysiology⁸. The facts that mutations or multiplications in the *SNCA* gene (encoding α -syn) cause familial PD⁹, and that experimental models of α -syn overexpression^{10,11} or artificially high loads of aggregated or preformed α -syn fibrils¹²⁻¹⁴ lead to synaptic dysfunction and neuron death, indicate that increased α -syn expression, accumulation or aggregation in DAn may be sufficient to promote nigral degeneration.

Despite intensive research, PD remains an incurable neurological condition. Currently available treatments are designed to compensate the loss of dopamine and dopaminergic function, but they are palliative and limited by side effects and lack of long-term efficacy^{15,16}. Unfortunately, current therapeutic strategies do not cure or block PD progression, emphasizing the critical need for finding new disease-modifying approaches that halt, slow or delay disease progression. Considering its importance in PD pathophysiology, α -syn could be an interesting therapeutic target for this purpose, and inhibition of α -syn aggregation using protein-solubilizing drugs a potential therapeutic strategy.

Nanotechnology has contributed to our understanding of PD pathogenesis by providing tools that promote the solubilization of α -syn fibers and prevent the formation of α -syn aggregates¹⁷. Different nanomaterials have been explored for their usefulness as antiparkinsonian, antioxidant and/or neuroprotective agents, including nanoparticles¹⁸, dendrimers¹⁹, and carbon nanotubes²⁰. Among them, dendrimers are especially interesting as they are highly branched polymers with well-defined geometry and unique properties that can be functionalized according to the specific application for which they are designed. A variety of

dendritic systems of different nature and topology have been tested in the context of neurodegenerative diseases, including PAMAM ²¹, polypropyleneimine dendrimers ²², glycodendrimers ²³, and phosphorous ²⁴ or carbosilane ²⁵ dendrimers, and shown to have an effect on α -syn aggregation ^{21, 24, 26, 27}. Moreover, dendrimers have also been reported to inhibit the aggregation of insoluble forms of amyloid ²⁸ and prion ²⁹ peptides, as well as to facilitate their degradation ^{30, 31}.

For the present studies, we focused on carbosilane dendrimers as they have proven to be effective in a variety of biomedical applications in a topology-dependent manner ^{25, 32-36}, and non-toxic at concentrations below 5 μ M when used as transfecting or anti-cancer agents ^{37, 38}. One of the main properties of carbosilane dendrimers is their inherent hydrophobicity, provided by their characteristic skeleton, which allows interaction with biological membranes in an efficient manner ³⁹. Moreover, low generations of carbosilane dendrimers are effective for most applications, unlike the case of other dendritic skeletons. Specifically in the context of protein misfolding diseases, cationic carbosilane systems have shown their ability to inhibit rotenone-induced α -syn fibrillation in mouse hippocampal cells ⁴⁰, and amyloidogenic islet amyloid polypeptide (IAPP) aggregation in a mouse model of type II diabetes ²⁵. In this work, we investigated how the topology of cationic carbosilane dendrimers (spherical, dendritic wedge or bow tie) could influence the interaction with α -syn to avoid aggregation. After biophysical characterization of the interaction between α -syn and the different dendritic systems, we selected the bow tie topology for testing effectiveness in an induced pluripotent stem cell (iPSC)-based experimental model of PD. Our results show that non-cytotoxic concentrations of cationic carbosilane dendrimers are effective at preventing abnormal accumulation and aggregation of α -syn in dopamine neurons from PD patients.

2. Results and discussion

2.1. Cationic carbosilane dendritic systems used in the studies

Previous work by some of our laboratories identified a strong influence of dendrimer topology in their capacity to interact with amyloid peptides, and therefore to inhibit beta-amyloid aggregation, in an animal model of type II diabetes²⁵. For this reason, in the current studies we first set out to investigate the influence of dendritic system topology on α -syn folding. For this purpose, we chose three cationic carbosilane dendritic systems with the same number of charges but different topologies: $G_1Si(SNMe_3I)_8$ (**I**) as a spherical dendrimer⁴¹, $(HOC_6H_4)_3Gn(SNMe_3I)_8$ (**II**) as a dendron⁴¹, and $(IMe_3NS)_4G_2[OC_6H_4O]G_2(SNMe_3I)_4$ (**III**) as a bow tie system²⁵ (Fig. 1).

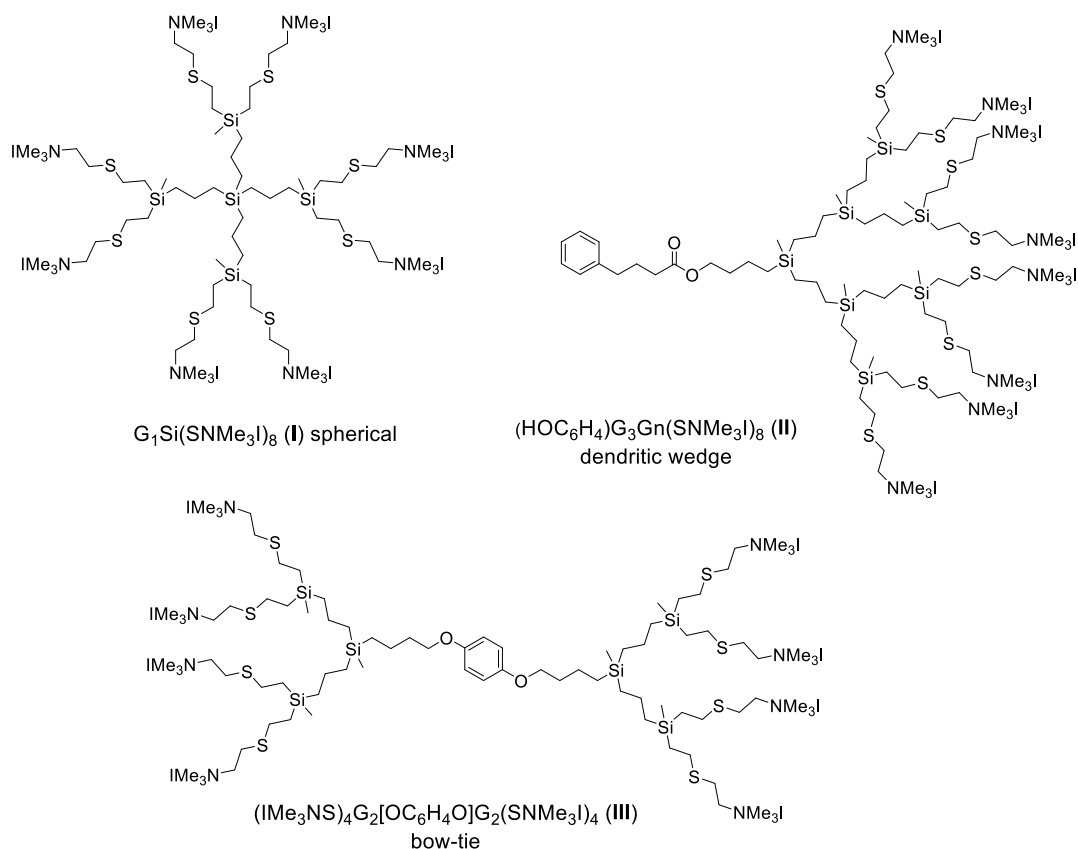


Figure 1. Dendritic structures of $G_1Si(SNMe_3I)_8$ (**I**) as a spherical dendrimer, $(HOC_6H_4)_3Gn(SNMe_3I)_8$ (**II**) as a dendron and $(IMe_3NS)_4G_2[OC_6H_4O]G_2(SNMe_3I)_4$ (**III**) as a bow tie system.

2.2. Study of interaction between α -Syn and dendritic system: biophysical characterisation.

The interaction of carbosilane dendrimers and α -syn and their effect on the aggregation status of synthetic peptides of α -syn have been thoroughly analyzed by different biophysical techniques such as zeta potential and circular dichroism ⁴⁰. To ascertain the influence of the system's topology on such interactions, we first measured changes in zeta potential (directly related to particle charge environment), to study the interaction between α -syn and different topologies of carbosilane dendrimers. α -Syn is a small acidic protein comprised of three well-differentiated domains: (i) a positively charged region called N-terminal lipid-binding α -helix, (ii) a central domain (non-amyloid β component, or NAC) that is involved in the process of aggregation to form cross β -structures, and (iii) a highly negative and hydrophobic domain known as C-terminal acidic tail ⁴². Due to the predominance of negative charges at pH= 7.4, α -syn zeta potential value is around -21 mV. However, upon addition of different concentrations of dendritic systems **I-III**, the zeta potential turned positive until reaching values of ~20 mV (Fig. 2A). Due to the cationic nature of dendritic structures, it is likely that nanoconjugates formed with α -syn have positive charge values independently of the system topology. These results indicate that the topology of the dendrimer system does not significantly influence the zeta potential of the resulting nanoconjugate with α -syn. Moreover, our results also show that nanoconjugates were formed by one molecule of dendrimer/dendron per one α -syn molecule (1:1 molar ratio), in all tested topologies (Fig. 2).

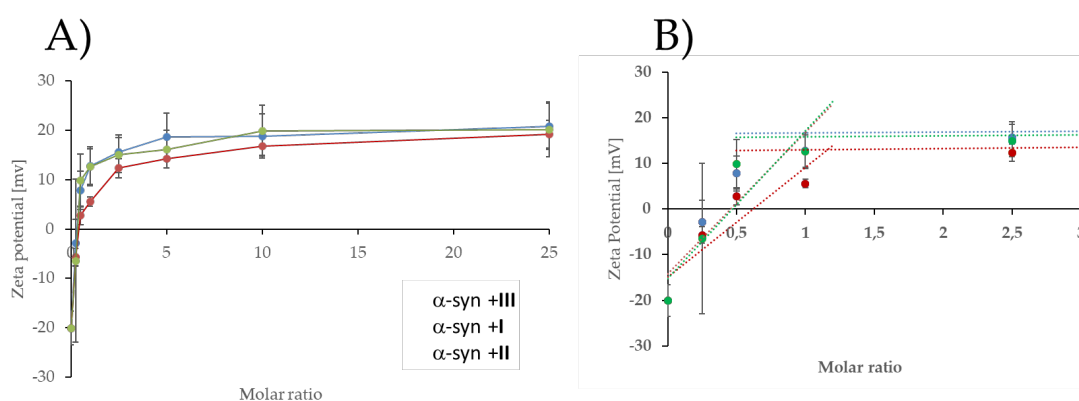


Figure 2. A) α -Synuclein zeta potential in the presence of carbosilane dendrimers spherical **I** (red), dendron **II** (green) and bow tie **III** (blue). B) Determination of number of dendritic system molecules attached to α -syn.

We next used circular dichroism (CD) to analyze conformational changes in the secondary structure of α -syn upon interaction with dendrimers **I-III** under nearly physiological conditions (in solution). The CD spectra of α -syn alone were typical of a substantially unfolded polypeptide chain, with a minimum negative peak characteristic of random coil observed at 200 nm, and a weaker and broader valley centered around 225 nm. Addition of dendrimers **I-III** over a concentration range spanning one order of magnitude (0.5 – 5 μ M) did not cause significant changes in CD spectra except for the largest concentration tested (Fig. 3). Taking these results into account, we then evaluated whether dendrimers **I-III** could inhibit the formation of the β structure of α -syn. Upon incubation at 37°C for 48 h, the CD spectra of α -syn showed a shift to positive values in the range 195-205 nm, indicating the conversion from a disordered structure into the β form (Fig. 4). The capacity of dendrimers **I-III** to inhibit this conversion was tested after 48 – 72h of incubation in the presence of two different concentrations of dendrimers (α -syn /dendrimer molar ratios of 1:2 or 1:4). All tested dendrimer topologies exhibited an overall inhibitory activity on the conversion of disordered α -syn into β structure (Fig. 4). However, dendron **II** elicited marked changes in α -syn CD spectra, which were more evident at the highest concentration tested, and thus was excluded from further studies. Between the spherical (**I**) and bow tie (**III**) systems, we chose the latter for downstream experiments in cells due to its relative stronger and more durable inhibiting activity at low concentration (Fig. 4), and also because previous tests of the spherical dendrimer **I** in a mouse α model of type II diabetes failed to observe anti-aggregation activity of the β -amyloid protein ²⁵.

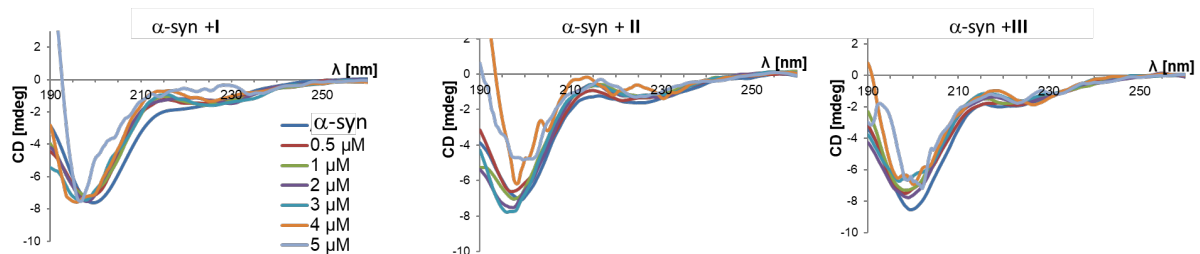


Figure 3. CD spectra of α -synuclein ($c = 1\mu$ M) alone and in the presence of dendritic systems **I-III**.

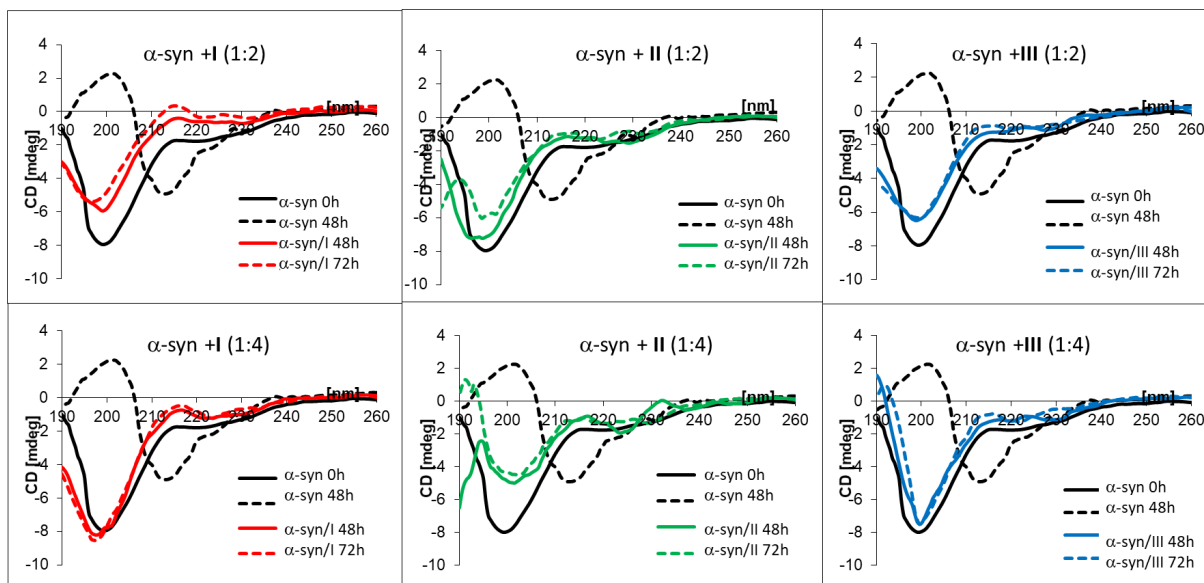


Figure 4. CD spectra of α -synuclein alone and in the presence of dendritic systems I-III after 48-72h of incubation.

2.3. Effect of cationic carbosilane dendrimers on the survival of PD patient dopamine neurons in culture.

Even though the interaction of carbosilane dendrimers and α -syn is well documented, as is their capacity to inhibit the aggregation of synthetic peptides of α -syn, to our knowledge, no studies have investigated the effect of carbosilane dendrimers on the endogenous α -syn present in disease-relevant cell types. The relevance of utilizing cell types as similar as possible to those affected by specific diseases when studying pathogenic mechanisms or testing potential therapies is widely recognized^{43,44}. In this respect, reprogramming patients' somatic cells to pluripotency for the generation of patient- or disease-specific iPSC has emerged as a powerful strategy to produce theoretically unlimited amounts of disease-relevant cell types⁴⁵. In the case of PD, several laboratories have generated patient-specific iPSC lines from idiopathic and genetic forms of the disease, and used them to differentiate DAN that recapitulated key hallmarks of the disease (reviewed in⁴⁶⁻⁴⁸). In particular, we have previously shown that DAN differentiated from iPSC obtained from PD patients carrying the G2019S mutation in the *LRRK2* gene (LRRK2-PD) exhibit abnormal accumulation of α -syn⁴⁹, as a consequence of impaired chaperone-mediated autophagy (CMA)⁵⁰. We have also shown that, while other LRRK2-PD iPSC-derived brain cells such as astrocytes abnormally accumulate α -syn as well, it is accumulation in DAN that ultimately leads to neurodegeneration⁵¹. For these reasons, in the current studies we set out to test whether cationic carbosilane dendrimers had

any effect in the abnormal, disease-related, accumulation of α -syn in LRRK2-PD iPSC-derived DAN. For this purpose, we used iPSC lines representing two LRRK2-PD patients and two healthy individuals as controls, generated and fully characterized in previous work ⁴⁹, and differentiated them into DAN following a robust directed protocol ⁵² (see Fig. 5A and Experimental section for details). Cationic carbosilane dendrimers (bow tie system, **III**) were added to DAN cultures at two different concentrations (1 and 3 μ M) and incubated for 48 h to first ascertain any effects on DAN survival. Under these conditions, the highest concentration of dendrimer resulted in ~60% decrease in DAN numbers, as measured by immunostaining for the DAN marker tyrosine hydroxylase (TH), when compared with parallel cultures treated with vehicle alone (Fig. 5B). In contrast, dendrimers were not toxic to DAN when used at 1 μ M for 48 h, at least in terms of cell viability (Fig. 5 B). Indeed, we even observed a slight increase (albeit not statistically significant) in DAN numbers in LRRK2-PD-DAN treated with 1 μ M **III** compared to untreated LRRK2-PD-DAN, which could be explained by the dendrimers' inhibitory effects on DAN α -syn accumulation (see below). Previous work in the context of Alzheimer's disease found that several dendrimers were efficient at preventing A β -induced cytotoxicity in human neuroblastoma SH-SY5Y cells ^{31, 53}. Our results so far demonstrate that human DAN derived from PD patient-specific iPSC can safely be cultured, for at least 48 h, in the presence of 1 μ M cationic carbosilane dendrimers of bow tie topology (**III**), and suggest that this treatment could be beneficial for their survival, even though culture times longer than 48 h might be necessary to unambiguously demonstrate the latter.

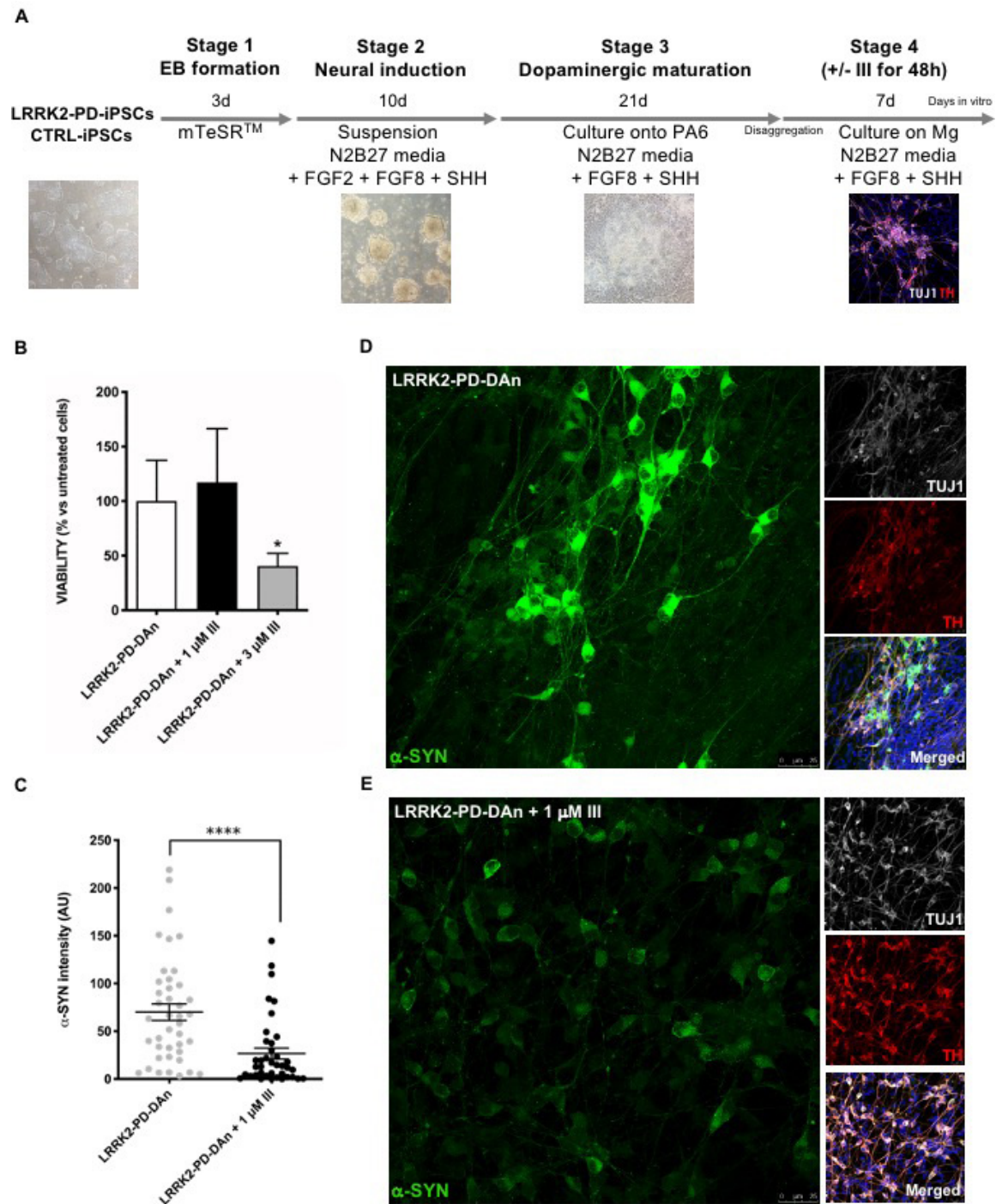


Figure 5. A. Schematic representation of the differentiation protocol implemented for the generation of dopaminergic neurons (DAn) from induced pluripotent stem cells (iPSCs) generated from two PD patients (LRRK2-PD) and two healthy age-matched controls (CTRL). B. Analyses of cell viability in an acute exposure (48h) testing two different doses of dendrimer III (1 μ M and 3 μ M). Bars represent means with SEM as error bars. (n=4, using 2 LRRK2-PD-DAn lines from two-independent experiments for each condition). C-E. Cytoplasmic accumulation of α -syn in LRRK2-PD-DAn and LRRK2-PD-DAn treated with 1 μ M III for 48h. Quantitative analyses of α -syn intensity (n=42-45, using 2 LRRK2-PD-DAn lines from two-independent experiments, ****p<0.0001) (C). Representative immunofluorescence images showing diffuse cytoplasmic accumulation of α -syn in LRRK2-PD-DAn (D) and LRRK2-PD-DAn treated with 1 μ M III for 48h (E). Scale bars, 25 μ m.

2.4. Effect of cationic carbosilane dendrimers on the abnormal accumulation of α -syn in dopamine neurons from PD patients.

Consistent with previous reports by our laboratories and others^{49, 50, 54, 55}, DAN differentiated from LRRK2-PD iPSCs showed evident cytoplasmic accumulation of α -syn after 30 days of differentiation, which could be quantitatively analyzed by anti- α -syn immunofluorescence intensity (Fig. 5C-D). We chose immunolabeling analysis with antibodies against α -syn because this technique has become the standard and most sensitive immunohistochemical method for neuropathological diagnosis of PD⁵⁶. Treatment of parallel cultures of LRRK2-PD iPSC-derived DAN with 1 μ M of **III** for the last 48 h of differentiation resulted in a statistically significant decrease in overall α -syn immunoreactivity when compared with untreated cultures (Fig. 5C-E).

Dendrimer effects were also evident when we quantified the percentage of DAN that stained positive for α -syn. In our differentiation and culture conditions, DAN from LRRK2-PD patients accumulate α -syn in their cytoplasm (and are thus scored as α -syn-positive DAN) at approximately twice the frequency when compared with DAN from control individuals (Fig. 6A). Using DAN differentiated from two independent LRRK2-PD patients, we found that treatment with 1 μ M of **III** for 48 h was sufficient to reduce the percentage of α -syn-positive DAN to values not statistically different to those measured in control DAN (Fig. 6A).

We next analyzed the capacity of dendrimers to reduce the intracellular accumulation of α -syn aggregates. Imaging by confocal microscopy of anti- α -syn immunostained DAN identifies α -syn aggregates as distinct puncta accumulated at the cell body⁵⁷. In our experiments, the percentage of LRRK2-PD DAN containing α -syn puncta at the cell body was significantly higher than that of control DAN ($51.8 \pm 8.7\%$ vs. $19.4 \pm 4.7\%$, $n = 5$ and 4 independent differentiations using 2 iPSC lines per condition; Fig. 6B-E). These results are in line with previous evidence⁴⁹⁻⁵¹, and highlight the advantage of using patient-specific iPSC-based models compared to other experimental systems where additional manipulations, such as treatment with exogenous α -syn fibrils or rotenone-induced α -syn fibrillation, are required^{22, 40}. Importantly, incubation of LRRK2-PD DAN with 1 μ M of **III** for 48h reduced the percentage of cells displaying α -syn puncta to values similar to those shown by control DAN (Fig. 6B-E). These results suggest that cationic carbosilane dendrimers of bow tie topology (**III**), when used

at non-toxic concentrations, are internalized by PD patients' DAN and directly interact with α -syn aggregates, probably helping to solubilize them into smaller aggregates that would be more easily degraded.

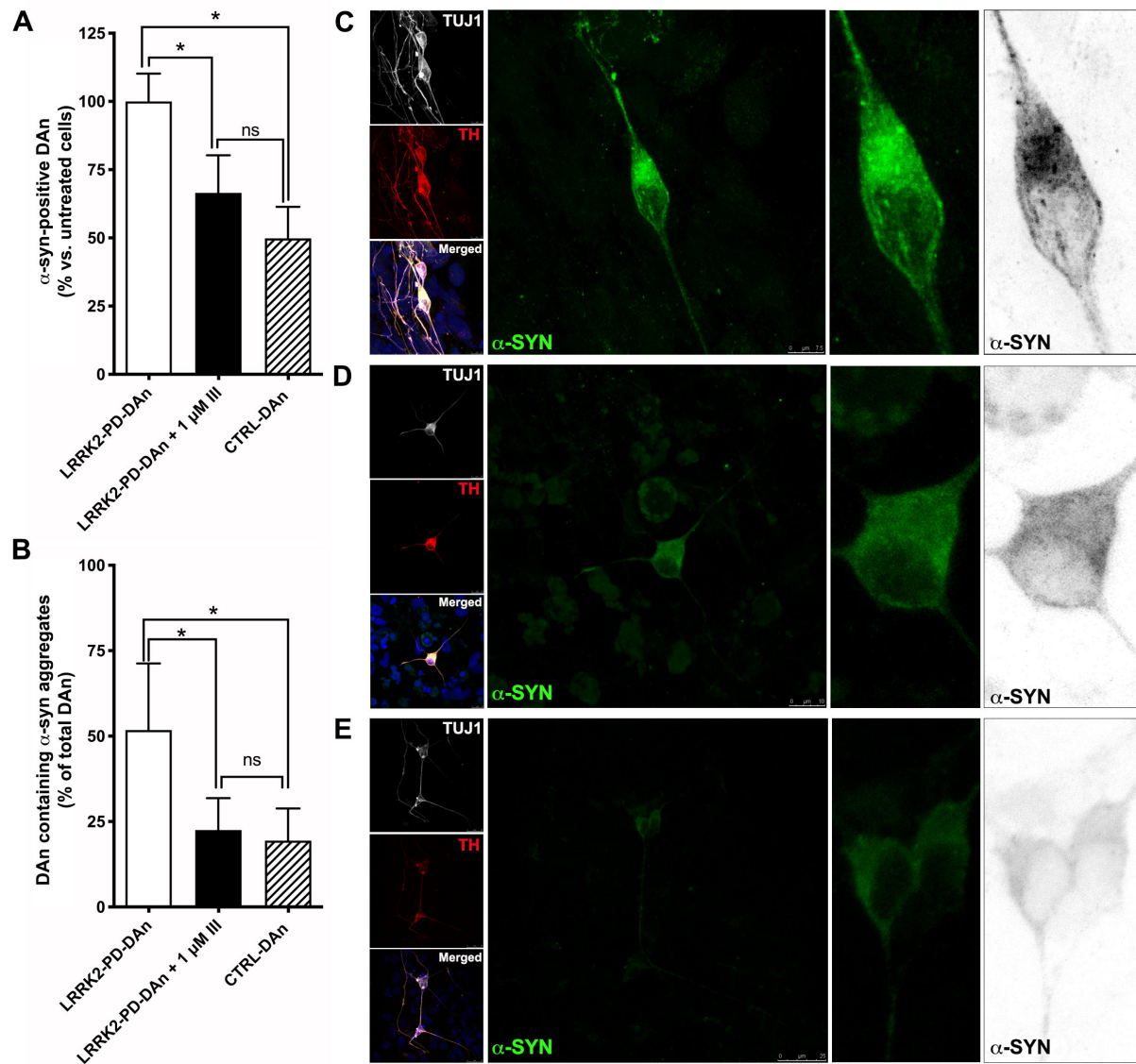


Figure 6. α -syn accumulation in DAN after 30 days of differentiation. A-B. Quantitative analyses of α -syn positive DAN (A) and α -syn aggregates (B). Bars represent means with SEM as error bars. (n=4, using 2 LRRK2-PD-DAN lines and 2 CTRL-DAN lines from two-independent experiments). The differences in both, diffuse cytoplasmic α -syn staining and α -syn aggregates accumulation, are statistically significant (*p<0.05) comparing LRRK2-PD-DAN + 1 μ M III or CTRL-DAN to untreated LRRK2-PD-DAN. No significant differences (ns) were observed between LRRK2-PD-DAN + 1 μ M III and CTRL-DAN. C-E. Representative immunofluorescence images showing α -syn aggregates accumulation in LRRK2-PD-DAN. Scale bar, 7.5 μ m (C), LRRK2-PD-DAN treated with 1 μ M III for 48h, Scale bar, 10 μ m (D) and CTRL-DAN. Scale bar, 25 μ m (E).

3. Conclusions

In summary, in this work we demonstrate the effectiveness of cationic carbosilane dendrimers toward key PD-related cell phenotypes using an experimental system based on disease-relevant cell types PD from patients. We further show the influence of dendritic topology on the interaction with α -syn and, therefore, on their ability to prevent the formation of α -syn aggregates. Bow tie dendrimers showed good performance and were well tolerated by DAN, a cell type especially susceptible to toxic insults (REF). The development of new therapeutic approaches for PD requires the use of appropriate experimental conditions mimicking the neurodegeneration that takes place in patients. In this sense, iPSC technology⁵⁸ has opened unprecedented opportunities for the generation of genuinely human models of disease⁴⁵. Dendrimers have been shown to interfere with the formation of amyloid fibrillary structures typically related with the onset and progression of protein misfolding diseases such as Alzheimer's disease^{28,29,53} and prion diseases^{59,60}. Thus, dendrimers as anti-neurodegenerative agents may have application in PD therapy in which disease progression is closely correlated to brain accumulation of insoluble α -syn. Numerous previous studies have explored the usefulness of dendritic systems to prevent and/or rescue α -syn aggregation in a variety of cell-free and cell-based experimental models of PD^{21,22,24,26,27}. Our results lend additional support to this line of study and, importantly, validate the development of dendrimer-based anti-PD therapies using DAN, the cell type most relevant for the disease, obtained from PD patients. At the cellular level, our results confirmed a significant decrease in cytoplasmic levels of α -syn as well as in α -syn aggregates in DAN from LRRK2-PD-iPSC treated during 48h with non-cytotoxic concentrations of bow tie dendrimer **III**. We believe that the results from our studies will help developing safe and efficient therapies for delaying or even halting PD progression.

4. Experimental section

4.1. Materials

Spherical dendrimer $G_1\text{Si}(\text{SNMe}_3\text{I})_8$ (I), dendritic wedge $(\text{HOC}_6\text{H}_4)\text{G}_3\text{Gn}(\text{SNMe}_3\text{I})_8$ (II) and bow tie system $(\text{IMe}_3\text{NS})_4\text{G}_2[\text{OC}_6\text{H}_4\text{O}]\text{G}_2(\text{SNMe}_3\text{I})_4$ (III) were synthesized according to previously described protocol^{25, 41}. For zeta potential and circular dichroism (CD) spectrometry, α -syn was purchased from Sigma-Aldrich (USA). For all experiments, α -syn and dendrimers were dissolved in phosphate-buffered saline (1.9 mM NaH_2PO_4 , 8.1 mM Na_2HPO_4 , pH=7.4).

4.2. Zeta Potential

The measurements of the zeta potential (electrokinetic potential) were performed with a Zetasizer Nano ZS from Malvern, which uses electrophoresis and LDV (Laser Doppler Velocimetry) techniques to allow measuring the electrophoretic mobility of the molecules in the solution. The zeta potential value was calculated directly from the Helmholtz-Smoluchowski equation using the Malvern software⁶¹. The measurements were performed at 37 °C with three repetitions. Increasing concentrations of the bow tie dendrimer in the range 0,25 μM - 25 μM were added to a 1 μM solution of α -syn and the zeta potential was measured.

4.3. Circular dichroism (CD) spectrometry

The CD spectra (190-260 nm) were recorded for α -syn in the presence/absence of dendritic systems on a Jasco J-815 CD spectropolarimeter, in 5-mm path length quartz cuvettes, with a wavelength step of 0,5 nm, a response time of 4 s and a scan rate of 50 nm/min, maintained at 37 °C. Each spectrum was the average of three repetitions. α -syn was used at a concentration of 2 $\mu\text{mol/l}$. Dendrimers/dendrons alone at the experiments' concentrations did not produce any discernible features in their CD spectra and were used as blanks for α -syn-dendrimers complex spectra.

4.4. iPSC-derived dopaminergic neurons (DAn) generation

The iPSC lines used in our study were previously generated and fully characterized⁴⁹. Specifically, we used iPSC generated from two patients carrying the G2019S mutation in the

LRRK2 gene (LRRK2-PD), and from two healthy age-matched controls (CTRL) (Table 1). The competent Spanish authorities (Commission on Guarantees concerning the Donation and Use of Human Tissues and Cells of the Carlos III National Institute of Health) approved the use of human iPSCs in this work. For DAN differentiation, iPSCs were transduced with LV.NES.LMX1A.GFP and processed as previously described⁵². Embryoid bodies (EBs) generated from iPSC colonies were cultured in nonadherent dishes for 3 days in the presence of mTeSR medium (Stem Cell Technologies) (Stage 1). At stage 2, EBs were culture for 10 days in suspension in N2B27 medium (DMEM/F12 medium (Gibco), Neurobasal medium (Gibco), 1% B27 (Gibco), 0.5% N2 (Gibco), 1% GlutaMAX (Gibco) and 1% penicillin/streptomycin) supplemented with 10ng/ml FGF2 (PeproTech), 100 ng/ml FGF8 (PeproTech) and 100 ng/ml SHH (PeproTech). For DAN maturation, at stage 3 the neural precursor cells (NPCs) were co-cultured with PA6 stromal cells for 3 weeks in N2B27 medium supplemented with FGF8 and SHH. Finally, at stage 4, dopaminergic neurons generated were dissociated using Accutase (Merck), re-plated on Matrigel-coated dishes and cultured for 1 week in N2B27 with FGF8 and SHH. Correct differentiation was judged by co-immunostaining with neuron-specific class III- β -tubulin (TUJ1), a widely used neuronal marker, and tyrosine hydroxylase (TH), the main neuronal marker for dopaminergic neurons.

Table 1. Summary of healthy controls and patients used in this study

Code	Status	Gender	Age at biopsy	Mutation
CTRL1	Control	M	66	No
CRTL2	Control	F	48	No
LRRK2-PD1	Parkinson's disease	M	66	G2019S (LRRK2)
LRRK2-PD2	Parkinson's disease	F	63	G2019S (LRRK2)

4.5. $(\text{IMe}_3\text{NS})_4\text{G}_2[\text{OC}_6\text{H}_4\text{O}]\text{G}_2(\text{SNMe}_3\text{I})_4$ (**III**) treatment

$(\text{IMe}_3\text{NS})_4\text{G}_2[\text{OC}_6\text{H}_4\text{O}]\text{G}_2(\text{SNMe}_3\text{I})_4$ (**III**) was added to DAN at day 3 of stage 4 of differentiation for 48 h. Then, dendrimers were removed and cells were incubated for a further 48 h with fresh media before analysis. To evaluate the effect of dendrimers on DAN survival, cells were treated with two different concentrations of **III**, 1 μM and 3 μM . The numbers of surviving DAN were calculated by counting tyrosine hydroxylase (TH)-positive neurons. For subsequent experiments, DAN were treated with 1 μM of **III** for 48 h, stained and α -syn accumulation analyzed.

4.6. Immunofluorescence

Cells were fixed with 4% paraformaldehyde in PBS at room temperature for 15 min. Cells were blocked and permeabilized with TBS with low triton (0.01% Triton X-100) and 3% donkey serum for 2h. Subsequently, cells were incubated for 48h at 4°C with the following primary antibodies: mouse anti-TUJ1 (1:500; T8660; Sigma), rabbit anti-TH (1:250; sc-14007; Santa Cruz) and mouse anti- α -syn (1:500; 610787; BD Biosciences). Samples were then incubated with secondary antibodies for 2 hours at 37°C: Alexa Fluor 647 anti-mouse IgG2a (1:100; A21241; Invitrogen), Cy3 anti-rabbit IgG (1:200; 711-165-152; Jackson) and Alexa Fluor 488 anti-mouse IgG1 (1:100; A21121; Invitrogen). To visualize nuclei, slides were stained with DAPI (1:5000; Invitrogen) and then mounted with PVA:DABCO. Images were taken using a Leica SP5 confocal microscope and analyzed with FIJI is Just ImageJ™. For quantification of cytoplasmic accumulation of α -syn, the fluorescence intensity in the green channel was corrected by the number of DAN (area green channel/area red channel). The scoring of α -syn-positive DAN was performed by researchers blinded to the experimental conditions.

4.7. Statistical analysis

Results are expressed as mean \pm SEM. Normal distribution of data was tested using D'Agostino & Pearson omnibus normality test. Student's t test or one-way ANOVA followed by Tukey's post hoc test were used for statistical analysis in normal distributions. Statistical analysis for non-normal distributions data was performed using the Mann-Whitney U test or

Kruskal-Wallis test. The software used was GraphPad Prism 6, and the value of $p < 0.05$ was considered statistically significant. The number of experiments (n) is indicated in the legends to the figures.

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Notes: The authors declare no competing financial interest

Author contributions

Conceptualization, R.F.-L., P.O., R.G., and A.R.; Methodology, R.F.-L., F.J.M., M.B., A.C., P.O., R.G., and A.R.; Investigation, R.F.-L., T.L.-C., I.F.-C., K.M., and P.O.; Writing – Original draft, R.F.-L. and P.O.; Writing – Review & Editing, K.M., M.B., A.C., R.G., and A.R.; Funding Acquisition, M.B., A.C., R.G., and A.R.; Supervision, R.G. and A.R.

Declaration of competing interests

The authors declare no competing financial interest

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