

GM1 oligosaccharide efficacy against α -synuclein aggregation and toxicity *in vitro*

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A B S T R A C T: Fibrillary aggregated α -synuclein represents the neurologic hallmark of Parkinson's disease and is considered to play a causative role in the disease. Although the causes leading to α -synuclein aggregation are not clear, the GM1 ganglioside interaction is recognized to prevent this process. How GM1 exerts these functions is not completely clear, although a primary role of its soluble oligosaccharide (GM1-OS) is emerging. Indeed, we recently identified GM1-OS as the bioactive moiety responsible for GM1 neurotrophic and neuroprotective properties, specifically reverting the parkinsonian phenotype both in *in vitro* and *in vivo* models.

Here, we report on GM1-OS efficacy against the α -synuclein aggregation and toxicity *in vitro*. By amyloid seeding aggregation assay and NMR spectroscopy, we demonstrated that GM1-OS was able to prevent both the spontaneous and the prion-like α -synuclein aggregation. Additionally, circular dichroism spectroscopy of recombinant monomeric α -synuclein showed that GM1-OS did not induce any change in α -synuclein secondary structure. Importantly, GM1-OS significantly increased neuronal survival and preserved neurite networks of dopaminergic neurons affected by α -synuclein oligomers, together with a reduction of microglia activation.

These data further demonstrate that the ganglioside GM1 acts through its oligosaccharide also in preventing the α -synuclein pathogenic aggregation in Parkinson's disease, opening a perspective window for GM1-OS as drug candidate.

Keywords: GM1 ganglioside, GM1 oligosaccharide, α -Synuclein, Parkinson's disease, Plasma membrane signaling

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1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder characterized by motor and non-motor clinical manifestations. The predominant motor symptoms of PD, including slow movement, resting tremor, rigidity, and gait disturbance are caused by the selective loss of dopamine (DA) releasing neurons from the *pars compacta* of the *substantia nigra* (SNpc) [2]. Five to 10 % of PD cases have a known genetic origin, while the vast majority, defined as idiopathic or sporadic PD (sPD), have only age as the main risk factor. Although the pathological mechanisms leading to neuronal degeneration are unknown, fibrillary aggregates of α -synuclein (α S) are considered as the PD neurological hallmark, supposed to play a causative role in the disease pathogenesis [3,4]. However, to date there is no explanation for what causes α S elevation and its aggregation in sPD.

α S is a 140-amino-acid protein abundant in brain, predominantly localized in nerve terminals, where it seems to be associated with plasma membrane microdomains [5–7]. Its physiological role is still poorly understood, although some evidence suggests its activity as regulator of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-complex and the release of neurotransmitters [4]. α S is a cytosolic protein and was initially defined as intrinsically disordered [8]. Subsequent studies revealed that, in living systems, α S exists in an α -helical tetrameric state which is more resistant to self-aggregation than the unfolded monomer [4,9,10]. *In vitro* studies showed that the α -helical folding of α S was mediated by binding to negatively charged lipids through its N-terminal acetyl group [11]. The destabilization of the helically folded tetramers produces α S oligomers (α SO),

which in turn tend to aggregate as small protofibrils and then larger insoluble fibrils enriched in β -sheets conformation [4]. The α SO have been reported to affect DA neurons health in several ways, including membrane disruption, mitochondria dysfunction, endoplasmic reticulum stress, synaptic impairment, and apoptosis [4]. Besides neuronal cells, α SO also affect glial cells, such as astrocytes and microglia, through the activation of toll-like receptors, which in turn lead to pro-inflammatory cytokine secretion, impairment of microglia phagocytic activity, and ultimately to the exacerbation of the neuroinflammation that plays a fundamental role in neuronal loss [4]. Additionally, it has been demonstrated that α SO are able to spread between different neural cells triggering the further aggregation of α S monomers in a prion-like manner, also including other proteins such as β -amyloid and tau [4]. This evidence highlights that α SO assume an essential key role in driving PD pathogenesis and that treatments aimed at decreasing their production, sustaining α S clearance or avoiding the spreading between cells, may be helpful to relieve PD phenotype.

Among the various membrane lipids interacting with the monomeric α S, physiologically preventing the α S misfolding, an important role has been attributed to GM1 ganglioside [11,12]. GM1 is abundant in the plasma membrane of neurons, highly concentrated in synaptic plasma membrane microdomains, regions where also α S localizes [10,13]. GM1 plays a fundamental role in sustaining neuronal development and homeostasis by acting through diverse mechanisms including the inhibition of inflammation, excitotoxicity, and oxidative stress reactions, the modulation of Ca^{2+} signaling, neurotrophic factors' pathways (*i.e.* activation of neurotrophin tyrosine kinase TrkA/TrkB/RET receptors) and the modulation of mitochondrial function [3]. It

¹ Ganglioside nomenclature is in accordance with IUPAC-IUBB recommendations [1].

has been reported that the specific binding of tetrameric α S to GM1 sustained its α -helical conformation at the expenses of the aggregation-prone β -sheet-rich state, typical of PD [11]. Along with this, recent evidence has shown a specific GM1: α S interaction which results in the inhibition of protein fibrillation [12]. A biological α S and GM1 connection was revealed by studying mice with the heterozygous disruption of *β4galnt1* gene (β 4galnt1^{+/-}), encoding for the enzyme required for GM1 biosynthesis: such mice with partial GM1 deficiency show α S aggregation both in central and peripheral tissues, which is specifically reversed by the GM1 administration [14–17].

GM1 was found to counteract α S fibrillation and toxicity in several additional preclinical models of α -synucleinopathy. In particular, it has been shown that GM1 induced α S clearance enhancing the autophagy machinery [18] and prevented the pro-inflammatory microglia activation both *in vitro* and *in vivo* [19]. Additionally, using rat models overexpressing human A53T α S mutant, it has been demonstrated that GM1 treatment reduced α S aggregation [20]. This evidence finally supports the GM1 role in favoring the correct α S folding and promoting clearance of pathologic α S aggregates.

In our attempts to dissect the molecular basis behind the neurotrophic properties of GM1 and to find a possible therapeutic strategy to counteract the PD pathogenesis, we have discovered that the penta-saccharide head of GM1 (GM1 oligosaccharide, GM1-OS) replicates the neurotrophic and neuroprotective properties of the entire GM1 molecule both *in vitro* and *in vivo* [21]. Specifically, we demonstrated that GM1 exerts its bioactivity through its hydrophilic head, which protruding in the extracellular space acts on the cell surface specifically activating neurotrophic Trk signaling. Thus, GM1-OS maintains the neurotrophic properties and, losing the amphiphilicity typical of GM1 ganglioside, it is able to efficiently access the central nervous system (CNS) [22]. Thus, it is likely that GM1 property in maintaining α S proper folding and levels could be ascribed to its oligosaccharide. An insight in this direction was obtained by the observation that GM1-OS systemic administration to *β4galnt1*^{+/-} parkinsonian mouse model, reduced α S aggregates in the SNpc [23].

In this work we assessed the capability of GM1-OS to directly influence α S proper folding, to prevent α SO toxicity in rat DA neurons and to alleviate α S related neuroinflammation.

2. Material and methods

2.1. Materials

Commercial chemicals were of the highest purity available, common solvents were distilled before use and water was doubly distilled in a glass apparatus.

Phosphate buffered saline (PBS), deuterium oxide (D₂O), glucose, paraformaldehyde (PFA), RNAase-free water, trypan blue, bovine serum albumin (BSA), poly-L-lysine, saponin, trypsin, Luria-Bertani (LB) medium, isopropyl- β -D-1-thiogalactopyranoside (IPTG), Tris-HCl, sodium chloride (NaCl), thioflavin T (ThT), cytarabine (Ara-c), BL21 *Escherichia coli* competent cells, HiPrep™ 26/60 Sephacryl® S-200 HR, Fast Protein Liquid Chromatography (FPLC) and ethylenediaminetetraacetic acid (EDTA) were from Sigma-Aldrich (St. Louis, MO, USA). Fetal Bovine Serum (FBS), Fetal Calf Serum (FCS), L-Glutamine and penicillin/streptomycin (P/S) were from EuroClone (Paignton, UK). Trypsin, Leibovitz L15 medium, Dulbecco's modified Eagle's medium (DMEM), Neurobasal medium, B27 supplement, brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) were from Life Technologies (St. Christophe, France). Dnase I was from Roche (Meylan, France). HiTrap Q Hp columns were from GE Healthcare (UK). α S for Nuclear Magnetic Resonance (NMR) spectroscopy was from Kerafast (Biozol, Calibre Scientific, UK), human α S (R-peptide) for cell culture was from Watkinsville (GA, USA), while human α S for Circular dichroism (CD) spectroscopy was produced by fermentation as above described (see Section 2.5).

2.1.1. Antibodies

For immunofluorescence analyses of DA neurons, the following antibodies were used: primary mouse monoclonal anti-tyrosine hydroxylase (TH)-

antibody (RRID:AB_477560) purchased from Sigma-Aldrich (St. Louis, MO, USA), primary rabbit polyclonal anti- α S antibody (RRID: AB_2192679) purchased from Cell Signaling Technology; primary rabbit polyclonal anti-TH antibody (RRID:AB_390204) purchased from Merck-Millipore (Darmstadt, Germany), primary mouse monoclonal anti-OX-41 antibody (RRID:AB_960866) purchased from Novus (Bio-Techne, Milano, Italy).

These antibodies were revealed with secondary antibodies: goat anti-rabbit IgG coupled with an Alexa Fluor 488 (RRID:AB_2532075) and goat anti-rabbit IgG coupled with an Alexa Fluor 568 (RRID not available, SAB4600084 catalog number) purchased from Sigma Aldrich (St. Louis, MO, USA), goat anti-mouse IgG coupled with an Alexa Fluor 488 (RRID:AB_2534069) purchased from Thermo Fisher Scientific (Cergy Pontoise, France).

2.2. Primary cultures

2.2.1. Primary culture of DA neurons

Rat DA neurons have been cultured as previously described [24,25]. Briefly, pregnant female rats (Wistar, Janvier Labs, France) at 15 days of gestation have been sacrificed using a deep anesthesia with CO₂ chamber and a cervical dislocation. The midbrains obtained from 15-day-old rat embryos have been dissected under a microscope. The embryonic midbrains have been removed and placed in ice-cold medium of Leibovitz (L15) containing 2 % P/S (penicillin 10,000 U/mL and streptomycin 10 mg/mL) solution and 1 % of BSA. The ventral portion of the mesencephalic flexure, a region of the developing brain rich in DA neurons, has been used for the cell preparations. The midbrains have been dissociated by trypsinization for 20 min at 37 °C (solution at a final concentration of 0.05 % trypsin and 0.02 % EDTA). The reaction has been stopped by the addition of DMEM containing Dnase I grade II (0.5 mg/mL) and 10 % of FCS. Cells have been then mechanically dissociated by 3 passages through a 10 mL pipette, centrifuged at 180 xg for 10 min at 4 °C on a layer of BSA (3.5 %) in L15 medium. The supernatant has been discarded and the cell pellets resuspended in a defined culture medium consisting of Neurobasal supplemented with B27 (2 %), L-glutamine (2 mM) and 2 % of P/S solution and 10 ng/mL of BDNF and 1 ng/mL of GDNF. Viable cells have been counted in a Neubauer cytometer using the trypan blue exclusion test. The cells were seeded at a density of 40,000 cells/well in 96 well-plates (pre-coated with poly-L-lysine) and maintained in a humidified incubator at 37 °C in 5 % CO₂/95 % air atmosphere. To obtain rat primary neuron-enriched cultures, 24 h after seeding the cells Ara-c was added to a final concentration of 7.5 μ M to suppress glial proliferation. Half of the medium was changed every 2 days with fresh medium.

2.2.2. Co-culture of DA neurons and microglia

Rat primary mesencephalic neuron-glia cultures were prepared as described by Zhang et al. (2005) and Callizot et al. (2019) with modifications [24,26]. Briefly, pregnant female rats of 14 days gestation (Rats Wistar; Janvier Labs, France) have been sacrificed using a deep anesthesia with CO₂ chamber and a cervical dislocation. Then, fetuses were removed from the uterus and immediately placed in ice-cold L15 Leibovitz medium with a 2 % P/S and 1 % BSA. The ventral portion of the mesencephalic flexure, a region of the developing brain rich in DA neurons, has been used for the cell preparations. The midbrains have been treated for 20 min at 37 °C with a trypsin-EDTA solution at a final concentration of 0.05 % trypsin and 0.02 % EDTA. The reaction has been stopped by the addition of DMEM containing Dnase I grade II (0.5 mg/mL) and 10 % FCS. Cells have been then mechanically dissociated by 3 passages through a 10 mL pipette. Cells have been then centrifuged at 180 xg for 10 min at 4 °C on a layer of BSA (3.5 %) in L15 medium. The supernatant has been discarded and the cell pellets suspended in a defined culture medium consisting of Neurobasal medium with a 2 % solution of B27 supplement, 2 mM L-glutamine, 2 % P/S, 10 ng/mL BDNF, 1 ng/mL GDNF, 4 % heat-inactivated FCS, 1 g/L glucose, 1 mM sodium pyruvate, and 100 μ M of non-essential amino acids. Viable cells have been counted in a Neubauer cytometer using the trypan blue exclusion test. The cells have been seeded at a density of 80,000 cells/well in 96 well-plates (pre-coated with poly-L-lysine) and maintained in a humidified incubator at 37 °C in 5 % CO₂/95 % air atmosphere. Half of the medium have been changed every 2 days with fresh medium.

2.3. Cell treatments

2.3.1. GM1-OS treatment

GM1-OS has been dissolved in PBS at a stock concentration of 2 mM and added to the culture medium at the final concentration of 100 μ M [27] on day 6 of culture for DA neurons without microglia and on day 7 of culture for the DA neurons with microglia.

2.3.2. α S administration

Human α S peptide has been reconstituted in defined culture medium at 4 μ M (stock solution) and slowly shacked at 37 °C for 3 days in dark to generate the α S oligomers (α SO) and protofibrils (PFF) [28]. The control medium was prepared in the same conditions. 1 h after GM1-OS incubation, the DA neurons have been intoxicated with the α S solution (250 nM containing ~60 % of α S) [28] for 96 h in the condition of absence of microglia and for 48 h in presence of microglia. α S (250 nM) has been diluted in control medium with or without GM1-OS.

2.4. Immunostaining

DA neurons were fixed by a solution of 4 % PFA in PBS, pH = 7.3 for 20 min at 23 °C. They were washed twice again in PBS. Cell membrane were permeabilized and non-specific sites have been blocked with a solution of PBS containing 0.1 % of saponin and 1 % of FCS, for 15 min at 23 °C. Cells were incubated for 2 h at 23 °C with:

- i) a rabbit polyclonal anti- α S (1:200) and a mouse monoclonal anti-TH (1:1000) in PBS containing 1 % FCS, 0.1 % saponin. This antibody recognizes specifically DA neurons and neurites, allowing the study of their cell survival and neurite network [24];
- ii) a rabbit polyclonal anti-TH (1:2000) and a mouse monoclonal antibody anti OX-41 (1:500) in PBS containing 1 % FCS and 0.1 % of saponin.

These antibodies were revealed with secondary antibodies goat anti-rabbit IgG coupled with an Alexa Fluor 488, goat anti-rabbit IgG coupled with an Alexa Fluor 568 and goat anti-mouse IgG coupled with an Alexa Fluor 488 at the dilution 1:400 in PBS containing 1 % FCS, 0.1 % saponin, for 1 h at 23 °C. For each condition, 20 pictures per well have been automatically taken using ImageXpress (Molecular Devices) at 20 \times magnification. All images were generated using the same acquisition parameters. From images, analyses will be directly and automatically performed by MetaXpress[®] (Molecular Devices).

The following read-outs were investigated: *i*) DA neuron survival (number of TH-positive neurons); *ii*) total neurite network of DA neurons (length of TH-positive neurites in μ m); *iii*) total microglia activation (area of microglial cells, μ m of OX-41 staining).

2.5. Purification of human recombinant α S for CD spectroscopy

The purification of human recombinant monomeric α S was done as previously described by Powers and Patel [29]. Briefly, pET21a plasmid encoding for the full-length human- α S was overexpressed in BL21 competent *Escherichia coli* cells. After overnight transformation, the bacteria were inoculated in LB medium containing ampicillin and grown at 37 °C until OD600 = 0.5–0.6. Once OD600 = 0.5–0.6 was reached, the α S expression was induced with 1 mM IPTG. After 4 h of culture growing, the cells were pelleted and frozen for 16 h, in order to lyse already most of the bacteria. The day after, the pellet was resuspended in IEX buffer A (20 mM Tris, 25 mM NaCl, 1 mM EDTA, pH = 8.00) and boiled for 15 min. Once the lysate cools down, it was spinned at 20,000 \times g for 45 min at 4 °C. The supernatant was then loaded into pre-equilibrated FPLC system into 2 \times 5 mL HiTrap Q HP columns with a flow rate of 1 mL/min and eluted in IEX buffer B (20 mM Tris, 1 M NaCl, 1 mM EDTA, pH = 8.00). Fractions were then analyzed with Western Blot to determine the fractions with the highest concentration of α S. The selected fractions were pooled together and purified by a size-exclusion chromatography (SEC). FPLC system was washed with 50 mM ammonium acetate (pH = 7.4). The proteins

were loaded into a HiPrep 26/60 Sephacryl S-200 HR. A flush of 50 mM ammonium acetate eluted the protein into several fractions. The protein content was analyzed by Coomassie-stained SDS-PAGE. The purest fractions were pooled together, quantified, batched, and lyophilized.

2.6. CD spectroscopy

α S samples were added to a 1 mM path length quartz cuvette for far-UV CD and analyzed using a J-1500 CD spectrometer (Jasco) at 25 °C. Temperature control with an accuracy of 0.1 °C was achieved with a heating/cooling accessory equipped with a Peltier element (PFD-425S) connected to a water thermostatic bath. Buffer spectra were recorded and subtracted. The helicity of the protein (*i.e.* the percentage of the entire protein sequence in an α -helical state) was obtained from the mean residue ellipticities, Θ_{222} , according to $f_{\text{helix}} = (\Theta_{222} - \Theta_{\text{coil}})/(\Theta_{\text{coil}} - \Theta_{\text{helix}})$ where % helicity = 100 f_{helix} . Here the mean residues ellipticities at 222 nm for the completely unfolded and completely folded peptides were obtained from $\Theta_{\text{coil}} = 640 - 45 T/^\circ\text{C}$ and $\Theta_{\text{helix}} = -40,000 (1 - 2.5/n) + 100 T/^\circ\text{C}$, where *n* is the number of amino acids in the polypeptide.

2.7. PFF generation for amyloid seeding aggregation assay (ASAA)

5 mg/mL recombinant α S in PBS was aggregated for 7 days at 37 °C with nutation to form ThT-positive fibrils. To generate soluble PFFs, α S fibrils were diluted to 1 mg/mL and tip-sonicated at power level 30 for 30 s (1 s off, 1 s on). After, the aggregation was checked by ThT and sedimentation assays. Aliquots of the resultant material were flash-frozen in liquid nitrogen and stored at -80 °C.

2.8. ASAA

Before each ASAA, lyophilized α S monomeric protein was dissolved in 40 mM phosphate buffer (pH = 8), filtered using a 0.22 μ m filter, and the concentration of recombinant protein was measured *via* absorbance at 280 nm using a Nanodrop One spectrophotometer. PFF was sonicated and added to a 96 well plate with 230 mM NaCl, 0.4 mg/mL α S and a 3 mm glass bead (Millipore Sigma 1040150500). The amyloid dye ThT was used to monitor the increase in fibrillar content. The 96 well plate is incubated at 42 °C in a BMG FLUOstar Omega plate reader. The generation of new fibril material was detected *via* fluorescence readings at 480 nm every 30 min until the signal plateaued towards the end of the amplification interval of 7 days.

2.9. NMR spectroscopy

NMR experiments were performed on 400 MHz Bruker Avance III and 600 MHz Bruker Avance spectrometers. GM1-OS (4 mM in deuterated or aqueous PBS, pH = 7.4) was characterized through one-dimensional (1D) hydrogen nuclear (1H), two-dimensional (2D) Correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), heteronuclear single quantum coherence (HSQC) and nuclear overhauser effect spectroscopy (NOESY) experiments acquired at 298 K and 283 K. When required, water suppression was achieved by excitation sculpting sequence from standard Bruker library.

α S (0.24 mM in PBS pH = 7.4) was characterized by 1D ¹H NMR experiments.

For interaction studies, the sample was prepared using a GM1-OS – α S ratio 10:1 (with a concentration of 0.8 mM and 0.08 mM for GM1-OS and α S, respectively).

Diffusion ordered NMR (DOSY) experiment were acquired at 298 K and NMR signals of different species were distinguished according to their diffusion coefficients. These values are inversely proportional to the hydrodynamic radius of the analyzed molecules, thus to their size.

DOSY spectra were acquired with the standard Bruker sequence with presaturation during relaxation delay for water suppression. For the determination of the diffusion coefficient *D* (log*D*), a big Δ of 250 ms and a little Δ of 3 ms were used. After acquiring the DOSY spectra, to determine the log*D* of the various species, Dynamics Center routine was used.

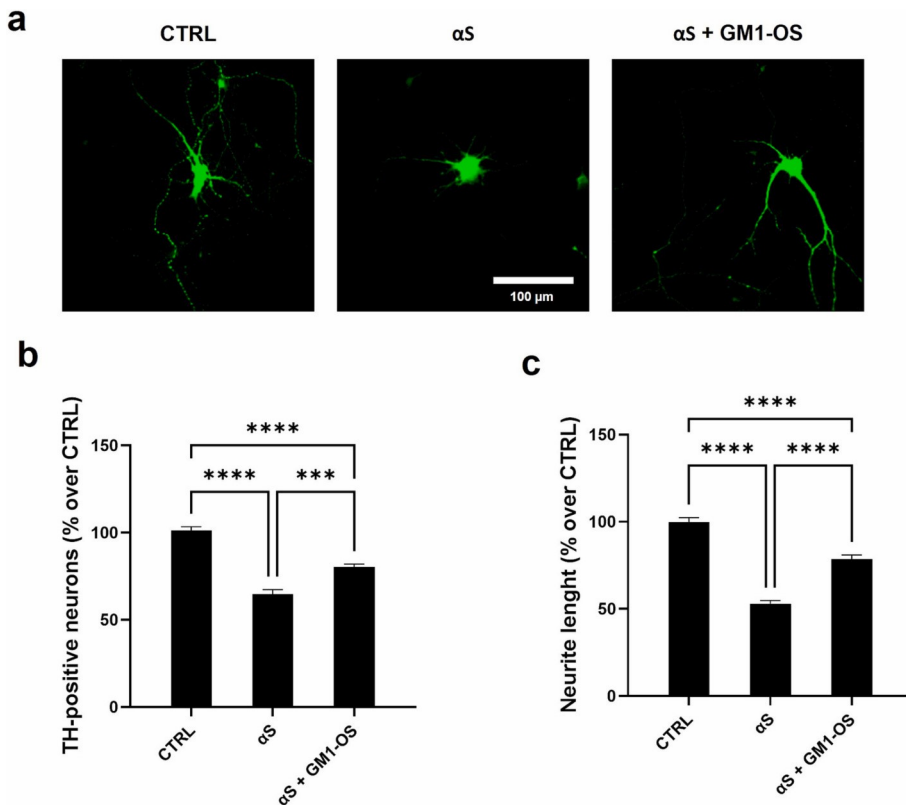


Fig. 1. GM1-OS neuroprotective effects in primary cultured rat DA neurons injured with α S. On day 6 of culture, primary DA neurons were pre-incubated with GM1-OS (100 μ M) for 1 h, before α S exposure. Next, α S solution (250 nM, containing ~60 % of α S) was added to the culture medium for 96 h. At the end of treatment, immunofluorescence evaluation of TH marker was performed as described in the methods section. (a) Representative immunofluorescence images of TH-positive neurons (20 \times magnification, scale bare 100 μ M); (b) Number of TH-positive neurons, as read-out of DA neurons' survival; (c) Length of TH-positive neurites in μ m, expressed as fold change over control (CTRL) to evaluate the total neurite network of DA neurons. All values are expressed as mean \pm SEM ($n = 6$, **** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; One-way ANOVA followed by Tukey's multiple comparisons test).

2.10. GM1-OS preparation

The GM1-OS was prepared by ozonolysis of GM1 followed by alkaline degradation as previously described [27,30,31]. GM1 ganglioside was purified from the total ganglioside mixture extracted from fresh pig brains collected at the slaughterhouse of the Galbani Company (Melzo, Italy) [32]. The ganglioside mixture, 5 g as sialic acid, was dissolved in pre-warmed (36 $^{\circ}$ C) 500 mL of 0.05 M sodium acetate, 1 mM CaCl₂ buffer (pH = 5.5). *Vibrio cholerae* sialidase (1 unit) was added to the solution every 12 h to transform the major part of polysialogangliosides into GM1 and obtain higher amount of GM1 [33]. Incubation at 36 $^{\circ}$ C was maintained for two days with magnetic stirring, and the solution dialyzed at 23 $^{\circ}$ C for 4 days against 10 L of water changed 5 times a day. The sialidase treated ganglioside mixture was subjected to 150 cm \times 2 cm silica gel 100 column chromatography equilibrated and eluted with chloroform/methanol/water, 60:35:5 by vol. The fractions containing GM1, identified by high-performance thin layer chromatography (HPTLC), were pooled, dried and submitted to a further column chromatographic purification using the above experimental conditions. Fractions containing pure GM1 were collected and dried. The residue was dissolved in chloroform/methanol (2:1 v/v) and precipitated by adding 4 volumes of cold acetone. After centrifugation (15,000 \times g) the GM1 pellet was separated from the acetone, dried, dissolved in 50 mL of deionized water and lyophilized giving 1.350 mg of white powder which was stored at -20 $^{\circ}$ C. To obtain GM1-OS, GM1 was dissolved in methanol and slowly saturated with ozone at 23 $^{\circ}$ C. The solvent was then evaporated under vacuum and the residue brought immediately to pH = 10.5–11.0 by addition of tri-ethylamine. After 3 days, solvent was evaporated and GM1-OS was purified by flash chromatography using chloroform/methanol/2-propanol/water 60:35:5:5 by volume as eluent. The oligosaccharide was dissolved in methanol and stored at 4 $^{\circ}$ C. Altogether, nuclear magnetic resonance, mass spectrometry and HPTLC analyses showed a homogeneity over 99 % for the prepared oligosaccharide (data not shown).

2.11. Statistical analysis

All values are expressed as mean \pm SEM (standard error of the mean). Statistical analysis was performed by one-way ANOVA, followed by Tukey's multiple comparisons test. $p < 0.05$ was considered significant.

3. Results

3.1. GM1-OS protects DA neurons from α S toxicity

Recently, using the *β4galnt1^{-/-}* parkinsonian mice model, it has been demonstrated that GM1-OS significantly reduced the α S levels and its phosphorylation at serine 129 (Ser129) residue, which is enriched in α S aggregates, in the SNpc [23]. Given the evidence of a potential role of GM1-OS in counteracting the α S toxicity, we investigated whether GM1-OS was capable to protect the DA releasing neurons, directly injured with α S. As shown in Fig. 1, α S exposure for 96 h induced neurotoxicity, as assessed by a significant reduction in TH-positive neurons and by the disruption of the neurite network. When GM1-OS was added to the culture medium, TH immunosignal loss and neurite disruption, induced by α S, was alleviated (Fig. 1), suggesting a GM1-OS ability to counteract long-term α S neurotoxicity, the main pathological hallmark of PD.

3.2. GM1-OS modulates α S-derived glia inflammation

α S-induced inflammation represents another distinctive mechanism implicated in PD pathophysiology, which seems to be involved both at the beginning and during the exacerbation of the neuronal damage [34,35]. Even if a series of possible events have been proposed, the exact biochemical/molecular mechanism that explains how and when the inflammation process starts following α S toxic aggregation is far to be completely elucidated. Recently it has been reported that GM1 is able to reduce the microglial and neuroinflammatory responses to α S in the A53T α S PD rat model [36]. Thus, to understand if the GM1 effect on α S-induced inflammation could be mediated by its oligosaccharide portion, we investigated a possible GM1-OS protective effect against α S chronic neurotoxicity, in a mixed culture of DA neurons and microglia.

As shown in Fig. 2, in presence of microglial cells, a large and fast neuroinflammation with microglial activation was observed and was associated with the loss of DA neurons. Importantly, GM1-OS significantly prevented the neuronal loss and the neurite network disruption (Fig. 2b, c) in this pro-inflammatory model. Additionally, GM1-OS partially reduced the

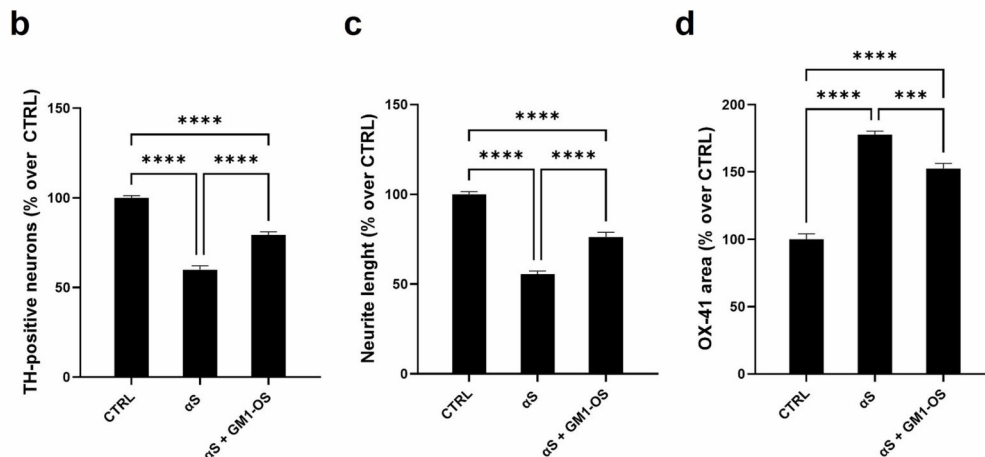
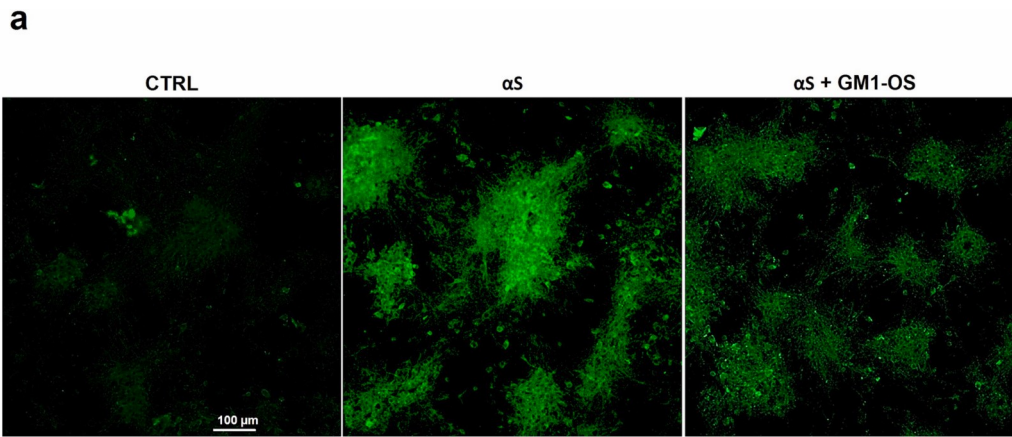


Fig. 2. GM1-OS neuroprotective effects in primary cultured rat DA neurons co-cultured with microglia and intoxicated with α S. On day 7 of culture, primary DA neurons were pre-incubated with GM1-OS (100 μ M) for 1 h, before α S exposure. Next, α S solution (250 nm, containing ~60 % of α S) was added to the culture medium for 48 h. At the end of α S exposure, immunofluorescence analysis against TH and OX-41 was performed as described in the methods section. (a) Representative immunofluorescence images of OX-41 signal (20 \times magnification, scale bare 100 μ M); (b) Number of TH-positive neurons, as read-out of DA neurons survival; (c) Length of TH- positive neurite in μ m, expressed as fold change over CTRL to evaluate the total neurite network of DA neurons; (d) Area of microglial cells, μ m of OX- 41 staining, as read-out of total microglia activation. All values are expressed as mean \pm SEM ($n = 6$, **** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; One-way ANOVA followed by Tukey's multiple comparisons test).

microglia activation, as demonstrated by the reduction in OX-41 signal (Fig. 2d). These results suggest an effect on the activation of microglial cells, and a remarkable GM1-OS neuroprotective effect in a pro-inflammatory context, suggesting that the GM1 capability to mitigate inflammation is mediated by its oligosaccharide portion.

3.3. GM1-OS inhibits α S aggregation *in vitro*

Previously, it has been shown that GM1 directly interacts with α S inhibiting its aggregation [12]. Specifically, it has been demonstrated that the α S-GM1 interaction and the consequent inhibition of fibrillation depends on the presence of the GM1 pentasaccharide, as the same effect was not obtained by glycosphingolipids with different oligosaccharide structure like GM3, GM2, or asialo-GM1 [11,12].

To assess the direct interaction between GM1 oligosaccharide and α S, we investigated whether GM1-OS could prevent α S aggregation and fibrillation by the ASAA [11]. As expected, ThT fluorescence increased over time as a function of α S self-aggregation (Fig. 3a). Notably the addition of α S PFF strongly accelerated α S aggregation as assessed by ThT increase and fluorescence peak after 25 and 50 h with PFF 10 ng and 10 pg, respectively (Fig. 3a). When GM1-OS was added to the reaction mixture, it abolished any changes in ThT fluorescence of α S alone (Fig. 3b) or in the presence of PFF (Fig. 3c). These data demonstrate that GM1-OS suppressed both the spontaneous and PFF-induced α S aggregation suggesting that the GM1 oligosaccharide directly interacts and prevents the protein misfolding, possibly explaining the mechanism underlying GM1-OS protection against α S neurotoxicity.

In order to understand whether GM1-OS influences the α S secondary structure, we performed CD spectroscopy experiments. As shown in Fig. 4, we found that GM1-OS does not induce any change in α S secondary structure (~ 5 % helical, 95 % random coil), suggesting that the mechanism by which GM1-OS prevents α S aggregation does not depend on the secondary structure, in

contrast to what has been reported for GM1, which induces the formation of a specific secondary structure (~ 50 % helical) [11].

To confirm this data, we acquired 1D NMR spectra for α S in absence and in presence of GM1-OS: the analysis of the amidic regions, where no signals of GM1-OS are present, showed the same distribution of backbone amide protons (NH), suggesting that a similar protein folding is maintained. This means that GM1-OS is not able to induce any modification in the α S secondary structure, confirming CD spectroscopy experiments (Fig. 5a).

Then, the interaction between GM1-OS and α S was analyzed using ligand based NMR techniques such as DOSY [37–39]. The analysis of the GM1-OS logD showed a decreasing after the addition of α S in a 10:1 ratio (from 3.71 10^{-10} m²/s to 3.3 10^{-10} m²/s) suggesting that GM1-OS is interacting with α S (Fig. 5b).

Finally, the spontaneous aggregation of α S was followed over 120 h: the α S logD in the freshly prepared sample (considered as its monomeric form) is 1.58 10^{-10} m²/s while after 60 h the logD decreases to 0.79 10^{-10} m²/s, indicating that the protein is undergoing the aggregation process (*i.e.* the α S molecular mass increases and its logD decreases) as shown in the Fig. 5c. The addition of GM1-OS (10:1 ratio) shown an α S logD of 1.51 10^{-10} m²/s, thus quite unchanged for 60 h (Fig. 5c), suggesting that GM1-OS prevents the α S aggregation and confirming the ASAA results.

4. Discussion

The accumulation and aggregation of α S have a central role in the onset of PD, but to date there is no explanation on what causes this elevation and the resultant aggregation [3,34]. *In vitro* experiments showed that the interaction of α S with GM1 ganglioside prevents the α S misfolding and the consequent aggregation [12]. Recently, a specific functional connection between α S and GM1 ganglioside *in vivo* was revealed by studying mice with the heterozygous disruption of β 4galnt1 gene, which codes for the GM2/GD2 synthase, an enzyme required for GM1 biosynthesis [14,15]. Mice with partial GM1

deficiency fully recapitulate the human sPD phenotype, spontaneously manifesting both motor and non-motor symptoms as aging proceed. Specifically, α S aggregation was found at the level of TH-positive neurons in the SNpc, in the myenteric plexus, and in heart ventricles, suggesting that the systemic GM1 deficiency correlates with the systemic α S aggregation [14,17,40]. In these mice, GM1 replacement therapy showed a recovery of the motor phenotype and a significant reduction in α S aggregates [14,17,40].

In line with these findings, subsequent detailed analysis on PD human tissues revealed significant reduction in GM1 content in SN, occipital cortex, and peripheral tissues [15,16]. From here, a theory has emerged defining a central role for the ganglioside GM1 in the establishment of neurodegeneration. Indeed, it has been reported that brain GM1 levels vary significantly among individuals of the same age and that they decline progressively along aging [41,42]: in subjects with lower basal level of the ganglioside, GM1 amount may not reach the threshold amount required to maintain the homeostasis of DA and forebrain neurons, presenting consequences also on the correct folding of α S, thereby gradually leading to the development of sPD [3,13,41]. As the population age increases and GM1 continues to decrease, the number and percentage of people with this condition will multiply. Despite this suggestive evidence, the use of GM1 replacement therapy in clinical trials is severely hampered due to its low brain penetrance through the blood brain barrier (BBB) [43–45].

Recently, we discovered that the neurotrophic and neuroprotective GM1 functions are actually attributed to its oligosaccharide portion: the soluble and hydrophilic saccharide core that, without entering the cell, remains in the extracellular environment [46,47] and interacts with the neurotrophin receptor TrkA mediating protective and restorative signaling typical of GM1 [21]. On the other hand, GM1-OS, lacking the hydrophobic ceramide, gains the advantage to efficiently cross the BBB, showing a 20-fold higher crossing rate than GM1 and a paracellular time- and concentration-dependent transport. Moreover, after barrier crossing, GM1-OS remains intact and maintains its neurotrophic properties [48]. Thus, GM1 exerts its bioactivity through its hydrophilic

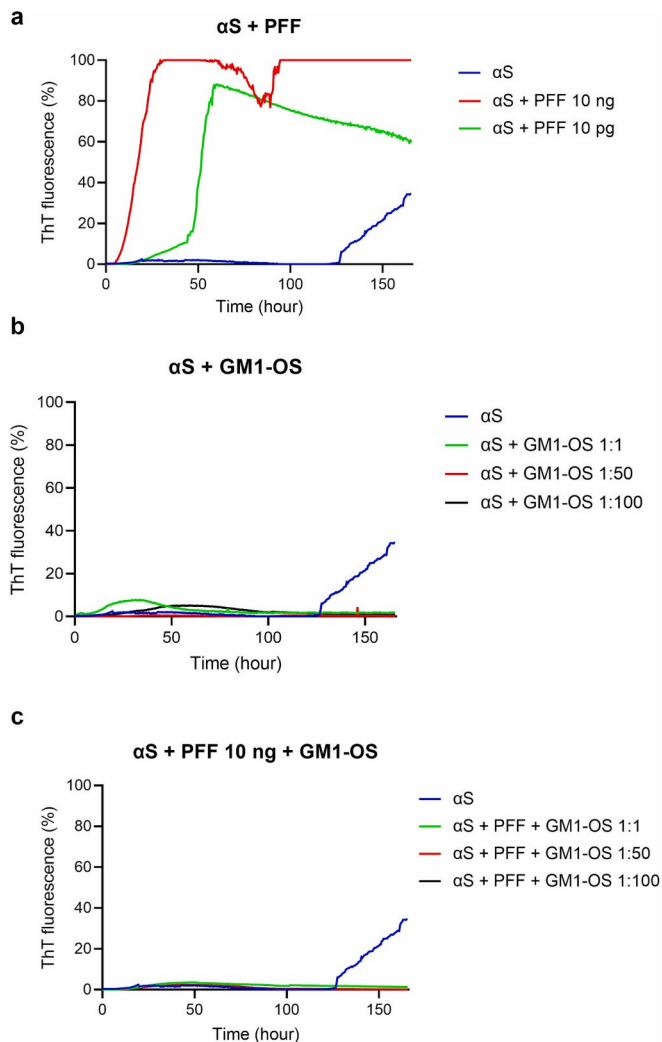


Fig. 3. Kinetics of α S fibrillation monitored by ThT fluorescence. (a) α S alone (0.4 μ g/mL) or in presence of PFF at different concentrations (10 ng or 10 pg); (b) α S alone or in presence of GM1-OS in the following α S:GM1-OS ratios: 1:1 nM (green line), 1:50 nM (red line), 1:100 nM (black line); (c) α S alone (0.4 μ g/mL) or in presence of both PFF (10 ng) and GM1-OS in the following α S:GM1-OS ratios: 1:1 nM (green line), 1:50 nM (red line), 1:100 nM (black line).

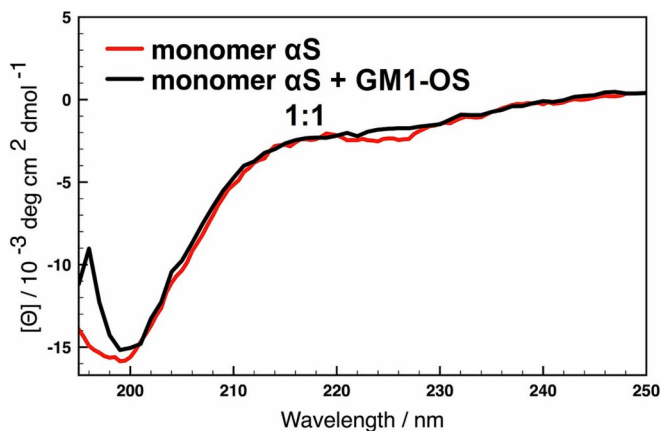


Fig. 4. Effect of GM1-OS on the α S secondary structure. Far-UV CD spectra of 5 μ M α S alone (red line) or in the presence of GM1-OS in ratios: 1:1 (black line).

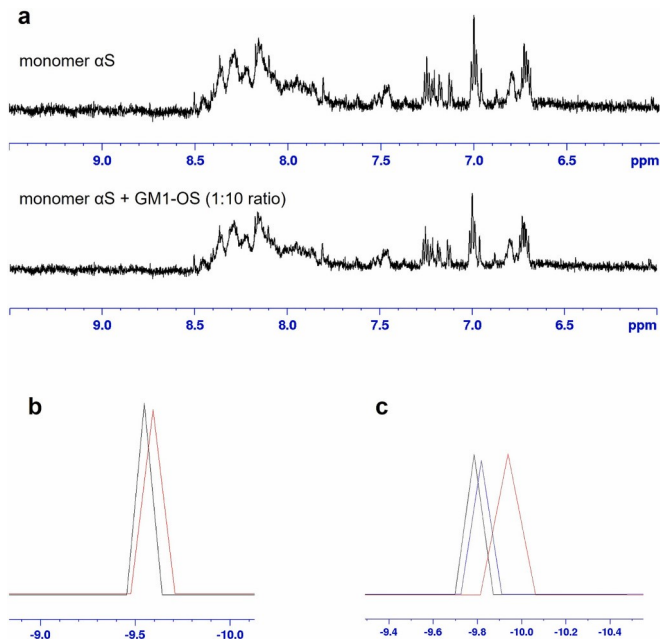


Fig. 5. NMR interaction studies between GM1-OS and α S. (a) 1D NMR region of the backbone NHs of α S alone (top) and α S in presence of GM1-OS (bottom): the two regions show the same spread of NHs chemical shift, suggesting that no changes in the secondary structure occurs; (b) logD values calculated for GM1- OS alone (black) and after the addition of α S in a 10:1 ratio (red): the diffusion coefficient is slightly decreased indicating the presence of an equilibrium with a bound form to α S; (c) logD values obtained for α S alone (black), α S alone after 60 h (red) indicating a spontaneous aggregation process and α S in presence of GM1-OS after 60 h (blue) where no aggregation phenomena can be observed.

head, which protrudes into the extracellular environment and therefore acts at the cell surface by interacting with plasma membrane-resident proteins [21].

These data suggested that the specific role of GM1 in maintaining α S clearance may also be mediated by its oligosaccharide portion. Accordingly, using the β 4galnt1^{-/-} parkinsonian mouse model, we found that GM1-OS-treated mice show a significant reduction of the abnormal α S content in the SNpc compared to untreated animals, as well as a reduced α S phosphorylation at the level of Ser129 residue [23].

In the present study we better characterized the mechanism by which the GM1-OS could account for the GM1 neurotrophic function in counteracting the α S toxicity. To this purpose, we exploited an *in vitro* model, represented by rat primary DA neurons alone or in presence of microglia challenged with α S [24,26]. In the absence of microglial cells, α S induced neurotoxicity over 96 h (Fig. 1). When microglia was co-cultured with DA neurons, α S-mediated neurotoxicity was observed right after 48 h, accompanied by microglia proinflammatory activation (Fig. 2). These data corroborate the role of the damage associated microglia (DAM) in the exacerbation of α S toxicity [49]. Notably, GM1- OS was able to lower α S-mediated neuronal loss and neurite network damage (Fig. 1), and to limit microglia activation (Fig. 2). These findings agree with the observed *in vivo* effect of GM1-OS in counteracting α S aggregation and toxicity [23]. Although the mechanism by which GM1- OS limited microglia activation is not elucidated here, our idea is that GM1-OS activates the TrkA receptor on the microglia plasma membrane, triggering the activation of intracellular anti-inflammatory pathways [50]. This hypothesis would seem to be supported by a proteomic analysis performed on neuroblastoma cells treated with GM1-OS, showing the upregulation of proteins involved in the modulation of inflammatory response [51]. Furthermore, by preventing the α S aggregation [23], GM1-OS could lead to a reduction of microglia activation, since extracellular aggregated α S is known to activate microglia [52]. Finally, GM1-OS by protecting DA neurons from α S-mediated toxicity (Fig. 1) indirectly reduces the microglial component activated by suffering neurons [53].

Following, we partially explained the mechanisms underlying GM1- OS protection against aberrantly folded α S. Our *in vitro* data demonstrate that GM1-OS directly interacts with α S and prevents self-aggregation and PFF-mediated fibrillation (Fig. 3). NMR data demonstrated the binding between GM1-OS and α S, confirming that in presence of GM1- OS the protein does not follow its typical aggregation pathway (Fig. 5).

Moreover, contrarily to GM1, no changes in secondary α S structure were observed in the presence of GM1-OS by CD- and NMR- spectroscopy. This different functioning should not be surprising, as GM1 and its oligosaccharide have different chemical and physical characteristics. Ganglioside GM1 is an amphiphilic lipid, composed by a hydrophobic component, the ceramide tail, and a hydrophilic component, the saccharide head. On the contrary, GM1-OS is composed only by the hydrophilic-soluble pentasaccharide. Their chemical composition well correlates within the different behavior in solution: GM1 forms micelles while GM1-OS remains in a monomeric state. Consequently the way they interact with α S may be different. However, how GM1-OS- α S interaction inhibits protein misfolding remains to be addressed.

It was demonstrated that aggregated α S can be released from the cytosol of a cell to the extracellular matrix, and then taken up by neighboring cells, leading to the further aggregation of endogenous intracellular α S, favoring the spreading of pathology [54]. Moreover, extracellular α S has the ability to interact with the negatively-charged membrane lipids [11,55,56] through the positively-charged lysine residues present in the N-terminus of α S. Considering the data shown in this work, our hypothesis is that GM1-OS, which, differently from the entire GM1, does not penetrate the cells and works on the cells surface [27,46], interacts with membrane-linked α S and with the extracellular α S aggregates [4], favoring the correct protein folding and aggregates clearance.

The α S-membrane interaction could alter multiple membrane properties such as curvature and thickness, leading to cytotoxicity [57,58]. Specifically, α SO have been proposed to mediate cytotoxicity by the permeabilization and disruption of membranes *via* the formation of membrane pores [59–61], enabling unrestricted calcium influx and consequently excitotoxicity. Regarding this latter, a possible GM1-OS protective mechanism is that the extracellular binding of α S with GM1-OS could prevent the association of α S with the plasma membrane, hindering the cytotoxicity. Importantly, the capability of GM1-OS, to modulates the calcium homeostasis in a TrkA-dependent fashion [62] could provide another possible mode by which it can counteract the α S cytotoxicity.

Moreover, it has been demonstrated that α S PFF administered to primary DA neurons are internalized by endocytosis, leading to progressive cytotoxicity associated with mitochondrial dysfunction, oxidative stress, and calcium imbalance [28]. By proteomic analysis, GM1-OS administered to neuroblastoma cells induced a reduced expression of proteins involved in the endocytosis [51], suggesting another possible protective mechanism, by which GM1-OS limits the internalization of aggregated α S. Additionally, GM1-OS specifically counteracted mitochondria dysfunction and oxidative imbalance, typical features of PD triggered by α S aggregation [31,51].

Further, another possible mechanism underlying GM1-OS protection against intracellular α S might involve the GM1-OS regulation of autophagy and lysosomal function, leading to an enhanced clearance of aggregated α S, as already demonstrated for the entire ganglioside GM1 [63].

Finally, the proteomic profile of neuroblastoma cells exposed to GM1-OS revealed a significant up-regulation of β -synuclein (β S), a protein belonging to the synuclein family [64]. Contrarily to the homologous α S, the β S does not aggregate in fibrils and it has been described to be capable of blocking α S fibrillation protecting cells from neurotoxic effects of α SO [65]. Thus, the induction of β S expression could represent an indirect mechanism underlying GM1-OS capability to counteract α S aggregates.

Although the mechanism by which GM1-OS prevents α S aggregation and α S induced-inflammation has not been fully elucidated yet and needs to be further investigated, in the present study we show new insights on the protective properties of GM1-OS against α S aggregation and toxicity, strengthening its use as a possible therapeutic strategy for α -synucleinopathies and PD.

5. Conclusions

In line with the evidence reported from independent groups [66–68], we recently proposed that the GM1-OS represent the bioactive portion of GM1 and that alone it could replicate the neurotrophic and neuroprotective properties of the entire GM1 molecule.

GM1-OS, without entering into the cells, restores and maintains the correct neuronal homeostasis, through the activation of the specific TrkA trophic plasma membrane signaling [27,46]. As suggested by proteomic analysis and confirmed by biochemical evidences, this event leads to the modulation of several mechanisms leading to protection against oxidative stress *via* mitochondrial regulation [31,64], to control of intracellular calcium flux and signaling [30], and to the protection from α S accumulation/aggregation [23].

Although the proposed mechanism needs to be confirmed in an *in vivo* model specific for α S toxicity and inflammation, here we further proved a direct involvement of GM1-OS in preventing α S aggregation and in protecting DA neurons from α S induced toxicity and inflammation. All together the data here presented suggest that GM1-OS could counteract the α S mediated toxicity/inflammation by two separate mechanisms: first, by activating the neuroprotective signaling *via* Trk receptors and secondly, by a direct interaction with the monomeric or aggregated α S.

These concepts bring out the GM1-OS as a cutting-edge molecule able to recover and cope with all aspects of neurodegenerative diseases, for which there is no effective therapy to date.

CRedit authorship contribution statement

Conceptualization, methodology, investigation, analysis, and draft of the manuscript, G.L., E.D.B.; E.V.C., M.F., A.H., N.C., T.B., L. Z., M.C., F. V., L.A.M., E.C.; Supervision, conceptualization, draft and revision of the manuscript, T.B., G.L., E.C.; GM1-OS chemical synthesis, L.A.M., M.G.C.; Revision of the manuscript, G.L., E.D.B.; E.V.C., M.F., A.H., N.C., T.B., L. Z., N.L., M.A., S.S., L.A.M., L.U.M, M.C., F.V., E.C; All authors have read and agreed to the published version of the manuscript.

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Institutional review board statement

The animal procedures involving dopaminergic neurons were supervised and approved by the local direction of the veterinary services of the Bouches-du-Rhone and by the ministry of higher education and research (APAFIS#26727). All experiments have been carried out by trained personnel in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and followed current European Union regulations (Directive 2010/63/EU).

Informed consent statement Not

applicable.

Declaration of competing interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Data availability

The data presented in this study are available upon reasonable request to the corresponding authors.

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