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Drp-1 dependent mitochondrial fragmentation and protective autophagy in dopaminergic SH-SY5Y cells overexpressing alpha-synuclein

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ABSTRACT

Parkinson's disease is a neurodegenerative movement disorder caused by the loss of dopaminergic neurons from substantia nigra. It is characterized by the accumulation of aggregated α-synuclein as the major component of the Lewy bodies. Additional common features of this disease are the mitochondrial dysfunction and the activation/inhibition of autophagy both events associated to the intracellular accumulation of α-synuclein. The mechanism by which these events contribute to neural degeneration remains unknown. In the present work we investigated the effect of α-synuclein on mitochondrial dynamics and autophagy/mitophagy in SH-SY5Y cells, an in vitro model of Parkinson disease. We demonstrated that overexpression of wild type α-synuclein causes moderated toxicity, ROS generation and mitochondrial dysfunction. In addition, α-synuclein induces the mitochondrial fragmentation on a Drp-1-dependent fashion. Overexpression of the fusion protein Opa-1 prevented both mitochondrial fragmentation and cytotoxicity. On the other hand, cells expressing α-synuclein showed activated autophagy and particularly mitophagy. Employing a genetic strategy we demonstrated that autophagy is triggered in order to protect cells from α-synuclein-induced cell death. Our results clarify the role of Opa-1 and Drp-1 in mitochondrial dynamics and cell survival, a controversial α-synuclein research issue. The findings presented point to the relevance of mitochondrial homeostasis and autophagy in the pathogenesis of PD. Better understanding of the molecular interaction between these processes could give rise to novel therapeutic methods for PD prevention and amelioration.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder affecting 2–3% of the population ≥ 65 years old. The characteristic features of PD are the degeneration of dopaminergic neurons in the *Substantia Nigra pars compacta* (SNpc), and the presence of intraneuronal protein aggregates designated as Lewy Bodies (LB). The latter represent one of the most prominent pathological hallmarks in PD. The presynaptic neuronal protein alpha-synuclein (αS) has been reported to be the major component of LB, suggesting that it plays a relevant role in PD pathogenesis (Spillantini et al., 1997).

The damage to dopaminergic neurons is caused by a complex interplay between toxic αS aggregates (owing to impaired protein degradation), oxidative stress, mitochondrial dysfunction, altered intracellular calcium homeostasis and neuroinflammation (Perfeito et al., 2014; Xie, H. et al., 2010; Roberts and Brown, 2015; Roberts et al., 2016).

It has been reported that excessive accumulation of αS can lead to its oligomerization or aggregation interfering with normal cellular processes or increasing the vulnerability to neuronal degeneration (Xie, W. et al., 2010). In the last two decades, many studies were aimed to shed light on the mechanisms that trigger αS oligomerization and mitochondrial membrane interaction in order to explain its cytotoxicity (Roberts and Brown, 2015).

Mitochondria are the powerhouses of the cell, consuming oxygen to generate energy for the maintenance of normal cellular processes. Under physiological conditions, mitochondrial electron transport chain generates superoxide anion ($O_2^{\bullet-}$). However, oxidative stress increases

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reactive oxygen species (ROS) production with deleterious consequences on cellular survival (Roberts et al., 2016). The disturbance in the balance between the ROS production and antioxidant defenses it has been considered a central feature in the development of degenerative diseases (Manoharan et al., 2016).

Several studies have demonstrated that a portion of endogenous or overexpressed αS resides within mitochondria (Li et al., 2007; Devi et al., 2008; Nakamura et al., 2008; Liu et al., 2009). At this location it could cause a decrease in complex I activity, thus linking αS with the effects of mitochondrial toxins, and potentially, sporadic PD. Remarkably, the amount of mitochondria- associated αS increases dramatically in PD, in the specific brain regions undergoing degeneration (Devi et al., 2008; Nakamura et al., 2008; Liu et al., 2009). By complex I inhibition, αS could potentially induce ROS production, oxidative stress, and neuronal death. In fact, there is some evidence describing neuroprotection mediated by molecular and pharmacological antioxidant strategies (Liu et al., 2011; Clark et al., 2010). All this data suggest that αS may have a specific functional role in mitochondrial homeostasis, with important implications for disease progression.

Mitochondria are morphologically dynamic organelles that continuously undergo two opposing processes, fusion and fission, which operate in equilibrium to form small individual units or interconnected networks (Sheridan and Martin, 2010). The interplay between these mitochondrial events controls mitochondrial morphology and properties. The key molecular factors involved in mitochondrial dynamics are Mitofusin 1 and 2 (Mfn-1 and Mfn-2) which together with Optic atrophy 1 (Opa-1) regulate fusion, whereas Fis-1 and Drp-1 are responsible for fission (Ni et al., 2015). Mitochondrial morphology and movement inside the cells' cytoskeleton are relevant for their function. This is particularly crucial for neurons as mitochondria are constantly transported between the cell bodies and nerve terminals, presumably according to local energy request (Xie and Chung, 2012). Thus, the maintenance of a healthy mitochondrial network is fundamental to guarantee cell viability (Cieri et al., 2017). Impairment of normal mitochondrial dynamics has been linked to neurodegenerative disorders (Itoh et al., 2013; McCoy and Cookson, 2012).

It has been reported that αS small oligomers, interacting with mitochondrial membranes are responsible for the subsequent mitochondrial dysfunction and fragmentation and neuronal demise (Nakamura et al., 2011). Fragmentation is required for the efficient mitochondrial degradation by autophagy (mitophagy). This process is usually associated to the maintenance of cellular homeostasis promoting the removal of dysfunctional mitochondria. However, there is controversial information about both the involvement and role of autophagy/mitophagy in dopaminergic neurons exposed to αS (Choubey et al., 2011; Vives-Bauza and Przedborski, 2011).

In summary, although it has been accepted that mitochondrial dysfunction is a central feature of PD, the connection between αS, mitochondrial dysfunction and autophagy is poorly understood (Nakamura, 2013).

This work was aimed to shed light on the effects of αS on mitochondrial integrity and autophagy employing αS transfected SH-SY5Y cells. Our results suggest that αS impairs cell viability and ATP synthesis while increases mitochondrial ROS production. An imbalance in mitochondrial dynamics is orchestrated by an increase in Drp-1 recruitment to mitochondria and Opa-1 processing. In addition, αS overexpression triggers the autophagic/mitophagic machinery in order to exert cellular protection. Our findings point to mitochondrial homeostasis and mitophagy as critical events in the pathogenesis of PD.

2. Materials and methods

2.1. Cell culture and treatments

Human neuroblastoma SH-SY5Y cell line (ATCC® CRL-2266™) (passages 6–25) is a thrice cloned (SK-N-SH - > SH-SY - > SH-SY5 - > SH-SY5Y) subline of the neuroblastoma cell line SK-N-SH (see ATCC HTB-11) which was established from a human metastatic bone tumor. It was kindly provided by the Max Planck Institute for Biophysical Chemistry (Göttingen, Germany). Cells were grown in Dulbecco's modified Eagle's medium/nutrient F12 (DMEM/F12) (Gibco, Thermo Fisher Scientific, Inc., MA, USA); supplemented with 10% fetal bovine serum (FBS) (Internegocios, S.A.; Buenos Aires, Argentina), 50 U/ml penicillin and 50 μg/ml streptomycin (Gibco). Cultures were maintained in a humidified incubator at 37 °C with 5% CO_2 and 95% air.

Differentiated and undifferentiated SH-SY5Y cells have been widely used as a cell model of dopaminergic neurons for PD research since they express tyrosine hydroxylase and dopamine-beta-hydroxylase, as well as the dopamine transporter (Xie, W. et al., 2010; Xicoy et al., 2017). Mdivi-1 (10 μM) (Santa Cruz Biotechnology, CA, USA) was added 24 h after transfection. Bafilomycin A1 (Baf A1) (100 nM) (Fermentek, Jerusalem, Israel) was delivered 2 h before cell lysis.

2.2. Plasmids and transfection

The pcDNA3-αS wild-type, pcDNA3-αS wild-type-GFP and pcDNA3 empty vector (EV) were kindly provided by the Max Planck Institute for Biophysical Chemistry. Both pcDNA3 wild type Opa-1 (Opa-1wt) and pcDNA3 Q297V Opa-1 (Opa-1Q297V) were gently provided by Dr. Donald D. Newmeyer (La Jolla Institute for Allergy and Immunology, USA). The pCI-neo mAtg5WT and mAtg5K130R expression plasmids were kindly provided by Dr. Noburu Mizushima (University of Tokyo, Japan) and Tamotsu Yoshimori (Osaka University, Japan). Transfections were performed according to the procedure described in Gorojod et al., (Gorojod et al., 2015) using the branched 25-kDa polyethylenimine (PEI) (Sigma-Aldrich, Saint Louis, MO, USA). Briefly, cells were seeded in 96, 12 and 6 well plates and allowed to grow for 24 h until 50–60% confluence. Transfection complexes were prepared in serum-free Opti-MEM media in a ratio PEI: DNA (3.75: 1). Mixtures were vortexed, incubated 10 min at RT (room temperature) and then drop-wise added to the cells in serum media. After 5 h, transfection medium was replaced with fresh complete DMEM and cells were again returned to the incubator for additional 48 h. To measure transfection efficiency cells were transfected with pcDNA3-GFP together with the plasmid of interest. Expressing GFP cells were visualized by fluorescence microscopy. The transfection efficiency was measured by scoring the number of fluorescent cells in a total of 300 cells per sample. The obtained efficiency, ranged from 35 to 40%.

2.3. Assessment of cell viability

LDH release assay was carried out by using the CytoTox-ONE Homogeneous Membrane Integrity Assay kit (G7890, Promega, WI, USA). Briefly, for quantitative analysis, 100 μl of the reagent was added to each well. After 2 h of incubation in the dark at RT, fluorescence intensity was measured at 560/70 nm excitation and 590 nm emission using an Optima FLUOstar microplate reader (BMG LABTECH, Ortenberg, Germany). The obtained values were normalized to those of the maximum LDH released (completely lysed).

2.4. ATP levels determination

Cellular ATP levels were measured using Cell Titer-Glo® Luminescent cell viability assay kit (G7570, Promega) according to the manufacturer's instructions. Briefly, cells were plated on 96-well white-plates. After treatment, 100 μl of CellTiter-Glo® reagent was added to each well. The contents were mixed for 2 min to induce cellular lysis followed by incubation at RT for 10 min to stabilize the signal. Then, the luminescence derived from Luciferase reaction was immediately recorded. Measurements were performed using an Optima FLUOstar microplate reader.

2.5. ROS generation

2.5.1. Total ROS levels

This assay was carried out according to the protocol previously described by Gara et al., (Gara et al., 2012) with slight modifications. Intracellular ROS generation was measured by the oxidation of the reagent 2′, 7′- dichlorodihydrofluorescein diacetate (DCFH-DA) to the fluorescent compound 2′,7′-dichlorofluorescein (DCF). αS tranfected cells were loaded with 15 μM DCFH-DA and incubated for 30 min at 37 °C. Next, medium was removed, placed into a black 96 well plate and fluorescence was recorded. The resulting cells were incubated with hypotonic solution (KCl 7.5 mM, pH = 7) for 1 h at 37 °C. Lysate was homogenized and centrifuged at 12800 × *g*, 15 min. The supernatant was placed into a black well plate and fluorescence was recorded. The obtained data, corresponding to the sum of the measurements, was relativized to total protein amount determined by Bradford assay (Bradford, 1976). Fluorescence was measured at λ_{ex} = 480 nm and λ_{em} = 530 nm by using Optima FLUOstar microplate reader.

2.5.2. Mitochondrial ROS levels

MitoSOX Red, a cationic derivative of dihydroethidium (DHE), is a fluorogenic dye employed for selective detection of mitochondrial $O_2^{\bullet-}$ in live cells. MitoSOX Red reagent is live-cell permeant and is rapidly and selectively targeted to the mitochondria where reacts with $O_2^{\bullet-}$ to produce 2-hydroxymitoethidium, which excites and emits at 510 and 580 nm, respectively. Despite its proposed specificity, it has been demonstrated that MitoSOX Red can also undergo unspecific reactions with other oxidants to form mito-ethidium, which overlaps the 2-hydroxymitoethidium fluorescence peak (510 nm). Then, it has been proposed another specific excitation peak at 400 nm for 2-hydroxymitoethidium (Robinson et al., 2008; Wojtala et al., 2014). Therefore, two different excitation wavelengths are used to distinguish the $O_2^{\bullet-}$. Then, cells were loaded with MitoSox Red 5 μM for 10 min at 37 °C in the dark. Fluorescence intensity of the cell lysates was measured in a FLUOstar OPTIMA fluorometer (λ_{ex}: 510 nm or 400 nm; λ_{em}: 590 nm). Values were normalized to the total amount of proteins determined by Bradford assay.

2.6. Western blotting

Western blots were performed according to the protocol described by our group (Alaimo et al., 2014). Slight modifications were performed: 1) cells were suspended in RIPA lysis buffer, 2) PVDF blotting membrane 0.45 μm (Amersham Hybond GE Healthcare Life Sciences, Germany) were used. Equal amount of proteins (60–80 μg) from each treatment were separated on 7.5–13.5% SDS-polyacrylamide gel electrophoresis (SDS PAGE) and blotted onto nitrocellulose membranes (Hybond ECL, GE Healthcare, Piscataway, NJ). The following primary and secondary antibodies were used: mouse anti-α-synuclein cat 610,786 (1:1000) (BD Biosciences, USA), mouse anti-LC3 mAb clone 5F10 (1:1000) (NanoTools, Germany), rabbit anti-MnSOD 06-984 (1:1000) (Millipore, California, USA). Mouse anti- β-Actin (C4) sc-47,778 (1:10,000), mouse anti-Cytochrome c (A-8) sc-13,156 (1:1000), rabbit IgG-HRP sc-2030 (1:1000), mouse IgG-HRP sc-2031 (1:1000) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Mouse anti-complex III subunit core 1-OxPhos (459140) (1:5000) was obtained from Molecular Probes (Invitrogen, Eugene, OR, USA) and mouse anti- Opa-1 (612607) (1:1000) and mouse anti- Drp-1 (611113) (1:400) were from BD Pharmingen (San Diego, CA, USA). Images were captured with the luminescent LAS 1000 plus Image Analyzer employing LAS 1000 pro software (Fuji, Tokyo, Japan). Quantitative changes in protein levels were evaluated employing ImageJ software (NIH).

2.7. Mitochondrial integrity and morphometric analysis

Cells overexpressing αS grown on coverslips were incubated with the cell-permeant mitochondria-specific red fluorescent probe MitoTracker Red CMXRos (75 nM in serum free media, 30 min, 37 °C). Afterwards, cells were washed twice with PBS and fixed with 4% paraformaldehyde/4% sucrose (4% PFA-S) (20 min at RT). Finally, cells were washed with PBS and mounted on glass slides. Samples were examined under a fluorescence microscope Olympus IX71 equipped with objective lens 60 × /1.43 oil (λ_{ex}: 543/20 nm; λ_{em}: 593/40 nm). Images were captured with a Hamamatsu Photonics ORCA-ER camera. Digital images were optimized for contrast and brightness using Adobe Photoshop 7.0 Software. To quantify mitochondrial morphologies, 200 cells/sample were scored and classified as cells exhibiting **tubular** mitochondria (normal, filamentous), **intermediate mitochondria** (tubular with fragmented regions), **intermediate with donuts** (tubular & fragmented morphology + ring-shaped mitochondria) and **fragmented** (cells with globular organelle), according to Alaimo et al., 2011 and Alaimo et al., 2014 (Alaimo et al., 2014; Alaimo et al., 2011), with slight modifications. Cells classified as “loss of mitochondrial membrane potential” (Δφm) referred to those showing loss of probe retention within mitochondria.

Morphometric parameters were determined by using the Mito-Morphology Macro of ImageJ software previously described (Dagda et al., 2009). MitoTracker Red CMXRos images were thresholded to optimally resolve individual mitochondria. This Macro traces mitochondrial outlines using analyze particles tool. The area/perimeter ratio was employed as an index of mitochondrial interconnectivity and inverse roundness was used as a measure of mitochondrial elongation.

2.8. Statistical analysis

Experiments were carried out at least in triplicate unless otherwise stated. Results are expressed as mean ± SEM values. Experimental comparisons between treatments were made by Student's *t*-test and one-way ANOVA, followed by Student Newman-Keuls post hoc test with statistical significance set at $p < 0.05$; $p < 0.01$ and $p < 0.001$. All analysis were carried out with GraphPad Prism 4 software (GraphPad Sofware, San Diego, CA, USA).

3. Results

3.1. α-synuclein decreases cell viability and ATP production in SH-SY5Y cells

Accumulated evidence have supported the use of αS overexpression in cells and tissues as an experimental model of PD. Models using this strategy develop inclusions of aggregated αS and/or αS-mediated neuronal loss replicating the pathological hallmarks of this disease (Visanji et al., 2016). The neuroblastoma SH-SY5Y cell line is an in vitro model widely employed in PD research. These cells are frequently chosen because of its human origin, dopaminergic (though no strictly) neuronal properties, and ease of maintenance (Xicoy et al., 2017). In the present work we used SH-SY5Y cells overexpressing wt-αS as an in vitro model of PD.

First, we prompted us to perform studies aiming to determine the cytotoxic effect of over-expressing αS in human SH-SY5Y cells. Employing LDH release assay as a parameter of toxicity we observed a significant increase in cell death after αS transfection (31 ± 7%, $p < 0.01$) (Fig. 1A). Under these conditions αS aggregates were generated (Fig. S1). Accordingly, we found that intracellular levels of ATP were decreased indicating the occurrence of mitochondrial damage (49 ± 5%; $p < 0.01$) (Fig. 1B).

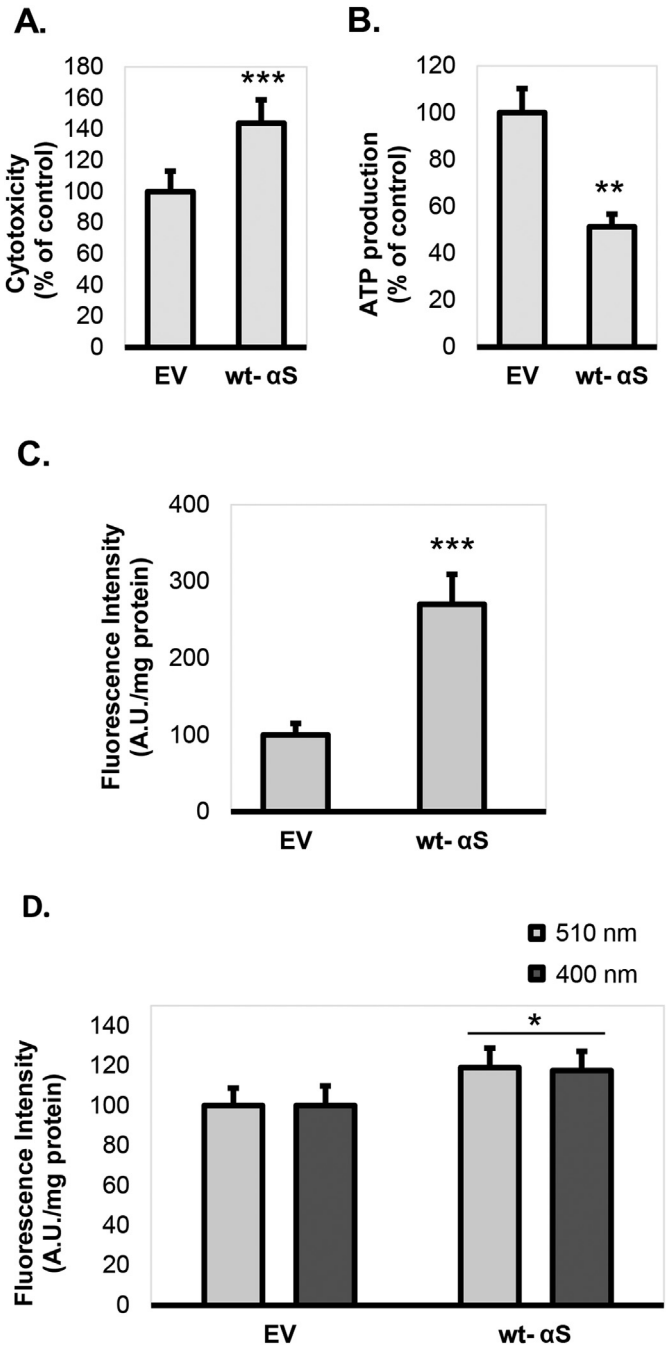

Fig. 1. αS induces cytotoxicity and increases ROS generation. SH-SY5Y cells were transfected with pcDNA3 empty vector (EV) or pcDNA3-αS wild type (wt- αS). 24 h post-transfection cytotoxicity was measured by LDH assay (A) and ATP production (B) was measured using Cell Titer-Glo Luminescent Kit G7890. Cytotoxicity was calculated as: % of cytotoxicity = 100 x (experimental values – medium background/ maximal LDH release – medium background). C. Total ROS: cells were loaded with DCDHF-DA (15 μM) and fluorescence intensity was measured by spectrofluorometry. λ_{em}: 480 nm, λ_{ex}: 530 nm. (D) Mitochondrial ROS: cells were loaded with MitoSOX (5 μM) and fluorescence intensity was measured by spectrofluorometry. λ_{ex}: 400 or 510 nm, λ_{em}:590 nm. Data were expressed as percentage versus control (100%). Results are average ± SEM of three individual experiments. ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$ vs control.

3.2. α-synuclein increases ROS production

Mitochondrial dysfunction often causes an increase in ROS generation which, in turn, contributes to an overall cellular oxidative damage. These events were described in different experimental models of PD (Esteves et al., 2010). Thus, we next analyzed the effects of αS overexpression on ROS production. Cells overexpressing αS exhibited increased levels of both reactive peroxides (170 ± 39%, $p < 0.001$) and $O_2^{\bullet-}$ anion (17 ± 3%, $p < 0.05$) measured with DCFH-DA and MitoSOX Red respectively (Fig. 1 C–D). Particularly, we quantified similar MitoSOX Red values for the both λex 510 and λex 400 nm indicating that $O_2^{\bullet-}$ was specifically detected under our conditions. Altogether, our results provide evidence about the occurrence of oxidative stress in our experimental model.

3.3. α-synuclein induces mitochondrial fragmentation and Δφm collapse

Impairment of normal mitochondrial dynamics has been linked to PD. Although previous studies have shown that αS affects the mitochondrial dynamics in cellular and animal models of PD, its role in the fusion/fission balance is not clear yet (Xie and Chung, 2012; Nakamura et al., 2011). In order to determine whether αS impacts on mitochondrial morphology in our model, we evaluated this parameter in αS transfected cells employing MitoTracker Red CMXRos staining. The morphological analysis was carried out according to the classification described in Section 2.7 (Fig. 2A). When different mitochondrial morphologies were scored (Fig. 2C) we found that in control cells (EV) < 10 ± 2% of the cells presented mitochondrial fragmentation. αS reduced the number of cells with tubular mitochondria (67.6 ± 4.6%, $p < 0.001$) while increased of cells with fragmented mitochondria (469 ± 61%, $p < 0.001$). On the other hand, no significant differences among the number of cells presenting intermediate or intermediate with donuts mitochondria were observed. Finally, αS increased the number of cells exhibiting Δφm collapse (392 ± 47%, $p < 0.001$). To deepen this analysis, additional parameters such us elongation, circularity and interconnectivity were calculated (Koch et al., 2015; Xilouri et al., 2016). Elongation, a parameter related with filamentous mitochondria, decreased in αS transfected cells while circularity, associated with fragmented and donut shape mitochondria, was increased. Interconnectivity is related with the network profile of this organelle. Thus, high values of interconnectivity suggest that mitochondria are connected while low values indicate the occurrence of fragmented mitochondria. Results obtained showed that interconnectivity is diminished in cells overexpressing αS. These data support the fact that αS increases the number of cells exhibiting fragmented mitochondria (Table 1). Together, these results demonstrate that αS impairs balance between fusion and fission promoting the mitochondrial network fragmentation and loss of Δφm.

3.4. Alterations in mitochondrial morphology are associated with $O_2^{\bullet-}$ generation

Next, we evaluated mitochondrial morphology and its relationship with $O_2^{\bullet-}$ generation by fluorescence microscopy employing MitoSOX Red. Under increased $O_2^{\bullet-}$ generation conditions, associated with overexpressed αS or rotenone treatment (positive control), mitochondria acquiring mainly donut and fragmented morphologies were observed (Fig. S2). These findings link changes in mitochondrial shape to ROS generation in αS transfected cells.

3.5. α-synuclein induces changes in the expression levels of Opa-1 and Drp-1

It has been widely described that alterations in mitochondrial morphology, regulated by mitochondrial fusion and fission proteins, may be involved in the pathophysiology of neurodegenerative conditions (Zorzano and Claret, 2015). Thus, we prompted us to analyze the expression levels of mitochondrial fusion (Opa-1) and fission (Drp-1) proteins which are critical in the maintenance of fission/fusion equilibrium (Campello and Scorrano, 2010; Pernas and Scorrano, 2016). Immunoblot analysis of cytosolic and mitochondrial fractions revealed the presence of large isoforms of Opa-1 (100 and 94 kDa) according to

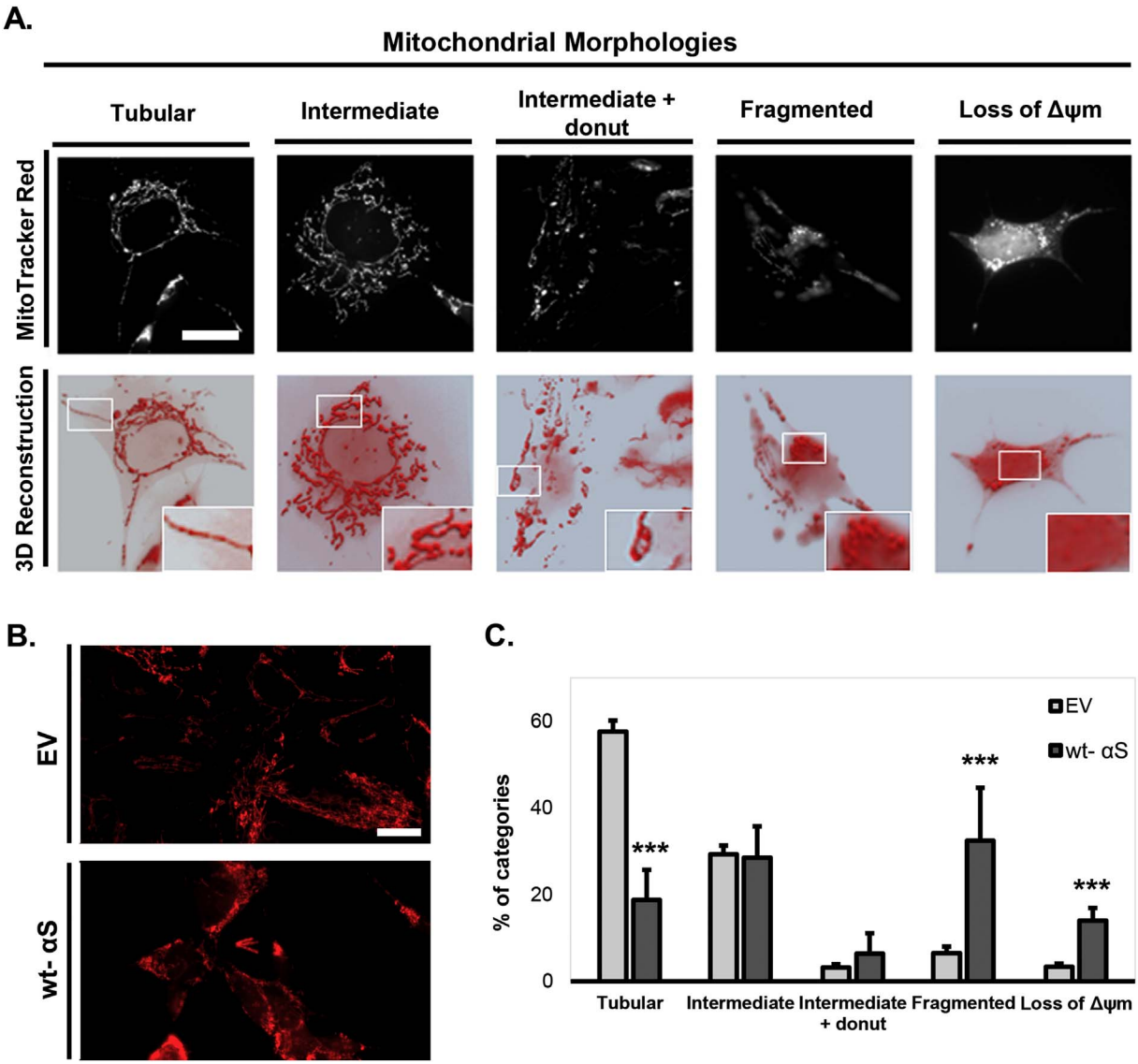

Fig. 2. αS alters mitochondrial morphology and Δφm. A. Classification of cellular categories based on the mitochondrial morphology. Mitochondria were visualized with MitoTracker Red CMXRos (75 nM) by fluorescence microscopy λ_{ex}: 543/20 nm; λ_{em}: 593/40 nm. Representative images were acquired and 3D reconstructions obtained with Huygens Deconvolution Professional Software. Five categories of cells exhibiting different mitochondrial morphology were scored: tubular mitochondria (normal), intermediate mitochondria (filamentous with swelling regions), Intermediate plus donuts, fragmented and "loss of Δφm". The last classification refers to cells exhibiting nuclear condensation and Δφm collapse. The inset represents a magnification of 2×. B. Representative images for Mitotracker Red staining in EV or wt- αS transfected cells C. Quantification of cellular categories: 200 cells/ sample were scored. Results represent average ± SEM of two independent experiments. Statistical significance,***p < 0.001 vs control. Scale bar: 10 μm.

Table 1
Mitochondrial morphology parameters. 100 cells were scored for EV and αS respectively. Results are expressed as average ± SEM. ***p < 0.001, **p < 0.01 αS vs. control.

Treatment	Elongation (1/circularity)	Interconnectivity (μm)
EV	40.22 ± 7.57	1.79 ± 0.91
αS	29.25 ± 2.95***	0.57 ± 0.12**

Alaimo et al. (Alaimo et al., 2014). These bands presented no differences in their expression levels between control and αS transfected cells. Moreover, in the mitochondrial fractions we found cleaved forms of Opa-1 with estimated MW of 70 and 40 kDa. αS decreased their intensity 6-folds (p < 0.001) and 71 ± 7% (p < 0.01) respectively. Interestingly, αS overexpression also induced the appearance of a lower MW (~35 kDa) Opa-1 isoform (Fig. 3A). Despite the presence of Opa-1 cleavage products, we were unable to detect the release of this protein into the cytoplasm. Accordingly, cytochrome c did not translocate to the cytosol (Fig. 3C). On the other hand, Drp-1 exhibited an 83 kDa band in accordance with the previously established MW for brain isoform (Alaimo et al., 2014; Park et al., 2011). αS overexpression induced an increase of Drp-1 levels in the cytosolic (96 ± 14%, p < 0.01) and mitochondrial (30 ± 2,8% p < 0.01) fractions. These results point to Opa-1 and Drp-1 as possible players of mitochondrial fragmentation triggered by αS.

3.6. Opa-1 protects against α-synuclein- induced mitochondrial disruption and cytotoxicity

To assess the role of Opa-1 in both the αS-induced mitochondrial dysfunction and cytotoxicity we analyzed the effect of transfecting SH-SY5Y cells with either wt or Q297V Opa-1 cDNAs on mitochondrial morphology and cell viability. Opa-1^{Q297V} mutant, characterized by lacking of GTPase activity (Yamaguchi et al., 2008), mimics the GTP-bound form of Opa-1, thereby preventing the disassembly of the mitochondrial cristae complexes structure (Alaimo et al., 2014; Satoh et al., 2003). Employing Mitotracker Red CMXRos staining we first

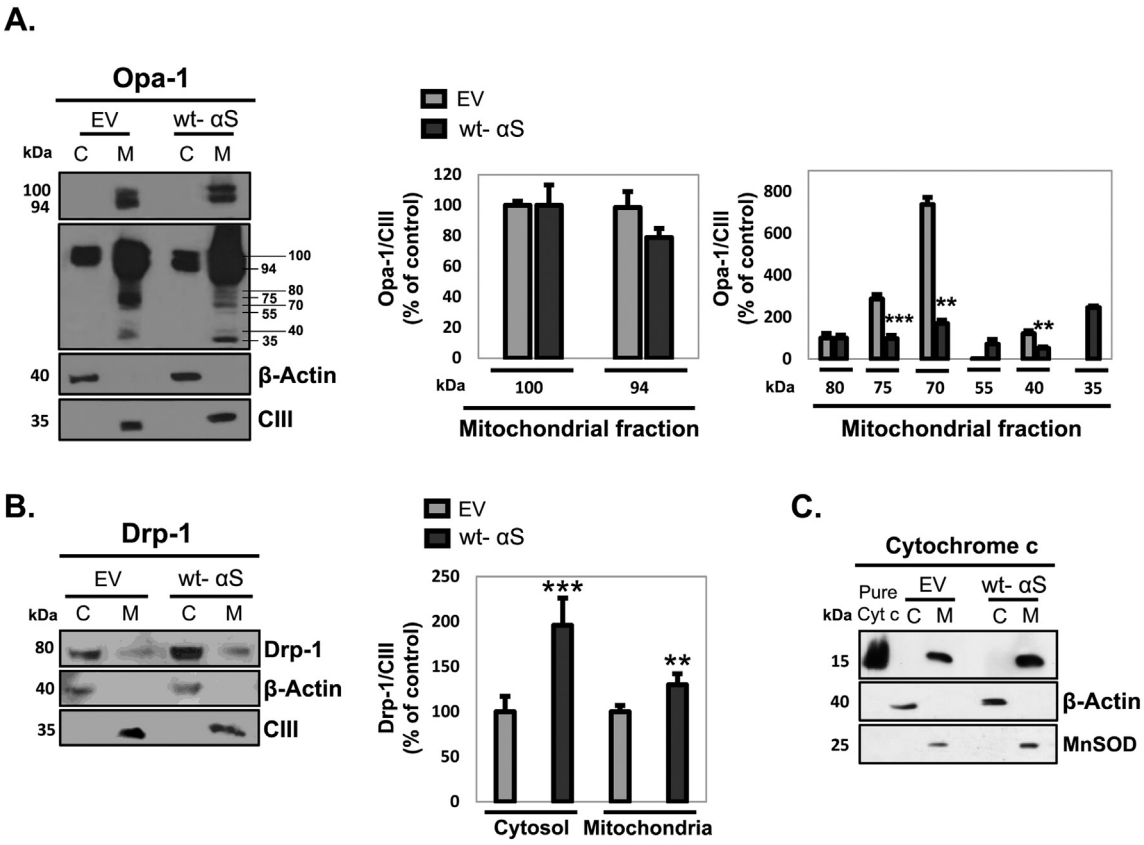

Fig. 3. Expression and subcellular localization of mitochondrial fusion/fission proteins. A. Representative immunoblot of Opa-1 levels. Large isoforms of Opa-1 (100 and 94 KDa) and shorts isoforms (70, 40 and 35 kDa) were revealed after 30s and 10 min of exposition respectively. B. Representative immunoblots of Drp-1 levels. C. Immunoblot against cytochrome C. Reprobing with an anti-β-Actin (for cytosolic fraction) and Complex III subunit core 1-OxPhos (for enriched mitochondrial fraction) antibodies was performed to normalize for protein loading. Signals were quantified with the FIJI (Image J) software. Images correspond to one representative experiment (n = 3). Results are expressed as a percentage of the respective control considered as 100%. C = cytosolic fraction, M = mitochondrial fraction, a: purified cytochrome c used as a standard; $^{**}p < 0.01$, $^{***}p < 0.001$.

analyzed the mitochondrial network morphology and Δφm dissipation (Fig. 4A). Images revealed that Opa-1^{wt} and Opa-1^{Q297V} rescued the tubular phenotype of mitochondria disrupted by αS (Fig. 4A). These results were confirmed by quantification of the mitochondrial categories (Fig. 4B). However, Opa-1 over-expression had no effect on Δφm dissipation. Considering that increased fragmentation implies a risk of neuronal death (Nakamura et al., 2011) we next investigated the effect of Opa-1 overexpression on cell viability (Fig. 4C). Results showed that Opa-1^{wt} (αS-Opa-1^{wt} 70 ± 10 vs αS 120 ± 14% $p < 0.001$) but not Opa-1^{Q297V} protected against αS-induced cytotoxicity. These results suggest that both αS-induced mitochondrial abnormalities and cell damage are modulated by Opa-1.

3.7. Drp-1 is required by αS- induced mitochondrial fragmentation

To ascertain whether αS -induced mitochondrial fragmentation is mediated by components of the mitochondrial fission machinery we performed experiments employing the mitochondrial division inhibitor-1 (Mdivi-1). Mdivi-1 specifically inhibits Drp-1 attenuating the early stages of fission, which involve the self-assembly of Drp-1 molecules (Cassidy-Stone et al., 2008). Thus, employing Mitotracker Red CMXRos and fluorescence microscopy we observed that incubation with Mdivi-1 (10 μM) prevent αS-induced mitochondrial network fragmentation (31.02 ± 2.2%, $p < 0.05$), as well as the loss of Δφm (6.95 ± 0.53%, $p < 0.05$) (Fig. 5).

3.8. Autophagy protects SH-SY5HY cells against αS-induced damage

Accumulated evidence in cellular and animal models of PD have linked αS accumulation to alterations in the autophagy lysosomal degradation pathway (Xilouri et al., 2016). To study the autophagic response we evaluated the conversion of LC3-I to LC3-II by immunoblotting (Klionsky et al., 2012). We found that αS increased (1.8 ± 0.11, fold, $p < 0.001$) LC3-II levels. Moreover, pre-incubation with Bafilomycin A1 (Baf A1), an inhibitor of vacuolar-type H^+-ATPase which blocks the acidic degradation step in autophagy, resulted in a more pronounced increment in LC3-II levels (4.5 ± 0.04 fold, $p < 0.001$). These results indicate that αS increases the autophagic flux (Fig. 6A).

In order to determine a possible role for autophagy in the removal of αS-injured mitochondria, we studied the occurrence of mitophagy by immunocytochemistry. The degree of co-localization between LC3 and TOM20 (a central component of the TOM, translocase of outer membrane receptor complex) was evaluated by confocal microscopy (Fig. 6B). Mander's analysis revealed that αS increases colocalization between LC3 and TOM20 (M1 = 0.73 ± 0.05, $p < 0.01$) suggesting that damaged mitochondria are being removed by mitophagy (Manders et al., 1993).

The proper elimination of dysfunctional mitochondria is essential for cellular survival (Ashrafi and Schwarz, 2013). Thus, we next investigated whether autophagy activation was cytoprotective. For this purpose, we inhibited the autophagic process interfering with Atg5-Atg12 conjugation. Cells were transfected with plasmids containing or Atg5^{wt} or Atg5^{K130R} and cell viability was assessed through LDH release assay. Results showed that autophagy inhibition increased αS cytotoxicity (36 ± 6%, $p < 0.01$). Surprisingly, Atg5^{wt} overexpression was cytoprotective (21 ± 3%, $p < 0.01$), probably by stimulating the autophagic process (Fig. 6D). Overall, our results demonstrate

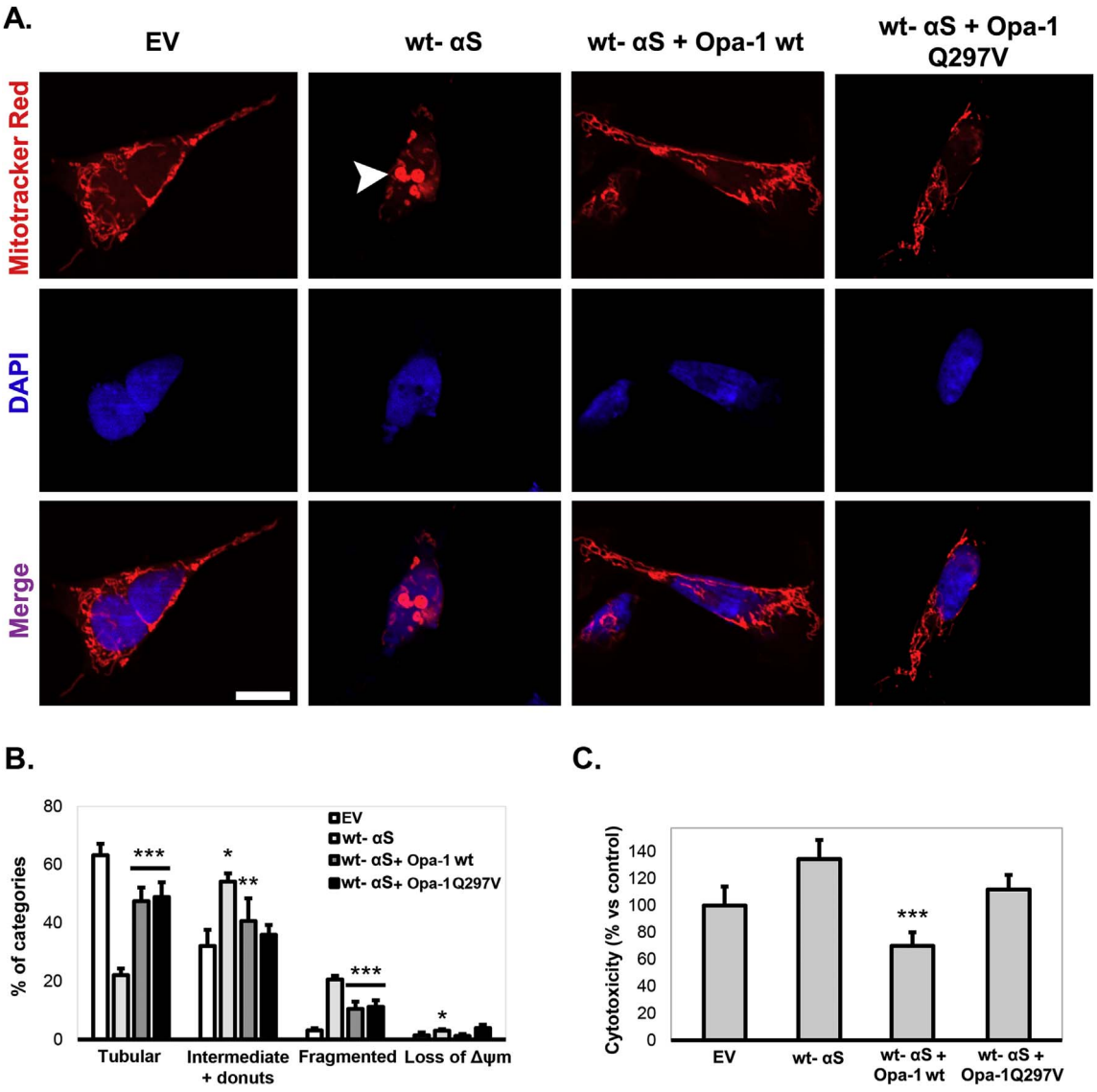

Fig. 4. Opa-1 protects against αS- induced mitochondrial disruption and cytotoxicity SH-SY5Y cells were transiently transfected with empty pcDNA3 vector (EV, control) or vectors encoding wt or Q297V Opa-1 and wt- αS. A. Confocal images of transfected SH-SY5Y cells labeled with MitoTracker Red CMXRos and DAPI. Images were acquired with an objective PLAPON W 60×, NA = 1.42. Scale bar = 10 μm B. Quantification of cells based on the categories described above for mitochondrial morphology. C. Cytotoxicity was measured by LDH assay and calculated as described in Fig.1. Results are average ± SEM of three individual experiments. Statistical significance *p < 0.05, **p < 0.01, ***p < 0.001 vs. control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

autophagy is activated as a response to counteract αS-induced cellular damage.

4. Discussion

Several observations demonstrate a central role for the protein αS in both sporadic as well as familial forms of PD. Furthermore, αS gene duplication/triplication causes a severe, highly penetrating form of PD (Spillantini et al., 1997), suggesting a dose-dependent pathogenic role for the overexpressed wild type protein. Thus, it has been proposed that the accumulation of αS in sporadic PD is the primary pathogenic event (Nakamura et al., 2011; Kamp et al., 2010). Accumulating evidence have shown that aggregation of αS is necessary for its pathogenicity. In model systems employing both wild type and mutant αS overexpression, the aggregation and deposition of this protein precedes neuronal cell death. Furthermore, strategies focused on reducing the aggregative process were shown to decrease neurodegeneration (Michel et al., 2016). Particularly, both differentiated and undifferentiated SH-SY5Y cells are sensitive to extracellular aggregated αS-induced toxicity (Emadi et al., 2009). In the present study, we observed that wt-αS overexpression in SH-SY5Y cells results in a decrease of 31% and 49% in cell viability and ATP production respectively (Fig. 1A and B). Previous results employing SH-SY5Y cells transfected with wt- αS have also reported a middle (34%, (Emadi et al., 2009)) or low (17%, (Emmanouilidou et al., 2010)) values of cytotoxicity suggesting a moderate effect of this protein in cell physiology and functionality.

Mitochondrial dysfunction is known to be a major player in PD. Accumulation of wt- αS in the mitochondria of human dopaminergic neurons reduced mitochondrial complex I activity and increased ROS production (Devi et al., 2008). These events ultimately result in Δφm collapse and decreased OXPHOS capacity. Previous results (Martinez et al., in preparation) have described the Δφm dissipation in isolated mitochondria of SH-SY5Y cells incubated with 10 μM αS. Accordingly, we now demonstrated that αS-transfected cells exhibit Δφm dissipation (Fig. 2C) as well as decreased ATP production (Fig. 1B). While there is considerable consensus about αS reducing Δφm, there is a controversial report from Nakamura et al. (Nakamura et al., 2011). These authors have shown that Δφm does not decline even at the highest levels of αS

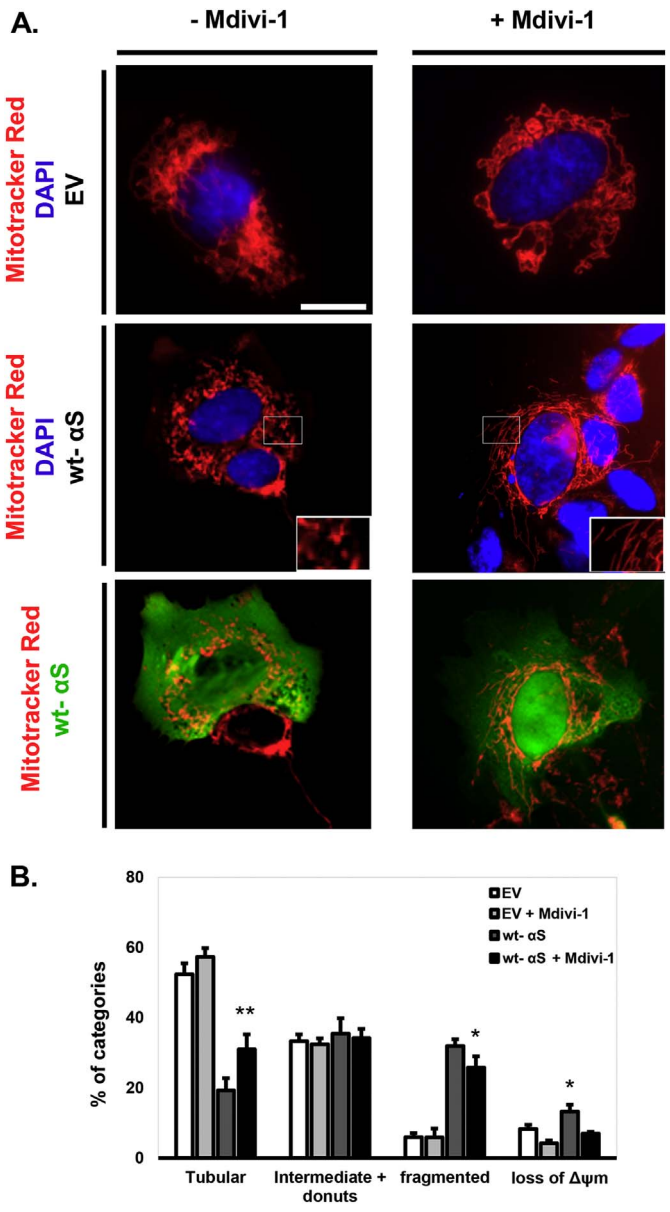

Fig. 5. Mdivi-1 inhibits αS- induced fragmentation. A. SH-SY5Y cells were transfected with EV or wt- αS-GFP and incubated with 10 μM Mdivi-1. Confocal Images were acquired with a fluorescence microscope Olympus IX71 with an objective PLAPON O 60×, NA = 1,42. Scale bar = 10 μm. B. Quantification of cells based on the categories described above for mitochondrial morphology. Results were expressed as % of categories ± SEM. Statistical significance *$p < 0.05$ and **$p < 0.01$ vs. control.

expression. We hypothesized that the differences in their results are attributable to the employed cell line (HeLa cells). In accordance with this proposal, they showed that αS has no effect on $O_2^{\bullet-}$ generation while we have demonstrated an increase of 17% in this parameter (Fig. 1D).

Mitochondrial dynamics is a key process regulating mitochondrial function and quality. It refers not only to changes in mitochondrial shape, but also to their movement along the cytoskeleton. Mitochondrial dynamics is tightly regulated by mitochondrial fusion and fission events (Chan, 2012). It has been reported that increased αS expression in cultured cells results in αS binding to mitochondria and mitochondrial fragmentation whereas αS silencing leads to elongated organelles (Kamp et al., 2010). However, while a number of key mitochondria-shaping proteins have been identified, the precise mechanisms affecting mitochondrial morphology in neuronal cells remain elusive. In this work we investigated the role of αS in mitochondrial dynamics and its impact in the cell fate.

In the first set of experiments, changes in both mitochondrial morphology and fusion/fission protein levels in αS transfected SH-SY5Y cells were analyzed. We observed that αS increases mitochondrial fragmentation (Fig. 2B–C) in accordance with Kamp et al. (Kamp et al., 2010). Opa-1 is a dynamin-related GTPase residing in the inner mitochondrial membrane that participates in both the regulation of mitochondrial fusion and the pro-apoptotic remodeling of mitochondria (Frezza et al., 2006). Western blot analysis showed the presence of large (100 and 94 kDa) and cleaved (~35-80 kDa) Opa-1 isoforms (Fig. 3A). Cleaved isoforms were localized only in the enriched mitochondrial fraction of αS transfected cells. In this regard, it has been suggested that the number and MW of bands detected by western blot analysis is dependent of tissue and specie under study (Olichon et al., 2002). We were not able to detect the release of the Opa-1 isoforms from the mitochondria to the cytosol in αS transfected cells as was previously reported by our group (Alaimo et al., 2014). Similarly, cytochrome c was not released to the cytosol (Fig. 3C). However, it is noteworthy that no agreement has been reached yet on whether the permeabilization event precedes or not the Opa-1 cleavage (Ramonet et al., 2013).

Taken together these results suggest that although αS induces Δφm dissipation, mitochondrial membrane permeabilization could not be taking place in our experimental conditions. Anyway, the occurrence of Opa-1 cleavage would explain the increased mitochondrial fragmentation observed. Our findings suggest that despite αS affects the fusion/fission balance; this mitochondrial dysfunction represents an early stage, perhaps prior to the plasmatic membrane permeabilization and the execution of the potential apoptotic process.

In order to establish the role of Opa-1 in fusion/fission balance and cell viability, SH-SY5Y cells were transfected with wt and Q297V Opa-1 plasmids. According to previous reports Q297V Opa-1 would contribute to the prevention of the dismantling of the mitochondrial cristae structure stabilized by Opa-1 oligomers allowing to assess the cristae structural defects (Alaimo et al., 2014; Yamaguchi et al., 2008; Maltecca et al., 2012). We showed that Opa-1^{wt} transfection prevents both mitochondrial fragmentation and cytotoxicity while it has no effect on Δφm dissipation (Fig. 4A, B). These results confirm the involvement of Opa-1 in mitochondrial fusion in αS transfected SH-SY5Y cells. The fact that Opa-1^{Q297V} mutant prevents mitochondrial fragmentation but not cell death suggests that the magnitude of Opa-1 complexes disassembly induced by αS, if occurs could be irrelevant for cell survival. This hypothesis is supported by the fact that no release of cytochrome c was observed in αS-transfected cells. This situation could be possible under no severe cytotoxic conditions as occurs in our model. On the other hand, the relevance of cristae tightness in both cell death and neurodegeneration remains to be elucidated (Pernas and Scorrano, 2016).

The dynamin-related protein 1 (Drp-1), identified as the master regulator of mitochondrial fragmentation, is a typical cytosolic protein. During fission, Drp-1 translocates to mitochondria where it constricts and cleaves this organelle (Ni et al., 2015; Corrado et al., 2012). Western blot analysis revealed that αS increased Drp-1 levels in the enriched mitochondrial fraction suggesting a role for this protein in our model (Fig. 3B). Interestingly, also the cytosolic levels of Drp-1 were augmented. This result suggests that αS, besides inducing the Drp-1 translocation to the mitochondria, increases the expression levels of Drp-1. Mitochondrial morphology analysis employing Mdivi-1, a small specific inhibitor of Drp-1, indicated the preservation of mitochondrial network (Fig. 5). These data revealed the requirement of Drp-1 for αS-induced mitochondrial fission. Altogether, these results demonstrate that αS induces an imbalance in the fusion/fission mitochondrial equilibrium resulting in a mitochondrial fragmentation. Our findings are in accordance with previous evidence demonstrating Drp-1-dependent mitochondrial fragmentation in SH-SY5Y cells treated with 6-OHDA (Gomez-Lazaro et al., 2008). However, Nakamura et al. (Nakamura et al., 2011) employing HeLa cells transfected with αS

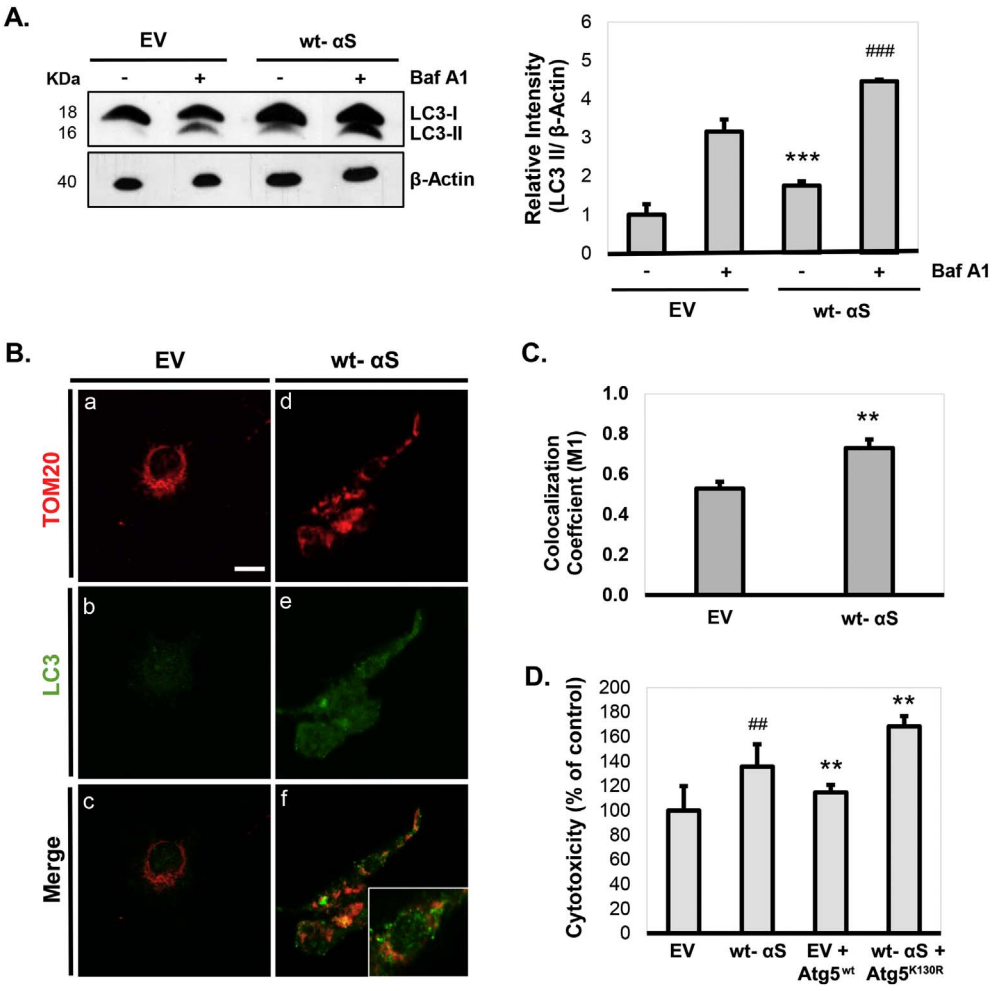

Fig. 6. Autophagy protects SH-SY5HY cells against αS-induced damage. A. Representative immunoblot showing LC3-II levels in SH-SY5Y cells control and overexpressing αS in presence or absence of Baf A1. Densitometry analysis for LC3-II ***$p < 0.001$ αS vs EV (without Baf A1); ###$p < 0.001$ αS vs EV (with Baf A1). B. Cells were transfected with EV or αS and immunocytochemistry employing TOM-20 (red; a,d) and LC3 (Green; b,d) was performed to analyze mitophagy. Both channels (red and green) were merged (c, f). C. A Mander's co-localization analyses among TOM-20 (mitochondria) and LC3 (autophagosome) was done to evaluate LC3 and mitochondria co-localization. Images were acquired with a confocal microscope, objective PLAPON O 60×, NA = 1.42. Coefficient M1 was calculated with FIJI ImageJ, plugin "Coloc2" and a Macro specially designed to obtain Mander's coefficients. Scale bar = 10 μm. D. Cells were co-transfected with EV or αS and $Atg5^{WT}$ or $Atg5^{K130R}$. The effect of the overexpression of these proteins was analyzed by LDH assay. Statistical significance **$p < 0.01$ αS + Atg5wt vs αS and ##$p < 0.01$ αS vs EV. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

found that mitochondrial fragmentation does not require Drp-1 but is involved in a direct interaction of αS with mitochondrial membranes. These controversial findings could be attributed to the usage of a non-neuronal cell line as mentioned above. In support of our results, recent evidence demonstrated the neuroprotective role of Mdivi-1 in the A53T-α-synuclein rat model of PD (Bido et al., 2017). On the other hand, Xie et al. (Xie and Chung, 2012) reported that wt- αS expression in SH-SY5Y cells did not alter mitochondrial fission and fusion proteins levels. However, these authors have studied these parameters in total lysates of SH-SY5Y cells, thus explaining the apparent difference with our results.

The fission events are relevant for autophagic clearance of dysfunctional mitochondria. It has been widely recognized that autophagy and cell death are intimately associated. However, the molecular pathways connecting these processes may be intricate. Under different conditions, autophagy may promote or inhibit cell death (Green and Levine, 2014).

Accumulated evidence has shown that the inhibition of autophagy results in the accumulation and increased toxicity of αS whereas the activation of this process could have therapeutic effects in PD models. However, the direct effects of αS on autophagy depends on the experimental model. Besides, the role of defective autophagy in neurodegeneration remain poorly understood (Xilouri et al., 2016; Spencer et al., 2009; Friedman et al., 2012; Xilouri et al., 2009).

We therefore evaluated the occurrence and impact of autophagy on αS-transfected SH-SY5Y cells. In accordance with Choubey et al. (Choubey et al., 2011) we found that αS increased the LC3-II expression levels in both the presence and absence of Baf-A1 indicating an active autophagic flux (Fig. 6A). Moreover, this increased autophagy seems to offer an advantage in our model by preventing cell death (Fig. 6C). While the damaging effect of αS on lysosomal integrity has been documented (Mazzulli et al., 2016), our results show that even if lysosomes in this model are damaged, their activity is still enough to complete the autophagic process. Moreover, as we previously proposed in a Parkinsonism model (Gorojod et al., 2015), only a pool of lysosomes may be affected, while the population of healthy ones would be able to complete the autophagic degradation process. It is probably that a sustained αS overexpression will induce a stronger lysosomal dysfunction and prevent the proper autophagic clearance.

Mitophagy is a specialized form of autophagy designed to remove and dispose dysfunctional mitochondria. Mitochondrial depolarization and fission, both observed in the present report, have been proposed as a requisite for mitophagy execution (MacVicar, 2013; Twig and Shirihai, 2011). In accordance with this evidence, we demonstrated the occurrence of mitophagy in our experimental model (Fig. 6B). There are scarce and discrepant information about both the involvement and role of autophagy/mitophagy in dopaminergic neurons exposed to αS (Koch et al., 2015; Xilouri et al., 2016). The end result depends on the particular αS levels, species, conformations, and cellular milieu (Xilouri et al., 2016).

In summary, our findings demonstrate for the first time that a deregulation in Opa-1 and Drp-1 expression levels in favor of mitochondrial fragmentation is implicated in αS toxicity in SH-SY5Y cells. In addition, αS overexpression triggers the autophagic/mitophagic machinery in order to exert cellular protection. We propose that these molecular pathways are activated in SH-SY5Y cells expressing αS levels which result in low or middle toxicity. Under conditions of excessive αS concentration, dysfunctional inhibition of autophagy/mitophagy is

prone to occur. Our results suggest the relevance of autophagy and mitochondrial homeostasis in the pathogenesis of PD. Better understanding of the molecular interaction between these processes could give rise to novel therapeutic methods for PD prevention and amelioration.

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Declarations of interest

None.

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