Video Article

Exogenous Administration of Microsomes-associated Alpha-synuclein Aggregates to Primary Neurons As a Powerful Cell Model of Fibrils Formation

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Abstract

For years, the inability of replicating formation of insoluble alpha-synuclein (αS) inclusions in cell cultures has been a great limitation in the study of αS aggregation in Parkinson's Disease (PD). Recently, the development of new animal models through the exogenous inoculation of brain extracts from diseased αS transgenic mice or PD patients has given new hopes to the possibility of creating more adequate cell models of αS aggregation. Unfortunately, when it comes to cells in cultures, administration of raw brain extracts has not proven as successful as in mice and the source of choice of exogenous aggregates is still *in vitro* preformed αS fibrils.

We have developed a method to induce the formation of intracellular αS inclusions in primary neurons through the exogenous administration of native microsomes-associated αS aggregates, a highly toxic αS species isolated from diseased areas of transgenic mice. This fraction of αS aggregates that is associated with the microsomes vesicles, is efficiently internalized and induces the formation of intracellular inclusions positive for aggregated and phosphorylated αS. Compared to *in vitro*-preformed fibrils which are made from recombinant αS, our method is faster and guarantees that the pathogenic seeding is made with authentic αS aggregates extracted from diseased animal models of PD, mimicking more closely the type of inclusions obtained *in vivo*. As a result, availability of tissues rich in αS inclusions is mandatory.

We believe that this method will provide a versatile cell-based model to study the microscopic aspects of αS aggregation and the related cellular pathophysiology *in vivo* and will be a starting point for the creation of more accurate and sophisticated cell paradigm of PD.

Video Link

The video component of this article can be found at https://www.jove.com/video/57884/

Introduction

Accumulation of alpha-synuclein (αS) proteinaceous inclusions is a prominent and important feature of Parkinson's Disease (PD) and alpha-synulceinopathies[1]. Unfortunately, while animal models are able to provide a sufficient cellular and biochemical environment to induce the aggregation steps necessary for the formation of protein fibrils[2], replicating formation of complex Lewy Body (LB)-like aggregates in cell cultures is difficult and challenging.

Here we describe a method to induce the formation of αS inclusions, similar to protein aggregates obtained in animal models and PD patients, in cultured cells, using brain isolated mouse primary neurons. Our protocol is based on the exogenous administration of microsomes-associated αS aggregates isolated from αS symptomatic transgenic (Tg) mice to mouse hippocampal or cortical primary neurons. This method takes advantage of the spreading and propagation ability of αS toxic species which, once added to the culture medium, are able to become internalized, and induce the formation of mature αS-positive aggregates[3,4,5,6,7,8].

Originally, standard methods to obtain formation of αS fibrils in cell cultures were based on the overexpression of the corresponding αS cDNA through the regular transfection protocols or viral-mediated infection[9]. While in the first case obtaining LB-like αS aggregates were fortuitous, showed low efficiency, and depended on the cell type, the second protocol led to the formation of insoluble fibrils, including high molecular weight (HMW) species in 24 - 48 h from infection[10]. In these methods, the formation of aggregates was probably due to an excessive and unbalanced in αS protein amount that becomes insoluble rather than a pathological conversion of the αS conformation that dictates aggregation. Instead, the technique that we are presenting here does not alter the αS expression level but induces widespread protein aggregation due to the internalization of exogenous fibrils. Moreover, the formation of αS aggregates through the administration of exogenous fibrils is a lengthy process that requires days or weeks to become exhaustive allowing us to study early and intermediate stages of αS inclusions formation in a time-lapse fashion and to correlate it with the cellular biochemical changes. Thus, our method is a valuable application to create cellular models of αS aggregation that are helpful to study αS fibril formation microscopically in relation to cellular pathophysiology.

In addition although administration of raw brain extracts from diseased αS Transgenic (Tg) mice[11,12] or human PD brains[6,13] is able to induce αS deposition in Tg or wild-type (WT) animals, application of the same procedure to cell cultures has not proven to be as successful, possibly because of the low amount of aggregates in the samples used and the lack of a standard procedure to isolate native αS toxic species[14]. Because of this, *in vitro* preformed fibrils (PFFs) of αS have been the aggregates source of choice until now for the induction of αS inclusions in cells and animal models[3,4,6,7,15,16]. With our protocol, however, we show that microsomes-associated αS aggregated species isolated from αS Tg mice can efficiently induce accumulation of intracellular LB-like αS inclusions in primary neurons.

In our lab, microsomes-associated αS aggregated species are isolated from the spinal cord (SpC) tissue of the diseased Tg mice expressing human A53T αS gene under the control of the mouse prion protein (PrP) promoter [Prp Human A53T αS Tg mice, Line G2-3[17]]. These mice show an age-dependent neurodegenerative phenotype that includes robust motor dysfunction and formation of inclusions in the central nervous system made of phosphorylated, ubiquitinated, and insoluble αS, starting after 9 months of age. Once motor dysfunction appears, the phenotype rapidly evolves into paralysis, starting from posterior limbs, that leads to the death in 2 - 3 weeks. Accumulation of αS aggregates parallels disease manifestation. Mice sacrificed at the onset of the motor dysfunction show a robust degree of αS aggregation in the SpC, brain stem and cerebellum. There is no need to wait until paralysis sets to sacrifice the mouse. Presymptomatic mice are taken at 9 months-old animals that do not display motor dysfunction.

Protocol

The use of WT and Tg animals was approved and complied in full by the national and international laws for laboratory animal welfare and experimentation (EEC council directive 86/609, 12 December 1987 and Directive 2010/63/EU, 22 September 2010). All the protocols described in this paper follow the animal care guidelines of our institution.

1. Isolation of Microsomes-associated αS Aggregates from Diseased A53T αS Tg Mice

1. Prepare the homogenization buffer which is composed of 250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 2 mM EDTA, and 1x phosphatase/protease-inhibitors. Keep the buffer on ice.
2. Homogenize the fresh or frozen tissue in a 1:10 (w/v) volume of ice-cold homogenization buffer using a Teflon pestle homogenizer, with 10 - 15 strokes.
3. Transfer initial homogenate (1 - 2 mL) to a microcentrifuge tube and centrifuge at 1,000 × g for 10 min at 4 °C using a refrigerated centrifuge in order to remove nuclei and unbroken cells in the resulting pellet (P1). Discard P1.
4. Transfer the supernatant (S1) to a clean microcentrifuge tube and centrifuge S1 at 10,000 × g for 20 min at 4 °C using a refrigerated centrifuge in order to obtain the second supernatant (S10) and the pellet (P10).
   NOTE: P10 is a crude membrane pellet that contains mitochondria and synaptosomes. Discard P10.
5. Transfer the supernatant (S10) to a polycarbonate bottle (>1 mL) and centrifuge S10 at 100,000 × g, for 1 h at 4 °C using an ultracentrifuge and a fixed angle rotor (90 Ti).
   NOTE: The supernatant is the pure cytosol fraction while the pellet, P100, contains microsomes-associated αS aggregates.
6. Resuspend the P100 pellet with 500 μL of the homogenization buffer.Transfer P100 to a clean microcentrifuge tube and centrifuge at 10,000 × g for 20 min at 4 °C in a refrigerated centrifuge.
7. Discard the supernatant and resuspend P100 with 100 μL of homogenization buffer.
   NOTE: This fraction is the microsomes-associated αS aggregates.
8. Sonicate samples for 2 s on ice [set output power 1 watt (RMS)]. Store samples at -80 °C.
9. The day after, determine the protein amount using BCA analysis.

2. Western Blot

Note: Biochemical characterization of the microsomes-associated αS aggregates is evaluated by Western Blot.

1. Cast a gradient 4 - 20% Tris-glycine polyacrylamide gel on a vertical electrophoresis apparatus.
2. Load 1 μg of microsomes-associated αS fractions, dissolved in the denaturing sample buffer.
3. In a different well, load 5 μL of protein standard marker.
4. Run the gel at 100 V in a Tris/glycine/SDS running buffer until protein marker reaches the end of the gel.
5. Transfer proteins to a nitrocellulose membrane using a basic carbonate buffer (10 mM $NaCO_3$, 3 mM $NaHCO_3$, 20% Methanol) O/N at 4 °C, at 200 mA, constant.
6. Block the membrane with PBS-nonionic surfactant 0.05% (PBS-T) with 5% non-fat dry milk on an orbital shaker for 30 min at RT.
7. Briefly, rinse the membrane with PBS-T.
8. Incubate the membrane with Syn-1 (1:5,000) or pSer129-αS antibody (1:5, 000) in 2.5% non-fat dry milk in PBS-T, O/N at 4 °C on an orbital shaker.
9. Wash the membrane for 10 min at RT with PBS-T on an orbital shaker.
10. Incubate the membrane with anti-mouse or anti-rabbit HRP-conjugated secondary antibody (1:3,000) in 2.5% non-fat dry milk in PBS-T for 1 h at RT.
11. Wash the membrane for 10 min at RT with PBS-T on an orbital shaker. Repeat 3x.
12. Obtain the signal through the regular chemiluminescence kits.

3. Primary Neuronal Cultures

Note: Primary neuronal cultures were prepared from the WT newborn (P0) mouse hippocampus or cortex (Line C57BL/6). The entire procedure, minus the centrifugation steps, is carried out under a cell culture hood, in sterile conditions.

1. Treat coverslips (18 mm Ø) with 65% nitric acid solution for at least 12 h at RT.
2. Remove the nitric acid. Rinse coverslips twice with PBS 10x and several times with distilled water.
3. Insert coverslips in 24-well dishes and coat them with poly-D-lysine (0.1 mg/mL in sterile distilled water or PBS 1x) for 1 h at 37 °C.
4. Remove poly-D-lysine and wash the coverslips three times with distilled water. Store them at 4 °C until needed.
5. Euthanize the mouse pups by decapitation and separate the heads from the bodies. Place the heads on dishes and gently dissect the skin.
6. By using fine scissors, open the skull by making an incision at the bases of the brains. Separate the two halves of the skulls and remove them carefully.
7. By using forceps, pinch off the brains from the bases and isolate the cortex and the hippocampus. Transfer them into two separate dishes containing Hank's Balanced Salt Solution (HBSS) medium containing 1% penicillin/streptomycin.
8. Repeat steps 3.6 and 3.7 for each animal. Be aware that the entire process should not take more than 30 - 45 min.
9. Mince the collected tissues and transfer them into two 50 mL conical tubes (one for hippocampus and one for cortex) with 10 mL of HBSS medium containing Trypsin 0.1%. Incubate in the water bath for 7 min at 37 °C.
   NOTE: No agitation is required.
10. Add 1 mL of DMEM containing 10% fetal bovine serum (FBS) and 10 µg/mL DNase to the homogenate to block trypsin activity.
11. Centrifuge the resulting dissociated tissue at 200 × g for 5 min at RT on a centrifuge and remove the supernatant.
    NOTE: The pellet represents the cells dissected from the tissue.
12. Resuspend the cells in the plating medium, containing 2% B27, 2 mM glutamine, 6 mg/mL glucose, 10% FBS, 12.5 µM glutamate and 10 µg/mL gentamicin.
13. Plate dissociated neurons on poly-D-Lysine coverslips in 24 well plates according to the ratio: 1 cortex/12 wells and 1 hippocampus/6 wells, resulting in approximately 150,000/200,000 cells per well. Maintain the cells in the incubator at 37 °C.
14. The day after (day *in vitro*, DIV 1), replace the plating medium with the medium containing 2% B27, 10 µg/mL gentamicin and 2 mM glutamine.
15. At DIV 2, remove 1/3 of medium and add 1/3 of the fresh medium containing 2.5 µM cytosine arabinoside for 48 h to reduce the glial contamination.
16. Maintain neurons at 37 °C and replace half of the medium every three days.

4. Neurons Treatment

Note: The treatment has been performed at DIV 7. All the steps are carried on under a cell culture hood in sterile conditions. An example of cortical neuronal culture density at DIV 7 is illustrated in **Figure 1**.

1. Pool microsomes-associated αS aggregates obtained from the spinal cord of three different diseased Tg mice in order to have a 1 µg/µL solution, using the original homogenization buffer for the dilution.
2. Remove 1/3 of the medium and replace it gently with the fresh medium containing 2% B27, 1x gentamicin and 2 mM glutamine.
3. Add 1 µg of pooled microsomes-associated αS aggregates to the cell medium. Return neurons in the incubator at 37 °C.
4. Every 3 days for 1 week, add 1/3 of fresh medium. Do not replace the medium. Just add it.
5. After 1 week of treatment, remove 1/3 of the medium and replace it gently with the fresh medium containing 2% B27, 10 µg/mL gentamicin and 2 mM glutamine. Repeat every 3 days.
6. Fix neurons after 2 weeks of treatment (DIV 21).

5. Immunofluorescence

1. Fix neurons with 2% paraformaldehyde in PBS 1x and 5% sucrose solution for 15 min at RT without shaking, under a chemical fume hood.
2. Remove the fixing solution. Briefly, wash with PBS 1x, 3 times.
   NOTE: Perform all the washing steps gently because primary neurons do not stick firmly to poly-lysine coverslips.
3. Permeabilize neurons with 0.3% of nonionic surfactant in PBS 1x for 5 min at RT.
4. Briefly, wash with PBS 1x, 3 times.
5. Incubate neurons with 3% FBS in PBS 1x for 30 min at RT, to block unspecific binding sites, on an orbital shaker.
6. Incubate neurons with appropriate primary antibody dissolved in 3% FBS in PBS 1x, O/N at 4 °C on an orbital shaker.
   NOTE: syn303 (1:1,000), mouse αS (1:200), pser129-αS (1:1,000) and Tau (1:10,000) antibodies were used.
7. Remove the antibody solution and wash, briefly, with PBS 1x, 3 times.
8. Incubate neurons with appropriate fluorescent secondary antibody dissolved in 3% FBS in PBS, for 1 h at RT in the dark on an orbital shaker.
9. Remove the antibody solution and wash, briefly, with PBS 1x, 3 times.
10. Stain neurons with DAPI solution (0.1 µg/mL in PBS 1x) for 15 min at RT in the dark on an orbital shaker.
11. Remove the antibody solution and wash, briefly, with PBS 1x, 3 times.
12. Mount coverslips on a slide using antifade mounting medium.

Representative Results

Following the protocol described above and summarized in **Figure 2**, we purified microsomes-associated αS aggregates from three diseased A53T αS Tg mice (**Figure 3**). Microsomes are crude membrane pellet fractions that contain the endoplasmic reticulum, Golgi, and small synaptic vesicles. The degree of purity of the microsomal pellet compared to other fractions was previously evaluated using specific organelle markers[18].

Once isolated, biochemical characterization of αS aggregates is assessed through denaturing SDS-Page, followed by incubation with Syn-1 or phosphorylated αS at Serine 129 (pSer129-αS) antibody. Compared to presymptomatic (PreS) and age-matched non-Tg (nTg) mice, microsomes isolated from sick mice co-precipitate with αS aggregates. Microsomes-associated αS species show the typical features of αS aggregates, such as accumulation of HMW detergent-resistant species, phosphorylation at serine 129[19] and C-and N-terminal truncated fragments (for a full characterization of microsomes-associated αS aggregates see reference[18,20,21]). These are fundamental requirements since monomeric αS does not get efficiently internalized and does not induce αS deposition[7,15,22]. It is important not to use any detergent (ionic or non-ionic) to resuspend P100 pellets since it can be harmful to cells. Also, in order to avoid sample variability, microsomes-associated αS aggregates from three different diseased mice will be pooled for neuronal treatment.

Administration of 1 μg of pooled microsomes-associated αS aggregates from diseased A53T mice to the culture medium of cortical or hippocampal neurons induces a time-dependent formation of αS inclusions, positive for aggregates-specific αS antibodies such as Syn303 (**Figure 4**, **5**) or pser129-αS (**Figure 5**). After two days (2d) of treatment these aggregates appear as small, scattered puncta that will become more abundant at later time points. After two weeks, αS inclusions resemble long and mature beads-like structures, heavily spread throughout the neuronal cultures, following a neurite pattern and partially co-localizing with presynaptic and neurites markers (**Figure 5**). Occasionally, newly formed αS inclusions can be seen to co-localize and stain the cell soma or cover the entire process, resembling necrotic neurites.

Although microsomes-associated αS aggregates fractions can efficiently spread in neuronal cultures, their amount has to be finely tuned to the number of neurons plated (**Figure 1**). In fact, exceeding the recommended ratio, μg of microsomes-associated aggregates: number of neurons, will induce premature cell death within a few days (**Figure 6**), while an insufficient amount of microsomes-associated αS aggregates will lead to a scarce and reduced number of inclusions after two weeks of treatment, similar to what was obtained at earlier time points (**Figure 4A**).
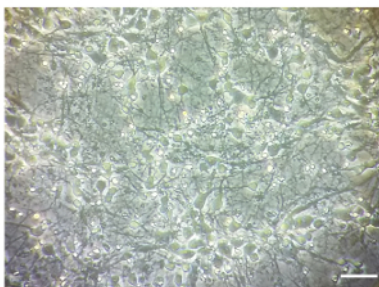

Figure 1. Cortical neuronal cultures. Representative image showing density at DIV 7 of cortical neuronal cultures. Images were taken with an inverted light microscope, 10X objective. Scale bar = 100 μm. Please click here to view a larger version of this figure.
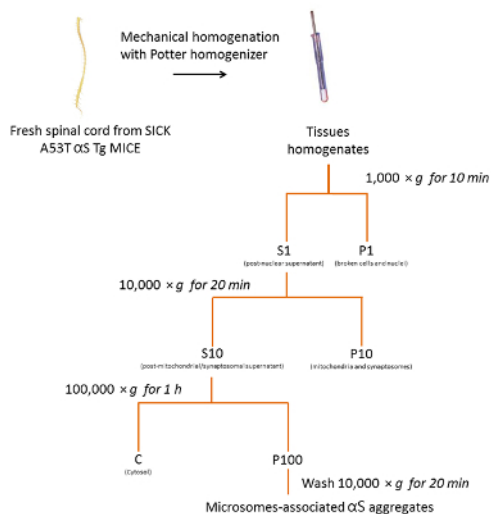

Figure 2. Isolation of microsomes-associated αS aggregates from mouse SpC. Flowchart of the purification protocol of microsomes-associated αS aggregates from SpC of diseased mice. Please click here to view a larger version of this figure.
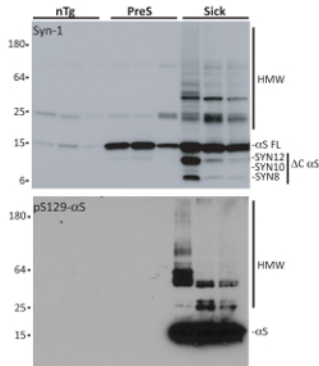
Figure 3. Isolation of microsomes fractions from diseased, presymptomatic A53T αS Tg and nTg mice. Western blot analysis showing purified microsomes-associated αS aggregates isolated from three diseased (Sick), presymptomatic (PreS) and aged-matched nTg mice. Diseased mice are A53T αS Tg mice that show the motor and neurological dysfunction, including the accumulation of αS inclusions, while presymptomatic animals are healthy A53T αS Tgs of 9 months of age that do not show yet any αS pathology related phenotype. nTg mice are littermates of diseased mice which do not carry the αS transgene and therefore do not develop αS induced pathology. 1 μg of each purified fractions were run on a denaturing SDS-Page, transferred on a nitrocellulose membrane and blotted with Syn-1 or pSer129-αS antibody. Only microsomes fractions isolated from sick mice contained HMW detergent-resistant αS aggregates that were phosphorylated at serine 129 and showed C-and N-terminal truncation. This figure has been adapted from Colla et al.[18]. Please click here to view a larger version of this figure.
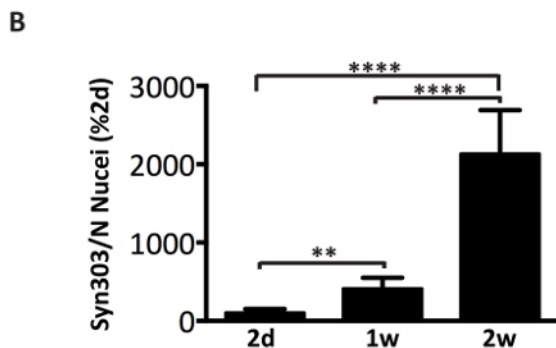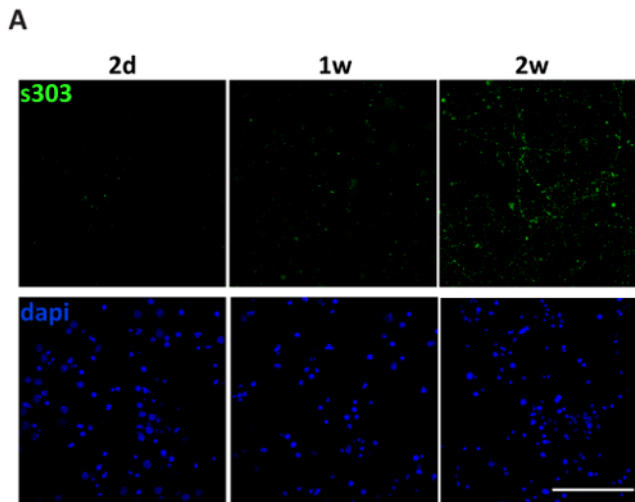

Figure 4. Time-dependent induction of αS deposition after administration of microsomes-associated αS aggregates. (A) Immunofluorescence of primary hippocampal neurons treated with 1 μg of microsomes-associated αS aggregates fractions pooled from three different diseased mice. Neurons were fixed at 2 days (2d), 1 week (1w) or 2 weeks (2w) of treatment and immunostained with syn303 (S303, 1:1000), an antibody specific for oxidized and aggregated αS. Cells were counterstained with DAPI. Confocal images were taken using a laser scanning confocal microscope, 63X objective. Scale bar = 50 μm. **(B)** Quantitative analysis of total fluorescence, after background subtraction, was done using the particles count plugin of the Image J software. Values were normalized for the number of nuclei per field (DAPI count) and expressed as the percentage of the S303 fluorescence signal at 2D. Values are given as the mean ± SD (n = 5). **p <0.001, ****p <0.00001, One-way ANOVA, followed by Fisher's LSD post-hoc test. Please click here to view a larger version of this figure.
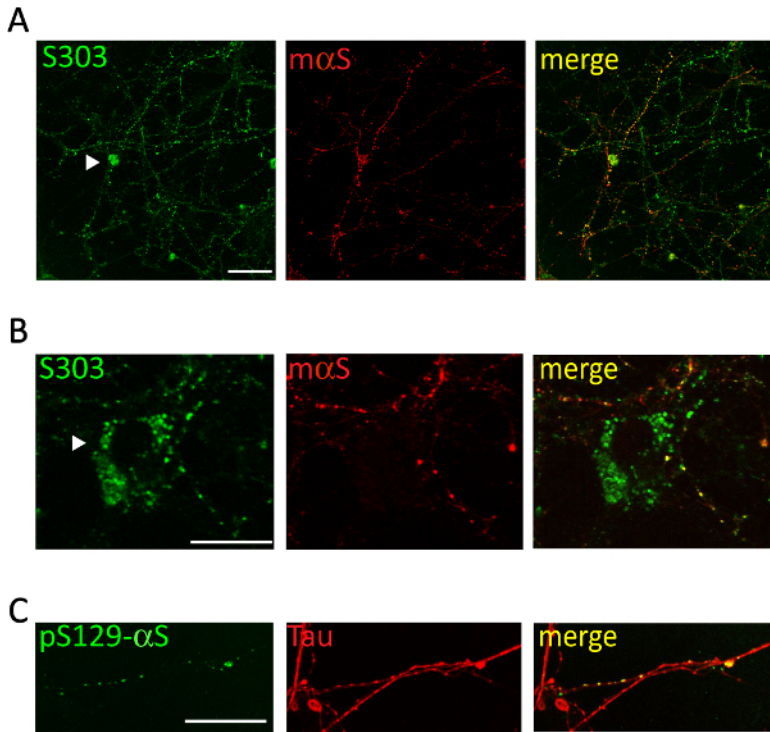
Figure 5. Intracellular αS inclusions colocalize with cortical neurites network. Representative confocal images of cortical neurons treated with microsomes-associated αS aggregates obtained from diseased Tg mice. After 2 weeks of treatment neurons were fixed and double stained with aggregates-specific antibodies [S303 **(A, B)** or pser129-αS, 1:1,000 (C)] and neurite markers [mouse αS, 1:200 (A,B) or Tau, 1:10,000 **(C)**]. Co-labelling of the fluorescent signals demonstrated partial co-localization of newly formed αS bead-like structures with the neurites network. Occasionally αS inclusions accumulate within the neuronal soma (A, B, arrowheads). Stacked images were acquired with a laser scanning confocal microscope, 63X objective. Scale bar = 50 µm. This figure has been modified from Colla *et al.*[20]. Please click here to view a larger version of this figure.

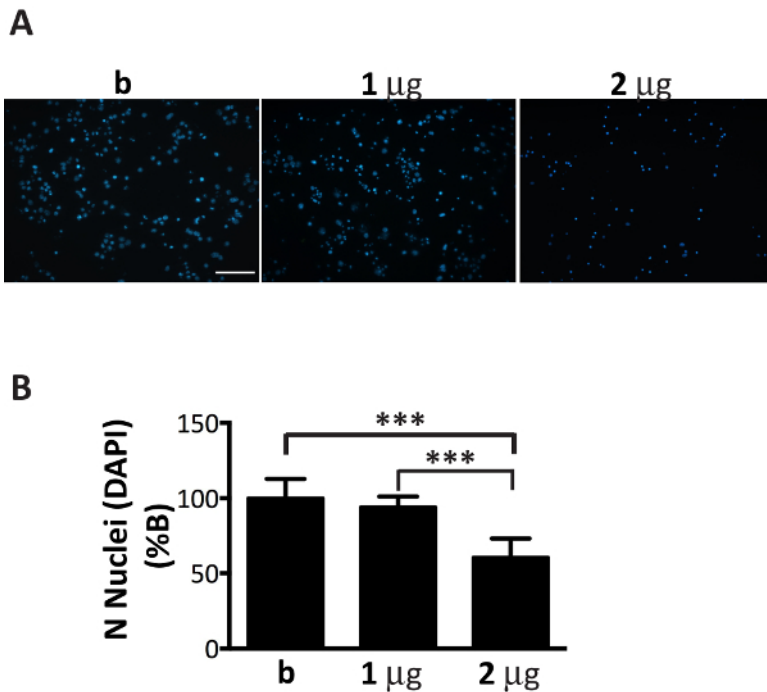June 2018 | 136 | e57884 | Page 6 of 9

Figure 6. Addition of suboptimal amount of microsomes-associated αS aggregates is toxic to neurons. DAPI staining of neurons treated with increasing concentration of microsomes-associated αS aggregates leads to cell death. **(A)** Cortical neurons were treated with 1, 2 µg of microsomes-associated αS aggregates extracted from diseased mice or only buffer that does not contain aggregates **(B)** and stained with DAPI. Fluorescent images were acquired with an epi-fluorescence microscope using a 20X objective. Scale bar = 100 µm.**(B)** DAPI-positive cells were counted using the Image J software. The graph shows a reduction in the number of nuclei with increasing concentration of microsomes-associated αS aggregates added to the neuronal media. Values are expressed as % of b and are given as the mean ± SD (n = 5), ***p <0.0001, One-way ANOVA, followed by Fisher's LSD post-hoc test. Please click here to view a larger version of this figure.

Discussion

We described a method to obtain formation of αS inclusions in brain-derived primary neuronal cultures from WT mice, through the addition of purified microsomes-associated αS aggregates isolated from αS Tg animal models.

Critical steps of this protocol are the following: the ratio of µg of microsomes-associated αS aggregates/neurons and the source of αS aggregates. As shown in the results session, it is crucial to optimize the ratio of µg of microsomes-associated αS aggregates/number of neurons since working in suboptimal conditions can lead to the premature cell death or too scarce intracellular aggregation (see the Representative Results section). Because of this, it is very important to assess the density of the neuronal culture at DIV 7 (shown in **Figure 1**) before starting the treatment. Additionally, αS aggregates have to be purified from diseased αS Tg mice, *i.e.* from animal models that accumulate LB-like inclusions characterized by phosphorylated and detergent insoluble αS HMW fibrils.

Possible modifications to this protocol regard the tissue, frozen or fresh, from which microsomes can be isolated and the starting amount. While we used SpC of Tg mice because of the high content of αS insoluble aggregates, the protocol is suitable to isolate microsomes-associated aggregates from any tissues, provided that the area has a high content in αS aggregates. Frozen samples can also be used since freezing does not affect the purification steps of microsomes-associated aggregates or the aggregates *per se*. While the recommended starting weight for tissues is about 100 - 150 mg, this protocol is suitable for obtaining microsomes from as low as 50 mg of raw material (no maximum weight limit). In the case of the amount lower than 100 mg, however, the appropriate homogenization ratio will be 1:20 (w/v) in order to have at least 1 mL of supernatant S10 to load on the polycarbonate bottle for the ultracentrifuge precipitation. In fact, loading volumes smaller than 1 mL may result in the collapse of the tube and sample loss. Increasing the homogenization volume will lead to a more diluted supernatant but the concentration of the microsomal pellet will remain unaffected.

A limitation of this protocol concerns the inefficient cross-seeding in the formation of αS inclusions that have been recently reported in the case administration of αS PFFs of human origin to murine neuronal cultures as opposed to mouse αS PFFs[24]. Since increasing the amount of exogenous αS fibrils given to murine cultures can bypass this issue, we recommend to finely tune the amount of microsomes-associated aggregates in the case of administration of fibrils obtained from other αS variants or from different species to mouse neuronal cultures than what we described.

Exogenous αS aggregates added to the culture media can be from different sources. *In vitro* αS PFFs have been previously used as seeding template of intracellular αS aggregates in cell cultures, primary neurons, and animal models[3,7,8,15]. Compared to our method where microsomes-associated αS aggregates can be isolated in few hours, the formation of PFFs is lengthy and laborious, requiring multiple steps of purifications, followed by additional assays to check αS aggregates conformations[25]. In addition, PFFs being obtained from bacterial-expressed human or mouse αS, *i.e.* lacking posttranslational modifications typical of eukaryotes, can present different conformations, with selective seeding and pathogenic properties, according to the nucleation protocols followed (*i.e.* ribbons vs fibrils)[5,8] leading to different results and conclusions.

Instead, single administration of *in vivo* purified αS aggregates guarantees the transmission of more authentic pathogenic templates, mimicking closely the process of formation of αS inclusions in animal models and PD patients.

As a future application of this technique, we believe that this protocol can be successfully used to isolate αS pathogenic seeds from the brain of PD patients or other αS Tg animal models, provided that diseased area from which microsomes are isolated are rich in αS inclusions.

To our knowledge, this is the first method that allows the purification of native toxic species of αS from *in vivo* PD models to be used as seeding template for obtaining formation of αS inclusions in primary neurons.

We believe that this method is extremely versatile and can provide an exceptional cell-based model to study the different aspects of αS aggregation and its influence on the cell pathophysiology. Because the formation of αS inclusions represent a complex process that has been difficult to replicate in cultured cells, we are hopeful that this model will provide great insights in acute pathogenic mechanisms, hard to identify in chronic and more elaborate systems such as are animal models.

Disclosures

The authors have nothing to disclose.

Acknowledgements

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