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Toxic properties of microsome-associated alpha-synuclein species in mouse primary neurons

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ABSTRACT

α-synuclein (αS) is a small protein that self-aggregates into α-helical oligomer species and subsequently into larger insoluble amyloid fibrils that accumulate in intraneuronal inclusions during the development of Parkinson's disease. Toxicity of αS oligomers and fibrils has been long debated and more recent data are suggesting that both species can induce neurodegeneration. However while most of these data are based on differences in structure between oligomer and aggregates, often preassembled *in vitro*, the *in vivo* situation might be more complex and subcellular locations where αS species accumulate, rather than their conformation, might contribute to enhanced toxicity. In line with this observation, we have shown that αS oligomers and aggregates are associated with the endoplasmic reticulum/microsomes (ER/M) membrane *in vivo* and how accumulation of soluble αS oligomers at the ER/M level precedes neuronal degeneration in a mouse model of α-synucleinopathies. In this paper we took a further step, investigating the biochemical and functional features of αS species associated with the ER/M membrane. We found that by comparison with non-microsomal associated αS (P10), the ER/M-associated αS pool is a unique population of oligomers and aggregates with specific biochemical traits such as increased aggregation, N- and C-terminal truncations and phosphorylation at serine 129. Moreover, when administered to murine primary neurons, ER/M-associated αS species isolated from diseased A53T human αS transgenic mice induced neuronal changes in a time- and dose-dependent manner. In fact the addition of small amounts of ER/M-associated αS species from diseased mice to primary cultures induced the formation of beads-like structures or strings of fibrous αS aggregates along the neurites, occasionally covering the entire process or localizing at the soma level. By comparison treatment with P10 fractions from the same diseased mice resulted in the formation of scarce and small puncta only when administered at high amount. Moreover, increasing the amount of P100/M fractions obtained from diseased and, more surprisingly, from presymptomatic mice induced a significant level of neuronal death that was prevented when neurons were treated with ER/M fractions immunodepleted of αS high molecular weight (HMW) species. These data provide the first evidence of the existence of two different populations of αS HMW species *in vivo*, putting the spotlight on the association to ER/M membrane as a necessary step for the acquisition of αS toxic features.

1. Introduction

Accumulation of α-synuclein (αS) aggregates in intracellular proteinacious inclusions called Lewy Bodies (LB) or Lewy neurites, according to their subcellular location, is a classical hallmark of Parkinson's disease (PD) and α-synucleinopathies (Goedert et al., 2012). αS is a small, soluble acidic protein highly expressed throughout the nervous system and with a well-described presynaptic localization (Iwai et al., 1995; Maroteaux et al., 1988). Point mutations in the αS gene (Appel-Cresswell et al., 2013; Krüger et al., 1998; Lesage et al., 2013; Polymeropoulos et al., 1997; Zarranz et al., 2004) and gene amplifications (Chartier-Harlin et al., 2004; Singleton, 2003) have been found in family pedigrees affected by autosomal dominant, early onset PD, although αS neurotoxicity contributes to both genetic and sporadic

Abbreviations: αS, alpha-synuclein; PD, Parkinson's disease; ER/M, endoplasmic reticulum/microsomes; HMW, high molecular weight; SpC, spinal cord; Ctx, cortex; PreS, presymptomatic; Tg, transgenic; nTg, non-Tg

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forms of PD (Shulman et al., 2011).

Aggregation of αS in insoluble inclusion is a complex nucleation reaction that includes at least two key steps: the transition from an unfolded monomer which has a naturally intrinsic unfolded conformation [(Burré et al., 2013; Chandra et al., 2003; Fauvet et al., 2012; Theillet et al., 2016; Weinreb et al., 1996), although lately these data have been questioned (Bartels et al., 2011; Dettmer et al., 2015; Wang et al., 2011)] to an oligomer-type of structure and the transition to an insoluble β-sheet rich protofibril (Cremades et al., 2012; Deleersnijder et al., 2013). Many variables are thought to influence these transitions: point mutations, such as those associated with genetic PD (Conway et al., 1998); C-terminal truncation (Li et al., 2005); protein level and stability (Li, 2004); environmental factors (Uversky et al., 2002); post-translational modifications, such as ubiquitination, phosphorylation, nitration/oxidation (Oueslati et al., 2010).

Membrane interaction appears to be another condition that can influence formation of αS fibrils (H.-J. Lee et al., 2002). In fact, αS is believed to shift between a free and a membrane-bound state in a dynamic equilibrium with the membrane-bound state accounting for 10–15% of the total protein amount. αS can bind synaptic vesicles and its binding is believed to mediate its synaptic function (Burré, 2015). αS has been implicated in a broad range of presynaptic functions that includes binding and promoting SNARE complex assembly to favor docking of synaptic vesicles to the cell membrane (Burré et al., 2010, 2014), lipid transport and metabolism (Golovko et al., 2005, 2009), neurotransmitters release and brain plasticity (Bendor et al., 2013). Association with membranes is mediated by αS N-terminal seven 11-amino-acids repeats that are predicted to form an amphipathic α-helix. αS has been found to bind high-curvature membranes such as in presynaptic vesicles, through the acquisition of a multimeric structure with a defined orientation (Burré et al., 2014). Thus, under normal conditions αS can shuffle between a native unfolded structure to a multimeric vesicle-bound conformation at the presynaptic terminals. However it is not clear how the transition from these native conformations to a toxic type of aggregates occurs.

We have recently described the presence of toxic αS high molecular weight (HMW) species (oligomers and aggregates) associated with the endoplasmic reticulum/microsomal vesicles (ER/M) *in vivo*, in diseased Prp-A53T transgenic (Tg) mice (Colla et al., 2012a). αS HMW species were sensitive to protease degradation suggesting that these αS species were associated with the microsomal membrane on the cytosolic side. Importantly, the appearance of αS oligomers at the ER/M level temporally preceded the onset of neurodegeneration and ER stress-induced cell death in a Tg mice, suggesting the microsomal membranes might be a unique place to foster the accumulation of toxic species of αS (Colla et al., 2012b).

In this paper we took a deeper look into the ER/M-associated αS HMW species, comparing them to the rest of αS aggregates purified through low speed centrifugation. Our findings provide evidence of the existence of a toxic species of αS *in vivo*, associated with the ER/M vesicles that has unique biochemical traits and is more aggressive in spreading and inducing cell death in neuronal primary cultures.

2. Materials and methods

2.1. Animal models

Transgenic mice expressing human A53T αS under the control of the mouse prion protein (PrP) promoter [line *G2-3(A53T)*] have been described previously (Colla et al., 2012a, 2012b; M. K. Lee et al., 2002; Li et al., 2005; Martin et al., 2006). This model develops fatal neurological disease at ~12 months of age which rapidly progresses to end state within 14–21 days of onset. Diseased mice show a drastic reduction in motor function, accumulation of intracellular αS inclusions and neuronal death. For this study, sick mice at 12–14 months of age, pre-symptomatic mice at 9 months and age-matched nTg littermates were used. Presymptomatic animals did not show any motor dysfunction or αS pathology in the central nervous system. All animal studies were 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).

2.2. Membrane fractions preparation

Membrane pellet fractions were obtained as previously described (Colla et al., 2012a). Briefly, fresh tissues were homogenized in a 1:10 (wt/vol) volume of lysis buffer (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 2 mM EDTA, protease and phosphate-inhibitors cocktails) using a Teflon pestle homogenizer. Initial homogenates were centrifuged at 1000 × *g* to remove nuclei and unbroken cells. The resulting supernatant was centrifuged at 10,000 × *g* for 20 min at 4 °C to obtain the first membrane pellet (called P10), while the supernatant was centrifuged at 100,000 × *g* for 1 hr at 4 °C to obtain the microsomal pellet (called P100). Both pellet fractions were washed with homogenization buffer once and centrifuged again at the same speed as previously mentioned. P10 and P100 were then re-suspended in 200 or 100 μl of lysis buffer, respectively, and their protein content was determined.

2.3. Western blot

Immunoblot and dot blot analyses were performed as previously described (Colla et al., 2012a, b). The following antibodies were used: syn1 (1:5000; BD Transduction Laboratories, Franklin Lakes, NJ); LB509 (1:5000; Abcam, Cambridge, MA); mouse pser129-αS (1:1000; DAKO, Glostrup, Denmark), syn303 (1:1000; Biolegend, San Diego, CA); A11 (ThermoFisher Scientific, Eugene, OR). Quantitative analysis of immune detected bands was done using ImageLab Software (Bio-rad, Hercules, CA).

2.4. Primary hippocampal and cortical neurons preparation and P10 and P100 fractions treatment

Primary neuronal cultures were prepared from wild-type (WT) newborn (P0) hippocampus and cortex of mouse strain B6.129, according to a procedure by Beaudoin et al., (2012). Hank's Balanced Salt Solution was used as mechanical dissection medium. Tissues mechanically dissected were treated with 0.1% trypsin for 7 min at 37 °C and then collected with regular DMEM medium containing fetal bovine serum (FBS) and DNase. Preparations were centrifuged at 1000 rpm for 5 min and pellet was resuspended in Neurobasal medium containing 2% B27, 1X Glutamax, 6 mg/ml glucose, 10% FBS, 12.5 μM glutamate and 1X Gentamicin. Dissociated neurons were plated on poly-D-lysine coated coverslips placed in 24 well dishes at a concentration of about 100,000 cells/cm^2. At day *in vitro* (DIV) 1 the medium was replaced with new fresh Neurobasal medium containing 2% B27, 1X gentamicin and 1X Glutamax. At DIV2, 1/3 of the medium was removed and replaced with fresh medium containing 2.5 μM cytosine arabinoside for 48 hrs to reduce glial contamination.

Neurons were treated at DIV7 with 0.5, 1 or 2 μg of various pellet fractions according to the experiment. To avoid sample variability, 4–5 pellet fractions isolated from different mice with the same phenotype were pooled. For internalization experiments, neurons were fixed after 2 days, 1 week or 2 weeks of treatment.

2.5. Cell lines

Human neuroblastoma SH-SY5Y cells were transfected with a pcDNA3.1 plasmid carrying human WT αS cDNA tagged at the C-terminal with Myc. Cells stably carrying the plasmid were kept polyclonal and routinely cultured in DMEM medium containing 10% FBS,