α-Synuclein regulates the partitioning between tubulin dimers and microtubules at neuronal growth cone

Daniele Cartelli and Graziella Cappelletti

ARTICLE ADDENDUM

α-Synuclein (Syn) is a presynaptic unfolded protein, which participates to many neuronal functions and whose mutations are associated to synucleopathies, neurodegenerative disorders including Parkinson’s disease (PD). Among the multitude of cell processes in which Syn is involved there is the modulation of synaptic function and plasticity, which strictly depend on the fine tuning of the cytoskeleton. Interestingly, it has been shown that Syn modulates actin assembly, but controversial evidence has been published about the interaction between Syn and microtubules (MTs). Indeed, A lim and colleagues showed that wild type (WT) Syn promotes MT assembly whereas Chen and colleagues claimed that neither monomeric nor oligomeric Syn influences MT polymerization in vitro. Very recently, we cleared the effects of WT Syn and PD-linked mutants on the MT system. We demonstrated that WT Syn acts as a foldable MT dynamase which regulates the nucleation, the growth velocity and the catastrophe frequency (the shift from a polymerizing to a shrinking phase) of individual MTs, both in a purified system and in neuronal cells; furthermore, we showed that PD-linked point mutations corrupt these functions and lead to tubulin aggregation instead of proper MT assembly. Due to the good alignment of Syn with the members of the Stathmin (STMN1) family, the same binding mode to tubulin tetramer and the partial overlapping functions in inducing MT catastrophes, we also proposed a functional redundancy of the roles played by Syn and STMN1. STMN1 is ubiquitously expressed in vertebrates and, through the induction of MT catastrophes, it regulates the partitioning of tubulin, the building block of MTs, between unassembled and polymerized forms. Accordingly, STMN1 loss resulted in higher MT mass and in increased nucleation rate from centrosomes but little changes in MT dynamics. Noteworthy, mutations of STMN1 lead to loss of axonal MT integrity and to several neurological phenotypes in Drosophila, as well as reduced levels of STMN1 are associated to MT instability and to neurological disorders like Down syndrome and Alzheimer’s disease.

Keeping in mind all these intriguing evidences, here we wonder if Syn is able to regulate the partition between tubulin dimers and MTs at neuronal growth cone, the region

CONTACT
Daniele Cartelli: daniele.cartelli@gmail.com; G. Cappelletti: graziella.cappelletti@unimi.it; Department of Biosciences, Universit degli Studi di Milano, Via Celoria 26, Milano, Italy; Center of Excellence on Neurodegenerative Diseases, Università degli Studi di Milano, Milano, Italy.

ARTICLE HISTORY
Received 7 November 2016
Accepted 23 November 2016

KEYWORDS
α-Synuclein; microtubules; neuron; Parkinson’s disease; tubulin

© 2017 Daniele Cartelli and Graziella Cappelletti. Published with license by Taylor & Francis. This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivatives License (http://creativecommons.org/licenses/by-nc-nd/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited, and is not altered, transformed, or built upon in any way.
where it is highly expressed.\textsuperscript{10} We assessed Syn ability in modulating MT assembly in such neuronal compartment using differentiated rat PC12 cells, which naturally express rat Syn starting from 7th day of NGF treatment;\textsuperscript{11} therefore, we transfected human WT or human mutant Syn and we performed experiments at 5th day of NGF-induced differentiation. Thereby, we analyzed the distal neurite of differentiated PC12 cells expressing either WT GFP-Syn or GFP alone under basal conditions (cells kept at 37°C), after MT-destabilizing cold-treatment (30 min at 4°C), and during MT (re)nucleation (15 and 60 min at 37°C). α tubulin staining shows the presence of MTs in cells overexpressing WT Syn after 15 min of re-warming unlike control cells (Fig. 1A), suggesting that the recovery of MT network occurs at earlier time point in the presence than in the absence of WT Syn. In order to substantiate our observations, we next removed the pool of unassembled tubulin and we measured the fluorescence of total α tubulin (Fig. 1B), which is proportional to MT mass, and of tyrosinated (Tyr) tubulin (Fig. 1C), which is associated with dynamic and neo-synthetized MTs\textsuperscript{12} and for which Syn has shown a high affinity.\textsuperscript{5} The quantification was performed in the distal part of the neurite, roughly the growth cone, measuring the total fluorescence intensities inside comparable areas (data not shown). Our analysis reveals that, after 15 min of re-warming, the presence of Syn significantly increases both total α tubulin and Tyr tubulin fluorescence, highlighting that Syn favors MT (re)nucleation. In parallel, we performed biochemical analyses on cytoskeletal fractions obtained from cells following destabilization and (re)nucleation of MTs, as described above. We measured the amount of α tubulin and Tyr tubulin associated to cytosolic dimers and to polymeric MTs under basal conditions, after MT-destabilizing cold-treatment and during MT (re)nucleation (Fig. 1D-E). Our results show a significant Syn-dependent increase of α tubulin and Tyr tubulin incorporated into MTs. Since pathological point mutations reduce the ability of Syn to interact with tubulin and abolish its ability in inducing MT assembly,\textsuperscript{5} we expect that PD-associated Syn mutants lead to tubulin aggregation during the recovery after cold-induced depolymerization. Indeed, the analyses of the distal neurite of differentiated PC12 cells expressing either A30P or A53T PD-linked Syns reveal that they cause MT bundling and aggregation (Fig. 2) instead of the proper regrowth as we observed with WT Syn (Fig. 1A). Thus, the present results confirm that WT Syn promotes MT nucleation in neuronal cells and indicate that WT Syn modulates the partition of tubulin between dimers and MTs whereas mutant Syns compromise MT (re)assembly and, probably, growth cone remodelling under stress condition, like aging is supposed to be.

Collectively our data show that Syn acts as foldable dynamase\textsuperscript{5} which is able to modulate the partitioning of tubulin at neuronal growth cone, either by setting the number of MTs or regulating their length, and reinforce our hypothesis of some redundancy between Syn and STMN1 actions. Indeed, both STMN1\textsuperscript{13} and Syn\textsuperscript{14} are involved in the regulation of neuronal process outgrowth, during which the fine tuning of tubulin/MTs partitioning is of crucial importance, and both Syn knockout\textsuperscript{15} and STMN1 knockout\textsuperscript{16} mice develop normally and only with aging they show neuronal defects or pathological phenotypes, such as axonopathy and neuronal degeneration. Thus, Syn and STMN1 can really be involved in the same process, namely tubulin subunit partitioning, which may be either global or spatially restricted. Indeed, STMN1 is ubiquitously expressed, even though it is brain enriched and developmentally regulated,\textsuperscript{17} whereas Syn is much more confined to the distal part of the axons,\textsuperscript{10} a compartment where the regulation of MT assembly becomes even more fundamental.

It has been proposed that the functions of MT associated protein 4 (MAP4) and STMN1 are regulated by cognate kinase systems that mediate phosphorylation and the activation of the counteractive activities of the proteins, which regulate tubulin subunit partitioning during interphase of the cell cycle.\textsuperscript{18} In neurons could be there the same situation for Syn and Tau, two MT-interacting proteins with partially redundant and partially counteractive actions and which are partner in crime in the induction in neurodegenerative processes.\textsuperscript{19} Furthermore, in human brain cytosol, they interact each other in a tubulin concentration-dependent manner,\textsuperscript{20} which can be a regulator of their actions and, thus, of MT assembly itself. As for MAP4 and STMN1, both Syn and Tau are phosphorylated by the same enzyme, namely glutathione synthase kinase 3 β (GSK-3β), a well-known regulator of MT assembly throughout the modulation of MT interacting proteins.\textsuperscript{21} Indeed, in transgenic mice expressing a point mutant (S9A) of human GSK-3β there are elevated levels of phospho-Syn and phospho-Tau, as well as it has been demonstrated that recombinant human GSK-3β directly phosphorylates Syn and Tau.\textsuperscript{22}

Together, these data establish a novel role for Syn in the regulation of partitioning between tubulin dimers and MTs at neuronal growth cone, process in which it can be helped by STMN1 or Tau. The system could be regulated by free tubulin concentration, in a sort of vicious/virtuous circle as we have already proposed,\textsuperscript{5} or by several kinases. Abnormalities in the biological properties of recipient proteins, i.e. tubulin or Syn, as well as in the regulators, as GSK3β, would lead to alterations of the system, defective regulation of MT assembly and growth cone remodelling and, possibly, to neurological diseases including PD.
**Material and methods**

**Cell cultures and transfection**

PC12 cells were maintained in cultures and differentiated as previously described. PC12 cells were transiently transfected using Lipofectamine 2000 (Invitrogen) (1:3 DNA to Lipofectamine ratio), with GFP-fused WT or mutated Syns or with GFP-containing control vector; the quantity of DNA was chosen according to previously reported data, as the Syn expression level was low and

*Figure 1.* WT Syn regulates the partitioning between tubulin dimers and MTs in differentiated PC12 cells. (A) Fluorescence microscopy micrographs of PC12 cells differentiated 5 d with NGF expressing GFP-Syn chimera (Syn) or GFP control vector (GFP), fixed before (BASAL), immediately after the MT destabilizing cold-treatment, 30 min at 4 °C (0), or at various times after rewarming (15 and 60 min at 37 °C) and stained for α tubulin (α TUB). Insets represent the GFP channel, and the yellow boxes the magnified areas shown on the right (GFP channel in green and α tubulin in red). Scale bar, 10 μm. Quantification of total fluorescence α tubulin (α TUB, B) and tyrosinated tubulin (Tyr TUB, C) in PC12 cells expressing GFP-Syn chimera (Syn) or GFP (GFP), extracted and fixed after the treatment described in (A). Values are expressed as mean ± SEM, and the cells analyzed are at least 12 for each experimental condition. *p < 0.05 vs CONT, according to Student’s t-test. Western blotting (D) and densitometric analyses (E) of α tubulin (α TUB) and tyrosinated tubulin (Tyr TUB) associated to tubulin dimers (Dim) or to MTs (MT) in PC12 cells expressing GFP-Syn chimera (Syn) or GFP (GFP), treated as described in A. In (E), values (mean ± SEM) represent ratio between MTs and dimers of α tubulin (α TUB, white bars) and tyrosinated tubulin (Tyr TUB, black bars) of Syn-transfected PC12 cells, and are expressed as control percentage (GFP-expressing PC12 cells). Data are obtained from at least three independent experiments. *p < 0.05, according to Student’s t-test, performed on the rough data.
comparable to the average physiological levels of the protein in the brain.2

**Western blotting**

Triton X-100 soluble and insoluble fractions of PC12 cells were made as previously reported.23 After SDS-PAGE, proteins were transferred onto polyvinylidene difluoride membranes and immunostained with the following antibodies: anti-α tubulin mouse IgG (clone B-5–1–2, Sigma-Aldrich); anti-tyrosinated tubulin mouse IgG (clone TUB-1A2, Sigma-Aldrich). Immunostaining was revealed by enhanced chemiluminescence (Super-Signal West Pico Chemiluminescent, Pierce). Quantification was performed by Image J software (NIH) and subtracting the background around bands.

**Immunofluorescence**

Transfected PC12 cells were fixed with cold methanol (6 min at −20°C); to remove unassembled tubulin, before fixation, some slides were extracted in PEM buffer (80 mM K-Pipes, 5 mM EGTA, 1 mM MgCl2, pH 6.8, containing protease inhibitors) with 0.5% Triton X-100, 0.2 M NaCl and 10 μM Paclitaxel (Sigma-Aldrich). PC12 cells were stained with anti-α tubulin mouse IgG or anti-tyrosinated tubulin mouse IgG and Alexa Fluor™ 568 donkey anti-mouse. By using Image J software, total fluorescence intensity was measured on the growth cone area. The region of interest was manually drowned and the analyses of the surface extension revealed that there were no significant differences between control cultures and WT-expressing PC12 cells (data not shown).

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

**Acknowledgements**

The authors are grateful to all the members of the lab and to all the people which contribute in delineating the Syn functions.

**Funding**

This work was supported by Fondazione Grigioni per il Morbo di Parkinson, Milan, Italy [to G.C.]; “Dote ricerca,” FSE, Regione Lombardia [to DC].

**References**

[1] Lashuel HA, Overk CR, Oueslati A, Masliah E. The many faces of α-synuclein: from structure and toxicity to therapeutic target. Nat Rev Neurosci 2013; 14:38-48; PMID:23254192; http://dx.doi.org/10.1038/nrn3406

[2] Sousa VL, Bellani S, Giannandrea M, Yousuf M, Valtorta F, Meldolesi J, Chieregatti E. {alpha}-synuclein and its A30P mutant affect actin cytoskeletal structure and dynamics. Mol Biol Cell 2009; 20(16):3725-39; PMID:19553474; http://dx.doi.org/10.1091/mbc.E08-03-0302

[3] Alim MA, Ma QL, Takeda K, Aizawa T, Matsubara M, Nakamura M, Asada A, Saito T, Kaji H, Yoshi M, et al. Demonstration of a role for alpha-synuclein as a functional microtubule-associated protein. J Alzheimer’s Dis 2004; 6:435-42; PMID:15345814

[4] Chen L, Jin J, Davis J, Zhou Y, Wang Y, Liu J, Lockhart PJ, Zhang J. Oligomeric alpha-synuclein inhibits tubulin polymerization. Biochem Biophys Res Commun 2007; 356:548-53; PMID:17374364; http://dx.doi.org/10.1016/j.bbrc.2007.02.163

Figure 2. Pathological Syn mutations corrupt the partitioning tubulin dimers and MTs. Fluorescent microscopy photographs of PC12 cells differentiated 5 d with NGF expressing mutated (A30P and A53T) GFP-Syn chimeras, fixed before (BASAL), immediately after the MT destabilizing cold-treatment, 30 min at 4 °C (0), or at 15 min after rewarming (37 °C), and stained for α tubulin. Insets show the GFP channel. Scale bar, 10 μm.
[5] Cartelli D, Aliverti A, Barbiroli A, Santambrogio C, Ragg EM, Casagrande FV, Cantele F, Beltramone S, Marangoz J, De Gregorio C, et al. α-Synuclein is a Novel Microtubule Dynamin. Sci Rep 2016; 6:33289; PMID:27628239; http://dx.doi.org/10.1038/srep33289

[6] Belmont LD, Mitchison TJ. Identification of a protein that interacts with tubulin dimers and increases the catastrophe rate of microtubules. Cell 1996; 84:623-631; PMID:8598048; http://dx.doi.org/10.1016/S0092-8674(00)81037-5

[7] Ringhoff DN, Cassimeris L. Stathmin regulates centrosomal nucleation of microtubules and tubulin dimer/polymer partitioning. Mol Biol Cell 2009; 20(15):3451-8; PMID:19515833; http://dx.doi.org/10.1091/mbc.E09-02-0140

[8] Duncan JE, Lytle NK, Zuniga A, Goldstein LS. The Microtubule Regulatory Protein Stathmin Is Required to Maintain the Integrity of Axonal Microtubules in Drosophila. PLoS One 2013; 8(6):e68324; PMID:23840848; http://dx.doi.org/10.1371/journal.pone.0068324

[9] Cheon MS, Fountoulakis M, Cairns NJ, Dierssen M, Ringhoff DN, Cassimeris L. Stathmin regulates centrosomal nucleation of microtubules and tubulin dimer/polymer partitioning. Mol Biol Cell 2009; 20(15):3451-8; PMID:19515833; http://dx.doi.org/10.1091/mbc.E09-02-0140

[10] McLean PJ, Ribich S, Hyman BT. Subcellular localization of alpha-synuclein in primary neuronal cultures: effect of missense mutations. J Neural Transm Suppl 2001; (58):53-63; PMID:11128613

[11] Stefanis L, Kholodilov N, Rideout HJ, Burke RE, Greene LA. Synuclein-1 is selectively up-regulated in response to nerve growth factor treatment in PC12 cells. J Neurochem 2001; 76:1165-76; PMID:11181836; http://dx.doi.org/10.1046/j.1471-4159.2001.00114.x

[12] Janke C, Bulinski JC. Post-translational regulation of the microtubule cytoskeleton: mechanisms and functions. Nat Rev Mol Cell Biol 2011; 12:773-86; PMID:22086369; http://dx.doi.org/10.1038/nrm3227

[13] Di Paolo G, Pellier V, Catsicas M, Antonsson B, Catsicas S, Grenningloh G. The phosphoprotein stathmin is essential for nerve growth factor-stimulated differentiation. J Cell Biol 1996; 133:1383-90; PMID:8682872; http://dx.doi.org/10.1083/jcb.133.6.1383

[14] Liu G, Wang P, Li X, Li Y, Xu S, Ueda K, Chan P, Yu S. Alpha-synuclein promotes early neurite outgrowth in cultured primary neurons. J Neural Transm 2013; 120:1331-43; http://dx.doi.org/10.1007/s00702-013-0999-8

[15] Abeliiovich A, Schmitz Y, Fariñas I, Choi-Lundberg D, Ho WH, Castillo PE, Shinsky N, Verdugo JM, Armanini M, Hynes M, et al. Mice lacking alpha-synuclein display functional deficits in the nigrostriatal dopamine system. Neuron 2000; 25:239-52; PMID:10707987; http://dx.doi.org/10.1016/S0896-6273(00)80886-7

[16] Liedtke W, Leman EE, Fyffe RE, Raine CS, Schubart UK. Stathmin-deficient mice develop an age-dependent axonopathy of the central and peripheral nervous systems. Am J Pathol 2002; 160:469-8; PMID:11839567; http://dx.doi.org/10.1016/S0001-8742(01)64866-3

[17] Ozon S, El Meštikawy S, Sobel A. Differential, regional, and cellular expression of the stathmin family transcripts in the adult rat brain. J Neurosci Res 1999; 56(5):553-64; PMID:10369222; http://dx.doi.org/10.1002/(SICI)1097-4547(19990601)56:5<553::AID-JNR11%3e3.0.CO;2-J

[18] Holmfeldt P, Sellin ME, Gullberg M. Predominant regulators of tubulin monomer-polymer partitioning and their implication for cell polarization. Cell Mol Life Sci 2009; 66:3263-76; PMID:19585080; http://dx.doi.org/10.1007/s00018-009-0084-5

[19] Taymans JM, Baekelandt V. Phosphatases of α-synuclein, LRRK2, and tau: important players in the phosphorylation-dependent pathology of Parkinsonism. Front Genet 2014; 5:382; PMID:25426138; http://dx.doi.org/10.3389/fgene.2014.00382

[20] Jensen PH, Hager H, Nielsen MS, Hojrup P, Gliemann J, Jakès R. α-synuclein binds to Tau and stimulates the protein kinase A-catalyzed tau phosphorylation of serine residues 262 and 356. J Biol Chem 1999; 274(36):25481-9; PMID:10464279; http://dx.doi.org/10.1074/jbc.274.36.25481

[21] Credle JJ, George JL, Wills J, Duka V, Shah K, Lee YC, Rodrigo O, Simkins T, Winter M, Moechars D, et al. GSK-3β dysregulation contributes to parkinsonian-like pathophysiology with associated region-specific phosphorylation and accumulation of tau and α-synuclein. Cell Death Differ 2015; 22(5):838-51; Epub 2014 Nov 14; PMID:25394490; http://dx.doi.org/10.1038/cdd.2014.179

[22] Buttrick GP, Wakefield JG. PI3-K and GSK-3: Acting together with microtubules. Cell Cycle 2008; 7 (17):2621-5; PMID:18728390; http://dx.doi.org/10.4161/cc.7.17.6514

[23] Cartelli D, Ronchi C, Maggioni MG, Rodighiero S, Giavini E, Cappelletti G. Microtubule dysfunction precedes transport impairment and mitochondria damage in MPP+-induced neurodegeneration. J Neurochem 2010; 115:247-58; PMID:20649848; http://dx.doi.org/10.1111/j.1471-4159.2010.06924.x