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

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ABSTRACT

The partitioning between tubulin dimers and microtubules is fundamental for the regulation of several neuronal activities, from neuronal polarization and processes extension to growth cone remodelling. This phenomenon is modulated by several proteins, including the well-known microtubule destabilizer Stathmin. We recently demonstrated that α-Synuclein, a presynaptic protein associated to Parkinson's disease, shares structural and functional properties with Stathmin, and we showed that α-Synuclein acts as a foldable dynamase. Here, we pinpoint the impact of wild type α-Synuclein on the partitioning between tubulin dimers and microtubules and show that Parkinson's disease-linked mutants lose this capability. Thus, our results indicate a new role for α-Synuclein in regulating microtubule system and support the concept that microtubules and α-Synuclein are partners in the modulation of neuronal health and degenerative processes. Furthermore, these data strengthen our hypothesis of the existence of a functional redundancy between α-Synuclein and Stathmin.

α-Synuclein (Syn) is a presynaptic unfolded protein,[1] 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 showed that Syn modulates actin assembly,[2] but controversial evidence has been published about the interaction between Syn and microtubules (MTs). Indeed, Alim and colleagues showed that wild type (WT) Syn promotes MT assembly[3] whereas Chen and colleagues claimed that neither monomeric nor oligomeric Syn influences MT polymerization in vitro.[4] Very recently, we cleared the effects of WT Syn and PD-linked mutants on the MT system.[5] 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.[5] 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.[6] Accordingly, STMN1 loss resulted in higher MT mass and in increased nucleation rate from centrosomes but little changes in MT dynamics.[7] Noteworthy, mutations of STMN1 lead to loss of axonal MT integrity and to several neurological phenotypes in Drosophila,[8] as well as reduced levels of STMN1 are associated to MT instability and to neurological disorders like Down syndrome and Alzheimer's disease.[9]

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

where it is highly expressed.[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;[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[12] and for which Syn has shown a high affinity.[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,[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[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[13] and Syn[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[15] and STMN1 knockout[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,[17] whereas Syn is much more confined to the distal part of the axons,[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.[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.[19] Furthermore, in human brain cytosol, they interact each other in a tubulin concentration-dependent manner,[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 (GSK3β), a well-known regulator of MT assembly throughout the modulation of MT interacting proteins.[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.[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,[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.
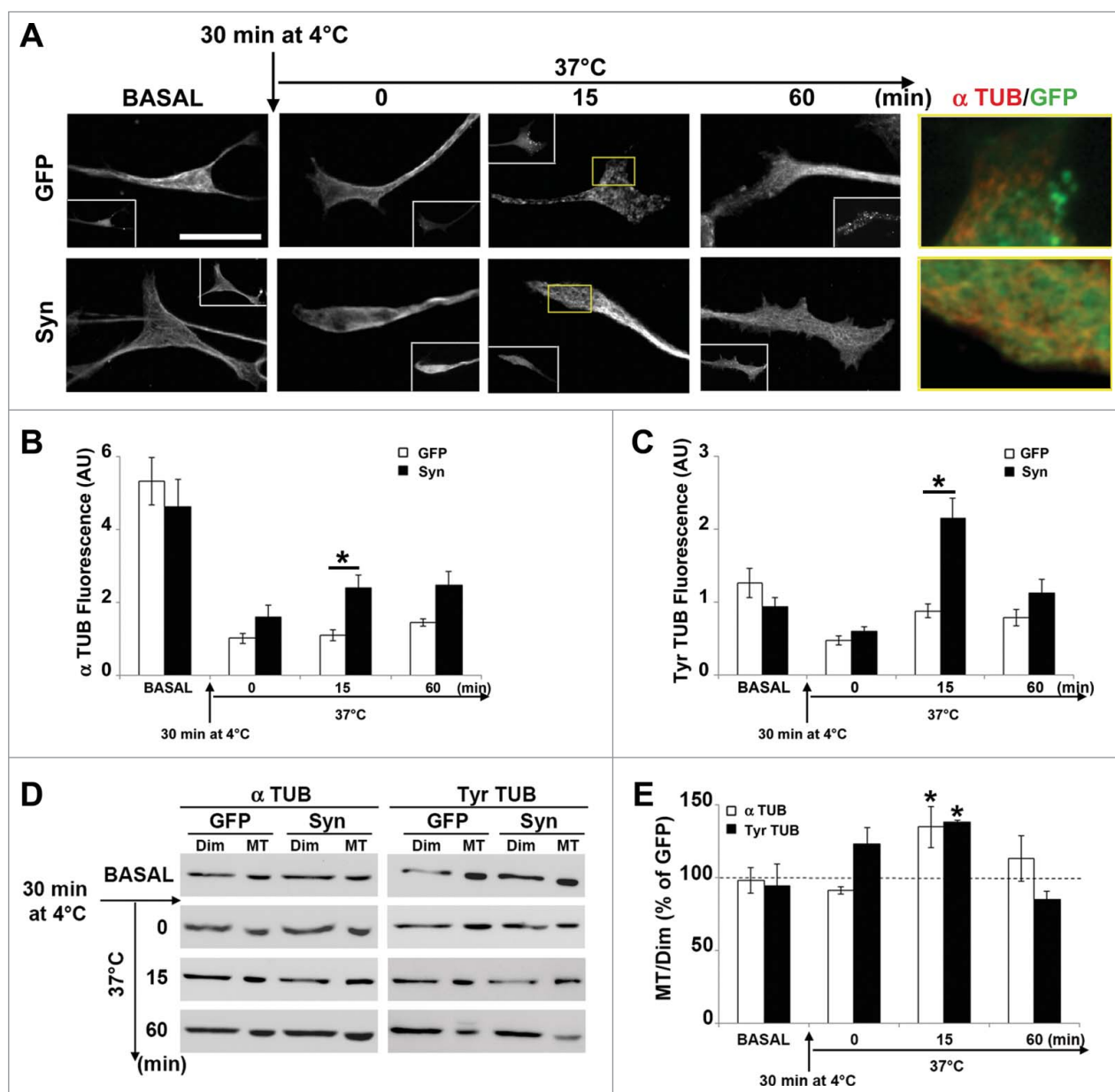
Figure 1. WT Syn regulates the partitioning between tubulin dimers and MTs in differentiated PC12 cells. (A) Fluorescence microscopy micrographs of PC12 cells differentiated 5 d with NGF expressing GFP-Syn chimera (Syn) or GFP control vector (GFP), fixed before (BASAL), immediately after the MT destabilizing 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 $\pm$ 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 $\pm$ 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.

Material and methods

Cell cultures and transfection

PC12 cells were maintained in cultures and differentiated as previously described.[5] 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
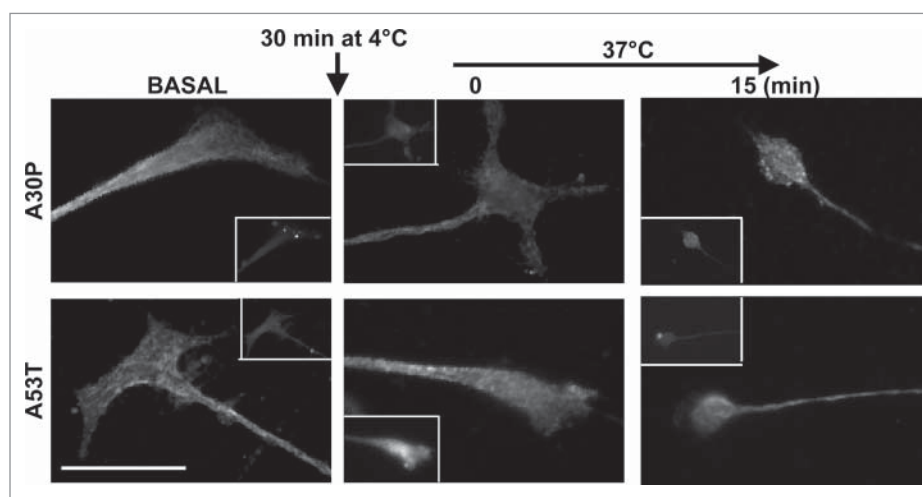
Figure 2. Pathological Syn mutations corrupt the partitioning tubulin dimers and MTs. Fluorescent microscopy photographs of PC12 cells differentiated 5 d with NGF expressing mutated (A30P and A53T) GFP-Syn chimeras, fixed before (BASAL), immediately after the MT destabilizing cold-treatment, 30 min at 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.

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 $MgCl_2$, 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).

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