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Dimerization propensities of Synucleins are not predictive for Synuclein aggregation

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ABSTRACT

Aggregation and fibril formation of human alpha-Synuclein (αS) are neuropathological hallmarks of Parkinson's disease and other synucleinopathies. The molecular mechanisms of αS aggregation and fibrillogenesis are largely unknown. Several studies suggested a sequence of events from αS dimerization via oligomerization and pre-fibrillar aggregation to αS fibril formation. In contrast to αS, little evidence suggests that γS can form protein aggregates in the brain, and for βS its neurotoxic properties and aggregation propensities are controversially discussed. These apparent differences in aggregation behavior prompted us to investigate the first step in Synuclein aggregation, *i.e.* the formation of dimers or oligomers, by Bimolecular Fluorescence Complementation in cells. This assay showed some Synuclein-specific limitations, questioning its performance on a single cell level. Nevertheless, we unequivocally demonstrate that all Synucleins can interact with each other in a very similar way. Given the divergent aggregation properties of the three Synucleins this suggests that formation of dimers is not predictive for the aggregation of αS, βS or γS in the aged or diseased brain.

1. Introduction

Since the discovery of alpha-Synuclein (αS) as main constituent of the Lewy body in 1997 [1], this protein has been put under the spotlight of intensive investigation in the field of neurodegenerative diseases. αS is highly abundant in the mammalian brain, with subcellular localization in the nucleus, presynaptic terminals and mitochondria of neurons [2,3]. Its physiological role is still enigmatic, but a multitude of putative functions have been suggested, *i.e.* involvement in vesicle regulation (*e.g.* vesicle biogenesis, trafficking, and fusion), chaperone activity for synaptic proteins, and modulation of synaptic transmission [4].

In solution αS appears to be a protein without ordered tertiary structure [5], but it forms distinct alpha-helical structures upon membrane binding [6,7], and β-sheets during aggregation processes [8,9]. Fibril formation appears to take place via intermediate states of dimers, oligomers, and protofibrils, finally resulting in the formation of Lewy bodies, which are the pathological hallmark of a variety of synucleinopathies including Parkinson's disease [10]. Whether αS physiologically exists as a monomer or adopts a stable tetrameric state is controversially discussed [11,12]. Increases in αS protein levels which are observed in genomic multiplications of the αS gene (SNCA) were suggested to result in abnormal aggregate formation and neuronal degeneration *in vitro* [13–15] and *in vivo* [16,17]. Human αS wild type and disease-associated αS mutants readily assemble into filaments *in vitro* [18] and disease-associated mutants of αS show accelerated aggregation and fibril formation *in vitro* [19,20].

The Synuclein protein family consists of two further proteins very closely related to αS, with also unresolved physiological functions. Beta-Synuclein (βS) is an abundant protein in mammalian CNS, while gamma-Synuclein (γS) is predominantly expressed in PNS. Comparison of the aggregation propensities of αS, βS, and γS revealed that αS forms fibrils most rapidly whereas βS and γS fibrillize much slower or not at all under the same experimental conditions *in vitro* [8,21,22]. These differences were attributed to structural characteristics of the Synuclein isoforms, in particular the hydrophobic NAC domain in αS which was proposed to be intimately involved in αS aggregation processes [23–25]. βS lacks most part of the NAC domain which was suggested to slowdown its aggregation and to cause βS interference with αS fibril formation [26,27,21]. γS, although structurally similar to αS, was proposed to be protected from aggregation due to increased structural stability in the amyloid forming region [28]. However, posttranslational modifications, *e.g.* oxidation, seem to drive oligomerization and aggregation of αS *in vitro* [4] and similar observations were made for γS [29].

Lewy bodies and Lewy neurites are immunopositive for αS [1]. Although some extranigral neuritic pathology was shown, human βS and γS have not been detected in Lewy structures or glial cytoplasmic inclusions of multiple system atrophy so far [30–32]. The general consensus in the field is that aggregation is a main pathogenic feature of

Abbreviations: αS, alpha-Synuclein; βS, beta-Synuclein; γS, gamma-Synuclein; BiFC, bimolecular fluorescence complementation; VN, N-terminal part of Venus; VC, C-terminal part of Venus; CTCF, corrected total cell fluorescence

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human αS. Aggregation of αS was shown in post-mortem brain [33], in animal models using transgene expression [34] or viral delivery [35], and in cellular models [13,36]. Some reports also point to a role of endogenous αS, βS, and γS which hampers the interpretation of results from animal studies [37–40]. Wild-type βS has been found to be protective in various settings against αS-mediated neurodegeneration [26,27,41]. However, recent evidence was provided for proteinase K-resistant human wild type βS aggregates and toxicity in dopaminergic neurons *in vivo* upon overexpression [42]. Interestingly, expression of human βS with a point mutation that was described in rare cases with DLB induced neurodegeneration in a transgenic mouse model of DLB [43,44]. Very recently, pathological aggregation of human γS was suggested to contribute to the pathogenesis of ALS [32].

The evident differences in protein domain structure, aggregation behavior and toxicity among the three family members αS, βS, and γS prompted us to investigate the initial step in aggregation, namely protein dimerization, using the Bimolecular Fluorescence Complementation (BiFC) technique. In the BiFC system, expression of two non-fluorescent fragments of a fluorophore fused to potential interaction partners was used to visualize the location of protein interactions in living cells [45,46]. BiFC was primarily applied for the investigation of heterodimeric protein interactions. Subsequently, several studies employed this technique also for the analysis of protein homodimerization and oligomerization [14,47]. Initial studies used the fluorophore GFP for BiFC. Later, YFP/mVenus, CFP and mCherry, served as alternative fluorophores, and were used in multicolor BiFC to enable the visualization of interactions between different proteins in the same cell and comparison of the efficiencies of complex formation with alternative interaction partners [48]. In the current study we show that the BiFC assay shows some Synuclein-specific limitations, but nonetheless allowed to demonstrate that all Synucleins show almost identical dimerization propensities, suggesting that Synuclein dimerization is not predictive for eventual Synuclein aggregation.

2. Material and methods

2.1. Bimolecular Fluorescence Complementation (BiFC)

The BiFC technique in the current study is based on the expression of two non-fluorescent fragments of the Venus fluorophore fused to human Synuclein isoforms α, β, and γ in order to visualize the localization of protein dimerization in cells. To that end, Venus cDNA was amplified by PCR to generate the N-terminal (VN) and C-terminal (VC) parts of the fluorophore. VN was introduced via NheI and AflII restriction sites, VC via XhoI and XbaI restriction sites into the pcDNA3.1 vector (Life Technologies, Carlsbad, CA, USA) to generate VN and VC controls. Synuclein cDNA was introduced 3′ terminally of VN via AflII and XhoI restriction sites including a linker of 36 nucleotides between VN and Synuclein to generate plasmids encoding VN-αS, VN-βS, and VN-γS. The linker encodes for a glycine-rich sequence that serves as flexible tether between the C-terminus of the VN fragment and the N-terminus of Synuclein. Synuclein cDNA was introduced 5′ terminally of VC via AflII and XhoI restriction sites to generate αS-VC, βS-VC, and γS-VC. Similarly, a small linker between the C-terminus of Synuclein and the N-terminus of the VC fragment provides flexibility of the two segments for fluorophore assembly as well as dimer formation. VC was excised via XhoI and XbaI restriction sites from the αS-VC plasmid, and the vector was blunted and religated to generate untagged αS control.

Cells were transfected with the different BiFC constructs and the internal control pDsRed2-Mito (BD Biosciences, Franklin Lakes, NJ, USA) as described in Section 2.2. Cells were fixed and stained 36 h post-transfection (Section 2.4). For all quantifications in the current study, images were acquired under non-saturating conditions using an Axioplan 2 equipped with Plan Neofluar 10×/0.30 Ph1, AxioCam HRm camera and AxioVision SE64 Rel. 4.9 software (Carl Zeiss, Jena, Germany). Images were analyzed using ImageJ 1.45 s software. Corrected total cell fluorescence (CTCF) was calculated as *Integrated Density of selected cells* subtracted by the multiplication of *Area of selected cells* and *Mean fluorescence of background readings* [49,50]. CTCF of the complemented Venus fluorescence (BiFC) was referred to the CTCF of the internal control DsRed2 in order to exclude artifacts from differences in transfection efficiency, protein expression levels, and cell coverage. For each condition, the area (901 μm × 675 μm) of approximately 200 transfected cells per image from 10 images per experimental condition was quantified. Statistical analysis was carried out by one-way ANOVA followed by Tukey's post hoc test from N ≥ 3 independent experiments.

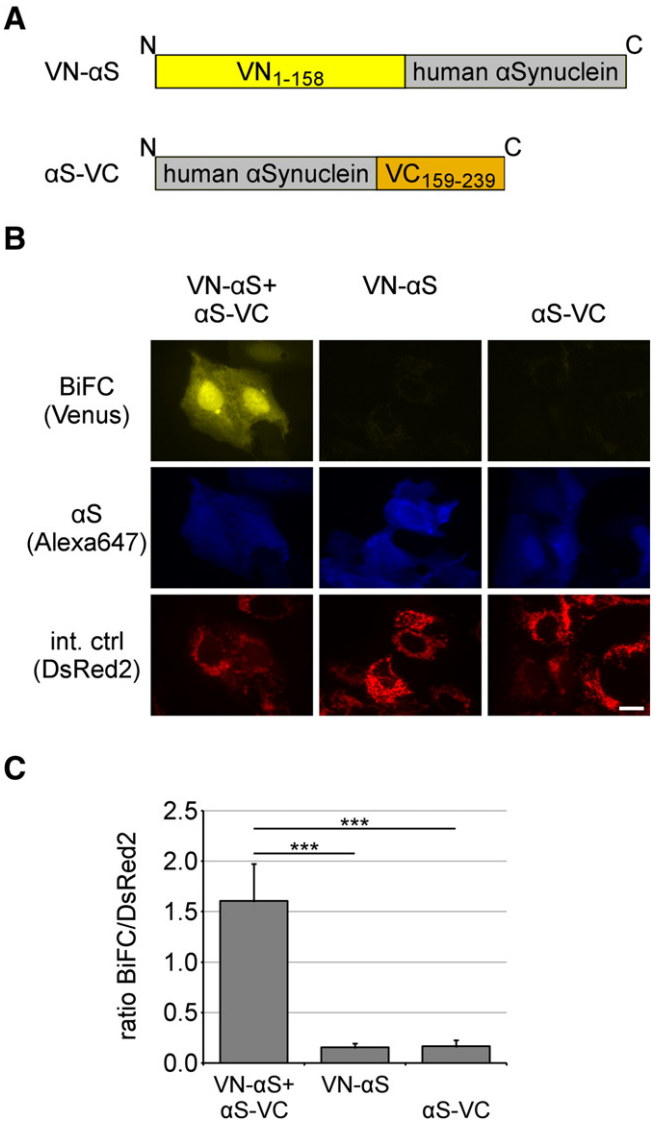

Fig. 1. Generation of cellular models based on Bimolecular Fluorescence Complementation (BiFC). (A) Schematic diagram of Venus-based BiFC constructs. VN-αS, N-terminal part of Venus (VN, amino acids 1 to 158) fused to the N-terminus of full-length human alpha-Synuclein (αS). αS-VC, C-terminal part of Venus (VC, amino acids 159 to 239) fused to the C-terminus of full-length αS allowing reconstitution of Venus fluorescence in case of antiparallel orientation of the two αS molecules. (B) Transfection of HEK293 cells with the indicated constructs and DsRed2-mito as internal control. ICC with αS-specific primary antibody and Alexa647-conjugated secondary antibody. Representative images show the complemented Venus signal (BiFC), αS protein (Alexa 647), and the internal control (DsRed2). Scale bar 20 μm. (C) Determination of the corrected total cell fluorescence (CTCF) of the BiFC signal *versus* internal control DsRed2 for all samples from 10 images of N ≥ 5 independent experiments. The BIFC signal in VN-αS + αS-VC is significantly higher than in the controls. Mean values ± SD; ***, P < 0.001.