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Comparative study of the effect of selected mutations on alpha-synuclein oligomerization in living cells using Bimolecular Fluorescence Complementation (BiFC)

Eva Sofia Ferreira Rodrigues

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica do Professor Doutor Tiago Fleming Outeiro (Universidade de Medicina de Göttingen) e do responsável académico no Departamento de Ciências da Vida, Professora Doutora Emília Duarte (Universidade de Coimbra)

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Abbreviations

α-syn – Alpha-synuclein

AD – Autosomal dominant

AR – Autosomal recessive

BiFC – Bimolecular fluorescence complementation

CK – Casein kinase

DA – Dopamine

DAQ – Dopamine Quinone

GRK – G-protein receptor kinase

LBs – Lewy bodies

NAC – Non-Abeta component

PD – Parkinson’s disease

PLD2 – Phospholipase D2

PLK – Polo-like kinase

PTK – Protein-tyrosine kinase

PTMs – Post-translational modifications

ROS – Reactive oxygen species

SNpc – *Substantia nigra pars compacta*

SUMO – Small ubiquitin-like modifier

TH – Tyrosine hydroxylase

TP – Triple proline (A30P/A56P/A76P)

Ub – Ubiquitin

UPS – Ubiquitin-proteasome system

VMAT2 – Vesicular monoamine transporter 2

WT – Wild-type

Resumo

A doença de Parkinson (DP) é a segunda doença neurodegenerativa mais comum que afecta cerca de 2% da população acima dos 60 anos de idade. Patologicamente é caracterizada pela perda progressiva de neurónios dopaminergicos na *substantia nigra* e pela presença de inclusões proteicas designadas por corpos de *Lewy*. Os corpos de *Lewy* são predominantemente compostos por fibrilhas de alfa-sinucleína. Várias mutações genéticas têm sido identificadas e implicadas no começo e desenvolvimento da DP. Mutações no gene da alfa-sinucleína são responsáveis por formas familiares dominantes da DP, demonstrando um papel importante desta proteína na progressão da neurodegeneração. Estudos mais recentes indicam que as espécies oligomericas de alfa-sinucleína, possíveis espécies intermediárias que precedem à formação de inclusões, são mais tóxicas que as espécies fibrilhares. No entanto, ainda pouco é conhecido relativamente aos determinantes moleculares da oligomerização em doenças neurodegenerativas. O estudo de interações proteína-proteína é crucial para perceber os determinantes moleculares da oligomerização da alfa-sinucleína na DP bem como a sua distribuição pela célula. Para este efeito neste estudo geramos mutações pontuais na alfa-sinucleína e investigámos o seu comportamento em células vivas através da técnica complementação de fluorescência bimolecular. Esta técnica permite analizar *in vivo* a dimerização/oligomerização da proteína de interesse através da reconstituição de fluorescência, permitindo-nos avaliar o efeito das mutações na oligomerização, atravez do aumento ou diminuição de fluorescência. Os resultados obtidos mostram que embora a maioria dos mutantes tenham mostrado um aumento na formação de oligómeros comparando com a alfa-sinucleína *wild-type*, o maior aumento de oligomerização foi observado precisamente nos mutantes conhecidos por

terem uma maior propensão para formar oligómeros e uma reduzida abilidade para formar fibrilhas. Assim, o uso desta poderosa técnica permitir-nos-á investigar a natureza das espécies oligoméricas de alfa-sinucleína na presença de várias mutações, bem como conduzir aos mecanismos moleculares subjacentes à neurodegeneração. Por último, este conhecimento será essencial para o desenvolvimento de novas estratégias terapêuticas para intervenção na DP.

Abstract

Parkinson's disease (PD) is the second most common neurodegenerative disorder affecting approximately 2% of the population over 60 years of age. Pathologically is characterized by the progressive loss of nigrostriatal dopaminergic neurons and the presence of protein inclusions termed Lewy bodies within dopaminergic neurons, which are predominantly composed of fibrillar alpha-synuclein (α-syn). Although several genetic mutations have been implicated in the onset of the disease, mutations in the α-syn gene are responsible for familial forms of PD demonstrating a crucial role for α-syn on the progression of neurodegeneration. Recently, it has been suggested that oligomers, possible intermediary species thought to precede fibrillar inclusions, are more toxic than the α-syn fibrils. However little is still known about the molecular determinants of oligomerization in neurodegenerative diseases. The study of protein-protein interactions is crucial for understanding the molecular determinants of oligomerization in PD and their distribution throughout the cell. For this purpose we generated selected mutations on α-syn and investigated their behavior in living cells through bimolecular fluorescence complementation (BiFC) assay. Since the BiFC stabilizes the interaction between the protein of interest upon the reconstitution of the fluorescence moiety, it allowed us to determine whether the formation of oligomers by the selected mutations are increased or decreased compared with wild type (WT) α-syn. Although almost all the α-syn mutants showed an increase formation of oligomers compared with WT, the strongest increase of oligomerization was observed with α-syn mutants known to have an increased propensity to form oligomers and a reduced ability to form fibrils. Thus, the use of this powerful assay will allow us to investigate the nature of the α-syn oligomeric species and to shed light into the

molecular mechanism underlying neurodegeneration. Ultimately, this knowledge will be essential for the development of novel therapeutic strategies for intervention in PD.

Keywords: alpha-synuclein; oligomerization; mutations; bimolecular complementation assay.

Chapter 1. Introduction

1.1 Parkinson's Disease: clinical and pathological characteristics

Parkinson's disease (PD) is a movement disorder that affects approximately 2% of the population over 60 years of age (de Lau and Breteler, 2006). First described in 1817 by James Parkinson in his "An essay on the Shaking Palsy", this chronic and irreversible neurodegenerative disease is clinically characterized by postural rigidity, resting tremor, postural instability and bradykinesia (Moore et al., 2005). These symptoms result from a progressive loss of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc), which promotes a depletion of striatal dopamine (DA) and subsequently signaling disruption in the posterior putamen and motor circuit (Rodriguez-Oroz et al., 2009). This loss of dopaminergic neurons is observed by depigmentation in the SNpc (Figure 1) (Chinta and Andersen, 2005; Dauer and Przedborski, 2003; Girault and Greengard, 2004; Korchounov et al., 2010).

During the disease, patients also suffer cognitive deficits and psychiatric problems due to the degeneration of wider neuronal circuits (Rodriguez-Oroz et al., 2009).

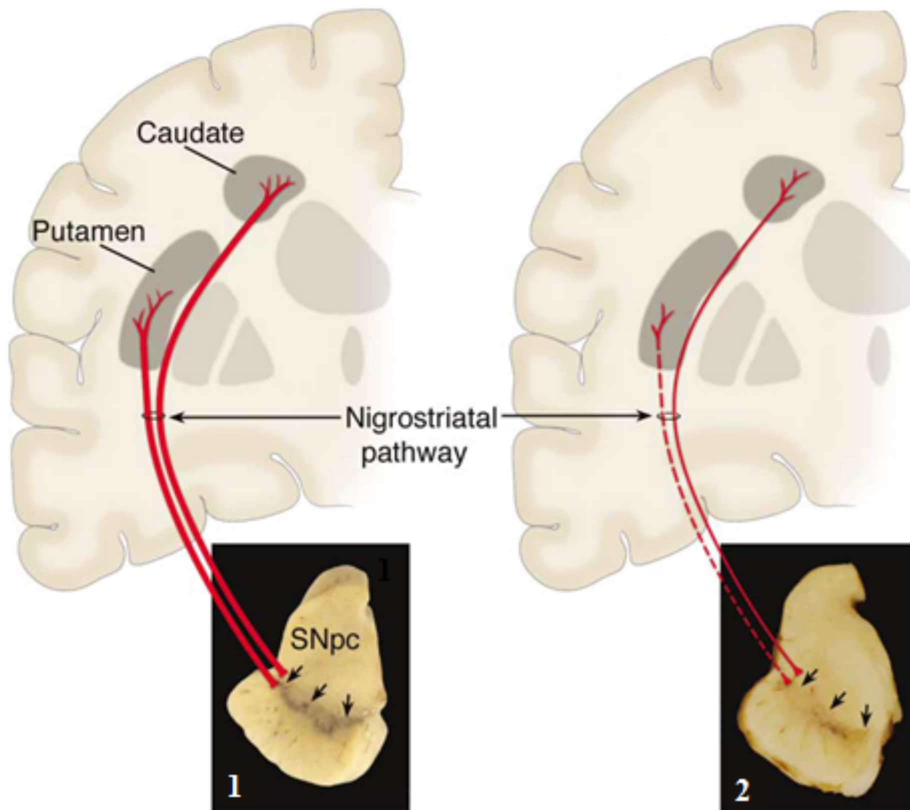


Figure 1. Schematic representation of nigrostriatal pathway that degenerates in PD.

(A) In normal nigrostriatal pathway, dopaminergic neurons of SNpc have a dark-brown pigmentation (1) and project to the putamen and caudate nucleus. (B) In PD, degeneration of the nigrostriatal pathway occurs due to progressive loss of dopaminergic neurons in the SNpc (2). Figure adapted from (Dauer and Przedborski, 2003).

Together with dopaminergic cell loss, PD is also pathologically characterized by the presence of neuronal intracellular inclusions termed Lewy Bodies (LBs) (Figure 2), mainly composed by alpha-synuclein (α-syn) and other proteins such as ubiquitin (Ub), synphilin-1, 14-3-3 protein (Goedert, 2001; Maries et al., 2003; Mezey et al., 1998; Spillantini et al., 1998; Weintraub et al., 2008).

LBs are a common pathological feature of several neurodegenerative disorders designed as *synucleinopathies* including PD, dementia with LBs and multiple system atrophy (Fink, 2006; Galvin et al., 2001; Mezey et al., 1998; Spillantini et al., 1998).

LBs are also found in other affected brain areas besides the SNpc, such as the locus coeruleus, the dorsal motor nucleus of the vagus, basal nucleus of Meynert and peripheral catecholaminergic neurons (Fahn and Sulzer, 2004; Mezey et al., 1998). Although their role is still unknown, the presence of these inclusions in the surviving neurons may represent a protective mechanism of the cells by the accumulation of misfolded and dysfunctional proteins that can be toxic and responsible for neurodegeneration (Chandra et al., 2005; Outeiro et al., 2008; Tanaka et al., 2004; Tompkins and Hill, 1997).

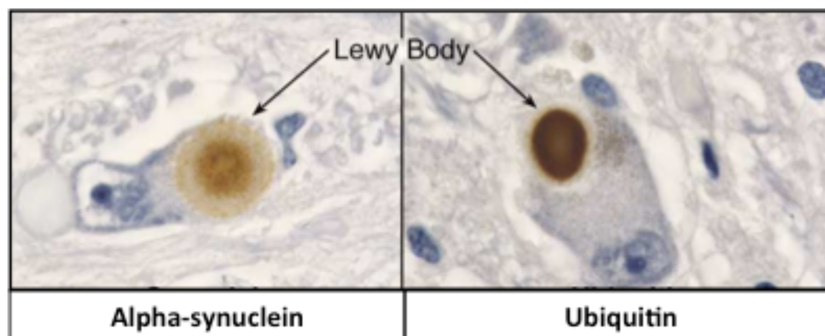


Figure 2. Lewy Bodies in PD human brain tissue.
Immunohistochemistry for α-syn (left) and Ub (right) shows the presence of LBs in the SNpc. Figure from (Dauer and Przedborski, 2003).

1.1.1 PD: A sporadic and familial disease

Most PD cases are idiopathic and have complex etiology. Both genetics and environmental factors play an important role in the disease onset. In both sporadic and familial PD, the development of the disease has been associated with protein misfolding, mitochondrial dysfunction, oxidative stress and ubiquitin-proteasome system (UPS) dysfunction, (Auluck et al., 2010; Dawson and Dawson, 2003).

Aging is the major risk factor for sporadic PD and is associated with increasing levels of α-syn misfolding and aggregation (Dawson and Dawson, 2003; Moore et al., 2005). Only a small percentage (±10%) of PD cases have a familiar genetic origin that is linked to genetic mutations.

In 1997 the first PD-associated mutation was identified in the SNCA gene, encoding α-syn. Currently, three point mutations in SNCA have been identified (A30P, A53T and E46K) (Kruger et al., 1998; Polymeropoulos et al., 1997; Zarranz et al., 2004). In addition, gene duplications and triplications of the SNCA gene were associated with rare familial PD cases (Chartier-Harlin et al., 2004; Farrer et al., 2004; Ibanez et al., 2004; Singleton et al., 2003).

While mutations in the SNCA and LRRK2 genes result in autosomal-dominant forms of PD, mutations in DJ1, PINK1 and ATP13A2, among others genes, result in an autosomal-recessive forms of PD (Table 1).

Table 1. Known genetic loci and genes linked with PD

Locus	Gene	Inheritance	References
PARK1	*SNCA*	AD	(Polymeropoulos et al., 1997)
PARK2	*PRKN*	AR	(Bonifati, 2012)
PARK3	Unknown	AD	(Pankratz et al., 1993)
PARK4	*SNCA*	AD	(Singleton et al., 2003)
PARK5	*UCHL1*	AD	(Leroy et al., 1998)
PARK6	*PINK1*	AR	(Valente et al., 2004)
PARK7	*DJ-1*	AR	(Bonifati et al., 2003)
PARK8	*LRRK2*	AD	(Zimprich et al., 2004)
PARK9	*ATP13A2*	AR	(Ramirez et al., 2006)
PARK11	*GIGYF2*	AD	(Lautier et al., 2008)
PARK13	*OMI/HTRA2*	AD?	(Strauss et al., 2005)
PARK14	*PLAG2G6*	AR	(Paisan-Ruiz et al., 2009)
PARK15	*FBXO7*	AR	(Di Fonzo et al., 2009)
PARK16	*Unknown*	AD	(Satake et al., 2009; Tan et al., 2010)

Abbreviations: AD - autosomal dominant; **AR** - autosomal recessive

1.2 Structure and localization of α-syn

α-syn is a small and highly conserved protein of 140 amino acids and a molecular weight of 14.5 kDa that, together with β-synuclein and γ-synuclein, completes the synuclein family (Lucking and Brice, 2000). α-syn was first isolated from the electric fish *Torpedo californica* using antiserum against synaptic vesicles. The protein acquired this name due to its localization at the nuclear envelope neurons and at presynaptic nerve terminals (Cheng et al., 2011; Lavedan, 1998; Maroteaux et al., 1988; Moore et al., 2005). α-syn is expressed in the brain, more specifically in the neocortex, hippocampus, olfactory bulb, nigrostriatal pathway, thalamus and cerebellum (Brooks, 2010).

The SNCA gene is located on chromosome 4q21-q22 and the coding region consists in five of the seven exons that comprise the gene (Bekris et al., 2010; Spillantini et al., 1995). In aqueous solution, α-syn has been described as a natively unfolded protein that can adopt α-helical structure upon binding to phospholipid vesicles. Indeed, α-syn can adopt different conformations or remains unstructured depending on the environment such as low pH, increase in temperature, increase in concentration and time-dependent manner (Auluck et al., 2010; Davidson et al., 1998; Moore et al., 2005). Recently, this notion was challenged by a study that demonstrated that α-syn is present as a helical folded tetramer when isolated under non-denaturing conditions. The native tetramers also showed a lower aggregation capacity and based on this, the destabilization of the native helical tetramer precedes α-syn misfolding and aggregation (Bartels et al., 2011; Wang et al., 2011). Structurally, the sequence of human α-syn can be divided into three distinct regions:

the N-terminal amphipathic region (residues 1–65), the central hydrophobic NAC (non-Abeta component) region (residues 66–95), and the C-terminal acidic region (residues 96–140) (Figure 3). The N-terminal half of α-syn contains seven 11-amino acid repeats with a highly conserved motif KTKEGV. This domain is responsible for protein-protein interactions and for forming structural apolipoprotein-like class A2 amphipathic α-helical by binding of α-syn to phospholipid vesicles (Davidson 1998). The central hydrophobic NAC region of α-syn is highly amyloidogenic and this confers the ability to undergo a conformational change from random coil to β-sheet structure, protofibrils and fibrils. The acidic C-terminal region remains unfolded and does not associate with vesicles. This region contains serine 129 (S129) and tyrosine 125 (Y125) residues that can play a role in α-syn fibrillization. (Davidson et al., 1998; Dev et al., 2003; Eliezer et al., 2001; Ulrih et al., 2008; Venda et al., 2010; Weinreb et al., 1996).

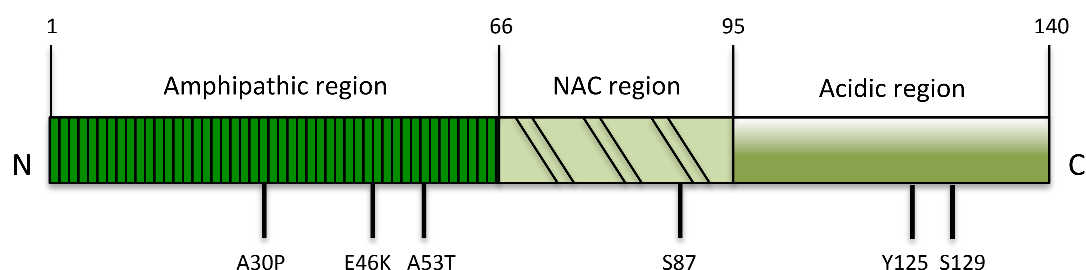


Figure 3. Schematic representation of α-syn domains.

The N-terminal region contains the three point mutations that cause familial PD. The NAC region is associated with increase propensity of α-syn aggregation. Phosphorylation site at Serine 87 (S87) is located in NAC region and phosphorylation sites at S129 and Y125 are located in acidic C-terminal region.

1.3 Proposed functions for α-syn

The normal function of α-syn is poorly understood, however several hypothesis have been proposed and suggest a role in presynaptic nerve terminals where it associates with presynaptic vesicles and membranes (Abeliovich et al., 2000; Maroteaux et al., 1988). Indeed, reduction of α-syn decreases the presynaptic vesicles in the reserve pool, suggesting that under normal conditions α-syn regulates the vesicle recycling at presynaptic terminals, including trafficking, docking, fusion and recycling after exocytosis (Abeliovich et al., 2000; Ben Gedalya et al., 2009; Cabin et al., 2002; Fortin et al., 2004; Kahle et al., 2000; Murphy et al., 2000) .

Through the interaction with proteins at presynaptic terminals α-syn regulates the vesicle recycling and DA homeostasis. This is supported by *in vitro* observations that showed a capability of α-syn to bind to vesicle phospholipid membranes and also to phospholipase D2 (PLD2) inhibiting its activity resulting in vesicle trafficking impairment. PDL2 is an enzyme involved in lipid-mediated signaling cascades, vesicle trafficking and endocytosis (Lotharius and Brundin, 2002; Outeiro and Lindquist, 2003).

DA has been described as an important neurotransmitter involved in movement control due to the association between the depletion of striatal DA and motor deficits observed in PD.

Synthesis of DA is limited by the activity of enzyme tyrosine hydroxylase (TH). Overexpression of α-syn in cells showed a significantly reduced TH activity and DA synthesis. These suggest that α-syn plays a role in the regulation of DA biosynthesis,

since it interacts with TH reducing phosphorylation and inhibiting its activation (Alerte et al., 2008; Perez et al., 2002).

Since DA is easily oxidized in the cytoplasm (Chinta and Andersen, 2005), during the synthesis it is stored in vesicles by the action of the vesicular monoamine transporter 2 (VMAT2). Impaired DA storage lead to its accumulation in the cytoplasm generates reactive oxygen species (ROS) promoting oxidative stress and other mechanisms which can trigger nigral dopaminergic cell death that are present in PD. Besides TH, α-syn also interacts with specific dopaminergic markers such as VMAT2, leading to the accumulation of DA in the cytosol (Lotharius et al., 2002).

Several studies using dopaminergic models showed that a deletion of α-syn reduces vesicles in distal reserve pool and increases DA release to synaptic cleft, and, on the other hand, α-syn mutants overexpression decrease the release of DA. Due to the reduction of synaptic vesicle recycle exocytosis, it was proposed that α-syn can work as an actively-dependent negative regulator of DA release during synaptic transmission (Abeliovich et al., 2000; Fortin et al., 2004; Lotharius and Brundin, 2002).

Among other symptoms, patients with PD can also suffer cognitive disturbances, mostly related with learn difficulty and memory. Thus, it has been postulated that α-syn might also have a role in synaptic plasticity modulating long-term potentiation and long-term depression (Cheng et al., 2011).

Mitochondrial dysfunction has been associated with pathogenesis of PD because α-syn accumulation inhibits mitochondrial complex I and increases production of ROS. Inhibition of complex I leads to the opening of mitochondrial permeability transition pore, releasing cytochrome c which induces caspase activity leading to apoptotic cell death (Esteves et al., 2011; Orth and Schapira, 2002; Schapira, 1999).

1.4 Oligomerization and aggregation of α-syn

The aggregation progress of α-syn into LBs is believed to play a crucial role in the pathogenesis of PD. The role of LBs in PD pathogenesis has been linked with toxicity in dopaminergic neurons however, *in vitro* studies evidenced that inclusion formation may act as a protective mechanism since it reduces the toxic effects promoted by α-syn oligomeric (Chandra et al., 2005; Kazantsev and Kolchinsky, 2008; Tanaka et al., 2004; Tompkins and Hill, 1997). The formation of α-syn inclusions has been reported in several models of PD such as cell cultures, *Drosophila melanogaster,* worms and mice (Feany and Bender, 2000; Karpinar et al., 2009; Winner et al., 2011). However, the exact role of α-syn aggregation on the progress of neurodegeneration remains controversial and uncertain (Bellucci et al., 2012).

When proteins do not adopt proper structure and function they are recognized and targeted to degradation by the cellular quality-control systems. Protein aggregation is also associated with defects in UPS and autophagy, which are unable to effectively degrade misfolded proteins. The accumulation of this misfolded proteins form β-sheet structures that can lead to neurodegeneration (Ross and Pickart, 2004).

α-syn aggregation, progresses from monomers to fibrils in a nucleation-dependent manner involving diverse prefibrillar species that differ in size, morphology and toxicity (Conway et al., 2000b; Fink, 2006; Wood et al., 1999) (Figure 4).

Although fibrillar aggregates of α-syn are the major component of LBs, strong evidence implicate soluble oligomers and prefibrillar species, also known as intermediate species, rather than mature fibrils as the more toxic α-syn species mediating neuronal death both *in vitro* and in animal models acting as the principal

pathogenic event in early progress of PD (Danzer et al., 2007; Karpinar et al., 2009; Kim et al., 2009; Outeiro et al., 2008; van Rooijen et al., 2008; van Rooijen et al., 2010a; van Rooijen et al., 2010b; Winner et al., 2011).

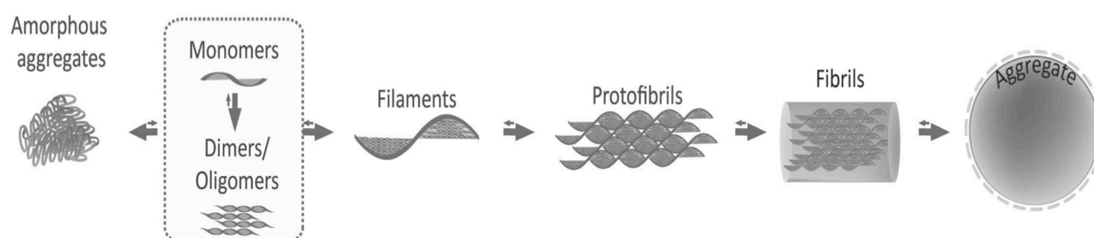


Figure 4. α-syn oligomerization and aggregation
LBs are spherical eosinophilic intracellular protein inclusions composed mainly of amyloid fibrils of α-syn in some surviving neurons. Aggregation of fibrillar α-syn into LBs progresses from unfolded monomers to fibrils through the formation oligomeric species. Figure adapted from (Goncalves et al., 2010).

In dopaminergic neurons, the cytosolic accumulation of DA generates ROS and forms DA-quinone (DAQ). DAQ interact with α-syn blocking α-syn fibril formation and lead to accumulation of the oligomeric species. In this context, the interaction of DA with α-syn induces a conformational change in α-syn, which leads to increased oligomerization (Outeiro et al., 2009).

Moreover, recent studies demonstrated that the presence of extracellular oligomers, which are secreted from neuronal cells, may represent a crucial way of α-syn propagation mediating toxicity (Danzer et al., 2007; Danzer et al., 2009; Danzer et al., 2011; Emmanouilidou et al., 2011; Mollenhauer et al., 2008).

Mutations of α-syn associated with familial PD (A53T, A30P and E46K) and molecular factors such as post-translational modifications, oxidative stress, association with lipids membranes, impairment of UPS, interaction with proteins and

with lipids and also environmental factors as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and pesticides can alter the structure of α-syn leading to accelerate fibrillization and/oligomerization (Auluck et al., 2010; Kazantsev and Kolchinsky, 2008).

1.4.1 Influence of mutations on α-syn oligomerization/aggregation

As mentioned above, point mutations in the gene encoding α-syn are associated with rare cases of autosomal dominant early-onset of PD namely.

The increased propensity of α-syn to aggregate into fibrillar inclusions is associated with a toxic gain of function, and enables molecular studies towards the understanding of the mechanisms that drive α-syn aggregation and its role in the neurodegeneration of PD (Rajagopalan and Andersen, 2001).

Since misfolded proteins can adopt different conformations, these mutations can promote different kinetics of α-syn aggregation due to their different propensities rates of oligomer and fibril formation (Conway et al., 1998; Conway et al., 2000a; Conway et al., 2000b; Li et al., 2002; Narhi et al., 1999). Furthermore, as α-syn mutants also increase the formation of oligomeric species that are considered to be more toxic than mature fibrils the oligomer-membrane interactions can disrupt membranes through the formation of pore-like structures and destabilization of bilayer integrity which result in membrane permeabilization. This may be a mechanism of cytotoxicity for neurodegeneration in PD and other neurodegenerative diseases (van Rooijen et al., 2008; van Rooijen et al., 2010a; van Rooijen et al., 2010b).

The A53T mutation extends the hydrophobic domain destabilizing the α-helical domain. This expanded hydrophobic core facilitates the protein ability to adopt the β-sheet structure, which is involved in the initial intermolecular contacts leading to the formation of oligomers (Conway et al., 1998; Conway et al., 2000a; Conway et al., 2000b; Giasson et al., 1999; Hashimoto et al., 1998; Narhi et al., 1999).

A53T and E46K mutants share increased rates of self-assembly and fibril formation (Bussell and Eliezer, 2001; Conway et al., 1998; Conway et al., 2000a; Conway et al., 2000b; Fredenburg et al., 2007; Giasson et al., 1999; Greenbaum et al., 2005) compared with both A30P mutant and WT α-syn. This is also consistent with studies showing that E46K exposes the hydrophobic surfaces for potential intermolecular interactions (Rospigliosi et al., 2009) and the A30P mutant disrupts the first α-helical domain reducing both amyloid formation and its affinity for phospholipids (Yonetani et al., 2009).

Although the three mutants display different rates of fibrillization, the formation of oligomers is enhanced by all of them in comparison to the WT α-syn (Conway et al., 1998; Conway et al., 2000a; Conway et al., 2000b; Fredenburg et al., 2007; Greenbaum et al., 2005; Lashuel et al., 2002; Outeiro et al., 2008; Rospigliosi et al., 2009). In addition A30P and A53T α-syn mutants form aggregates with faster oligomerization than WT (Karpinar et al., 2009).

To further understand the different aggregation properties and correlate the ability to form amyloid fibrils with the oligomer formation, artificial proline mutants [(A56P, A30P/A56P/A76P (TP)] were generated and verified to display an impaired propensity to fibril formation (Karpinar et al., 2009). The same effect was observed in mutants disrupting salt bridges between β-strands of α-syn (E35K and E57K)

(Winner et al., 2011), which increased the formation of oligomers when compared with WT α-syn.

1.4.2 Post-translational modifications of α-syn

Protein post-translational modifications (PTMs) play an important role in the regulation of their structure and function. These modifications lead to alterations in protein size, charge, structure and folding, which may interfere with their activity, binding affinity and hydrophobicity of proteins (Clark et al., 2005).

Given that α-syn has several residues that can be affected by PTMs characterizing the effects of PTMs on oligomerization/fibrillization may provide new insights into these molecular processes.

1.4.2.1 - Phosphorylation

Protein phosphorylation is considered the most important PTM and the most studied.

Evidence from immunohistochemical and biochemical studies demonstrated that the majority of α-syn within LBs isolated from PD patients and others synucleinopathies α-syn is phosphorylated at S129 and this PTM is believed to be intrinsically linked to PD pathogenesis (Fujiwara et al., 2002; Okochi et al., 2000; Paleologou et al., 2008). Furthermore the detection of phosphorylated S129 has been one of the main criteria to identify LBs in human brains as well as in animal models (Fujiwara et al., 2002; Okochi et al., 2000).

Besides S129, α-syn can be phosphorylated in other residues including S87 (Okochi et al., 2000; Paleologou et al., 2010; Paleologou et al., 2008; Pronin et al., 2000) and three tyrosine residues (Y125, Y133 and Y136) in the C-terminal domain (Chen et al., 2009).

The identity of kinases responsible for α-syn phosphorylation has been subject of study in order to understand its role in modulation of the physiological and pathogenic activities of α-syn. Casein kinase (CK1 and CK2) (Okochi et al., 2000), G-protein coupled receptor kinases (GRK2 and GRK5) (Pronin et al., 2000) and Polo-like kinase (PLK 1, PLK2 and PLK3) mediate α-syn phosphorylation at serine residues (Mbefo et al., 2010).

Recent studies *in vitro* demonstrated that α-syn phosphomimic S129E/D have different biophysical and aggregation properties of α-syn compared to phosphorylated WT α-syn because they do not disrupt the long-range interactions between N- and C-terminal domains that stabilize WT α-syn. These results demonstrated that phosphorylation at S129 inhibits fibril formation whereas phosphomimics S129D/S129E form fibrils similar to WT α-syn (Paleologou et al., 2008).

In a *Drosophila melanogaster* model of PD, it was shown that phosphorylation at S129 enhances α-syn neurotoxicity *in vivo*. Using α-syn phospho-mutants that mimic the un-phosphorylated (S129A) and phosphorylated (S129D, S219E) state of the protein are useful to determine the toxic effects and aggregation caused by their expression, compared with WT α-syn. The S129A phosphomimic decreased toxicity but increased the number of α-syn inclusions. In contrast, compared to overexpression of WT α-syn, the phosphomimic S129D or WT α-syn phosphorylated

by GRK2 increase the toxicity but did not increase α-syn inclusions (Chen and Feany, 2005; Oueslati et al., 2010; Paleologou et al., 2008).

In a rat model of PD, overexpression of α-syn phospho-mutants showed opposite effects namely that S129A increase toxicity whereas S129D produces no toxicity (Gorbatyuk et al., 2008).

It was proposed that phosphorylation at S129 may act as a protective strategy increasing inclusions formation to avoid α-syn toxicity (Chen and Feany 2005). In addition, α-syn phosphorylation at S129 occurs after fibril formation and/or during LBs formation since it was demonstrated that α-syn aggregates were substrates of the same kinases involved in phosphorylation of α-syn at S129 (Paleologou et al., 2010).

Although the mechanism of neurotoxicity remains unclear, these findings suggest that phosphorylation play a role in modulation of α-syn aggregation, LBs formation and toxicity in vivo (Paleologou et al., 2008)

A recent study showed that in synucleinophaties, S87, located in NAC region of α-syn (El-Agnaf et al., 1998a), is phosphorylated by CK1 and inhibits oligomerization. S87 phosphorylation induces conformational change in lipid-biding affinity of α-syn that alters its aggregation (Paleologou et al., 2010).

Phosphorylation of α-syn at tyrosine residues is mediated by the Src family of protein-tyrosine kinase (PTKs), which includes c-Src and Fyn (Ellis et al., 2001), as well as $p72^{syk}$ (Syk) (Negro et al., 2002).

PTKs play a role in regulation of signal transduction pathways and regulate cell growth, differentiation and synaptic function. Phosphorylation of α-syn at tyrosine 125 has been the most reported and has different effects (Ellis et al., 2001; Nakamura

et al., 2001). More recently, in was described that phosphorylation of α-syn at Y125 by Syk has an impact on α-syn oligomerization preventing it (Negro et al., 2002).

Furthermore, phosphorylation of α-syn at Y125 decreases oligomeric species and during aging this phosphorylation is reduced once LBs from patients with PD showed less Y125 phosphorylated (Chen et al., 2009).

1.4.2.2 – UPS and SUMOylation of a-syn

The UPS is the major intracellular system for degradation of misfolded and damaged proteins that cannot be properly folded by molecular chaperones.

The impairment of UPS has been considered one of the mechanisms that leads to accumulation of misfolded α-syn, which increases fibrillization and LB formation.

Recent studies demonstrate that soluble oligomers are targeted to 26S proteasome and inhibit it, supporting the interaction between impaired proteasome activity and aggregated α-syn (Emmanouilidou et al., 2010; Kim et al., 2011).

Besides ubiquitination, another PTM found in α-syn is SUMOylation, a process that shares similarities with ubiquitination. The small ubiquitin-like modifier (SUMO) proteins are covalently linked to its subtracts regulating both protein functions and cellular processes (Ross and Pickart, 2004).

SUMOylation represents an important PTM that has a number of functional consequences for the target proteins due to its highly dynamic and reversible process as specific proteases that can rapidly remove SUMO from their substrates. Indeed, SUMOylation controls protein-protein interactions, affects subcellular localization,

stability and solubility of target proteins. (Dorval and Fraser, 2006; Krumova et al., 2011).

SUMO proteins display similarities to Ub in both the structure and the biochemistry of their conjugation and, in some cases may antagonize the proteasome pathway by competing with Ub (Dorval and Fraser, 2006).

Although SUMOylation has been traditionally considered as a nuclear process, proteomic studies revealed that a large fraction of cytosolic proteins is targeted for SUMO modification (Dorval and Fraser, 2006). Recently, many proteins involved in neurodegenerative diseases have proved to be targets of SUMOylation, implicating this process in neurodegeneration. α-syn is modified preferentially by SUMO1 at lysine residues within the N-terminal (Dorval and Fraser, 2006).

Recent studies found SUMOylated α-syn in aggregates and inclusions that were formed as a result of proteasome impairment. Thus, proteasome inhibition induces SUMOylation of α-syn and subsequent oligomerization (Kim et al., 2011). Other studies provided a comprehensive role of α-syn SUMOylation in aggregation both *in vitro* and *in vivo* showing that SUMOylation delays the fibril formation (Krumova et al., 2011).

Although the molecular mechanisms of neurodegeneration in the SNpc, present in PD cases, remain unknown several studies believe that misfolding, oligomerization and fibrillization followed by protein deposition n LBs are the central event in the onset and progression of the disease.

1.4.3 Bimolecular Fluorescence Complementation assay to monitor α-syn oligomerization

Oligomers are currently considered the most toxic α-syn species but the underlying mechanisms of α-syn aggregation in PD remain to be determined. Thus, the main goal of this project was to compare, for the first time, the effect of different mutations on α-syn oligomerization using the same cell based model, bimolecular fluorescence complementation (BiFC), that enables us to directly visualize the formation of α-syn oligomeric species in living cells. This powerful assay is based on the association between two non-fluorescent fragments of a fluorescent protein, which are fused with the protein of interest (Figure 5). When the protein of interest dimerizes it facilitates the association between the protein fragments and forms a bimolecular fluorescent complex (Kerppola, 2006a; Kerppola, 2006b; Kerppola, 2008; Kerppola, 2009). Since the BiFC assay stabilizes the interactions between the protein of interest due to the reconstitution of the fluorescent protein moiety, we believe that the use of this model recapitulates the initial events of α-syn aggregation, the oligomerization, constituting a powerful tool in the study of molecular basis of neurodegenerative diseases because allows us to investigate how manipulations on α-syn can affect oligomerization and aggregation progress over time as well as their subcellular localization.

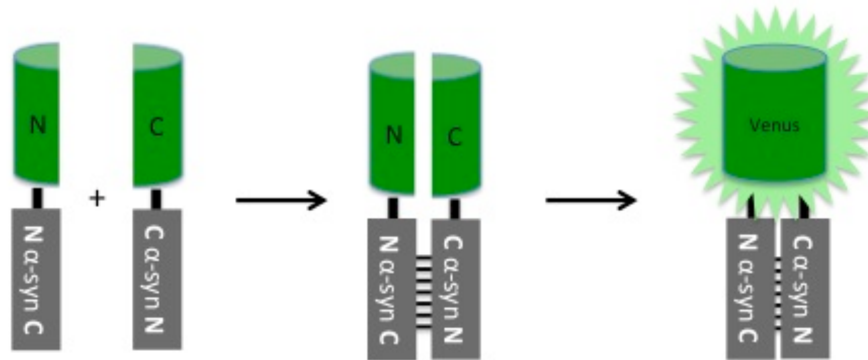


Figure 5. Schematic representation of the BiFC assay. A parte of Venus protein is fused with N-terminal of α-syn and the other parte is fused with C-terminal of α-syn. When the two constructs are both overexpressed in the cells, due to α-syn dimerization, the fluorophore is reconstitute.

Chapter 2 - Material and methods

Generation of BiFC constructs

The two α-syn BiFC constructs utilized to generate the selected mutations on α-syn were VN-link- α-syn and α-syn -VC using Venus the fluorescent protein reporter (Outeiro et al., 2008). The mutants were generated using the QuikChange site-directed mutagenesis kit (Stratagene, Agilent Technologies) employing the designed oligonucleotide mutagenic primers (Table 2). The primers A30P, A53T, E35K, E57K, A56P, A76P, K96R, K102R, S129G, Y125F and Y125D were designed according to the manufacturer's instructions using the QuikChange Primer Design Program and the web-based program Primer X. The following mutations were generated: A30P, A53T, E46K, E35K, E57K, A56P, A76P, A30P/A76P, A30P/A56P/A76P, S129A, S129D, S129G, S87A, S87E, Y125F, Y125D, K96R/K102R. All mutations were confirmed by DNA sequencing.

Table 2. List of primers used in this study.

Mutation	Primer
A30P	Forward: 5'-GGGTGTGGCAGAAGCACCAGGAAAGACAAAAGA-3' Reverse: 5'-TCTTTTGTCTTTCCTGGTGCTTCTGCCACACCC-3'
A53T	Forward: 5'-GAGTGGTGCATGGTGTGACGACAGTGGCTGAGAAGAC-3' Reverse: 5'-GTCTTCTCAGCCACTGTCGTCACACCATGCACCACTC-3'
E46K	Forward: 5'-TAGGCTCCAAAACCAAGAAGGGAGTGGTGCATGG-3' Reverse: 5'-CCATGCACCACTCCCTTCTTGGTTTTGGAGCCTA-3'
E35K	Forward: 5'-CAGAAGCAGCAGGAAAGACAAAAAAGGGTGTTCTCT-3' Reverse: 5'-AGAGAACACCCTTTTTTGTCTTTCCTGCTGCTTCTG-3'
E57K	Forward: 5' GTGGCAACAGTGGCTAAGAAGACCAAAGAGC 3' Reverse: 5' GCTCTTTGGTCTTCTTAGCCACTGTTGCCAC 3'
A56P	Forward: 5'-GGTGTGGCAACAGTGCCTGAGAAGACCAAAG-3' Reverse: 5'-CTTTGGTCTTCTCAGGCACTGTTGCCACACC-3'
A76P	Forward: 5'-TGACGGGTGTGACACCAGTAGCCCAGAAG-3' Reverse: 5'-CTTCTGGGCTACTGGTGTCACACCCGTCA-3'
S129A	Forward: 5'CTTATGAAATGCCTGCTGAGGAAGGGTATC-3' Reverse: 5'GATACCCTTCCTCAGCAGGCATTTCATAAG-3'
S129D	Forward: 5'-GGCTTATGAAATGCCTGATGAGGAAGGGTATCAAG-3' Reverse: 5'-CTTGATACCCTTCCTCATCAGGCATTTCATAAG CC-3'
S129G	Forward: 5'-GACAATGAGGCTTATGAAATGCCTGGTGAGGAAGGGTATC-3' Reverse: 5'-GATACCCTTCCTCACCAGGCATTTCATAAGCCTCATTGTC-3'
S87A	Forward: 5'-AAGACAGTGGAGGGAGCAGGGGCCATTGCAGCAG-3' Reverse: 5'-CTGCTGCAATGGCCCCTGCTCCCTCCACTGTCTT-3'
S87E	Forward: 5'-ACAGTGGAGGGAGCAGGGGAAATTGCAGCAGC-3' Reverse: 5'-GCTGCTGCAATTTCCCCTGCTCCCTCCACTGT-3'
Y125F	Forward: 5'-GGATCCTGACAATGAGGCTTTTGAAATGCCTTCTGA-3' Reverse: 5'-TCAGAAGGCATTTCAAAAGCCTCATTGTCAGGATCC-3'
Y125D	Forward: 5'-GATCCTGACAATGAGGCTGATGAAATGCCTTCTGAGG-3' Reverse: 5'-CCTCAGAAGGCATTTCATCAGCCTCATTGTCAGGATC-3'
K96R	Forward: 5'-GCCACTGGCTTTGTCAGAAAGGACCAGTTGGGC-3' Reverse: 5'-GCCCAACTGGTCCTTTCTGACAAAGCCAGTGGC-3'
K102R	Forward: 5'-AAGGACCAGTTGGGCAGGAATGAAGAAGGAGCC-3' Reverse: 5'-GGCTCCTTCTTCATTCCTGCCCAACTGGTCCTT-3'

Site-directed mutagenesis was performed with 2 ng of DNA template, two oligonucleotide primers (10 μM) (containing the desired mutation) and *PfuTurbo* DNA polymerase (2,5 U/μL) in a 25 μL volume reaction. The thermal cycling conditions were 1 min. at 95°C, followed by 16 cycles of 50s at 95°C, 50s at 58/60°C and 6,30 min. at 68°C, and a final extension step 6 min. at 60°C.

Parental DNA template was digested with *DpnI* for 1h at 37ºC. The mutated DNA plasmid was transformed into XL1-Blue supercompetent cells according to manufacture's instructions. After plasmid DNA purification, the inoculated bacterial colonies were digested with XhoI/AflII (Fermentas) and the presence of the insert was confirmed in Agarose gel electrophoresis.

Cell culture

HEK (Human Embryonic Kidney) 293 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (PAN) containing 4,5g/L Glucose, L-Glutamine, 3,7 g/L $NaHCO_3$ and supplemented with 10% fetal calf serum (FCS) (Biochrom AG) and 1% of Penicillin Streptomycin (PAN). Cells were kept at 37ºC in a humidified atmosphere with 5% CO_2.

Cell transfections

HEK 293 cells were plated 24h prior to transfection with confluence of 80 000 cells/well in a 24-well plate. Cells were transfected with equimolar amounts of the plasmids (0,25 μg of each plasmid) using Metafectene (Biotex) according to the manufacture's instructions and optimized protocol. 4 μL Metafectene/μg of plasmid was prepared in Opti-MEM (Gibco) with neither serum nor antibiotics and the cells were also incubated in the same medium for 30 min before transfection. 5h after transfection complexes addition, the transfection medium was replaced by growth medium to minimize the toxicity of Metafectene.

Live Hoescht nuclear staining

Hoescht 33258 (pentahydrate, bis-benzimide) dye (Invitrogen) in DMEM was applied to HEK 293 cells at 1 mg/mL and incubated for 30 min at 37°C. The solution was then removed and the cells were washed and imaged in DPBS.

Live cell imaging and Quantification of intensities

At 24h post-transfection cells were imaged on an Olympus IX81-ZDC microscope system (Olympus Germany, Hamburg, Germany) and observed with a 10x objective. 16 images/well/condition were collected randomly for quantification analysis (Scan^R). Venus fluorescence was analyzed using the Olympus Scan^R Image Analysis Software. Values of each condition were then averaged and statistical analysis was performed. For subcellular localization studies, cells were observed with 40x and 63x objectives.

Western Blot analysis

After cell live imaging, cells were lysed with Radio-Immunoprecipitation Assay (RIPA) lysis buffer (50 mM Tris pH 8.0, 0.15 M NaCl, 0.1% SDS, 1% NP40, 0.5% Na-Deoxycholate), 2 mM EDTA and a protease inhibitor tablet (Roche diagnostics, Germany) (1 tablet/10 mL)). Cell lysates were kept at -80°C until the next day, scraped, sheared by passing through a 27-gauge 1 mL syringe 4-6 times and collected

in tubes. The lysates were centrifuged for 1 min. (13,000xg, 4°C) and the supernatant was transferred to a new tube. Protein concentration was determined using the Bradford assay (Biorad) Before loading on the gel, samples of 30 µg were denatured for 10 min at 99°C in protein sample buffer (PBS 4x, 1 M Tris-HCl pH 6.8, β-Mercaptoethanol, 20% SDS, Glicerol 100%, Bromophenol blue), separated on 12% SDS-polyacrylamide gel with a constant voltage of 110V using Tris-Glycine SDS 0,5% running buffer. Proteins were transferred to nitrocellulose membrane (Whatman, Germany) for 1:30h with constant amperage of 0.2A using Tris-Glycine transfer buffer. Membranes were blocked for 1h at room temperature in 5% skim milk (Fluka, Sigma) in TBS-T and washed 1 time in TBS-T for 10 min. Membranes were then incubated with primary antibodies (mouse anti-α-syn, 1:1000, BD Transduction Laboratories; rabbit anti-α-syn (C-20), 1:1000 (Santa Cruz Biotechnologies) diluted in 5% BSA (ROTH) overnight at 4°C with gentle agitation. After wash in in TBS-T, membranes were incubated at room temperature for 2h with secondary antibody (goat anti-mouse or anti-rabbit, 1:10000, horseradish peroxidase labeled secondary antibody, GE Healthcare, UK). After washing, immunoblots were analyzed using the ECL chemiluminescent detection system (Milipore, USA) in AlphaImager software (AlphaInnotech, Germany).

For reprobing, membranes were stripped of antibody with Stripping buffer (0.2 M Glycerin, 0.5 M NaCl, pH 2.8) for 30 min at room temperature with gentle agitation. After washing, membranes were treated as described above, but were incubated with anti-β-actin antibody (mouse anti-β-actin, 1:2500, Sigma-Aldrich).

Protein levels were quantified using ImageJ (http://rsbweb.nih.gov/ij/) and normalized to the β-actin levels.

Flow Cytometry

Cells were plated as previously described. 24h post-transfection, cells were imaged as described above and then were trypsinized, neutralized with growth medium, centrifuged (500 xg, room temperature) and the pellet was reconstituted in PBS (Phosphate-buffered saline). The resulting suspensions were transferred to a 96-well plate. The fluorescence was measured using a microcapillary system (Guava easyCyte HT system, Millipore Corporation). 80 000 events were count.

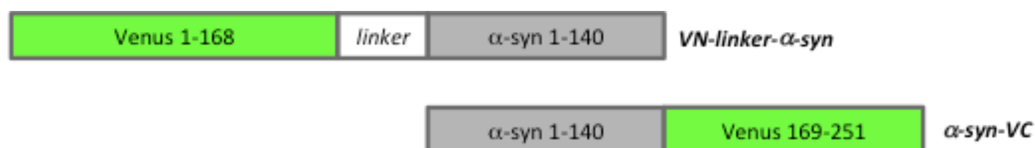
Statistical analysis

Data were analysed using GraphPad Prism 5 software and are expressed as the mean ± SEM. The values of α-syn mutations from either microscopy or flow cytometry were normalized to WT and mean values for each experiment were determined. Statistical differences from WT were calculated using unpaired Student *t*-test.

Chapter 3. Results

Accumulating evidence suggests that α-syn oligomers might be the toxic species. (Karpinar et al., 2009; Outeiro et al., 2008; Winner et al., 2011). To further investigate the effect of selected mutations on α-syn oligomerization, we used the BiFC assay that has to be an effective method for visualizing and monitoring α-syn oligomerization in living cells (Outeiro et al., 2008). BiFC involves the fusion of two non-fluorescent fragments of Venus with α-syn. The functional Venus fluorophore is reconstituted upon α-syn - α-syn interaction, which brings the two fragments together. We assessed α-syn oligomerization by transfecting HEK 293 cells with the BiFC constructs, VN-linker- α-syn and α-syn -VC, carrying the α-syn mutations in both constructs (Fig 6A). 24 hours after transfection, Venus fluorescence could be detected in approximately 80% of the cells, indicating dimerization/oligomerization. All the mutations formed dimers/oligomers in HEK 293 cells, as assessed by Venus fluorophore reconstitution (Fig. 6Bi). When only one of the constructs was expressed alone protein complementation (green cells) was not observed (Fig. 6Bii and iii).

A



B

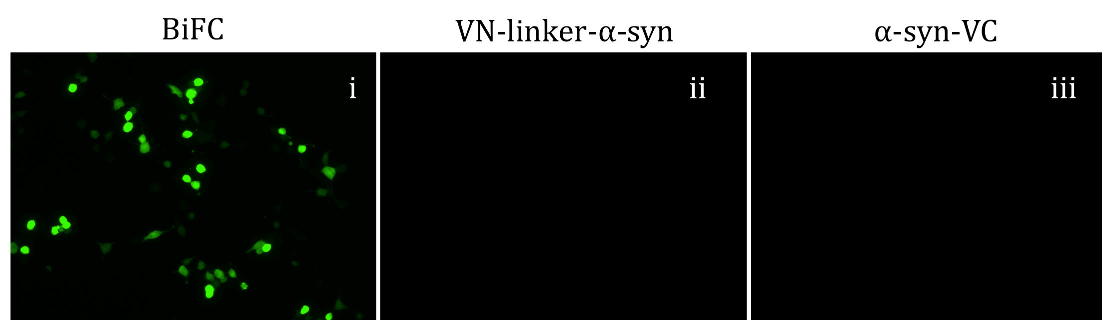
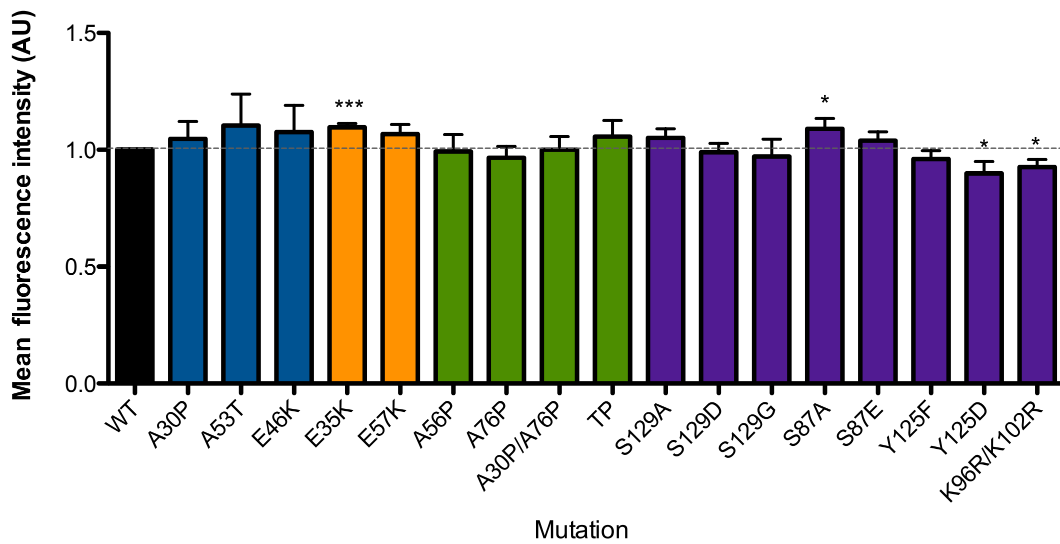


Figure 6. α-syn oligomerization in living cells. A. Schematic representation of the α-syn BiFC constructs. B. Fluorescence images showing the bimolecular complementation driven by α-syn - α-syn interactions twenty-four hours after transfection.

The effect of the selected mutations was first assessed by epifluorescence microscopy and then by flow cytometry on the same cells. To investigate the effects of the different mutants on α-syn oligomerization, we compared control (WT α-syn) BiFC constructs with four groups of mutations: 1) the familial PD mutations (A30P, A53T and E46K); 2) mutants designed with the substitution of Glu-to-Lys (E35K and E57K) which species form toxic oligomers and strongly decrease the tendency of fibril formation (Winner et al., 2011); 3) the artificial proline mutants (A56P, A76P, A30P/A76P and TP) that consist of alanine replacement by prolines, are known to

interfere with fibril formation and result in enhanced formation of toxic oligomers (Karpinar et al., 2009); and 4) the PTM mutations which mimic phosphorylation of serines 87 (S87A and S87E), 129 (S12A, S129D and S129G) and tyrosine 125 (Y125F and Y125D), as well as the mutation with impaired α-syn SUMOylation (K96R/K102R) (Krumova et al., 2011).

A



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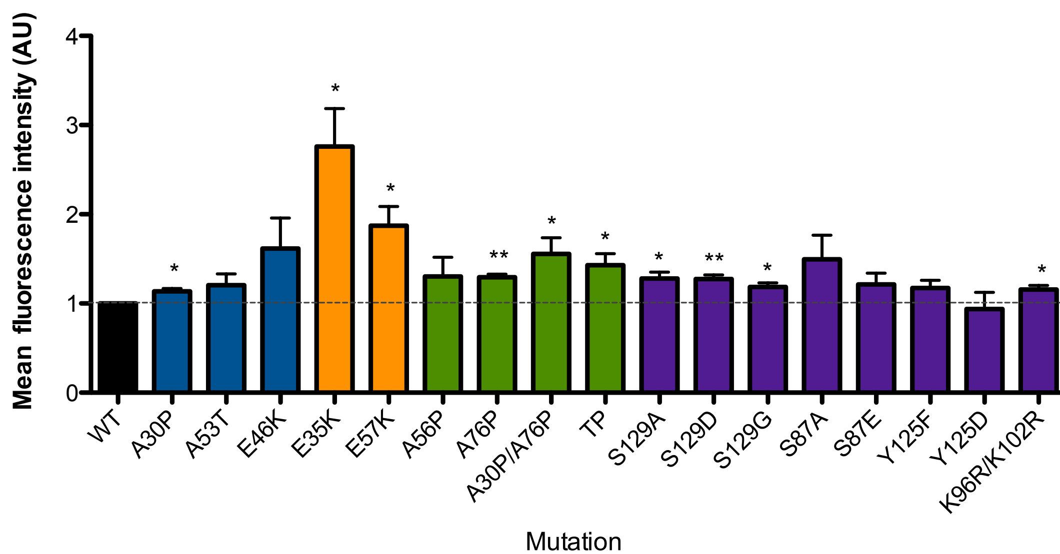


Figure 7. Assessment of α-syn oligomerization using BiFC. A. HEK 293 cells were transfected with WT or α-syn mutants and analyzed by epifluorescence microscopy to quantify fluorescence intensity (n=6). B. HEK 293 cells transfected with WT or α-syn mutants were analyzed by flow cytometry to quantify fluorescence intensity (n=3). Results were normalized to WT. Data represents mean ± SEM. Student's *t* test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$)

Using fluorescence microscopy, we did not detect statistically significant differences in the oligomerization of the three mutant forms of α-syn associated with familial PD compared with WT α-syn, despite a tendency towards an increase (Fig. 7A). Using flow cytometry, we found that the A30P mutant showed a significant increase in oligomerization when compared to WT α-syn (Fig. 7B).

Next, we investigated whether the subcellular distribution of oligomeric species formed by WT α-syn and each α-syn mutant were differentially distributed throughout the cell. Interestingly, cells carrying A30P α-syn tended to display more oligomers in the cytosol, though some cells displayed a similar distribution in the nucleus and cytosol (Fig. 8). The A53T mutation caused a pronounced localization of oligomers in the nucleus in the majority of the cells (Fig. 8). Cells transfected with the E46K mutant showed oligomers in the nucleus and others in the cytosol (Fig. 8). These results suggest that the familial PD mutations may cause different effects in the cell, depending on the subcellular compartment where they accumulate.

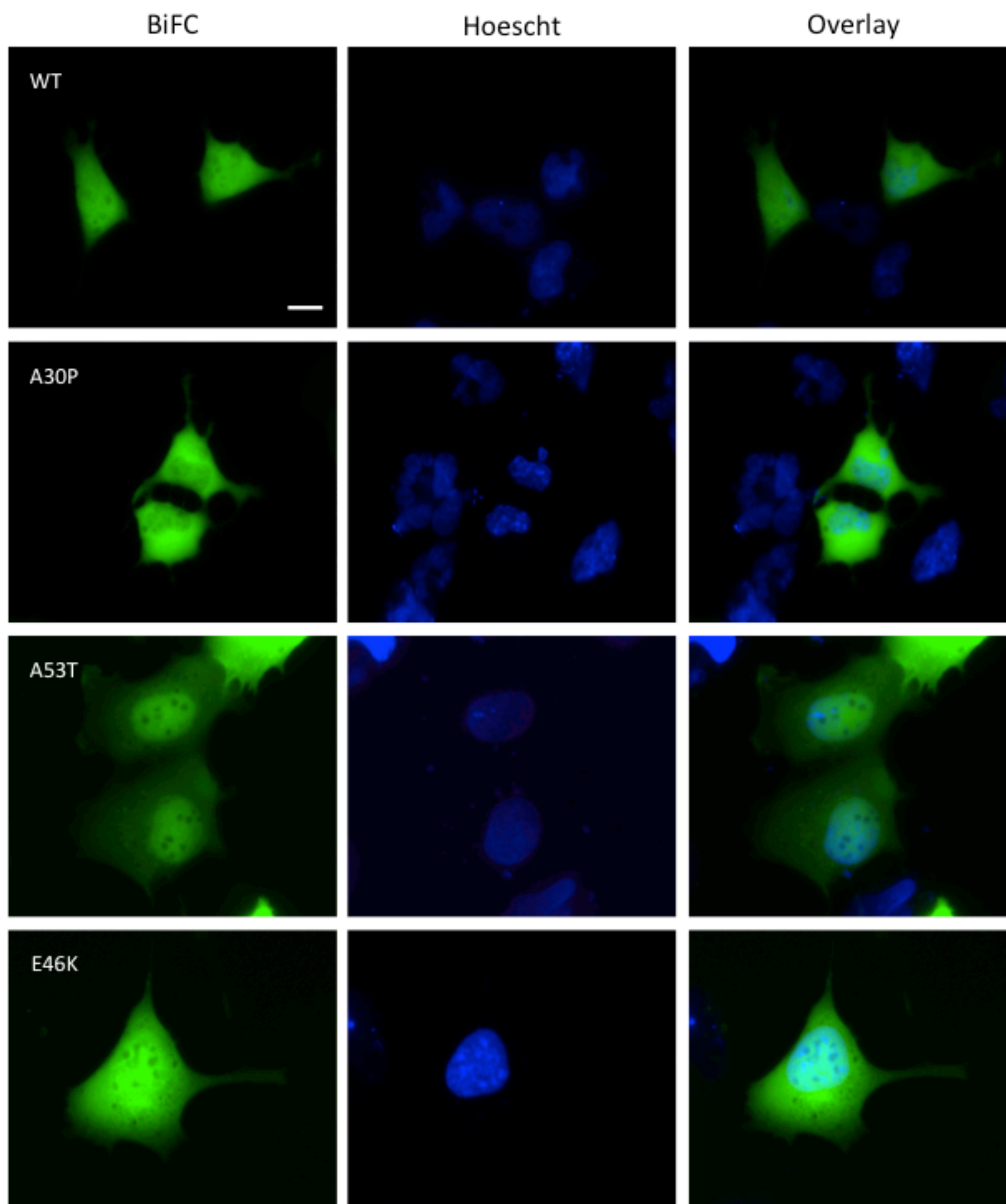


Figure 8. Effect of familial PD mutations on α-syn oligomerization in living cells. Subcellular distribution analyzed, by fluorescence microscopy, of WT and familial PD mutants in HEK 293 cells showing subcellular distribution of α-syn oligomers. (Scale bar: 15 μm)

The E35K and E57K mutants displayed increased oligomerization as assessed by the increase fluorescence, compared with WT α-syn. While the E35K mutant showed

a significant difference by both analysis methods, the E57K mutant showed a significant difference only by flow cytometry (Fig. 7). As expected, these results are consistent with the hypothesis that these mutants promote oligomerization (Winner et al., 2011), explaining the observed correlation between oligomerization and toxicity (Outeiro et al., 2008; Winner et al., 2011) given that the BiFC assay traps α-syn in specific oligomeric species. The subcellular distribution of oligomers formed by these mutants was identical (Fig. 9).

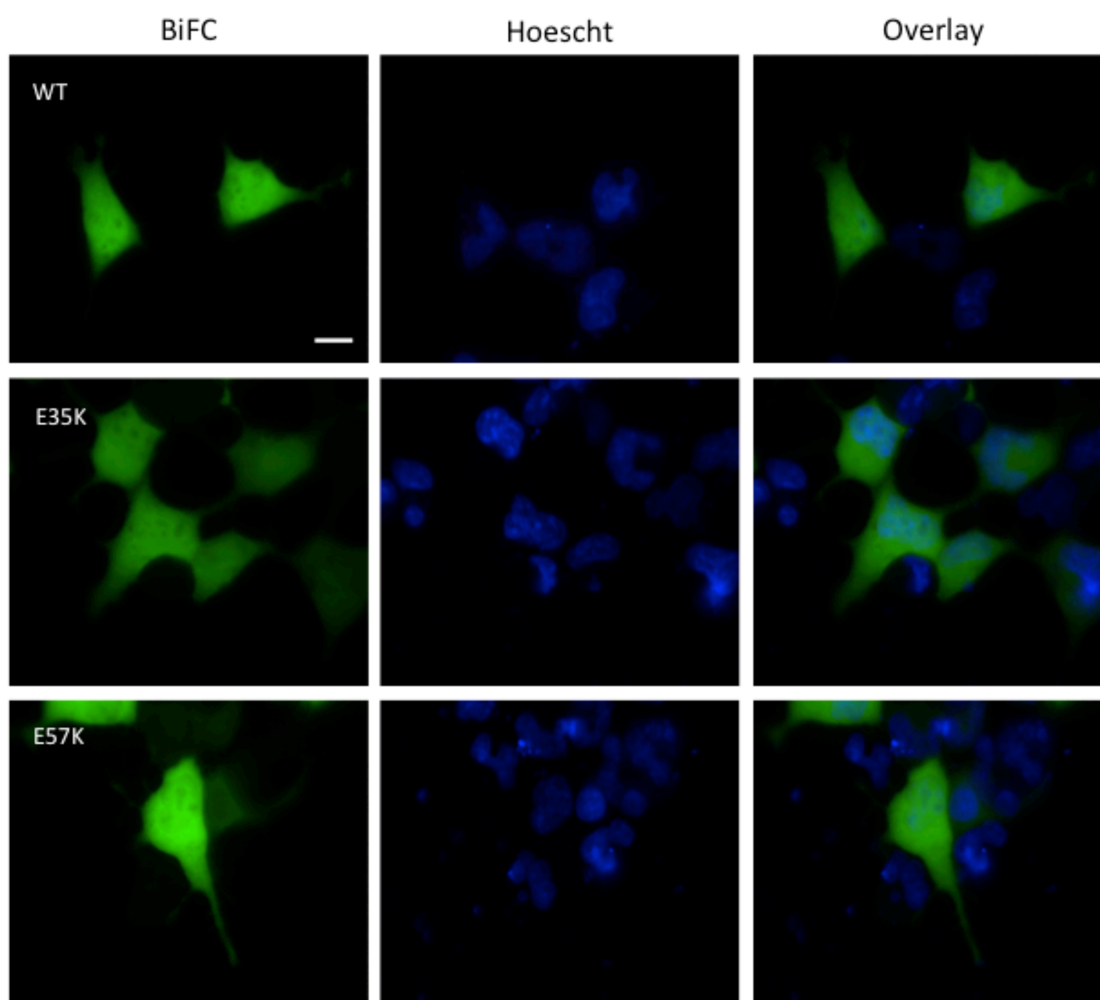


Figure 9. Effect of oligomer-forming mutations on α-syn oligomerization in living cells. Subcellular distribution analyzed, by fluorescence microscopy, of WT and oligomer-forming mutants in HEK 293 cells showing subcellular distribution of α-syn

oligomers. (Scale bar: 15 μm)

No significant differences in α-syn oligomerization were observed for the artificial proline mutants via microscopy (Fig. 7A). However, flow cytometry analysis enabled us to detect increased oligomerization by the A76P, A30P/A76P and TP mutants (Fig. 7B). In addition, these mutants accumulated more in the cytosol than the nucleus (Fig. 10). Consistent with previous results, (Karpinar et al., 2009) the TP mutant also increased α-syn oligomerization using the BiFC assay.

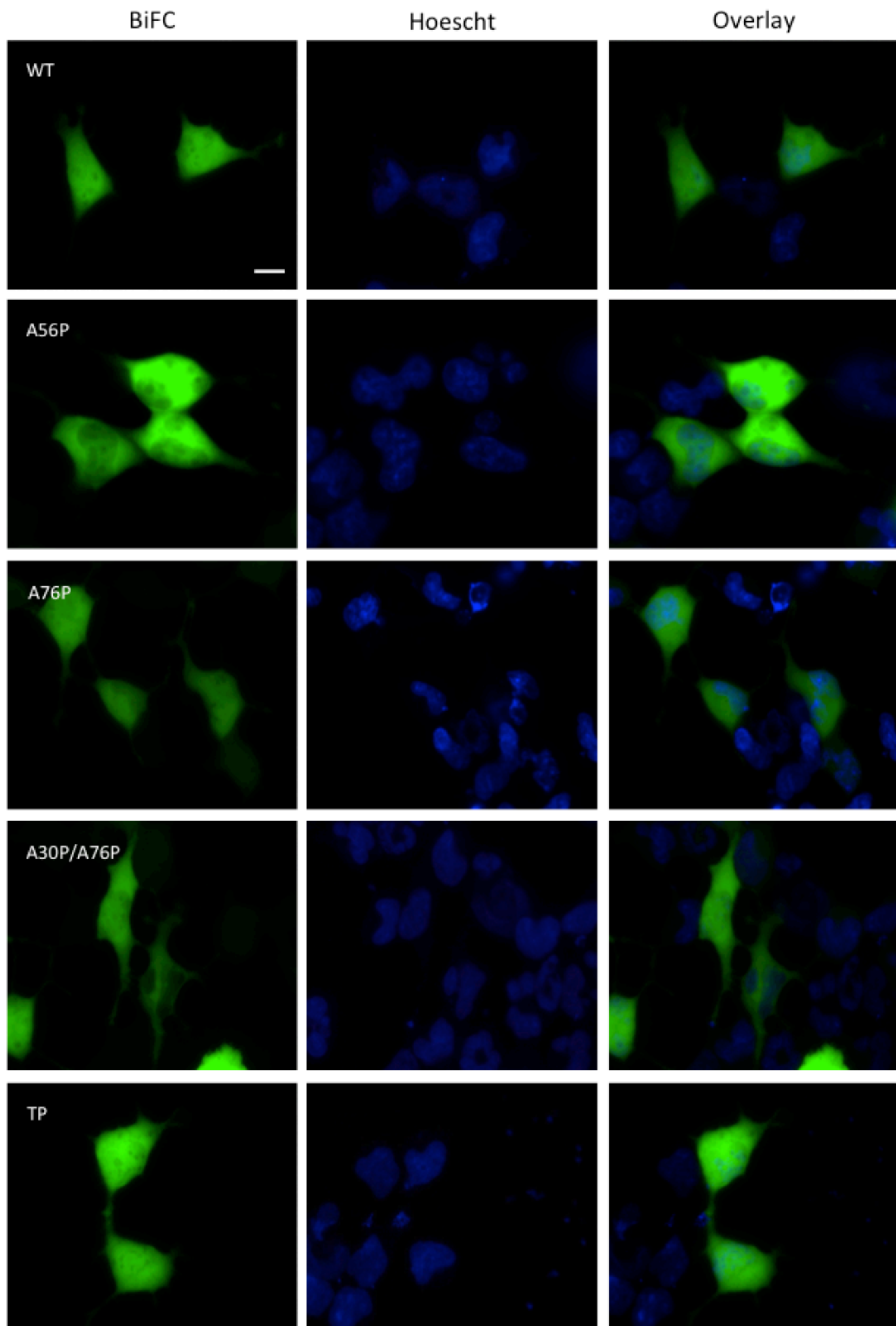


Figure 10. Effect of artificial proline mutations on α-syn oligomerization in living cells. Subcellular distribution analyzed, by fluorescence microscopy, of WT and

artificial proline mutants in HEK 293 cells showing subcellular distribution of α-syn oligomers. (Scale bar: 15 μm)

To investigate the effect of α-syn phosphorylation on residues S87, Y125, and S129 on oligomerization, a panel of α-syn substitution mutants were generated to block (S129A, S129G, S87A and Y125F) or mimic (S129D, S87E and Y125D) phosphorylation of these residues.

Several studies suggest that phosphorylation at S129 may play an important role in regulating α-syn fibrillization, aggregation, LB formation and toxicity (Fujiwara et al., 2002; Oueslati et al., 2010; Paleologou et al., 2008) in different model systems. However, whether phosphorylation enhances or decreases α-syn oligomerization and toxicity remains unknown. Using fluorescence microscopy, we found, that the S129A, S129D and S129G did not significantly change the oligomerization properties of α-syn compared to the WT form (Fig. 7A). In contrast, flow cytometry analysis revealed that α-syn oligomerization was significantly increased by the three S129 mutants (Fig. 7B). The subcellular distribution of oligomers was similar in all the three mutants (Fig. 11). Altogether, these results suggest that oligomerization of α-syn may not be affected by S129 phosphorylation.

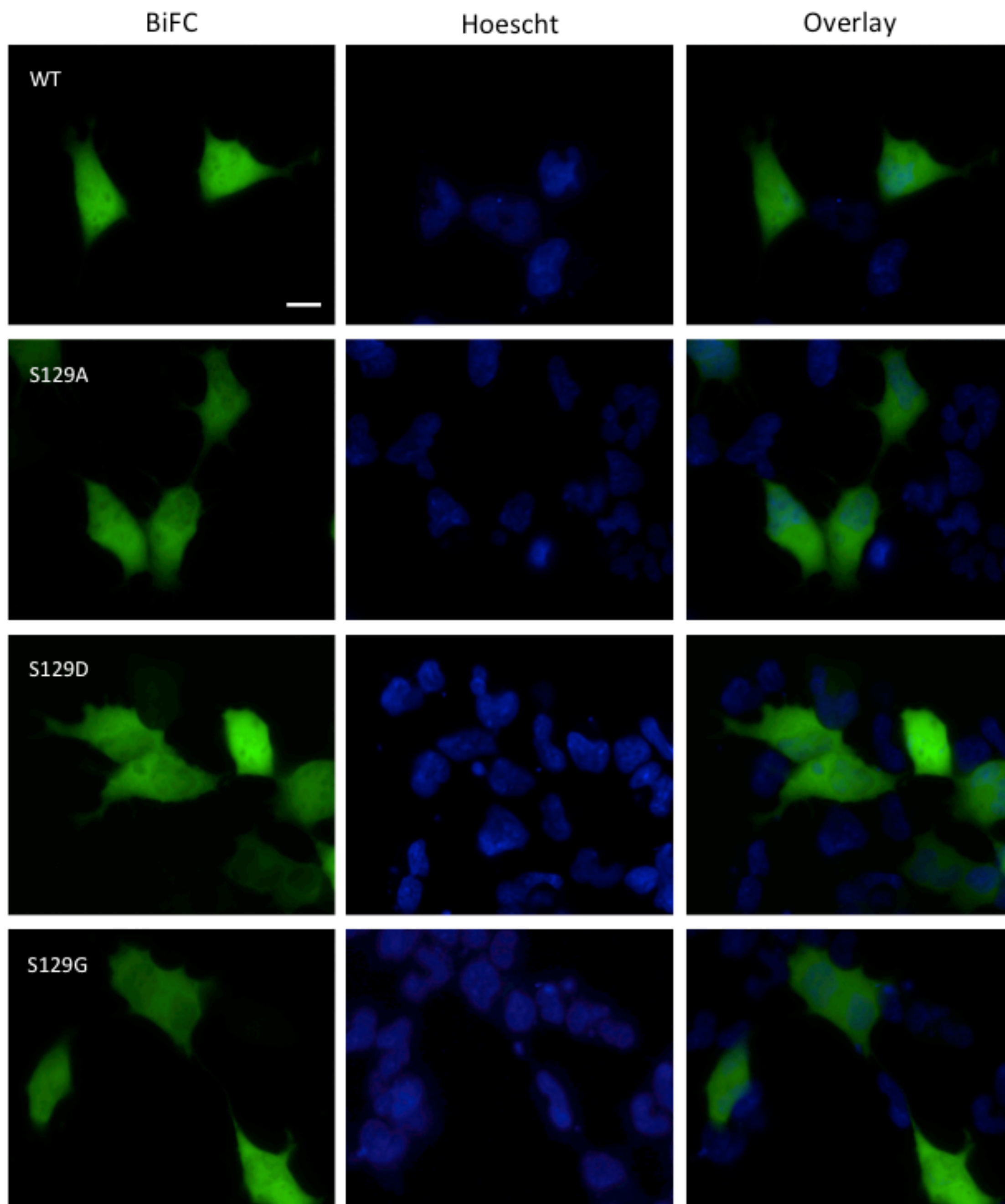


Figure 11. Effect of mutations at S129 on α-syn oligomerization in living cells. Subcellular distribution analyzed, by fluorescence microscopy, of WT and S129 mutants in HEK 293 cells showing subcellular distribution of α-syn oligomers. (Scale bar: 15 μm)

Next, we observed that the S87A mutant resulted in a significant increase in oligomerization (Fig. 7A). In contrast, the S87E mutation did not change the oligomerization properties of α-syn. Both α-syn mutants displayed a similar subcellular distribution of oligomers (Fig. 12).

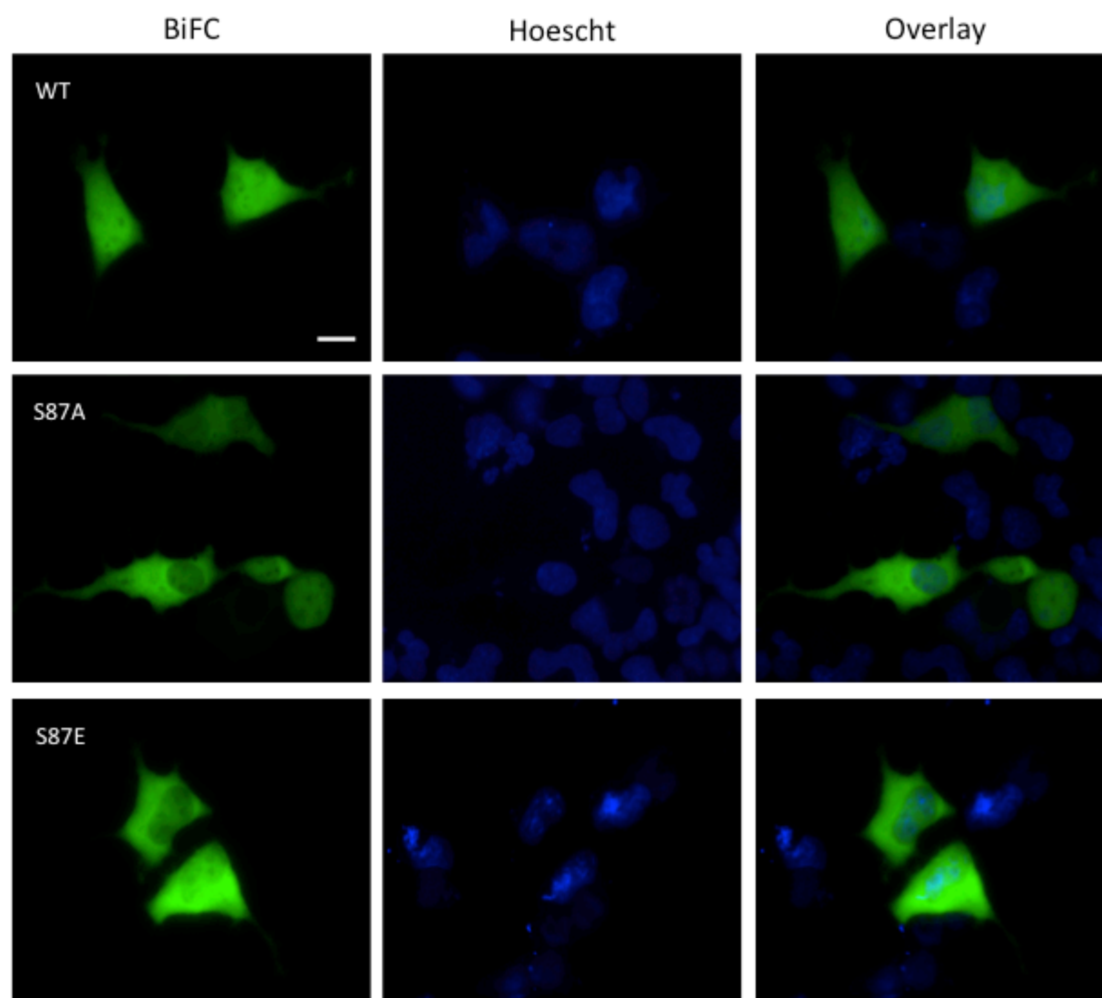


Figure 12. Effect of mutations at S87 on α-syn oligomerization in living cells. Subcellular distribution analyzed, by fluorescence microscopy, of WT and S87 mutants in HEK 293 cells showing subcellular distribution of α-syn oligomers. (Scale bar: 15 μm).

Phosphorylation at Y125 has been suggested to inhibit the formation of soluble oligomeric species (Chen et al., 2009). Similarly, we found that Y125D resulted in a

significant decrease of oligomerization, while the Y125F mutant did not induce significant differences compared to WT α-syn (Fig. 7A). The fluorescence level of the Y125D mutant was also confirmed to be lower by flow cytometry analysis (Fig. 7B). In any case, phospho-mutants at S87, Y125, and S129 did not seem to alter the subcellular localization of α-syn oligomers (Fig. 11, 12 and 13).

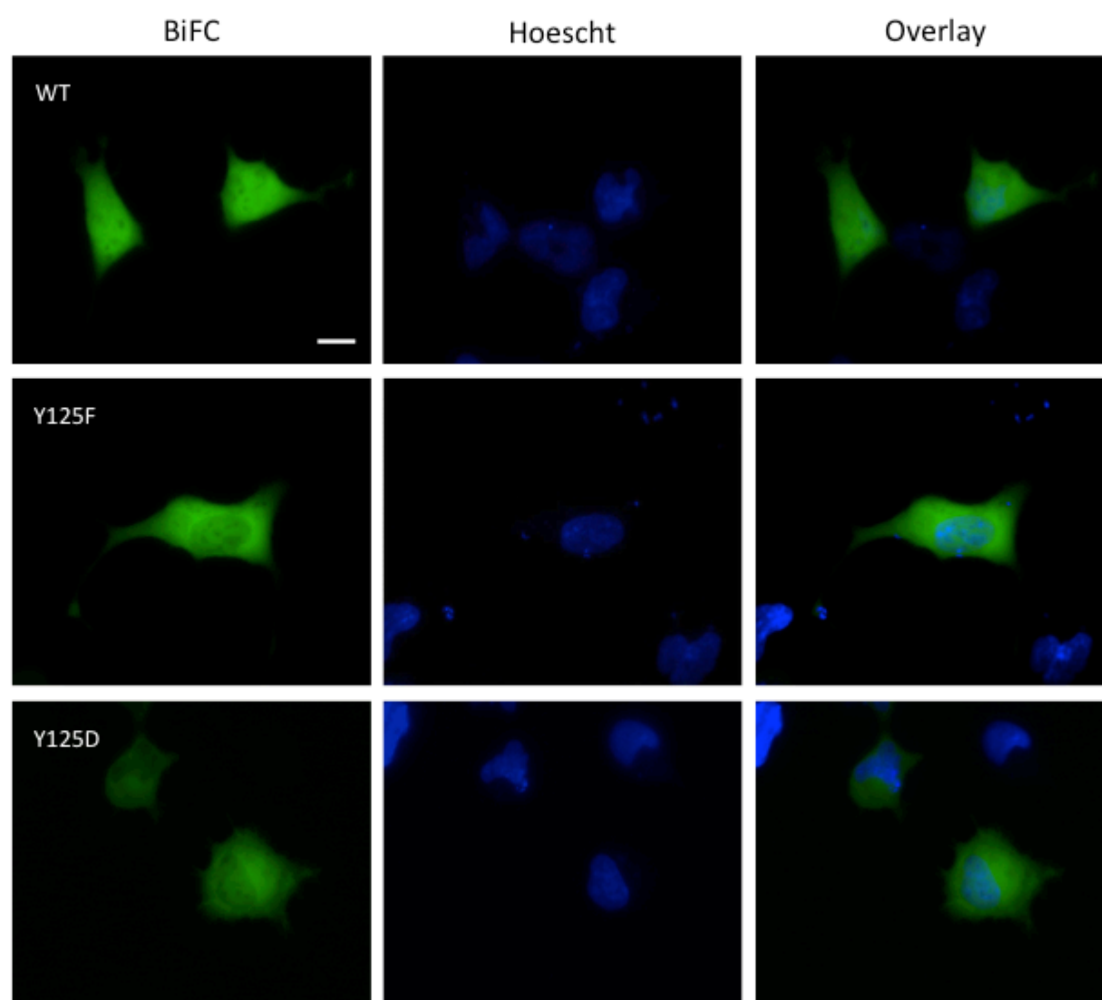


Figure 13. Effect of mutations at Y125 on α-syn oligomerization in living cells. Subcellular distribution analyzed, by fluorescence microscopy, of WT and Y125 mutants in HEK 293 cells showing subcellular distribution of α-syn oligomers. (Scale bar: 15 μm)

SUMOylation is known to affect the stability and solubility of proteins and recent evidence suggests that SUMOylation of α-syn inhibits its aggregation and related toxicity (Krumova et al., 2011). Even though the K96R/K102R mutant is reported to impair α-syn SUMOylation and to increase propensity for aggregation, our microscopy results demonstrated a small but significant decrease of oligomerization (Fig. 7A). In contrast, using flow cytometry analysis, we detected a significant increase of α-syn oligomerization (Fig. 7B).

When we assessed subcellular distribution of oligomers formed by this mutant, we found circumstances in which cells displayed oligomers predominantly in the nucleus or predominantly in the cytosol, while some displayed a more homogeneous distribution between the nucleus and the cytosol (Fig. 14). Together, these results suggest that an impairment of α-syn SUMOylation leads to an increase of oligomerization, which is dependent upon the cellular environment. This may explain the differential results obtained between fluorescence microscopy and flow cytometry.

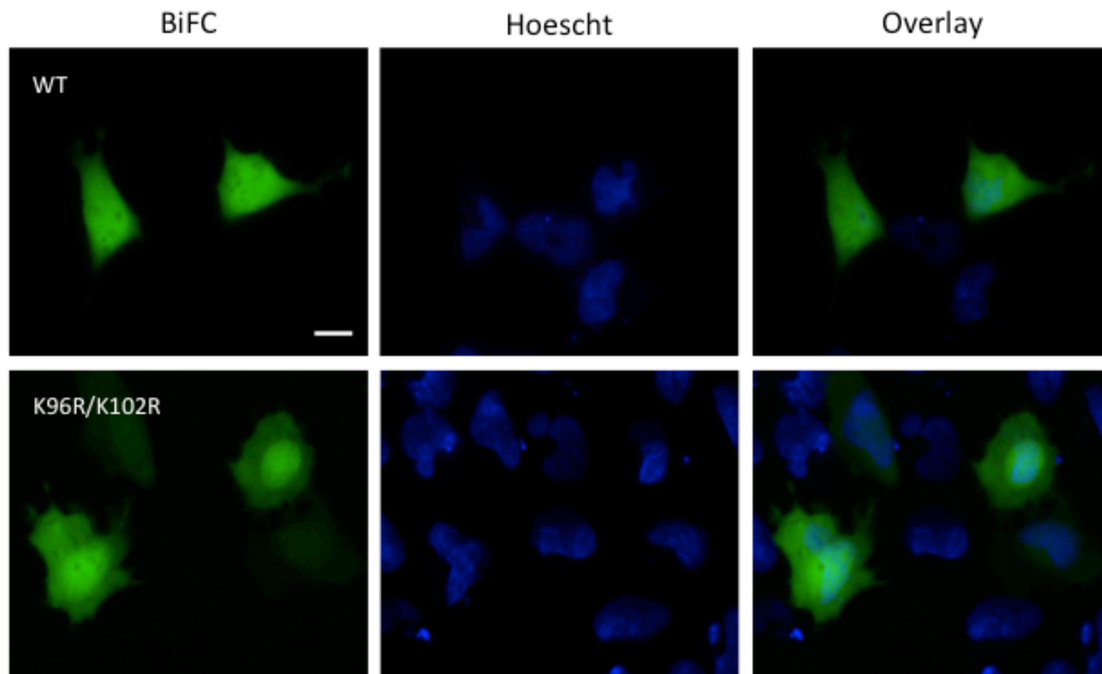
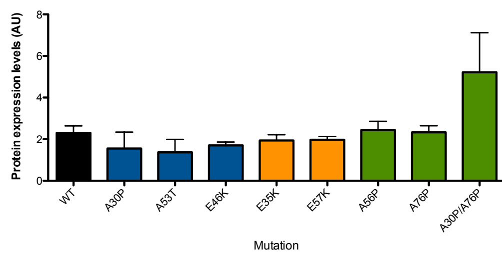


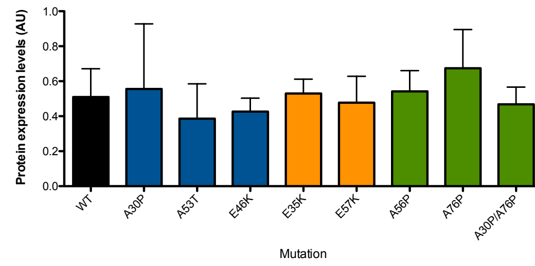
Figure 14. Effect of SUMO mutation on α-syn oligomerization in living cells. Subcellular distribution analyzed, by fluorescence microscopy, of WT and SUMO mutant in HEK 293 cells showing subcellular distribution of α-syn oligomers. (Scale bar: 15 μm)

We next assessed the expression pattern of the various α-syn mutants. The S129G, Y125 and K96R/K102R mutants showed decrease levels of expression, compared with WT α-syn and all the other α-syn variants demonstrated equal levels of expression (Fig. 15). Since no signal was detected for K96R/K102R mutant with the monoclonal α-syn antibody, we used a polyclonal antibody for this mutant instead (Fig. 15).

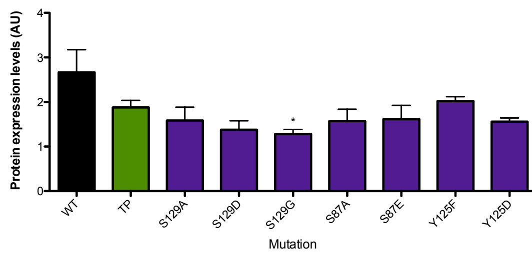
A.



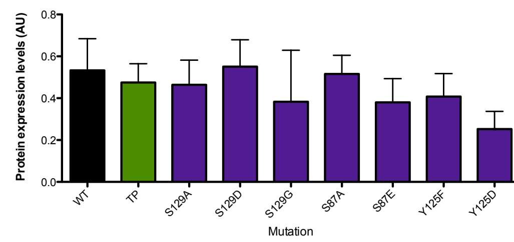
B.



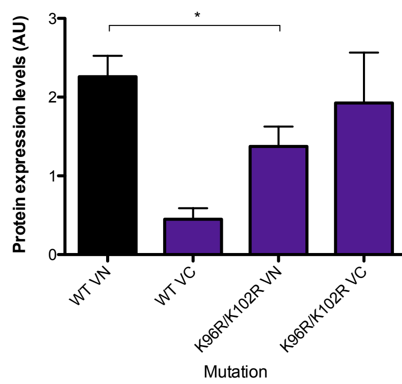
C.



D.



E.



F.

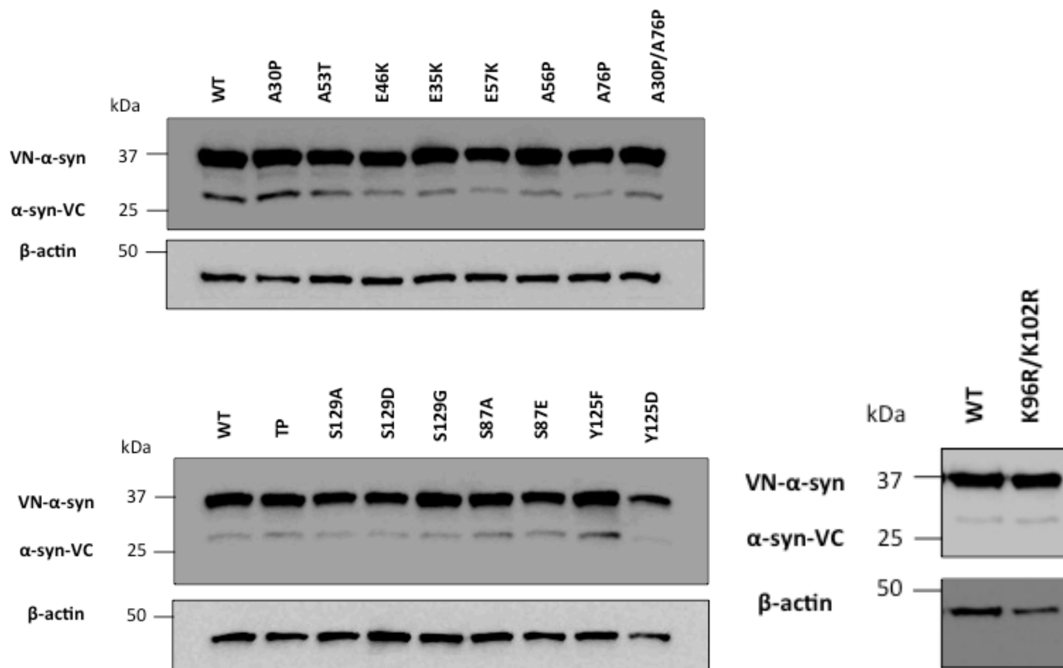


Figure 15. Immunoblot analysis of the levels of different α-syn mutants. A-B. Quantification of expression levels of VN-linker- α-syn. C-D. Quantification of expression levels of α-syn-VC. E. Quantification of expression levels of K96R/K102R mutant. F. Representative western blots. Data was normalized to β-actin levels (n=6, Student's *t* test, $*P < 0.05$).

Chapter 4. DISCUSSION

Aggregation of α-syn is thought to be a causal contribution to the pathogenesis of PD and several other neurodegenerative diseases (Chandra et al., 2005; Tanaka et al., 2004; Tompkins and Hill, 1997).

Recent evidences studies suggest that oligomeric and prefibrillar species of α-syn, rather than mature fibrils, are the most toxic and pathogenic species in PD (Karpinar et al., 2009; Outeiro et al., 2008; Winner et al., 2011). Nevertheless, the precise nature of the toxic species is still under debate.

Conventional techniques, including the use of specific antibodies, atomic force microscopy, nuclear magnetic resonance and various *in vitro* aggregation models, have been applied for studying protein-protein interactions (Danzer et al., 2007; Ding et al., 2002; Engelender et al., 1999; Karpinar et al., 2009; Kayed et al., 2003; Lashuel et al., 2002; McLean et al., 2001; Opazo et al., 2008; Winner et al., 2011). However, until recently, it had not been possible to directly visualize interaction between α-syn molecules in living cells. The BiFC assay affords the possibility of direct visualization of α-syn oligomerization in living cells, using different fluorescent proteins as reporters (Kerppola, 2006b; Outeiro et al., 2008).

The BiFC assay allows us to quantitatively analyze protein-protein interactions using the intensity of the fluorescent signal as the readout. In the present study, oligomerization of α-syn was studied using fluorescence microscopy and flow cytometry. Interestingly, using flow cytometry, we were able to detect more significant interactions between the mutant and WT α-syn species. The difference between the results observed using the two different methods might be due to the number of cells analyzed: 80,000 cells/condition were analyzed by flow cytometry

and approximately 1000 cells/condition were analyzed by microscopy. Another explanation can be, that some cells might be overexposed during the automatic microscopy scan and those cells were not quantified with the method of analysis utilized.

In the present study, we found that the A30P mutation increased α-syn oligomerization. Although WT and A30P α-syn both form oligomers (Karpinar et al., 2009; Outeiro et al., 2008) many studies demonstrated, that A30P fibrillizes more slowly than WT (Conway et al., 2000a; Conway et al., 2000b; Li et al., 2001). This might explain, at least in part, the differences in the increase of oligomeric species, suggesting that the nucleation-dependent process from oligomers to fibrils is a kinetically slow process that results in accumulation of oligomers.

The effect of familial PD mutations on α-syn oligomerization was assessed in a previous study, which also used the BiFC assay (Outeiro et al., 2008). In this study, no significant differences were detected in the fluorescence signal or in the subcellular distribution of oligomers. However, our results show that the oligomeric species formed by the WT α-syn and each PD mutant displayed different subcellular distributions. These different results may now be apparent due to the more sensitive methods of analysis utilized in our study.

Among the familial PD mutants, A30P and E46K showed α-syn oligomers formed throughout the cell whereas the A53T showed α-syn oligomers in the nucleus. My results are consistent with the study that also demonstrated the presence of α-syn in the nucleus, promoting neurotoxicity by directly binding to histones (Kontopoulos et al., 2006). Thus, differences in the subcellular distribution, with respect to the familial PD mutations, result in the hypothesis that the mutations have different effects in subcellular environments, which can promote more oligomerization.

Further studies will be necessary to quantify the formation of α-syn oligomers - both in the nucleus and in the cytosol - in order to understand and explain the different patterns of oligomerization and consequent aggregation.

In a recent study, it was shown that mutants (E35K and E57K) disrupt salt bridges between the β-strands of α-syn and favor the formation of more oligomers than WT α-syn and the familial PD mutations (Winner et al., 2011). As expected, our results are in line with previous data, showing considerably more oligomerization and a similar subcellular distribution. Combining these results with the A30P mutant, the increased accumulation of oligomers may be caused by the impaired or slowed fibrillization of α-syn.

Another group of structure-based designed mutants (A56P, A76P, A30P/A76P and TP) was studied in four established model systems for PD (Karpinar et al., 2009). The overexpression of these α-syn mutants, in all the model systems, showed a stronger enhancement of oligomerization and impaired fibrillization. In agreement with these previous data, our study showed that three of the four proline mutants (A76P, A30P/A76P and TP) increased oligomer formation, compared with WT α-syn, but without differences between them. Moreover, these mutants promoted the formation of oligomers with preferential cytosolic localization. The idea suggested above is corroborated by these results, which show that the α-syn variants, with reduced fibrillization and β-structure formation, enhance the formation of oligomers. This may be a possible relationship between impaired fibril formation, cytosolic distribution and cytotoxicity of the soluble oligomers.

Phosphorylation of S129 has been associated with the pathogenesis of PD (Okochi et al., 2000; Paleologou et al., 2008). However, the exact role of phosphorylation on α-syn oligomerization and subsequent aggregation remains unclear. In contrast with a previous report (Paleologou et al., 2008), all of the phosphomutants (S129D and S129G or S129A) showed a similar increase of oligomerization, compared with WT α-syn. One possible explanation for these observations is that phosphorylation at S129 does not influence the formation of oligomers. Phosphorylation is an important PTM or proteins, which, in the case of α-syn, might also regulate its physiological activities. Several studies have tried to address the role of phosphorylation in modulating oligomerization and aggregation (Fujiwara et al., 2002; Okochi et al., 2000; Paleologou et al., 2010; Paleologou et al., 2008; Pronin et al., 2000). Although the S87E mutant did not show significant differences compared with WT α-syn, it showed a tendency to reduce α-syn oligomerization when compared with the non-phosphorylated form. Thus, because S87 is located in the NAC region of α-syn, which is an important region for α-syn aggregation and fibrillization (El-Agnaf et al., 1998b), it makes sense that phosphorylation at S87 results in less interaction between α-syn and decreasing α-syn oligomerization.

Moreover, the observation that α-syn phosphorylation at Y125 results in decreased formation of oligomeric species (Chen et al., 2009) is supported by our results. Since the Y125 residue is located before the S129 residue, a similar interpretation to that put forward for S129A, S129G and S129G might be considered. This is further supported by the notion that phosphorylation at S129 and Y125 may be related (Chen et al., 2009). Thus, one possibility is that when α-syn is phosphorylated at S129, other kinases may be activated and, at the same time, may begin

phosphorylating Y125 as well. Such a cascade would result in a reduction of oligomers that aggregate quickly into into LBs.

When the most significant SUMO acceptor sites (K96 and K102) of α-syn were modified, replacing K96 and K102 with arginine (K96R/K102R), the SUMOylation of α-syn was strongly impaired, leading to increased inclusion formation and toxicity (Krumova et al., 2011). The different results obtained by the methods used in the present study can be partly explained by different subcellular distribution of oligomers. When random fields were visualized by microscopy, the cells that were analyzed may have possessed increased oligomerization in the nucleus or in the cytosol. Perhaps few cells with similar distributions were analyzed and this may explain the reduced oligomerization shown by fluorescence microscopy.

Altogether, the present results suggest that an impairment of α-syn SUMOylation may occur in the nucleous or in the cytosol, forming toxic oligomers which may or may not enhance by different cellular pathways can enhance or not the propensity of α-syn to aggregate taking us to believe that SUMOylation has an important relevance for neurodegeneration in PD. Recent data reveals that impaired SUMOylation of overexpressed α-syn contributes to pathological characteristics of α-syn (Krumova et al., 2011). The results of this study demonstrate that impaired SUMOylation results in increased levels of oligomerization. Moreover, SUMOylation is a known modulator of solubility and of protein-protein interactions (Dorval and Fraser, 2006; Fei et al., 2006; Janer et al., 2010; Mukherjee et al., 2009; Palacios et al., 2005). As is known that the oligomers are soluble species, it is possible that SUMOylation of α-syn may increase oligomerization by keeping more oligomers in solution. Indeed, a recent study showed that proteosomal impairment promoted the formation and accumulation

of SUMOylated α-syn oligomers and aggregates (Kim et al., 2011). However, whether SUMOylation, per se, can act as a protective or toxic mechanism, still remains to be determined. Even if, as it has been reported, small amount of SUMOylated α-syn are enough to delay fibril formation (Krumova et al., 2011), several cellular pathways may be involved during the process of α-syn aggregation.

Even though the protein levels were similar between all α-syn variants, the levels of S129G, K96R/K102R and Y125D were slightly lower than WT α-syn. This may explain some of the observed differences in oligomerization. Nevertheless, the A30P/A76P mutant also showed a parallel increase between protein levels and α-syn oligomerization, which, in part, may explain the increase in oligomerization.

Final remarks

Our study constitutes the first report where the effects on oligomerization of different mutant forms of α-syn were assessed in parallel in the same cellular system.

Our data revealed that oligomerization occurs in all circumstances of α-syn overexpression, regardless of mutation but can have different behaviors depending on α-syn modifications. In some cases increased formation of oligomers is correlated with impaired fibril formation, but additional biochemical studies will be essential to confirm the state of α-syn. It is conceivable that different types of oligomers may exist throughout the process of oligomerization, explaining differential cytotoxicity. Oligomers may possess different states of seeding throughout the cells and the site at which oligomers are located may impact the process of oligomerization as well.

In some cases, the decrease of oligomerization may be explained by a fast aggregation progress, resulting in a circumstance in which the oligomeric species immediately form large aggregations.

Thus, it will be interesting to investigate the presence of different types of oligomers, which occur throughout the pathway in which oligomers become fibrils aggregates. In conclusion, understanding the molecular mechanisms of subcellular distribution and oligomer-induced α-syn toxicity might open novel avenues for therapeutic intervention in PD.

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