Elucidating the role of post-translational modifications of alpha-synuclein using semisynthesis: phosphorylation at Tyrosine 125 and monoubiquitination at Lysine 6

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To my parents

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Abbreviations

α	Alpha
Å	Angstrom
Ala/A	Alanine
Asp/D	Aspartate
CD	Circular dichroism
Cys/C	Cysteine
Da	Dalton
°C	Degree Celsius
GuHCl	Guanidinium hydrochloride
HSQC	Heteronuclear single quantum coherence
E.coli	Escherichia coli
g	Gram
Glu/E	Glutamate
h	Hour
HEPES	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HPLC	High pressure liquid chromatography
IPTG	Isopropyl b-D-thiogalactopyranoside
k	Kilo
L	Liter
LB	Luria broth medium
M	Molar
m	milli

min	Minutes
MALDI	Matrix-assisted laser desorption/ionization
MESNa	2-mercaptoethane sulfonate
µ	micro
NaCl	Sodium chloride
NMR	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
rpm	Revolutions per minute
s	Seconds
Ser/S	Serine
SDS	Sodiumdodecylsulphate
SEC	Size-exclusion chromatography
TOF	Time of flight
Tris	Tris(hydroxymethyl)aminomethane
Tyr/Y	Tyrosine
UV	Ultra-violet
v/v	Volume per volume
w/v	Weight per volume
WT	Wild type

Abstract

Alpha-synuclein (α-syn) is a natively unfolded protein that is closely linked to Parkinson's disease (PD) by genetic, neuropathologic and biochemical evidence. Aggregated and fibrillar forms of α-syn are the main components of intracellular protein inclusions found in PD patients' brains, termed Lewy Bodies (LB). Both in animal models and *in vitro,* α-syn forms fibrillar aggregates that resemble those observed in PD brain tissues. Although disease-associated mutations have been shown to promote the fibrillization of α-syn, the exact mechanisms responsible for triggering α-syn aggregation and toxicity in sporadic PD remain unknown. Addressing this gap of knowledge is crucial for understanding the molecular basis of the disease and developing effective therapies for the treatment of PD and other synucleinopathies. This project was initiated on the basis of the working hypothesis that post-translational modifications (PTM) may play important roles in modulating α-syn function and/or regulating its aggregation and toxicity. More specifically, α-syn is ubiquitously N-terminally acetylated, and phosphorylated (serines 87 and S129), ubiquitinated (lysines 12, 21 and 23) and truncated forms of α-syn have been observed in association with wild-type α-syn in LB and in brain tissues from PD patients and transgenic animals. Other modifications, such as phosphorylation at tyrosine 125 (Y125), were significantly reduced in diseased brains. Despite the discovery of candidate enzymes that mediate α-syn phosphorylation, ubiquitination and truncation, little is known about how each of these modifications alters α-syn structure, function, aggregation and toxicity *in vivo*. This is primarily due to the lack of tools that allow site-specific introduction of these modifications and the lack of natural mutations that can mimic the effect of these modifications, including phosphorylation.

The primary focus of this thesis was to develop strategies to overcome these limitations so as to elucidate the effect of phosphorylation at Y125 and monoubiquitination at lysine 6 (K6) on α-syn structure, fibril formation, membrane binding and subcellular localization. Towards this goal, we developed two semisynthetic strategies that allow site-specific introduction of PTM in α-syn based on the ligation of synthetic peptides containing the desired modified amino acid with recombinantly expressed proteins using Expressed Protein Ligation. This approach enables the introduction of single or multiple PTM in the N- or C-terminal regions of α-syn, and the preparation of modified α-syn in milligram quantities. Using these approaches, we were able to show for the first time that ubiquitination stabilizes the monomeric form of the protein and inhibits, rather than promotes, α-syn aggregation, while phosphorylation at Y125 does not significantly change the structure and aggregation propensity of α-syn. With the semisynthetic

pY125 α-syn in our hands, we were also able to investigate for the first time the sub-cellular localization of pY125 α-syn through its microinjection into primary neurons. This was not previously possible due to technical limitations related to the absence of appropriate antibodies against pY125 α-syn for immunocytochemical studies and difficulties in generating site-specifically modified α-syn. Furthermore, we were able to investigate the effect of α-syn phosphorylation on its interactions with other proteins, by probing the effect of pS129 and pY125 on the binding to a nanobody that was specifically selected by phage-display to tightly bind to the C-terminal domain of α-syn. Accordingly, we demonstrated that phosphorylation at a single residue is capable of disrupting the binding of full-length α-syn to another protein. These results have wide-ranging implications for the potential role of phosphorylation and other PTM in regulating α-syn's function(s).

We also exploited our ability to combine semisynthetic and enzymatic approaches to investigate potential cross-talk between different PTM, namely phosphorylation at Y125 and S129 and monoubiquitination at K6. These advances would eventually allow investigating the effect of cross-talk between other N and C-terminal modifications on α-syn's properties and to investigate PTM-dependent protein-protein and protein-ligand interactions *in vitro* and in cellular models of synucleinopathies.

Together, our semisynthetic approaches provide novel means for the introduction of site-specific modifications in the N- and C-termini of α-syn. Current efforts in our laboratory are focused on extending these approaches to investigate the dynamics of PTM through the use of photocaged modified amino acids and to prepare novel fluorescently labeled α-syn variants to investigate its folding at the single-molecule level in living cells. The results presented in this thesis and preliminary studies from our group demonstrate that semisynthetic α-syn can provide unique opportunities to investigate structure-function of α-syn and the role of PTM in the biology of α-syn in health and disease.

Keywords: Parkinson's disease (PD); alphα-synuclein (α-syn); Lewy Bodies (LB); Aggregation; Amyloid; Oligomers; Dopamine (DA); Solid-phase peptide synthesis (SPPS); Native Chemical Ligation (NCL); Expressed Protein Ligation (EPL); Intein; Circular Dichroism (CD); Nuclear Magnetic Resonance (NMR); Transmission Electron Microscopy (TEM); Microinjection; Immunocytochemistry; Fluorescent Microscopy; Nanobodies; Isothermal Titration Calorimetry (ITC).

Resumé et Mots-clés

La protéine alpha-synuclein (α-syn) est impliquée dans la maladie de Parkinson suite à des données génétiques, neuropathologiques and biochimiques. Sous forme agrégée, α-syn constitue la composante majoritaire des inclusions cytoplasmiques trouvées dans les neurones des patients de la maladie de Parkinson, communément appelées Corps de Lewy. Dans les modèles animaux ou dans des expériences *in vitro*, α-syn forme des fibrilles ressemblant aux inclusions qui ont été identifiées dans les cerveaux des patients. Des mutations pathogéniques ont été identifiées dans la séquence d'α-syn et sont capables d'engendrer une augmentation de l'agrégation de la protéine. Cependant, la majorité des patients de la maladie n'ont pas de mutations dans leurs gènes et l'identité des facteurs qui induisent l'agrégation d'α-syn dans les cas sporadiques est inconnue. L'étude approfondie de ces facteurs est nécessaire afin de mieux comprendre les bases moléculaires de la maladie et pour mieux développer des thérapies effectives pour le traitement de la maladie. Ce projet a été initié suite à la spéculation que les modifications post-traductionnelles (MPT) jouent probablement un rôle important dans la régulation de la fonction d'α-syn, de son agrégation et de sa toxicité.

α-syn est acétylée (N-terminale), et des formes phosphorylées (sur les serines 87, 129), ubiquitinées (aux lysines 12, 21 et 23) et tronquées (1-134, 1-133, 1-126, 1-122, 1-119, 1-115 and 1-96) ont été observées dans les corps de Lewy. La quantité d'autres modifications, la phosphorylation à la tyrosine 125 par exemple, diminuent avec la progression de la maladie. Malgré la découverte de plusieurs enzymes responsables de la phosphorylation, ubiquitination et troncation d'α-syn, nous n'avons pas d'indications sur les effets de ces modifications sur la structure, fonction, agrégation et toxicité *in vivo*, probablement dû au fait que ces enzymes ne sont pas spécifiques par rapport aux acides aminées d'intérêt et que les mutants utilisés pour mimer ces modifications peuvent modifier intrinsèquement la structure de la protéine.

Le but ultime de ma thèse était de développer des stratégies qui visent à surmonter ces limitations et à utiliser ces stratégies pour élucider l'effet de la phosphorylation à Y125 et la mono-ubiquitination à la lysine 6 (K6) sur la formation des fibrilles, l'interaction avec les membranes et la localisation dans la cellule d'α-syn. Nous avons développé deux stratégies semi-synthétiques qui permettent l'introduction de MPT à des positions spécifiques de la protéine en se basant sur une combinaison de synthèse peptidique pour la flexibilité d'acides aminés non naturels qui peuvent être introduits et la production de protéine recombinante dans *E.coli*. Cette méthodologie est connue sous le nom de Ligation de Protéine Exprimée. Grace à cette approche, nous pouvons introduire des MPT dans le domaine N-terminal ou C-terminal

d'α-syn. Nous pouvons montrer pour la première fois, que la protéine mono-ubiquitinée inhibe l'agrégation alors que la phosphorylation à Y125 n'induit pas de changements significatifs sur la structure et la capacité d'agrégation de la protéine.

De plus, nous avons pu déterminer la localisation de la protéine phosphorylée à Y125 dans la cellule grâce à la micro-injection de la protéine dans des neurones primaires de rat. Les études précédentes n'avaient pas pu accéder à ce type d'informations sur la localisation à cause de difficultés techniques liées à la cross-réactivité de l'anticorps anti-pY125, ce qui élimine son utilisation pour des études d'immunocytochimie. Nous avons aussi étudié l'effet de la phosphorylation sur l'interaction d'α-syn avec d'autres protéines. Nous avons choisi d'étudier l'effet de la phosphorylation à S129 et Y125 sur l'interaction de la protéine avec un « nanobody » qui a été spécifiquement sélectionné par la méthode de « phage-display » pour interagir avec le domaine C-terminal d'α-syn. Les études précédentes d'interaction d'α-syn étaient basées sur l'utilisation de fragment de peptides C-terminaux. Nous démontrons pour la première fois que la phosphorylation d'un résidu unique est capable d'empêcher l'interaction de la protéine entière à une autre protéine. Ces résultats ont plusieurs implications concernant le rôle de la phosphorylation et d'autres MPT qui régulent la fonction et la dysfonction d'α-syn.

En résumé, notre nouvelle approche semi-synthétique permet d'introduire des MPT dans le domaine N-terminal et C-terminal d'α-syn. La richesse de cette approche réside dans sa capacité d'étudier l'effet de modifications de résidus qui n'étaient pas accessibles précédemment avec les méthodes génétiques et chimiques standards. Nos résultats devraient permettre de préparer d'autres formes modifiées d'α-syn à travers l'utilisation de l'approche semi-synthétique et l'étude de l'influence de ces MPT d'α-syn sur la fonction physiologique et pathologique d'α-syn.

Mots-clés : Maladie de Parkinson ; alphα-synuclein ; Corps de Lewy ; agrégation ; fibres amyloïdes ; Modifications post-traductionnelles ; Oligomères ; Dopamine ; Synthèse peptidique en phase solide ; Ligation chimique native ; Ligation de Protéine Exprimée ; Inteine ; Dichroïsme circulaire ; Résonnance Magnétique Nucléaire ; Microscopique Electronique à Transmission ; Microinjection ; Immunocytochimie ; Microscopie de Fluorescence ; Titration Calorimétrique Isotherme

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I. INTRODUCTION

I.2. Parkinson's disease

Parkinson's Disease (PD) is the second most prevalent neurodegenerative disease affecting 1-2% of the population older than 60 years (de Lau and Breteler, 2006). The major symptoms of PD are bradykinesia, resting tremor or postural instability and the disease is characterized histologically by a progressive loss of neuromelanin-containing dopaminergic neurons of the substantia nigra (SN) *pars compacta* (Figure I-1.A) and the presence of intracellular proteinaceous inclusions termed Lewy Bodies (LB) (Figure I-1.B). The proteins alpha-synuclein (α-syn) and ubiquitin are major components of the LB (Spillantini et al., 1997, Wakabayashi et al., 1997, Spillantini et al., 1998b). Motor dysfunction in PD patients only manifest at a late-age after substantial dopaminergic neuron loss, suggesting a strong compensatory system in the early stages of the disease that counteracts the consequence of early neurodegeneration (Bernheimer et al., 1973).

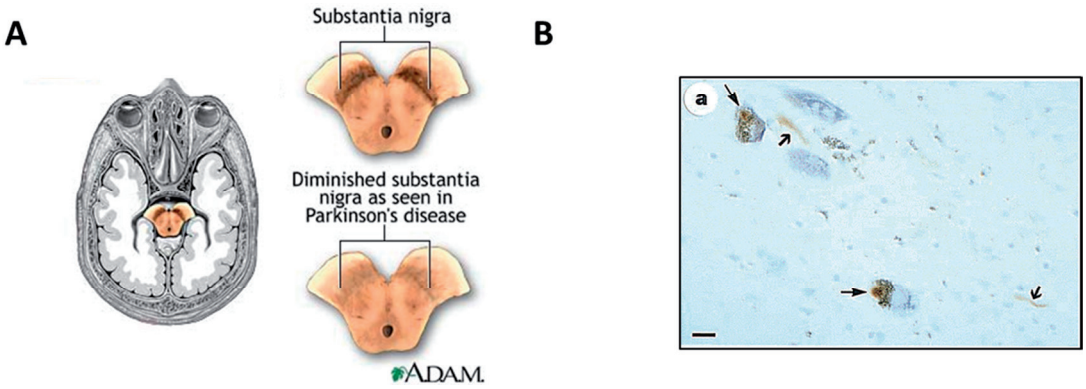


Figure I-1. Lewy Bodies and dopaminergic lesions in PD
A. Schematic depiction of the loss of neuromelanin-containing neurons in the substantia nigra in PD patients. Image adapted from A.D.A.M images (http://www.adamimages.com/Illustration/SearchResult/1/parkinson) and B. Immunohistochemical analysis of dopaminergic neurons containing Lewy Bodies that are immunopositive for α-syn (thick arrow). Figure from Spillantini *et al.* (Spillantini et al., 1997).

The symptoms of PD appear chronologically during the different stages of the disease: non-motor symptoms characterized by olfactory deficits, insomnia and constipation precede the motor symptoms whose diagnosis allows checking more precisely for the existence of PD

(bradykinesia and rigidity) (Section I.2.3.1). Late stages of the disease are accompanied by cognitive decline and psychiatric syndromes (Braak et al., 2004).

I.2.1. Genetic causes

While the majority of PD patients with idiopathic or late-onset disease do not seem to have inherited the disease, gene mutations account for 5% of total PD cases (Hatano et al., 2009). A large number of genes have been implicated in PD but in the focus of the thesis, we will only mention the most common gene mutations (Figure I-2). Two genes are responsible for autosomal dominant inherited causes of PD: the SNCA/PARK1 gene coding for the protein α-syn and the PARK8 gene coding for the protein LRRK2 (Gasser, 2009). Autosomal recessive inherited PD is triggered by mutations in the genes PARK2, PARK6 and PARK7 coding respectively for Parkin, PINK-1 and DJ-1 (Gasser, 2009).

PARK locus	Gene	Map position	Clinical phenotype	Pathology
PARK1/4	*SNCA*	4q21	Parkinsonism with common dementia	Lewy bodies
PARK2	*parkin*	6q25-q27	Early-onset, slowly progressing parkinsonism	Lewy bodies
PARK3	Unknown	2p13	Late-onset parkinsonism	Lewy bodies
PARK5	*UCHL1*	4p14	Late-onset parkinsonism	Unknown
PARK6	*PINK1*	1p35-p36	Early-onset, slowly progressing parkinsonism	One case exhibiting Lewy bodies
PARK7	*DJ-1*	1p36	Early-onset parkinsonism	Unknown
PARK8	*LRRK2*	12q12	Late-onset parkinsonism	Lewy bodies (usually)
PARK9	*ATP13A2*	1p36	Early-onset parkinsonism with Kufor–Rakeb syndrome	Unknown
PARK10	Unknown	1p32	Unclear	Unknown
PARK11	*GIGYF2*	2q36-q37	Late-onset parkinsonism	Unknown
PARK12	Unknown	Xq	Unclear	Unknown
PARK13	*Omi/HTRA2*	2p13	Unclear	Unknown
PARK14	*PLA2G6*	22q13.1	Parkinsonism with additional features	Lewy bodies
PARK15	*FBX07*	22q12-q13	Early-onset parkinsonism	Unknown
PARK16	Unknown	1q32	Late-onset parkinsonism	Unknown
FTDP-17	*MAPT*	17q21.1	Dementia, sometimes parkinsonism	Neurofibrillary tangles
SCA2	*Ataxin 2*	12q24.1	Usually ataxia, sometimes parkinsonism	Unknown
SCA3	*Ataxin 3*	14q21	Usually ataxia, sometimes parkinsonism	Unknown
Gaucher's locus	*GBA*	1q21	Late-onset parkinsonism	Lewy bodies

Figure I-2. Major genes and loci associated with PD
A summary of the different genes that are implicated in PD. The most studied are: SNCA and LRRK2, whose mutations induce autosomal dominant familial PD and Parkin, DJ-1 and PINK1 that are linked to autosomal recessive PD. Figure from Martin *et al.* (Martin et al., 2011)

I.2.1.1. Alpha-synuclein

α-syn was the first identified gene to cause PD (Polymeropoulos et al., 1996). α-syn contains three types of disease-causing mutations: single-point mutations, whole-gene multiplication (duplication and triplication) (Singleton et al., 2003) and gene polymorphism (Xia et al., 1996).

- Single-point mutations

The A53T mutation is the most commonly found mutation in PD patients and was first identified in Greek/Italian families (Polymeropoulos et al., 1997). A30P and E46K mutations were first reported in families of German and Spanish origin respectively (Kruger et al., 1998, Zarranz et al., 2004). Depending on the nature of α-syn mutation, PD patients exhibit different clinical symptoms (Corti et al., 2011). Patients with A53T mutation do not have a single phenotype but present largely diverse symptoms ranging from typical PD with late-onset disease to atypical PD with earlier-onset, faster disease progression or dementia (Golbe et al., 1990, Papapetropoulos et al., 2003, Ki et al., 2007, Puschmann et al., 2009). Patients with A30P mutation present the symptoms of idiopathic PD (late onset and mild progression) while the patients with E46K mutations show severe parkinsonism phenotypes (early-onset and dementia) (Kruger et al., 2001, Zarranz et al., 2004).

- Gene multiplication

In 2003, triplication of the SNCA gene was identified in the Iowa kindred where patients with four copies of the gene had a very early onset (mean age: 38 years) and a fast progression of the disease (Singleton et al., 2003). Later, patients of French and Italian origins with SNCA duplication showed clinical features similar to typical idiopathic PD with a late-onset (mean age of onset of 48) (Chartier-Harlin et al., 2004, Ibanez et al., 2004). The Lister Swedish/American family presents a good example of how the number of SNCA gene copies affects the phenotypic expression of the disease within a single family: one branch of the family diagnosed with a duplication of the gene showed a typical late-onset PD while the other branch, which had a triplication of the gene, exhibited early-onset PD with dementia (Fuchs et al., 2007). There is hence a proven correlation between the number of copies of the gene and hence protein expression levels and the severity of the disease. Aside from the impact of single-point mutations on the pathogenesis, this is the first time that altered levels of α-syn expression were speculated to lead to the progression of PD (Eriksen et al., 2005) (Figure I-3).

• Allele polymorphism

Genetic variability has been reported in the α-syn promoter where a dinucleotide repeat polymorphic marker REP1 with an allele-length variability was mapped upstream of the transcription start site of the gene (Xia et al., 1996). This gene variability was suggested to show an association to PD in some cases (Kruger et al., 1999, Tan et al., 2000, Farrer et al., 2001b), while this was not confirmed in other studies (Parsian et al., 1998, Khan et al., 2001). The reason behind the discrepancy has been attributed to small population studies with potential biases. Hence, a collaborative program of 18 research sites was launched to investigate whether SNCA haplotypes are associated with PD and whether REP1 variability modifies the age of disease-onset (Maraganore et al., 2006). The results showed association of the allele-length variability in REP1 with PD: individuals that had the 263 base pair (bp) allele, not the 259 bp allele, were associated with a high risk for PD and the genotypes did not change the age of disease-onset (Maraganore et al., 2006). The effect of allelic variation in REP1 on transcription was assessed in cellular models where expression level increased 3-fold (Chiba-Falek and Nussbaum, 2001). Thus, α-syn levels are influenced by genetic variability in the promoter region. In addition to polymorphism in the promoter region, genetic variability was also identified in the coding (especially in exons 5 and 6) and non-coding sequence of α-syn with an increased tendency for PD (Mueller et al., 2005).

I.2.1.2. Other genes involved in Parkinson's Disease

• LRRK2

While α-syn mutations are rare, mutations in the gene coding for the protein LRRK2 (Leucin rich repeat kinase 2) are more common and occurs in 5-15 % of patients with autosomal dominant PD (Berg et al., 2005). One particular mutation – G2019S – is very frequent. The gene encodes a large proteins of 2,527 amino acids that harbors several domains: a leucine rich domain LRR, a GTPase domain and a kinase domain (Mata et al., 2006) (Figure I-3).

Several missense mutations are distributed among the sequence of LRRK2 and are present in patients with typical late-onset familial and sporadic PD (Paisan-Ruiz et al., 2004, Zimprich et al., 2004). The G2019S mutation was shown to increase the kinase activity of LRRK2 and mutations occurring in its GTPase domain resulted in a decrease of activity (Cookson, 2010). Unlike α-syn, no gene dosage effect has been observed in the case of LRRK2. Despite numerous studies that would link LRRK2 to the proteins α-syn and tau and to the impairment of protein translation, the physiological function of the protein is still unknown (Cookson, 2010).

• Parkin

Autosomal recessive forms of PD can be identified by the mapping of consanguineous families. Parkin mutations are the most common cause of early-onset PD and occur in 50% of the autosomal recessive familial forms of PD (Kitada et al., 1998, Periquet et al., 2003). Clinically, patients with Parkin mutations show similar phenotype as sporadic PD but also possess some particular clinical and neuropathological features: earlier-age of onset, slower disease progression but complicated early-motor fluctuations (Dawson and Dawson, 2010) and the absence of ubiquitinated LB (Farrer et al., 2001a, Hardy et al., 2003) (Figure I-3).

A large number and diversity of mutations, ranging from large deletions and multiplications to small deletions and insertions and single-point mutations have been identified (Corti et al., 2011). The Parkin protein is an E3 ubiquitin ligase of 465 residues with different known substrates such as synphilin, α/β tubulin, the p38 subunit of an aminoacyl tRNA synthase, and Parkin-associated endothelin receptor-like receptor (Shimura et al., 2000). Mutations in Parkin were hypothesized to lead to the loss of the E3 ligase activity and the accumulation of non-ubiquitinated substrates. However, *in vitro* studies using single-point mutations of Parkin do not affect its ligase activity but rather its solubility (Matsuda et al., 2006).

• PINK1

PINK1 (Phosphatase and tensin homologue (PTEN)-induced novel kinase 1) mutations are the second most common cause for early-onset PD with clinical symptoms in patients closely linked to patients with Parkin mutations (Valente et al., 2004a, Valente et al., 2004b, Ibanez et al., 2006) (Figure I-3). Missense mutations in PINK1 have been reported and induce a loss-of-function of the protein (Valente et al., 2004a). The PINK1 protein is a tumor suppressor that contains several domains: an N-terminal mitochondrial targeting motif, a trans-membrane region and a Ser/Thr kinase domain. Endogenous PINK1 has been reported to be localized within the inner and outer mitochondrial membrane while over-expressed PINK1 is present on the mitochondrial cristae (Silvestri et al., 2005). The kinase domain has been shown to face the cytosol while the N-terminal domain is attached inside the mitochondria (Zhou et al., 2008).

• DJ-1

Mutations in the DJ-1 gene are the least occurring in autosomal recessive PD (1% of the cases) (Bonifati et al., 2003a, Bonifati et al., 2003b) (Figure I-3). They include large deletion, missense mutations, frame shift and deletions of exons (Corti et al., 2011). The DJ-1 protein is a molecular chaperone that is activated during oxidative stress and translocates to the outer mitochondrial membrane (Dawson et al., 2010).

Studies on mutations in the different genes that induce PD when mutated provided critical insights into potential mechanisms through which the encoded proteins could cause PD. Those genes could yield therapeutic targets in the future if their normal and pathogenic functions could be unraveled. Until then, they shed light on the implication of cellular pathways such as mitochondrial dysfunction and oxidative stress on the pathogenesis, mainly due to data collected via the expression and genetic manipulations of these genes in cellular and animal models (example of Parkin and PINK1).

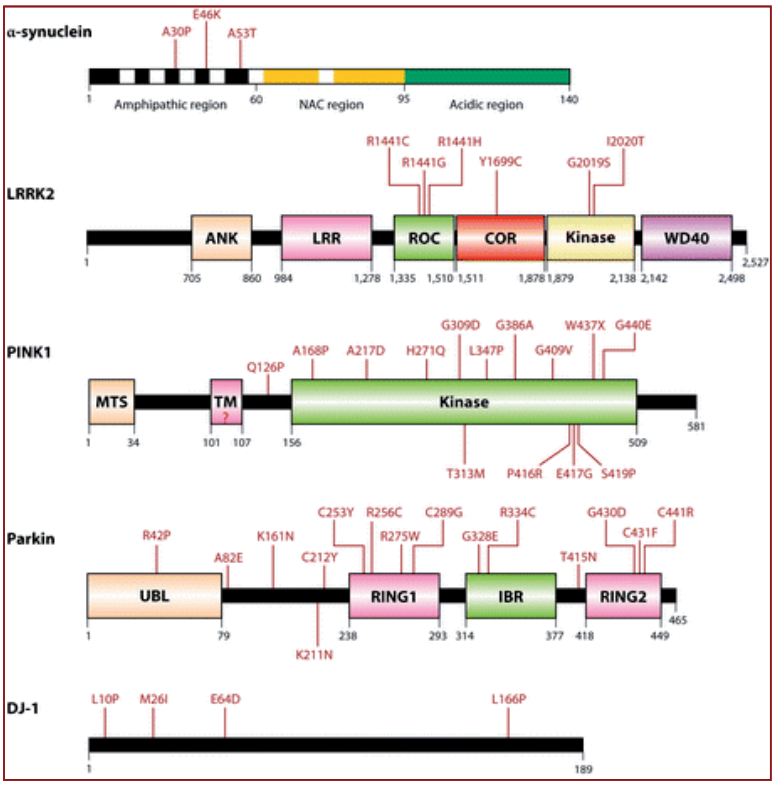


Figure I-3. Pathogenic mutations in the proteins involved in PD
Structural and functional domains of the proteins involved in PD. LRRK2 domains: ANK (Ankyrin repeat), LRR (Leucine Rich Region), ROC (homologous sequence to Ras-related superfamily of GTPases), COR (domain responsible for dimerization) and WD40 (structural motif). PINK1 domains: MTS (mitochondria targeting sequence), TM (transmembrane domain) and kinase domain. Parkin domains: UBL (ubiquitin-like domain), RING1 (Really Interesting New Gene 1), IBR (in between RING fingers) and RING2. Figure from Martin *et al.*(Martin et al., 2011).

I.2.2. Environmental factors that trigger Parkinson's Disease:

95% of PD cases are sporadic (Hatano et al., 2009). The environmental and molecular factors that trigger the pathogenesis are still unknown. Some studies have suggested that individuals who are exposed to manganese and copper or work on a farm using pesticides have a higher risk of developing PD (Gorell et al., 1999, Priyadarshi et al., 2001). The only confirmed study on the

link between environmental factor and PD pathogenesis is based on the discovery of toxin-induced phenotypes.

I.2.2.1. MPTP

In 1982, an outbreak of parkinsonism cases emerged in drug-abusers in northern California that was traced back to the injection of an illicit drug, 1-methyl-4-phenyl-4-proprionoxy-piperidine (MPPP). The compound MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a side product of the chemical synthesis reaction of MPPP, was found to induce the PD phenotype in these individuals (Langston et al., 1983). The selectivity of MPTP-induced toxicity to dopaminergic neurons is due to the conversion of this agent to MPP+ pyridinium ion by the enzyme monoamine oxidase B (MAO-B), which is subsequently taken up by DA transporters where it inhibits the complex I in DA neurons (Moore et al., 2005) (Figure I-4). MPTP exposure in drug users caused damage to the nigrostriatal dopaminergic pathway (Bove et al., 2005). Oxidative stress and decline in the activity of mitochondrial complex I of the respiratory chain in the SN of PD patients suggested the involvement of mitochondrial dysfunction as one possible pathway for neurodegeneration (Schapira et al., 1990, Jenner, 2003).

I.2.2.2. Paraquat

Another agent whose chemical structure closely resembles MPTP is the herbicide paraquat. Paraquat does not easily cross the blood-brain barrier but is still capable of inducing severe damage to the brain of individuals that were exposed to it (Bove et al., 2005) (Figure I-4).

I.2.2.3. Rotenone

Rotenone is also a major complex I inhibitor (Sherer et al., 2002a). It is a flavonoid found in the roots and stems of several plants and was commonly used as a pesticide and insecticide. Data on the stability and half-life suggest it may not have been involved in an epidemiologically-induced PD (Bove et al., 2005). Rotenone easily crosses the blood-brain barrier, but unlike MPP+, it is not selectively taken up by DA neurons (Talpade et al., 2000, Sherer et al., 2002a) (Figure I-4).

It is important to note that the link between the inhibitors of the complex I of the respiratory chain, oxidative stress, and neuronal loss is still missing. Current efforts are underway to resolve this issue through the generation of toxin-based animal models of PD (section I.2.4.2).

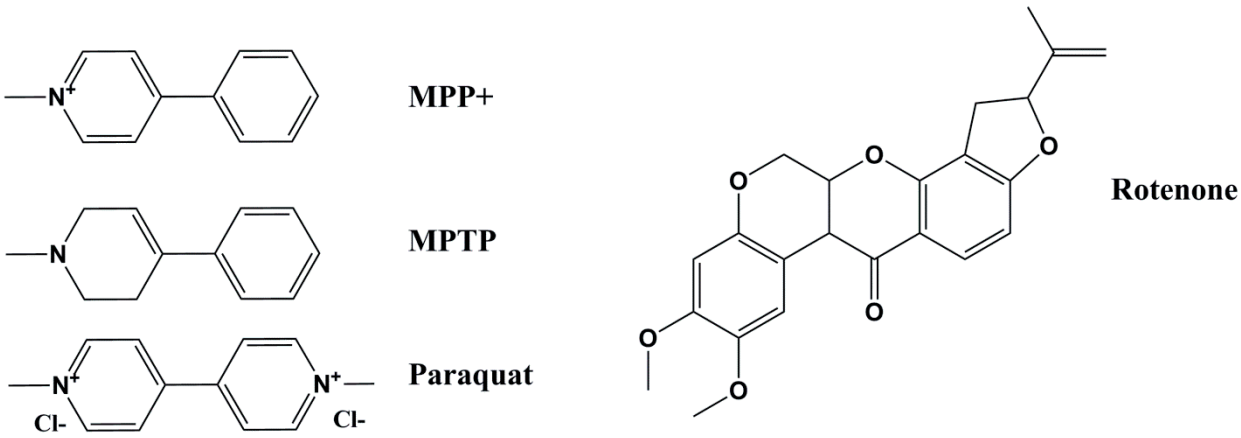


Figure I-4. Chemical structure of MPTP, MPP+, paraquat and rotenone

I.2.3. Diagnosis and treatment of PD

I.2.3.1. Diagnosis of PD

- Motor symptoms

The features that best characterize PD are tremor at rest, bradykinesia, rigidity, and postural instability (Vingerhoets et al., 1997, Bohnen et al., 2008). Rating scales have hence been used to assess motor impairment in PD patients although with varying reliability (Jankovic, 2008). The Hoehn and Yahr scale and the Unified Parkinson's Disease Rating Scale (UPDRS) are the most widely used for assessing disability and impairment (Ebersbach et al., 2006, Jankovic, 2008).

- Imaging techniques

The most prominent diagnosis currently available for PD is brain imaging (Brooks and Pavese, 2011). Brain abnormalities can be visualized using Transcranial ultrasonography (TCS) which detects increased echogenicity due to an augmentation of ferritin quantities in PD patients' brains (Becker et al., 1995, Berg et al., 2001) and Magnetic Resonance Imaging (MRI) which reports changes (atrophy) in brain volume (Geng et al., 2006).

Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT) allow investigation of dopamine terminal dysfunction through the use of radiolabelled tracers. A 50% reduction of ^{18}F-dopa uptake has been reported in PD patients (Morrish et al., 1998) and uptake was inversely correlated with the degree of rigidity and bradykinesia (Vingerhoets et al., 1997) (Figure I-5). Other radiolabelled tracers are also used to monitor the transport of DA from the cytoplasm into secretory vesicles (Bohnen et al., 2006).

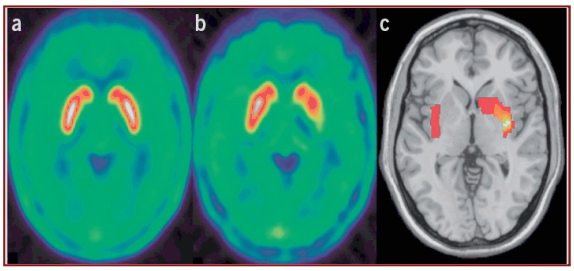


Figure I-5. PET images assessing the uptake of ^{18}F-dopa
Control brain (a) shows high uptake in the striatum while PD patient (b) exhibit marked reduction. The difference in uptake between (a) and (b) is shown in (c). Figure adapted from Obeso *et al.* (Obeso et al., 2010).

• Non-motor symptoms

Some non-motor symptoms such as depression, sleep disorders and constipation occurs in PD patients (Chahine and Stern, 2011) precede motor dysfunction in PD patients. Olfactory dysfunction (identification, and discrimination of smells) is also present in more than 90% of PD patients and standardized tests are starting to be used to detect these olfactory impairment (Ansari and Johnson, 1975, Doty et al., 1995, Bohnen et al., 2008). Genetic testing for disease-linked mutation could present an appealing method to detect PD since several mutations have been identified. However, the variable penetrance of those mutations prevents the wide use of genetic testing as a diagnostic tool. Direct analysis of the blood plasma could be a useful method to determine if modified forms of α-syn (phosphorylated and oligomeric) are present (El-Agnaf et al., 2006, Foulds et al., 2011).

While clinical diagnosis of PD patients currently relies mainly on the observation of motor symptoms, the detection of the histopathological hallmark of PD, i.e. the LB, still relies on *post-mortem* observation of PD brain samples. New molecules that would bind to α-syn selectively in the LB and allow its detection in PET and SPECT could provide a drastic improvement in PD patient diagnosis.

I.2.3.2. Treatment of PD

All the treatments mentioned herein are symptomatic and no current treatment that is able to reverse the pathology of PD has been discovered.

• Oral medications

L-Dopa

In 1960, Hornykiewicz and colleagues showed a prominent loss of dopamine (DA) in the putamen and the caudate nucleus of PD patients (Ehringer and Hornykiewicz, 1960). Indeed, it is now widely accepted that the loss of DA as the result of the degeneration of dopaminergic

neurons in the SN *pars compacta* is the major pathological hallmark of PD. DA-replacement therapy emerged after the isolation of L-dopa, the naturally-occurring isomer of the amino acid D-dopa (L-dihydroxyphenylalanine) from the bean of *Vicia faba*, and a successful clinical trial of its use in patients with a dramatic anti-akinesia effects has been reported (Birkmayer and Hornykiewicz, 1961) (Figure I-6). L-dopa has since been considered a "gold-standard" in drug therapy for PD patients. However, it has been shown that, as the disease progresses, involuntarily muscle movements termed dyskinesia are being seen in the treated patients. Around 90% of the patients experience these motor response fluctuations after 10 years of treatment (Ahlskog and Muenter, 2001). PET imaging studies have showed increased DA levels correlated with dyskinetic symptoms in the caudate-putamen (de la Fuente-Fernandez et al., 2004). The increase of unnaturally high levels of extracellular DA may be the cause of the emergence of dyskinesia (Carta and Bezard, 2011). The obscure relationship between L-dopa administration and the clinical response has to be elucidated to design L-dopa-like drugs with better pharmacokinetics properties.

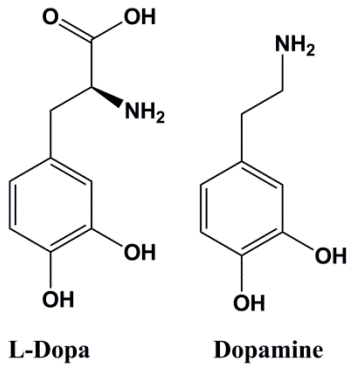


Figure I-6. Structure of dopamine and L-dopa

Anticholinergic drugs

Anticholinergic drugs were the first treatment option for PD patients but have been supplanted by the use of L-dopa in patients (Brocks, 1999). The rationale for the use of anticholinergic drugs in therapy was based on the report of the loss of DA in PD patients and the subsequent imbalance between dopaminergic and cholinergic pathways (Brocks, 1999). Anticholinergic drugs such as beperiden and benztropine have been used in young PD patients but the treatment was stopped due to side-effects that were reported in the elderly (constipation and tachycardia). Other side-effects were reported for use of anticholinergic drugs and the inductions of

cholinergic deficit that could result in hallucinations in treated patients (Zahodne and Fernandez, 2008).

Antiglutamatergic drugs

Excessive activation of N-methyl-D-aspartate (NMDA) receptors in PD patients is due to the depletion of striatal DA and the loss of its inhibitory effect on these receptors. As a consequence, a massive influx of Ca^{2+} in the neurons triggers neuronal death (Koutsilieri and Riederer, 2007). Amantadine and Memantine are two antiglutamatergic drugs that were used for the treatment of PD but their efficiency is not comparable to DA-replacement medication (Koutsilieri and Riederer, 2007). Also, excessive inhibition of glutamatergic function has been shown to be associated with schizophrenia, rendering the administration of these drugs to patients quite risky (Lange et al., 1997).

- Surgical interventions

Ablative surgery

Advances in imaging techniques used to monitor PD progression in patient brains along with the growing amount of data that shows that oral medications do not revert the disease has triggered the development of surgical interventions in the following brain regions: motor thalamus, the *globus pallidus*, and the subthalamic nucleus (STN) (Starr et al., 1998). The choice of these brain regions is based on the occurrence of motor dysfunction in PD patients and the abnormal physiology of the *globus pallidus* and the STN. The patients that have undergone ablative surgery exhibited improvement of motor symptoms with major relief from tremors in the majority of cases. However, some side-effects were reported in these patients such as lip and hand numbness and confusion (Starr et al., 1998, Walter and Vitek, 2004).

Deep brain stimulation

Deep Brain Stimulation (DBS) potentially offers an alternative to the motor-complication side-effects of DA-replacement therapy and the potential complications related to ablative surgery. To relieve PD patients of the symptoms of the disease, DBS relies on the use of high-frequency stimulation via surgically implanted electrodes in specific regions in the brain (the subthalamic nucleus STN and globus pallidus), with each region of the brain having different intra- and inter-network connectivities. It has subsequently been used to treat bradykinesia and rigidity and offers the promise to become the method of choice for the treatment of PD (Hamani et al., 2005, Fasano et al., 2012). The molecular mechanism through which DBS alleviates the symptoms is still unknown but it was speculated that it acts as a reversible local inactivation of an ion

channel or by blockage of neuronal membrane depolarization (Benazzouz et al., 1993). Apathy, sleep deficiency, mood disorders and anxiety, pathological gambling and compulsive eating can occur as side effects after stimulation of the STN (Anderson and Mullins, 2003, Guehl et al., 2003, Fasano et al., 2012). Even though DBS can be applied to treat PD patients, it is still regarded as an experimental method that requires further study and optimization for the choice of brain area, type of electrodes to be used according to the different brain regions and better criteria for the selection of the patients according to their age and disease progression (Fasano et al., 2012).

- Cell-replacement therapy

Stem cell transplantation

The aforementioned treatments above have proved effective to a point, especially when applied in the early stages of the disease, but they present adverse effects and do not counteract the progression of the disease. Grafts of human fetal ventral mesencephalic tissue have arisen as a way to replace dying dopaminergic neurons in the striatum (Politis and Lindvall, 2012). The source of the transplanted stem cells is mostly from pluripotent embryonic or somatic cells or from neuronal stem cells. However, due to the poor survival of the pluripotent stem cells in the brain and the significant risk of tumor formation, stem cells derived from fetal or adult nervous system have been used (Pardal and Lopez-Barneo, 2012). Excellent results were shown for PD patients with a dramatic reduction in rigidity and bradykinesia (Lindvall et al., 1990, Bjorklund et al., 2003, Mendez et al., 2008, Olanow et al., 2009). The main limitation for this technique resides in the availability of the donor tissue. The dissection step that precedes the transplantation generates a mixture of neural population with different resistance to cell death. Hence, due to the high cell mortality, a large number of human embryos will be needed for cell preparation. Due to the origin of these stem cells, ethical, moral, and legal issues will be faced in many countries. Motor complications such as dyskinesia were also reported in some patients, due to aberrant synaptic connections between transplanted and endogenous neurons. In summary, major research efforts will be required prior to generalizing the procedure of cell transplantation in PD patients. Additionally, the effect of immune reactions, the risk of tumor progression and the generation of motor complications will need to be investigated.

I.2.4. Animal models of PD

PD is a neurodegenerative disease. Considering that human brain is not an easily accessible organ for biochemical analysis, animal models have to be generated with the corresponding phenotype of the disease. While cellular models offer an easy and affordable way to identify

specific pathways and molecular targets, the results derived from those studies cannot be extended to the progression of the disease *in vivo* and in patients. Hence, animal models are needed as the next step to validate the findings, especially in the context of neurodegenerative diseases where complex neuronal circuitry direct the progression of the disease. Therefore, the availability of animal models would permit scientists to study disease mechanisms, validate potential molecular targets and test novel therapeutic strategies.

I.2.4.1. Criteria for the perfect PD model

A PD model needs to fulfill several criteria: i) the degeneration of neurons should be age-dependent and progressive, ii) motor dysfunction such as rigidity and postural instability should be present and reversible in presence of dopamine-replacement therapy, iii) Lewy bodies and Lewy neurites should be reported in the brain slices (Dawson et al., 2010).

I.2.4.2. Toxin-based models

- 6-Hydroxydopamine

6-Hydroxydopamine (6-OHDA) is the hydroxyl form of DA. It has been used as a neurotoxin for catecholaminergic neurons in animal models, due to the induced release of radicals and the inhibition of complex I and IV of the mitochondrial respiratory chain (Schober, 2004, Alvarez-Fischer et al., 2008b). 6-OHDA does not cross the blood-brain barrier and thus stereotactic injections are required for the administration of the toxin to the SN or the striatum. Unilateral injection of the toxin is carried out in rats (where the non-injected hemisphere acts as a control) resulting in the loss of more than 80% of nigrostriatal neurons (Kirik et al., 1998, Schober, 2004) and motor impairment (Lindner et al., 1999, Alvarez-Fischer et al., 2008b). However, the absence of protein inclusions and the acute and non-progressive neuronal damage were the major drawbacks of these models (Schober, 2004).

- MPTP

MPTP is an inhibitor of the complex I of the mitochondrial respiratory chain that was implicated in the emergence of PD phenotypes in drug users (Langston et al., 1983) (section I.2.2.1). Administration of the toxin to animal can be carried out by stereotactical injection or by intravenous or intraperitoneal injection in which case it rapidly reaches the blood brain barrier (Schober, 2004). Treatment of animals with MPTP became the basis for animal model of PD because of its selective uptake by dopaminergic neurons and the precedent proofs of its effect in humans (section I.2.2.1). Non-human primates that were treated with MPTP showed α-syn positive LB-like structures and presented similar clinical, pathological, neurochemical and

pharmacological response responses as idiopathic PD (Markey et al., 1984, Forno et al., 1988). Mouse models using mild continuous MPTP intoxication showed upregulation of α-syn expression within DA neurons, cell loss and damage in the nigrostriatal dopamine pathway (Vila et al., 2000, Fornai et al., 2005). However, the issue of the presence of LB-like inclusions in these models remains controversial (Shimoji et al., 2005, Alvarez-Fischer et al., 2008a). It is interesting to note that few rodent models of MPTP-induced PD exist because of the resistance of rats to the toxin, even mouse models have exhibited less sensitivity than primates and were able to recover after 48 hours of treatment (Schober, 2004).

- Paraquat and rotenone

Paraquat and rotenone are used as herbicide and pesticide and were shown to induce PD symptoms in individuals that were exposed to them (Sherer et al., 2002a, Bove et al., 2005) (section I.2.2.2 and I.2.2.3). Upon systemic injection of paraquat to mice, DA neuron loss, reduced motor activity and most importantly an up-regulation of α-syn are seen and could potentially suggest that the paraquat animal model reproduce the major aspects of PD (Brooks et al., 1999, Manning-Bog et al., 2002). Later studies have shown that the combined administration of paraquat and manganese exacerbated the toxicity and caused more deficits in the dopaminergic system (Thiruchelvam et al., 2000).

In the rotenone-based rodent model, the toxin was administered via inhalation, subcutaneous injections or oral delivery (Uversky, 2004). The rats elicited motor dysfunctions (rigidity and postural instability) that were reversed by L-Dopa treatment (Betarbet et al., 2000, Bove et al., 2005) along with dopaminergic neuronal loss (Uversky, 2004). Among the toxin-based models, rotenone-infused rats were the sole model showing LB-like inclusions that were positive for α-syn and ubiquitin (Betarbet et al., 2000, Sherer et al., 2003).

The use of toxin-induced animal models represents a good model of PD: substantial nigrostriatal degeneration is observed, along with good replication of PD motor symptoms. However, the absence of LB-like inclusions (except in the rotenone models) prevents the use of these models to study aggregation-dependent neurodegeneration.

I.2.4.3. Genetic models

Several animal models have relied on the expression of proteins that are involved in PD (LRRK2, Parkin, DJ-1 and PINK1) and the data that emerged from their use have been reviewed extensively (Dawson et al., 2010, Martin et al., 2011). In the scope of the manuscript we will focus on α-syn-based models.

- Transgenic models

Mouse models

The first transgenic (Tg) mouse model that over-expressed WT α-syn was generated by Masliah and colleagues, under the platelet-derived growth factor (PDGFβ) promoter (Masliah et al., 2000). The mice showed motor impairment on the rotarod assay and the progressive accumulation of non-fibrillar neuronal inclusions which are positive for α-syn and ubiquitin. However, this model showed neither the loss of dopaminergic neurons nor the formation of LB. Subsequent studies suggested that the nature of the promoter used to drive the transgene expression dictates the pattern of expression of α-syn in the brain and the region of the brain that are targeted, hence defining the phenotypes of these models (Chesselet, 2008).

The Thy1 promoter induces the expression of high levels of α-syn in cortical and subcortical neurons, including the SN *pars compacta* (Rockenstein et al., 2002). In some of the Thy1 Tg mice, α-syn- and ubiquitin-positive inclusions were reported, along with age-dependent motor impairment. However, those inclusions lacked the amyloid-like characteristic of the LB (van der Putten et al., 2000). In other studies, fibrillar inclusions were detected in mice over-expressing WT and A30P α-syn, but the localization of these inclusions was different than in PD patients; indeed, in old mice, the inclusions were localized to the spinal cord, amygdala and the brain stem (Freichel et al., 2007).

The use of the Prion (Prp) promoter drives widespread high expression of α-syn in the extranigral region, and some of the phenotypes that resulted are not consistent with human PD (glial pathology, motor neuron degeneration). The A30P mouse model under Prp promoter only showed some motor dysfunction, as well as the presence of truncated species (Gomez-Isla et al., 2003). The PD-associated mutations such as A53T have been investigated in mouse models, revealing protein inclusions in the spinal cord, brain stem and thalamus, leaving the hippocampus and the SN spared (Giasson et al., 2002, Lee et al., 2002c). Motor impairment was also reported for A53T compared to WT and A30P model (Lee et al., 2002c) (Figure I-7).

The animal models that relied on the use of the tyrosine hydroxylase (TH) promoter (which directs expression in dopaminergic neurons) are the only ones which present nigrostriatal neurodegeneration. PD is undeniably a complex disease and nigrostrital degeneration may only represent one aspect of the disease pathology. But for these models again, only the double-mutated variant of α-syn A53T/A30P was able to show loss of TH-positive neurons as well as motor impairment and age-dependent DA level reduction but without the presence of inclusions(Thiruchelvam et al., 2004). The other models expressing A53T, A30P or WT α-syn failed to show any dopaminergic neuron degeneration (Matsuoka et al., 2001, Richfield et al.,

2002). However, the physiological relevance of the doubly-mutated variant is not clear since no report of PD patients carrying both A53T and A30P mutations is known.

Truncated variants of α-syn 1-120 and 1-130 were also investigated in mouse models under the TH promoter and the results have been variable: over-expression of α-syn 1-130 yielded selective but non-progressive loss of DA neurons, as well as motor dysfunction in the mice, whereas the mice that over-expressed α-syn 1-120 presented some protein inclusions but not neuron loss (Tofaris et al., 2006, Wakamatsu et al., 2008). The mouse model produced by Tofaris et al. (1-120 truncation expressed in α-syn KO mice) was the first to show presence of inclusions (Tofaris et al., 2006) and importantly, this was done in the absence of endogenous murine α-syn . Small amounts of mouse α-syn were capable of inhibiting the fibrillization of human α-syn *in vitro* (Rochet et al., 2000) and the presence of endogenous mouse α-syn in Tg mice over-expressing human α-syn would potentially impair its fibrillization propensity *in vivo*. The correlation between the inhibition of α-syn fibril formation *in vitro* and the decreased number of inclusions *in vivo* was also confirmed with studies involving β-syn, another member of the synuclein family: *in vitro* studies have revealed the inhibitory effect of β-syn on α-syn fibrillization and protofibril formation (Uversky et al., 2002b, Park and Lansbury, 2003) and a reduced number of inclusions was reported in double-transgenic mice for α-syn and β-syn compared to α-syn Tg mice (Hashimoto et al., 2001).

A mouse strain expressing an α-syn conditional transgene under a tetracycline promoter was generated by Nuber and colleagues (Nuber et al., 2008). The model failed to present fibrillar inclusions but showed loss of DA neurons in the SN as well as a progressive motor decline. Most importantly, when the expression of the transgene was turned off, the progression of the disease was halted but the symptoms were not reversed.

Drosophila models

While no single rodent model can fulfill all the criteria for a perfect PD model, *Drosophila* models over-expressing WT and mutant α-syn exhibit an age-dependent and progressive DA neurons loss, α-syn positive fibrillar inclusions and a decline of climbing ability (Feany and Bender, 2000) (Figure I-7). However, the loss of DA neurons that was initially reported by Feany and colleagues was not reproduced by other groups (Pesah et al., 2005) and the variability could be attributed to technical considerations (methods of fixing brain slices mainly) (Drobysheva et al., 2008). Due to the ease of genetic manipulation in *Drosophila*, several factors that could potentially influence α-syn-induced toxicity were examined (Mizuno et al., 2010):

studies of post-translational modifications of α-syn confirmed the presence of cross-talk between phosphorylation at residue 125 and 129 (Chen and Feany, 2005, Chen et al., 2009), expression of Parkin and PINK1 in Tg flies was shown to suppress motor impairment and retinal degeneration (Haywood and Staveley, 2004, 2006, Todd and Staveley, 2008) and dopaminergic neuronal loss was prevented by the expression of the chaperone Hsp70(Auluck et al., 2002) which interacts with α-syn *in vitro* and *in vivo* (section I.3.7) .

Caenorhabditis elegans models

Nematode models over-expressing α-syn presented accelerated dopaminergic neuron loss but no LB-like inclusions (Lakso et al., 2003, Kuwahara et al., 2006) (Figure I-7) .The effect of PD mutations on DA neurons loss was also probed in these models but did not show major differences with respect to the WT. Nevertheless, a reduction in DA levels was observed in the case where A53T or A30P were expressed (Kuwahara et al., 2006). *C.elegans* models do not allow accurate assessment of motor impairment but they have been used to perform genetic screenings. More than 900 gene targets were screened via RNAi knockdown and 20 genes were identified to potentially affect the folding of α-syn and the degeneration of neurons (Hamamichi et al., 2008). The effect of environment and toxins was also probed in this model confirming data observed in mouse and fly models on the increase of α-syn toxicity in presence of rotenone and 6-OHDA (Calahorro and Ruiz-Rubio, 2011) .

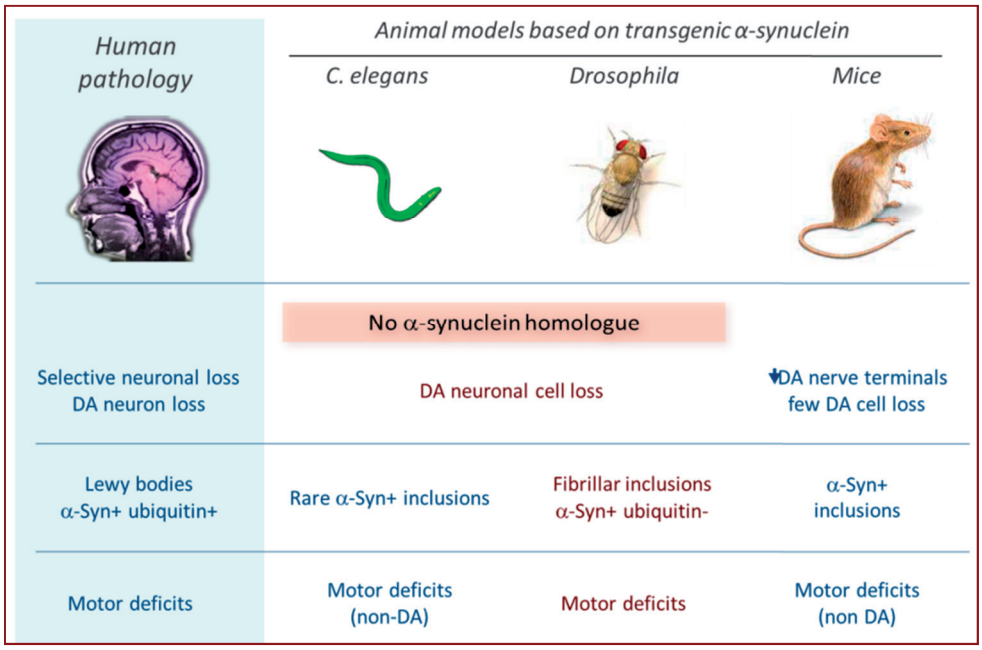


Figure I-7. Summary of the different transgenic PD models
Table summarizing the findings that were reported from the use of *C.elegans*, *Drosophila* and mouse models. Figure courtesy of Dr. Bernard Schneider, EPFL

- AAV-delivery models

To develop a rat model of PD, lentiviral vectors expressing α-syn were injected into the SN of rats. In contrast to transgenic mice models, a selective loss of nigral dopaminergic neurons associated with a dopaminergic denervation of the striatum was observed in rats expressing WT or mutant forms of α-syn (Lo Bianco et al., 2002). Expression of WT and A53T α-syn in adult rats resulted in 30-80% loss of the nigral dopaminergic neurons and the presence of α-syn-positive inclusions (Kirik et al., 2002, Klein et al., 2002, Lo Bianco et al., 2002). However, the expression of rat α-syn resulted in aggregation but no cell loss, suggesting that α-syn inclusions are not cytotoxic (Lo Bianco et al., 2002). The use of lentiviruses and adeno-associated viruses is more troublesome than the generation of transgenic mice since it requires stereotactic injection within the cell bodies of the SN *pars compacta*. Experts in virology are also needed to ensure proper handling of the virus and to confirm reproducibility (Chesselet, 2008). However, this methodology has major advantages: since the injection can be done in adult rats, the effects of early developmental expression of α-syn as well as the possibility of activation of compensatory mechanisms are circumvented (Waxman and Giasson, 2009). It also allows testing for genetic modifiers and other targets more rapidly at a lesser cost. The use of viral vector delivery in rat allowed the screening of phosphomimetic mutations of α-syn aiming at investigating the role of phosphorylation on inclusion formation and toxicity (Gorbatyuk et al., 2008, Azeredo da Silveira et al., 2009, Oueslati et al., 2012) as well as the study of proteins and toxins on the rescue (Yamada et al., 2005) or the exacerbation of α-syn toxicity(Sato et al., 2011, Mulcahy et al., 2012) (by Parkin and rotenone and GRK-6-mediated phosphorylation respectively).

C.elegans and *Drosophila* have been used for the rapid genetic and pharmacological screening compounds and for the easy and quick genetic manipulation. The major limitation for the use of these models is their failure to exhibit complex muscle complication which are key for PD pathogenesis, such as bradykinesia, rigidity and postural instability. Mouse models are considered as a close model to humans, because of the short life-span (2 years) of the mice which can allow modeling a disease in a short time(Dawson et al., 2010).

- Cellular models

Saccharomyces cerevisiae models

In the *S.Cerevisiae* model, no ortholog of α-syn is known but it provides a readily manipulable system to screen genes. Studies in *S.cerevisiae* have shown that α-syn localizes to the plasma membrane and forms cytoplasmic inclusions when its concentration increases (Outeiro and

Lindquist, 2003). A53T α-syn expression revealed the same phenotype but A30P mutant did not form inclusions (Outeiro and Lindquist, 2003). These inclusions however were not constituted of amyloid but rather α-syn associated with vesicles (Soper et al., 2008). Toxicity levels were also assessed and apoptotic signals, such as the accumulation of reactive oxygen species and the release of cytochrome c, were reported after the expression of WT or mutants α-syn (A30P and A53T) (Flower et al., 2005). The yeast model has been widely used for genetic screening and yielded novel findings such as the contribution of α-syn to the impairment of endocytosis and vesicle trafficking (Zabrocki et al., 2008); the α-syn-induced inhibition of ER-Golgi trafficking (Cooper et al., 2006) and the modulation of the phospholipase D activity (Outeiro and Lindquist, 2003). Moreover, different subcellular localizations of α-syn were revealed since A30P α-syn mutant localized to the cytoplasm (lower binding affinity to lipid vesicles *in vitro*) whereas WT and A53T bound preferentially to the plasma membrane (Sharma et al., 2006, Zabrocki et al., 2008). Interestingly, the localization of α-syn to the membrane was associated with phosphorylation by the CK kinase (Zabrocki et al., 2008) and initiation of aggregation (Zabrocki et al., 2005).

Mammalian cells models

The mere expression of α-syn in cells does not result in the formation of fibrillar inclusions, except for a few reports using adenoviral infection of COS7 cells (Gosavi et al., 2002, Lee and Lee, 2002). Co-expression of other proteins or submitting the cells to oxidative stress are essential for the formation of cellular aggregates. Co-expression of the protein synphilin (which was shown to be present in the LBs) and α-syn promotes the formation of cytoplasmic inclusions (Engelender et al., 1999, Smith et al., 2005). The expression of the enzyme transglutaminase (that catalyzes the cross-linking of α-syn *in vitro* and *in vivo*) was responsible for the formation of inclusions in 8% of the cells (Junn et al., 2003) and a construct consisting of the fusion of GFP to the C-terminus of α-syn was shown to undergo truncation (in the GFP domain) in 5% of the cells and to induce aggregation of α-syn (McLean et al., 2001, Badiola et al., 2011). The low percentage of inclusion-positive cells is a major drawback of these cellular models. Other cellular models for α-syn aggregation were reported using toxins. Oxidative stress seems to promote the formation of α-syn aggregates in 5-20% of the cells that were treated with peroxynitrite (Paxinou et al., 2001), rotenone (Lee et al., 2002b) and $FeCl_2$ (Hasegawa et al., 2004, Shendelman et al., 2004, Roberti et al., 2007).

Other cellular models relied on the uptake of extracellular α-syn that would act as a seed for the fibrillization of the intracellular protein. Uptake was mediated by calcium phosphate (Waxman

and Giasson, 2010), microinjection (Roberti et al., 2007), Lipofectamine (Nonaka et al., 2010) and cationic-liposomes (Luk et al., 2009). Extracellular α-syn was shown to be toxic to neighboring cells and to activate apoptosis signaling cascade (Danzer et al., 2011) and to activate microglia (Zhang et al., 2005). In one report, the toxicity was shown to be dependent on the concentration of the protein: extracellular α-syn fused to the TAT cell-penetrating peptide was able to protect the cells against H_2O_2 insult in the nanomolar range but gained toxicity at micromolar concentration (Albani et al., 2004). Cell culture studies reveal also numerous data discrepancies concerning the toxicity of intracellular α-syn: in some assays, the expression of WT α-syn rendered the cells more sensitive to cellular stress (Saha et al., 2000, Iwata et al., 2001, Petrucelli et al., 2002), whereas in other studies, α-syn was shown to be protective (da Costa et al., 2000, Hashimoto et al., 2002). These discrepancies probably arise from the use of different cell lines and transfection methods, of different promoters and tags on the protein (Waxman and Giasson, 2009).

To date, while no animal models are able to mimic all the pathological and neurological features of PD, each model has its own advantages and limitations. The toxin-induced model (MPTP, paraquat and rotenone) shows a good replication of motor dysfunction, along with neuronal loss but no LB-formation. In contrast, LB have been observed in transgenic models in some cases but a consistency in neuronal loss is still missing. As the scientific community is waiting for the the perfect PD model, transgenic animal models are used to investigate the role of particular proteins and molecular pathways through genetic screening and the toxin-induced models would on the other hand facilitate the screening for a therapeutic treatment.

I.3. Alpha-synuclein

I.3.1. A brief historical overview

The first synuclein gene was discovered in 1988 by Maroteaux *et al.* while screening an expression library with an antiserum against cholinergic vesicles from the electric lobe of the Pacific electric ray *Torpedo Californica* (Maroteaux et al., 1988) (Figure I-8). A cDNA coding for a 140 amino acid-protein was identified and was named synuclein because of the protein's localization in both the nucleus and at the presynaptic terminals. The protein was found to express only in nervous system tissue and it was hypothesized that synuclein could be involved in regulating events at the synaptic cleft via structural or regulatory pathways (Maroteaux et al., 1988).

In 1993, the analysis of all protein sequences of an amyloid-plaque sample by Ueda *et al.* resulted in the identification of unknown short peptides (termed NAC for Non-Amyloid Beta Component) (Ueda et al., 1993). The two NAC sequences, which exhibited high propensity to form amyloid fibrils (Ueda et al., 1993) corresponded to residue 61-80 and 81-95 of a 140 amino acid precursor known to be especially expressed in the brain that was initially given the name NACP and later called alpha-synuclein (α-syn). Two additional proteins with a very high sequence homology were later identified in human and rat brains; β-synuclein (134 residue, also called phosphoneuroprotein 14) (Nakajo et al., 1993) and γ-synuclein (127 amino acids) (Jakes et al., 1994, Lavedan et al., 1998).

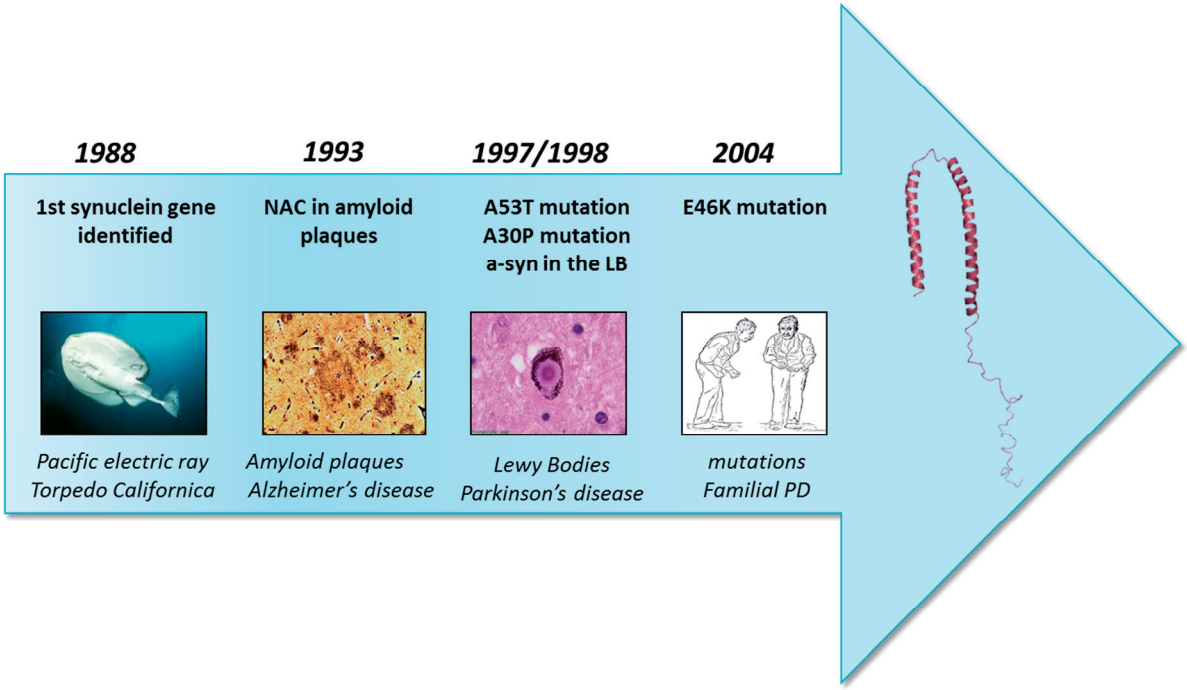


Figure I-8. Schematic representation of the major discoveries on α-syn
1988: discovery of the first synuclein gene in the Pacific electric ray *Torpedo Californica*. 1993: the NAC fragment of α-syn was identified in amyloid plaques. 1997: discovery of the first disease mutation in α-syn gene A53T. 1998: report of the second mutation A30P and the identification of α-syn as the main component of the LB. 2004: discovery of the third mutation E46K.
Photo credits: http://www.visualphotos.com/image/1x6470358/pacific_electric_ray_torpedo_californica
http://www.liv.ac.uk/researchintelligence/issue30/alzheimers.html
http://frontalcortex.com/?page=oll&topic=24&qid=413
http://www.biotek.uio.no/english/research/news-and-events/news/2010/VNM_VB_artikkel_HD.html

In 1997, the first α-syn mutation A53T was discovered in one Italian and three Greek families with an autosomal dominant inheritable form of PD (Polymeropoulos et al., 1997). The second mutation, A30P, was discovered very shortly after in German families (Kruger et al., 1998). In the meantime, while the link between α-syn and the genetics of PD was starting to emerge, the

missing link between the protein and the neuropathological hallmark of PD – the Lewy Bodies – was identified. Using antibodies against α-syn, immunohistochemical studies revealed the presence of the protein in neuronal cytoplasmic inclusions (NCIs) and oligodendrocytic glial cytoplasmic inclusions (GCIs) in multiple system atrophy (MSA) patients' brains, and in the Lewy Bodies (LB) from PD patients and dementia with Lewy Bodies (DLB) patients (Spillantini et al., 1997, Arima et al., 1998, Spillantini et al., 1998b). A third single-point mutation in α-syn gene, E46K, was discovered in 2004 in a Spanish kindred and duplication and triplication of the gene were also reported to be associated with autosomal dominant forms of familial PD (Singleton et al., 2003, Chartier-Harlin et al., 2004, Zarranz et al., 2004) (Figure I-8).

I.3.2. α-syn gene, transcript and protein

The human α-syn gene SNCA lies on chromosome 4q21.3-q22 and spans a region of 111kb (Campion et al., 1995, Chen et al., 1995, Shibasaki et al., 1995). It is organized in 7 exons (of which 5 are coding) (Lavedan et al., 1998). The ATG starting codon is encoded by exon 2, the stop codon by exon 6 and the NAC region (that was identified in amyloid plaques in AD patients) is encoded by exon 4 (Figure I-9).

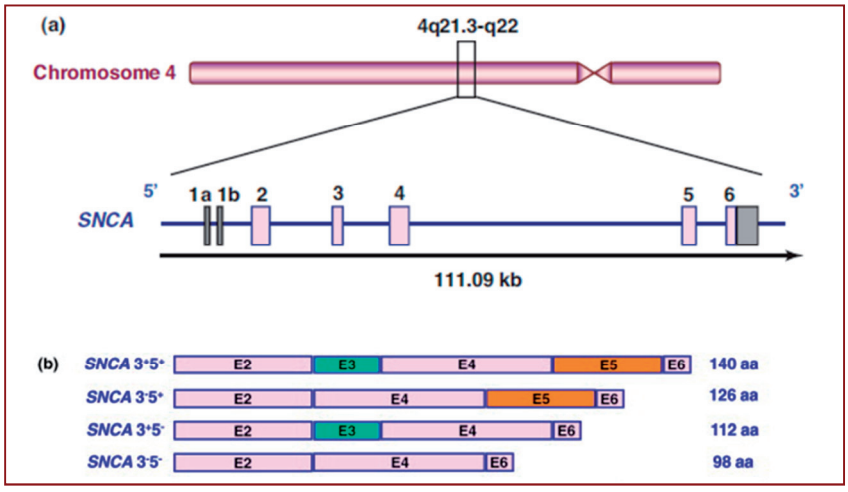


Figure I-9. Organization of the α-syn gene and alternative splicing isoforms
The α-syn gene is organized in 7 exons: the initiating methionine is encoded by exon 2, exon 3 encodes the NAC domain and the stop codon is present in exon 6. b) Alternative splicing of α-syn's exons 3 and 5 results in proteins of 140, 126, 112 and 98 residues. Adapted from Venda *et al.* (Venda et al., 2010)

The overall organization of the gene is conserved across animal species. Patients with duplication and triplication of SNCA have amplified exon 3 and 4 probably due the intrinsic instability of the region 4q21-23 on chromosome 4 (Chartier-Harlin et al., 2004). As for the

other synuclein genes, β-syn gene has been mapped to chromosome 5q35 with a total of 6 exons (of which 5 are coding); and the γ-syn gene lies on chromosome 10q23.2-q23.4 and has 5 exons (all protein-coding) (George, 2002) (Figure I-10).

The predominant α-syn isoform consists of 140 amino acids and corresponds to the transcription of exons 2,3,4,5 and 6. Other isoforms of α-syn caused by alternative splicing of exons 3 and 5 results in the production of shorter proteins. SNCA126 is produced by deletion of exon3, SNCA112 has a deletion of exon 5 and SNCA96 is produced by deletion of both exons 3 and 5. SNCA112 is the second most abundant isoforms and has been found in the brains of patients with LB disease (Venda et al., 2010).

```
alpha     1   MDVFMKGLSKAKEGVVAAAEKTKQGVAEAAGKTKEGVLYVGSKTKEGVVHGVATVAEKTK   60
beta      1   MDVFMKGLSMAKEGVVAAAEKTKQGVTEAAEKTKEGVLYVGSKTREGVVQGVASVAEKTK   60
gamma     1   MDVFKKGFSIAKEGVVGAVEKTKQGVTEAAEKTKEGVMYVGAKTKENVVQSVTSVAEKTK   60

alpha    61   EQVTNVGGAVVTGVTAVAQKTVEGAGSIAAATGFVKKDQLGKNEEG_____AP_QEGILE  114
beta     61   EQASHLGGAVFSG_____________AGNIAAATGLVKREEFPTDLKPEEVAQEA_AEEPLI  108
gamma    61   EQANAVSEAVVSSVNTVATKTVEEAENIAVTSGVVRKEDLRPSAPQQEGEASKEKEEVAE 120

alpha   115  DMPVDPDNEAYEMPSEEGYQDYEPEA 140
beta    109  EPLMEPEGESYEDPPQEEYQEYEPEA 134
gamma   121  EAQSG--GD---------------- 127
```

Figure I-10. Amino acid sequences of alpha, beta and gammα-synuclein
The main characteristic of β-syn is the absence of 11 residues of the NAC domain, while γ-syn exhibits deletion of residues in the C-terminal domain compared to α-syn.

The molecular mass of α-syn is 14460 Da and its sequence can be divided into three domains (Figure I-11): the amphipathic N-terminal domain and the hydrophobic NAC regions that are both highly conserved between species and the C-terminal domain that is variable in size and sequence (Surguchov, 2008).

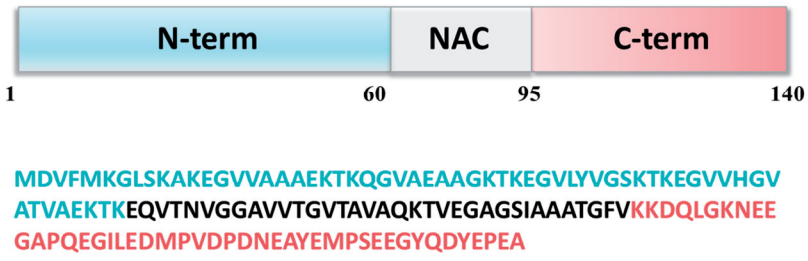


Figure I-11. Amino acid sequence of α-syn and its structural domains

The N-terminal domain spans from residue 1 to 60 and is characterized by six imperfect repeats of the consensus motif KTKEGV and forms amphipathic α-helices when bound to membranes

and synthetic lipid vesicles (Bussell and Eliezer, 2003, Jao et al., 2004, Bisaglia et al., 2006). The central NAC region from residues 60 to 95 is highly hydrophobic and is essential for fibril formation (Giasson et al., 2001). The C-terminal domain is highly disordered and acidic (rich in glutamate and aspartate residues). The α-syn protein is highly conserved among mammals and does not exist in invertebrates (Figure I-12). The comparison of the N-terminal domain of α-syn between mammal and bird species shows only 4 differences within 84 residues, while the majority of amino acids differences reside mainly in the C-terminal domain of the protein. Interestingly, in the majority of vertebrates α-syn contains the A53T disease-linked mutation (A53T), while α-syn in primates and human has an alanine at this position (Surguchov, 2008).

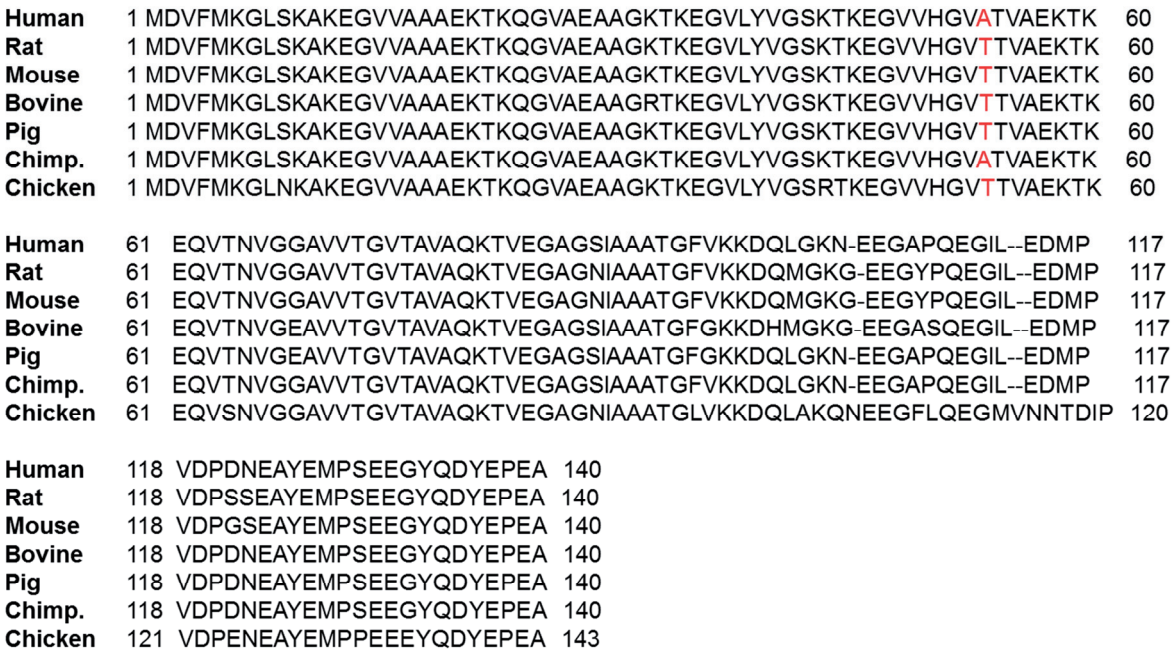


Figure I-12. Comparison of α-syn amino acid sequence between species
Sequence alignment of α-syn protein from human, rat, mouse, bovine, pig, primate and chicken species. The A53T mutation has been highlighted in red. Alignment was based on Uniprot sequences P37840, P37377, O55042, Q3T0G8, Q3I5G7, P61144 and Q9I9H1.

I.3.3. alpha-synuclein's structure

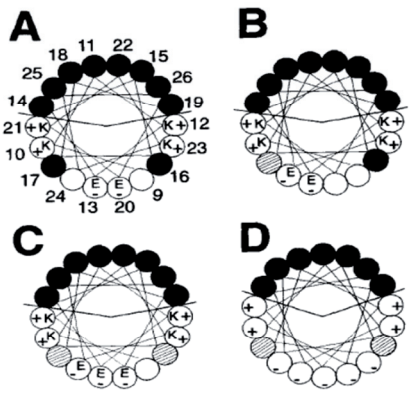
I.3.3.1. In solution

Since α-syn does not adopt stable secondary structures in solution, it has been referred to as a natively unfolded protein (Weinreb et al., 1996, Fauvet et al., 2012b). It be is also heat-resistant and the boiling step that is commonly used in purification procedures to remove contaminating proteins does not affect α-syn (*i.e.* heating does not cause the protein to precipitate). The Stokes radius of α-syn is estimated to be 34 Å, which would correspond to a 58kD globular protein (α-

syn is 14kD) (Weinreb et al., 1996). When run on SDS-PAGE, the band corresponding to α-syn migrates at an apparent mass of around 19kD. The slow migration of α-syn in SDS-PAGE has been attributed to the high density of negative charges in the C-terminal domain which could lead to reduction in SDS binding.

I.3.3.2. In presence of membranes

The imperfect 6-mer repeats present in the N-terminal domain of α-syn (Figure I-13) were shown to be "homologous" to other repeats of 11-residues in a plant protein (LEA76, a member of the family of late-embryo-abundant (LEA) group III) that shifts from random coil to α-helical conformation upon fast drying (Wolkers et al., 2001, George, 2002). Other 11-residue imperfect repeats also encode a class-A2 lipid binding motif that was described in exchangeable apolipoproteins (Segrest et al., 1992). A theoretical wheel projection of the α-helix of α-syn was constructed in 1995 after the discovery of the zebra finch homologue of α-syn (synelfin). The representation of residues 1-18 of canary synelfin (which are identical to the corresponding sequence of human and rat α-syn) showed a segregation of polar and nonpolar residues in opposite faces of the helix in order to form hydrophobic and polar faces, similar to the consensus A2 amphipatic α-helix (George et al., 1995) (Figure I-13).



MDVFMKGLSKAKEGVVAAAEKTKQGVAEAAGKTKEGVLYVGSKTKE
GVVHGVATVAEKTKEQVTNVGGAVVTGVTAVAQKTVEGAGSIAAATG
FVKKDQLGKNEEGAPQEGILEDMPVDPDNEAYEMPSEEGYQDYEPEA

Figure I-13. Comparison of helical wheel projection between α-syn and other proteins
Top: Helical wheel projection of A: Human α-syn, B: synelfin (zebra finch homolog), C: apolipoprotein, D: LEA76. Apolar residues are shown in black. Figure from George *et al.*(George et al., 1995). Bottom: The imperfect 6 repeats in α-syn N-terminal domain were highlighted in red.

These results suggested that α-syn can adopt a conformation that is likely to associate with lipids via an α-helical conformation. Indeed, it was later shown that α-syn does bind to anionic phospholipid vesicles and lipid-membrane mimetics (SDS micelles) (Eliezer et al., 2001) and adopts an α-helical conformation (Davidson et al., 1998). The N-terminal domain of α-syn is responsible for the interaction with phospholipids. Among the disease-associated mutations, only A30P exhibited weaker membrane binding affinity *in vitro* (Perrin et al., 2000) and in yeast (Outeiro and Lindquist, 2003). The loss of the A30P mutant's vesicle-binding property was also confirmed *in vivo* as well the implication of the N-terminal domain of α-syn for the binding via the fractionation of adult rat brain extracts (Jensen et al., 1998).

The region of α-syn that mediates the interaction with the micelles has been mapped to residue 1-102/103, with the C-terminal domain of α-syn remaining unbound and unstructured (Eliezer et al., 2001, Jao et al., 2004). NMR-based structural studies revealed that α-syn forms two antiparallel broken helices (helix 1: residue 3-37 and helix 2: residue 45-92) when bound to SDS micelles or lipid vesicles (Bussell and Eliezer, 2003, Chandra et al., 2003, Bortolus et al., 2008, Drescher et al., 2008) (Figure I-14). However, it was later shown that the curvature of the micelles dictates the conformation of SDS-bound α-syn, i.e. whether it forms an extended single helix or two broken helices (Trexler and Rhoades, 2009). FRET studies have shown the prevalence of an extended helix in presence of large vesicles (100nm), whereas the two helix conformation predominates in the presence of highly-curved micelles (Trexler and Rhoades, 2009).

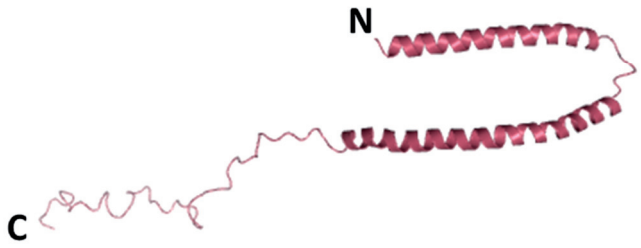


Figure I-14. Solution NMR structure of α-syn forming two broken helices when bound to SDS-micelles
PDB structure: 1XQ8

I.3.4. Fibrillization of alpha-synuclein

The conversion of α-syn from soluble monomers to insoluble aggregates has been implicated in the formation of LB in PD and other synucleinopathies. α-syn-positive fibrillar inclusions were detected in the brain of patients with neurodegenerative diseases, such as PD, DLB, MSA and

AD using immunohistochemistry (Forno, 1996, Polymeropoulos et al., 1997, Arima et al., 1998, Kruger et al., 1998, Spillantini et al., 1998b, Hamilton, 2000, Cookson, 2005). The fibrillar nature of these α-syn-positive inclusions was confirmed by immunogold-labelling and by staining with fluorescent dyes that specifically bind to fibrillar amyloid-like structure (Thioflavin S and Congo Red dyes) (Surguchov, 2008). *In vitro*, α-syn readily forms long mature amyloid fibrils that resemble closely α-syn fibrils from *post mortem* PD brains (Giasson et al., 1999, Conway et al., 2000a) (Figure I-16).

Although the molecular determinants of α-syn fibril formation remain unknown, studies during the past two decades have consistently shown that the NAC region is essential for α-syn aggregation (Giasson et al., 2001, Qin et al., 2007). This hypothesis is supported by the findings that 1) deletion of residues 71-82 from α-syn completely abolished α-syn fibril formation (Giasson et al., 2001); 2) the NAC region occurs within the sequence that forms the core of α-syn fibrils as revealed by solid-state NMR studies (more specifically residues 38-95) (Heise et al., 2005, Kloepper et al., 2007) by limited proteolysis studies (residues mapped: 32-100) (Qin et al., 2007) and 3) deletion of the C-terminal domain, which results in further exposure of the NAC region, increases the fibrillization of α-syn *in vitro*, with the fragments 1-87 and 1-120 showing the fastest aggregation rates (Crowther et al., 1998, Serpell et al., 2000, Murray et al., 2003).

- Mechanism of aggregation and structure of intermediates

α-syn fibril formation follows a nucleation-dependent mechanism (Wood et al., 1999) characterized by an initial lag phase that is associated with the formation of a fibrillization competent nuclei (oligomers) followed by a rapid growth phase associated with fibril formation and growth, via addition of monomers until an equilibrium (plateau) is reached between the monomers and fibrils (Figure I-15). Consistent with a nucleated polymerization mechanism, the addition of preformed shortened fibrils to monomeric α-syn solutions results in elimination of the lag phase (Wood et al., 1999).

I.3.4.2. Fibrils

α-syn forms amyloid fibers that are usually 10 nm wide and 0.1-10 μm long as seen by transmission electron microscopy (TEM) and atomic force microscopy (AFM) (Giasson et al., 1999, Conway et al., 2000a). These fibrils possess a cross-β-structure as discerned by X-ray fiber diffraction, which shows an intense reflection parallel to the axis of the fibril at around 4.7-4.8 Å (equal to the distance between two β-strands of the same β-sheet) and a signal

perpendicular to the axis of the fibril at around 10 Å (distance between two adjacent β-sheets) (Figure I-16) (Serpell et al., 2000).

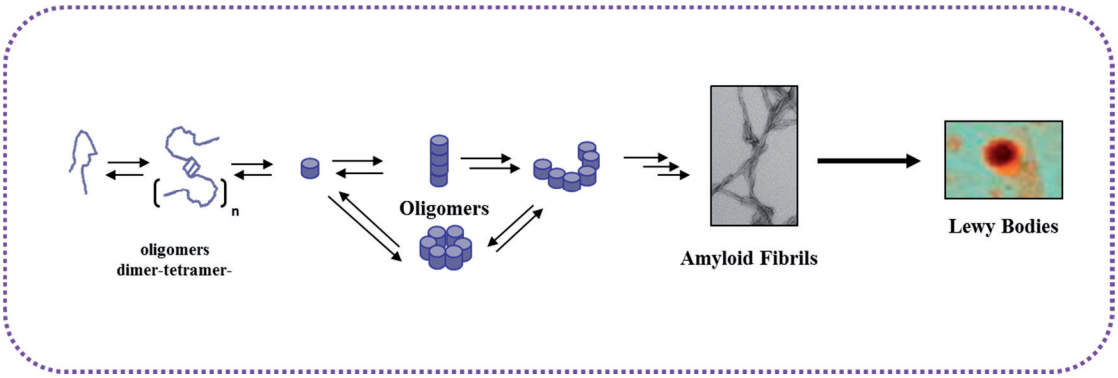


Figure I-15. Proposed mechanism of α-syn aggregation
α-syn's aggregation has been suggested to proceed via discrete intermediates. α-syn monomers assemble to form oligomers, some of them have pore-like properties, and further polymerize to form amyloid fibers. *In vivo*. the assembly result in the formation of LB. Adapted from a figure courtesy of Bruno Fauvet, EPFL.

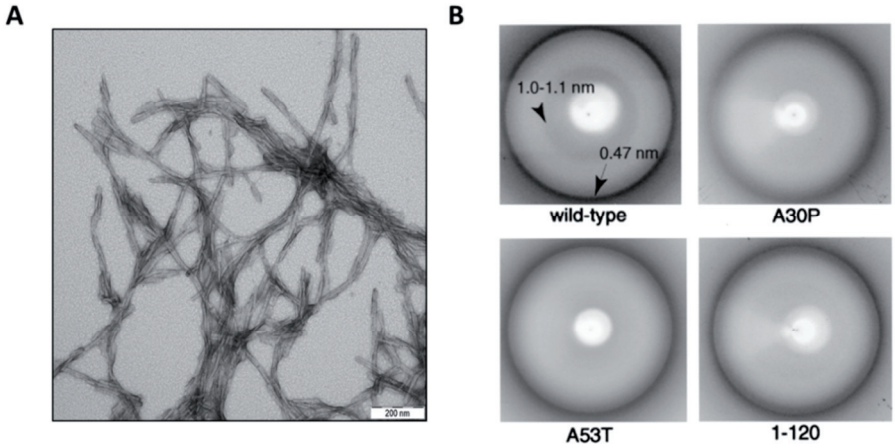


Figure I-16. Fibrillization of α-syn
A: TEM image of α-syn fibrils. The scale bar represents 200nm. B: X-ray diffraction of WT, A30P, A53T and C-terminally truncated α-syn. A reflection at 0.47 nm parallel to the fiber axis depicts the distance between two β-strands of the same β-sheet while the weaker reflection at 1-1.1nm is perpendicular to the fiber axis and represents the distance between two neighboring β-sheets of the cross-β-structure. Figure B is from Serpell *et al.*(Serpell et al., 2000).

I.3.4.3. Oligomers

In vitro aggregation studies revealed that α-syn fibril formation proceeds through a series of transient distinct oligomers. These oligomers are short-lived and very heterogenous in size (from 35 to 125 nm) and morphology (Hong et al., 2011). TEM and AFM studies have shown that α-syn can form spherical, amorphous or pore-like oligomers depending on the conditions

used to induce α-syn oligomerization and/or fibrillogenesis (Lashuel et al., 2002, Wright et al., 2009, Hong et al., 2011). Although direct evidence for oligomers in the brain remains lacking, biochemical analysis of brain homogenates and CSF samples from PD patients (El-Agnaf et al., 2006) and animal models of synucleinopathies (Chen et al., 2009, Colla et al., 2012) have revealed the presence of SDS-resistant oligomers using western blots and oligomer-specific ELISA assays and antibodies (Kayed et al., 2003, El-Agnaf et al., 2006) (Figure I-17).

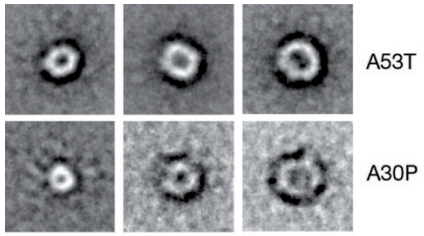


Figure I-17. Pore-forming oligomers of α-syn
TEM images of oligomers of A53T and A30P α-syn. Figure from Lashuel *et al.* (Lashuel et al., 2002).

I.3.4.4. The PD-linked mutations promote α-syn oligomerization and fibril formation

In vitro studies have showed that the three disease associated-mutants A30P, A53T and E46K promote α-syn oligomerization compared to the WT protein (Conway et al., 1998, Lashuel et al., 2002, Fredenburg et al., 2007), however only the A53T mutant was shown to increase the rate of fibril formation (Conway et al., 1998, Giasson et al., 1999, Conway et al., 2000b). The effect of PD-linked mutations was also shown to influence the size distribution and final morphology of α-syn oliogmers (Ding et al., 2002, Lashuel et al., 2002). For example, the A30P and E46K mutant promoted the formation of annular, pore-like protofibrils, whereas A53T formed both annular and tubular protofibrillar structures (Lashuel et al., 2002). The WT protein formed annular oligomers, but only after longer incubation times and to a much lesser extent that the PD-linked mutants (Ding et al., 2002).

I.3.4.5. Several factors influence α-syn fibril formation

- Lipids

α-syn binds to synthetic phospholipid vesicles *in vitro* and to synaptic vesicles *in vivo* (Davidson et al., 1998, Jensen et al., 1998). Studies by Lee et al showed that membrane-bound α-syn had a stronger propensity to aggregate and suggested that the α-syn fibril formation could be initiated on neuronal membranes (Lee et al., 2002a). This hypothesis has been supported by *in vitro*

studies that showed enhanced aggregation of α-syn in presence of vesicles composed of polyunsaturated fatty acid acyl groups (Perrin et al., 2001, Ding et al., 2002).

• Oxidation

Oxidative stress has been implicated in the pathogenesis of PD (Jenner et al., 1992) and is thought to play a major role in α-syn-mediated toxicity. Several studies have shown that conditions that induce oxidative stress (treatment with peroxynitrite, rotenone and $FeCl_2$) enhance α-syn aggregation and fibril formation in cell-based models (Paxinou et al., 2001, Lee et al., 2002b, Hasegawa et al., 2004). This oxidative-stress-induced aggregation correlates with increased α-syn toxicity in rat models of synucleinopathies (Sherer et al., 2003, Yu et al., 2010). Among the α-syn residues are susceptible to oxidation, methionine is readily oxidized to a sulfoxide and tyrosine to nitrotyrosine. However, α-syn does not contain cysteine residues. *In vitro*, methionine oxidation inhibits the formation of fibrils of α-syn and the addition of a 4-fold molar excess of oxidized α-syn completely inhibited the fibrillization of the WT protein (Uversky et al., 2002c). Aside from methionine oxidation, α-syn's tyrosine nitration is induced by oxidative stress (Danielson et al., 2009) and was shown to be present in α-syn aggregates both in LB and in rat brain samples (Giasson et al., 2000, Yu et al., 2010) and to seed the aggregation of the WT protein (Hodara et al., 2004).

• Metals

α-syn is a metal-binding protein and several studies have identified different residues within the C-terminus (119-124) that are involved in α-syn binding to different metal ions was reported (Binolfi et al., 2006, Santner and Uversky, 2010, Lu et al., 2011). Different cations can bind to α-syn and exhibit variable effects on the rate of α-syn fibrillization and the structure of the final α-syn fibrils (Santner and Uversky, 2010). Copper (II) was shown to bind to the C-terminal domain of α-syn and to increase its fibrillization (Paik et al., 1999, Binolfi et al., 2006) and a comparative study of different di- and tri-valent cations showed that Al^{3+} was the most effective in the stimulation of fibrillization (Uversky et al., 2002a). α-syn oligomerization was also showed to be induced by metal-binding and more specifically a report by Wright *et al.* showed the presence of a copper-induced oligomeric specie that, once separated from the monomers and the fibrils, was responsible for cytotoxicity (Wright et al., 2009) while Fe^{3+}-induced oligomers of α-syn were able to form permeating pores at a lipid-bilayer (Kostka et al., 2008).

I.3.5. Functions of alpha-synuclein

While the propensity of α-syn to form fibrils, a property that is enhanced by disease-associated mutations, provides a direct link between the protein and PD pathology, very little is known about the physiological function(s) of α-syn and how alteration in its function(s) could contribute to the pathogenesis of PD and other synucleinopathies. α-syn KO animal and cell-based models have been generated in the hopes of deciphering the normal function of the protein. As a result, hypotheses have emerged concerning the role of α-syn in several physiological processes such as synaptic maintenance, DA release, axonal transport, neurotransmission, and inhibition of the enzyme phospholipase D activity.

I.3.5.1. Synaptic maintenance

Synaptic plasticity is intimately regulated by vesicle trafficking. At rest, the majority of the synaptic vesicles are clustered in a reserve pool. Upon increase of neuronal activity, the vesicles exit from the pool, are directed to the site of fusion and are prepared for exocytosis. The transition of the vesicles from the pool to other sites is regulated by complex cellular signals (Bellani et al., 2010).

The first clue to the involvement of α-syn in maintenance of the synaptic function came when researchers observed the strong presence of α-syn in presynaptic terminals, its association with the membranes of synaptic vesicles and the increased expression of the zebra finch homolog of α-syn in the brain region implicated in song learning (Maroteaux et al., 1988, George et al., 1995, Irizarry et al., 1996). Experiments looking at complete or partial down-regulation of α-syn in animal models or cell culture hinted towards the regulation of vesicle trafficking. In particular, α-syn knock out (KO) mice showed a lack of abnormalities in the synaptogenesis but exhibited altered DA release (Abeliovich et al., 2000). Reports in primary hippocampal neuronal cultures confirmed that α-syn was not involved in synapse formation since its expression was delayed after synapse formation (Murphy et al., 2000). Using anti-sense oligonucleotides, a significant reduction in the size of the distal presynaptic vesicular pool was observed upon knockdown of α-syn (Drobysheva et al., 2008). Another report using α-syn KO mice showed significant impairment in synaptic response and a slower depletion of vesicles from the reserve pool compared to WT α-syn mice (Cabin et al., 2002). Together, these findings led to the hypothesis that α-syn is implicated in the process of synaptic vesicles trafficking from the reserve pool to the release sites (Bellani et al., 2010). Larsen *et al.* reported that the level of docked vesicles was increased in cells over-expressing WT α-syn but that the level of secreted

catecholamines was reduced and hence proposed that α-syn inhibits the vesicle priming step that would prepare them for exocytosis (Larsen et al., 2006).

I.3.5.2. Inhibition of phospholipase D activity

α-syn has been shown to bind preferentially to acidic phospholipids *in vitro* and *in vivo* (Davidson et al., 1998, Jensen et al., 1998, Perrin et al., 2000). On the other hand, the hydrolysis of phosphatidylcholine to phosphatidic acid is regulated by the enzyme phospholipase D (PLD) that has been shown to be inhibited by WT and mutant α-syn *in vitro* (Jenco et al., 1998, Ahn et al., 2002). More specifically, tyrosine phosphorylation at residue 125 increases the inhibitory effect of α-syn . However, the finding that α-syn inhibits PLD was challenged by a recent report (Rappley et al., 2009). PLD activation is linked to cell growth and neurotransmitter release (Ahn et al., 2002) thus, a normal balance of lipids is required for regulating a variety of signals at the plasma membrane and for proper synaptic function. Therefore, alteration in lipid composition may play a role in an α-syn -dependent toxicity.

I.3.5.3. Chaperone role

α-syn, a protein with no defined secondary structure, is able to act as a chaperone in order to inhibit the aggregation of other proteins such as esterases that are protected against heat and pH-stress; and alcohol dehydrogenase whose activity is preserved even from heat-induced denaturation. α-syn was also shown to protect against chemical-induced, heat-induced and DTT-induced precipitation of the proteins insulin, glutathione S-transferase (GST) and bovine serum albumin respectively (Kim et al., 2000, Souza et al., 2000b, Ahn et al., 2006). Studies by Kim *et al.* showed that the presence of the unstructured C-terminal domain of α-syn is responsible for its thermal stability (Park et al., 2002) and hence is important for its chaperone activity. Indeed, the fragment 2-97 of α-syn has been shown to be completely devoid of chaperone-like activity (Souza et al., 2000b).

I.3.5.4. Neuroprotective role

Although the majority of studies focus on the possible toxic mechanisms of α-syn, some reports have suggested that it may have neuroprotective properties. The over-expression of WT α-syn in neuronal and mammalian cell lines protected against H_2O_2-induced oxidative stress (Lee et al., 2001b, Hashimoto et al., 2002) but not against MPP^+ (Lee et al., 2001b). Interestingly, A30P and A53T mutants did not show this protective ability (Lee et al., 2001b). A subsequent study showed that the protective effect of α-syn against oxidative stress induced by MPP^+ but not H_2O_2 nor 6-OHDA could be reproduced in cellular models of α-syn toxicity (Jensen et al.,

2003). Moreover, WT but not A53T α-syn mutant was able to prevent the increase of caspase-3-like (acetyl-Asp-Glu-Val-Asp-aldehyde-sensitive caspase) after pro-apoptotic stimulation of neurons by staurosporine and etoposide (da Costa et al., 2000).

Some Tg animal models over-expressing WT or A53T α-syn were also shown to resist paraquat-induced toxicity while the control mice exhibited severe neuronal loss (Manning-Bog et al., 2003). Moreover, no correlation was found between the presence of apoptotic neurons and α-syn expression in rat model of 6-OHDa-induced insult (Kholodilov et al., 1999). In addition to the protective effect against toxin-based insults, α-syn was shown to rescue cells from the lethal degeneration induced by the deletion of the co-chaperone Cysteine-string protein-α (CSPα) in Tg mice, while A30P α-syn was unable to show any protective effect (Chandra et al., 2005). This protective effect may have been mediated by the rescue of the synaptic transmission impairment that resulted from CSPα KO.

I.3.6. Mechanisms of α-syn toxicity

I.3.6.1. Why is α-syn toxic to Dopaminergic neurons?

α-syn is widely expressed in the brain but neurodegeneration occurs mainly in the SN, *i.e.* in the dopaminergic neurons, in PD (Obeso et al., 2010). Motor deficit in PD has been attributed to the high susceptibility and death of dopaminergic neurons in the SN, although the molecular basis underlying this increased susceptibility of DA neurons remain unknown. Several studies suggest α-syn plays an important role in regulating the production, storage, release and reuptake of DA; impairment in each of these processes by α-syn could contribute to neuronal death in PD (Figure I-18).

- <u>α-syn and DA Production</u>

The activity of Tyrosine Hydroxylase (TH), the rate-limiting enzyme for the conversion of tyrosine to L-dopa, is negatively modulated by α-syn both at the gene expression level and/or by direct interaction with the enzyme (Perez et al., 2002, Baptista et al., 2003). Hence, under physiological conditions, α-syn attenuates the amount of cytoplasmic DA. When monomeric α-syn is getting scarce due to aggregation, the inhibitory mechanism exerted by α-syn on TH activity is impaired, resulting in an increase of cytoplasmic DA.

- <u>α-syn and DA Storage</u>

The storage of DA in vesicles is modulated by the activity of the vesicular monoamine transporter 2 (VMAT2), which triggers the uptake of cytoplasmic DA into vesicles for storage

(Venda et al., 2010). An impairment of synaptic vesicle formation could trigger the accumulation of DA in the cytoplasm, resulting in cell death. This was shown in PD patients' brains where levels of VMAT2 were reported to be decreased in PET studies (Lee et al., 2000). Moreover, the overexpression of A53T α-syn has been shown to decrease the levels of VMAT2, which would results in an increase of cytoplasmic DA (Lotharius et al., 2002, Lotharius and Brundin, 2002). Under physiological conditions, α-syn is thus able to regulate the availability of synaptic vesicles and the storage by VMAT2, but under pathological conditions, dysfunction of DA storage could lead to the accumulation of toxic dopamine which could contribute to neurodegeneration and cell death.

- α-syn and DA Release

The link between the physiological function of α-syn and synaptic vesicle trafficking was detailed earlier in section I.3.5.1. Over-expression of the α-syn mutants A53T and E46K, but not A30P, was moreover shown to induce an impairment of synaptic vesicle exocytosis (Nemani et al., 2010).

- α-syn and DA Uptake

The reuptake of DA into the presynaptic neuron is mediated by the dopamine transporter DAT. α-syn binds to DAT and accelerates DA uptake by increasing the number of transporters at the membrane. Parkin disrupts this interaction and rescues the cell from increased DA-induced toxicity (Lee et al., 2001a, Moszczynska et al., 2007).

In summary, the impaired activity of α-syn on several levels of DA synthesis, storage and release may contribute to the increased vulnerability of dopaminergic neurons in PD (Figure I-18). However, the toxic effect on dopaminergic neurons does not only result from α-syn nor DA alone, but could result from the interaction or the synergistic toxic effect of both molecules. Consistent with this hypothesis, several groups have shown that oxidized DA strongly inhibits α-syn fibrillization and promotes the accumulation of potentially toxic oligomers (Conway et al., 2001, Rochet et al., 2004).

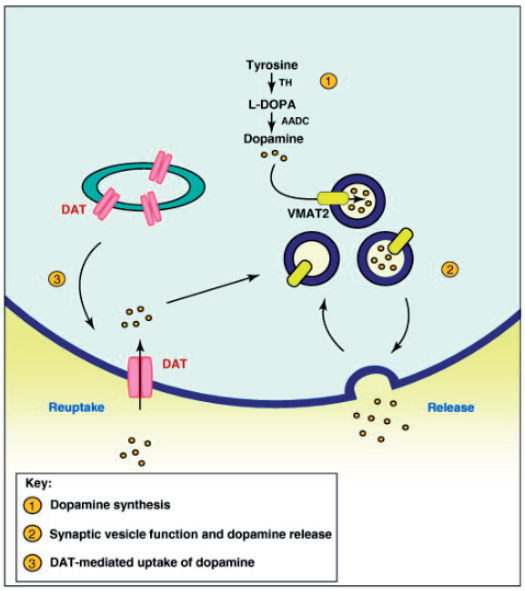


Figure I-18. Effects of α-syn on DA synthesis, storage, release and uptake
α-syn's toxic effects can be reflected in several steps of DA biosynthesis, storage into vesicles, release at the synapse and reuptake by DAT. Figure from Venda *et al.* (Venda et al., 2010).

I.3.6.2. Lewy Bodies: toxic or protective?

Identification of fibrillar α-syn as the main component of the LB in PD brains led to the hypothesis that α-syn fibril formation is the primary cause of neurodegeneration in PD. This hypothesis was supported by findings demonstrating that PD-linked disease mutations accelerate and increase the extent of α-syn fibril formation *in vitro* (section I.3.4) and in animal models of synucleinopathies (Giasson et al., 2002, Kirik et al., 2002, Lee et al., 2002c, Lo Bianco et al., 2002). However, this hypothesis failed to explain the following experimental observations; i) some of the surviving neurons in PD patients contain LB (Spillantini et al., 1997); ii) there is a lack of correlation between the extent of LB formation and neurodegeneration in humans. Moreover, LB have been detected in the brain of patients who died without showing signs of PD or other neurodegenerative diseases (Harding and Halliday, 1998, Richard et al., 2002). These findings were later better understood by *in vitro* data demonstrating that α-syn fibril formation, like amyloid-β in Alzheimer's disease (AD), proceed through a series of distinct oligomers that disappear upon fibril formation (section I.3.4). The accumulated evidence in support of the toxic oligomer hypothesis in AD and observation of a toxic effect induced by α-syn in the absence of fibril formation led to the hypothesis that α-syn oligomers, rather than the fibrils, are the primary toxic species and fibril formation results in the sequestration of toxic α-syn species. Consistent with this hypothesis, two independent studies recently showed that mutations

(A30P/A56P/A76P and E57K/E35K) that preferentially promote the formation of or stabilize α-syn oligomers exacerbated α-syn toxicity in *C.elegans*, *Drosophila* (Karpinar et al., 2009) and rat models of synucleinopathies (Karpinar et al., 2009, Winner et al., 2011). However, the molecular factors that induce the formation of α-syn oligomers *in vivo* remain unknown, but interactions with metals (Wright et al., 2009), polyunsaturated fatty acids (Sharon et al., 2003a, Sharon et al., 2003b, Assayag et al., 2007), DA (Conway et al., 2001, Rochet et al., 2004) and lipids (Lee et al., 2002a) have been shown to trigger and/or stabilize α-syn toxic oligomers *in vitro* with some of these oligomers inducing cytotoxicity (Assayag et al., 2007, Wright et al., 2009).

I.3.6.3. Mitochondrial dysfunction

PD pathogenesis is associated with the impairment of mitochondrial function and most specifically of complex I of the electron transport chain. A defect in complex I activity was detected in the SN of PD patients (Schapira et al., 1990, Sherer et al., 2002a) and known inhibitors of complex I – rotenone and MPTP – cause neurodegeneration in humans and animal models (section I.2.2.1 and I.2.2.2). This was shown to be mediated by inhibition of ATP production and generation of reactive oxygen species (ROS) (Sherer et al., 2002b). Furthermore, pathogenic mutations in PINK1, a kinase that localizes to the mitochondrial membrane, result in a decrease of the complex I activity, while DJ-1, a protein that acts as an antioxidant, is mutated in autosomal recessive PD and is translocated to the mitochondria upon oxidative stress insult.

The link between α-syn aggregation and complex I inhibition was initially suggested based on cell culture studies where rotenone treatment triggered α-syn aggregation and increased both DNA and protein damage (Sherer et al., 2002b). Later, it was shown that MPTP treatment of cells resulted in α-syn aggregation and release of caspase 3 (Kalivendi et al., 2004).

In addition, α-syn was also shown to impair mitochondria's structure and activity through direct binding. Several reports have revealed that α-syn localizes to the mitochondria in human brains (Devi et al., 2008) and cell-culture models (Cole et al., 2008, Nakamura et al., 2008, Shavali et al., 2008) and is able to bind purified mitochondria *in vitro* (Cole et al., 2008). The translocation of α-syn from the cytosol to the mitochondria was reported to increase during the progression of the disease (Devi et al., 2008) and was hypothesized to be triggered by acidification of the intracellular milieu as a result of oxidative insults (Cole et al., 2008). α-syn's import into the mitochondria causes fission of the organelles, a decrease of intracellular ATP, and elevated level of ROS resulting in cell death (Devi et al., 2008, Nakamura et al., 2011).

I.3.6.4. Oxidative stress

Impairment of the complex I of the mitochondria results in oxidative stress. Elevated level of oxidation markers such lipid peroxidation (Dexter et al., 1989), accumulation of the DNA damage product 8-hydroxy-2'-deoxyguanosine (Alam et al., 1997b), protein carbonyl formation (Alam et al., 1997a), and α-syn nitration (Giasson et al., 2000) were reported in the SN of PD patients. The increase of ROS in the SN could arise from several causes (Sherer et al., 2002a): i) the synthesis of DA by TH and the catabolism of DA by monoamine oxidase B generate H_2O_2 and ii) the spontaneous auto-oxidation of dopamine in the cytoplasm that also yields H_2O_2.Oxidative stress is hence one of the major contributors to the pathogenesis of PD (Jenner et al., 1992) and could even be an early feature in the pathogenesis.

I.3.6.5. Dysfunction of the ubiquitin/proteasome pathway

Genetic evidence on the potential impairment of the proteasomal degradation pathway in PD arises from the discovery of mutations in the gene coding for Parkin, an E3 ligase, that were linked to autosomal recessive PD and mutations in the gene for UCHL-1, a ubiquitin C-terminal hydrolase, which are linked to autosomal-dominant PD (Leroy et al., 1998, Shimura et al., 2000). It was hypothesized that defective proteasomal-degradation of α-syn would result in its accumulation and further aggregation in cells. One cell-based study confirmed this hypothesis and showed accumulation of non-ubiquitinated α-syn upon inhibition of the proteasome, while other studies could not reproduce this finding (Paxinou et al., 2001).

On the other hand, proteasome activity was shown to be impaired by α-syn mutations. Stable PC12 lines expressing A53T α-syn but not cells expressing the WT protein showed inhibition of the proteasome activity which resulted in the accumulation of ubiquitinated α-syn aggregates, along with an increase of non-apoptotic death (Stefanis et al., 2001). Furthermore, mice over-expressing a double-mutant form of α-syn (A30P/A53T) showed significant decline in proteasomal activity and an increase in the level of ubiquitinated proteins (Chen et al., 2006).

It has been proposed that α-syn -mediated inhibition of the proteasome results from the direct binding of the protein to the subunit S6' in the proteasomal cap or to the catalytic core of the 20S proteasome, thus disrupting its degradation activity (Snyder et al., 2003, Lindersson et al., 2004). Aggregated forms of α-syn exhibited stronger affinity to the 19S subunit of the proteasome with an IC_{50} in the range of 1 and 500nM (respectively for ubiquitin-independent and ubiquitin-dependent degradation). The monomeric form of α-syn did not inhibit the ubiquitin-dependent degradation and only showed partial inhibition of the ubiquitin-independent pathway (Snyder et al., 2003). Other reports have shown a preferential binding of the fibrils and

oligomers to another subunit of the proteasome (20S) which resulted in a marked inhibition of the degradation activity (Lindersson et al., 2004).

I.3.6.6. Dysfunction of the lysosomal pathway

Previous studies have shown that only a small fraction of α-syn is degraded by the proteasome (Ancolio et al., 2000, Paxinou et al., 2001, Lee et al., 2004, Vogiatzi et al., 2008, Ebrahimi-Fakhari et al., 2011) and that the majority of the protein is readily transported into lysosomes for degradation (Cuervo et al., 2004, Mak et al., 2010). Treatment of cells with 3-methyladenine dramatically increased the steady-state levels of α-syn (Webb et al., 2003). Chaperone-mediated autophagy was reported to be affected by PD-linked mutations: α-syn mutants A30P and A53T were able to block the uptake of receptors located on the lysosomal membrane, which would prevent their degradation and the degradation of other substrates (Cuervo et al., 2004).

I.3.6.7. Cell to cell transmission

The molecular mechanisms that determine how α-syn pathology spreads in the brain remain unknown. Recent studies suggest that α-syn pathology spreads through cell-to-cell transmission and propagation mechanisms whereby α-syn aggregates are secreted by one cell, cross the membrane of the recipient cell where they then induce/seed the formation of α-syn aggregates and/or exert their toxic effects *via* multiple mechanisms.

Studies on *post mortem* tissue of PD patients who had undergone embryonic neuron transplantation as a cell-replacement therapy revealed the presence of LB in 12 year-old and 16-year-old fetal mesencephalic neuron grafts (Li et al., 2008, Chu and Kordower, 2010, Li et al., 2010). It was hence speculated that the host-to-graft disease propagation was probably due to the transmission of α-syn from cell to cell (Li et al., 2008). Cell-to-cell transmission of α-syn was also reported in Tg mice over-expressing human α-syn that had mouse cortical neuronal stem cell transplants in the hippocampus. After 4 weeks, 2.5 to 15% of the grafts were positive to human α-syn, indicating that α-syn was transferred from the host brain to the grafts (Desplats et al., 2009). The transmitted α-syn had a capacity to seed aggregation and inclusions were shown in the grafted neurons (Hansen et al., 2011). Transmission of α-syn from neurons to glia was also reported in Tg mice over-expressing human α-syn under the control of a neuronal promoter. α-syn was detected both in neurons and glial cells and was causative of an inflammatory response (Lee et al., 2010).

α-syn has also been detected in the cerebrospinal fluid, blood plasma and in the saliva of PD patients (El-Agnaf et al., 2003, El-Agnaf et al., 2006, Tokuda et al., 2006, Devic et al., 2011). It is secreted from cells by exocytosis (Lee et al., 2005) and stress factors such as serum

deprivation and H_2O_2 treatment, proteasomal or lysosomal inhibition can enhance its secretion to the cell culture medium (Lee et al., 2005, Steiner et al., 2011). Once released in the extracellular milieu, α-syn can associate with membranes thanks to its lipid-binding properties (section I.3.3.2). However, monomeric and fibrillar α-syn display different preferred routes of entry into cells: monomeric α-syn penetrates cells through passive diffusion or by association to lipid rafts while aggregated α-syn is uptaken through endocytosis (as suggested from data where low temperature, dynamin inhibitors and trypsin treatment were applied to cells) (Lee et al., 2008a). The toxicity of extracellular α-syn is dependent on its internalization since treatment of cells with endocytosis inhibitors was protective (Sung et al., 2001). In cell-based models, extracellular α-syn can induce the formation of α-syn aggregates inside cells if it is introduced via cationic liposomes, calcium phosphate, Lipofectamine or without any transfection agent (Luk et al., 2009, Nonaka et al., 2010, Waxman and Giasson, 2010, Steiner et al., 2011). However, *in vivo*, α-syn does not need agents to cross the cellular membrane and is able to seed the aggregation of endogenous α-syn (Li et al., 2008, Desplats et al., 2009).

I.3.7. Interaction between α-syn and other proteins

α-syn has been shown to interact with a large panel of proteins with different roles in the cellular function and homeostasis such as membrane trafficking, ion channel modulation, redox metabolism, and gene regulation (Zhou et al., 2004, Woods et al., 2007). More than 70 potential interacting proteins were reported to interact with α-syn in the LB (Wakabayashi et al., 2007). Proteomics studies using C-terminal peptide fragments of α-syn and pull-down assays from mouse brain synaptosomes revealed that α-syn interacts mainly with mitochondrial proteins. Interestingly, the phosphorylated forms of this peptide are bound preferentially to cytoskeletal proteins and proteins involved in the endocytotic pathways (McFarland et al., 2008) (section I.4.2.2). Below is a brief overview of some of the key proteins that have been shown to interact with α-syn: tau, synphilin, β- and γ-syn, Rab, histones and chaperones.

I.3.7.1. Tau

The presence of neurofibrillary tangles, composed of hyperphosphorylated forms of the microtubule-associated protein Tau, is one of the pathological hallmarks of AD (Querfurth and LaFerla, 2010). Data from *post mortem* analysis of PD brain samples showed the presence of phosphorylated Tau at serine 262 in the striatum (Wills et al., 2010). The same group showed an increase of Tau phosphorylation and the absence of binding to the microtubules in an A53T transgenic mouse model of PD (Wills et al., 2011). Mice over-expressing the A30P α-syn mutant elicited the same findings (Frasier et al., 2005). Aside from PD-related mutations,

neurotoxins (such as MPTP) were shown to trigger the hyperphosphorylation of tau in cellular models (Qureshi and Paudel, 2011). *In vivo* and *in vitro* evidence for a direct interaction between α-syn and tau came from affinity purification (Jensen et al., 1999) and a mammalian two-hybrid assay (Benussi et al., 2005) and from the observation that α-syn induces and enhances the fibrillization of tau, which does not usually proceed readily *in vitro* but requires the addition of polyanionic cofactors such as heparin (Giasson et al., 2003, Kotzbauer et al., 2004). This finding was successfully reproduced in cell-based models where exogenous α-syn fibrillar seeds induced the intracellular aggregation and hyperphosphorylation of tau (Waxman and Giasson, 2011).

I.3.7.2. Synphilin

A yeast-two hybrid screen for α-syn interactants yielded synphilin as a hit (Engelender et al., 1999). It was later reported that synphilin is a component of the LB and was linked to PD after the discovery of a single-point mutation - R621C - in sporadic PD patients (Marx et al., 2003). These findings suggested a potential role in LB formation and PD (Wakabayashi et al., 2000). Furthermore, the two proteins were shown to interact *in vivo* (Engelender et al., 1999) and the interaction was shown to occur via the C-terminal domain of α-syn (Kawamata et al., 2001). Engelender and colleagues developed a cellular model of α-syn aggregation by co-expression of α-syn and synphilin and showed that expression of synphilin was required for α-syn aggregation (Engelender et al., 1999, McLean et al., 2001) (section I.2.4.3). These findings suggested that impairment of synphilin's degradation could potentially trigger the aggregation of α-syn.

I.3.7.3. β-and γ-syn

The two other members of the synuclein family β-syn and γ-syn are also expressed in the brain (Li et al., 2002). α-syn and β-syn were shown to interact in transgenic animal brain samples or in cell lines expressing both proteins (Hashimoto et al., 2001). Both β- and γ-syn bind to α-syn *in vitro* and are able to inhibit its aggregation (Hashimoto et al., 2001, Uversky et al., 2002b, Park and Lansbury, 2003). Moreover, crossbreeding Tg mice that over-expresses β-syn with mice that over-express α-syn inhibited α-syn aggregation and resulted in improved motor symptoms (Hashimoto et al., 2001), thus providing evidence for the interaction between the two proteins *in vivo*. These findings suggest that β-syn may act as a negative regulator of α-syn aggregation *in vivo*. Furthermore, supplementing the medium for *Drosophila* with a β-syn-derived peptide composed of D-amino acids was sufficient to improve locomotor activites of A53T α-syn -over-expressing flies (Shaltiel-Karyo et al., 2010).

I.3.7.4. Rab

Rab GTPase proteins regulate membrane trafficking processes and alternate between cytosolic (inactive) and membrane-bound (active) states. Consistent with the localization of α-syn at the synapse and the proposed role as a regulator of synaptic vesicle trafficking (section I.3.5.1), it was postulated that α-syn may interact with Rab proteins. Indeed, higher-molecular weight species of α-syn were shown to interact with Rab3a (presynaptic localization), Rab5, and Rab8 in DLB brain samples while the monomeric form of the protein interacts weakly (Dalfo et al., 2004, Dalfo and Ferrer, 2005). Moreover, extracellular α-syn interacts with Rab11a to ensure its internalization in cells (Liu et al., 2009). In a yeast model where α-syn expression impaired ER-Golgi trafficking (specifically by affecting the vesicle docking step), the expression of Ypt1p (the homologue of Rab1) rescued from α-syn toxicity and similar observations were made for Rab1, Rab3a and Rab8 both in neuronal and fly models (Cooper et al., 2006, Gitler et al., 2008).

I.3.7.5. Histones

The nuclear localization of α-syn has been confirmed after toxin insults by several reports (Goers et al., 2003a, Surguchov, 2008) and was shown to promote cell death as evidenced by findings used a fusion of α-syn to a nuclear localization signal (Kontopoulos et al., 2006). The molecular determinants that were responsible cytotoxicity involved a binding of α-syn to histones. α-syn was shown to bind to Histone 1 and 3 (Goers et al., 2003a, Kontopoulos et al., 2006) and to inhibit the acetylation of Histone 3 in cell-based and *in vitro* assays with reports of toxicity in which resulted in enhanced toxicity in cell-culture and *Drosophila* models (Kontopoulos et al., 2006). Furthermore, due to interaction of α-syn with Histone 1, the fibrillization rate of α-syn was dramatically increased (Goers et al., 2003a).

I.3.7.6. Chaperones

α-syn interacts with the chaperones Hsp27, Hsp70, and Hsp90 (Outeiro et al., 2006). Overexpression of Hsp27 reduced α-syn-induced toxicity in cells by 30% and the other chaperone Hsp70 was shown to strongly inhibit α-syn fibrillization through the preferential binding to α-syn oligomers (Dedmon et al., 2005). The co-chaperone carboxyl terminus of Hsp70-interacting protein (CHIP) co-localizes with Hsp70 and α-syn in LB and decreases the fibrillization rate of α-syn (Shin et al., 2005). CHIP ubiquitinates α-syn (Kalia et al., 2011) and directs it for degradation via the proteasomal and the lysosomal pathways (Shin et al., 2005).

I.4. Post-translational modifications of alpha-synuclein

The molecular factors that are responsible for triggering α-syn aggregation and mediating its toxicity in sporadic PD remain unknown. The identification of different post-translational modifications (PTM) of α-syn within the LB suggested that these modifications could play an active role in the initiation of α-syn aggregation and LB formation. This hypothesis was supported by a landmark study by Fujiwara *et al*. that demonstrated for the first time that the majority of α-syn within LB is phosphorylated at S129. This study also suggested that phosphorylation at S129 enhances α-syn fibril formation (Fujiwara et al., 2002). A subsequent and more comprehensive analysis of α-syn PTM using mass spectrometry confirmed this finding and revealed the presence of additional modifications, including N-terminal acetylation, ubiquitination and truncation (Anderson et al., 2006) (Figure I-19). The effect of some of these modifications on the structure, aggregation and toxicity of α-syn has been investigated both *in vitro* and *in vivo* and the search for enzymes regulating these PTM remains an active area of investigation, as these could be potential therapeutic targets for the treatment of PD and related synucleinopathies (Oueslati et al., 2010).

I.4.1. Phosphorylation at Serine residues

α-syn contains four serine residues (two in the N-terminal domain - S9 and S42 -, S87 in the NAC region and S129 in the C-terminus) (Figure I-20). To date, only S129 and S87 have been linked to PD through phosphorylation. Okochi *et al*. demonstrated that α-syn expressed in cell lines is constitutively phosphorylated at multiple sites. By expressing different S→A mutants of α-syn, they identified serine 129 and serine 87 as the main phosphorylation sites of α-syn (Okochi et al., 2000). Phosphorylation of α-syn at S87 was later discovered to be present within the LB (Paleologou et al., 2010).

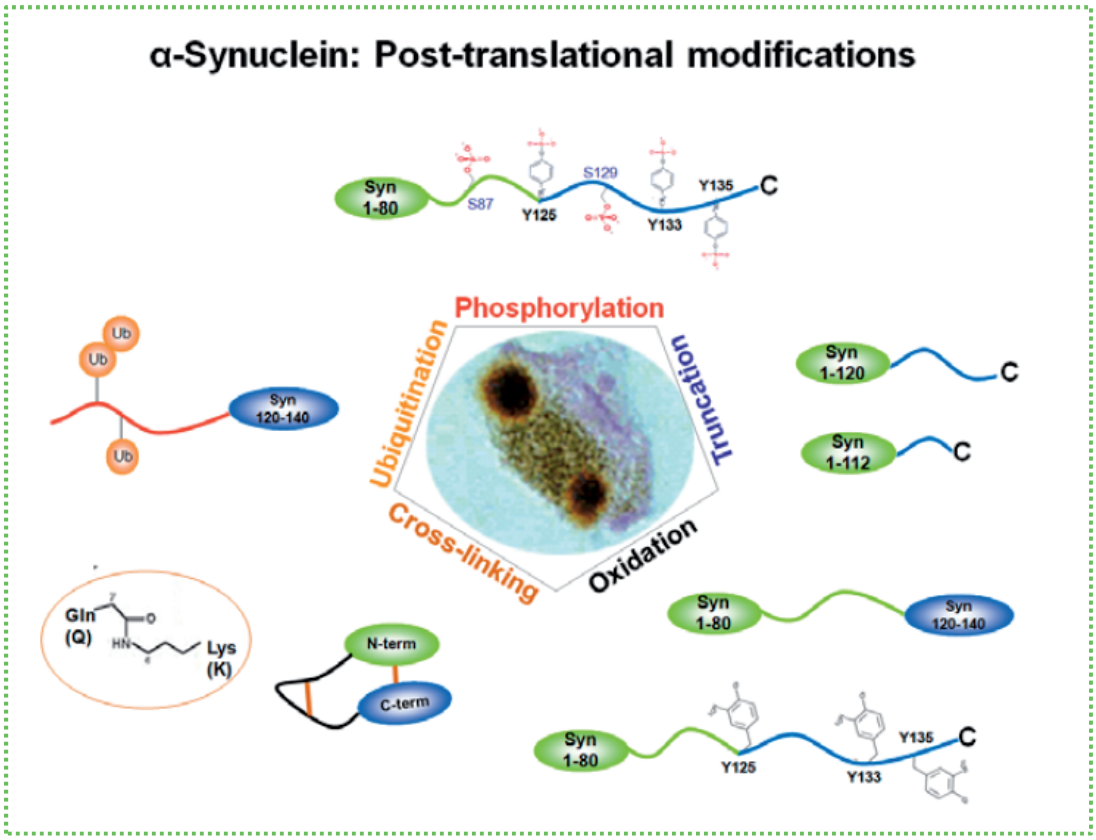


Figure I-19. Most studied PTM occurring in α-syn
Scheme summarizing α-syn's modifications that have been shown to occur in the LB. The majority of the PTM are clustered in the C-terminal domain of the protein (except for ubiquitination and phosphorylation at S87). Figure from Oueslati *et al.* (Oueslati et al., 2010)

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Human     1 MDVFMKGLSKAKEGVVAAAEKTKQGVAEAAGKTKEGVLYVGSKTKEGVVHGVATVAEKTK  60
Rat       1 MDVFMKGLSKAKEGVVAAAEKTKQGVAEAAGKTKEGVLYVGSKTKEGVVHGVTTVAEKTK  60
Mouse     1 MDVFMKGLSKAKEGVVAAAEKTKQGVAEAAGKTKEGVLYVGSKTKEGVVHGVTTVAEKTK  60
Bovine    1 MDVFMKGLSKAKEGVVAAAEKTKQGVAEAAGRTKEGVLYVGSKTKEGVVHGVTTVAEKTK  60
Pig       1 MDVFMKGLSKAKEGVVAAAEKTKQGVAEAAGKTKEGVLYVGSKTKEGVVHGVTTVAEKTK  60
Chimp.    1 MDVFMKGLSKAKEGVVAAAEKTKQGVAEAAGKTKEGVLYVGSKTKEGVVHGVATVAEKTK  60
Chicken   1 MDVFMKGLNKAKEGVVAAAEKTKQGVAEAAGKTKEGVLYVGSRTKEGVVHGVTTVAEKTK  60

Human    61 EQVTNVGGAVVTGVTAVAQKTVEGAGSIAAATGFVKKDQLGKN-EEGAPQEGIL--EDMP  117
Rat      61 EQVTNVGGAVVTGVTAVAQKTVEGAGNIAAATGFVKKDQMGKG-EEGYPQEGIL--EDMP  117
Mouse    61 EQVTNVGGAVVTGVTAVAQKTVEGAGNIAAATGFVKKDQMGKG-EEGYPQEGIL--EDMP  117
Bovine   61 EQVTNVGEAVVTGVTAVAQKTVEGAGSIAAATGFGKKDHMGKG-EEGASQEGIL--EDMP  117
Pig      61 EQVTNVGEAVVTGVTAVAQKTVEGAGSIAAATGFGKKDQLGKN-EEGAPQEGIL--EDMP  117
Chimp.   61 EQVTNVGGAVVTGVTAVAQKTVEGAGSIAAATGFVKKDQLGKN-EEGAPQEGIL--EDMP  117
Chicken  61 EQVSNVGGAVVTGVTAVAQKTVEGAGNIAAATGLVKKDQLAKQNEEGFLQEGMVNNTDIP  120

Human   118 VDPDNEAYEMPSEEGYQDYEPEA 140
Rat     118 VDPSSEAYEMPSEEGYQDYEPEA 140
Mouse   118 VDPGSEAYEMPSEEGYQDYEPEA 140
Bovine  118 VDPDNEAYEMPSEEGYQDYEPEA 140
Pig     118 VDPDNEAYEMPSEEGYQDYEPEA 140
Chimp.  118 VDPDNEAYEMPSEEGYQDYEPEA 140
Chicken 121 VDPENEAYEMPPEEEYQDYEPEA 143
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Figure I-20. Conservation of the α-syn phosphorylation sites among species.
Sequence alignment of α-syn protein from human, rat, mouse, bovine, pig, primate and chicken species. The phosphorylation sites that have been linked to the pathology (S87, S129 and Y125) are highlighted in red while the other potential phosphorylation sites are in green. Alignment was based on Uniprot sequences P37840, P37377, O55042, Q3T0G8, Q3I5G7, P61144 and Q9I9H1

The casein kinases CK1 and CK2 were implicated in phosphorylating these sites. Subsequent studies showed that the G-protein-coupled receptor kinases (GRKs) also phosphorylate α-syn *in vitro* and *in vivo* at S129 (Pronin et al., 2000, Sato et al., 2011).

I.4.1.1. Phosphorylation at S129

Fujiwara *et al.* reported that > 90% of α-syn in LB is phosphorylated at S129 compared to 4% of phosphorylation in total α-syn (Fujiwara et al., 2002). A comprehensive proteomics study carried out by Anderson *et al.* confirmed this finding in DLB, MSA and PD patients (Anderson et al., 2006). Moreover, pS129 α-syn was detected in Tg mice over-expressing α-syn (Kahle et al., 2002, Neumann et al., 2002, Wakamatsu et al., 2007, Rieker et al., 2011), in rat models expressing α-syn (Yamada et al., 2004), and in Tg flies expressing WT and mutants of α-syn (Takahashi et al., 2003, Chen and Feany, 2005). Interestingly, the detection of pS129 correlated with the formation and progression of α-syn pathology.

Although the natural kinases and phosphatases involved in regulating the phosphorylation/dephosphorylation of α-syn at S129 and S87 remain unknown, some potential candidates have been recently identified *in vitro* and eventually validated *in vivo* (Oueslati et al., 2010) (Figure I-21). Some of the kinases that have been identified are specific for S129: G-coupled-receptor kinase 1,2,5 and 6 (GRK) (Pronin et al., 2000, Liu et al., 2010) and the Polo-like kinases 2 and 3 (PLK) (Inglis et al., 2009, Mbefo et al., 2010). On the other hand, CK1 and CK2 have been shown to phosphorylate α-syn both at S87 and S129 (Okochi et al., 2000). CK2 and GRK-5 have been identified in the LB (Arawaka et al., 2006, Ryu et al., 2008). It is important to note that not all identified kinases exhibit the same phosphorylation efficiency, and that some enzymes are able to phosphorylate fibrillar α-syn more efficiently than others. Furthermore, some kinases have been shown to phosphorylate fibrillar α-syn more preferentially than the monomeric form of the protein (Waxman and Giasson, 2008, Mbefo et al., 2010). The only phosphatase that has been identified and implicated in the dephosphorylation of pS129 α-syn *in vivo* is Phosphoprotein Phosphatase 2A (PP2A) (Lee et al., 2011), whereas *in vitro* PP2C has been shown to be much more efficient (Waxman and Giasson, 2008).

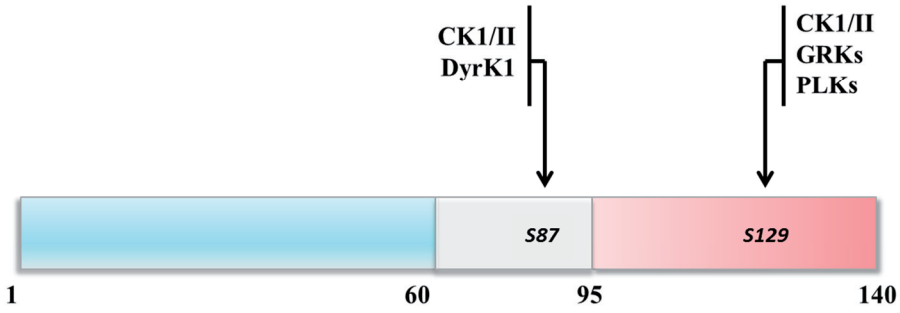

Figure I-21. Kinases implicated in the phosphorylation of α-syn at Serine residues.
Figure adapted from Oueslati *et al.* (Oueslati et al., 2010)

The impact of phosphorylation at S129 on fibril formation was probed *in vitro* and *in vivo*, which yielded various results. *In vitro* phosphorylation assay using CK2 demonstrated an increase in α-syn aggregation (Fujiwara et al., 2002) while studies done by Paleologou *et al.*, using CK1 , showed an inhibition of fibrillization (Paleologou et al., 2008). The discrepancy is probably due to the difference in kinases and in the extent of phosphorylation in each study since CK1 phosphorylates α-syn efficiently at S129 but CK2 induces only partial (< 5%) phosphorylation of the protein at S129.

The expression of the kinase GRK6 in a rat model resulted in a significant increase in the levels of pS129 α-syn but had no effect on the fibrillization of the protein (Sato et al., 2011). Studies using Tg mice over-expressing α-syn, with pharmaceutically-enhanced PP2A activity, revealed reduced pS129 levels that were accompanied with a decrease of α-syn aggregation (Lee et al., 2011). AAV-mediated overexpression of S129A α-syn in the SN of rats resulted in enhanced aggregation and loss of DA neurons (Azeredo da Silveira et al., 2009). Although these findings suggest that blocking phosphorylation may enhance α-syn toxicity, the increased propensity of S129A fibrillization *in vitro* (Paleologou et al., 2008) and *in vivo* (rats and *Drosophila*) (Chen and Feany, 2005, Gorbatyuk et al., 2008, Azeredo da Silveira et al., 2009) suggest that the enhanced toxicity is a result of the enhanced aggregation of this mutant rather than of the blocking of phosphorylation at S129. Interestingly, while the effect of S→A substitution at S129 on α-syn aggregation can be correlated to toxicity in the rat model of PD, the S129A mutant exhibited reduced toxicity in the *Drosophila* model compared to WT and S129E (Chen and Feany, 2005, Azeredo da Silveira et al., 2009). The major causes of the discrepancies between the results remain unknown, but may be explained by the fact that *Drosophila* do not express α-syn and that the interaction between human and rat α-syn may play an important role in regulating its aggregation and toxicity (Rochet et al., 2000).

- Effect of pS129 on toxicity

While the effects of phosphorylation at S129 on α-syn aggregation were not identical in the different reports and animal models, the same discrepancies were observed for the toxicity effects. The co-expression of the GRK6 kinase with A53T α-syn in a rat model was shown to exacerbate A53T-induced toxicity while co-expression of GRK6 and the S129A mutant did not result in major changes in toxicity relative to the WT protein (Sato et al., 2011). Attenuating or blocking phosphorylation at S129 through the induction of phosphatase activity or expression of the S129A mutant respectively resulted in opposing effects in mice and rat models. Tg mice over-expressing α-syn, with an enhanced PP2A activity, revealed a better motor performance and reduced microglial activation, suggesting that phosphorylation at S129 might induce neurodegeneration (Lee et al., 2011). However, overexpression of S129A, which cannot be phosphorylated, in rat SN resulted in increased loss of DA neurons compared to expression of the WT protein (Gorbatyuk et al., 2008, Azeredo da Silveira et al., 2009).

In invertebrate models, the effect of phosphorylation at S129 on toxicity was not consistent: the over-expression of the *Drosophila* homolog of the kinase GRK2 induced degeneration of dopaminergic neurons (Chen and Feany, 2005) while the expression of PLK2 suppressed α-syn -induced neuronal loss in *C.elegans* (Gitler et al., 2009). While both models do not have α-syn homologues the discrepancies between the *Drosophila* and *C.elegans* models could result from the different extent of phosphorylation by the two kinases since PLK2 was shown to phosphorylate α-syn more efficiently than GRK2 *in vitro* and cell-based studies (Mbefo et al., 2010).

The use of the phosphomimicking mutant S129D to investigate the effect of constitutive phosphorylation in the rat and *Drosophila* models of PD also yielded contradictory results. Over-expression of S129D α-syn resulted in an accelerated loss of dopaminergic neurons compared to WT α-syn in fly model (Chen and Feany, 2005), while no additional toxic effect was reported for this mutant in the rat model (Gorbatyuk et al., 2008, Azeredo da Silveira et al., 2009).

I.4.1.2. Phosphorylation at S87

Unlike S129 which is highly conserved among species, a serine is present at position 87 of α-syn in primates whereas in mice, rats and other species, this residue is an asparagine (Figure I-20). Recently, our group developed an antibody that is specific for pS87. Using this antibody, Paleologou *et al.* demonstrated the presence pS87 α-syn in LB and showed that the level of pS87 is increased in brain samples of AD, MSA and PD in comparison to healthy individuals

(Paleologou et al., 2010). In a Tg mouse model of PD, the levels of pS87 were also shown to be increased compared to controls (Paleologou et al., 2010). Interestingly, pS87 α-syn seemed to be preferentially present in membrane fractions.

Only CK1/2 (Okochi et al., 2000) and the dual specificity tyrosine-regulated kinase Dyrk1A have been shown to phosphorylate α-syn at S87 (Kim et al., 2006). pS87 and CK1 co-localize in inclusions in Tg mice and PD brains (Paleologou et al., 2010). No specific phosphatase that regulates pS87 dephosphorylation has been reported to date.

- Effect of pS87 on the conformation and aggregation of α-syn

S87 is the only phosphorylation site to lie within the NAC region and thus phosphorylation of this residue was expected to attenuate the fibrillization of α-syn. Indeed, phosphorylation at S87 or S→E/D substitution at this residue was shown to inhibit the fibrillization of α-syn *in vitro* (Waxman and Giasson, 2008, Paleologou et al., 2010). This finding was also confirmed *in vivo* where S→E substitution was shown to inhibit α-syn aggregation and to protect against toxicity compared to WT and S87A expression that resulted in 28% loss of DA neurons and formed proteinase-K-resistant and ThS-positive aggregates (Oueslati et al., 2012).

I.4.2. Phosphorylation at Tyrosines

Previous proteomics data on α-syn phosphorylation in LB did not report phosphorylation at tyrosine residues (Anderson et al., 2006), as it could not be detected under the experimental conditions used at that time. Indeed, the tyrosine phosphorylation/dephosphorlation cycle of proteins is a very dynamic process and phosphotyrosine containing proteins have been reported to be rare (Yousefi et al., 1994).

I.4.2.1. Phosphorylation at Y125

Comparison of the amino acid sequences of α-syn and its homologues revealed that the four tyrosine residues are conserved between species (Figure I-20). Among these tyrosine residues, three lie in the C-terminal domain (Y125, Y133 and Y136) whereas Y39 is in the N-terminus. Ellis *et al.* were the first to demonstrate that α-syn is phosphorylated at tyrosines in cell-culture models (Ellis et al., 2001). Upon treatment with pervanadate, a general phosphatase inhibitor, Y125 was identified as the major phosphorylation site (Ellis et al., 2001, Ahn et al., 2002). Y125 was also the major phosphorylation site using *in vitro* phosphorylation assays with tyrosine kinases (Nakamura et al., 2001, Nakamura et al., 2002, Negro et al., 2002). Chen *et al.* also showed that inhibition of phosphatases with general inhibitors enhances the detection of pY125. Up to 30% of total α-syn in *Drosophila* brain homogenate was reported to be phosphorylated at

Y125, suggesting that phosphorylation at this residue occurs at low levels and/or is tightly regulated (Chen et al., 2009).

• pY125 is a pathophysiological marker of PD

Given that the α-syn PD-linked mutations A30P and A53T do not affect phosphorylation at Y125 in cells treated with phosphatase inhibitors (Ellis et al., 2001) as well as upon *in vitro* phosphorylation by the Fyn kinase (Nakamura et al., 2001), this suggests that this phosphorylation may not be directly linked to the pathogenesis of familial PD (Ellis et al., 2001). However, Nakamura *et al.* reported that phosphorylation of α-syn at Y125 by Src and Pyk2/RAFTK kinases does not occur spontaneously but is induced by hyperosmotic-stress (Nakamura et al., 2002). Similar observations were reported in rotenone-treated cells where pY125 was reported among other modifications, suggesting that this PTM is induced by mitochondrial dysfunction (Mirzaei et al., 2006). Interestingly, in the only study from human brain samples that seems to stress a physiological role for pY125, the levels of pY125 α-syn were shown to decrease with age and with the pathology, suggesting that the loss of pY125 is associated with pathogenesis (Chen et al., 2009).

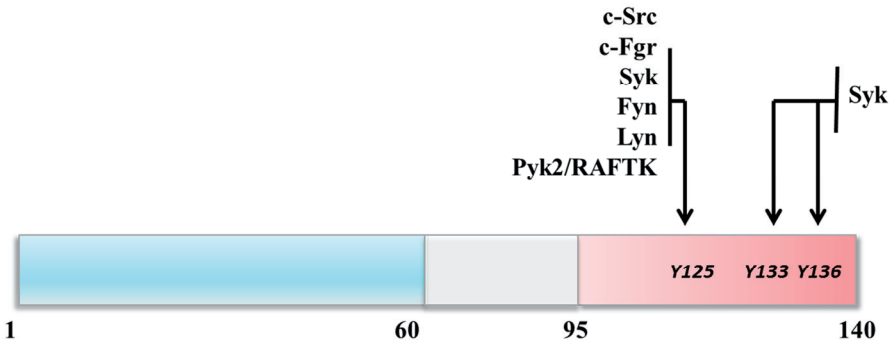

Figure I-22. Kinases implicated in the phosphorylation of α-syn at tyrosines

In vitro and cell culture studies have shown that α-syn is phosphorylated at Y125 by Fyn (Ellis et al., 2001, Nakamura et al., 2001), Pyk2/RAFTK (Nakamura et al., 2002), c-Src (Ellis et al., 2001, Nakamura et al., 2002), and c-Fgr (Negro et al., 2002); and at the three tyrosine residues Y125, Y133 and Y136 by Syk (Negro et al., 2002) (Figure I-22).

Only the Syk kinase was shown to efficiently phosphorylate α-syn at Y125, whereas phosphorylation by Fyn and c-Frg is largely substoichiometric, (Negro et al., 2002). Nevertheless, although Syk and α-syn have been reported to co-localize in mouse brain sections

(Negro et al., 2002), the natural phosphatases involved in regulating α-syn tyrosine phosphorylation *in vivo* remain unknown.

• Effect of pY125 on the conformation and aggregation of α-syn

Although the site-specific effect of phosphorylation at each of these tyrosine residues has not been fully investigated, inhibition of α-syn oligomerization and inclusion formation has been observed under conditions where all three C-terminal tyrosine residues are phosphorylated, *i.e.* pY125/pY133/pY136 (Negro et al., 2002). Partial phosphorylation was not sufficient to inhibit α-syn fibrillization (Negro et al., 2002). The combined effect of triple tyrosine phosphorylation was also investigated in the fly model (through the expression of Shark kinase, a homolog of Syk) and the same inhibitory effect of this modifications on α-syn oligomerization was reported (Chen et al., 2009).

• Effect of pY125 on toxicity

To determine the functional consequences of Y125 phosphorylation in a *Drosophila* model, a mutant form of α-syn (α-syn^{YF}) in which all C-terminal tyrosines was mutated to phenylalanine (Y125F/Y133F/Y136F) was generated (Chen et al., 2009). The flies over-expressing this mutant exhibited substantial loss of dopaminergic neurons and a reduction in climbing ability compared to flies over-expressing WT α-syn. These findings suggest a protective for α-syn phosphorylation at its C-terminal tyrosine residues and was confirmed in Tg flies over-expressing the Shark kinase (the *Drosophila* homolog of Syk) and α-syn S129D mutants to mimic for phosphorylation at this residue: a rescue of the neurotoxicity-induced by the phosphomimic was reported. These findings, together with the decrease of the level of pY125 in humans with age and pathology progression, confirm a protective role for Y125 phosphorylation. However, it is important to note that the effect of site-specifically-modified α-syn at Y125 was not investigated.

I.4.2.2. Functional roles of phosphorylation

Although the majority of studies on α-syn phosphorylation have focused on elucidating the effects of this modification on α-syn aggregation, pathology and toxicity, increasing evidence suggest that PTM, including phosphorylation, may play important roles in regulating α-syn physiological and cellular properties by modulating its subcellular localization, its degradation and its interaction with other proteins and ligands.

- Subcellular localization

The subcellular localization of proteins is an important determinant of their function and dysfunction. Several studies have recently shown that phosphorylation at S129 enhances the nuclear localization of α-syn (Wakamatsu et al., 2007, Schell et al., 2009, Mbefo et al., 2010). Studies in the A30P mouse model of α-syn revealed the presence of nuclear pS129 only in cortical regions of the brain. The nuclear localization of phosphorylated α-syn was absent in control littermates and increased with age in Tg mice (Schell et al., 2009). Similar observations were made in the A53T Tg mouse model (Wakamatsu et al., 2007). These findings were also confirmed in cell culture studies: HEK cells over-expressing α-syn and the kinase GRK5 showed a nuclear localization for pS129 while the WT α-syn remained predominantly cytosolic (Schell et al., 2009, Mbefo et al., 2010). Although the mechanism of α-syn translocation from the cytosol to the nucleus, as well as the functional consequences of S129 phosphorylation in the nucleus remain unknown, it has been suggested that α-syn may play a role in transcriptional regulation in the nucleus through its interaction with histones (section I.3.7), but further studies are needed to test this hypothesis.

- Targeting to the proteasome

α-syn is degraded both by the proteasomal and lysosomal pathways (Webb et al., 2003). The hypothesis that specific PTM of α-syn could result in a preferential degradation was confirmed in studies by two independent groups who reported that proteasomal inhibition in cell-culture and primary neurons resulted in an increase of phosphorylated α-syn at S129, suggesting that phosphorylation at S129 may target α-syn to the proteasome for degradation (Chau et al., 2009, Machiya et al., 2010). Interestingly, this degradation was reported to be independent of ubiquitination (Machiya et al., 2010).

- Inhibition of phospholipase D

Studies by Payton *et al.* suggested potential functional roles for α-syn phosphorylation at Y125. α-syn was previously identified as an inhibitor of phospholipase D isoforms 1 and 2 (PLD) (Jenco et al., 1998, Ahn et al., 2002), although this finding was contradicted by another study (Rappley et al., 2009). Tyrosine phosphorylation at residue 125 appears to specifically play a role in the modulation of the activity of PLD since pY125 α-syn (obtained by treatment of the cells with pervanadate) rescued the activity of the enzyme (Ahn et al., 2002, Payton et al., 2004). PLD is thought to play a role in exocytosis and α-syn could potentially regulate the activity of the enzyme via its own phosphorylation.

• Protein-protein interactions

Other potential roles for Y125 phosphorylation may be mediated by phosphorylation-dependent protein-protein interactions. Peptide-based proteomic studies have identified several proteins from mouse synaptosomes that interact preferentially with a pY125 C-terminal α-syn peptide compared to the non-phosphorylated form. The increased interaction between pY125 and proteins from the cytoskeleton and clathrin heavy subunits could suggest that phosphorylation at Y125 could induce the binding of α-syn to microtubules and play a role in stabilizing synapses. The same interacting proteins were reported in pull-down studies using a pS129 α-syn peptide (McFarland et al., 2008). Together, these studies point to a potential role for phosphorylation in synaptic vesicle trafficking. Interestingly, the pY125 peptide interacted more preferentially with CK1 than the corresponding pS129 peptide, despite the fact that CK1 phosphorylates α-syn at S129 and not Y125 (Okochi et al., 2000). These findings point to a mechanism of cross-talk between the two phosphorylation sites where pY125 could be involved in a cooperative manner in the phosphorylation S129.

• Binding to metal ions

Phosphorylation at Y125 was shown to increase the binding to bivalent cation such as Cu^{2+}, Pb^{2+} and Fe^{2+} and to induce the transfer of the binding sites of the metal ions from the N- to the C-terminal domain of α-syn to residues close to the phosphorylation sites. Similar findings were observed for S129 phosphorylation (Lu et al., 2011).

I.4.3. Ubiquitination

The discovery that LB were immunoreactive for both α-syn and ubiquitin suggested that ubiquitination of α-syn may play a role in LB formation (Spillantini, Crowther et al. 1998). Biochemical analysis of the insoluble fractions of brain samples from PD brains revealed the presence of higher-molecular-weight bands (~ 22-29kD) that were immunoreactive to α-syn and ubiquitin, thus providing the first evidence that α-syn in LB is actually ubiquitinated and does not simply colocalize with ubiquitin (Hasegawa et al., 2002).

I.4.3.1. Ubiquitinated α-syn is a pathological hallmark of PD

The link between ubiquitinated α-syn and PD pathogenesis was provided by studies showing that this PTM was only present in samples from PD patients and more specifically in insoluble fractions of PD and DLB brain samples but not in controls (Hasegawa et al., 2002, Tofaris et al., 2003, Anderson et al., 2006). Furthermore, α-syn was shown to be mono-, di- and to a lesser extent tri-ubiquitinated (Hasegawa et al., 2002, Sampathu et al., 2003, Tofaris et al., 2003,

Anderson et al., 2006). Interestingly, some of the ubiquitinated forms of α-syn were also shown to be phosphorylated at S129 (Hasegawa et al., 2002, Tofaris et al., 2003). The sites of ubiquitination within α-syn have been identified using mass spectrometry analysis of LB-derived materials and were identified as K12, K21 and K23 (Anderson et al., 2006).

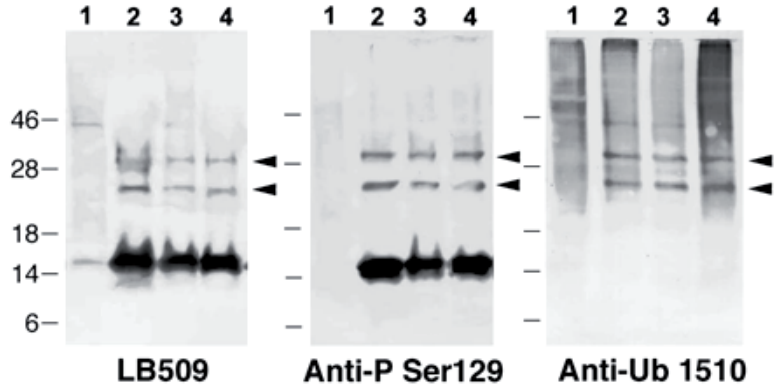

Figure I-23. Immunoblots revealing that mono- and di-ubiquitinated α-syn is phosphorylated at S129.
Immunoblot analysis of PD/DLB brain samples using antibodies for α-syn (LB509), pS129 α-syn and ubiquitin. Figure from Hasegawa *et al.* (Hasegawa et al., 2002)

• E3 ligases and DUBs

The conjugation of a single ubiquitin moiety or polyubiquitin chains on lysine residue relies on the combined enzymatic activity of a E1 ligase (Ubiquitin-activating enzyme), a E2 ligase (Ubiquitin-carrier enzyme) and a E3 ligase, that through substrate recognition, determines the site of modification (Pickart, 2001, David et al., 2011). Several E3 ligases have been reported to ubiquitinate α-syn *in vitro* and *in vivo*. α-syn is monoubiquitinated *in vitro* and in cell-culture models by Seven in absentia homologue-1 (SIAH-1) at residues 12, 21, 23 (same residues as in the LB) but also at lysines 10, 34, 43, and 96 (Lee et al., 2008b, Rott et al., 2008). Nedd4 polyubiquitinates α-syn through K63 ubiquitin chains mainly on K12 and K96, and on K23, 45 and 58 to a lesser extent (Tofaris et al., 2011) (Figure I-24). Other E3 ligases such as TRAF6 and CHIP also ubiquitinate α-syn (Zucchelli et al., 2010) (Kalia et al., 2011). The USP9X enzyme was recently reported to deubiquitinate α-syn (Rott et al., 2011).

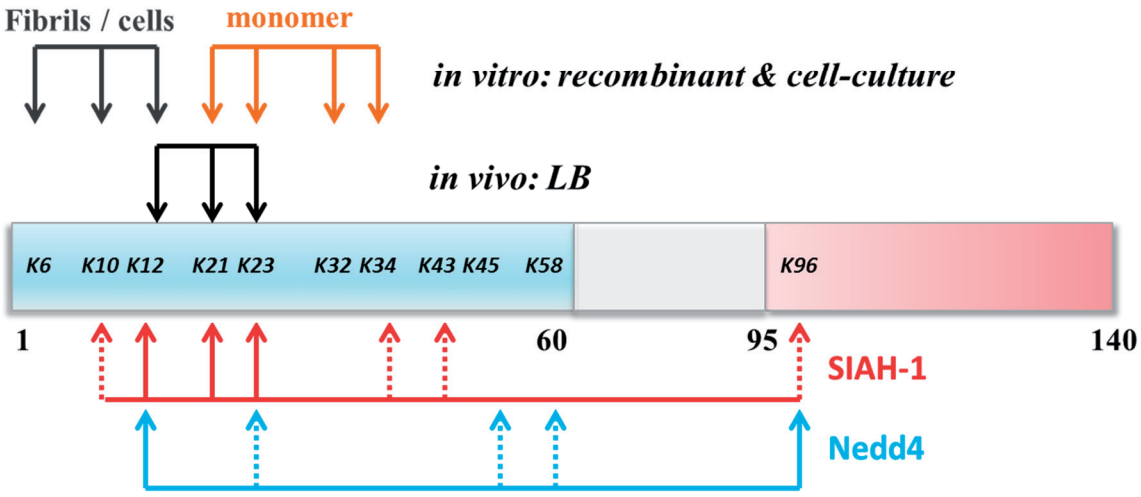

Figure I-24. Ubiquitination sites *in vivo* and *in vitro*
SIAH-1 and Nedd4 ligases ubiquitinate α-syn *in vitro* at major sites (thick arrows) and at other sites to a lesser extent (dotted arrows). *In vivo*, α-syn is ubiquitinated at K12, K21 and K23 while in cell-based studies where α-syn and ubiquitin were co-expressed, α-syn shows ubiquitination at K6, K10 and K12. *In vitro* ubiquitination assays using rabbit reticulocytes of monomeric or fibrillar α-syn also results in heterogeneous ubiquitination sites.

• Ubiquitinated α-syn and aggregation

The fact that many α-syn inclusions in PD patients and Tg mice brains are not immunoreactive to anti-ubiquitin antibodies suggests that ubiquitination of α-syn is not required for its aggregation *in vivo* and that aggregation of α-syn precedes its ubiquitination (Sampathu et al., 2003). Consistent with this hypothesis, *in vitro* ubiquitination experiments conducted with pre-formed α-syn fibrils resulted in mono- and di-ubiquitinated forms that recapitulated the same ubiquitination pattern of α-syn obtained from brain samples, while *in vitro* ubiquitination of monomeric α-syn resulted predominantly in polyubiquitinated species (Sampathu et al., 2003). The sites of ubiquitination were furthermore dependent on the aggregation state of the protein. Nonaka *et al.* showed that the sites of ubiquitination of fibrillar α-syn resembled those occurring *in vivo* (Figure I-24) (Nonaka et al., 2005). Initially, *in vitro* and cell culture studies suggested that ubiquitination of α-syn promotes its aggregation. Cell-based studies using over-expressed E3 ligase SIAH-1 reported an increase in α-syn aggregation. SIAH-1-ubiquitinated α-syn was only present in Triton-insoluble fractions of cell lysates and an increase in inclusion formation was reported in these cells (Lee et al., 2008b, Rott et al., 2008). *In vitro* aggregation assays also showed increased fibrillization of α-syn that had been ubiquitinated by SIAH-2 (Rott et al., 2008). These results would lead to the speculation that α-syn ubiquitination might trigger LB formation.

- Ubiquitinated α-syn and toxicity

Very few reports have investigated the relation between ubiquitination of α-syn and cell death. In one report, Lee *et al.* have shown that ubiquitination of α-syn by SIAH-1 enhances apoptotic cell death (Lee et al., 2008b). Different findings were reported from yeast models where an ortholog of the Nedd4 ligase rescued α-syn-induced toxicity (Tofaris et al., 2011).

I.4.3.2. Functional role of ubiquitination

Ubiquitination of proteins represents an important mechanism not only for targeting proteins for degradation *via* the proteasome, but also for regulating protein subcellular localization and interaction with other proteins. In the case of α-syn, several studies have shown that the ubiquitin-proteasome pathway is not the main pathway involved in regulating the degradation of α-syn (Ancolio et al., 2000, Cuervo et al., 2004, Mak et al., 2010). No accumulation of ubiquitinated α-syn was observed upon pharmacologically-induced impairment of proteasomal function (Rideout and Stefanis, 2002, Tofaris et al., 2003) while earlier reports were able to reveal the presence of ubiquitinated α-syn upon proteasomal inhibition (Rideout et al., 2001). Co-expression of α-syn and SIAH-1 did not result in an increase of α-syn steady-state levels in the presence of MG132, a widely used proteasome inhibitor (Lee et al., 2008b). However, incubation of α-syn with SIAH-2 in presence of purified 26S proteasomes resulted in its degradation while WT α-syn was not degraded (Rott et al., 2011). Interestingly, two independent groups have shown that non-ubiquitinated α-syn can be degraded by the proteasome (Bennett et al., 1999, Webb et al., 2003, Machiya et al., 2010). Recently, Goldberg and colleagues demonstrated that Nedd4-mediated ubiquitination accelerates the degradation of α-syn via the lysosomal-endosomal pathway (Tofaris et al., 2011). Together, these studies suggest that ubiquitination may play a role in regulating the turnover of α-syn, but the extent of α-syn clearance and the pathways involved are dictated by the site and pattern of ubiquitination as well as the E3 ligase that is used.

I.4.4. Truncation of α-syn

I.4.4.1. Truncation of α-syn occurs in healthy and PD individuals

Western-blot and Mass Spectrometry (MS) analysis of purified LB from patients with synucleinopathies revealed the presence of truncated forms of α-syn (Baba et al., 1998, Campbell et al., 2001, Liu et al., 2005, Anderson et al., 2006). These species were also observed in several Tg mouse models of PD (Giasson et al., 2002, Lee et al., 2002c, Liu et al., 2005). The observation that truncation of the C-terminal domain increases α-syn aggregation *in vitro*

(Crowther et al., 1998, Serpell et al., 2000, Murray et al., 2003, Hoyer et al., 2004) led to the hypothesis that proteolytic cleavage of α-syn at the C-terminus may be required for or involved in the initiation of α-syn aggregation *in vivo*. This hypothesis was supported by the finding that expression of the PD-linked mutations resulted in increased levels of truncated α-syn in SH-SY5Y cells and in Tg mouse models of PD (Li et al., 2005). However, truncated forms of α-syn (of 12, 10 and 8 kD masses) were shown to also exist in samples from healthy individuals (Li et al., 2005) with the 12 and 10 kD masses representing around 10-25% of total α-syn levels. The 8 kD species was however only found in insoluble fractions of mouse and human brains. Proteomic studies of α-syn isolated from LB has identified the following proteolytic products of α-syn in LB: 1-134, 1-133, 1-126, 1-122, 1-119, 1-115 and 1-96 (Anderson et al., 2006).

• <u>Proteases involved in the truncation of α-syn</u>

A number of proteases have been identified to cleave α-syn at its C-terminal domain: Neurosin generates a 1-80 residues fragment as a major truncated variant. Three additional minor species were observed in these studies: 1-97, 1-114 and 1-121 (Iwata et al., 2003, Kasai et al., 2008), Calpain I cleaves monomeric α-syn within the NAC region and fibrillar α-syn after residue 120 (Mishizen-Eberz et al., 2003, Mishizen-Eberz et al., 2005), whereas Cathepsin D, a lysosomal enzyme, cleaves α-syn at its C-terminus (Sevlever et al., 2008). The following α-syn fragments were observed upon treatment with matrix metalloproteases: 1-54, 1-57, 1-78 and 1-79 (Sung et al., 2005, Levin et al., 2009). The 20S proteasome also cleaves α-syn and generates truncated forms, but only when α-syn is in a free, non-vesicle bound state (Liu et al., 2005) (Figure I-25)

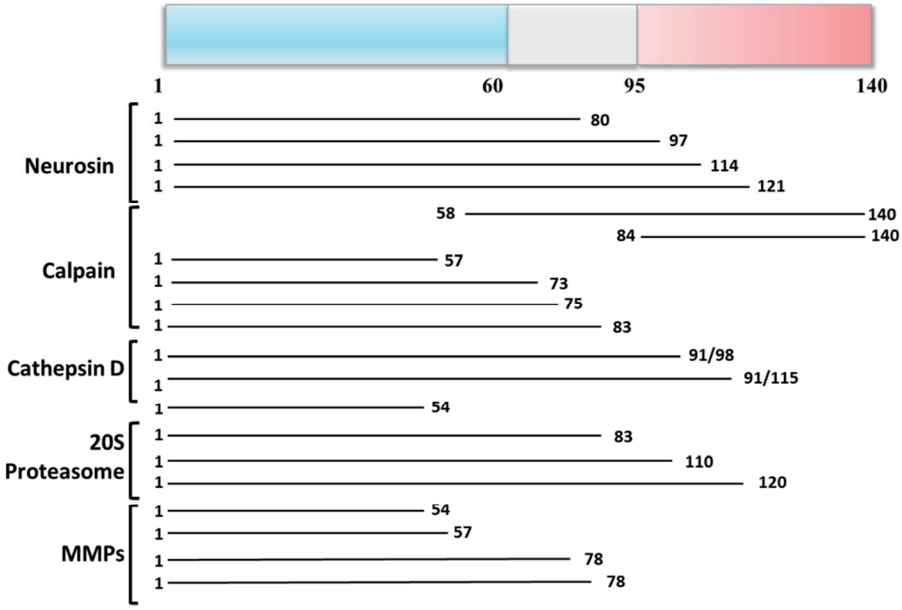

Figure I-25. Truncated variants of α-syn generated by proteolysis

- Effect of truncations on aggregation and toxicity

In vitro aggregation assays of α-syn 1-87 (Serpell et al., 2000), 1-110 (Crowther et al., 1998, Murray et al., 2003, Liu et al., 2005), 1-108 (Hoyer et al., 2004), 1-120 (Liu et al., 2005) (Crowther et al., 1998, Serpell et al., 2000, Murray et al., 2003, Li et al., 2005), 1-123 (Li et al., 2005), 1-124 (Hoyer et al., 2004) and 1-130 (Crowther et al., 1998, Murray et al., 2003) have consistently showed that removal of the C-terminal acidic residues enhances α-syn fibril formation and that aggregates formed by truncated variants can seed the aggregation of the full-length protein (Liu et al., 2005).

In cell culture studies, overexpression of the truncated variants 1-110 and 1-120 resulted in increased vulnerability of cells to oxidative stress compared to the full-length protein, even when substoichiometric amounts of truncated variants were present (Liu et al., 2005).

The effect of α-syn truncation on aggregation and toxicity was also investigated in various animal models of synucleinopathies. *Drosophila* models expressing the truncated 1-87 form showed increased accumulation of α-syn into large inclusion bodies and enhanced neurotoxicity (Periquet et al., 2007). Tg mice over-expressing the fragment 1-120 in α-syn KO mice showed the presence of α-syn-positive inclusions in the SN and olfactory bulb and increased motor dysfunction (Tofaris et al., 2006). Finally, Tg mice overexpressing 1-130 α-syn exhibited selective DA neuron loss, albeit without cytoplasmic inclusions (Wakamatsu et al., 2008). This could be due to the interaction between mouse and human α-syn in this model, which could be preventing its fibrillization as suggested by *in vitro* data (Rochet et al., 2000). Moreover, the over-expression of truncated α-syn in animal models reflects neither the physiological nor the pathological conditions in PD since only a small fraction of α-syn is truncated in the brain of PD patients (Anderson et al., 2006).

I.4.5. Other PTM: acetylation, nitration, sumoylation

I.4.5.1. Acetylation

α-syn is ubiquitously acetylated at its N-terminal Met residue (Anderson et al., 2006). Recent studies from our group and others have shown that N-terminal acetylation α-syn does not significantly influence its secondary structure, aggregation propensity or subcellular localization as compared to the WT protein (Fauvet et al., 2012a, Kang et al., 2012, Maltsev et al., 2012, Trexler and Rhoades, 2012).

I.4.5.2. Nitration

Oxidative stress is one of the major pathways that have been implicated in α-syn-induced toxicity and neurodegeneration (section I.3.6.4). Released reactive oxygen and nitrogen species modify α-syn to generate species containing 3-nitrotyrosine, a modification that has been identified in LB (Giasson et al., 2000). Oxidative stress-induced covalent cross-linking of α-syn via o-o′-dityrosine formation was also detected *in vitro* and shown to contribute to the formation of stable α-syn oligomers (Souza et al., 2000a).

I.4.5.3. Sumoylation

α-syn has been shown to be sumoylated at lysines 96 and 102. Recent studies suggested that α-syn sumoylation is induced by proteasomal impairment (Kim et al., 2011, Krumova et al., 2011). The conjugates responsible for this modification were identified as SUMO1, and to a lesser extent SUMO2 (Dorval and Fraser, 2006). Although the consequences of α-syn SUMOylation remain unexplored, recent studies suggest that α-syn sumoylation blocks α-syn aggregation and that small amounts of the sumoylated proteins (around 10%) are sufficient to inhibit aggregation (Krumova et al., 2011).

I.5. α-syn studies of Tyr phosphorylation and ubiquitination: limitations and proposed solutions

I.5.1. Studies of α-syn phosphorylation

I.5.1.1. Use of phosphomimics

Previous studies that aimed at investigating the phosphorylation of α-syn at Serine or Tyrosine residues relied primarily on the use of natural amino acids that mimic the phosphorylation state of these residues. When the identity of the natural kinases is not known, substitution of Serine and Threonine by Glutamate or Aspartate is used to mimic the phosphorylated states of both residues (pS/pT) in cell culture and *in vivo* studies (Figure I-26). Whereas the structure and charge similarity between pS/pT and E/D allows for mimicking some aspects of phosphorylation, there are no natural amino acids that mimic the phosphorylated state of tyrosine residues (pY). Therefore, the results from studies where E/D substitution is used to mimic tyrosine phosphorylation should be interpreted with caution.

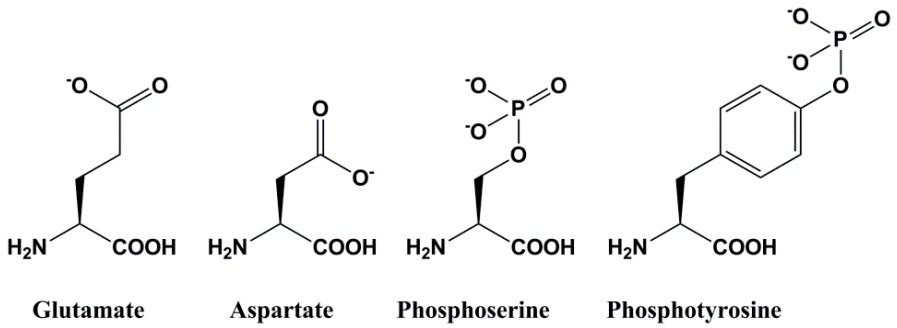

Figure I-26. Structure of pSer, pTyr and their phosphomimics : Glu and Asp
Due to charge similarity (net charge of -1 for E/D and -2 for pS), natural amino acids mimicking phosphorylation at serine have been used.

Advantages and limitations of using phosphomimics

One major advantage of using phosphomimics is their easy generation by site-directed mutagenesis. When the phosphomimicking substitutions reproduce key features of true phosphorylation, they provide a valuable tool to investigate cross-talk between different PTM and between phosphorylation and disease-associated mutations. In addition, the use of S/T → E/D substitution allows for mimicking constitutive phosphorylation. However, it has become increasingly clear that phosphomimics do not reproduce all aspects of phosphorylation, as the substitution of Ser by Glu or Asp in different proteins and model systems has yielded different results. Herein, I will present a brief overview of previous studies that investigated α-syn phosphorylation through the use of phosphomimicking substitutions and our current understanding of the impact of these substitutions on α-syn aggregation and toxicity *in vitro* and *in vivo*.

- Phosphomimics do not fully reproduce phosphorylation

The first detailed study that compared the structure and aggregation propensity of phosphomimics and truly-phosphorylated α-syn at S129 (pS129) was carried out by Paleologou *et al.* (Paleologou et al., 2008). The results from this study demonstrated that the phosphomimicking mutations (S129E/D) do not reproduce all aspects of phosphorylation *in vitro* and in cell culture. Indeed, while the secondary structure of WT, S129E/S129D and pS129 was random-coiled and the binding of these proteins to lipid vesicles was unaffected, differences emerged from high-resolution NMR studies that showed the disruption of long-range interactions between the N- and C-terminal domains of α-syn. Moreover, a more extended conformation for the phosphorylated protein, but not S129E/D, was reported as calculated by its hydrodynamic radius (WT: 28.2 Å, S129D: 28.3 Å, S129E: 27.8 Å, pS129: 35.3 Å). In addition,

in vitro fibrillization assays revealed a marked difference between the phosphomimics and pS129 α-syn: the S129A mutant, which is commonly used to block phosphorylation *in vivo*, aggregated more readily than the WT protein while S129E and S129D exhibited similar fibrillization propensities to WT α-syn. On the other hand, natively phosphorylated α-syn at S129 completely abolished α-syn aggregation, even in presence of 80% of WT α-syn. Interestingly, the effect of α-syn phosphorylation at S87 on its aggregation properties was reproduced by S87D or S87E. Both the phosphorylation at S87 and the substitution of S with E/D resulted in a marked inhibition of α-syn fibril formation *in vitro* and *in vivo* (Paleologou et al., 2010, Oueslati et al., 2012).

The S129A and S129D α-syn mutants have been used in animal models to elucidate the effect of phosphorylation at this residue on α-syn aggregation and toxicity. Phosphorylation of α-syn at S129 was initially thought to block its aggregation since the overexpression of S129A α-syn in *Drosophila* and rat models resulted in an increased aggregation (Chen and Feany, 2005, Azeredo da Silveira et al., 2009). However, it later became clear that the increased aggregation propensity of this protein is due to the S→A mutation rather than to the blockade of phosphorylation at this residue; (Paleologou et al., 2008). Whether the increased toxicity of this mutant, which was reported by two independent studies using the rat model of PD, is also caused by enhanced aggregation of this mutant remains to be determined. Nevertheless, the increased effect on toxicity that was shown for S129A in the rat model was not reported in *Drosophila*, where S129A was not toxic. On the other hand, expression of the phosphomimic S129D in fly models or co-expression of WT α-syn and GRK kinases in rats resulted in the loss of DA neurons and increased motor deficits (Chen and Feany, 2005, Sato et al., 2011). This discrepancy in terms of the toxicity of these mutants may arise from the fact that the fly, unlike the rat, does not express a homolog of α-syn, which could potentially influence the aggregation of the human protein. Indeed, *in vitro* studies have shown that mouse α-syn (one amino acid difference with rat α-syn) had a higher fibrillization propensity than human α-syn and that mixing both α-syn proteins resulted in the formation of more oligomers and less fibrils (Rochet et al., 2000). Bearing in mind recent findings that support the toxic effects of oligomers, the interaction between rat and human α-syn could exacerbate α-syn toxicity in rodent models and therefore studies using α-syn KO animals are needed to verify this hypothesis. Moreover, to investigate the extent of aggregation in the different animal models, different approaches were followed such as the use of proteinase K digestion in *Drosophila* in some studies and Thioflavin

S staining of rat brain sections in others, thus making a direct comparative assessment of aggregation quite difficult (Chen et al., 2005, Azeredo da Silveira et al., 2009).

To summarize, phosphomimics exhibit the same fibrillization rate as WT α-syn both *in vitro* and *in vivo* while pS129 was reported to inhibit α-syn aggregation *in vitro* and to alter its structure and subcellular localization. It is thus difficult to extrapolate the relationship between phosphorylation and aggregation *in vivo* using phosphomimics. Hence, the identification of the natural kinases that specifically phosphorylate α-syn at this residue is crucial for accurate determination of the effect of this modification on α-syn aggregation and toxicity in animal models of synuleinopathies.

I.5.1.2. Use of kinases

Several kinases have been reported to phosphorylate α-syn at S129, including CK1 , CK2, GRKs and PLKs (sections I.4.1.1and I.4.1.2). However, only CK1 and PLKs phosphorylate α-syn efficiently *in vitro* and in cell culture and co-localize with α-syn in DA neurons. While PLK2 and PLK3 phosphorylate α-syn specifically at S129, CK1-mediated phosphorylation occurs at S129 and S87. Therefore, without the use of mutations that block phosphorylation at S87 or S129, only PLK2 or PLK3 can be used today to induce specific and efficient phosphorylation of α-syn *in vitro* and *in vivo*. Recent studies suggest that expression of PLK2 in *C.elegans* and primary neuron models is neuroprotective (Gitler et al., 2009). Similarly, the tyrosine kinase Syk phosphorylates α-syn at the three tyrosines Y125, Y133 and Y136 *in vitro*. Other tyrosine kinases are more specific but much less efficient. Therefore, site-specific phosphorylation at tyrosine residues is only possible today by using Syk combined with mutation of the remaining tyrosine residues to phenylalanine.

The use of a specific but non-efficient kinase is possible but one has to always take into consideration the level of phosphorylation achieved and the resulting heterogeneity of α-syn species when interpreting the results. Therefore, there is an urgent need to identify the natural kinases and phosphatases that are responsible for regulating α-syn phosphorylation at different residues. These advances will facilitate studies aimed at determining the effects of phosphorylation *in vivo* and validating these kinases as potential targets for the treatment of PD.

I.5.1.3. Use of nonsense suppression methodology

An alternative approach for the study of protein phosphorylation is the use of nonsense suppression methodology. This methodology was pioneered by P. Schultz's group and is based on the generation of a pair of modified tRNA and aminoacyl-tRNA synthetase that has been

evolved to incorporate a modified amino acid at the position of an amber stop codon in the gene where the modified amino acid is to be incorporated. To date, more than 50 amino acids have been incorporated into proteins, spanning the range of naturals, D-amino acids, phosphorylated, and photocaged amino acids (Young and Schultz, 2010). The genetic code was expanded in 2011 with the addition of phosphoserine (Park et al., 2011). This approach allowed site-specific incorporation of two phosphoserine residues in the activated form of human mitogen-activated ERK activating kinase 1 (MEK1). However, one major drawback of this approach remains the low yields of modified proteins obtained. In the case of MEK1, a yield of 25 micrograms of protein per liter of culture medium was reported. Phosphotyrosine residues have not been introduced in proteins using this methodology but nonhydrolyzable phosphorylated tyrosine analogues were successfully incorporated into the enzyme serotonin N-acetyltransferase (Zheng et al., 2005b, Young and Schultz, 2010). Another major limitation of the nonsense suppression method is the requirement for large quantities of aminoacylated tRNAs and the restricted number of unnatural amino acids that can be co-incorporated in a protein.

I.5.2. Studies of α-syn ubiquitination

In comparison to protein phosphorylation, investigating the effect of site-specific ubiquitination (mono- and poly-ubiquitination) *in vitro* is even more challenging, mainly due to the lack of methodologies that allow the site-specific introduction of these modifications without the introduction of mutations into the protein of interest. Furthermore, no natural amino acid can be used as a mimic for ubiquitinated lysines. In LB, α-syn has been shown to be mono-, di-, and sometimes triubiquitinated at K12, K21 and K23, raising questions about the nature of the molecular mechanisms that regulate this process *in vivo*.

I.5.2.1. Methods generating native isopeptide bonds

- E3 ligases

The E3 ligases SIAH-1 and SIAH-2 have been shown to monoubiquitinate α-syn at K12, K21 and K23 but also at other lysines (section I.4.3). Results from two independent groups revealed that SIAH-mediated ubiquitination results in an increase of α-syn fibrillization but yielded contradictory results on the effect of SIAH-1/2 on α-syn degradation (Lee et al., 2008b, Rott et al., 2008, Rott et al., 2011). The authors did not investigate the roles of ubiquitination at each lysine residue because of the requirement of multiple single-point mutations in the sequence of α-syn (which contains 15 lysine residues) to achieve site-specific ubiquitination. Recently, it was reported that Nedd4 polyubiquitinates α-syn mainly at K12 and K96, as well as at other lysines; however, the identified sites and the nature of the ubiquitin conjugates are not the same

as in the LB (K12, K21 and K23 are ubiquitinated *in vivo*). The data presented by these studies clearly show that only a small percentage of α-syn is ubiquitinated in presence of E3 ligases as evidenced by the requirement for immunoprecipitation to detect ubiquitinated forms of α-syn by western blotting (Rott et al., 2008, Tofaris et al., 2011). The investigation of the sub-cellular localization of ubiquitinated α-syn is impossible for the moment due to the absence of antibodies that specifically discriminate ubiquitinated α-syn from the WT protein.

- Use of rabbit reticulocytes

To study ubiquitination *in vitro* and to circumvent the use of E3 ligases, rabbit reticulocyte fractions that contain the enzymatic machinery necessary for ubiquitin conjugation are commonly used (Hasegawa et al., 2002, Nonaka et al., 2005). While this approach yielded mainly mono-and di-ubiquitinated forms of α-syn, similar to what is observed in the human brain, the sites of these modifications were not the same and were highly dependent on the polymerization state of the protein: when α-syn was monomeric, rabbit reticulocyte-based ubiquitination resulted in modification of K21, K23, K32 and K34, while fibrillar α-syn was ubiquitinated at K6, K10 and K12 (Nonaka et al., 2005). This methodology is also restricted for *in vitro* studies and cannot be applied for cell-based studies or *in vivo*.

I.5.2.2. Non-native isopeptide bonds

To circumvent the use of enzymatic reactions for the generation of ubiquitinated proteins, chemists have developed an array of chemical reactions for the conjugation of ubiquitin to proteins through isopeptidic-like bonds. Site-specificity was achieved but non-native isopeptide bonds were generated in the ligation reaction: Oxime (Shanmugham et al., 2010), triazole (Yin et al., 2000, Rostovtsev et al., 2002, Weikart et al., 2012) and disulfide-based (Chatterjee et al., 2010, Chen et al., 2010) isopeptide-like bonds allowed the conjugation of a ubiquitin moiety to the protein of interest through either irreversible or unstable bonds (

Figure I-27). These approaches have been successfully applied to generate monoubiquitinated proteins such as histone 2B (H2B) and proliferating cell nuclear antigen (PCNA) as well as di-ubiquitin chains (section I.5.3.3). Recently, a disulfide-bond strategy was used to generate several forms of monoubiquitinated α-syn at different lysine residues and enabled the site-specific investigation of monoubiquitination on its aggregation propensity (Meier et al., 2012). Cysteine mutations were introduced in the sequence of α-syn as substitutions of lysines 6, 10, 12, 21, 23, 32, 34, 46 and 96. The effect on aggregation was dependent on the site of ubiquitination since monoubiquitination at K23 and K10 slightly affected the rate of fibrillization but not its extent, while monoubiquitionation at other residues completely inhibited

the aggregation (Meier et al., 2012). However, it is important to stress that the use of disulfide linkages does not mimic the native state of the protein and may influence its structure. Furthermore, the unstable nature of the disulfide bond prevents further use of these monoubiquitinated proteins in cellular assays or introducing these proteins into cellular environments, where they would be readily reduced back to free proteins through reduction by glutathione or other cellular reducing agents.

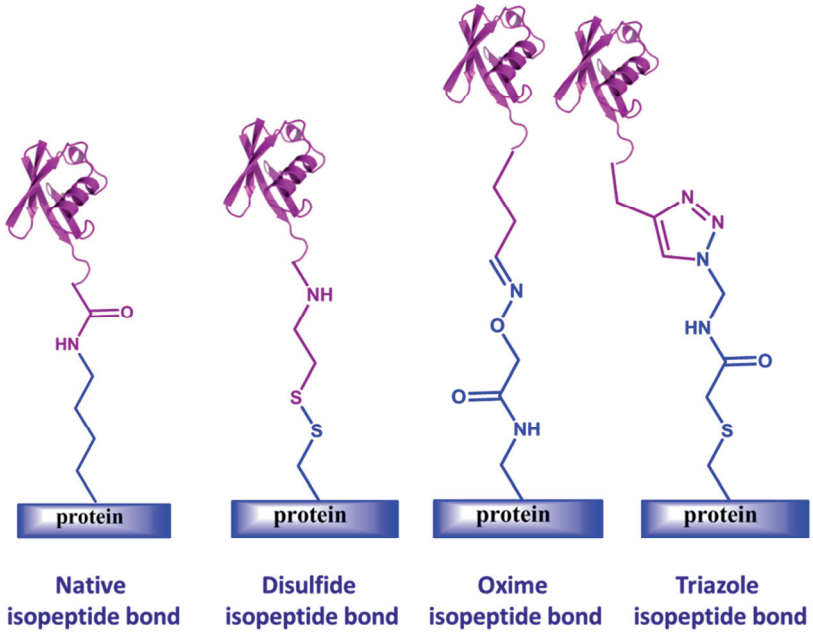

Figure I-27. Different approaches for the generation of isopeptide bonds for ubiquitin conjugation
Several strategies have been used to generate monoubiquitinated proteins through the ligation of ubiquitin to the protein of interest via disulfide, oxime and triazole isopeptide-like bonds.

I.5.3. Protein semisynthesis

To overcome the limitations associated with the use of phosphomimicking mutations or the requirement for efficient and specific kinases to study the structural, functional and pathologic consequences of phosphorylation, a phosphorylated serine or tyrosine residue can be site-specifically incorporated into the protein of interest using different chemical strategies. One obvious approach is based on the use of solid-phase peptide synthesis (SPPS), which was developed by Merrifield (Merrifield, 1986). This approach allows the incorporation of any unnatural amino acids in the sequence of a peptide of around 50-70 residues at maximum (Kent, 1988). SPPS involves the attachment of the protected C-terminal amino acid on a polymer-based resin and the assembly of the subsequent amino acids stepwise from the C-terminus to the N-terminus. Two types of protecting groups are needed in SPPS: a temporary protecting group of

the α-amino group of the residues, such as Fluorenylmethyloxycarbonyl (Fmoc) or tert-butyloxycarbonyl (Boc) groups that are removed with base or acid treatment respectively, after the formation of each peptide bond; and side-chain protecting groups that are removed after assembly of the complete peptide chain (Figure I-28). Peptide bond formation is carried out by activating the carboxylic acid moiety of the residue to be attached using coupling agents such as O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (known as HBTU) in presence of HOBt (Hydroxybenzotriazole) to eliminate side-reactions such as racemization at the alpha carbon. Cleavage of Fmoc-based peptides is carried out in Trifluoroacetic acid (TFA) in the presence of scavengers to trap reactive byproducts. Boc-based peptides are cleaved at low temperature with Hydrogen fluoride (HF) or with TFA/TFMSA (trifluoromethanesulfonic acid). The peptide is subsequently purified by reversed-phase HPLC and characterized by mass spectrometry. Recent advances in chemistry have allowed for efficient coupling conditions, minimization of side-reactions and enhanced peptide solubility, all of which have contributed to the development of fully-automated machines for the synthesis of peptides.

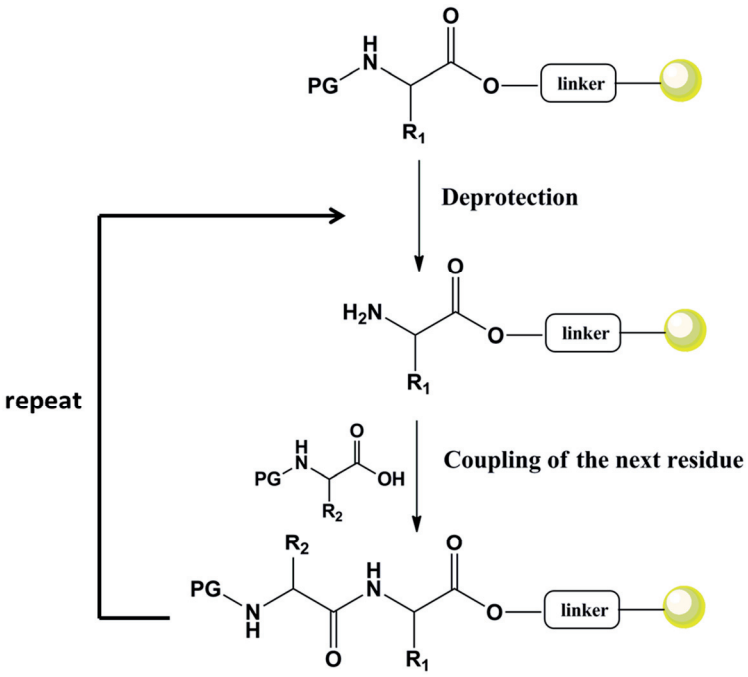

Figure I-28. General SPPS scheme
The last residue in the desired sequence is attached via its C-terminal carboxylic group to a polymer resin. Deprotection of the amine and activation of the carboxylic acid of the next residue allows elongation of the peptide and formation of a peptide bond. The same cycle is repeated for the remaining residues. Final deprotection allows cleaving the peptide from the resin and deprotection of the side-chains. Figure adapted from CEM website

I.5.3.1. Native Chemical ligation

SPPS allows the assembly of peptides on solid support of up to 50-70 amino acids (depending on the sequence) (Kent, 1988, Dawson and Kent, 2000). To overcome this limitation, chemical ligations method were developed in the early 1990s, which have enabled the preparation of proteins and peptides from the ligation of unprotected peptide fragments (Kent, 1988). In 1994, Dawson and colleagues reported the ligation of two unprotected peptides to synthesize human Interleukin-8 (Dawson et al., 1994). The reaction was termed "Native Chemical Ligation" (NCL) and had the following characteristics: it was chemoselective and was performed in aqueous solutions at physiological pH, but compared to previous chemical ligation strategies, which generated thioesters (Schnolzer and Kent, 1992), thioethers, (Englebretsen et al., 2002), oximes (Thumshirn et al., 2003), thiazolidines (Liu and Tam, 1994) and disulfide bonds at the ligation sites (Chatterjee et al., 2010), this new reaction allowed for the generation of a native peptide bond at the ligation site. The reaction occurs between a peptide bearing a C-terminal thioester with a peptide containing an N-terminal cysteine. The first step of the reaction is mediated by a reversible thiol-thioester exchange between an electrophilic thioester at the C-terminus of peptide 1 and the nucleophilic thiol of the cysteine residue at the N-terminus of peptide 2 (Figure I-29). This transthioesterification reaction is followed by a spontaneous S-N acyl shift to generate an amide bond at the ligation site. The ligation occurs hence at a cysteine residue and the presence of other cysteines in the sequence does not interfere since the irreversible S-N acyl rearrangement can only occur with an N-terminal cysteine (Hackenberger and Schwarzer, 2008).

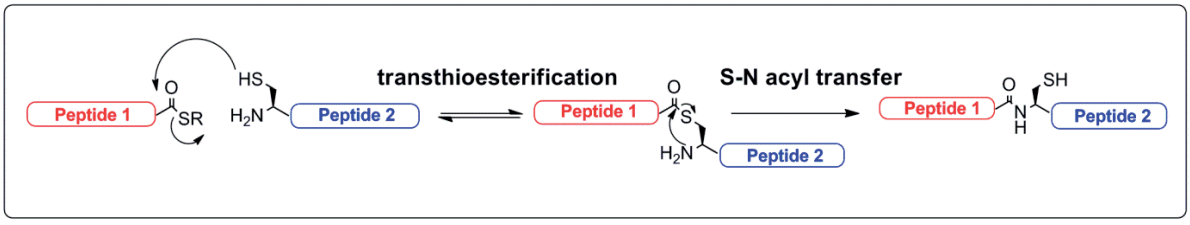

Figure I-29. Native Chemical Ligation Mechanism.

• Catalytic thiol cofactors

The nature of the thioester is very important for NCL. Indeed, aryl thioesters are more reactive than alkyl thioesters but they are also more prone to hydrolysis. The common procedure that is widely followed is based on the preparation of peptide thioesters as alkyl form for better handling and storage and the *in situ* conversion to aryl thioesters during the NCL reaction

through the addition of a thiol catalyst. The most common thiol catalysts used to date are thiophenol, benzyl mercaptan and 4-mercaptophenylacetic acid (MPAA) (Johnson and Kent, 2006). The addition of the thiol catalyst also helps to maintain the cysteine at the N-terminus of peptide 2 in a reduced state.

- Ligation at Alanine

Native chemical ligation requires cysteine residues and lacking such residues in proteins requires the introduction of cysteine residues at the ligation site. To circumvent the introduction of unnatural cysteine residues in such proteins, Yan and colleagues have developed a desulfurization method that allow for the conversion of cysteine residues back to alanine (Yan and Danishefsky, 2001), which then enable carrying out ligation reactions at alanine residues, which occur more frequently in proteins. Hydrogenolytic desulfurization using Raney nickel was also used; however prolonged treatment with Raney nickel was found to result in demethylthiolation of methionine residues. A metal-free desulfurization step was developed in 2007 by Wan and colleagues, which relied on the reaction between tert-butyl mercaptan and trialkylphosphite in presence of a radical initiator (Wan and Danishefsky, 2007). This reaction accommodates methionine and different cysteine protecting groups such as Acm and Thz. These advances expanded the use of NCL to prepare proteins of increasing size and sequence complexity.

- Ligation at other residues

Chemical advances have allowed synthesizing β-thiol-containing amino acid analogues, which in turn have enabled performing NCL at sites other than cysteine and alanine. After desulfurization, phenylalanine, leucine, valine, threonine and glutamine can be generated at the ligation site (Hackenberger and Schwarzer, 2008, Butterfield et al., 2012a) (Figure I-30).

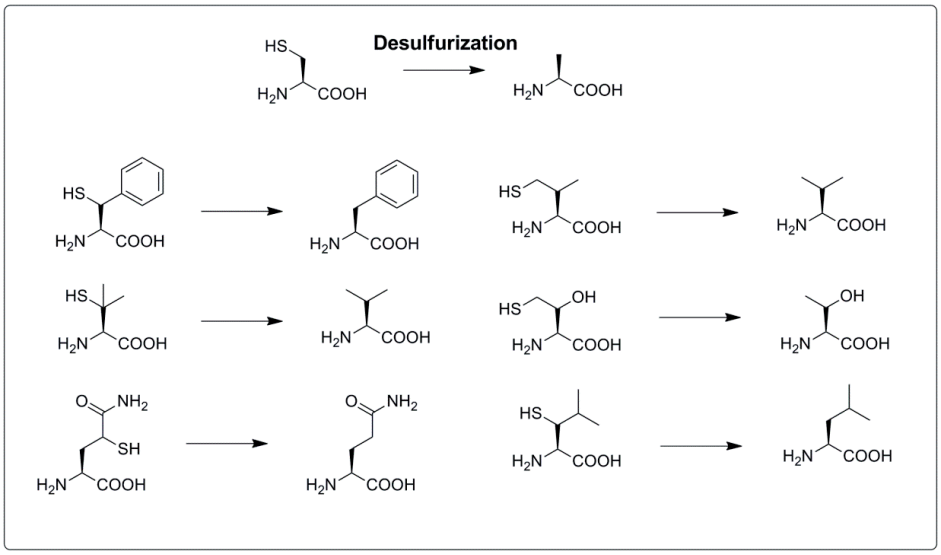

Figure I-30. Desulfurization allows performing ligations at Ala, Phe, Thr, Leu, Val and Gln

• Synthesis of peptide thioesters by SPPS

Peptide thioesters can be prepared using Fmoc and Boc-based SPPS (Hackenberger and Schwarzer, 2008). For the purpose of this thesis, I will only describe thioester formation on Fmoc-based peptides. The thioester is usually introduced at the C-terminus of the protected peptide after cleavage from a 2-chlorotrityl resin under mild acidic conditions. The carboxylic group at the C-terminus is activated by a strong coupling reagent such as PyBOP, which is then attacked by a thiol to form a thioester. A final deprotection step releases the side-chain protections. However, using this method, there is a high risk of epimerization at the C-terminal residue (Hackenberger and Schwarzer, 2008) (Figure I-31.A).

Another Fmoc-based strategy relies on the *in-situ* synthesis of the thioester. The peptide is assembled on a resin containing a protected thiol group on β-mercapto-α-hydroxypropionic acid. After cleavage from the resin and deprotection of the thiol, an intramolecular O-S acyl shift generates the thioester (Botti et al., 2004, Hackenberger and Schwarzer, 2008) (Figure I-31.B).

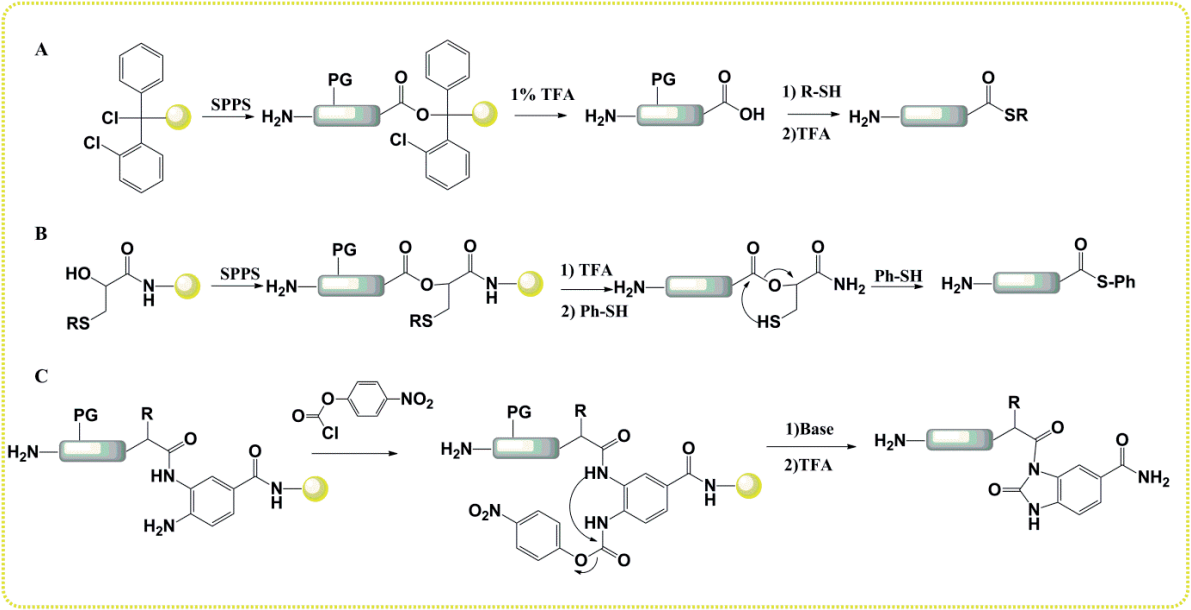

Figure I-31. Generation of peptide thioesters

Thioester formation using an N-acyl urea-based C-terminal group has been reported recently (Blanco-Canosa and Dawson, 2008). A 3,4-diaminobenzoic acid group (Dbz) is added onto a Rink amide MBHA resin before the coupling of the first residue. After peptide assembly, the C-terminus is activated through acylation with *p*-nitrophenylchloroformate followed by the addition of a base to promote the intramolecular attack of the anilide to form the resin-bound N-benzimidazolinone (Nbz). The peptide is subsequently cleaved in TFA. The Nbz group is a good leaving group which can be used in place of a thioester during NCL, however it is not as stable as alkyl thioesters, therefore thiolysis of the Nbz group is often used to store the peptides as alkyl thioesters (Figure I-31.C).

I.5.3.2. Expressed protein ligation

To date, a large number of proteins of various sizes (up to 300 amino acids) have been prepared using NCL (Hackenberger and Schwarzer, 2008, Kumar et al., 2011). Catalytically active enzymes and membrane proteins have also been successfully generated (Hackenberger and Schwarzer, 2008). However, despite major advances in ligation reaction methodologies, the size limitation in SPPS and the requirement of multiple NCL reactions to prepare large proteins resulted in low yields and thus limited the utility of NCL to mostly small proteins. To overcome these limitations, two independent groups developed a strategy that combines the chemoselective ligation of NCL with the possibility to produce longer protein fragments via recombinant expression of proteins in *E. coli* (Evans et al., 1998, Muir et al., 1998). The

Expressed Protein Ligation approach (EPL) is based on the reaction between a protein fragment containing a thioester and another fragment harboring a cysteine at its N-terminus. Overcoming the size restriction in NCL through the development of EPL allowed the introduction of PTMs, fluorescent probes, unnatural amino acids, and stable isotopes in proteins (Muir, 2003a). Ion channels and membrane proteins were successfully produced using semisynthesis (Valiyaveetil et al., 2002, Olschewski and Becker, 2008) and the reaction could also take place in living cells (Giriat and Muir, 2003).

Depending on the position of the modification, two strategies are to be followed: if the modified amino acid is in the N-terminal part of the protein, a peptide thioester, containing the modified amino acid, will be produced by SPPS and ligated to a protein fragment expressed in *E. coli* with an N-terminal cysteine (Figure I-32). If the modification is in the C-terminal domain, a protein thioester is produced in bacteria using intein-mediated splicing (Figure I-33) and the C-terminal fragment containing the modified residue is prepared by SPPS (with an N-terminal cysteine) (Figure I-32).

Although a wide variety of proteins can be prepared by semisynthesis, practical considerations have to be considered before designing an EPL strategy (Szewczuk et al., 2009): i) the modified amino acid to be introduced in the protein needs to be positioned within 50 amino acids from the N- or C-terminus of the protein of interest due to the size limitation imposed by SPPS, ii) the folding and the solubility of the truncated protein segments – to be expressed in fusion with the intein or with an N-terminal cysteine – might be different from the full-length protein and iii) the ligation site has to be wisely chosen so as not to disturb the native sequence of the protein and not to disrupt the kinetics of the NCL reaction. Indeed, the identity and steric hindrance induced by the amino acid that is adjacent to the ligation site influences the ligation kinetics (Hackenberger and Schwarzer, 2008).

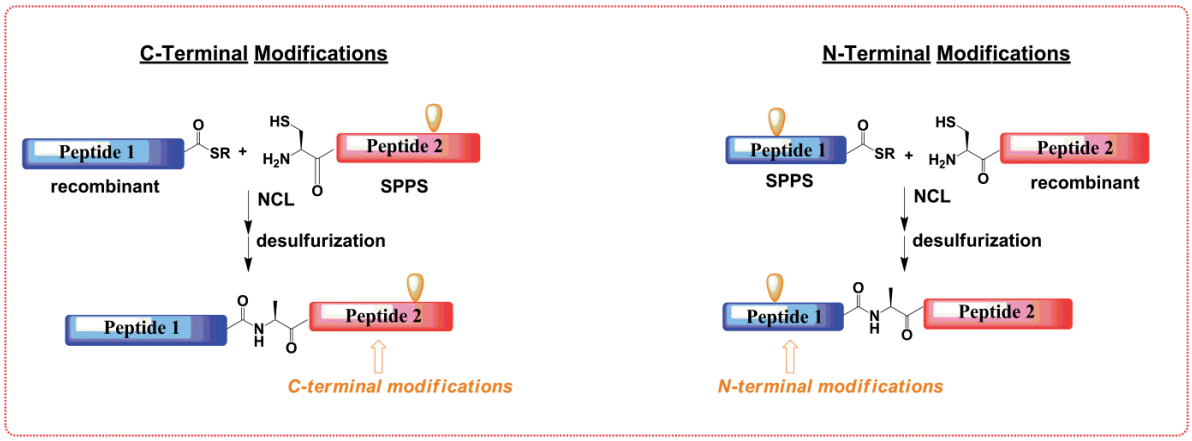

Figure I-32. Strategies for the semisynthesis of proteins: N- and C-terminal modifications

• Generation of protein thioesters

Synthetic peptide thioesters or peptides containing an N-terminal cysteine are readily prepared by SPPS and the generation of recombinant protein fragments with an N-terminal cysteine residue relies on cloning and molecular biology (Figure I-32). The missing piece of the puzzle was the generation of recombinant protein thioesters. The answer came from nature itself and protein engineering approaches that allowed using protein splicing for EPL.

In 1990, it was observed that the open-reading frame for the gene of *Saccharomyces cerevisiae* vacuolar ATPase (Sce VMA) was twice as large as the actual encoded protein and that 454 amino acids that were derived from the translation of the protein had no similarity to vacuolar ATPases (Noren et al., 2000). Compared to RNA splicing that removes introns from the coding sequence, protein splicing involves the removal of intein sequences from a protein precursor. Inteins should be seen as around 100 amino acid enzymes that break two peptide bonds and ligate the two excised exteins. Over 300 inteins have been identified with low sequence similarity, however the key residues in the splicing event, which are situated at the junction between the intein and the extein, are conserved (Saleh and Perler, 2006).

• Mechanism of protein splicing

The sequence of the majority of inteins begins with a Cys or Ser residue and ends with an Asn while the first residue of the C-extein is Cys, Ser or Thr. The first step in intein-mediated protein splicing is an N-S or an N-O acyl shift of Cys/Ser1 of the intein (Figure I-33). To overcome the high energy-demanding rearrangement of the peptide bond to an ester or thioester bond, a conformational change was reported in inteins that pushed the equilibrium toward the thioester bond (Klabunde et al., 1998, Romanelli et al., 2004). In addition to the strained geometry, deprotonation of the nucleophilic thiol or hydroxyl of Cys/Ser1 is required as well as the protonation of the leaving amino group. A basic residue was identified in proximity of Cys1 that fulfills this role (Saleh and Perler, 2006).

The second step of the process is a transthioesterification that involves the nucleophilic attack of the thiol or hydroxyl group of the first residue of the C-extein (Cys/Ser/Thr) termed +1 on the thioester at the junction between the N-extein and the intein. In this step, the N-extein is brought to the C-extein through the Cys/Ser +1. A conformational change of the intein as well as the low pKa of Cys+1 (estimated to be around 5.8) were suggested to favor the nucleophilic attack (Saleh and Perler, 2006).

The third step involves the cyclization of the last residue of the intein (Asn) to release the intein with a succinimide C-terminus. In the last step, the amide bond is restored with a Cys or Ser

between the N-extein and the C-extein after a S-N or O-N acyl shift (Noren et al., 2000, Muir, 2003a, Saleh and Perler, 2006).

• Intein engineering for EPL

Mutation of the last residue of the intein (Asn) to Ala promotes only the first step of the protein splicing and blocks the succinimide formation. Addition of thiols such as DTT or MESNa to the mutated intein results in the thiolysis of the thioester bond that was formed in the first step of the protein splicing, and the release of the N-extein as a thioester. Commercially available vectors from New England Biolabs contain the modified mini-inteins *Saccharomyces cerevisiae* vacuolar ATPase (Sce VMA1) and the *Mycobacterium xenopi* DNA Gyrase A (Mxe GyrA) in fusion with chitin-binding domain for purification purpose (Muir, 2003a).

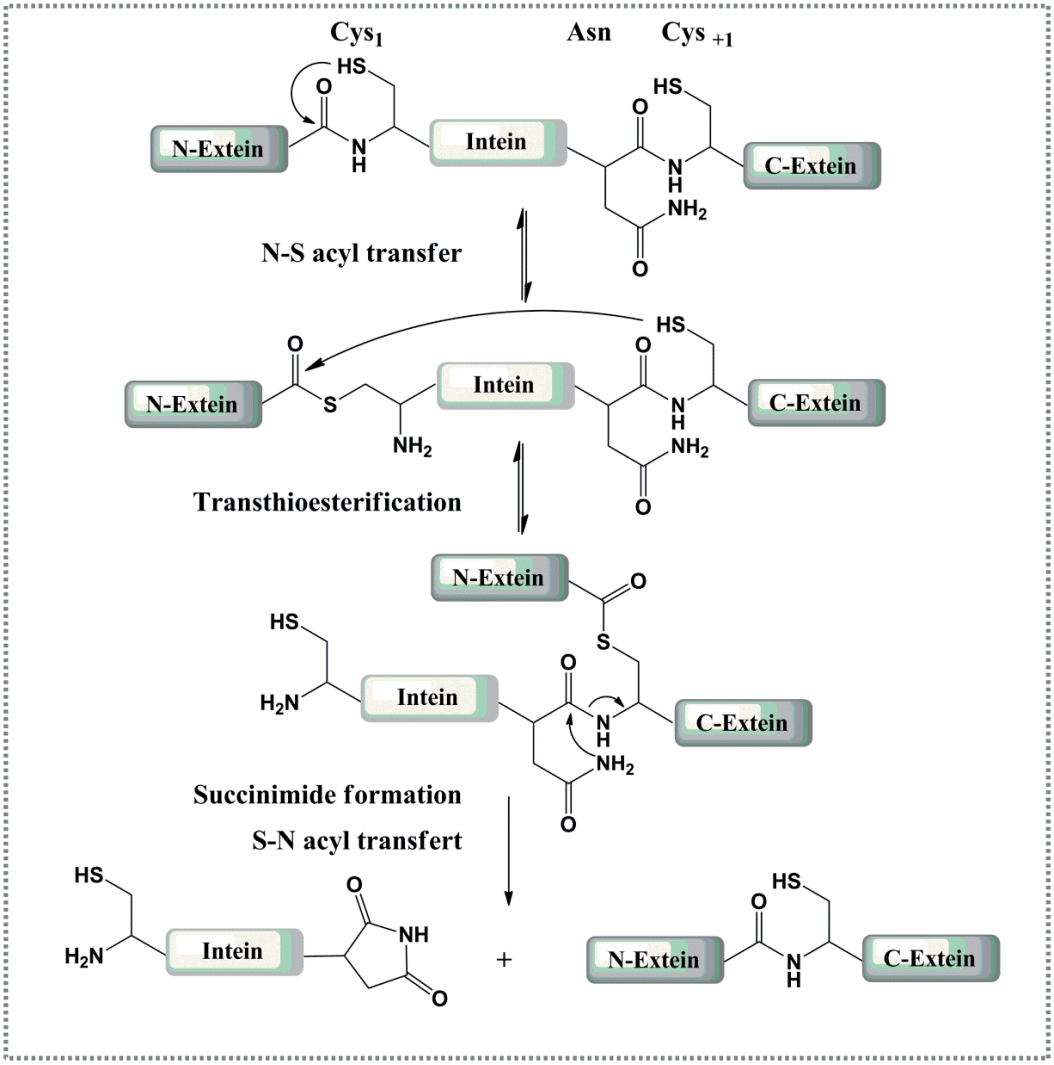

Figure I-33. Mechanism of intein splicing

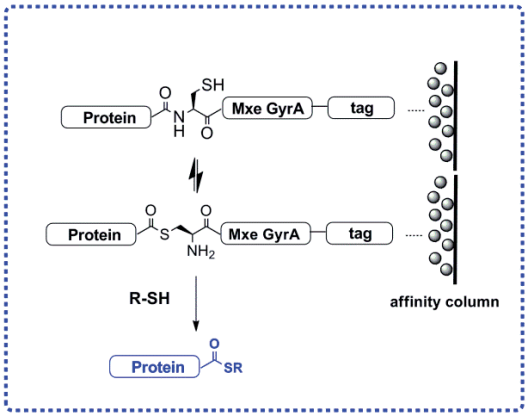

Figure I-34. Production of protein thioesters using modified inteins

• Generation of proteins with an N-terminal Cys

Recombinant proteins with an N-terminal cysteine residue can be prepared using different approaches with typically high yields. The most straightforward approach is the introduction of a Cys codon after the initiating ATG codon in the gene of the protein of interest. The initiating Met will be cleaved by endogenous methionine aminopeptidases during translation, releasing a Cys at the N-terminus (Muir, 2003a). However, the activity of the enzymes depends on the identity and bulkiness of the amino acid that is adjacent to the initiating Met residue (Hirel et al., 1989). To overcome this limitation, a protease recognition sequence can be added upstream of the Cys residue. Factor Xa and Tobacco Etch Virus (TEV) recognition sequences have been used to generate a free Cys after incubation with the protease (Muir, 2003a). In a recent study, Bartke and colleagues elegantly designed a plasmid that expressed their protein of interest with the modified TEV recognition under the control of the T7 promoter, along with the gene encoding the TEV protease under the control of an AraC promoter. Coexpression of the TEV-cleavable truncated protein with TEV protease in *E. coli* leads to processing into a truncated histone *in vivo* (Bartke et al., 2010). To improve the solubility of the protein fragment, some reports have relied on the fusion with the SUMO tag (11kD). To demonstrate the feasibility of this approach, fragments of a membrane protein have been prepared with the SUMO tag upstream of the Cys residue and subsequent incubation with SUMO protease released the free Cys (Komarov et al., 2009).

An alternative approach to generate proteins with an N-terminal Cys residue is based on the fusion to an intein that is engineered to undergo cleavage at its C-terminus as a result of a pH or temperature change. Plasmids encoding the intein *Methanobacterium thermoautotrophicum (Mth)* RIR1 or *Mxe GyrA* or *Ssp DnaB* are commercially available from New England Biolabs

and allow releasing the N-terminal Cys of a protein of interest without the use of a protease (Evans et al., 1999, Muir, 2003a).

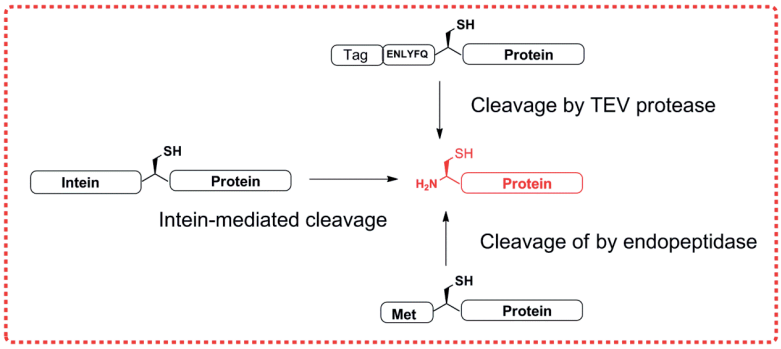

Figure I-35. Different approaches to generate protein fragments with N-terminal Cys.

I.5.3.3. Applications of protein semisynthesis for the generation of ubiquitinated proteins

A large panel of natural modifications (phosphorylation, acetylation, oxidation, D-amino acids) and unnatural modifications (photocleavable groups, non-hydrolyzable phosphonates, fluorescent probes, cross-linking groups) were readily incorporated into proteins via semisynthesis (Muir, 2003a). However, other naturally-occurring modifications such as glycosylation and ubiquitination present additional challenges for protein semisynthesis. Indeed, ubiquitination requires the conjugation of a 75-residue protein residues via an isopeptide bond; and different types of sugar moieties of varying complexity can be added onto the side-chains of asparagine (N-glycosylation) or serine/threonine (O-glycosylation). In the case of ubiquitination, since the isopeptide bond could not be readily produced via standard EPL, different approaches were followed to generate ubiquitinated proteins with an unstable or irreversible isopeptide bond (Spasser and Brik, 2012).

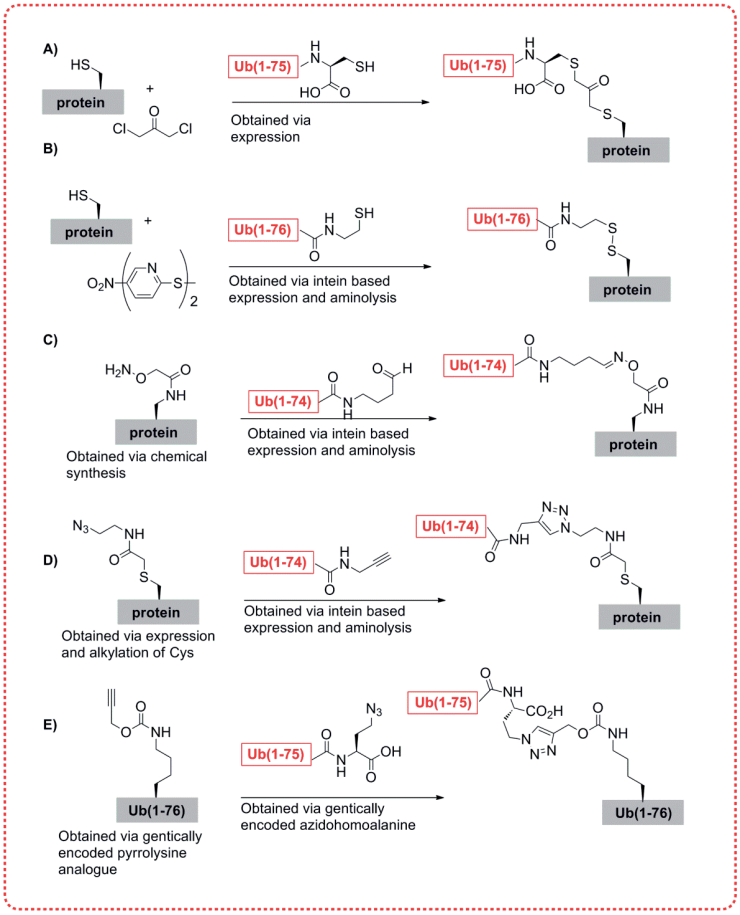

Figure I-36. Chemical approaches to generate monoubiquitinated proteins via isopeptide-like bonds
A: dichloroacetone-based approach (Yin et al., 2000) ,B: disulfide-based approach (Chatterjee et al., 2010, Chen et al., 2010, Meier et al., 2012), C: oxime-based approach (Shanmugham et al., 2010) , D and E: oxime-based approach (Eger et al., 2010, Weikart and Mootz, 2010, Weikart et al., 2012). Figure adapted from Spasser and Brik (Spasser and Brik, 2012).

• Dichloroacetone-based isopeptide bond

This approach is based on the cross-linking of two cysteine residues with dichloroacteone and was used for the generation of diubiquitin chains with K63, K48, K29 and K11 isopeptidic linkage (Yin et al., 2000). The lysines residues to be ubiquitinated in one ubiquitin were replaced by Cys while the C-terminal Gly residue of the other ubiquitin was also mutated to Cys. Addition of dichloroacetone resulted in the formation of ubiquitin dimers that were able to selectively inhibit the activity of DUB (Figure I-36.A)

• Disulfide-based isopeptide bond

Monoubiquitinated Histone 2B (H2B) at K120 was prepared through a disulfide-bond linkage. H2B was expressed in *E.coli* with a Lys → Cys mutation at residue 120 (Chatterjee et al., 2010). Ubiquitin was expressed in fusion with an intein and addition of cysteamine yielded the

generation of ubiquitin with a C-terminal aminoethanethiol linker. Subsequent activation of H2B with a more reactive thiol (2,2′-dithiobis(5-nitropyridine) (DTNP)) in presence of the C-terminally activated ubiquitin resulted in the generation of ubiquitinated H2B (Chatterjee et al., 2010). Independently, Chen *et al.* described the generation of monoubiquitinated proliferating cell nuclear antigen (PCNA) using the same strategy (Chen et al., 2010). This approach allowed to generate milligram quantities of ubiquitinated proteins with good yields (80%) and to perform various structural studies (Chatterjee et al., 2010) or protein-protein interaction assays (Chen et al., 2010). The methodology can be easily extended to other proteins through the introduction of cysteines by single-point mutagenesis (Figure I-36.B) and was recently applied to the study of site-specific impact of monoubiquitination of α-syn on its fibrillization rate (Meier et al., 2012).

- Oxime-based isopeptide bond

The oxime-based approach relies on the condensation of an aldehyde-containing peptide with a peptide harboring an aminooxy group. The methodology was used to prepare peptide fragments derived from the diubiquitin chain (K63 and K48-linked) (Shanmugham et al., 2010). Using SPPS, a ubiquitin peptide where lysines were modified by aminoxyacetyl-l-diaminopropionic acid was prepared. Since the isopeptide bond between the two ubiquitin fragments could not be hydrolyzed by deubiquitinating enzymes, they were used as probes to investigate the specificity of the enzymes toward the substrate and it was revealed that the peptide sequence flanking the isopeptide bond is important for the recognition and the selectivity (Figure I-36.C).

- Click chemistry-based isopeptide bond

Click-chemistry allows the cycloaddition of azide-containing peptide onto an alkyne-containing peptide to produce a triazole at the ligation site (Rostovtsev et al., 2002). In a study by Weikart and colleagues, recombinant SUMO2 (a ubiquitin-like protein) and Ubc9 (an E2 ligase) were functionalized respectively with an alkyl and azide group. SUMO2 (with a deletion of the diglycine motif at the C-terminus) was fused to an intein and subsequently released as a thioester with MESNa. Reaction with propargylamine introduced the alkyne moiety while alkylation of the side chain of a cysteine residue in the substrate to be modified with iodoacetamide ethyl azide introduced the azide group (Figure I-36.D). The same group reported later the preparation of diubiquitin chains through click-chemistry and nonsense suppression methodology: *p*-azidophenylalanine (AzF) is incorporated in place of lysine residues, while SUMO2 is prepared as discussed earlier. By using this approach, conjugation of SUMO2 to the enzyme Ubc9 was reported (Weikart et al., 2012).

Other groups have also used click-chemistry, in addition to the nonsense suppression methodology, to generate diubiquitin chains where the azide function was introduced via the methionine analogue azidohomoalanine (Aha) and the alkyne function via a pyrrolysine analogue, the propargyl-protected lysine derivative Plk (Eger et al., 2010). The methionine analogue Aha was introduced after substituting the codon for the C-terminal Gly by a Met codon. Pyrrolysine analogues were incorporated at lysine sites through the amber stop codon UAG via a pyrrolysyl-tRNA synthetase/tRNA CUA pair (Eger et al., 2010). All Lys-linked diubiquitin chains were prepared and were shown to be recognized by other E3 ligases and subsequently polyubiquitinated (Figure I-36.E).

• Expressed protein ligation

McGinty and colleagues pioneered the generation of site-specifically ubiquitinated proteins with a native isopeptide bond using a semisynthetic approach (McGinty et al., 2008). Monoubiquitinated H2B at K120 was prepared via two EPL reactions: for the first ligation, fragment 117-125 of H2B with a photocleavable protecting auxiliary of the N-terminal Cys residue and another photocleavable auxiliary that was masking the amino group of the Lys residue at position 120 was prepared by SPPS. Ubiquitin thioester produced by thiolysis of an intein fusion was added to the synthetic peptide to yield a protected ubiquitinated H2B fragment. Upon photoirradiation, the thiol group at position 117 was released and a NCL reaction was carried out with the N-terminal fragment of H2B thioester (produced by intein-mediated splicing) spanning residues 1-116 (McGinty et al., 2008) (Figure I-37).

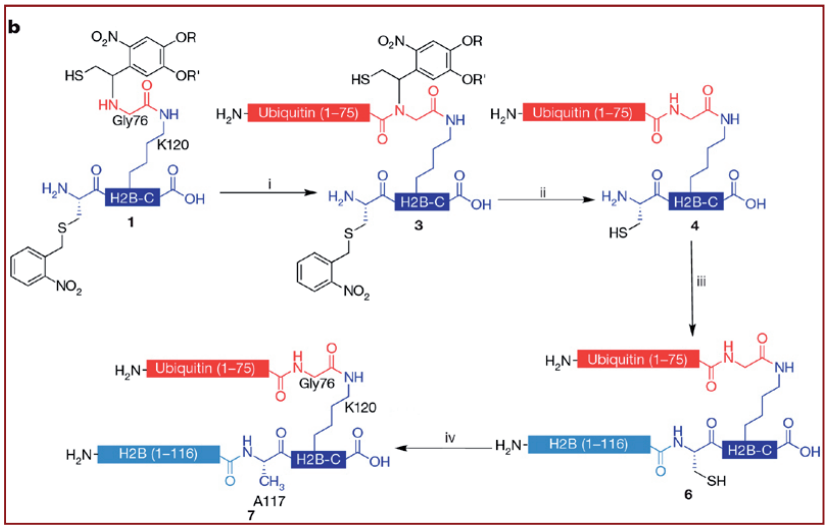

Figure I-37. Generation of monoubiquitinated H2B via a native isopeptide bond
Figure from Ginty *et al.* (McGinty et al., 2008)

The monoubiquitinated histone H2B was able to induce the methylation of Histone 3 in the nucleosome and provided a proof of histone PTM cross-talk *in vitro*. The EPL reactions that resulted in the first report of a monoubiquitinated protein relied on slow ligations that take up to 5-7 days and low yields reported (20%) (McGinty et al., 2008), rendering this methodology less amenable for the study of other proteins.

I.6. Objective of the PhD study

One of the major research interests in the Lashuel laboratory is to elucidate the effect of PTM in regulating α-syn's function in health and disease. Prior to 2008, phosphomimicking substitutions of S129 and S87 were routinely used in transgenic animals and cell-culture without prior investigation of the effect of these mutations on the structure of the protein; and it took a study conducted in our laboratory in 2008 to demonstrate that the phosphomimics do not reproduce all aspects of phosphorylation (Paleologou et al., 2008). Serine substitution by glutamate was shown to mimic the effect of phosphorylation at S87 on α-syn's aggregation, but pS87 and S87E exhibited different membrane binding properties, whereas the S129E/D mutants did not reproduce the effect of phosphorylation on the structure, aggregation and subcellular localization of α-syn (Paleologou et al., 2008, Paleologou et al., 2010).

At the beginning of this PhD study, the majority of PTM in α-syn were shown to localize in the N-terminal (ubiquitination) and the C-terminal domain of the protein (phosphorylation, truncation and nitration). The primary focus of my PhD project focused on investigating the effect of α-syn's phosphorylation at Y125 and monoubiquitination at K6. Given that the kinases that phosphorylate α-syn at Tyr residues are either non-specific or inefficient and that the E3 ligases that are responsible for the conjugation of ubiquitin to α-syn result in a mixture of the non-ubiquitinated form of α-syn along with mono- and di-ubiquitination on several sites, we decided to pursue the development of two semisynthetic approaches: one strategy to allow the introduction of modifications in the N-terminal domain of α-syn and which was further optimized to introduce any unnatural amino acid within the first 29 residues and another strategy for the introduction of modifications in the C-terminal region of α-syn (in the last 40 residues). The first strategy allowed the production of pY125 α-syn and the second one generated monoubiquitinated α-syn at K6 in milligram quantities (in collaboration with the group of Prof. Ashraf Brik). With these homogeneously modified proteins in hand, we were able to investigate the effect of these modifications on the structure, aggregation, subcellular localization and protein-protein interactions of α-syn. These studies have provided novel insight into the role of these PTM in the mechanism of α-syn fibril formation, and the tools generated are proven to be very valuable in studies aimed at identifying novel therapeutic targets and development of diagnostic tools based on quantitative assessment of the levels of these modifications in Cerebrospinal Fluid and blood. Further optimization of this methodology along with the

development of effective means to introduce the proteins in cells and animal models would pave the way for novel approaches to investigate α-syn's biology and pathogenesis.

II. **Chapter 1: Elucidating the role of C-terminal post-translational modifications using protein semisynthesis strategies: α-synuclein phosphorylation at tyrosine 125**

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II.2. Abstract

Despite increasing evidence that supports the role of different post-translational modifications (PTM) in modulating α-synuclein (α-syn) aggregation and toxicity, relatively little is known about the functional consequences of each modification and whether or not these modifications are regulated by each other. This lack of knowledge arises primarily from the current lack of tools and methodologies for the site-specific introduction of PTM in α-syn. More specifically, the kinases that mediate selective and efficient phosphorylation of C-terminal tyrosine residues of α-syn remain to be identified. Unlike phospho-serine and phospho-threonine residues, which in some cases can be mimicked by serine/threonine → glutamate or aspartate substitutions, there are no natural amino acids that can mimic phospho-tyrosine. To address these challenges, we developed a general and efficient semisynthetic strategy that enables the site-specific introduction of single or multiple PTM and the preparation of homogeneously C-terminal modified forms of α-syn in milligram quantities. These advances have allowed us to investigate, for the first time, the effects of selective phosphorylation at Y125 on the structure, aggregation, membrane binding and subcellular localization of α-syn. The development of semisynthetic methods for the site-specific introduction of single or PTM represents an important advance

toward determining the roles of such modifications in α-syn structure, aggregation and functions in health and disease.

II.3. Introduction

α-synuclein (α-syn) aggregation and fibrillogenesis in the brain are the hallmark of Parkinson's disease (PD) and have been linked to the pathogenesis of several neurodegenerative diseases that are collectively referred to as "synucleinopathies" (Trojanowski and Lee, 2003). PD is characterized by the formation of intraneuronal inclusions termed Lewy bodies (LB), which are composed primarily of insoluble fibrillar and aggregated forms of α-syn (Spillantini et al., 1997, Dawson, 2000). Although three mutations in the α-syn gene A30P (Kruger et al., 1998), A53T (Polymeropoulos et al., 1997), and E46K (Zarranz et al., 2004)) have been linked to early-onset forms of the disease, they account for only 1% of all PD cases. Therefore, a better understanding of the molecular determinants that govern the structural, aggregation and functional properties of α-syn is crucial to elucidating its role in the pathogenesis of sporadic and inherited forms of PD.

The primary sequence of α-syn can be divided into three domains, which are: 1) The N-terminal domain which contains the sites of three disease-associated mutations and the imperfect 6 repeats of a conserved sequence (KTKEGV) that play an important role in regulating α-syn secretion (Desplats et al., 2009), internalization (Emmanouilidou et al., 2010, Jang et al., 2010) and binding to membranes; 2) The central fragment, also known as the NAC - non amyloid component - (residues 61-95) region, is highly hydrophobic and is essential for α-syn oligomerization and fibrillogenesis (Del Mar et al., 2005); 3) The C-terminal region comprising residues 96-140. This region is highly disordered and negatively charged (10 glutamate and 5 aspartate residues). The C-terminal region has been proposed to play a role in regulating α-syn transport to the nucleus and has been shown to mediate α-syn interactions with other proteins (Jensen et al., 1999, Giasson BI, 2003, Goers J, 2003, Cherny et al., 2004), metal ions (Paik et al., 1999, Brown, 2007) and natural ligands (e.g. dopamine and polyamines) (Goers et al., 2003b). The majority of α-syn PTM (truncation, phosphorylation, methionine oxidation, and nitration) occurs within the C-terminal region. The clustering of PTM in this functional domain of the protein suggests that these modifications may be involved in the regulation of α-syn's function in health and disease. This hypothesis is further supported by the fact that the majority of these post-translational modification sites are highly conserved across different species.

Furthermore, the close proximity of these different modifications suggests that they may act in concert, and cross-talk between these modifications may constitute an additional molecular switch for regulating the dynamics of α-syn function and aggregation (Figure II-1). Previously, our group identified the polo-like kinases, PLK2 and PLK3, which phosphorylate α-syn efficiently *in vitro* and are major contributors to α-syn phosphorylation *in vivo* (Mbefo et al., Inglis et al., 2009). However, the kinases that carry out selective and efficient phosphorylation of the other known and putative phosphorylation sites (S87, Y125, Y133, and Y136) remain to be identified.

Protein semisynthesis (Muir et al., 1998, Muir, 2003b, Hackenberger and Schwarzer, 2008, Berrade and Camarero, 2009, Chiang et al., 2009, Komarov et al., 2009, Broncel et al., 2012b, Spasser and Brik, 2012), which combines the powers of recombinant protein expression, peptide chemical synthesis and native chemical ligation (NCL) has successfully been used to study post-translational modifications on a variety of proteins (Muir, 2003b, Szewczuk et al., 2009) and offers means of generating site-specifically modified forms of α-syn (Hejjaoui et al., 2011, Butterfield et al., 2012b) (Figure II-2).

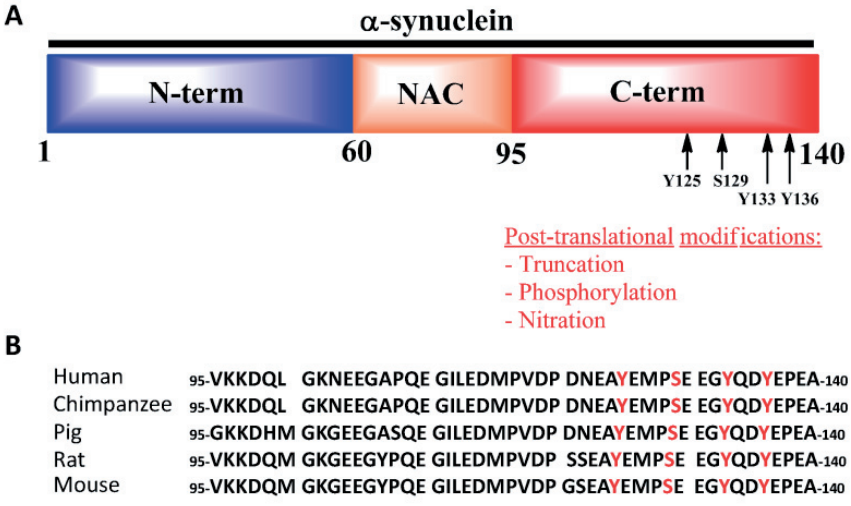

Figure II-1. The clustering of C-terminal post-translational modifications of α-syn
A: Schematic depiction of the sequence of α-syn with its 3 domains. The putative and identified sites of C-terminal phosphorylation are indicated with arrows. B: Sequence alignment of the sequences of the C-terminal domain of α-syn from 5 different species. The conserved positions of the phosphorylation sites are highlighted in red.

To address the aforementioned challenges and to facilitate the investigation of the structural and functional consequences of specific C-terminal PTM of α-syn, we developed a general and efficient semisynthetic strategy that permits the site-specific introduction of single or multiple PTM. Despite mounting evidence supporting the role of C-terminal phosphorylation in

modulating α-syn aggregation and toxicity, relatively little is known about the functional consequences of tyrosine phosphorylation and whether or not tyrosine and serine phosphorylation in this region are regulated by each other (Chen et al., 2009). This is partly due to the lack of tools and methodologies for the site-specific introduction of single or multiple PTM in α-syn. This strategy also enables the preparation of milligram quantities of homogeneous forms of α-syn modified at the C-terminus that are sufficient for carrying out detailed biochemical, biophysical and cellular studies. We used this strategy to produce α-syn phosphorylated at tyrosine 125 (pY125 α-syn). We choose to investigate Y125 phosphorylation for the following reasons: 1) the levels of pY125 α-syn have been reported to decrease with age (Chen et al., 2009); 2) studies by Feany and colleagues suggest that phosphorylation at Y125 acts as a counter-regulator of pS129-induced toxicity in the fly model of PD (Chen et al., 2009), and provide evidence for a potential cross-talk between these two modifications; 3) because there are no effective mutagenesis-based strategies for mimicking tyrosine phosphorylation and thus very little is known about the structural and functional consequences of selective phosphorylation of α-syn at Y125.

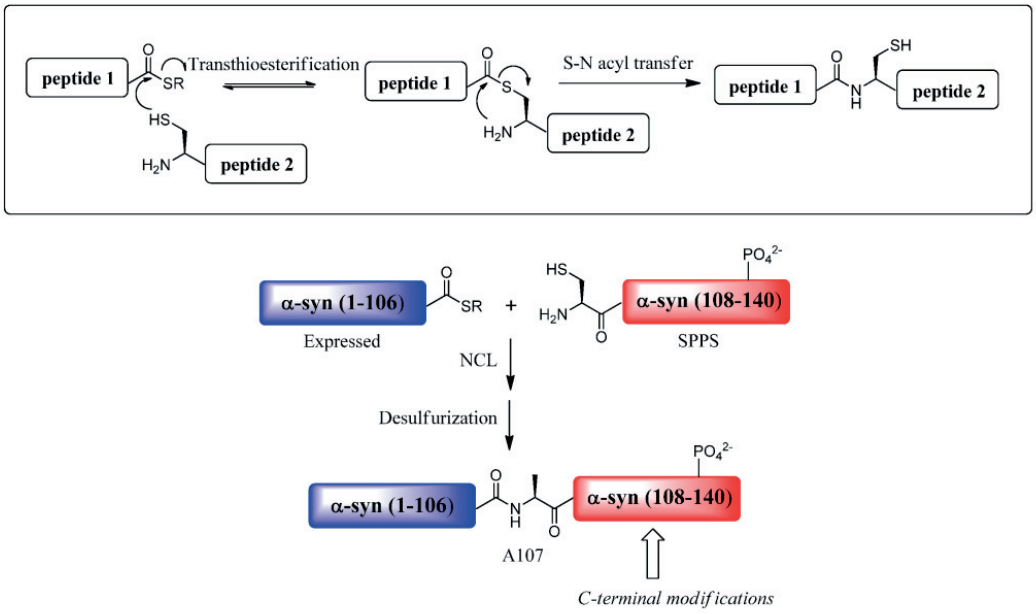

Figure II-2. Mechanism of Native Chemical Ligation
Top: A peptide thioester and a peptide fragment bearing an N-terminal Cys undergo a thioester exchange followed by an S to N acyl migration to create an amide bond between two unprotected peptides in aqueous solution. Bottom: Semisynthetic strategy to generate derivatives of α-syn site-specifically phosphorylated within the C-terminal region. The protein thioester α-syn(1-106)SR generated by recombinant protein expression is ligated by NCL to the synthetic C-terminal fragment α-syn(A107C-140) bearing the desired phosphorylated side chain. A final desulfurization step restores the native Ala-107.

More specifically, we determined the effect of phosphorylation at Y125 on the structure, fibril formation, and phospholipid membrane association-induced conformational shifts *in vitro*. In addition, we further probed cross-talk between phosphorylation at S129, Y125, and S87 residues by determining the relative activity of each phosphorylated form of α-syn as a substrate for the kinases that phosphorylate α-syn at other residues within the C-terminus. We also assessed, for the first time, the subcellular localization of pY125 α-syn in mammalian cells and rat hippocampal neurons. Our work demonstrates the power and potential of applying chemical approaches to elucidate the role of C-terminal PTM in regulating α-syn function in health and disease.

II.4. Material and Methods

II.4.1. Materials

9-Fluorenylmethoxycarbonyl (Fmoc)-amino acids, t-butyloxycarbonyl (Boc)-amino acids, 2 - (1H - benzotriazole - 1 - yl) - 1,1,3,3 - tetramethyluronium hexafluorophosphate (HBTU), were purchased from Novabiochem or Anaspec. The side-chain functionalities of the Fmoc-amino acids were chemically protected as shown in parentheses: Asp(OtBu), Glu(OtBu), Lys(Boc), Thr(tBu), Ser(tBu), Tyr(tBu), Gln(Trt), and Asn(Trt). Phosphorylated Tyr residues were purchased from Novabiochem or Anaspec as protected Fmoc-Tyr[PO(OBzl)]-OH building blocks. All unlisted amino acids were not protected. The Boc-amino acid was Boc-Cys(Trt). *N*,*N*-diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA), triisopropylsilane (TIPS), sodium 2-mercaptoethanesulfonate (MESNa), ammonium iodide, 1,2-ethanedithiol (EDT), 2-methyl-2-propanethiol (*tert*-butyl mercaptan, *t*BuSH) dimethylsulfide, guanidine hydrochloride (GuHCl) and tris(2-carboxyethyl)-phosphine hydrochloride (TCEP) were purchased from Sigma-Aldrich. Piperidine and diethyl ether were purchased from Acros. 2-2'-Azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (VA-044) was purchased from Wako. The solvents *N*-methylpyrrolidone (NMP), dichloromethane, and HPLC-grade acetonitrile were purchased from VWR.

II.4.2. General Solid Phase Peptide Synthesis and Purification

Solid phase peptide synthesis was performed on an automated CS 336X peptide synthesizer from CS Bio using standard Fmoc protocols including the *in situ* neutralization protocol described by Alewood *et al.* (Alewood et al., 1997). A preloaded Fmoc-Ala Wang resin was

used. Syntheses were carried out on a 0.2 mmol scale and coupling steps were performed with 5 eq. Fmoc-amino acid, 5 eq. HBTU/HOBt, and 5 eq. DIPEA in NMP. The N-terminal Cys residues were attached with Boc protecting groups on their N-terminus. Fmoc-group deprotections were carried out with 20% piperidine in DMF. Peptide side chain deprotections, removal of N-terminal Boc group and cleavage from the resin was performed by treatment with 81.5:5:3:5:2.5:2:1.5 of TFA:water:phenol:thioanisole:EDT:Me_2S:NH_4I for 4 hours on a rotating wheel to generate peptides with a C-terminal acid functionality. The crude product was subsequently precipitated three times with a ten-fold excess volume of cold diethyl ether, dissolved in water and lyophilized. Peptides were then purified by reversed-phase HPLC (Waters 600 system) using a Cosmosil C18 preparative column (20x250 mm, 5µm particles, 300Å pores) with a linear gradient of 20-70% B over 30 minutes (solvent A was water/0.1% TFA and solvent B was acetonitrile/0.1% TFA). Peptide elution was monitored by UV absorbance at 214 nm and 280 nm. Peptide masses were confirmed by MALDI-TOF-MS and ESI-MS analysis. Peptide purity was confirmed by re-injection on an analytical RP-HPLC column (Cosmosil C18, 4.6x250 mm, 5µm particles, 300Å pores).

II.4.3. Expression and Purification of Recombinant α-Syn(1-106)SR

C-terminally truncated human α-synuclein was initially cloned into the pT7-7 vector in fusion with the GyrA Mini-Intein from *Mycobacterium Xenopii* and a chitin binding domain (CBD) using the following primers*:* Forward: 5'-GGG CAA GAA TGA AGA AGG ATG CCG AAG AGC AGG AAT TCT G-3' and reverse: 5'-CAG AAT TCC TGC TCT TCG GCA TCC TTC TTC ATT CTT GCC C-3'. The α-syn fragment (1-106) was isolated by NdeI and SapI digest. The intein was isolated from the pTXB1 vector (New England Biolabs) by SapI and BamHI digest. Both fragments were ligated into pT7-7 digested by NdeI and BamHI to generate the plasmid pT7-7 α-syn-Mxe-CBD. To insert a polyhistidine tag at the C-terminus of the intein instead of CBD , an EcoRI site was introduced using site-directed mutagenesis using the following primers: Forward: 5' CTG GCC TCA CCG GTC TGG AAT TCG GCC TCA CGA CAA ATC 3' and reverse 5' GAT TTG TCG TGA GGC CGA ATT CCA GAC CGG TGA GGC CAG 3'. An oligonucleotide containing six histidines and two stop codons with the digested recognition sequences of EcoRI and BamHI sites was ordered: Forward 5'AA TTC CAC CAC CAC CAC CAC CAC TGA TAA G 3' and reverse 5' GA TCC TTA TCA GTG GTG GTG GTG GTG GTG G 3'. The annealed oligonucleotides were ligated into the plasmid pT7-7 α-syn-Mxe-CBD digested with EcoRI and BamHI. Expression was carried out in *E.coli* BL21(DE3). Protein expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside

(IPTG) and by growing the cells for 4h at 37°C. The cells were harvested by centrifugation and lysed by ultra-sonication in 40 mM Tris-acetate, 5 mM EDTA pH 8,3. The supernatant was separated from the cell debris by centrifugation (23, 000 x g, 30 min, 4°C). 4 equivalents of $MgCl_2$ were added prior to the loading on the Ni-NTA column. The soluble fraction was loaded on a HisPrep FF 16/60 (Amersham) pre-equilibrated with 50 mM HEPES pH 7.5. A washing step of 50 mM HEPES 25 mM imidazole, pH 7.5 for 5 column volumes was included to remove non-specific binding. The protein α-syn-Mxe-$His_{(6)}$ was eluted with a step elution of 50 mM HEPES, 350 mM Imidazole pH 7.5. The splicing reaction was carried out directly in the eluate from the Ni-NTA column for 12 h under agitation at RT in presence of 0.25 M MESNa. The splicing efficiency was around 90% (by SDS-PAGE analysis, data not shown). The sample was subsequently purified by reverse-phase HPLC using a preparative C4 column (VYDAC, 20x250 mm,5µm particles, 300Å pores) with a linear gradient of 20-70% B in 30 min at a flowrate of 10 ml/min (solvent A was water/0.1% TFA and solvent B was acetonitrile/0.1% TFA). The pure protein thioester was obtained in a yield of 10 mg per liter of BL21 (DE3) expression medium.

II.4.4. Expression and purification of ^{15}N labeled proteins

α-syn A107C: The α-syn mutant A107C was generated using single-point mutagenesis with the following primer pairs to generate the plasmid pT7-7 α-syn A107C: forward primer 5' GCA AGA ATG AAG AAG GAT GCC CAC AGG AAG GAA TTC TG 3' and reverse primer 5' CAG AAT TCC TTC CTG TGG GCA TCC TTC TTC ATT CTT GC 3'. BL21 (DE3) cells were transformed with the plasmid and grown in M9 minimal media with ^{15}N labeled ammonium chloride. Protein expression was induced by the addition of 1 mM IPTG for 4 h at 37°C. After harvesting the cells by centrifugation at 6000 x g for 15 min, the cell pellet was resuspended in 40 mM Tris-acetate and 5mM EDTA (pH 8.3) and lysed by ultra-sonication. The insoluble material was removed by centrifugation at 23,000 x g for 40 min at 4°C. The supernatant was boiled for 15 min in a water bath at 100°C followed by centrifugation at 23,000 x g for 40 min, which precipitates most cellular proteins but not α-syn A107C. The final supernatant was filtered through a PVDF membrane with 0.22 µm pores. Anion-exchange chromatography was performed on a Pharmacia AKTA FPLC system using a 20 mL HiPrep 16/10 Q FF column (Amersham). The protein was eluted with a linear gradient of 0 to 1 M NaCl (the protein elutes at approximately 300 mM NaCl) and subsequently purified on a Superdex 200 26/60 size-exclusion chromatography column (Amersham) using a mobile phase of buffer containing 50 mM Tris, 150 mM NaCl, pH 7.5. The purified protein was dialyzed extensively against de-ionized water and lyophilized and stored at -20°C until used. From 1 L of expression

medium, the average yield of lyophilized material was 30 mg. To remove the glutathatione disulfide adduct, the lyophilized powder was dissolved in a buffer containing 50 mM Tris 150mM NaCl (pH 7.5), with 30eq of TCEP and the solution was incubated for 10 min at RT. The protein was subsequently purified on a C4 preparative column (Vydac) with a linear gradient of 20-70% B in solvent A over 30 min at a flow rate of 10 mL/min (solvent A was water/0.1% TFA and solvent B was acetonitrile/0.1% TFA). The ^{15}N labeled protein was obtained in a final yield of 10 mg per liter of bacterial expression.

α-syn (1-106)SR: The expression of ^{15}N labeled α-syn(1-106) SR was carried out in a similar manner as for the non-labeled protein with an overall yield of pure protein of ~3.5 mg per liter.

II.4.5. Semisynthesis and desulfurization of α-syn phosphorylated at Tyr 125

The semisynthesis of α-syn site-specifically phosphorylated within the C-terminus (residues 107-140) involved an NCL reaction between recombinant α-syn(1-106)SR and synthetic peptides α-syn (A107C-140) phosphorylated at Tyr-125 or the WT peptide. NCL reactions were carried out in 6 M GuHCl, 200 mM sodium phosphate buffer, pH 7.0 using 1.5 mM of α-syn(1-106)SR and 2 molar excess of C-terminal peptide A107C-140 pY125 or A107C-140 WT peptide in the presence of 30 eq. TCEP in a volume of 1 mL at 37°C with shaking at 1000 rpm. Three ligation reactions were carried out in parallel. To monitor the NCL reactions, aliquots of the reaction mixture (4µl) were removed and quenched with 38 µl of water containing 0.1% TFA and subsequently analyzed by SDS-PAGE (15% polyacrylamide) and MALDI-TOF-MS. RP-HPLC was not used due to the co-elution of α-syn(1-106)SR and the ligated product under all conditions tested in our laboratory. Based on SDS-PAGE analysis, the desired ligated product was produced within 1-2 hrs.

Free radical-based desulfurization of the semi-synthetic α-syn mutant was carried out as previously described by Danishefsky and colleagues (Wan and Danishefsky, 2007). In our case, the ligation and the desulfurization were carried out in one-pot. 1mL of a solution of 1 M of TCEP (in 6 M GuHCl, 200 mM sodium phosphate pH 7.0) was added to the ligation reaction. 160 µl of 2-methyl-2-propanethiol and 100 µl of a 0.1 M solution of 2-2'-Azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (VA-044) free radical initiator (in 6 M GuHCl, 200 mM sodium phosphate pH 7.0) were added under argon. The reaction was incubated at 37°C with agitation at 1000 rpm in a ventilated fume hood for 3.5 h. The progress of desulfurization was followed by MALDI-TOF-MS.

II.4.6. Purification of the semisynthetic pY125 α-syn

Prior to purification, the ligated desulfurized products from the 3 ligation/desulfurization reactions (that were carried out in parallel) were desalted through a 5mL HiTrap desalting column (Amersham) with 20 mM sodium dihydrogen citrate pH 4.0 as the mobile phase. The fractions containing the protein were subsequently pooled and manually injected on a 5 mL cation-exchange column HiTrap SP HP (Amersham). We took advantage of the charge difference between the full-length protein, the fragment α-syn(1-106)OH (hydrolyzed thioester) and the C-terminal 107-140 peptide for a single-column purification. At pH 4.0, the peptide is negatively charged (net charge: -2) and will not bind to the column while the full-length protein and the fragment (1-106) are positively charged and hence will bind to the column. At pH 5.0, the charge of the full-length protein is -1 and the fragment (1-106) has a charge of 6 and can be subsequently separated using a gradient of salt. We initially allow the peptide to pass in the flow through by washing the column after injection with 10 column volumes (CV) of 20 mM sodium citrate pH 4 then with a linear gradient of NaCl from 0 to 100% B (where A is a solution of 20 mM sodium citrate pH 4, B the same solution supplemented with 0,5 M NaCl) over 4 CV on a Akta Purifier system. To elute bound proteins, we applied a linear gradient of NaCl from 0 to 70% B (where A is B is a solution of 20 mM sodium citrate pH 5 and B is the same solution supplemented with 0,25 M NaCl) over 30 CV at a flow of 1 mL/min. The full-length protein elutes around 20% B and the fragment (1-106) around 30% B. The fractions containing the protein were extensively dialyzed against de-ionized water and lyophilized. The final yield of pure lyophilized protein is around 19% (9 mg).

II.4.7. Preparation and purification of pS129 and pS87 α-syn

Recombinant WT α-syn (3 mg per reaction, 10 µM) was phosphorylated at S129 *in vitro* by PLK3 (10 µg per reaction, 4.8 µg/mL) at 30°C in 20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 2 mM DTT, and 1 mM ATP. The phosphorylated protein was purified by reversed-phase HPLC using a semipreparative C8 column (InertSil 7.6 x 250 mm, 5 µm particles, 300Å pores). The proteins were eluted with a linear gradient from 20% to 40% of solvent B in solvent A over 40 min at a flowrate of 5 mL/min (solvent A was water/0.1% TFA and solvent B was acetonitrile/0.1% TFA). The collected fractions were pooled according to purity assessed by ESI-MS and MALDI-TOF-MS. After purification, pS129 α-syn was lyophilized and stored at -20°C until use. pS87 α-syn was prepared as previously described (Paleologou et al., 2010) .

II.4.8. Preparation of α-syn S129A, S129E and Y125F

The expression and purification of α-syn S129A, S129E and Y125F were performed as described (II.4.4) (Paleologou et al., 2008).

II.4.9. Circular dichroism measurements

Protein samples (10 μM) in pH 7.5 buffer containing 50 mM Tris buffer and 150 mM NaCl were analyzed at 25°C using a Jasco J-815 CD spectrometer. An average of 10 spectra was collected in the range of 195-250 nm using a 0.1 mm path length quartz cuvette. Data points were acquired every 0.2 nm in the continuous scanning mode at a speed of 50 nm/min with a digital integration time of 2 s, and a bandwidth of 1 nm. Processed spectra were obtained by subtracting the baseline signal due to the buffer and cell contribution from the protein spectra and a final smoothing was applied (Savitzky-Golay filter; convolution width of 25 data points).

II.4.10. *In vitro* Phosphorylation assay

pY125 α-syn (6 μM) was incubated in 50 mM Tris pH 7.5, 10 mM DTT, 1 mM ATP, 1 mM MgCl2 in presence of 0,42 μg (1μL) of PLK3 (Invitrogen) in a total volume of 50 μL. For the phosphorylation by CK1, 6 μM of protein were incubated in CK1 buffer 1X supplemented with 1 mM ATP and 1000 units of CK1 (1 μL) in a final volume of 50 μL. The reaction was allowed to proceed for 12 h at 30°C with no agitation. The extent of phosphorylation was monitored by MALDI-TOF-MS and western-blotting using pS87, pS129 and pY125-specific antibodies.

For phosphorylation by tyrosine kinases, WT α-syn, -pS87, and -pS129 were incubated at a concentration of 3 μM in a final volume of 30 μL with the appropriate tyrosine kinase. Kinases were added at a final concentration of 50 nM. The reaction buffers were the following: Syk / Lyn: 50 mM Tris and 5 mM $MnCl_2$ (50μL); Fyn / Src: 20 mM Tris and 5 mM $MnCl_2$ (pH 7.4); Fgr: 50 mM Tris and 5 mM $MgCl_2$ (pH 7.5). The reactions were started by the addition of freshly prepared ATP (1 mM), and incubated for 14 h at 30 °C before analysis.

II.4.11. NMR spectroscopy

Work carried out by Igor Diky (Prof. David Eliezer's group)

NMR studies were performed as previously described (Paleologou et al., 2008). Briefly, lyophilized ^{15}N-labeled α-syn was dissolved in sample buffer (100 mM NaCl, 10 mM Na_2HPO_4, in 90% H_2O, 10%, D_2O at pH 7.4 to a concentration of 0.1-0.3 mM. For spectra in the presence of SDS, the sample buffer also contained 40 mM SDS. All ^{1}H, ^{15}N HSQC spectra were collected at 10 °C in the absence of SDS and at 40 °C in the presence of SDS on either a Varian INOVA 600 MHz spectrometer (Weill Cornell Medical College) or a Bruker Avance 800 MHz

spectrometer (New York Structural Biology Center) equipped with cryogenic probes. NMR data were processed by NMRPipe (Delaglio et al., 1995) and analyzed in NMRViewJ (Johnson, 2004) . Spectra were referenced indirectly to sodium 2,2-dimethyl-2-silapentane-5-sulfonate and ammonia using the known chemical shift of water. Assignments for the spectra of mutant and phosphorylated α-syn at Y125 were estimated by transferring each previously assigned cross-peak in the HSQC spectrum of WT α-syn to the closest unassigned cross-peak in each spectrum. Normalized per residue, the chemical shift change was calculated as $\Delta\delta = \sqrt{(\Delta\delta_{H1}^2 + \Delta\delta_{N15}^2/25)}/2$). For PRE experiments, α-syn was dissolved in sample buffer without SDS, a 10-fold excess of MTSL spin-label was added and the resulting solution was incubated for 30 minutes. A buffer-exchange column was used to remove unbound spin-label and the sample was divided into diamagnetic and paramagnetic samples. DTT (final concentration 2 mM) was added to the diamagnetic sample to reduce off the spin-label. Matched two-dimensional HSQC spectra were collected on the two samples, and the ratio of the intensity of each cross-peak was determined.

II.4.12. *In vitro* fibrillization assay

pY125 α-syn and WT α-syn were incubated at 15 µM in a volume of 450 µL under constant agitation at 1200 rpm for 48h or 7 days at 37°C. ThT fluorescence reading was carried out with a ThT concentration of 10 µM and a protein concentration of 1.5 µM in a volume of 70 µL in a pH 8.5 buffer containing 50 mM Glycine. A TECAN spectrometer was used to measure ThT fluorescence at an excitation wavelength of 450 nm and an emission wavelength of 485 nm. Aliquots taken at 0, 24 and 48 h were measured in triplicates. Values were expressed as the ThT fluorescence of three measurements (mean ± S.D.).

II.4.13. Transmission electron microscopy (TEM)

7 µL of proteins were deposited on Formvar-coated 200 mesh copper grids (Electron Microscopy Sciences). Grids were washed with two drops of double-distilled H_2O and stained with two drops of freshly prepared 2% (w/v) uranyl acetate (Electron Microscopy Sciences) and then vacuum dried from the edge of the grids. Specimens were viewed on a Philips CIME 12 electron microscope, operated at 80 kV. Digitized photographs were recorded with a CCD camera (Digital Camera Morada by Soft Imaging System, 4008 X 2672 pixels).

II.4.14. Western-blot analysis

Protein samples were separated at 80 V on 15% polyacrylamide 1 mm-thick gels over 1-2 h. The proteins were transferred onto nitrocellulose membranes (Omnilab SA) using a semi-dry transfer system (Bio-Rad) under constant current (~200 mA per membrane) and the membranes

were blocked for 1 h at room temperature with Odyssey blocking buffer (Li-COR Biosciences) diluted three-fold in PBS buffer. The membranes were probed with the relevant primary antibodies: mouse anti-α-syn (1:1000 dilution, BD Transduction Laboratories), rabbit anti-α-syn ab51252 (1:1000 dilution, Abcam), rabbit anti-α-syn SA3400 (1:1000 dilution, Biomol) mouse monoclonal anti-phosphorylated α-Syn at Tyr-125 (1:1000 dilution; BD Transduction Laboratories), homemade rabbit anti-pY125 α-syn (1:1000); mouse monoclonal anti-pS129 α-syn (dilution 1:5000; Wako), rabbit anti-pS87 α-syn (1:200 dilution,(Mbefo et al., 2010)) overnight at 4°C. After three washes with PBS buffer containing 0.1% (v/v) Tween 20 (Fluka), the membrane was incubated with secondary goat anti-mouse or anti-rabbit antibodies conjugated to Alexa 680 (dilution 1:5000; Invitrogen). The membranes were then washed three times with PBS buffer containing 0.01% (v/v) Tween 20 and once with PBS buffer and scanned on a LiCOR scanner at 700 nm.

II.4.15. pY125 α-syn dephosphorylation assay

HeLa cells were grown to 90% confluency in a 10cm Petri dish and lysed by occasional vortexing on ice over 30 min in 600 µL of lysis buffer (50 mM tris pH 7.4, 150 mM NaCl, 5% glycerol, 25 mM sucrose, 1% NP-40, and 0.25% sodium deoxycholate with 100 mM PMSF and protease inhibitor cocktail from Sigma). The lysate was clarified by centrifugation at 20800 g for 30 min at 4°C. Incubation of pY125 α-syn (1.2 µg in 100 µL) with HeLa lysate was carried out for 0, 30 min and 1 h at 37°C. The reaction was quenched by the addition of Laemmli sample buffer. For inhibition by sodium orthovanadate, different concentrations of the activated inhibitor (0.1, 1 and 10 mM) were incubated with samples of lysate containing 1.2 µg of pY125 α-syn for 2 h. Lysate from each incubation (300 ng) was loaded onto a SDS-PAGE gel and analyzed by Western-Blot with antibodies against WT α-syn and pY125 α-syn .

II.4.16. Cell culture and transient transfection

Human embryonic kidney cells (HEK 293T) and HeLa cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum and 5% penicillin/streptomycin in a humidified incubator with 5% CO_2 at 37 °C. For HEK 293T cells, transient transfection was performed in 6-well plates at a cell confluence of 70–80%, using a standard calcium phosphate ($CaPO_4$) transfection protocol. For HeLa cells, transient transfection using Lipofectamine^{TM} 2000 (Invitrogen) was performed according to the manufacturer's instructions. The total amount of plasmid used in the transfection was 2 µg/well for 6-well-plates and 0.8 µg/well for 24-well plates.

II.4.17. Detection of pY125 α-syn in HEK 298T and HeLa cells

HeLa and HEK 293T cells were grown in 6-well-plates and transfected with the plasmid pAAV α-syn WT and pAAV α-syn Y125F. The cells were treated with pervanadate (100 µM sodium orthovanadate and 4 mM H_2O_2) 24 h post-transfection for 5, 10 and 20 min, as reported previously (Ellis et al., 2001). The medium containing the inhibitor was removed, and the cells were subsequently harvested by pipetting in PBS or by scraping. The cells were pelleted by centrifugation at 100 g for 3 min, lysed by the addition of 100 µL of Laemmli buffer and boiled at 95°C for 30 min. 30 µL of sample was loaded on SDS-PAGE and analyzed by Western-Blot with antibodies for WT α-syn and pY125 α-syn

II.4.18. Subcellular fractionation

HEK 293 cells were grown in 6-well-plates, transfected with the plasmid pAAV WT α-syn and treated with pervanadate (100 µM sodium orthovanadate and 4 mM H_2O_2) 24h post-transfection. Subcellular fractionation was carried out using the kit from Calbiochem according to the manufacturer's instructions and as previously described (Mbefo et al., 2010). Pervanadate (100 µM sodium orthovanadate and 4 mM H_2O_2) was also added during the extraction steps.

II.4.19. Primary neuronal cultures

Work carried out by Jia Cui (Prof. Yan Zhang's group)

Rat primary neurons were cultured from newborn Sprague Dawley (SD) rat hippocampus, following the regulations of Peking University Animal Care and Use Committee. In brief, rat hippocampal tissues were dissociated with 0.25% trypsin (Invitrogen), which was then inactivated by 10% decomplemented fetal bovine serum (FBS, HyClone). The mixture was triturated through a pipette to produce a homogenous mixture. After filtering the mixture through 0,7 µm sterilized filters, the flow-through was centrifuged. The pellet was then washed once with PBS followed by one wash with Dulbecco's Modified Eagle Medium (DMEM) in Earle's balanced salt solution containing 0.225% sodium bicarbonate, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1% dextrose and antibiotic Pen-Strep with 5% FBS (all from Invitrogen). Cells were then plated on poly-L-lysine (Sigma) coated plates or on glass coverslips at a density of $1x10^6$ cells/ml. Neurons were incubated at 37°C in DMEM without phenol red with 5% FBS and 5% circulating CO_2. Cytarabine was added to culture media 24 hours after plating to a final concentration of 10 µM to inhibit cell growth. The culture medium was changed every 48 hours. Cells were used for experiments at day 7 of culture.

II.4.20. Subcellular localization of pY125 α-syn in HeLa cells

HeLa cells were seeded on coverslips (100 000 cells in a 24-well-plate) pre-coated for 30 min at RT with Poly-L-lysine. The cells were transfected with the plasmid pAAV α-syn WT (0.8 µg) and subsequently treated with pervanadate prepared as described above for 30 min 24 h post-transfection. The cells were subsequently fixed in 4% paraformaldehyde in PBS for 30 min, washed three times in PBS, blocked for 1 h with 3% BSA, 0.1% Triton X-100 in PBS and incubated overnight at 4°C with a cocktail of primary antibodies (mouse α-syn pY125, BD Pharmingen, 558246, 1:1000; rabbit α-syn, ab51252, Abcam, 1: 1000) in 1% BSA, 0.1% Triton X-100 in PBS. The cells were then washed three times with 1% BSA, 0.1% Triton X-100 in PBS and incubated for 1 h at RT with a cocktail of secondary antibodies (goat anti-mouse coupled to Alexa Fluor 568, Molecular Probes Inc., 1:1000; goat anti-rabbit coupled to Alexa Fluor 488, Molecular Probes Inc., 1:1000). After three washes in PBS, coverslips were counter-stained with 4,6-diamidino-2-phenylindole (DAPI, 1:5000), washed 3 times with PBS and mounted on glass slides with Vectashield mounting medium (Vector). Cells were imaged using a Zeiss LSM700 confocal microscope.

II.4.21. Microinjection and immunocytochemistry

Work carried out by Jia Cui (Prof. Yan Zhang's group)

Thin-walled Borosilicate glass capillaries (outer diameter=1.0 mm, inner diameter=0.5 mm) with a microfilament (MTW100F-4, World Precision Instrument) were pulled with a Flaming/Brown Micropipette Puller (P-97, Sutter) to obtain injection needles with a tip diameter of approximately 0.5 µm. Microinjections into the cytosol of each were performed cell using an Eppendorf Microinjector FemtoJet and Eppendorf Micromanipulator (Eppendorf). Neurons were injected with 25 fl/shot at an injection pressure of 100 hPa, a compensation pressure of 50 hPa, and an injection time of 0.1 s. pY125 α-syn was diluted for a final concentration of 1 µM in phosphate-buffered saline (PBS, 0.14 M NaCl, 0.003 M KCl, 0.01 M Na_2HPO_4, 0.002 M KH_2PO_4, pH7.2). The solutions were injected with 100 µg/ml dextran Texas Red (DTR, MW: 3000, Molecular Probes) as a fluorescent marker or with Fast Green as a visible marker to enable the identification of injected cells. The injected cells were treated either with control DMEM medium or with pervanadate (100 µM orthovanadate and 4 mM H_2O_2) for 30 min. For immunocytochemistry, cells were fixed, permeabilized and blocked with 10% donkey serum at room temperature followed by incubation with anti- α -synuclein antibody (mouse α-syn pY125, BD Pharmingen, 558246, 1:1000; rabbit α-syn, ab51252, Abcam, 1: 1000) at 4°C for 24 h. Cy2 or Cy3-conjugated donkey anti-rabbit antibody was used as the secondary antibody. Nuclei were

subsequently stained by Hoechst 33258 (1 μg/ml, Sigma) for 15 minutes in the dark. The coverslips were mounted on glass slides with Immunon™ mounting medium (Shandon) and imaged by fluorescence microscopy (Olympus BH2-RFCA, Olympus) with a digital camera (Olympus DP70 Digital Microscope Camera, Olympus).

II.4.22. Isolation, Expression and Purification of NbSyn87

Work carried out by Farah El Tuk and Tim Guilliams (Prof. Christopher Dobson's group)

The antibody fragment, NbSyn87, was isolated and identified by the Dobson group using phage display selection following the immunization of a llama with α-syn, according to previously published protocol (Decanniere et al., 1999, Conrath et al., 2001). The expression and purification of NbSyn87 was performed as described in Guilliams *et al.* 2012.

II.4.23. Isothermal Titration Calorimetry

Work carried out by Tim Guilliams (Prof. Christopher Dobson's group)

Calorimetric data were recorded using an iTC200 calorimeter (MicroCal, LLC, Northampton, MA, USA). A solution of 40 μl of NbSyn87 at a concentration of 150 μM was titrated in 2 μl aliquots with a 150 s intervals into the calorimetric cell containing a standard volume (203 μl) of 10 μM monomeric α-syn in solution. Experiments were performed at 25 °C in 10 mM phosphate and 150 mM sodium chloride at a pH of 7.4. The thermodynamic analysis was performed using the Microcal analysis software (Origin 7.0) using a simple 1:1 bimolecular binding model.

II.5. RESULTS

The semisynthesis of α-syn site-specifically phosphorylated within the C-terminus (residues 107-140) involved an NCL reaction between the recombinant thioester α-syn(1-106)SR and the synthetic peptide α-syn (A107C-140) phosphorylated at Tyr-125 or the corresponding nonphosphorylated peptide. Because the primary sequence of α-syn does not contain any cysteine residues, we mutated alanine residue 107 to a cysteine to allow the NCL reaction to occur with the juxtaposed residue at position 106 being an unhindered residue (Gly).

II.5.1. Generation of semisynthetic pY125 α-syn

Recombinant α-syn(1-106)SR was expressed in *E.coli* utilizing the intein approach, which exploits the mechanism of intein self-splicing to generate recombinant proteins with a C-terminal thioester (Figure II-3.A) (Muir et al., 1998). In the case of natural intein splicing, a series of transthioesterification and succinimide formation events that involve an arginine

residue at the C-terminus of the intein mediates the splicing of the intein and the linkage of the N-extein and the C-extein by an amide bond (Evans and Xu, 1999). Briefly, truncated α-syn(1-106) was expressed in fusion with the GyrA mini-intein (*Mxe GyrA*) which contained a C-terminal hexahistidine purification tag to facilitate purification from bacterial lysate.

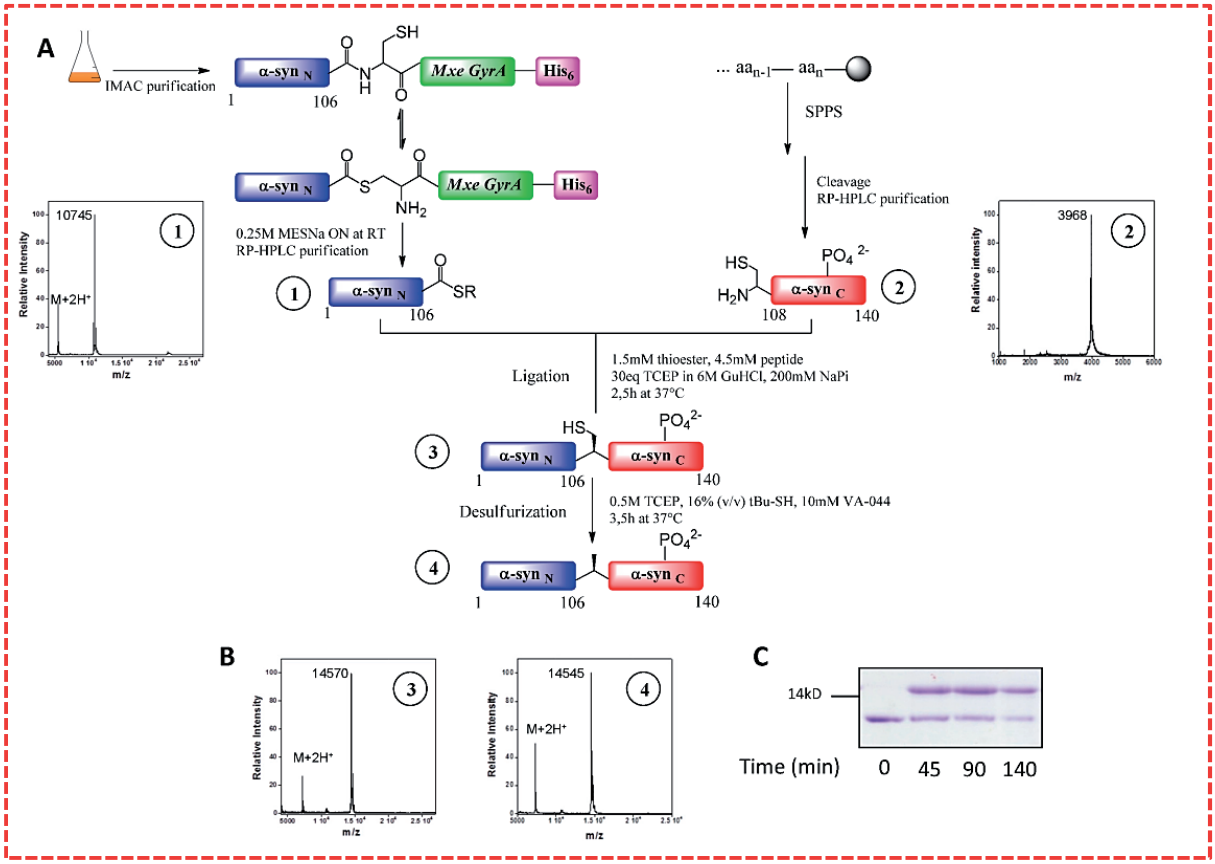

Figure II-3. Semisynthesis and characterization of phosphorylated α-syn derivatives
A: Expressed Protein Ligation (EPL) approach to generate WT α-syn and pY125 α-syn. The α-syn(1-106)SR fragment was generated using intein-mediated thioester formation. MALDI-TOF analyses of 1) α-syn(1-106)SR (expected mass: 10744 Da [M+H]); The synthetic peptides α-syn (A107C-140) and α-syn(A107C-140)_pY125 were generated by Fmoc-based SPPS. MALDI-TOF analyses of 2) α-syn(A107C-140)_pY125 (expected mass: 3970 Da [M+H]);. Full-length semisynthetic α-synA107C and α-synA107C_pY125 were generated by a NCL step between α-syn(1-106)SR and synthetic peptide α-syn(A107C-140)_pY125 or α-syn(A107C-140). Radical desulfurization of the purified ligation product generated pY125 α-syn and α-syn WT. The final purification step involved a cation-exchange chromatographic step. B: MALDI-TOF analyses of 3) α-synA107C_pY125 (expected mass: 14573 Da [M+H]); and 4) pY125 α-syn (expected mass: 14541 Da ([M+H])). C: Coomassie blue stained SDS-PAGE analysis of the NCL reaction to generate pY125 α-syn. Lane 1, 2, 3, and 4 represent α-syn(1-106)SR; ligation reaction after 45 min, 90 min, and 140 min, respectively.

Initially, we used the chitin binding domain from the commercially available IMPACT system (New England Biolabs) as a purification tag for the attachment of the fusion protein to the chitin beads followed by splicing on the column with 0.25 M MESNa and subsequent purification of the thioester by reversed-phase HPLC. We then added a C-terminal histidine tag on the intein to facilitate the purification of the protein from larger volumes of *E.coli* lysates .The purification

procedure was therefore modified and cell lysates were passed through a Ni-NTA column to immobilize the fusion protein and remove undesired proteins (Figure II-3.A). The α-syn(1-106)SR was released by thiolysis with an excess of MESNa. The protein thioester was then purified by RP-HPLC and characterized by MALDI-TOF mass spectrometry and RP-HPLC (Figure II-3.A and Figure S II-3). The peptide fragments α-syn (A107C-140) *p*Y125 and α-syn (A107C-140) WT, each with an N-terminal cysteine, were generated by stepwise SPPS (Figure S II-1 and Figure S II-2).

The expressed fragment α-syn(1-106)SR was then subjected to two separate NCL reactions under denaturing conditions with 2 molar excess synthetic C-terminal peptides α-syn (A107C-140) pY125 and α-syn (A107C-140) (Figure II-3.A), generating two variants of full length α-syn A107C selectively phosphorylated at Tyr-125 (pY125 α-syn) and the wild-type (WT) protein, respectively. Hydrolyzed α-syn(1-106)-OH was generated as a minor side product, even when the NCL reaction was carried out at lower pH values. Due to the co-elution of α-syn(1-106)OH and the full-length protein by reverse phase HPLC under all conditions tested in our laboratory, we could not quantitatively assess the yield at each step of the semisynthesis. The final yield of the purified protein is 19%. (Figure II-3.C and Figure S II-5). Radical-initiated desulfurization of Cys-107 in the ligated proteins re-established the native Ala-107 residue in the phosphorylated variant of α-syn and its WT counterpart with minimal loss of starting material. A loss of 32 mass units from each protein was observed by MALDI-TOF-MS in each protein, consistent with the loss of a sulfur atom (Figure II-3.B, Figure S II-6 and Figure S II-8). Separation of the ligated fragment from the hydrolyzed thioester and excess C-terminal peptide was initially achieved by using a combination of different purification methods, including RP-HPLC, anion-exchange, liquid isoelectric focusing and size exclusion chromatography. Only a combination of two or more of these techniques resulted in pure semi-synthetic proteins. However, this approach resulted in very low final yield. Later, the ligation products were purified using cation-exchange chromatography, which permitted single-step chromatographic separation of the full-length fragment, the hydrolyzed thioester, the C-terminal peptide and other contaminants (Figure S II-9, Figure S II-10 and Figure S II-11). Using optimized NCL, desulfurization and purification conditions, we obtained milligram quantities of the purified ligated proteins. In the case of α-syn phosphorylated at Y125, MALDI-TOF analysis of the purified products demonstrated a major peak with m/z = 14545 corresponding to the desired $[M+H^+]$ ions (Figure II-4.A and Figure S II-6 andFigure S II-8). Western-blot analysis using

anti-α-syn and anti-pY125 α-syn antibodies further confirmed the identity and purity of the semisynthetic WT α-syn and pY125 α-syn proteins (Figure II-4.B).

II.5.2. Semisynthetic pY125 α-syn adopts an α-helical structure upon binding to membrane

The secondary structure of the semisynthetic pY125 α-syn was investigated using circular dichroism in solution (50 mM Tris buffer and 150 mM NaCl, pH 7.5,) and in the presence of POPG vesicles (with a lipid:protein mass ratio of 20:1). As shown in Figure II-4.C, pY125 α-syn exhibits a virtually identical CD spectrum to that of the WT recombinant α-syn in solution (random coil) and adopts an α-helical structure upon binding to the lipid vesicles.

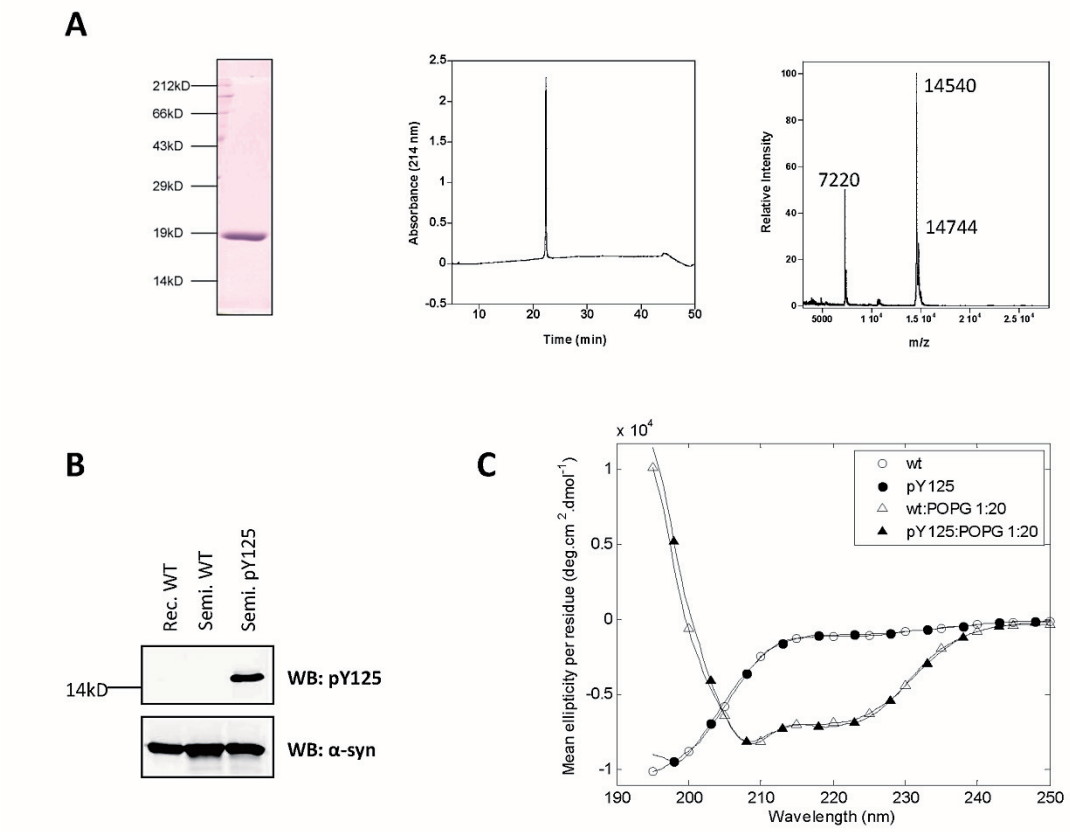

Figure II-4. Biochemical and biophysical characterization of semisynthetic WT and pY125 α-syn.
A: SDS-PAGE, RP-HPLC and MALDI-TOF analyses of the pure semisynthetic pY125 α-syn. MALDI analysis pY125 α-syn (expected mass: 14541 Da ([M+H]), a sinapinic matrix adduct is also observed as well as the double-charged peak. **B:** Western blot analyses of WT and pY125 α-syn confirmed that the phosphorylated semisynthetic protein could be detected by the anti-phosphorylated α-syn primary antibody, as well as by a non-phospho-specific antibody **C**: Circular dichroism spectra of pY125 α-syn in the absence (circles) and presence (triangles) of POPG vesicles. Open and closed symbols indicate WT recombinant α-syn and pY125 α-syn, respectively.

II.5.3. Phosphorylation at Y125 does not alter the structure of monomeric α-syn

To determine the effect of Y125 phosphorylation on the structural properties of monomeric α-syn, ^{15}N labeled A107C α-syn and A107C/pY125 α-syn were generated for solution NMR studies (Figure S II-12 and Figure S II-13). The NMR spectra of the free proteins were essentially identical and similar to the equivalent spectrum of the WT protein (not shown), indicating that both A107C and A107C/pY125 α-syn remain in the highly disordered ensemble characteristic of this protein (Eliezer et al., 2001).

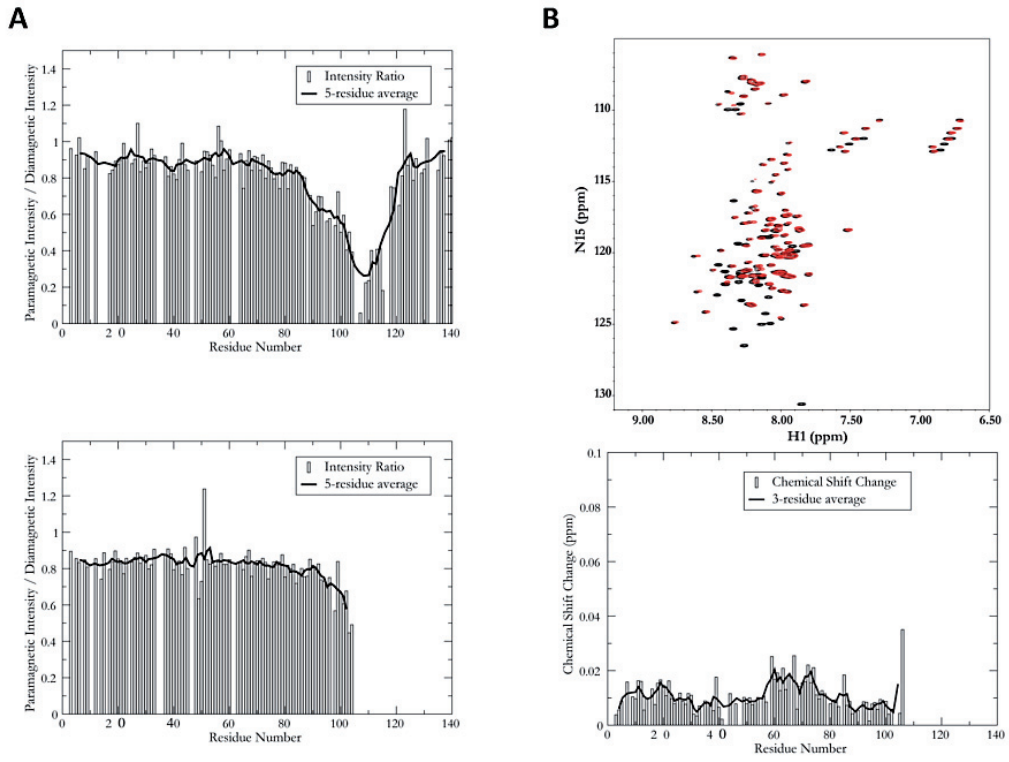

Figure II-5. NMR characterization of free and micelle-bound WT and pY125 α-syn.
A: Paramagnetic relaxation enhancement profiles for A107C (upper panel) and A107C/pY125 (lower panel) α-syn, derived from the intensity ratios of equivalent resonances between ^{1}H, ^{15}N HSQC spectra collected with and without an attached spin-label. A ratio of 1 indicates no PRE effect, while smaller ratios indicated a PRE effect, resulting from the proximity of the corresponding residue to the site of spin-label attachment. B: Overlaid ^{1}H, ^{15}N HSQC spectra of SDS micelle bound WT (black) and A107C/pY125 (red) α-syn (upper panel) and plots of the normalized chemical shift changes between the two spectra as a function of residue number (lower panel). The solid line in panel B is a three-residue average. Note that each red peak corresponds closely to a black peak, indicating that the structures of the two variants are highly similar in the micelle-bound state. Black peaks without corresponding red peaks arise from residues 108 and beyond, where the semisynthetic A107C/pY125 protein is not ^{15}N-labeled and therefore does not give rise to signals in this spectrum.

To assess the potential influence of phosphorylation on the transient long-range structure of the protein, we collected paramagnetic relaxation data for phosphorylated and unphosphorylated α-syn modified at Cys 107 by the spin label MTSL. The paramagnetic spin label increases the

relaxation rate and thus broadens the resonance of any residue within ~20 Å. Intramolecular contacts can thus be discerned by measuring the intensity ratio of resonances in a paramagnetically-labeled sample to the equivalent resonances in a diamagnetic control.

Plots of the intensity ratio as a function of residue number for A107C and A107C/pY125 α-syn, shown in Figure II-5.A reveal two interesting results. First, even in the unphosphorylated sample, there is little indication of the previously reported long-range contacts between the C- and N-terminal regions of the protein (Paleologou et al., 2008). This observation is surprising, but suggests that position 107 may not be highly sensitive to these contacts. However, there is a significant difference in the data between the phosphorylated and unphosphorylated proteins in the regions proximal to the labeling site. Residues close to the labeling site are broadened because they are covalently constrained to close proximity with the spin label, resulting in a well (or minimum) around position 107.

It has been argued that the breadth of this well reflects the local compactness of the protein. Despite the fact that only residues prior to the position 107 in A107C/pY125 α-syn are ^{15}N labeled give rise to data, it is evident that the well around position 107 is narrower for the phosphorylated protein. This result indicates that in the phosphorylated protein, the C-terminal region samples a more expanded ensemble of conformations.

Because the PRE data could not be used to monitor changes in long-range contacts due to phosphorylation at position 125, we also measured and compared the hydrodynamic radii of the phosphorylated and unphosphorylated proteins using pulsed field gradient NMR methods. The hydrodynamic radius of A107C/pY125 α-syn was found to be identical, within experimental error, to that of both A107C and WT α-syn, indicating that no significant overall compaction or expansion of the monomer occurs upon Y125 phosphorylation. These observations contrast with observations made in the case of S129 phosphorylation, where a significant degree of expansion occurs (Paleologou et al., 2008).

We also examined the potential effects of phosphorylation at Y125 on the micelle-bound form of the protein; this form is thought to closely resemble one of several membrane-bound forms of the protein. We observed a high degree of overlap between the $^{1}H,^{15}N$-HSQC spectra of WT A107C/pY125 α-syn in the presence of SDS micelles (Figure II-5.B), indicating that no gross structural changes in the protein occur upon phosphorylation at Y125. To more closely assess the effects of Y125 phosphorylation, we plotted the chemical shift changes between the two spectra as a function of residue number (Figure II-5.B). The results show that while the changes are generally small (on the order of 0.01 ppm), they are larger than those we observed for pS129 (Paleologou et al., 2008). Furthermore, these changes appear to be slightly greater in regions

corresponding to the center of each of the two previously characterized helices of the micelle-bound form of the protein (Bisaglia et al., 2005).

II.5.4. Phosphorylation at Y125 does not affect the fibrillization of α-syn

Previously, work from our group and others (Paleologou et al., 2008, Levitan et al., 2011) has shown that phosphorylation at S129 inhibits the fibrillization of α-syn *in vitro*. To determine whether phosphorylation at Y125 influences the aggregation properties of α-syn, we optimized conditions for α-syn fibril formation at low protein concentrations (15 µM) and compared the extent and kinetics of fibrillization of semisynthetic pY125 α-syn to those of the semisynthetic WT α-syn and recombinant WT proteins. Each protein was incubated with agitation at 37°C and the extent of fibrillization was assessed by ThT fluorescence and TEM after 72 h of agitation. As evidenced by the increase in ThT signal shown in Figure II-6, WT and pY125 α-syn forms mature fibrils after 24 h of incubation. Next, we assessed the amount of monomeric protein using a filtration assay and used TEM to characterize the structural properties of the aggregates formed by each protein. On the structural level, both WT and pY125 α-syn formed mature fibrillar structures (Figure II-6.C and Figure S II-14).

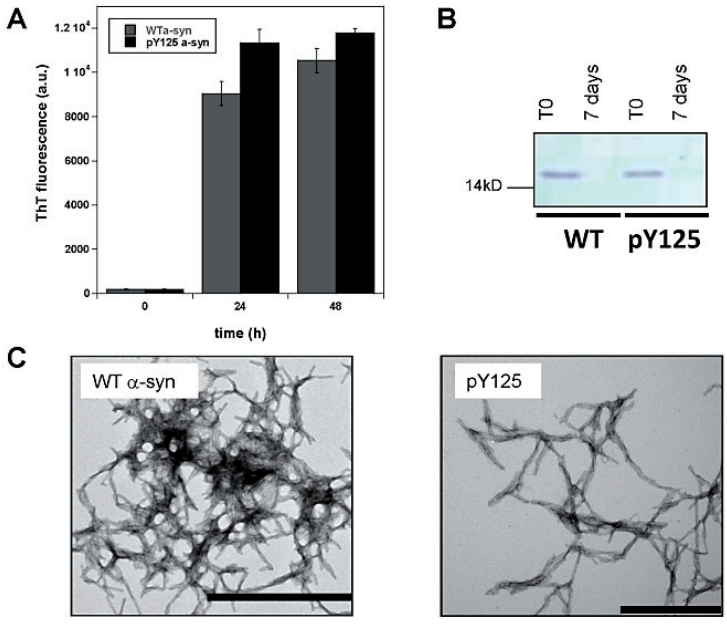

Figure II-6. Fibrillization of pY125 α-syn.
A: ThT fluorescence of recombinant WT and pY125 α-syn monitored over 48 h. B: SDS-PAGE analysis of the remaining WT and pY125 α-syn monomers prior to and after aggregation for 72 h and filtration through a 100 kD membrane. C: TEM images of WT and pY125 α-syn after 7 days of aggregation. The scale bar represents 500 nm. The images are representative of 5 independent experiments. The scale bar represents 500 nm.

After filtration of the samples through a 100 kDa membrane filter, the amount of monomeric α-syn was assessed by SDS-PAGE analysis. No significant amounts of monomers were detected for WT and pY125 α-syn after 7 days (Figure II-6.B). Together, these data suggest that phosphorylation at Y125 does not alter the aggregation of α-syn.

II.5.5. pY125 α-syn is dephosphorylated within minutes *in vitro* and *in vivo*

To investigate the subcellular localization *in vivo*, pY125 α-syn was microinjected into hippocampal primary neurons at a concentration of 1 µM. Because microinjected pY125 α-syn might be rapidly dephosphorylated, we fixed the neurons either immediately or 30 min after microinjection and probed for pY125 α-syn and total α-syn using the appropriate antibodies. Interestingly, in the injected cells, the signal for pY125 α-syn dramatically decreased within 30 min compared to the signal for WT α-syn (data not shown).

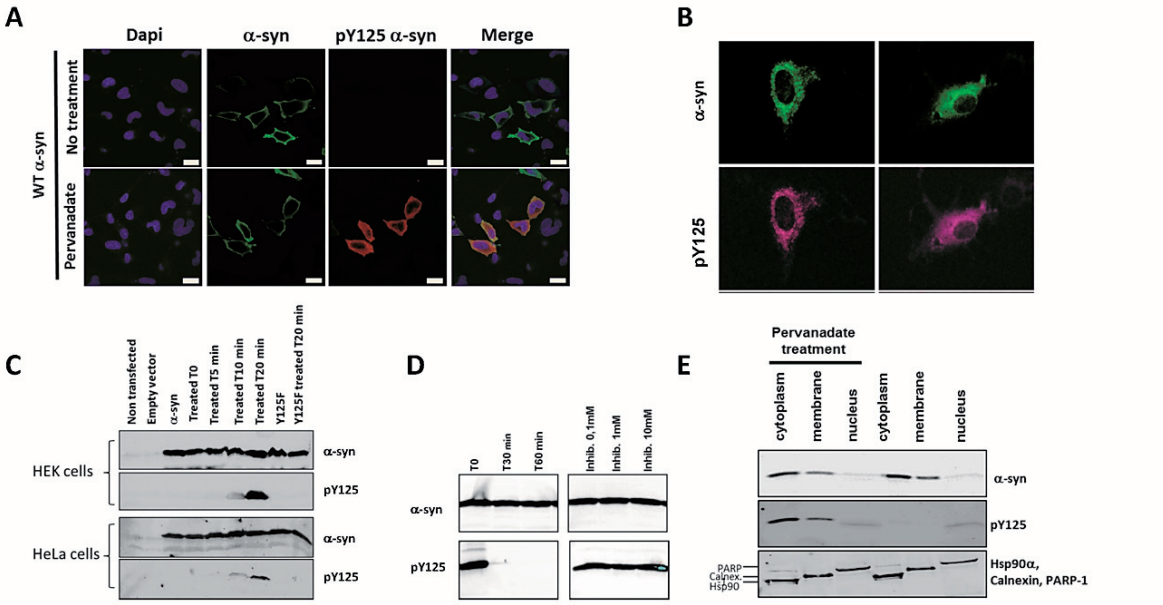

Figure II-7. Dephosphorylation and detection of pY125 α-syn in cells
A: Confocal images of HeLa cells transfected with WT α-syn plasmid and treated or not with pervanadate for 30min. The scale bar represents 20 µm. Subsequently, the cells were fixed and stained with the indicated antibodies (respectively rabbit anti-α-syn from Abcam ab51252 and mouse anti-pY125 α-syn from BD Pharmingen). B: Detection of pY125 α-syn in primary neurons. Confocal images of rat hippocampal neurons microinjected with 1 µM pY125 α-syn (25 fl/injection) together with the visible marker dye Faster Green (100 µg/ml) to identify the injected cells, the cells were treated with medium containing pervanadate for 30 min. Subsequently, the cells were fixed and stained with the indicated antibodies (respectively rabbit anti-α-syn from Abcam ab51252 and mouse anti-pY125 α-syn from BD Pharmingen). C: Immunoblots of HEK and HeLa cell lysates untreated or treated with pervanadate for 0, 5, 10 and 20min. The membranes were probed with total α-syn and pY125-specific antibodies (respectively rabbit anti-α-syn ab51252 from Abcam and mouse anti-pY125 α-syn from BD Pharmingen) D: Immunoblots of pY125 α-syn dephosphorylated from HeLa cell lysate. Membranes were probed with total α-syn and pY125-specific antibodies. The dephosphorylation was inhibited using different concentrations of sodium orthovanadate. E: Immuboblots of the subcellular fractionation of HEK cells over-expressing WT α-syn and treated with pervanadate for 20 min. Markers for cytoplasmic, membrane and nuclear fraction were used to assess for the purity of each fractions (Hsp90α, calnexin and PARP-1).

To confirm these observations and to test for rapid dephosphorylation *in vitro*, a HeLa cell lysate was spiked with pY125 α-syn and incubated for 0, 30 and 60 min. A loss of signal from the phosphorylated protein but not from the total α-syn protein was observed, indicating that pY125 α-syn is completely dephosphorylated by endogenous phosphatases within 30 min of incubation (Figure II-7.D). These results indicate tight regulation of the phosphorylation state of α-syn at Y125 by endogenous tyrosine phosphatases. In these experiments, dephosphorylation was effectively inhibited using sodium orthovanadate, a general tyrosine-phosphatase inhibitor.

II.5.6. pY125 α-syn is localized into the cytoplasm in HeLa cells and primary neurons

Previous attempts to characterize the possible effect of Y125 phosphorylation on the subcellular localization of α-syn were unsuccessful, in part due to lack of pY125-specific antibodies that are suitable for immunocytochemistry (Chen et al., 2009). In an attempt to overcome this limitation, we generated an antibody specific for pY125 α-syn and used it to immunolocalize of pY125 α-syn in HeLa cells and rat hippocampal neurons. The pattern of pY125 α-syn immunoreactivity produced by this antibody was compared with the pattern obtained using a commercially available antibody from BD Pharmingen.

For these experiments, we first identified conditions that prevent rapid dephosphorylation of pY125 α-syn. Previous reports indicated that pY125 α-syn is detectable in HEK293 cells that overexpress WT α-syn upon treatment with pervanadate (Ellis et al., 2001, Ahn et al., 2002). We validated these findings and found by Western-blot analysis of lysates from HEK293 and HeLa cells overexpressing human WT α-syn, that treatment with pervanadate results in the upregulation of pY125 α-syn in these cells (Figure II-7.C).

In cells over-expressing WT α-syn, but not the mutant Y125F α-syn, we observed a significant increase in the level of pY125 α-syn when the duration of treatment with pervanadate was increased. Non-specific bands of higher molecular weight were also detected with both the commercial (BD Pharmingen) and homemade anti pY125 α-syn antibodies after pervanadate treatment (Figure S II-15). This precludes the use of these antibodies to accurately assess in more details the subcellular localization of pY125 α-syn in specific cellular organelles. We were able to partially circumvent this problem by studying the subcellular localization of pY125 α-syn in HeLa cells overexpressing α-syn (Figure II-7.A). Quantification of the average fluorescence intensity of 65 cells (data not shown) treated with pervanadate showed that the increasing levels of cytosolic pY125 α-syn correlated well with increasing α-syn levels, and indicated a predominantly cytosolic localization of pY125 α-syn. We did not observe pY125 α-syn immunoreactivity in the nucleus nor was any cytosolic increase in pY125 α-syn

immunoreactivity observed in HeLa cells over-expressing the mutant Y125F α-syn (Figure S II-16). When semisynthetic pY125 α-syn was microinjected into rat hippocampal neurons at a concentration of 1 μM, it showed a cytoplasmic distribution similar to the distribution of WT α-syn immunoreactivity in HeLa cells (Figure II-7.B). To more accurately assess the subcellular localization of pY125, we preformed subcellular fractionation studies on HEK 293T cells overexpressing α-synuclein in the presence and absence of pervanadate. Using this approach, we can demonstrate conclusively that pY125 localizes mainly in the cytosolic fraction and to a small extent in the membrane fraction, consistent with the results from immunocytochemistry (Figure II-7.E).

II.5.7. Phosphorylation at Y125 does not block the phosphorylation at S129 and S87 *in vitro* and vice-versa

The proximity of the three C-terminal tyrosine residues in α-syn suggests the possibility that the phosphorylation of one of these residues may trigger or modulate the extent of phosphorylation at the other residues. In a recent study, Y125 phosphorylation was suggested to protect against α-syn toxicity (Chen et al., 2009). Using a transgenic *Drosophila* model overexpressing the S129D phosphomimetic mutant of human α-syn in retinal and dopaminergic neurons, Chen *et al.* demonstrated that co-expression of Shark, a *Drosophila* homolog of the human tyrosine kinase Syk, which was previously reported to efficiently (although non-specifically) phosphorylate α-syn at Y125 (Negro et al., 2002), significantly alleviates neurodegeneration due to S129D α-syn expression. To investigate potential cross-talk between Y125 and S129 phosphorylation sites, pY125 α-syn was incubated with PLK3, CK1 kinases, which are known to phosphorylate α-syn specifically and efficiently at S129 (PLK3) or at S87 and S129 (CK1). Phosphorylation at Y125, S129 and S87 was detected using the appropriate phosphospecific antibodies and confirmed by MALDI-TOF MS (Figure II-8.A-B).

These results indicated that phosphorylation of α-syn at Y125 does not prevent recognition of the protein by PLK3 or CK1, affect the subsequent phosphorylation of pY125 α-syn at S129 and S87, or prevent recognition of the double-phosphorylated species (pY125/pS87 and pY125/pS129) by the appropriate antibodies. To determine whether phosphorylation at Y125 influences S129 phosphorylation by endogenous kinases, we assessed the levels of pS129 in HeLa and HEK293 cells before and after treatment with pervanadate. Despite the significant increase in pY125 α-syn levels observed upon treatment with pervanadate, we did not observe any significant changes in pS129 levels (Figure II-9).

A recent study by Chen and colleagues suggested that phosphorylation at S129 does not affect the tyrosine phosphorylation of α-syn (Chen et al., 2009). However, these results were based on expression of a phosphomimetic mutant (S129D) that does not reproduce all aspects of phosphorylation at this site (Paleologou et al., 2008). Therefore, we sought to determine whether prior phosphorylation at S129 effects further phosphorylation at Y125 by the non-receptor tyrosine kinases Syk and members of the Src family (Lyn, Fyn, c-Fgr and c-Src), which have been previously shown to phosphorylate tyrosine residues in the C-terminus of α-syn.

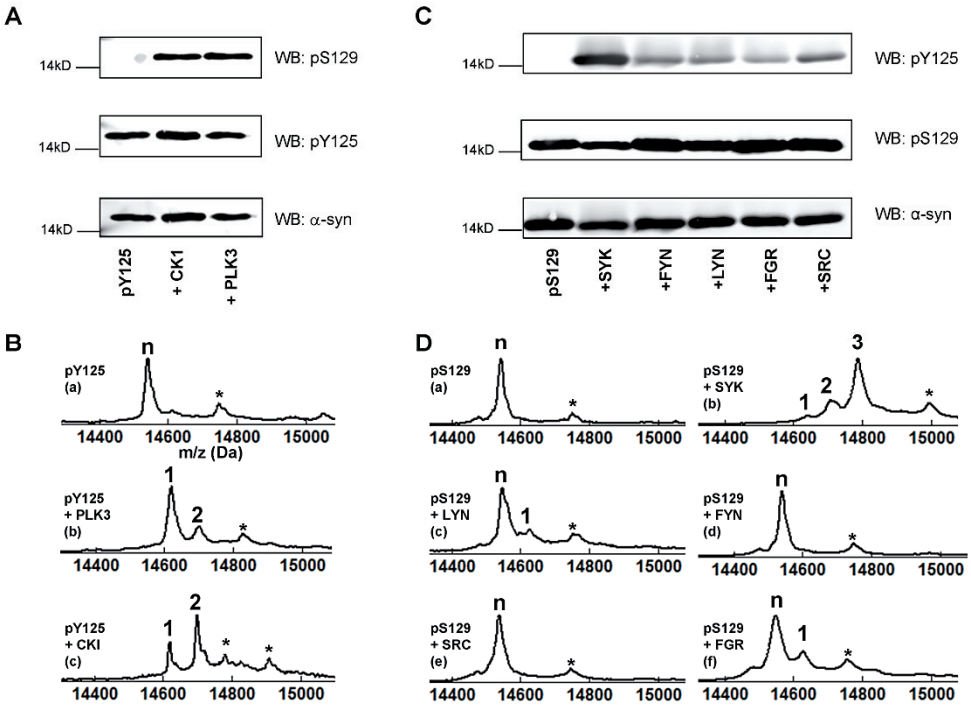

Figure II-8. Phosphorylation of pY125 and pS129 α-syn.
A: Immunoblots of pY125 α-syn phosphorylated by CK1 and PLK3. Membranes were probed using pS129, pY125, and α-syn antibodies. B: MALDI-TOF-MS analysis of the phosphorylation reaction after 12h. C: Immunoblots of pS129 α-syn phosphorylated by Syk, Lyn, Fyn, c-Src, and c-Fgr. Membranes were probed for pY125, pS129, and α-syn immunoreactivity. D: MALDI-TOF analysis of pS129 α-syn phosphorylated by (a) Syk, (b) Lyn, (c) Fyn, (d) c-Src, (e) and c-Fgr (f) after 14h of reaction. In all MALDI-TOF-MS spectra, the symbol 'n' indicates the peak corresponding to the starting material, the numbers above the other peaks correspond to the number of phosphorylation events, which were each detected by a +80 Da mass shift. The symbol '*' indicates a sinapinic acid matrix adduct.

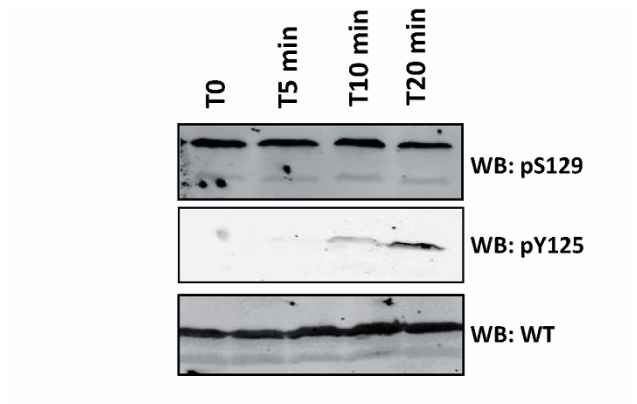

Figure II-9. Phosphorylation of pY125 α-syn and pS129 α-syn.
Immunoblots of HeLa cells over-expressing WT α-syn and treated with pervanadate for 0, 5, 10 and 20min. Membranes were probed with pS129 and pY125 α-syn and WT α-syn antibodies.

The protein was first quantitatively phosphorylated using PLK3, purified by reversed-phase HPLC and incubated at 3 µM for 14 h at 30°C in the presence of 100 nM of each individual tyrosine kinase. Western blot and MALDI-TOF-MS analyses (Figure II-8.C-D), showed the same efficiency and site-specificity of phosphorylation by these kinases as reported in previous *in vitro* studies. Of these kinases, Syk is by far the most efficient at phosphorylating α-syn with a reported K_{cat}/K_m of around 30, it also phosphorylated multiple tyrosines within the C-terminal region of α-syn (Ellis et al., 2001, Negro et al., 2002). We consistently observed phosphorylation at Y125 and at two other sites by MALDI-TOF MS (Figure II-8.D (b)). The other kinases were more specific but lacked efficiency. For example, the K_{cat}/K_m for Lyn is around 0.5. Most importantly, we observed that S129 phosphorylation did not block the activity of any of the tyrosine kinases. As a control, WT α-syn was incubated under the same conditions, a similar phosphorylation pattern was obtained (data not shown). Western-blot-based detection of Y125 phosphorylation appeared to be more sensitive than MALDI-TOF-MS because pY125 α-syn could be detected in all of the enzymatic reactions (Figure II-8.C). Together, these data suggest that S129 phosphorylation of α-syn does not interfere with further modifications of the protein by tyrosine kinases. Moreover, because the consensus recognition sequences of tyrosine kinases such as Syk includes acidic residues (Brunati et al., 1995), pS129 might act to stabilize the electrostatic interactions between the kinases and α-syn.

We performed a similar experiment aimed at determining whether phosphorylation at S87, another disease-related phosphorylation site within α-syn that was recently identified by our group (Paleologou et al., 2010), influence the recognition and processing of α-syn by the soluble tyrosine kinases mentioned above. We observed that phosphorylation of α-syn at S87 does not

significantly influence the protein's activity as a substrate for the non-receptor tyrosine kinases Syk, Fyn, Ly, c-Fgr, and c-Src or the site-specificity of its phosphorylation by these enzymes (Figure S II-17).

II.5.8. Phosphorylation at Y125 disrupts the binding of nanobody Nb87 to α-syn C-terminus

Phosphorylation of α-syn at Y125 has been previously shown to affect α-syn protein-protein interactions as described in reports using C-terminal fragments of α-syn (McFarland et al., 2008). We sought to probe the effect of phosphorylation at S129 and Y125 on the interaction of full-length α-syn with proteins or peptides that are known to bind tightly to α-syn via specific interactions involving the C-terminal region of the protein. After careful review of the literature, we decided to focus on Nanobodies, which are single antibody variable domains obtained by immunization of camels (Dumoulin et al., 2003, Vincke et al., 2009, De Genst et al., 2010) . More specifically, we took advantage of the use of Nanobody Nb87 that was previously identified by the Dobson group using phage-display and was shown to bind with high affinity (Kd in the range of nM) to α-syn (De Genst et al., 2010)(Guilliams *et al*, 2012). NMR studies have shown that Nb87 binds sites comprising α-syn C-terminal residues 118-132 (Guilliams *et al*, 2012). To assess the influence of α-syn phosphorylation at S129 and Y125, we generated the corresponding forms of the protein either via *in vitro* phosphorylation of WT α-syn with PLK3 (pS129) or by semisynthesis (pY125) and evaluated their binding to Nb87 using Isothermal Titration Calorimetry technique (in collaboration with the Dobson group). Given the common use of phosphomimics, we also sought to compare the binding of pS129 and S129E to Nb87. S129A and Y125F were also included as controls.

Phosphorylation of α-syn at Y125 and the mutation of Tyr125 to Phe resulted in 638-fold and 23-fold lower affinity to Nb87 respectively compared to the WT protein (Figure II-10.C and Figure II-11.A). These results suggest that the epitope of α-syn recognized by Nb87 is most likely situated around residue 125 and that phosphorylation of α-syn at Y125 strongly impaired α-syn-Nb87 interactions. Aside from the disruption of the binding to Nb87, phosphorylation at Y125 also induced higher enthalpy of binding (5-fold higher), compared to WT and Y125F α-syn (Figure II-11.A), which suggests a binding event that has an entropic contribution.

We were also able to assess for the first time, the effect of site-specific phosphorylation of full-length α-syn on its interactions with proteins. Phosphorylation at S129 did not affect significantly the binding to Nb87 and even showed a slight increase of binding affinity in respect to WT or S129A α-syn (Kd = 10nM) (Figure II-10. D,E,F and Figure II-11. B).

However, some differences emerged in the case of the phosphomimic: the binding of S129E to Nb87 was 4 fold weaker than pS129 (Figure II-10. D,E,F and Figure II-11. B), consistent with previous results from our lab highlighting major differences between phosphomimics S129E and S129D and phosphorylated α-syn pS129 on the structure and fibrillization rate of α-syn (Paleologou et al., 2008). The binding enthalpies were similar though for all α-syn constructs (Figure II-11.B). These results demonstrate that the phosphomimics are not suitable for investigating α-syn phosphorylation-dependent protein-protein interactions.

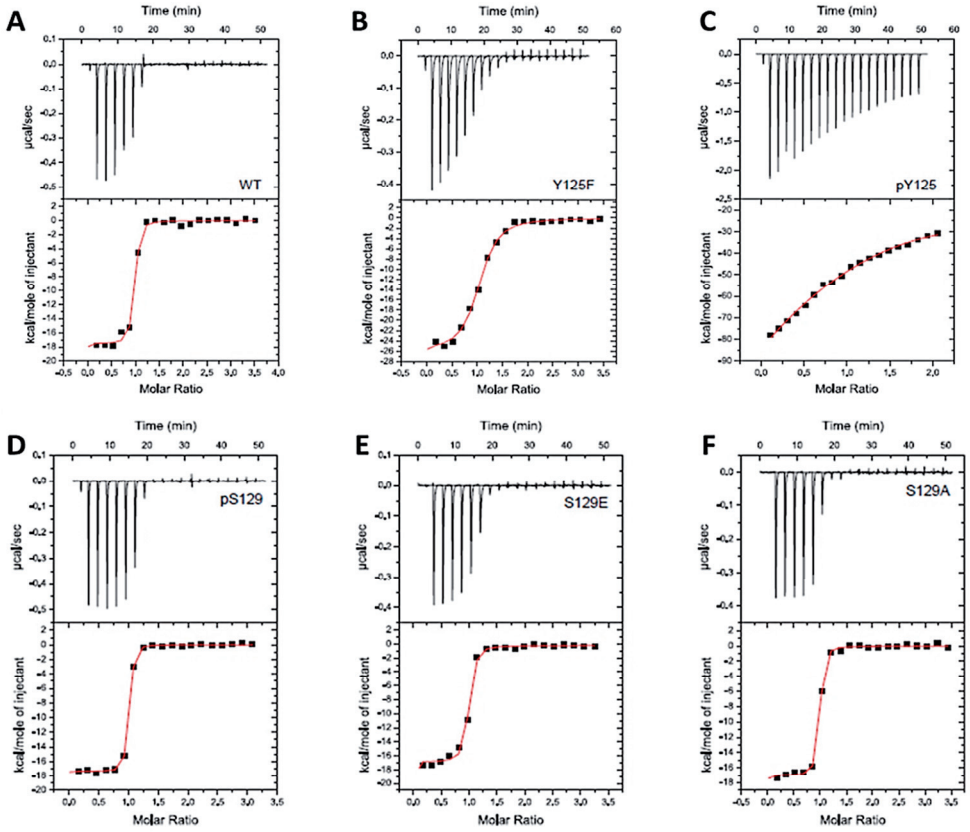

Figure II-10. Raw ITC thermograms of Nb87 binding to α-syn variants
Raw ITC thermograms of Nb87 binding to WT (A), Y125F (B), pY125 (C), pS129 (D), S129E (E), S129A (F) α-syn. Data were recorded in PBS at 25°C.

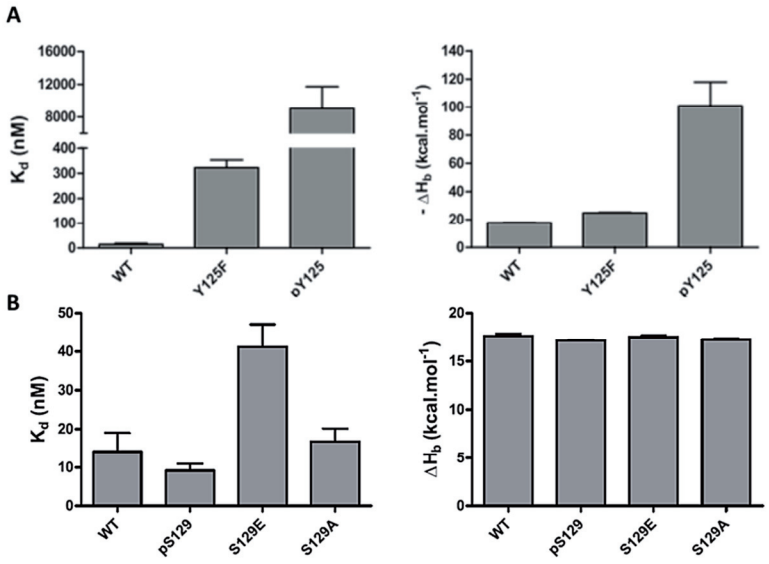

Figure II-11. Dissociation constants and binding enthalpies of Nb87 binding to α-syn variants
Dissociation constants and binding enthalpies of α-syn-Nb87 binding were calculated for WT, Y125F and pY125 α-syn (A) and WT, pS129, S129E and S129A α-syn (B)

II.6. Discussion

Recent studies indicate that α-syn phosphorylation at serine residues S87 and S129 can dramatically influence α-syn structure (Paleologou et al., 2008, Paleologou et al., 2010), turn-over (Chau et al., 2009), membrane binding (Paleologou et al., 2008, Paleologou et al., 2010), aggregation (Paleologou et al., 2008, Paleologou et al., 2010) and protein-protein interactions (McFarland et al., 2008). Notably, phosphorylations at S87 and S129 have emerged as a defining hallmark of PD and other synucleinopathies (Fujiwara et al., 2002, Anderson et al., 2006). The use of phosphomimetic mutations (S→E/D) combined with the identification of kinases that specifically phosphorylate α-syn *in vitro* and *in vivo* has facilitated detailed studies that aimed at investigating the structural and functional consequences of phosphorylation of the protein at these residues *in vitro* and *in vivo* (Okochi et al., 2000, Pronin et al., 2000, Chen and Feany, 2005, Gorbatyuk et al., 2008, Azeredo da Silveira et al., 2009, Chen et al., 2009, Inglis et al., 2009, Mbefo et al., 2010). In addition to serine residues, α-syn has also been shown to undergo phosphorylation at its C-terminal tyrosine residues Y125, Y133, Y136 *in vitro,* with a preference for Y125 as the major phosphorylation site (Ellis et al., 2001, Nakamura et al., 2001, Ahn et al., 2002, Negro et al., 2002). However, only phosphorylation at Y125 has been detected *in vivo* (Chen et al., 2009).

Several kinases have been reported to phosphorylate tyrosine residues in the C-terminus of α-syn. However, all known kinases are either inefficient or lack specificity to phosphorylate α-syn at specific tyrosine residues in its C-terminus. The tyrosine kinase Syk phosphorylates α-syn at multiple tyrosine residues (Y125, Y133 and Y136) (Negro et al., 2002). In contrast, Fyn, Lyn, c-Fgr and c-Src phosphorylate α-syn specifically at Y125, but these kinases are less efficient (Negro et al., 2002). The lack of specificity and efficiency of tyrosine phosphorylation of α-syn has hampered efforts to obtain homogenous preparations of phosphorylated α-syn and to elucidate the structural and functional consequences of phosphorylation at Y125. Unlike phospho-serine and phospho-threonine, which in some cases can be mimicked by serine → glutamate or aspartate substitutions, there are no natural amino acids that can mimic phospho-tyrosine. Inspired by the pioneering work of Muir and Cole on the use of expressed protein ligation to study the effect of site-specific phosphorylation and other post-translational modifications (Muir et al., 1998), we developed, for the first time, an efficient semisynthetic approach based on a one-pot native chemical ligation and desulfurization followed by a single chromatographic purification step for the production of pY125 α-syn. This strategy enabled us to obtain preparations of α-syn protein site-specifically modified at single or multiple residues within the C-terminal region comprising residues 107-140, the region that contains the majority of the reported PTM of the protein. Using this approach, we were able to obtain milligram quantities of semisynthetic WT and pY125 α-syn and to carry out detailed studies aimed at elucidating the effect of this modification on the structure, membrane binding, aggregation and subcellular localization of α-syn.

The results presented here demonstrate that phosphorylation at Y125 does not significantly alter the structure of monomeric α-syn in solution (Figure II-4.B and Figure II-5). Although the hydrodynamic radius of pY125 α-syn is virtually identical to that of the WT protein, the paramagnetic relaxation data presented here indicate that the C-terminal region of pY125 α-syn samples a more expanded ensemble of conformations than in the WT protein. Interestingly, these observations contrast with the case of S129 phosphorylation, where a significant degree of expansion occurs (Paleologou et al., 2008). Phosphorylation at Y125 did not interfere with the ability of α-syn to form an α-helical structure upon binding to synthetic phosphatidylglycerol (POPG) or SDS micelles as assessed by CD spectroscopy. These results are consistent with previous observations regarding pS129 and with the fact that both S129 and Y125 lie outside the region involved in α-syn binding to membranes. However, the $^{1}H,^{15}N$-HSQC spectra of the protein reveal small structural changes that are larger than those observed for the case of pS129

(Paleologou et al., 2008). Furthermore, the structural changes in the protein that result from Y125 phosphorylation appear to be slightly greater in regions corresponding to the centers of each of the two previously characterized helices of the micelle-bound form of the protein (Bussell and Eliezer, 2003). Thus, while phosphorylation at Y125 does not result in large-scale structural changes in the micelle-bound form of the protein, it may cause slight rearrangements of the two micelle-bound helices. Such small changes may be consistent with results that indicate that modifications occuring in the C-terminal tail of the protein can influence the N-terminal lipid-binding domain (Sevcsik et al., 2011).

Previous studies by Negro *et al.* showed that co-incubation of α-syn with Syk kinase inhibits α-syn aggregation *in vitro* (Negro et al., 2002). Co-expression of Syk and α-syn was also reported to inhibit α-syn oligomerization and inclusion formation in cell culture (Negro et al., 2002) and in *Drosophila* expressing Shark, the fly homolog of Syk (Chen et al., 2009). However, it is difficult to discern the relative contribution of phosphorylation at Y125 in these studies, because the Syk kinase phosphorylates all three C-terminal tyrosine residues (Y125, Y133 and Y136) to different extents. In subsequent studies by Negro and colleagues, it was reported that the addition of other tyrosine kinases (Lyn and c-Fgr) did not influence the aggregation of α-syn *in vitro* (Negro et al., 2002). However, the significantly reduced efficiency of these kinases for the phosphorylation of α-syn makes it difficult to draw any conclusions regarding the effect of phosphorylation at Y125 on α-syn aggregation and fibrillization. In this work, the availability of semisynthetic pY125 α-syn allowed us to address this question directly for the first time. WT α-syn and semisynthetic pY125 α-syn exhibited similar aggregation properties as discerned by TEM and ThT fluorescence, demonstrating that pY125 α-syn behaves similarly to the recombinant protein produced in *E. coli*, unlike phosphorylation at S129, which inhibits α-syn fibrillization. Interestingly, pY125 α-syn exhibited similar aggregation properties as WT α-syn, consistent with the NMR results demonstrating that phosphorylation at Y125 induces only subtle structural changes on monomeric α-syn.

The availability of semisynthetic pY125 α-syn provided unique opportunities to assess the subcellular localization of pY125 α-syn and to explore the potential cross-talk between phosphorylation at S129 and Y125. Recent studies by Chen *et al.* suggested that Y125 phosphorylation may protect against α-syn toxicity by decreasing α-syn phosphorylation at S129 (Chen et al., 2009). To investigate possible cross-talk between phosphorylation at S129 and Y125, we combined semisynthetic approaches and enzymatic phosphorylation assays. Employing optimized procedures for the *in vitro* phosphorylation of α-syn, we used

semisynthetic pY125 α-syn to examine the effect of phosphorylation at Y125 on S129 phosphorylation by subjecting pY125 α-syn to phosphorylation by kinases that phosphorylate α-syn efficiently at S129 (PLK3) and/or S87 and S129 (CK1) (Paleologou et al., 2008, Mbefo et al., 2010). To determine whether phosphorylation at S129 influence Y125 phosphorylation, we prepared pS129 α-syn by *in vitro* phosphorylation with PLK3 (Mbefo et al., 2010) and examined the extent of Y125 phosphorylation (pS129/pY125) by the tyrosine kinases Fyn, Lyn, Src, c-Fgr and Syk (Ellis et al., 2001, Nakamura et al., 2001, Ahn et al., 2002, Negro et al., 2002). Our results demonstrate that phosphorylation at Y125 does not attenuate α-syn phosphorylation at S129 or S87 *in vitro* and vice-versa. These findings are consistent with the results of studies from Feany and colleagues in *Drosophila* suggesting that S129 phosphorylation of α-syn does not influence Y125 phosphorylation. The levels of pY125 α-syn were similar in flies overexpressing WT, S129A or S129D, suggesting that S129 phosphorylation does not influence phosphorylation at Y125. This study confirms these findings in mammalian cells (HeLa and HEK 293T), where no changes in pS129 levels were observed after treatment with phosphatase inhibitors that significantly enhance pY125 α-syn levels.

The lack of significant interest in phosphorylation of α-syn at Y125, compared to phosphorylation at S87 and S129 can be attributed to difficulties in detecting significant levels of pY125 α-syn in human brain tissues and lack of evidence for a direct correlation between pY125 α-syn levels and disease progression. Proteomic and mass spectrometry analyses of α-syn from Lewy bodies failed to detect phosphorylation at Y125 (Anderson et al., 2006). The rapid dephosphorylation of α-syn in *post-mortem* tissues may explain the limited success of previous studies in detecting pY125 α-syn in human brains and by biochemical analysis of brain-derived materials using mass spectrometry. Due to the dynamic regulation of tyrosine phosphorylation *in vivo*, detection of α-syn phosphorylation at Y125 appears to be dependent on the use of tyrosine phosphatase inhibitors or on the time elapsed prior to *post-mortem* analyses. Recently, Y125 phosphorylation was shown to occur both in human and *Drosophila* brains and pY125 α-syn levels was reported to decrease with aging and in PD brains, suggesting a possible protective role for this modification (Chen et al., 2009). Interestingly, in the presence of phosphatase inhibitors, 30% of α-syn in *Drosophila* appears to be phosphorylated at Y125 supporting the role of Y125 phosphorylation in modulating α-syn properties. In this study, we showed that upon addition of pY125 α-syn to cell lysates or microinjection into primary neurons, pY125 α-syn is almost completely dephosphorylated within 5 min. This effect is blocked in the presence of pervanadate. These results are consistent with a very rapid

dephosphorylation of α-syn both *in vitro* and *in vivo*. Phosphorylation at Y125 appears to be tightly regulated such that the levels of pY125 α-syn are maintained at very low levels but increases significantly in cell culture, primary neurons, and brain tissues, upon treatment with the phosphatase inhibitor pervanadate (Ellis et al., 2001, Ahn et al., 2002, Chen et al., 2009). Together, these studies demonstrate that α-syn undergoes phosphorylation at Y125 *in vivo* and suggest that experiments aimed at evaluating and correlating specific PTM with disease progression must take into account the stability and dynamics of these modifications, and that the protocols used for the analysis and preparations of samples must be modified accordingly.

Nakamura *et al* reported that the levels of pY125 α-syn increases by approximately 15 fold within the first few minutes in response to hyperosmotic stress, but then declines rapidly after 10 min. These observations indicate the existence of efficient dephosphorylation mechanisms that regulate pY125 α-syn levels (Nakamura et al., 2002). However, the identities of the phosphatases responsible for regulating pY125 α-syn phosphorylation remain unknown. The availability of semisynthetic pY125 α-syn as a substrate should facilitate the development of assays to screen focused libraries of tyrosine phosphatase inhibitors and identify natural phosphatases that are involved in regulating Y125 phosphorylation *in vivo*. To prevent the rapid dephosphorylation of pY125 α-syn, we will replace phosphotyrosine by nonhydrolyzable phosphonate analogues (Berkowitz et al., 1996, Desmarais et al., 1999) that , once incorporated into α-syn, will render it resistant to phosphatase activity (Zheng et al., 2005a). Cole and colleagues have successfully introduced non-hydrolyzable tyrosine analogues into proteins using expressed protein ligation strategies and demonstrated their utility to investigate the role tyrosine phosphorylation in regulating the activity of different tyrosine phosphatases and their interaction with other proteins (Lu et al., 2003, Zheng et al., 2003, Shen et al., 2005, Zheng et al., 2005a, Schwarzer et al., 2006, Szewczuk et al., 2009).

Whether phosphorylation at Y125 plays an important role in regulating α-syn aggregation and toxicity *in vivo* remains unknown. This question can only be answered by experimental conditions that permit site-specific regulation of phosphorylation at Y125 *in vivo*. To determine the functional consequences of Y125 phosphorylation, Feany and colleagues overexpressed WT α-syn, and mutant (α-syn ^{YF}) in which all C-terminal tyrosines were mutated to phenylalanine (Y125F/Y133F/Y136F) and compared the effects of the mutations on α-syn aggregation, toxicity, and locomotor deficit in flies. The mutant (α-syn ^{YF}) exhibited enhanced toxicity and caused a significant reduction in climbing ability compared to flies overexpressing the WT

protein. Additionally, overexpression of the Shark kinase, the *Drosophila* homolog of Syk, increased the levels of pY125, decreased oligomer formation and rescued the neurotoxicity of WT α-syn and the more toxic S129 phosphomimetic S129D. It is important to note that in both approaches, the three tyrosines were either mutated or phosphorylated to different extent, making it difficult to decipher the effects of selective modification at Y125. Identification and expression of the kinases that allow selective modification of pY125 and/or coexpression of such kinases with Y133F/Y136F is crucial for gaining insight into the role of Y125 phosphorylation in regulating α-syn aggregation and toxicity *in vivo*. The availability of semisynthetic pY125 α-syn and our improved understanding of the dynamics of Y125 phosphorylation should aid in the development of assays to identify the natural kinases and phosphatases involved in the regulation of Y125 phosphorylation and will facilitate a quantitative evaluation of the extent of pY125 α-syn during disease progression.

The role of phosphorylation in regulating α-syn interactions with other proteins and ligands remains unexplored. Recently McFarland *et al*. attempted to address this knowledge gap using C-terminal-derived α-syn peptides (WT and phosphorylated at S129 and Y125) to immunoprecipitate interactants from mouse brain synaptosomes (McFarland et al., 2008). Phosphorylation at S129 and Y125 reduced the affinity of α-syn peptides to proteins of the mitochondrial electron transport complex and preferentially directed the interaction toward proteins involved in the cytoskeletal architecture and the endocytosis pathway. Kinases were also identified among the interactants of phosphorylated peptides (McFarland et al., 2008). Furthermore, phosphorylation of α-syn has major impacts on its binding to metal. The binding of α-syn to bivalent cations ($Cu^{2+,}$ Pb^{2+} and Fe^{2+}) was reported to be increased in presence of C-terminal peptides phosphorylated at S129 or Y125 (Lu et al., 2011). Phosphorylation also affected the repartition of the binding sites with a clear preference toward the C-terminal domain (Lu et al., 2011). Although these studies provided important insights into the potential role of phosphorylation in regulating α-syn's interactions with proteins and metal ions, they were based on C-terminal peptide fragments which are likely to eliminate potential candidates that would require the binding to N-terminal and NAC region of α-syn. Therefore, the extent to which these studies reflect the impact of phosphorylation on the interaction of the full-length protein remains unknown.

We probed here, for the first time, the effect of full-length pS129 and pY125 α-syn on its interactions with other proteins. When comparing the affinity of the different phosphorylation variants of α-syn on the binding to nanobody Nb87, it was revealed that pS129 had a slightly

stronger affinity to Nb87 while pY125 exhibited a 638-fold lower affinity. Phosphorylation of α-syn has been shown to play a major role in aggregation, with phosphorylation of S87 and/or S129 inhibiting its fibrillization (Paleologou et al., 2008) while phosphorylation of Y125 has no effect on α-syn aggregation. In addition, our binding studies confirm previous findings from our lab that suggested that phosphomimics do not reproduce all structural aspects of α-syn phosphorylation (Paleologou et al., 2008). We report here that the effect of mimicking phosphoserine by a glutamate also influences protein-protein interaction since the binding affinity of pS129 and S129E to Nb87 was not identical. We show for the first time that phosphorylation of a single residue of α-syn is able to disrupt strong interactions, with definitely strong implications for the complex of proteins interacting with α-syn *in vivo* and the cellular mechanism that are modulated as a direct consequence.

II.7. Conclusions

We recently reported a semisynthetic approach that enabled site-specific modification of the N-terminus of α-syn and used this approach to prepare and characterize an α-syn form that is mono-ubiquitinated at Lysine 6 (Hejjaoui et al., 2011). In this study, we describe a general and efficient semisynthetic strategy that enables the site-specific introduction of single or multiple PTM of α-syn and the preparation of homogeneously modified forms of α-syn at its C-terminus in milligram quantities. These two approaches make it possible to prepare all the known disease-associated PTM of α-syn, except pS87, and to study potential cross-talk between these modifications. These advances have allowed us to investigate for the first time the effect of selective phosphorylation at Y125 α-syn on the structure, aggregation, membrane binding, subcellular localization of the protein and protein-protein interaction. In addition, these advances provide unique opportunities to investigate PTM-dependent protein-protein interactions involving α-syn and to study the cross-talk between different PTM. Furthermore, the availability of these proteins is crucial for advancing current research programs with the following aims: 1) characterization of PTM in humans and in animal models of PD; 2) development of biomarkers and target identification and 3) development of sensitive assays for the detection and quantitative assessment of specific PTM *in vivo* and in biological fluids. Therefore, the development of these semisynthetic strategies represents an important advance toward unraveling the roles of PTM in determining α-syn structure, aggregation and functions in health

and disease. We hope that this work will encourage other research groups to pursue similar approaches to elucidate the role of PTM in regulating the function of other proteins, particularly proteins linked to neurodegenerative diseases.

II.8. Supporting information

II.8.1. Synthesis and characterization of α-syn C-terminal peptides

Peptide: α-Syn(A107C-140)
sequence: CPQEGILEDMPVDPDNEAYEMPSEEGYQDYEPEA
synthetic specifications: couplings used HBTU; double couplings performed at Cys107, Glu109, Met-116, Asp-119, and Met-127
yield: 25%
expected mass: 3886 Da

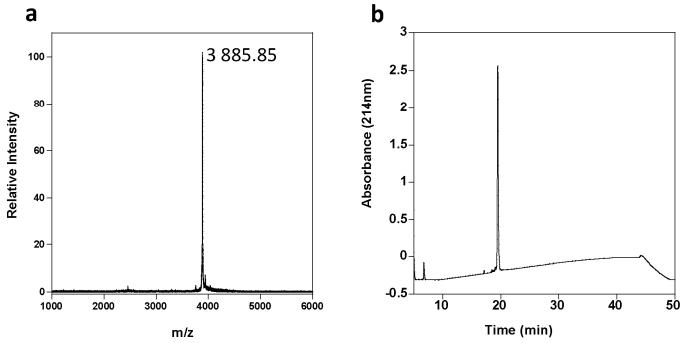

Figure S II-1. Analysis of peptide α-syn A107C-140
a) MALDI-TOF analysis (observed mass=3885.85Da) and b) RP-HPLC analysis of purified α-syn A107C-140 on a C_{18} analytical column (CosmoSil Protein-R, 4.6x250 mm, 5µm particle size) with a linear gradient of 0 to 80%B over 30min (A: water/0.1%TFA and B: acetonitrile/0.1% TFA).

Peptide: α-syn(A107C-140)_*p*Y125
sequence: CPQEGILEDMPVDPDNEA-*p*Y-EMPSEEGYQDYEPEA
synthetic specifications: see above
yield: 25%
expected mass: 3969 Da

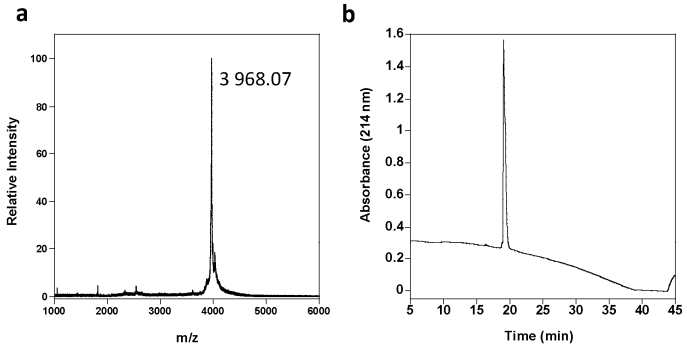

Figure S II-2. Analysis of peptide α-syn A107C-140_*p*Y125
a) MALDI-TOF analysis (observed mass= 3968 Da). b) RP-HPLC analysis of purified α-syn A107C-140_*p*Y125 on a C_{18} analytical column (CosmoSil Protein-R, 4.6x250 mm, 5µm particle size) with a linear gradient of 0 to 80%B over 30min (A: water/0.1%TFA and B: acetonitrile/0.1% TFA).

II.8.2. Generation of 15N-labeled α-syn(1-106)SR and non-labeled α-syn(1-106)SR

Non-labeled α-syn(1-106)SR

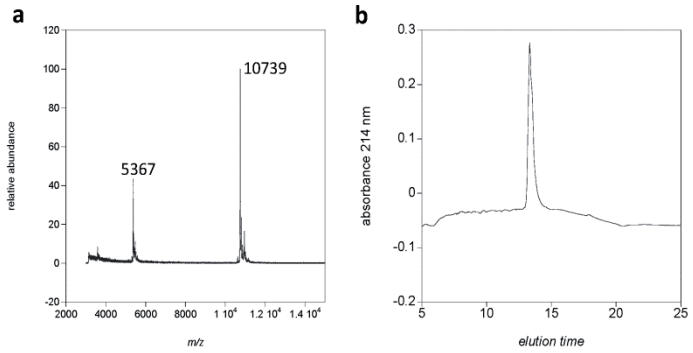

Figure S II-3. Analysis of α-syn(1-106)SR
a) MALDI-TOF analysis (observed mass= 10739). The mass 5367 corresponds to the double-charged. b) RP-HPLC analysis of purified α-syn(1-106)SR on a C_4 analytical column (CosmoSil $5C_4$) with a linear gradient of 0 to 80%B over 15min (A: water/0.1%TFA and B: acetonitrile/0.1% TFA).

^{15}N labeled α-syn (1-106)SR

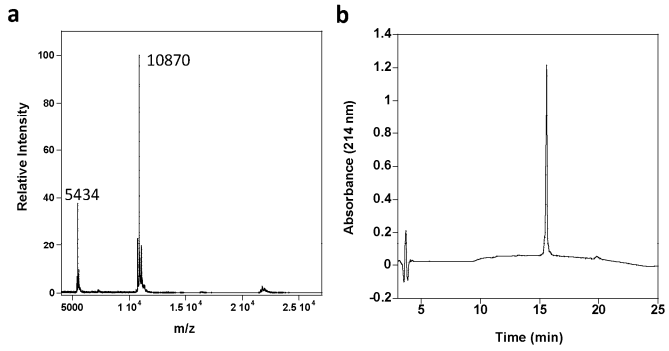

Figure S II-4. Analysis of ^{15}N α-syn(1-106)SR
a) MALDI-TOF analysis (observed mass= 10870). The mass 5434 corresponds to the double-charged. b) RP-HPLC analysis of purified α-syn(1-106)SR ^{15}N labeled on a C_4 analytical column with a linear gradient of 0 to 80%B over 30min (A: water/0.1%TFA and B: acetonitrile/0.1% TFA)

II.8.3. Semisynthesis and purification of WT α-syn and pY125 α-syn

α-SynA107C wild-type

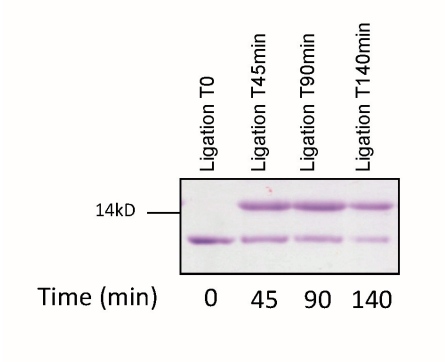

Figure S II-5. SDS-PAGE analysis of the ligation reaction
SDS-PAGE analysis of native chemical ligation reaction between α-syn(1-106)SR and α-syn(A107C-140).

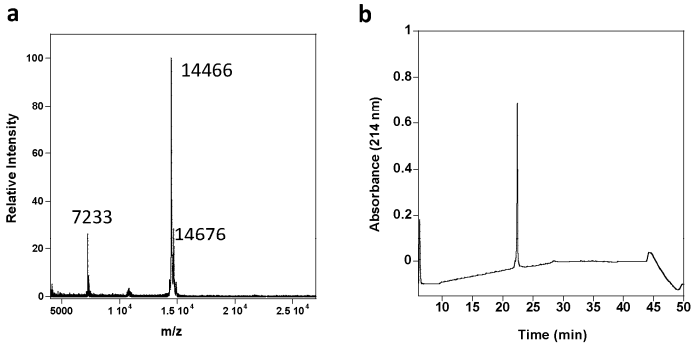

Figure S II-6. Analysis of semi-synthetic WT α-syn
a) MALDI-TOF analysis of the desulfurized product (expected mass: 14461). The mass of 14676 corresponds to a sinapinic matrix adduct and the mass of 7233 to the double-charged. b) RP-HPLC of semi-synthetic WT α-syn on an analytical C18 with a linear gradient of 0 to 80%B over 30min (A: water/0.1%TFA and B: acetonitrile/0.1% TFA).

α-synA107C_*p*Y125

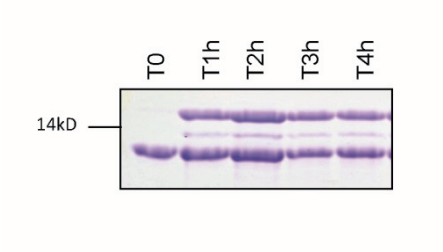

Figure S II-7. SDS-PAGE reaction of the ligation reaction
SDS-PAGE analysis of native chemical ligation reaction between α-syn(1-106)SR and α-syn(A107C-140_pY125). Note: the ligation corresponding to the gel was done with two molar excess of α-syn(1-106)SR.

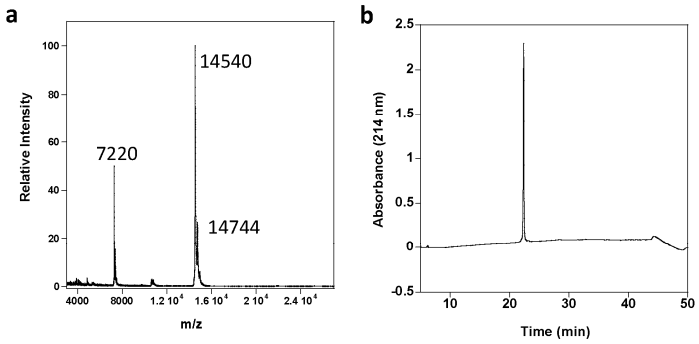

Figure S II-8. Analysis of of semi-synthetic pY125 α-syn
a) MALDI-TOF analysis of the desulfurized product (expected mass: 14541). The mass of 14744 corresponds to a sinapinic matrix adduct and the mass of 7220 to the double-charged. b) RP-HPLC of semi-synthetic pY125 α-syn on an analytical C18 with a linear gradient of 0 to 80%B over 30min (A: water/0.1%TFA and B: acetonitrile/0.1% TFA).

General scheme of ligation and purification

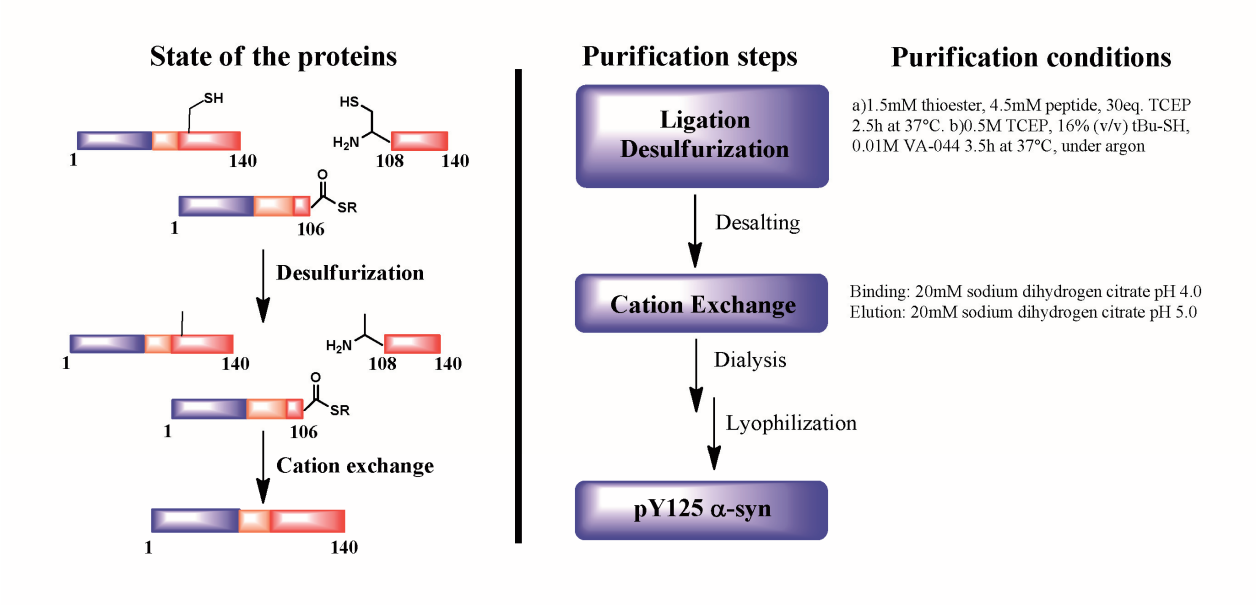

Figure S II-9. Scheme of the main step for the generation of pY125 α-syn
Scheme of the main steps for the generation of pY125 α-syn: ligation, desulfurization and purification by chromatographic methods.

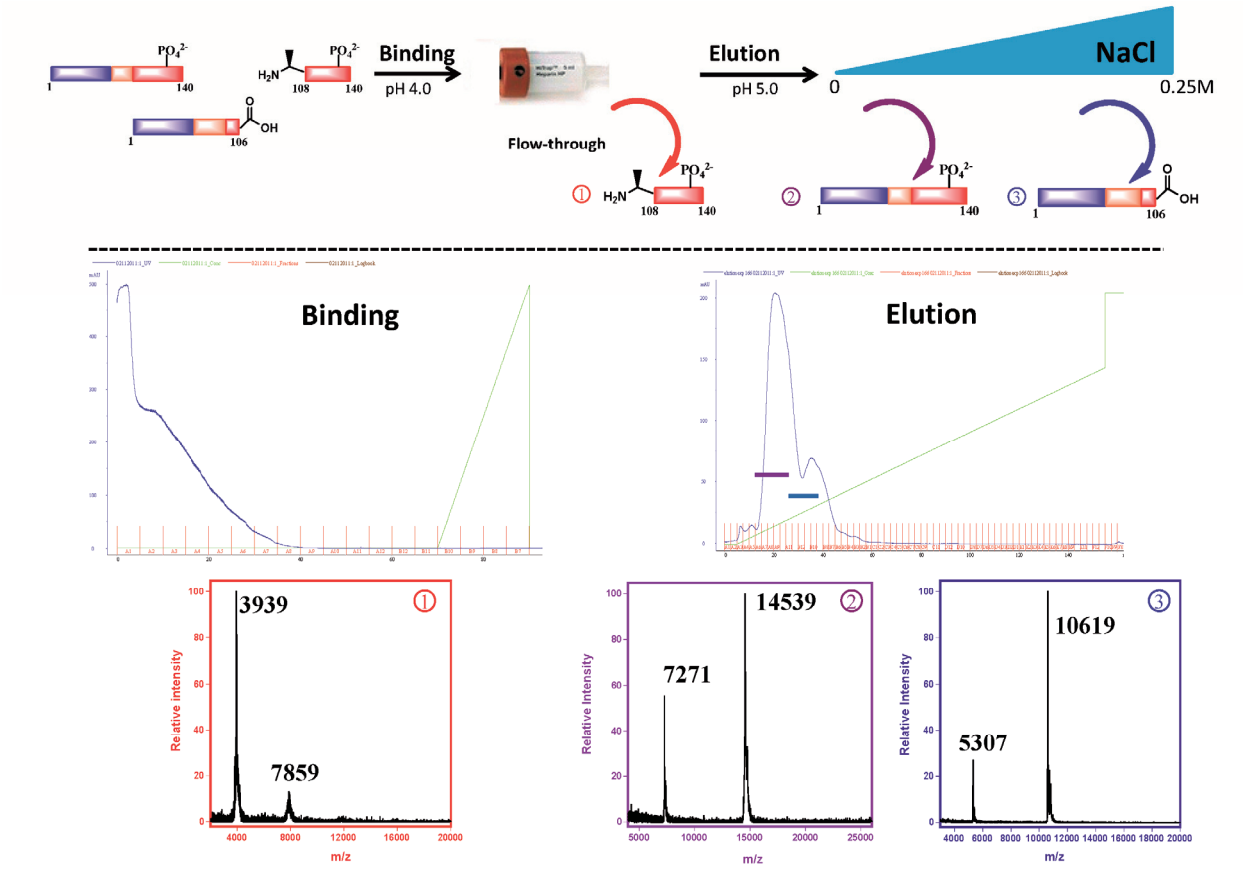

Figure S II-10. Scheme of the cation-exchange chromatography purification of pY125 α-syn.

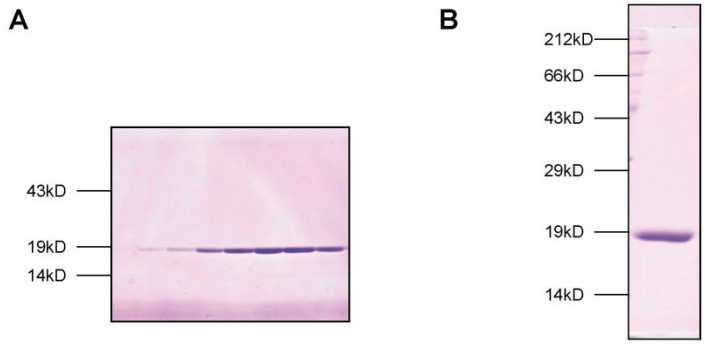

Figure S II-11. SDS-PAGE of the fractions of the cation exchange column
A: SDS-PAGE analysis of the fractions containing pY125 α-syn (eluting from the cation exchange column at pH 5). B: SDS-PAGE analysis of the pure lyophilized pY125 α-syn.

II.8.4. Generation of α-syn A107C N15 labeled and Semisynthesis of α-syn A107C_pY125 N15 labeled

α-synA107C ^{15}N labeled

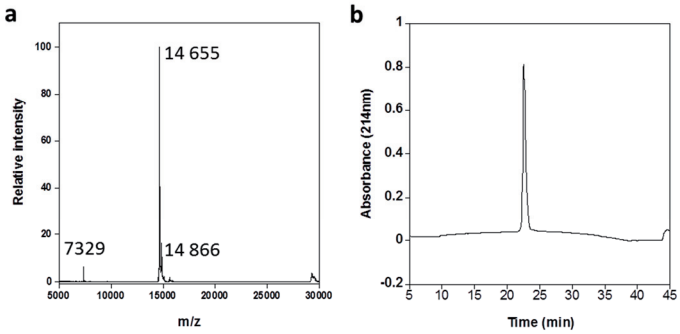

Figure S II-12. Analysis of ^{15}N labeled α-syn A107C
a) MALDI-TOF analysis of the protein (expected mass: 14659). The mass of 14866 corresponds to a sinapinic matrix adduct and the mass of 7329 to the double-charged. b) RP-HPLC of ^{15}N labeled α-syn A107C on an analytical Protein-R C_{18} column with a linear gradient of 0 to 80%B over 30min (A: water/0.1%TFA and B: acetonitrile/0.1% TFA).

α-synA107C_*p*Y125 ^{15}N labeled

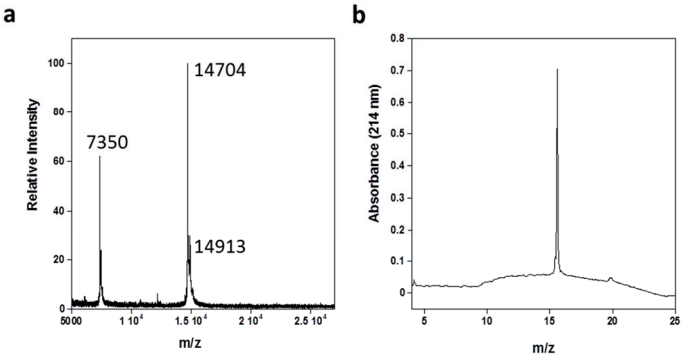

Figure S II-13. Analysis of semi-synthetic pY125 α-syn ^{15}N labeled
a) MALDI-TOF analysis of the ligated product (expected mass: 14701). The mass of 14913 corresponds to a sinapinic matrix adduct and the mass of 7350 to the double-charged. b) RP-HPLC of semi-synthetic pY125 α-syn ^{15}N labeled on an analytical Protein-R C_{18} column with a linear gradient of 0 to 80%B over 30min (A: water/0.1%TFA and B: acetonitrile/0.1% TFA).

II.8.5. TEM images of recombinant and semisynthetic WT α-syn

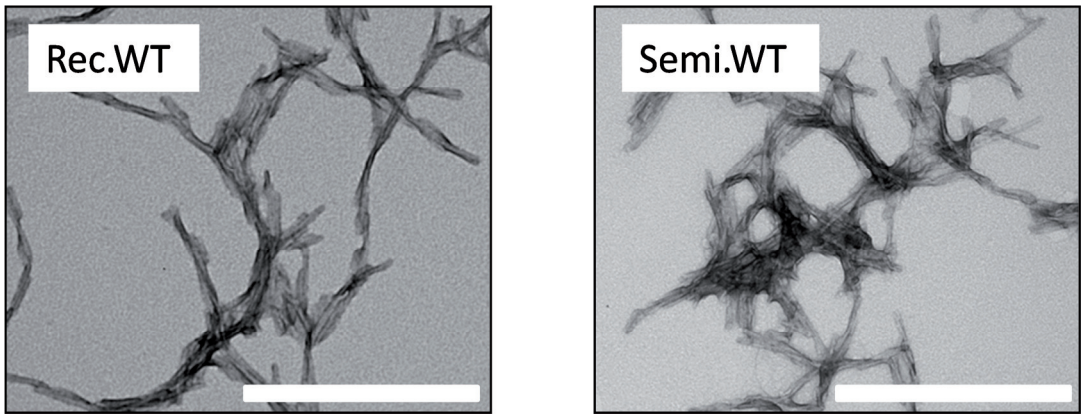

Figure S II-14. TEM images of semisynthetic and WT α-syn
TEM images of recombinant WT and semisynthetic α-syn incubated for 7 days on a rotating wheel at 37°C. The images are representative of 3 independent experiments. The scale bars represent 200nm.

II.8.6. Analysis of HEK and HeLa cells and mouse neurons treated with pervanadate: detection of non-specific bands

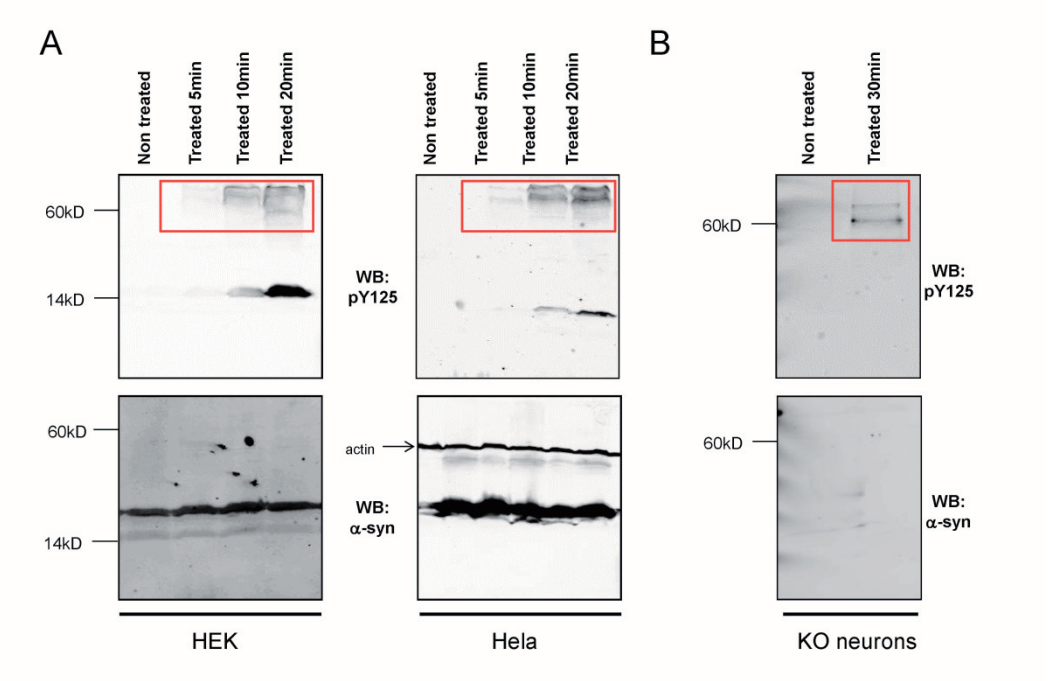

Figure S II-15. Detection of non-specific bands using pY125 antibodies
A. Immunoblots of HEK293 (left) and HeLa (right) cells over-expressing WT α-syn and treated with pervanadate for 5, 10 and 20min. Membranes were probed with WT α-syn (Biomol SA3400) and pY125 antibodies (BD Pharmigen). B. Immunoblots of mouse α-syn KO primary neurons treated with pervanadate for 30min. Membranes were probed with WT α-syn (BD transduction laboratory) and pY125 antibodies (BD Pharmigen).

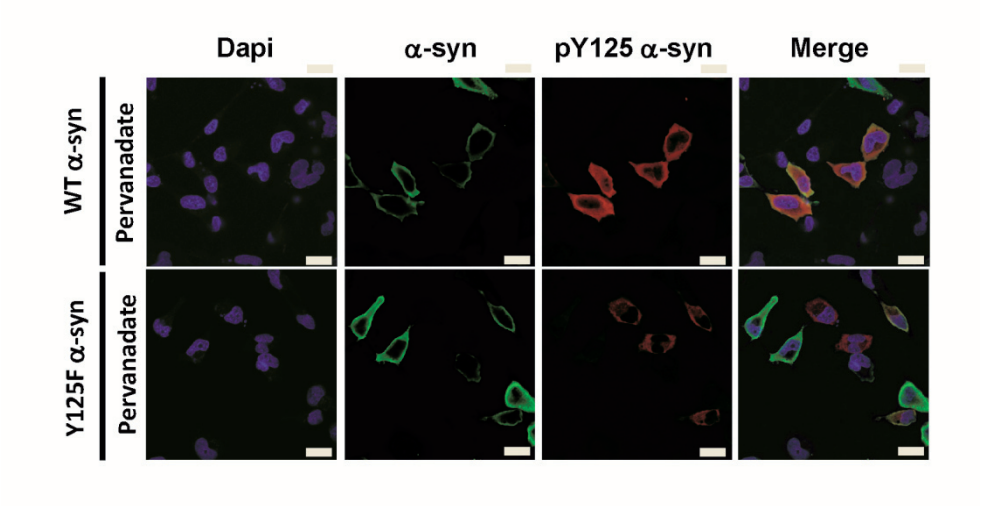

Figure S II-16. Detection of non-specific signal by immunocytochemistry
Confocal images of HeLa cells over-expressing WT or Y125F α-syn and treated with pervanadate for 30min. The cells were fixed and stained with anti-pY125 antibody (BD pharmigen) and anti-α-syn (abcam).

II.8.7. Cross-talk between α-syn phosphorylation at S87 and Y125: *in vitro* phosphorylation assay

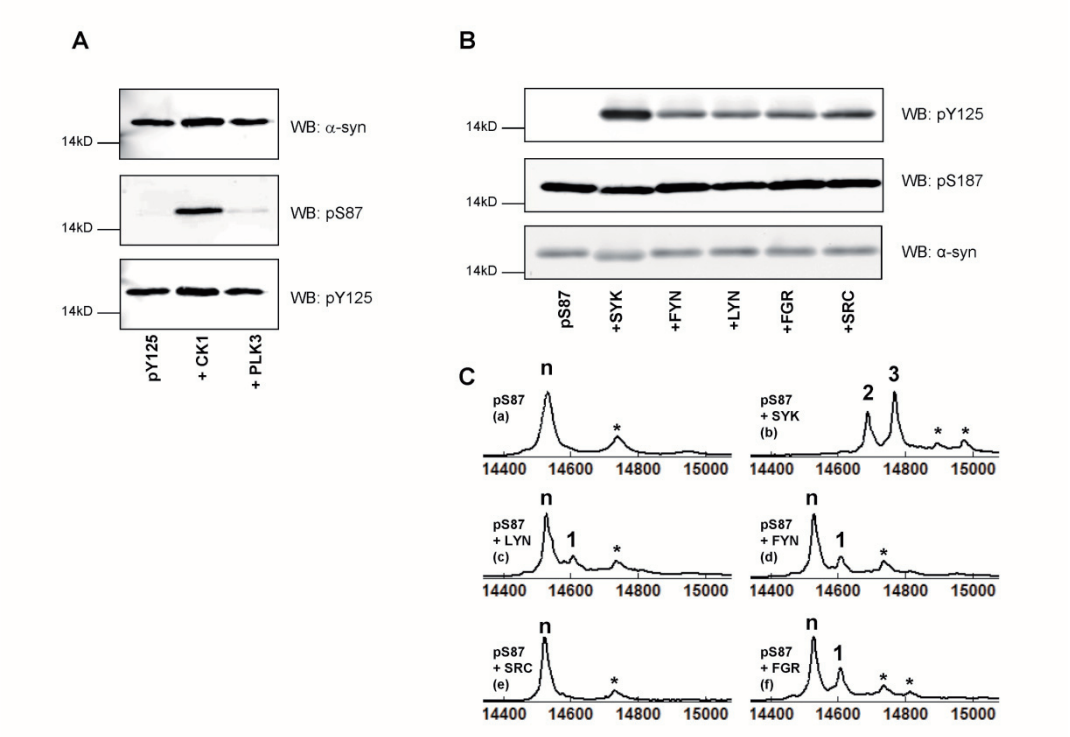

Figure S II-17. Immunoblots of pY125 α-syn phosphorylated using CK1 and PLK3
A: Immunoblots of pY125 α-syn phosphorylated using CK1 and PLK3. Membranes were probed using pS87, pY125, and α-syn antibodies. **B**: Immunoblots of pS87 α-syn phosphorylated using Syk, Lyn, Fyn, c-Src, and c-Fgr. Membranes were probed for pY125, pS87, and α-syn immunoreactivity. **D**: MALDI-TOF analysis of pS87 α-syn (a) phosphorylated by Syk (b), Lyn (c), Fyn (d), c-Src (e), and c-Fgr (f) after 14h of reaction. In all MALDI-TOF-MS spectra, the symbol 'n' indicates the starting material peak, while the numbers above other peaks correspond to the number of phosphorylation events, each detected by a +80 Da mass shift. The symbol '*' indicates a sinapinic acid matrix adduct.

Supporting information for the figure S17:
To obtain site-specific phosphorylation at S87, S129A α-syn was incubated with CKI until phosphorylation at S87 was complete, then pS87 α-syn was purified by reversed-phase HPLC, and incubated with the tyrosine kinases in the same conditions as in the experiment with pS129 α-syn. Using first the mutant S129A α-syn, we showed that the S129A mutation itself did not influence phosphorylation at Y125 (not shown). Using the purified pS87 α-syn, we observed that the behavior of pS87 α-syn is similar to that of pS129 α-syn and WT α-syn: phosphorylation at Y125 by Syk is very efficient as judged by Western Blot (Figure S II-17.B) but again not specific as shown by MALDI-TOF MS (Figure S II-17. C (b)). We noted, however, that pS87 α-syn might be more efficiently phosphorylated at Y125 by Fyn than pS129 α-syn, since a single phosphorylation event could be seen with pS87 α-syn but not pS129 α-syn. However, Western-Blot data does not support a large effect.

III. Chapter 3- Towards elucidating the role of ubiquitination in the pathogenesis of Parkinson's disease using semisynthetic ubiquitinated α-synuclein

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III.2. Abstract

Herein we report, for the first time, the semisynthesis and characterization of site-specific (K6) mono-ubiquitinated form of α-syn. These advances allowed us to investigate the effect of ubiquitination on the membrane binding, oligomerization and fibrillogenesis. The ability to site-specifically introduce ubiquitin modifications into α-syn represents a major advance towards elucidating the role of ubiquitination in regulating α-syn's function(s) in health and disease.

III.3. Introduction

Ubiquitination of lysine residues has emerged as an important mechanism for regulating a variety of cellular processes such as cell signaling pathways, DNA repair and protein degradation by the 26S proteasome (Pickart and Fushman, 2004, Ikeda and Dikic, 2008). Until recently, studies aiming at deciphering the effect of ubiquitination on protein function have relied primarily on biochemical approaches to reconstitute the ubiquitinated protein *in vitro*. As a result, the progress in the field has been very much dependent on the discovery of the E2-E3 enzymatic machinery that is highly specific to the protein of interest (Pickart, 2001). The lack of effective and general method that allows site-specific incorporation of ubiquitin (Ub) or

polyubiquitin chains has hampered efforts to elucidate the molecular and structural basis underlying the effects of ubiquitination on regulating protein function. Several groups have recently reported elegant chemical methods to facilitate site-specific peptide and protein ubiquitination. Several of these methods are based on the attachment of ubiquitin through non-native isopeptide bonds to generate enzymatically stable Ub conjugates of peptides or recombinant proteins (Yin et al., 2000, Li et al., 2009, Chatterjee et al., Chen et al., 2010, Shanmugham et al., 2010, Weikart and Mootz, 2010). Site-specific ubiquitination of peptides and proteins via formation of native isopeptide bond is now possible thanks to the work of Muir, Liu and our group (Chatterjee et al., 2007, McGinty et al., 2008, Ajish Kumar et al., 2009, Yang et al., 2009).

We recently developed a highly efficient and chemoselective method to facilitate site-specific ubiquitination of peptides and proteins (Ajish Kumar et al., 2009). Our approach utilizes δ-mercaptolysine residue to mediate transthioesterification with ubiquitin thioester, followed by S-N acyl transfer to form the native isopeptide bond between ubiquitin and lysine residues. The thiol handle of δ-mercaptolysine is then desulfurized to furnish the desired native isopeptide linkage without altering the native sequence of the target peptides/proteins. To facilitate the use of this unique residue in Fmoc-, Boc-SPPS and in sequential ligation, δ-mercaptolysine with different protecting groups was also synthesized (Haj-Yahya et al., 2010). Using these (advanced) synthetic tools, herein we describe, for the first time, an efficient strategy for the semisynthesis and characterization of monoubiquitinated α-synuclein at Lys6 (T7-Ub-α-syn(K6)).

α-synuclein (α-syn) is a natively unfolded 140 amino acid presynaptic protein that is implicated in the pathogenesis of Parkinson's disease (PD) and related neurodegenerative diseases that are collectively termed, "synucleopathies" (Spillantini et al., 1998a, Gai et al., 1999, Campbell et al., 2001). The pathology of PD is characterized by the loss of dopaminergic neurons and the presence of intracellular inclusions, known as Lewy Bodies (LB), composed primarily of α-syn (Spillantini et al., 1998a). Several posttranslational modifications, including phosphorylation, C-terminal truncations and ubiquitination, have been shown to be closely associated with PD pathology and were identified in α-syn within LB isolated from PD brains (Hasegawa et al., 2002, Sampathu et al., 2003, Tofaris et al., 2003, Anderson JP, 2006). Understanding the role of these modifications in regulating α-syn aggregation, LB formation and toxicity is crucial to understanding the biology of α-syn, elucidating its role in the pathogenesis of PD, and may lead to the identification of novel therapeutic targets to treat the disease.

The majority of α-syn species found in Lewy bodies are mono- or diubiquitinated at multiple lysine residues (Hasegawa et al., 2002, Sampathu et al., 2003, Anderson JP, 2006). Directed site-specific ubiquitination of α-syn at a single or multiple lysine residues has not been possible. For example, the co-expression of α-syn with ubiquitin ligases results in predominantly mono and di-ubiquitination at multiple sites (Lee et al., 2008b, Rott et al., 2008). Similarly, ubiquitination of recombinant monomeric or fibrillar α-syn using rabbit reticulocytes fraction II or rat-brain extracts revealed that ubiquitination occurs at multiple, but distinct lysine residues (Nonaka et al., 2005), thus precluding the possibility of investigating the effect of ubiquitination at specific lysine residues. To address this problem, we developed a semisynthetic strategy, combining Cys- and δ-mercaptolysine based native chemical ligation methods (NCL) (Dawson et al., 1994) which allows for site specific incorporation of ubiquitin and preparation of highly homogenous monoubiquitinated α-syn forms.

III.4. Material and Methods

III.4.1. Synthesis of α-syn (1-18)(K6mK)- thioester (1)

Work carried out by Mahmood Haj-Yahya (Prof. Ashraf Brik's group)

α-syn (1-18)(K6mK)- thioester was prepared by solid phase peptide synthesis (SPPS) on MBHA resin (0.59 mmol/g) following the *in situ* neutralization protocol for Boc chemistry developed by Kent and coworkers.

III.4.2. Cleavage and deprotection

Work carried out by Mahmood Haj-Yahya (Prof. Ashraf Brik's group)

To the dried resin (250 mg) in a round bottom flask equipped with stirring bar, 750 μL of thioanisole/EDT (2:1) was added. The reaction mixture was cooled to 0 ºC, using ice bath, followed by the addition of TFA (5 mL) and stirred for 5-10 min. To this solution, TFMSA (500 μL) was added slowly with constant stirring. The reaction was allowed to reach room temperature (RT) over 40 min and stirred for an additional 45 min. Resin was removed by filtration and washed with TFA. To precipitate the peptide, the combined filtrate was added drop-wise to 10-fold volume of cold ether, centrifugation, decanting of ether, followed by dissolving the residue in acetonitrile-water. RP-HPLC purification was carried out on C18-column (Jupiter 5 micron, 300A, 250 x 21.2 mm) using 10-60% B over 30 min afforded the corresponding peptide **1** in ~10% yield.

III.4.3. Generation of α-syn N-terminal cysteine (2)

Starting with a plasmid encoding for α-syn Δ2-17 A18C, we performed single-point mutagenesis to generate the N-terminal truncated fragment with alanine at position 19 mutated to cysteine (α-syn Δ2-18 A19C). The following primers were used: forward 5' GAG ATA TAC ATA TGT GTG AGA AAA CCA AAC AGG 3'OH and reverse 5' CCT GTT TGG TTT TCT CAC ACA TAT GTA TAT CTC 3'OH. For protein expression, *E.coli* BL21 DE3 cells were transformed with the plasmid and grown in LB medium until OD600 = 0.6. Protein expression was induced by adding 1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) and the culture was incubated for 4 h at 37°C. After harvesting the cells, by centrifugation at 6000 g for 15 min, the pellet was resuspended in 40 mM Tris-acetate and 5mM EDTA pH 8.3 and lysed by ultra-sonication. The insoluble material was removed by centrifugation at 23000 g for 40min at 4°C. The supernatant was boiled for 15min followed by centrifugation at 23000 g for 40min and the final supernatant was filtered through a PVDF membrane with a 0.22 μm cut-off. Anion-exchange chromatography was performed on a Pharmacia AKTA FPLC system using a 25 mL High-TrapQ column (Amersham). The protein was eluted with a NaCl gradient (the protein elutes at around 300mM NaCl) and subsequently purified on a Superdex 200 26/60 size-exclusion chromatography column (Amersham) using 50 mM Tris 150mM NaCl pH 7.5 as the mobile phase. The purified protein was dialyzed extensively against di-ionized water and then lyophilized and stored at -20°C until use. From two liters expression, the average yield of pure lyophilised material is 25 mg. The purity and identity of the protein was assessed by SDS-PAGE and MALDI-TOF analyses. The mass obtained had +26Da and +70Da thiazolidine adducts as previously reported by other groups (Gentle et al., 2004, Chiang et al., 2009).

III.4.4. Deprotection of thiazolidine

Work carried out by Mahmood Haj-Yahya (Prof. Ashraf Brik's group)

The expressed fragment A19C-140 (50mg, 3.94 mmol) was dissolved in 1 ml Gn.HCl (pH=7.2) followed by the addition of methoxylamine (1ml, 0.4 M) containing 30 eq of TCEP. The reaction was kept for 3.5 h at 37 C in which HPLC and mass spectrometry analysis indicated complete removal of the thiazolidine adducts.

III.4.5. Ligation of peptide thioester (1) and α-syn19-140(A19C) (2)

Work carried out by Mahmood Haj-Yahya (Prof. Ashraf Brik's group)

Peptide thioester, **1** (2.4 mg, 1.00 mmol) and **2**, (10.5 mg, 8.3 x 10^{-4} mmol) were dissolved in 400 μL of 6 M guanidine**.**HCl, 200mM phosphate buffer, pH 7. To this solution 8 μL each of

benzylmercaptan (2% v/v) and thiophenol (2% v/v) were added and incubated for 24 h at 37 ºC. The progress of reaction was monitored by RP-HPLC using an analytical C4 column (C-4, 300 A, 5 micron, 150 x 4.6 mm) and a gradient of 5-60 %B over 30 min. Preparative HPLC was carried out on semiprepartive column (Jupiter 5 micron, 300A, 250x 10 mm) using a gradient of 5-60% B over 30 min to afford 5.2 mg of pure ligation product **3**.

Acm removal: The purified peptide **3** (5.2 mg, 3.56 x 10^{-4} mmol) was dissolved in 15% acetic acid (648 µL) under nitrogen, and $Hg(OAc)_2$ (3.4 mg, 0.01 mmol, 30 eq.) was added and kept at RT for 3 h. DTT (6.6 mg, 0.04 mmol, 120 eq.) was added and the reaction was left for another 12 h. The reaction mixture was filtered and purified over a semi-prep HPLC column (C4, 300A, 10 micron, 250 x 10 mm) using a gradient of 5-60% B over 30 min to give 3.5 mg of peptide **4**.

III.4.6. Generation of T7 ubiquitin thioester (5)

The T7-tagged ubiquitin was amplified by PCR using the following primers: forward 5'GGC AGC CAT ATG GCT AGC ATG AC 3'OH and reverse 5' CCG CAT CGC TCT TCG GCA GCC GCC GCC ACG CAG 3'OH. After digestion with NdeI and LguI (Promega and Fermentas), it was ligated into a similarly digested pT7-7 vector containing the mini intein Mxe GryA followed by a chitin-binding domain. The resulting plasmid encoding T7-Ub fused to its C-terminus to the intein was verified by DNA sequencing. For protein expression, *E.coli* BL21 DE3 cells were transformed with the plasmid and grown in LB medium until OD = 0.6, before induction with with 0.5 mM IPTG for 4 h at 37°C. After harvesting the cells by centrifugation at 6000 g for 15 min, the pelett was resuspended in 40 mM Tris-acetate 5 mM EDTA (pH 8.3) lysis buffer and the cells were lysed by ultra-sonication (5 cycles of 1 min non-stop pulse at 13W and 30s rest). The insoluble material was removed by centrifugation at 23000g for 40min at 4°C followed by filtration through a PVDF membrane with a 0.22µm cut-off (Millipore). Initially, chitin beads (NEB) were used for affinity purification of the fusion proteins. However, we later observed that a two-step chromatographic purification protocol gave higher purity and yield of T7-Ub-SR. In this protocol, the supernatant was applied first to a 25 mL High-TrapQ anion-exchange column (Amersham) and separation was performed on a Pharmacia AKTA FPLC system. The protein eluted with a NaCl gradient (the protein elutes at around 200mM NaCl) and subsequently purified on a Superdex 200 26/60 size-exclusion chromatography column using 50mM Tris 150mM NaCl pH 7.5 as the mobile phase. The fractions containing the proteins were pooled and splicing was induced by addition of 2-mercaptoethanesulfonic acid (Sigma) at a final concentration of 0.5M and incubation for 12 h at room temperature. The splicing efficiency was determined as 60%. T7-Ub-SR was further purified by reverse phase

HPLC using a C4 column (Vydac) and eluted using a linear gradient of acetonitrile/0,1% TFA in water/0.1% TFA. The fractions containing the thioester were pooled, evaporated and lyophilised and kept at -20°C until used. The protein purity was assessed by analytical reverse phase HPLC and mass spectrometry. From 2 liters expression pelett, the average yield of lyophilised pure product was 6 mg.

III.4.7. Ligation of peptide (4) with ubiquitin thioester (5)

Work carried out by Mahmood Haj-Yahya (Prof. Ashraf Brik's group)

Purified peptides, **4** (3.5 mg, 2.41 x 10^{-4} mmol) and **5** (3.2 mg, 3.13 x 10^{-4} mmol), were dissolved in 130 μL of 6 M guanidine**.**HCl, 200 mM phosphate buffer pH ~7. To this solution 2 μL each of benzyl mercaptan and thiophenol were added and incubated for 13 h at 37 ºC. The progress of the reaction was monitored by HPLC using an analytical C4 column and a gradient of 5-60% B over 30 min. The product was purified using semi-prep HPLC column (C4, 300A, 10 micron, 250 x 10 mm) and a gradient of 5-60% B over 30 min to give 3.2 mg of pure ligation product **6**.

III.4.8. Procedure for desulfurization of ubiquitylated αsyn(1-140) (6)

Work carried out by Mahmood Haj-Yahya (Prof. Ashraf Brik's group)

To a solution of peptide **6** (3.2 mg, 1.30 x 10^{-4} mmol, 1.30 mM) in 100 μL 6 M guanidine**.**HCl, 200 mM phosphate buffer pH 7.9, 0.5 M TCEP (26.67 mg, 0.10 mmol) in the same buffer and 20 μL of *t*-BuSH was added. To this reaction mixture, 10 μL of 0.1 M radical initiator VA-044 in the same buffer was added and incubated at 37 ºC for 3 h. The progress of the reaction was was monitored by HPLC using an analytical C4 and a gradient of 5-60% B over 30 min. The product was purified using semi-prep HPLC column (C4, 300A, 10 micron, 250 x 10 mm) and a gradient of 5-60% B over 30 min to give 2.5 mg of pure desulfurized monoubiquitinated α-syn, **7**.

III.4.9. Final purification by liquid-based isoelectric focusing

The final product after desulfurization contained some unmodified full-length α-syn which appears to co-elute with T7-Ub-α-syn. The best separation of T7-Ub-α-syn from WT α-syn was achieved using a micro-rotofor system (Biorad), which allows for liquid-based isoelectric focusing of protein mixtures. T7-Ub-α-syn (125μg) was dissolved in 8M urea, 4% glycerol, 2% ampholytes pH range 4-6 for a total volume of 2,5mL. Constant power of 1 Watt was applied and the purification was allowed to proceed until voltage remained constant (~ 2h and 30min). The different fractions were analyzed by SDS-PAGE electrophoresis and the fraction containing

pure T7-Ub-α-syn was extensively dialyzed against 50mM Tris 150mM NaCl pH 7.5 and concentrated through a 30kD cut-off membrane (Amicon).

III.4.10. SDS-PAGE and Western-Blot analysis

Proteins were solubilized in 50 mM Tris 150 mM NaCl, pH 7.5. Gels were prepared using standard Laemmli techniques and stained with Coomassie Blue stain or with Silver staining (Invitrogen). For Western blot analysis, the proteins were transferred to a nitrocellulose membrane (Omnilab). The membranes were blocked for 15 min at RT under constant rocking using Odyssey blocking buffer (Li-COR Biosciences) and diluted 1:3 in phosphate-buffered saline (PBS). For revelation of T7-Ub-α-syn by anti-Ub antibodies, membranes were boiled in PBS prior to blocking and the primary antibody was incubated at RT overnight. Membranes incubated with anti-α-syn and anti-T7 antibodies were incubated with the following primary antibodies at 4 °C with constant rocking overnight: mouse anti-α-syn (1:1000 dilution; BD Transduction Laboratories), mouse anti-T7 (1:15000 dilution; Novagen), mouse anti-pS129 α-syn (1:5000 dilution; Wako), rabbit anti-pS87 α-syn (1:200 dilution,(Paleologou et al., 2010)), mouse anti-ubiquitin (1:250 dilution; Zymed) and rabbit anti-ubiquitin (1:500 dilution; Dako). Membranes were washed three times with PBS-T (PBS containing 0.01% Tween), followed by incubation with secondary antibodies goat anti-mouse and anti-rabbit IgM conjugated to Alexa 680 (Invitrogen). Finally, membranes were washed three times with PBS-T and once with PBS and scanned in a Li-COR scanner (Li-COR Biosciences).

III.4.11. Ubiquitin hydrolysis assay

15 µM of T7-Ub-α-syn was incubated with UCH-L3 (BostonBiochem) and the de-ubiquitination reaction was carried out as previously described (McGinty et al., 2009). The T7-Ub-α-syn contained small amount of WT α-syn which served as a reference for the formation of WT α-syn after UCH-L3 mediated hydrolysis of T7-Ub-α-syn. Briefly, UCH-L3 was diluted to 10 µM in 50 mM Tris 150 mM NaCl 15 mM DTT pH 7.5 and allowed to be reduced for 15min at RT. From the reduced UCH-L3, 5 µL were taken into 20µL of T7-Ub-α-syn in a 37°C water bath. Aliquots of 5µL were taken after 2 and 10min and the reaction was quenched by addition of 5µL Laemmli buffer 2X. The amount of enzyme used was doubled after 10min. Aliquots were taken after 30 and 60min. The reaction was monitored by the appearance of WT α-syn and T7-Ub using SDS-PAGE electrophoresis.

III.4.12. *In vitro* phosphorylation assay

A mixture of WT α-syn and T7-Ub-α-syn (28μM) was incubated with 10mM DTT, 1mM MgCl2 and 1mM ATP in 49μL of 50mM Tris pH 7.5. G-protein-coupled-receptor kinase 5 GRK5 (1μL, 0,38μg, Invitrogen) and Polo-like kinase 3 PLK3 (1μL, 0,42μg, Invitrogen) were added to the sample. For phosphorylation with Casein kinase 1 CK1, 28μM of proteins were mixed with CK1 buffer (NEB), 1mM ATP in 49uL of Tris 50mM pH 7.5. 1000 units (1μL) of CK1 (NEB) were added to the sample. The reaction mixtures were incubated overnight at 30°C and the extent of phosphorylation was monitored by western-blotting using anti-pS129 and anti-pS87 antibodies.

III.4.13. Lipid binding assay

1-Palmitoyl-2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] sodium salt POPG (Avanti Polar Lipids) was purchased as a chloroform solution, and the solvent was removed by evaporation and lyophilization. The phospholipid was resuspended to a final concentration of 10 mg/ml in a solution of 50 mm HEPES 150 mm NaCl pH 7.5. To form large unilamellar vesicles, 10 cycles of freezing in dry ice and thawing in a 37 °C water bath were carried out. Small unilamellar vesicles were prepared by extrusion through a 100-nm polycarbonate membrane (Avestin) according to the manufacturer's instructions. The vesicles were stored at 4 °C and used within 5 days. For lipid binding assay, 15μM of protein (WT α-syn and T7-Ub-α-syn) were incubated with freshly prepared POPG vesicles at a 1/10 mass ratio in 50mM Tris 150mM NaCl, pH 7.5. The extent of membrane binding of the proteins was analyzed by monitoring the extent of α-helical structure in membrane-bound proteins using CD spectroscopy.

III.4.14. Circular Dichroism (CD) Measurements

CD spectra were obtained on a Jasco J-815 CD spectrometer using protein concentrations of 15μM. Bovine ubiquitin was purchased from Sigma. Spectra were recorded in 0.1 cm cells from 250 to 195 nm with a step size of 0.2 nm, a bandwidth of 1 nm, and a response time of 8s. For all spectra, an average of ten scans was obtained and the CD spectrum of the buffer (50mM Tris 150 mM NaCl pH 7.5) was recorded and subtracted from the protein spectra.

III.4.15. *In vitro* aggregation studies

Protein samples (14μM in 450μL) were incubated at 37°C with constant rotation for 10 days. The extent of fibril formation was assessed using the Thioflavin T (ThT) fluorescence assay after 5, 7, and 10 days of incubation. Readings were carried out with a ThT concentration of 5

μM and a protein concentration of 1.4 μM in 70 μl of 50 mM glycine (pH 8.5) and fluorescence measurements were recorded on an "Analyst AD" spectrometer (Bucher Biotec) at an excitation wavelength of 450 nm and an emission wavelength of 485 nm. All samples were analyzed in triplicate and corrected for the fluorescence level of freshly solubilized α-syn. Values are expressed as the ThT fluorescence of three measurements (means ± S.D.).

III.4.16. Transmission Electron Microscopy (TEM)

7μL of proteins were deposited on Formvar-coated 200 mesh copper grids (Electron Microscopy Sciences). Grids were washed with two drops of double-distilled H_2O and stained with 2 drops of freshly prepared 2% (w/v) uranyl acetate (Electron Microscopy Sciences) followed by vacuum drying from the edge of the grids. Specimens were viewed on a Philips CIME 12 electron microscope, operated at 80 kV and digitized photographs were recorded with a CCD camera (Digital Camera Morada (Soft Imaging System) 4008 X 2672 pixel).

III.5. Results and discussion

III.5.1. Generation of monoubiquitinated α-syn at K6 (T7-Ub-α-syn (K6))

We focused our efforts on monoubiquitinated α-syn at K6 *(T7-Ub-α-syn(K6))* because *in vitro* ubiquitination of fibrillar α-syn was shown to occur primarily at K6, K10 and K12 (Nonaka et al., 2005). However, our approach can be applied to prepare monoubiquitinated α-syn involving any lysine residues within the N-terminal region 1-18 and can be modified to allow ubiquitination at other lysine residues. The semisynthetic strategy of *T7-Ub-α-syn(K6)* is based on expressed protein ligation (
Figure III-1). Our design is based on linking two fragments using native chemical ligation (NCL): a recombinantly expressed α-syn fragment comprising residues 19-140 bearing an N-terminal Cys *α-Syn(19-140)* and a synthetic peptide thioester comprising the N-terminal residues 1-18 bearing δ-mercaptolysine, α-Syn(1-18)-SR, in which K6 is replaced with the acetamidomethyl (Acm) protected δ-mercaptolysine (Haj-Yahya et al., 2010). Notably, the sequence of α-syn lacks Cys residues. Therefore, the ligation site was selected at Ala19, which is temporarily replaced with Cys to allow NCL and the assembly of full length α-syn. Conversion of Cys19 to Ala19 and removal of the thiol handle of the δ-mercaptolysine is simultaneously achieved under desulfurization conditions to afford the native monoubiquitinated protein *T7-Ub-α-syn(K6)* (Yan and Dawson, 2001). With this design in mind, the C-terminal fragment with an N-terminal free cysteine α-syn(19-140) was expressed in E. Coli (Figure S III-1). The α-syn(1-18)-SR peptide was prepared using solid phase peptide

synthesis (SPPS) according to the *in situ* neutralization protocol for Boc-chemistry (Schnolzer et al., 1992), wherein the Acm-protected δ-mercaptolysine was coupled instead of K6.

Having both fragments in hand, we then turned our attention to the ligation and ubiquitination steps. The NCL of α-syn(19-140) and the synthetic α-Syn(1-18)-SR peptide thioester was carried out under denaturing conditions (i.e. 6 M GuHCl, 200 mM phosphate buffer, pH 7) and in the presence of 2% v/v thiophenol/benzylmercaptan. The backbone ligation reaction was followed by RP-HPLC and mass spectrometry, both of which indicated a nearly complete ligation after 24 h, and gave the desired product in 43% isolated yield. Following the ligation step, the Acm protecting group was removed quantitatively by incubation with $Hg(OAc)_2$ in 15% acetic acid for 3 h, followed by treatment with DTT for an additional 12 h.

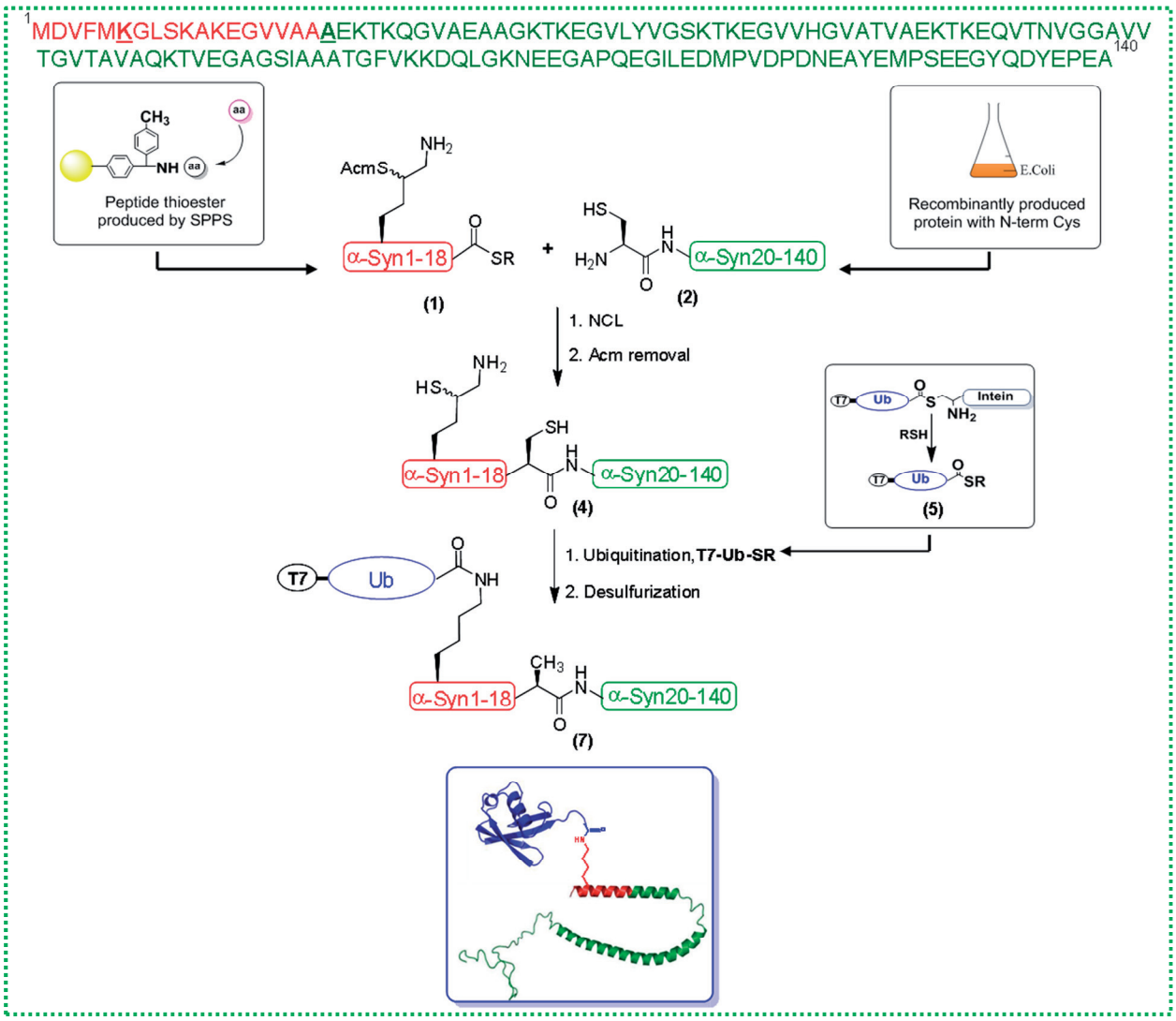

Figure III-1.Schematic depiction of the semisynthetic strategy for the ubiquitination of α-syn at K6.

Next, we focused on the ubiquitination between the T7-Ub-SR and full length α-Syn(1-140)

presenting the free δ-mercaptolysine. Using the above described ligation conditions, a complete ligation of both proteins was observed after 12 h and gave the ubiquitinated product in 54% isolated yield (

Figure S III-3). Finally, the isolated product was subjected to the metal free desulfurization conditions for 3 h to give the native monoubiquitinated α-Syn *T7-Ub-α-syn(K6)* in 78 % isolated yield (Figure III-2) (Wan and Danishefsky, 2007). SDS-PAGE analysis of *T7-Ub-α-syn(K6)* revealed a slow migrating band at ~ 26 kDa, consistent with the mass spectrometry data and conjugation of one molecule of ubiquitin (Figure III-3.A). Western-blot analysis of this sample showed that this band is detectable using anti-T7, anti-α-syn and anti-ubiquitin antibodies (Figure III-3.A). To verify the formation of a native isopeptidic bond between ubiquitin and α-syn, we incubated *T7-Ub-α-syn(K* 6) with ubiquitin C-terminal-carboxyhydrolase (UCH-L3) to see if it can be hydrolyzed. Upon addition of UCH-L3, we observed the generation of T7-ubiquitin monomers and WT α-syn (Figure S III-5).

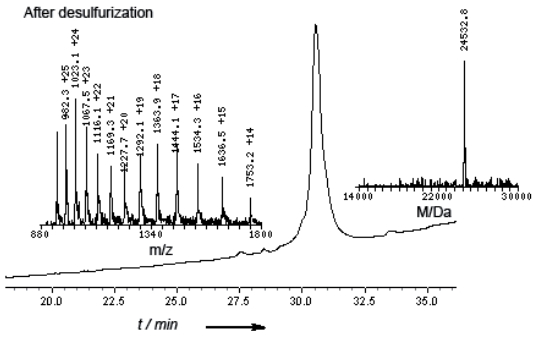

Figure III-2. Analytical HPLC of the crude desulfurization reaction peak
Analytical HPLC analysis corresponding to the desired monoubiquitinated α-Syn with the observed mass of 24532.8 Da (calcd m/z 24531.7 Da). The progress of the reaction was analyzed using RP-HPLC (C4 column) with a gradient of 5%-60% B over 30 min. Reported mass is for total protein.

III.5.2. Effect of monoubiquitination at K6 on the secondary structure

Having verified the chemical integrity of *T7-Ub-α-syn(K6)*, we then focused on elucidating the effect of ubiquitination on the secondary structure, membrane binding and aggregation properties of α-syn *in vitro* using circular dichroism (CD), thioflavin T binding assay, and transmission electron microscopy (TEM). As expected, WT α-syn exhibited a CD spectrum consistent with a random coil structure, while T7-Ub-SR showed a spectrum that reflects a mixture of α-helices, β-sheets and random coil, consistent with the structure of ubiquitin

(Jenson et al., 1980). *T7-Ub-α-syn(K6)* showed a CD spectrum that is virtually identical to that obtained from a solution containing an equimolar mixture of T7-Ub-SR and WT α-syn, suggesting that the native structures of ubiquitin and α-syn are preserved in the ubiquitinated α-syn, *T7-Ub-α-syn(K6).*

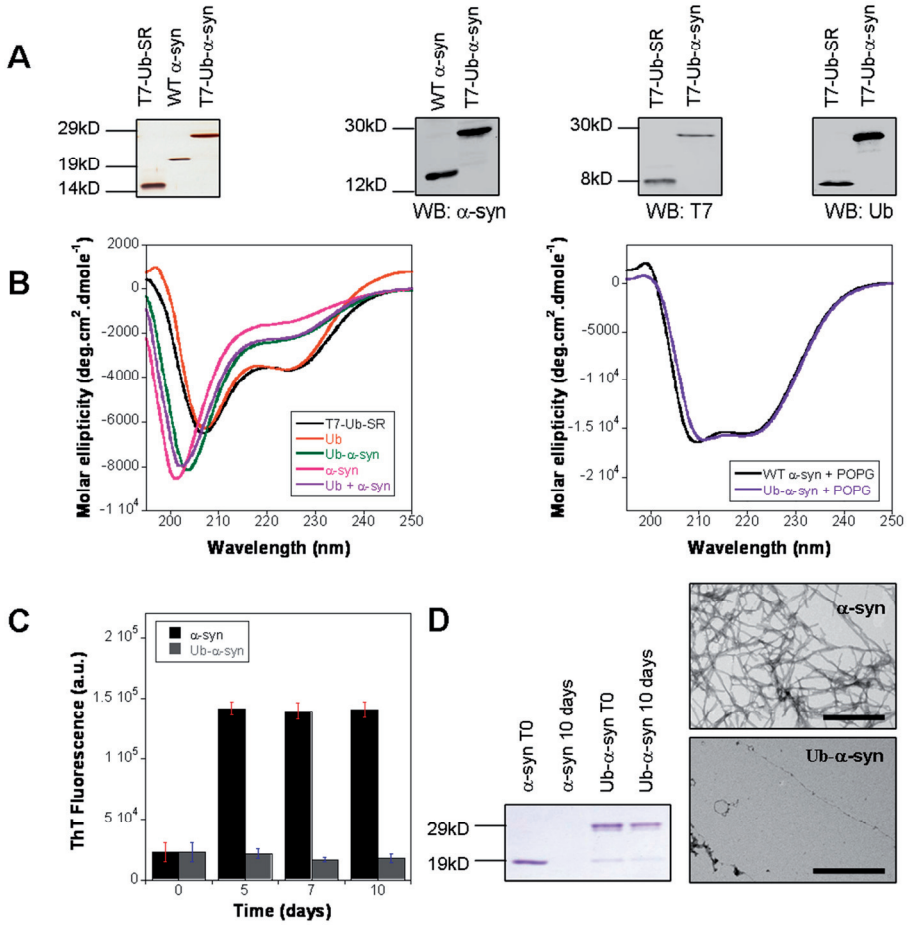

Figure III-3. Characterization of monoubiquitinated α-syn at K6
A) SDS-PAGE analysis of T7-Ub-SR, α-syn WT and T7-Ub-α-syn. 15% gel, Silver staining. Immunoblots of T7-Ub-SR and T7-Ub-α-syn using anti-T7 and anti-ubiquitin antibodies (Zymed) and of α-syn WT and T7-Ub-α-syn using anti-asyn antibody. B) CD data of T7-Ub-SR, ubiquitin (Sigma), mix of ubiquitin and α-syn WT, α-syn WT and T7-Ub-α-syn. CD spectra of α–syn WT and T7-Ub-α-syn bound to POPG vesicles. C) ThT fluorescence assay of samples taken at time 0, 5, 7 and 10 days. D) Quantification of monomeric α-syn using SDS-PAGE analysis at time 0 and 10 days after filtration through a 100 kDa membrane. EM images of α-syn WT and T7-Ub-α-syn at day 7; scale bar represents 200 nm. The figures are representative of 2 independent experiments

In neurons, α-syn shows a cytoplasmic and membrane localization. The N-terminal region comprising residues 1 to ~ 100 (Bisaglia et al., 2005, Ulmer et al., 2005, Bodner et al., 2010) has been shown to mediate α-syn interaction with membranes and adopt an α-helical structure upon interaction with lipid vesicles *in vitro*. The effect of ubiquitination on α-syn membrane binding has not been investigated. Figure III-3.B shows that α-syn WT and monoubiquitinated α-syn

adopt a similar α-helical structure upon binding to POPG vesicles, suggesting that the conjugation of a ubiquitin moiety on K6 does not induce major changes in the structure of α-syn in solution or alter its interaction with synthetic lipids.

III.5.3. Effect of monoubiquitination at K6 on fibril formation

To determine the effect of N-terminal ubiquitination on α-syn fibril formation, we compared the fibrillization of the monoubiquitinated α-syn at K6 to that of the WT protein using the ThT fluorescence assay and TEM. Figure III-3.C and D demonstrate that ubiquitination at K6 results in significant inhibition of α-syn fibril formation as evidenced by the absence of any changes in the ThT signal after incubation at 37 ºC for 10 days under rotating conditions (Figure III-3.C). Consistent with the ThT data, WT α-syn formed extensive mature fibrillar structures, whereas no fibrils were detected in samples containing the monoubiquitinated protein. These findings are consistent with previous reports demonstrating that not all α-syn inclusions in transgenic mouse models are ubiquitinated (van der Putten et al., 2000, Sampathu et al., 2003), which suggests that ubiquitination is not required for inclusion formation and could be a late event that occurs after synuclein fibrillization (Sampathu et al., 2003).

On the other hand, our data contradict other studies reporting that ubiquitination enhances α-syn aggregation *in vitro* and in cell cultures (Lee et al., 2008b, Rott et al., 2008). However, it is noteworthy that none of these studies examined the aggregation of homogenous preparations of mono-ubiquitinated α-syn, instead the samples used contained heterogeneous mixtures of unmodified as well as α-syn ubiquitinated at different lysine residues.

III.5.4. Effect of monoubiquitination at K6 on phosphorylation

The semisynthesis of α-syn provides unique opportunities to explore cross-talk between different post-translational modifications and how it influences α-syn aggregation and LB formation in synucleinopathies. For example, several studies have reported close association between ubiquitination and phosphorylation of α-syn at S129 within LB (Hasegawa et al., 2002, Tofaris et al., 2003, Anderson JP, 2006), suggesting that S129 phosphorylation may play a role in regulating α-syn ubiquitination and degradation. Studies by Nonaka *et al* suggested that the effect of S129 phosphorylation on α-syn ubiquitination *in vitro* is minor (Nonaka et al., 2005). However, the effect of ubiquitination at each of the putative lysine residue on α-syn phosphorylation has not been investigated.

To explore the interplay between these two modifications, we investigated the effect of

ubiquitination at K6 on the extent of α-syn phosphorylation at S87 and S129 by three kinases, caseine kinase I, polo like kinase 3 and G-protein-coupled receptor kinase 5, which are known to phosphorylate α-syn at these residues (Okochi M, 2000, Arawaka et al., 2006, Mbefo et al., 2010). We took advantage of the difference in size between WT and *T7-Ub-α-syn(K6)* and included both proteins in the phosphorylation reaction to assess the activity of the kinases and allow for direct comparison between the two proteins under identical phosphorylation conditions. We show that ubiquitination at K6 does not influence significantly the extent of α-syn phosphorylation at S87 (by CK1) and S129 (by CK1, GRK5 and PLK3) (Figure S III-6).

III.6. Conclusion

In summary, we report, for the first time, the semisynthesis and characterization of site-specific (K6) monoubiquitinated form of α-syn (T7-Ub-α-syn(K6)). The strategy has yielded sufficient quantities that allowed us to perform biochemical and biophysical studies to elucidate the effect of this modification on the structure, membrane binding, and fibrillization of α-syn. In addition, we were able for the first time to investigate the cross-talk between two disease-associated post-translational modifications (ubiquitination and phosphorylation at specific residues). The ability to site-specifically introduce ubiquitin modifications into α-syn represents a major advance towards elucidating the role of ubiquitination in regulating α-syn's function(s) in health and disease. Our findings present strong evidence in the support of the hypothesis that N-terminal ubiquitination of α-syn stabilizes the monomeric form of the protein, thus preventing its oligomerization and fibrillogenesis *in vitro*.

The results presented within highlight the potential of applying advances in semisynthesis of proteins approaches to dissect the role of post-translational modifications in modulating α-syn function in health and disease. Current efforts in our laboratories are focused on elucidating the effect of ubiquitination at other lysine residues as well as other post-translational modifications and extending our studies to examine the consequences of these modifications on the subcellular localization, life-time, and toxicity of α-syn in cellular models of synucleinopathies.

III.7. Supporting information

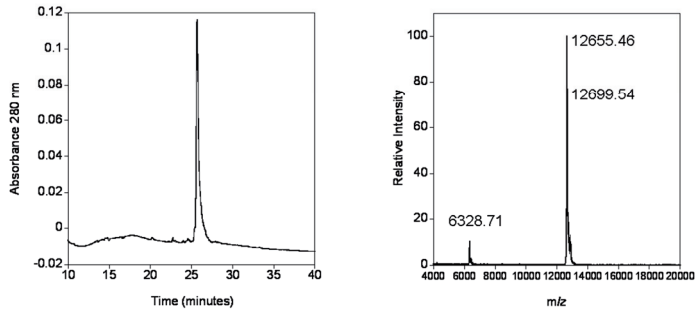

Figure S III-1. Analysis of α-syn fragment A19C-140
Analytical RP-HPLC and MALDI-Tof analysis of the purified N-terminal truncated α-syn fragment A19C-140. The purity of the protein was analyzed by RP-HPLC (C4 column) using a gradient of acetonitrile/0.1%TFA 5% to 80% over 30min. The MALDI spectrum shows the observed mass 12657 and 12699 Da (calc. m/z 12631 Da) and the double charged 6332 Da. The addition of +26 and +70Da corresponds to the formation of thiazolidine adducts that form upon the spontaneous protection of the N-terminal cysteine residue during protein expression in *E. Coli*.

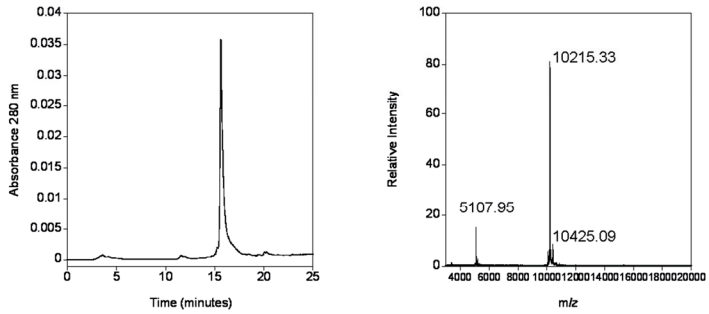

Figure S III-2. Analysis of T7-ubiquitin-thioester
Analytical RP-HPLC and MALDI-Tof of purified T7-ubiquitin-thioester. The purity of the protein was analyzed by RP-HPLC (C4 column) using a gradient of acetonitrile/0.1%TFA 5% to 80% over 30min. The MALDI spectrum shows the observed mass 10215 Da (calc. m/z 10216 Da) and the double charged 5107 Da.

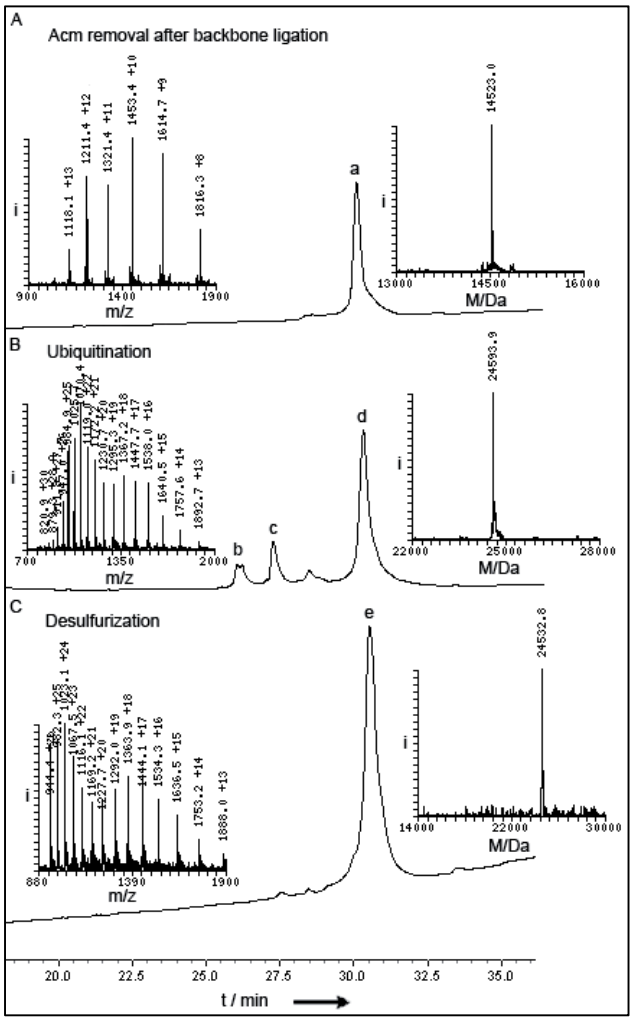

Figure S III-3. Analytical data on the semisynthesis of monoubiquitinated α-Syn
A) Analytical HPLC of the Acm removal reaction after 15 hr ; peak a corresponds to the desired product with the observed mass 14523 Da (calcd m/z 14524 Da); B) Analytical HPLC of the ubiquitination reaction after 12 hr; peak b corresponds to the hydrolyzed ubiquitin thioester and the remaining expressed T7-Ub-SR (methansulfonate derivative), peak c corresponds to benzylmercaptan exchange thioester, peak d corresponds to the desired product with the observed mass 24593.9 Da (calcd m/z 24595.8 Da), ; C) Analytical HPLC of the crude desulfurization reaction peak; peak e corresponds to the desired monoubiquitinated α-Syn with the observed mass of 24532.8 Da (calcd m/z 24531.7 Da). The progress of the reaction was analyzed using RP-HPLC (C4 column) with a gradient of 5%-60% B over 30 min. Reported mass is for total protein.

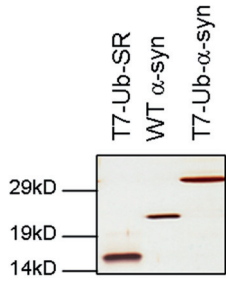

Figure S III-4. SDS-PAGE analysis of rotofor fractions.
15% SDS-PAGE silver stained gel (Invitrogen) illustrating the separation of T7-Ub-α-syn from unmodified α-syn using the micro-rotofor system. WT α-syn eluted pure in fraction 5 of the micro-rotofor chamber and mixed with T7-Ub-α-syn in fraction 6. T7-Ub-α-syn eluted pure in fraction 7.

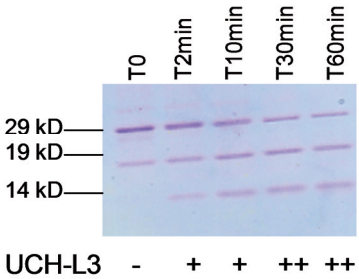

Figure S III-5. De-ubiquitination of T7-Ub-α-syn
15% SDS-PAGE gel, coomassie staining.

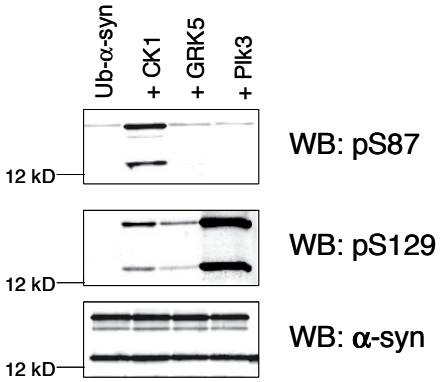

Figure S III-6. Phosphorylation of monoubiquitinated α-syn
Immunoblots of monoubiquitinated α-syn in presence of CK1, GRK5 and Plk3 or no kinase. Detection was made with anti-α-syn antibody to detect total α-syn proteins and with antibodies against phospho Ser129 and phospho Ser87.

IV. **Conclusion**

Despite more than two decades of intensive resarch on α-syn, our knowledge about its physiological functions and the mechanisms by which it contributes to neurodegeneration in PD and other synucleinopathies remains limited. That being said, the conceptual and technical advances that were made by the scientific community have certainly brought us closer to addressing this knowledge gap and are paving the way for exciting discoveries to be made in the coming years.

A better understanding of the molecular determinants that trigger α-syn aggregation is essential for developing effective therapeutic strategies to inhibit this process and treat or protected against neurodegeneration in PD and related synucleinopathies. The discovery that some of α-syn's PTM (phosphorylation at S87, S129 and ubiquitination) were present mainly in LB suggests that these modifications play important roles in inducing α-syn aggregation and LB formation. Several approaches have been applied to determine the effect of these modifications in regulating α-syn aggregation and toxicity in different animal models of synucleinopathies. However, most of these approaches relied on the use of natural mutations to mimic phosphorylation or on the co-expression of α-syn with inefficient or non-specific kinases, in the case of phosphorylation. Similarly, studies aimed at addressing the role of ubiquitination relied on the use of E3 ligases that lack specificity and ubiquitinate α-syn at multiple lysine residues. While these studies have been informative, they do not allow addressing the roles of specific modifications in regulating α-syn's biology in health and disease.

Motivated by these challenges and the desire to develop novel strategies to study post-translational modifications in neurodegenerative diseases, we sought to develop efficient methods for the generation of homogenous, site-specifically modified α-syn for the investigation of α-syn serine/tyrosine phosphorylation and ubiquitination.

Inspired by the pioneering work of Dawson, Muir and others on the native chemical ligation of proteins (section I.5.3.1), we developed two strategies based on expressed protein ligation for the introduction of PTM in the N-terminal or the C-terminal domain of α-syn that allowed us to produce milligram quantities of pY125 α-syn (section II) and monoubiquitinated α-syn at K6 (section III). These advances have allowed us to investigate, for the first time, the effects of selective phosphorylation at Y125 and ubiquitination at K6 on the structure, aggregation, membrane binding and subcellular localization of α-syn. The development of semisynthetic methods for the site-specific introduction of single or multiple PTM represents an important

advance towards determining the roles of such modifications on α-syn structure, aggregation and functions in health and disease. In addition, we were able to investigate cross-talk between phosphorylation at residues S129, Y125, and S87 by determining the relative activity of each phosphorylated form of α-syn as a substrate for the kinases that phosphorylate at the other residues. In the case of monoubiquitinated α-syn at K6, our studies demonstrated for the first time that ubiquitination, which was thought to enhance α-syn aggregation and LB formation, stabilizes instead the monomeric protein and inhibits its fibrillization. It is noteworthy that the semisynthesis of monoubiquitinated α-syn represents the second successful attempt to prepare ubiquitinated proteins *via* a native isopeptide bond.

The different strategies outlined in this thesis represent powerful methodologies that can be extended to other proteins implicated in neurodegenerative diseases (e.g. Tau, Huntingtin) and allow the introduction of site-specific chemical modifications and/or PTMs. These approaches can also be used to site-specifically introduce fluorescent probes, fluorescence resonance energy transfer (FRET) fluorophores pairs, or affinity handles to allow for selective labeling at specific residues using small probes or the covalent immobilization or cross-linking of proteins using the handles (for review: (Muir, 2003a)). Non-hydrolyzable phosphonate amino acids have been synthesized and incorporated into proteins to prevent rapid dephosphorylation at Ser or Tyr and facilitate the investigation of the consequences of phosphorylation in living cells or complex biological samples. Furthermore, the availability of semisynthetic homogeneous post-translationally modified forms of α-syn as substrates should aid in the development of assays to identify the natural enzymes (kinases, phosphatases, E3 ligases, deubiquitinases) involved in the regulation of these modifications and will facilitate the development of quantitative assays to measure the levels of these modification during disease progression. These studies are likely to lead to the identification of novel therapeutic targets for the treatment of PD and the development of diagnostic tools for early detection and monitoring of neurodegeneration in PD and other neurodegenerative diseases.

Future Directions

We are currently applying our semisynthetic strategy for the generation of monoubiquitinated α-syn at K12 and K23 and, taking advantage of the native isopeptide bond in our system, we are investigating the effect of monoubiquitination at K12 on the degradation of α-syn by the proteasome, in collaboration with Aaron Ciechanover's group. Preliminary data using purified proteasomes reveal an ubiquitination-dependent proteasomal degradation of α-syn (Shabek et

al., 2012), in contrast to previous reports which suggested that unmodified α-syn can be degraded by the proteasome (Bennett et al., 1999, Tofaris et al., 2001, Machiya et al., 2010). Recent advances and novel synthetic tools developed by our collaborators in Ashraf Brik's group have enabled for the first time the generation of diubiquitin and tetraubiquitin chains by total chemical synthesis. These advances have enabled the generation of tetraubiquitinated α-syn through K48 linkage, which is currently being investigated as part of a joint collaborative project between the two groups. The ability to investigate mono and poly-ubiquitinated forms of α-syn will eventually allow us to decipher the code of α-syn ubiquitination.

The next challenge that is currently being tackled by our group involves the development of efficient strategies for the delivery of semisynthetic and chemically synthesized α-syn into living cells to assess the effect of PTM on the cellular properties of α-syn in their natural environment. Our laboratory is currently investigating the use of cell-penetrating peptides to facilitate the delivery of the proteins into cells. TAT-peptide fusion to α-syn has been previously shown to transduce efficiently in cell-based and animal models (Albani et al., 2004, Recchia et al., 2008). If successful, our method, combining chemistry, biochemistry and biology, would definitely pave the way to the systematic study of all α-syn PTM *in vivo*.

To expand our methodology for cell-based and animal applications for the study of cross-talks between phosphorylation at these two residues, we were inspired from the seminal work of Imperiali's group who reported the synthesis of two photocleavable protecting groups of phosphorylated residues that can be cleaved using different wavelengths (Goguen et al., 2011). We thus plan to introduce photocaged phosphorylated serine and tyrosine residues that can, through irradiation at 420 and 365 nm respectively, be exposed sequentially. Moreover, the use of other photo-sensitive amino acid derivatives is very appealing since the near-UV wavelengths that are commonly used do not alter the cell's DNA. A large panel of photo-caged and photo-crosslinking amino acids can thus be used in cell culture and animal studies. The latter could furthermore benefit from the use of two-photon excitable photosensitive amino acids, due to the deeper tissue penetration of the lower-wavelength light used.

In summary, now that the methodology has been developed for the introduction of any α-syn modification at any residue, its potential will be best harvested from the generation of tailor-made variants to answer specific questions. This can only be achieved through the optimization of methodologies to introduce these proteins into cells and animal brains along with

development of novel chemistries for the modification of specific residues; and most importantly through the fruitful interactions between scientists from different disciplines.

While semisynthetic approaches were previously used for the study of enzymes and other proteins (Muir, 2003a), the field is just starting to bloom for amyloid proteins and proteins involved in neurodegenerative diseases with recent reports of the semisynthesis of the Prion protein (Olschewski et al., 2007, Becker et al., 2008, Olschewski and Becker, 2008), Tau (Broncel et al., 2012a) and Amyloid−β (Bockhorn et al., 2010). We hope that the methodology will quickly expand to other laboratories for the study of proteins involved in neurodegeneration and that the shadows masking the impact of the different PTM on the pathogenesis of neurodegenerative disorders will be removed.

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VI. **Table of Figures**

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- Shabek N., Herman-Bachinsky Y., Buchsbaum S., Lewinson O., Haj-Yahya M., Hejjaoui M., Lashuel H., Sommer T., Brik A., Ciechanover A., **Molecular Cell**, 2012 (in press)
- Butterfield S., Hejjaoui M., Fauvet B., Awad L., Lashuel H., Chemical Strategies for Controlling Protein folding and Elucidating the Molecular Mechanisms of Amyloid Formation and Toxicity, **Journal of Molecular Biology**, 2012, 421(2-3)
- Hejjaoui M., Butterfield S., Fauvet B., Vercruysse F., Cui J., Dikiy I., Prudent M., Olschewski D., Zhang Y., Eliezer D., Lashuel H., Elucidating the role of C-terminal post-translational modifications using protein semisynthetic strategies: α-synuclein phosphorylation at tyrosine 125, **Journal of the American Chemistry Society**, 2012, 134(11).
- Hejjaoui M., Haj-Yahya M., Kumar A., Brik A. and Lashuel H., Towards Elucidation of the Role of Ubiquitination in the Pathogenesis of Parkinson's Disease with Semisynthetic Ubiquitinated α-Synuclein, **Angewandte Chemie International Edition**, 2011, 50(2)

Poster presentations

Society of Neuroscience — **Washington, 2011**
22nd American peptide symposium — **San Diego, 2011**
4th International peptide symposium — **Kyoto, 2010**
EPFL-ETH-Tokyo tech joint symposium — **Lausanne, 2010**

Oral presentation

Brain Mind institute — **Lausanne, 2012**

Professional Experiences

Swiss Federal Institute of Technology Lausanne EPFL **Lausanne, Switzerland**
Doctoral assistant 10.08-09.12
Advisor: Prof. Hilal Lashuel

- Pioneered the design of efficient strategies for the semisynthesis of α-synuclein to elucidate the impact of post-translational modifications on aggregation, structure and subcellular localization
- Supervised the projects of undergraduate students and assisted in teaching courses

Sanofi-Aventis Pharmaceuticals **Labège, France**
Research internship 03.08-08.08
Advisor: Dr. Francois Autelitano

- Developed a protocol for the identification of proteins interacting with Lymphotoxin β receptor
- Work resulted in the identification of novel target proteins for treatment of inflammatory diseases and allowed for the present continuation of the project by colleagues at Sanofi-Aventis

Harvard Medical School **Boston, USA**
Research assistant 06.07-09.07
Advisor: Prof. Charles Richardson/Dr.Udi Qimron

- Optimized a high-throughput screening assay on a generated library of 7000 transductant strains to detect for resistance to phage infection.
- In 3 months, delivered a system typically requiring more than 6 months and identified the bacterial gene responsible for the resistance

Swiss Federal Institute of Technology Lausanne EPFL **Lausanne, Switzerland**
Summer Research Scholar 07.06-08.06
Advisor: Prof. Hilal Lashuel

- Optimized an assay for the limited proteolysis of α-synuclein bound to membranes still used by an entire core facility at the EPFL

Technical Skills

Chemistry: Solid phase peptide synthesis, Native chemical ligation.

Molecular biology: Single-point mutagenesis, Classical cloning, PCR.

Biochemistry: Expression of proteins in E.Coli, Purification of proteins using: affinity chromatography, anion and cation exchange, reverse-phased chromatography.

Biophysics: Protein oligomerization studies, Circular dichroism

Cellular biology: Cell culture, transfection using calcium phosphate and lipofectamine, subcellular fractionation, immunoprecipitation, immunocytochemistry.

Microscopy: Transmission electron microscopy, Wide-field fluorescence microscopy

Leadership Activities & Personal Strength

Swiss Federal Institute of Technology EPFL **Lausanne, Switzerland**
Representing the EPFL at Beirut Science Fair 2010-2011

- Launched a collaboration between the fair organizers and the EPFL direction
- Budgeted the project, organized the participation of 4 students and prepared materials for kids

Ecole Nationale Supérieure de Chimie de Montpellier **Montpellier, France**
Head of the Gala society 03.06-12.07

- Organized the prestigious graduation of my peers (over 500 attendees)
- Supervised a team of 15 persons and managed the financial budget (around 20,000 □)

Interests: Swimming, basket-ball (ENSCM team 2005), karate Shotokan, salsa dance and travelling

Languages: French (bilingual), Arabic (bilingual), English (fluent), German (basic)