Scuola Internazionale Superiore di Studi Avanzati - Trieste

Molecular approaches to study the function and dysfunction of α-synuclein.

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1.1 Parkinson's Disease

1.1.1 Brief history, clinical symptoms and etiology of PD

Parkinson's disease (PD) is a slowly progressing degenerative disorder of the central nervous system which affects the motor functions. Its cardinal symptoms are *bradykinesia*, resting tremor, postural instability, and/or rigidity.

It was first described in the Western world in 1817 by James Parkinson, a British physician who published an essay on what he called "the shaking palsy".

Long before that, accounts of a clinical syndrome consisting of tremor and *akinesia* were present in Indian medical Ayurvedic texts written more than 3000 years ago (Manyam, 1990) (Katzenschlager *et al.*, 2004). Remarkably, this syndrome, termed *kampavata* in Sanskrit, was treated with natural products from *Mucuna pruriens*, a plant now known to contain levodopa (L-DOPA), the drug that revolutionized PD therapy in the mid-20th century.

The biochemical changes underlying the disease were elucidated in the 1950s: the discovery of dopamine as a neurotransmitter in the brain by Arvid Carlsson, and the subsequent insight provided by Paul Greengard into the cellular signaling mechanisms triggered by dopamine, gained these researchers the Nobel Prize for Medicine in 2000.

The discovery of dopamine was followed very quickly by reports of markedly depleted levels of dopamine in the basal ganglia of individuals dying from Parkinson's disease. Ehringer and Hornykiewicz (Ehringer and Hornykiewicz, 1960) in Vienna were the first to report such findings in 1960, and Birkmayer and Hornykiewicz (Birkmayer and Hornykiewicz, 1961) published the first positive results with L-DOPA treatment one year later. Cotzias (Cotzias, 1968) in the USA developed the first practical dosage regime for the routine use of L-DOPA in the treatment of Parkinson's disease – still one

of the major achievements in the rational development of novel therapies for neuropsychiatric diseases.

Despite these breakthroughs, society still pays an enormous price for PD, since L-DOPA therapy is only symptomatic and is able to slow but not to reverse the course of the disease.

At least 220,000 people in Italy currently have PD, although some estimates are much higher. The annual cost of a patient to the nation is estimated around 8000 euros. The risk of PD increases with age, so analysts expect the financial and public health impact of this disease to increase as the population gets older. The average age of onset is 60 years, and the prevalence rises significantly from 0.3 % in the entire population to about 1% in people over 60 years old and up to 5% in over 85 years old (de Lau and Breteler, 2006). However, about 5 to 10 percent of people with PD have "early-onset" disease that begins before the age of 50. In very rare cases, parkinsonian symptoms may appear before the age of 20 (juvenile parkinsonism).

While PD occurs throughout the world, a number of studies have found a higher incidence in developed countries, possibly because of increased exposure to environmental toxins. An increased risk in people who live in rural areas or work in certain professions has been observed, although these studies are not conclusive and the reasons for the increased risks are not clear.

The idea that Parkinson's disease might be caused by chemicals in the environment got a major boost in 1982 when Dr. J. William Langston discovered several young people who developed Parkinson's symptoms literally overnight after using tainted heroin (Langston *et al.*, 1983). He found that the symptoms were caused by a contaminant called MPTP, which bears a strong chemical similarity to many pesticides and other environmental chemicals.

The environmental toxin hypothesis was dominant for most of the 20th century, and until 10 years ago there was considerable skepticism about the existence of a genetic etiology of PD.

The report of the first pathogenic SNCA mutation in familial PD in 1997 (Polymeropoulos *et al.*, 1997), together with the finding that the gene's product α-synuclein is the principal component of filamentous Lewy bodies (the neuropathological hallmark of the disease) (Spillantini *et al.*, 1997), brought a second revolution in the field. Since then, about ten additional genes and genetic *loci* have been implicated as causes of Lewy body PD or other familial Parkinson syndromes.

1.1.2 Neuropathological features

The pathologic examination of brains from PD patients demonstrates depigmentation of the substantia nigra (SN), caused by the selective and progressive loss of dopaminergic neurons projecting in the striatum, and the presence of intraneuronal proteinaceous inclusions known as Lewy bodies within the surviving neurons of the SN and other brain regions (dorsal motor nucleus of the vagus, locus ceruleus, raphe and reticular formation nuclei, thalamus, amygdala, olfactory nuclei, pediculopontine nucleus, and cerebral cortex, among others). These inclusions are enriched in filamentous α-synuclein and other proteins that are often highly ubiquitinated (Fig. 1).
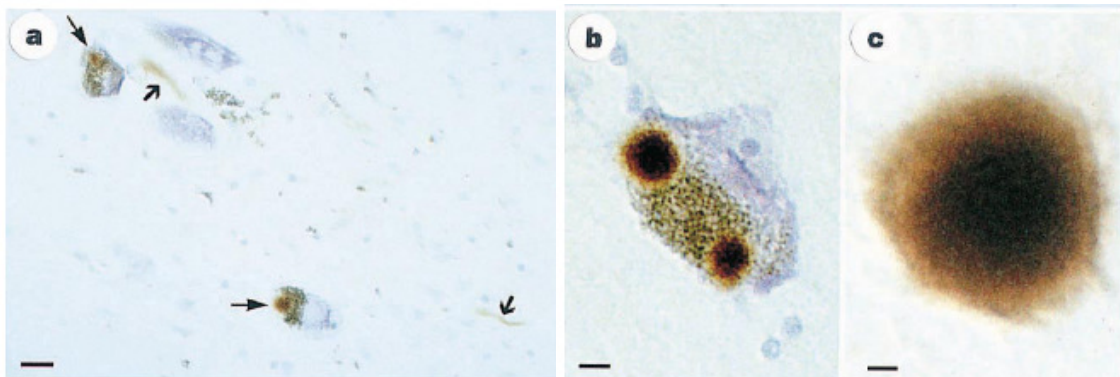
Figure 1. Substantia nigra from patients with Parkinson's disease (from the MRC Cambridge Brain Bank) immunostained for α-synuclein. **a**, Two pigmented nerve cells, each containing an α-synuclein positive Lewy body (thin arrows). Lewy neurites (thick arrows) are also immunopositive. Scale bar, 20 mm. **b**, A pigmented nerve cell with two α-synuclein-positive Lewy bodies. Scale bar, 8 mm. **c**, α-synuclein positive, extracellular Lewy body. Scale bar, 4 mm. (From Spillantini *et al.*, 1997).

Approximately 60-80% of dopaminergic neurons are lost before the motor signs of PD emerge.

Depletion of dopamine within the striatum causes dysregulation of the motor circuits that project throughout the basal ganglia, resulting in the clinical manifestations of PD.

1.1.3 Motor circuits in Parkinson disease

The basal ganglia motor circuit modulates cortical output necessary for normal movement: signals from the cerebral cortex are processed through the basal ganglia-thalamocortical motor circuit and return to the same area via a feedback pathway.

The basal ganglia are composed of four principal nuclei: the striatum, the globus pallidus (GP) consisting of external and internal segments (GPi and GPe), the substantia nigra (SN) consisting of pars compacta and reticulata (SNc and SNr), and the subthalamic nucleus (STN).

The striatum is the major recipient of input to the basal ganglia from the cerebral cortex, thalamus and brainstem. Its neurons project to the GP and SNr. The output nuclei of the basal ganglia are GPi and SNr. Their inhibitory output is directed to the thalamocortical pathway and suppresses movement.

Two pathways exist within the basal ganglia circuit, referred to as the direct and indirect pathways.

In the direct pathway, outflow from the striatum directly inhibits the GPi and the SNr, resulting in an activation of the thalamus and ultimately the cortex, thus facilitating movement. The indirect pathway comprises inhibitory connections between the striatum and the GPe and between the GPe and the STN. The subthalamic nucleus exerts an excitatory influence on the GPi and SNr. Activation of the indirect pathway increases inhibition of the thalamus and cortex, thus inhibiting movement. Striatal neurons containing D1 receptors constitute the direct pathway and project to the GPi/SNr. Striatal neurons containing D2 receptors are part of the indirect pathway and project to the GPe.

Dopamine is released from nigrostriatal (SNc) neurons and, through its different effect on D1 and D2 receptors, activates the direct pathway and inhibits the indirect pathway, facilitating movements.

In PD, decreased striatal dopamine causes increased inhibitory output from the GPi/SNr. This increased inhibition of the thalamocortical pathway suppresses movement. Via the direct pathway, decreased striatal dopamine stimulation causes decreased inhibition of the GPi/SNr. Via the indirect pathway, decreased dopamine inhibition causes increased inhibition of the GPe, resulting in disinhibition of the STN. Increased STN output increases GPi/SNr inhibitory output to the thalamus (Fig. 2).

Basal ganglia circuitry in normal conditions   **Basal ganglia circuitry in PD**
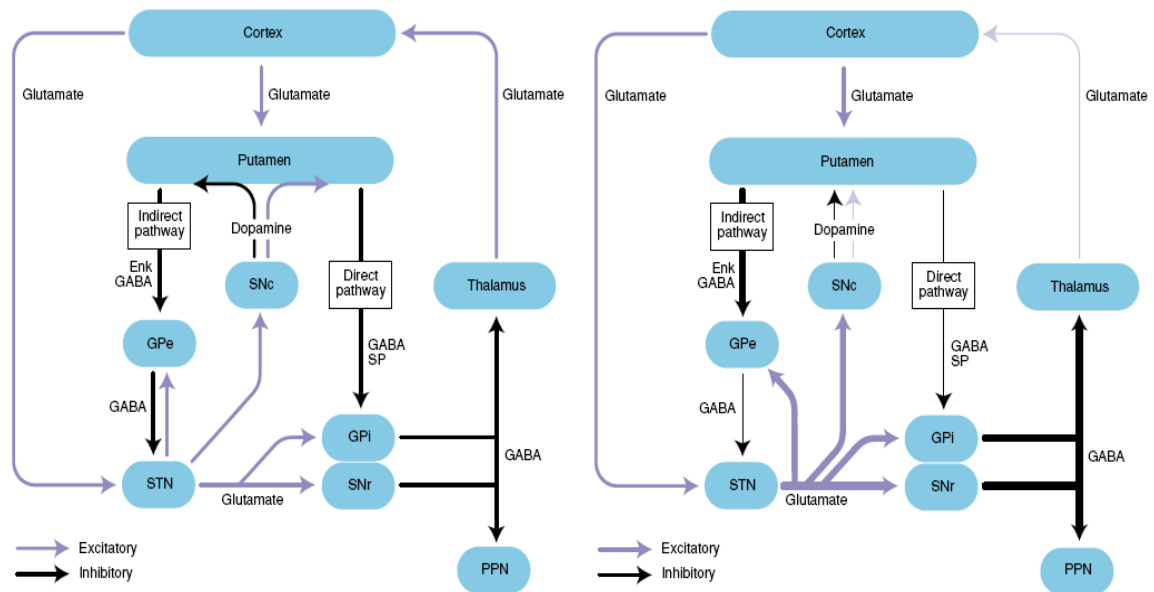
Figure 2. Basal ganglia circuitry in normal conditions (left) and in Parkinson's disease (right). Adapted from: Expert Reviews in Molecular Medicine, 2003, Cambridge University Press.

1.1.4 The PARK genes

Familial cases of Parkinson's disease account for about 10% of the total number of cases. To date 13 forms of familial PD have been mapped to different chromosome *loci*, of which 8 genes have been identified as the causative genes, i.e., α-synuclein (SNCA), parkin, UCH-L1, PINK1, DJ-1, LRRK2, ATP13A2 and OMI/HtrA2 (Table 1). For UCH-L1, additional families with this mutation are needed before concluding that UCH-L1 is the definite causative gene for PARK5, as only one family so far has been reported. SNCA, UCH-L1, and LRRK2 mutations cause autosomal dominant PD and the remaining gene mutations autosomal recessive PD. Age of onset tends to be younger in familial PD compared with sporadic PD, particularly so in autosomal recessive PD.

Name	Locus	Gene	Function	Inheritance	Lewy Bodies
PARK1	4q21-23	α-synuclein	Unknown	AD	yes
PARK2	6p25.2-27	Parkin	E3 ubiquitin ligase	AR	no
PARK3	2p13	Unknown	-	AD	yes
PARK4	4p14-16.3	α-synuclein	Unknown	AD	yes
PARK5(?)	4p14	UCH-L1	Ubiquitin ligase and hydrolase	AD	?
PARK6	1p35-36	PINK-1	Mitochondrial Ser-Thr kinase	AR	?
PARK7	1p36	DJ-1	Chaperone/ Transcription regulator	AR	?
PARK8	12p11.2-13.1	LRRK2	Multi domain kinase	AD	yes
PARK9	1p36	ATP13A2	Lysosomal type 5 P-type ATPase	AR	?
PARK10	1p32	Unknown	-	NP	?
PARK11	2q36-37	Unknown	-	AD	?
PARK12	Xq23-25	Unknown	-	XR	?
PARK13	2p13	Omi/HtrA2	Serine protease	AD?	?

Table 1. Genetic *loci* identified in familial PD.

1.1.5 Pathogenesis of PD

Fundamental clues to understand PD pathogenesis arise from the analysis of PD patients *post mortem* brains, studies of animal models of the disease and investigations of the function of the familial PD genes. The main hypotheses indicate misfolding and aggregation of proteins and/or mitochondrial dysfunction and oxidative stress as the culprits of the demise of the dopaminergic neurons in the brain of PD patients.

Misfolding and aggregation of proteins

A common feature of neurodegenerative disorders is the aggregation and accumulation of misfolded proteins in inclusions. Those are present in specific populations of neurons and in brain areas physiologically linked to the observed clinical manifestations. Each disorder has a unique histopathological signature defined by the constituent protein and the distribution and cellular localization of the deposits. The contribution of these aggregated proteins to neuronal death has been debated; for example it has been proposed that the nuclear deposition of huntingtin in Huntington's disease may be neuroprotective, while soluble oligomeric forms of the amyloid β peptide found in senile plaques in Alzheimer's disease are thought to be the primary cytotoxic species in the disease. PD and other synucleinopathies (DLB, MSA) are characterized by the accumulation of filamentous aggregates of AS in Lewy bodies, which co-stain with ubiquitin, pointing to a role of the ubiquitin-proteasome system in the disease. Selective loss of the 20S subunit and of the functional activity of the proteasome is also evident in PD *post mortem* brains. Beside AS, other genes involved in the pathogenesis are closely linked to the degradation system: UCH-L1 gene, mutated in rare familial cases of PD and also found in Lewy bodies of sporadic PD, encodes an ubiquitin hydrolase and ligase, while Parkin, the most common cause of familial cases of PD, appears to function as an ubiquitin E3 ligase.

Parkin is a 465 amino acid protein containing two RING fingers separated by an in-between RING (IBR) domain at the carboxyl terminus. The amino terminus bears a ubiquitin-like domain that binds to the RPN10 subunit of the 26S proteasome. It has been proposed that parkin dysfunction might lead to the toxic accumulation of its substrate. Many disparate substrates for parkin have been discovered, among which the septin CDCREL1 and PAEL-R (von Coelln *et al.*, 2004). Over-expression of CDCREL1 and PAEL-R *in vivo* mediates dopaminergic neurodegeneration (Dong *et al.*, 2003; Yang *et al.*, 2003), and both also accumulate in the brains of patients with parkin-related autosomal recessive Parkinson's disease (ARPD) (Imai *et al.*, 2001; Choi *et al.*, 2003), but not in *Drosophila* or mammalian parkin knockout models. By contrast, the parkin substrate aminoacyl-tRNA synthetase cofactor p38 is upregulated in the midbrain of parkin null mice as well as in the brains of patients with ARPD and idiopathic PD and its adenovirus-mediated over-expression in the substantia nigra of mice induces loss

of dopaminergic neurons (Ko *et al.*, 2005). Interestingly, it has been observed that parkin can rescue neurons from α-synuclein-induced proteasomal dysfunction (Petrucelli *et al.*, 2002).

Mitochondrial dysfunction

Mitochondrial dysfunction has long been implicated in the pathogenesis of PD.
An important feature of the PD *post mortem* brains is a reduction of mitochondrial complex I activity, discovered in 1983 when the accidental exposure of drug abusers to MPTP resulted in acute and irreversible parkinsonian syndromes (Langston *et al.*, 1983). MPTP inhibits the first enzyme complex of the mithochondrial electron-transfer chain. After systemic administration, it crosses the blood brain barrier in minutes and, once in the brain, is oxidized to $MPDP^+$ by monoamine oxidase B (MAO-B) in glia and serotoninergic neurons. It is then converted to the active toxic molecule MPP^+, probably by spontaneous oxidation. MPP^+ is a high affinity substrate for DAT and for norepinephrine and serotonin transporters. It therefore accumulates in dopaminergic neurons, where it confers toxicity and cell death through complex I inhibition and the resulting energy crisis and generation of free radicals. Furthermore, other environmental neurotoxins and inhibitors of complex I such as rotenone and paraquat are also able to induce dopaminergic loss. Indeed, rotenone-administered rats develop a PD-like syndrome characterized by neuronal degeneration and the formation of α-synuclein-rich inclusion bodies.
Recently, strong evidences in support of the role of mithochondrial dysfunction in PD came from the discovery of some of the familial genes: although α-synuclein and parkin mutations confirm that protein misfolding and UPS dysfunction play a major role in dopaminergic degeneration, these genes seem to be also implicated in mitochondrial dysfunction. Furthermore, the discovery of PINK1, HtrA serine peptidase 2 (Omi/HtrA2) and DJ-1 mutations confirmed that mitochondrial dysfunction is a main upstream pathway to parkinsonism.

AS has been found to interact with cytochrome C oxidase (complex IV), the last protein in the mitochondrial electron transport chain, in a yeast two-hybrid screening (Elkon *et al.*, 2002). In transgenic mice, over-expression of AS impairs mitochondrial function, increases oxidative stress and enhances nigral pathology induced by MPTP (Song *et al.*,

2004a). Moreover, in a recent study of mice over-expressing A53T mutant α-synuclein, degenerating mitochondria were immunostained for AS, raising the possibility that mutant AS might damage mitochondria directly (Martin *et al.*, 2006). Whereas over-expression of α-synuclein increases sensitivity to MPTP, AS null mice are resistant to MPTP (Fornai *et al.*, 2005) as well as to other mitochondrial toxins such as malonate and 3-nitropropionic acid (Klivenyi *et al.*, 2006). Thus, AS seems to mediate some of the toxic effects of MPTP.

Parkin deficiency or mutations have been shown to lead to oxidative stress and mitochondrial dysfunction. Leukocytes from individuals with parkin mutations present a selective impairment in complex-I activity (Muftuoglu *et al.*, 2004). Parkin can associate with the outer mitochondrial membrane and prevent mitochondrial swelling, cytochrome C release and caspase activation, and this protective effect is abrogated by proteasome inhibitors and parkin mutations (Darios *et al.*, 2003). Parkin has also been localized to mitochondria in proliferating cells, where it has been shown to associate with mitochondrial transcription factor A and to enhance mitochondrial biogenesis (Kuroda *et al.*, 2006).

A *Drosophila* model has revealed a role for parkin in maintaining mitochondrial function and preventing oxidative stress. Parkin null mutants had severe mitochondrial pathology associated with reduced lifespan, apoptosis, flight muscle degeneration and male sterility (Greene *et al.*, 2003). Microarray analysis in these flies revealed upregulation of genes involved in oxidative stress and electron transport, including a homologue of the mammalian peripheral benzodiazepine receptor, which has been linked to the regulation of mitochondrial integrity and is thought to be involved in the regulation of mitochondrial swelling, respiration, transmembrane potential and the prevention of oxidative damage to the mitochondria. Genes involved in the innate immune response were also induced in parkin mutants. A genomic screen for modifiers of lifespan in the parkin null flies found the strongest modifier to be loss-of-function mutations of glutathione S-transferase (GSTS1) (Greene *et al.*, 2005). Reanalysis of the same flies revealed progressive degeneration of a select cluster of dopaminergic neurons and evidence of increased oxidative damage with increased protein carbonyls compared with controls. Furthermore, neurodegeneration was enhanced in GSTS1 null mutants, whereas GSTS1 over-expression significantly rescued the parkin phenotype (Whitworth *et al.*, 2005).

Mammalian models also support a role for parkin in maintaining mitochondrial function. Deletion of exon 3 of parkin in mice results in nigrostriatal dysfunction and reduced expression of several proteins involved in mitochondrial function and oxidative stress, including subunits of complexes I and IV. The mice had also decreased mitochondrial respiratory capacity and showed evidence of increased oxidative damage (Palacino *et al.*, 2004). Intriguingly, parkin deficiency in these mice did not cause dopaminergic degeneration, which is also observed in exon 2 and other exon 3 deletion models (Goldberg *et al.*, 2003; Itier *et al.*, 2003; Perez and Palmiter, 2005). It will therefore be interesting to determine whether neurodegeneration in these models requires an environmental insult such as an oxidative stressor.

Very recently, several mono- and double-mutant mouse lines expressing high levels of doubly mutated human AS and/or a targeted deletion of Parkin have been generated. These mice show genotype-, age- and region-dependent morphological alterations of mitochondria in neuronal somata, which are restricted to the brain and lacking in skeletal muscle cells. These alterations are not accompanied by alterations of the number or the size of the mitochondria or by leakage of cytochrome C, Smac/DIABLO or Omi/HtrA2, but in the SN coincide with a reduced complex I capacity. None of the transgenic animals developed overt motor disabilities or any gross histopathological abnormalities in the analyzed brain regions.

PINK1 encodes a ubiquitously expressed 581 amino acid protein, which consists of an N-terminal mitochondrial targeting motif, a highly conserved serine/threonine kinase domain and a C-terminal autoregulatory domain. Loss of PINK1 function adversely affects mitochondrial function and cell viability under stress. Mitochondrial membrane potential ($\Delta\psi$m) and levels of cell death were measured in a neuroblastoma cell line over-expressing G309D PINK1 after exposure to an exogenous source of cellular stress, MG-132, a proteasome inhibitor. Cells over-expressing G309D PINK1 had significantly reduced $\Delta\psi$m compared with the wild type and increased levels of cell death following exposure to stress, but not under basal conditions. Moreover, cells over-expressing wild-type PINK1 had higher $\Delta\psi$m and lower levels of cell death than cells transfected with vector alone (Valente *et al.*, 2004). Consistent with these results, over-expression of wild-type PINK1 was subsequently shown to reduce the release of cytochrome C from mitochondria under basal conditions and staurosporine-induced stress.

Omi/HtrA2 protein has been previously implicated in neurodegeneration, and recently associated with predisposition to PD (Strauss *et al.*, 2005). It is a PDZ domain-containing serine protease, and also contains an N-terminal mitochondrial targeting motif and a reaper-like motif. Omi/HtrA2 is thought to localize to the mitochondrial inter-membrane space, where it is released into the cytosol during apoptosis to relieve the inhibition of caspases by binding to inhibitor of apoptosis proteins (IAPs). Omi/HtrA2 is also able to induce cell death through its proteolytic activity. OMI/ HTRA2 knockout mice have been shown to display parkinsonian phenotypes, including rigidity and tremor (Martins *et al.*, 2004). Mutations or polymorphisms found in sporadic PD cases have been shown to increase susceptibility to stress, as shown by decreased $\Delta\psi$m after exposure to staurosporine. It is therefore tempting to speculate that PINK1 and Omi/HtrA2 share a common pathway in the mitochondrial response to cellular stress and modulation of apoptosis.

DJ-1 is a multifunctional member of the ThiJ/PfpI/DJ-1 superfamily. Its subcellular distribution of is primarily cytoplasmic with a smaller pool of mitochondrial- and nuclear-associated protein (Zhang *et al.*, 2005).

DJ-1 mutations disrupt protein activity by either destabilizing the protein or affecting its subcellular localization. The L166P, M26I and D149A mutations all show reduced nuclear localization in favour of mitochondrial localization (Bonifati *et al.*, 2003; Xu *et al.*, 2005). As the mitochondrial function of DJ-1 remains to be determined, it is not clear whether the toxicity associated with increased mitochondrial localization is due to a mitochondrial gain of function or to a loss of access to binding partners in different cellular compartments.

Interestingly, the complex multi-domain protein LRRK2, recently shown to cause auto-somal dominant PD in 5-6% of cases with a positive family history, might also be associated with the outer mitochondrial membrane (OMM) (West *et al.*, 2005; Gloeckner *et al.*, 2006) and is able to bind parkin (Smith *et al.*, 2005b).

Oxidative stress

A most noticeable feature of the PD *post mortem* brains is the presence of oxidative stress: elevated levels of lipid peroxidation markers such as HNE and malondialdehyde,

evidences of oxidative damage to proteins (such as nitration of tyrosine residues) and nucleic acids, reduced levels of glutathione and oxidized glutathione. Inhibition of complex I increases the production of reactive oxygen species (ROS) which can react with proteins, nucleic acids and lipids causing cellular damage. Dopaminergic neurons may be a particularly fertile environment for the generation of ROS, as the metabolism of dopamine (DOP) produces hydrogen peroxide and superoxyde radicals, and auto oxidation of dopamine produces DOP quinone and other species which can react with proteins and damage them. Mitochondrial dysfunction may disrupt vesicular storage of DOP, causing the free cytosolic concentration of DOP to rise allowing harmful DOP-mediated reaction to damage cellular macromolecules.

Among a variety of environmental factors potentially toxic to nigral cells, iron is supposed to play an important role in the pathogenesis of PD (Gotz *et al.*, 2004). It is generally accepted that iron accumulates in the brain as a function of age. However, this process is quite specific and involves the accumulation of iron-containing molecules in certain cells, particularly in brain regions that are preferentially targeted in neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. An increase in total iron concentration in the substantia nigra in PD cases has been reported consistently (Gotz *et al.*, 2004). Iron is an essential cofactor for many proteins that are involved in the normal function of neuronal tissue, such as the non-heme iron enzyme tyrosine hydroxylase, which is required for dopamine synthesis. Large amounts of iron are sequestered in neuromelanin granules in the dopaminergic neurons of the substantia nigra and the noradrenergic neurons of the locus ceruleus. Neuromelanin is synthesized by the oxidation of excess cytosolic catechols that are not accumulated in synaptic vesicles by vesicular monoamine transporter-2 (VMAT2) and binds iron avidly.

Iron imbalance contributes to the enhanced generation of ROS. The ferrous iron (Fe II) catalyzes the conversion of hydrogen peroxide into a hydroxide ion and a hydroxyl free radical with the concurrent oxidation of ferrous iron to ferric iron.

In neuroblastoma cells, iron has been shown to produce oxidative stress (Sangchot *et al.*, 2002) and to contribute to the aggregation of α-synuclein. In the presence of iron and free-radical generators, such as dopamine or hydrogen peroxide, BE-M17 human neuroblastoma cells that over-express wild-type, A53T or A30P AS produce intracellular aggregates that contain AS and ubiquitin (Ostrerova-Golts *et al.*, 2000). This is prevented by the action of the iron chelator desferrioxamine. Such aggregates disturb the

cytosolic environment and interact with vesicles and their dopamine transporters and intraneuronal mitochondria, and these disturbances might result in activation of cell-death cascades. Furthermore, mutant AS enhances vulnerability to the ferrous iron in the neuroblastoma cells suggesting close relationships between iron and AS metabolism. *In vitro* fibrillization studies also indicate that iron accelerates AS aggregation (Uversky *et al.*, 2001) and that iron-catalyzed oxidative reactions mediated by the heme-containing cytochrome c and hydrogen peroxide might be crucially involved in promoting AS aggregation, a process that is inhibited by desferrioxamine (Hashimoto *et al.*, 1999).

The familial gene DJ-1 has been ascribed various biological and biochemical functions (Nagakubo *et al.*, 1997; Hod *et al.*, 1999), including a potential role in oxidative stress response, either as a redox sensor or antioxidant protein (Mitsumoto and Nakagawa, 2001; Canet-Aviles *et al.*, 2004). In mammalian cells exposed to an oxidative stressor, such as paraquat or H_2O_2, DJ-1 undergoes an acidic shift in pI-value by modifying its cysteine residues, which quench ROS and protect cells against stress-induced death. Its *Drosophila* homologues DJ-1α and DJ-1β are also oxidized at their cysteine residues after exposure to the same stresses both *in vivo* and in culture (Yang *et al.*, 2005; Meulener *et al.*, 2005).

Further evidence has been obtained from various model systems. Mammalian cell cultures, and mouse and *Drosophila* knockouts all indicate that the ablation of functional DJ-1 either by small interfering RNA (siRNA) or gene deletion sensitizes cells to oxidative stress. These cells can be rescued by over-expression of wild-type but not mutant (L166P) DJ-1 (Yokota *et al.*, 2003). Therefore, loss of DJ-1 increases levels of intracellular ROS and increases susceptibility to dopaminergic neuron degeneration *in vivo* following exposure to exogenous sources of oxidative stress (Kim *et al.*, 2005). However, it is unlikely that DJ-1 exerts its protective function through simple antioxidation. Its ability to quench ROS is modest (Yang *et al.*, 2005; Junn *et al.*, 2005), and several lines of evidence indicate that it is more likely implicated in the regulation of apoptosis. It is plausible that, through modification of its cysteine residues on exposure to H_2O_2, DJ-1 is acting as a sensor of cellular ROS levels and an effector of apoptotic/survival pathways. DJ-1 has also been shown to function as a redox sensitive molecular chaperone that is capable of preventing the aggregation of α-synuclein and of the neurofilament subunit NFL (Shendelman *et al.*, 2004).

1.2 α-synuclein

The gene encoding α-synuclein (SNCA) was the first causative gene of familial PD to be identified in 1997 (Polymeropoulos *et al.*, 1997). Three missense mutations (A53T, A30P and E46K) in the SNCA gene, as well as duplication and triplication of the locus containing the SNCA gene, have been identified to date in familial forms of PD (Singleton *et al*., 2003; Chartier-Harlin *et al*., 2004; Kruger *et al*., 1998; Zarranz *et al*., 2004). Patients with gene duplications have a less severe phenotype and a later age of onset than those with triplications, suggesting that the level of expression of α-synuclein correlates with disease severity. In addition, polymorphisms within the α-synuclein promoter are associated with an increased PD risk, further implicating altered α-synuclein expression as a disease mechanism.

Familial PD caused by α-synuclein missense mutations is extremely rare. Most importantly, α-synuclein was demonstrated to be the major component of Lewy bodies and Lewy neurites in idiopathic or sporadic PD (Spillantini *et al.*, 1997).

Since these two important discoveries, the role of α-synuclein in neurodegenerative disorders has become evident and drug discovery efforts have been focusing on targets related to α-synuclein misfolding and fibrillization.

α-synuclein (AS) is an abundant, natively unfolded neuronal protein. It was first discovered as a neuron-specific presynaptic protein in *Torpedo californica* and rat (Maroteaux *et al.*, 1988) and then recognized as a component of amyloid fibrils in Alzheimer's disease (and so termed NACP, non-A beta component of AD amyloid precursor) (Ueda *et al.*, 1993).

It is highly soluble and its localization is both cytosolic and nuclear and enriched at the synaptic terminals, where it is associated with synaptic vesicles.

The AS sequence comprises 140 aminoacids, lacks both cysteine and tryptophan residues and can be divided into three different regions (Fig. 3):

   (i)   The positively charged N-terminal region (amino acids 1-60), comprising seven imperfect 11 amino acids repeats containing the *consensus* sequence KTKEGV;

   (ii)   the non-aβ-amyloid component (NAC) (amino acids 61-95),

(iii)   the negatively charged C-terminal region 96-140, which contains several sites of post-translational modifications and metal binding.
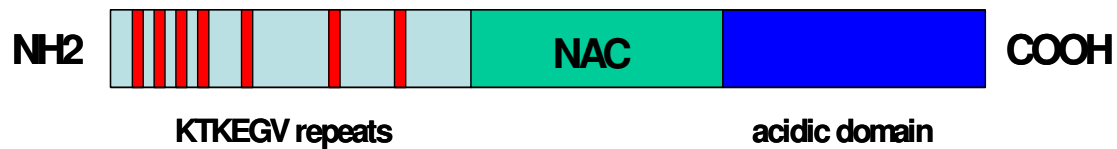
Figure 3. Scheme of the different regions in AS sequence.

The exact functions of normal AS remain to be fully elucidated, although data support a role in synaptic plasticity (George *et al.*, 1995), regulation of dopamine synthesis, uptake and release (Abeliovich *et al.*, 2000), lipid metabolism and vesicle-mediated transport (Lotharius and Brundin, 2002; Willingham *et al.*, 2003) trafficking within the endoplasmic reticulum/Golgi network (Cooper *et al.*, 2006). A chaperone activity (strengthened by its homology to 14-3-3 proteins) has also been proposed based on the ability of AS to inhibit thermally- or chemically-induced aggregation *in vitro* (Souza *et al.*, 2000). More recently, a genetic study in mice suggests that α-synuclein may function as a co-chaperone with cysteine-string protein α in protecting nerve terminals against injury (Chandra *et al.*, 2005).

α-synuclein is a member of a family (not found outside vertebrates) of three synaptic proteins that share considerable sequence homology and include α-synuclein, β-synuclein, and γ-synuclein. Synucleins have in common a highly conserved α-helical lipid-binding motif with similarity to the class-A2 lipid-binding domains of the exchangeable apolipoproteins. The α- and β-synuclein proteins are found primarily in brain tissue, where they are localized mainly in presynaptic terminals. The γ-synuclein protein is found primarily in the peripheral nervous system and in the retina, but its expression in breast tumors is a marker for tumor progression. Although normal cellular functions have not been determined for any of the synuclein proteins, there is evidence that the expression of β-synuclein may regulate the levels or the metabolism of α-synuclein, since β-synuclein inhibits α-synuclein aggregation in transgenic mice (Hashimoto *et al.*, 2001). Furthermore, more recent studies indicate that increased expression of β-synuclein can decrease the levels of α-synuclein by mechanisms that do

not appear to affect α-synuclein mRNA levels (Fan *et al.*, 2006), but it is likely that the functions of β-synuclein extend beyond regulating the expression of α-synuclein.

AS is able to form fibrillar structures *in vitro*. AS contains a highly amyloidogenic domain within its midregion that, by itself, fibrillizes more readily than the holoprotein (Giasson *et al.*, 2004b), which may explain why γ-synuclein forms fibrils much more slowly than AS, and β-synuclein does not form fibrils under typical *in vitro* or *in vivo* conditions. When monomeric AS is incubated at 37 °C, pH 7.4, it forms fibrils with a condition-dependent rate. Agitation, in particular, greatly accelerates the process. Typically, at 100 µM protein concentration, fibrillation will be completed within 3 days with agitation, whereas without agitation it will take months. The *in vitro* kinetics of AS fibril formation show an initial lag phase followed by an exponential growth phase and a final plateau, usually attributed to a nucleation-dependent polymerization. Increasing concentrations of AS lead to increased rate of fibrillation. AS fibrils grown *in vitro* typically vary in length from about 500 nm to 3 µm.
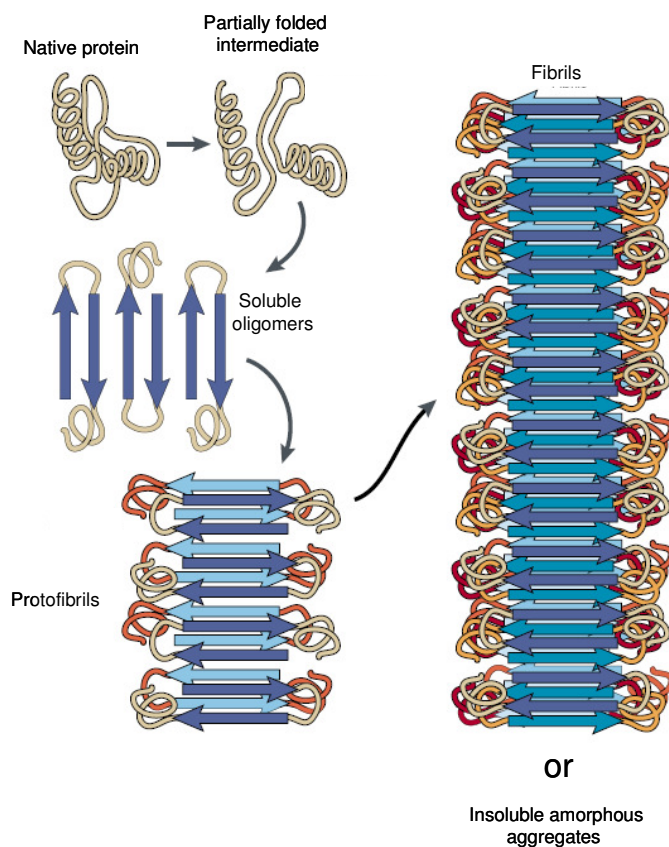

Figure 4. The AS fibrillization process (adapted from Soto, 2003).

Although the full details of the aggregation process remain to be elucidated, partially folded intermediates are critical species in the early stages of most if not all pathological aggregation and especially fibrillation reactions. All fibrils, independent of the original structure of the given amyloidogenic protein, have a common cross-β structure consisting of β-sheets in which the β-strands are perpendicular to the axis of the fibril. In order to attain this structure, folded proteins have to undergo significant conformational rearrangements to allow the needed topological changes. This suggests a simple kinetic model of fibrillation involving conversion of monomeric AS into the critical, partially folded intermediate, which leads, depending on the conditions, to the formation of soluble oligomers, insoluble amorphous aggregates, or insoluble fibrils, as shown by electron microscopy and AFM analysis (Fig. 4). The soluble oligomers may represent an early step leading to the formation of mature fibrils or an off-pathway end product. Extensive data support the hypothesis that "pathological" or abnormal aggregation arises from the key partially folded intermediate precursor.

Many factors have been reported to affect *in vitro* fibrillization of AS. Two out of three identified familial pathological mutations, namely A53T and E46K, accelerate fibril formation (Conway *et al.*, 2000; Greenbaum *et al.*, 2005), while A30P has been proposed to accelerate oligomerization (Conway *et al.*, 2000). Other factors that accelerate AS fibrillation are pesticides (such as rotenone, dieldrin and paraquat), metals, polycations (polyamines such as spermine and histones), glycosaminoglycans (GAGs) and membranes. In particular di- and trivalent metal ions cause significant accelerations in the rate of AS fibril formation *in vitro*. The most effective cations *in vitro* include aluminum(III), copper(II), iron(III), and lead(II) showing a strong correlation with increasing ion charge density. The mechanism for this presumably involves the metal ions binding to negatively charged carboxylates, thus masking the electrostatic repulsion and facilitating collapse to the partially folded conformation. Specific binding sites for some metals, such as copper, are present.

A variety of flavonoids and polyphenols can instead inhibit the fibril formation of AS and in some cases also disaggregate existing fibrils (Li *et al.*, 2004; Zhu *et al.*, 2004). These compounds bind to monomeric AS and lead to formation of stable AS oligomers. Another interesting class of compounds able to inhibit fibrillization of AS is that of dopamine and related catecholamines (see section 1.2.2).

In vivo, membrane-bound AS has been suggested to play an important role in the function of AS.

In vitro, AS preferentially interacts with membranes containing acidic phospholipids. The nature of the interaction of AS with vesicles is highly dependent on the phospholipid composition, the ratio of AS to phospholipid, and the size of the vesicles. The presence of membranes has been reported to both accelerate and inhibit fibrillation, and this probably reflects the varying conditions used in the experiments. Interaction with membranes involves formation of helical structure in AS, and a strong correlation is observed between the induction of α-helix in AS and the inhibition of fibril formation. Protofibrillar or fibrillar AS caused a much more rapid destruction of the membrane than soluble monomeric AS, indicating that protofibrils (oligomers) or fibrils are likely to be significantly neurotoxic.

Another important issue relevant for *in vivo* condition is that of oxidative/nitrative challenge and post translational modifications (phosphorylation, ubiquitination, nitration, C-terminal truncation, oxidation). AS is constitutively phosphorylated at serin 129 and extensive phosphorylation has been found in Lewy bodies of different synucleinopathies (Anderson *et al.*, 2006; Fujiwara *et al.*, 2002). It is not clear if this modification constitutes a normal physiological or a pathological process, although some studies in *Drosophila* (ref) and in cellular models suggest that phosphorylation increases aggregate formation (Smith *et al.*, 2005a).

AS has also been found mono- or di-ubiquitinated in pathological lesions of *post mortem* brains of PD and different synucleinopathies (Tofaris *et al.*, 2003; Sampathu *et al.*, 2003; Shimura *et al.*, 2001). Interestingly, ubiquitination of AS requires previous phosphorylation (Hasegawa *et al.*, 2002).

Some of the AS in Lewy bodies is also covalently modified by tyrosine nitration (Giasson *et al.*, 2000). Whether this occurs before or after fibril formation and whether nitration of the monomer accelerates or retards fibrillation are controversial. *In vitro* studies have shown that fibril formation is completely inhibited by nitration, due to the formation of stable soluble oligomers (Yamin *et al.*, 2003; Uversky *et al.*, 2005). In addition, the presence of substoichiometric concentrations of nitrated AS prevented fibrillation of non-modified AS. *In vivo,* nitration of tyrosine occurs via reaction with oxidative derivatives of NO, and under these conditions additional products of AS oxidation could arise, for example, di-tyrosine cross-linked oligomers.

C-terminally truncated AS is normally generated from full-length AS in brains and in cultured cells and is enriched in pathological aggregates in transgenic mice and human synucleinopathies (Li *et al.*, 2005b). *In vitro*, recombinant C-terminally truncated AS enhances fibril assembly (Murray *et al.*, 2003; Hoyer *et al.*, 2004).

Other modifications can occur *in vivo* due to the oxidative environment.

Oxidation of methionines has been described inhibiting AS fibrillization at neutral pH and forming relatively stable oligomers (Uversky *et al.*, 2002; Hokenson *et al.*, 2004).

Another possible modification is the formation of a covalent adduct with 4-hydroxy-2-nonenal (HNE), one of the most important products of lipid oxidation which has been implicated in the pathogenesis of Parkinson disease. Incubation of HNE with AS *in vitro* results in covalent modification of the protein and HNE modification of AS leads to inhibition of fibrillation in an HNE concentration-dependent manner, due to the formation of stable soluble oligomers (Qin *et al.*, 2007).

1.2.1 Animal models

Several α-synuclein animal models have been generated in flies, worms, and mice adding further support to the view that dysfunctional AS is linked to mechanisms of neurodegeneration in PD.

Genetic models:

Yeast:

The budding yeast *Saccharomyces cerevisiae* recapitulates many of the findings made with higher eukaryotes. For example, yeast cells expressing AS accumulate lipid droplets, have vacuolar/lysosomal defects, and exhibit markers of apoptosis, including the externalization of phosphatidylserine, the release of cytochrome C, and the accumulation of reactive oxygen species. AS associates with the plasma membrane in a highly selective manner, before forming cytoplasmic inclusions through a concentration-dependent, nucleated process (Outeiro and Lindquist, 2003).

Genome-wide screens were performed in yeast to identify genes that enhance the toxicity of AS. Of 4850 haploid mutants containing deletions of nonessential genes, 86 were

identified that were sensitive to AS, clustering in the processes of lipid metabolism and vesicle-mediated transport (Willingham *et al.*, 2003).

Drosophila:

Results from transgenic *Drosophila* also support a causative role of pathological AS in the mechanisms of PD (Bilen and Bonini, 2005). For example, panneuronal over-expression of wild-type or mutant AS resulted in age-dependent degeneration of a sub-set of dopaminergic neurons and of the ommatidial array, formation of filamentous AS inclusions, and locomotor dysfunction such as loss of climbing ability (Feany 2000 nature). Phosphorylation of Ser129 residue of AS characteristic of synucleinopathy lesions is recapitulated in AS transgenic *Drosophila* (Takahashi *et al.*, 2003). Mutation of Ser129 to alanine to prevent phosphorylation completely suppresses dopaminergic neuronal loss produced by expression of human AS. In contrast, altering Ser129 to the negatively charged residue aspartate, to mimic phosphorylation, significantly enhances toxicity. The G protein-coupled receptor kinase 2 (Gprk2) phosphorylates Ser129 *in vivo* and enhances AS toxicity. Blocking phosphorylation at Ser129 substantially increases aggregate formation. Thus Ser129 phosphorylation status is crucial in mediating AS neurotoxicity and inclusion formation and, because increased number of inclusion bodies correlates with reduced toxicity, inclusion bodies may protect neurons from AS toxicity (Chen and Feany, 2005).

Interestingly, co-expression of parkin with AS in the dopaminergic neurons suppresses the AS-induced premature loss of climbing ability, while directed expression of parkin in the eye counteracts the AS-induced degeneration of the ommatidial array (Haywood and Staveley, 2004).

Furthermore, bigenic flies expressing human AS and heat shock protein 70 or Rab1 GTPase were protected from neurodegeneration despite the formation of Lewy body-like AS inclusions (Cooper *et al.*, 2006; Bilen and Bonini, 2005).

Gene expression profiling of A30P transgenic flies revealed dysregulation of lipid processing, membrane transport and energy genes that might indicate perturbed vesicle membrane fusion and permeability with early mitochondrial damage (Scherzer *et al.*, 2003). Recently, a lifetime proteomic profiling of A30P transgenic flies provided evidence for a dysregulation of a group of proteins associated with the actin cytoskeleton and mitochondrion at presymptomatic and early disease stages that may presage the development of later symptoms (Xun *et al.*, 2007).

These *Drosophila* models exhibit key features of PD and may serve as powerful tools in the discovery of genetic factors or pharmacologic agents that influence the pathogenic process.

C. elegans

Over-expression of both WT and A53T human AS by transgenic injection with a pan-neuronal or motor neuron promoter into *Caenorhabditis elegans* causes motor deficits. Neuronal and dendritic loss are accelerated in all three sets of *C. elegans* dopaminergic neurons when human AS is over-expressed under the control of a dopaminergic neuron or pan-neuronal promoter, but not with a motor neuron promoter. There is no significant differences in neuronal loss between over-expressed WT and A53T forms or between worms of different ages (Lakso *et al.*, 2003). These results demonstrate neuronal and behavioral perturbations elicited by human AS in *C. elegans* that are dependent upon expression in specific neuron subtypes. This transgenic model in *C. elegans*, an invertebrate organism with excellent experimental resources for further genetic manipulation, may help facilitate dissection of pathophysiologic mechanisms of various synucleopathies.

A genome wide expression screen was performed in *C. elegans* over-expressing both WT and A53T human AS. 433 genes were up- and 67 genes down-regulated. Gene ontology (GO) categories within the regulated gene lists indicated over-representation of development and reproduction, mitochondria, catalytic activity, and histone groups. Seven genes (pdr-1, ubc-7, pas-5, pas-7, pbs-4, RPT2, PSMD9) with function in the ubiquitin–proteasome system and 35 mitochondrial function genes were up-regulated. Nine genes that form histones H1, H2B, and H4 were down-regulated (Vartiainen *et al.*, 2006). These results demonstrate the effects of AS on proteasome and mitochondrial complex gene expression and provide further support for the role of these complexes in mediating neurotoxicity. The results also indicate an effect on nuclear protein genes that suggests a potential new avenue for investigation.

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Mice:

Expression of wild-type and mutant AS in mice has yielded differing results depending on the promoter used (platelet-derived growth factor, Thy-1, PrP). These mice provide a model that exhibits AS inclusion formation (although different from the PD Lewy bodies) and associated motor phenotype, but their substantia nigra is unaffected.

The PrP A53T mice spontaneously develop a disease phenotype at approximately 9–11 months of age with behavioral symptoms such as ataxia, rigidity, postural instability, and brainstem neuropathology including Lewy body-like inclusions. The prion promoter drives strong expression of the transgene in multiple brain regions, but pathology appears most severe in the mouse brainstem. Interestingly, the PrP A53T mice develop mitochondrial pathology in the brainstem and spinal cord (Martin *et al.*, 2006). Transgenic mice that express either wild-type or doubly A53T and A30P mutated human α-synuclein under the control of the tyrosine hydroxylase (TH) promoter (Richfield *et al.*, 2002) display several phenotypic variations from non-transgenic animals that are consistent with PD. For instance, the double mutant mice have swollen, dystrophic TH-positive neurites emigrating from the substantia nigra (SN). Behaviorally, the mice are hyperactive at a young age, but show age-related deficits in locomotion following MPTP or amphetamine treatment. A neurochemical profile of dopamine and its metabolites demonstrates an early (pre-symptomatic) alteration of the ratio of dopamine to its metabolites, DOPAC and HVA. Recently, it was shown that age-related neuronal decline, though more severe in the double mutant mice, occurs nonetheless in WT AS mice (Thiruchelvam *et al.*, 2004).

More recently, transgenic mice were developed expressing a truncated form of AS that fibrillizes more readily than the full-length protein *in vitro*, which show evidence of substantia nigra Lewy bodies and neurodegeneration (Tofaris *et al.*, 2006).

Rats and primates:

Rat and primate models have been developed by vector-mediated over-expression of WT and mutant AS, showing a great deal of variability in the degree of neuropathological changes and motor impairment, probably due to variations in the levels of AS expression and to species-specific differences in the processing of this protein.

Intoxication models:

Several models using neurotoxins have been used to investigate the functions of α-synuclein, in particular its potential role in the pathogenesis of PD.

MPTP

The parkinsonian effects of MPTP are due primarily to the loss of ATP in nigrostriatal neurons, secondary to inhibition of mitochondrial complex I. This loss results in elevated concentrations of ROS, which can cause cell death. A crucial interaction between MPTP and α-synuclein is supported by the inability of MPTP to induce neurodegeneration in α-synuclein-knockout mice (Dauer *et al.*, 2002). MPTP treatment has also been used successfully on mice and primates.

Rotenone

Rotenone-treated rats have emerged as another promising animal model of syncleinopathies and PD. Epidemiological studies have revealed a correlation between the onset of PD and exposure to pesticides and insecticides such as rotenone. *In vitro*, rotenone induces degeneration of dopaminergic neurons by inhibiting complex I and generating ROS, which can cause oxidative damage to proteins and DNA, leading to apoptotic death of DA neurons.

The MPTP and rotenone models of PD are valuable because they exhibit dopaminergic neurodegeneration and a pattern of motor impairment that is characteristic of PD. However, the MPTP-lesioned animals do not always have α-synuclein aggregates, and only a percentage of rotenone-treated rats survive treatment with the toxin and develop the appropriate syndrome. Despite these limitations, these models show that inhibition of complex I results in the accumulation of excess α-synuclein and selective degeneration of dopaminergic neurons.

1.2.2 The role of dopamine

The functional interplay between AS and dopamine (DOP) is of paramount interest because it provides an attractive explanation for the selective loss of dopaminergic neurons in PD.

In dopaminergic neurons, DOP is synthesized by tyrosine hydroxylase (TH) in the cytosol. Like all catecholamines, DOP is unstable and prone to autoxidation, with production of ROS and reactive DOP derivatives such as quinones (DAQ) and dopaminochrome (DACHR) which are cytotoxic species able to cause oxidative damage to cellular proteins and DNA. This toxicity is avoided by rapid sequestration of DOP in synaptic vesicles by vesicular monoamine transporter 2 (VMAT2). Interestingly, mice that express low levels of VMAT2 display age-associated nigrostriatal dopamine dysfunction that ultimately results in neurodegeneration (Caudle *et al.*, 2007). After release in the synaptic cleft, DOP is re-uptaken in the cytosol by the dopamine transporter (DAT).
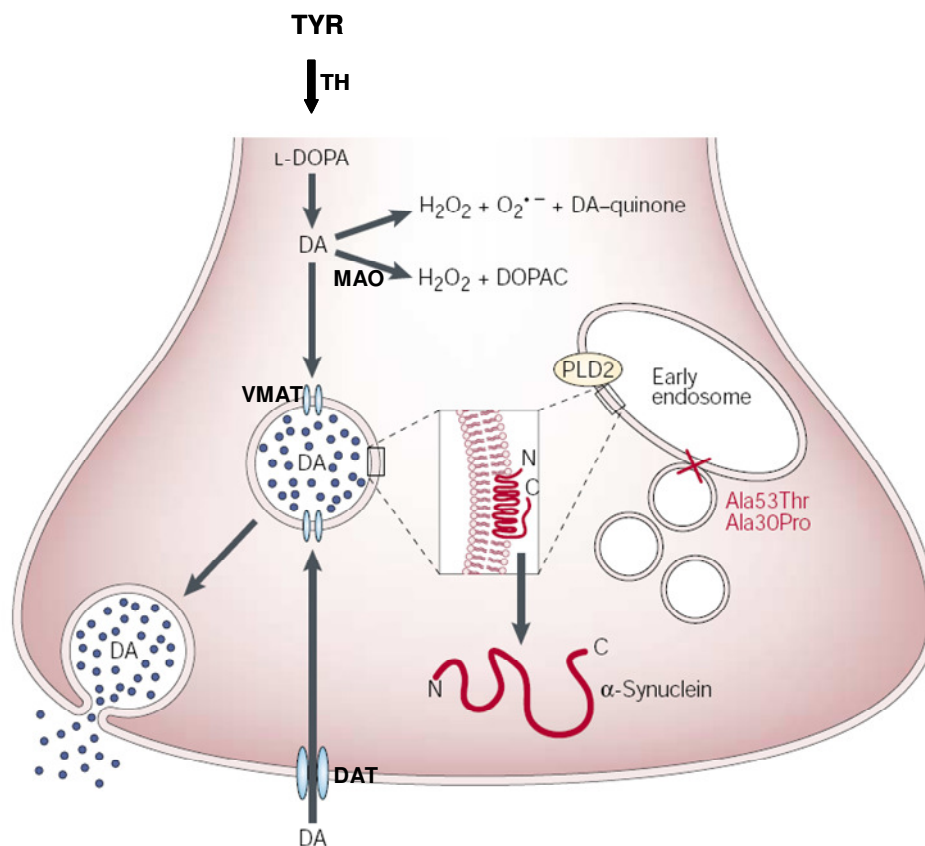

Figure 5. The potential function of AS in intracellular dopamine storage in a presynaptic terminal from a dopaminergic neuron (adapted from Lotharius and Brundin, 2002).

Several evidences point to an important role of AS in regulating DOP metabolism at multiple levels (Fig. 5): AS interaction with TH inhibits synthesis of DOP (Perez *et al.*, 2002), AS is involved in vesicles formation and recycling through interaction with PLD2 (Jenco *et al.*, 1998) and binds fatty acids in vesicles with different affinities among the pathological mutants (Sharon *et al.*, 2001); VMAT2 expression is decreased by AS A53T over-expression (Lotharius *et al.*, 2002); AS binds to DAT and regulates its trafficking (Lee *et al.*, 2001; Fountaine and Wade-Martins, 2007).

It is possible that small imbalances in these processes due to pathological mutations, environmental factors and aging can lead to accumulation of DOP in the cytosol; interaction of cytosolic DOP and its derivatives with AS, in turn, can further disregulate DOP metabolism and exacerbate oxidative stress in a circular process.

Indeed, over-expression of AS in cultured dopaminergic neurons causes cell death while it is protective in non-dopaminergic neurons (Xu *et al.*, 2002). Furthermore, the pro-apoptotic properties of AS in dopaminergic neurons are strictly dependent on endogenous DOP and are abolished by an inhibitor of DOP synthesis.

In a cellular model of AS aggregation, the increase in the steady-state dopamine levels, obtained by expressing different mutant forms of TH, inhibits the formation of AS aggregates and induces the formation of non-toxic oligomeric intermediates (Mazzulli *et al.*, 2006).

In vitro, chatechol derivatives, including dopamine, have been shown to inhibit AS fibrillogenesis causing accumulation of oligomeric species (Cappai *et al*., 2005; Conway *et al*., 2001; Li *et al*., 2005a; Bisaglia *et al*., 2007).

In a screening of a library of 169 compounds to identify drug-like molecules able to block AS fibrillization *in vitro*, all but one of the 15 inhibitors found were catecholamines related to dopamine. The inhibitory activity of dopamine depended on its oxidative ligation to AS and was selective for the protofibril-to-fibril conversion, causing accumulation of the AS protofibril (Conway *et al.*, 2001).

DOP induces AS to form soluble, SDS-resistant oligomers. The DOP/AS oligomeric species are not amyloidogenic as they do not react with thioflavin T and lack the typical amyloid fibril structures as visualized with electron microscopy. Circular dichroism studies indicate that in the presence of lipid membranes DOP interacts with AS, causing an alteration to the structure of the protein. Furthermore, DOP inhibits the formation of

iron-induced AS amyloidogenic aggregates, suggesting that DOP acts as a dominant modulator of AS aggregation (Cappai *et al.*, 2005).

DOP modulates differently the stability of protofibrils and fibrils composed of wild-type or variants of AS (A30P and A53T) as shown by a high hydrostatic pressure (HHP) study. Although fibrillization of AS was inhibited by DOP, long incubation times (18 days) eventually led to the formation of fibrils with a decreased stability. As for the protofibrils, in the absence of DOP they all exhibited identical stability, while in its presence, the mutant ones acquired a greater stability, which can shed light onto why these mutations are so aggressive. Indeed the A30P protofibrils, when added to mesencephalic and cortical neurons in culture, decreased the number and length of neurites and increased the number of apoptotic cells. The toxic effects were abolished by HHP treatment, which is able to break the protofibrils into smaller aggregates, as seen by atomic force microscopy. This suggests that strategies aimed at breaking and/or clearing these aggregates is promising in alleviating the symptoms of PD (Follmer *et al.*, 2007).

The exact mechanism of DOP/AS interaction has not been fully elucidated yet, and given its potential pharmacological and therapeutical relevance, is the object of many ongoing research efforts.

There is strong debate about the covalent (Bisaglia *et al.*, 2007; Conway *et al.*, 2001; Li *et al.*, 2005a) or non-covalent (Norris *et al.*, 2005) nature of the DOP/AS complexes.

The experimental data of Conway *et al.* were consistent with the formation of covalent adducts between DOP quinone (DAQ) and α-synuclein.

Li *et al.* tested the inhibitory abilities of DOP and several analogs including chemically synthetic and natural polyphenols *in vitro*. The MS and NMR characterizations demonstrated that DOP and its analogs inhibit AS fibrillization by a mechanism where the oxidation products (quinones) of DOP analogs react with the amino groups of AS chain, generating AS/quinone adducts. NMR experiments suggest that at least three amino groups of lysine residues in a AS molecule are modified by DOP to generate imino groups that link to the aromatic ring of quinone and the side chains of the protein. The covalently cross-linked AS adducts by DOP are primarily large molecular mass oligomers, while those by catechol and p-benzoquinone (or hydroquinone) are largely monomers or dimers, probably because DAQ reacts with the amino group of DOP molecule to form melanin that further covalently cross-links DOP/AS adducts to form more complicated DOP/AS oligomers.

The DOP quinoprotein retains the same cytotoxicity as the intact AS, suggesting that the oligomeric intermediates are the major elements that are toxic to the neuronal cells (Li *et al.*, 2005a).

In another study, Bisaglia *et al.* carried out a kinetic and structural analysis of the early oxidation products of dopamine. Specifically, considering the potential high toxicity of dopaminochrome for both cells and mitochondria, they focused on its rearrangement to 5, 6-dihydroxyindole. After the spectroscopic characterization of the products derived from the oxidation of dopamine, the structural information obtained was used to analyze the reactivity of quinones toward AS. The results suggest that indole-5, 6- quinone (IQ), rather than DAQ or DACHR, is the reactive species (Bisaglia *et al.*, 2007).

Norris *et al.* proved that dopamine inhibition of AS fibrillization generated exclusively spherical oligomers that depended on dopamine autoxidation but not AS oxidation, because mutagenesis of Met, His, and Tyr residues in AS did not abrogate this inhibition. However, truncation of AS at residue 125 restored the ability of AS to fibrillize in the presence of dopamine. Mutagenesis and competition studies with specific synthetic peptides identified AS residues 125–129 (i.e. YEMPS) as an important region in the dopamine-induced inhibition of AS fibrillization. Significantly, the dopamine oxidation product dopaminochrome was identified as a specific inhibitor of AS fibrillization. DACHR promotes the formation of spherical oligomers by inducing conformational changes, as these oligomers regained the ability to fibrillize by simple denaturation/renaturation (Norris *et al.*, 2005).
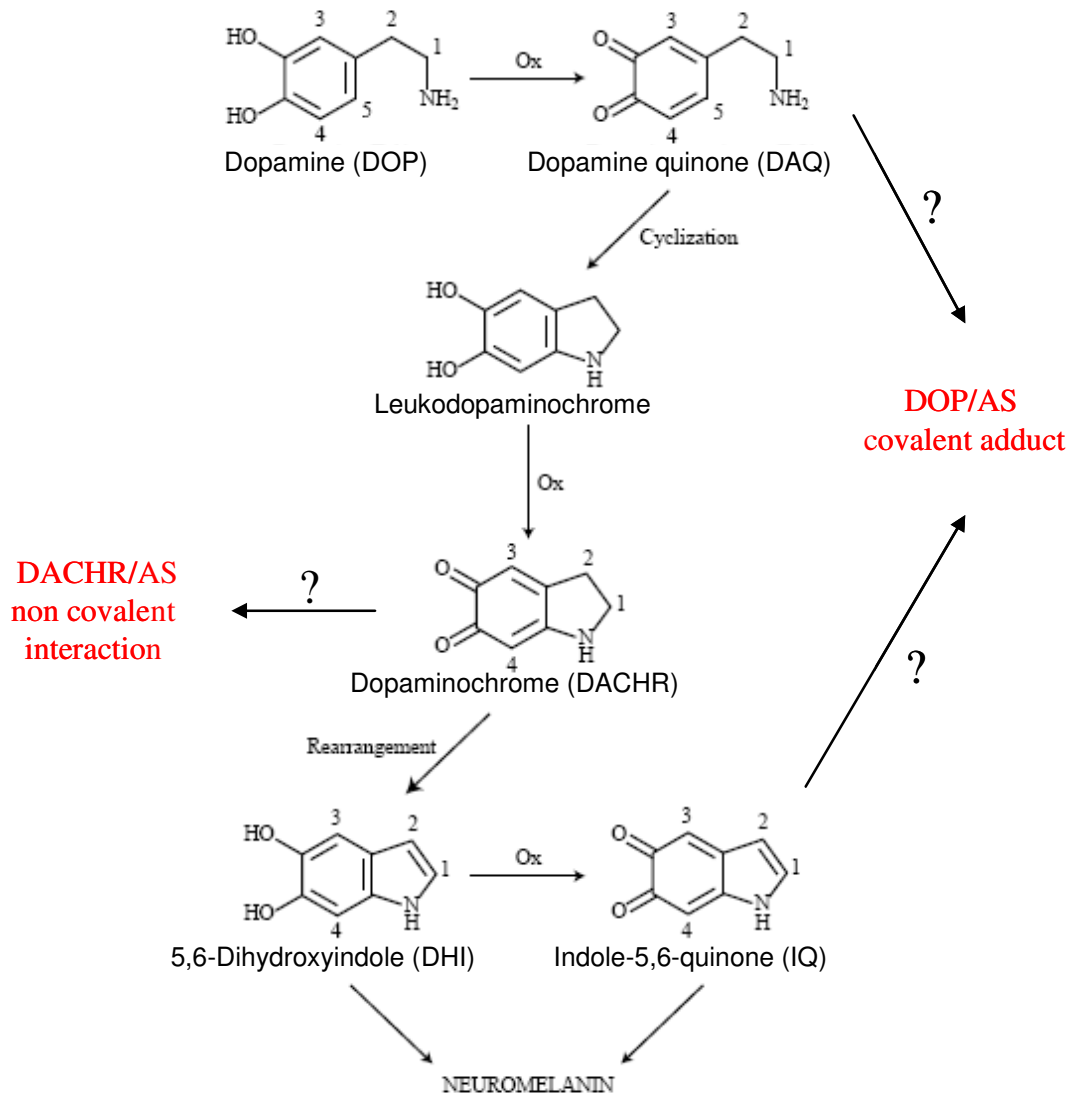
Figure 6. The oxidative pathway of neuromelanin synthesis and possible interactions with AS. (Adapted from Bisaglia *et al.*, 2007).

A summarizing scheme of the oxidative pathway of neuromelanin synthesis from DOP and of the proposed interactions with AS is shown in Fig. 6. The initial reaction involves the oxidation of dopamine (DOP) to yield the corresponding dopamine-o-quinone (DAQ). The two following steps are the cyclization of the quinone to give leukoaminochrome and its subsequent oxidation to dopaminochrome (DACHR). Then, dopaminochrome rearranges to 5, 6-dihydroxyindole (DHI) which can be oxidized to indole- 5, 6-quinone (IQ) and polymerize to form neuromelanin (Bisaglia *et al.*). Interactions have been proposed between AS and either DAQ or IQ (covalent) or DACHR (non covalent). Altogether, these studies suggest that dopamine may play a key role in modulating AS aggregation and toxicity and may be linked to the selective vulnerability of dopaminergic neurons in PD.

1.3 SUMO modification in neurodegenerative disorders

SUMO proteins have been detected within inclusions in various neurodegenerative disorders such as multiple system atrophy (MSA), Huntington's disease and other related polyglutamine disorders. In addition, several proteins in the brain have been identified as SUMO targets. Given the pleiotropic roles of SUMO modification on cellular processes as well as its relationship with the ubiquitin pathway, there are a number of possible mechanisms by which SUMOylation can contribute to the underlying neurodegenerative process.

PolyQ diseases

Polyglutamine (polyQ) diseases are neurodegenerative disorders associated with the genomic expansion of a CAG triplet repeat in the disease-specific genes resulting in an abnormal extension of glutamine residues in the product protein that can range from 35 to over 140 pathogenic repeats. They are characterized by the formation of cytoplasmic and/or intranuclear protein inclusions in selective populations of neurons and include Huntington's disease (HD), spinal and bulbar muscular atrophy (SBMA), dentatorubral-pallidoluysian atrophy (DRPLA) and a collection of spinocerebellar ataxias (SCA-1, -2, -3, -6, -7, and -17).

Enhanced immunoreactivity for SUMO-1 was found in affected neurons of DRPLA, SCA-1, Machado–Joseph disease (MJD, also known as SCA-3) and Huntington's disease patients (Ueda *et al.*, 2002).

In addition, co-localization of SUMO and polyQ protein aggregates has been demonstrated in histological studies of individuals with DRPLA as well as for cellular models of atrophin-1 inclusion bodies. In this cellular model, co-expression of wild-type SUMO-1 with polyQ-atrophin-1 significantly accelerated the formation of nuclear aggregates and promoted apoptosis. Conversely, co-expression of SUMO-1 lacking the C-terminal diglycine motif required to conjugation reduced, but did not abolish, the accumulation of polyQ-atrophin-1 aggregates. Expression of the conjugation-deficient SUMO promoted cell survival, as compared to the mutant polyQ protein alone or in the presence of wild-type SUMO-1 (Terashima *et al.*, 2002).

SUMO-1 immunostaining has been demonstrated in selected nuclear aggregates associated with MJD, although this does not always overlap with ataxin-3 and is often located in different populations of inclusions (Pountney *et al.*, 2003). In contrast, neuronal mod-

els of huntingtin (Htt) deposition indicated an association with SUMO-1 (Steffan *et al.*, 2004). Similar co-localization of SUMO-1 with both wild-type and mutant ataxin-1 has been observed in SCA1 (Riley *et al.*, 2005).

The mutant protein involved in SBMA is a pathogenic polyQ fragment of the androgen receptor (AR).

Wild-type AR is a known SUMO-1 substrate and SUMOylation negatively regulates its transcriptional activity (Poukka *et al.*, 2000). However, it is not known if or to what extent the pathogenic polyQ-AR is SUMOylated and how SUMOylation might contribute to the development of SBMA. In a *Drosophila* model of SBMA, expression of a pathogenic androgen receptor fragment with an expanded polyQ repeat results in nuclear and cytoplasmic aggregates as well as progressive neurodegeneration (Chan *et al.*, 2002). Expression of a catalytic-deficient C175S mutant form of the SUMO activating enzyme (E1) subunit Uba2 (equivalent to the human SAE2) greatly enhanced degeneration in this model. Similar observations have been made in an MJD model using a disease-related ataxin-3, which suggests that downregulation of SUMO modification is detrimental to cells.

Direct SUMOylation of disease-related proteins has also been implied to play a role in neurodegeneration, potentially by mediating changes in protein solubility or levels of toxic soluble oligomers, or disregulating the protein's nuclear/cytoplasm trafficking.

One of the first identified polyQ-related SUMO substrates was a pathogenic fragment of huntingtin (Htt) (Steffan *et al.*, 2004). Expression of a permanent, non-hydrolyzable Htt-SUMO conjugate in a neuronal cell line resulted in a more stable protein and increased Htt-mediated transcriptional repression. This also resulted in decreased formation of inclusions and potentially in an increase of the levels of toxic oligomers. The SUMO-1 conjugation sites are located within the N-terminal domain of the Htt peptide and overlap with the lysine residues targeted by ubiquitin, which may lead to competition between the two modifiers. The relative contribution of ubiquitin and SUMO to pathogenesis has been investigated in *Drosophila* models. Expression of mutant Htt led to progressive degeneration, which was reduced in flies heterozygous for SUMO and modestly worsened in flies with genetically reduced ubiquitination. Furthermore, expression of a mutant Htt in which the conjugation lysines were mutated substantially decreased degeneration.

Another SUMOylation substrate is mutant ataxin-1, which accumulates in SCA1 and is covalently modified by SUMO-1 via at least five different target lysines (Riley *et al.*,

2005). An increase in the length of the polyQ stretch negatively regulates ataxin-1 SUMOylation. The SUMO modification is modulated by phosphorylation and is also dependent upon translocation mediated by a nuclear localization signal.

These studies cumulatively demonstrate the presence of SUMO in neuronal inclusions in a variety of polyQ disorders and suggest a potential role for SUMO in polyQ pathogenesis.

Neuronal intranuclear inclusion disease

Neuronal intranuclear inclusion disease (NIID) is a rare neurodegenerative disorder characterized by the presence of widespread eosinophilic intranuclear inclusions. Although the histopathology is comparable to polyQ-related disorders, there is no evidence for CAG expansions in NIID. Interestingly, ataxin-1 and ataxin-3 are detected within the NIID aggregates.

Extensive SUMO-1 immunostaining has been observed in nuclear inclusions in cases of familial, juvenile and sporadic NIID (Pountney *et al.*, 2003; Takahashi-Fujigasaki *et al.*, 2006).

The identity of the SUMO targets within NIID aggregates has yet to be determined. The transcriptional co-repressor, histone deacetylase HDAC4, is associated with nuclear inclusions and represents a potential candidate substrate. Another possibility is that the aggregates in NIID arise from promyelocytic leukaemia (PML), since it has been shown that neuronal intranuclear inclusions in sporadic and familial NIID cases contained PML along with SUMO-1 and ubiquitin. That provides a further link to SUMOylation: PML nuclear bodies are subnuclear structures that participate in various nuclear processes including intranuclear protein degradation mediated by the proteasome (Lallemand-Breitenbach *et al.*, 2001; Lafarga *et al.*, 2002). Evidence suggests the convergence and integration of both ubiquitin and SUMOylation pathways in nuclear bodies (Bailey and O'Hare, 2005).

Alzheimer's disease

Alzheimer's disease (AD) is the most common cause of dementia in the elderly and is associated with cognitive and memory dysfunction. This progressive disease is neuropathologically characterized by the presence of senile plaques and neurofibrillary tangles.

Senile plaques result from the extracellular accumulation of aggregated amyloid-beta (Aβ) peptides, which are generated by β- (BACE) and γ-secretase cleavage of the transmembrane amyloid precursor protein (APP). An *in vitro* expression and SUMOylation approach has identified APP as a SUMO-1 target (Gocke *et al.*, 2005). In addition, an unbiased screening of a human brain cDNA library has led to the identification of SUMO-3 as a new regulator of Aβ generation (Li *et al.*, 2003). The study showed that SUMO-3 over-expression reduced Aβ production whereas expression of SUMO mutants that cannot be conjugated or form polymeric chains increased the production of these amyloidogenic peptides. In contrast, a more recent study investigating the effects of over-expression of the three major SUMO isoforms on the APP processing pathway showed that SUMO-3 increased the generation of Aβ (Dorval *et al.*, 2007). This was accompanied by a reduced APP turnover and an increase in APP holoprotein, C-terminal fragments and BACE levels. In agreement with the study by Li *et al.*, conjugation- and polymerization-deficient mutants also stimulated Aβ secretion.

AD is also characterized by the presence of neurofibrillary tangles, which are intracellular aggregates of tau (Goedert *et al.*, 1988) and strongly immunoreactive for ubiquitin [103, 104]. Tau is a phosphoprotein involved in the regulation of microtubule stability and is responsible for axonal development. Tau pathology is also associated with other neurological disorders collectively called tauopathies, which include frontotemporal dementia (FTD), Pick's disease (PiD), progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD). It has recently been demonstrated that tau was covalently modified by SUMO-1, and to a lesser extent by SUMO-2 and SUMO-3 (Dorval and Fraser, 2006). The major modification site, lysine K340, maps to the microtubule-tubule binding domain.

ALS

Amyotrophic lateral sclerosis (ALS) affects motor neurons in the spinal cord and the brain. The disorder occurs mostly sporadically, but it can also be inherited. Genetic mutations in the gene encoding superoxide dismutase 1 (SOD1), an abundant copper/zinc-containing protein, account for some cases of familial ALS cases. This cytoplasmic metalloenzyme is an important cellular antioxidant and plays a vital role in detoxifying cells during oxidative stress. Several independent proteomic studies identified yeast SOD1 as being covalently modified by SUMO-1 (Zhou *et al.*, 2004; Wykoff and

O'Shea, 2005; Hannich *et al*., 2005; Denison *et al*., 2005) on multiple lysines, under normal and stress conditions (Zhou *et al.*, 2004). SUMOylation of human SOD1 has also been observed but, in contrast to yeast, only a single lysine was targeted (Fei *et al.*, 2006). SUMO could be conjugated to both wild-type and ALS-related mutant SOD1 which resulted in decreased turnover and enhanced aggregation. Inclusion bodies could be detected in these cell culture models that co-localized with SUMO-1 which is consistent with the accumulation of modified SOD1.

MSA

Multiple system atrophy (MSA) belongs to the group of synucleinopathies, neurological disorders characterized by the accumulation of filamentous aggregates of α-synuclein in cytoplasmic inclusion bodies. SUMO-1 immunoreactivity has been reported in MSA oligodendroglial cytoplasmic inclusions as well as punctuated staining at the nuclear membrane level (Pountney *et al.*, 2005).

Parkinson's disease

In PD, several familial genes including α-synuclein and UCH-L1 are closely linked to the ubiquitin–proteasome system, which is thought to have significant cross-talk with the SUMOylation pathway.

Parkin is an ubiquitin E3 ligase and mutations in this protein cause the majority of familial PD cases. It has been shown that parkin regulates the turnover of the SUMO E3 ligase RanBP2 by catalyzing its ubiquitination and promoting proteasome degradation (Um *et al.*, 2006). More recently, a functional non-covalent interaction between parkin and SUMO-1 has been reported in neuronal cells as well as in rat brain cortex (Um and Chung, 2006), which resulted in parkin nuclear localization and autoubiquitination.

Another familial gene, DJ-1, is a multifunctional protein involved in transcriptional regulation (Takahashi *et al.*, 2001) and plays a role in the cellular response to oxidative stress (Taira *et al.*, 2004), a known contributing factor to dopaminergic neuron degeneration in PD. Loss-of-function DJ-1, either by deletion or point mutation, leads to early onset PD. Interestingly, oxidative stress has been shown to regulate global SUMOylation. Initial reports demonstrated that high oxidative stress upregulated SUMOylation (Saitoh and Hinchey, 2000; Bossis and Melchior, 2006). However, subsequent investi-

gations showed that high but physiological concentrations of reactive oxygen species had the opposite effect. Under mild oxidative conditions, SUMO E1 and E2 enzymatic activities are reversibly inhibited by the formation of a disulfide bond between their catalytic cysteines, which results in a downregulation of SUMO conjugation (Bossis and Melchior, 2006).

DJ-1 is modified by SUMO-1 (Shinbo *et al.*, 2006) and also interacts with members of the PIAS family (Takahashi *et al.*, 2001), which act as SUMO E3 ligases (Johnson and Gupta, 2001; Kahyo *et al.*, 2001). Substitution of the DJ-1 SUMO acceptor lysine, K130, abolishes its activity but does not affect subcellular localization. It has been shown that the PD-related DJ-1 missense mutant, L166P, can be mis-SUMOylated as compared to its wildtype counterpart. This was evident by the presence of higher molecular weight SUMO/DJ-1-positive bands and suggested that the mutant protein may have been polySUMOylated on selected lysine residues or SUMOylated on multiple target residues. Alternatively, SUMO conjugation of the L166P may promote aggregation leading to these high molecular weight species. These changes in SUMOylation of the L166P mutant can potentially explain its insolubility, change in subcellular localization and instability, which may be due to its rapid degradation by the proteasome. The DJ-1 L166P mutant and to a lesser extent the SUMO site K130R mutant were more sensitive to oxidative stress induced by UV irradiation which implicates SUMOylation in the DJ-1-mediated response to the cell insult (Shinbo *et al.*, 2006).

The relationship between SUMO, DJ-1 and oxidative stress appears to be increasingly complex: the emerging picture could be one of an integrated positive feedback pathway where both DJ-1 and SUMO are involved in the cellular response to oxidative stress.

Other SUMOylation targets

Proteomic approaches have led to the identification of a large number of SUMO substrates from yeast and mammalian cells under a variety of conditions. Although many of these have yet to be validated in cellular models or *in vivo*, further investigation of these candidate SUMO targets may provide additional insights into the involvement of SUMOylation in neurodegenerative diseases. For example, E2-25K is an unusual ubiquitin conjugating enzyme that also functions as an ubiquitin E3 ligase to produce diubiquitin and free polyubiquitin chains (Chen and Pickart, 1990). E2-25K is a SUMOylation target and conjugation impairs the formation of ubiquitin thioester and, consequently, the

generation of free ubiquitin chains (Pichler *et al.*, 2005). E2-25K is also expressed at high levels in the brain and interacts with huntingtin (Htt). This interaction is independent of the length of the polyQ stretch but requires an intact Htt N-terminal domain (Kalchman *et al.*, 1996) which contains the target lysines for SUMO and ubiquitin conjugation (Steffan *et al.*, 2004). Another target of interest is dynamin, which interacts with SUMO-1, Ubc9 and PIAS1 through its GTPase effector domain. Dynamin is a key element in the secretory pathway which has been associated, for example, with APP processing in Alzheimer's disease (Chyung and Selkoe, 2003). Although no SUMOylated dynamin has been detected, it has been shown that expression of SUMO-1 or Ubc9 inhibited lipid-dependent oligomerization and decreased dynamin-mediated endocytosis of transferrin (Mishra *et al.*, 2004).

Molecular chaperones are essential components of the protein quality control in cells. They play critical roles in the folding of newly synthesized polypeptides and targeting of misfolded proteins for proteasome-mediated degradation. Chaperones are also involved in aggresome formation and are associated with protein inclusions in neurodegenerative diseases. The majority of proteomic studies have identified molecular chaperones as SUMO substrates, including HSP90 and HSP70 (Denison *et al.*, 2005; Hannich *et al.*, 2005; Panse *et al.*, 2004; Rosas-Acosta *et al.*, 2005; Zhao *et al.*, 2004; Zhou *et al.*, 2004). SUMOylation could potentially affect chaperones activity, their recruitment to specific subcellular localizations as well as interactions with substrates or other components involved in protein quality control. Finally, several yeast proteasome subunits have been identified as SUMO substrates which raises the possibility that SUMOylation may participate in proteasome organization or regulation (Denison *et al.*, 2005; Panse *et al.*, 2004; Wohlschlegel *et al.*, 2004). Chaperones and the proteasome are two important systems that have been closely linked to neurodegenerative diseases and represent another potential connection to the SUMOylation pathway.

1.3.1 The SUMO proteins

SUMO proteins belong to the family of the Ubiquitin-like proteins (Ubls), composed by about a dozen related members that are conjugated to substrate proteins, altering their properties and hugely increasing the complexity of the proteome in eukaryotic cells. SUMO is distantly related to ubiquitin (20% identity) and was first identified in mammals, where it was found to be covalently linked to the GTPase activating protein Ran-GAP1 (Mahajan *et al.*, 1997; Matunis *et al.*, 1996). Vertebrates express three paralogs, designated as SUMO-1, SUMO-2 and SUMO-3. Recently a fourth member of the SUMO family was reported to be expressed in kidney cells, although previous reports indicated that the intronless SUMO-4 was likely to be a non-expressed pseudogene. The conjugated forms of SUMO-2 and SUMO-3 only differ from one another by three N-terminal residues and have yet to be functionally differentiated. They form a distinct subfamily known as SUMO-2/-3 and are 50% identical in sequence to SUMO-1. While virtually all of the SUMO-1 is engaged in conjugates, there is a free pool of SUMO-2/-3 that is utilized when cells are exposed to a variety of cellular stress (Saitoh and Hinchey, 2000).

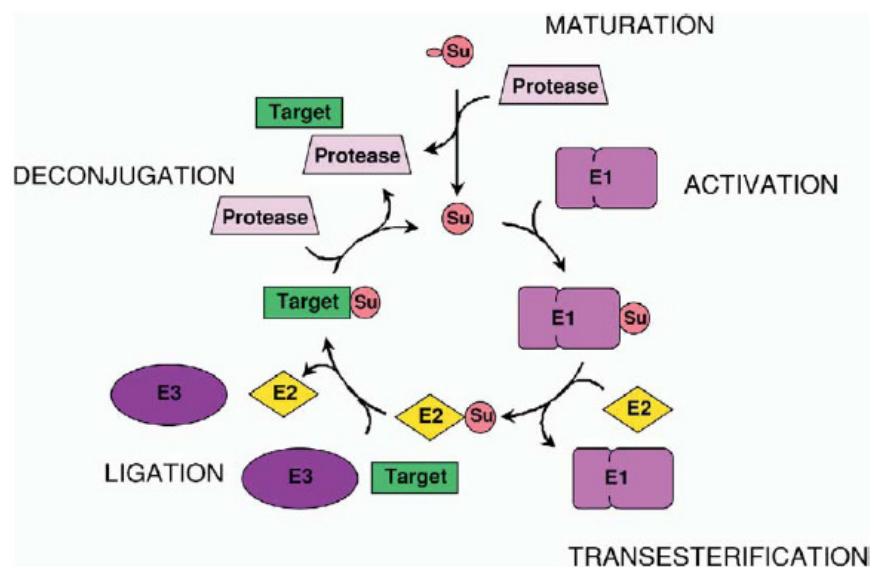## 1.3.2 Mechanism of SUMO conjugation

Figure 7. SUMO conjugation pathway. (Adapted from Hay, 2005).

SUMO proteins are conjugated to target proteins by an enzymatic cascade (Fig. 7) involving:

i.   a SUMO activating enzyme (E1) which is an heterodimer containing SAE1 and SAE2 subunits

ii.   a SUMO conjugating enzyme (E2), Ubc9

iii.   possibly, a SUMO ligase (E3)

Like most other Ubls, SUMO is synthesized as a lager precursor that must be processed to reveal the C-terminal glycine residue that is linked to lysine side chains in target proteins. This processing is carried out by SUMO-specific proteases that also remove SUMO from modified substrates. In yeast two SUMO specific proteases (Ulp1 and Ulp2) have been characterized and belong to the family of cysteine proteases. In the human genome eight genes have been identified with significant sequence homology to yeast Ulp1, and seven of them have been shown to function as SUMO specific proteases. Each of them appears to have a distinct subcellular localization.

To initiate the SUMO modification reaction, SAE1/SAE2 catalyzes the formation of adenylated SUMO in which the C-terminal carboxyl group of SUMO is covalently linked to AMP. Breakage of the SUMO-AMP linkage is followed by formation of a covalent intermediate in which the C-terminal carboxyl group of SUMO forms a thioester bond with the sulphydryl group of a cysteine residue in SAE2. In the second step of the reaction, SUMO is transesterified from SAE2 to cysteine 93 in the E2 enzyme Ubc9. Ubc9 (differently from other conjugating enzymes) is able to directly recognize substrate proteins. Thus Ubc9-SUMO thioester can catalyze the formation of an isopeptidic bond between the C-terminal carboxyl group of SUMO and the ε-amino group of a lysine in the substrate protein. Typically, but not always, lysine residues subject to SUMO modification are found within a SUMOylation *consensus* motif, ψKxE (where ψ is a large hydrophobic residues and x is any residue). SUMO-2/-3 each possess exposed SUMOylation *consensus* motifs that can be utilized to form polymeric SUMO chains, whose function is unknown. The ψKxE motif is directly recognized by Ubc9, however, with the exception of RanGAP1, SUMO modification with only SAE1/SAE2 and Ubc9 is rather inefficient. The discovery of proteins that increase the efficiency of SUMO conjugation has indicated the existence of SUMO E3 ligases. They all bind Ubc9 and accelerate the rate of SUMO modification in a substrate specific fashion. Furthermore

they appear to target multiple proteins with few recognizable similar feature. In higher eukaryotes, PIAS proteins family, RanBP2, Pc2, TOPORS and TRAF7 have been identified as SUMO E3 ligases.

1.3.3 Conjugation-independent functions of SUMO

An important emerging aspect in SUMO biology is their ability to regulate biochemical pathways independent of their conjugation to target substrates. Generic SUMO-binding motifs (SBM) have been identified and consist of a hydrophobic core which is adjacent to a series of acidic residues and contains potential phosphorylation sites (Hannich *et al.*, 2005; Hecker *et al.*, 2006; Minty *et al.*, 2000; Song *et al.*, 2004b). Structural studies have demonstrated that, unlike the helical ubiquitin-binding motifs, the SBM are conformationally flexible and are located between α-helical and β-strand structures (Hecker *et al.*, 2006). Different examples are known in which a non-covalent SUMO interaction is able to regulate the substrate function and cellular localization: besides the already cited interaction of SUMO-1 with parkin, which results in its nuclear localization and autoubiquitination, other examples are provided by dynamin, DNA glycosylase, the androgen receptor and PML, whose non-covalent association with SUMOylated proteins constitutes the nucleating event for the formation of nuclear bodies (Shen *et al.*, 2006; Lin *et al.*, 2006).

1.3.4 Biological function

The pathway of SUMO conjugation affects many biological processes and is required for cell viability in yeast, nematodes and higher eukaryotes. In *S. cerevisiae*, mutation in genes for the SUMO E1 and E2 display cell cycle defects and arrest at the G2/M boundary (Johnson and Blobel, 1997; Seufert *et al.*, 1995), while in S. *pombe* mutations in the homologous genes results in cells with mitotic defects and severely impaired growth (al-Khodairy *et al.*, 1995; Shayeghi *et al.*, 1997). This indicates that SUMOylation is important for passage through the cell cycle. Recent studies have also highlighted a critical

role for SUMO deconjugation in various aspects of mitosis (Azuma *et al.*, 2003; Bachant *et al.*, 2002; Dieckhoff *et al.*, 2004; Stead *et al.*, 2003).

The initial genetic experiments also indicated that cells lacking components required for SUMO modification had an impaired ability to repair damaged DNA. In *S. pombe*, disruption of the SUMO homolog gene is not lethal, but cells display aberrant mitoses, sensitivity to DNA damaging agents, loss of minichromosomes and chromosomes with greatly elongated telomers (Tanaka *et al.*, 1999).

In S. *cerevisiae*, Ubc9 is necessary for damage tolerance and damage induced interchromosomal homologous recombination (Maeda *et al.*, 2004). In eukaryotic cells Ubc9 is involved in controlling the activity of the post-replicative DNA repair pathway through modification of PCNA (Hoege *et al.*, 2002), in synergy with the ubiquitin conjugating system that targets the same lysine residue (Stelter and Ulrich, 2003).

Another proposed cellular function for SUMO modification is the regulation of the subcellular localization of its substrates. Although cytoplasmatic proteins can be SUMOylated, in most cases nuclear targeting is required for SUMO modification *in vivo*. Among others, one example is RanGAP1: unmodified RanGAP1 is cytoplasmic, whereas SUMO-1 modification on a target lysine directs it to the nuclear pore complex, where it interacts with RanBP2 and is recruited to the mitotic spindle (Joseph *et al.*, 2002).

The observation that a number of proteins (IκBα, NEMO, PCNA, Hungtintin) can be modified by both SUMO and Ubiquitin on the same lysine residues raises the possibility that SUMOylation may act as an antagonist of proteasome-mediated degradation, with the competition between both conjugation pathways representing a means of modulating substrate stability. However, there are evidences that the relation of SUMO and Ubiquitin conjugating systems is not only antagonistic and the picture is much more complex, as in the case of the ubiquitin conjugating enzyme E2-25K whose activity is inhibited by SUMOylation (Pichler *et al.*, 2005) and PML bodies which are thought to integrate both system (Bailey and O'Hare, 2005).

Finally, SUMO modification modulates the transcriptional activity of a large number of transcription factors, such as p53, the androgen receptor, heat shock transcription factors, p300, Elk-1 and others. In most cases reported to date, SUMO is associated with transcriptional repression, but, in a limited number of instances, an increased transcriptional activity was observed.

1.4 Aims of this work

While familial PD caused by α-synuclein mutations is extremely rare, PD characterized by α-synuclein containing Lewy bodies accounts for >90% of sporadic Parkinsonian disorders.

New insights into the role of α-synuclein pathology in PD are reorienting the design of drug discovery efforts to focus on targets related to α-synuclein misfolding and fibrillization into filamentous Lewy bodies and neurites, as well as other potential pathogenic pathways involving α-synuclein post-translational modifications and degradation.

Thus, clarity about the relationship of α-synuclein-mediated neurodegenerative mechanisms to PD will have a powerful impact on the development of disease-modifying therapies for PD.

The purpose of this thesis is to elucidate these molecular mechanisms by a multifaceted approach:

1. the development of a cellular model to study α-synuclein dysfunction *in vitro*;

2. the identification of small peptides able to bind and interfere with the pathological function of mutant α-synuclein by a yeast two-hybrid screening of a random library of aptamers;

3. the study of the interaction between dopamine and α-synuclein;

4. the study of the role of post-translational modification of α-synuclein by SUMO.

2.1 Plasmids, cloning and mutagenesis

AS expression in mammalian cells:

PcDNA3.1 vector containing:

1) Human WT and A53T AS (with/without FLAG tag or Myc-HIS$_6$ tag)

2) Single lysine to arginine mutants: K12R, K96R and K102R AS (FLAG tag)

3) Triple K12R/K96R/K102R AS (FLAG tag)

The mutants were generated using site-directed mutagenesis employing mutagenic primers and two-steps PCR. All constructs were confirmed by DNA sequencing.

Expression of SUMO machinery in mammalian cells:

pcDNA3.1 vector containing:

1) Human WT SUMO-1/2/3-HA (conjugable)

2) Mutant SUMO-1/2/3-HA (unconjugable)

3) SUMO-1-GFP conjugable and unconjugable

4) Human Ubc9-HA

Bacterial expression of WT and mutant AS:

pET-11a vector containing:

WT, E83A, E126A, S129A, Triple (E83A/E126A/S129A) AS.

Human WT AS cDNA was cloned into the bacterial expression vector pET-11a at the *Nde*I site. The mutants were generated using site-directed mutagenesis employing

mutagenic primers and two-steps PCR. All constructs were confirmed by DNA sequencing.

2.2 Cell culture

All cell lines were grown at 37°C in a humified atmosphere containing 5% CO_2.

HEK (human embryonic kidney) 293T cells were cultured in D-MEM (Dulbecco's modified Eagle's medium), 10% FBS (fetal bovine serum), 100 U/ml penicillin, 100 µg/ml streptomycin.

SH-SY5Y (human neuroblastoma) cells were cultured in 1:1 volume ratio of F12 Nutrient Mixture (Ham) and EMEM (modified Eagle's medium), 2 mM glutamine, 1% NEAA (non essential aminoacids), 15% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin.

2.3 Transfection

The process of introducing nucleic acids into cells by non-viral methods is defined as "transfection". Transfection is accomplished either by microinjection of the foreign DNA into the cell, or through chemical or biological reagents such as a calcium ions or liposomes that create a "gate" in the cell membrane allowing the uptake of the foreign DNA.

Calcium phosphate method:

HEK 293T cells were transfected through the calcium phosphate method.

Cells were plated the day before transfection at a density of 250000 cells per well in 6-wells multiwells so to reach about 50% confluency at the time of transfection.

A transfection mix composed by 0.25 mM $CaCl_2$, 1-3 µg plasmid DNA and H_2O to a final volume of 100 µl was added dropwise with bubbling to a tube containing 100 µl HBS 2X (140 mM NaCl, 1.5 mM $Na_2HPO_4*2H_2O$, 50 mM HEPES pH 7.1). After 20 minutes incubation at room temperature the mixture was added to the cells. Cells were incubated overnight with the precipitates and the medium was changed the day after. Cells were collected and assayed 36-48 hours after transfection.

Lipofectamine:

SH-SY5Y cells were transfected accordingly to the Lipofectamine 2000 (Invitrogen) protocol.

Briefly, cells were plated in growth medium without antibiotics in order to be 90-95% confluent at the time of transfection. Complexes were prepared as follows (for 100 mm dishes):

24 μg DNA were diluted in 1.5 ml medium without serum. Separately 60 μl Lipofectamine were diluted in medium without serum and incubated for 5 minutes at room temperature. Then the DNA and Lipofectamine solutions were combined, mixed and incubated for 20 minutes at room temperature. Complexes were added dropwise to the cells. Changes in the medium were not necessary after transfection. Cells were collected and assayed 36-48 hours after transfection.

2.4 Creation of stable cell lines

For a cell's genetic material to retain the gene introduced through transfection, a stable transfection must occur. Integration of DNA into the chromosome or stable episomal maintenance of genes occurs with a relatively low frequency. The ability to select for these cells is made possible using genes that encode resistance to a lethal drug. An example of such a combination is the marker gene for neomycin phosphotransferase with the drug G418. Individual cells that survive the drug treatment expand into clonal groups that can be individually selected, propagated and analyzed.

The pcDNA3.1 vector carries the Neomycin resistance gene which confers resistance to the G418 drug, an antibiotic which interferes with eukaryiotic ribosomes function.

The minimal concentration of G418 for selection of stable transfectants depends on the cell line and through a titration experiment was determined to be 300 μg/ml for SH-SY5Y neuroblastoma cells.

SH-SY5Y cells were plated on a 100 mm Petri dish at 90% confluency and transfected with 24 μg of linearized pcDNA3.1 vector carrying WT or A53T α-synuclein gene using the Lipofectamine method. A plate of cells transfected with the empty vector was included as control.

The following day the cells were split into 2 dishes. 48 hours after transfection, the medium was changed and the antibiotic for selection (300 μg/ml G418) was added. The medium was changed every 3 days. After 5 days of selection a massive cell death was

observed and only a few single stably transfected cells survived. After 15 days the G418-resistant colonies reached a convenient size and some of them were collected and transferred to 24-well plates. All the remaining cells were pooled, expanded, assayed for transgene expression by western blot and immunofluorescence and frozen. Once the small monoclonal cultures had grown to confluency, they were passed to larger plates progressively, assayed for transgene expression by western blot and immunofluorescence and frozen.

2.5 Protein extraction, electrophoresis and western blot

Quick extraction in Sample Buffer:
HEK 293T or SH-SY5Y cells plated in 6-well multiwells were rinsed in PBS and harvested in 200 µl Sample Buffer 2X (100 mM TRIS-HCl pH 6.8, 1.4% β-mercaptoethanol, 4% SDS, 20% Glycerol, 0.2% bromophenol blue) heated at 100°C. Samples were boiled 5 minutes and sonicated before loading on SDS-polyacrylamide gel.

Soluble / Insoluble fractionation:
SH-SY5Y cells plated in 100 mm dishes or 6-well multiwells were rinsed with room temperature PBS and harvested in cold PBS. Cells were centrifuged briefly at 4°C at 2000 rpm and supernatant was discarded. Cells were lysed in Lysis Buffer (50mM TRIS pH 7.5, 150mM NaCl , 0.2% TRITON X-100) plus protease inhibitors cocktail for 15-20 minutes on ice, then centrifuged for 30 minutes at 4°C at 13000 rpm. The supernatant (soluble fraction) was recovered, protein concentration was determined by Bradford assay using a calibration curve built with standard amounts of BSA (bovine serum albumine) and Sample Buffer 2X was added.
The pellet (insoluble fraction) was resuspended in 100 µl Sample buffer 2X and sonicated.
Samples were boiled for 5 minutes before loading on SDS-polyacrylamide gel.

Extraction from yeast cells
1.5 ml from a 5 ml culture at OD 0.6-0.7 were centrifuged at 13000 rpm for 3 minutes to harvest yeast cells. The pellet was resuspended in 50 µl Sample buffer 2X, put in dry

ice until frozen, thawed at RT and sonicated for 30 seconds. Samples were boiled for 5 minutes before loading on SDS-polyacrylamide gel.

Extraction from mouse brain

2 adult C57/BL mice were sacrificed, brains were dissected and midbrain and striatum regions were isolated, washed in PBS and weighted. The samples were divided in two groups (with and without the cysteine protease inhibitor NEM) and homogenized in RIPA buffer containing protease inhibitors cocktail. After 30 minutes centrifugation at 4°C at 13000 rpm the supernatant was recovered and the protein concentration was determined by Bradford assay. Protein extracts were subsequently used for Western blot analysis or immunoprecipitation.

2.6 Immunoprecipitation

HEK 293T cell plated on 100 mm plates were rinsed with room temperature PBS and harvested in cold PBS. Cells were centrifuged briefly at 4°C at 2000 rpm and supernatant was discarded. Cells were lysed in RIPA buffer (50 mM TRIS-HCl pH 8, 300 mM NaCl, 0.5% DOC, 1% NP40, 0.1% SDS, 5 mM EDTA) plus protease inhibitors cocktail and 20 mM N-ethylmaleimide (NEM) for 15 minutes on ice, then sonicated and centrifuged 20 minutes at 4°C at 13000 rpm. Protein concentration was determined by Bradford assay using a calibration curve built with standard amounts of BSA (bovine serum albumine). The same amount of total proteins (1-2 mg) was used for each sample. The extracts were incubated with the immunoprecipitating antibody (1 µg) for 2 hours at 4°C with rocking, then 30 µl of Protein A or Protein G Sepharose 50% slurry were added and incubated at 4°C with rocking for 1 additional hour. Beads were pelleted briefly at low rpm and washed 3 times with RIPA buffer, then dried out with a syringe. Samples were boiled in 20 µl Sample Buffer 2X for 5 minutes before loading on SDS-polyacrylamide gel.

For the immunoprecipitation experiments from mouse brain extracts, 1 mg of total protein extract was used with 2 µg anti AS antibody and incubation was done overnight at 4°C.

2.7 Immunofluorescence

Cells were seeded onto 13 mm coverslips and allowed to attach for 24 hrs. After medium removal and washing in PBS cells were fixed in 4% paraformaldehyde for 10 minutes at room temperature. After fixation, cells were rinsed in PBS and incubated 5 minutes with glycine 100 mM to quench autofluorescence. After rinsing in PBS cells were permeabilized with Triton X-100 0.1% for 5 minutes, washed again in PBS and incubated in BSA 0.2% for 10 minutes to block non specific sites before primary antibody incubation.

Both primary and secondary antibodies were incubated in 0.2% BSA, 1% NGS, 0.1% Triton X-100.

After primary antibody incubation, cells were washed 2 times in PBS and subjected to secondary antibody incubation. Nuclei were labeled with DAPI (1 µg/ml) added during secondary antibody incubation. Cells were washed 2 times in PBS and mounted on slides using Vectashield mounting medium.

Antibodies:
The following antibodies were used:
Anti Myc tag mouse monoclonal, Cell Signaling, 1:1000
Anti FLAG tag mouse monoclonal, SIGMA, 1:2000
Anti HA tag mouse monoclonal, kindly provided by L. Collavin, LNMCIB, 1:1000
Anti α-synuclein rabbit polyclonal, Santa Cruz, 1:500
Anti SUMO-2 or -3, mouse monoclonal, Southwestern, 1:200
Anti SUMO 2/3 rabbit polyclonal, Zymed, 1:250

2.8 Treatments

MPP$^+$: SH-SY5Y cells were treated with MPP$^+$ 1-50 mM for 30 minutes to 1 hour.

HNE: SH-SY5Y cells were treated with HNE 10-500 µM for 30 minutes to 1 hour. Stably transfected SH-SY5Y cells were treated with HNE 1-50 µM for 72 hours.

FeCl$_2$: Stably transfected SH-SY5Y cells were treated with FeCl$_2$ 1-5 mM for 72 hours.

RA: Stably transfected SH-SY5Y cells were treated with all-trans retinoic acid (RA) 10 or 50 µM for 6 days changing medium and adding fresh RA every other day.

Ammonium chloride: Stably transfected SH-SY5Y cells were treated with ammonium chloride (NH_4) 20 or 50 mM for 24 hours.

2.9 MTT assay

Determination of cell growth rates is widely used in the testing of drug action, cytotoxic agents and screening other biologically active compounds. Several methods can be used for such determinations, but indirect approaches using fluorescent or chromogenic indicators provide the most rapid and large scale assays. Among such procedures, the MTT assay developed by Mossman [ref] is still among one of the most versatile and popular assays for determination of cell number.

The MTT assay involves the conversion of the water soluble MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) to an insoluble formazan. The formazan is then solubilized, and the concentration determined by optical density at 570 nm.

A stock solution of MTT 5 mg/ml was prepared in PBS, filtered through a 0.22 μm filter and added to the cell colture (a confluent well of a 6-well multiwell) together with fresh medium to a final concentration of 0.5 mg/ml. Cells were incubated at 37°C for 3 hours, harvested and pelleted at 1600 rpm for 3 minutes. The supernatant was discarded and the pellet was resuspended in 2 ml isopropanol-HCl 0.1 M and centrifuged at 13000 rpm for 2 minutes. The supernatant was used for the absorbance measurement at 570 nm on a fluorimeter.

2.10 Yeast two-hybrid screening

Many eukaryotic transacting transcriptional regulators are composed of physically-separable, functionally independent domains. Such regulators often contain a DNA-binding domain (DNA-BD) that binds to a specific promoter sequence and an activation domain (AD) that directs the RNA polymerase II complex to transcribe the gene down-stream of the DNA-binding site (Keegan *et al*., 1986; Hope and Struhl, 1986; Ma and Ptashne, 1987). Both domains are required to activate a gene and, normally (as in the case of the native yeast GAL4 protein), the two domains are part of the same protein. If physically separated by recombinant DNA technology and expressed in the same host cell, the DNA-BD and AD peptides do not directly interact with each other and thus cannot activate the responsive genes (Ma and Ptashne, 1988; Brent and Ptashne, 1985). However, if the DNA-BD and AD can be brought into close physical proximity in the promoter region, the transcriptional activation function will be restored (Fig. 8). In prin-ciple, any AD can be paired with any DNA-BD to activate transcription, with the DNA-BD providing the gene specificity (Brent and Ptashne, 1985). The yeast two-hybrid is a sensitive assay for protein-protein interactions based on co-transformation of host yeast strains with two plasmids, one containing an in frame fusion of the coding sequences for a DNA-BD and a protein of interest (bait), and the other containing a fusion of the cod-ing sequences of a transcription AD and a DNA library (prey). The interaction between the hybrid proteins generates an easily detectable phenotype by placing one or more suitable reporter genes under the control of the prey dependent promoter.
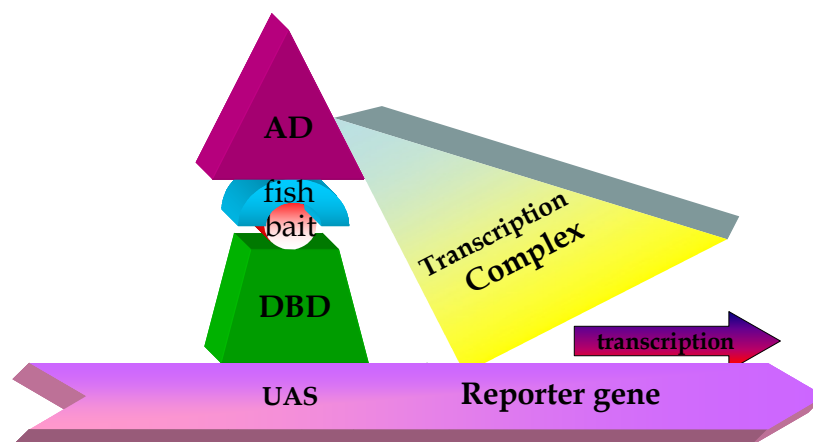

Figure 8. The yeast two-hybrid assay principle.

2.10.1 LexA system:

The bait is expressed as a fusion with the full length prokaryotic LexA protein (which normally functions as a repressor of SOS genes in *E. coli*) in a yeast strain containing 6 LexA binding sites ("operators") upstream of a selectable marker gene, LEU2, which allows growth on leucine-deficient medium.

The GAL1 inducible promoter in pJG4-5 is used to drive expression of cloned or library-encoded proteins fused to the B42 AD, an 88-residue acidic *E. coli* peptide that activates transcription in yeast. Transformants containing the AD fusion plasmids must be grown in medium containing galactose (and raffinose) as the carbon source when one induces expression of the fusion protein; otherwise, such transformants are grown in glucose-containing medium to keep expression repressed. Inducible expression means there is less opportunity for the foreign fusion proteins to have a toxic effect on the yeast host and thus be eliminated from the pool of potentially interacting proteins. Bait-prey interaction activates transcription of LEU2 and allows selection of interactors on media lacking leucine. The yeast strain also contains an autonomously replicating, high copy number plasmid containing the lacZ reporter gene under the control of 8 LexA operators. The gene product β-galactosidase confers a blue color to the colonies grown in presence of the substrate X-Gal. The amount of the β-galactosidase activity is so easily quantified and provides an indication of the strength of the interaction.

2.10.2 The library

A set of yeast and mammalian expression vectors has been developed that contains the *E. Coli* Thioredoxin (Trx) gene as a scaffold protein (Colas *et al.*, 1996). The vector (pJG4-5) designed for the two-hybrid presents the Thioredoxin gene fused to the VSV tag inserted downstream the B42 transactivation domain. A random library of 16 aminoacids long peptides has been inserted into the active site of the Thioredoxin to screen for peptide aptamers that bind to a particular "bait" (kindly provided by Prof. Del Sal, University of Trieste, Italy). The complexity of the library is estimated to be about 10^9 (Fig. 9).
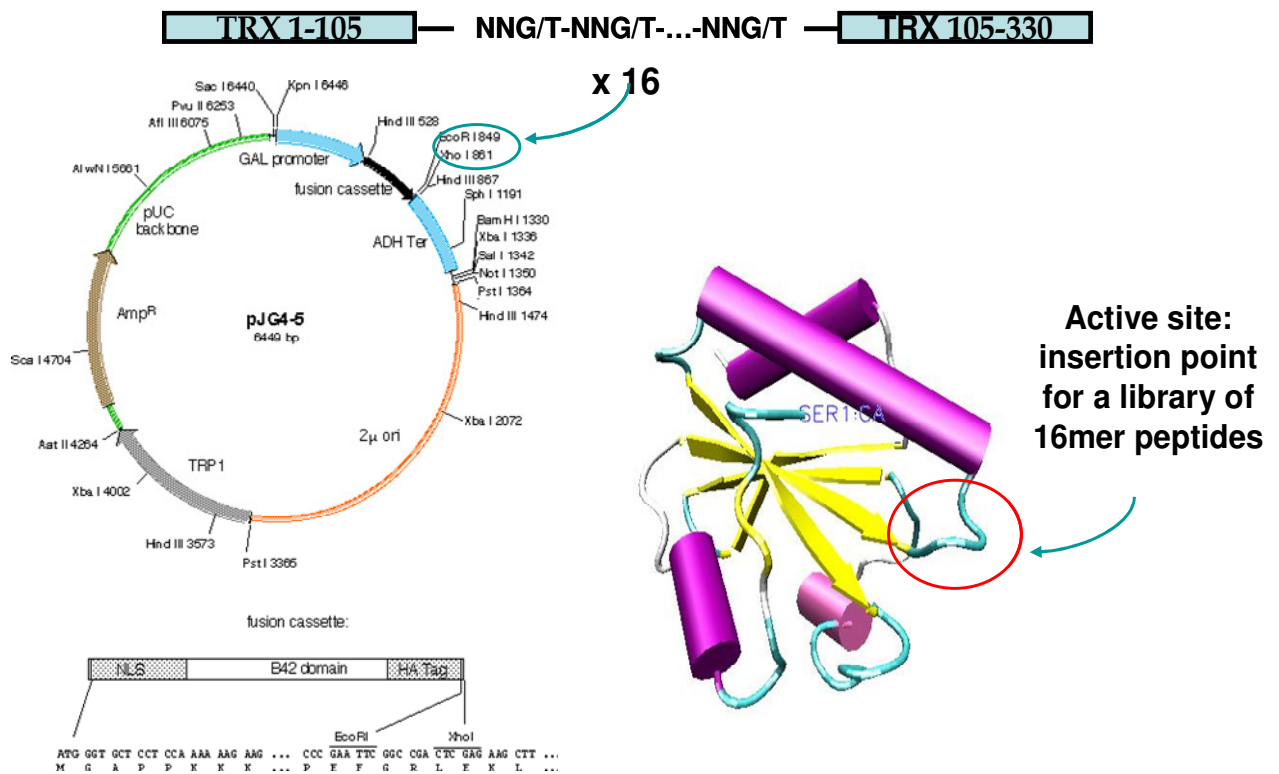
Figure 9. Scheme of the library vector pJG4-5 encoding a random library of 16 amino-acids-long peptides inserted in the active site of the Thioredoxin scaffold.

2.10.3 Procedure overview

The yeast two-hybrid screening consists of different steps.

1) The bait is cloned in the LexA vector (pEG202) and is tested in yeast for expression and lack of autoactivation of the reporter genes.

2) The bait vector and the LacZ reporter plasmid (pSH18-34) are co-transformed in the yeast strain.

3) The library is transformed in the yeast cells from the previous step and transformants are selected on medium lacking tryptophan (to select for library vector pJG4-5), histidine (to select for pEG202) and uracil (to select for pSH18-34).

4) Interactors are selected on medium containing galactose and lacking leucine (activation of LEU2 reporter gene) and assayed for β-galactosidase activity using the colony-lift filter assay (activation of LacZ reporter gene).

5) Library plasmids are isolated from the positive clones, amplified in *E. coli*, characterized by restriction enzyme digestion and sequenced.

6) Interactions are confirmed by co-transformation of the rescued library plasmids and the bait in the original yeast strain containing the lacZ reporter plasmid, with the appropriate controls (empty vectors and unrelated baits).

2.10.4 Transformation protocol

Day 1

EGY48 yeast containing the bait and lacZ reporter plasmid (EGY48/pSH18-34/pLexA-A53T AS strain) was grown at 30°C ON in 5 ml SDGlu/-UH.

Day 2

1 ml of the ON culture was inoculated in 50 ml SDGlu/-UH and incubated at 30°C ON with shaking (250 rpm).

Day 3

An aliquot of the ON culture was diluted into 500 ml pre-warmed YPD to produce an OD600 = 0.2, then incubated at 30°C with shaking for 3-4 hrs until the OD600 reached 0.7. Cells were centrifuged at 1500 x g for 15 min at RT. The cell pellet was washed by vortexing in sterile H_2O and was centrifuged at 1500 x g for 15 at RT and resuspended in 2 volumes of freshly prepared sterile 1X TE/LiAc.

9 tubes were prepared each containing 5 µg library DNA plus 20 µl carrier (10 µg/µl of salmon sperm DNA) . A control tube without library DNA was added as a control.

200 µl of competent cells were added into each tube and mixed by vortexing.

1.2 ml PEG/LiAc sterile solution was added to each tube, mixed and incubated at 30°C for 30 minutes with shaking.

DMSO was added to a final concentration of 10% (140 µl) and mixed.

Cells were transformed by heat shock for 15 min in a 42°C water bath, rapidly cooled on ice for 2 minutes and centrifuged at 1500 rpm for 5 minutes at RT.

Cells were resuspended in 200 ml of pre-warmed YPD, incubate at 30°C for 1 hr with shaking and centrifuged at 1500 x g for 5 minutes at RT.

The cell pellet was resuspended in 20 ml of pre-warmed SDGlu /-UHW.

Dilutions (100 µl of 1:10, 1:100 and 1:1000 dilution) of the total culture were spread on SDGlu/–UHW 10-cm plates to check the transformation efficiency. The remaining culture was incubated at 30°C ON with shaking.

Day 4

Cells were centrifuged at 1, 500 x g for 5 min at RT and resuspended in 1.5 ml SD/-UWH.

Dilutions of the total culture were spread on SDGlu/-UWH to compare to the number of primary transformants. All the remaining sample was spread onto 25 X 25-cm SDGal-Raf/-UHWL plates for interactors selection.

2.10.5 Colony-lift filter β-galactosidase assay

Nitrocellulose filters were leaned onto the SDGAlRaf/-UHWL master plates containing the Leu+ clones to be tested, then lifted and incubated for 10 minutes at -80°C to permeabilize yeast colonies. They were then soaked in Z buffer containing 0.5 mg/ml X-Gal and incubated at RT. Blue staining of the colonies with strongest interactions appeared after about 30 minutes. Colonies that became blue by two hours were considered positive and collected for further analysis.

2.10.6 "4 selective plates" test

The master plates were manually replicated to the following 4 plates:

1. SDGlu/-UHW+X-gal

2. SDGal-Raf/-UHW+X-gal

3. SDGlu/-UHWL

4. SDGal-Raf/-UHWL

Plates were incubated at 30°C and results were examined after 1, 2 and 3 and 4 days.

Colonies were considered positive when showing the Leu+ and lacZ+ phenotypes only on galactose: library transformants containing DNAs that encode peptides that interact with the bait will exhibit galactose-dependent growth on media lacking leucine (Leu+) and galactose-dependent β-galactosidase activity (LacZ+).

2.10.7 Reagents and media

Saccharomyces cerevisiae **EGY48** (MATα, his3 , trp1, ura3, LexAop(x6) -LEU2) re-porter host strain; carries a wild-type LEU2 gene under the control of LexA operators. The strain is deficient for HIS, LEU, TRP and URA which are markers that respectively allow selection of the bait plasmid (HIS3), the lacZ reporter plasmid (URA3) and the library plasmid (TRP1).

E. coli **strain KC8** (hsdR, leuB600, trpC9830, pyrF::Tn5, hisB463, lacΔX74, strA, galU, K). KC8 has a defect in trpC, which can be complemented by TRP1 from yeast. Thus, KC8 cells are used to rescue library plasmids (which carry TRP1) from yeast co-transformants that also contain the pLexA-BAIT plasmid. The mixed plasmid DNA is isolated from yeast and introduced into KC8 competent cells, which are then plated on M9 minimal medium/–W. Due to incompatibility of the plasmid replication origins, only one plasmid construct will propagate in a given KC8 transformant. Only those transformants with a library plasmid will survive on the selection medium.
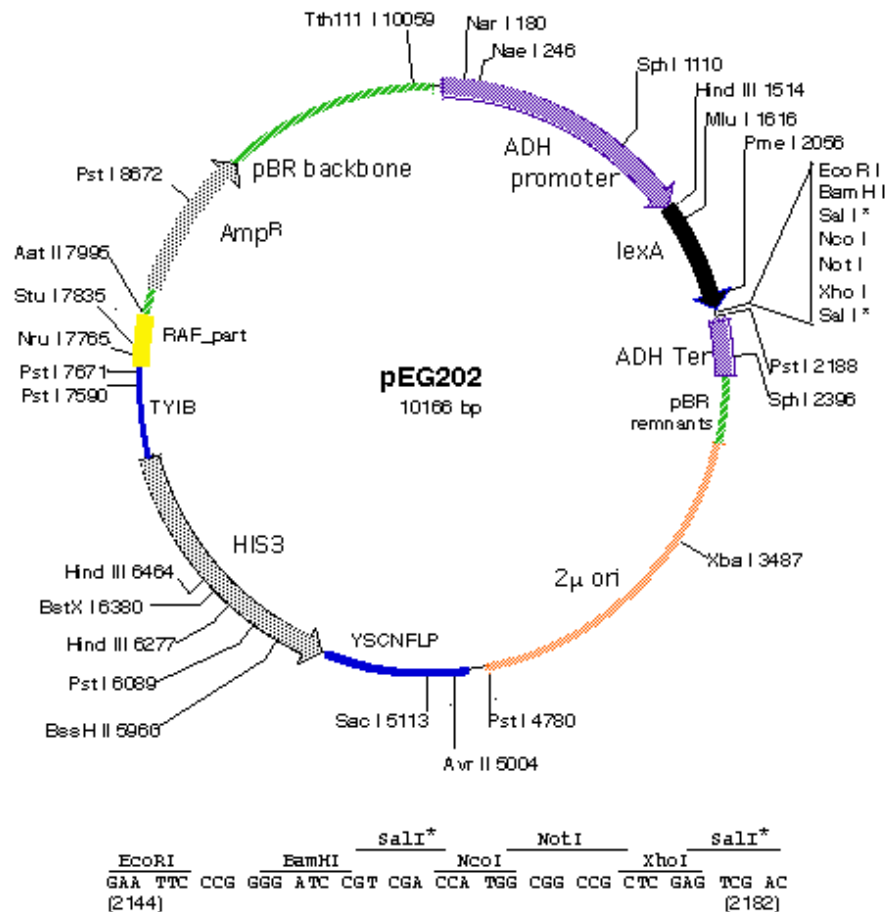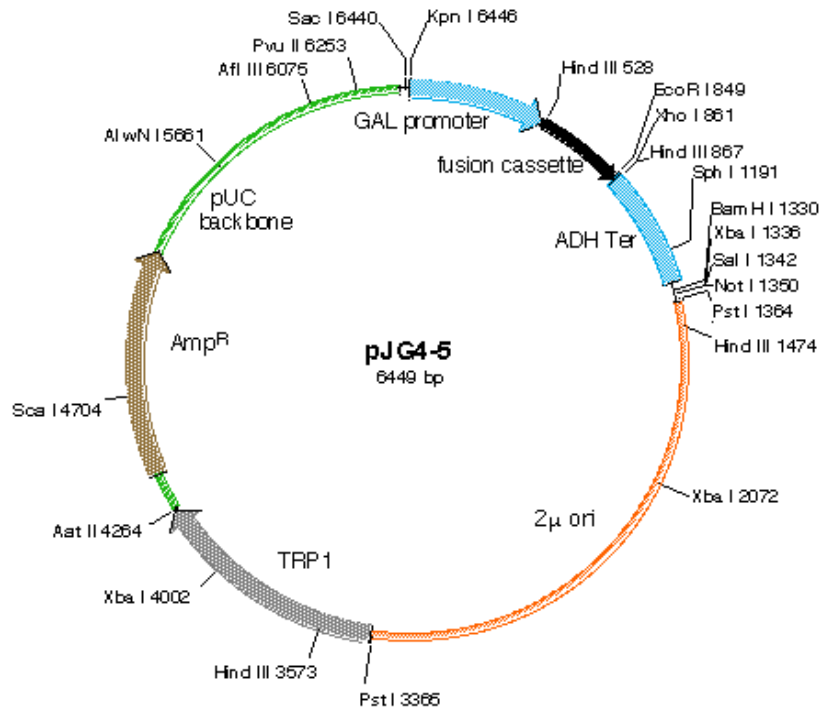
Plasmids

Figure 10. pEG202 (similar to pLexA) is a yeast - *E. coli* shuttle vector that contains a yeast expression cassette that includes the promoter from the yeast ADH1 gene (PADH1), sequences that encode amino acids 1 to 202 of the bacterial repressor protein LexA, a polylinker, and the transcription terminator sequences from the yeast ADH1 gene (TADH1). The plasmid also contains a *E. coli* origin of replication (pBR ori), the ampicillin resistance gene (AmpR), a yeast selectable marker gene (HIS3), and a yeast origin of replication (2 mm ori). pEG202 confers upon a his3- yeast strain the ability to grow in the absence of histidine and directs the constitutive expression of LexA (fused to approximately 17 amino acids encoded by the polylinker).
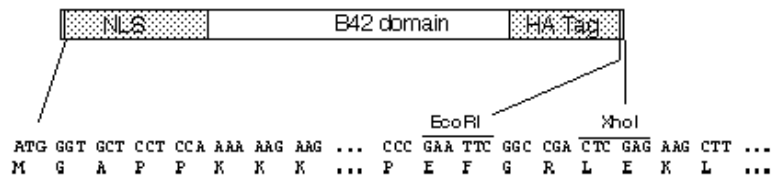

fusion cassette:


```
ATG GGT GCT CCT CCA AAA AAG AAG ... CCC GAA TTC GGC CGA CTC GAG AAG CTT ...
 M   G   A   P   P   K   K   K  ...  P   E   F   G   R   L   E   K   L  ...
```

Figure 11. pJG4-5 is a yeast - *E. coli* shuttle vector that contains a yeast expression cassette that includes the promoter from the yeast GAL1 gene (PGAL1), followed by sequences that encode the 106 amino acid fusion moiety or activation tag, and the transcription terminator sequences from the yeast ADH1 gene (TADH1). cDNAs or other protein coding sequences can be inserted into the unique *Eco*RI and *Xho*I sites so that encoded proteins are expressed with the fusion moiety at their amino terminus. The fusion moiety includes the nuclear localization signal from the SV40 virus large T antigen (PPKKKRKVA), the B42 transcription activation domain, and the hemagglutinin (HA) epitope tag (YPYDVPDYA). The plasmid also contains an *E. coli* origin of replication (pUC ori), the ampicillin resistance gene (AmpR), a yeast selectable marker gene (TRP1), and a yeast origin of replication (2 mm ori).

Figure 12. pSH18-34 contains the wild-type GAL1 promoter fused to lacZ. The GAL1 upstream activation sequences (UASg) have been deleted. Four high affinity overlapping type colE1 LexA operators which bind two LexA dimers have been inserted in place of UASg. The vector also contains an *E. coli* origin of replication (pBR ori), the ampicillin resistance gene (AmpR), a yeast selectable marker gene (URA3), and a yeast origin of replication (2μ ori).
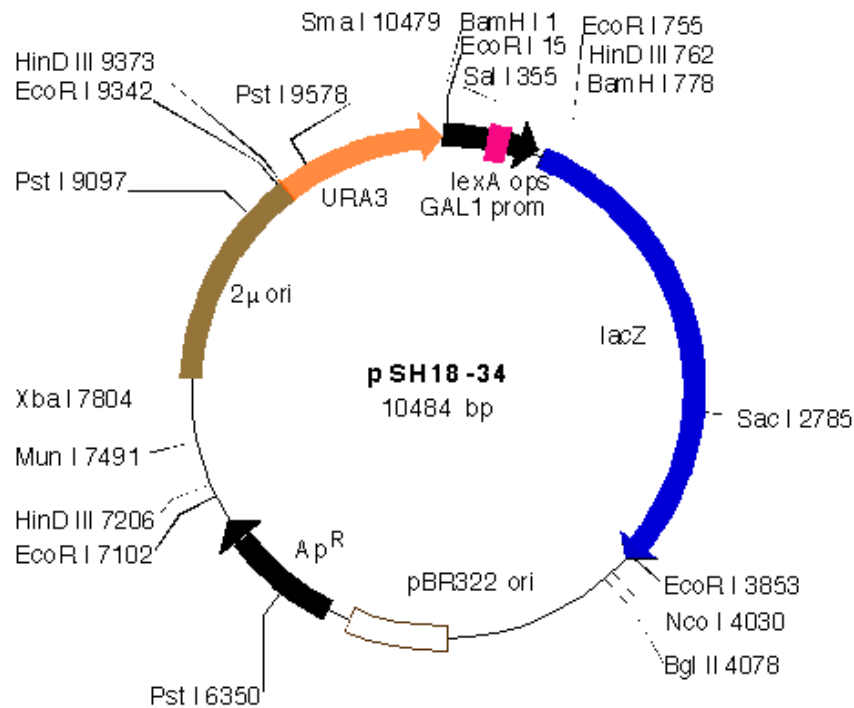
YEAST MEDIA

YPD medium

20 g/L Difco peptone

10 g/L Yeast extract

20 g/L Agar (for plates only)

pH 6.5

SD (synthetic dropout) medium

1.2 g Yeast nitrogen base without amino acids

5 g Ammonium Sulfate

20 g Agar (for plates only)

850 ml H2O

100 ml of the appropriate sterile 10X Dropout Solution

pH 5.8

SDGlu media contains 2% glucose as the carbon source; SDGal-Raf induction media contains 2% galactose and 1% raffinose as the carbon source (raffinose is included to improve growth properties of the cells).

SDGal-Raf/X-gal plates

SD media as described above

Galactose 2%

Raffinose 1%

BU salts 1X

X-Gal 40 mg/L (stock 20 mg/ml in DMF)

10X BU Salts

Dissolve the following components in 1 L (total) of H2O:

70 g Na2HPO4• 7H2O

30 g NaH2PO4

Adjust to pH 7, then autoclave and store at room temperature.

10X Dropout (DO) Solution

L-Adenine hemisulfate salt 200 mg/L

L-Arginine HCl 200 mg/L

L-Histidine HCl monohydrate 200 mg/L

L-Isoleucine 300 mg/L

L-Leucine 1000 mg/L

L-Lysine HCl 300 mg/L

L-Methionine 200 mg/L

L-Phenylalanine 500 mg/L

L-Threonine 2000 mg/L

L-Tryptophan 200 mg/L

L-Tyrosine 300 mg/L

L-Uracil 200 mg/L

L-Valine 1500 mg/L

Z buffer

$Na_2HPO_4 \bullet 7H_2O$ 16.1 g/L

$NaH_2PO_4 \bullet H_2O$ 5.50 g/L

KCl 0.75 g/L

$MgSO_4 \bullet 7H_2O$ 0.246 g/L

pH 7.0

E. coli MEDIA

M9 minimal medium (1 L)

1X Minimal Salts

1 mM $MgSO_4$

0.2 % Glucose

50 µg/ml ampicillin

1 mM thiamine-HCl

1X DO solution

pH 7.0-7.4

M9 minimal medium is used for nutritional selection of *E. coli* transformants comple-
mented by the wild-type yeast gene. For optimal recovery of KC8 add a 1X mixture of
amino acids lacking the specific nutrient (tryptophan) that will allow selection of the
desired plasmid. The same DO supplements used for yeast SD medium can be used to
supplement M9 minimal medium. In addition, KC8 requires thiamine for growth on
minimal medium.

10 X Minimal salts (1 L):

105 g K2HPO4

45 g KH2PO4

10 g (NH4)2S04

5 g NaCitrate

Transformation reagents

10X TE buffer

100 mM TRIS-HCl

10 mM EDTA

pH 7.5

PEG/LiAC solution

PEG 4000 40%

TE buffer 1X

LiAc 1 mM

pH 7.5

65% glycerol/MgSO4 solution

Glycerol 65% v/v

MgSO4 100 mM

Tris-HCl, pH 8.0 25 mM

2.11 Expression and purification of recombinant α-synuclein

Expression:

E. coli strain BL21 cells were transformed with the WT and mutant AS constructs. One bacterial colony was inoculated into 5 ml SOC broth containing 70 µg/ml ampicillin (Q-Biogene, Serva), and incubated overnight at 37°C with continuous shaking. Over-expression of the protein was achieved by transferring 2.5 ml pre-culture to 500 ml LB medium supplemented with 70 µg/ml ampicillin. Cells were grown at 37°C, with continuous shaking to an OD_{600} nm of about 0.4-0.6 followed by induction with 1 mM iso-propyl-β-thiogalactopyranoside (IPTG) for 3 h. After induction, the bacterial cells were harvested by centrifugation at 5000g for 10 min and stored at -20°C.

Lysis and clearing of lysate:

The cell pellet was re-dissolved in 50 mM Tris (Applichem), 50 mM KCl (Applichem), 5 mM MgAc (Applichem), 0.1% Sodium Azide (Applichem), pH 8.5 (250 mg pellet/ml of buffer). The cell suspension was sonicated for 10 minutes, and the lysate was centrifuged at 8000g for 30 min. The supernatant was separated from the pellet and the former was first boiled for 20 min, then centrifuged at 8000g for 30 min, and finally filtered through a 0.22 µm filter (Millipore).

Purification:

The protein was firstly purified through anion exchange chromatography (HiPrep Q FF column, Amersham) in 20 mM Tris, pH 8.0/ 20 mM Tris, 1 M NaCl, pH 8.0 followed by injection onto a size exclusion chromatography column (Superdex 200 10/300 or Superdex 200 16/60, Amersham) in 50 mM Tris/HCl, pH 7.5. Purified preparations were dialyzed against water for approximately 24 h, then lyophilized and stored at -20°C until use.

2.11.1 Ion Exchange (IEX) Chromatography

IEX separates proteins with differences in charge to give a very high resolution separation with high sample loading capacity. The separation is based on the reversible interaction between a charged protein and an oppositely charged chromatographic medium. Proteins bind as they are loaded onto a column. Conditions are then altered so that bound substances are eluted differentially. This elution is usually performed by increases in salt concentration or changes in pH. Changes are made stepwise or with a continuous gradient. Most commonly, samples are eluted with salt (NaCl), using a gradient elution (Fig. 13). Target proteins are concentrated during binding and collected in a purified, concentrated form.
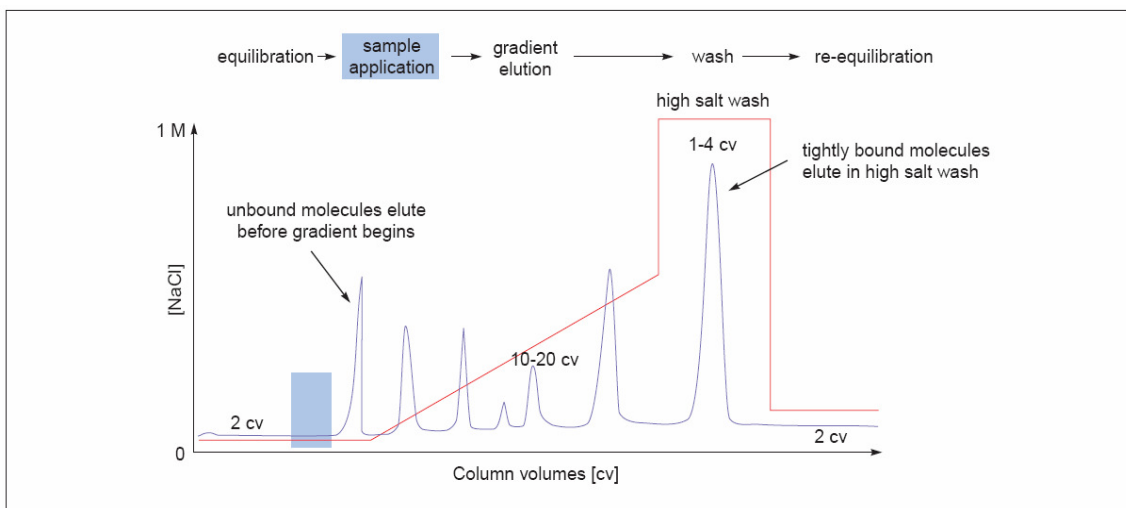
Figure 13. The ion exchange chromatogram.

The net surface charge of proteins varies according to the surrounding pH. When above its isoelectric point (pI) a protein will bind to an anion exchanger, when below its pI a protein will bind to a cation exchanger. Typically IEX is used to bind the target molecule, but it can also be used to bind impurities, if required. IEX can be repeated at different pH values to separate several proteins which have distinctly different charge properties.

2.11.2 Gel Filtration (GF)

GF separates proteins with differences in molecular size. The technique is ideal for the final polishing steps in a purification when sample volumes have been reduced (sample volume significantly influences speed and resolution in gel filtration). Samples are eluted isocratically (single buffer, no gradient, Fig. 14). Buffer conditions are varied to suit the sample type or the requirements for further purification, analysis or storage step, since buffer composition does not directly affect resolution. Proteins are collected in purified form in the chosen buffer.
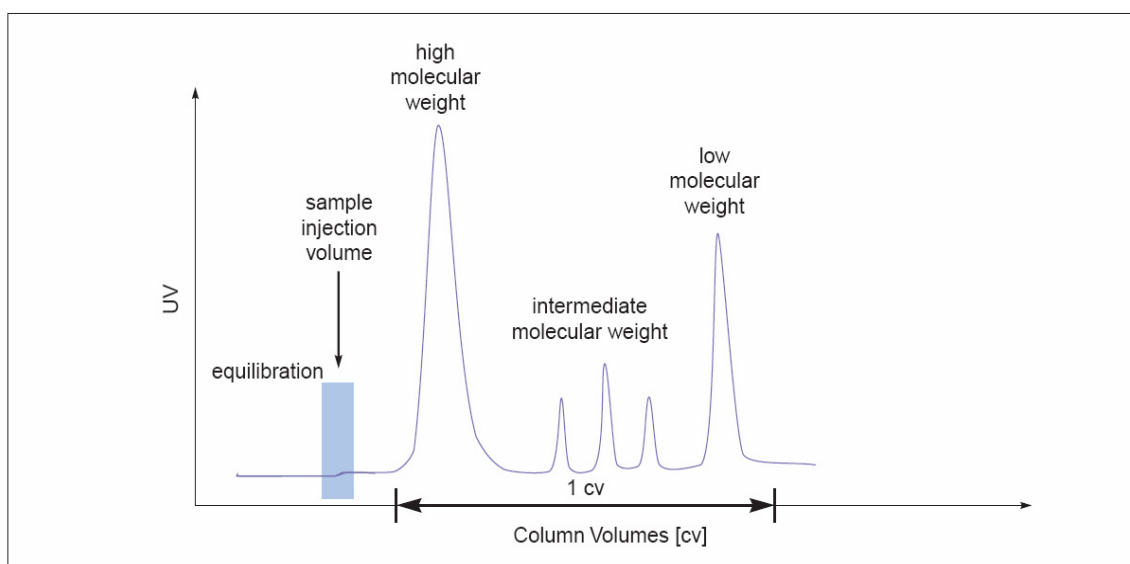

Figure 14. The gel filtration chromatogram.

MS analysis:

Mass spectrometry (MS) is an analytical technique used to measure the mass-to-charge ratio of ions. Recombinant WT and mutant AS proteins were analyzed to assess MW and purity of the samples. Intact proteins were ionized by matrix-assisted laser desorption/ionization (MALDI) before mass analysis.

Sample preparation

- o Matrix: Sinapinic acid (SA)
- o Matrix solution #1: SA saturated in MeOH
- o Matrix solution #2: SA (14 mg/mL) in 0.1% TFA/ACN 1/1
- o Sample deposition: 2 layers sample preparation
- o Sample diluted 1/1 with 0.1% TFA
- o Layer 1: 200 nL solution 1 fast evaporation on target
- o Layer 2: mixture 1/1.5 with matrix – 0.3 µL deposited on target on top of layer1

Analysis

- o Linear positive ion mode on the ABI 4700 (PAF Lausanne)

Calibration

- o one point calibration on WT α-synuclein [M+H]+: 14'461.19

2.12 Fibrillization studies of WT and mutant α-synuclein

2.12.1 Fibrillization conditions:

To characterize the aggregation properties of WT and mutant AS, proteins were dissolved in 20 mM Tris (Aldrich) / 150 mM sodium chloride (Aldrich), pH 7.4, at a concentration of 100 µM (500 µl total volume in a 1, 5 ml test-tube). The concentration of AS was determined using its molar extinction coefficient at 280 nm (i.e. ε_{280}=5120) on a Cary 100 Bio spectrophotometer. The purified proteins were then subjected to fibrillization conditions in absence or presence of an equimolar quantity of dopamine hydrochloride (Fluka) at 37 °C with continuous shaking for the indicated time points.

2.12.2 ThT assay.

Fibril formation was monitored by the ThT assay, which was performed by combining 10 µl of aggregated AS with 80 µl of 50 mM Glycine-NaOH (Fluka), pH 8.5, and 10 µl of 100 µM ThT (Sigma) in water. Fluorescence measurements were recorded in an Analyst Fluorescence instrument (LJL Biosystems). The excitation and emission wave-

lengths were set at 450 nm and 485 nm, respectively. The relative fluorescence at 485 nm was used as a measure of the amount of fibrillar aggregates formed in solution.

2.12.3 Circular Dichroism (CD).

The average secondary structure of WT and mutant AS was determined by CD spectroscopy using a Jasco J-815 Spectrometer (Omnilab). The Far UV-CD spectra (195-250 nm, integration time of 2 seconds for 0.2 nm) were collected at RT in a 1 mm path length quartz cuvette containing AS (20 µM in water).

2.12.4 Gel Electrophoresis (SDS-PAGE).

The AS samples were filtered through 0.22 µm filters, diluted in loading buffer [4% (w/v) Sodium dodecyl sulphate (Fluka), 60mM Tris, pH 6.8, 10% (v/v) Glycerol (Fluka), 5% (v/v) α-mercaptoethanol (Fluka), 8 % (w/v) bromophenol blue, 45.8% (v/v) distilled water] and separated on 12% SDS [(31.3% (v/v) Acrylamide N-N'-methylenebisacrylamide 37, 5:1 solution (Fluka), 25% (v/v) 1.5M Tris, pH 8.8 (Sigma), with 0.4% (v/v) SDS (Sigma), 42.7% (v/v) distilled water, 0.3% (v/v) ammonium persulfate (APS) and 0.12% (v/v) N, N, N', N'-tetramethylenediamine, (TEMED)], 1mm gel. Gels were stained with Simply Blue safe stain (Invitrogen) according to manufacturer's instructions.

2.12.5 Transmission Electron Microscopy (TEM).

For EM studies WT and mutant AS samples were deposited on Formvar-coated 200 mesh copper grids (Electron Microscopy Sciences) at a concentration of 35 µM. Grids were washed with two drops of water and stained with two drops of freshly prepared 0.75% (w/v) uranyl acetate (Electron microscopy sciences). Specimens were inspected on a Philip CIME 12 electron microscope, operated at 80 kV. Digitized photographs were recorded with a slow scan CCD camera (Gatan, Model 679).

3.1 A cellular model to study AS function in PD

We set up a cellular model to study AS function in PD by stably over-expressing WT and A53T AS in SH-SY5Y neuroblastoma cells. These cells express tyrosine hydroxylase (TH) and produce dopamine (Biedler *et al.*, 1978), express dopamine receptors (Farooqui, 1994), and are able to sequester dopamine as well as the parkinsonian toxin MPP$^+$ (Richards and Sadee, 1986). They however express a very low amount of AS which is not detectable with most of the available anti AS antibodies.

SH-SY5Y cells were transfected with pcDNA3-AS (either WT or A53T) and clones were selected with G418. A stable cell line transfected with the empty vector was established as a negative control. Clones were isolated and expression of AS protein was assessed by Western blot and immunofluorescence with an anti AS antibody. As shown in Fig. 15, different clones showed different AS expression levels in Western blots and immunofluorescence. The cellular localization of WT and A53T AS was similarly diffused in the nucleus and in the cytoplasm, while only a very faint staining was visible in the control cell line (data not shown).
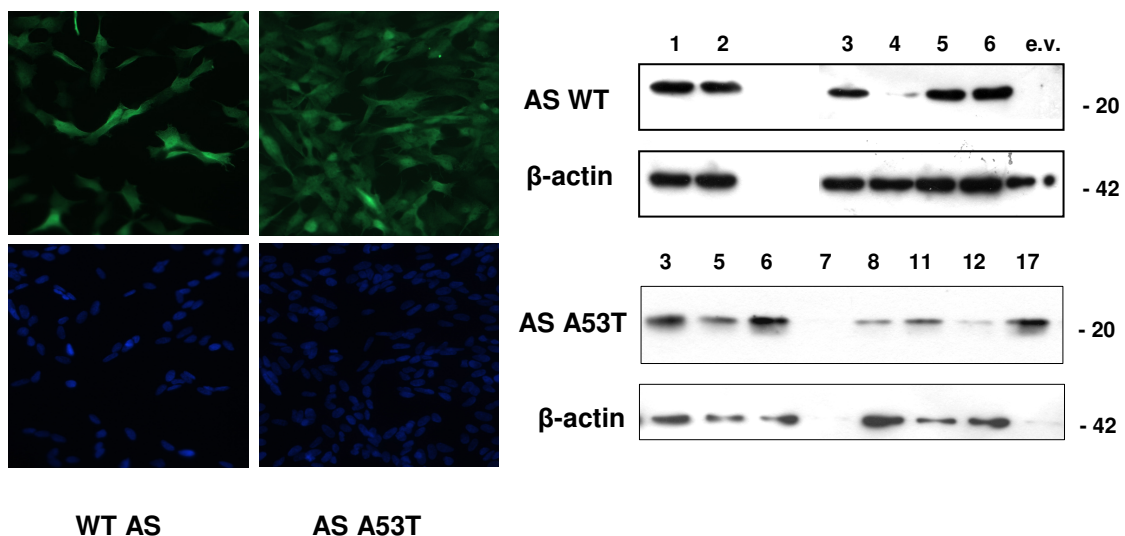
Figure 15. Anti AS immunofluorescence (left) or WB (right) analysis of different clones of SH-SY5Y cells stably over-expressing WT or A53T AS.

In order to better investigate the subcellular localization of AS in the stable cells, we also tried different fixation protocols for immunofluorescence. As shown in Fig. 16, 4% paraformaldehyde fixation yielded a diffuse nuclear and cytoplasmic localization, while other methods such as methanol/acetone or ethanol fixations revealed a localization of AS to the cellular membrane.

para 4%

MeOH/Ac

EtOH
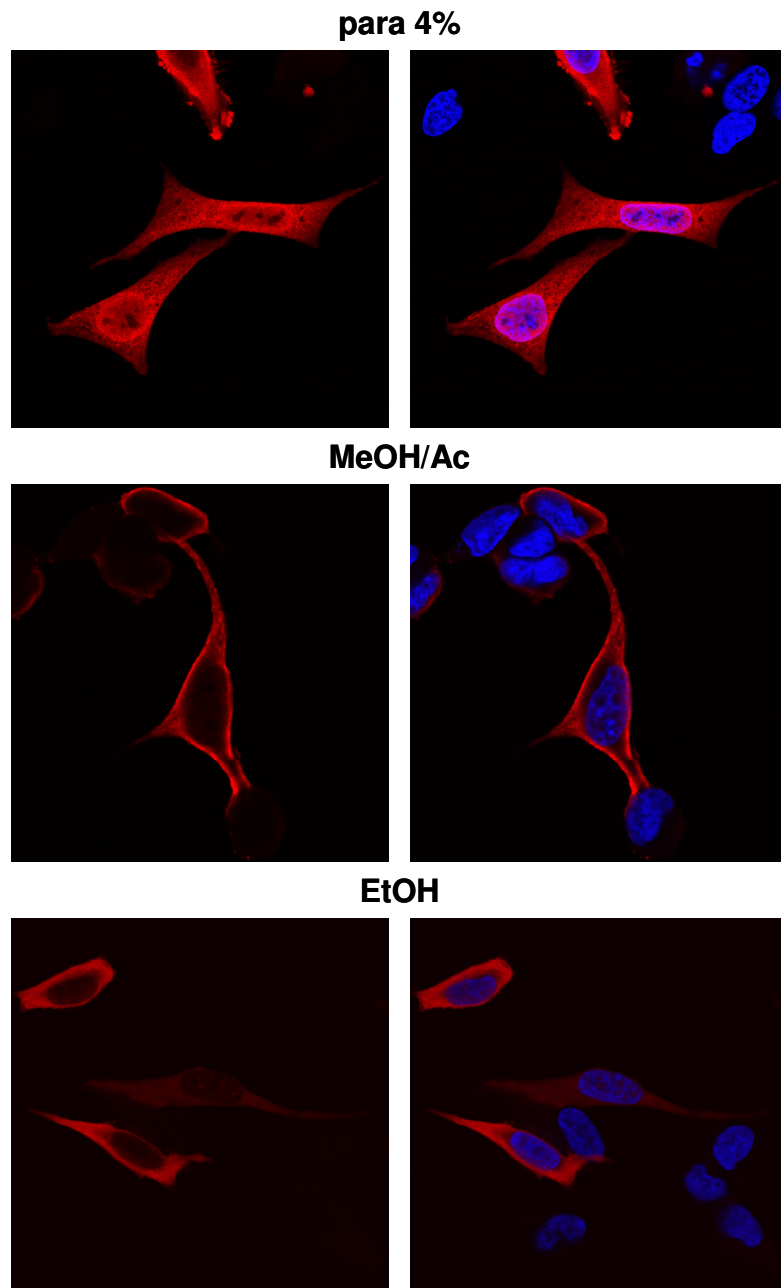
Figure 16. Anti AS immunofluorescence of SH-SY5Y cells stably expressing WT AS using different fixation protocols.

3.1.1 Stably over-expressed AS sensitizes SH-SY5Y cells to iron in-
duced toxicity

We examined the effects of PD-related neurotoxic stimuli on our cellular model through MTT viability assays. SH-SY5Y cells stably over-expressing WT or A53T AS were treated for 72 hours with 1, 2 and 5 mM $FeCl_2$ or 1, 10 and 20 µM HNE. As shown in Fig. 17, the highest iron concentration caused significant cell death in cells over-expressing either WT or mutant AS, but not in the control cell line stably transfected with the empty vector. On the contrary, HNE treatment caused cytotoxicity at the highest concentration with no significant difference between the AS over-expressing clones and the control.

Figure 17. MTT assay of viability of SH-SY5Y cell lines over-expressing WT or A53T AS or the empty vector. The experiment was performed in duplicate.

3.1.2 Iron induces AS aggregation in SH-SY5Y cells stably over-expressing WT or A53T AS

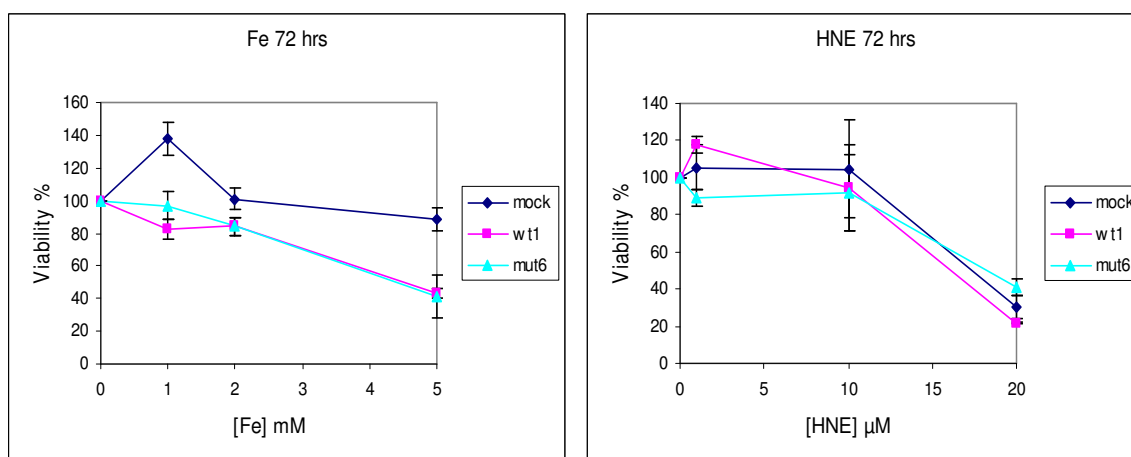We then investigated the effect of iron on AS protein in our cellular model. To this purpose, we treated different clones over-expressing WT or A53T AS with 5 mM $FeCl_2$ for 72 hours and we performed a separation of the Triton-X100 soluble and insoluble fractions of the cellular lysates before subjecting them to Western blot analysis with an anti AS antibody. As shown in Fig. 18, no difference was evident in the soluble fraction of treated and untreated cells. On the contrary, iron induced an increase in the high molecular weight AS reactivity present in the insoluble fraction only in cells over-expressing WT or mutant AS, but not in the empty vector control. These high molecular weight signals likely represent oligomeric AS complexes which are Triton-X100 insoluble but SDS soluble. The intensity of these signals varied among the different clones, even though the expression level of the protein was similar.
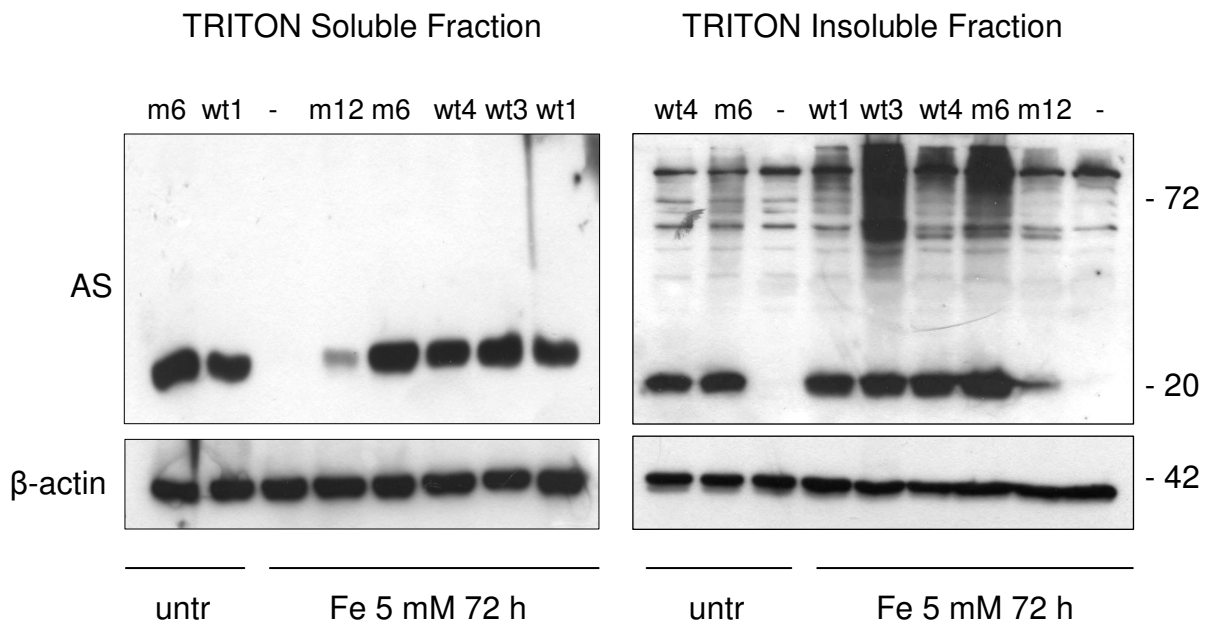

Figure 18. Anti AS immunoblot of Triton-X100 soluble (left) or insoluble (right) fractions of lysates of treated (5 mM $FeCl_2$ for 72 hours) or untreated SH-SY5Y cell lines stably over-expressing WT or A53T AS or the empty vector. Bottom: anti β-actin WB is shown as loading control.

We then performed immunofluorescence experiments with an anti AS antibody on the stable cell lines treated with iron. 5 mM $FeCl_2$ treatment for 72 hours (i.e. the same conditions as the WB fractionation experiment described above) did not change the normal AS diffuse cellular localization in SH-SY5Y cells stably over-expressing WT or A53T AS (data not shown). A higher concentration of iron (10 mM $FeCl_2$ for 24 hrs) was able to induce AS aggregates visible at the fluorescence microscope in both WT and A53T cell lines, causing extensive cell death and a change in the cells' morphology which appeared "shrunk" at the light microscopy (Fig. 19). We also tried other treatments related to oxidative stress and proteasomal inhibition (5µM MG132 for 24 hrs, 5 µM MG132 plus 5 mM $FeCl_2$ for 24 hrs, 100 µM H_2O_2 plus 5 mM $FeCl_2$ for 24 or 60 hrs, 50 µM dopamine plus 1 mM $FeCl_2$ for 4 days) but none of them resulted in visible AS aggregation.

WT AS

AS A53T

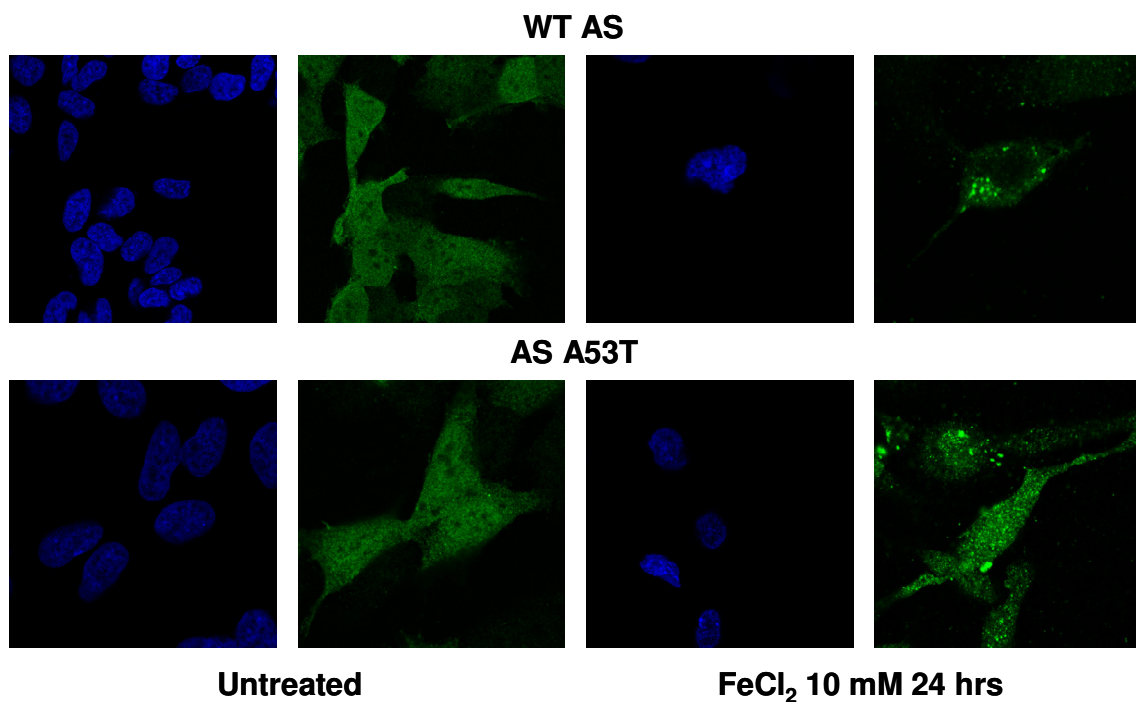**Untreated**   **FeCl$_2$ 10 mM 24 hrs**

Figure 19. Anti AS immunofluorescence of SH-SY5Y cells stably expressing WT or A53T AS untreated (left) or treated with 10 mM $FeCl_2$ for 24 hrs (right).

3.1.3 RA differentiation of SH-SY5Y cells stably over-expressing WT or A53T AS induces AS aggregation

SH-SY5Y can be differentiated by prolonged exposure to all-trans retinoic acid (RA) in a neuronal-like phenotype with attenuation of proliferation rate, extension of neuritic processes and presence of neuronal markers (NSE, GAP43). It was shown that RA treatment induces TrkB expression (Kaplan *et al.*, 1993), the brain-derived neurotrophin (BDNF) receptor.

We treated the stable cell lines over-expressing WT or A53T AS or the empty vector with 2 different concentrations of RA (10 and 50 µM) for 6 days changing medium and adding fresh RA every other day. AS shown in Fig. 20, the cells acquired an elongated morphology with long neuritic processes. The AS cellular localization did not change significantly with respect to the untreated cells.

WT AS   **AS A53T**

RA 10 µM 6 days
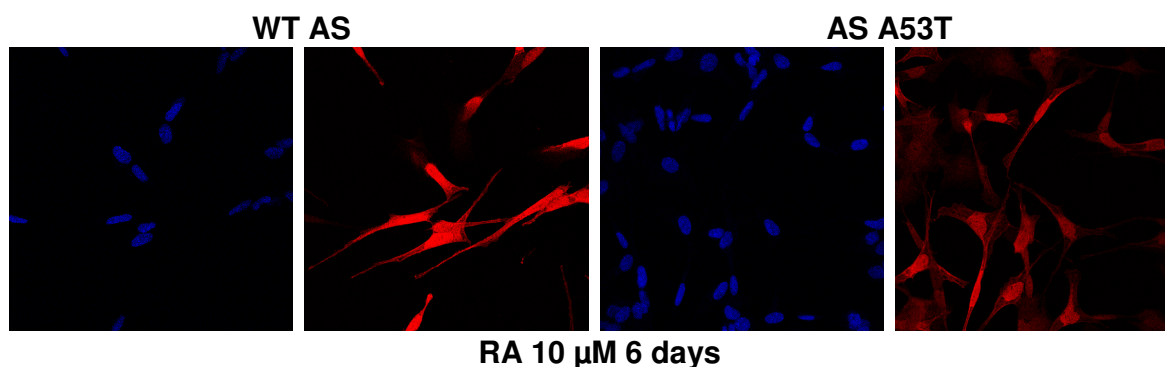
Figure 20. Anti AS immunofluorescence of RA differentiated SH-SY5Y cells stably over-expressing WT or A53T AS.

We performed a separation of the Triton-X100 soluble and insoluble fractions of the cellular lysates before subjecting them to Western blot analysis with an anti AS anti-body. As shown in Fig. 21, no difference was evident in the soluble fraction of differentiated and undifferentiated cells. On the contrary, differentiation induced an increase in the high molecular weight AS reactivity present in the insoluble fraction only in cells over-expressing WT or mutant AS, but not in the empty vector control. These signals

are similar to the one induced by the iron treatment described in section 3.1.2 and likely represent oligomeric AS complexes.
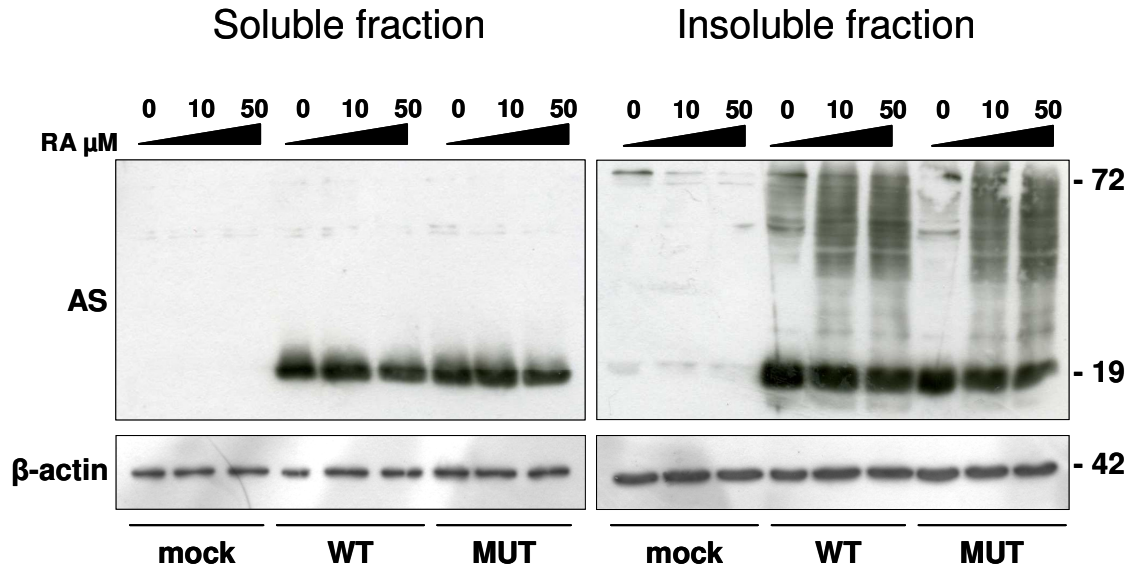
Figure 21. Anti AS immunoblot of Triton-X100 soluble (left) or insoluble (right) fractions of lysates of undifferentiated or differentiated (10 or 50 µM RA for 6 days) SH-SY5Y cell lines stably over-expressing WT or A53T AS or the empty vector. Bottom: anti β-actin WB is shown as loading control.

3.1.4 Effects of lysosomal proteolysis inhibition on SH-SY5Y cells stably over-expressing WT or A53T AS

The idea that abnormalities in proteasomal degradation of AS underlie PD has been prevalent for years, yet some studies failed to show alteration of AS levels by proteasomal inhibition (Ancolio *et al.*, 2000; Rideout *et al.*, 2001; Rideout and Stefanis, 2002) suggesting that there are alternative forms of AS degradation.

Whereas proteins with short half-lives are mostly broken down by the proteasome, most cytosolic proteins with long half-lives (>10 hours) are degraded by autophagic pathways within lysosomes (Glickman and Ciechanover, 2002; Cuervo, 2004; Dice, 2000). AS has been shown to be internalized and degraded in lysosomes by chaperone-mediated autophagy (CMA) (Cuervo *et al.*, 2004), a particular form of autophagy in which following binding of the chaperone-substrate complex to a lysosomal membrane receptor, lamp2a (Cuervo and Dice, 1996), substrate proteins are translocated into the lumen for degradation by hydrolases (Cuervo and Dice, 1998; Cuervo *et al.*, 1997). A30P and A53T mutations have been shown to inhibit both their own degradation and that of other substrates by CMA.

Impaired CMA of pathogenic AS may favor toxic gains-of-functions by contributing to its aggregation or additional modifications, such as nitrated or dopamine-adduct formation that could further underlie PD and other synucleinopathies.

To investigate if a lysosomal degradation pathway of AS was involved in our cellular model, we treated SH-SY5Y stable cell lines over-expressing WT / A53T AS or the empty vector with ammonium chloride (NH_4Cl), an inhibitor of lysosomal proteolysis. After 24 hrs the cells were lysed in Sample Buffer 2X and protein extracts were analyzed by Western blot with an anti AS and an anti β-actin antibody.

At the used concentrations (20 and 50 mM) the treatment resulted in a significant amount of cell death, as shown by the decrease in β-actin signal (interpreted as a measure of the number of cells) with increasing NH_4Cl concentrations (Fig. 22). Additional experiments, like MTT and Caspase-3 activation assays, are needed to better quantify the ammonium chloride-induced, AS-dependent cell death.
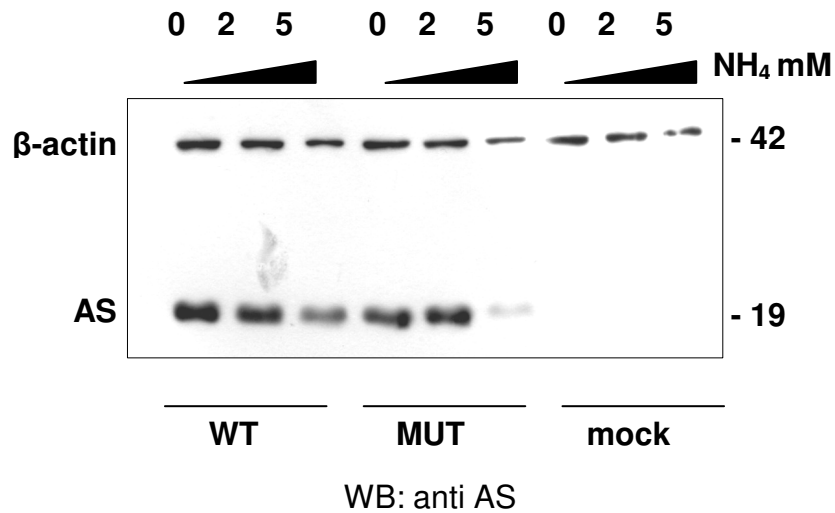
Figure 22. Anti AS immunoblot of protein extracts from SH-SY5Y cell lines stably over-expressing WT or A53T AS or the empty vector treated with 0, 20 or 50 mM ammonium chloride (NH_4) for 24 hrs. The membrane was then probed with an anti β-actin antibody.

We performed immunofluorescence studies with an anti AS antibody on the SH-SY5Y stable cell lines over-expressing WT / A53T AS or the empty vector treated with different concentrations of NH_4Cl (0, 10, 25, 50 mM) for 24 hours. The highest concentration caused a decrease in the nuclear and an increase in the cytosolic localization of both WT and mutant AS (Fig. 23), while no significant effect was seen at the lowest concentrations (data not shown).
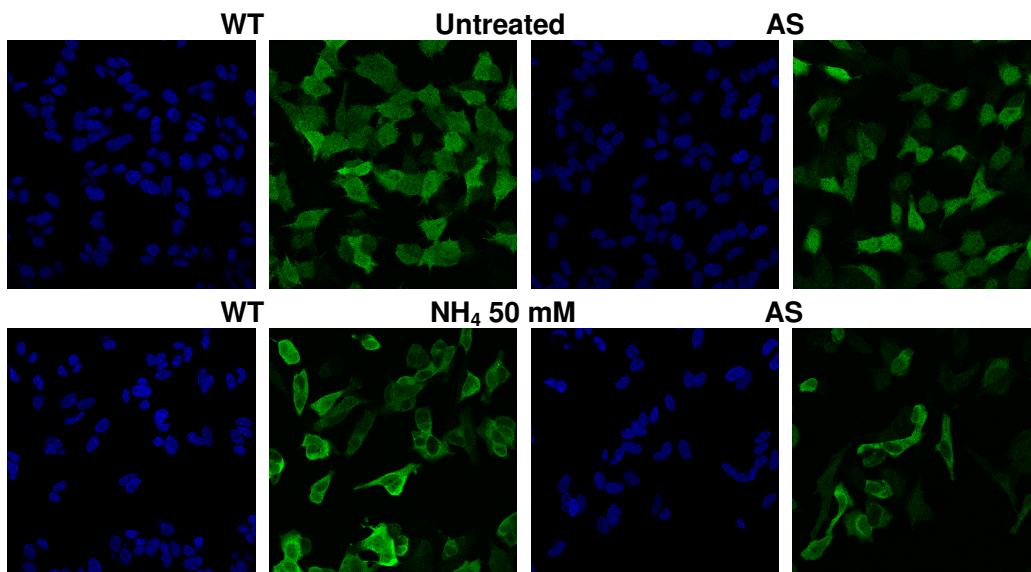

Figure 23. Anti AS immunofluorescence of SH-SY5Y cells stably over-expressing WT or A53T AS treated or untreated with 50 mM ammonium chloride (NH_4) for 24 hrs.

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3.2 A yeast two-hybrid screening for aptamers binding AS pathologic mutant A53T

Peptide aptamers are combinatorial proteins that specifically bind intracellular proteins and modulate their function. They are powerful tools to study protein function within complex regulatory networks and to guide small-molecule drug discovery. The design of peptide aptamers is inspired directly from the structure of immunoglobulins or T cell receptors, where variable peptidic loops are displayed by constant framework regions. Two fundamental differences distinguish peptide aptamers from these naturally occurring recognition molecules. First, peptide aptamers are extremely simple, consisting of a single variable peptide loop constrained within a constant scaffold protein. Second, the double constraint of the variable loop does not depend on disulfide bonds but is simply ensured by an insertion between two closely located residues of a rigid scaffold protein. This design renders peptide aptamers less vulnerable to proteases and enhances the average binding affinity by reducing the conformational freedom of the variable loops. So far, the scaffold protein most often used has been Thioredoxin (LaVallie *et al.*, 1993) but other scaffolds such as green fluorescent protein (Abedi *et al*., 1998), staphylococcal nuclease (Norman *et al.*, 1999) or the protease inhibitor Stefin A (Woodman *et al.*, 2005) have also been explored. For all of the above-mentioned reasons, peptide aptamers are ideally suited to bind intracellular target proteins, and they can be conveniently selected using intracellular techniques. Yeast two-hybrid methods are most often employed to select peptide aptamers for their ability to recognize target proteins in cells; a first application of this technology led to the identification of Trx-based peptide aptamers able to bind and inhibit the activity of the cyclin-dependent kinase (Cdk) 2, a kinase controlling the G1 to S phase transition of the cell cycle (Colas *et al.*, 1996).

We therefore decided to perform a yeast two-hybrid screening of a library of random peptide aptamers to individuate small peptides able to interact with the pathologic mutant A53T of the AS protein.

3.2.1 Preparation of the AS A53T bait plasmid

The first step was the preparation of a construct in which our protein of interest, AS A53T, was fused to LexA. A pcDNA3.1 plasmid containing the WT AS sequence was kindly provided by P. T. Lansbury. The point mutation was generated by site-directed mutagenesis employing mutagenic primers and two-steps PCR, and confirmed by DNA sequencing. The A53T AS sequence was then subcloned in pEG202 bait vector at the *Nco*I and *Not*I sites.

3.2.2 Preparation and testing of the bait strains

EGY48 MATα yeast strain was co-transformed with the lacZ reporter plasmid (pSH18-34) and the bait expressing plasmid (pLexA-AS) and grown in appropriate selective medium.

A series of control experiments was performed to check the expression and stability of the bait protein in yeast and to exclude autoactivation of the reporter genes by the bait itself or by its interaction with the empty library vector pJG4-5 TRX.

To test bait expression, cell lysates were used to perform Western blot analysis using an anti-LexA antibody (Fig. 24). A protein of the expected size was detected only in the bait strain.

To test the autoactivation potential of the bait, EGY48/pSH18-34/pLexA-AS strains were grown in SDGlu/-UH plates in presence of X-Gal (to test for auotoactivation of LacZ) or SDGlu/-UHL (to test for autoactivation of LEU2). The result was growth of white colonies in the first test and no growth in the second test, as expected in absence of autoactivation.

To test for autoactivation due to interaction of the bait with the empty library vector containing the Thioredoxin scaffold, pJG4-5 TRX was transformed in EGY48/pSH18-34/pLexA-AS strains and the transformants were plated on medium containing galactose to induce library vector expression, in particular SDGal-Raf/-UHW plates in presence of X-Gal (to test for activation of lacZ) or SDGal-Raf/-UHWL plates (to test for activation of LEU2). The result was growth of white colonies in the first test and no growth in the second test, as expected in absence of activation.
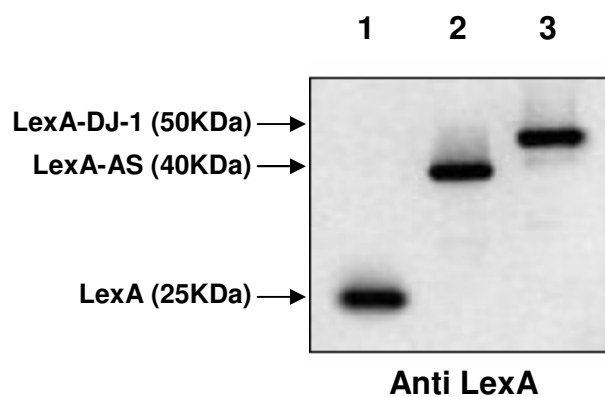
Figure 24. WB analysis of the bait expression in yeast. LexA alone and LexA-DJ-1 were loaded as controls. **Lane 1**: LexA, ; **lane 2**: LexA-AS A53T; **lane 3**: LexA-DJ-1.

3.2.3 Transformation of the library

We took advantage of a peptide library (kindly provided by Prof. G. Del Sal, University of Trieste) encoding 16 aminoacids-long random peptides inserted in the active site of the *E. coli* Thioredoxin scaffold (Colas *et al.*, 1996). The estimated complexity of the library was 10^9.

A pilot transformation experiment was performed using 5 µg of the library DNA. The yeast strain containing the LacZ reporter plasmid and the bait AS A53T (EGY48/pSH18-34/pLexA-AS A53T) was transformed by the lithium acetate method with the library plasmid and cells were plated on SDGlu/-UHW plates for selection. A transformation efficiency of $1.1 * 10^4$ colonies/µg DNA was achieved.

A subsequent transformation of 45 µg of the library DNA yielded a better transformation efficiency of $7.5 * 10^5$ transformants/µg DNA, for a total of $3.4 * 10^7$ transformants. The amplified transformants were collected after 4 days incubation at 30°C, aliquoted and stored at -80°C.

3.2.4 Screening for interactors

Interactors were selected growing an aliquot of transformed yeast cells in YPD medium containing galactose to induce library expression (4 hrs) and plating them on SDGal-Raf/-UHWL selective plates at high seeding density. Bait-prey interaction allows growth in absence of leucine. Colonies were manually collected after 4-7 days and

SDGal-Raf/-UHWL master plates were prepared. From those plates, colonies were lifted on a nitrocellulose filter and subjected to the β-galactosidase assay. Positive blue colonies were then collected on SDGlu/-UHW plates and streaked twice every 2 days to allow plasmids segregation, and finally were subjected to the "4 selective plates" test. Plasmid DNA was extracted from the positive clones according to QUIAGEN-miniprep protocol and electroporated in KC8 *E. coli* cells. Transformed cells were plated in M9 minimal medium plates. DNA was extracted from the KC8 cells and analized by restriction enzyme digestion, then sequenced.

The library transformants from the 5 µg and the 45 µg transformations were screened extensively.

Screening of 9 aliquots of the 5 µg transformation (for a total of $9 * 10^9$ cells) yielded 2 putative interactors, 1 aliquot of the 45 µg (10^9 cells) yielded 33 putative interactors, and 5 aliquots of the 45 µg transformation ($5 * 10^9$ cells) yielded 63 putative interactors which resulted to belong to 2 different peptidic sequences.

3.2.5 Validation

A first step of validation consist in a re-transformation of the isolated library plasmid together with the bait plasmid in a yeast strain containing the LacZ reporter. The interaction is then assessed by the "4 selective plates" assay. Of the collected putative interactors, only the 2 clones individuated in the largest screening passed this test. They were then subjected to a second validation test assessing the specificity of the interaction with the bait. To this end, they were co-transformed in a yeast strain containing the LacZ reporter together with different unrelated baits (RrsI, p53, DJ-1, glutamate transporter) or the empty LexA vector. Unfortunately, the two peptide interactors resulted to activate the reporter genes independently of the presence of the AS bait. Activation of the reporter genes by the peptides were modulated by the presence of the bait, resulting for example in a stronger activation in presence of the empty LexA vector, a weaker activation in presence of the AS bait, and no activation at all in presence of the RrsI bait.

3.3 A computational and *in vitro* study of dopamine-AS interaction

The interplay between dopamine and AS plays a central role in Parkinson's disease. To clarify the structural determinants of the interactions between dopamine and AS, we started a collaboration with the computational group of Prof. Carloni.

They docked dopamine and two of its derivatives onto conformers representative of an NMR ensemble of AS and predicted two moieties of AS involved in non-covalent inhibition of fibrillation, the [125]YEMPS[129] region, which forms non-specific hydrophobic contacts with the dopamine aromatic ring, and E83, which forms a water-mediated hydrogen bond with the ligands.

We then constructed mutants designed to alter the properties of these moieties and investigated *in vitro* the effects of dopamine on their aggregation properties, thanks to a collaboration with the group of Prof. H. Lashuel at EPFL in Lausanne.

We found, as suggested by modeling, that the E83A mutation abolishes the ability of dopamine to inhibit AS aggregation. The strategy that we outlined, which enables ligands to be devised that mimic the interactions between dopamine and AS, will help develop therapeutics for PD and related synucleinopathies.

3.3.1 Docking of dopamine and its derivatives on AS.

This part of the work was performed by Fernando Herrera. The clustering of AS NMR structures was done by Adriana Munoz (Herrera, Chesi *et al.*, submitted).

A set of 18 structures representing about 50% of the total number of conformers were selected from the NMR ensemble using a cluster methodology. Non-covalent dopamine-AS complexes were then obtained by docking dopamine (DOP), its protonated (DOP-H) and oxidized (DCH) forms (Fig. 25 A) on these structures. Similar results were obtained by considering all the resulting 5, 400 complexes (Fig. 25 A) or only the 54 most populated clusters, of the position of the ligands, for each docking experiments (3 for each of the 18 representative structures).

Ligands bind mostly to C-terminal region that includes the residues [125]YEMPS[129], a result that was obtained by considering the total number of hits or contacts and by including the most populated complexes (or clusters), which was usually one of the lowest energy in the AUTODOCK calculation (Fig. 25 A). This result is consistent with previ-

ous *in vitro* biochemical and biophysical studies implicating these residues in dopa-mine-induced inhibition of AS fibrillation *in vitro* (Norris *et al.*, 2005). A significant but much smaller number of hits are also found at the N-terminal region of AS for DOP and DCH (Fig. 25 A).

3.3.2 Molecular dynamics simulations of the docked structures:

This part of the work was performed by Fernando Herrera (Herrera, Chesi *et al.*, sub-mitted).

To investigate the structural stability of the docked structures, we performed 6 ns MD simulations in aqueous solution for the top 6 AS conformers in complex with the three ligands (18 complexes). The 6 studied AS conformers represent the 25% of the total number of conformations. The most populated complexes (or clusters) from the docking procedure were considered as starting complexes.

In 11 MD structures (out of the 18 that we investigated) the ligand binds to the C-terminal region that includes ^{125}YEMPS^{129} during the timescale investigated, consistent with previous *in vitro* experiments (Norris *et al.*, 2005). The complexes that we obtained show that the binding involves hydrophobic interactions between the aromatic ring of the ligand and hydrophobic side chains of AS (Table 2 and Fig. 25 B-C). In addition, the OH group of DOP and DOP-H forms hydrogen bonds (H-bonds) to polar groups in the same region of the AS protein. Because in the DCH molecule such group is absent, this interaction is not found in the DCH/AS complexes. We conclude that the ligands share a similar binding mode in which the most frequent contacts involve the aromatic ring moieties shared by the different ligands. In fact, DOP (or DOPH) and DCH share a remarkable similarity, as suggested by the calculation of the Tanimoto coefficients for the electrostatic potential (Te) and shape (Ts) (Willett *et al.*, 1998) between each of them. The results for DOP (or DOPH) and DCH are Te=0.71 and Ts=0.85. In 6 cases out of the 11, the ligands form rather stable contacts with E83 within the NAC region (Fig. 25 C, Table 2); these interactions are "water-mediated", since both E83 and the ligand H-bond to the same water molecule. Because of the lack of explicit solvent in the docking calculations, the water mediated contacts could be found only after the MD simulations. In fact, our docking simulations results (Fig. 25 A) showed that most of the interactions occur on the C-terminal part of the protein and a very few of them on the

E83 residue. Consistently, the contacts maps of the final MD structures show close contact between the C-terminal and the NAC region.

In all the 7 remaining simulations, the ligands do not bind in a stable manner with the protein. As an example, in the MD of conformer #13 with DOP, the ligand moves from its starting binding region (residues 92, 93 and 103) towards residues 99, 105 and 106 after 3 ns, and does not form stable interactions with the protein during the time scale of the MD simulation.

An analysis of the structural properties of the protein suggests that the C-terminal binding region assumes a specific conformation accommodating the ligand (Fig. 25 B). To quantify this property, we calculated the standard deviation (SD) of the n, n+1, n+4 angles among the $C\alpha$'s of the protein for the 11 stable and 7 unstable complexes. In the 11 stable complexes the smallest values are those of the C-terminal binding region (SD of the angles 125-126-129 and 128-129-132 are 11° and 13° respectively; to be compared to the value of 30° averaged over all the angles' SD). In addition, these deviations are smaller than any of the correspondent angles in the 7 unstable complexes (average 28°). The Ramachandran angles ϕ, Ψ for E126 in the 11 stable complexes are equal to -110° ± 30°, 0° ± 20°, while those of the other 7 have more spread values around the same average -110° ± 40°, 0° ± 70°.

Taken together, our calculations suggest that DOP, DOP-H and DCH bind mostly to the C-terminal part of the protein that includes the [125]YEMPS[129] region, fully consistent with previous *in vitro* experiments (Norris *et al.*, 2005). Within this region, the ligands form H-bond to E126 and S129 in same cases and nonspecific hydrophobic interactions with all of the five residues (Table 2). In all cases, AS always assumes a similar, kinked conformation in its binding region (Fig. 25 B). In the event of such binding, it may form water-mediated interactions with E83. Binding to other regions of AS do occur, but to a lesser extent and much more weakly.

DOP•AS

Persist	Hydrogen Bonds	Hydrophobic contacts	Water-mediated contacts
80 %	- Ser129(O)-DOP(O1) (D: 2.7±0.1 Å; A: 149°±23°)	- Gln134 (D: 4.4±0.4 Å) - Lys43 (D: 5.2±0.5 Å)	
100 %	- Glu123(OE2)-DOP(O1) (D: 2.6±0.3 Å; A: 65°±11°)	- Gln134 (D: 4.7±0.7 Å)	- Glu83 (D: 6.0±1.2 Å)
100 %	- Glu131(OE2)-DOP(O2) (D: 3.0±0.9 Å; A: 159°±24°)	- Tyr125 (D: 7.3±1.8 Å)	- Glu83 (D: 10.1±1.9 Å)

DOP-H•AS

Persist	Hydrogen Bonds	Hydrophobic contacts	Water-mediated contacts
100 %	- Glu130(OE2)-DOP-H(O1) (D: 2.9±0.5 Å; A: 152°±17°)	- Met127 (D: 4.4±0.4 Å)	
45 %	-	- Met127 (D: 6.6±0.6 Å)	
100 %	- Pro128(O)-DOP-H(O2) (D: 2.8±0.2 Å; A: 145°±33°)		- Glu83 (D: 9.2±0.9 Å)
100 %	- Glu126(O)-DOP-H(N1) (D: 2.8±0.1 Å; A: 104°±45°) - Glu126(OE2)-DOP-H(O2) (D: 2.7±0.4 Å; A: 155°±23°)		- Glu83 (D: 19.5±2.2 Å)*

DCH•AS

Persist	Hydrogen Bonds	Hydrophobic contacts	Water-mediated contacts
100 %		- Ile112 (D: 6.7±1.1 Å) - Glu126 (D: 7.7±0.5 Å) - Tyr136 (D: 4.3±0.5 Å)	
75 %		- Glu126 (D: 5.9±1.5 Å) - Gln134 (D: 6.6±1.3 Å)	
65 %		- Glu123 (D: 4.3±0.4 Å)	- Glu83 (D: 8.7±0.6 Å)
70 %		- Thr81 (D: 5.7±0.6 Å) - Pro128 (D: 4.4±0.6 Å)	- Glu83 (D: 8.3±0.9 Å)
100 %		- Val66 (D: 5.2±1.1 Å) - Glu126 (D: 4.0±0. 5 Å)	

Table 2. Persistence (Persist.), hydrogen bonds and hydrophobic contacts for 12 out of 18 complexes forming stable interactions with the [125]YEMPS[129] region (only 11 complexes show more than 65% of persistence). The distance (D) in the hydrogen bond column was measured between the heavy atoms while the angle (A) was defined as the donor-H…acceptor angle. The hydrophobic and water-mediated contacts with Glu83 were measured as the distance (D) between the center of mass of the ligand and the specific amino acid. O1 and O2 in DOP and DOP-H are the hydroxyl oxygen in the para- and meta- position relative to the ethylamine group. * In this case the water-mediated contact involved also the Glu 126 and it is formed at the end of the molecular dynamics simulation (Herrera, Chesi *et al.*, submitted).
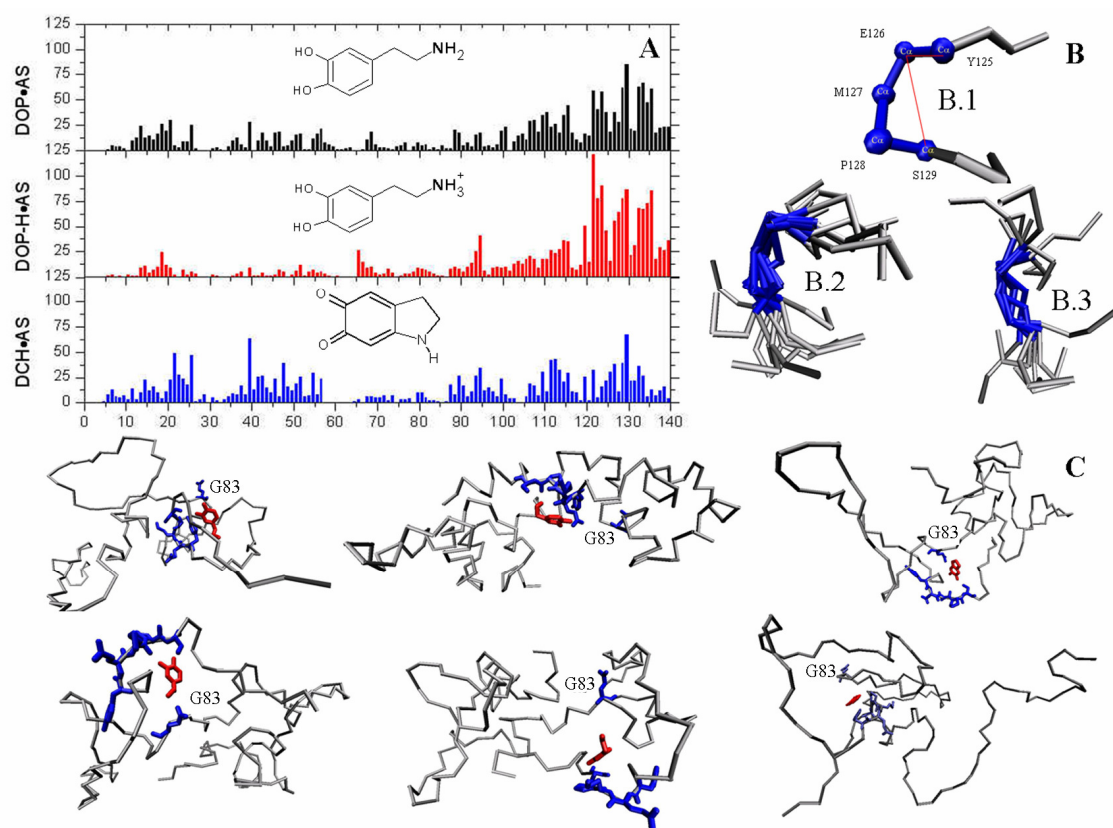
Figure 25. **Molecular docking and MD simulations of dopamine and its derivatives onto AS**: A) Number of hits between AS and dopamine (DOP), protonated dopamine (DOP-H) and dopaminochrome (DCH) as obtained in the 5, 400 docking calculations performed in this work. A hit is defined as a contact within a cutoff of 5 Å between the Cα of each amino acid and the center of mass of each ligand. B) Close up on the [125]YEMPS[129] Cα carbons (B.1) after the MD simulation. If the ligands bind to this region (B.2), the angle between Cα 125-126-129 shows a relatively small spread (79°±11°). The remaining 7 conformations is also shown (B.3) (105°±20°). C) In 11 simulations out of 18, the ligands bind to the [125]YEMPS[129] region. Here we show the six structures (among those) which form an additional (water-mediated) contact with E83. The 125-129 residues and E83 are colored in blue, the ligand is colored in red (Herrera, Chesi *et al.*, submitted).

3.3.3 Testing the structural model by *in vitro* aggregation studies.

The calculations point to the key role of H-bonding, water-mediated ligand-E83 interactions and suggest that H-bonds also contribute to the C-terminal dopamine interactions (Table 2). To test the validity of these conclusions, we investigated the aggregation properties of four alanine mutants of AS *in vitro*: namely E83A, E126A, S129A and E83A/E126A/S19A in the presence and absence of dopamine. The replacement of Glu and Ser by an Ala will result in the loss of the H-bonding functionality of the residues' side chain. Thus the E83A mutation is expected to dramatically affect dopamine-AS interactions (and therefore the fibrillation process), because E83 forms a specific water-mediated H-bonds with the ligands. Our model shows that ligand-AS interactions at the C-terminal are dominated by nonspecific hydrophobic interactions and predict that the E126A and S129A mutations would not significantly alter ligand-AS interaction. In addition, in the case of S129A, the Ser to Ala mutation might not affect H-bonding with the ligand as it involves the backbone.

Human WT AS cDNA was cloned into the bacterial expression vector pET-11a at the *Nde*I site. Mutants were generated using site-directed mutagenesis employing mutagenic primers and two-steps PCR. All constructs were confirmed by DNA sequencing.

Recombinant human WT, E83A, E126A, S129A and E83A/E126A/S129A AS proteins were expressed and purified as described in the Material and Methods section. The molecular weight and purity of the samples was checked by mass spectrometry (Fig. 26).
The aggregation properties of the five proteins were determined by incubating 100 μM of protein in TRIS buffer (20 mM Tris, 150mM NaCl, total volume = 500 μl in 1.5 ml plastic tubes) at 37 °C with continuous shaking for 72 hrs in the absence or presence of equimolar dopamine. At regular intervals, aliquots were removed and subjected to analysis by ThT fluorescence assay, SDS-PAGE gel, circular dichroism (CD) and electron microscopy (EM). All the mutants show increased aggregation relative to the WT (Fig. 27 A). Interestingly, the two AS variants containing the E83A mutation retain the ability to form fibrils in the presence of an equimolar quantity of dopamine (Fig. 27 A), whereas fibril formation by the WT, E126A and S129A variants was abolished in the presence of dopamine. This result clearly suggests that the nature of dopamine-AS interactions in the C-terminal region are distinct from those in the NAC region, consistent

with our predictions. A consistent picture is obtained by monitoring the loss of soluble protein during the fibrillization reaction by SDS-PAGE. Aliquots of the samples at various time points were diluted (factor 1:10) and filtered through 0.22 µm PVDF filters to eliminate fibrillar and insoluble aggregates, before analysis in 12% PAA gels with Coomassie Blue staining. The signal corresponding to the protein in the flow through (monomer and soluble oligomers) was quantified using the software ImageJ. In agreement with ThT data, we see a decrease of soluble content that is proportional to the degree of aggregation by each protein (Fig. 27 B, blue rectangles). In the presence of dopamine, the majority of WT, S129A, and E126A protein remain in solution, whereas very little soluble protein remains in the case of the two E83A-containing mutants (Fig. 27 B, red rectangles). CD spectra show that all proteins adopt predominantly random coil conformation (data not shown) but form β-sheet rich structures after incubation for 72 hrs at 37°C. In the absence of dopamine and after incubation for 72 hrs, the majority of WT, E126A, and S129A precipitates out of solution and the CD spectra of the remaining material exhibits a predominantly random coil structure, except for S129A which exhibits a spectra consistent with species (soluble aggregates) rich in β-sheet structure (Fig. 27 C). Co-incubation with dopamine prevents the transition from random coil to β-sheet in the case of WT, S129A, E126A, but not in the case of E83A or E83A/E126A/S129A, further confirming that dopamine is able to prevent fibrillization of the WT, E126A and S129A mutants but not the E83A E83A/E126A/S129A mutants (Fig. 27 C).

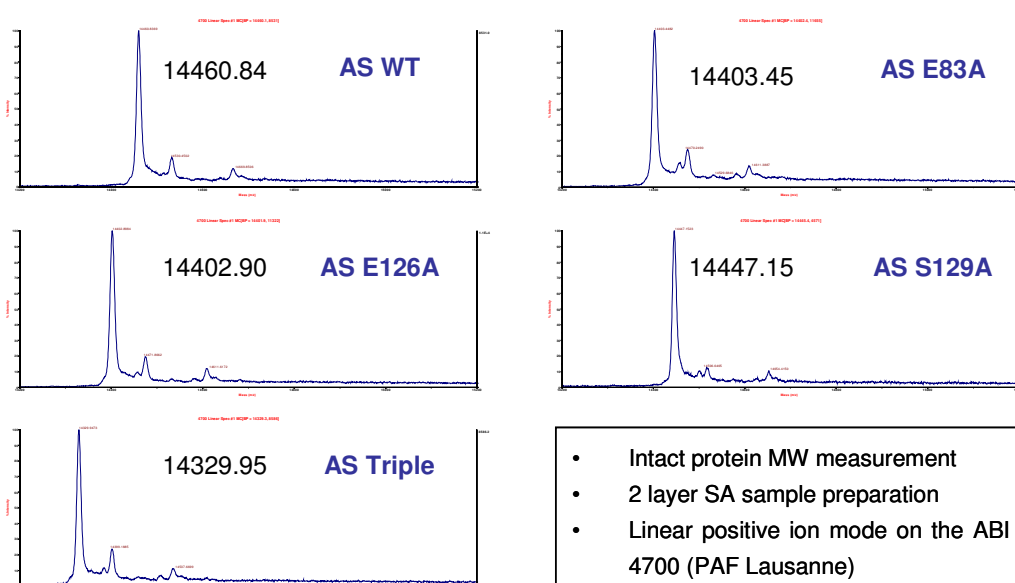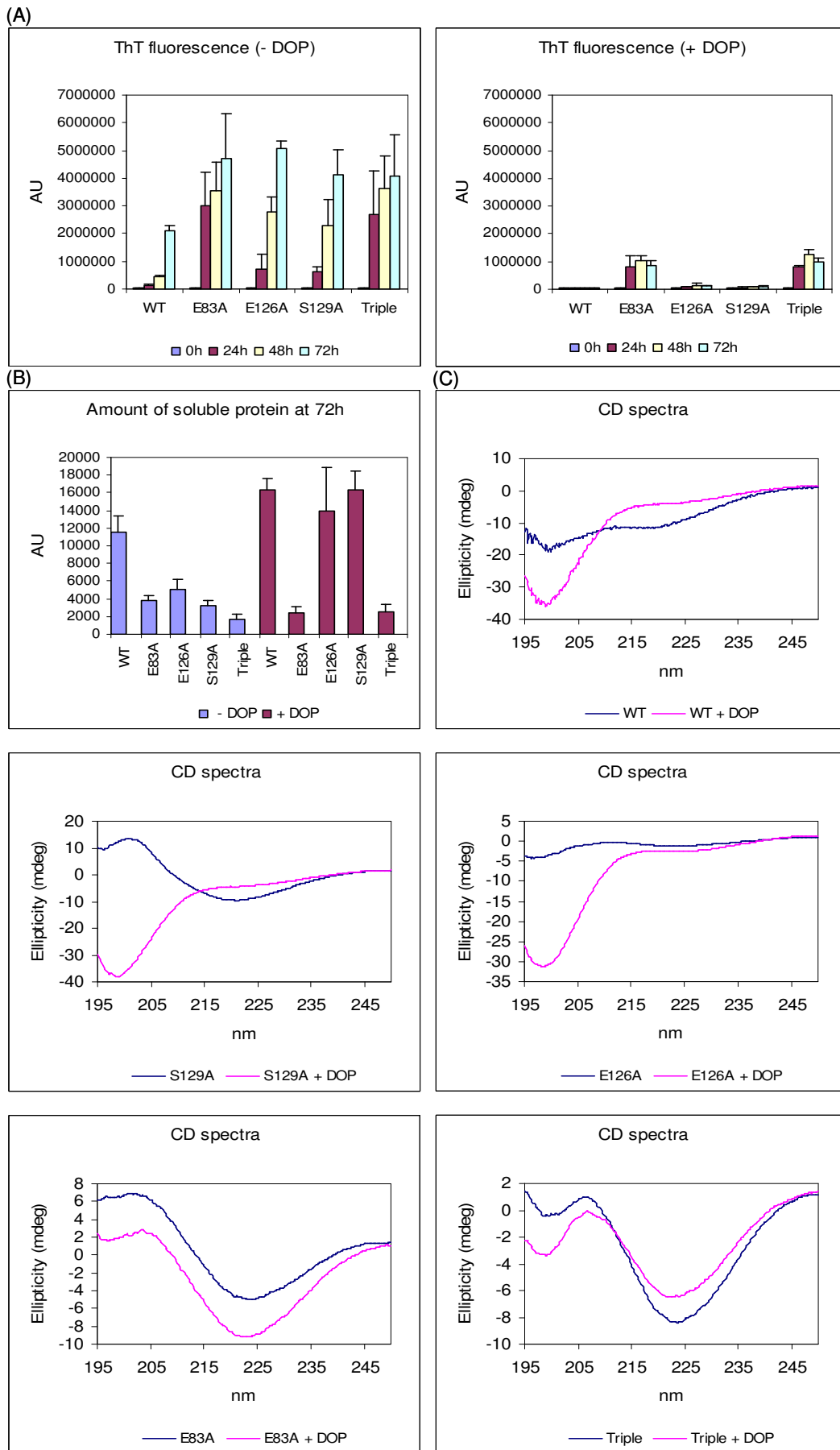**Figure 26.** Mass spectrometry analysis of the WT and mutant AS samples.

(A)

ThT fluorescence (- DOP)

ThT fluorescence (+ DOP)

(B)

Amount of soluble protein at 72h

(C)

CD spectra

CD spectra

CD spectra

CD spectra

CD spectra

Figure 27. *In vitro* **fibrillization of AS:** (A) Kinetics of fibrillization of WT and mutant (E83A, E126A, S129A and Triple) AS under assembly conditions in absence or presence of an equimolar quantity of dopamine (DOP) as monitored by the enhancement of Thioflavin-T (ThT) fluorescence intensity over time. Data are expressed as the mean ± SEM (Standard Error of the Mean) of 2 or 3 independent experiments. (B) Amount of soluble protein remaining after 72 hrs of WT and mutant AS under assembly conditions in absence or presence of an equimolar quantity of DOP monitored by means of SDS-PAGE. (C) CD spectra of WT and mutant AS in incubated under assembly conditions for 72 hrs in absence (red line) or presence (blue line) of an equimolar quantity of DOP (Herrera, Chesi *et al.*, submitted).

To characterize the extent of aggregation and the effect of dopamine on the structural properties of the AS aggregates, we performed EM studies on AS samples incubated for 72 hrs in the presence or absence of dopamine. In the absence of dopamine, after 48-72 hrs the mutant proteins showed abundant fibrils resembling those formed by WT AS (Fig. 28 left panels). In the presence of dopamine, WT AS, E126A and S129A formed predominantly soluble aggregates and no mature fibrils could be detected in these samples, consistent with previously reported data on the WT protein. On the contrary, addition of dopamine did not inhibit fibril formation or change the structure of the fibrils formed by both E83A containing mutants (Fig. 28 right panels).
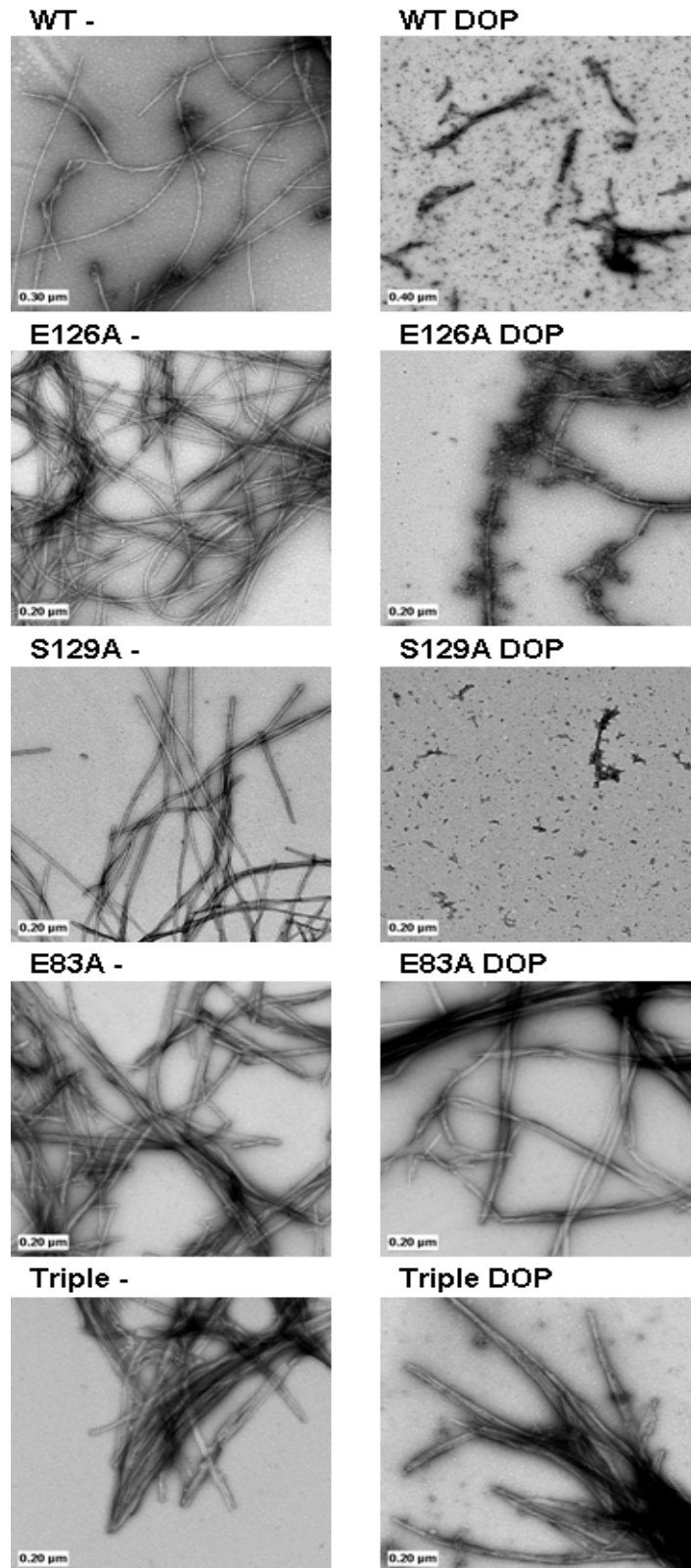
Figure 28. TEM analysis of WT and mutant AS (E83A, E126A, S129A and Triple) filament assembly in absence or presence of an equimolar quantity of dopamine (DOP). Proteins were incubated under assembly conditions for 72 hrs and analyzed by negative staining EM as described in Materials and Methods (Herrera, Chesi *et al.*, submitted).

3.4 SUMO modification of AS

AS presents three standard SUMOylation *consensus* sites. Given the important role that SUMOylation might play in neurodegenerative diseases, we decided to investigate if AS could be a target of SUMO modification. To this end, we took advantage of constructs expressing proteins of the SUMOylation pathway (SUMO-1/2/3, Ubc9) as well as of SUMO mutants in the C-terminal diglycine motif that abolish conjugation.

3.4.1 AS is SUMOylated in HEK and SH-SY5Y cells

HEK293T cells were co-transfected with pcDNA3-AS, pcDNA3-HA-SUMO (-1, -2 or -3) or their unconjugable mutants and pcDNA3-HA-Ubc9 by the calcium phosphate method.

The cells were lysed in Sample Buffer 2X 36 hrs after transfection and the lysates were analyzed by Western blot with an anti AS antibody or an anti HA antibody to check expression and conjugation efficiency of the SUMO/Ubc9 constructs.
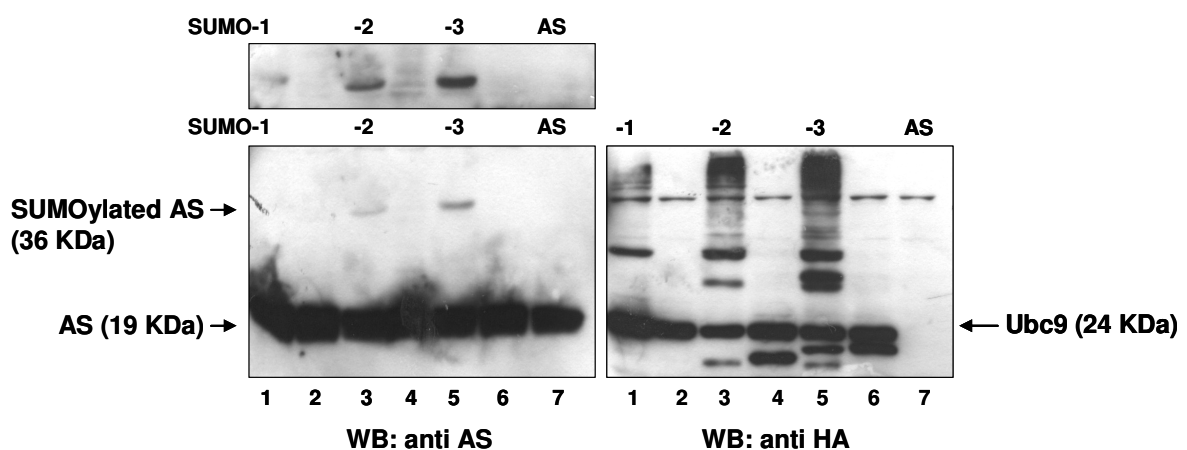
Figure 29. AS is modified by SUMO-1, -2 and -3 in HEK cells. WB analysis of HEK293T cells transfected with AS alone (lane 7) or plus Ubc9 and SUMO-1, -2, or -3 (lanes 1, 3, 5) or their unconjugable forms (lanes 2, 4, 6). Left panel: anti AS antibody (top, longer exposure). Right panel, anti HA antibody.

As shown in Fig. 29, over-expressed AS appears as a band running at about 19 KDa (lane 7). In the presence of conjugable isoform of SUMO plus Ubc9, an extra AS immunoreactive band appears at about 36 KDa, corresponding to the weight of SUMOylated AS (lanes 1, 3, 5 and top panel). The band is not present in the controls over-expressing the respective unconjugable form of SUMO plus Ubc9 (lanes 2, 4, 6). In the right panel the same samples were analyzed with an anti HA antibody: a ladder of bands corresponding to SUMO conjugates is present only in the lanes corresponding to samples expressing the conjugable forms of SUMO plus Ubc9, and not in the controls.

The rank of the intensities of the modified AS bands is: SUMO-1 < SUMO-2 < SUMO-3 and is reflected in the respective amount of total conjugation in the anti HA Western blot, so that it is not possible to say if AS has a greatest affinity for SUMO-3 modification or if the SUMO-3 machinery is more effective in this cellular model system. However, we decided to concentrate in the characterization of SUMO-3 modification of AS.

To this purpose, we performed the same experiment in a different cell lines (HEK293T and SH-SY5Y) with a different AS constructs (FLAG-AS and AS-Myc-His$_6$) and a different antibodies (anti AS and anti Myc).

HEK293T cells were co-transfected with pcDNA3-FLAG-AS, pcDNA3-HA-SUMO-3 or its unconjugable mutant and pcDNA3-HA-Ubc9 by the calcium phosphate method.

The cells were lysed in Sample Buffer 2X 36 hours after transfection and the lysates were analyzed by Western blot with an anti AS antibody or an anti HA antibody to check expression and conjugation efficiency of the SUMO/Ubc9 constructs.

SH-SY5Y cells were co-transfected with pcDNA3-AS-Myc-His$_6$, pcDNA3-HA-SUMO-3 and pcDNA3-HA-Ubc9 by the lipofectamine method. The cells were lysed in Sample Buffer 2X 36 hours after transfection and the lysates were analyzed by Western blot with an anti Myc antibody.
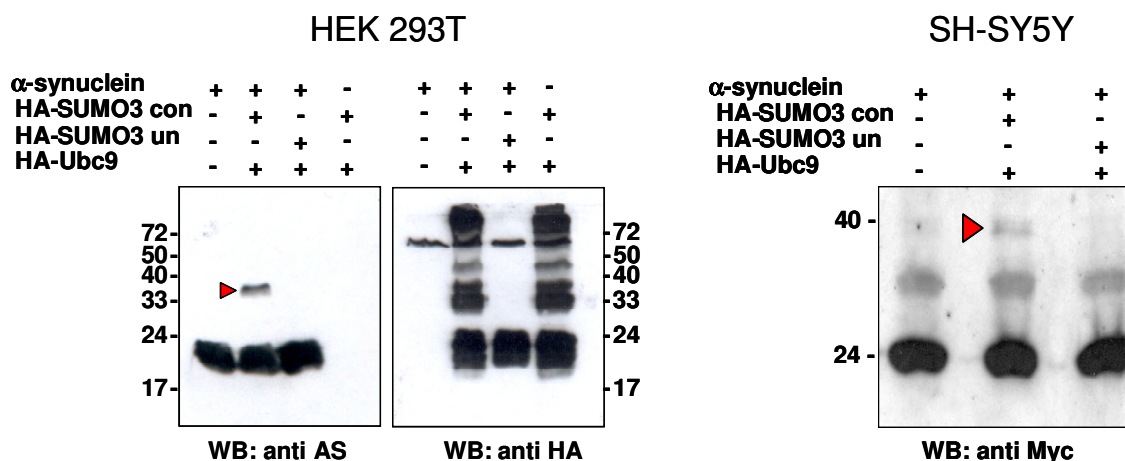
Figure 30. AS is SUMOylated in HEK and SH-SY5Y cells. Left: WB analysis of HEK293T cells transfected with the indicated constructs (left panel: anti AS antibody; right panel, anti HA antibody). Right: WB analysis of SH-SY5Y cells transfected with the indicated constructs (anti Myc antibody).

As shown in Fig. 30 (left), in HEK cells over-expressed FLAG-AS appears as a band running at about 20 KDa (lane 1). In the presence of conjugable SUMO-3 and Ubc9, an extra AS immunoreactive band appears at about 38 KDa, corresponding to the weight of SUMOylated AS (lane 2). The band is not present in the control over-expressing an un-conjugable form of SUMO-3 and Ubc9 (lane 3). As an additional control, it is shown that in presence of conjugable SUMO-3 and Ubc9 but in absence of AS no reactivity is present (lane 4). In the right panel the same membrane is probed with an anti HA anti-body, showing a ladder of bands corresponding to SUMO conjugates only in the lanes corresponding to samples expressing the conjugable form of SUMO and Ubc9.

On the right the same experiment performed in SH-SY5Y cells with a different con-struct (AS-Myc-His$_6$) and a different antibody (anti Myc) is presented. In this case, too, the extra band corresponding to a modified form of AS is present only when a func-tional SUMOylation machinery is over-expressed.

We confirmed the identity of the modified form of AS as a SUMOylated form by immunoprecipitation experiments. HEK293T cells were co-transfected with FLAG-AS, HA-SUMO-3 (conjugable or unconjugable) and HA-Ubc9, and were lysed 36 hours after transfection in RIPA buffer containing NEM, an inhibitor of the cysteine proteases family to which the SUMO de-conjugating enzymes belong. Immunoprecipitation of the protein extracts was performed with an anti HA antibody and samples were analyzed by Western blot with an anti AS antibody. As shown in Fig. 31, the modified AS band is present in the input lysate and in the IP performed with the conjugable form of SUMO-3, but not in the controls.
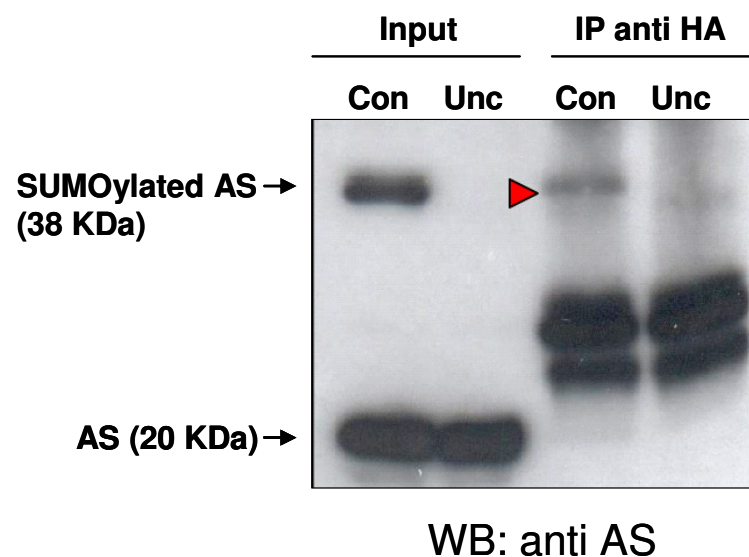
Figure 31. Immunoprecipitation of SUMO3ylated AS in protein extracts from HEK293T transfected cells with FLAG-AS, HA-SUMO-3 conjugable/unconjugable and HA-UBC9 immunoprecipitated with anti HA and revealed with anti AS.

3.4.2 The WT AS and the pathological mutant A53T do not show differential SUMO3ylation in HEK cells.

We then investigated if the AS familial mutant A53T presented a different amount of SUMO-3 modification in the HEK cellular model.

HEK293T cells were co-transfected with pcDNA3-FLAG-AS either WT or A53T, pcDNA3-HA-SUMO-3 or its unconjugable mutant and pcDNA3-HA-Ubc9 by the calcium phosphate method.

The cells were lysed in Sample Buffer 2X 36 hours after transfection and the lysates were analyzed by Western blot with an anti AS antibody. As shown in Fig. 32, there were no significant differences in the amount of modification of the two forms.

	WT			A53T			
α-synuclein	+	+	+	+	+	+	-
HA-SUMO3 con	-	+	-	-	+	-	+
HA-SUMO3 un	-	-	+	-	-	+	-
HA-Ubc9	-	+	+	-	+	+	+

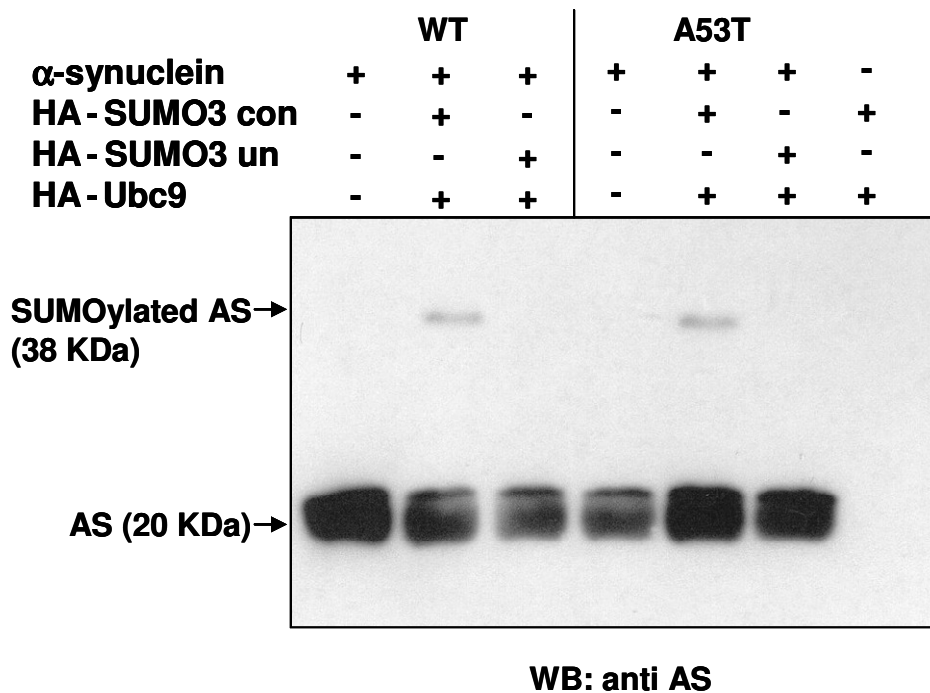SUMOylated AS→ (38 KDa)

AS (20 KDa)→

WB: anti AS

Figure 32. Anti AS immunoblot of protein extracts from HEK293T transfected with FLAG-AS WT or pathological mutant A53T, HA-SUMO-3 conjugable/unconjugable and HA-Ubc9.

3.4.3 SUMOylation of AS in mouse brain

We investigated the possibility of an *in vivo* SUMO modification of AS, particularly in the brain regions relevant to PD. Two adult mice C57/BL were sacrificed and midbrain and striatum regions were dissected, homogenized and lysed in presence or absence of the cysteine protease inhibitor NEM, which is known to inhibit SUMO proteases activity. Protein extracts were analyzed by Western blot or subjected to immunoprecipitation with an anti AS antibody. As shown in Fig. 33, AS is abundantly expressed in midbrain and striatum and appears in Western blots as a band running at 19 KDa. A higher molecular weight immunoreactive band running at about 30 KDa is evident only in the samples treated with NEM. However, the AS antibody used in this experiment could not enrich this band, while it could successfully immunoprecipitate the band corresponding to unmodified AS at 19 KDa. Even though not conclusive, this experiment raises the possibility of an *in vivo* modification of AS. Further studies are necessary to identify the nature of the modification, which is compatible with a covalent attachment of one SUMO moiety.

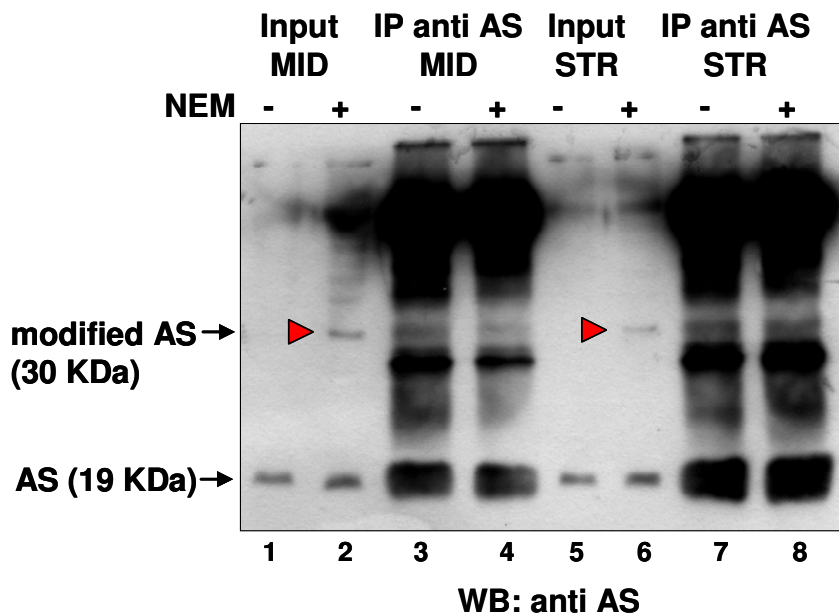**Figure 33.** Anti AS immunoblot of protein extracts from midbrain and striatum of adult mice. Lanes 1, 2, 5, 6: total extracts from midbrain (1, 2) or striatum (5, 6) processed in presence (2, 6) or absence (1, 5) of the cysteine protease inhibitor NEM. Lanes 3, 4, 7, 8: anti AS immunoprecipitated samples from midbrain (3, 4) or striatum (7, 8) in presence (4, 8) or absence (3, 7) of NEM.

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3.4.4 Identification of the lysine residue target of SUMOylation

The SUMO moiety is covalently attached to lysine residues in the substrate protein. The SUMOylation *consensus* motif ψKxE (where ψ is a large hydrophobic residues and x is any residue) is found in many, but not all the substrate proteins. Substrate specificity appears to derive directly from Ubc9 and the respective substrate motif. AS aminoacidic sequence comprises 15 lysine residues, and 3 of them, namely K12, K96 and K102, are identified as putative SUMOylation targets by the free software SUMOplot (http://www.abgent.com/doc/sumoplot). K12 is located in the N-terminal repeats, while K96 and K102 belong to the C-terminal region of AS (Fig. 34).
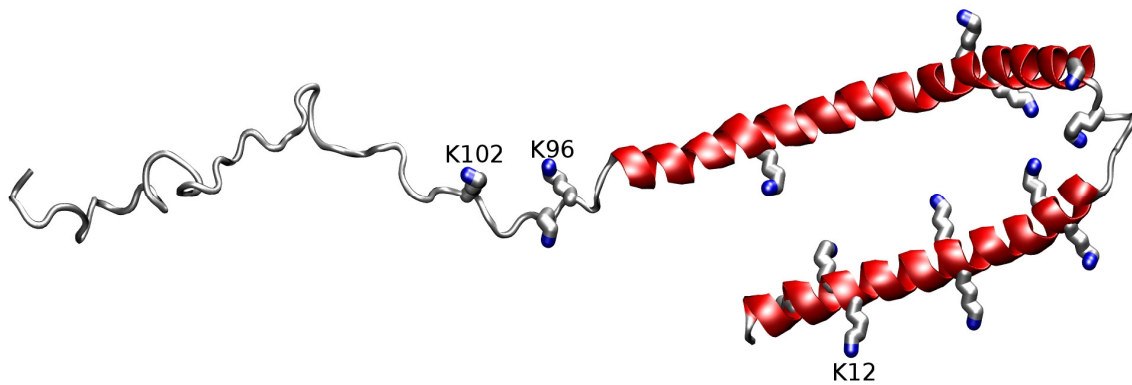
Figure 34. The 3 lysines belonging to SUMO *consensus* sites identified by SUMOplot. The AS structure is taken from PDB entry 1XQ8 (Ulmer *et al.*, 2005).

In order to identify the target lysine we mutagenized the 3 lysines to arginine, a residue with the same charge but unable to be conjugated by SUMO. Three single mutants (K12R, K96R and K102R) and one triple mutant (K12R/K96R/K102/R) were generated.
The single mutants were constructed starting from the WT AS DNA template by a two step PCR with a couple of internal oligos bearing the mutation and a couple of external oligos. The triple mutant was constructed starting from the K12R template using a couple of mutagenic primers bearing the two C-terminal mutations. The amplified PCR fragments were cloned in pcDNA3-FLAG vector and sequenced.

HEK293T cells were co-transfected with each of the 4 AS mutants plus pcDNA3-HA-SUMO-3 (or its unconjugable form) and pcDNA3-HA-Ubc9 by the calcium phosphate method. Proteins were extracted as described before and samples were analyzed by Western blot with an anti AS antibody.
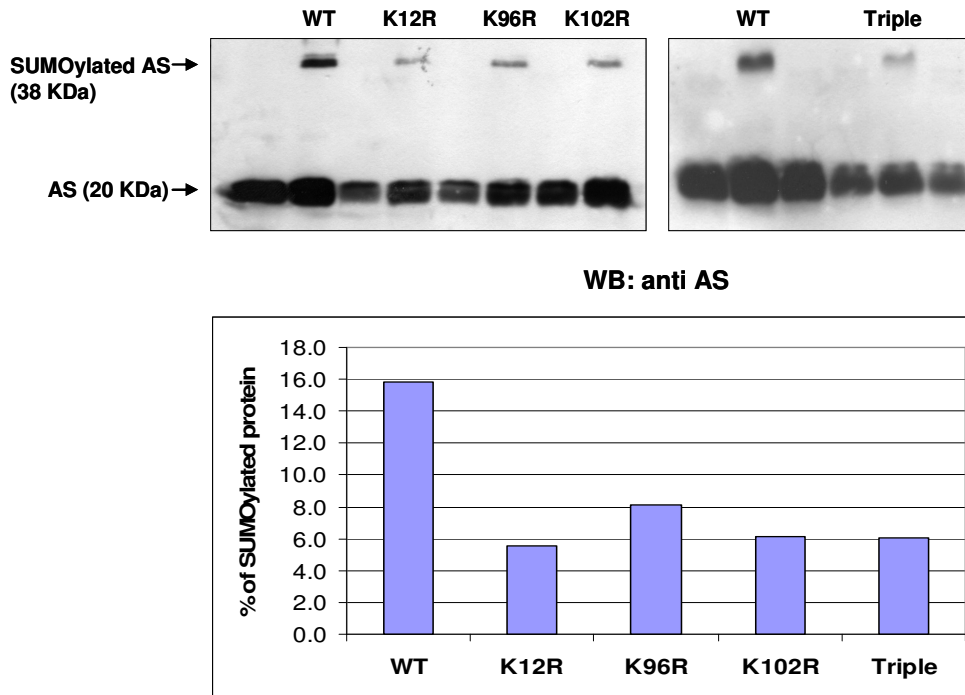
Figure 35. Lysine mutations decrease but do not abolish AS SUMO-3 modification. **Top panel**: Anti AS immunoblot of protein extracts from HEK293T cells transfected with FLAG-AS (WT or K-mutant) plus HA-SUMO-3 (conjugable or unconjugable) and HA-UBC9. **Bottom panel**: quantification of the percentage of SUMOylated protein (ImageJ).

AS shown in Fig. 35, the lysine mutations are able to decrease the normal level of SUMO-3 modification of AS to about 40% of the WT value, which in this assays is about 16%, but are not able to completely abolish it. This result suggests that more than one lysine can be SUMOylated alternately and probably a non standard *consensus* site is involved.

3.4.5 Effect of PD related treatments on global SUMO2/3ylation in SH-SY5Y cells

We investigated the effect of treatments relevant to PD pathogenesis on endogenous SUMOylation (in particular by SUMO-2/3) in SH-SY5Y cells.

1-methyl-4-phenylpyridinium (MPP$^+$) treatment of SH-SY5Y cells is a classic *in vitro* model to study PD. MPP+ enters inside the cells through dopamine or norepinephrine transporters and inhibits mitochondrial complex I, leading to generation of ROS and ATP depletion.

4-hydroxynonenal (HNE) is highly reactive aldehyde which is a toxic end-product of the free radical-stimulated peroxidation of cellular membrane lipids, or circulating lipo-protein molecules. The presence of HNE is increased in brain tissue and cerebrospinal fluid of different neurodegenerative disorders such as AD, PD, ALS and DLBD. Immu-nohistochemical studies show presence of HNE-modified proteins in the inclusions characteristic of these diseases. HNE concentration is increased in the brainstem of MPTP treated mice (Selley, 1998).

Ferrous iron clorure (FeCl$_2$): iron is believed to play an important role in aging and neurodegeneration, since during ageing the total iron concentration increases in some brain regions that are targeted by degenerative diseases such as AD, PD and Hunting-ton's disease. The ferrous (Fe II) oxidative state of iron is free to participate in Fenton chemistry and is a major source of oxidative stress. The reaction involves the ferrous iron catalyzed conversion of hydrogen peroxide into a hydroxide ion and a hydroxyl free radical with the concurrent oxidation of ferrous iron to ferric iron. In dopaminergic cells, intracellular redox and iron imbalance results in aberrant oxidation of dopamine to 6-hydroxydopamine which in turn can undergo autoxidation to the corresponding do-pamine quinone concomitant with generation of superoxide. Oxidative cascade leads to further generation of ROS which induce oxidative damage of DNA, increase in protein carbonyls, protein nitration and increased lipid peroxidation.

Given the highly dynamic nature of the SUMOylation response to oxidative stress, we choose to treat the cells acutely for a short time (30 minutes to 1 hour) with elevated concentrations of the stressors.

For the Western blot analysis, SH-SY5Y cells were treated with 10, 100 and 500 μM HNE or 1, 10 and 50 mM MPP$^+$ or 0.5, 1, 5 and 10 mM FeCl$_2$ for 1 hour. In the case of iron, a longer treatment of 24 hours was also performed. Cells were lysed in Sample Buffer 2X and the lysates were analyzed by Western blot with an anti SUMO2/3 antibody. The same membranes were probed with an anti actin antibody for equal loading. As shown in Fig. 36, global SUMO-2/3ylation is evident as a high molecular weight immunoreactive smear corresponding to the signal of the SUMO-2/3 modified proteins. Lower molecular weight bands at 20-30 KDa may represent the free unconjugated SUMO-2/3 pool. 1 hour HNE treatment at 100 or 500 μM concentration was able to increase the global SUMO-2/3 conjugation as shown by the increased signal in the high molecular weight smear and the disappearance of the low molecular weight bands. On the contrary, MPP$^+$ treatment at all the tested concentrations was able to decrease SUMO-2/3 conjugation as shown by the decreased signal in the high molecular weight smear and the increase of the low molecular weight bands. FeCl$_2$ treatment for 1 or 24 hours did not affect global SUMO2/3ylation. The highest iron concentration induced cell death at 24 hours as shown by the decrease in β-actin reactivity and by analyzing cell morphology at the light microscopy.
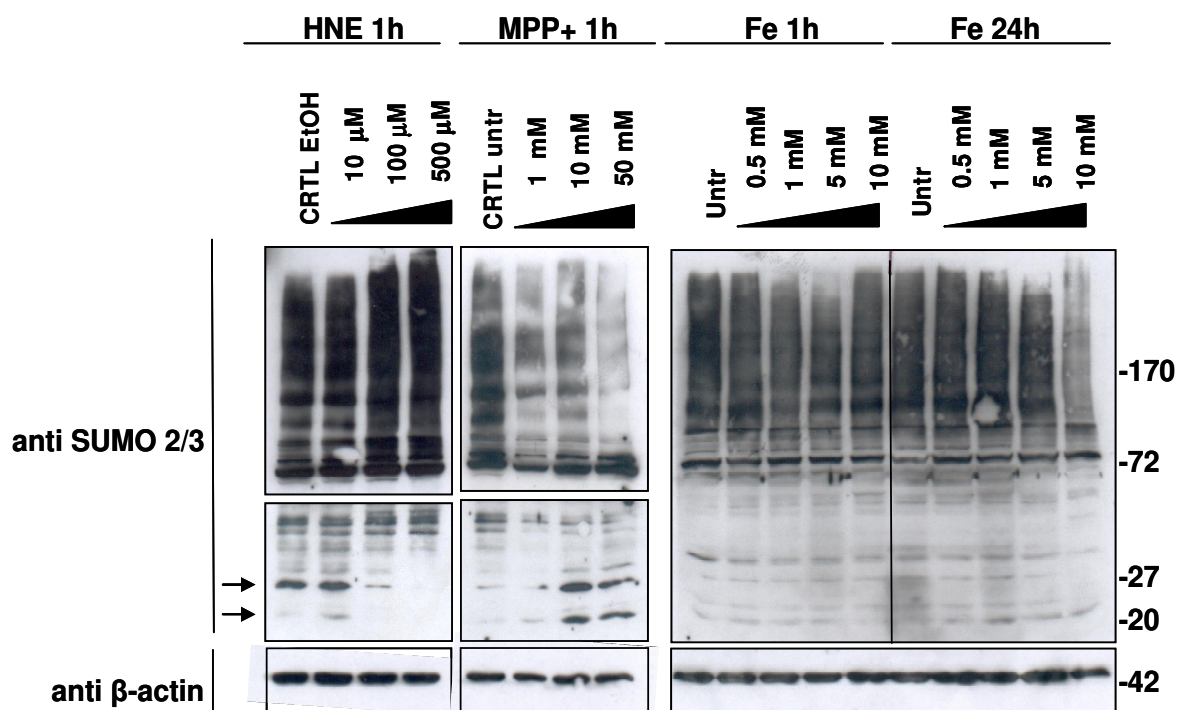
Figure 36. Anti SUMO-2/3 immunoblot analysis of protein extracts from SH-SY5Y cells treated with HNE, MPP$^+$ or FeCl$_2$ for the indicated times.

We then moved to immunofluorescence studies to investigate if these treatments could cause a cellular re-localization of SUMO-2/3.

The endogenous localization of SUMO-2 and -3 in untreated SH-SY5Y cells was first investigated taking advantage of 2 different antibodies. As shown in Fig. 37, SUMO-2 and -3 are mainly localized in the nucleus, both in the nucleoplasm and in small bright nuclear dots. SUMO-3 also shows a faint cytoplasmic localization.

Anti SUMO-2

Anti SUMO-3
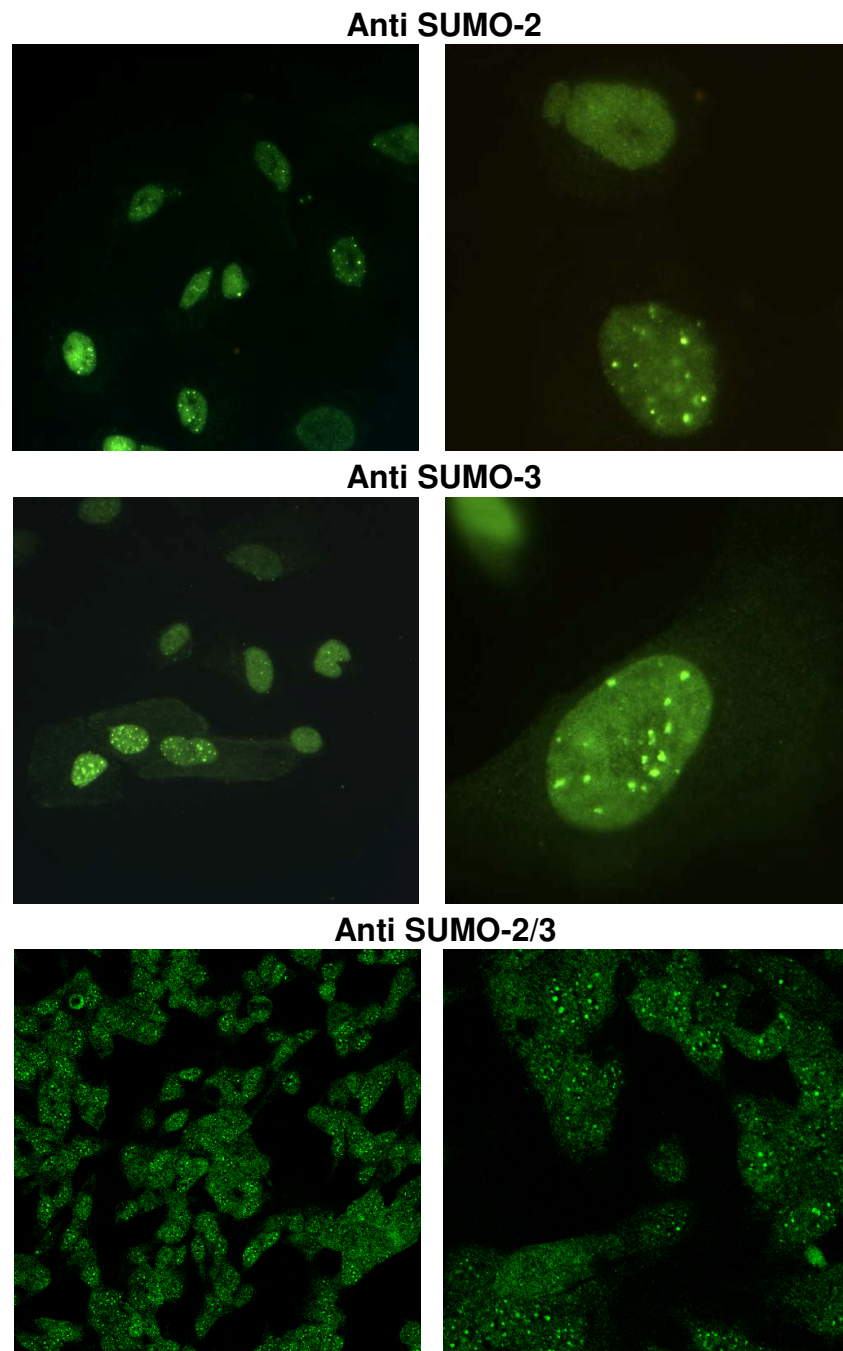
Anti SUMO-2/3

Figure 37. Immunofluorescence showing endogenous localization of SUMO-2 or SUMO-3 in SH-SY5Y cells.

Cells were then treated for 30 minutes with 50 mM MPP$^+$ or 100 µM HNE and then analyzed by immunofluorescence with the anti SUMO-2/3 antibody. Images were taken at the confocal microscopy.

As shown in Fig. 38, MPP$^+$ treatment causes a strong reduction in the nucleoplasmic localization of SUMO-2/3 and an increase in perinuclear reactivity. The nuclear dots are still present. HNE treatment did not cause any evident relocalization of SUMO-2/-3, but caused a change in the morphology of the cells which appear less adherent and more roundish. Indeed, HNE has been previously shown to disrupt microtubules in neuronal cells through tubulin modification (Neely *et al.*, 1999).
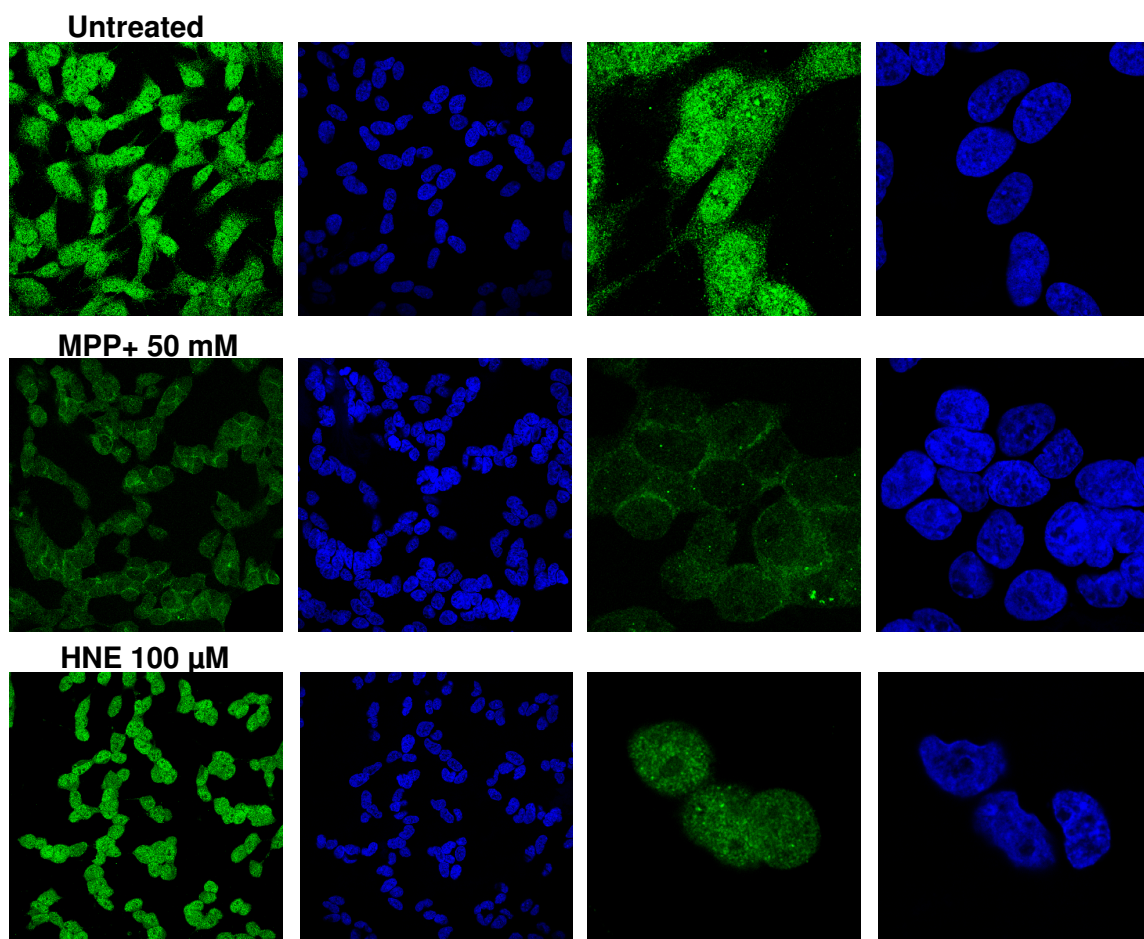
Figure 38. Anti SUMO-2/3 immunofluorescence showing the effect of 30 minutes MPP$^+$ and HNE treatment on endogenous SUMO-2/3 localization in SH-SY5Y cells.

4.1 Discussion

Despite the fact that the first detailed clinical description of Parkinson's disease (PD) dates back to nearly two centuries ago, little is known about the molecular etiology of the disease, and a cure able to halt or revert the course of its progression is still lacking. Although the dopamine precursor L-DOPA has proven to be a powerful PD treatment for the first stages of the disease, its ability to facilitate an acceptable quality of life for patients wanes with time, due to the development of motor complications. Clearly, the current symptomatic therapies cannot completely ameliorate later-stage symptoms, nor can they address the ongoing degeneration in the dopaminergic and non-dopaminergic systems. For this reason, a good deal of current research has focused on finding the cause of dopaminergic cell loss and on exploring protective, restorative, and replacement therapies.

The recent discovery of single-gene mutations that are responsible for heritable forms of PD offer a big opportunity to gain insight into the molecular mechanisms of the disorder and provide novel targets for drug therapy.

Among the PD familial genes, a central role is played by α-synuclein (AS). This protein is the cause of rare familial forms of the disease when mutated or over-expressed and constitutes the principal component of the characteristic proteinaceous inclusions (Lewy bodies) in sporadic PD cases. Thus, AS seems to represent an ideal drug target for PD and those related neurodegenerative disorders (synucleinopathies) that are characterized neuropathologically by accumulations of AS inclusions in the central nervous system.

As several studies suggest, AS deposition plays a central role in the pathogenesis of PD and other synucleinopathies, and inhibition of AS aggregation may be a feasible strategy for therapeutic intervention. The fact that AS belongs to a class of natively unfolded proteins, which have little or no ordered structure under normal physiological conditions, makes the rational design of compounds that can stabilize the native, non-toxic

conformation of AS a challenging task. However, the identification of the critical binding region in the AS molecule responsible for its self-association (residues 69–72) (El-Agnaf *et al.*, 2004) enabled the design of peptide-based inhibitors of AS aggregation and toxicity containing part of the binding region. On the other hand, screenings for molecules able to inhibit fibril formation *in vitro* have been performed and a number of inhibitory compounds have been identified (Conway *et al.*, 2001).

The Drosophila AS over-expression model proved particularly useful for drug screening and validation. The locomotor response of WT AS transgenic flies was tested to prototypes of the major classes of drugs currently used to treat PD. A time course study was first conducted to determine when impaired locomotor activity appeared relative to wild-type flies. A climbing or negative geotaxis assay measuring the ability of the organisms to climb up the walls of a plastic vial was used. Based on the results obtained, normal and transgenic flies were treated with each of the drugs in their food for 13 days and then assayed. The activity of transgenic flies treated with L-DOPA was restored to normal. Similarly, the dopamine agonists pergolide, bromocriptine, and 2, 3, 4, 5-tetrahydro-7, 8-dihydroxy- 1-phenyl-1H-3-benzazepine (SK&F 38393) were substantially effective. Atropine, the prototypical muscarinic cholinergic receptor antagonist, was also effective but to a lesser extent than the other antiparkinsonian compounds. p-Chlorophenylalanine, an inhibitor of serotonin synthesis, was without beneficial effect as was α-methyl-p-tyrosine, an inhibitor of tyrosine hydroxylase, the rate-limiting step in catecholamine biosynthesis (Pendleton *et al.*, 2002).

In another study, directed expression of Hsp70, a chaperone up-regulated in stress responses that refolds misfolded proteins and is able to suppress the neuronal toxicity of abnormal polyglutamine proteins (Warrick *et al.*, 1999), was shown to prevent dopaminergic neuronal loss associated with AS in Drosophila, while interference with endogenous chaperone activity accelerated AS toxicity.

Interestingly, Lewy bodies in human *post mortem* tissue immunostain for molecular chaperones, also suggesting that chaperones may play a role in Parkinson's disease progression.

Treatment with geldanamycin (a naturally occurring benzoquinone ansamycin that specifically binds to and interferes with the activity of the molecular chaperone Hsp90, a negative regulator of heat-shock factor) also resulted in complete protection against α-synuclein toxicity, despite the continued presence of Lewy body–like inclusions (like in transgenic expression of Hsp70) (Auluck and Bonini, 2002).

Very recently, inhibitors of the histone deacetylase Sirtuin 2 (SIRT2) have been shown to rescue AS–mediated toxicity in cellular models of PD and in the AS *Drosophila* model by promoting the formation of enlarged AS inclusions (Outeiro *et al.*, 2007). Human SIRT2 is involved in cell cycle regulation via the deacetylation of α-tubulin at Lys 40 (North *et al.*, 2003). However, the identification of p53 and histones H3 and H4 as additional substrates for SIRT2 suggests a broader regulatory role in the cell (Heltweg *et al.*, 2006; Vaquero *et al.*, 2006). Small-molecule inhibitors targeting HDACs ameliorate several models of neurodegeneration (Langley *et al.*, 2005). Rescue via inclusion enlargement, and the concomitant reduction in total surface area of inclusions, agrees with a cytoprotective role of aggregates and suggests a mechanistic basis for the effect of SIRT2 inhibition - that it reduces aberrant interactions of aggregates with cellular proteins. Conceivably, coalescence of misfolded proteins into larger inclusions may lower the concentration of toxic, submicroscopic AS oligomers, thereby leading to the rescue of proteasome dysfunction. Indeed, the formation of large β-amyloid aggregates is protective against proteotoxicity in *Caenorhabditis elegans* (Cohen *et al.*, 2006). The exact mechanism whereby SIRT2 inhibition affects AS aggregation remains uncertain. Increased α-tubulin acetylation is associated with microtubule stabilization, and AS has been reported to interact with α-tubulin as well as the microtubule-binding proteins MABP1 and tau (Alim *et al.*, 2004; Jensen *et al.*, 2000). One possibility is that the increase in acetylated α-tubulin resulting from SIRT2 inhibition may stimulate aggregation of AS through its affinity to microtubules. Moreover, microtubule stabilization itself could be an important factor contributing to neuroprotection. Interestingly, a neuroprotective role for another microtubule deacetylase, HDAC6, was recently proposed, although the protective mechanism is unclear (Dompierre *et al.*, 2007; Iwata *et al.*, 2005; Kawaguchi *et al.*, 2003).

4.1.1 A cellular model to study AS function in PD

To gain insight into α-synuclein's function in PD, we developed a cellular model stably over-expressing AS. We stably transfected WT or A53T AS in a SH-SY5Y neuroblastoma cell line, since these cells express tyrosine hydroxylase (TH) and produce dopamine (Biedler *et al.*, 1978), express dopamine receptors (Farooqui, 1994), and are able

to sequester dopamine and the PD toxin MPP$^+$ (Richards and Sadee, 1986). They however express a very low amount of endogenous AS which is not detectable with most of the available anti AS antibodies.

In our stable cell lines, AS is expressed at high level both in the cytoplasm and nucleus and associated to the cell membrane. We showed that, in agreement with published data on neuroblastoma cell lines (Lev *et al.*, 2006; Ostrerova-Golts *et al.*, 2000), AS overexpression sensitizes our cells to the toxicity induced by ferrous iron, an oxidative stress generator which is believed to play an important role in aging and neurodegeneration, in particular in PD. Differently from those studies, we did not observe an increased toxicity of the mutant respect to the WT form of AS.

Iron treatment induced aggregation of WT or A53T AS, visible in Western blot as high molecular weight reactivity in the Triton-insoluble fraction of the cellular protein extract. The high molecular weight signals likely represent oligomeric AS complexes which are Triton-insoluble but SDS soluble.

At higher concentration, iron treatment was able to induce formation of AS aggregates visible at the fluorescence microscope in both WT and A53T cell lines, causing extensive cell death and changes in the cells' morphology. At the concentration used for the Western blot experiment, however, no aggregates were visible in immunofluorescence at the light microscope. It is likely that the oligomeric complexes visible in Western blot are too small to be visible at the light microscope, where only larger aggregates can be detected.

We also showed that prolonged retinoic acid (RA) treatment, which is known to provoke SH-SY5Y cells differentiation into a neuronal-like phenotype with attenuation of proliferation rate, was able to induce AS aggregation that was visible in the Triton-insoluble fraction in Western blot, but not in immunofluorescence.

We are interested in inducing aggregation of AS in this cellular model because it provides a mean of screening compounds that are able to revert this phenotype. Many *in vitro* screenings of inhibitors of AS aggregation have been performed, but the cellular models provide the advantage of more physiological conditions and of the presence of the cellular protein interaction network. In principle, it is possible to hypothesize a high-throughput screening on these cells based on different readouts: viability (MTT assay), detergent insolubility, microscopy, and FRET (Desai *et al.*, 2006; Pollitt *et al.*, 2003) or FLIM (Esposito *et al.*, 2007) assays. It is important to appreciate how these approaches are complementary: a large body of evidence has supported the idea that early amyloid

aggregates are soluble oligomers, and it is these, rather than the mature amyloid fibrils, that are the toxic and pathogenic species that drive neurodegeneration and eventually neuronal cell death. Thus, simply blocking the formation of the mature fibrils may not represent the best therapeutic strategy and may even enhance AS toxicity *in vivo*. Indeed, the different readouts can distinguish between the late big aggregates (microscopy) and the early small oligomers (detergents, FRET or FLIM), while the resulting cytotoxicity can be measured easily with the MTT assay.

Our cellular model will also prove useful for investigation of the physiological AS function and the basic cellular processes related to its over-expression.

Autophagy, the process by which cells recycle cytoplasm and dispose of excess or defective organelles by lysosomal degradation, has entered the research spotlight largely owing to the discovery of the protein components that drive this process. Autophagy has been linked to disease processes, particularly in cancer and neurodegeneration. Whether autophagy protects from or causes disease is unclear. In neurodegenerative disease, it may exert a protective function allowing the removal of protein aggregates before they become toxic, or on the other hand it may be toxic inducing cell death in neurons that accumulate aggregated proteins.

In a recent study in *Drosophila*, Pandey *et al.* showed that induction of autophagy is sufficient to rescue degeneration associated with UPS impairment, illustrating the compensatory relationship between autophagy and the UPS (Pandey *et al.*, 2007). Interestingly, the histone deacetylase activity of HDAC6 was essential for autophagy to compensate for impaired UPS function, and ectopic expression of HDAC6 alone was sufficient to rescue degeneration caused by proteasome mutations and polyQ toxicity, in an autophagy-dependent manner. This study suggests that impairment of autophagy (for example, associated with ageing or genetic variation) might predispose to neurodegeneration. Moreover, these findings suggest that it may be possible to intervene in neurodegeneration by augmenting HDAC6 to enhance autophagy.

AS has been recently described as a substrate of chaperone-mediated autophagy (Cuervo *et al.*, 2004). In our cell lines, we observed an accumulation of the mutant, but not of the WT AS protein upon treatment with ammonium chloride, an inhibitor of lysosomal proteolysis. Furthermore, the cells expressing AS seem to be more vulnerable to ammonium chloride toxicity, with the mutant lines more sensitive than the WT. These data are just very preliminary and need to be repeated and confirmed with a viability assay such as MTT, but are in agreement with published data showing that the

mutant A53T AS impairs autophagy (Cuervo *et al.*, 2004). Our cellular model will therefore prove useful to study the mechanism of autophagy in relation to AS over-expression and clarify if it exerts a protective or a harmful role in disease.

4.1.2 A yeast two-hybrid screening for aptamers binding AS pathologic mutant A53T

Peptide aptamers comprise a new class of molecules, with a peptide moiety of random-ized sequence, which are selected for their ability to bind to a given target protein under intracellular conditions. They have the potential to inhibit the biochemical activities of a target protein, can delineate the interactions of the target protein in regulatory networks, and identify novel therapeutic targets, and thus represent a new basis for drug design and protein therapy, with implications for basic and applied research, for a broad variety of different types of diseases.

In this work, we choose to perform a yeast two-hybrid screening of a random library of peptide aptamers to identify small peptides able to bind and interfere with the patho-logical function of the α-synuclein familial mutant A53T.

We exhaustively screened a library with an estimated complexity of 10^9 different se-quences of 16-aminoacids random peptides inserted in a Thioredoxin scaffold. Quite surprisingly, we could isolate only two peptide interactors which, at further analysis, resulted to activate the reporter genes independently of the presence of the AS bait.

The lack of identification of positive peptide interactors from the screening was some-how unexpected.

AS does seem to be able to interact with other proteins in yeast two-hybrid screenings of cDNAs libraries, although only a few interactors were reported (Elkon *et al.*, 2002; Engelender *et al.*, 1999; Ghee *et al.*, 2000). It is possible that, in our case, the Thiore-doxin scaffold is not the optimal one, somehow preventing peptide-AS interaction. In-deed, the choice of the protein scaffold has been shown to influence the outcome of yeast two-hybrid screenings (Klevenz *et al.*, 2002). Other protein scaffolds for aptamer screening are available (green fluorescent protein, staphylococcal nuclease, stefin A) and might be worth trying, together with the unconstrained peptides. Another possible explanation is that AS requires recognition sequences longer than 16 aminoacids, al-

though in general peptide screenings reported in literature are consistent with a recognition motif of only 4 aminoacids (the fact that AS is an intrinsically disordered protein might affect the modality of interaction with its binding partners).

An alternative approach to the yeast two-hybrid is the screening for peptides or chemical compounds able to disrupt a particular phenotype in a model system. In the case of AS, this can be done for example in a cellular model of AS toxicity or aggregation such as the one described in section 3.1.

4.1.3 A computational and *in vitro* study of dopamine-AS interaction

Unraveling structural determinants of dopamine binding may shed light on the mechanisms by which this molecule modulates AS fibrillization and toxicity, thus providing new clues for therapeutics intervention in PD and related diseases.

To clarify the structural determinants of the interactions between dopamine and AS, we performed a computational and *in vitro* study of the interaction between dopamine and α-synuclein, which allowed to identify and characterize dopamine binding sites in the C-terminus and NAC region of α-synuclein.

Dopamine and two of its derivatives were docked onto conformers representative of an NMR ensemble of AS. We predicted two moieties of AS involved in non-covalent inhibition of fibrillation, the [125]YEMPS[129] region, which forms non-specific hydrophobic contacts with the dopamine aromatic ring, and E83, which forms a water-mediated hydrogen bond with the ligands. The C-terminal [125]YEMPS[129] region had been already implied in DOP and DOP derivatives binding by an experimental work (Norris *et al.*, 2005), while the E83 residue is a completely novel site of interaction.

We then investigated *in vitro* the effects of dopamine on the aggregation of mutants designed to alter the properties of these moieties. We found, as suggested by modeling, that the E83A mutation abolishes the ability of dopamine to inhibit AS aggregation. The strategy that we outlined, which enables ligands to be devised that mimic the interactions between dopamine and AS, will help develop therapeutics for PD and related synucleinopathies. In particular, we identified structural determinants of AS conformations that bind dopamine which can be exploited for *in silico* screening of libraries of compounds to identify novel inhibitors of AS aggregation.

The effect of E83A mutation could be investigated *in vivo* creating a stable cellular model such as the one described in section 3.1, comparing the resulting phenotype with the one of the WT AS cells.

The effect of dopamine could be easily assessed adding DOP to the culture medium or modulating the synthesis of endogenous DOP production by over-expressing different TH mutant forms as in the work by Mazzulli *et al.* (Mazzulli *et al.*, 2006).

3 or 4 lysine residues in AS have been proposed to be target of covalent modification by DOP derivatives (Li *et al.*, 2005a). It is possible to hypothesize a mechanism in which DOP is first bound non covalently to AS and then reacts with a target lysine. Interestingly, a lysine (K80) is present very close to residue E83. It would be appealing to investigate by computational and mutagenesis studies the role of that lysine in relation to E83 and DOP inhibition of AS fibrillization.

Intriguingly, lysine residues are also target of post-translational modifications such as acetylation, ubiquitination and SUMOylation. We proved that AS is a substrate of SUMOylation (see section 3.4), and further studies could clarify if there is a link between this modification and AS aggregation and its inhibition by dopamine.

4.1.4 SUMO modification of AS

An important issue in the physiological and pathological function of AS is that of its post-translational modification.

In this work, the role of post-translational modification of α-synuclein by SUMO and of SUMOylation in general was investigated in the contest of PD.

We proved that AS is a target of SUMOylation when over-expressed together with the SUMO machinery in two different cell lines, HEK and SH-SY5Y cells. We found that, in our conditions, AS can be modified by SUMO-1, -2 and -3. We then focused on SUMO-3 modification and confirmed the modification by immunoprecipitation in HEK cells. We found no difference in the amount of modification in WT or A53T AS.

As this work was in progress, SUMO modification of AS in a similar over-expression cell system was reported by another group (Dorval and Fraser, 2006). The target lysine, however, was not identified.

To identify the target lysine residue in AS, we mutated the three lysines belonging to standard SUMOylation *consensus* motifs to arginine. We observed a reduction in the amount of modification in all the three single and in the triple mutants, but SUMOylation was not completely abolished. This result suggests that more than one lysine can be SUMOylated alternately and probably a non standard *consensus* site is involved. Indeed, non-*consensus* SUMOylation sites have been reported for several SUMO targets (Chung *et al.*, 2004; Denison *et al.*, 2005). It is also possible that the Lys to Arg substitution is not the best choice. The SUMO *consensus* motif is recognized by the E2 conjugating enzyme Ubc9. Lys to Arg mutation might not completely abolish Ubc9 binding. To overcome this problem, more than one residue or the entire *consensus* site could be mutated. Computational studies could also aid in identifying the target lysine, by docking studies of the Ubc9-SUMO complex (of which a crystallographic structure has been published (Reverter and Lima, 2005)) with peptides containing the AS putative SUMOylation sites.

An alternative approach to identify the target lysine is mass spectrometry (MS). MS has successfully been used to identify the lysines target of ubiquitination in proteins (Peng *et al.*, 2003).

Unlike ubiquitin whose carboxyl-terminal sequence is RGG, the tripeptide at the carboxyl terminus of human SUMO proteins is TGG. The presence of the arginine residue at the carboxyl terminus of ubiquitin allows tryptic digestion of ubiquitin conjugates to yield a signature peptide containing a diglycine remnant attached to the target lysine residue and rapid identification of the ubiquitination site by mass spectrometry. The absence of lysine or arginine residues in the carboxyl terminus of mammalian SUMO makes it difficult to apply this approach to mapping SUMOylation sites. However, Knuesel *et al.* have shown that a SUMO variant terminated with RGG can be conjugated efficiently to its target proteins under normal SUMOylation conditions and have developed a computational program which allows efficient identification of the modified peptides (Knuesel *et al.*, 2005). This approach could be applied profitably to the identification of the AS target lysine(s). More recently, an automated pattern recognition tool has been developed, which is able to identify modified peptides and modification sites within complex MS/MS spectra (Pedrioli *et al.*, 2006) without the need for a mutant SUMO form.

The individuation of the lysine is important because the SUMOylation incompetent AS mutant would be useful to shed light into the biological function of the modification.

For example, it could be used to establish a stable transfected cell line whose phenotype could be compared with that of the WT AS cell line.

A fundamental point is whether SUMOylation of AS occurs *in vivo*. We investigated this possibility, particularly in the brain regions relevant to PD, by dissecting the midbrains and striata of WT adult mice and performing Western blot and immunoprecipitation experiments with an anti AS antibody in presence or in absence of the cysteine protease inhibitor NEM, which is considered an inhibitor of the SUMO de-conjugating enzymes.

We observed a modified form of AS with a molecular weight compatible with a modification by one SUMO moiety, which was enriched in the samples treated with NEM. We could not, however, confirm by immunoprecipitation that it was indeed a SUMO conjugate. Even though not conclusive, this experiment raises the possibility of an *in vivo* modification of AS, but further studies are necessary to identify the nature of the observed modification.

Different biological functions for an endogenous SUMO modification of AS can be hypothesized.

SUMOylation could play a role in aggregation and/or degradation of misfolded AS. In this respect, it could exert a protective or a toxic function. Interestingly, SUMOylation has been shown to reduce aggregates formation in cellular models and to enhance neurotoxicity in a *Drosophila* model of Huntington's disease (Steffan *et al.*, 2004).

SUMO modification could facilitate or inhibit aggregation of AS. *In vitro* fibrillization experiments (like the ones described in section 3.3.3) with SUMOylated AS represent a simple way to investigate this issue. A tri-cistronic plasmid for over-expression of SUMOylation enzymes and SUMO-1/2 in *E. coli* has been described (Uchimura *et al.*, 2004), which could be used along with the pET-AS vector and a suitable purification strategy to make preparative levels of SUMO-1- and SUMO-2-conjugated AS recombinant protein.

SUMOylation could also contribute to the stabilization of the AS aggregates or early oligomers by competing with ubiquitin for lysine modification, impairing proteasomal degradation of misfolded AS.

SUMO modification could play a role in the subcellular localization of AS. In mammalian cells, SUMOylation of several substrates has been linked to nuclear import. Unmodified RanGAP is cytoplasmic, whereas SUMO-modified RanGAP is associated with the nuclear pore (Mahajan *et al.*, 1997; Matunis *et al.*, 1996). In the case of the IKB

kinase regulator NEMO, for example, fusion of NEMO to SUMO was sufficient for localization to the nucleus (Huang *et al.*, 2003).

A nuclear function of AS has been recently proposed, based on evidences that a substantial fraction of AS appears to be localized in the nucleus in cellular models and *in vivo*. Indeed, in our cellular model, over-expressed AS clearly stains the nucleus of SH-SY5Y cells.

A recent study showed that targeting of AS to the nucleus promotes toxicity, whereas cytoplasmic sequestration is protective in both cell culture and transgenic *Drosophila*. AS mutations that cause familial Parkinson's disease, A30P and A53T, exhibit slightly increased nuclear targeting in cell culture. Interestingly, toxicity of AS can be rescued by administration of histone deacetylase inhibitors in both cell culture and transgenic flies. AS binds directly to histones, reduces the level of acetylated histone H3 in cultured cells and inhibits acetylation in histone acetyltransferase assays (Kontopoulos *et al.*, 2006). The fibrillation rate of AS is dramatically accelerated in the presence of histones *in vitro*. AS co-localizes with histones in the nuclei of nigral neurons from mice exposed to injections of the herbicide paraquat (Goers *et al.*, 2003). All these clues (SUMO modification, physical interaction with histones, sensitivity to HDAC inhibitors) might indicate a previously unappreciated role of AS in regulation of transcription and chromatin structure.

Further investigations regarding its toxic/protective role in PD and neurodegeneration are needed to establish whether SUMOylation of AS could represent a drug target, similarly to other post translational modification of AS. For instance, the finding that phosphorylation enhances AS toxicity has indicated the kinases responsible for phosphorylation as therapeutic targets for small-molecule inhibitors. SUMOylation of AS could also represent an easily detectable biomarker of the disease.

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