Review

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Alpha-synuclein at the intracellular and the extracellular side: functional and dysfunctional implications

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Abstract: Alpha-synuclein (α-syn) is an abundant neuronal protein whose physiological function, even if still not completely understood, has been consistently related to synaptic function and vesicle trafficking. A group of disorders known as synucleinopathies, among which Parkinson's disease (PD), is deeply associated with the misfolding and aggregation of α-syn, which can give rise to proteinaceous inclusion known as Lewy bodies (LB). Proteostasis stress is a relevant aspect in these diseases and, currently, the presence of oligomeric α-syn species rather than insoluble aggregated forms, appeared to be associated with cytotoxicity. Many observations suggest that α-syn is responsible for neurodegeneration by interfering with multiple signaling pathways. α-syn protein can directly form plasma membrane channels or modify with their activity, thus altering membrane permeability to ions, abnormally associate with mitochondria and cause mitochondrial dysfunction (i.e. mitochondrial depolarization, Ca^{2+} dys-homeostasis, cytochrome c release) and interfere with autophagy regulation. The picture is further complicated by the fact that single point mutations, duplications and triplication in α-syn gene are linked to autosomal dominant forms of PD. In this review we discuss the multi-faced aspect of α-syn biology and address the main hypothesis at the basis of its involvement in neuronal degeneration.

Keywords: alpha-synuclein; familial mutations; neurodegeneration; Parkinson's disease.

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Introduction: structure of α-synuclein – monomer, oligomers or aggregates?

α-syn is a small protein (140 amino acids) encoded by the *SNCA* gene. It contains a N-terminal α-helical region, a highly hydrophobic central stretch of 12 amino acids referred as non-amyloid component (NAC) and an acidic negatively charged C-terminal region. The N-terminal domain participates in lipid binding, the hydrophobic central region in oligomerization and the C-terminal domain possibly acts in recruiting additional proteins to the membrane and is required for chaperone-like activity (Figure 1).

In solution, α-syn does not adopt a consistent secondary structure and is generally considered to belong to the intrinsically unstructured proteins group. However, *in vitro* and in the presence of lipid membranes it assumes an α-helical secondary structure (Davidson et al., 1998; Eliezer et al., 2001). Recently, it has been shown that it can be also purified as tetramers (Bartels et al., 2011; Wang et al., 2011; Dettmer et al., 2015), but the issue is debated because others failed to reproduce these data (Fauvet et al., 2012; Burre et al., 2014).

Both *in vitro* and *in vivo* α-syn can form fibrillar aggregates that have a morphology similar to the amyloid fibrils found in Alzheimer's disease. As in other amyloidogenic diseases, the early hypothesis that the aggregation of α-syn has a causative role in the pathogenesis of PD has attracted a lot of interest. Huge efforts were directed to the study of the process of aggregation and of the mechanisms that elicit 'aggregates' toxicity in the cells. A general consensus has been reached by the fact that the neurotoxic species are the soluble oligomeric forms rather than fibrillar end deposits, suggesting that aggregation *per se* is a protective attempt to sequestrate toxic species (Zuchner and Brundin, 2008; Karpinar et al., 2009; Winner et al., 2011; Kalia et al., 2013). However, the question is still

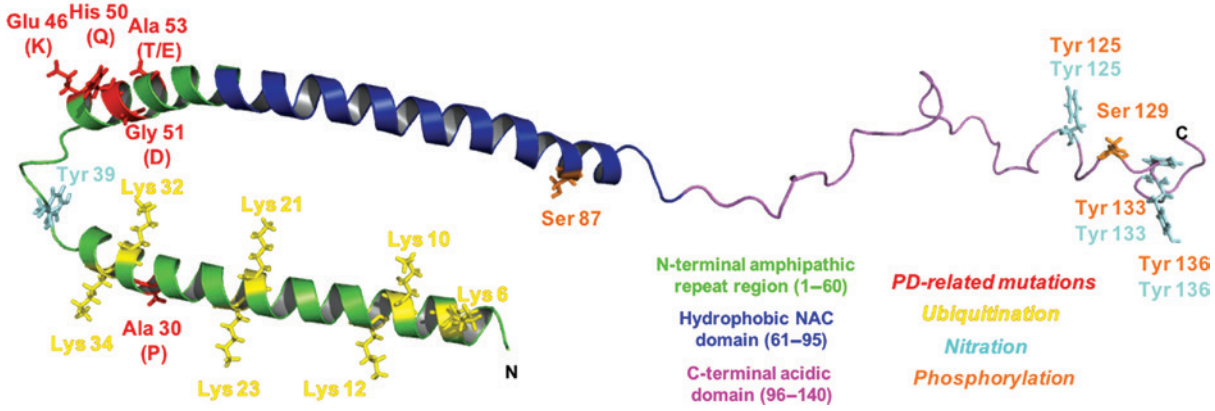

Figure 1: Schematic representation of α-syn structure.
The N-terminal domain (green) contains KTKEGV repeats where human missense mutations (indicated in red) are associated. The central hydrophobic domain (NAC) is in blue and the C-terminal acidic domain is in purple. Ubiquitination (lys), nitration (tyr) and phosphorylation (ser and tyr) sites are indicated in yellow, pale blue and orange, respectively.

open, and, in particular, the pathological mechanisms and the reasons for the selective vulnerability of specific brain regions are far from elucidated.

The characterization of α-syn oligomers started from *in vitro* experiments in which recombinant α-syn was found to spontaneously aggregate (Conway et al., 1998). Circular dichroism, NMR spectroscopy and atomic force microscopy have revealed the formation of various oligomeric structures during α-syn aggregation *in vitro*, consistent with a progressive increase in β sheet structure (Pfefferkorn et al., 2012; Lashuel et al., 2013) and have also made it possible to quantify the dimension of the oligomers, which range in size from 4 to 24 nm. Their detection in living cells, instead, is very hard, being limited by the resolution of conventional or electron microscopy techniques, and the dimension of the aggregates. The overexpression of α-syn in cellular models does not lead to the formation of detectable aggregates in the majority of cases, unless exogenous α-syn fibrils were added to trigger their nucleation. However, the detectable large aggregates represent the final step of the process. Recently, bimolecular fluorescence complementation (BiFC) and number and brightness fluctuation spectroscopy analysis were successfully used to directly visualize α-syn oligomerization in living cells, allowing the initial events to be studied (Outeiro et al., 2008; Plotegher et al., 2014). By these techniques it was possible to visualize the formation of dimers/oligomers of α-syn and to study their distribution in the cellular environments. Oligomers have been found both in the cytosol and in the nucleus, and, interestingly, their distribution was not homogeneous, confirming the prediction that they could be bound to intracellular structures such as lysosomes, exosomes or mitochondria (Nakamura et al., 2008; Emmanouilidou et al., 2010a; Mak et al., 2010).

The process of α-syn aggregation is modulated by various internal and external factors. Missense mutations or duplication/triplication in α-syn encoding gene (Deng and Yuan, 2014), post-translational modification (PTM) including ser129 phosphorylation (Chen et al., 2009) as well as the exposure to mitochondrial toxins, nitrative and oxidative insults (Paxinou et al., 2001; Hodara et al., 2004) are likely aggregation promoting stimuli. On the contrary, α-syn sumoylation (Krumova et al., 2011) or ubiquitination (Nonaka et al., 2005; Rott et al., 2014) negatively regulate aggregates formation. It has been also suggested that a dynamic equilibrium between monomer and tetramer may regulate aggregation process of α-syn by contributing to controlling the amount of available aggregating species (Gurry et al., 2013). The development of novel super-resolution microscopy techniques has very recently made it possible to follow α-syn self-assembly process at nanoscale level, and it will certainly contribute to clarify the mechanisms of α-syn seeding and toxicity in neurons (Pinotsi et al., 2016).

α-synuclein mutations

Several point mutations in the *SNCA* gene were identified and linked to the autosomal dominant inherited form of PD. Among them, those harboring the amino acid substitutions A53T (Polymeropoulos et al., 1997), A30P (Kruger et al., 1998) and E46K (Zarranz et al., 2004), were identified first and thus their effects on protein properties are

better characterized. A53T mutation is the most frequent and the only one associated with a specific phenotype: the carriers develop PD symptoms in the fourth–sixth decades and undergo a rapid progression of the disease in the next 10 years (Spira et al., 2001). Three novel missense mutations – H50Q (Appel-Cresswell et al., 2013; Proukakis et al., 2013), G51D (Kiely et al., 2013), and A53E (Pasanen et al., 2014) – have recently expanded the family of PD-associated mutants. H50Q mutation is prevalently associated with late onset of PD and cognitive impairment (Khalaf et al., 2014); G51D mutation is associated with an unusual PD phenotype characterized by early disease onset, moderate response to levodopa, rapid progression and frequently psychiatric symptoms (Lesage et al., 2013) and A53E is also associated with atypical PD that starts early with numbness and hyperreflexia and then results in parkinsonism associated with severe spasticity, myoclonic jerks and psychiatric disturbances (Pasanen et al., 2014); see Table 1.

Big effort has been made to understand the mechanisms by which the mutations alter the physiological properties of α-syn since their comprehension could provide critical insights into the molecular basis underlying the pathogenesis of PD, and aid in the development of strategies to treat or prevent the disease. Overall, α-syn mutants are more inclined to accumulate than wild-type proteins because of their structural modifications, but also because their presence compromise the activity of the pathways involved in protein turnover and clearance. PD-linked mutations differentially affect α-syn propensity to aggregate *in vitro*. The A53T, E46K, and H50Q mutations enhance α-syn fibrillization, instead the G51D and A53E mutations attenuate it and favor the formation of amorphous aggregates (Fares et al., 2014; Ghosh et al., 2014; Rutherford and Giasson, 2015). The A30P mutant exhibits enhanced oligomerization rate compared with wild-type α-syn (Conway et al., 2000; Fredenburg et al., 2007; Khalaf et al., 2014). Functional studies showed that G51D α-syn oligomerizes more slowly and its fibrils are more toxic than those of the wild-type protein (Lesage et al., 2013). Notably, whereas the A30P and G51D mutants exhibit defective interaction with membranes (Jo et al., 2002), the E46K mutation increases the binding affinity of α-syn to phospholipids (Choi et al., 2004; Fares et al., 2014). The relevance of these findings *in vivo* is still to be elucidated. Interestingly, it was recently reported that the A30P, E46K, H50Q, G51D and A53T mutants exhibited identical propensities to oligomerize in living cells, but had distinct abilities to form inclusions. While the A30P mutant reduced the percentage of cells with inclusions, the E46K mutant had the opposite effect (Lazaro et al., 2014), thus offering different experimental models to study oligomers/aggregates-induced cell toxicity.

It is worth noting that despite the effect of mutations on α-syn structure and propensity to aggregate being different, the overexpression of the mutant forms in cell models results in enhanced susceptibility to toxicity and cell death after exposure to mitochondrial toxins, thus suggesting that distinct structural and biophysical properties converge to a similar phenotype in the process of pathogenicity.

α-synuclein cell toxicity

Numerous answers are still pending on the mechanism of cell toxicity induced by α-syn: both cellular and animal models have revealed multi-faced, and sometime contradictory, aspects. As multiplication of the whole *SNCA* gene has been recognized as causative of familial PD (Singleton et al., 2003; Chartier-Harlin et al., 2004), many studies focused on dissecting the propensity of α-syn molecules to aggregate and give rise to intracellular inclusions in respect with their abundance. The budding yeast *Saccharomyces cerevisiae* has been extensively used as a powerful system to study the α-syn-mediated cytotoxicity (Auluck

Table 1: Clinical phenotypes of α-syn mutations.

Mutation	Age of onset (years)	Disease duration (years)	Cognitive impairment	Psychiatric distrurbances	Myoclonus	Epilepsy	References
A53T	40–60	10–15	Occasionally	Occasionally	Occasionally	No	Polymeropoulos et al., 1997
A30P	>60	10–15	Occasionally	No	No	No	Kruger et al., 1998
E46K	>60	<10	Occasionally	Occasionally	No	No	Zarranz et al., 2004
H50Q	>60	10–15	Yes	Occasionally	No	No	Appel-Cresswell et al., 2013
G51D	>40	10–15	Occasionally	Yes	Yes	Yes	Kiely et al., 2013
A53E[a]	40–60	>15	Occasionally	Occasionally	Occasionally	No	Pasanen et al., 2014

[a]Only one family reported.

et al., 2010). This model has allowed the establishment of a linear correlation between the amount of overexpressed α-syn and toxicity. At low expression level, wild-type and A53T α-syn did not impair cell growth and viability, but at higher levels both of them redistributed from the cell surface to cytoplasmic foci and induced cellular toxicity by causing endoplasmic reticulum (ER) stress and mitochondrial dysfunction (Smith et al., 2005a; Chung et al., 2013). Intriguingly, similar effects have been described also in mammalian cells. Fragmented and dysfunctional mitochondria (Hsu et al., 2000; Devi et al., 2008; Chinta et al., 2010; Loeb et al., 2010; Nakamura et al., 2011; Mullin and Schapira, 2013), altered mitophagy (Norris et al., 2015), ubiquitin-proteasoma dysfunction (Stefanis et al., 2001; Tanaka et al., 2001; Petrucelli et al., 2002) as well as chaperone-mediated autophagy (CMA) impairment (Cuervo et al., 2004; Winslow et al., 2010; Petroi et al., 2012) have been reported as common features in cells overexpressing α-syn at high levels. However, it must be noticed that the absence of α-syn (both in silenced and in knocked down cells) is also responsible for mitochondrial dysfunction and augmented autophagic process (Calí et al., 2012; Lashuel et al., 2013) thus suggesting that a proper amount of α-syn is required to guarantee cell wellness: both an increase and a reduction in its expression levels have detrimental consequences.

How α-syn oligomers induce cellular toxicity is not completely clear: a better knowledge of the biological function of α-syn is necessary to profoundly understand the mechanisms. Different functional roles for α-syn have been proposed considering its action at the intracellular and extracellular sides. They will be discussed in the following sections.

α-synuclein function at the intracellular side

α-Syn in vesicle trafficking and synaptic transmission

α-syn is particularly enriched in neurons at the presynaptic terminal (Maroteaux et al., 1988; Iwai et al., 1995). This preferential localization justified the suggestions that have been made about 20 years ago on its role in the synaptic plasticity during song learning in birds (George et al., 1995). Additional papers have appeared later supporting α-syn function in shaping synaptic transmission, however the absence of overt phenotype in transmitter release in mice lacking α-syn (Abeliovich et al., 2000; Cabin et al., 2002; Chandra et al., 2004) maintained the situation unclear until direct interaction of α-syn and synaptobrevin 2 was shown by immunoprecipitation assay (Burre et al., 2010) and synaptic defects were revealed in synucleins triple knock out mice (Burre et al., 2010; Greten-Harrison et al., 2010). Although no orthologue of α-syn exists in yeast, its heterologous expression in *S. cerevisiae* was reported to inhibit cell growth and to finally result in cell death (Outeiro and Lindquist, 2003; Soper et al., 2008). As previously mentioned, heterologous α-syn expression induces neurotoxicity that correlates with its expression level. When expressed at low levels α-syn localized at the plasma membrane, but when expressed at high levels it formed cytosolic inclusions (Auluck et al., 2010). Interestingly, one of the first detectable defect in α-syn overexpressing yeast cells is in ER to Golgi vesicle trafficking. α-syn interfered with secretory pathways by inhibiting the activity of Rab GTPase YPT1 (a yeast hortolog of murine Rab1): vesicles efficiently budded from the ER but failed to dock and fuse with Golgi membranes, and thus accumulated (Cooper et al., 2006). Similarly, α-syn-induced defects in vesicle trafficking were shown in *Caenorhabditis elegans*, *Drosophila melanogaster* and mammals. Interestingly, in animal models of PD, toxicity was rescued by the introduction of Rab1 GTPase (Cooper et al., 2006; Gitler et al., 2008; Thayanidhi et al., 2010). The pioneer observation that the overexpression of non-toxic levels of α-syn led to a modest accumulation of vesicles in yeast cells (Gitler et al., 2008), was extended to mammalian glutamatergic hippocampal pyramidal and in mesencephalic dopaminergic primary neurons, where it was found that a moderate increase in α-syn expression inhibited neurotransmitter release without generating toxicity and cellular inclusions, thus suggesting that this defect may precede detectable neuropathology (Nemani et al., 2010). By monitoring the synaptic vesicle cycle, it was established that defective vesicles re-clustering after endocytosis events was responsible for a reduction of density at the active zone and consequently for impaired neurotransmitter release (Nemani et al., 2010). The precise role of α-syn in synaptic transmission has been fully recognized when Sudof and co-workers demonstrated that it physiologically acts as chaperone in the formation of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-complex, a machinery necessary for vesicle fusion and neurotransmitter release at the synapse (Burre et al., 2010). Vesicle fusion is mediated by the interaction between the SNARE proteins localized at the vesicles, i.e. synaptobrevin (vesicle-associated

membrane protein, VAMP2) and the proteins at the presynaptic plasma membrane (i.e. SNAP25 and syntaxin). The SNARE complex undergoes cycles of assembling and disassembling during each release action, an operation that requires a strict control by molecular chaperones and protein folding quality control mechanisms. α-syn directly interacts with VAMP2 and promotes SNARE complex assembly (Burre et al., 2010). The finding that triple-knock out mice lacking α, β and γ synucleins developed age-dependent neurological impairment and died prematurely indicated that, during aging, the sustaining of the presynaptic SNARE complex assembly by α-syn is essential for neuronal survival (Burre et al., 2010; Greten-Harrison et al., 2010). Intriguingly, it was also observed that the re-introduction of α-syn in cultured neurons from triple-knock out mice caused an increase in SNARE complex assembly in a dose-dependent manner (Burre et al., 2010). More recently, the same group has demonstrated that the functional role of α-syn in promoting SNARE complex assembly at the presynaptic plasma membrane is exerted only by α-syn molecules which assemble in multimeric form upon membrane binding. Cytosolic unfolded α-syn was instead ineffective to this function (Burre et al., 2014). At the presynaptic terminal, α-syn is in equilibrium between a soluble and a membrane-bound form: physiological multimeric, α-helical, and membrane-bound species act as a SNARE-complex chaperone, but monomeric, natively unfolded forms and amyloid-like aggregates fail to do it and instead lead to neurotoxicity (Burre et al., 2015). These observations perfectly fit with the finding that blocking α-syn membrane binding significantly enhanced its neurotoxicity *in vivo*, and suggest that α-syn-mediated neurotoxicity could operate through a 'folding pathway'.

α-syn in channel formation and regulation

As mentioned above, the soluble monomeric form of α-syn develops an α-helical conformation upon binding to membranes containing anionic lipids or phosphatidylethanolamine (PE) (for a model of insertion see, for example, Tsigelny et al., 2007 and Cramer et al., 2011). According to a well-accepted hypothesis, oligomeric species are able to form transmembrane proteinaceous channels. However, experimental evidence has been obtained of an alternative possibility, i.e. an increase in membrane permeability due to a thinning of the hydrophobic core of the lipid bilayer (Stockl et al., 2013). In this latter case, incorporation of oligomers between the tightly packed lipids would facilitate the diffusion of small molecules. Beside its ability to directly increase membrane permeability to small molecules, a more indirect, endogenous channel-modulating role for α-syn is also emerging.

One of the first indications for channel-forming ability of α-syn has been obtained in the frame of a study aimed at understanding the mechanism of action of several, pathologically relevant non-fibrillar amyloid proteins (Quist et al., 2005). In this work, α-syn has been shown by atomic force microscopy to assemble in channel-like structures with up to eight subunits forming a ring, and in accordance, the preparation gave rise to ion channel activity upon reconstitution to planar lipid bilayers, displaying heterogeneous single channel conductances (up to approximately 300 pS in 100 mM KCl). Formation of 'doughnut-shaped' protein particles was confirmed also by Tsigelny et al. (2012). Subsequent studies using *in vitro* systems provided a more detailed biophysical characterization of the channel formed by α-syn. In 2007, Zakharov and colleagues reported that oligomeric α-syn induced ion conductivity, showing non-resolvable single channel events, but only in the presence of anionic lipids in the membrane, while the monomeric form, under the same conditions, gave rise to different conductance levels ranging up to more than 1 nS in 100 mM KCl, with 400 pS being the prevalent value (Zakharov et al., 2007). Interestingly, application of 1 mM Ca^{2+} decreased both channel conductance and the probability of being open. The authors also studied the cases α-syn mutants, concluding that E46K and A53T substitutions enhanced channel activity, in contrast to A30P which suppressed channel formation. Di Pasquale and colleagues also observed that E46K mutation generated enhanced, non-stop activity in planar lipid bilayers (Di Pasquale et al., 2010) while the ganglioside GM3 was able to counteract this effect. However, in their system the wt protein and the E46K mutant (applied at 200 nM concentration) displayed main conductance levels of 150 pS, and 35 pS in 100 mM KCl, respectively. In the work by Schmidt et al. (2012), the addition of preformed oligomers at 2 μM concentration to a planar lipid membrane resulted in the insertion of multiple uniform pores displaying 100 pS conductance each (in 250 mM KCl, thus this conductance would correspond to 40 pS in 100 mM KCl supposing that no saturation occurs in the range from 0 to 250 mM KCl). Importantly, these authors identified two different substances able to prevent membrane permeabilization: baicalein (Schmidt et al., 2012) and anle138b (Wagner et al., 2013). This latter molecule is able to slow down Parkinson progression in an *in vivo* model, linking thus the ability of α-syn to form oligomers and channels to pathogenesis (Levin et al., 2014). Interestingly, (-)-epigallocatechin gallate, a well-known green

tea catechin, is also able to block channel activity induced by α-syn oligomers (Kim et al., 2009). Another remarkable work dealing with the single channel properties of α-syn from the group of Dalla Serra provided evidence for the formation of well-behaved channels with a single channel conductance of 35 pS in 100 mM KCl using 0.1 μM α-syn in planar lipid bilayer membrane. This value is in good agreement with that obtained by Schmidt and colleagues. In addition, to this conductance value, they observed two additional levels, suggesting that different values could correspond to pores formed by independent α-syn molecule clusters, favoring thus a barrel-stave pore model (Tosatto et al., 2012). Altogether, several groups arrived at the same conclusion, namely that α-syn used at pathologically relevant concentrations (about 100 nM in the monomeric form; Hall et al., 2012) is able to form non-selective cation channels when inserted into artificial membranes containing negatively charged lipids.

Evidence has been found in favor of channel formation by α-syn not only in artificial membranes, but in cell membranes as well (Furukawa et al., 2006; Tsigelny et al., 2007). For example, Feng and colleagues generated an immortalized dopaminergic cell line (MN9D) expressing human wild-type α-syn in a doxycycline-regulated manner and observed that expression of the protein resulted in a voltage-independent, leak-like current (Feng et al., 2010). Application of 50 μM paraquat and 100 μM dopamine, known to induce strong oxidative stress, caused a 2.5-fold increase of the membrane conductance and an 8-fold increase in cell death (Feng and Maguire-Zeiss, 2011). In addition, a recent work by Mironov highlighted that the extracellular or intracellular application of 50–100 nM α-syn in patched hippocampal neurons formed non-selective cation channels whose properties depended on the side of application. The pores were equally permeable for Na^+, K^+ and Ca^{2+} and exhibited multiple levels of conductance (30, 70 and 120 pS at –100 mV), but the channels formed by intracellular (provided through the pipette in the whole-cell configuration) or extracellular α-syn were characterized by different activities at resting membrane potential, implicating a different physiological outcome, i.e. depolarization was induced only by the extracellularly applied α-syn (Mironov, 2015).

In addition to the above-described features, α-syn might alter ion homeostasis by impacting the activity of endogenous channels. Interestingly, α-syn was found to physically interact with the outer mitochondrial membrane voltage-dependent anion-selective channel protein 1 (VDAC1) in co-immunoprecipitation assay performed on substantia nigra of α-syn overexpressing rat (Lu et al., 2013), and VDAC1 expression level was significantly decreased in neurons from brain of PD patients containing α-syn positive inclusions (Chu et al., 2014). A few studies suggest that VDAC1 (or in general, VDAC isoforms) activity might impact on disease progression by affecting mitochondrial function. A very recent report shows that nanomolar concentration of monomeric α-syn is able to reversibly block VDAC1 channel activity in planar lipid bilayer, suggesting that α-syn translocation through the channel may enhance cell toxicity (Rostovtseva et al., 2015), similarly to the previously reported interaction with α-hemolysin which is also a β-barrel pore like VDAC (Gurnev et al., 2014). The authors suggest that once in the intermembrane space, α-syn directly targets complexes of the mitochondrial respiratory chain, thereby inducing enhanced ROS production and a consequent amplifying mechanism leading to transformation of α-syn into the fibrillary form (Rostovtseva et al., 2015). In relation to mitochondrial physiology, it has to be mentioned that overexpression of the N-terminal of α-synuclein in dopaminergic MN9D cells and primary cortical neurons seems to induce opening of the permeability transition pore (PTP) and thus to reduce cell survival (Shen et al., 2014). Furthermore, recent *in vivo* data also link mutant A53T α-syn overexpression to PTP (Martin et al., 2014), whose components possibly comprise VDAC (but see Bernardi et al., 2015). Further experiments are needed to understand the exact role of these interactions in the context of autophagy, apoptosis and mitochondrial metabolism/energetics.

VDAC is not the only channel-forming protein which directly interacts with α-syn: the properties of different types of plasma membrane channels have been shown to be affected by the interaction with it. Direct binding of α-syn to ATP-dependent potassium channel (KATP) channels has been demonstrated in insulin-secretory granules (Geng et al., 2011) and two proteins, ENSA and sorcin, well known as KATP channel modulators have been shown to interact with α-syn by bacteriophage display assay (Woods et al., 2007). Furthermore, recent electrophysiological evidence points to the ability of α-syn to stimulate the activity of KATP channels (Mironov, 2015). In this scenario, KATP opening could dampen the toxic effect of extracellular α-syn, by opposing the depolarization induced by the protein applied from outside of the cells. Whether α-syn interacts with the mitochondria-located mitoKATP remains to be determined, but in light of the numerous observed effects of this protein on mitochondrial function, an interaction cannot be excluded. Oligomeric α-syn selectively inhibited plasma membrane-located human nicotinic acetylcholine receptors (in particular α4β2-nAChR-mediated currents) in a dose-dependent, non-competitive manner.

This inhibition may contribute to cholinergic signaling deficits observed in PD patients (Liu et al., 2013). The voltage-gated Ca^{2+} channel Cav2.2 instead becomes activated by extracellular α-syn, as shown in patch clamp experiments on rat neurons, causing increased dopamine release (Ronzitti et al., 2014). Interestingly, Cav2.2 undergoes translocation out of the rafts to cholesterol-poor region of the plasma membrane upon α-syn treatment (Ronzitti et al., 2014), thus suggesting a role for α-syn also in the remodeling of plasma membrane microdomains.

In summary, channel-modulating action of both extracellular and intracellular α-synuclein might alter cells' physiology, but a contribution by the pores formed by the protein itself seems of relevance as well. Future experiments addressing the exact mechanisms of interaction and the consequences of α-syn-formed pores in intact cells or *in vivo* will certainly help a better understanding of the causative role of α-syn in PD pathogenesis.

At the end of this paragraph, it is worth mentioning a novel aspect that further links α-syn to ions homeostasis perturbation and which has recently emerged from a study showing that oligomeric α-syn may perturb Na^+ and Ca^{2+} homeostasis by reducing the activity of the plasma membrane Na^+/K^+ ATPase. By interacting and sequestering the Na^+/K^+ pump in clusters, α-syn oligomers inhibit Na^+ efflux from the neurons thus favoring Ca^{2+} influx induced by glutamate application and in turn enhancing excitotoxicity (Shrivastava et al., 2015).

α-syn in mitochondrial/endoplasmic reticulum function and calcium regulation

Alterations of mitochondrial function have been documented by several groups in different cellular and animal models overexpressing wt or mutant α-syn. α-syn accumulation has been associated with mitochondrial complex I dysfunction (Devi et al., 2008; Chinta et al., 2010; Loeb et al., 2010; Luth et al., 2014) and mitochondrial defects including altered morphology, loss of mitochondrial membrane potential, increased mitochondrial ROS (Devi et al., 2008; Chinta et al., 2010). Reduced complex I activity and cytochrome c release were observed both in transgenic mice expressing mutant A53T or A30P α-syn (Martin et al., 2006) and in cells overexpressing α-syn (Hsu et al., 2000; Parihar et al., 2008; Shavali et al., 2008). It has also been shown that α-syn regulates mitochondria dynamics by participating in the fusion/fission process and autophagy (Kamp et al., 2010; Winslow et al., 2010; Nakamura et al., 2011). The direct interaction between α-syn and mitochondria has been proposed in a number of papers (Li et al., 2007; Devi et al., 2008; Parihar et al., 2008) thus postulating that the physical association of α-syn with the mitochondrial membranes may cause membrane damage (Kamp et al., 2010). Many lines of evidence have shown that α-syn binds preferentially to high-curvature, detergent-resistant membranes enriched in cholesterol, sphingolipids, and acidic phospholipids (Middleton and Rhoades, 2010; Jensen et al., 2011) as well as cardiolipin, a lipid that is present almost exclusively in mitochondria (Kubo et al., 2005).

Recently, we and others have found that α-syn has a biological role in modulating ER/mitochondria relationship and associated functions (Calí et al., 2012; Guardia-Laguarta et al., 2015). Low levels of wt α-syn overexpression positively regulated mitochondrial Ca^{2+} handling by favoring ER-mitochondria tethering. However, enhanced accumulation of α-syn caused its redistribution to localized foci, and, similar to its silencing, reduced the ability of mitochondria to accumulate Ca^{2+} and impaired mitochondrial morphology. The absence of Ca^{2+} transfer from the ER to mitochondria resulted in augmented autophagic fluxes suggesting that, in the long range, this situation may compromise cell bioenergetics and survival (Calí et al., 2012). Later, our data have been reinforced by the finding that wild-type α-syn was present in mitochondria-associated ER membranes (MAM), a structural and functional compartment where mitochondria and ER membranes are in close physical and biochemical contact (Guardia-Laguarta et al., 2014) and that regulates a number of key metabolic functions. Remarkably, pathogenic α-syn point mutations resulted in its reduced association with MAM, and consequently in lower degree of apposition of ER with mitochondria and increased mitochondrial fragmentation. The overexpression of wild-type α-syn in mutant α-syn-expressing cells, as well as the overexpression of the mitochondrial fusion/MAM-tethering protein Mitofusin 2, reverted the fragmentation phenotype, implying that the role of α-syn in enhancing ER-mitochondrial apposition is crucial to mitochondria physiology in agreement with our previous suggestions (Calí et al., 2012; Guardia-Laguarta et al., 2014). It thus appears that α-syn should be present at a threshold level, not too much but also not too low, and that mutations/conditions that favor its aggregation or its depletion may have detrimental consequences through a loss of function mechanism.

This biological function of α-syn is particularly interesting because, alterations in MAM are emerging as a common element in a number of neurodegenerative diseases, including AD (Zampese et al., 2011; Area-Gomez

et al., 2012; Hedskog et al., 2013) and amyotrophic lateral sclerosis (Stoica et al., 2014).

α-synuclein at the extracellular side: action, secretion and spreading

Extracellular α-syn

α-syn is a cytosolic protein but numerous studies have convincingly reported that it can be present extracellularly in different animal or cellular models.

The presence of α-syn outside the cell body is traced back to 1993, when Uèda and colleagues, identified and cloned an apparently unknown protein from amyloid preparations obtained from the cortex of AD patients. The protein was named NAC (Non-Aβ component of AD amyloid) (Uèda et al., 1993), and only soon after it was recognized as a fragment of α-syn (Jakes et al., 1994). The presence of α-syn in AD plaques was later confirmed by numerous studies and it has been proposed that it could act as co-aggregation factor and favor the plaques formation through a process of cross-seeding (Giasson et al., 2003; Waxman and Giasson, 2011; Guo et al., 2013). However recently, and unexpectedly, it has been found that the intracerebral injection of α-syn in the brains of AD mice double transgenic for APP and presenilin1 mutants failed to cross-seed Aβ plaques *in vivo*, but rather, in mice also co-expressing the A30P α-syn mutant, wild-type α-syn inhibited the amyloid-β plaques formation (Bachhuber et al., 2015), thus suggesting that the interaction between Aβ and α-syn leads to inhibition of Aβ deposition.

Since 15 years ago, when the presence of α-syn at nanomolar concentration in the cerebrospinal fluid (CSF) and plasma of PD patients was first described (Borghi et al., 2000; El-Agnaf et al., 2003), a big effort was made to quantify α-syn in these biological fluids and establish a link between the amount of the protein and the pathology, thus proposing that α-syn could represent a biomarker for PD. The issue is still debated as no univocal correlation has been established (Paleologou et al., 2009; Parnetti et al., 2014; Lleo et al., 2015).

α-syn, a prion-like protein?

In 2003 the idea started to emerge that α-syn could have a prion-like behavior. Similarly to prion protein, the conversion from α-helix to β-sheet in α-syn structure could be implicated in the pathogenicity of the protein (Wood et al., 1999; Angot and Brundin, 2009; Yonetani et al., 2009; Angot et al., 2010). Despite the precise molecular mechanisms that induce α-syn aggregation being unknown, the most accepted hypothesis suggests a nucleated polymerization process starting from changes in the protein secondary structure. The protein is prevalently unfolded or in α-helix conformation and the monomers initiate to self-assembly through an accretion mechanism, generating amyloid-like fibrils (Wood et al., 1999; Uversky et al., 2001; Chu and Kordower, 2015) that consequently promote their deposition as lewy bodiest (LB). As for the majority of amyloidogenic proteins, differences in ion strength or in the concentration of metals and lipids to which α-syn is exposed *in vitro* give rise to different oligomeric/aggregate species harboring distinct properties in terms of toxicity, propagation and seeding (Xin et al., 2015). It has been hypothesized that α-syn-associated pathologies could be due to specific 'strains' that display 'aggressive' characteristics (Bousset et al., 2013). In 2003 Braak and co-workers proposed that the progression of PD clinical symptoms could directly correlate with the diffusion of α-syn pathology throughout the brain (Braak et al., 2003a). The so-called Braak's stages define with precision the progression of α-syn lesions, that first appeared in brainstem and olfactory bulb and then proceeded caudo-rostrally to midbrain and cortical areas (Braak et al., 2006). According to this theory, it has been found that the intra-gastric administration of rotenone in rats resulted in Lewy pathology that first appeared in the enteric nervous system and then spread to the substantia nigra of the animals (Pan-Montojo et al., 2010). This scenario led to consideration that α-syn could act as an exogenous pathogenic agent in the onset of PD, and it was proposed, for the first time, the possibility that it could act like prions not only in the structural changes that undertake its deposition in intracellular aggregates but also in its propagation and infection mechanisms (Braak et al., 2003b; Hawkes et al., 2009), see Olanow and Brundin (2013), for a comprehensive review.

In 2008, the finding that human embryonic dopaminergic cells transplanted in the striatum of PD patients developed LB-inclusions positive to both α-syn and ubiquitin (Kordower et al., 2008; Li et al., 2008) suggested the possible cell-to-cell transmission of α-syn, and indicated that α-syn could spread from affected cells to healthy neurons. Interestingly, the grafted cells displayed a neurodegenerative phenotype with α-syn inclusions with the same characteristic of those existing in dopaminergic neurons of the substantia nigra of the patients (Chu and Kordower, 2010; Li et al., 2010; Kurowska et al., 2011). A seeding mechanism for α-syn deposition was thus

proposed and supported by experiments in rodents overexpressing human α-syn, where grafted cells were found to display puncta of exogenous α-syn, in some cases surrounded by endogenous protein (Hansen et al., 2011; Kordower et al., 2011; Angot et al., 2012).

Rodent models have been widely used to study the process of propagation of α-syn *in vivo*. A clear PD-like pathology can be induced by injecting asymptomatic mice with brain homogenates obtained from animals overexpressing human A53T α-syn (Luk et al., 2012b; Mougenot et al., 2012), or with recombinant mouse or human α-syn fibrils (Luk et al., 2012a; Masuda-Suzukake et al., 2013; Paumier et al., 2015). In both cases, the exogenous protein was found in brain areas distant from the injection site, thus suggesting that injected α-syn is able to propagate. α-syn inclusions in the brain and spinal cord together with the appearance of motor impairments have been observed in mice after intramuscular and gastric injection of α-syn (Holmqvist et al., 2014; Sacino et al., 2014) and after α-syn oligomers injection in the olfactory bulb of mice or intravenously in rats, thus indicating that the protein is able to cross the blood-brain barrier and accumulate in cortical neurons and spinal cord inducing diffuse microglial activation (Peelaerts et al., 2015). Interestingly, human α-syn derived from LB of PD patients was able to induce synucleinopathy when it was inoculated in the substantia nigra of monkeys (Recasens et al., 2014). However, there is no evidence for an inter-organism infectivity due to α-syn, thus, at the moment, and according to actual definition of prion, it cannot be established that α-syn completely follows a prion-like mechanism to induce pathology (Irwin et al., 2013).

α-syn secretion, propagation and uptake

To sustain the process of α-syn spreading and propagation of infectivity it is necessary to figure out at least four different steps: the release from the affected cells, the spreading, the uptake by the healthy cells and the induction of pathology (Figure 2). All these aspects are currently the object of intensive investigation. The observation that stressing conditions as dopamine treatment or the presence of mutations in LRRK2 and SNCA genes can enhance α-syn secretion further increases the level of interest (Lee et al., 2005; Jang et al., 2010; Fares et al., 2014; Khalaf et al., 2014).

α-syn secretion

Several pathways have been proposed to account for this first step. The membrane leakage from dying cells during PD progression is the most obvious, but it has been excluded as no accompanying release of other cytosolic proteins such as ubiquitin or lactate dehydrogenase has been revealed in the same conditions (Lee et al., 2005). Furthermore, it has been shown that α-syn released from the damaged neurons did not enhance protein propagation

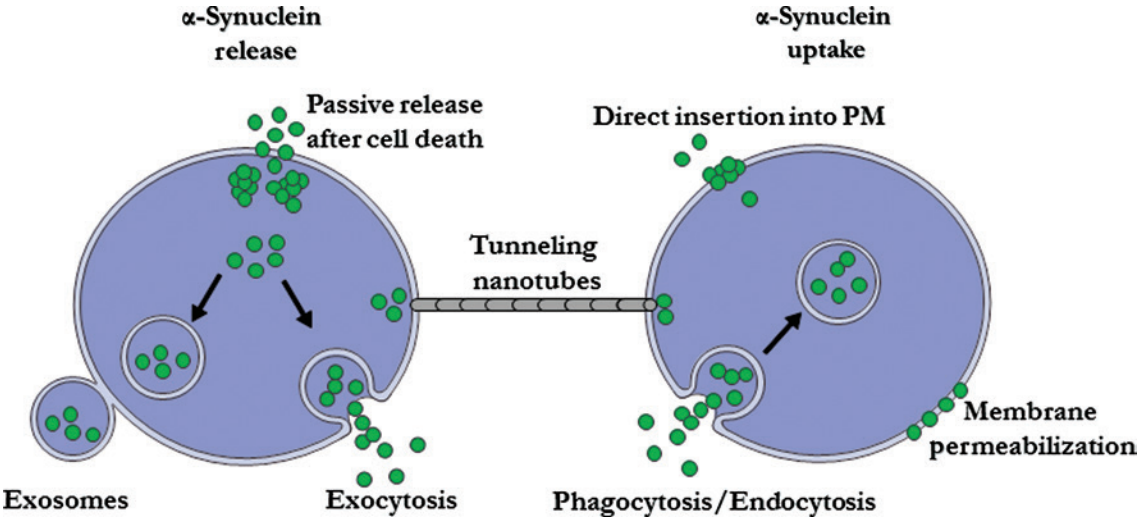

Figure 2: Schematic cartoon illustrating the pathways for α-syn release and uptake by the cells.
α-syn can be released by the cells through membrane disruption, conventional exocytosis or exosomes. Its uptake can occur by simply diffusion to the plasma membrane or by endocytosis. Tunneling nanotubes has been also proposed for α-syn cell-to-cell transfer but no solid evidence for this mechanism has been provided so far.

(Ulusoy et al., 2015). Thus, considering the involvement of α-syn in the regulation of vesicles trafficking (see above), it has been hypothesized that the protein could take advantage from this function to escape or to be taken up by neurons (Emanuele and Chieregatti, 2015). However, as the amount of secreted α-syn in neuroblastoma overexpressing cells was reduced by low temperature but not by the incubation with the canonical inhibitor of exocytosis Brefeldin A, it has been suggested that a non-conventional vesicular pathway rather than a ER-Golgi mediated secretion could be involved (Lee et al., 2005). The possibility that the so-called exosome-mediated transmission could have a role in α-syn secretion has recently emerged. Even if exosome-associated α-syn represents a minor fraction of secreted α-syn, this route is considered the main mechanism that accounts for α-syn extrusion and spreading (Emmanouilidou et al., 2010a; Danzer et al., 2012). Exosomes are small vesicles, generated from the late endosomes or multi vesicular bodies (MVBs) (Piper and Katzmann, 2007), and released from the cells into extracellular space. MVBs contain cellular protein, small RNAs and miRNAs and they are normally directed to lysosomes to be degraded. However, under particular conditions or stimuli, they can form the so-called exosomes, small vesicles detectable by the presence of specific markers, that are conveyed to the plasma membrane and the extracellular ambient. They have been isolated from the CSF (Street et al., 2012) but also from adult human brain (Banigan et al., 2013) and are now considered important not only to remove waste material from the cells but as mediators of intercellular communication (Rajendran et al., 2014; Mittelbrunn et al., 2015). Exosomes obtained from plasma of PD patients displayed higher level of α-syn than those from control groups (Schneider and Simons, 2013; Shi et al., 2014) and interestingly, it has been recently described that exosomes lipids enhance the aggregation propensity of α-syn (Grey et al., 2015). In addition, α-syn secretion is augmented upon genetic or pharmacological-induced impairment of lysosomal and proteasomal functions (Lee et al., 2013) and its release is promoted through exosomes.

Once released, the protein could undergo two different destinies: the degradation by extracellular proteases or the internalization by other cells. Extracellular α-syn by binding to the plasma membrane of neurons could induce toxicity due to membrane permeabilization and alteration of ions homeostasis and synaptic function (Gallegos et al., 2015; Pacheco et al., 2015), and it can also serve as nucleation site for the aggregation process (Mahul-Mellier et al., 2015). Furthermore, it has been shown that α-syn can be absorbed by non-neuronal cells such as astrocytes and microglia, suggesting that it could contribute to neuroinflammation process observed in PD brain. It is interesting to note that there is no α-syn mRNA in healthy brain oligodendroglia, but α-syn-positive inclusions have been found in oligodendrocytes and astrocytes from multiple system atrophy (MSA, a synucleinopathy) patients, suggesting that the protein found in these cells has an exogenous origin, possibly from the extracellular ambient (Tu et al., 1998; Reyes et al., 2014). The targeting of extracellular α-syn to microglia could be protective at first stage, as it promotes its removal by phagocytes activation (Lee et al., 2008b), but in the long range, excessive inflammation could turn in α-syn accumulation as a consequence of enhanced post-translational nitration (Giasson et al., 2000) and further contributing to PD pathogenesis by triggering neuronal loss (Dzamko et al., 2015; Stojkovska et al., 2015).

α-syn propagation

The pathways for α-syn propagation are still amply debated. The general consensus agrees on the fact that the spreading of α-syn aggregating over long distances requires axonal transport (George et al., 2013; Ubeda-Banon et al., 2014). At the same time, it has been shown that this pathway is dysfunctional in PD (De Vos et al., 2008; Millecamps and Julien, 2013), thus rising an apparent contradiction. To understand the question, it is necessary to distinguish between the transport of newly synthetized α-syn from the site of its production (the cell body) to the site of its physiological action (the distal axon and nerve terminals where the synapsis occurs) and the transport of α-syn from one neuron to the other. The first type of movement, as for other synaptic proteins, occurs following the anterograde transport (Utton et al., 2005), the second, instead, is mediated both by anterograde and retrograde axonal transport (Jang et al., 2010; Freundt et al., 2012) or by trans-synaptic transmission (Masuda-Suzukake et al., 2014).

Several studies suggest that slowed axonal transport of α-syn to distal site could account for its accumulation and aggregation at the cell body (Roy, 2009), and that, in turn, the formation of α-syn oligomers could directly interfere with microtubules and kinesin motors, thus affecting mainly the anterograde transport and enhancing deficit in axonal transport of α-syn at the synaptic site (Prots et al., 2013), but leaving the dynein mediated retrograde transport essentially unaffected and prompting it to act as propagation pathway for PD pathology, according to the model proposed by Braak (see above). The issue

of PD pathology spreading is obviously attracting a lot of interest. Understanding more about these mechanisms is necessary to develop possible therapies targeting the progression of PD symptoms. For a more comprehensive view refer to (Lamberts et al., 2015; Valera and Masliah, 2016).

α-syn uptake

The question on how α-syn could be internalized and transported across the cell membranes is also under debate: the simple diffusion across the plasma membrane appears to be the privileged pathway for the uptake of monomeric α-syn (Lee et al., 2008a), instead the internalization of oligomeric and fibrillary species seems more likely to involve simple endocytosis, since the treatment with canonical inhibitors of this pathway consistently reduces the acquisition of α-syn by the cells (Desplats et al., 2009; Hansen et al., 2011). Even if non-conventional endocytosis has been proposed as alternative pathway (Holmes et al., 2013), the search for α-syn binding receptor responsible for an α-syn internalization failed to identify any plausible candidate.

More recently, the possibility that α-syn can follow the prion behavior and pass from cell to cell by tunneling nanotubes, i.e. F-actin containing tubes that connect the cytoplasm of two cells, has been proposed, even if no direct evidence has been provided so far (Agnati et al., 2010).

How intracellular and extracellular α-syn levels are controlled by the cell

Considering that the different aspects of α-syn (patho) biology mentioned above are strictly related to both α-syn abundance and aggregation propensity, extensive work has been done to dissect the mechanisms deputed to the control of its intracellular levels and to define PTM that could impact on its stability. Phosphorylated, ubiquitinated and nitrated forms of α-syn have been found in LB from patients (Good et al., 1998; Hasegawa et al., 2002; Anderson et al., 2006; Beyer and Ariza, 2013). Other modifications such as sumoylation (Kim et al., 2011; Krumova et al., 2011; Shahpasandzadeh et al., 2014) and N-terminal acetylation (Maltsev et al., 2012; Bartels et al., 2014; Dikiy and Eliezer, 2014) have also been reported. It has been suggested that PTM could drive both the behavior and the intracellular levels of α-syn. The intracellular turnover of α-syn must be tightly controlled, not only because abnormally aggregated/accumulated α-syn is responsible for cell proteostasis stress but also because, as described above, an adequate amount of α-syn is required for to the proper cell functioning. The clearance of α-syn is guaranteed by two main systems: the ubiquitin proteasome system (UPS) and the autophagy-lysosome system (ALS). Impairment of these pathways might trigger alternative elimination routes, eventually leading to α-syn secretion from the cell and thus contributing to enhancing the spreading of pathology (see above). In the following paragraphs the role of PTM and impaired α-syn turnover in PD pathogenesis will be discussed in detail.

α-syn post-translational modifications (PTM)

Phosphorylation

α-syn phosphorylation occurs both at serine and tyrosine residues. The main site of phosphorylation is Ser 129 located in the C-terminal region. Different kinases have been reported to be responsible for its phosphorylation, among them the caseine kinase I and II (Okochi et al., 2000; Ishii et al., 2007; Dzamko et al., 2014), the G-protein coupled receptor kinases (GRK2, GRK3, GRK5, and GRK6) (Pronin et al., 2000), the Polo-like kinases 1–3 (Inglis et al., 2009), LRRK2 (Qing et al., 2009) and recently also the Drosophila serine/threonine kinase LK6 (and its mammalian homologous Mnk2a) which are activated by the ERK signaling (Zhang et al., 2015). Numerous studies suggest that phosphorylation at Ser 129 accelerates inclusions formation, toxicity and neuronal death in different mammalian cell models (Smith et al., 2005b; Sugeno et al., 2008) and in Drosophila (Chen and Feany, 2005). Others have shown that Ser 129 phosphorylation is protective in yeast and rat models because it reduces the concentration of toxic oligomers by favoring the formation of aggregates (Azeredo da Silveira et al., 2009; Mbefo et al., 2010). Thus, the precise relevance of phosphorylation in both physiological and pathological context is still elusive (Sato et al., 2013; Tenreiro et al., 2014). Ser 129 is approximately 90% phosphorylated in α-syn accumulated in LB, but, phosphorylated Ser 129 has been found also in the soluble, non-fibrillar fraction of α-syn in PD brain (Anderson et al., 2006). In normal brain, instead, no more than 4% of α-syn is phosphorylated at this residue (Fujiwara et al., 2002; Sato et al., 2013; Walker et al., 2013). Intriguingly, very recently it has been shown that different PTM could contribute to

generate different α-syn conformational states harboring distinct subcellular localization. Interestingly, the Ser 129 phosphorylated α-syn is targeted to the mitochondria (Nam et al., 2015). Phosphorylation can also occur at Ser 87, Tyr 125, Tyr 133 and Tyr 136 (Oueslati et al., 2010). A study reported that phosphorylation in Ser 97 has a role in keeping α-syn unfolded, reducing its oligomerization and binding to membranes (Paleologou et al., 2010), thus underlining that the role of α-syn phosphorylation in the modulation of aggregation propensity and disease progression or initiation is rather complex.

The scenario is further complicated by the observation that other PTM as well as the presence of mutations appear to counteract or concur to phosphorylation effects. Interestingly, whereas Ser(P)-129 levels were reduced in the A30P case, both A53T and E46K mutants consistently exhibited increased Ser 129 phosphorylation levels compared with wild-type α-syn (Mbefo et al., 2015).

Nitration

Oxidative damage is a contributing factor in PD pathogenesis (Cardoso et al., 2005; Sherer and Greenamyre, 2005) and multiple elements participate to increase oxidative stress. Dopaminergic neurons of the substantia nigra undergo oxidative injury, which can result in the formation of peroxynitrite (due to the reaction of superoxide radical with the nitric oxide) that reacts with the tyrosine residues producing 3-nitrotyrosine. Different forms of 3-nitrotyrosine-modified α-syn have been found in LB: Tyr 39, Tyr 125, Tyr 133, and Tyr 136 are the main targets of this modification (Giasson et al., 2000). Nitrated α-syn shows increased propensity to form dimers and oligomers as a consequence of the crosslinking between two tyrosines (Souza et al., 2000) and reduced fibril formation ability (Yamin et al., 2003). Furthermore, by reducing monomer degradation via the UPS and inhibiting the binding to lipids (Hodara et al., 2004), it promotes mitochondrial impairment and cell death (Yu et al., 2010; Liu et al., 2012).

Ubiquitination

Ubiquitination regulates protein degradation, protein-protein interaction, and subcellular localization (Sadowski and Sarcevic, 2010). Poly- but not mono-ubiquitinated substrates are targeted for proteasome-mediated degradation (Nemani et al., 1996; Petroski and Deshaies, 2005). α-syn ubiquitination can occur at the Lys 6, Lys 10, Lys 12, Lys 21, Lys 23, Lys 32 and Lys 34 residues, mainly in a mono- and di-ubiquitinated manner and, analogously to the phosphorylated forms, ubiquitinated α-syn has been isolated from LB (Hasegawa et al., 2002; Tofaris et al., 2003; Nonaka et al., 2005). The functional significance and molecular mechanisms regulating α-syn ubiquitination are still unclear. Parkin (Chung et al., 2001; Shimura et al., 2001), the mammalian homologues of Drosophila seven in absentia (SIAH-1 and SIAH-2) (Liani et al., 2004; Lee et al., 2008c; Rott et al., 2008), and CHIP (Murata et al., 2001; Kalia et al., 2011) have been indicated among the E3 ubiquitin ligases involved in α-syn ubiquitination. Interestingly, the SIAH-1 and the CHIP-mediated mono- and poly-ubiquitination was shown to exert opposite effects on α-syn self-assembly. The former promoted α-syn aggregation and apoptotic cell death (Lee et al., 2008c), while the latter targeted the oligomeric species and reduced their levels via lysosomal and proteasomal degradation (Shin et al., 2005; Tetzlaff et al., 2008; Kalia et al., 2011).

Sumoylation

This process is similar to ubiquitination and requires the action of specialized enzymes that covalently attach SUMO-1 (small ubiquitin-like modifier) to lysine residues of a wide range of substrate proteins, among which α-syn. SUMO-1 was found to be associated with α-syn immunopositive inclusion bodies in cases of dementia with LB (Pountney et al., 2005) and with lysosomes clustered around α-syn intracellular inclusions in brain of mouse and rat PD models (Weetman et al., 2013; Wong et al., 2013). It has been shown that sumoylation negatively regulates α-syn aggregation by promoting its solubility and thus it has been suggested that this PTM could have a potential neuroprotective role (Krumova and Weishaupt, 2013).

N-terminal acetylation

N-terminal acetylation is a common PTM of mammalian α-syn. NMR spectroscopy and circular dichroism studies have revealed that this modification increases the propensity of α-syn to fold in α-helical structures and reduces fibrils formation (Maltsev et al., 2012; Bartels et al., 2014; Dikiy and Eliezer, 2014), but scarce information are available on its impact at the cellular level.

The interplay between α-syn, the ubiquitin proteasome and the autophagy-lysosome systems

The mechanisms deputed to the control of α-syn intracellular levels are extensively studied: as mentioned above, both proteasomes and lysosomes-mediated pathways participate in α-syn turnover (Liu et al., 2003; Cuervo et al., 2004) and compensate each other if one of the two is defective (Yang et al., 2013); see Figure 3. It has been proposed that proteasomes and lysosomes might be alternatively involved in the degradation of α-syn according to the cell proteostasis stress level: the former engaged under normal conditions to control endogenous α-syn levels (Abeliovich et al., 2000) and the latter under conditions of abnormal α-syn expression (Lee et al., 2004b; Ebrahimi-Fakhari et al., 2011). The scenario is further complicated by the fact that α-syn affects itself both the proteolytic systems involved in its degradation. Evidence supporting a role for the UPS in α-syn degradation comes from studies showing α-syn accumulation upon proteasome inhibition (Bennett et al., 1999; Webb et al., 2003). Detergent-resistant oligomeric species of endogenous α-syn in cultured cortical neurons after proteasomal inhibition (Bedford et al., 2008) and in brain neurons in mice that, upon depletion of an essential subunit of the 26S proteasome, developed neurodegeneration with Lewy-like inclusions (Rideout et al., 2004) have been detected, even if alternative pathways (e.g. the 20S proteasome) for the selective degradation of non-ubiquitinated and Ser129 phosphorylated α-syn species have been proposed (Tofaris et al., 2001; Machiya et al., 2010) and consistent evidence also points to a role for ALS, mainly macroautophagy and CMA, in the regulation of α-syn levels. Indeed, α-syn has been found within lysosomal compartments and rapamycin, i.e. a well-known macroautophagy enhancing agent, promotes its lysosome-mediated degradation (Tofaris et al., 2011). Accordingly, the treatment with macroautophagy and lysosome inhibitors attenuated it (Paxinou et al., 2001; Webb et al., 2003; Spencer et al., 2009). These studies have also shown that ALS pathway accounts for the degradation of oligomeric intermediates, but not of mature fibrillar inclusion bodies (Lee et al., 2004a); and later, it has been shown that it may also control the regular turnover of α-syn and not exclusively the removal of oligomeric species (Vogiatzi et al., 2008; Mak et al., 2010).

Interestingly, the work by Cuervo and colleagues has identified a selective route for lysosomes-mediated degradation of wild-type versus mutant α-syn. The discrimination between the two is mediated by the pentapeptide VKKDQ sequence in α-syn, which is consistent with a CMA recognition motif. Differently than in the case of wt α-syn, the binding of the A30P and A53T mutants to the CMA receptor at the lysosomal membrane inhibited their CMA-mediated degradation as well as that of other substrates (Cuervo et al., 2004), thus resulting in the toxic gain of function.

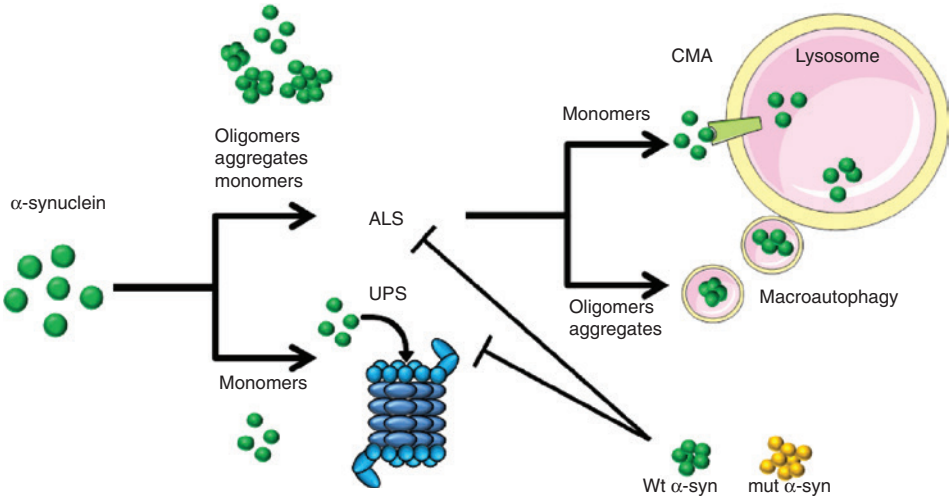

Figure 3: Schematic cartoon illustrating the interplay between α-syn, the ubiquitin proteasome (UPS) and the autophagy-lysosome systems (ALS).

Intracellular α-syn levels are controlled by the balanced activity of the proteasoma and autophagy pathways. In turn, it has also been shown that the presence of oligomeric/aggregates forms of α-syn, as well as of mutations and PTM, can modify the efficiency of the mechanisms controlling the cellular proteostasis equilibrium (see the text). The cartoon shows a simplified view of α-syn action in UPS and ALS.

As mentioned above, in addition to be the routes for α-syn turnover, the UPS and the ALS are also two important targets of its action. Indeed, the oligomeric and aggregated forms of α-syn have been reported to exert an inhibitory effect on the UPS. In particular, α-syn A30P and A53T mutants inhibit the activity of both the 20S and the 26S proteasome (Tanaka et al., 2001; Snyder et al., 2003; Lindersson et al., 2004; Chen et al., 2005) and soluble α-syn oligomers, by binding to 26S proteasome, impede other proteosomal substrates to be degraded and thus cause a general impairment of the machinery for protein turnover and quality control, extending proteostasis stress (Emmanouilidou et al., 2010b). Macroautophagy and CMA dysfunction has been linked to α-syn accumulation both in cellular and animal models (Cuervo et al., 2010). Overexpression of either wild-type or mutant α-syn led to consistent dysfunction of the CMA (Xilouri et al., 2009): defects in lysosomal acidification and the accumulation of autophagic vacuoles were observed in several cell models (Cuervo et al., 2004; Spencer et al., 2009; Yu et al., 2009; Crews et al., 2010). It is interesting to note that PTM, such as phosphorylation, prevented α-syn CMA-dependent degradation (Martinez-Vicente et al., 2008), suggesting that targeting PTM may represent a potential therapeutic approach. However, the situation is rather complicated as a compensatory up-regulation of macroautophagy has been shown to occur upon α-syn induced CMA dysfunction, thus suggesting the existence of consistent crosstalk between these two routes (Massey et al., 2006; Choubey et al., 2011). Interestingly, more recently, it has been proposed that ALS pathway may also control the amount of extracellular α-syn species. Indeed, ALS inhibition not only limits intracellular degradation of misfolded proteins but increases the secretion of small α-syn oligomers that exacerbate inflammatory response and cellular damage (Poehler et al., 2014). These findings have important relevance for the understanding of PD pathogenesis as they argue in favor of the possibility that extracellular α-syn has a major toxic role and support the hypothesis for a protective role of intracellular α-syn aggregation.

Conclusions

Numerous aspects of α-syn biological role require further investigation in order to fully understand its implication in PD pathogenesis. If on the one hand, α-syn seems to be dispensable, i.e. α-syn null mice are viable and show no evident phenotype, on the other its participation in mediating toxic effects is suggested by the observation that the same mice display striking resistance to the neurotoxin MPTP-induced degeneration of dopaminergic neurons and dopamine release (Dauer et al., 2002; Klivenyi et al., 2006). A consistent number of reports has indicated a protective role for overexpressed α-syn, which appeared to counteract various environmental toxic agents or cellular stresses (da Costa et al., 2000; Hashimoto et al., 2002; Colapinto et al., 2006; Kim et al., 2012), but duplication and triplication of its gene are unequivocally linked to the development of PD. The original idea that α-syn could account for synucleopathies through toxic gain of function has been recently challenged by an increasing number of findings that underline possible loss of function mechanisms and attribute to α-syn an important signaling role. For these reasons the scientific community has recently made a lot of effort to dissect the contribution of α-syn in multiple pathways involved in signal transduction, with the hope that the modulation of these pathways could represent a way to counteract the disease.

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