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# Protein Transmission, Seeding and Degradation: Key Steps for $\alpha$ -Synuclein Prion-Like Propagation

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Converging lines of evidence suggest that cell-to-cell transmission and the self-propagation of pathogenic amyloidogenic proteins play a central role in the initiation and the progression of several neurodegenerative disorders. This “prion-like” hypothesis has been recently reported for  $\alpha$ -synuclein, a presynaptic protein implicated in the pathogenesis of Parkinson's disease (PD) and related disorders. This review summarizes recent findings on  $\alpha$ -synuclein prion-like propagation, focusing on its transmission, seeding and degradation and discusses some key questions that remain to be explored. Understanding how  $\alpha$ -synuclein exits cells and propagates from one brain region to another will lead to the development of new therapeutic strategies for the treatment of PD, aiming at slowing or stopping the disease progression.

**Key words:** secretion, seeding, turnover, extracellular proteins, disease propagation

## INTRODUCTION

Abnormal accumulation of aggregated proteins is a common neuropathological feature of several neurodegenerative disorders, including Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD) and many other disorders [1]. Over the past few years, an increasing body of evidence suggests that the amyloidogenic proteins implicated in these neurological diseases can propagate from one brain region to another in a “prion-like” manner and contribute to the disease progression [2, 3].

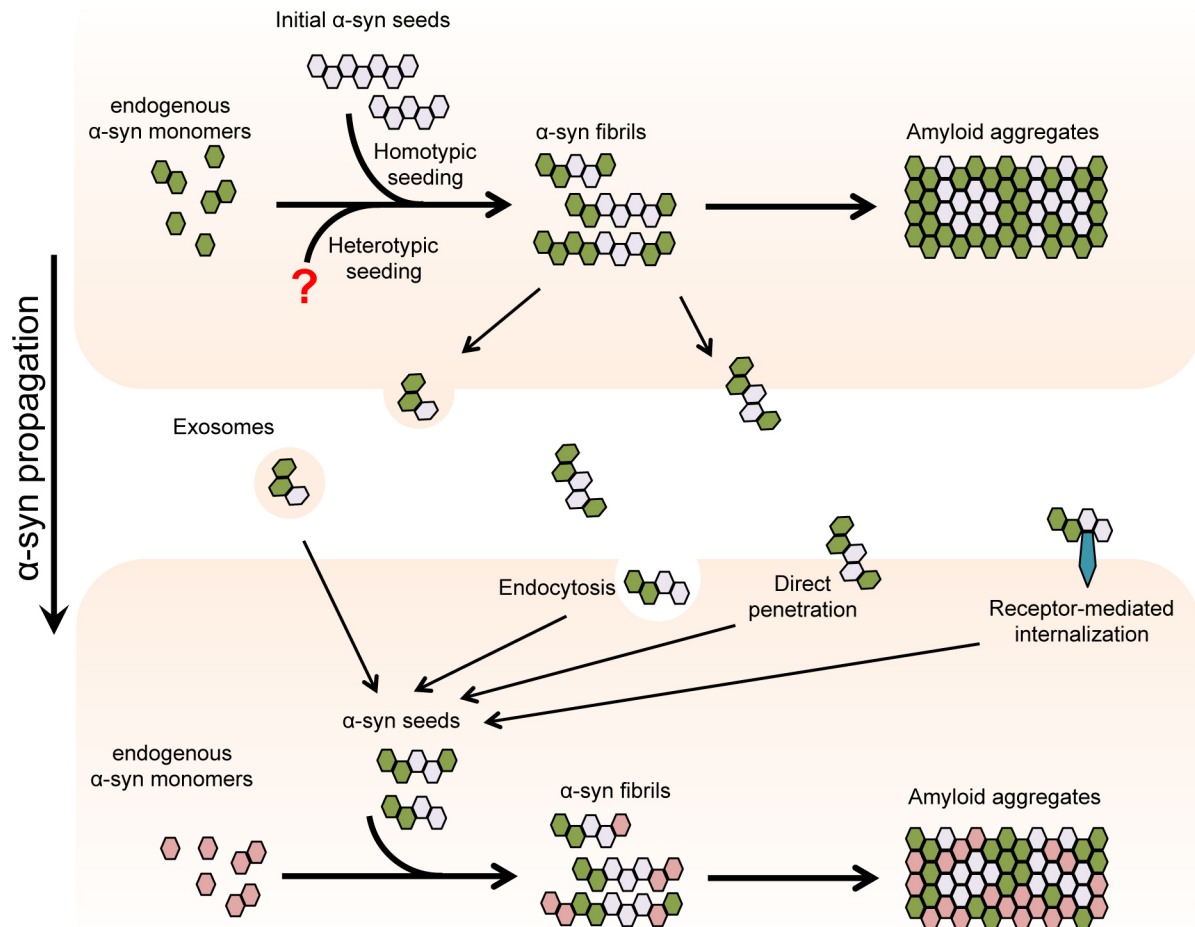
This concept of “prion-like propagation” was recently described for the presynaptic protein,  $\alpha$ -synuclein ( $\alpha$ -syn), which is the

major component of Lewy bodies (LB) and a key player in the pathophysiology of PD and other diseases, collectively termed synucleinopathies [4, 5]. This prion-like paradigm comprises a cascade of cellular events including: the secretion, the uptake and the protein seeding by the recipient cells. This process results on self-perpetuating states and the formation of pathogenic protein strains capable of recruiting endogenous soluble proteins (Fig. 1). Finally, the formed pathogenic species induce axonal degeneration and neuronal death.

Understanding how and where  $\alpha$ -syn propagation is initiated and the characterization of the major factors playing a role in the modulation of intracerebral  $\alpha$ -syn spreading will lead to the identification of new therapeutic targets aiming at slowing or stopping the disease progression. In this review, we explore the recent findings from cell-based assays and animal models of  $\alpha$ -syn prion-like propagation and discuss how and where the pathological  $\alpha$ -syn species could be initiated and the key mechanisms related to its intercellular transmission.

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**Fig. 1.** Schematic representation of the different steps of  $\alpha$ -syn seeding and propagation: Initial  $\alpha$ -syn seeds recruit endogenous soluble monomers, in a homotypic seeding manner, and enhance the formation of amyloid fibrils and aggregates. Up to date, no heterotypic seeding in vivo, catalyzed by other proteins, has been described for the aggregation of  $\alpha$ -syn. After the seeding, the newly formed amyloidogenic fibrils are transmitted to the recipient cells via different routes, including exosomal transport, endocytosis, direct penetration and receptor-mediated internalization. Once in the recipient cells, the internalized newly formed seeds enhance the aggregation of the endogenous soluble protein and the process of seeding and transmission continue in an uninterrupted manner. A potential strategy to slow/stop  $\alpha$ -syn prion-like propagation is to facilitate  $\alpha$ -syn clearance, at the intracellular and/or extracellular milieu, by the identification and modulation of the activity of the key enzymes implicated in  $\alpha$ -syn turnover.

## $\alpha$ -SYNUCLEIN TRANSMISSION

### *Extracellular $\alpha$ -synuclein*

A crucial tilting point in the PD field was the discovery of  $\alpha$ -syn secretion.  $\alpha$ -syn was thought at first to be an exclusively intracellular protein [6], as it lacks a canonical endoplasmic reticulum signal peptide that would direct it to the secretory pathway. However, this notion was challenged when  $\alpha$ -syn was detected in biological fluids, such as blood plasma and Cerebrospinal fluid [7, 8]. Gradually, it became evident that  $\alpha$ -syn secretion is a physiologic process in the protein's life cycle and that a proportion of  $\alpha$ -syn is located in the extracellular milieu [8-12]. Although, the mechanism of  $\alpha$ -syn release has not been fully elucidated, data point towards non-classic secretory pathways [13]

that involve vesicle trafficking through exocytotic vesicles [12] and exosomes (Fig. 1) [9].

The discovery of  $\alpha$ -syn secretion prompted the examination of the paracrine role of extracellular  $\alpha$ -syn in brain homeostasis, which under pathological conditions could contribute to the cascade of events leading to neuronal degeneration [14, 15]. It is therefore possible that certain abnormalities or deficits affecting  $\alpha$ -syn proteostasis [16] could lead to increased extracellular  $\alpha$ -syn accumulation and aggregation, and subsequently to its concomitant detrimental effects. In support of this, a growing number of reports have demonstrated that various species of  $\alpha$ -syn when applied extracellularly are potent to induce multiple neurotoxic [9, 11, 17] and inflammatory responses [18-24], as well as, to cause deleterious seeding effects [25].

The hypothesis of  $\alpha$ -syn prion-like propagation and its potential implication in the progression of PD was first proposed by two independent studies showing that intra-striatal transplants of fetal ventral mesencephalic progenitors in PD patients developed  $\alpha$ -syn-positive LB-like inclusions, a decade after transplantation, suggesting a direct transfer of pathogenic  $\alpha$ -syn from host-to-grafted tissue [26, 27]. Subsequent *in vivo* studies have also provided evidence for the cell-to-graft transmissibility of pathogenic  $\alpha$ -syn in  $\alpha$ -syn-transgenic mice [28, 29]. More recently, a growing body of studies reported that the intracerebral inoculation of brain homogenate prepared from PD-diseased brains [30] or symptomatic  $\alpha$ -syn-transgenic mice [31], as well as the *in vitro*  $\alpha$ -syn preformed fibrils (Pffs) [29, 32-37] are sufficient to cause all the major pathological changes observed in PD, including aggregate deposition, neurodegeneration and neuroinflammation.

Although the precise mechanism(s) of  $\alpha$ -syn exocytosis, the nature of extracellular  $\alpha$ -syn in terms of its aggregation state, the physiologic role(s) of extracellular  $\alpha$ -syn, as well as, its potential association with other molecules (e.g. chaperones, lipids) remain unresolved issues, these biological observations opened up new avenues for investigations to uncover the pathophysiological significance of extracellular  $\alpha$ -syn in synucleinopathies.

#### **Routes and mechanisms of $\alpha$ -synuclein transmission:**

The exact molecular mechanisms by which the newly synthesized  $\alpha$ -syn amyloidogenic species are released from “diseased” (donor) cells and transmitted to “healthy” bystander (recipient) cells are still elusive. Over the last years, various species-specific and cell-type dependent mechanisms have been suggested and hypothesized [38].  $\alpha$ -syn can be actively secreted via non-classic exocytotic vesicles [12, 39] and exosomes [9], but could also be directly released from injured/dying neurons. Once in the extracellular space,  $\alpha$ -syn could either gain access to neighboring cells via active processes involving conventional endocytosis [17, 28, 40], exosomal transport [9, 41], or receptor-mediated internalization [17]. Alternatively, extracellular  $\alpha$ -syn could be transmitted to neighboring cells via direct penetration of the plasma membrane (Fig. 1) [40, 42, 43].

Among these proposed mechanisms for  $\alpha$ -syn cell-to-cell spreading, the transmission of pathogenic  $\alpha$ -syn through exosomes necessitates further exploration. Exosomes enable the transfer of membrane and cytosolic components from donor cells to the extracellular matrix and recipient cells by various mechanisms, such as endocytosis, receptor-ligand binding, or fusion with the plasma membrane [44]. As such, these small vesicles could serve as a system to facilitate cell-to-cell communication *in vivo*, rather

than merely providing a way of disposing of unwanted proteins. It is therefore possible, that under pathological conditions, exosomes that contain aberrant forms of  $\alpha$ -syn might also enable its accumulation and spreading, similarly to what has been proposed for other aggregation-prone proteins, like prion protein (PrP<sup>Sc</sup>), the amyloid precursor protein (APP), and phosphorylated tau [45]. Although, the total amount of exosome-associated  $\alpha$ -syn is small, the fact that it was found in both lumen and surface of these vesicles [9] argues in favor of a Trojan-horse hypothesis and future studies need to prove or disprove this theory.

Another question that still remains to be answered is whether the PD-linked  $\alpha$ -syn mutations affect the secretion of  $\alpha$ -syn. Future studies need to explore the possibilities that these mutants might have higher affinities for exosome-association, thus altering the total amount of  $\alpha$ -syn that is packed in these vesicles, or whether they affect the total number or composition of exosomes *per se*. Given that oxidative stress and inhibition of the major degradation mechanisms (proteasome and lysosomal pathways) have been shown to increase both vesicular translocation and release of  $\alpha$ -syn [39, 46], and considering also that the PD-associated proteins, LRRK2 and GBA, have been shown to affect  $\alpha$ -syn secretion [47] and transmission [48], future studies also need to investigate whether other PD-associated proteins or their mutant variants could affect  $\alpha$ -syn seeding and propagation and which are the underlying mechanisms.

Collectively, all these novel findings support the idea for common mechanisms of neurodegeneration in which the critical events are protein misfolding, aggregate deposition and propagation [2, 3]. As such, development of novel therapies that would prevent and/or reverse the protein's conformational changes and that would halt the onset and progression of several neurodegenerative diseases require a precise understanding not only of why proteins misfold and accumulate locally, but also of how this pathology spreads through-out the brain [2, 3]

#### **$\alpha$ -SYNUCLEIN SEEDING AND DISEASE PROPAGATION**

##### ***Seeding and nucleation***

The initiation step of protein propagation consists of the formation of pathogenic species, which are able of self-amplification by the recruitment of soluble proteins. The resulting malicious strains are then transferred from one cell to another via different routes [4] and thus the process of self-amplification continues in an uninterrupted manner (Fig. 1).

This process, termed seeding, is a nucleation-dependent mechanism in which the seed provides a template for the assembly of other soluble monomers and leads to the formation of highly

ordered protein aggregates defined by their insolubility and  $\beta$ -sheet structure. The seeding mechanism, that was first described for prion protein (PrP) and later on for  $\beta$ -amyloid (A $\beta$ ), provided an explanation on the conversion of soluble proteins to amyloid fibrils *in vitro* [49, 50]. A similar process was recently reported for  $\alpha$ -syn, where the addition of  $\alpha$ -syn Pffs acts as a seed and accelerates the aggregation of soluble monomers. This seeding effect was characterized by reduction of the lag phase and the rapid formation of  $\alpha$ -syn-rich fibrils [51, 52].

In mammalian cell lines, the addition of a small amount of exogenous Pffs has been shown to seed the formation of intracellular  $\alpha$ -syn aggregates that recapitulate the main features of LBs [53, 54]. Using tagged Pffs, Luk et al., showed that the exogenous fibrils form the core of the inclusions, which are surrounded by non-tagged endogenous proteins, thus demonstrating that the Pffs play the role of the template and recruit soluble monomers (Fig. 1) [53]. A similar phenomenon was also observed in primary neuronal cultures [33, 55, 56]. For instance, the addition of human or mouse Pffs in primary cultures of hippocampal neurons induced the formation of LB-like structures [33, 55]. Moreover, using microfluidics systems, Volpicelli-Daley et al., and Tran et al., demonstrated that the formed  $\alpha$ -syn inclusions are able to propagate from one neuron to another and to induce axonal degeneration and neuronal toxicity [33, 55].

Thereafter, several independent groups, using various well-characterized *in vivo* models, also described the seeding effects and the propagation capacity of  $\alpha$ -syn in mouse brain [34, 57, 58]. The reported results revealed that a unique and a single inoculation of a small amount of  $\alpha$ -syn Pffs is sufficient to initiate the conversion of normal endogenous protein to pathogenic aggregated forms in the brain of adult [34, 58] and newborn mice [59]. The formed pathogenic species recapitulate the main features of pathogenic  $\alpha$ -syn present in LB (hyper-phosphorylation at Ser129, ubiquitination and  $\beta$ -sheet structure) and are able to propagate from the injection site to other interconnected brain regions [34, 57-59].

It is worth noting that the inoculation of tissue homogenate from brains of PD patient [30] or symptomatic  $\alpha$ -syn-transgenic mice [31] are also able to seed the aggregation of the endogenous protein and to initiate the formation of  $\alpha$ -syn prion-like strains. Importantly, a recent study by Schweighauser et al., reported that the injection of  $\alpha$ -syn-transgenic brain extracts, either fresh or fixed with formaldehyde, are able to induce  $\alpha$ -syn pathology in mice brains. This observation demonstrates that homogenate of  $\alpha$ -syn extracted from transgenic animals share a common and important prion propriety, which is the resistance to inactivation

by formaldehyde [60].

Together, these observations demonstrate that seeding is an important step in  $\alpha$ -syn propagation and represents a rate-limiting factor for the initiation of this process. However, the question of whether  $\alpha$ -syn Pffs alone are capable of self-seeding or whether other proteins/factors are also required to augment this process is still under debate.

#### **Homotypic versus heterotypic seeding**

Although the central role of the seeding process on the initiation of  $\alpha$ -syn cell-to-cell propagation has been demonstrated, the identity of the seed(s) that initiates the formation of pathogenic  $\alpha$ -syn species in PD-diseased brains remains elusive.

Up to date, the available experimental data demonstrate that the exogenous  $\alpha$ -syn amyloid-like fibrils are able to induce the aggregation of counterpart cytosolic soluble proteins in cell culture [33, 53] and *in vivo* [35, 57-59]. This process that is also referred to as self-seeding or homotypic seeding, suggests that a sequence-specific templating and a typical cross  $\beta$ -sheet conformation are required for the seeding process (Fig. 1) [36, 54, 61]. This hypothesis was confirmed in cell lines stably overexpressing  $\alpha$ -syn that lacked the NAC region ( $\Delta$ 71-82), the hydrophobic sequence essential for  $\alpha$ -syn aggregation [62]. In these cells, the addition of  $\alpha$ -syn Pffs failed to induce the formation of intracellular inclusions, conforming the requirement of the sequence-specific templating and the importance of the  $\beta$ -sheet conformation for seeding [53]. The importance of the sequence-specific templating for the initiation of protein aggregation was also reported for tau [54], another amyloidogenic protein, thus suggesting common mechanisms in the conversion of soluble amyloidogenic proteins to pathogenic strains. Interestingly, in a recent study, Sacino et al., showed that the intra-hippocampal injection of the non-aggregating form of  $\alpha$ -syn ( $\Delta$ 71-82) was also able to induce the formation of a mature  $\alpha$ -syn pathology that spread from the injection site to other brain regions [57]. The fact that this soluble form of  $\alpha$ -syn is unable to induce the seeding *in vitro* and *in vivo* suggests that other factors could be implicated in the initiation of endogenous seeding, for example the induction of the endogenous  $\alpha$ -syn expression and accumulation.

Together these observations suggest that the sequence-specific templating is important for  $\alpha$ -syn seeding *in vivo*, and probably other factors might also be implicated in the initiation of the seeding process in the central nervous system.

Moreover, the fact that other amyloidogenic proteins, such as tau, Huntingtin (Htt) and A $\beta$ , are able to promote  $\alpha$ -syn aggregation suggests that these proteins could also initiate the heterotypic seeding of pathogenic  $\alpha$ -syn. Indeed, tau and  $\alpha$ -syn synergistically

promote the fibrillization of each other *in vitro* [63] and in cell culture systems [64]. Moreover, both proteins co-localize in brains of PD patients and in transgenic animal models [36, 63], thus suggesting that tau could promote the formation of pathogenic  $\alpha$ -syn species. Htt, a protein that is implicated in Huntington's disease (HD), is also capable of seeding  $\alpha$ -syn aggregation. Several studies showed that Htt and  $\alpha$ -syn co-aggregate *in vitro* [65] and in cell-based assays [66] and co-localize in brain tissues from transgenic mouse models of HD [67] and from HD patients [68]. Finally, addition of A $\beta$  peptides promotes  $\alpha$ -syn aggregation *in vitro*, enhances its accumulation *in vivo*, and induces neuronal dysfunction in transgenic animal models [69] and in brains of LB disease patients [70]. Collectively, these observations clearly suggest that the aggregation and propagation of  $\alpha$ -syn *in vivo* could also be initiated by heterotypic seeding, involving other amyloidogenic proteins. Nevertheless, the heterotypic seeding hypothesis has been challenged by a recent work by Sander et al. [54]. They used stable cell lines overexpressing either tau, Htt, A $\beta$  or  $\alpha$ -syn and showed that the addition of exogenous amyloid-like fibrils, generated from different amyloidogenic proteins, exclusively induced homotypic seeding rather than cross-seeding [54]. Together, these data suggest that several candidate proteins, including the amyloidogenic proteins, are potent to enhance  $\alpha$ -syn aggregation both *in vitro* and *in vivo*. However, their relative involvement on the seeding of "prion-like"  $\alpha$ -syn species remains to be explored.

#### **Where does the seeding process start?**

The idea of a possible propagation of pathogenic  $\alpha$ -syn between the brain regions during the progression of PD was first suggested in the seminal work by Braak et al. [71]. In this study, the authors performed several longitudinal analyses to evaluate the neuroanatomical changes in the brain of PD patients and proposed a model in which the disease stages are correlated with the regional distribution of Lewy neurites (LN) and LB in the central nervous system. According to the Braak's model, LB and LN formation starts early in the disease (even before the motor symptoms emerge) and originate in the olfactory bulb and in the brainstem, specifically at the dorsal motor nucleus of the vagus nerve [72]. In parallel to disease progression, LB and LN are detected in other brain regions and appear to propagate through brain structures, in a stereotypic pattern, to reach the other regions including the midbrain and, at later stages, the cerebral cortex [38, 72]. Moreover, subsequent neuroanatomical investigations reported the presence of  $\alpha$ -syn-positive inclusions in other organs, including the gastro-intestinal track, notably the gastric myenteric plexus [73] and colon [74], at the early stages of the

disease, thus suggesting that the formation of pathogenic  $\alpha$ -syn species could start outside the central nervous system. Together, these neuropathological observations suggest that the process of  $\alpha$ -syn prion-like spreading could start in the brain, as well as in the peripheral nervous system.

Although the Braak's model remains under debate [75, 76], recent *in vivo* studies sought to investigate  $\alpha$ -syn spreading through the routes proposed in Braak's scheme, namely the vagus nerve and the olfactory bulb [38, 72]. Using an adeno-associated viral delivery system, Ulusoy et al., showed that peripheral  $\alpha$ -syn overexpression in the vagus nerve, resulted in a stereotypical propagation of the exogenous protein to central nervous system (the pons, midbrain and forebrain) [77], thus suggesting that the pathogenic  $\alpha$ -syn species can travel long distances and can propagate from the peripheral to the central nervous system. In the olfactory bulb, Rey et al. showed that inoculation of  $\alpha$ -syn monomers and oligomers resulted in a rapid uptake by the neurons at different layers, including the glomerular layer, mitral cell layer and in granule cell layer. Interestingly, few hours post-injection, the authors detected the exogenous  $\alpha$ -syn far from the injection site, within brain regions interconnected with the olfactory bulb [78]. Together, these results provide *in vivo* evidence supporting Braak's hypothesis and demonstrate that  $\alpha$ -syn propagation could be initiated in the central and the peripheral nervous systems.

In the recently developed animal models of  $\alpha$ -syn prion-like propagation, based on the inoculation of exogenous  $\alpha$ -syn Pffs, several intracerebral injections sites have been tested. In 2012, Luk et al., showed that the intra-cerebral delivery of Pffs induced the formation of intra-neuronal inclusions in naïve mouse brains. In this study, the authors inoculated  $\alpha$ -syn Pffs in different brain regions, including the striatum, the cortex and the hippocampus and in all cases the delivery of the exogenous fibrils induced the formation of pathogenic inclusions and promoted their spreading from the injected site to different interconnected brain structures [34]. Other groups reproduced these observations in wild type and in M20 transgenic animal model of PD and confirmed that the intracerebral inoculation of Pffs in the hippocampus [59, 63] as well as in the midbrain (substantia nigra) [58] induced  $\alpha$ -syn inclusion formation and protein propagation. Together, these observations demonstrate that any region in the brain represents a potential area where  $\alpha$ -syn propagation could start.

Interestingly, a recent study by Sacino et al., reported that hind limb intramuscular injection of  $\alpha$ -syn Pffs was sufficient to induce pathology in brains of transgenic mouse models of PD [37]. Two to four months post-injection, the authors detected pathogenic  $\alpha$ -syn aggregates in the brains as well as in the spinal cord. Transection of the sciatic nerve innervating the injected muscle

significantly delayed the appearance of the pathology in the brain, thus suggesting the implication of long-distance retrograde transport from the periphery to the central nervous system.

Taken together, neuropathological and experimental data demonstrate that the initiation of  $\alpha$ -syn seeding and spreading could start at any structure in both peripheral and central nervous system. However, the possibility that the transmission of  $\alpha$ -syn pathology described in the above studies might be attributed to the simple diffusion of the initial injected  $\alpha$ -syn strains cannot be excluded. Therefore, future studies need to determine the relative contribution of local versus trans-synaptic transmission of pathogenic  $\alpha$ -syn and which mechanisms are responsible for their long-distance axonal transportation.

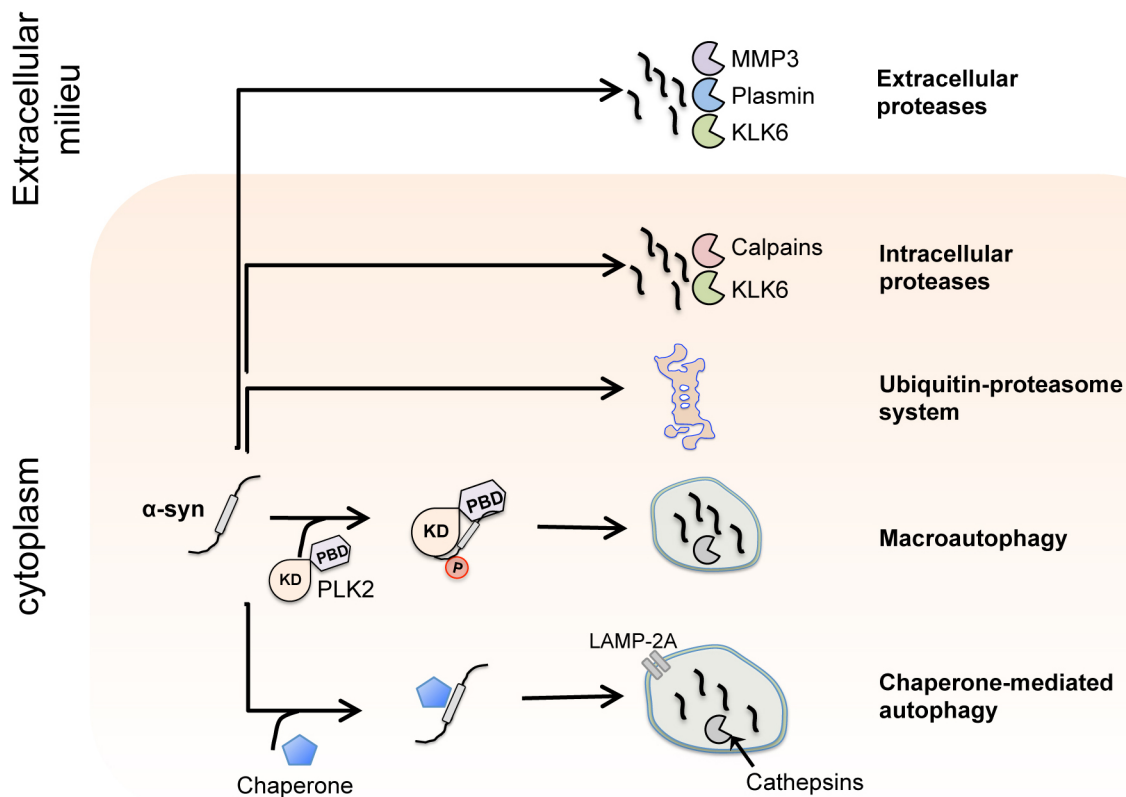
### $\alpha$ -SYNUCLEIN DEGRADATION AND POTENTIAL THERAPEUTIC TARGETS

Although the extent to which  $\alpha$ -syn is involved in synucleinopathies is not yet clear, it is of high importance to elucidate the underlying mechanisms that regulate its protein levels. Considering that the levels of  $\alpha$ -syn depend on a dynamic balance between the rate of

protein synthesis and the rate of clearance mechanisms [4, 79, 80], an imbalance between these mechanisms caused by dysfunction of one or more of these pathways can result in abnormal levels of  $\alpha$ -syn that might favor the formation and/or accumulation of oligomeric and fibrillar species that, as mentioned above, promote the pathology [4, 81].

Consequently, strategies aspiring to reduce the total protein burden of  $\alpha$ -syn could be promising and could be proven therapeutic and beneficial to patients suffering from PD or other synucleinopathies [4, 81, 82]. One of these potential strategies would be the triggering of proteins involved in the clearance of  $\alpha$ -syn [79]. Therefore, elucidation of the exact mechanisms that regulate the protein levels of  $\alpha$ -syn is of high importance.

However, the simple question of how  $\alpha$ -syn is degraded has caused much controversy and a body of conflicting results. Although the exact mechanisms are still unclear, overall, it has been demonstrated that intracellular  $\alpha$ -syn monomers and aggregates are predominantly degraded by the ubiquitin-proteasome system (UPS) [83-86] and the autophagy-lysosome pathway [85, 87-89], including chaperone-mediated autophagy (CMA) [90, 91]. Lysosomal cathepsins [92-94] and cytoplasmic calpains [95-98] are



**Fig. 2.** Summary of the different intra and extracellular  $\alpha$ -syn degradation pathways. Intracellular  $\alpha$ -syn is predominantly degraded by the ubiquitin-proteasome system and the autophagy-lysosome pathway, including macroautophagy and chaperone-mediated autophagy. Intracellularly, lysosomal cathepsins, and cytoplasmic calpains are considered to be major  $\alpha$ -syn degrading enzymes, while KLK6 has also been implicated in the proteolysis of intracellular  $\alpha$ -syn. At the extracellular level, MMP3, KLK6 and plasmin have been reported to degrade extracellular  $\alpha$ -syn.

considered to be major  $\alpha$ -syn degrading enzymes, while kallikrein-related peptidase 6 (KLK6) has also been shown to play a pivotal role in proteolysis of intracellular  $\alpha$ -syn (Fig. 2) [99, 100].

Over the recent years, an increasing body of evidence suggests that extracellular  $\alpha$ -syn is quickly removed from the brain parenchyma by efficient cleaning and disposal mechanisms. Such clearance mechanisms include cell-mediated uptake and degradation, as well as proteolysis by extracellular proteases [79, 81]. In the context of cell-mediated uptake (endocytosis and phagocytosis), it has been suggested that adjacent cells, including neurons [40] and astrocytes [21], as well as, the innate (microglia) [101, 102] and adaptive immune system (infiltrating lymphocytes and autoantibodies) [103-105] are crucially involved. While, in the context of extracellular degradation, studies in cell-free systems and in cultured cells have implicated that certain matrix metalloproteinases (MMPs) - particularly MMP3 [10, 106], KLK6 [107-109] and plasmin [110] could degrade extracellular  $\alpha$ -syn (Fig. 2).

Despite more than a decade of investigation, it is evident that the exact mechanisms and key players responsible for the clearance of  $\alpha$ -syn are less studied. Accordingly, several questions need further investigation in the coming future. For example, only a small number of  $\alpha$ -syn-degrading enzymes have been identified and our understanding of those characterized so far is incomplete. It is worth mentioning that there are still no studies available to ascertain whether the aforementioned  $\alpha$ -syn-degrading enzymes are indeed able to catabolize extracellular  $\alpha$ -syn *in vivo*.

Little is known also about the ability of  $\alpha$ -syn-degrading enzymes to cleave/degrade both monomeric and aggregated (oligomeric and fibrillar)  $\alpha$ -syn forms that are increasingly assuming pathogenic significance promoting the protein spreading. For instance, studies in AD field showed the existence of conformation specific enzymes, as insulin-degrading enzyme (IDE), one of the major degrading enzymes of A $\beta$ , selectively degrades the monomeric but not the oligomeric or fibrillar forms [111], thus suggesting that  $\alpha$ -syn-degrading enzymes might also have differences in their substrate specificities. This is further supported by recent studies, which demonstrated the existence of diverse fibrillar  $\alpha$ -syn strains with substantial differences in their proteolysis by proteinase K [36, 52]. Another critical query here is whether the produced proteolytic fragments are innocuous or even more toxic promoting further aggregation. For example, the proteolytic processing of  $\alpha$ -syn by KLK6 [99] or plasmin [110] generates fragments that inhibit  $\alpha$ -syn aggregation. On the other hand, the processing of  $\alpha$ -syn by MMP3 [10, 112] or calpain I [95, 97] leads to fragments that promote  $\alpha$ -syn aggregation, suggesting that these proteases may participate in the disease-linked  $\alpha$ -syn

aggregation in synucleinopathies and that cannot be promising therapeutic targets.

This is an area that merits more detailed exploration and in coming years the complete set of  $\alpha$ -syn-degrading enzymes remains to be established and critically assessed. Although, up-regulation of these enzymes in well-characterized mouse models of PD could prove effective in the clearance of  $\alpha$ -syn aberrant forms, complete elimination of extracellular  $\alpha$ -syn is not desirable as it probably has a hitherto unidentified physiological role in brain parenchyma. In addition, another potential complication would be that the excessive protein degradation could be proven detrimental to neuronal integrity, since enzymes hydrolyze not only the targeted protein but also a range of other physiological substrates.

Another attractive direction of future research is to study human samples for differences in enzymatic activities between healthy and diseased individuals. However, a major obstacle in deciphering roles played by extracellular enzymes in the brain function and dysfunction is the apparent cellular and molecular complexity of the system. Indicatively, recent studies have revealed that certain proteases are also associated with exosomes, thus suggesting that these proteases may also contribute to extracellular proteolysis of their target substrates [113]. It is also known that many enzymes are often partnered with their specific inhibitors and act in very intricate cascades of enzymatic activities. In support of this, the degrading enzymes of extracellular  $\alpha$ -syn, MMPs, KLK6 and plasmin, have been shown to cross talk each other in various systems [114-116]. Taking this into consideration, future studies should investigate the plausible assumption that  $\alpha$ -syn-degrading enzymes work synergistically in complex proteolytic cascades [117, 118], along with other eliminative processes, in order to regulate the catabolism of extracellular  $\alpha$ -syn and perhaps this of other amyloidogenic proteins, such as A $\beta$ .

## FUTURE DIRECTIONS

Studies of protein prion-like propagation have important implications in the understanding of the etiology and the progression of PD and other neurodegenerative disorders and the development of disease-modifying treatments aiming at stopping or slowing the disease progression. However, some important questions remain unexplored.

Over the recent years a broad spectrum of data have been accumulated pointing to an involvement of extracellular  $\alpha$ -syn in PD and other synucleinopathies. The experimental models based on the inoculation of exogenous  $\alpha$ -syn Pffs provide an important tool to explore the cellular and the molecular mechanisms of

$\alpha$ -syn prion-like spreading. However, "Parkinson's disease is not a disorder in which somebody injects synuclein into your brain," (Prof. Ted Dawson, <http://www.nature.com/news/misfolded-protein-transmits-parkinson-s-from-cell-to-cell-1.11838>) and an important question remains to be elucidated concerning the origin and the identity of the endogenous seed(s).

Moreover, the majority of the existent evidence stems from experiments where primarily purified or synthetic forms of the proteins were used. Evidence from our laboratory for a differential susceptibility between recombinant and cell-secreted  $\alpha$ -syn forms to KLK6-proteolysis raises new questions regarding their structural and functional properties [109]. The interaction of  $\alpha$ -syn with lipids and other factors seems to alter its behavior and provide a biological context to understand that such modifications may force the protein to adopt multiple conformations and exert different functions. In support of this, it has been already demonstrated that cell-secreted  $\alpha$ -syn forms are strong agonists of Toll-like receptor 2 (TLR2), which activates inflammatory responses in microglia, while the recombinant  $\alpha$ -syn forms exhibited much less potency [102]. Given that the *in vivo* environment differs considerably from the *in vitro* situation, one should be more skeptical when interpreting results derived from studies that used only recombinant or synthetic  $\alpha$ -syn forms, as some of the findings may not be physiological. In this respect, use of purified naturally secreted  $\alpha$ -syn forms in similar studies would be beneficial to the field.

In view of the importance of  $\alpha$ -syn-degrading enzymes in  $\alpha$ -syn-clearing mechanisms and in PD progression and given that  $\alpha$ -syn may accumulate because of dysfunctional degradation, several genetic association studies should also be attempted to find potential links between defective proteases (e.g. polymorphisms or mutations) and PD pathology.

The future challenges for synucleinopathies are 1) to identify the origin of the pathogenic entities that initiate  $\alpha$ -syn prion-like propagation; 2) to decipher the underlying propagation mechanisms, 3) to elucidate how and where this pathogenic process starts, and 4) to develop novel targeted strategies that facilitate the clearance of unwanted proteins but without disturbing the balance in protein homeostasis. Advanced understanding of the implications of protein degradation pathways will lead to diverse strategies for up-regulation of specific  $\alpha$ -syn-degrading enzymes, either by means of gene therapy or by pharmacological modulation using small compounds that would modify the activity of the endogenous enzymes (or their inhibitors) at the transcriptional or protein level.

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