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REVIEW

Release and uptake of pathologic alpha-synuclein

Veselin Grozdanov[1] · Karin M. Danzer[1]

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Abstract
Parkinson's disease (PD) is a chronic progressive neurodegenerative disease, which is characterized by severe loss of dopaminergic neurons and formation of Lewy bodies, which are rich in aggregated alpha-synuclein (α-syn). Two decades of intensive research have compiled a massive body of evidence that aggregation of α-syn is a critical process in PD and other synucleinopathies. The dissemination of Lewy body pathology throughout the central nervous system strongly suggests a cell-to-cell transmission of α-syn. Although in vitro and in vivo evidence has convincingly demonstrated that aggregation-prone α-syn can spread from cell to cell, the exact mechanisms and the role for the disease pathology remain elusive. Except for cases of direct contact, the transmission of α-syn from cell to cell requires that α-syn is released to the extracellular space and taken up by recipient cells. Furthermore, internalized α-syn needs to gain access to the cytoplasm and/or target organelles of the recipient cell. Here, we review the current state of knowledge about release and uptake of α-syn and discuss the key questions that remain unanswered.

Keywords Cell-to-cell transmission · Parkinson's disease · Alpha synuclein · Exosomes · Spreading

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease, affecting up to 0.3% of the entire world population (de Lau and Breteler 2006). It is a chronic progressive disease, which leads to a high degree of disability and a compromise in the quality of life. PD is characterized by a devastating loss of neurons in affected areas, foremost dopaminergic neurons in the substantia nigra pars reticulata (Gibb 1991; Gibb and Lees 1991). Pathologically, PD is characterized by the accumulation of large intracellular inclusions termed 'Lewy bodies' and 'Lewy neurites' (Lewy 1912). Since the discovery that the small protein alpha-synuclein (α-syn) is the main constituent of Lewy bodies 20 years ago (Spillantini et al. 1997), a lot of attention and research has been focused on the aggregation of α-syn. A seminal study by Braak and colleagues elegantly demonstrated that Lewy body pathology spreads throughout the central nervous system (CNS) following a uniform pattern along anatomically interconnected areas (Braak et al. 2003). This and follow-up studies gave rise to a hypothesis that aggregation of α-syn, or α-syn itself, can spread from cell to cell by uptake and release of aggregation-prone α-syn, further promoting seeded aggregation of endogenous α-syn in a template fashion. Despite a compelling body of evidence that α-syn is transmitted from cell to cell both in vitro and in vivo, many questions remain open and hinder the development of therapeutic strategies that could prevent the spread of the disease throughout the CNS.

Aggregation of α-syn

α-syn is a small, well-conserved protein that is expressed in many tissues and cell types (Shibayama-Imazu et al. 1993; Iwai et al. 1995; Lavedan 1998; Villar-Pique et al. 2016). Increased levels of α-syn are associated with synucleinopathies, as exemplified by patients with α-syn gene duplication and triplication, the latter presenting with early-onset PD (Singleton et al. 2003; Chartier-Harlin et al. 2004; Ibanez et al. 2004). Inside the cell, α-syn localizes mainly to the cytoplasm; however, it has also been reported to be located in the nucleus, attached to mitochondria or at the endoplasmic reticulum, while in the CNS, it is located at the pre-synaptic termini (McLean et al. 2000b; Goers et al. 2003; Li et al. 2007; Devi et al. 2008; Guardia-Laguarta et al. 2014). Although the

✉ Karin M. Danzer
karin.danzer@uni-ulm.de

[1] Neurology, Ulm University, Ulm, Germany

physiological function of α-syn is still unclear, its strong conservation, ubiquitous expression and high abundance (up to 1% of the total protein in CNS) indicates its importance for the cell. In aqueous solutions, α-syn is suggested to have an intrinsically disordered structure, adopting many possible conformations, whereas in association to membranes it acquires a helical conformation (Weinreb et al. 1996; Davidson et al. 1998; McLean et al. 2000a; Chandra et al. 2003). Which form of α-syn represents the physiologic form inside mammalian cells is still a matter of debate. Some studies argued that the predominant form of α-syn in the cell is a disordered monomer (Fauvet et al. 2012; Burre et al. 2013); others provide evidence for membrane-associated α-syn homotetramers to be the physiologically relevant form (Bartels et al. 2011; Dettmer et al. 2013). Despite the discussion on the physiological form of α-syn, it is clear that aggregated α-syn is central to the pathology of synucleinopathies. This became obvious after the discovery that α-syn is the main component of Lewy bodies and Lewy neurites (Spillantini et al. 1997). The investigation of these and further types of α-syn-positive inclusions (e.g., glial cytoplasmic inclusions) has provided helpful insights into α-syn pathology in the human brain and suggested that α-syn aggregation is a key early process in inclusion formation (Kuusisto et al. 2003; Saito et al. 2003) and synaptic dysfunction (Kramer and Schulz-Schaeffer 2007). Furthermore, the observation of glial α-syn immunoreactivities hinted at the possibility that α-syn is transferred between different cell types and that this process may play a role in the degradation of pathologic α-syn (Braak et al. 2007; Kovacs et al. 2014; Rohan et al. 2016; Loria et al. 2017).

In intracellular inclusions, α-syn is found as large, beta-sheet-enriched fibrils (Spillantini et al. 1998). Recently, it has been demonstrated that different strains of aggregated α-syn fibrils exist, which exhibit specific effects on cells, have different seeding properties and can induce distinct synucleinopathies in vivo (Bousset et al. 2013; Peelaerts et al. 2015; Pieri et al. 2016). α-syn fibrils are formed in a multistep-process, involving numerous intermediate states including protofibrils, large oligomers and small oligomers (Hoyer et al. 2002; Caughey and Lansbury 2003; Munishkina et al. 2003). These intermediate species are termed "on-(fibrillary)-pathway" and are metastable, spontaneously forming large fibrils upon incubation (Cappai et al. 2005). In addition, α-syn can also associate to "off-pathway" oligomers, which do not turn into large fibrillar assemblies (Ehrnhoefer et al. 2008). On- and off-pathway oligomers are toxic to cells, can compromise membrane integrity and seed the aggregation of endogenous α-syn (Danzer et al. 2007, 2009; Winner et al. 2011). The exact molecular events that result in the initial aggregation of α-syn remain largely disputed and many mechanisms have been proposed: a dose-dependent effect of α-syn expression, saturation of membranes with α-syn, stabilization of intermediate species by the environment, impact by bacterial and eukaryotic amyloid proteins, truncation and other post-translational modifications, as well as prion-like, template-driven aggregation. All of these can result in the formation of aggregation-prone α-syn, leading to self-aggravating pathology.

Once a cell contains aberrant α-syn, there are three possibilities for handling it: degradation, deposition in a specialized compartment (inclusion), or release into the extracellular space. Both deposition inside the cell and release into the extracellular space are tightly related to failure of proper degradation. Degradation of α-syn can occur both via autophagic clearance and by proteasomal degradation (Cuervo et al. 2004; Rideout et al. 2004; Emmanouilidou et al. 2010b; Dargemont and Ossareh-Nazari 2012). Moreover, it has been demonstrated that depending on the conformation of α-syn, it can be targeted to the proteasome or to the lysosome (Shin et al. 2005). Both endogenous (cytoplasmic) and internalized α-syn aggregates can be targeted to the lysosome, the former via autophagy and the later via the endosome (Lee et al. 2004, 2008a). Failure of autophagic clearance can result in the secretion via non-classical exocytosis and drastically promote release of aggregated α-syn (Alvarez-Erviti et al. 2011).

Release into the extracellular space (Fig. 1)

α-syn lacks a classical secretory signal and was initially believed to be an exclusively intracellular protein. However, measurable quantities of α-syn have been detected in CSF, plasma and cell culture supernatant (El-Agnaf et al. 2006; Mollenhauer et al. 2008). The possibility of neuron-to-neuron transmission of misfolded α-syn in PD was highlighted by the appearance of aggregated α-syn in naïve transplanted embryonic stem cells in PD brains (Kordower et al. 2008; Li et al. 2008). Recombinant α-syn oligomers can be taken up by neurons in culture and trigger cell death (Danzer et al. 2007, 2009). Furthermore, Desplats et al. (2009) demonstrated that α-syn can be directly transmitted from neuronal cells overexpressing α-syn to transplanted embryonic stem cells both in tissue culture and in transgenic animals, supporting the idea that a prion-like mechanism could be responsible for the host-to-graft transfer of PD pathology (Brundin et al. 2008). In cell culture, α-syn is released by cell lines overexpressing α-syn and both by wild-type and α-syn-overexpressing primary neurons (El-Agnaf et al. 2003; Lee et al. 2005; Sung et al. 2005). Interestingly, the amount of released α-syn appears to correlate with the levels of intracellular α-syn (Reyes et al. 2015). The first important question, therefore, is whether α-syn is released passively or actively from the cell. Passive release can occur by two different ways: diffusion through the cell membrane and release through compromised cell membranes. Only monomeric but not oligomeric and aggregated α-syn can pass the cell membrane by

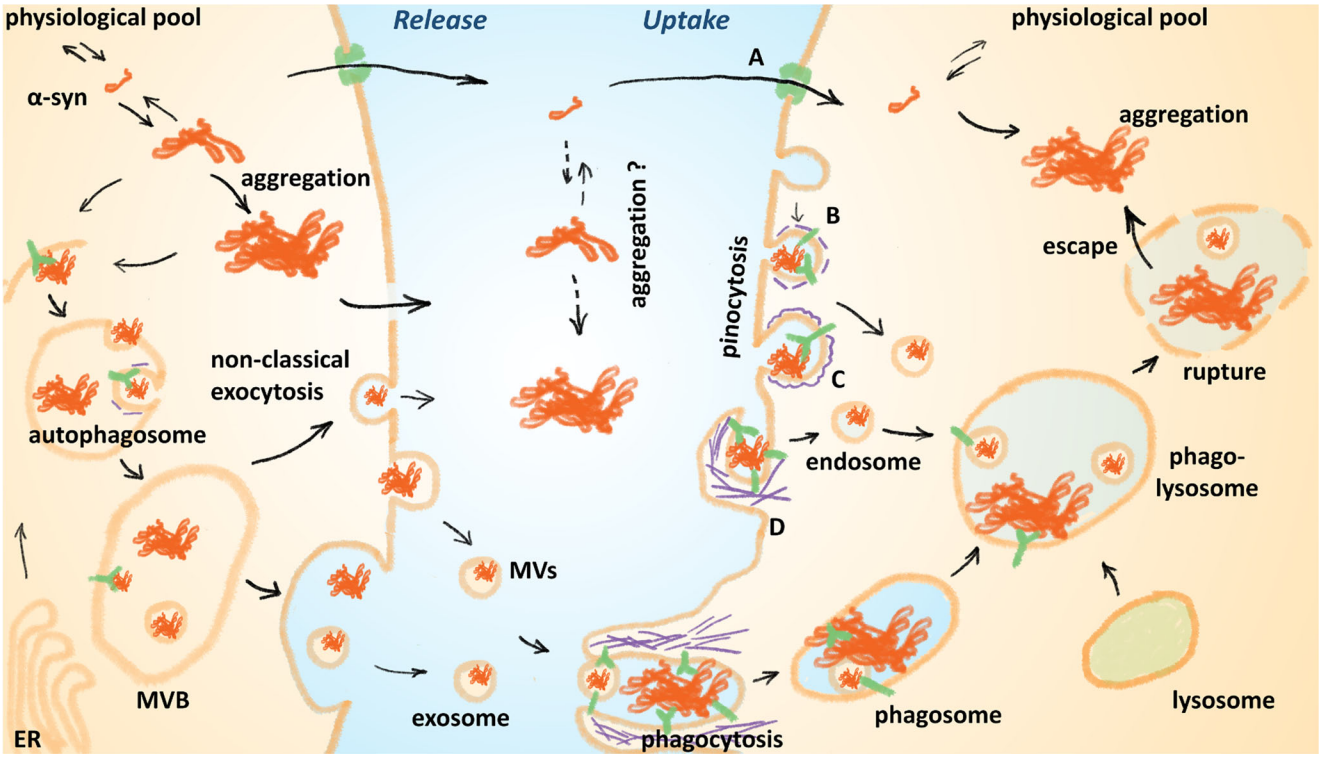

Fig. 1 Release and uptake of α-synuclein. Monomeric α-syn can aggregate to different molecular weight species. Aggregated α-syn that fails to be degraded via the autophagic system can be secreted by non-classical exocytosis and by release of multivesicular bodies' (*MVB*) contents to the extracellular space. α-syn can also be released in association to vesicles, either through membrane shedding ('microvesicles', MVs), or by incorporation into vesicles of the MVB ('exosomes" when in the extracellular space). Passive release of α-syn can occur through leaky cell membranes or by a putative translocator for monomeric α-syn, which can also facilitate passive uptake of monomeric α-syn (*A*). In the extracellular space, α-syn can be modified (e.g., by proteases) and/or aggregate further. Extracellular α-syn can be taken up by caveolin-dependent pinocytosis (*B*), clathrin-dependent pinocytosis (*C*) or actin-dependent macropinocytosis (*D*). LAG3 and further binding partners (Na/K-ATPase, neurexin) and receptors can mediate the uptake process. Large α-syn particles and vesicles are also engulfed by actin-mediated phagocytosis in phagocytic cells. Internalized material is routed by the endosomes and phagosomes to the phagolysosome for degradation. Failure of lysosomal degradation and lysosomal rupture can release α-syn into the cytoplasm, where it can seed the aggregation of endogenous α-syn from the physiological pool

diffusion (Ahn et al. 2006; Lee et al. 2008a). Diffusion of monomeric α-syn can occur in both directions and relies on a so-far unidentified membrane translocator, since α-syn cannot simply pass the lipid bilayer or escape from vesicles (Lee et al. 2005, 2008a). Remarkably, exogenously applied α-syn monomers can enter and exit the cell by diffusion, while α-syn synthesized by the cell in the cytoplasm remains trapped inside the cell (Lee et al. 2008a). This suggests that rapid formation of physiological multimers (e.g., tetramers) or post-translational modifications prevent nascent α-syn from passively exiting the cell. There is little evidence that α-syn is released through compromised cell membranes and cell death. An in vivo study by Ulusoy and colleagues suggested that transmission of α-syn in a rat model does not result from neuronal death or injury and even requires healthy functioning neurons (Ulusoy et al. 2015). Importantly, it is possible that considerably less α-syn is available outside the cell in vivo, and therefore cell death may contribute more to the extracellular pool. However, evidence that α-syn is released passively in vivo is largely missing. Although much progress has been made in the establishment of extracellular α-syn as a biomarker, a systematic increase in PD and other synucleinopathies remains a matter of debate (El-Agnaf et al. 2003; Llorens et al. 2016; Shahnawaz et al. 2017). Different species of α-syn can be found in blood, CSF and saliva not only of PD patients but also of healthy controls with no indications of manifest or prodromal neurological disease (El-Agnaf et al. 2003; Llorens et al. 2016; Simonsen et al. 2016). Thus, it remains to be clarified if neuron death contributes to α-syn release in vivo and if this α-syn has diagnostic value, as well as relevance for the spread of disease.

If α-syn is released actively by the cell, several important questions arise: What is the mechanism of α-syn secretion? How is α-syn secretion regulated? What is the function of α-syn secretion in cells? The answers to these questions are closely associated with the question which species of α-syn are secreted. Several active mechanisms have been demonstrated for the release of monomeric, oligomeric and aggregated α-syn: ER-Golgi-dependent exocytosis and non-classical exocytosis, as well as in association with vesicles (Lee et al.

2005; Emmanouilidou et al. 2010a; Jang et al. 2010; Alvarez-Erviti et al. 2011; Danzer et al. 2011). Both monomeric and aggregated α-syn can be secreted through non-classical exocytosis by neurons in cells culture (Lee et al. 2005). Monomeric, oligomeric and aggregated α-syn can also be found in exosomes from cells overexpressing α-syn (Emmanouilidou et al. 2010a; Alvarez-Erviti et al. 2011; Danzer et al. 2012; Bliederhaeuser et al. 2016). Interestingly, secretion of α-syn is increased by stress, α-syn aggregation, lysosomal dysfunction, inhibition of the proteasome and mitochondrial dysfunction (Lee et al. 2005; Jang et al. 2010). Thus, α-syn secretion is actively regulated by conditions that promote α-syn aggregation. The function of α-syn release is likely also specific for different species of α-syn. For example, the secretion of α-syn monomers and small oligomers can aim to prevent the recruitment of endogenous α-syn by an aggregation seed or could have a purely physiologic function, like enhancement of vesicle recycling (Chandra et al. 2005). In contrast, secretion of aggregated α-syn can protect neurons from its toxicity and aim at the uptake and degradation by other cell types like professional phagocytes and astrocytes, or the degradation by extracellular enzymes. Indeed, microglia degrade efficiently extracellular α-syn (Lee et al. 2008b) and several extracellular proteases (Calpain 1, neurosin) can degrade extracellular α-syn and are active in brains from PD and DLB patients (Ogawa et al. 2000). Importantly, low-molecular weight α-syn released from cells does not necessarily have to be aggregation-prone per se but can be rendered so by extracellular processing like truncation. By contrast, aggregated α-syn released from neighboring cells probably resists proteolytic degradation outside the cell and is internalized by recipient cells.

As evident from the above-mentioned studies, release of α-syn seems to be dependent on the conformation of α-syn. This notion is further supported by the observation that familial PD mutations increase the secretion of α-syn in vitro (Lazaro et al. 2014). Thus, if the proteins responsible for the specific recognition and secretion of aberrant α-syn are identified, novel approaches to prevent disease spread may emerge. Unfortunately, it has been difficult to study the secretion of different conformations of α-syn since it is impossible to let the cell produce only one species of α-syn. This technical hurdle has prevented the efficient identification of the mechanism responsible for the specific secretion of pathologic but not physiologic α-syn.

Uptake from the extracellular space (Fig. 1)

The uptake of α-syn from the extracellular space has been intensively studied in recent years. Uptake of pathologic α-syn results in toxicity and seeding of aggregation of endogenous α-syn and is possibly a critical step necessary for the cell-to-cell transmission of α-syn pathology.

As with release, uptake of extracellular α-syn can occur passively (by diffusion), or actively, i.e., by endocytosis. Diffusion of monomeric but not oligomeric and aggregated α-syn through the plasma membrane has been observed, as discussed in the previous section but it has not been investigated whether it contributes to the transmission of pathology. Otherwise, extracellular α-syn is taken up by different types of endocytosis. Two main types of endocytosis can be distinguished: pinocytosis (often referred to simply as endocytosis) and phagocytosis. These two main types utilize different protein machineries and are responsible for the engulfment of different types of extracellular material: large particles are engulfed by phagocytosis, while macromolecules, small molecules and extracellular fluid are internalized by pinocytosis. While pinocytosis occurs constitutively in virtually all mammalian cells, phagocytosis is carried out mainly by professional phagocytes like macrophages and microglia. Thus, the uptake mechanism and fate of extracellular α-syn vary depending on the species of α-syn and on the type of recipient cell.

Pinocytosis is constitutive and accounts for the internalization of extracellular material, which is trapped in endocytic pits. Solutes and small molecules are internalized this way by clathrin-, caveolae- or actin-dependent processes. In addition, larger molecules can be efficiently internalized by pinocytosis through binding to a specific receptor, a process that is termed 'receptor-mediated endocytosis' and is clathrin-mediated. Uptake of α-syn by pinocytosis has been demonstrated in many studies with different cell types: differentiated SH-SY5Y, COS-7 and primary neurons. Hansen et al. observed that exogenously applied α-syn is internalized in a dynamin-dependent process also in vivo (Hansen et al. 2011). Interestingly, blocking the internalization of endocytic vesicles by dynamin inhibition resulted in an accumulation of α-syn oligomers and aggregates on the cell membrane (Lee et al. 2008a, b). The same was observed when proteins on the outer side of the cell membrane were trimmed by treatment with an unspecific protease (Lee et al. 2008a, b). This strongly suggests that aggregated α-syn is taken up by receptor-mediated endocytosis. Indeed, α3-subunit of Na+/K + -ATPase has been reported to be a cell surface partner of α-syn assemblies (Shrivastava et al. 2015). Furthermore, Mao et al. recently identified LAG3 as a receptor that binds specifically α-syn pre-formed fibrils (PFFs), initiating α-syn transmission and toxicity (Mao et al. 2016). Interestingly, α-syn PFFs also bound to neurexin 1a (Mao et al. 2016), which was also found by Shrivastava et al. to be a cell surface binding partner for α-syn fibrils (Shrivastava et al. 2015). Mao and colleagues screened a large panel of transmembrane proteins for their affinity to bind α-syn PFFs and found three receptors, of which LAG3 showed the highest specificity for α-syn PFFs.

Not only did LAG3 exhibit high affinity for aggregated and not for monomeric α-syn but it also did not bind tau and amyloid beta aggregates. In addition, ablation of LAG3 in neurons disrupted transmission of α-syn pathology and alleviated dopaminergic cell loss and disease phenotype in an animal model (Mao et al. 2016). The identification of receptors for aggregated α-syn on neurons opens interesting possibilities; however, much remains to be elucidated. For example, it should be investigated whether LAG3 is also responsible for the internalization of α-syn oligomers, whether it binds aggregates produced from the cells and whether it binds different strains of α-syn fibrils. Interestingly, Lee et al. demonstrated that different types of brain cells take up exogenous synuclein with different kinetics (Lee et al. 2008b). In particular, microglia were much more efficient than neurons and astrocytes, both in uptake and degradation of extracellular α-syn. This findings suggest that either the same uptake pathways are differentially regulated in different cell types or that different cell types are equipped with distinct receptors for extracellular α-syn. Liu and colleagues identified ciliary neurotrophic factor receptor as a mediator of neurotoxicity induced by α-syn (Liu et al. 2010) but independent of α-syn endocytosis; in another study, inhibition of TLR2 partially ablated uptake of neuron-released α-syn by microglia (Kim et al. 2013). Therefore, it remains to be elucidated which exact receptors are involved in the uptake not only of different species of α-syn but also by different brain cells. The picture is further complicated by the fact that α-syn produced in mammalian cells may be modified such that its interaction with receptors and uptake is totally different compared with recombinant α-syn. This hypothesis is suggested by two independent observations: first, Lee and colleagues noticed that while monomeric recombinant α-syn can freely move into and out of the cell through the cell membrane, cell-produced monomeric α-syn remains trapped inside the cell (Lee et al. 2008a). Second, Delenclos and colleagues demonstrated that α-syn oligomers purified from a neuroglioma H4 cell line stably overexpressing α-syn are attached to the outer side of the cell membrane, although it has been shown numerous times that recombinant α-syn oligomers are readily internalized by different cell types (Delenclos et al. 2017). Thus, the solid body of knowledge about the uptake of different recombinant α-syn species cannot be simply translated to cell-produced α-syn without extensive testing.

The studies discussed above have all investigated the uptake of α-syn, which is not enclosed in vesicles. However, we and others have previously shown that a portion of α-syn is secreted in association to exosomes, small membrane-enclosed vesicles (Emmanouilidou et al. 2010a; Danzer et al. 2012). Although a relatively small amount of extracellular α-syn is associated with exosomes, their potential as a biomarker of PD, as well as their role in the pathogenesis of PD, are gaining increasing attention (Tofaris 2017). Using a well-established luciferase complementation system, it has been shown that both neurons and microglia take up α-syn oligomers much more efficiently in association with exosomes (Danzer et al. 2012; Bliederhaeuser et al. 2016). Interestingly, inhibition of phagocytosis or micropinocytosis of exosome-associated α-syn oligomers by cytochalasin D was not found in neuroglioma H4 cells (Delenclos et al. 2017), while we have previously found that microglia and monocytes efficiently take up exosomes via actin- and PI3K-dependent pathways, strongly indicative of phagocytosis (Bliederhaeuser et al. 2016). Virtually all types of uptake have so far been described for exosomes; however, there is still no consensus in the literature which uptake mechanism is responsible for exosome internalization and it seems that the uptake mode is dependent not only on the recipient cell type but also on the cell type the released the exosomes, as well as on the cargo (Feng et al. 2010; Mulcahy et al. 2014). Thus, uptake of exosome-associated α-syn likely occurs through different mechanisms in neurons and professional phagocytes. It remains to be investigated whether uptake by different mechanisms results in different intracellular trafficking and effects on cells.

A further prerequisite for the prion-like spread of misfolded α-syn is that the transmitted α-syn gains access to the cytoplasm of the recipient cell, where the pool of the endogenous protein is localized. Elegant studies by Freeman et al. and Flavin et al. demonstrated that aggregated α-syn can evade degradation after uptake from the extracellular space and can escape into the cytoplasm from endosomal–lysosomal compartments (Freeman et al. 2013; Flavin et al. 2017). Following rupture of lysosomes, α-syn aggregates can induce mitochondrial dysfunction, inflammatory activation and oxidative stress. Therefore, it is also possible that α-syn that escapes the lysosome can directly seed the aggregation of cytoplasmic α-syn.

Conclusions

In recent years, much progress has been made in our understanding of the molecular processes that underlie the transmission of α-syn pathology from cell to cell. New knowledge is available regarding mechanisms that increase the secretion of α-syn, extracellular modifications of α-syn, receptors involved in the uptake and the involvement of vesicles. The contribution of different cell types and the differences between different α-syn species have also gained appreciation. Although our knowledge has improved significantly, there are still numerous technical difficulties that need to be overcome and questions that need to be answered, before we understand cell-to-cell transmission of α-syn well enough to tailor efficient therapies for PD and other synucleinopathies.

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