How is alpha-synuclein cleared from the cell?

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Abstract

The levels and conformers of alpha-synuclein are critical in the pathogenesis of Parkinson's Disease and related synucleinopathies. Homeostatic mechanisms in protein degradation and secretion have been identified as regulators of alpha-synuclein at different stages of its intracellular trafficking and transcellular propagation. Here we review pathways involved in the removal of various forms of alpha-synuclein from both the intracellular and extracellular environment. Proteasomes and lysosomes are likely to play complementary roles in the removal of intracellular alpha-synuclein species, in a manner that depends on alpha-synuclein post-translational modifications. Extracellular alpha-synuclein is cleared by extracellular proteolytic enzymes, or taken up by neighbouring cells, especially microglia and astrocytes, and degraded within lysosomes. Exosomes, on the other hand, represent a vehicle for egress of excess burden of the intracellular protein, potentially contributing to the transfer of alpha-synuclein between cells. Dysfunction in any one of these clearance mechanisms, or a combination thereof, may be involved in the initiation or progression of Parkinson's disease whereas targeting these pathways may offer an opportunity for therapeutic intervention.

In this review we discuss systems, intracellular and extracellular, responsible for the clearance of alpha-synuclein (α-Syn). Given the prevailing idea of the varying degrees of "alpha-synuclein burden" underlying PD pathogenesis, it is important to understand the pathways through which α-Syn is not only produced, but also cleared, as dysfunction of such systems may lead to aberrant accumulation of α-Syn and instigate or influence the risk of developing Parkinson's Disease (PD) and other synucleinopathies (Figure 1a). It is especially important to understand if there are different clearance systems for removing different forms of α-Syn, occurring at physiological circumstances or under pathological conditions. Understanding the systems involved in α-Syn clearance may pave the way for novel therapeutic approaches that aim to boost such endogenous mechanisms in order to restore neuronal homeostasis (Figure 1b).

Intracellular protein degradation systems

There are main systems for degradation of intracellular proteins; the proteasomal and lysosomal pathways. The 26S proteasome degrades intracellular proteins mainly through the ubiquitin system, following the conjugation of at least four ubiquitin molecules in a chain-like fashion on lysine residues of substrate proteins. The conjugation of the ubiquitin chain on substrate proteins occurs through a regulated series of enzymatic reactions, with the selectivity determined by E3 ligases specific for particular substrates. Degradation within the barrel-like 20S proteasome occurs following recognition of the ubiquitinated substrate protein by the ATPase subunits of the 19S proteasome regulatory particle. Ubiquitin-independent proteolysis through the proteasome may also occur, involving recognition of substrates through non-ATPase complexes, such as the PA28 activator, or even in the absence of recognition complexes, as may occur for natively unfolded proteins (Demartino and Gillette, 2007, Gallastegui and Groll, 2010).

The lysosome degrades intracellular proteins through the endosomal and three autophagic pathways: macroautophagy, chaperone-mediated autophagy (CMA) and microautophagy. In microautophagy, invaginations of the lysosomal membrane allow pinched-off vesicles with their constituents to be degraded within the lysosomes; microautophagy can either occur in a bulk fashion or it can be selective, in the form of endosomal microautophagy (eMI) (Tekirdag and Cuervo, 2018). Macroautophagy entails the creation of double membrane structures that engulf intracellular organelles or proteins, creating autophagosomes, which then fuse with lysosomes as autophagolysosomes and degrade their constituents. It is now recognized that apart from bulk macroautophagy there are many forms of selective

macroautophagy. CMA entails the selective recognition of a pentapeptide motif on target proteins by the cytosolic chaperone Heat Shock Cognate Protein of 70 kDa (hsc70), the translocation of this complex to the lysosomal membrane, with binding to the rate-limiting step in the pathway, the lysosome-associated transmembrane protein LAMP2A, and subsequent threading and degradation within the lysosomal lumen. Lysosomal hsc70 and other lysosomal proteins regulate this process; in fact, lysosomal hsc70 is essential for this process, and only hsc70-positive lysosomes are capable of CMA. The endocytic and exocytic pathways interact with the macroautophagic pathway, creating amphisomes through the fusion of multivesicular bodies (MVBs) or late endosomes (LEs) with autophagosomes (Xilouri et al., 2016b, Tekirdag and Cuervo, 2018). There is also an interaction of these pathways with microautophagy, in particular eMI. In this process, substrate proteins are selectively targeted through the same pentapeptide motif as in CMA, *via* binding to Hsc70 into late endosomes or MVBs, where they can be degraded following fusion with lysosomes. Alternatively, such proteins may be targeted for secretion within extracellular vesicles, termed exosomes (Tekirdag and Cuervo, 2018) (see below).

Our current knowledge about the intracellular degradative pathways involved in the clearance of α-Syn stems largely from their genetic or pharmacological manipulation in various model systems. These approaches have some inherent limitations: a) it is now known that inhibition of one proteolytic pathway leads to alterations in one or more of the other pathways, which may influence the clearance of the particular protein under study, in this case α-Syn, which is degraded by multiple pathways; b) pharmacological inhibition may not be selective leading to cellular changes that influence the levels of proteins independently of the particular targeted degradation system; for example pharmacological proteasome inhibitors may upregulate Cytomegalovirus (CMV)-driven transcription, thus leading to high levels of expression of genes that are expressed in a heterologous fashion in cells, through alterations in their rate of production and not clearance; this has been demonstrated in the case of α-Syn (Biasini et al., 2004); c) when the protein is not in a steady state within the cell, such as when transiently overexpressed, or is otherwise artificially altered, for example through the addition of tags of various lengths, its subcellular localization and clearance pathways may be different compared to its physiological state. This is especially true for α-Syn, as its normal predominant presynaptic localization in neuronal cells is difficult to reproduce in cell culture conditions. Thus, multiple experimental approaches, including *in vivo* experimentation, are needed in order to arrive at safe conclusions regarding α-Syn clearance mechanisms.

The role of intracellular protein degradation systems in α-Syn clearance

Initial studies using pharmacological inhibitors showed that the proteasome degrades α-Syn in cells and both 20S and 26S proteasomes degrade α-Syn in purified systems (Bennett et al., 1999, Tofaris et al., 2001, Webb et al., 2003, Tofaris et al., 2011, Shabek et al., 2012). However, other studies did not detect significant accumulation of endogenous or overexpressed α-Syn following pharmacological proteasomal inhibition in cell lines and primary neurons (Rideout et al., 2001, Rideout and Stefanis, 2002, Vogiatzi et al., 2008). Emmanouilidou et al. (2010b) (Emmanouilidou et al., 2010b), on the other hand, found a robust accumulation, upon proteasomal inhibition, of soluble intermediate oligomeric species of α-Syn that co-eluted with the 26S proteasome on gel filtration. In contrast, higher order α-Syn oligomers or the monomeric form were not altered upon proteasomal inhibition. It is therefore likely that the proteasome degrades a fraction of α-Syn, the abundance and species of which may vary between cell types and experimental conditions.

Lysosomes have been extensively investigated with regards to α-Syn clearance. The first paper that advanced the possibility that the lysosome could be important for α-Syn degradation was the work of Harry Ischiropoulos' group in 2001, in which they showed that a general pharmacological lysosomal inhibitor, ammonium chloride, increased the amount of stably overexpressed human α-Syn in HEK 293 cells, whereas proteasomal inhibition had no effect (Paxinou et al., 2001). Since then, most pieces of work have confirmed a role for lysosomal processes in α-Syn degradation, although the particular components of the lysosomal involvement and the type of α-Syn affected differ across studies. Webb et al. (2003), used an inducible system in PC12 cells to overexpress Wild Type (WT) or mutant A53T or A30P human α-Syn, and pharmacological tools to inhibit the proteasome, the lysosome, more generally with bafilomycin, or macroautophagy specifically, using 3-Methyladenine (3-MA). They showed that mutant A53T α-Syn was degraded through both the proteasome and macroautophagy, whereas WT and A30P were mainly degraded through the proteasome. Pharmacological induction of macroautophagy with rapamycin enhanced the clearance of all three α-Syn forms when overexpressed, but did not promote the clearance of endogenous WT protein under baseline culture conditions (Webb et al., 2003). In partial agreement, Cuervo et al. (2004) found that general lysosomal inhibition with ammonium chloride led to a very significant prolongation of the half-life of endogenous α-Syn in rat midbrain dopaminergic cultures and of overexpressed WT human α-Syn in PC12 cells, whereas 3-MA had little or no effect, suggesting that a non-macroautophagy-dependent lysosomal process was responsible for WT α-Syn degradation; it

was indeed confirmed that WT α-Syn could be degraded in isolated liver lysosomes through the selective process of CMA in a fashion dependent on the pentapeptide recognition motif $_{95}$VKKDQ$_{99}$ present within α-Syn (Cuervo et al., 2004). Vogiatzi et al. (2008) showed that molecular downregulation of LAMP2A, the rate-limiting step in CMA, led to accumulation of α-Syn protein, but not mRNA, in cultured neuronal cells and primary cortical and ventral midbrain dopaminergic neurons. Oligomeric α-Syn forms also accumulated, likely secondary to the effect on monomeric α-Syn levels. Furthermore, an artificial mutant form of α-Syn lacking the targeting pentapeptide motif was degraded more slowly than the WT protein in neuronal cells, again indicating that CMA is important for the degradation of endogenous WT α-Syn in this neuronal cell context. At the same time, pharmacological inhibition of macroautophagy *via* 3-MA led to a more modest accumulation of α-Syn in such cellular systems, indicating that macroautophagy is also a pathway for endogenous α-Syn degradation in neuronal cells (Vogiatzi et al., 2008). Notably, α-Syn mutants, such as the originally identified in rare familial PD A30P and A53T, were not efficiently degraded by CMA (Cuervo et al., 2004). Oligomerization or dopamine modification also rendered WT α-Syn resistant to CMA-dependent degradation in isolated lysosomes; in fact, apart from monomeric α-Syn, only dimeric α-Syn could also be degraded by CMA (Martinez-Vicente et al., 2008). Alvarez-Erviti et al. (2010) corroborated that LAMP2A downregulation led to a slowing down of WT α-Syn degradation in a dopaminergic cell line. Similarly to Cuervo et al. (2004) and Webb et al. (2003), they found that 3-MA did not influence WT α-Syn levels, but led to decreased clearance of the A53T mutant. However, it should be noted that LAMP2A is a late-endosomal and lysosomal protein that is involved in multiple trafficking steps to the lysosome (Alvarez-Erviti et al., 2010).

An interesting observation that requires further validation is that pharmacological inhibition of the lysosome but not macroautophagy inhibition *via* 3-MA, led to the stabilization of intermediate SDS-stable α-Syn oligomeric forms, but no change in more mature α-Syn aggregates or monomeric α-Syn (Lee et al., 2004). Assuming that CMA cannot degrade α-Syn oligomeric forms, as mentioned above, this suggests that eMI or some other endosomal-lysosomal pathway may be responsible for the selective clearance of these α-Syn oligomers. Interestingly, the same intermediate oligomeric species showed a slight accumulation with proteasomal inhibition, suggesting that some of the α-Syn oligomers, as reported also by Emmanoulidou et al. (2010), can be degraded by the proteasome. Tanik et al. (2013) used a model of seeding of intracellular α-Syn by exogenous α-Syn fibrils, and observed that the formed aggregates were resistant to proteolytic clearance even by macroautophagy, in agreement with the study of Lee et al. (2004) (Tanik et al., 2013). In a twist that links α-Syn

degradation to its secretion, the microtubule-binding protein p25a induced the aggregation of α-Syn in autophagosomes and amphisomes, which led to their enhanced secretion due to concurrent partial block of autophagosome to lysosome fusion; this type of secretion mediated through autophagosomes/amphisomes is termed exophagy (Ejlerskov et al., 2013).

Only a few studies have investigated the relative contribution of proteasomes and lysosomes in animal studies. Ebrahimi-Fakhari et al. (2011), using an elegant cranial window approach, showed that *in vivo* application of proteasomal inhibitors to superficial mouse cortical regions led to accumulation of both endogenous mouse and overexpressed human α-Syn (Ebrahimi-Fakhari et al., 2011). In contrast, general lysosomal inhibition only led to accumulation of α-Syn in the setting of transgenic overexpression, suggesting that the ALP was activated to handle accumulated α-Syn. In these experiments, only the monomeric form of α-Syn was assessed. Genetic inhibition of macroautophagy at the level of the mouse midbrain in a conditional knock-out of Atg7, a gene involved in autophagosome formation, led to the accumulation of ubiquitinated inclusions that did not contain α-Syn. These mice had significantly higher levels of α-Syn than controls, without however any evidence of α-Syn aggregation (Ahmed et al., 2012). Using a similar animal model, Friedman et al. (2012) observed that aberrant α-Syn, as assessed by the Syn303 antibody that recognizes oxidized forms of the protein, accumulates only along nigrostriatal axons, in areas of axonal swellings, and only in animals of advanced age (Friedman et al., 2012). This finding could be interpreted as showing that axonal/synaptic α-Syn is degraded in part by macroautophagy. Mak et al. (2010) showed that LAMP2A-positive puncta colocalized with α-Syn *in vivo* in the mouse substantia nigra following a variety of stressors, and that the uptake and internalization of α-Syn in lysosomes was enhanced under these conditions (Mak et al., 2010). Xilouri et al. (2016a) showed that Adeno-Associated Virus (AAV) shRNA targeting LAMP2A along the nigrostriatal axis, led to the cytoplasmic accumulation of α-Syn in small aggregates, suggesting that lysosomes, in part *via* the CMA pathway, degrade α-Syn in nigral neurons (Xilouri et al., 2016a). However, α-Syn accumulation was detected rather late in the process, when axonal degeneration in particular was already established, leaving open the possibility that altered cellular localization of α-Syn or other secondary effects may be at play. A further note of caution is suggested by the finding that knock-out of LAMP2, which also includes other LAMP2 isoforms in addition to LAMP2A, in mouse brain did not lead to overall changes in α-Syn levels, or other purported CMA substrates (Rothaug et al., 2015). Analysis at the subcellular level was not performed and a selective increase in certain neuronal cell types or compartments was not ruled out, while the possibility

exists that compensatory responses, such as the induction of other protein degradation systems, may have played a role for such negative findings.

Taken together, despite some conflicting results, the picture that is emerging is that a combination of pathways, including CMA, contribute to the degradation of α-Syn under homeostatic conditions and especially when α-Syn accumulates in cells. Altered forms, such as mutant α-Syn or intermediate level oligomers, may be cleared by the proteasome or macroautophagy. α-Syn is also found within endosomes (Hasegawa et al., 2011, Boassa et al., 2013) and previously shown to be targeted to the endosome by ubiquitination (Tofaris et al., 2011). Endosomal α-Syn is either degraded by lysosomes (Tofaris et al., 2011) or enters the recycling endosome and is released in a process involving Rab11a and Hsp90 (Liu et al., 2009, Hasegawa et al., 2011). It is therefore probable that different fractions of α-Syn are processed by multiple pathways depending on localization and pathological state of the cell. Another major determinant appears to be post-translational modifications that may target α-Syn to particular degradation pathways.

Role of post-translational modifications in α-Syn clearance

Ubiquitination of protein substrates is the predominant post-translational modification that targets proteins for degradation. Conjugation of a specific type of ubiquitin chain to protein substrates is a highly regulated process, which mediates trafficking of selective protein cargoes primarily to the proteasome but also to lysosomes *via* the endosomal or autophagic pathway. As mentioned, this post-translational modification occurs in a three-step catalytic process, involving a ubiquitin activating enzyme E1, a ubiquitin conjugating enzyme E2, and a ubiquitin ligase E3. There are approximately 30 E2s and 650 E3s in mammalian cells. At the proteasome and during endosomal uptake, ubiquitin chains are disassembled by one of the 100 de-ubiquitinating enzymes (DUBs) so that the ubiquitin molecules can be reused in subsequent rounds of degradation, but this action of deubiquitinases can also serve to prevent the degradation of substrates. The way ubiquitin molecules are linked together to form chains determines the degradative pathway to which the substrate is targeted. Lysine-48 (K48) or Lysine-11 (K11) linked chains are recognized by specific adaptor proteins such as Rad23 and target proteins to the 26S proteasomes, whereas polyubiquitin chain linked *via* lysine-63 (K63) trigger the assembly of the endosomal complex required for transport (ESCRT) or autophagy receptors (Nathan et al., 2013). ESCRT complexes are comprised of four distinct assemblies (ESCRT 0, I, II, or III), which recognize the cargo, associate with the endosomal membrane, and sort protein substrates in intraluminal vesicles (Raiborg and Stenmark, 2009). Autophagy receptors

TAX1BP1, NDP52, NBR1, p62 (SQSTM1), and optineurin recruit LC3-coated phagophores to mediate selective autophagy (Stolz et al., 2014).

Studies in human post-mortem brain have shown that a fraction of α-Syn accumulates in Lewy bodies in an ubiquitinated form (Tofaris et al., 2003, Anderson et al., 2006), suggesting that ubiquitin ligases are relevant to its trafficking and turnover. Given the central role of lysosomal trafficking in Parkinson's pathogenesis (Tofaris, 2012), it is possible that ubiquitination may act as a signal to selectively direct monomeric or aggregated forms of α-Syn to this pathway. In this context, a potent E3 for α-Syn is the ubiquitin ligase NEDD4 (neuronally expressed developmentally down-regulated gene 4), which serves a role in the endosomal or lysosomal targeting of substrates (Tofaris et al., 2011). NEDD4 is upregulated in response to oxidative stress (Hoshikawa et al., 2003), traumatic head injury (Sang et al., 2006), and neurodegeneration (Kwak et al., 2012), indicating that its expression in neurons is tightly regulated and may serve broader protective functions. In a purified system, NEDD4 and its yeast orthologue Rsp5 robustly ubiquitinate α-Syn by recognizing its proline-rich carboxy-terminus (Tofaris et al., 2011). This leads to the conjugation of uniform K63-linked ubiquitin chains on specific lysine residues of α-Syn (Lys-21 and Lys-96). Interestingly, Lys-96 of α-Syn has been identified as one of its primary ubiquitination sites in rat brain by an antibody-based proteomic analysis (Na et al., 2012) and K63-linked conjugates are present in Lewy bodies (Alexopoulou et al., 2016). In mammalian cells, NEDD4 overexpression promoted the degradation of α-Syn by an ESCRT-mediated lysosomal route (Tofaris et al., 2011). In addition, NEDD4 also ubiquitinates cytosolic misfolded proteins following heat stress (Fang et al., 2014) and misfolded α-Syn assemblies (Davies et al., 2014, Mund et al., 2018), suggesting that it may also promote the clearance of aggregated α-Syn. NEDD4 overexpression protects against α-Syn accumulation in yeast, *Drosophila* and rat models of α-synucleinopathy (Davies et al., 2014). In agreement with these observations, a chemical genetic screen has identified a small molecule that binds to and activates NEDD4 as a neuroprotective agent in yeast and iPSC-derived cortical neuronal models of α-Syn toxicity (Chung et al., 2013). The finding that NEDD4 and its yeast orthologue Rsp5 directly ubiquitinate α-Syn (Tofaris et al., 2011, Davies et al., 2014) has been confirmed and extended by recent studies (Wijayanti et al., 2014, Sugeno et al., 2014). CHIP (Carboxyl terminus of Hsp70-interacting protein) is an E3 ligase with chaperone activity able to target misfolded proteins for degradation. This mode of action makes it a candidate E3 in the context of neurodegeneration associated with neuronal inclusions. Indeed, two studies have shown that overexpression of CHIP targets α-Syn oligomers for degradation (Shin et al., 2005, Tetzlaff et al., 2008). It remains unclear whether CHIP has a direct action on

α-Syn, as in a purified system it does not ubiquitinate α-Syn even in the presence of its co-factor Hsp70 (Tofaris et al., 2011).

Ubiquitination of α-Syn has also been proposed as a modification that regulates its aggregation. In this context, monoubiquitination by the E3 ligases SIAH (seven in absentia homolog) 1 and 2 were shown to promote aggregation (Rott et al., 2008, Lee et al., 2008c). In a purified system, unlike NEDD4, SIAH ligases conjugate ubiquitin on multiple lysine residues (K10, K12, K21, K23, K34, K43, and K96), akin to the pattern seen with reticulocyte lysates (Nonaka et al., 2005), raising the possibility that there is no specificity. Why monobiquitination promotes aggregation is currently unclear. Interestingly, removal of SIAH-mediated monoubiquitination by the deubiquitinase USP9x prevents the proteasomal degradation of α-Syn (Rott et al., 2011) and in a purified system monoubiquitinated α-Syn can be efficiently degraded by isolated 26S proteasomes (Shabek et al., 2012). The degradation of mono-, di- or tetra- K48-linked ubiquitinated α-Syn synthesized *in vitro* was also investigated using cell extract enriched in 26S proteasomes and the entire cohort of deubiquitinases (Haj-Yahya et al., 2013). This study showed that di- and tetra- ubiquitinated α-Syn is degraded efficiently by the proteasome, whereas in monoubiquitinated α-Syn, the single ubiquitin moiety is efficiently cleaved well before the molecule is degraded. These findings suggested that the role of poly-Ub chains of K48-linkage is to protect α-Syn from deubiquitinase activity and to enable a long enough residence time of the conjugated substrate on the proteasome to allow its efficient degradation. It is therefore likely that monoubiquitination such as the one observed by SIAH may prime α-Syn for ubiquitin chain extension by yet unidentified E3s and degradation by the 26S proteasome.

Besides USP9x, additional deubiquitinating enzymes have been shown to act on α-Syn. The abundance of K63-linked conjugates in Lewy bodies was found to inversely correlate with the localisation of deubiquitinase USP8, which directly deubiquitinates α-Syn in a purified system and mammalian cells, preventing its lysosomal degradation (Alexopoulou et al., 2016). Knockdown of USP8 in the *Drosophila* model reduced α-Syn levels and toxicity suggesting that both in cells and simple model organisms, USP8 acts as a negative regulator of α-Syn clearance (Alexopoulou et al., 2016). The endoplasmic reticulum (ER)-associated deubiquitinase USP19 was shown to recruit misfolded proteins, including α-Syn, to the ER surface for deubiquitination and encapsulation into ER-associated late endosomes for secretion to the cell exterior (Lee et al., 2016).

α-Syn is also conjugated to small ubiquitin-like modifier (SUMO) at lysine residues, but the role of this modification in α-Syn clearance or aggregation is not fully understood. A

proportion of Lewy bodies in nigral neurons stain positive for SUMO1 (Rott et al., 2017), suggesting a role of this modification in Parkinson's pathogenesis. Some studies demonstrated increased α-Syn aggregation by SUMOylation upon proteasomal inhibition (Oh et al., 2011, Kim et al., 2011), but SUMOylation itself had limited or no effect on aggregation (Dorval and Fraser, 2006, Krumova et al., 2011). The SUMO ligase PIAS2 promoted SUMOylation of α-Syn, which in turn reduced α-Syn ubiquitination by SIAH and NEDD4 ubiquitin ligases and increased aggregation and α-Syn release from the cells (Rott et al., 2017). In this context, it was previously shown that SUMOylation utilizes the ESCRT complex to sort α-Syn into extracellular vesicles for secretion (Kunadt et al., 2015). It is therefore possible that SUMOylation occurs secondarily to impaired ubiquitin-mediated degradation to promote α-Syn secretion in a process that may not be efficient enough to prevent aggregation.

The particular case of phosphorylated (pS129) α-Syn

Phosphorylation at S129 seems to play a major role in most synucleinopathies since a large proportion of α-Syn is phosphorylated in LBs and GCIs. Phosphorylation at S129 has been documented to play a pivotal role in α-Syn stability, toxicity and pathophysiology, although the direction of the effect is hotly debated (reviewed in (Oueslati, 2016)). Recent studies in cell culture demonstrated that overexpression of PLK2 kinase affected total α-Syn levels. This significant decrease of α-Syn levels could only be reversed upon lysosomal inhibition (Oueslati et al., 2013, Dahmene et al., 2017). In contradiction to these findings, a study in cell models and mouse brains proposed that PLK2-mediated regulation of α-Syn reflected a modulation in mRNA levels of α-Syn, rather than a degradative effect through autophagy (Kofoed et al., 2017).

Provided that modulation of pS129 α-Syn levels could affect α-Syn toxicity and disease progression in synucleinopathies, the degradation mechanism(s) involved could possibly represent a viable therapeutic target. Waxman and Giasson (2008) first showed in cultured cells and in primary neurons that pS129 α-Syn is increased upon proteasomal inhibition (Waxman and Giasson, 2008). Further research has proposed that the increased levels of pS129 α-Syn could be related either directly to proteasomal turnover or indirectly to increased CK2 kinase activity (Waxman and Giasson, 2008, Chau et al., 2009). Studies in SH-SY5Y neuroblastoma cells and rat primary cortical cultures further demonstrated the involvement of the proteasomal system in the degradation of pS129 α-Syn (Machiya et al., 2010). Upon inhibition of protein biosynthesis by cycloheximide, pS129 α-Syn half-life was significantly shorter than that of the non-phosphorylated form, indicating that the phosphorylated form is selectively targeted for degradation. Proteasomal inhibition stabilized pS129 α-Syn and increased its half-life,

suggesting that the proteasomal pathway is responsible for pS129 α-Syn turnover. This appeared to occur in an ubiquitin-independent manner. Long exposure to lysosomal inhibitors led to accumulated levels of pS129 α-Syn, but this paralleled the effects on monomeric α-Syn (Machiya et al., 2010). Collectively, these data indicate that the proteasome may be the primary site for the degradation of monomeric pS129 α-Syn. More recent findings have demonstrated that insoluble pS129 α-Syn is also degraded by the proteasome, but the lysosome may play a complementary role (Arawaka et al., 2017, Peng et al., 2018). Extensively aggregated forms of pS129 α-Syn can no longer be degraded by the proteasome and eventually accumulate within LBs (Arawaka et al., 2017).

Crosstalk among different post-translational modifications (PTMs), i.e. phosphorylation, ubiquitination, sumoylation, has been reported to affect protein stability. *In vitro* studies demonstrated that the length of ubiquitin chain and the crosstalk between N-terminal polyubiquitination and C-terminal phosphorylation of α-Syn regulates α-Syn fibril formation, phosphorylation and clearance (Haj-Yahya et al., 2013). Studies in yeast cells showed that the interplay between sumoylation and phosphorylation appears to differentially degrade α-Syn in an autophagic- or proteasomal- dependent manner, respectively (Shahpasandzadeh et al., 2014). Seemingly, the degradation mechanism(s) involved in pS129 α-Syn turnover could be related to the differential conformational states of α-Syn, cellular stress or the interplay among distinct PTMs.

Clearance mechanisms of extracellular α-Syn

The discovery of α-Syn secretion prompted the examination of the paracrine role of extracellular α-Syn in brain homeostasis, which under pathological conditions could contribute to the cascade of events leading to neuronal degeneration (Vekrellis et al., 2011, Marques and Outeiro, 2012). Extracellular α-Syn aggregates can be neurotoxic (Sung et al., 2001), can cause neuroinflammatory effects (Zhang et al., 2005), and are capable of transmission from neuron to neuron *via* the extracellular milieu, thus propagating pathology by a “transfer” and “seeding” mechanism (Danzer et al., 2007). The level of extracellular α-Syn depends both on the rate of α-Syn release from neuronal cells and the rate of its removal through various clearance pathways (Figure 1). It is therefore possible that certain abnormalities or deficits affecting α-Syn clearance could lead to increased extracellular α-Syn accumulation and aggregation, and subsequently to its concomitant detrimental effects. The exact mechanisms and key players responsible for the clearance of extracellular α-Syn species are not clear. However, there is increasing evidence suggesting that such clearance mechanisms might include cell-mediated uptake and

degradation, as well as proteolysis by extracellular proteases (Lee et al., 2008b, Lee et al., 2014).

Extracellular Proteolytic enzymes

Among extracellular proteolytic enzymes, KLK6 (neurosin) is the most studied regarding its effects on α-Syn proteolysis. KLK6 is a secreted serine protease that is predominantly expressed in the CNS (Yamashiro et al., 1997), mostly by oligodendroglia (Yamanaka et al., 1999), and has been detected at high levels in human CSF (Diamandis et al., 2000). KLK6 is a typical member of the KLK family. It is synthesized as pre-pro-enzyme of 244 amino acids containing a signal peptide of 16 amino acids and an activation peptide of 5 amino acids that is cleaved prior to its activation in the extracellular milieu. Recent evidence suggests that its activation may occur *in vivo* by other proteases, such as kallikrein-related peptidase 5 (KLK5) or plasmin and possibly to a lesser degree through autoactivation (Bayes et al., 2004). A role of KLK6 in PD and other Synucleinopathies was first proposed when it was found to co-localize with α-Syn in LB of PD patients (Ogawa et al., 2000, Iwata et al., 2003), although this has not been confirmed in more recent studies (Leverenz et al., 2007, Xia et al., 2008). KLK6 was further identified as capable of catalyzing the limited proteolysis of recombinant α-Syn, thus preventing its aggregation (Iwata et al., 2003). It was suggested that KLK6 is localized to mitochondria and under stress conditions it is released into the cytoplasm where it can cleave intracellular α-Syn (Iwata et al., 2003). Endogenously secreted KLK6 could also cleave the extracellular α-Syn. In the same study, it was also shown that KLK6 cleaves the A53T mutant α-Syn less efficiently, suggesting a differential specificity of KLK6 for wild-type α-Syn. Conversely, later studies showed that intracellularly it is expressed predominantly in the ER-Golgi system and that it exhibits activity only in the extracellular space (Tatebe et al., 2010), while it can cleave both recombinant WT and A53T α-Syn forms equally well (Kasai et al., 2008). Interestingly, KLK6 precursors localize to the ER before secretion, meaning that disruptions in the endosomal-sorting pathway could interfere with this process and contribute to PD pathogenesis. Despite the fact that these findings suggested that KLK6 could also function in the cytoplasm of neuronal cells and could accordingly be involved in intracellular α-Syn catabolism, more work is necessary for the confirmation of this hypothesis.

Significantly, in a study by the Masliah group, KLK6 expression was inversely correlated with α-Syn accumulation in brains with DLB, and the lentiviral-driven expression of mouse KLK6 reduced intracellular and neuropil aggregates of α-Syn and reversed the pathology in WT but not in A53T α-Syn transgenic mice (Spencer et al., 2013). In addition, KLK6 can efficiently

degrade recombinant as well as naturally secreted oligomeric forms of α-Syn (Pampalakis et al., 2017). These studies suggest the potential therapeutic benefit of targeting KLK6 in synucleinopathies.

Of the other human proteases, Matrix Metalloproteinases (MMPs) and plasmin have also been shown to cleave extracellular α-Syn *in vitro*. Sung et al. (Sung et al., 2005) demonstrated that oxidative injury induces the cleavage of extracellular α-Syn released from neuronal cells by MMPs. However, the cleavage of α-Syn by MMP-3, which is induced upon α-Syn overexpression, further induces its aggregation, and the resulting aggregates exhibit enhanced cytotoxicity (Levin et al., 2009). In order of decreased efficiency, MMP14, 2, 1 and 9 can also cleave α-Syn (Sung et al., 2005). MMP1 promotes α-Syn aggregation, but MMP9 does not alter it (Vekrellis et al., 2011).

Plasmin, an extracellular serine protease, is also able to cleave and degrade extracellular α-Syn (Kim et al., 2012). Contrary to MMP3 that cleaves at the C-terminus (Sung et al., 2005, Levin et al., 2009), plasmin mainly cleaves at the N-terminus. C-terminally truncated α-Syn has a greater tendency for aggregation (Crowther et al., 1998, Serpell et al., 2000) and approximately 15% of α-Syn in LBs is C-terminally truncated (Tatebe et al., 2010). Plasmin is derived from its inactive form, plasminogen, by tissue type plasminogen activator (tPA) or urokinase plasminogen activator (uPA) (Schaller and Gerber, 2011). Although plasmin is synthesized mainly in the liver, it has also been detected in the CNS and is mainly expressed in neurons and astrocytes. Plasmin cleaves monomeric and further oligomeric and fibrillar forms of α-Syn irrespective of the familial type of point mutation. However, tPA, uPA and thrombin do not cleave α-Syn. Extracellular α-Syn has also been shown to increase plasminogen activator inhibitor-1 (PAI-1) expression in neurons, astrocytes and microglia and thus may inhibit plasmin activity (Joo et al., 2010), suggesting that the plasmin system may be dysregulated in PD.

Other Clearance Systems for extracellular α-Syn

In addition to proteolytic degradation, other clearance pathways could represent potential therapeutic targets. More specifically, in the context of cell-mediated uptake (endocytosis and phagocytosis), it has been suggested that adjacent cells, including neurons (Lee et al., 2008a) and astrocytes (Lee et al., 2010), as well as the innate (microglia) (Lee et al., 2008b, Kim et al., 2013) and adaptive immune system (infiltrating lymphocytes and autoantibodies) (Masliah et al., 2005) are crucially involved. Cell-mediated clearance pathways including endocytosis or phagocytosis have been reported to clear extracellular α-Syn (Lee et al., 2008a, Konno et al., 2012). Aggregated α-Syn was found to be internalized into neuronal

cells *via* clathrin-dependent endocytosis, then move into the lysosome through the recycling endosomal pathway. Oligomeric extracellular α-Syn may also enter the cell through three potential receptors, HSPG (Ihse et al., 2017), LAG3 (Mao et al., 2016), and α3-NKA (Shrivastava et al., 2015). In contrast, the monomeric α-Syn may be transported into the cytosol through direct translocation across the plasma membrane (Lee et al., 2008a). These data suggest that only aggregated forms of extracellular α-Syn can be cleared by cell-mediated uptake and degradation, and this might represent a mechanism of preventing neurons from exposure to potentially toxic α-Syn. Once inside the cell, the protein may undertake multiple pathways. Endocytic vesicles are directed to the autophagic system for degradation becoming rapidly acidified within hours of treatment before finally co-localizing with markers of late endosomal and lysosomal compartments (Konno et al., 2012, Karpowicz et al., 2017). Microglia rapidly internalize extracellular α-Syn by receptor-mediated endocytosis in a fashion that depends on the activation state of the microglial population (Konno et al., 2012). The internalized α-Syn is directed to the lysosome for degradation, which in turn leads to changes in their phenotype. In fact, α-Syn is shown to interact with toll-like receptor-2 (TLR2) on the microglial cell surface resulting, at least to a great extent, in the subsequent endocytosis and clearance of the imported α-Syn (Kim et al., 2013).

The case of exosomes

Previous work using cell culture systems indicated that oligomeric and monomeric α-Syn follow a calcium-regulated pathway of release that involves, at least in part, the externalization of exosomes, small extracellular vesicles of endosomal origin (Emmanouilidou et al., 2010a). α-Syn can thus be exported within exosomes, suggesting a mechanism through which α-Syn shuttles between the host neurons and the neighboring cells (Danzer et al., 2012, Karpowicz et al., 2017). Exosome-associated α-Syn oligomers are more likely to be taken up by recipient cells, and to induce toxic effects, as compared with free α-Syn oligomers (Danzer et al., 2012). In the brain, exosomes are implicated in intercellular communication of neurons, by modulating local protein expression, triggering localized signaling events and regulating synapse formation (Chivet et al., 2013). Along with the paracrine contact through the exchange of secreted molecules, glia and neurons can communicate by releasing and receiving extracellular vesicles, which allows a coordinated regulation of action across long distances. In addition, exosomes have been assigned with the important role of removing unwanted molecular material as means for cell maintenance. In the CNS, such waste elimination is made possible *via* the endocytosis of exosomes by glial cells, including microglia and astrocytes. Given these fundamental

functions of exosomes, it is now suggested that exosome-associated α-Syn could facilitate two different processes: the spreading of pathology *via* a cell-to-cell transfer mechanism or the disposal of selected toxic α-Syn species to maintain neuronal homeostasis. These possible hypotheses have only recently started to be investigated. However, they seem to depend on two critical parameters: the uptake mechanism of exosomal α-Syn and the particular type of recipient cell (Fig. 2).

The potential of exosome-associated α-Syn to facilitate the transmission of aberrant α-Syn species through synaptically connected neuronal networks requires the endocytosis of pathological α-Syn by neurons and has recently been reviewed elsewhere (Valdinocci et al., 2017, Yuan and Li, 2019). On the other end, the exosome-mediated removal of aggregated α-Syn species is thought to be accomplished *via* the internalization of exosomes by microglia and astrocytes, the brain cells that are responsible for maintaining brain microenvironment homeostasis (Filippini et al., 2019). Even though the uptake mechanism still remains a matter of debate, its kinetics seem to be dependent on the particular recipient cell type; microglia are more efficient in the uptake of exosome-associated extracellular α-Syn compared to neurons or astrocytes (Danzer et al., 2012). In all cases, the encapsulation of α-Syn cargo inside exosomes serves to accommodate the entry across specific recipient cell lipid bilayers. Once internalized, exosomal α-Syn escapes the endosomal compartment and is targeted to the lysosome for degradation. Although receptor-mediated endocytosis has been proposed as a main route of exosome internalization, a more recent study using pharmacological inhibitors failed to confirm this (Delenclos et al., 2017). According to this study, the entry of exosome-associated α-Syn in recipient H4 neuroglioma cells could be attributed neither to caveolin-mediated endocytosis nor macropinocytosis. These findings suggest that different uptake pathways or distinct molecular components could operate in different cell systems for exosomal α-Syn. Regarding microglia the predominant hypothesis suggests that the exosomes containing α-Syn are sorted due to the specific lipid composition of their cell membrane. This seems to be a common pathway in the microglia-mediated clearance of toxic aggregated proteins including, amyloid-β and α-Syn (Paolicelli et al., 2018).

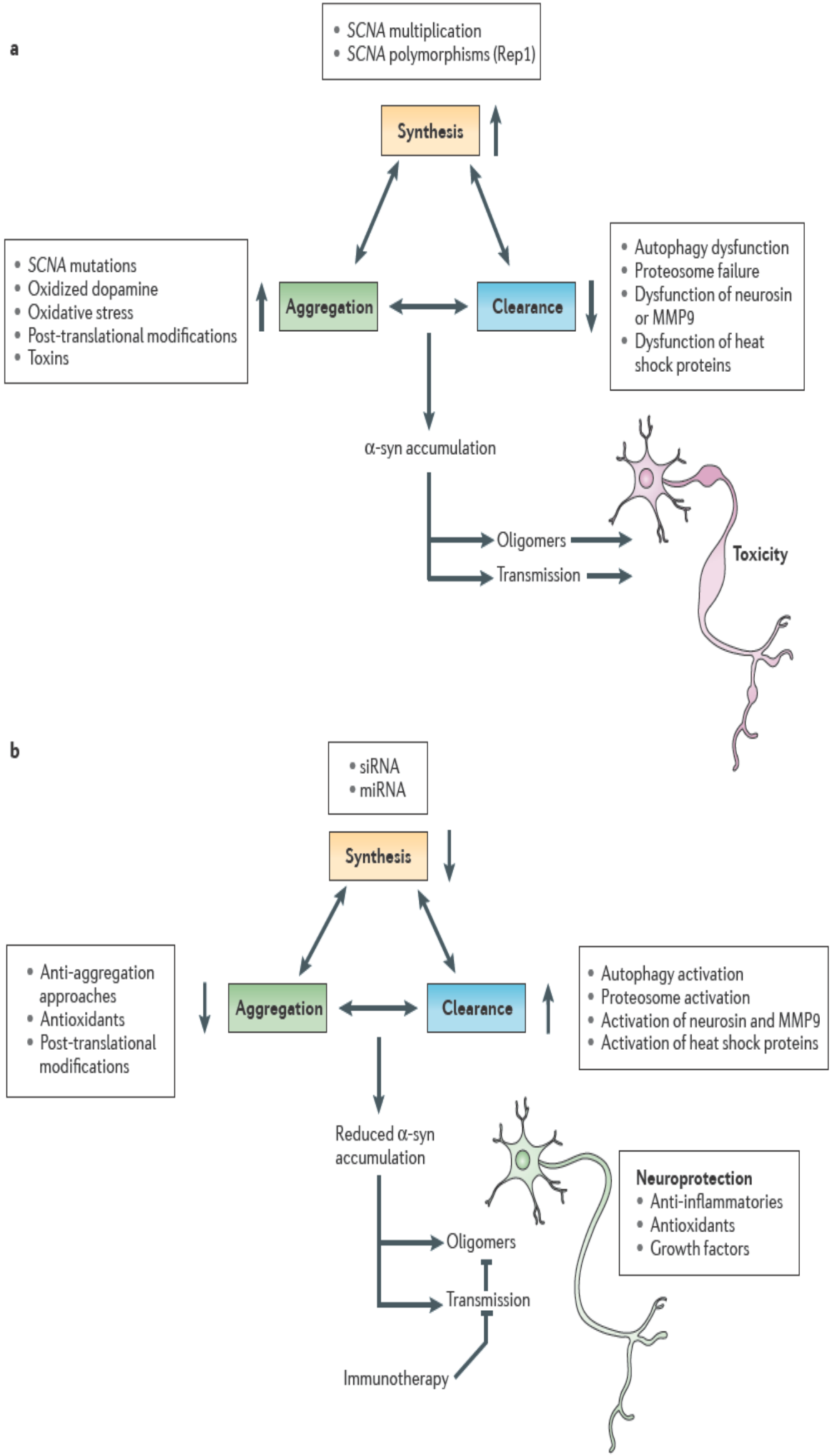
Like microglia and neurons, astrocytes are capable of taking up extracellular α-Syn, which induces the production of pro-inflammatory cytokines and chemokines by these cells (Lee et al., 2010). Internalized α-Syn aggregates are also thought to be efficiently degraded by astrocytic lysosomes. Several studies have indicated that astrocytes have the ability to secrete and also to receive exosomes from other cell sources (Verkhratsky et al., 2016). The mechanism of internalization is reported to be receptor-independent, and could involve

phagocytosis, clathrin-mediated or lipid raft-mediated endocytosis. As in the case of microglia, selective vesicle targeting requires efficient sorting signals such as the presence of specific proteins or lipids in the exosomal membrane. Importantly, Ngolab et al. demonstrated recently that administration of human brain-derived exosomes containing pathological forms of α-Syn in mouse brain was able to incite α-Syn aggregation in astrocytes even though this occurred to a very low extent compared with neurons (Ngolab et al., 2017).

It is more than likely that several of the aforementioned mechanisms work together to clear extracellular α-Syn, and deficiency in any one of them could potentially destabilize this delicately balanced system, leading to further aggregation and propagation of α-Syn.

Concluding remarks

As made clear throughout this review, the manner of α-Syn clearance is complex and multifaceted. Clearance mechanisms operate both within and outside neurons to remove α-Syn conformers, maintaining an equilibrium under homeostatic conditions. A deeper understanding of how this delicate balance may be perturbed in disease states, and may contribute to disease pathogenesis will be needed in order to harness such pathways for therapeutic benefit.


a
• SCNA multiplication
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Synthesis
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• Oxidized dopamine
• Oxidative stress
• Post-translational modifications
• Toxins
Aggregation
Clearance
• Autophagy dysfunction
• Proteosome failure
• Dysfunction of neurosin or MMP9
• Dysfunction of heat shock proteins
α-syn accumulation
Oligomers
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Synthesis
• Anti-aggregation approaches
• Antioxidants
• Post-translational modifications
Aggregation
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• Autophagy activation
• Proteosome activation
• Activation of neurosin and MMP9
• Activation of heat shock proteins
Reduced α-syn accumulation
Oligomers
Transmission
Immunotherapy
Neuroprotection
• Anti-inflammatories
• Antioxidants
• Growth factors

Figure 1 | Cellular events controlling α-Syn levels and possible therapeutic strategies to combat α-Syn accumulation and transmission. **(a)** α-Syn levels are tightly regulated by the balance between the rates of α-Syn synthesis, clearance and aggregation. Abnormalities affecting α-Syn synthesis and stability or clearance deficits may increase the propensity of α-Syn to aggregate and accumulate. **(b)** Strategies to reduce α-Syn accumulation include decreasing α-Syn synthesis, activating mechanisms/proteins involved in clearance, using antibody-mediated immunotherapy and using anti-aggregating, antioxidant or post-translational modification approaches. Adapted from (Lashuel et al., 2013).

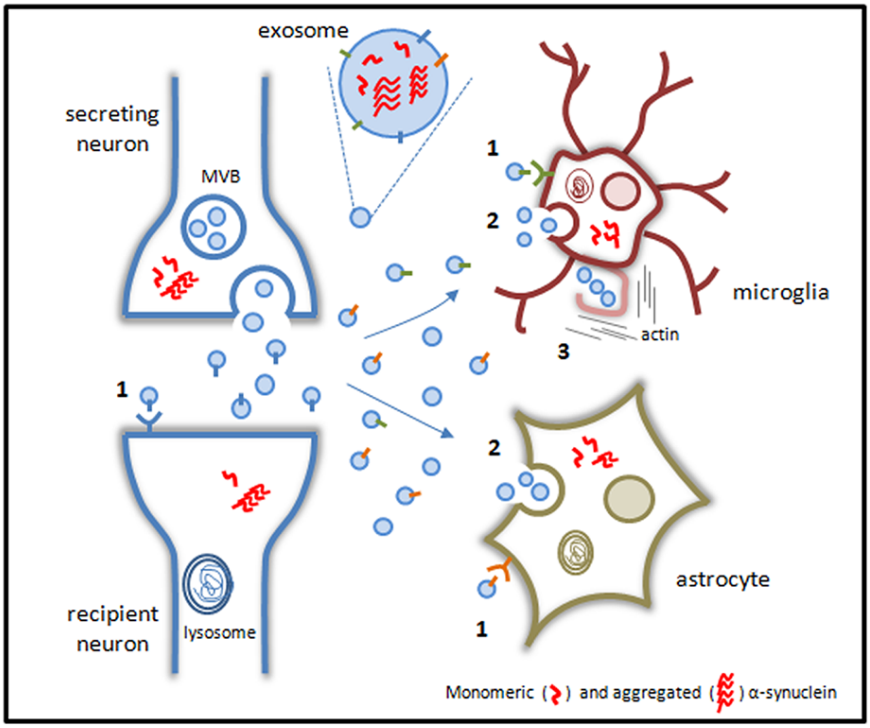

Figure 2. Mechanisms involved in exosome-mediated clearance of α-Syn. Exosomes that contain α-Syn conformers (monomers, oligomers, aggregates) are secreted by neurons and are uptaken by neurons, astrocytes and/or microglial cells where they are thought to be delivered to lysosomes for degradation. The cell type of destination for these exosomes is defined by the specific lipid composition of exosomal membrane and the presentation of specialized receptor molecules in the exosome surface. Astrocytes could internalize exosome-associated α-Syn by receptor-mediated endocytosis (1) and phagocytosis (2) whereas microglia could also utilize actin cytoskeleton-induced macropinocytosis (3).

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