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Review

Alpha-synuclein: from secretion to dysfunction and death

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The aggregation, deposition, and dysfunction of alpha-synuclein (aSyn) are common events in neurodegenerative disorders known as synucleinopathies. These include Parkinson's disease, dementia with Lewy bodies, and multiple system atrophy. A growing body of knowledge on the biology of aSyn is emerging and enabling novel hypotheses to be tested. In particular, the hypothesis that aSyn is secreted from neurons, thus contributing to the spreading of pathology not only in the brain but also in other organs, is gaining momentum. Nevertheless, the precise mechanism(s) of secretion, as well as the consequences of extracellular aSyn species for neighboring cells are still unclear. Here, we review the current literature and integrate existing data in order to propose possible mechanisms of secretion, cell dysfunction, and death. Ultimately, the complete understanding of these processes might open novel avenues for the development of new therapeutic strategies.
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Facts

- Alpha-synuclein is associated with both familial and idiopathic cases of Parkinson's disease.
- The precise function of alpha-synuclein remains equivocal.
- Alpha-synuclein misfolds and forms protein aggregates in Parkinson's disease and in various *in vitro* and *in vivo* models of synucleinopathies.
- Alpha-synuclein has been detected in human and mouse CSF and in the media of cultured cells.

Open Questions

- Is alpha-synuclein normally secreted from cells or is it released from dying/damaged cells? How is it secreted?
- What are the effects of secreted forms of alpha-synuclein?
- What is the toxic genus of alpha-synuclein?
- Is the spreading of alpha-synuclein pathology a prion-like phenomenon?

Alpha-synuclein and Synucleinopathies

Alpha-synuclein (aSyn) is a 140 amino-acid protein that was originally identified in association with synaptic vesicles in the presynaptic nerve terminal[1] and has been shown to interact with membranes both *in vitro* and *in vivo*.[2–4] It is highly abundant in the brain and also present in other tissues, including red blood cells.[5–8] aSyn is a member of a protein family of synucleins, together with beta (β)- and gamma (γ)-synuclein. These proteins share a characteristic consensus sequence (KTKEGV) that is repeated about six times at the N-terminal part of the protein. β-synuclein shares the closest homology (90% homology in the N-terminus and 33% homology in the C-terminus) with aSyn.[5]

Point mutations in the *SNCA* gene, encoding for aSyn, and multiplications of the *SNCA* locus have been identified in families with autosomal-dominant forms of Parkinson's disease (PD).[9] Genome-wide association studies linked single-nucleotide polymorphisms in the *SNCA* gene with increased susceptibility to sporadic PD.[10] Moreover, *SNCA* gene polymorphisms have also been associated with increased risk of multiple system atrophy (MSA).[11]

In PD, aSyn is found as a major component of Lewy bodies and Lewy neurites, the hallmark protein inclusions made up primarily of insoluble and fibrillar aSyn protein.[12] aSyn also accumulates in dementia with Lewy bodies (DLB) and MSA.[13] In MSA, aSyn is found predominantly within oligodendrocytes as cytoplasmic inclusions.[14] These disorders share the accumulation of aSyn aggregates as a pathological feature and are collectively known as synucleinopathies. Additionally, aSyn was also identified as a component of amyloid from brain tissues of Alzheimer's disease (AD) patients.[15]

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Abbreviations: aSyn, alpha-synuclein; PD, Parkinson's disease; DLB, dementia with Lewy bodies; SNP, single-nucleotide polymorphisms; MSA, multiple system atrophy; AD, Alzheimer's disease; Aβ, amyloid-beta peptide; PRA1, prenylated Rab acceptor protein 1; GDI, GDP dissociation inhibitors; CSPα, cysteine-string protein alpha; LTP, long-term potentiation; CSF, cerebrospinal fluid; VPS, vacuolar protein sorting
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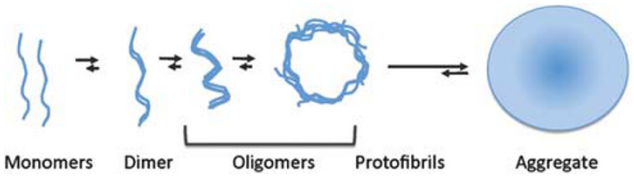

Figure 1 Schematic representation of the aSyn aggregation process. Monomeric forms of aSyn associate to form dimers and oligomers that grow into protofibrils and, finally, form mature fibrillar structures

The presence of a hydrophobic 12 amino-acid sequence in the central part of the protein is required for the oligomerization and fibrillization of aSyn.[16] Deletion or disruption of this domain blocks the capacity of aSyn to form amyloid fibrils. The process of aSyn aggregation (Figure 1) has been studied in detail in an attempt to identify the toxic species responsible for neuronal dysfunction and death. However, it is still unclear what is/are the toxic forms of the protein. There is evidence showing that inhibition of aSyn aggregation process is associated with a decrease of aSyn toxicity.[17,18] However, similarly to the case of amyloid-beta (Aβ) plaques in AD, it was suggested that the fibrillar forms of aSyn might not represent the most toxic aSyn species. Instead, pre-fibrillar, soluble oligomeric species (comprising multiple aSyn molecules) are now suggested to be the main toxic aSyn species, with amyloid aggregates possibly serving as a reservoir for these oligomeric species (Figure 1). *In vitro* studies showed that the acceleration of oligomerisation, and not fibrilization, is the distinctive shared property of the A53T and A30P aSyn mutations linked to early-onset PD.[19] The neurotoxic effects of aSyn oligomers were also studied *in vivo*, using animal models of synucleinopathies. In these studies, aSyn mutant variants that promote oligomer formation were designed and tested for toxicity *in vivo*. In one of these studies, overexpression of aSyn variants with impaired fibril formation ability caused increased toxicity in *C. elegans* and *Drosophila* models. Furthermore, the increasing inability of the mutants to form fibrils was directly correlated with toxicity and neurodegeneration.[20] In another study, aSyn variants that were shown to promote oligomer formation caused most prominent dopaminergic cell death upon lentiviral injection into rat *substantia nigra* (SN).[21] Together, these studies provide evidence for the importance of soluble oligomers as the prominent toxic species in synucleinopathies, although the precise size and type of the toxic oligomeric species remains to be determined.

Recently, it was hypothesized that PD, as well as other neurodegenerative disorders associated with protein misfolding and aggregation, might be a prion-like disease where pathological forms of aSyn spread throughout the brain. In particular, the spreading of aSyn pathology could contribute to the progression of neurodegeneration and clinical symptoms.[22–24] Nevertheless, the molecular determinants underlying aSyn secretion, extracellular effects and transmission of pathology are still unclear.

aSyn Function in Vesicle Trafficking

Accumulating evidence from studies involving different model organisms, including yeast, worm, fly, and mammalian PD models, shows an association between aSyn and defects in vesicle trafficking.

aSyn was shown to induce aggregation of several yeast Rab GTPase proteins and this aggregation was more pronounced in yeast mutants that produce high levels of acidic phospholipids.[25] Furthermore, aSyn overexpression in yeast causes cytoplasmic lipid droplet and vesicle accumulation. This effect is associated with an aSyn-induced block of ER-to-Golgi trafficking.[26–28] Importantly, results from a genetic screen in yeast identified modifiers of ER-to-Golgi trafficking block as the most prominent suppressors of aSyn toxicity.[27] The Rab GTPase Ypt1p (orthologue of the mammalian Rab1) was among the strongest suppressors, restoring normal ER-to-Golgi complex trafficking and aSyn localization to the plasma membrane.[27] The protective effect of Rab1 against aSyn toxicity was further verified in rat primary midbrain neuronal cultures and in a *Drosophila* and a *C. elegans* model of PD, where Rab1 overexpression significantly reduced dopamine (DA) neuron cell death.[27]

An inhibitory effect of aSyn on ER-to-Golgi complex trafficking was verified also in mammalian kidney and neuroendocrine cells, with the A53T aSyn mutant causing stronger inhibition then the wild-type form.[29] The aSyn-elicited trafficking defect can be rescued by the co-overexpression of Ykt6p, a vesicle-associated SNARE that promotes vesicle fusion, and has been amongst the suppressors of aSyn toxicity previously identified in yeast.[27,29]

The mechanism of the aSyn-induced trafficking block was further addressed in a recent study showing that in neuronal cells, aSyn co-localizes and interacts with prenylated Rab acceptor protein 1 (PRA1).[30] PRA1 is conserved from yeast to human and localizes predominantly in the Golgi and late endosomes.[31–33] It was shown to interact with multiple prenylated Rab GTPases and regulate their recycling through inhibiting the removal of Rab from the membrane by GDP dissociation inhibitors.[34] In addition to the previously described accumulation of cytoplasmatic vesicles upon aSyn overexpression, these vesicles localize in the periphery of the cytosol when aSyn is co-expressed together with PRA1.[30] These results suggest a synergistic action of aSyn and PRA1 in interfering with vesicle trafficking and recycling.

In addition, aSyn was recently shown to induce vulnerability to perturbations in retrograde endosome–Golgi transport pathway in a yeast model.[25]

Together these studies suggest that aSyn causes defects in multiple vesicle trafficking steps.

Effects of aSyn on Neuronal Function and Synaptic Transmission

Although the physiological function of aSyn is not fully understood, there is evidence suggesting that aSyn has a role in neurotransmitter release[35–38] and in the maintenance of the reserve pool of synaptic vesicles in primary hippocampal neurons.

Mice lacking the synaptic co-chaperone cysteine-string protein alpha (CSPα) present progressive neurodegeneration and impairment in synaptic function. Interestingly, transgenic expression of aSyn rescued neurodegeneration and motor impairment resulting from CSPα deficiency. Moreover, aSyn

was able to reverse the SNARE-complex assembly impairment observed in the CSPα knockout mice.[36] These data indicate a physiological neuroprotective function of aSyn at the synapse.

Recently, a direct interaction between aSyn and SNARES was demonstrated.[39] aSyn was found to bind the N-terminus of SNARE protein synaptobrevin-2 directly by its C-terminus. This study also brought additional evidence that aSyn enhances SNARE-complex assembly both *in vitro* and *in vivo*.[39] Loss of synuclein function leads to an age-dependent impairment of SNARE-complex assembly and loss of neuronal function as shown in alpha-, β- and γ-synuclein triple knockout mice.[40]

However, another study suggested an indirect inhibitory effect of aSyn on SNARE-complex assembly through sequestering of arachidonic acid. Arachidonic acid was shown to stimulate SNARE-complex formation and exocytosis and these effects were negatively regulated by aSyn both *in vitro* and *in vivo*.[41]

Together, these studies suggest a physiological function of aSyn at the synapse in modulating SNARE-complex assembly.

The role of aSyn in neurotransmitter release is mainly based on its regulation of synaptic vesicle recycling. aSyn knockout mice display altered DA release in response to paired stimuli, reduction in striatal DA and an attenuation of DA locomotor response to amphetamine.[42] Another *in vivo* study showed that lack of aSyn leads to a permanent increase of the vesicle refilling rate in the DA readily releasable pool, maintaining stable DA release during stimulation in contrast to decline of DA release observed in normal conditions.[43] Together, these findings suggest that aSyn is an activity-dependent, negative regulator of DA neurotransmission. Moreover, aSyn has also been shown to regulate synaptic glutamate release. Electrophysiology studies in brains from aSyn knockout mice suggest that lack of aSyn impairs mobilization of glutamate from the reserve pool.[44]

Studies in animal models of PD pathology implicate aSyn in functional synaptic deficits. In a transgenic mouse model overexpressing A30P mutant aSyn, a reduction of locomotor activity accompanied by a reduced size of the DA storage pool was observed.[45] In the striatum of a transgenic mouse model expressing truncated human aSyn, synaptic accumulation of aSyn was accompanied by an age-dependent redistribution of the synaptic SNARE proteins as well as by reduction in DA release.[46] This redistribution of SNAREs is also observed in PD brain samples. In cultured neurons from another PD mouse model, aSyn overexpression leads to loss of several presynaptic proteins and neurotransmitter release deficits.[47] A modest overexpression of aSyn that does not lead to overt toxicity causes significant inhibition of neurotransmitter release through inhibition of vesicle reclustering after endocytosis.[48] These results indicate that increased levels of aSyn lead to a defect in synaptic vesicle recycling that precedes detectable neuropathology.

Imaging studies in patients also point to synaptic dysfunction and neurotransmitter deficiencies in synucleinopathies including PD and DLB.[49] Recently, small aSyn detergent-insoluble aggregates with presynaptic localization were detected abundantly in patients with DLB, together with significant synaptic pathology with almost complete loss of dendritic spines at the postsynaptic area.[50,51] It has been suggested that it is the presynaptic aSyn aggregation-linked synaptic dysfunction, rather than cell death, that leads to neurodegeneration in DLB and PD.[51]

Recently, the impact of different aSyn species (monomers, oligomers, and fibrils) on synaptic transmission was explored. Interestingly, oligomeric forms of aSyn were shown to increase synaptic transmission and to impair long-term potentiation,[52] a paradigm that mimics the process of memory formation. The effects of aSyn were mediated by NMDA receptor activation, in similarity to what is also known for Aβ oligomers (Diogenes and Outeiro, personal communication).

Altogether, the effects of aSyn on neuronal function are emerging and will provide important insight into the molecular basis of PD and other synucleinopathies.

Secretion of aSyn and Disease Propagation

The absence of a secretory signal peptide sequence in aSyn suggested it might be purely an intracellular protein and its pathological function was, therefore, studied in a cell autonomous context. However, this view was challenged by the presence of aSyn in biological fluids such as cerebrospinal fluid (CSF) and blood plasma of both PD and normal subjects.[53–55] Recently, aSyn release mechanisms as well as possible functions of extracellular aSyn have been intensively studied.

aSyn was shown to be secreted into the culture medium in various cell lines overexpressing aSyn.[53,56,57] Moreover, secretion of endogenous aSyn from rat embryonic cortical neurons was also demonstrated.[57] Both monomeric and aggregated forms of aSyn were found to be secreted (Figure 2).[57,58]

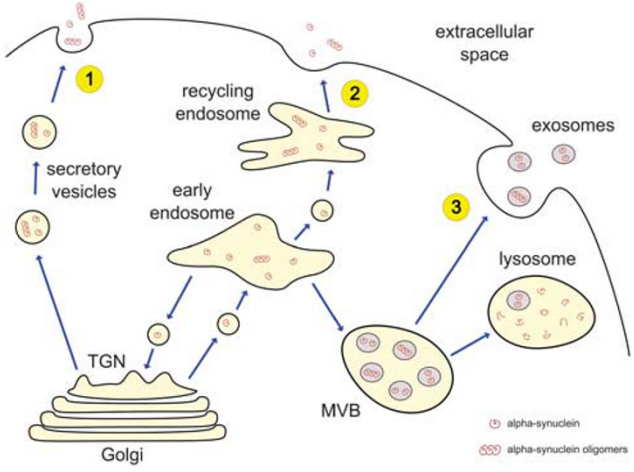

Figure 2 Proposed mechanisms of aSyn secretion. aSyn can be secreted both in its monomeric or aggregated forms by non-classical exocytic or endocytic pathways. aSyn can be directly integrated into secretory vesicles and subsequently released by exocytosis (**1**). Alternatively, aSyn can be translocated to early endosomes. From early endosomes, aSyn protein can either be released to the extracellular space through the recycling endosome (**2**) or incorporated to intraluminal vesicles of multivesicular bodies (MBV). MVB cargo including aSyn can be directed to degradation by fusion with lysosomes or to secretion by fusion with the plasma membrane and release of exosomal vesicles (**3**). TGN = trans-Golgi network

The mechanisms of aSyn secretion are not fully understood, but there is evidence showing that aSyn can be secreted by non-classical, ER/Golgi-independent protein export pathway (Figure 2).[57] Upon overexpression in SH-SY5Y cells, aSyn localizes in large dense core vesicles. Interestingly, it was shown that this intravesicular fraction of aSyn is more prone to aggregation compared with cytosolic aSyn protein.[57] In another study, DA was shown to induce aSyn aggregation in the vesicle fraction and to increase aSyn secretion levels.[59] It has also been suggested that the release of aSyn is upregulated under cellular stress conditions that cause accumulation of misfolded/damaged proteins.[60]

Additionally, a fraction of aSyn was shown to be secreted in association with exosomes by a calcium-dependent mechanism (Figure 2)[61] and exosomal aSyn release was found to be increased upon lysosomal dysfunction.[62] However, a very recent study reports no enrichment of aSyn in exosomal pellet of neuronal cells overexpressing aSyn as well as an absence of aSyn-positive signal in exosomal fraction derived from CSF of PD patients and control subjects.[63] This study further shows that in neuronal cells aSyn is present in endosomal compartments (Figure 2), and that aSyn secretion and lysosomal targeting is regulated by vacuolar protein sorting 4. Moreover, it was suggested that the recycling pathway regulated by Rab11 has functional relevance in aSyn secretion (Figure 2).[63]

In recent years, extracellular aSyn has received a lot of scientific attention due to its potential role in disease initiation and progression. Considering the progressive nature of neurodegenerative disorders as well as the defined, stepwise spreading of Lewy body pathology in PD,[22] the idea of extracellular aSyn as a pathogenic 'prion-like' agent is extremely appealing. This scenario is strengthened by observations describing the presence of aSyn-positive Lewy-body-like inclusions in long-term mesencephalic transplants in PD patients.[23,64] These findings suggest host-to-graft propagation of aSyn-associated pathology. Subsequent *in vitro* experiments showed inter-neuronal transmission of aSyn (Figure 3).[58,65] Moreover, the host-to-graft transmission of aSyn has also been observed *in vivo* in aSyn transgenic mouse models.[24,65] This transmission process seems to rely on endocytic uptake of aSyn by the recipient neurons.[24,66]

All of these findings indicate that aSyn can be both secreted and transmitted between neuronal cells. Moreover, there is evidence suggesting that aSyn secretion is stimulated by cellular stress conditions including lysosomal dysfunction.

aSyn-induced Neuroinflammation and Cell Death

Although many studies showed secretion and cell-to-cell transmission of aSyn, this phenomenon might not, *per se*, be sufficient to explain the spread of aSyn Lewy-body-like pathology and progressive neurodegeneration observed in PD. There is additional evidence, however, that aSyn can act as a nucleation/seeding agent for aggregation and can affect the viability of the recipient neurons (Figure 3).

Exogenous aSyn fibrils were shown to seed the formation of Lewy-body-like intracellular inclusions in cultured cells.[67] Furthermore, there is evidence of inclusion formation via aSyn cell-to-cell transmission both in cell co-cultures and

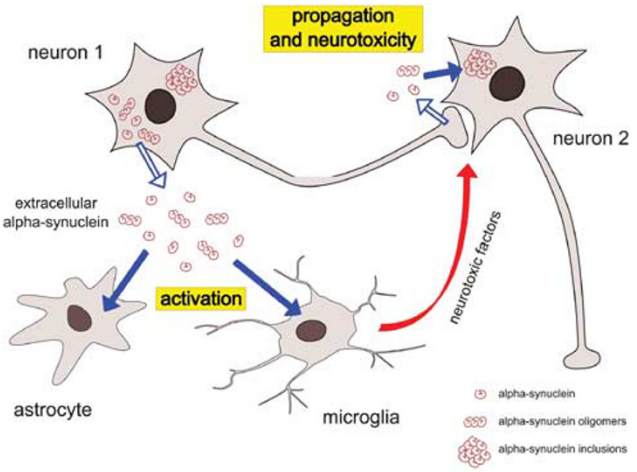

Figure 3 The role of extracellular aSyn in neuroinflammation, neurotoxicity, and spread of pathology. aSyn can be actively secreted or released by dying neurons to the extracellular space. Extracellular aSyn can then activate surrounding astrocytes and microglia, eliciting glial pro-inflammatory activity. Upon activation microglia produce pro-inflammatory cytokines, nitric oxide, and reactive oxygen species, which may be toxic to neurons. aSyn can be directly transferred between neurons, leading to propagation of aSyn aggregation process and compromising the viability of the recipient neuron

in vivo in grafted cells (Figure 3).[24,65] Importantly, in addition to seeding aSyn aggregation, neuron-derived extracellular aSyn triggered apoptotic process in recipient neurons.[65]

The neurotoxic effect of extracellular aSyn oligomeric species was addressed in several studies. Recombinant aSyn oligomers can be taken up by neurons in culture and trigger cell death.[68,69] Furthermore, aSyn oligomers secreted from neuronal cells were also shown to induce toxicity in recipient neurons,[58] an effect that was inhibited by Hsp70. Moreover, treatment of the conditioned media containing neuron-derived aSyn with oligomer-interfering compounds rescued the recipient neuronal cells from toxicity.[61] Together, these studies indicate an important role of extracellular aSyn in the progression of PD-linked pathological processes, propagation of inclusion bodies and neurodegeneration.

The importance of extracellular aSyn in PD pathology is further supported by the role of aSyn in triggering neuroinflammatory glial responses. Several studies indicate that an inflammatory process in the SN, characterized by the presence of activated microglia and the secretion of pro-inflammatory and neurotoxic factors, may contribute to either initiation and/or aggravation of neuronal degeneration.[70–72] Studies of biological fluids from PD patients further support a role for neuroinflammatory processes in PD, as increased levels of pro-inflammatory cytokines such as interleukin-1 β (IL1-β), interleukin-2 (IL-2), interleukin-6 (IL-6) or tumor necrosis factor-alpha (TNF-α) were found in CSF or serum of PD patients.[73–75]

Various other studies focused on the investigation of the extracellular effects of aSyn on glial cell activation (Figure 3).[76–80] Upon aSyn exposure *in vitro*, microglia cells produce increased levels of NO, TNF-α and IL1-β[77,78]. The A30P and E46K mutants of aSyn were shown to elicit stronger pro-inflammatory and immunoregulatory responses when

compared with wild-type aSyn.[81] In addition, the induction of microglia classical activation by extracellular aSyn also includes modulation of toll-like receptor expression.[77] Extracellular aggregated aSyn added to neuron-glia culture lead to microglia activation and enhanced dopaminergic neurodegeneration (Figure 3).[76] In this study, microglial enhancement of aSyn-mediated neurotoxicity was shown to be dependent on phagocytosis of aSyn and activation of NADPH oxidase with production of reactive oxygen species.[76] Recently, direct activation of the inflammatory response in a microglial cell line by neuron-derived extracellular aSyn was described.[82]

A direct effect of extracellular aSyn on microglia activation *in vivo* was described in mice injected with human wild-type aSyn protein into the SN. This treatment induced expression of pro-inflammatory cytokines, the expression of endothelial markers of inflammation and microglial activation.[78] Moreover, it was demonstrated that the presence of extracellular aSyn made microglia more susceptible to systemic pro-inflammatory challenge.[78]

In addition to the effects on microglia, aSyn has also been shown to induce astrocytic responses (Figure 3). Transfer of aSyn from neurons to astrocytes was demonstrated in culture as well as *in vivo* in brains of transgenic mice expressing human aSyn.[79] The transmission of neuronal aSyn led to intra-astrocytic aSyn accumulation and triggered astrocytic inflammatory response.

Together these different studies provide evidence for the role of extracellular aSyn in modulating glial function and neuroinflammation, further supporting the importance of the study of the mechanisms by which aSyn reaches extracellular compartments.

Therapeutic Intervention and Future Perspectives

The process of aSyn aggregation is thought to be associated with both a gain of toxic function and to haploinsufficiency, by sequestering the wild-type protein and therefore reducing the amount of functional aSyn available in the cell. Although accumulating evidence suggests oligomeric forms of aSyn might constitute the toxic genus, it is still currently unclear what is the precise form of the toxic aSyn species. Thus, one possible intervention strategy might be to interfere with aSyn aggregation in order to reduce the levels of the toxic species.

Recently, aSyn was suggested to exist, physiologically, as a folded tetramer.[83] Moreover, it was shown that the tetrameric form of aSyn resists aggregation. Interestingly, the stabilization of the native structure of proteins, preventing their initial misfolding, is an appealing intervention strategy. For oligomeric proteins, such as transthyretin, for example, compounds capable of stabilizing the functional tetrameric structure hold great potential and are in the process of being developed.[84,85] In light of the recent findings for aSyn, and despite the current controversy in the field, it is tempting to speculate that a similar strategy might be envisioned for PD and other synucleinopathies.

Another line of possible intervention is linked with the emerging role of extracellular aSyn in disease progression. Interfering with either the process of aSyn secretion or with the neuronal uptake of extracellular aSyn might prove beneficial for stopping or slowing down the propagation of aSyn pathology in PD and other synucleinopathies. In addition, removal of aSyn from the extracellular space might decrease detrimental inflammatory processes. One promising approach is immunotherapy, as immunization with human aSyn reduces the accumulation of aggregated aSyn and reduces neurodegeneration in transgenic mice overexpressing aSyn.[86] Thus, immunotherapy with antibodies promoting the removal of extracellular aSyn species might delay the progression of the disease.

Altogether, the recent progress in our understanding of the molecular mechanisms underlying aSyn secretion and cytotoxicity might pave the way for the development of novel strategies for therapeutic intervention in PD and other synucleinopathies.

Conflict of Interest

The authors declare no conflict of interest.

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