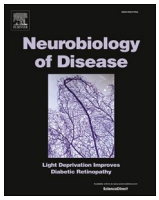
Neurobiology of Disease 168 (2022) 105696

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi



Review

Extracellular alpha-synuclein: Sensors, receptors, and responses

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ARTICLE INFO

Keywords:
Extracellular alpha synuclein
Spreading
Inflammation
Immune responses

ABSTRACT

Synucleinopathies are a group of progressive neurodegenerative diseases known for the accumulation of insoluble aggregates containing the protein alpha-synuclein (aSyn). Recently, it has been assumed that pathology spreads in the brain during disease progression, implying that, at some point in the process, aSyn may exist outside of cells. In this context, extracellular-aSyn (e-aSyn) might transduce signals to the inside of the cells it interacts with, and/or be internalized by different types of cells through the extracellular matrix. Both negatively charged lipids and membrane receptors have been hypothesized as modulators of the loss of cellular homeostasis and cytotoxicity, and of the internalization of e-aSyn. Internalized e-aSyn causes the disruption of multiple cellular processes such as the autophagy lysosomal pathway (ALP), mitochondrial function, endoplasmic reticulum (ER)-stress, UPR activation, or vesicular transport. These processes happen not only in neurons but also in glial cells, activating inflammatory or anti-inflammatory pathways that can affect both neuronal function and survival, thereby affecting disease progression.

In this review, we explore possible effects e-aSyn, all the way from the extracellular matrix to the nucleus. In particular, we highlight the glial-neuronal relationship as this is particularly relevant in the context of the spreading of aSyn pathology in synucleinopathies.

1. Introduction

Synucleinopathies, including Parkinson's disease (PD), dementia with Lewy bodies (DLB), or multiple system atrophy (MSA), are a group of neurodegenerative diseases characterized by the abnormal deposition of aggregated forms of alpha-synuclein (aSyn), known as Lewy bodies (LBs), Lewy neurites (LNs), or glial cytoplasmic inclusions (GCIs) (Spillantini et al., 1997; Tu et al., 1998), in different cell types in the brain (Brás et al., 2020a). These aggregates are enriched in phosphorylated aSyn in serine 129 (paSyn) (Anderson et al., 2006) and in truncated forms of the protein (Liu et al., 2005; Kellie et al., 2014), suggesting that conformational changes in aSyn may explain the cytotoxicity found in some aggregated states and subsequent progression and differences in the severity of the different pathologies. In addition, the contribution of aSyn to synucleinopathies is further supported by familial forms of the diseases associated with point mutations and multiplications of the gene encoding for aSyn (*SNCA*) (Chartier-Harlin et al., 2004; Polymeropoulos et al., 1997; Krüger et al., 1998; Zarranz et al., 2004; Lázaro et al., 2014; Fujioka et al., 2014; Lesage et al., 2013).

In physiological conditions, intracellular aSyn is thought to be mainly located in the presynaptic terminals (Maroteaux et al., 1988) of neurons, binding to the synaptic vesicle membrane (Man et al., 2021). The physiological function of aSyn is often associated with the recycling

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https://doi.org/10.1016/j.nbd.2022.105696
Received 6 December 2021; Received in revised form 28 February 2022; Accepted 15 March 2022
Available online 20 March 2022

and release of synaptic vesicles (Cabin et al., 2002; Gedalya et al., 2009; Murphy et al., 2000). Intracellular aSyn interacts with anionic lipids and vesicle-associated membrane protein 2 (VAMP2), a protein that participates in neurotransmitter release, promoting synaptic vesicle clustering (Diao et al., 2013). aSyn can also modulate SNARE-mediated vesicle fusion by interacting directly with the lipid membranes (Dewitt and Rhoades, 2013).

Although our current understanding of the molecular underpinnings of synucleinopathies remain elusive, several factors are thought to play a role in the aggregation of aSyn including aging (Van Den Berge et al., 2021), environmental factors such as toxins (Jang et al., 2009; William Langston et al., 1983; William Langston et al., 1984), mutations (Chartier-Harlin et al., 2004; Polymeropoulos et al., 1997; Krüger et al., 1998; Zarranz et al., 2004; Lázaro et al., 2014; Fujioka et al., 2014; Lesage et al., 2013), and chemical alterations, such as posttranslational modifications (PTMs) in aSyn (de Oliveira et al., 2017; Vicente Miranda et al., 2017; Beyer and Ariza, 2013; Oueslati et al., 2010).

2. Prion-like spreading of aSyn

Different aggregated forms of aSyn have been shown to spread from cell to cell (Henrich et al., 2020; Henderson et al., 2019; Prusiner et al., 2015), possibly in a prion-like manner, thereby affecting different tissues and anatomically-connected brain areas (Braak et al., 2003). In this context, the study of the interaction between extracellular aSyn (here abbreviated as e-aSyn) and different cell types, not only provides us key information regarding the physiological function of aSyn but also about its effects upon entry into the cell. E-aSyn can play physiological and also pathological roles, but this is still not well understood. During the spreading of pathology aSyn can be released from the cytosol of a cell to the extracellular matrix, and then taken up by neighboring cells. Thus far, studies focusing on the spreading and consequent deleterious effects of aSyn make use of approaches to mimic what are presumed to be pathological forms of e-aSyn, such as pre-formed fibrils (PFFs). To this end, recombinantly aSyn PFFs are typically added to cells or injected into different tissues in animal models (Delenclos et al., 2019; Gerdes et al., 2020).

The spreading of aSyn can take place from cell-to-cell, from region-to-region, and also between organs (Foulds et al., 2013; Chang et al., 2020; Challis et al., 2020). From the gut, where some types of PD are hypothesized to start (Challis et al., 2020), to the blood (Abd Elhadi et al., 2019), the spreading of extracellular aSyn may affect multiple cell types in the body. Thus, by investigating systemic effects of e-aSyn, we might obtain additional insight into the origin, progression, and plethora of symptoms present in the synucleinopathies, thereby identifying potential therapeutic targets.

A variety of pathways and receptors have been implicated in the transfer of aSyn between neurons and immune cells. The discovery of receptor-mediated endocytosis and neuronal damage caused by e-aSyn opened a field investigation of pathological mechanisms. Membrane proteins, like the cellular prion protein (PrP^C) (Ferreira et al., 2017), and receptors like the lymphocyte-activation gene 3 (LAG3) (Mao et al., 2017), may play crucial roles in the toxicity and pathological spreading of e-aSyn. Since these proteins are also expressed in immune cells, they may impact on the interplay between these cells and other tissues and organs (Anderson et al., 2016; Brown et al., 1998; Liu et al., 2015).

If pathological forms of e-aSyn are internalized, they may seed the aggregation of endogenous aSyn present inside the cells, and also directly damage organelles (Ganjam et al., 2019; Colla et al., 2012; Senol et al., 2021). In fact, mitochondrial impairments are thought to be one of the consequences of aSyn internalization (Ganjam et al., 2019; Ugalde et al., 2020; Park et al., 2020). In turn, mitochondrial dysfunction potentiates the aggregation of aSyn (Lee et al., 2002a), possibly causing a feedback loop that can eventually collapse on the cell death. Inside the cell, aggregated aSyn is believed to be degraded by the autophagy-lysosome pathway (ALP) (Webb et al., 2003). However, high levels of aggregated aSyn can impair lysosomal function and may escape degradation (Freeman et al., 2013), accumulating in the cytosol, and seeding the aggregation of endogenous aSyn (Senol et al., 2021). Aggregated e-aSyn was also shown to cause endoplasmic reticulum (ER) stress and ion imbalance (Paiva et al., 2018), vesicular transport alterations (Cooper et al., 2006), and genetic modifications in cells (Kontopoulos et al., 2006), promoting cell stress.

Glial cells are known to maintain homeostasis in the brain and protect and support neurons (Graeber and Streit, 2010; Sofroniew and Vinters, 2010). In a pathological setting, the function of glial cells becomes even more substantial. In synucleinopathies, glial cells such as microglia, can take up and process neuron-released aSyn, and respond by releasing cytokines and chemokines thereby mounting a pro- or anti-inflammatory response in the brain (George et al., 2019; Austin et al., 2006; Choi et al., 2020). This can then modulate astrocytic function, playing a role in the progression of the diseases (Rothhammer et al., 2018; Liddelow et al., 2017).

Interestingly, the presence of glial cytoplasmic inclusions composed mainly of aggregated aSyn in oligodendrocytes is a pathological hallmark of MSA. Although there is still an ongoing debate as to whether oligodendrocytes express aSyn, it is possible that aSyn accumulation in these cells may result from the transfer of aSyn from donor neurons, suggesting that oligodendrocytes may play an important role in aSyn pathology spreading in MSA. In vitro, oligodendrocytes are able to internalize e-aSyn monomers, oligomers and, although to a lesser extent, fibrils (Reyes et al., 2014) .

In similarity to its effects in glial cells, pathological e-aSyn is also hypothesized to modulate the inflammatory state of the immune system. In fact, with aging, low-grade inflammation may emerge, thereby increasing the propensity to develop neurodegenerative disorders (Domingues et al., 2020).

Herein, we discuss the effects of e-aSyn on different cell types, from neurons and glia to immune cells.

3. Interaction of extracellular aSyn with membrane lipids

As mentioned above, misfolded aSyn is thought to spread between neuroanatomically-connected regions of the brain in synucleinopathies (Braak et al., 2003). Several pathways and cellular responses have been hypothesized to play a role in this cell-to-cell transmission, leading to neuronal death and, therefore, progressive tissue damage (Vicente Miranda et al., 2017; Ferreira et al., 2017; Ugalde et al., 2020; Brás et al., 2020b).

The extracellular matrix is likely the first barrier that interacts with e-aSyn (Fig. 1). Similarly to intracellular aSyn, e-aSyn has also the ability to interact with phospholipids and fatty acids from the membrane through its N-Terminal (Eliezer et al., 2001). The positively charged lysine residues present in the N-terminus of aSyn bind preferentially to the negatively charged membranes (Zarbiv et al., 2014), altering multiple membrane properties such as curvature and thickness (Braun et al., 2012). In turn, this interaction may also affect the structure of aSyn itself (Davidson et al., 1998). Intracellular N-terminal-acetylated aSyn was shown to bind to membranes and to have reduced aggregation propensity when compared to the non-acetylated form of the protein (Bartels et al., 2014; O'Leary et al., 2018). Phosphorylation of aSyn was also shown to inhibit interactions with membranes (Kuwahara et al., 2012; Fiske et al., 2011). Therefore, it is possible that these and other posttranslationally modified forms of e-aSyn may affect the way the protein interacts with the extracellular side of the plasma membrane.

The binding of the N-terminus of aSyn with membranes allows multiple partners, such as the SNARE complex (Lou et al., 2017), to interact with the intrinsically disordered C-terminal region. This suggests that this interaction may be highly relevant when it comes to the normal function of aSyn. The binding of aSyn with membranes is dependent on both membrane composition (Galvagnion et al., 2016) and potential mutations or post-translational modifications (PTMs)

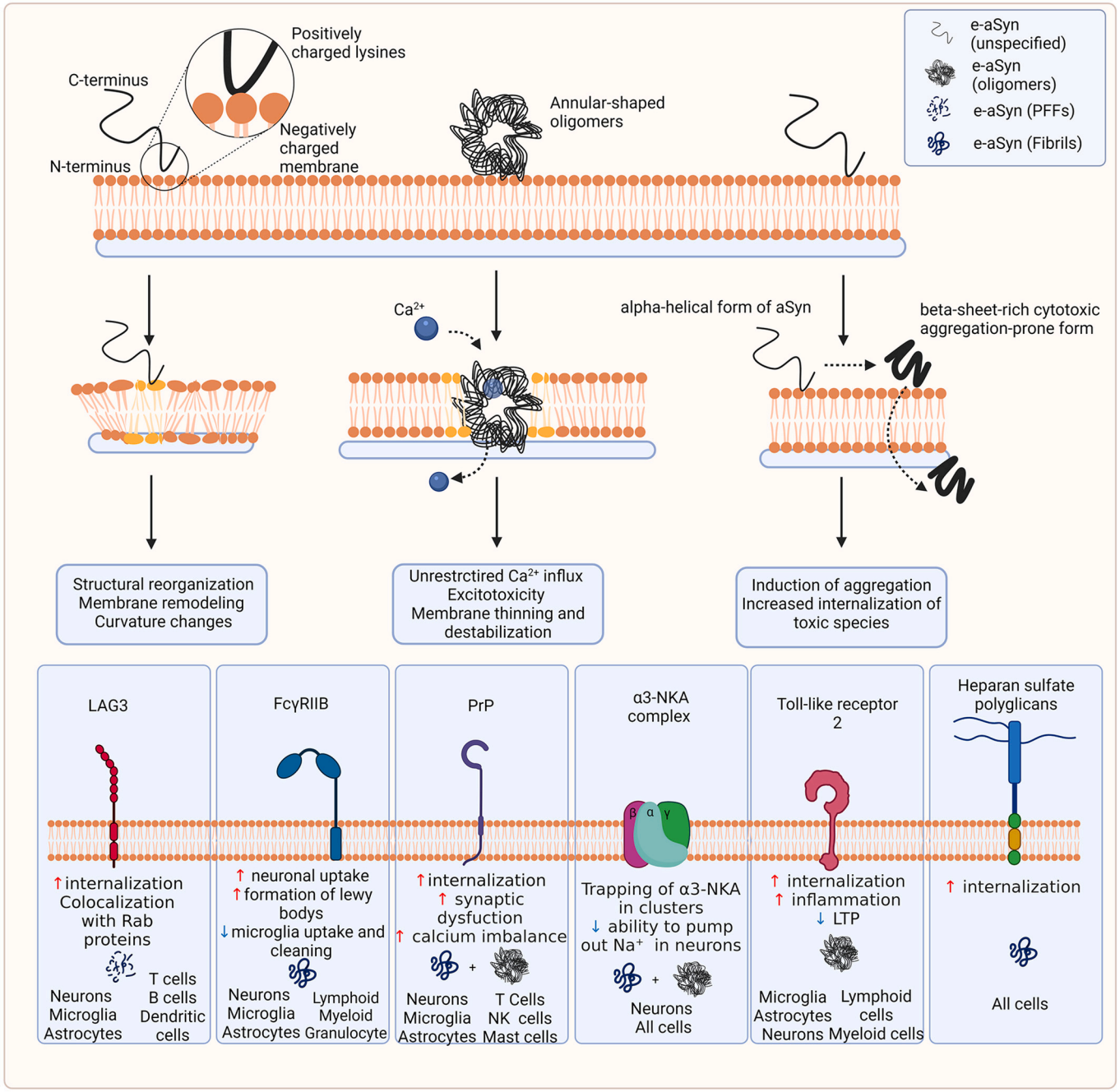

Fig. 1. Interaction between e-aSyn and the extracellular matrix. e-aSyn may induce lipid membrane perturbations and also seed the aggregation of monomeric aSyn. In the plasma membranes of different cells, different receptors/proteins may have the ability to interact with e-aSyn, causing a multitude of responses.

(O'Leary et al., 2018; Fares et al., 2014; Ghosh et al., 2014; Robotta et al., 2017) in aSyn. aSyn mutants associated with familial forms of PD, such as the A30P or E46K mutants, show altered binding to anionic lipids, possibly affecting membrane interactions (Stöckl et al., 2008). In physiological conditions, aSyn can be occur as soluble unfolded monomers or as membrane-bound multimeric forms (Eliezer et al., 2001). The pathological forms of aSyn are thought to consist of beta-sheet rich oligomeric or fibrillar assemblies (Ghosh et al., 2015).

Oligomeric forms of aSyn have been shown to cause membrane remodeling (Jiang et al., 2013), and curvature changes of the cellular membrane (Varkey et al., 2010), possibly leading to membrane permeabilization (Zhu et al., 2003; Kayed et al., 2004). Fibrillar aSyn aggregates have been associated with abnormal accumulation of raft domains (Bucciantini et al., 2012). These direct effects of aSyn on membranes may play a pivotal role in neuronal damage in pathological conditions. Intracellular aSyn has been hypothesized to associate with the mitochondrial membrane, affecting mitochondrial curvature and the rate of fusion (Pozo Devoto and Falzone, 2017).

Another proposed mechanism for membrane-mediated cytotoxicity is the permeabilization and disruption of membranes via the formation of pores by oligomeric aSyn. This hypothesis was based on the finding of annular-shaped aSyn structures (Lashuel et al., 2002) that could be incorporated into the lipid membrane, enabling unrestricted calcium influx and consequently excitotoxicity. Therefore, these pores could act as protein channels (Quist et al., 2005; Zakharov et al., 2007) that destabilize membrane permeability (Zhu et al., 2003). The incorporation of aSyn pores may also cause damage and thinning of the membrane (Chaudhary et al., 2016), which, by itself, could modulate diffusion

transport across the membrane. Consistently, it has been shown that different aSyn mutations (Lashuel et al., 2002; Zakharov et al., 2007; Volles and Lansbury, 2002) and PTMs may affect the efficiency of the leakage, supporting the idea that distinctive organizational assemblies of aSyn oligomers may mediate different pathological pathways.

Certain lipid compositions of membranes may also affect the way that aSyn multimerizes, and promote a transition from an alpha-helical form into a beta-sheet-rich form that may be cytotoxic (Varkey et al., 2010; Volles and Lansbury, 2002; Sharon et al., 2003; Necula et al., 2003; Bodner et al., 2009; Bodner et al., 2010; Perrin et al., 2001; Terakawa et al., 2018). Membrane interactions have been shown to induce the formation of dimeric aSyn (Drescher et al., 2010). Importantly, membrane-bound aSyn has higher aggregation propensity in brain fractions and can also seed the aggregation of cytosolic aSyn (Lee et al., 2002b).

Interestingly, it was shown that GM1 and GM3, lipids present in exosomes, can catalyze aSyn aggregation (Marie et al., 2015). Membranes in the brain are known to have an increased number of fatty acids, such as polyunsaturated fatty acids (PUFAs), that may play a role in neurological disorders. These fatty acids promote the formation of soluble aSyn oligomers (Sharon et al., 2003) that can play a role in disease modulation (Perrin et al., 2001; Fecchio et al., 2013).

The use of aSyn knock-out mice revealed a link between aSyn and lipid metabolism. These mice display altered metabolism of brain PUFA's in neurons and astrocytes (Guschina et al., 2021; Castagnet et al., 2005), suggesting that altered levels or function of aSyn in the brain may affect lipid composition and, thereby, affect the seeding or e-aSyn.

4. Interactions of extracellular aSyn with membrane receptors and membrane proteins

Several membrane protein receptors have been shown to mediate the effects of e-aSyn (Fig. 1). Consistently, in vitro incubation of cells with unspecific proteases aiming to trim cell surface proteins reduce aSyn internalization (Lee et al., 2008a).

A cell surface protein that was shown to modulate cell-to-cell spreading of exogenous aSyn is LAG3 (Mao et al., 2017). This immunoglobulin family member is expressed in neurons, microglia, and peripheral immune cells, like natural killer cells, B cells, T cells, and dendritic cells (Anderson et al., 2016; Liu et al., 2018). Although the role of LAG3 has been extensively investigated in immune-mediated diseases such as cancer (Andrews et al., 2017) and autoimmune disorders (Hu et al., 2020), its role in the CNS remains elusive. LAG3 has been shown to bind recombinant mouse aSyn PFFs, but not monomers (Mao et al., 2017). LAG3 has four Ig-like domains (Triebel et al., 1990), and the 52-109 region of the D1 domain was found to be responsible for this interaction (Mao et al., 2017).

LAG3-aSyn colocalizes with multiple Rab proteins, such as the early endosomal marker Rab5, and with other endosomal GTPases (Mao et al., 2017). These endosomal markers have been strongly implicated in the internalization of e-aSyn (Sung et al., 2001). LAG3 deletion or inhibition decreases the endocytosis of aSyn PFFs, and reduces dopaminergic loss *in vivo*, inhibiting both cell-to-cell transmission and aSyn pathology (Mao et al., 2017). This reduced neurotoxicity was also found in neuronal cell cultures expressing A53T, where blockade of LAG3 function reduces the levels of aSyn phosphorylated on serine 129 (paSyn) and PFF toxicity, suggesting that this interaction may also play a role in familial forms of synucleinopathies (Mao et al., 2017).

LAG3 can be cleaved by metalloproteases and exist as a soluble form (soLAG3) (Li et al., 2007). This form is increased in the serum of PD patients and is associated with an increase in the severity of non-motor symptoms (Cui et al., 2019; Guo et al., 2019). LAG3 was also identified as a significant predictor of regional brain atrophy in PD patients in a study that compared genetic data from genome-wide association studied (GWAS) and high-quality regional imaging from magnetic resonance imaging (MRI) (Freeze et al., 2018). In any case, although several studies support an important role of LAG3 in synucleinopathies, there is still controversy regarding its function and expression in the nervous system (Emmenegger et al., 2021). Therefore, additional studies will be necessary to clarify the role of LAG3 in synucleinopathies.

The Na+ /K+ -ATPase (NKA) is an enzyme found in the membrane of all animal cells, exporting three sodium ions and importing two potassium ions using ATP, affecting numerous processes, most notably the control of neural activity (Clausen et al., 2017). Its discovery culminated in a Nobel Prize in Chemistry in 1997 (Skou, 1998). NKA complex is composed of three subunits (Geering, 2008). The α3 subunit of the NKA complex has been associated with neurodegenerative diseases, such as Alzheimer's disease (AD) (Petrushanko et al., 2016; Ohnishi et al., 2015), PD (Shrivastava et al., 2015), and amyotrophic lateral sclerosis (ALS) (Ruegsegger et al., 2016). This subunit interacts with amyloid-b (Ohnishi et al., 2015), SOD1 (Ruegsegger et al., 2016), and aSyn, possibly mediating the endocytosis of these proteins into neuronal cells (Shrivastava et al., 2015). This cell surface partner of aSyn was identified, along with neurexin 1a and 2a, using a proteomic-based analysis (Shrivastava et al., 2015). The interaction traps α3-NKA within clusters of aSyn, ultimately leading to α3-NKA redistribution and decreased efficiency in the sodium export after action potential affecting the neuronal refractory period (Shrivastava et al., 2015). Mutations within this subunit are associated with diseases such as rapid-onset dystonia Parkinsonism (De Carvalho Aguiar et al., 2004), a rare disease that is characterized by PD-like symptoms that are not improved by levodopa (Brashear et al., 1997).

More recently, the cellular prion protein (PrP^C) has been shown as a promising therapeutic target. This cell surface glycoprotein anchored via glycophosphatidylinositol (Stahl et al., 1987) is expressed in most cells but increased in the CNS (Wulf et al., 2017), and is thought to regulate metal homeostasis (Brown et al., 2001), cell proliferation (Bribián et al., 2012), adhesion and differentiation (Prodromidou et al., 2014), survival (Doeppner et al., 2015), and death (Carulla et al., 2011), to maintain myelin (Bremer et al., 2010), and to contribute to T cell activation (Mattei et al., 2004), and inflammation (Liu et al., 2015). Nevertheless, it is mostly known for its role in multiple neurodegenerative diseases ranging from prion diseases to Alzheimer's disease (Schwarze-Eicker et al., 2005; Laurén et al., 2009; Takahashi et al., 2011). In fact, the term *prion* derives from "*proteinaceous infectious particle*", as the protein was initially identified in prion diseases (Prusiner, 1982). The physiological form of the protein PrP^C is rich in alpha-helical structure, and converts into a protease-resistant misfolded beta-sheet rich form known as PrP^{Sc} that aggregates into amyloids and plaques and causes cell death (Cohen et al., 1993). PrP^{Sc} is able to catalyze the conversion of PrP^C into its pathological form, and this is our current understanding of its infectious nature (Wang et al., 2018).

The interaction between PrP^C and aSyn was shown to promote not only the transfer of aSyn in multiple cells (Urrea et al., 2018; Urrea et al., 2017; Aulić et al., 2017; Thom et al., 2021), but also to cause synaptic dysfunction and impairment of long-term potentiation (Ferreira et al., 2017). PrP^C increases the uptake of fibrillary forms of aSyn in cell lines and in primary mouse neurons (Urrea et al., 2018). This interaction takes place through the N-terminal region of PrP, which is known to interact with amyloid structures (Rösener et al., 2020). Oligomeric aSyn has been shown to interact with PrP^C, leading to Fyn kinase-mediated phosphorylation of mGluR5, thereby activating the NMDAR2B and, ultimately, leading to an increase in intracellular calcium and synaptic dysfunction causing long-term potentiation abnormalities (Ferreira et al., 2017). This interaction has been hypothesized to promote the formation of cofilin/actin rods by rearranging the cytoskeleton and affecting actin dynamics, blocking axonal transport (Brás et al., 2018). Similarly to LAG3, there is also debate regarding the role of PrP^C on aSyn-pathology, as other studies suggested that PrP does not interact with or modulate neuronal death in the presence of aSyn oligomers (La Vitola et al., 2019) . A major issue is whether the same types of

oligomers were being used in the different studies.

The FcγRIIB receptor is known for its role in the immune system, where it is mainly expressed (Anania et al., 2019), playing a pivotal role in phagocytosis and cytokine stimulation (Tridandapani and Anderson, 2008; Nimmerjahn and Ravetch, 2008). This receptor is also expressed in neurons, binding specifically to aSyn fibrils, and mediating the transmission of aSyn and Lewy body-like inclusion formation (Choi et al., 2018). This receptor does not act as a direct receptor but rather as a sensor of aSyn, activating lipid raft-dependent endocytosis (Choi et al., 2018). FcγRIIB is also present in microglia, where it mediates inhibition of aSyn phagocytosis (Choi et al., 2015), suggesting it may play a role in the spreading of aSyn pathology from neuron to neuron while simultaneously inhibiting the microglial cleansing activity.

Heparan sulfate (HS) in proteoglycans can function as a receptor, mediating the uptake of e-aSyn fibrils in neuronal and non-neuronal cell lines (Holmes et al., 2013). The interaction between the negative sulfated HS and aSyn fibrils seems to be mediated by the positive charges of the protein. Besides, addition of soluble heparin to cells in culture competitively inhibited cell binding and uptake of aSyn fibrils into primary neurons (Karpowicz et al., 2017; Ihse et al., 2017).

More recently, a CRISPR-based screen in HEK293 identified SLC35B2 and myosin-7B (MYO7B) as critical regulators of e-aSyn PFFs endocytosis. Since SLC35B2 is a regulator of HS proteoglycan (HSPG) biosynthesis, SLC35B2 KO cells were unable to internalize e-aSyn PFFs, since HSPG was shown to be essential for the interaction and recruitment of PFFs to the cell surface. Again, these interactions were shown to be mediated by negative charges of HS and the clustered K-T-K motifs in the aSyn PFFs structure. MYO7B regulates e-aSyn PFFs cell entry by maintaining a plasma membrane-associated actin network, which controls membrane dynamics (Zhang et al., 2020).

The role of glial cells in aSyn-associated neurodegeneration cannot be overstated. Microglia have been shown to take up and release aSyn, thereby being a relevant player in the cell-to-cell spreading (George et al., 2019) and clearing of aSyn (Choi et al., 2020). Glial membrane receptors have also been shown to potentially mediate the effects of e-aSyn. Toll-like receptors (TLR) are membrane receptors expressed mainly in innate immune cells, where they have the ability to recognize pathogenic molecules (El-Zayat et al., 2019). Once these receptors recognize a pathogen they can activate or suppress inflammatory responses, transcriptional factors, and phagocytosis (Kawasaki and Kawai, 2014; Doyle et al., 2004). In synucleinopathies, TLR2 (La Vitola et al., 2018) and 4 (Choi et al., 2020) mediate the inflammatory response by microglia in the presence of e-aSyn, affecting cytokine and chemokine release (Choi et al., 2020). They also mediate phagocytosis and glial-mediated cleaning of toxic form of aSyn. In microglia, TLR2 enables the internalization of aSyn oligomers released from neuronal cells. This receptor has been associated with PD and is significantly increased in PD patient brains. Interestingly, TLR2 levels were correlated with the accumulation of pathological aSyn in Lewy bodies. Its activation in neurons also showed to increase the levels of endogenous aSyn in neuronal models, resulting in an increase in the levels of autophagy markers (Dzamko et al., 2017). These results suggested a promising therapeutic potential for this receptor, which culminated in the exploitation of immunotherapy-based strategies targeting TLR2, which showed to ameliorate accumulation of aSyn, neuroinflammation, behavioral problems and neurodegeneration in PD/DLB mouse models, bocking both transmission and neurodegeneration associated with the interaction between the receptor and e-aSyn. It was also suggested that TLR2 may play a role in the gut-brain interaction, relevant in PD (Gorecki et al., 2021).

Interestingly, many of the membrane proteins mentioned above are present both in the nervous and immune systems. LAG3 is also a known immune checkpoint receptor involved in immunoregulation and T cell function and immune homeostasis, inhibiting T cell proliferation and activation (Anderson et al., 2016), suggesting it might play a role in potential extracellular aSyn responses in immune cells.

It remains unclear whether different forms of e-aSyn (such as oligomers, fibrils, or exosome-packed protein) are internalized via distinct receptors or different endocytic mechanisms. Understanding how e-aSyn gets access to the cell interior, especially into neurons, will help to elucidate the mechanisms of PD pathology and will open new frontiers for therapeutic interventions.

5. Extracellular aSyn in packages: exosomes and endocytosis

Different aSyn species have been found in exosomes, suggesting this may be another route for the transfer of aSyn between cells (Emmanouilidou et al., 2010; Danzer et al., 2012). It has been reported that neurons and microglia take up e-aSyn oligomers very efficiently when they are associated with exosomes (Bliederhaeuser et al., 2016). A recent study has shown that monomeric and oligomeric aSyn were found in exosomes purified from the plasma of PD patients. These exosomes were rapidly engulfed by BV2 mouse microglial cells, inhibiting autophagy, thereby leading to its accumulation in the cytosol and, eventually, to its release to the extracellular space in exosomes, suggesting microglia play a role in the transmission of aSyn pathology. Further *in vivo* studies in mice, after unilateral injection into the striatum of human plasma exosomal aSyn demonstrated the localization of exogenous human exosomal aSyn in nigral neurons, and the presence of high molecular weight aSyn species, suggesting that exogenous human exosomal aSyn seeded the aggregation of endogenous aSyn *in vivo* after engulfment of exosomes by microglia cells (Xia et al., 2019).

Additional evidence on the importance of the endocytic pathway on aSyn internalization comes from *in vitro* and *in vivo* studies, where inhibition of the endocytic pathway, by lowering temperature, by using dynamin-1 defective mutants or by inhibiting endocytosis with dynasore, reduces the uptake of monomeric, oligomeric and PFF e-aSyn (Lee et al., 2008a; Cipollini et al., 2008; Desplats et al., 2009; Samuel et al., 2016; Sacino et al., 2017; Lee et al., 2008b). Internalization of e-aSyn, independent of the aggregation state, in neurons in *in vivo* models depends on dynamin activation and can be inhibited by endocytosis inhibitors (Hansen et al., 2011).

6. Transfer of aSyn via tunneling nanotubes

aSyn can exploit different pathways to spread between cells. Using quantitative fluorescence microscopy in co-cultured neurons, aSyn fibrils were found to be transferred from a donor to an acceptor cell inside lysosomal vesicles via tunneling nanotubes (TNTs). In particular, aSyn fibrils were able to seed soluble aSyn aggregation in the cytosol of acceptor cells. This suggests that neuronal cells overloaded with aSyn aggregates in lysosomes can dispose of this material by TNT-mediated intercellular (Abounit et al., 2016).

More recently, the mechanism of lysosome-TNT-mediated transfer was further detailed. e-aSyn fibrils seem to subvert lysosomal structure and function, using them as Trojan horses for seeding and propagation between cells. Using super-resolution microscopy, e-aSyn fibrils were found to affect the morphology of lysosomes, to induce lysosomal membrane permeabilization (LMP), and to impair lysosomal function. e-aSyn fibrils also induce peripheral redistribution of lysosomes, probably mediated by transcription factor EB (TFEB), since e-aSyn fibrils induce TFEB nuclear translocation. This increases the efficiency of aSyn transfer to neighbor cells. It was also shown that seeding of soluble aSyn in the "acceptor" cells takes place mainly inside lysosomes from donor cells, since they displayed LMP. Furthermore, by using a heterotypic coculture system, donor cells bearing aSyn fibrils were found to transfer damaged lysosomes to acceptor cells, while receiving healthy lysosomes by TNTs (Senol et al., 2021).

Recently, using co-culture and monoculture systems of differentiated THP-1 and SH-SY5Y cells, aSyn was found to bind migrating mitochondria within TNTs, suggesting that this transfer mechanism may also contribute to cell-to-cell spread of aSyn aggregates and disease

propagation (Valdinocci et al., 2021).

7. Extracellular aSyn can induce lysosomal and autophagy dysfunction

Whether produced inside the cell, or taken up from the extracellular environment, it is believed that the main pathway for degradation of aggregated forms of aSyn is the autophagy-lysosome pathway (ALP) (Lee et al., 2004; Cuervo et al., 2004) (Fig. 2). Upon internalization of e-aSyn, the protein co-localizes with markers of the endocytic pathway (such as EEA1 and Rab5), and hours after internalization, it can assemble into high-molecular-weight oligomers and co-localize with markers of late endosomal and lysosomal compartments (Lamp-1) (Karpowicz et al., 2017; Konno et al., 2012).

ALP dysfunctions have been implicated in the accumulation of aSyn aggregates leading to their release and uptake by adjacent cells, in a

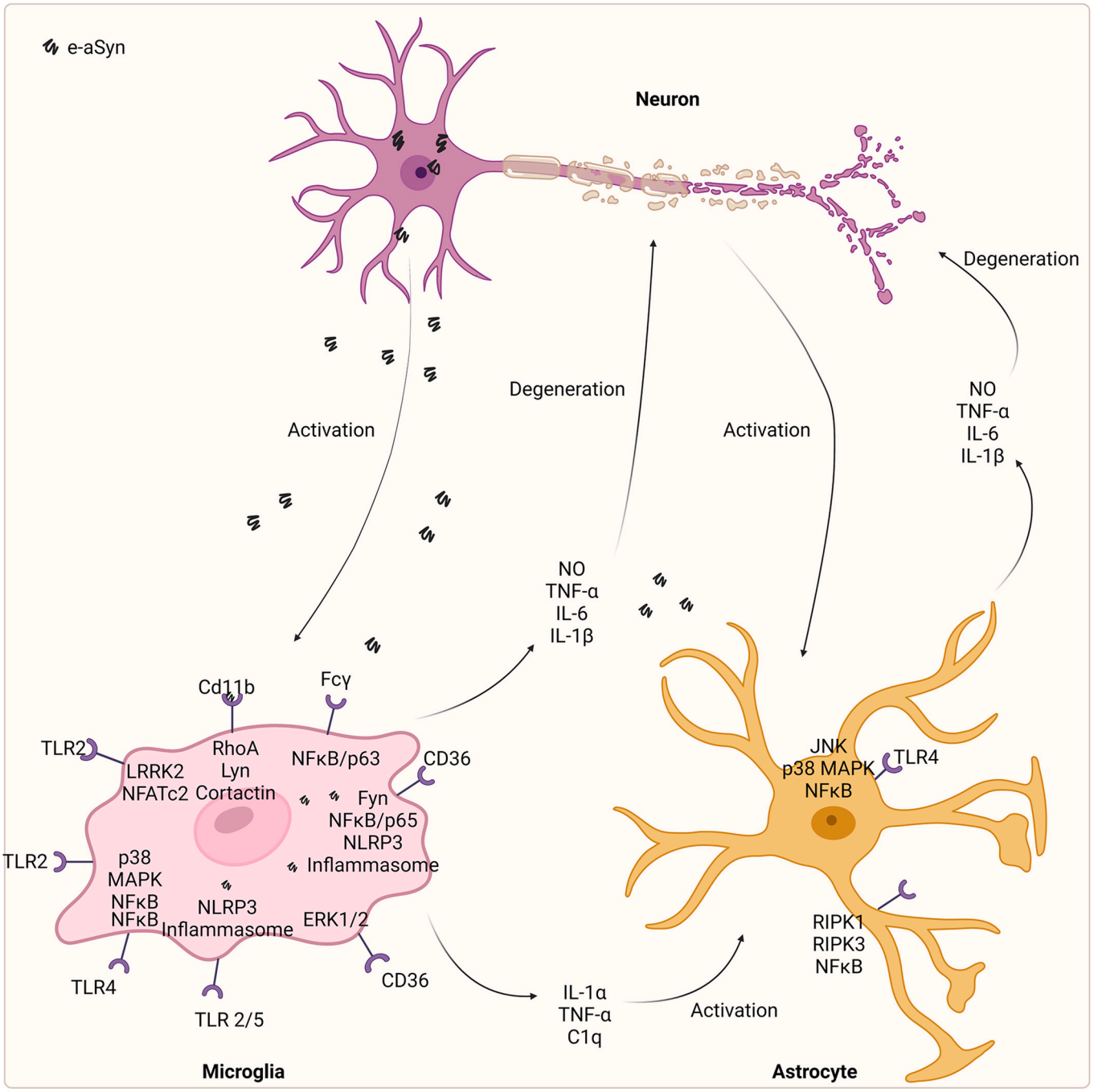

Fig. 2. Extracellular-aSyn (e-aSyn) induces different pathological responses. e-aSyn species (fibrils, PFFs, oligomers, or others, shown in red) can be found free, or associated with exosomes or ectosomes in the extracellular space. e-aSyn may then be internalized via endocytosis and/or receptor-mediated endocytosis. e-aSyn gains access to lysosomes from the endocytic pathway, and may cause lysosomal dysfunction, rupture, permeabilization and the release of species that may seed the aggregation/toxic conversion of endogenous aSyn (green). e-aSyn or intracellular aSyn may interact with mitochondria and induce their dysfunction, increased production of ROS and, ultimately, fragmentation. At ER membranes, e-aSyn may form toxic oligomers, induce stress-mediated UPR, raise cytoplasmatic Ca^{2+} concentration, and trigger apoptosis. e-aSyn may also inhibit vesicular ER-Golgi traffic, induce chromatin acetylation interacting with TADA2a, and induce DNA damage.

prion-like cycle of propagation (Sacino et al., 2017; Steiner et al., 2011; Jang et al., 2010; Bae et al., 2015). Strikingly, the levels of plasma oligomeric aSyn are higher in patients with different lysosomal storage diseases (LSD) then in matched controls (Pchelina et al., 2014; Shachar et al., 2011; Suzuki et al., 2007), and examination of postmortem substantia nigra from PD patients showed that key lysosomal enzymes such as cathepsin D (CtsD), lysosomal-associated membrane protein (LAMP-2A), and the lysosomal hydrolase acid β-glucosidase (known as GCase) are reduced or less active (Chu et al., 2009; Gegg et al., 2012). Mutations in *GBA1*, that encodes for GCase, also known as glucocerebrosidase, are among the most common known genetic risk factors for LB disorders (Mazzulli et al., 2011; Bultron et al., 2010).

In primary neurons, pharmacological inhibition of GCase activity causes a dose-dependent increase in the pathological burden of LB-like inclusions initiated by treatment with e-aSyn PFFs. GCase activity also modulates neuronal susceptibility to pathological e-aSyn insult, when endogenous aSyn aggregation was induced by very low concentrations of e-aSyn PFFs (Henderson et al., 2020).

Furthermore, in mouse cortical neurons and differentiated dopaminergic cells, GCase activity is inhibited by e-aSyn PFFs, but not by e-aSyn monomers, suggesting a positive feedback loop in aSyn aggregation and accumulation regulated by GCase (Gegg et al., 2020).

A possible explanation for the involvement of GCase deficiency/inactivity in PD is the accumulation of its substrate, glucosylceramide, that strongly induces the formation of toxic aSyn oligomers, as shown in an iPSC-derived midbrain culture model, where GCase activity was inhibited pharmacologically.

Accumulation of intra-lysosomal aSyn species might induce their release to the cytoplasm, leading to LB formation, mitochondrial dysfunction, endoplasmic reticulum stress, cell-to-cell transmission by exocytosis etc (Fig. 2). Indeed, treatment of neuronal cells with aSyn aggregates (mimicking putative e-aSyn), results in the accumulation of aSyn in the lysosome, inducing its rupture. This has also been observed upon cell-to-cell transfer of aSyn aggregates (Freeman et al., 2013; Flavin et al., 2017).

Furthermore, after the endocytosis of e-aSyn species (oligomers or fibrils), these can colocalize with lysosomal markers (LAMP-1 and LAMP-2A), causing lysosome enlargement and reduction of CtsD enzyme activity (Hoffmann et al., 2019). Furthermore, e-aSyn PFFs added to HEK293 cells or cultured primary neurons, seed the formation of inclusions by endogenous that cannot be effectively degraded (Tanik et al., 2013; Vasili et al., 2022).

8. Extracellular aSyn can induce mitochondrial dysfunction

Mitochondrial dysfunction is a hallmark of PD and other synucleinopathies. Neurons in general, and dopaminergic neurons in particular, have high-energy demands, and dysfunction in mitochondria highly impacts their physiology.

Different species of aSyn affect mitochondrial biology and dynamics (fission and fusion, mitophagy, mitochondria retrograde/anterograde transport), protein importing and bioenergetics (Bose and Beal, 2016; Smith et al., 2005) (Fig. 2). aSyn has been shown to interact directly with mitochondrial membranes and several proteins, such as the translocases of the outer membrane 20 (TOM 20) and complex I (CI) proteins among others. Since mitochondria is also involved in apoptosis, perturbations in their function can trigger cell death (Di Maio et al., 2016; Buhlman, 2016; Devi et al., 2008; Reeve et al., 2015). In agreement, e-aSyn oligomers induce selective oxidation of the ATP synthase beta subunit and mitochondrial lipid peroxidation in rat primary neurons. This oxidation increases the opening of the permeability transition pore (PTP), triggering mitochondrial swelling and, ultimately, cell death (Ludtmann et al., 2018).

Oligomers of aSyn were also shown to impair mitochondria through interactions with cardiolipin, an anionic phospholipid present mostly in the inner mitochondrial membrane (Hoch, 1992). aSyn oligomers were found to induce robust permeabilization of mitochondrial-like vesicles and to trigger cytochrome c release from isolated mitochondrial upon cardiolipin binding (Camilleri et al., 2013).

In a primary neuronal model where PFFs induce the endogenous neuronal accumulation of paSyn (Volpicelli-Daley et al., 2011), the majority of the paSyn (considered pathogenic aSyn) was shown to associate with mitochondria. Importantly, PFFs display a stronger binding to purified mitochondria than monomers, revealing a preferential mitochondrial binding by aggregated aSyn (Wang et al., 2019).

Another mechanism of mitochondrial dysfunction induced by e-aSyn oligomers is by interfering with mitochondrial protein import systems. e-aSyn oligomers can localize to the outer mitochondrial membrane and bind to TOM20, somehow leading to increased ROS production (Di Maio et al., 2016).

Mitochondrial dynamics, including fission/fusion, morphology, mitophagy and cellular transport is also affected by oligomeric e-aSyn. Oligomeric e-aSyn induces mitochondrial damage by downregulating the protein Parkin, a PD-associated E3 ubiquitin ligase that mediates the degradation of defective mitochondria, being a key regulator of mitophagy. Loss of parkin function leads to the accumulation of damaged mitochondria (Narendra et al., 2008; Hammerling et al., 2017; Chung et al., 2020; Chen and Dorn, 2013). In PC12 cells, e-aSyn oligomers induce oxidative/nitrosative stress and cause parkin *S*-nitrosylation. This PTM induces an increase in the autoubiquitination and degradation of parkin which, in turn, results in cell death. On the other hand, parkin overexpression protects PC12 against toxicity induced by e-aSyn oligomers (Wilkaniec et al., 2019).

Also, e-aSyn impairs mitochondrial biosynthesis due to the parkin-dependent reduction of PGC-1α levels, the master regulator of mitochondrial biogenesis, leading to the accumulation of abnormal mitochondria (Wilkaniec et al., 2021). The authors claimed that these findings provide the first compelling evidence for the direct association of e-aSyn-mediated parkin depletion and impaired mitochondrial function in PD, thus linking parkin dysfunction and e-aSyn signaling in PD pathophysiology.

Astrocytes perform a neuroprotective role by taking up e-aSyn oligomers, but long-term storage of these species can affect their mitochondrial integrity (Lindström et al., 2017). Treatment of mouse astrocytes with different e-aSyn species (monomers, oligomers, or fibrils) activates astrocytes and increases cytokine levels and markers of oxidation. However, only oligomeric species induce mitochondrial dysfunction and increase extracellular hydrogen peroxide production (Chavarría et al., 2018). Also, treatment of astrocytic cultures with anti-aSyn oligomer antibodies prevents aSyn accumulation and mitochondrial damage (Gustafsson et al., 2017).

e-aSyn can also induce mitochondrial dysfunction via stimulation of outer cellular membrane receptors. Aggregated e-aSyn interacts with neuronal purinergic P2X7R and induces intracellular calcium mobilization (Wilkaniec et al., 2017). More recently, treatment of SH-SY5Y cells with oligomeric e-aSyn was found to lead to a P2X7R-dependent decrease in mitochondrial membrane potential as well as to an elevation of mitochondrial ROS production, resulting in breakdown of cellular energy production. Moreover, e-aSyn induces P2X7R-dependent deregulation of AMP-activated protein kinase, and reduces parkin levels. Activation of pathways of programmed cell death were observed after P2X7R stimulation in response to e-aSyn (Wilkaniec et al., 2020). In fact, several other studies have suggested a role for the purinergic receptor P2X7R in aberrant signaling in PD (Van Weehaeghe et al., 2019; Lee et al., 2011).

9. Extracellular aSyn can induce endoplasmic reticulum (ER)-stress and UPR activation

Several studies have implicated ER-stress and hipper-activation of unfolded protein response (UPR) signaling pathways in PD. There are several mechanisms through which aSyn aggregates might induce ER-

stress, such as via the direct binding to ER membranes, dysregulation of intracellular vesicular transport , ion imbalance (e.g. Ca^{2+} and iron), and UPR activation, all of which can ultimately cause neuronal cell death (Fig. 2).

Aggregated forms of e-aSyn, as well as aSyn overexpression, can induce programmed cell death in neurons through either caspase-dependent or -independent pathways, due to chronic activation of the UPR caused by a sustained ER-stress in response to the accumulation of aggregated proteins (Lin et al., 2007; Walter and Ron, 2011). e-aSyn oligomers induce ER-stress in differentiated SH-SY5Y cells, leading to upregulation UPR related and programmed cell death genes (Song et al., 2017).

ER stress was also reported in brain slices from a transgenic rat model of PD treated with aSyn aggregates. aSyn activates ER stress mediators associated with PERK that activate ATF6 signaling, leading to the expression of GRP78, eIF2a and the pro-death apoptotic proteins CHOP and caspase-12, thereby inducing neurodegeneration (Gorbatyuk et al., 2012).

Ion imbalance is another mechanism through which aSyn can induce ER-stress. ER calcium release is triggered after inositol-1,4,5-triphosphate receptor (IP3R) stimulation by IP3. The inositol-1,4,5-triphosphate kinase B (ITPKB) inactivates IP3 and has a variant (thymine-to-cytosine substitution) associated with reduced risk of sporadic PD (Chang et al., 2017). In primary neurons, knockdown or pharmacological inhibition of ITPKB increases the levels of phosphorylated, insoluble aSyn pathology induced by e-aSyn PFFs. Conversely, ITPKB overexpression reduces e-aSyn PFF-induced aSyn aggregation. Also, ITPKB inhibition or knockdown increased intracellular Ca^{2+} levels in neurons, leading to an accumulation of Ca^{2+}in mitochondria that show increased respiration, suggesting e-aSyn-induced pathology is mediated by ITPKB by the regulation of ER-to-mitochondria Ca^{2+}transport. Furthermore, the effects of ITPKB on mitochondrial Ca^{2+}import and respiration can be prevented by pretreatment with pharmacological inhibitors of the mitochondrial Ca^{2+}uniporter complex, which is also enough to reduce e-aSyn PFF induced pathology in neurons. Taken together, ITPKB may act as a negative regulator of e-aSyn induced aSyn aggregation and, highlighting the importance of functional ER-to-mitochondria Ca^{2+}flux in PD (Apicco et al., 2021).

aSyn aggregates, but not monomers, have been shown to bind to and activate SERCA, the most important ER-Ca^{2+} pump, and to induce the release of Ca^{2+} to the cytosol in neuronal cells. Treatment of cell cultures with SERCA inhibitors normalize cytosolic Ca^{2+} levels, protect cells from stress, and improve viability. The interaction between aSyn aggregates and SERCA was also detected in human DLB brain tissue (Betzer et al., 2018).

Finally, e-aSyn may affect cellular iron metabolism mediated by ER-stress. Treatment of neuronal cells with e-aSyn PFFs changes the expression of key proteins involved in iron metabolism, such as the divalent metal transporter 1 (DMT1), the iron transporter (FPN), the iron regulatory protein 1 (IRP1) and hepcidin. Pretreatment of cells with the endocytosis inhibitor dynasore reverses these effects of e-aSyn (Mi et al., 2021).

Microgliosis is considered an important event in PD (Wu et al., 2002). ER-stress and mitochondria disfunction work together, via protein kinase C delta (PKCδ), in promoting e-aSyn-induced microglia activation. PKCδ is activated in PD brains and in PD models, where it participates in reactive microgliosis (Zhang et al., 2007; Gordon et al., 2012). In particular, e-aSyn PFFs induce increased levels of PKCδ, oxidative stress, mitochondria dysfunction, upregulation of the ER-stress classical markers ATF4, IRE1-a and eLF2a, and activation of NLRP3 inflammasome in primary microglia cells and in the SN of mice after e-aSyn PFF injection into the striatum.

Primary microglia cells treated with e-aSyn PFFs display increased expression of thioredoxin-interacting protein (TXNIP), an endogenous inhibitor of the thioredoxin (Trx) pathway, a major antioxidant protein system, and a known inducer of NLRP3 inflammasome activation. Knockdown of PKCδ in cells challenged with e-aSyn PFF is enough to reduce ER-stress, reduce expression of TXNIP and activation of NLRP3 inflammasome. Moreover, attenuation of mitochondrial ROS via mito-apocynin and inhibition of ER-stress signaling with Salubrinal reduces the induction of the ER-stress/TXNIP/NLRP3 signaling axis, suggesting that mitochondrial dysfunction and ER-stress act in concert to promote microglial activation in response to e-aSyn PFF (Samidurai et al., 2021).

10. Effects of extracellular aSyn in the nucleus

Recently, it was shown that e-aSyn PFFs induce changes in the levels of histone 3 acetylation in the striatum and in the SN of mice injected with PFFs. Those changes seemed to be related to decreased transcriptional adapter 2-alpha (TADA2a) levels. TADA2a is a component of the p300/CBP-associated factor and is important in the process of histone H3/H4 acetylation. Consistently, using the BioID system in SH-SY5Y cells overexpressing WT or A53T-aSyn, the authors identified TADA2a as strong binding partner of aSyn (Roux et al., 2012). Furthermore, the levels of TADA2a and acetylated histone H3 are decreased in the SN of PD brains (Lee et al., 2021).

Furthermore, e-aSyn was shown to alter the expression of sirtuins, DNA-bound poly(ADP-ribose) polymerases (PARPs), and other stress response and pro-survival proteins in PC12 cells. Sirtuins and PARPs are nicotinamide adenine dinucleotide (NAD)-dependent enzymes, involved in histone deacetylation and in poly-ADP-ribosylation processes, respectively (Mendoza-Alvarez and Alvarez-Gonzalez, 1993; Houtkooper et al., 2010; Strosznajder et al., 2010). Oligomeric e-aSyn also enhances expression of the pro-apoptotic protein Bax, reduces the anti-apoptotic protein Bcl2, and induces free radical production, decreases mitochondria membrane potential and activates programmed cell death (Motyl et al., 2018) (Fig. 2).

In different cell types (N27, primary neurons, astrocytes and microglia) and in mice, treatment with e-aSyn PFFs induces toxicity and reduces the levels of Lamin B1 and HMGB1, both nuclear proteins and established markers of cellular senescence (Verma et al., 2021), further confirming effects of e-aSyn in the nucleus of cells.

11. Contribution of extracellular aSyn to neuroinflammation

Neuroinflammation involves the participation of microglia and astrocytes. It is well-established that a vicious cycle operates in PD, where the neuronal death taking place in early stages of PD triggers the activation of microglia, which, in turn, causes additional neuronal death by releasing several pro-inflammatory molecules (Fig. 3). Neuroinflammation is also present in MSA and DLB, and correlates with the density of inclusions and disease duration (Ozawa et al., 2004; Ahmed et al., 2012a; Amin et al., 2020).

The detection of activated microglial cells in animal models (Sanchez-Guajardo et al., 2013), in human *postmortem* brains samples (Harms et al., 2021), and in *in vivo* positron emission tomography (PET) imaging studies (Gerhard et al., 2006; Ouchi et al., 2005) supports the possibility that microglia become activated even before neuronal death. In fact, aggregated forms of aSyn released by neurons (e-aSyn) can activate microglia directly (Sanchez-Guajardo et al., 2013; Zhang et al., 2005; Alvarez-Erviti et al., 2011; Su et al., 2008), or indirectly, via the induction of reactive A1 astrocytes (Chou et al., 2021; Lee et al., 2010; Mavroeidi and Xilouri, 2021) (Fig. 3).

As explained above, it has been shown that oligomeric e-aSyn interacts with TLR2, activating p38 MAPK and NFκB, resulting in the production of NO, IL-1β, TNFα and IL-6 (Kim et al., 2013). Neuron-released e-aSyn also activates the leucine-rich repeat kinase 2 (LRRK2) via TLR2, promoting the nuclear translocation of NFATc2 and the release of TNFα and IL-6 cytokines (Kim et al., 2020). e-aSyn monomers and oligomers interact with TLR2 and TLR5, activating the NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3) inflammasome (Scheiblich et al., 2021), activating caspases and, thereby, the

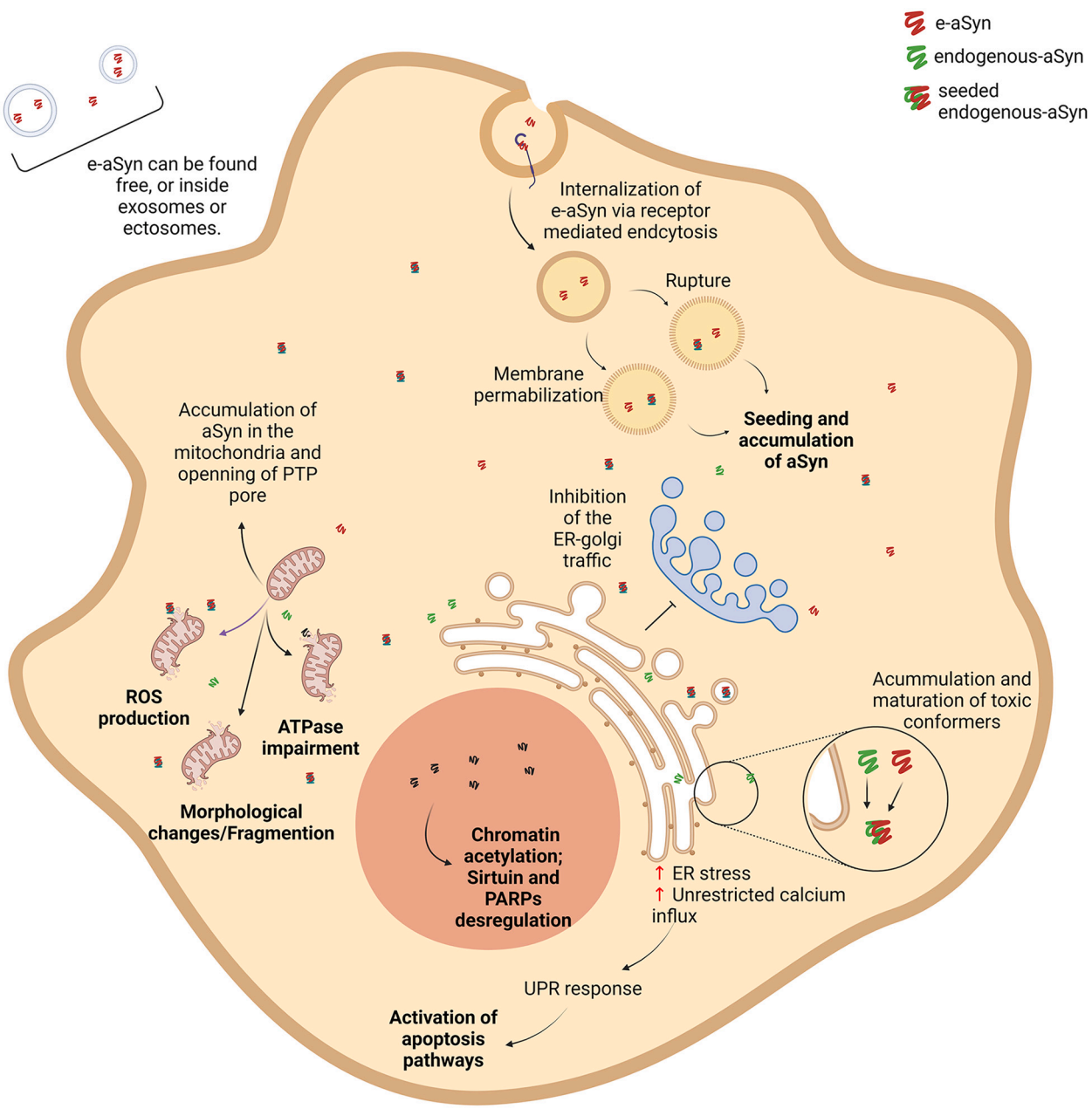

Fig. 3. Crosstalk between e-aSyn released from neurons and glial cells. Neurons produce different forms of aSyn and secrete them to the extracellular space. These e-aSyn forms act through distinct receptors and can activate microglia. Activated microglia release a plethora of factors, some of which lead to neuronal degeneration and others to astrocyte activation. Reactive astrocytes secrete pro-inflammatory factors and reduce the secretion of neuroprotective molecules, and take up glutamate, thereby promoting the degeneration of neurons. Secreted pathological e-aSyn forms can also interact with astrocytic receptors, inducing the activation of glial cells. Microglia and astrocytes internalize e-aSyn forms but may be unable to digest them, accumulating these proteins intracellularly and possibly favoring their spread through other healthy CNS sites.

processing of pro-IL1 into IL-1β. Monomers, oligomers and fibrils of aSyn also interact with TLR4, inducing the nuclear translocation of NFκB and secretion of TNFα, chemokine CXCL1 and NO (Fellner et al., 2013) (Fig. 3).

Another possible microglial receptor for aggregated e-aSyn is the Fcγ receptor for IgG antibodies, and this interaction promotes nuclear accumulation of NFκB/p65 and IL-1α release (Cao et al., 2012). e-aSyn aggregates also interact with CD11b (a marker for CD8+ cytotoxic T cell activation and memory in virus infection) activating RhoA, and Rho-associated protein kinase (ROCK), thereby inducing NADPH oxidase (Nox2) to generate H_2O_2. This leads to phosphorylation of Lyn, a Src family kinase, and cortactin, an F-actin–associated protein, culminating in the reorganization of actin filaments and the migration of microglia towards the source of e-aSyn (Wang et al., 2015; Hou et al., 2018).

e-aSyn aggregates also bind CD36, inducing Fyn kinase activity, PKCδ and NFκB/p65 nuclear translocation which, in turn, promotes NLRP3 inflammasome activation (Panicker et al., 2019).

Overall, these signaling pathways triggered by e-aSyn aggregates in microglia culminate in the generation of pro-inflammatory mediators such as NO, TNFα, IL-6 and IL-1β, which induce neuronal degeneration (Copas et al., 2021), while C1q, IL-1α and TNFα induce astrocyte activation (Liddelow et al., 2017) (Fig. 3). Astrocyte activation, in turn, produces more NO, IL-1α, IL-1β and IL-6 (Copas et al., 2021). Furthermore, the secretion of neuroprotective molecules, such as neurotrophic factors and glutamate uptake, is decreased in activated astrocytes (Copas et al., 2021; Takaki et al., 2012), aggravating this toxic scenario.

As mentioned before, astrocytes can also become active or reactive by the direct contact between TLR4 with recombinant e-aSyn, inducing the expression of pro-inflammatory cytokines and NO production (Rannikko et al., 2015).

Fibrillar e-aSyn can also interact with a yet unidentified receptor activating the receptor-interacting protein kinases-1 (RIPK1) and −3 (RIPK3) followed by NFκB signaling (Chou et al., 2021).

Pathogenic aSyn also functions as a chemoattractant factor binding to CD11b and attracting microglia to sites where it is heavily produced by neurons (Wang et al., 2015). Moreover, it is speculated that the total amount of misfolded aSyn released by neurons increases during disease progression. This may direct impact on the immune response mounted, as studies have shown that the ability of e-aSyn to initiate a pro-inflammatory response is greater when the protein is misfolded (as oligomers or fibrils) (Hoffmann et al., 2016), or influenced by PD-associated mutations (Hoenen et al., 2016; Roodveldt et al., 2010).

Considering the major role and influence of neuroinflammation in PD pathogenesis, several therapeutic approaches aimed at modulating neuroinflammation are being considered, such as inhibition of A1 astrocytes, anti-TNF or anti-NFκB therapies (Copas et al., 2021; Marogianni et al., 2020; Cardinale et al., 2021), as discussed below.

12. Peripheral immune responses in PD: a field in its infancy

The identification of early, specific predictors of PD is being eagerly pursued by the PD scientific community, as this would enable earlier diagnosis and, possibly, earlier therapeutic intervention. Assuming toxic aSyn species exist, as postulated by the gain of toxic function theory, toxic aSyn species may not only affect CNS cells, but also peripheral immune cells. In this way, immune cells could sense and respond to toxic e-aSyn species in the gastrointestinal tract, blood, or derived from the CNS, by adjusting the activation pattern, receptor expression, and/or the secretion of specific cytokines/chemokines. Although our understanding of the precise mechanisms that connect peripheral immune responses, innate and adaptative, and PD has advanced in recent years, there are still several gaps to be filled. Below, we focus on responses from cells other than glia.

Only a few reports have investigated the immune response against e-aSyn at the periphery once neuroinflammation and brain injuries are already in progress or in latency. This means that we need to investigate further what happens outside the CNS, as this may lead to the identification of alarm signs and targets for intervention. This is particularly relevant if we think that PD may begin in the olfactory bulb or in the gut, and not necessarily in the brain.

aSyn aggregates can activate several immune cells outside the CNS, such as T-cells, macrophages, monocytes, and neutrophiles. Injecting e-aSyn PFFs in the striatum of WT mice, results in an increase in the number of leukocytes, B, T, NK cells, and monocytes in the spleen when compared to e-aSyn monomer injected mice. A similar increase was observed in the inguinal lymph nodes, although no changes were observed in the number of circulating cells in the blood of these mice. Instead, a decrease in the total number of monocytes, neutrophils, and NK cells was detected (Earls et al., 2019). Since this was an exploratory study, further studies will be necessary to understand the peripheral responses in synucleinopathies.

In blood samples from PD patients, stored 10 years before the onset of the motor symptoms, in combination with samples from other PD cohorts with patients at different times of motor diagnosis, an inverse correlation between the number of years after disease onset and T cell reactivity was observed (Lindestam Arlehamn et al., 2020). A very specific CD25-CD127- T cell subset that produces IL-10 in response to aggregated e-aSyn suggests a compensatory anti-inflammatory mechanism operating in early stages of PD. These findings indicate that specific T cell reactivity to aggregated aSyn is a feature of premotor and early motor PD and, if combined with other symptoms that have been linked to PD, are good predictors of the disease.

Ageing progressively decreases the ability of macrophages (and microglia) to phagocytose e-aSyn (Bliederhaeuser et al., 2016). Macrophages can be directly activated by e-aSyn leading to increased inflammatory response and e-aSyn uptake and clearance. Both the N-terminal and C-terminal domains of aSyn, but not the NAC region, are necessary for macrophage activation, which is accompanied by ERK activation (Lee et al., 2009). Furthermore, the scavenger role of macrophages is important for e-aSyn clearance outside the CNS. More recently, in iPSC-derived macrophages from PD patients were found to clear fibrillar e-aSyn in an actin-dependent pathway. However, this phagocytic capacity was compromised by an excess of endogenous or exogenous aSyn (Haenseler et al., 2017).

The evidence supporting the involvement of neutrophils in PD is still more limited at the present moment. Our group investigated the participation of neutrophil extracellular traps (NETs) in amyloid diseases using an *in vitro* approach. Fibrils of aSyn can induce the release of NETs, and the proteases associated with these traps can digest the amyloid fibrils into toxic aggregates (Azevedo et al., 2012). Whether neutrophils and their traps can play a role in PD, in the gut or even in the brain, will require additional investigation.

Phagocytosis of e-aSyn by monocytes depends on the receptors they express. Therefore, characterization of receptor expression profiling of phagocytic cells will be necessary to allow a detailed understanding of the mechanisms used by these cells to clear e-aSyn. Interestingly, impairment of lysosomal function caused either by GBA mutations or by decreased enzymatic activity, as observed in monocytes from PD patients, leads to failure of e-aSyn clearance (Wijeyekoon et al., 2018). HLA-A (MHC class I) and HLA-DR and HLA-DQ (MHC class II) molecules from monocytes bind to e-aSyn and induce high levels of T cell responses to e-aSyn in the CNS (Sulzer et al., 2017; Williams et al., 2018; Ahmed et al., 2012b; Fiszer et al., 1994; Schröder et al., 2018).

In animal models, intravenous administration of e-aSyn PFFs and ribbon oligomers after LPS priming, results in an increase in the population of brain resident microglia and in leukocytes recruited to the spinal cord and to the brain (Peralta Ramos et al., 2019).

Clearly, additional studies are necessary for to address the involvement of peripheral immune responses in synucleinopathies.

13. Extracellular aSyn and autoimmune responses

Several genes associated with PD are also associated with different autoimmune diseases (e.g. rheumatoid arthritis, ulcerative colitis, or Crohn's disease) according to genome-wide association studies (Buhat and Tan, 2014; Foo et al., 2016; Hui et al., 2018; Witoelar et al., 2017). In addition, there is a network of interactions between the protein products of genes associated with autoimmune diseases and those of genes associated with PD (Witoelar et al., 2017). Here, we focus on the role of e-aSyn in a possible autoimmune response.

Autoimmunity can result from an abnormal processing of self-proteins, which can generate epitopes presented by MHC that are then recognized by specific T cells that escape tolerance during thymic selection. The production of autoantibodies against specific epitopes of aSyn, that could give PD an autoimmune component, has been controversial since there are studies showing either an increase or decrease of the levels of these antibodies in CSF, serum, and blood (Papachroni et al., 2007; Yanamandra et al., 2011; Majbour et al., 2016; Horvath et al., 2017; Abd-Elhadi et al., 2016). It is important to emphasize that endogenous damage-associated molecular patterns (DAMPs), such as epitopes in aSyn, can elicit an autoimmune response that transforms the dopaminergic neurons exposing these epitopes via MHCI as a target of CD8+ killer cells, thereby destroying them. Alternatively, the response may target the secreted e-aSyn, aiding in its clearance (Bae et al., 2012). These DAMPs in endogenous e-aSyn might emerge after conformational changes in its structure due to oligomerization and fibrillation. Based on this, immunotherapy is currently being explored as a possible strategy against PD, either by active or passive immunization (Bae et al., 2012;

El-Agnaf et al., 2017; Brudek et al., 2017).

A recent study focused on the study of aSyn epitopes that are capable of eliciting a humoral immune response, using a pool of peptides derived from aSyn (9-10 amino acid peptides - MHCI binders, and 15 amino acid peptides - MHCII binders) to simulate PBMCs from PD patients or from healthy controls (Sulzer et al., 2017). Different responses were identified and, interestingly, two antigenic regions in aSyn were identified - the first in the N-terminal region, containing the segment 31/32-45/46, and the second in region near the C-terminal, containing the segment 116-140, and requiring phosphorylation on S129 for its antigenicity. This elegant study revealed that peptides derived from two regions of aSyn induce an immune response in PD patients. Epitopes at the N-terminal end of the protein were specifically displayed by two MHCII beta chain alleles, DRB5*01:01 and DRB1*15:01, associated with 30% of PD patients, as well as an additional MHCII allele and an MHCI allele not previously associated with PD. This response is enacted mostly by IL-5 secreting $CD4^+$ T cells, as well as IFNγ $CD8^+$ cytotoxic T cells. Thus, immune responses to e-aSyn associated with PD have both MHCI and II components.

14. Conclusion

Synucleinopathies are thought to involve the spreading of e-aSyn between cells. Misfolded e-aSyn may cause a plethora of deleterious effects not only in dopaminergic neurons, associated with motor symptoms, but also in different cell types. The significant association of aging with synucleinopathies suggests that the concept of inflammaging may be highly relevant in these disorders. The misconception that neurodegenerative disorders arise exclusively due to damage in the nervous system arises from the clinical definition of these diseases. Nevertheless, it is important to consider that the CNS is an immune-privileged site. In this context, the spreading of pathological e-aSyn can have effects starting on the extracellular matrix all the way into the intracellular environment, affecting multiple organelles.

The full extension of the relationship between the immune system and synucleinopathies remains elusive but the effects that e-aSyn has on immune cells is starting to emerge. Misfolded e-aSyn can spread, damage the receiving cells, and cause direct cell death or damage-associated inflammation through both innate and adaptive immune activation. Therefore, studying the immune system in the context of neurodegenerative diseases is of high interest. Importantly, the use of the immune system's natural specificity against different targets, there is a great hope in immunotherapy for tackling multiple diseases, from cancer to PD. However, since the immune system can act as a double edge sword due to the differences between normal and uncontrolled inflammation, as present in multiple autoimmune diseases, it will be essential to thoroughly investigate its involvement in synucleinopathies in order to exploit it as a source for biomarkers and for targets for therapeutic intervention.

Declaration of Competing Interest

The authors have no conflict of interest to declare

Acknowledgements

TFO is supported by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) under Germany's Excellence Strategy (EXC 2067/1-390729940) and by SFB1286 (Project B8).

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