

α -Synuclein pathology as a target in neurodegenerative diseases

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Abstract

α -Synuclein misfolds into pathological forms that lead to various neurodegenerative diseases known collectively as α -synucleinopathies. In this Review, we provide a comprehensive overview of pivotal advances in α -synuclein research. We examine structural features and physiological functions of α -synuclein and summarize current insights into key post-translational modifications, such as nitration, phosphorylation, ubiquitination, sumoylation and truncation, considering their contributions to neurodegeneration. We also highlight the existence of disease-specific α -synuclein strains and their mechanisms of pathological spread, and discuss seed amplification assays and PET tracers as emerging diagnostic tools for detecting pathological α -synuclein in clinical settings. We also discuss α -synuclein aggregation and clearance mechanisms, and review cell-autonomous and non-cell-autonomous processes that contribute to neuronal death, including the roles of adaptive and innate immunity in α -synuclein-driven neurodegeneration. Finally, we highlight promising therapeutic approaches that target pathological α -synuclein and provide insights into emerging areas of research.

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Key points

- α -Synuclein can self-aggregate, oligomerize and fibrillize into different pathological strains that are associated with various α -synucleinopathies.
- Parkinson disease and related α -synucleinopathies are widely thought to be prion-like disorders, in which α -synuclein pathology spreads via a templating mechanism.
- Neurodegeneration that results from pathological α -synuclein is mediated by cell-autonomous and non-cell-autonomous mechanisms.
- Parthanatos is the main cell death pathway by which pathological α -synuclein induces neurodegeneration.
- Pathological α -synuclein molecular pathway analysis has created new therapeutic opportunities for Parkinson disease and related α -synucleinopathies.
- PET imaging and α -synuclein seed amplification assays will guide future clinical studies by enabling the monitoring of α -synuclein pathology and responses to therapy.

Introduction

The protein α -synuclein is predominantly expressed in the brain, and the accumulation of pathological forms of this protein characterizes the group of neurodegenerative disorders known as α -synucleinopathies¹. The most prominent of these diseases is Parkinson disease (PD), but others include dementia with Lewy bodies (DLB)² and multiple system atrophy (MSA)³. α -Synuclein pathology can also occur in Alzheimer disease (AD)⁴. The pathology results from aggregation of α -synuclein, leading to the formation of insoluble fibrils that are deposited in neurons, glia and nerve fibres, resulting in the formation of Lewy bodies and Lewy neurites^{2,5}. This aggregation of α -synuclein disrupts cellular function, leading to neuronal death and progressive loss of motor and cognitive functions.

Genetic mutations and environmental factors are thought to contribute to the misfolding and aggregation of α -synuclein. The underlying molecular mechanisms by which α -synuclein aggregation causes neurodegeneration are not fully understood, but several pathways have been proposed, including cell-to-cell transmission of pathological α -synuclein, defects in proteostasis, mitochondrial dysfunction, and oxidative and nitrosative stress leading to cell-autonomous and non-cell-autonomous neuronal death^{6–9}.

In this Review, we provide an overview of the pivotal discoveries regarding the role of pathological α -synuclein in PD and related α -synucleinopathies. In addition, we review the current understanding of the molecular mechanisms of neurodegeneration induced by pathological α -synuclein and the current and potential therapeutic approaches that target these mechanisms.

Early characterization of α -synuclein pathology

The *SNCA* gene, which encodes α -synuclein, and the protein itself were initially identified in a study published in 1988 (ref. 10), which demonstrated localization of the protein to the nucleus and synapses, hence the name synuclein¹⁰ (Fig. 1). Initially, in a study published in

1993, α -synuclein was identified as the non-amyloid- β component of amyloid plaques in AD¹¹ (Fig. 1), and several reports have confirmed the presence of pathological α -synuclein in AD and its contribution to the pathogenesis of AD¹².

α -Synuclein was first associated with PD in a study published in 1997, in which a mutation in *SNCA* that led to an amino acid substitution in the α -synuclein protein¹³ was identified in autosomal dominant PD¹³ (Fig. 1). Several further disease-associated mutations that result in amino acid substitutions in α -synuclein were subsequently reported^{14–22} (Fig. 2). Moreover, duplication and triplication of the *SNCA* locus has been associated with familial PD, and the effects of these mutations indicate a direct relationship between *SNCA* gene dosage and disease progression^{23,24}. The evidence that *SNCA* mutations are associated with autosomal dominant PD and that similar α -synuclein accumulation is observed in both familial and sporadic PD^{25,26} cemented the role of pathological forms of α -synuclein in neurodegeneration.

In the same year that a mutation in α -synuclein was identified as a cause of autosomal dominant PD, α -synuclein was also identified as a major component of Lewy bodies and Lewy neurites in idiopathic PD and DLB^{5,27} (Fig. 1). α -Synuclein immunoreactivity was also detected in glial cytoplasmic inclusions in MSA²⁸ and, shortly afterwards, in neurodegeneration with brain iron accumulation²⁹. Subsequent work showed that phosphorylation of α -synuclein at Ser129 is a major post-translational modification of α -synuclein that was enriched in Lewy bodies and Lewy neurites³⁰ (Fig. 1), and this modification has served as a marker of pathological α -synuclein³¹. Microscopy studies have revealed that α -synuclein-immunopositive Lewy pathology is associated with crowding of organelle components and cellular membranes, and cryo-electron tomography has revealed that pathological α -synuclein inclusions are composed of α -synuclein fibrils interwoven with cellular organelles^{32,33}.

The α -synuclein protein

Structure

α -Synuclein accounts for approximately 1% of the total cytosolic proteins in the CNS³⁴. The protein is made up of 140 highly charged amino acid residues that remain unstructured in aqueous solution¹¹ and has three distinct domains: the N-terminal, central and C-terminal domains (Fig. 2). The N terminus (residues 1–60) contains four imperfect KTGEGV repeats that have a crucial role in modulating interactions of α -synuclein with membranes. The central hydrophobic domain (residues 61–95), which is also called the NAC domain, confers a β -sheet structure to α -synuclein and is crucial for its aggregation. The C terminus (residues 96–140) is enriched in acidic residues, is unstructured and is thought to be essential for maintaining α -synuclein solubility. The C terminus is also the target of diverse post-translational modifications and metal binding^{10,11,35,36}.

Early studies of α -synuclein purified from *Escherichia coli* or mouse tissue under native or denaturing conditions indicated that the protein exists as natively unfolded monomers that adopt an α -helical secondary structure only when they bind to lipid vesicles^{37,38}. A study in which endogenous α -synuclein was purified from human erythrocytes under non-denaturing conditions, however, has indicated that it forms a folded tetramer of approximately 58 kDa that resists aggregation^{39,40}. Use of similar methods in further studies of the mouse brain has suggested that the predominant native conformation of α -synuclein is a large, unstructured monomer with a tendency to aggregate⁴¹. Additional evidence has suggested that α -synuclein exists as an equilibrium mixture of unstructured monomers and multimers and that modifications and interactions

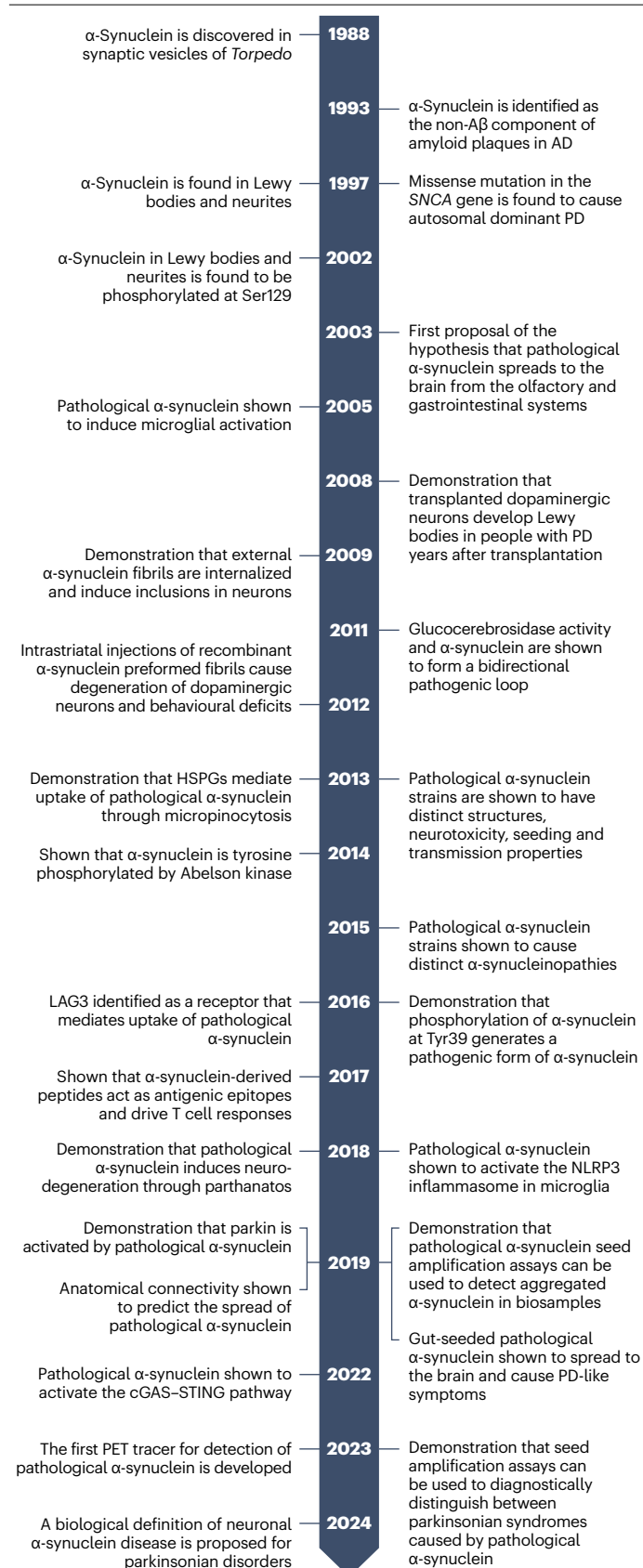


Fig. 1 | Timeline of key advances in the field of α-synuclein biology and pathobiology. Timeline of key discoveries since the initial purification and cloning of α-synuclein. Aβ, amyloid-β; AD, Alzheimer disease; cGAS, cyclic GMP–AMP synthase; HSPG, heparan sulfate proteoglycan; LAG3, lymphocyte-activation gene 3 protein; NLRP3, nucleotide-binding domain leucine-rich repeat-containing family pyrin domain-containing 3; PD, Parkinson disease; STING, stimulator of interferon genes.

with lipids, other proteins and small molecules transiently stabilize the different species^{42–44}. Collectively, the evidence has suggested that α-synuclein has conformational plasticity and structural flexibility that is likely to contribute to its different physiological functions^{45,46}.

Physiological function

The precise physiological function of α-synuclein remains unclear, but its predominant expression at presynaptic terminals implies a regulatory role within the synapse⁴⁷. Indeed, β-synuclein, another member of the vertebrate-specific synuclein family⁴⁸, has a similar presynaptic localization and, although γ-synuclein – the third member of the family – is expressed by glia and only specific neuronal populations⁴⁹, all three synucleins seem to support the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins in their role of mediating synaptic vesicle fusion and neurotransmitter release⁵⁰. In mice, triple knockout of the synucleins caused an age-dependent decrease in SNARE complex assembly, suggesting that these proteins are essential for maintaining normal SNARE complex assembly⁵⁰.

In line with these observations, evidence has suggested that α-synuclein has a direct role as a chaperone, similar to cysteine string protein-α (CSPα), a synaptic vesicle protein that is recruited to the outer membrane of presynaptic vesicles and contributes to neurotransmitter release⁵¹. Deletion of the gene encoding CSPα in mice resulted in a reduction of SNARE complexes and lethal neurodegeneration, but transgenic expression of α-synuclein restored the levels of the SNARE complex and abolished the degenerative phenotype⁵². In mice that lacked both α-synuclein and CSPα, neurodegeneration was exacerbated⁵². These observations revealed that α-synuclein can complement the function of CSPα as a molecular chaperone. The complex role of α-synuclein in exocytosis has been reviewed in detail elsewhere⁵³.

Involvement in neurodegeneration

Studies in numerous transgenic mice that express wild-type and mutant human α-synuclein⁵⁴ have provided evidence that α-synuclein is involved in neurodegeneration. Neuronal expression of wild-type α-synuclein results in a relatively modest phenotype with motor impairment; in some, but not all, cases, inclusions and age-dependent loss of dopaminergic terminals are also observed⁵⁴. In cultured neurons, modest overexpression of human α-synuclein led to impairments in neurotransmitter release and synaptic vesicle exocytosis^{55,56}, and similar effects were observed in mouse models that express human α-synuclein, demonstrating that α-synuclein overexpression alters synaptic physiology^{57,58}. Expression of mutant human α-synuclein in multiple transgenic mouse models has demonstrated that pathological accumulation of α-synuclein leads to substantial neurodegeneration throughout the nervous system^{54,59}. However, in most of these models, dopaminergic neurons were relatively spared from neurodegeneration^{54,59}, suggesting that mouse dopaminergic neurons are resilient to the toxic effects of transgenic overexpression of α-synuclein.

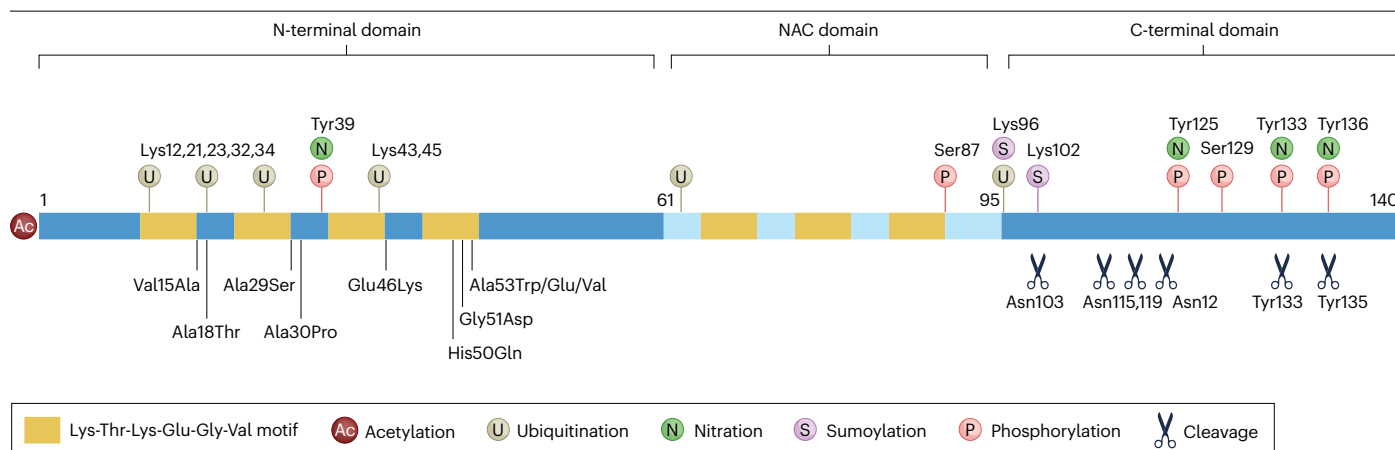


Fig. 2 | Structure of α -synuclein with alterations associated with Parkinson disease. The schematic shows the primary structure of α -synuclein and sites of point mutations, post-translational modifications and truncations that are

associated with α -synuclein pathology. NAC, non-amyloid- β component of amyloid plaques.

Post-translational modifications

α -Synuclein undergoes a number of post-translational modifications. These modifications include phosphorylation, nitration, sumoylation, truncation and ubiquitination^{60,61} (Fig. 2), all of which are thought to have crucial roles in disruption of the native state of the protein that leads to altered binding affinity for itself and/or other proteins, thereby contributing to its aggregation and toxicity^{60,61}. Other post-translational modifications of α -synuclein have been described, but their physiological and pathophysiological roles have yet to be determined^{60–62}.

Phosphorylation. Phosphorylation is the most common post-translational modification of α -synuclein. Under physiological conditions, phosphorylation of α -synuclein occurs at very low levels (approximately 4% of soluble, monomeric α -synuclein), but phosphorylation at serine residues 87 and 129 (refs. 30,31,63–66) and at tyrosine residues 39, 125, 133 and 136 (refs. 67–72) is associated with pathological aggregation of α -synuclein, suggesting that one or more of these modifications are involved in the development of α -synuclein pathology.

Several lines of evidence indicate that phosphorylation of α -synuclein at Ser129 is important in the development of α -synuclein pathology. First, phosphorylation of α -synuclein is prominent in α -synucleinopathies: more than 90% of α -synuclein aggregates are phosphorylated at Ser129 (refs. 30,31). Moreover, the extent of phosphorylation at this site correlates with the likelihood of disease progression^{30,31,73,74}. More directly, phosphorylation of α -synuclein at Ser129 promoted fibril formation³⁰, and in a *Drosophila* model of PD, phosphorylation at Ser129 led to formation of α -synuclein oligomers and accelerated neuronal loss⁷⁵. On the basis of this evidence, phosphorylation of α -synuclein at Ser129 has emerged as a marker of pathological α -synuclein in PD, although its exact role in driving α -synucleinopathy and neurodegeneration remains unclear⁷⁶. In addition, phosphorylation of Ser129 also has a physiological role in mediating synaptic function through protein–protein interactions^{77,78}, although only a small fraction of α -synuclein is phosphorylated at Ser129 under physiological conditions^{77,78}.

Evidence also indicates that phosphorylation of α -synuclein at Tyr39, which is mediated by Abelson kinase, also known as c-Abl^{67,69}, contributes to PD pathogenesis. The interaction of chaperones

with α -synuclein around the Tyr39 residue prevents α -synuclein aggregation⁷⁹, and phosphorylation at Tyr39 impairs these interactions, thereby promoting aggregation of α -synuclein and its relocalization to the mitochondria⁷⁹. Cryo-electron microscopy has shown that phosphorylation at Tyr39 promotes formation of the pY39 α -synuclein fibril core, which is associated with PD, supporting the hypothesis that phosphorylation at Tyr39 has a pathological role⁷². Furthermore, in models of α -synuclein-induced neurodegeneration, prevention of Tyr39 phosphorylation by deletion of the gene encoding Abelson kinase^{67,80} or pharmacological inhibition of Abelson kinase is protective^{81–85}, and increasing the activity of Abelson kinase promotes formation of toxic α -synuclein species and potentiates neurodegeneration⁶⁷.

Ubiquitination and sumoylation. Ubiquitinated α -synuclein is often seen in the Lewy bodies and Lewy neurite inclusions; mono-ubiquitinated, di-ubiquitinated and tri-ubiquitinated α -synuclein are present in both^{86,87}. Depending on the type of ubiquitin modification of α -synuclein, it can protect against pathological α -synuclein or promote its aggregation. The E3 ubiquitin ligases SIAH-1 and SIAH-2 mono-ubiquitinate α -synuclein at lysine residues 12, 21 and 23, which promotes α -synuclein aggregation^{88,89}. The C-terminal U-box domain of co-chaperone HSP70-interacting protein (CHIP), which is also an E3 ubiquitin ligase, also mediates mono-ubiquitination and polyubiquitination of α -synuclein⁹⁰ to regulate its degradation via the proteasomal and lysosomal pathways⁹¹. Immunoreactivity to CHIP is seen in Lewy bodies, and CHIP overexpression reduces α -synuclein aggregation and increases its degradation⁹¹. Polyubiquitination of α -synuclein at lysine residue 63 is mediated by neuronal precursor cell-expressed developmentally downregulated protein 4 (NEDD4) and promotes α -synuclein degradation via the endosomal–lysosomal pathway⁹². Ubiquitination by NEDD4 protects against pathological α -synuclein⁹².

α -Synuclein can also be conjugated to small ubiquitin-like modifier (SUMO). The E3 SUMO-protein ligase protein inhibitor of activated STATS (PIAS2) sumoylates α -synuclein, causing aggregation of α -synuclein that precedes formation of inclusions⁹³.

Nitration. α -Synuclein can be nitrated at tyrosine residues 125, 133 and 136 in the C terminus and at tyrosine residue 39 in the N terminus⁹⁴.

Nitration of α -synuclein contributes to its aggregation under conditions of oxidative and nitrative stress^{95,96}. Nitrated α -synuclein is present in brain regions that are affected in α -synucleinopathies^{95,96} and in α -synuclein transgenic models of PD⁹⁷. Nitration of α -synuclein accelerates degeneration of dopaminergic neurons by generating robust T cell proliferative and neuroinflammatory responses^{98,99}. Nitration (and oxidation) also impairs autophagy-mediated degradation, thereby increasing cellular vulnerability to stressors and contributing to neuronal degeneration^{100,101}.

Truncation. α -Synuclein that is truncated at the C terminus is present in Lewy bodies in people with PD^{2,102}. The C terminus has highly charged amino acids that promote disordered protein structure and thereby maintains solubility of the protein^{103,104}. Consequently, C-terminal truncation of α -synuclein promotes its aggregation and drives formation of pathological α -synuclein^{103,105–108}. In transgenic *Drosophila* and mice that express C-terminally truncated human α -synuclein, the α -synuclein aggregates and causes neurotoxicity^{109,110}. Several enzymes have been implicated in truncation of α -synuclein, including asparagine endopeptidase, calpain-1, caspase-1, neurosin, cathepsin D and matrix metalloproteinase 3 (refs. 111–117).

Spreading of pathological α -synuclein

In 2003, Braak et al.¹¹⁸ proposed that pathological α -synuclein spreads in a stereotypical manner from either the olfactory system or the gastrointestinal tract (Fig. 1). This hypothesis was based on post-mortem examination and localization of Lewy pathology by use of α -synuclein immunostaining¹¹⁸. The findings indicated that deposition of pathological α -synuclein tends to begin in either the anterior olfactory nucleus or in the dorsal motor nucleus of the vagus before spreading into the brain, with Lewy pathology developing in the substantia nigra only in the later mid-stage of PD pathogenesis¹¹⁸. The observation that Lewy pathology is present in the enteric nervous system (ENS) and the PNS in people with presymptomatic, incidental Lewy body pathology and subsequently develops in the midbrain and other regions during disease progression of PD led to the suggestion that pathological α -synuclein is transported from the ENS to the CNS via the vagus nerve^{119,120}. In support of this hypothesis, vagotomy in people with peptic ulcer disease markedly reduces the incidence of PD^{121,122}. Similarly, PD-associated behavioural and neuropathological findings that develop in mice after injection of preformed fibrils of pathological α -synuclein into the pylorus and duodenum are attenuated by vagotomy¹²³.

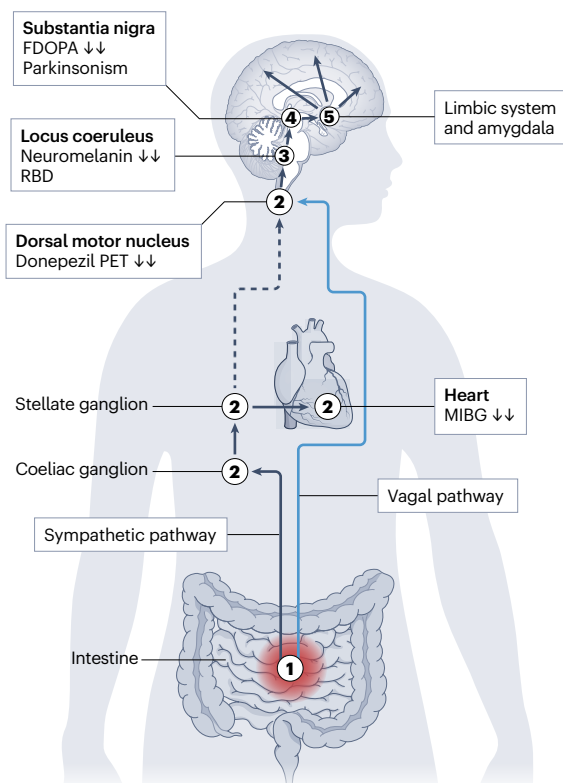
Evidence in the past 5 years, however, has given rise to the notion that PD can be categorized as either body-first PD, in which the pathology first develops in the gastrointestinal tract and spreads to the CNS as described above, or brain-first PD, in which the pathology first develops in the brain and spreads down the neural axis (Fig. 3). Rapid eye movement (REM) sleep behaviour disorder (RBD) is considered a prodromal symptom of body-first PD, as it strongly predicts development of PD but precedes motor symptoms¹²⁴, and use of multimodal imaging has shown that deficits in sympathetic and parasympathetic imaging precede dopaminergic deficits in RBD-positive (body-first) PD, whereas dopaminergic deficits preceded the sympathetic and parasympathetic imaging abnormalities in RBD-negative (brain-first) PD¹²⁵ (Fig. 3). This observation demonstrates that the pattern of pathological spreading differs between the two subtypes, and neuropathological evidence further supports the notion of body-first and brain-first PD¹²⁶. Exposure, and routes of exposure, to various environmental toxicants could drive the development of body-first or brain-first PD¹²⁷.

α -Synuclein is widely thought to spread through the nervous system via cell-to-cell transmission. According to this hypothesis, misfolded α -synuclein can act as a template for conversion of endogenous, monomeric α -synuclein to an abnormal conformation, thereby propagating the pathology intercellularly. The concept initially emerged following observations that grafted fetal mesencephalic dopaminergic neurons develop Lewy bodies in the brains of people with PD several years after transplantation^{128–131}. Evidence to support the hypothesis came from studies that showed that α -synuclein fibrils are internalized and induce formation of α -synuclein inclusions in cell lines and primary mouse neurons^{132–134}. Transmission of pathological α -synuclein pathology in vivo was, to our knowledge, first demonstrated in a study in which mouse neuronal precursor cells were transplanted into transgenic mice that expressed human α -synuclein; the transplanted cells acquired misfolded α -synuclein and α -synuclein inclusions subsequently formed in them¹³². In subsequent studies, direct injection of recombinant α -synuclein preformed fibrils^{135,136} or pathological α -synuclein from people with PD into the brains of wild-type mice¹³⁷ caused widespread formation of α -synuclein inclusions, resulting in neurodegeneration and parkinsonian deficits. Further supporting the notion that a prion-like mechanism is involved was the observation that endogenous α -synuclein is required for cell-to-cell transmission – mice that were deficient for α -synuclein and received injections of α -synuclein preformed fibrils did not develop α -synuclein inclusions or neurobehavioural abnormalities and neurodegeneration did not occur^{123,135}. Anatomical connectivity accurately predicts the pattern of α -synuclein pathology spreading^{138–140}, but some neuronal populations are spared from α -synuclein toxicity and instead serve as cellular conduits for spread along the neural network¹⁴¹.

Initially, α -synuclein was thought to be an intracellular protein, but it is now known that monomeric and pathological α -synuclein are secreted from cells via unconventional exocytosis^{142,143}. Extracellular α -synuclein is present in cerebrospinal fluid (CSF), plasma, saliva, tears, skin and urine in healthy individuals as well as in people with PD^{144–149}. Extracellular pathological α -synuclein can be transferred into cells through various mechanisms. For example, pathological α -synuclein binds to heparan sulfate proteoglycans (HSPGs), which mediate its uptake via micropinocytosis¹⁵⁰. Similarly, lymphocyte-activation gene 3 protein (LAG3) acts as a receptor for pathological α -synuclein^{151,152} and works with amyloid- β precursor-like protein 1 (APLP1) to mediate most neuronal endocytosis of pathological α -synuclein¹⁵¹. LAG3 and APLP1 are expressed on other cell types and neurons, suggesting that they could mediate uptake of pathological α -synuclein beyond neurons¹⁵¹. Toll-like receptor 2 is known to have a role in the uptake of pathological α -synuclein into microglia^{153,154}. Various other transmembrane proteins, including the Na⁺–K⁺ ATPase¹⁵⁵, neuroligins^{152,155,156}, cellular prion protein^{157–161}, Fc γ RIIB receptor¹⁶², LRP1 (ref. 163) and GPNMB¹⁶⁴, also bind to fibrillar and/or oligomeric forms of α -synuclein, suggesting that they could be involved in its cellular uptake. However, future studies are needed to determine their roles and whether they mediate uptake into specific cell types and of specific strains of pathological α -synuclein (see the section ‘Disease-specific α -synuclein strains’).

The mechanisms that follow cellular uptake of pathological α -synuclein and lead to misfolding of endogenous, unfolded α -synuclein are poorly understood. Some preliminary evidence indicates that fibrils exit endosomes via endolysosomal perforation¹⁶⁵. The mechanism by which pathological α -synuclein is released from cells is also not well understood, although exosomal release^{166,167},

Body-first PD



Brain-first PD

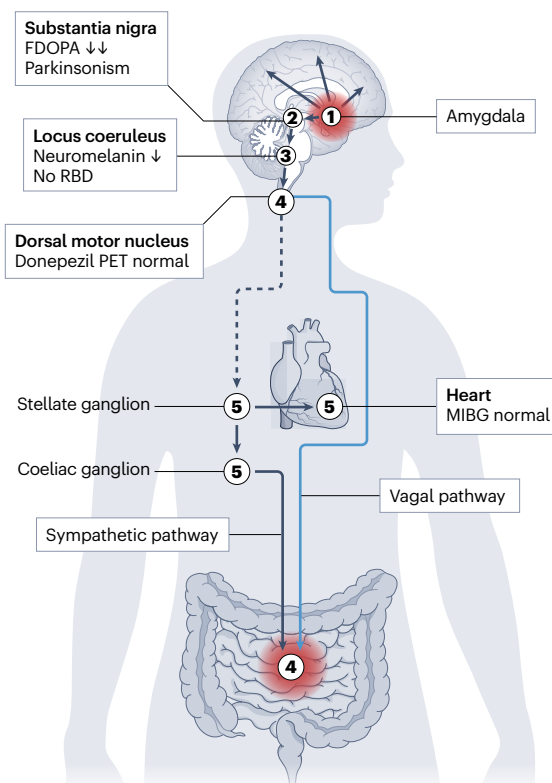


Fig. 3 | Characteristics of body-first and brain-first Parkinson disease. The arrows and numbers illustrate the progression of α -synuclein pathology. The boxes highlight the expected imaging findings and symptoms at the onset of motor symptoms. In the body-first subtype (left), α -synuclein initially aggregates in the gut before spreading through the autonomic nervous system to the spinal cord and brainstem. Prodromal signs include autonomic dysfunction and rapid eye movement sleep behaviour disorder (RBD). Conversely, in the brain-first subtype

(right), α -synuclein pathology begins in susceptible brain structures, such as the limbic system, or enters through the olfactory pathway before spreading down the brainstem. RBD and dysautonomia typically manifest after onset of parkinsonism. As the disease progresses, widespread pathology develops across these systems in nearly all individuals, leading to converging symptoms. FDOPA, fluorodopa; MIBG, meta-iodobenzylguanidine scan; PD, Parkinson disease. Figure adapted with permission from ref. 125, Oxford University Press.

direct cellular membrane penetration¹⁶⁸ and tunnelling nanotubes^{169,170} all contribute to the transfer of pathological α -synuclein between cells.

Disease-specific α -synuclein strains

Different conformations of pathological α -synuclein aggregates, referred to as strains, seem to drive the pathogenesis and clinical manifestations of the different α -synucleinopathies. The concept of strains derives from prion disease, in which distinct pathology, seeding and transmission of prions and distinct clinical symptoms are attributed to conformational differences between prion strains¹⁷¹. Pathological α -synuclein strains generated in vitro by repetitive seeded fibrillization under different conditions have distinct structures, neurotoxicity, and seeding and transmission properties^{172,173}. Furthermore, pathological α -synuclein strains derived from people with PD, MSA and DLB have different biophysical and biological properties, including seeding propensity, resulting α -synuclein pathology and neurotoxicity^{174–176}. These strains also have distinct structures as determined by nuclear magnetic resonance and cryo-electron microscopy^{177,178}. In MSA, the cellular milieu of oligodendrocytes drives the formation of pathological α -synuclein with distinct conformational and biological properties, differentiating

it from the α -synuclein strains found in other α -synucleinopathies¹⁷⁹. Distinct strains may even be present in brain-first and body-first PD¹⁸⁰. The biophysical and biological features of the different pathological α -synuclein conformations could be driving the different clinical manifestations and neuropathologies of the α -synucleinopathies.

α -Synuclein detection methods

Development of new methods to detect pathological α -synuclein species in biospecimens from people with α -synucleinopathies using seed amplification assays (SAAs) and to detect α -synuclein aggregation via brain imaging will improve the diagnosis of these diseases and monitoring of their progression. These methods are still in the early stages of development and validation, but hold great promise not only for diagnosis and monitoring but also for providing a deeper understanding of the pathophysiology of α -synucleinopathies.

Seed amplification assays

SAAs – protein misfolding cyclic amplification and real-time quaking-induced conversion – exploit the ability of pathological α -synuclein species to propagate to multiply them in vitro, thereby

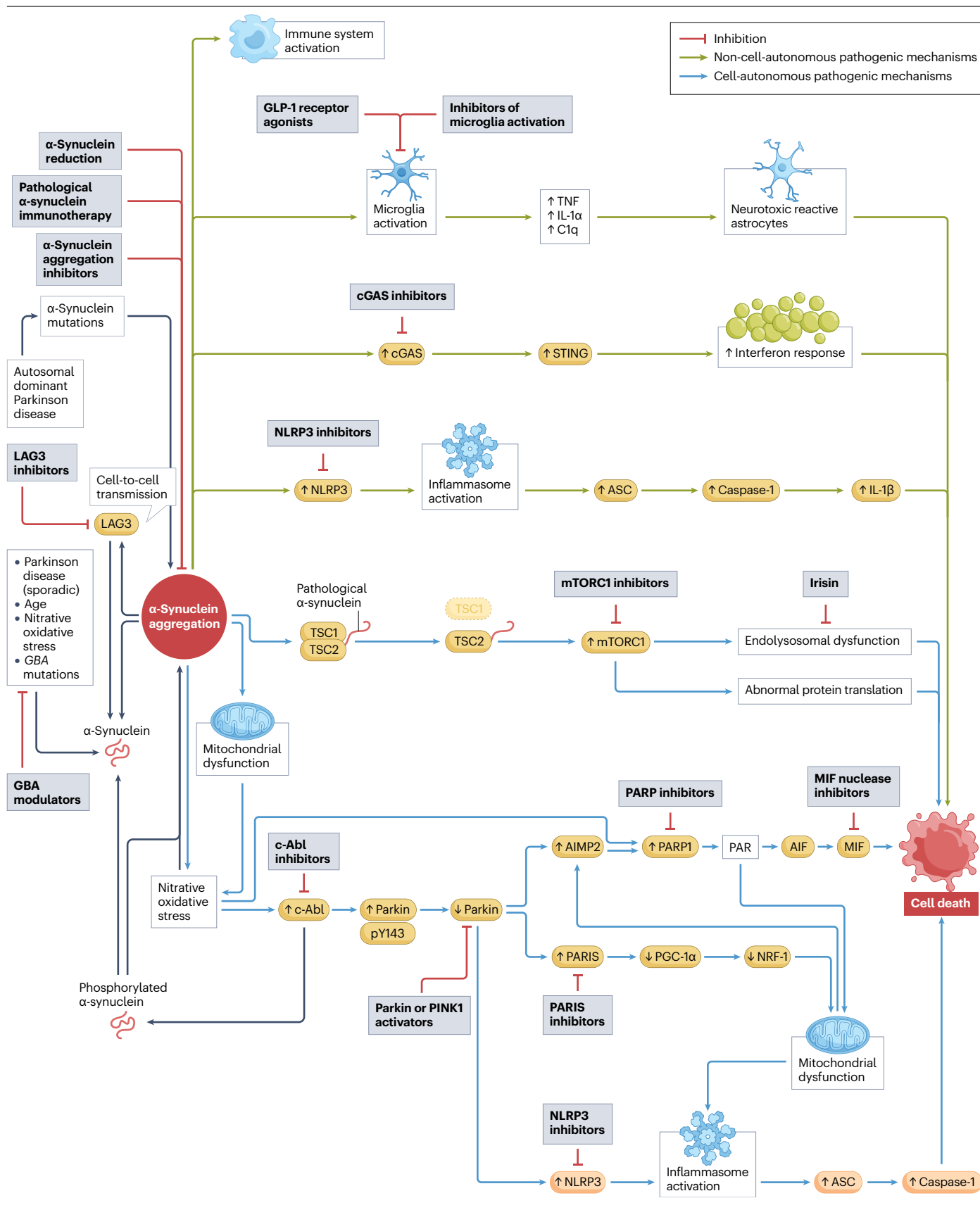


Fig. 4 | Pathways of α -synuclein-related neurodegeneration and possible interventions. Multiple factors contribute to the generation of pathological α -synuclein aggregates (left). Formation of these aggregates leads to activation of non-cell-autonomous pathogenic mechanisms, which involve innate and adaptive immunity, and cell-autonomous pathogenic mechanisms of neurodegeneration. Therapeutic interventions are represented by grey boxes. AIF, apoptosis-inducing factor; AIMP2, aminoacyl tRNA synthetase complex-interacting multifunctional protein 2; ASC, apoptosis-associated speck-like protein containing a CARD; c-Abl, Abelson kinase; cGAS, cyclic GMP-AMP

synthase; C1q, complement component 1q; GBA, glucosylceramidase β 1; GLP-1, glucagon-like peptide 1; LAG3, lymphocyte-activation gene 3 protein; MIF, macrophage migration inhibitory factor; mTORC1, mTOR complex 1; NLRP3, nucleotide-binding domain leucine-rich repeat-containing family pyrin domain-containing 3; NRF1, nuclear respiratory factor 1; PAR, poly(ADP-ribose); PARIS, parkin-interacting substrate; PARP, poly(ADP-ribose) polymerase; PGC-1 α , peroxisome proliferator-activated receptor- γ coactivator 1 α ; STING, stimulator of interferon genes; TNF, tumour necrosis factor; TSC, tuberous sclerosis protein.

enabling detection of small amounts in biological samples^{181,182}. Protein misfolding cyclic amplification enables detection of aggregated α -synuclein in biosamples^{174,183} and can discriminate PD from MSA with high sensitivity¹⁸⁴. The real-time quaking-induced conversion assay enables real-time monitoring of the kinetics of pathological α -synuclein aggregation and can also be used to study the biophysical properties of pathological α -synuclein strains¹⁸¹.

Use of SAAs will facilitate development of biological definitions of α -synucleinopathies and will facilitate observational and interventional disease-modifying trials in parkinsonian disorders¹⁸⁵. However, these assays also have great diagnostic potential, and the ability to detect pathological α -synuclein in the serum provides the opportunity to develop powerful blood-based diagnostic assays¹⁸⁶. SAAs can distinguish parkinsonian syndromes that involve α -synuclein pathology from those with other causes with sensitivities and specificities of more than 90%^{187–189}. Among people with newly diagnosed PD, SAA analysis of the CSF has a diagnostic sensitivity of 82.6% and a specificity of 88.2%¹⁸⁶. In the prodromal phase of PD, use of SAAs identifies pathological α -synuclein in the CSF¹⁸⁹ and in extracellular vesicles in the blood¹⁹⁰. Indeed, SAAs have detected pathological α -synuclein in the CSF¹⁹¹, serum¹⁹², olfactory mucosa, skin, saliva¹⁹³ and other biospecimens¹⁹⁴, providing multiple opportunities to study strains of pathological α -synuclein and to develop diagnostic platforms. To date, use of skin and CSF samples with SAAs results in the greatest diagnostic accuracy¹⁹⁵. Kinetic analysis of SAA profiles, such as determining thioflavin maximal fluorescence intensity, lag time and the time at which aggregation is 50% complete, has the potential to enable differentiation of PD, DLB, MSA¹⁸⁸ and cognitive decline in PD^{16,196}. Determining whether SAAs can be used in clinical practice to reliably differentiate PD, DLB and MSA and to monitor disease progression and response to therapeutic disease-modifying therapies will be important.

PET imaging

The development of radiopharmaceuticals for PET imaging of pathological α -synuclein could transform the diagnosis and treatment of α -synucleinopathies by enabling earlier and more accurate diagnoses, monitoring of disease progression. PET imaging findings could also serve as an outcome measure in clinical trials. PET ligands that are currently in development for imaging of pathological α -synuclein seem to have good selectivity for α -synuclein aggregates in models of α -synucleinopathies and in people with PD and related α -synucleinopathies^{197–199}. For example, the F0502B PET tracer binds to α -synuclein with high affinity and recognizes aggregated α -synuclein in brain tissue from mice, non-human primates and humans with PD¹⁹⁹. The ACI-12589 PET tracer shows specificity for pathological α -synuclein in tissue from people with PD and MSA, and binding in the brains of living people with MSA has been demonstrated¹⁹⁸. The C05-05 PET tracer enables visualization of α -synuclein aggregates in living people with PD

and DLB, and the signal density correlates with the severity of motor symptoms in PD. Future research needs to focus on their clinical utility. In addition, the differences in conformation and biophysical properties of different α -synuclein strains raise the possibility that radioligands could be developed to differentiate between the α -synucleinopathies.

Targeting pathological mechanisms in α -synucleinopathies

Many points in the cascade of pathological α -synuclein-induced neurodegeneration could be targeted to treat PD and other α -synucleinopathies (Fig. 4), from preventing its misfolding and aggregation to enhancing clearance mechanisms and inhibiting downstream toxic effects. In this section, we highlight potential intervention points that could reshape treatment strategies for PD and related disorders.

α -Synuclein aggregation

The most fundamental target in α -synucleinopathies is α -synuclein itself. One possible strategy is to reduce the expression of α -synuclein by use of small hairpin RNA, small interfering RNA or antisense oligonucleotides (ASOs) to reduce the expression of *SCNA* mRNA^{200–202}. Consistent with this strategy, use of ASOs to target α -synuclein in a mouse model of PD in which mice express human α -synuclein reduced neuropathology and early behavioural deficits^{203,204}. Other approaches to reducing levels of α -synuclein should be considered, such as viral vector delivery of α -synuclein CRISPR–Cas9 or RNAi²⁰⁵. However, the effects of knocking down α -synuclein after development are not known, so caution is warranted as reducing levels of α -synuclein could be associated with unintended toxicity. Beyond reducing expression of α -synuclein, inhibiting its aggregation could be an alternative approach to stopping the development of pathology (Fig. 4). In models of α -synucleinopathies, including transgenic human A30P α -synucleinopathy and a transgenic mouse model of MSA, administration of anle138b (3-(1,3-benzodioxol-5-yl)-5-(3-bromophenyl)-1H-pyrazole) prevents formation of pathological α -synuclein aggregates and protects against motor deficits^{206–210}. Similarly, administration of NPT100-18a to α -synuclein–GFP transgenic mice interferes with interactions of α -synuclein with membranes, reduces α -synuclein aggregation and protects against neurodegeneration²¹¹. Some agents that block α -synuclein aggregation are in preclinical or clinical trials for the treatment of PD^{212,213}. The current status of α -synuclein-based therapies and other disease-modifying treatments for PD has been reviewed in full elsewhere²¹⁴.

An alternative strategy for targeting pathological α -synuclein is immunization to remove aggregates that are already present^{215,216} (Fig. 4). In transgenic mouse models of α -synucleinopathies, active immunization (which stimulates the immune system) and passive immunization (direct antibody administration) have reduced neuropathology and neurobehavioural deficits^{217–220}. Immunization could also prevent cell-to-cell transfer of pathological α -synuclein (Fig. 4), and the

same principle could be used to prevent pathological α -synuclein from binding to its transmembrane receptors (Fig. 4). Indeed, in the striatal pathological α -synuclein preformed fibril model of PD, passive immunization with LAG3 antibodies reduced neuropathology and neurobehavioural deficits^{151,152}. Further studies are needed to explore the potential of interfering with cell-to-cell transmission of pathological α -synuclein in this way.

Despite success with passive and active immunization in animal models, passive immunization failed to alter the disease course in people with PD in the first two clinical trials^{221,222}. However, post-hoc analysis of results from one of these trials has suggested that the monoclonal antibody prasinezumab, which targets aggregated α -synuclein, slows motor progression in people with rapidly progressing PD²²³. Confirmation of this observation requires additional randomized clinical trials. In addition, the immunization approach might need to be optimized; for example, monoclonal antibodies that are specific to defined pathological α -synuclein strains might be needed to realize the potential of this approach.

Another approach to removing α -synuclein pathology that has already formed is to promote its degradation. Degradation of α -synuclein occurs via the ubiquitin–proteasome system and the autophagy–lysosome pathway²²⁴; the ubiquitin–proteasome system seems to be the main degradation pathway for non-pathological α -synuclein, whereas the autophagy–lysosome pathway seems to be the major pathway of degradation of pathological α -synuclein^{225–227}. Impairment of these clearance mechanisms can lead to increased intracellular pathological α -synuclein aggregates²²⁸. Therefore, strategies that increase degradation of α -synuclein aggregates, such as tagging aggregates for degradation²²⁹, or promoting macroautophagy²³⁰ and chaperone-mediated autophagy^{231,232}, offer opportunities to reduce the burden of pathological α -synuclein (Fig. 4).

Promotion of α -synuclein degradation seems to be one mechanism that contributes to the benefits of physical exercise, and particularly endurance exercise and training, in PD. Endurance exercise and training have been shown to reduce the risk of developing PD, improve the efficacy of symptomatic medication and improve quality of life^{233–235}. Administration of the myokine irisin, which is released from the muscles during exercise, has been shown to be beneficial in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) intoxication model of PD^{236,237} and the intrastratial α -synuclein preformed fibril mouse model of sporadic PD²³⁸, suggesting that the benefits of exercise could be mediated, at least in part, by irisin. In mice, the benefits of irisin seemed to result from increased autophagy–lysosome degradation of pathological α -synuclein²³⁸ (Fig. 4).

One possible drug target to promote macroautophagy of α -synuclein aggregates is mechanistic target of rapamycin (mTOR), which is a key regulator of cellular metabolism, autophagy and protein translation²³⁹. Pathological α -synuclein binds to tuberous sclerosis protein 2 (TSC2), which destabilizes the TSC1–TSC2 complex and activates mTOR complex 1 (mTORC1). Activation of mTORC1 increases mRNA translation and contributes to neurodegeneration induced by pathological α -synuclein²⁴⁰ (Fig. 4). Thus, selective inhibitors of mTORC1 could be used both to increase macroautophagy-mediated clearance of α -synuclein aggregates and to rectify the mRNA dysregulation that occurs in PD. Indeed, in an α -synuclein preformed fibril mouse model of PD, the mTOR inhibitor rapamycin has been shown to promote clearance of pathological α -synuclein aggregates and reduce mRNA dysregulation^{225,240–242}. Rapamycin itself is unlikely to be a good drug candidate in PD owing to its poor brain penetration,

its immunosuppressive effects and its off-target effects²⁴³, but development of brain-penetrant mTORC1 inhibitors could have potential for the treatment of PD and related α -synucleinopathies.

Impairment of α -synuclein degradation is a key mechanism in PD associated with mutations in *GBA*, which encodes the lysosomal enzyme glucocerebrosidase^{244,245}. Mutations in *GBA* account for 5–15% of PD cases, making it one of the most important risk factors²⁴⁶. The reduction of glucocerebrosidase activity that results from the mutation leads to accumulation of pathological α -synuclein²⁴⁷. In animal models of α -synuclein-induced neurodegeneration, treatment with *GBA* gene therapy, glucocerebrosidase enzyme replacement therapy, glucocerebrosidase substrate reduction therapy and glucocerebrosidase chaperones has been beneficial²⁴⁶ (Fig. 4). Several studies of therapies that target *GBA* and/or glucocerebrosidase are ongoing²⁴⁸ to determine the best treatment strategy for this form of PD²⁴⁶.

Cell-autonomous neuronal death

Emerging evidence indicates that poly(ADP-ribose) polymerase 1 (PARP1)-dependent cell death – also known as parthanatos – is the main cell death pathway by which pathological α -synuclein induces neurodegeneration²⁴⁹ (Fig. 4). First, genetic depletion or pharmacological inhibition of PARP1 prevents pathological α -synuclein-induced cell death in primary neuronal cultures, and prevents neurodegeneration and behavioural deficits in mouse models of sporadic and genetic α -synucleinopathies^{250,251} (Fig. 4). Second, apoptosis-inducing factor (AIF), which is a key mediator of parthanatos, is translocated to the nucleus in the ventral mesencephalon of people with PD²⁵². Finally, genetic knockout or selective inhibition of macrophage migration inhibitory factor (MIF) nuclease, which is crucial for parthanatos²⁵³, prevented neurodegeneration and behavioural deficits in two orthogonal pathological α -synuclein models of dopaminergic cell loss and in the MPTP intoxication model of PD²⁵⁴ (Fig. 4).

Initiation of the parthanatic death cascade involves nitrate and oxidative stress that is triggered by pathological α -synuclein aggregates²⁵⁰ and exacerbated by disruption to mitochondrial function by pathological α -synuclein aggregates²⁵⁵. This nitrate and oxidative stress leads to DNA damage that activates the DNA sensor PARP1, which increases the levels of poly(ADP-ribose) (PAR), thereby setting the cascade in motion²⁵⁶ (Fig. 4). PAR binds to hexokinase 1 on the mitochondria, driving mitochondrial dysfunction through impairment of glycolysis^{257–259} (Fig. 4). The nitrate and oxidative stress also activates the non-receptor tyrosine kinase Abelson kinase, which phosphorylates α -synuclein at the Tyr39 residue, thereby generating a pathogenic form of α -synuclein that further contributes to pathological α -synuclein aggregation⁶⁷ (Fig. 4). The E3-ubiquitin ligase parkin is also phosphorylated by Abelson kinase, and its ubiquitination activity is consequently inhibited²⁶⁰, leading to the accumulation of aminoacyl tRNA synthetase complex-interacting multifunctional protein 2 (AIMP2), parkin-interacting substrate (PARIS) and nucleotide-binding domain leucine-rich repeat-containing family pyrin domain-containing 3 (NLRP3)^{80,261}, all of which lead to increased cell death by parthanatos or inflammasome activation (Fig. 4).

The impairment of parkin activity in sporadic PD suggests that activators of parkin or PINK1, which itself activates parkin, could be used to treat PD²⁶² (Fig. 4). In multiple models of PD, inhibitors of Abelson kinase prevent neurodegeneration^{67,80,81,83,85,263–265} (Fig. 4), although two clinical trials of the Abelson kinase inhibitor nilotinib failed owing to poor brain penetration^{266,267}. Trials of Abelson kinase inhibitors with better brain penetration are in progress²⁶⁸, but their CNS penetration

is still limited and off-target effects could limit their clinical utility. Allosteric inhibitors of Abelson kinase, for example, neurotinib, hold particular promise because off-target effects are minimized and brain penetrance is superior²⁶⁹.

Targeting the downstream effects of parkin impairment could be an alternative therapeutic strategy to reduce parthanatos. AIMP2 directly activates PARP1 (refs. 270,271) (Fig. 4). Accumulation of PARIS leads to mitochondrial dysfunction^{272–274}, and AIMP2 levels are increased downstream of PARIS accumulation – AIMP2 levels do not increase in response to pathological α -synuclein in mice with a knockout of the gene encoding PARIS, indicating that the increase in AIMP2 levels is downstream in the PARIS pathogenesis cascade⁸⁰ (Fig. 4). In multiple models of PD, genetically reducing levels of PARIS or pharmacological inhibition of PARIS is protective^{80,273,275–277} (Fig. 4). Similarly, accumulation of NLRP3 leads to activation of the NLRP3 inflammasome activation, and downstream accumulation of apoptosis-associated speck-like protein containing a CARD (ASC) accumulation and activation of caspase-1 (ref. 278). This pathway is also dependent on PARIS-induced mitochondrial dysfunction²⁶¹ (Fig. 4). In models of PD, NLRP3 inflammasome inhibitors are also neuroprotective^{279,280}.

Non-cell-autonomous neuronal death

Adaptive immunity. Adaptive immunity is known to have a role in PD^{99,281}, and some evidence has suggested that α -synuclein-derived peptides act as antigens that are recognized by T cells²⁸² (Fig. 4). Furthermore, CD8⁺ and CD4⁺ T cells have been observed in the substantia nigra of post-mortem brains of people with PD^{283,284}. Genome-wide association studies have indicated that the major histocompatibility complex (MHC) class II alleles HLA-DRB5*01 and HLA-DRB1*15:01 are associated with PD^{285,286}, and that the non-HLA genes in the MHC class III region are also associated with an increased risk of PD²⁸⁷. In mouse models of PD, targeted overexpression of human α -synuclein leads to microglial activation and an adaptive immune response²⁸⁸, and CD4⁺ T cells mediate brain inflammation and neurodegeneration²⁸⁹. Expression of MHC class I molecules in catecholaminergic neurons also makes them vulnerable to T cell-induced degeneration²⁹⁰. Some evidence has also suggested that the adaptive immune system is involved in other α -synucleinopathies^{291,292}. This evidence has suggested that modulation of adaptive immunity could, therefore, form the basis of disease-modifying therapy for PD and related α -synucleinopathies.

Innate immunity. Pathological α -synuclein induces microglial activation^{293,294} (Fig. 4) – microgliosis is widely observed in rodent and non-human primate models of pathological α -synuclein-induced degeneration^{295–300} and in the substantia nigra in post-mortem brains from people with PD^{301,302}. Pathological α -synuclein can also activate myeloid cells in the periphery^{303,304}.

Microglial activation and pro-inflammatory responses to pathological α -synuclein are initiated, in part, by uptake of extracellular pathological α -synuclein³⁰⁵. Toll-like receptors (TLRs) are activated by pathological α -synuclein and contribute to neuroinflammation³⁰⁶. Expression of TLR2 and TLR4 is higher among people with PD than among healthy individuals³⁰⁶. However, activation of TLRs in α -synucleinopathies can have protective and deleterious effects³⁰⁶; antagonism of TLR2 attenuates pathological α -synuclein-induced neurodegeneration^{307,308}, whereas activation of TLR4 clears α -synuclein aggregates³⁰⁹. Pathological α -synuclein also binds to microglial Fc γ receptor IIb, which in turn inhibits microglial phagocytosis and

decreases the clearance of pathological α -synuclein in culture and in A53T α -synuclein transgenic mice³¹⁰. The response of microglia to pathological α -synuclein is regulated by Fyn kinase activity and the class B scavenger receptor CD36 (ref. 311). Ultimately, activation of NF- κ B pathways in microglia seems to be central to the inflammatory response^{300,306,311}, but other transmembrane receptors that bind to pathological α -synuclein also have roles in microglial activation²⁸¹.

Pathological α -synuclein induces release of IL-1 β from microglia via priming and activation of the NLRP3 inflammasome³¹¹ (Fig. 4). NLRP3 and downstream ACS and IL-1 β are all elevated in people with PD³¹², providing support for the need to evaluate NLRP3 inhibition as a disease-modifying therapy in PD. Pathological α -synuclein also activates the cyclic GMP–AMP synthase–stimulator of interferon genes (cGAS–STING)-dependent interferon response, leading to neurodegeneration^{313,314} (Fig. 4); this mechanism raises the prospect of cGAS inhibitors as a disease-modifying therapeutic approach in PD³¹⁵ (Fig. 4). IL-6 released from α -synuclein-activated microglia induces dysregulation of neuronal iron uptake, leading to toxic neuronal iron accumulation³¹⁶. Some evidence has suggested that pathological α -synuclein also stimulates microglial cytokine secretion and reactive oxygen species production, leading to activation of mitogen-activated protein (MAP) kinase pathways that contribute to neurodegeneration^{303,317–319}. Microglia also play a part in the spread of pathological α -synuclein²⁸¹.

Astrocytes also have a role in the innate immune response to pathological α -synuclein. Pathological α -synuclein can be transmitted from neurons to astrocytes³²⁰, and inclusions of α -synuclein are present in astrocytes in post-mortem brains of people with PD^{321,322}. Astrocytes produce multiple pro-inflammatory cytokines, including IL-1 α , IL-1 β , IL-6, IL-18, tumour necrosis factor (TNF), chemotactic cytokines, and CC, CXC and CXCL chemokines^{320,323}. In addition, the profile of growth factors that astrocytes produce and secrete changes in response to pathological α -synuclein³²⁴. These pro-inflammatory responses in astrocytes are dependent on TLR4, whereas this receptor does not seem to be required for uptake of pathological α -synuclein into astrocytes^{325,326}.

Besides neuroinflammation induced by astrocytic accumulation of pathological α -synuclein, reactive astrogliosis contributes to neurodegeneration and neurotoxicity. In cultures, treatment of astrocytes with pathological α -synuclein or its overexpression in astrocytes leads to neuronal and astrocytic Ca²⁺ flux, oxidative stress and astrocytic reductions in cholesterol^{327,328}, presumably leading to neurotoxicity. Selective expression of A53T α -synuclein in mouse astrocytes led to astrocytic dysfunction in glutamate uptake and alterations in the blood–brain barrier, and induced microglial activation in the midbrain, brainstem and spinal cord, suggesting that inflammatory responses elicited by reactive astrocytes contribute to non-cell-autonomous neurodegeneration³²⁹. Furthermore, IL-1 α , TNF and complement component 1q (C1q) released from activated microglia in response to α -synuclein drive the formation of neurotoxic reactive astrocytes^{300,330} (Fig. 4), which induce cell death, in part, via saturated lipids³³¹. Glucagon-like peptide 1 (GLP-1) receptor agonists inhibit the release of IL-1 α , TNF and C1q from activated microglia, thereby reducing numbers of neurotoxic reactive astrocytes³⁰⁰ (Fig. 4). Consequently, GLP-1 receptor agonists are neuroprotective because (among other mechanisms³³²) they prevent microglial reactivity and formation of neurotoxic reactive astrocytes³⁰⁰ (Fig. 4). Epidemiological studies have indicated that GLP-1 receptor agonists, which are used for

the treatment of obesity and diabetes mellitus type 2, lower the incidence of PD³³³. In clinical studies, the GLP-1 receptor agonist exenatide slowed the progression of PD^{334,335}, and lixisenatide slowed the progression of early-stage PD in people receiving stable anti-symptomatic treatment for PD³³⁶. A 36-week clinical trial of the brain-penetrant GLP-1 receptor agonist NLY01 failed to meet its global trial end points, but pre-specified analysis has shown that PD progression was slowed substantially in people under 60 years of age³³⁷. The benefits of exenatide³³⁸ and lixisenatide were also greatest in people under 60 years of age³³⁶. Together, these studies support future clinical trials of GLP-1 receptor agonists in PD; trials in people under 60 years of age that last for more than 12 months are most likely to demonstrate benefits^{338,339}.

Conclusions and perspectives

Extensive studies to understand the physiological and pathological role of α -synuclein started in 1997, when a mutation in *SNCA* was discovered as a genetic cause of PD. Distinct pathological mechanisms of degeneration induced by pathological α -synuclein, including cell-autonomous and non-cell-autonomous mechanisms, are giving rise to a spectrum of preclinical validation studies and disease-modifying clinical trials in humans with PD. Insights into the role of innate and adaptive immunity have opened up new therapeutic opportunities for disease modification in PD. Understanding of cell-to-cell transmission mechanisms, disease-specific strains of pathological α -synuclein and the development of biomarkers based on SSAs and PET imaging are changing the landscape of basic research and clinical practice in this area. Further studies are required to identify the origins of different pathological α -synuclein strains and how they lead to distinct α -synucleinopathies. Methods that enable reliable distinction between the α -synucleinopathies will guide future clinical studies, and neuroimaging agents that enable monitoring of α -synuclein pathology and responses to therapy will be crucial to validate disease-modifying therapies. Furthermore, PET imaging and SAAs could enable identification of individuals who are asymptomatic but at risk of α -synucleinopathies early in the disease course, when disease-modifying therapies could be implemented before disease manifestation³⁴⁰. PET imaging and SAAs could also be used to biologically stage α -synucleinopathies, thereby further facilitating drug intervention trials.

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Author contributions

H.P., T.-I.K. and T.M.D. researched data for the article and wrote the article. V.L.D. and T.M.D. reviewed and edited the manuscript before submission. All authors made substantial contributions to discussion of the content.

Competing interests

T.M.D. owns stock in Aevum Therapeutics and stock options in D&D Pharmatech, Inhibikase Therapeutics and Ventyx Biosciences; holds equity in D&D Pharmatech; is compensated for his role on the scientific advisory boards of Aevum and Ventyx; is entitled to royalties from AbbVie; and is a founder and inventor of technology for Neuraly, which is now a subsidiary of D&D Pharmatech. V.L.D. owns stock options in D&D Pharmatech and Inhibikase Therapeutics; holds equity in D&D Pharmatech; and is a founder and inventor of technology for Neuraly, which is now a subsidiary of D&D Pharmatech. The other authors declare no competing interests.

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