Journal of Parkinson's Disease 3 (2013) 415–459
DOI 10.3233/JPD-130216
IOS Press

Review

Limelight on Alpha-Synuclein: Pathological and Mechanistic Implications in Neurodegeneration

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Abstract. The pathogenesis of many neurodegenerative disorders arises in association with the misfolding and accumulation of a wide variety of proteins. Much emphasis has been placed on understanding the nature of these protein accumulations, including their composition, the process by which they are formed and the physiological impact they impose at cellular and, ultimately, organismal levels. Alpha-synuclein (ASYN) is the major component of protein inclusions known as Lewy bodies and Lewy neurites, which are the typical pathological hallmarks in disorders referred to as synucleinopathies. In addition, mutations or multiplications in the gene encoding for ASYN have also been shown to cause familial cases of PD, the most common synucleinopathy. Although the precise function of ASYN remains unclear, it appears to be involved in a vast array of cellular processes. Here, we review, in depth, a spectrum of cellular and molecular mechanisms that have been implicated in synucleinopathies. Importantly, detailed understanding of the biology/pathobiology of ASYN may enable the development of novel avenues for diagnosis and/or therapeutic intervention in synucleinopathies.

Keywords: Alpha-synuclein, synucleinopathies, neurodegeneration, Lewy body, aggregation

HISTORIC PERSPECTIVE OF THE DISCOVERY OF ALPHA-SYNUCLEIN

The initial implication of alpha-synuclein (ASYN) in neurodegeneration arose with the identification of its presence in amyloid plaques of Alzheimer's disease (AD) patients [1]. Though ASYN was discovered half a decade prior, in the electric organ of *Torpedo californica,* and initially named *synuclein* for its ostensibly restricted cellular localizations, within synaptic nerve terminals and within the nuclear envelope [2], its presence in AD plaques was intermittently referred to as non-amyloid-beta component precursor (NACP). Non-amyloid-beta component of AD amyloid (NAC) is the hydrophobic peptide of ASYN, which actually predominates in fibrillary depositions– second only to the amyloid-beta (Aβ) fragment, itself [2]. NAC involvement was suggested to be a core facet of AD amyloidoisis and has been postulated to promote the process, via seeding of a nucleation center. In this proposed paradigm, proteolysis of ASYN avails its hydrophobic region for paired helical filament (PHF)/amyloid fibril attachment, whereby it may influence the aggregation state of Aβ, by potentiating the conversion of soluble Aβ into insoluble amyloid in AD [2]. This detrimental effect of ASYN was contentious, however, since other studies observed a

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ISSN 1877-7171/13/$27.50

lack of ASYN immunoreactivity in amyloid plaques from AD patients, with ASYN only being found in Lewy bodies (LBs), dystrophic neurites and surrounding the core of the plaques [3–5]. Identical results were obtained in AD mouse models, in which ASYN staining was absent in the amyloid core, calling into question the association between ASYN and Aβ pathology [6].

The implication of ASYN as a causal factor of neurologic pathology expanded when the SNCA gene, by which it is encoded, was identified as the first Parkinson's disease (PD)-related gene. This occurred when linkage of a dominantly inherited form of early onset, familial Parkinson's disease and a single point mutation (Ala53Thr) of SNCA was established in a large, well-characterized Italian-American kindred and in three unrelated Greek pedigrees [7]. A second missense mutation (Ala30Pro) in the ASYN gene, which was subsequently found to co-segregate with progressive parkinsonism in a German kindred [8], provided further evidence of a connection between ASYN and the pathogenesis of PD. Elucidation of these familial mutations impelled researchers to examine brain tissue of PD patients for the presence of ASYN within LB lesions, which pathologically characterize the disease. These studies led to successful identification of wild-type ASYN within LBs of patients with sporadic PD and Dementia with Lewy Bodies (DLB), indicating a core level of involvement of ASYN in the pathology of these neurodegenerative disorders [9]. It was, furthermore, determined that selective deposition of ASYN into LBs is a phenomenon that occurs in a range of disorders, collectively referred to as synucleinopathies (Fig. 1).

THE SYNUCLEINS

The synuclein protein family is comprised of three members: alpha-, beta- (BSYN) and gamma-synuclein (GSYN), whose genes have been mapped to chromosome 4q21, 5q35 and 10q23, respectively [10, 11]. The sequences of these soluble, heat-resistant proteins are highly-conserved and they are relatively similar to one other (Fig. 2). Although the first 42 amino acids in the sequence of all three members are identical, ASYN and BSYN are more closely related to each other [12] and they are both more highly-conserved than GSYN. Of the trio, ASYN is most implicated in the pathogenesis of neurodegenerative disorders, though a recent report sheds new light on implications for a role of BSYN-induced pathology in neurodegeneration [13].

SYNUCLEINOPATHIES

The presence of LBs and Lewy neurite (LN) lesions, which are found, respectively, within the soma and processes of degenerating nerve cells, was initially described by F. Lewy, in 1912. Lewy identified the inclusions in brain tissue from PD patients and he defined them as eosinophilic and insoluble in ethanol, chloroform and benzene, indicative of a proteinacious constituent [14]. It is now well established that amyloidogenic filaments of ASYN are the primary component of LBs and LNs. Synucleinopathies comprise a class of neurodegenerative diseases that share a morphologic hallmark, which is pivotally characterized by the involvement of Lewy pathology in a subset of neurons and glia. The synucleinopathies include PD, DLB, Multiple System Atrophy (MSA) and Pure Autonomic Failure (PAF). PD and DLB are the most prevalent neurodegenerative disorders, after AD, and it is with these conditions that intracytoplasmic LBs and dystrophic LNs are most commonly associated.

PARKINSON'S DISEASE

PD is the second most common neurodegenerative disease whose classical clinical motor manifestations include postural instability, bradykinesia, resting tremor and muscular rigidity (Fig. 1). Additionally, many patients experience a range of non-motor symptoms, which usually precede onset of movement-related maladies, such as depression, dysautonomia, gastrointestinal disturbances and sleep disorders [15–17]. Pathologically, PD is largely characterized by loss of nerve cells within the *substantia nigra pars compacta* (SNpc) and by the consequential attenuation of dopaminergic innervation to the striatum. Surviving neurons bear signs of Lewy pathology and exhibit abrogated levels of neuromelanin pigmentation [17]. Most cases of PD are sporadic and, although environmental influences such as pesticides, cigarette smoking and coffee have been suggested to impact PD risk [18], aging represents the most dominant risk factor for development of the disease [19–22]. Thus, in the absence of therapeutic intervention, incidence of PD is predicted to rise as life expectancy continues to increase.

A genetic basis for the etiology of PD is also well established, as many distinct chromosomal regions have, to date, been linked to PD [23–25] (Table 1). A subset of these regions contain genetic mutations that are causally related to monogenic forms of the dis-

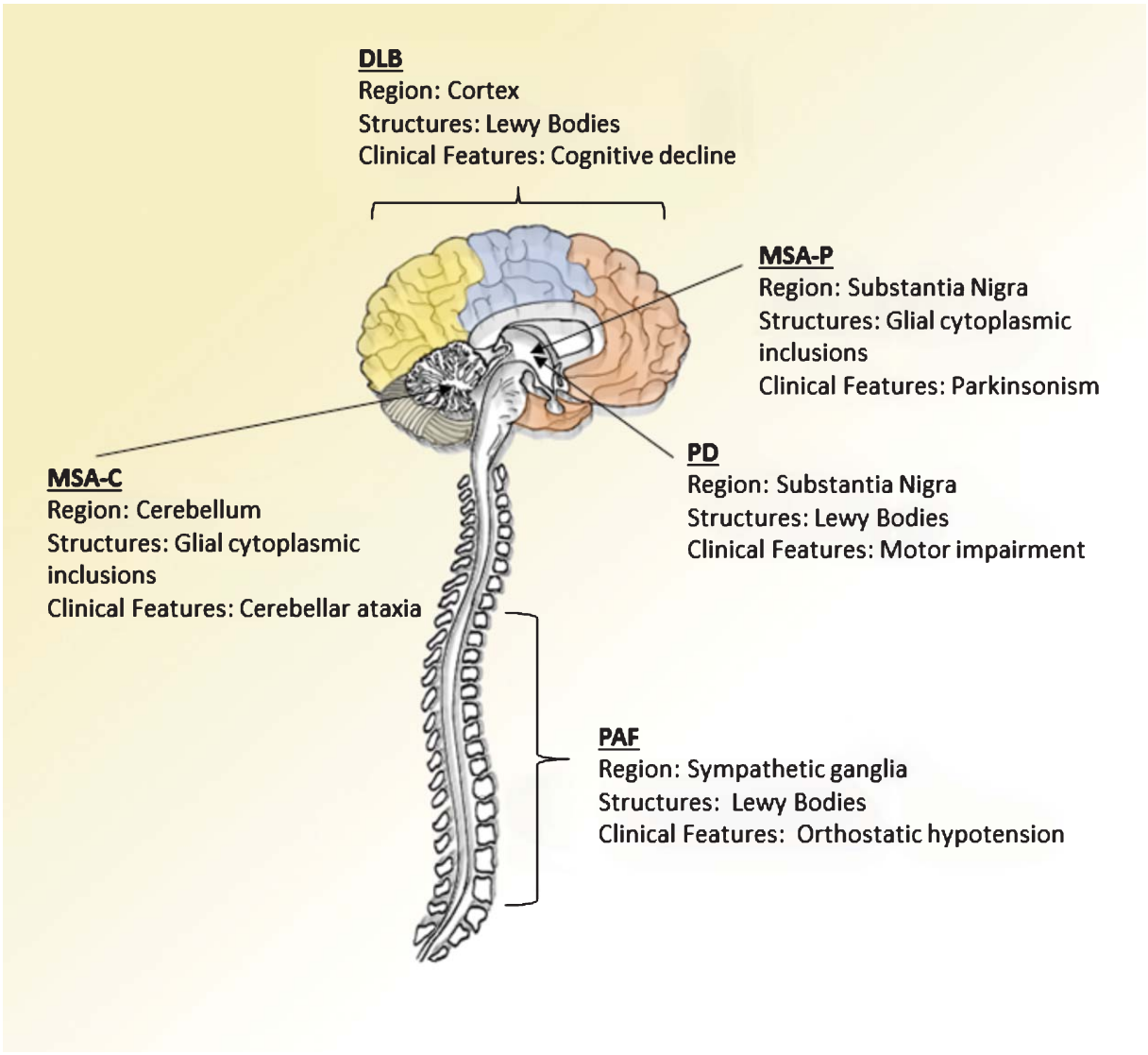

Fig. 1. Schematic overview of synucleinopathies. Major neuropathologic features of the synucleinopathies discussed in this review, including anatomical localization of most affected brain regions, histopathological changes and primary clinical manifestations. Note that the pathophysiology of these neurodegenerative disorders is deeply complex and only key hallmarks are represented here.

ease, which comprise approximately 10% of PD cases [26–28]. The most well-studied familial mutations are those in the *SNCA* gene. Missense mutations (A30P, E46K A53T, H50Q, G51D) [7, 8, 29–31] of ASYN, as well as duplication and triplication of the wild-type *SNCA* gene [32, 33], all appear to confer a gain of cytotoxic functional effect, implicating upregulated expression levels and qualitative alterations of ASYN in PD onset and progression [33–35]. While ASYN point mutations are considered rare occurrences, which have only been reported in a few isolated cases of multi-generational PD families [36], *SNCA* multiplications are more prevalent, such that 31 families have been reported across the globe [33, 37–41]. Clear indications of *SNCA* dosage effects have been ascertained from studies that assessed and/or compared disease states of multiplication carriers and described greater severity of symptoms from triplication, versus duplication, patients [33, 40, 42–44]. Further evidence for the importance of this protein in the etiology of PD has been gathered from recent genome-wide association studies, showing that polymorphic variations in non-coding regions of the SNCA locus represent a major factor to sporadic PD pathogenesis [45–47].

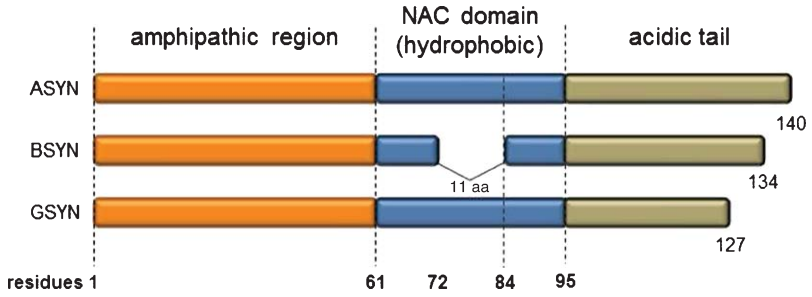

Fig. 2. Schematic structures of synuclein proteins. The synuclein family is composed by ASYN, BSYN and GSYN protein, which share high homology. The N-terminal region is highly conserved between the three proteins, while major differences are observed in the acidic C-terminal, with BSYN and GSYN having a shorter size when compared to ASYN. BSYN lacks 11 amino acids in the NAC domain, which is responsible for the amyloidogenic properties of the protein and this may influence the aggregation propensity of BSYN.

Sequencing of the SNCA gene has revealed that common variants, including a dinucleotide repeat sequence (REP1) polymorphism of the *SNCA* promoter region, which may result in upregulation of ASYN levels, are associated with increased risk for PD [48]. Nevertheless, others have found no association between SCNA REP1 variability and PD pathogenesis [49, 50].

DEMENTIA WITH LEWY BODIES

DLB is a highly debilitating cognitive condition of the elderly. It is the second most frequent cause of dementia [51, 52] and, due to a clinical profile that overlaps with AD and PD, it is often misdiagnosed in early stages [53, 54]. Fluctuating levels of consciousness, visual hallucinations and parkinsonism typify differential diagnosis of the disease [55–57]. Overall, DLB patients experience greater levels of neuropsychiatric symptoms than AD patients and these symptoms can present in early stages of disease progression [58, 59]. Motor symptoms are typically predominated by bradykinesia and rigidity and are often devoid of resting tremor [57].

Similar to PD, nigral-related pathology is also observed in DLB. A classical sign of the disorder is degeneration of the cortical areas of the brain with formation of cortical LBs (Fig. 1) [53] and, furthermore, the number of cortical LBs correlates with severity of dementia [60]. ASYN immunoreactivity is widely used to identify LB markers of pathology and, based on this method, three basic clinicopathological classifications of DLB have been defined: (1) brainstem-predominant, (2) limbic (transitional) and (3) diffuse neocortical [53]. Additional considerations of concomitant involvement of AD-like pathology and Lewy-related pathology (LRP) have resulted indications of a direct correlation between DLB and LRP and an inverse relationship between DLB syndrome and AD pathology [61–63]. Improved diagnostic criteria and retrospective analyses have revealed that, while LRP burden is largely concentrated in the amygdala of AD patients, a more widespread pattern of deposition is observed in DLB patients [64–67]. Detecting differences between PD, DLB and AD is challenging. Postmortem classifications are more accurate when LRP and AD pathology are both considered [63] and successful differentiation in the clinic is dependent on detailed assessments of attention, cognitive function and episodic memory [68].

MULTIPLE SYSTEM ATROPHY

MSA is a relatively rare but rapidly progressive nerve disorder with generally poor function and survival prognosis [69–72]. Patients present with dysautonomia and motor impairment. Though prominent symptoms may evolve throughout disease progression, two motor subtypes are classified by dichotomous profiles of movement dysfunction. A parkinsonian phenotype delineates the MSA-P variety from MSA-C, which is typified by cerebellar ataxia [72]. Progressive akinesia and rigidity are the most prevalent symptoms of MSA-associated parkinsonism, however, tremor is less common than in PD. Postural instability occurs in early stages but frequent falling is not typical at disease onset. MSA-C is characterized by cerebellar oculomotor dysfunction, ataxic gait, limb kinetic ataxia and ataxic dysarthria [73]. Nearly all MSA patients experience autonomic failure [74].

Three diagnostic categories have been established, consisting of possible, probable and definite diagnoses but glial cytoplasmic inclusions (GCIs), primarily composed of filamentous ASYN and found in oligodendrocytes of afflicted individuals, are recognized as

Table 1
Summary of PD-associated loci, genes and potential risk factors. AD: autosomal dominant; AR: autosomal recessive; KRS: Kufor Rakeb syndrome

Symbol	Gene	Locus	Status and inheritance	Disease onset	Lewy bodies	Mutations	Risk-confering variants	Refs.
PD causative loci and genes								
PARK1/PARK4	SNCA	4q21-q22	Validated; AD; rarely sporadic	Early	Yes	A30P, E46K, A53T; H50Q; G51D; genomic duplica-tions/triplications	Dinucleotide polymorphism (REP1) in the SNCA promoter increases risk for PD	[7, 8, 29, 31–34, 44, 48, 465–467]
PARK2	Parkin	6q25.2-q27	Validated; AR; sporadic	Juvenile; Early	No	≈ 170 mutations (different mutations accoss all 12 exons: point mutations; small detetions; deletions (exon 3); duplications of exons); ≈ 887 exonic rearrangements	Promoter polymorphisms increase risk for PD; heterozygous mutations may increase risk for late-onset PD	[23, 468–473]
PARK3	SPR (?)	2p13	Not validated; AD	Late	Yes	Not identified	Variants may increase risk for PD	[474, 475]
PARK5	UCHL1	4p13	AD		Yes	1 heterozygous missense mutation	S18Y variant may be a weak protective factor against early-onset PD	[476–483]
PARK6	PINK1	1p35-p36	Validated; AR; rarely sporadic	Early	Yes (recent report)	≈ 50 point mutations (missense/nonsense mutations), rare whole-exon deletions (exons 4–8, 6–8, 7; one heterozygous whole-exon deletion); exonic rearrangements	Heterozygous mutations may increase risk for late-onset PD	[473, 484–493]
PARK7	DJ-1	1p36	Validated; AR		?	≈ 15 point mutations (missense mutations in coding and promoter regions, frame-shift and splice site mutations), exonic deletions	Heterozygous mutations may increase risk for late-onset PD	[473, 494–500]
PARK8	LRRK2	12q12	Validated; AD; sporadic	Late	?	>80 missense variant, 7 pathogenic (R114C, A1437H, R1441G, R1441H, Y1699C, G2019S, I2020T); mostly missense mutations; rare splice site and nonsense variants	2 variants (G2385A, R1628P) increase risk for PD	[471, 501–506]

Table 1
(*Continued*)

Symbol	Gene	Locus	Status and inheritance	Disease onset	Lewy bodies	Mutations	Risk-confering variants	Refs.
PARK9	ATP13A2	1p36	Validated; AR	Complex phenotype; Early PD; Juvenile KRS		≈ 10 point mutations (missense/truncating mutations)	Heterozygous variants increase risk for PD	[507–510]
PARK10	(?)	1p32	Not validated; Unknown relevance - may represent a risk factor for PD	Late		Not identified	Unknown	[511–513]
PARK11	GIGYF2 (?)	2q36-q37	Not validated; Unknown relevance - may represent a risk factor for PD; AD			Missense variants		[514–519]
PARK12	?	Xq21-q25	Not vaLidated; Unknown relevance - confirmed as risk factor for PD			Not identified		[520, 521]
PARK13	Omi/HTRA2	2p12	Not validated; Unknown relevance - may represent a risk factor for PD; AD			2 missense variants	Regulatory variants may contribute to risk for PD	[522–524]
PARK16	?	1q32	Not vaLidated; Unknown relevance - confirmed as risk factor for PD	?		Not identified	Variants increase risk for PD	[45, 46]
PARK17	VPS35	16q11.2	Validated; AD			2 point mutations	Unknown	[525, 526]
PARK18	EIF4G1	3q27.1	Not validated; AD		Yes	Translation initiator mutations		[527]
Loci and genes associated with atypical parkinsonism								
PARK14	PLA2G6	22q13.1	Validated; AR	Juvenile levodopa-responsive dystonia-parkinsonism	?	2 missense mutations	Unknown	[528]
PARK15	FBXO7	22q12-q13		Early-onset parkinsonian-pyramidal syndrome		Point mutations		[529–531]
Recently identified genes and potential risk factors for PD								
SCA2 (12q24.1); GBA (1q21); MAPT (17q21.1); GAK (4p16); HLA-DRA (6p21.32); APOE (19q13); Synphilin-1 (5p23); NR4A2/Nurr1 (2q22-q23); Mortalin(HSPA9) (5q31); SYT11 (1q21.1); RAB7L1 (1q32); ACMSD (2q21.3); STK39 (2q24.3); MCCC1/LAMP3 (3q27); BST1 (4p15); SCARB2 (4q21.1); STBD1 (4q21.1); HLA-DRB5 (6p21.3); GPNMB (7p15); FGF20 (8p22); CCDC62/HIP1R (12q24); STX1B (16p11.2); SREBF1 (17p11.2)								[47, 532–546]

the defining histopathological marker of the disease (Fig. 1) [75–77]. Although autopsy analysis usually reveals widespread pathology of GCIs, olivopontocerebellar and striatonigral regions are most implicated in clinical involvement and neuronal loss [71, 78–82]. In general, patients are poorly responsive to dopaminergic therapy [70] and unresponsiveness has been correlated to neuronal loss in the putamen [83]. By convention, MSA is regarded as a sporadic disorder [84, 85], though a few familial descriptions have been reported [85–87] and, recently, the G51D SNCA mutation, which results in a parkinsonism-pyramidal syndrome, has been associated with MSA [31, 88].

PURE AUTONOMIC FAILURE (PAF)

PAF is a severe and gradually progressive, middle-to-late onset degenerative disorder of the autonomic nervous system, which is distinguished from PD and MSA by absence of typical neurologic features [89]. Progressive autonomic disturbances of the disorder result from the degeneration of the peripheral autonomic nervous system, without central involvement [90]. Orthostatic hypotension and anhidrosis/hypohidrosis are the defining clinical symptoms [91, 92]. Early stage MSA with prominent autonomic failure can be particularly difficult to distinguish from PAF, resulting in elevated incidence of inaccurate preliminary diagnoses [92]. Though late stage PAF patients may endure severe states of autonomic disturbances, life-expectancy and capacity for maintenance of daily living activities remains minimally affected [93]. The presence of LB pathology in the peripheral nervous system indicates that PAF is a member of the synucleinopathies (Fig. 1) [94].

ASYN: THE PURPORTED CULPRIT

ASYN is a small (14.5 kDa), highly charged and heat stable protein, with a sequence composed of 140 amino acids and functionally divided into three subdomains (Fig. 2). The N-terminal region (residues 1–60) is highly-conserved and contains a series of imperfect hexameric (KTKEGV)-containing repeats, which remain unstructured in solution and form amphipathic α-helices in the presence of lipids [95, 96]. The α-helical structures share strong homology to lipid-binding domains of class A_2 apolipoproteins [97] and they are hypothesized to impart lipid-binding capacity to ASYN. The central, hydrophobic (NAC) domain (residues 61–95) is responsible for amyloidogenic properties of the protein, rendering conformational transformation of random coil to β-sheet structure a possibility. The C-terminus (residues 96–140) is highly acidic [95], composed largely of glutamate, aspartate and proline residues and possesses a strongly negative charge. It is the least conserved region, among the synucleins, and it is postulated to confer chaperone-like activity [98–100].

The intrinsically disordered nature of ASYN requires the implementation of sophisticated assays to obtain structural information. This protein has not been crystallized and the leading technique to describe the features of ASYN monomers is spectroscopy. Despite absence of a well-defined secondary structure, it is possible to predict propensities of different regions of the protein for adopting secondary structural features. This is possible via comparison of the chemical shift of different nuclei of the protein with those corresponding to random coil peptides [101]. To that end, NMR chemical shift studies have identified that α-helical propensities are mostly localized to the N-terminal region and that extended, β-sheet conformations are more commonly observed within the C-terminus of ASYN [102, 103]. Helical propensity, which is inversely correlated with aggregation tendency [104], is diminished in A30P and A53T forms of the protein and A53T exhibits enhanced propensity for β-sheet conformations [105], while E46K differs least from that of wild-type ASYN [106].

A powerful approach to characterize the transient structures involves employment of paramagnetic relaxation enhancement measurements. In this way, it has been possible to detect important, long-range intramolecular interactions within monomeric forms of ASYN [107–109]. Most notably, a hydrophobic patch, amid the C-terminal region of the NAC domain (residues 85–95), has been found to interact with the C-terminus (residues 110–130) [107]. As well, intramolecular interactions have been described to occur between the C-terminus and the N-terminus. Both interactions are hypothesized to have stabilizing effects on monomeric forms of ASYN and, thereby, prevent oligomerization. These interactions are disrupted in A30P and A53T mutations [110] and are unaffected or enhanced in E46K forms of ASYN [106]. Moreover, within this context, phosphorylation at Ser-129 is inhibitory to *in vitro* fibrillization of ASYN [111].

In the final stages of aggregation, the insoluble, fibrillar species of ASYN are far too large to be assessed by solution NMR. Instead, high-resolution, solid-state magic angle spinning (MAS) NMR spectroscopic anal-

ysis can be used. In this regard, it has been shown that full-length ASYN fibrils are composed of a disordered N-terminal domain, a flexible C-terminus and a β-sheet rich core that is comprised, at minimum, of residues 38–95 [112]. Furthermore, this study elucidated that the C-terminus of ASYN begins at residue 107, if not sooner. Solid-state MAS NMR spectroscopy analysis of A53T ASYN reveals an increased capacity for β-sheet structures in a manner, which is likely to extend the core region [113]. Additionally, a likelihood for the occurrence of at least two distinct mechanisms of fibril nucleation has been determined by solid-state NMR analysis, as a result of observed differences in spectral profiles of the central core region between samples [114].

A plethora of protein [115–119] and metal ion [120, 121] interactions are thought to occur primarily through the C-terminal region of ASYN. A number of posttranslational modification (PTM) sites have also been mapped to this region, suggesting they may modulate the function/behavior of ASYN in the cell.

Of note, ASYN shares structural homology with the 14-3-3 family of chaperone proteins [122], binds to 14-3-3 proteins, themselves, and to the ligands of 14-3-3 proteins as well [123].

A splice variant of ASYN, of 112 amino acids, is expressed in cardiac, skeletal and pancreatic tissues but less is known about this variant [124].

BSYN: FRIEND OR FOE?

BSYN is very similar to ASYN in most respects. Slightly smaller in size, the sequence of BSYN consists of 134 amino acids, which are even more highly conserved than that of ASYN [12]. Interestingly, a putative phosphorylation site of a serine residue at position 118 has been conserved across species in BSYN but not in ASYN or GSYN. The most pertinent distinction from ASYN is a lack of 11 amino acids (residues 73–83) in the center of the amyloidogenic NAC domain (Fig. 2) [12, 125]. In adult rats, BSYN has a wide distribution throughout the brain [126].

While some evidence indicates a protective role for wild-type BSYN, as it exhibits low propensity to form fibrils and it is able to decrease ASYN aggregation [127–129], possibly by direct interaction with Akt [130], other studies point out that several cellular stressors are able to induce fast and efficient BSYN fibrillation [131]. Furthermore, BSYN was detected within hippocampal axonal brain lesions of PD and DLB patients [132]. Point mutations in BSYN protein have been described in rare cases of DLB [133] and have been shown to induce neurodegeneration in mice [134]. Interestingly, we have recently demonstrated that overexpression of wild-type BSYN protein leads to formation of proteinase K-resistant aggregates and dopaminergic neuronal loss in primary neurons and in rats [13], suggesting that this homologue may also play a role in the neurodegenerative process. Therefore, even if BSYN does, in fact, confer neuroprotective potential, it may also assume neurotoxic properties, under certain conditions, and this demands further investigation.

GSYN

GSYN is the least conserved and the smallest member of the synuclein family, with a sequence length of 127 amino acids (Fig. 2). Sequence alignment demonstrates that breast cancer specific gene (BCSG1), a putative marker of breast cancer progression [135], is equivalent to GSYN [136]. Though GSYN is most prevalently expressed in the brain, it is also expressed in the ovary, testis, colon and heart [136]. GSYN contains nearly twice as many amino acid substitutions with ASYN, as compared to BSYN [136]. So far, no direct correlation between GSYN and synucleinopathies has been identified.

AGGREGATION AND POSTTRANSLATIONAL MODIFICATIONS OF ASYN

Most neurodegenerative diseases can be seen as dynamic and triangulated processes, composed of interrelated aspects that influence each other, such as protein aggregation, neuronal dysfunction and neuronal death. Numerous research efforts have focused on understanding the mechanisms involved in the aggregation process. Understanding this process is crucial to identifying the toxic species, which result in disease. The central domain of ASYN is believed to be required for oligomerization and fibrillization of the protein [137]. Deleting or disrupting this region, for example, impedes the ability of ASYN to form amyloid fibrils. The C-terminal region of ASYN can also modulate aggregate formations, in a pH-dependent manner [138].

Misfolding of ASYN leads to aggregation-prone conformations, which ultimately evolve into amyloid-like fibrils (Fig. 3). An *in vitro* ASYN fibrillation pathway has been demonstrated to occur in a

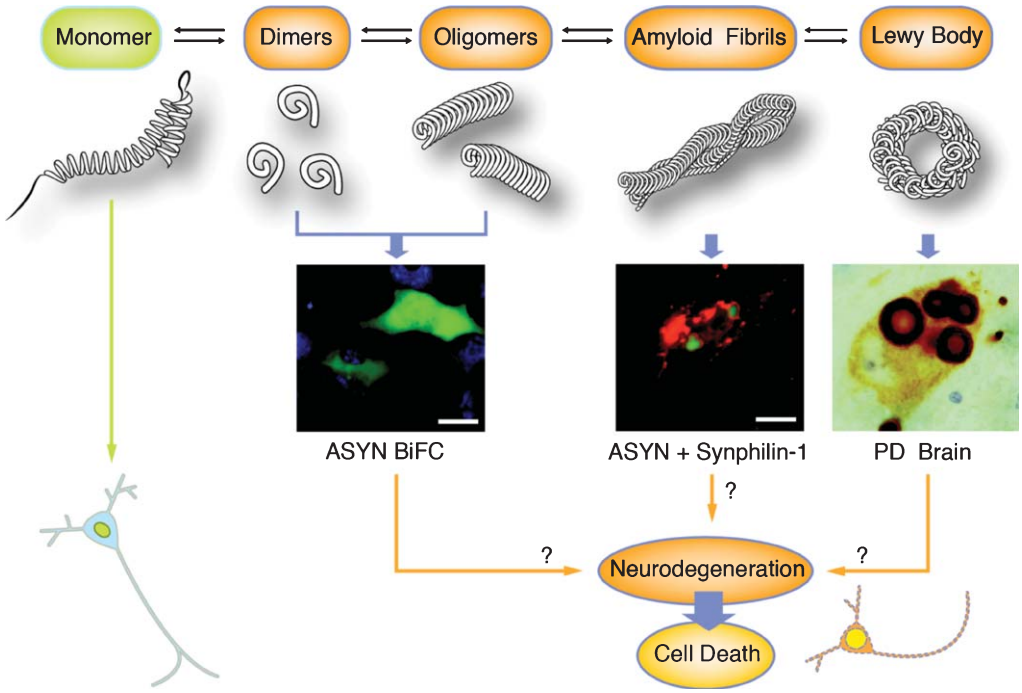

Fig. 3. Schematic model of ASYN aggregation. Cellular failure to degrade misfolded monomeric ASYN may favor a homo-interaction and promote the generation of unstable dimers and oligomers. Oligomers may associate with monomers and form a range of intermediary species, including amyloid fibrils, in a nucleation-dependent manner. The accumulation of amyloid fibrils leads to the formation of LBs, one of the primary hallmarks of synucleinopathies. Which of these species is the most responsible for cellular toxicity and neurodegeneration is still unknown. The bimolecular fluorescence complementation (BiFC) assay, which relies on formation of a fluorescent complex, from non-fluorescent components, enables visualization of ASYN dimers and oligomers (green) in living cells [258]. Assessment of ASYN aggregation is also possible by co-transfection of ASYN and Synphillin-1, which promotes inclusions that are comprised of ASYN (red) and are immunoreactive for ThioflavinS (green) [547].

nucleation-dependent manner, such that a range of intermediate species is produced [139]. Through the use of electron and atomic force microscopy, two distinct morphological forms of protofibrillar ASYN species have been defined - a spherical and an annular form. Though monomeric ASYN is natively unfolded in solution, it possesses a central hydrophobic region, from which it derives its tendency to oligomerize into spherical protofibrils - stabilizing β-sheet-rich conformations that resemble normal amyloid fibrils. Formation of fibrils and formation of an alternate, pore-like annular protofibril form of ASYN appears to be dependent on production of the rapidly-forming spherical protofibril [140]. It is believed that a similar process occurs *in vivo*, whose misregulation results in accumulations of annular protofibrillar structures, and that these species will pose a greater threat of toxicity to neurons than mature fibrils, themselves [140–142].

Two recent reports challenge a conventional understanding of the native conformation of ASYN [143, 144]. Though ASYN has, historically, been viewed as an "intrinsically disordered" protein, it has now been suggested to predominantly exist in a stable, tetrameric state, which possesses a low propensity for aggregation. The debate over the native conformation is ongoing. Although various posttranslational modifications (PTMs) of ASYN are hypothesized to influence aggregation events, underlying mechanisms remain elusive. The major disease-associated PTMs are phosphorylation, truncation and ubiquitination, and they will be discussed below in greater detail [115]. Other PTMs, such as N-terminal acetylation [143, 145] and sumoylation [146, 147], have also been described but their precise contributions to synucleinopathies are less clear.

PHOSPHORYLATION

A total of four serine, four tyrosine and ten threonine residues serve as sites for prospective phosphorylation events within the ASYN sequence. All of these sites are highly conserved across species and, with only one exception (serine 87), they are all localized to the C-terminus of the protein [115]. A large fraction of

the ASYN within LB inclusions, isolated from post-mortem brain tissue of PD, DLB and MSA patients, is phosphorylated at serine 129 (S129-P) [145, 148–151] and, as such, S129-P is considered to be a signifying marker of these disorders [145, 148, 150, 151]. Upregulated levels of serine 87 phosphorylation (S87-P) are also associated with synucleinopathies [152], while phosphorylation of tyrosine residues Y125, Y133 and Y135 have been inversely correlated with aggregation and S129-P-related toxicity, indicating a probability of posttranslational molecular cross-talk among various sites [153]. Evidence, derived from several *in vitro* studies, suggests that the ASYN fibrillization process is inhibited by phosphorylation of S129 [115, 152, 154, 155], implying that S129-P is non-essential and, perhaps, even counteractive to LB formation. Observations that fibrillated forms of ASYN become phosphorylated at S129, furthermore, suggest that S129-P may actually occur as a late-stage event, during the course of LB maturation, which follows ASYN fibrillization [152, 154].

Conclusions, drawn from *in vivo* studies, regarding effects of S129-P of ASYN are, in large part, conflicting to one another. While S129-P appears to be necessary for ASYN-induced toxicity in *Drosophila* [156], for example, the reverse of this has been cited in *Caenorhabditis elegans* [157]. Results in rodent models of PD, utilizing recombinant adeno-associated viral (rAAV) vectors to express the phosphorylation mimic (S129D) or nonphosphorylatable (S129A) version of ASYN have yielded discordant results as well. Whereas some studies have cited greater levels of dopaminergic cell loss, upon expression of S129A-ASYN, as compared to that of S129D-ASYN [158, 159], another group observed no significant differences between the two conditions [160]. A53T ASYN-induced toxicity in the rAAV-based rat model of PD is, however, enhanced by increased levels of S129-P [161]. In this model, temporal exacerbation of dopaminergic neuronal degeneration is specifically associated with expression of G-protein-coupled receptor kinase 6 (GRK6), which results in S129 phosphorylation of ASYN, such that neuronal loss begins at an earlier time-point, as compared to control. Along this line, a recent study has found that, though no differences are observed between S129D and S129A variants of ASYN in later stages of disease progression, in the rAAV-based rodent model, neuronal degeneration progresses at a slower rate in the mutant S129A ASYN animals [162], suggesting that S129 phosphorylation may play an important role in regulating the time course of disease progression.

TRUNCATED FORMS OF ASYN

Approximately 15% of ASYN in LBs is truncated [120, 163] and at least five species of C-terminally truncated forms of ASYN were initially identified by mass spectrometry [145]. Analysis of cytosolic and LB-derived fractions demonstrated that some truncated species are LB-specific. Subsequent to these findings, three additional truncations (two C-terminal isoforms and one N- and C-terminal isoform), detected only in aggregated forms of ASYN, have been identified in brain tissue of PD, DLB and MSA patients [164–166]. Though the isoforms are detected in normal brain tissue, they are highly enriched in SDS- and urea-soluble fractions of PD and DLB samples [164–166]. Several studies have demonstrated that truncated ASYN species promote fibril assembly [167, 168] and potentiate aggregation of the full-length version of the protein [164, 165, 167, 169, 170]. Taken together, these findings indicate that, though ASYN truncation may occur via normal cellular processing of the full-length protein, the products of truncation may represent a population of aggregation-prone species, which are capable of initiating fibrillogenic processes and, perhaps, even seeding aggregation *in vivo*. Several studies utilizing transgenic mouse models of Lewy body disease (LBD) have, indeed, reported that C-terminal truncation of ASYN results in enhancement of neuropathological features [171–173]. Furthermore, expression of C-terminally truncated ASYN leads to pathological accumulations of full-length ASYN in rats [170] and, as well, it is reported to accumulate within dystrophic neurites in the murine Thy-1-ASYN transgenic model of LBD [174]. Axonal accumulations of ASYN fragments may arise as a result of impaired axonal transport and/or may represent a causal factor of axonopathies in PD and DLB.

UBIQUITINATION AND DEGRADATION OF ASYN

The major degradation pathways responsible for eliminating misfolded, aggregated and otherwise damaged forms of proteins are the ubiquitin proteasome system (UPS) and the autophagy-lysosomal pathways (ALP) [175]. The UPS is conventionally believed to be responsible for processing short-lived, soluble proteins within the cell and polyubiquitination is recognized as the classical posttranslational marker, which signifies that a protein is destined for degradation by the 26S proteasome complex [176–179]. LBs of PD and DLB

are abundantly-laden with ubiquitinated structures and proteasome subunits have, as well, been positively identified as constituents of those environments [180–184]. Genetic mutations [185, 186] and down-regulated expression levels in enzymes [187], which mediate the proteasomal pathway, have, furthermore, been linked to familial and juvenile forms of PD. Moreover, the hydrolytic activities of proteasomes within the SNpc of PD patients are significantly reduced [188, 189], as compared to normal controls. For these reasons, degradation of ASYN was initially thought to occur exclusively via the UPS and earlier studies were primarily focused on evaluating impairments within this system [190–195]. Though investigative efforts, aimed at assessing the link between proteasomal impairment and ASYN accumulation, have largely concluded in support of a role for metabolism of ASYN by the proteasome, some results have been conflicting [190, 193, 196].

One inherent conundrum is posed by the observation that, though most LBs are highly immunoreactive to ubiquitin, approximately 10% are devoid of its presence [197–199]. Moreover, while many studies have found that inhibition of proteasomal function results in impaired clearance and accumulation of ASYN [190, 193, 196], others have reported the reverse of this, citing unlikeliness that ASYN is a substrate of the proteasome at all [190, 191, 193, 200, 201]. An early report cited that wild-type and A53T forms of ASYN are both degraded by the proteasome in SH-SY5Y neuroblastoma cells, that this degradation is selectively inhibited by β-lactone-imposed blockade of proteasomal activity and that the A53T mutant form of ASYN is more resistant to proteasomal degradation than wild-type ASYN [190], indicating a putative explanation for the pathologic effect of the mutant form of the protein. A counter report, utilizing HEK293 and murine neuronal cell models, described conflicting results, whereby, neither wild-type, nor A53T, forms of ASYN are degraded by the UPS [191].

Induction of endogenous ubiquitin/ASYN-positive, cytoplasmic inclusions, as a result of proteasomal inhibition, was reported to occur, however, in the *in vitro* PC12 dopaminergic cell line. This result was observed in differentiated cells, as well as in naïve cells that express minute levels of ASYN, demonstrating that relatively low ASYN expression levels can be recruited to ubiquitin-positive inclusions, during circumstances of impaired proteasomal function. Interestingly, only viable cells were found to be positive for ASYN immunoreactivity and for discretely, punctated patterns of ubiquitin, as opposed to diffuse patterns of ubiquitin staining that are observed within the cytoplasm of apoptotic cells. Findings of this study demonstrate a causal link between proteasomal inhibition and neuronal cell death. This study, furthermore, reported a solid correlation between UPS impairment and inclusion formations, which were positive for both ubiquitin and ASYN, but reported inconclusive findings regarding correlations between inclusion formation and neuronal cell death [200].

In a follow-up study, conducted by the same laboratory, results derived from PC12 cells [200] were examined further, within the context of primary cortical cultures [202]. Results from this study indicate that primary cortical neurons are similarly affected by proteasomal inhibition, as compared to cells of the PC12 dopaminergic line. Characterization by confocal imaging revealed co-localization of ASYN, thioflavin S and ubiquitin within inclusions of proteasomaly-impaired cells, compared to diffuse patterns of ASYN staining and diminished levels of thioflavin S in control cells. Examination of protein extracts showed that proteasomal impairment results in aggregated forms of detergent-insoluble ubiquitin within cytoplasmic inclusions, however, it was determined that ASYN protein content, itself, is not aggregated or ubiquitinated in this setting, suggesting that proteasomal impairment may not be the primary causal factor, underlying aggregation of ASYN [201]. Further investigations in this model determined that, while proteasomal inhibition results in accumulations of ASYN within ubiquitinated and detergent-insoluble inclusions, ASYN is not actually required for the formation or insoluble nature of such inclusions. Rather, it appears that ASYN is integrally involved in the formation of fibrillar and amyloid-like structures, which may characterize the inclusions [203]. Similarly, in conditional knock-out mice, 26S proteasomal depletion is sufficient to drive neurodegeneration and to induce formation of ubiquitinated Lewy-like inclusions that are positive for ASYN aggregation [204]. However, inclusion formation and neurodegeneration in mice, caused by genetic depletion of 26S proteasomes, both occur independently of ASYN [205].

Another study that identified ubiquitinated forms of ASYN in aggregate-containing, insoluble cellular fractions, derived from brain tissue of PD and DLB patients, found that impaired proteasomal activity was not detected in these regions, which possessed ubiquitin-positive ASYN [166]. This study demonstrated that only LB-derived ASYN from diseased tissue, as opposed to normal tissue, was ubiquitinated–to an extent, however, that is insufficient for driving

ubiquitin-dependent degradation by the UPS [178]. One explanation, supported by findings of others [193], is that, *in vivo*, ubiquitination might not be required for proteasomal degradation of ASYN; the full-length, unmodified version of ASYN may enter the 20S subunit of the proteasome in a manner that is ubiquitin-independent. It has also been proposed that ASYN is ubiquitinated in a non-canonical, ATP-independent manner, via ubiquitin C-terminal hydrolase-L1 (UCH-L1), [206, 207]. Mutations and variants of the UCH-L1 gene result in gain-of-cytotoxic effects, including intracellular buildup of ASYN, resulting from inhibition of its degradation [208].

Moreover, the ubiquitin-protein isopeptide ligase, seven in absentia homolog (SIAH), is also known to be responsible for monoubiquitination of ASYN at lysines 12, 21 and 23, which are reported to be monoubiquitinated in LBs [209]. SIAH is a direct interactor of ASYN that has been demonstrated to promote aggregation of ASYN, *in vitro* and *in vivo,* and monoubiquitination of ASYN, by SIAH, is believed to act as a trigger event for ASYN aggregation and LB formation [209]. Alternatively, it is possible that Ser-129 phosphorylated forms of ASYN may be degraded by the proteasome, in a ubiquitin-independent/dephosphorylation-dependent manner [210]. In this scenario, breakdown of the proteasomal system would explain the presence of accumulated P-129-ASYN in LBs. Furthermore, while it may be likely that the UPS plays a role in regulating ASYN, it is also possible that ubiquitinated forms of ASYN arise as a result of pathological circumstances, which exist independently of proteasomal function and may, rather, represent a cause of proteasomal impairment.

Further investigations have determined that ASYN may also be targeted to the lysosome by means of macroautophagy (often referred to as autophagy), chaperone-mediated autophagy (CMA) and endocytosis. Indeed, several lines of evidence suggest that the UPS and lysosomal processing may both be involved in regulation of ASYN [211–214]. Although autophagy has generally been regarded as a short-term response to acute conditions of nutrient-deprived cells, it is now believed to be important for normal recycling of cytoplasmic material [215–217]. Levels of autophagy-related protein 7 (Atg7) are, for example, reduced in brain tissue of DLB patients [218] and Atg7-conditional knockout mice, that lack expression of Atg7 in the central nervous system, suffer from motor deficits, tremor, neuronal loss and premature death [216]. Neuronal death occurs in a cell-autonomous fashion and presence of ubiquitin-positive inclusion bodies accumulate within autophagy-impaired neurons, in a time and age-dependent manner [216]. Abnormal expression levels of LC3 are observed in DLB patients, as well as in Atg7-deficient mice [216, 218]. Moreover, increased levels of free Atg5 are also observed in Atg7-deficient mice, while proteasomal functions are unaffected, further indicating impairment of autophagic function [216]. Interestingly, upregulated levels of mTOR correlate with ASYN accumulation in DLB patients and both inhibition of mTor and induction of Atg7 result in reduction of ASYN accumulation and attenuation of related pathologic effects [218].

The first report of differential degradative pathways for ASYN suggested that ASYN may be degraded by both proteasomal and autophagic pathways [211]. In this study, inducible cell lines for wild-type and mutant (A53T and A30P) species of ASYN were utilized in conjunction with inhibitors and activators, specific to proteasomal or autophagic function, to demonstrate that ASYN can be degraded by both systems. Inhibition of either degradation pathway results in accumulated levels of all three forms of the protein. Electron microscopy was also employed to visualize wild-type and mutant forms of ASYN, localized within autophagic vesicles. Consistent with a hypothesis that assumes insoluble, aggregated forms of the protein are directed to the autophagic pathway, while soluble forms are more prone to being degraded by the proteasome, A53T was found to be more sensitive to autophagic disturbances, as compared to wild-type and A30P. This observation echoes that of a former study, which also notated that A53T is less efficiently metabolized by the proteasome [190]. As molecules of A53T ASYN are theorized to possess a greater propensity to aggregate, they are probably less likely to be translocated into the proteolytic compartment of the proteasome. Such a model of differential degradative fates for aggregated and soluble forms of the same protein may help to reconcile inconsistencies between findings of earlier works.

Indications for a diversity of degradation fates of ASYN continue to emerge. One study found, for example, that a specific subset of ASYN oligomeric species are selectively targeted to the proteasome in an *in vitro* cell model and this targeting results in perturbations of proteasomal function [219]. On the other hand, supporting evidence of ASYN degradation, via the lysosome, has also been reported in a study, which examined aggregate-clearing capacities of neuronal and non-neuronal cells, in the presence and absence

of lysosomal function [220]. While large oligomeric species of ASYN are specifically degraded by the lysosome, fibrillar inclusion bodies are not [220]. In this setting, lysosomal inhibition results in an increased presence of ASYN accumulation and corresponds to upregulated levels of cytotoxic effects, implicating a direct role of the lysosomal pathway in the regulation of ASYN and, upon impairment, in the pathogenesis of synucleinopathies.

Strong evidence of cross-talk between the UPS and the ALP was further demonstrated in an elegant study, which employed intravital imaging and transgenic mouse models to demonstrate that, *in vivo*, while physiological levels of ASYN are degraded by the UPS, increased levels of ASYN result in proteasomal dysfunction and activation of autophagic functions [213]. Moreover, inhibition of either system – the UPS or the ALP - results in upregulated activity of the other [221]. It has been theorized that a molecular switch mechanism may be involved in mediating differential degradative targeting of ASYN between the UPS and the ALP. A prime candidate, likely to play such a role, is the carboxyl terminus of Hsp70-interacting protein (CHIP). CHIP has been postulated to facilitate ASYN degradation by acting as a molecular modulator between the proteasomal and the lysosomal pathways [222, 223]. CHIP co-localizes with Hsp70 and with ASYN in LB-like inclusions and reduces inclusion formation, in an *in vitro* model of ASYN aggregation. This study found that, though the U-box/ubiquitin ligase domain is sufficient for driving ASYN degradation by the lysosome, the tetratricopeptide/Hsp70 binding domain is required for proteasomal degradation of ASYN. It has also been reported that CHIP selectively reduces toxicity from stabilized oligomeric forms of ASYN but imparts no effect on cytotoxic manifestations that result from more transient interactions of ASYN, demonstrating particular affinity for targeting of certain conformations of ASYN [222, 223].

Another potential candidate that may act as a molecular switch is the deubiquitinase, USP9X, which is reported to interact with and deubiquitinate ASYN, *in vivo*. USP9X levels are diminished in cytosolic fractions of PD SNpc and DLB cortices. USP9X promotes accumulation of monoubiquitnated ASYN species and, upon proteolytic inhibition, induces toxic ASYN inclusion formation. Monoubiquinated ASYN is primarily degraded via the proteasome and deubiquitinated forms of ASYN are degraded by autophagy. Deubiquitination of ASYN leads to its accumulation, suggesting that autophagic means of degradation may be less efficient than the proteasomal pathway. Decreased levels of cytosolic USP9X and the accumulation and aggregation of monoubiquitination may represent a pivotal factor, involved in influencing the fate of ASYN [224].

Evidence also indicates that CMA is an important pathway, through which ASYN is delivered to lysosomes [225–228]. CMA is the highly-selective process, by which singular proteins bearing a specific pentapeptide (KFERQ) motif are recognized and bound by heat shock cognate protein 70 (HSC70) and are translocated across the lysosomal membrane for degradation by hydrolases [229–233]. Though it has been determined that ASYN contains a sequence that is consistent with a CMA recognition motif [225], it has also been reported that the presence of such a motif does not ensure degradation, via the CMA pathway [234]. A landmark study demonstrated, however, that purified ASYN is, indeed, able to translocate into intact lysosomes and undergo degradation in a CMA-dependent manner [225]. This study found that inhibition of lysosomal proteases increases levels of ASYN, associated with lysosomes, and that exogenously added proteases bear no effect on this association, demonstrating ASYN is, in fact, inside lysosome vesicles. Introduction of competitor CMA substrates interferes with entry of ASYN into the lysosome and the reciprocal phenomenon is also observed.

Importantly, assessment of ASYN mutants showed that A30P and A53T both exhibit greater binding affinities for lysosomal membranes and lower internalization efficiencies, as compared to wild-type ASYN [225]. Lysosomal binding of A30P and A53T are both more effective at blocking binding of alternative CMA substrates than wild-type ASYN. Further investigations have confirmed that CMA is a vital regulator of ASYN degradation in human and rodent neuronal systems and that interference with this system results in accumulation of insoluble oligomers of ASYN [226]. These results suggest a putative role for lysosomal degradation of ASYN, via the CMA pathway, and demonstrate that its mutants may act as inhibitors of degradation to other CMA substrates. Furthermore, upon circumstances in which this pathway becomes overloaded, as a result of upregulated levels of wild-type ASYN or in the presence of pathogenic variants of ASYN, compensatory macroautophagic functions become activated, in an attempt to manage the burden [225, 235].

Endocytic trafficking of ASYN to the lysosome has also been cited, via facilitation of the E3 ubiquitin protein ligase, Nedd4 [236]. Nedd4 is highly expressed in LB-containing neurons and functions in

the endosomal-lysosomal pathway by robustly ubiquitinating ASYN. Upregulated levels of Nedd4 led to increased ubiquitination of ASYN and to lysosomal degradation of ASYN [236]. Conversely, inhibition of Nedd4 is correlated with increased levels of ASYN [236]. Ubiquitination by Nedd4 may play an important role in targeting ASYN to the endosomal-lysosomal pathway and, by reducing intracellular levels of ASYN, may function as a neuroprotective agent.

Though several degradative routes have been purported to be responsible and/or involved in processing the various species and forms of ASYN, an effective mechanism for clearing mature, fibrillar aggregates remains unknown. This has recently been highlighted within the context of an established model [237, 238], which utilizes primary neuronal and HEK293 model systems for studying pre-formed fibril-derived aggregations of ASYN. In this setting, insoluble aggregates of ASYN localize to degradation machinery but they are impervious to proteolytic clearance mechanisms [239] and activating autophagy actually enhances associated effects of toxicity and cell death. The pathways involved in regulating ASYN clearance remain contentious, however, a culmination of evidence suggests a mechanism that is multifactorial, which likely involves a dynamic variety of degradation-related systems, under various physiologic and diseased conditions. Several lines of evidence, for example, link degradation defects to mitochondrial dysfunction [189, 240–244]. Moreover, autophagic dysfunction has recently been implicated in transcellular transmission of ASYN [245], thus, providing further evidence of a fundamental and wide-reaching effect of misregulated ASYN degradation.

NUCLEAR LOCALIZATION OF ALPHA-SYNUCLEIN: FACT OR FICTION?

Although ASYN was initially described as a presynaptic and nuclear protein [2], several studies have provided conflicting evidence, regarding its localization in the nuclear compartment. While some have observed ASYN expression exclusively within the cytoplasm and nerve terminals, others reported widespread expression throughout neuronal and other cellular systems [126, 149, 246–256]. Interestingly, endogenous ASYN has been detected in neuronal nuclei of PD patients, contrasting to a mainly cytoplasmic localization of the protein in healthy controls [257]. Aggregated ASYN has also been identified in oligodendroglial nuclear inclusions in patients with MSA [247] and both monomeric and oligomeric forms were detected in the nuclear compartment of mammalian cells [258–261].

Very few studies have addressed the subcellular localization of GSYN and BSYN. Nevertheless, GSYN has been reported to be mostly cytoplasmic in neuronal cells [248] or associated with discrete cytoplasmic structures, present in the perinuclear area [262]. Similarly, BSYN was described to be present in the nuclear outer membrane of human astrocytes [263].

NUCLEAR TRANSLOCATION OF ASYN

So far, it is still unclear how ASYN enters the nucleus. It was initially proposed that the C-terminal region of the protein is required for its nuclear translocation [248, 264] but two recent studies have demonstrated that this event is driven by the N-domain of the protein [265, 266]. Regardless of which mechanisms are involved in ASYN nuclear translocation, several factors have been proposed to play a role in this process. Enhanced oxidative stress may disturb subcellular localization of ASYN, as increased cytoplasmic oxidation promotes disruption of the nuclear membrane and facilitates the translocation of both monomeric and aggregated forms of ASYN to the nucleus [267]. Likewise, higher expression of nuclear ASYN is observed in mice, under oxidative stress [246, 257]. In dopaminergic cells, oxidative stress promotes the intranuclear accumulation of a 10 kDa, N-terminal-truncated fragment of ASYN [264] and increases neuronal susceptibility to oxidative stress-induced neurotoxicity [268].

Mutations and PTMs also have an impact on ASYN intracellular dynamics. In neuroblastoma cells and in animal models of ASYN overexpression, the familial mutations, A30P and A53T, exhibited increased intranuclear ASYN localization and neurotoxicity [257, 259]. On the other hand, a recent study using ASYN, tagged with photoactivatable GFP, revealed that A30P and E46K mutations have a faster shuttling into the nucleus, when compared to the wild-type protein or to the A53T mutant, which was enhanced in the presence of HSP70 [265]. Rotenone-treated rats have increased accumulation of monoubiquitinated ASYN within the nucleus of nigral neurons [269], while nuclear accumulation of phosphorylated ASYN (Ser129) is observed in the brain of A53T and A30P transgenic mice [270, 271]. However, nuclear localization may correlate more with enhanced toxicity of ASYN species, rather than phosphorylation,

as neurons expressing an ASYN mutation which prevents its phosphorylation, S129A, exhibited high nuclear ASYN signal [272]. ASYN glycation may also enhance its nuclear localization, as both monomers and oligomers may enter more easily into the nucleus, through their pore-forming ability [273].

PUTATIVE FUNCTIONS OF ASYN IN THE NUCLEAR COMPARTMENT

Nuclear localization of ASYN raises the possibility for a role in transcription regulation but consensus has not been reached, regarding the identities of the nuclear interactors of the protein. It is, furthermore, unclear if these putative associations trigger apoptotic or protective neuronal pathways. ASYN was reported to colocalize with histone 3, *in vivo*, and to form a tight 2 : 1 complex with purified histones, *in vitro*, which dramatically accelerated the rate of ASYN fibrillization [246]. Although it has been described that ASYN directly inhibits histone 3 acetylation [259], it was proposed that different expression levels of the protein or the presence of specific cellular stressors may shift effects of ASYN from inhibiting histone acetylation to promoting acetylation [266] (Fig. 4, 1A). The interaction between ASYN and histones may also decrease the pool of free histones available for DNA binding, leading to destabilization of nucleosomes and to subsequent transcription deregulation [246, 266, 268].

A question that remains unanswered is whether ASYN interferes with transcriptional regulation by direct binding to DNA (Fig. 4, 1B, 1C). Recent studies have demonstrated that ASYN can bind directly and preferentially to transcriptionally active and histone-free, single copy DNA and change the stability and conformation of the DNA [274–277]. In comparison to native protein, glycated ASYN forms a more stable complex with DNA and induces DNA nicking. Moreover, by increasing the generation of ROS in the nucleus, glycated ASYN induces histone glycosylation and subsequent DNA damage [278]. Recently, it was also reported that ASYN can modulate Noch1 expression by inducing conformational changes in chromatin, by facilitating the binding of p53 to its core elements or by direct interaction with p53 [279].

Besides random binding, ASYN may interact with specific promoter regions and regulate the expression of several genes, including the master mitochondrial transcription factor, PGC1 alpha, which was found to be down-regulated in PD brains [257], and other genes involved in PD-related pathogenic pathways, such as protein ubiquitination [280]. ASYN: chromatin binding is increased in mice, under oxidative stress conditions and in postmortem PD brains, when compared to age-matched controls [257]. Conversely, DNA also modulates ASYN folding and induces alpha-helical conformation [273], a common feature seen in prion-like proteins [281], strengthening the hypothesis that synucleinopathies belong to the group of prion-like disorders.

ASYN AND TRANSCRIPTIONAL DEREGULATION

Although transcriptional deregulation and epigenetic changes are recognized as important mechanisms in neurodegeneration [187, 282–285], very few studies have addressed the direct association between transcriptional deregulation and ASYN. ASYN accumulation was found to up-regulate caveolin-1 and to down regulate several signaling pathways, upstream of cyclic adenosine monophosphate (cAMP) response element binding protein (CREB), such as extracellular signal regulated protein kinase (ERK), protein kinase C (PKC) [286, 287], nuclear factor kappa B (NF-kB) [248, 288] and glycogen synthase kinase 3b (GSK3b) [260]. Consequently, downregulation of CREB leads to impairment of its downstream targets, namely Bcl-2 [260] and brain-derived neurotrophic factor (BDNF) expression [289], which impairs tyrosine hydroxylase (TH) expression [290]. However, nuclear ASYN also downregulates expression of the nuclear receptor, Nurr1, and its downstream target, GDNF receptor, Ret. These data explain, at least partially, why exogenous BDNF has failed to protect nigral dopamine neurons against ASYN-induced toxicity in some PD animal models [291, 292] and suggests Nurr1 as a promising new target of ASYN toxicity [291].

Overexpressing wild-type or mutant human ASYN promotes significant changes in the expression of many genes that are important for neurotransmission, stress responses, apoptosis and several transcription factors. Interestingly, changes in genes that regulate dopamine homeostasis, such as GTP cyclohydrolase and TH, were found to be downregulated by wild-type, but not mutant, ASYN overexpression [293].

At nanomolar concentrations, ASYN was described to exert a neuronal protective effect by activating prosurvival PI3K/Akt and Bcl-2 family signaling pathways. On the contrary, both micromolar and higher levels of ASYN resulted in a significant cytotoxic effect [294]. A neuroprotective role of physiological levels of ASYN in dopaminergic cells was recently

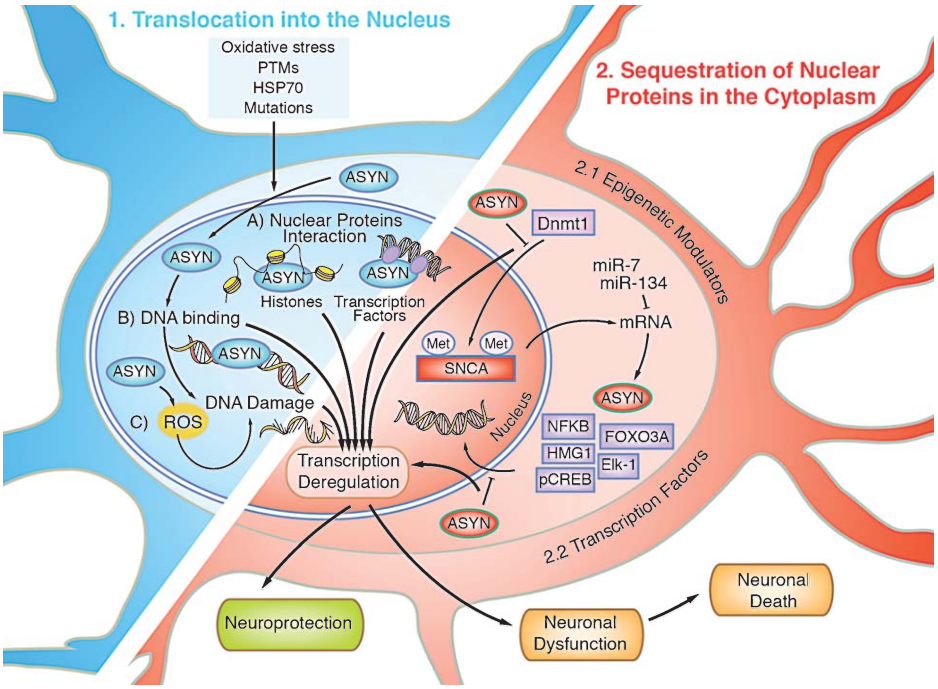

Fig. 4. Schematic representation of different pathways potentially involved in ASYN-induced transcriptional deregulation. ASYN effects on cellular transcription may occur by nuclear localization of the protein and interaction (1) with different substrates or by cytoplasmic trapping of transcription factors and chromatin methylation modifiers (2). Several factors have been proposed to enhance ASYN nuclear localization, such as oxidative stress, PTMs, HSP70 and familial mutations. Once inside the nucleus, the protein is able to interact with nuclear proteins, such as transcription factors or histones (1A), or with specific DNA promoter regions (1B) and, thereby, modulate gene expression. ASYN-induced toxicity may also involve genomic damage, through direct binding of DNA or by increasing levels of ROS in the nucleus (1C). ASYN may also mediate the cytoplasmic retention of DNA methyltransferase 1 (Dnmt1), creating a loop that leads to ASYN overexpression (2.1). The excessive production of ASYN mRNA can be balanced by the overexpression of miR7 and 134, which target and down-regulate ASYN mRNA and protein levels. Mislocalization of transcription factors into cytosolic ASYN inclusions may also lead to gene expression profiling changes (2.2). ASYN-induced transcription deregulation may be responsible for either activating neuroprotective genes or exacerbating neuronal pathology, through apoptotic genes, leading to neurodegeneration and neuronal death.

identified, via decreasing p300-mediated acetylation of NF-κB [295]. Similarly, overexpression of ASYN, but not BSYN, conferred resistance to hydrogen peroxide challenged neuronal cells, via up-regulation and interaction with Jun N-terminal Kinase-interacting protein (JIP)-1b and, consequently, by inactivation of the JNK stress-signaling pathway [296].

CYTOPLASMIC SEQUESTRATION OF NUCLEAR PROTEINS

Gene expression regulation also relies on the correct subcellular distribution of many transcription factors. A growing number of recent studies reported aberrant mislocalization of transcription factors and their regulatory kinases in cytosolic ASYN aggregates (Fig. 4, 2.1, 2.2). ASYN can mediate cytoplasmic retention of DNA methyltransferase 1 (Dnmt1), resulting in global DNA hypomethylation and up-regulation of PD-related genes, including SNCA. Reduction of nuclear levels of Dnmt1 and DNA methylation were also observed in human postmortem brains of PD and DLB patients [297].

Phosphorylated transcription factor, Elk-1, was shown to accumulate within ASYN glial cytoplasmic inclusions [298–300] and cytoplasmic aggregates of phosphoCREB were observed in SNpc neurons of PD brains [301, 302]. Nuclear localization of NF-kB is also absent in motor neurons from ALS patients [303] and it is present within neuronal intracellular LBs [304]. Furthermore, co-factor high mobility group protein 1 was also identified as a ligand for cytosolic ASYN filaments, which may explain the impairment of neuronal response to estrogen, in synucleinopathies [305].

The role of ASYN in transcription deregulation is still unclear, as it has been implicated in pathways that influence both neuronal protection and toxicity. In line with some studies suggesting a protective role for ASYN accumulation, transcription factor, FOXO3a,

was also detected in LBs and LNs in PD and DLB [306]. Intriguingly, the amount of ASYN in neurons seems to represent a key element in determining its tendency towards opposing roles, with lower levels being crucial for cell protection against specific insults and high levels promoting harmful effects [266].

ASYN AND MITOCHONDRIA DYSFUNCTION: CAUSE OR EFFECT?

Impairment of mitochondrial function has been associated with wild-type and mutant ASYN overexpression and has been proposed to play a role in the onset and progression of synucleinopathies [307–311]. Proper mitochondrial dynamics and intact electron transport chain activity are crucial to maintaining reactive oxygen species (ROS) homeostasis and to responding to high-energy demands of neurons, mostly via oxidative phosphorylation (OXPHOS).

Although general agreement has been reached on the fact that mitochondria: ASYN interaction is a major player in the complex neurodegenerative network, the exact mechanism underlying this deleterious interrelationship is mostly unknown. To date, three different scenarios have been drawn: i) ASYN promotes, either directly or indirectly, mitochondrial dysfunction; ii) ASYN-related toxicity is enhanced by mitochondrial impairment or iii) mitochondrial dysfunction and ASYN toxicity act together and, as partners, exacerbate their individual neurodegenerative properties and lead to cell death.

The existence of a synergistic interaction between ASYN and mitochondria is corroborated by studies showing that mice overexpressing ASYN and treated with MPTP, a complex I inhibitor, exhibit accumulation of ASYN and greater mitochondrial pathology, compared with wild-type mice [312–314], whereas ASYN-null mice display striking resistance to mitochondrial toxin-induced neuronal death [315–317]. Chronic treatment with another potent mitochondrial complex I inhibitor, rotenone, also leads to the specific death of dopaminergic neurons, along with accumulation of ASYN inclusions [318]. Interestingly, molecules that decrease rotenone toxicity are also able to suppress ASYN toxicity [319]. Moreover, abrogation of mitochondrial DNA in yeast inhibited both ROS production and apoptosis, induced by ASYN, suggesting that the mitochondrion is required in ASYN-induced toxicity [320].

Whether ASYN regulates mitochondrial-related proteins/pathways, or whether it is directly localized and bound to this organelle, is still unclear. However, mounting evidence suggests that, in a variety of model systems, ASYN is localized to or associated with mitochondria [321–327]. Mitochondrial ASYN has been detected in specific brain areas of the rodent brain, such as the hippocampus, striatum and SNpc [249] and several-fold increased levels of mitochondrial ASYN are observed in the SNpc of PD patients [321].

In addition to binding to the outer mitochondrial membrane, ASYN translocates into the mitochondria, where it might impair OXPHOS. ASYN contains a mitochondrial targeting signal, consisting of 32 N-terminal amino acids, and it translocates into the mitochondria, in a cardiolipin-dependent manner [328]. Interestingly, in ASYN knockout mice, a decrease in the amount of mitochondrial cardiolipin was observed [329]. Although the exact localization of ASYN inside the mitochondria is still unclear, some studies show a distribution throughout the inner membrane space and the matrix [321, 324, 330] and others report that its localization is exclusively in the inner-membrane space [321, 331].

Several studies have reported that ASYN predominantly associates with mitochondrial complex I. ASYN was, for example, found to be associated with mitochondrial complex I in cells and in mitochondria from the SNpc and from striatal neurons of PD patients. This association is, furthermore, correlated to decreased activity and to increased levels of oxidative stress. These effects were shifted to an early time point in neurons overexpressing the A53T mutation [321]. While most of the studies did not report alterations in other mitochondrial complexes, reflecting a high specificity of ASYN for complex I, a reduction in complex IV activity and in mitochondrial DNA damage was observed in A53T ASYN transgenic mice, together with mitochondrial morphological changes and ASYN inclusions within mitochondria [332]. Likewise, alterations in ATP production and a shift in membrane potential, without reduction of complex I activity, were described [333]. A recent study also demonstrated that neurons containing ASYN pathology express higher levels of the respiratory chain subunits, possibly reflecting a compensatory mitochondrial mechanism [334], whereas mice lacking ASYN also exhibit mitochondrial deficits, such as compromised activity of complexes I and III [329]. The exact timing of mitochondrial impairment during the development of synucleinopathies is unknown. Studies reporting decreased complex I activity of SNpc neurons from transgenic mice, overexpressing A30P or A53T ASYN, with no evident nigral degeneration, may

suggest that mitochondrial dysfunctional is an early event or even a trigger for neurodegeneration [335].

Mitochondria are remarkable, dynamic organelles that constantly undergo a process of fusion and fission [336, 337]. Changes in mitochondrial dynamics have been suggested to occur prior to mitochondrial dysfunction and have been observed in different ASYN overexpressing models [330, 331, 338]. Both endogenous and overexpressed ASYN induces mitochondrial fragmentation, while ASYN knockdown results in elongated mitochondria. Mitochondrial fragmentation is also increased in the presence of E46K and A53T mutants but controversial results have been described for A30P ASYN [330, 331]. Although it seems clear that ASYN induces mitochondrial fragmentation, whether it happens through inhibition of mitochondrial fusion [331] or as a result of increased fission [330], is matter of debate. Overexpression of mitochondrial fusion native proteins, such as Mfn2, Mfn1 and OPA1 and knockdown of fission protein, Drp1, were unable to rescue the complete phenotype, suggesting that mitochondrial fragmentation, induced by ASYN, is independent of mitochondrial fusion/fission machinery [330, 331]. Moreover, only oligomeric forms of ASYN (trimeric and tetrameric) induce fragmentation *in vitro* [330]. Conflicting results have been provided, regarding the impact of ASYN homologues on mitochondrial dynamics. While some studies describe a redundant function for A- and BSYN overexpression, with both similarly disrupting the mitochondrial network [331], others report that B- or G-isoforms cause less fragmentation than ASYN [330]. Additionally, BSYN-induced mitochondrial fragmentation did not result in mitochondrial malfunctioning [13]. Changes in the mitochondrial architecture were also observed in transgenic mouse models, overexpressing wild-type or mutant ASYN [330, 332], and in overexpressing cells [307, 324].

Proper control of mitochondrial turnover is also crucial to assure cellular energetics. Selective elimination of defective mitochondria occurs via autophagy (mitophagy), by delivering damaged mitochondria to the lysosome [339]. As previously described in this review, ASYN impairs macroautophagy [340], which could lead to a deficient clearance of damaged mitochondria. However, both the wild-type and, especially, the A53T mutant ASYN promote an up-regulation of mitophagy, resulting in massive mitochondrial degradation, bioenergetic deficits and neuronal death [341, 342].

Mitochondrial trafficking is reliant on microtubules and it is crucial to supplying energy, maintaining calcium (Ca^{2+}) homeostasis and regulating mitochondrial degradation throughout neurons. Reduced mitochondrial movement was observed in neuronal cells, overexpressing ASYN, and could be restored by co-expression of the fusion protein, Mfn2 [341]. Moreover, ASYN effects on mitochondria also include oxidation of mitochondrial-associated metabolic proteins, decreasing their enzymatic activity and increasing levels of nitric oxide and Ca^{2+} [310, 343].

ASYN, MITOCHONDRIA AND THE ENDOPLASMATIC RETICULUM: A CALCIUM-DEPENDENT CONVERSATION

The interplay between ASYN and mitochondria may also involve a third player: the endoplasmic reticulum (ER). The abnormal accumulation of misfolded proteins within the ER induces the unfolded protein response (UPR). If the stress is prolonged, ER can trigger ROS generation, activation of ER-stress apoptotic pathways and, eventually, neurodegeneration and cell death [344]. Recent studies have described high levels of various truncated and multimeric ASYN species in the ER of A53T ASYN transgenic mice and in brains of postmortem PD patients [345]. In mice, ASYN accumulation in the ER appears near the age of onset of pathology and increases as the disease worsens, suggesting ASYN levels in the ER may correlate with disease severity [345]. Postmortem evaluation of nigral dopaminergic neurons from sporadic PD patients, bearing ASYN inclusions, revealed activation of the UPR, suggesting a direct effect of ASYN accumulation/aggregation on ER stress [346, 347]. Moreover, it has recently been described that ASYN may exert its pathological effects, in part, by directly interacting and inactivating the UPR activator glucose-regulated protein 78/immunoglobulin heavy chain-binding protein (GRP78/BiP) [348] or by blocking ER to Golgi trafficking [349, 350]. Furthermore, ASYN-induced ER-stress was suggested to be dependent on ASYN phosphorylation at Ser129 [351].

Both monomeric and oligomeric ASYN can interfere with mitochondrial function, indirectly, by enhancing ER stress [345, 352, 353]. Enhanced levels of ER stress was proposed to precede mitochondrial disruption, caspase-3 activation [351] and induce ASYN aggregation [354]. The physical and functional interaction between the ER and mitochondria has been well described and it is crucial for cell survival and response to cellular stress. Although the ER can inter-

fere with mitochondrial fission, Ca^{2+} signaling is the main biological link between the two organelles and it is a key mediator of ER-mitochondria-induced apoptotic cell death. ASYN overexpression and ASYN oligomers can trigger Ca^{2+} influx in cells, leading to deregulation of a plethora of Ca^{2+}-mediated cellular processes [141, 355]. Moreover, increased Ca^{2+} levels, induced by ASYN, were described to lead to a selective death of SNpc neurons, which may be particularly relevant in the pathogenesis of PD [356]. Such high levels of intracellular Ca^{2+} may promote ASYN oligomerization and aggregation, by direct binding to the C-terminus of the protein [357–360] and interfere with the interaction of ASYN with other proteins, such as MAP1A [359].

Additionally, ASYN disrupts Ca^{2+} homeostasis, through impairment of the ER Ca^{2+} pump and/or transient receptor potential (TRP) channels, and increases glial cell vulnerability to oxidative stress and death in MSA [361]. In PD, ASYN may also deregulate ER-mitochondrial contact sites, impairing Ca^{2+} transference between the two organelles [362]. Increases in ER Ca^{2+} release to the intracellular space may activate several apoptotic pathways and induce mitochondria to trigger apoptotic cell death. Looking in the reverse way of this cross talk, mitochondrial dysfunction may also cause ER stress, enclosing a feedback loop, which leads to activation of cascade signaling. A deleterious effect of mitochondria, on ER homeostasis, is highlighted by several studies showing that OXPHOS inhibitors induce an upregulation of ER stress-related proteins [363–366]. Furthermore, Ca^{2+} chelation or blocking of Ca^{2+} release from mitochondria ameliorates ER stress, suggesting that impairment of mitochondrial Ca^{2+} buffering may be responsible for ER stress induction [365].

Elevated cytosolic Ca^{2+}, released from both dysfunctional mitochondria and the ER, lead to an increase of nitric oxide species (NOS) generation that can react with superoxide. The highly toxic species produced, as a result of this reaction, may induce cellular damage at the proteasomal, ER and mitochondrial level. Interestingly, in PD, ASYN within LBs was observed to be enriched with nitrated species [169, 367] and sporadic PD patients also exhibited polymorphisms in neuronal NOS and inducible NOS [368]. Additionally, abnormal ROS production, derived from dysfunctional mitochondria, can react with NOS and produce reactive nitrogen species, which can also alter the native conformation, lipid binding dynamics and aggregation pathway of ASYN [369, 370]. Ultimately, the cell can undergo a perpetuating cycle where aggregated ASYN would enhance mitochondrial dysfunction and ER stress and further production of more toxic ASYN species.

ASYN AND NEUROTRANSMISSION

Synaptic transmission is an essential and tightly regulated neuronal process that, invariably, deteriorates with disease progression of synucleinopathies. Substantial data indicates a role for ASYN in neurotransmitter release, as ASYN localizes to nerve terminals [2, 371–373], is loosely associated with the reserve pool of synaptic vesicles [149, 249, 374] and with lipid rafts [375] and interacts with lipid membranes *in vitro* [97, 103, 376, 377]). Furthermore, hippocampal neurons, deficient for ASYN expression, experience a reduction in the size of their distal pool of vesicles [378].

A particular emphasis has been placed on elucidating the potential role(s) ASYN plays in regulating recruitment and release of dopamine, within the nigrostriatal pathway [379–381]. ASYN-deficient mice display enhanced recovery of dopamine release, in response to paired-pulse stimuli of nigrostriatal terminals [379]. Upregulated levels of extracellular Ca^{2+} produce similar effects in wild-type animals. ASYN knockout mice also exhibit decreased levels of dopamine in the striatum and suffer defects in locomotor response to amphetamines [379]. These results suggest that ASYN may act as a negative regulator of dopamine transmission, by way of modulating a Ca^{2+}-dependent process.

It has, furthermore, been reported that absence of wild-type ASYN or presence of the mutated A30P form of ASYN both result in altered compartmentalization of presynaptic dopamine. ASYN-deficient mice display elevated refilling rates of the readily releasable pool, which is postulated to represent a compensatory mechanism for alterations of dopamine compartmentalization [381]. Conversely, elevated levels of wild-type ASYN in transgenic rodent models, reduces the size of the reserve and readily releasable pools [382]. Moreover, overexpression of ASYN leads to deficient synaptic vesicle exocytosis and diminished capacity of neurotransmitter release in hippocampal and dopaminergic neurons [382, 383]. Interestingly, though A53T and E46K mutants also impede neurotransmitter release in hippocampal neurons, A30P does not, suggesting a necessity of membrane binding for ASYN-induced impairment of vesicle exocytosis and neurotransmitter release. It has also been noted that,

while ASYN does not appear to impact endocytosis, directly, it does impact reclustering of synaptic vesicles after endocytosis occurs [382]. Upregulation of ASYN in rodent nigral neurons is correlated with significant loss of presynaptic vesicles in dopaminergic striatal terminals and with resultant motor defects [384]. In this study, too much ASYN resulted in deficient capacity for generating and recycling presynaptic vesicles, axonal degeneration and, consequently, diminished capacity for dopamine release– all of which, culminate in neuronal loss. Notably, the A30P mutant was found to be less toxic than wild-type ASYN in the rat nigrostriatal system [384]. ASYN expression has also been linked to significant reductions of dopamine release and reuptake and to signs and symptoms of degeneration, within nigrostriatal axons and terminals in rodents [385].

Strong evidence of ASYN involvement with SNARE-related aspects of neurotransmission emerged when its transgenic expression was reported to rescue neurodegenerative lethalities, which were induced by deletion of cysteine-string protein-α (CSPα) [386]. In this study, transgenic expression of ASYN functionally rescued the deleterious effects that inhibition of CSPα exerted on SNARE complex assembly and deletion of endogenous synucleins exacerbated this phenotype [386].

Further efforts have substantiated the hypothesis that ASYN is able to compensate for loss of CSPα, via functional recovery of SNARE-protein function [387]. This study reported decreased levels of SNARE-complex in ABG-SYN triple knockout mice. The study also illustrated the requirement for concurrent binding of ASYN to phospholipids, by its N-terminal domain, and to the SNARE protein, synaptobrevin-2 (also called VAMP2, vesicle associated membrane protein 2), by its C-terminal domain, to directly promote SNARE-complex assembly [387]. *In vitro* analyses of ASYN have demonstrated that ASYN induces clustering of vesicles containing VAMP2 alone or VAMP2 and synaptotagmin-1. This study also reported that specific binding to anionic lipid membranes and to VAMP2 was required for induction of clustering effects [388]. The mutant A30P form of ASYN resulted in reduced vesicle clustering ability of native ASYN, as compared to wild-type, E46K and A53T versions of ASYN [388]. The culmination of these results denote a potentially neuroprotective role of ASYN, under physiologic conditions. To that end, the relationship between pathological effects of asyn mutants on its function, as a SNARE-protein chaperone, have been elucidated within *in vitro* cell culture and neuronal systems. Findings demonstrate that non-functional ASYN is not necessarily toxic and that mutations do not significantly alter the physiological functions of the protein, suggesting that pathology is based on gain-of toxic-function, rather than loss of physiological function. Furthermore both N- and C-terminal domains of the protein are essential for its SNARE chaperone ability but point mutations in the central region of ASYN and familial PD mutations, which enhance neurotoxicity, do not affect its function, indicating that distinct sequences of the protein are required for its physiological and pathological roles [389].

Glutamate and some of the receptors it acts on, such as the N-methyl-D-aspartate receptor (NMDAR) and the non-NMDA, AMPA receptors (AMPAR), have also been associated with PD and other synucleinopathies. Presynaptic mobilization of the distal pool of glutamate vesicles is, for example, impaired in the hippocampus of ASYN knockout mice [390]. Alterations in expression levels and in the molecular, subunit-derived compositions of NMDAR are also observed in brain tissue of PD patients and in models of parkinsonism [390–394]. The levels of cell surface NMDAR are regulated through a clathrin-mediated endocytic process that requires involvement of RAB5B protein and is promoted by ASYN [392]. In particular, oligomeric forms of ASYN have been specifically demonstrated to impart disruptive effects on the glutamatergic system, in a rodent hippocampus model of long-term potentiation (LTP) [393]. In this model, evidence suggests that prolonged exposure to oligomeric forms of ASYN impairs LTP function, via effects of saturation, resulting in heightened levels of synaptic transmission and upregulated levels of both NMDA and AMPA receptors. Interestingly, these detrimental effects were not observed, upon exposure to monomeric or fibrillar forms of ASYN [393].

TRANSMISSION, SPREADING AND "PRION-LIKE" PROPERTIES OF ASYN PATHOLOGY

A provocative question has emerged in the field, regarding the transmissibility of pathological properties of synucleinopathies, which asks whether or not ASYN possesses "prion-like" properties (Fig. 5). Speculation that ASYN-pathology may arise as a function of a "prion-like" mechanism dawned when postmortem autopsy of PD patient brain tissue revealed that engrafted nigral neurons develop LB pathology [395, 396]. Nigral neural transplantation therapy has

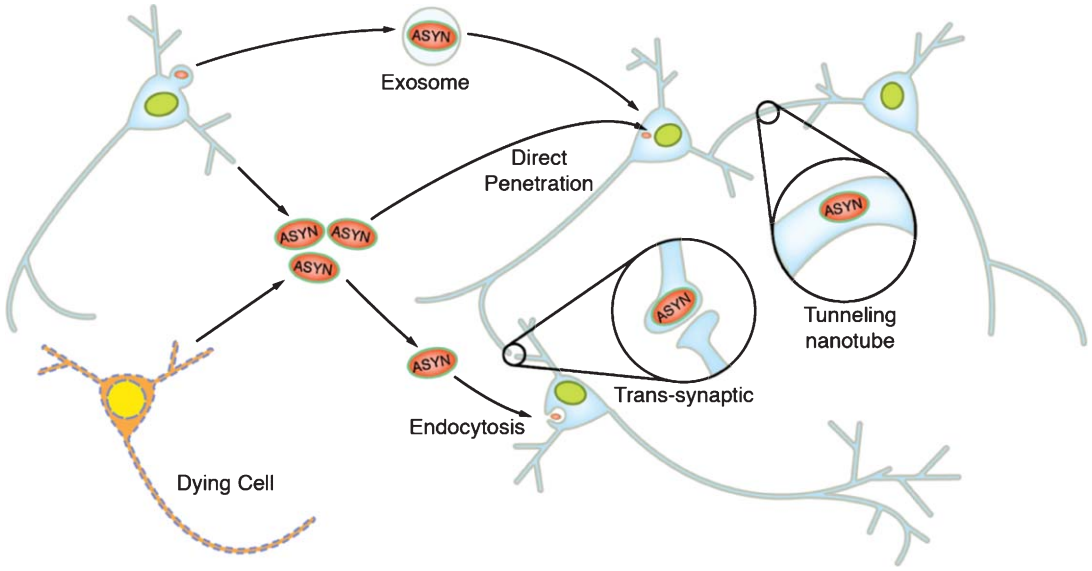

Fig. 5. Proposed mechanisms of ASYN propagation. Various ASYN species may be transferred between neurons and induce disease spreading to other brain regions. Once released from the host cell to the extracellular environment, via a non-classical exocytic pathway or via exosomes, these species can enter other neurons by direct penetration, if the cellular membrane is disrupted, or by using the cellular trafficking system of the target cell, via endocytosis. The other two mechanisms of propagation involve a direct interaction between the host and the target cell and can occur either by the formation of tunneling nanotubes or by trans-synaptic transmission.

been conventionally utilized as an attempt to counterbalance loss of degenerated neurons and, previous to this finding, it was believed that healthy grafts were unaffected by the disease process. These findings, derived from postmortem examinations of patients who died soon (18 months) or long (between 11 and 16 years) after transplantation, however, elucidate an opposing reality. Autopsy studies revealed that grafted neurons of patients who died soon after transplantation appeared healthy, non-afflicted and bore no evidence of LB pathology [397]. Some of the fetal nigral neurons, which had been ectopically grafted into the putamen of patients who died much later, however, bore signs of pathology that resembled diseased nigral neurons of the host tissue [398]. In the patients who survived for many years after transplantation, inclusions were detected in a percentage of grafted neurons that is consistent with the percentage of inclusion-bearing neurons found in PD patients. These findings demonstrate that the pathogenic process in PD is a dynamic one and that it is capable of inflicting young, healthy cells that are devoid of any predisposing features.

Whether or not ASYN operates as an infectious prion protein remains a matter of debate, however, converging lines of evidence suggest that ASYN is, indeed, released from and taken up by neuronal cells. It is well established, for example, that monomeric and oligomeric forms of ASYN protein are present in extracellular biological fluids, including cerebral spinal fluid, saliva and blood plasma of diseased and normal patients [399–402]. A multitude of ongoing efforts are aimed at deciphering qualitative and quantitative differences in extracellular levels of ASYN to better understand mechanisms of cellular release/uptake of ASYN and to establish potential biomarker standards, which may be predictive of susceptibility to disease onset [402–408]. In the meantime, the Braak staging method has emerged as a means of classifying neuropathological stages of disease progression, related to PD, based on a correlation between severity of symptoms and topographic localization of inclusion burden [409]. The basic premise, underlying Braak's hypothesis, postulates that neuronal damage does not arise randomly, that it, rather, follows a predetermined sequence and that various brain regions possess innately differing levels of vulnerability for acquiring disease-related attributes. Findings of Braak's staging study substantiated that, as with other neurodegenerative disorders, occurrence of inclusion body pathology precedes onset of clinical symptomology in PD. The study also categorically documented the developmental pattern by which pathological inclusions of ASYN spread throughout the brain. Pathological tracing results suggest that earliest stages of the disease begin in the brain stem and that later stages are exemplified by development of LBs within cortical regions.

Signs of pathology within the SNpc are actually witnessed at medial stages of disease progression and this is also when presentation of cardinal motor symptoms begin to arise.

Results from a series of *in vitro* and *in vivo* experiments have corroborated the notion that ASYN is transmitted between neuronal cells and that its pathogenic effects are conferred, via this transmission [410–414]. An early study reported that monomeric and aggregated forms of ASYN are constitutively secreted from cultured neuronal cells, via a non-classical exocytic pathway, and that secretion of both forms increases, in response to impairments of proteasomal and mitochondrial function [415]. Constitutive cellular release of ASYN from SH-SY5Y cells has also been demonstrated to occur, via exosomes, in a Ca^{2+}-dependent manner and exposure to secreted ASYN was associated with toxicity [416]. Toxic effects were exacerbated by increased presence of oligomeric species and were inversely correlated to internalization of ASYN. In this study, internalization of exported ASYN was observed in non-differentiated SH-SY5Y cells but not in differentiated SH-SY5Y or cortical neurons. Interestingly, cellular stressors, such as oxidative stress and proteasomal inhibition, did not impact ASYN secretion in this study [416].

Cell-to-cell transfer of ASYN, via exosomes, in SH-SY5Y cells was corroborated in a subsequent study that identified lysosomal dysfunction as a mediating factor, which results in higher levels of ASYN release and intracellular transmission, as well as presence of insoluble, ASYN-positive inclusions [417]. Contradictory to previous findings, however, this report cited detection of exosome-mediated transfer of ASYN in differentiated neurons. Release by exosomes was initially believed to be a mechanism that is restricted to cellular clearance [418, 419] but, more recently, it has become associated with roles that may be related to prion propagation [420–422]. It has also been established that aggregated forms of ASYN can be taken up by cells in an *in vitro* neuronal model, via endocytosis, and that they are then targeted for degradation, by means of the lysosomal pathway, though monomeric forms are not [423]. In this system, it appears that ASYN monomers may be capable of translocating the plasma membrane, directly, thereby evading proteolytic processing by the lysosome. It is believed that this may represent a neuroprotective mechanism, in which cells of the nervous system process toxic forms of ASYN. Furthermore, it has been demonstrated that neuronal cells and primary murine cortical stem cells uptake ASYN, derived from donor neurons, via endocytosis, within the context of co-culture systems [413], suggesting a similar mechanism of transmission may occur in human mesencephalic transplants. To explore this hypothesis further, murine stem cells were injected into the hippocampus of human ASYN-expressing transgenic mice and, upon examination, ASYN positivity was discovered in the engrafted cells, including signs of inclusion body formation [413]. Additionally, the same study found that impairment of lysosomal function results in accumulation of endocytosed ASYN and, ultimately, leads to inclusion formation. Substantiating results were obtained in a subsequent study, which demonstrated direct transfer of ASYN to dopaminergic neurons that were grafted into the striatum of ASYN-expressing transgenic mice–recapitulating, more closely, the clinical phenomena observed in human patients [410]. Templating effects of ASYN were also addressed in this study, providing additional evidence that misfolded ASYN may self-propagate and promote neurotropic spread of disease.

Furthermore, recent investigations continue to provide evidence of intraneuronal transmission of ASYN, *in vivo* [414, 424], including an elegant study conducted in non-transgenic, wild-type mice, in which a single striatal inoculation of synthetic ASYN fibrils resulted in intercellular transmission of ASYN pathology, a spreading progressive dissemination of degeneration across brain regions and a reduction in dopamine levels and motor symptoms, which are characteristically consistent with a PD phenotype [412]. Depositions of ASYN were initially detected at the site of injection and subsequently appeared in neighboring regions, which were directly connected to the striatum. LB-like inclusions, defined by accumulations of hyperphosphorylated ASYN, were observed in several regions, while other regions remained devoid of them throughout the duration of the study. Inclusions appeared in a temporal and spatial manner, which is in accordance with dissemination of the pathology along a neuronal network. Direct inoculation of an unaffected region (i.e. hippocampus) resulted in robust accumulation of ASYN inclusions, demonstrating that affliction is connectivity-dependent. As neither injection of monomeric ASYN into wild-type mice nor injection of ASYN fibrils into ASYN KD mice resulted in inclusion formation or any other signs of pathology, the derived implication suggests that ASYN fibrils act as a template, which promotes fibrillization of normal ASYN in the cell [412].

It is believed that intercellular transmission of ASYN is not limited to neurons, as evidence suggests that glial cells, which do not normally express

the protein [425–427], acquire ASYN exogenously, via uptake from the surrounding environment [428, 429]. This data suggests a potential mechanism whereby ASYN that is released from neurons may represent an underlying factor in the pathogenesis of MSA. The culmination of these findings strengthen the hypothesis, which asserts that extracellular seeds of ASYN impact disease onset and progression in a "prion-like" manner.

ASYN AS A TARGET FOR THERAPEUTIC INTERVENTION

Most attempted therapies for synucleinopathies have used PD as their main model. However, although some symptoms appear to be identical between PD, PFA, MSA and DLB, the success of a therapy in one may not be predictive of outcomes in the others. An example is illustrated in recent reports showing that deep brain stimulation provides therapeutic benefits for PD patients, while differential outcomes are observed in MSA patients [430, 431]. In 2006, it was reported that the success of this surgical intervention in MSA patients seems to be driven by the presence of ASYN inclusions, with positive results being observed in MSA patients that present with ASYN accumulation in the brain [430]. Conversely, subthalamic deep brain stimulation helps to improve motor symptoms in PD patients with ASYN duplication [432].

It is still unclear whether ASYN causes dysfunction of multiple cellular systems or whether organelle-specific dysfunction induces ASYN pathology. Although determining the initiator(s) and the contributor(s) in synucleinopathies remains a challenge, it is tempting to assume that modulating or inhibiting the impairment of different organelles, such as the ER and mitochondria, may aid in preventing ASYN accumulation and aggregation. It has been shown that pharmacological inactivation of ER stress diminishes accumulation of ASYN monomers and oligomers in the ER of A53T mice and, though it does not protect dopaminergic neurons from death, it significantly attenuates motor dysfunction and delays disease onset [433]. Moreover pharmacological inhibition of ER-stress, by Salubrinal, and deregulation of ER Ca^{2+} release, by knocking down homocysteine-inducible ER stress protein, protect cells from death induced by A53T ASYN overexpression [308, 434]. Along the same line of evidence, overexpression of GRP78/BiP reduces ASYN neurotoxicity, by down regulating apoptosis, and protects striatal dopaminergic neurons from cell death [435, 436]. Furthermore, modulation of mitochondrial-related proteins, such as Mnf2 and dominant negative Drp1, or the use of drugs aimed at inhibiting excessive mitochondrial degradation, partially protects against ASYN-induced neuronal death [341].

A therapeutic approach for synucleinopathies may, ultimately, involve modulation of ASYN expression. Abnormal levels of ASYN in the CNS are thought to be an important trigger for neurodegeneration and may arise from an imbalance between ASYN synthesis, aggregation and/or clearance. The impairment of one of these mechanisms may favor the formation and accumulation of intra- and extracellular toxic ASYN species.

CLEARANCE OF ASYN BY IMMUNOTHERAPY

Precedence has already been set for extensive development of immunotherapies that are targeted to other neurodegenerative diseases - most notably, in the case of AD. The fact that protein accumulations in AD are extracellular, makes them easier targets for antibody recognition and degradation. However, recent reviews, compiling several studies and the latest advances on immunotherapy development in AD, show that most of the clinical trials have failed to achieve satisfactory results [437, 438]. In synucleinopathies, ASYN accumulation is intracellular, adding an extra layer of complexity to antibody-based strategies. Despite these limitations, the idea of generating antibodies to specifically recognize oligomers or inclusions, at different stages of disease development, remains appealing. Targeting and degrading oligomers, which are considered, by some, to be the most toxic species [258, 439], could potentially prevent the onset or progression of disease. Furthermore, propagation capacity has been attributed to oligomers and small aggregates [413, 440] in a "prion-like" fashion [441, 442] and this could be used as an "open door", easing the path for immunotherapeutic targeting, since the species would be present at the membrane surface or within the extracellular space.

So far, several studies have auspiciously demonstrated ASYN oligomer/aggregate clearance, due to immunotherapy. In 2011, decreased levels of ASYN oligomerization in cells, within the context of the Bimolecular Fluorescence Complementation assay, were achieved by using a monoclonal antibody [443]. Active immunization has also been used to reduce ASYN accumulation in transgenic mice, preventing neurodegeneration without promoting an inflamma-

tory response [444]. As well, passive immunization with an ASYN antibody, in the same mouse model, effectively reduced neuropathological deficits. Interestingly, the antibody was detected in the lysosomal compartment, suggesting that it recognizes ASYN and drives the protein towards degradation [445].

Considering the relatively nascent stage of this research, particularly, as compared to the AD field, medical applications based on immunotherapies in PD are still a work-in-progress. Nevertheless, the first phase I clinical trial for immunotherapy against ASYN began last year. Developed by AFFiRiS, the AFFiTOPE, PD01, improved ASYN-induced pathology, including neuronal loss [446].

MODULATING ASYN LEVELS BY GENE THERAPY

ASYN gene duplication and triplication are known causes of PD, indicating that ASYN overproduction can play a crucial role in the onset of the disease. Besides genetic alterations, DNA methylation at intron 1 of SNCA may directly contribute to ASYN expression deregulation in sporadic PD cases [297, 447]. Down-regulation of a gene is normally achieved by utilization of adeno-associated virus (AAV), short hairpin RNA (shRNA) or micro RNA (miR) technology. An interesting field of research is the interplay between miR and ASYN. ASYN mRNA has a 3′UTR, which is double the size of its coding region, suggesting that the 3′UTR might play a role in mRNA stability and in the translation process [448]. Furthermore, it also implies that the mRNA can be controlled under a tight regulatory process, which has been corroborated by recent studies, proving that specific miRs modulate ASYN expression.

In MPTP treated mice, increase in ASYN expression was accompanied by a 50% decrease in miR-7 levels [449]. miR-7, a highly expressed miR in the SNpc and striatum, can alter ASYN protein synthesis by reducing the stability of the mRNA [450]. When overexpressed, miR-7 can target the ASYN3′-UTR, down regulate ASYN synthesis [449, 451], decrease cytotoxicity and rescue proteasomal impairment [449]. miR-153, along with miR-7, is highly expressed in the brain and can also target the 3′UTR region of ASYN mRNA [450, 451]. The miR-153 is able to reduce ASYN mRNA and protein levels but with a lower success rate, as compared to miR-7. Interestingly, miR-153 and miR-7 may have a complementary effect, as significant downregulation of ASYN is observed in the presence of both miRs [451].

ASYN knockdown has also been successfully accomplished by shRNAs, *in vitro* and *in vivo*, in rat and non-human primate brains [452, 453], though antagonistic effects have been described. While ASYN silencing confers a protective effect in the presence of PD- and MSA-inducing drugs [454, 455], it has also been described to induce nigrostriatal degeneration [456] and to increase toxicity in dopaminergic neurons [457]. These contradictory data suggest that ASYN gene silencing may have a dual role in cell homeostasis and, therefore, the applicability of this technique is still a ways away from reaching the clinic.

PHARMACOLOGICAL PREVENTION OF AGGREGATION AND ACCUMULATION OF ASYN

Presently, all drugs used to treat patients with synucleinopathies are merely palliative, as none are capable of reversing pathological conditions. Moreover, the available therapeutics do not target the actual hallmark of pathology in synucleinopathies – ASYN misfolding and subsequent accumulation. New treatments, directed toward important intermediary species within the ASYN aggregation pathway, are urgently needed. Recent studies suggest that ASYN may exist in a tetrameric form that is more stable and may exhibit lower propensity to aggregate. Therefore, it is appealing to think that the development of compounds that are able to stabilize this, native or not, ASYN structure may prevent its misfolding and aggregation. Another promising approach would consist of modulating PTMs known to increase ASYN aggregation propensity. The development of specific kinase inhibitors for Ser129 or compounds that block ASYN truncation may reduce ASYN pathology.

Regulation of ASYN expression can also be achieved at the protein degradation level. Impairment of protein folding machinery and/or degradation pathways are believed to lie at the epicenter of ASYN accumulation [458–463]. Preventing oligomer formation or promoting the clearance of previously formed, larger species of ASYN may be reached with drugs that are capable of activating the proteasome, modulating expression levels of lysosomal proteins and specific chaperones or enhancing ASYN binding to chaperone-like molecules. Indeed, enhancing macroautophagy, through rapamycin treatment [211] or overexpression of Beclin 1 [464], increases ASYN clearance and confers neuroprotection. It is worth noting that nanotechnology should be taken into consideration when

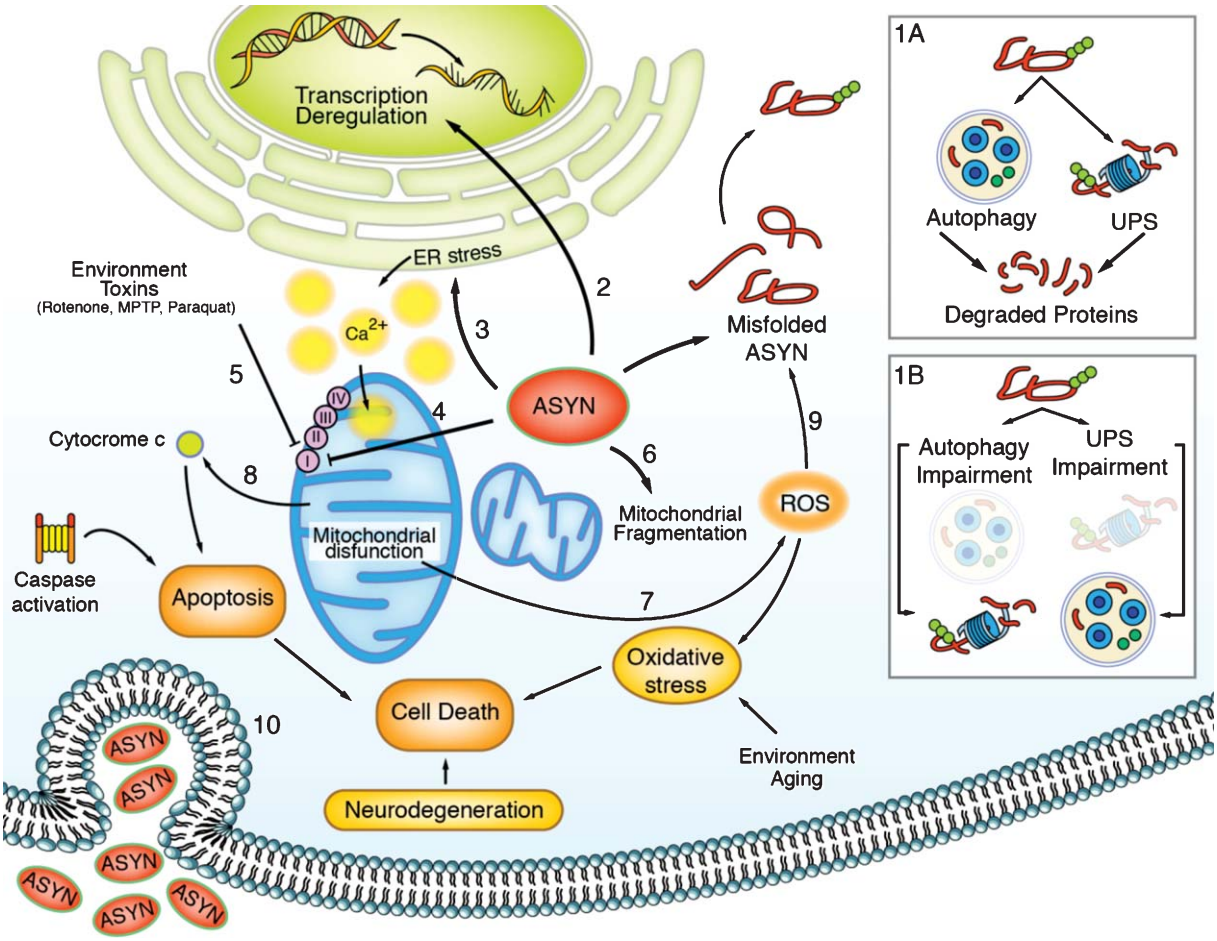

Fig. 6. Schematic summary of established ASYN pathogenic mechanisms. The correct folding of proteins and the targeting of misfolded protein, either by the proteasome or the lysosome, are vital processes for maintaining cellular homeostasis (1A). In synucleinopathies, the impairment of either degradation system leads to the accumulation of aberrant ASYN (1B) that can become toxic to the cell. Nevertheless, the mechanisms that lead to neurodegeneration are still unknown, with several organelles being affected. One of the less studied effects of ASYN is its translocation and role in the nucleus, where it can bind to DNA or to transcription factors and promote transcription deregulation (2). At the cytoplasmic level, ASYN can alter ER homeostasis (3), leading to an imbalance of intracellular Ca^{2+} that will promote changes at a mitochondrial level. ASYN, as well as several drugs known to cause PD, can act on the mitochondria to induce mitochondrial complex I dysfunction (4, 5). Besides complex I binding, ASYN may also alter mitochondrial dynamics and contribute to mitochondrial fragmentation (6). Mitochondrial impairment can promote cell damage through several pathways: by increasing ROS production (7), oxidative stress and inducing apoptotic pathways, via caspase activation (8). Increased ROS and oxidative damage, due to mitochondrial dysfunction, may further promote misfolded protein conformations (9). Different ASYN species may also be secreted into the extracellular space to induce disease transmission to neighboring neurons (10). Together, all of the above-mentioned mechanisms may culminate in neurodegeneration and cell death.

designing new drugs, since this may facilitate penetration of various compounds across the blood brain barrier, directing them to specific cellular localizations and, thereby, reducing potential systemic side effects.

Understanding of the role of ASYN and that of other players involved in these diseases is still quite limited. This limitation has led to many failures in clinical trials. Ultimately, only an in-depth understanding of the basic molecular pathways, which underlie the onset and progression of synucleinopathies, will allow the identification of novel therapeutic targets and the development of multidisciplinary neuroprotective strategies.

CONCLUDING REMARKS

The correlative link of ASYN and neurodegeneration was unearthed approximately 20 years ago, when it was discovered in AD plaques. Since its debut, within hallmark lesions of synucleinopathies and within the context of genetic mutations that segregate with related diseases, an array of functional relationships between

ASYN and several molecular and cellular pathways have been explored. As a result, ASYN-induced toxicity is associated with impairment of several neuronal systems. (Fig. 6) Preliminarily, efforts were largely focused on understanding the aggregation process, itself. The driving force, underlying this interest, has been propelled by the supposition that inhibiting the process of aggregation may impede or reverse disease progression. As studies that are aimed at elucidating the mechanisms, which give rise to aggregation of ASYN, continue to evolve, some fundamental concepts have, thus far, prevailed: the process is nucleation-dependent and the intermediary species may be more toxic than the mature amyloids. Furthermore, across a spectrum of cellular and subcellular foci of interests, most research efforts investigating synucleinopathies explore ASYN-derived effects that arise as a function of one, or more, of the following conditions: 1.) modulation of expression levels, 2.) genetic mutations, 3.) PTMs and/or 4.) species-specific variations. While a breadth of information has been generated, a number of gaps remain to be filled. Of note, particular enthusiasm is currently directed at targeting ASYN presence occurring outside of the cell, which presents a potential target for development of preventative biomarker technology, as well as small molecule, monoclonal antibody and nanobody therapeutic approaches. Further understanding of the mechanistic basis, by which ASYN-toxicity is propagated between cells, represents a critical research aim for attaining the ultimate goal of bridging a link between the bench and the bedside of ASYN-associated disorders.

ACKNOWLEDGMENTS AND DISCLOSURE STATEMENT

This work was supported by the DFG Center for Nanoscale Microscopy and Molecular Physiology of the Brain (CNMPB). RP is funded by Fundação para a Ciência e Tecnologia, Portugal (SFRH/BD/80884/2011). We thank Dr. Wei-Ping Gai, Flinders University, for the picture of the human Lewy body included in Fig. 3, and we thank Catarina Fernandes for her excellent support with figure preparation.

The authors have nothing additional to disclose and no conflicts of interest to declare.

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