α-Synuclein Misfolding and Neurodegenerative Diseases

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Abstract: α-Synuclein is an abundant presynaptic brain protein, misfolding, aggregation and fibrillation of which are implicated as critical factors in several neurodegenerative diseases. The list of the well-known synucleinopathies includes such devastating disorders as Parkinson's disease, Lewy body variant of Alzheimer's disease, diffuse Lewy body disease, dementia with Lewy bodies, multiple system atrophy, and neurodegeneration with brain iron accumulation type I. The precise functions of α-synuclein remain elusive, but there are evidence indicating its involvement in regulation vesicular release and/or turnover and synaptic function in the central nervous system. It might play a role in neuronal plasticity responses, bind fatty acids, regulate certain enzymes, transporters, and neurotransmitter vesicles, be involved in neuronal survival and even can act as a molecular chaperone. Structurally, α-synuclein is an illustrative member of the rapidly growing family of natively unfolded (or intrinsically disordered) proteins and considerable knowledge has been accumulated about its structural properties and conformational behavior. The molecular mechanisms underlying misfolding, aggregation and fibrillation of α-synuclein and the role of various environmental and genetic factors in stimulation and inhibition of these processes are relatively well understood. Here, the main structural features of α-synuclein, its functions, and involvement in various human diseases are summarized providing a foundation for better understanding of the biochemistry, biophysics and neuropathology of α-synuclein aggregation.

Keywords: α-Synuclein, synucleinopathies, aggregation, fibril, nerodegeneration, natively unfolded protein.

HOW TO BECOME A STAR

α-Synuclein was first described by Maroteaux *et al*. in 1988 as a neuron-specific protein localized to the nucleus and presynaptic nerve terminals of the Pacific electric ray *Torpedo californica* [1]. Only 24 papers mentioning α-synuclein have been published between 1988 and 1996, i.e. before the year 1997 when Polymeropoulos *et al*. identified a mutation in the α-synuclein gene in familial cases of early-onset PD [2] and Spillantini *et al*. showed the presence of α-synuclein in Lewy bodies [3]. More than 2500 papers on α-synuclein, now believed to be one of the major players in the PD pathogenesis, have been published since that time clearly marking 1997 as a turning-point in the re-assessment of the molecular basis of PD [4]. Fig. (**1**) illustrates this dramatic increase in the interest of researchers in α-synuclein over the past decade, and shows that the number of PubMed annotated publications dealing with this protein steadily increases, exceeding 365 papers per year for the last two years. This means that each day, at least one α-synuclein-related paper is published.

The major reason for this rising popularity is the involvement of α-synuclein in a diverse group of neurodegenerative disorders, known as synucleinopathies. Synucleinopathies share common pathologic proteinaceous lesions

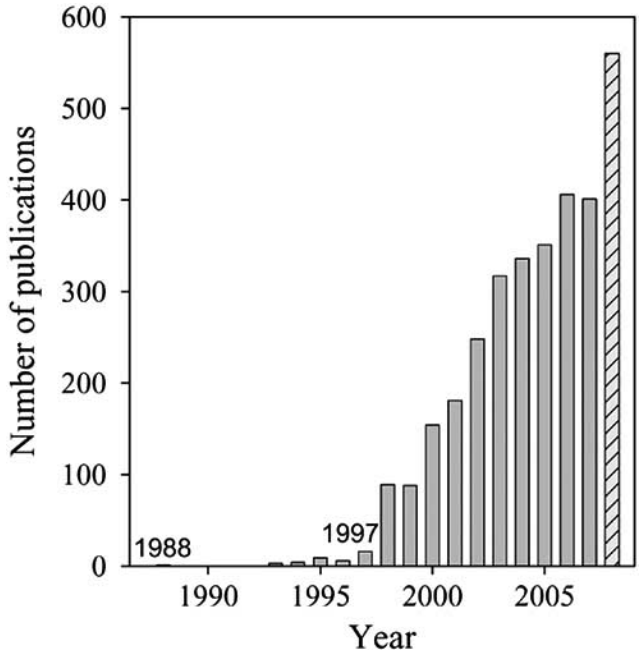

Fig. (1). The increase in the number of α-synuclein related publications annotated in PubMed. Gray dashed bar represents the projected number of papers dealing with α-synuclein in 2008. This projection was calculated based on the number of papers published during the first 3 months of the year (140).

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that are composed of aggregated α-synuclein and are deposited in the selectively vulnerable populations of neurons and glia [5-8]. The term synucleinopathies was introduced in 1998 (i.e., just one year after the discovery of α-synuclein deposition in PD) when it was recognized that filamentous α-synuclein deposits might represent a common hallmark linking multiple system atrophy (MSA) with Parkinson's disease (PD) and dementia with Lewy bodies (DLB) [9]. In addition to these three diseases, current list of the synucleinopathies includes (but is not limited to) neurodegeneration with brain iron accumulation, type I (also known as adult neuroaxonal dystrophy or Hallervorden-Spatz diseases, HSD), pure autonomic failure and several Lewy body disorders, including diffuse Lewy body disease (DLBD), the Lewy body variant of Alzheimer's disease (LBVAD) [3, 9-17]. Furthermore, even before α-synuclein detection as the major LB component in PD, the peptide derived from the central hydrophobic region of this protein (residues 61-95), known as NAC (non-Aβ component of AD amyloid), was found to represent a second major intrinsic constituent of the AD senile plaques [18, 19]. Intriguingly, subsequent work failed to confirm the presence of NAC in amyloid plaques [20]. Growing evidence associates the onset and progression of clinical symptoms as well as the degeneration of affected brain regions in these neurodegenerative disorders with the formation of abnormal filamentous aggregates containing α-synuclein. Therefore, it has been concluded that all aforementioned disorders are brain amyloidoses unified by pathological intracellular inclusions of aggregates having the α-synuclein protein as a key component [5-9, 17, 21, 22]. Some key facts linking α-synuclein aggregation with the pathogenesis of different synucleinopathies are outlined below. It is believed that understanding why α-synuclein pathology develops in these apparently unrelated conditions may shed light on the mechanisms operating in different synucleinopathies [23].

α-SYNUCLEIN AND SYNUCLEINOPATHIES: NEUROPATHOLOGY OF "FATAL ATTRACTION"

Synucleinopathies is a group of neurodegenerative disorders characterized by fibrillary aggregates of α-synuclein protein in the cytoplasm of selective populations of neurons and glia [5-8]. Clinically, synucleinopathies are characterized by a chronic and progressive decline in motor, cognitive, behavioral, and autonomic functions, depending on the distribution of the lesions. Because of clinical overlap, differential diagnosis is sometimes very difficult [24]. The neuropathological spectrum of synucleinopathies has been intensively discussed [5-8, 24-31], and the potential mechanisms linking the α-synuclein aggregation with the development of several of these diseases are the major focus of numerous studies. This idea is illustrated by Fig. (**2**) which links disease-specific deposits (LB (**A**), LNs (**B**), GCI (**C**), NCI (**D**) and axonal spheroids (**E**)) with amyloid-like fibrils (**F**) comprised of natively unfolded α-synuclein (**G**). Table **1** lists

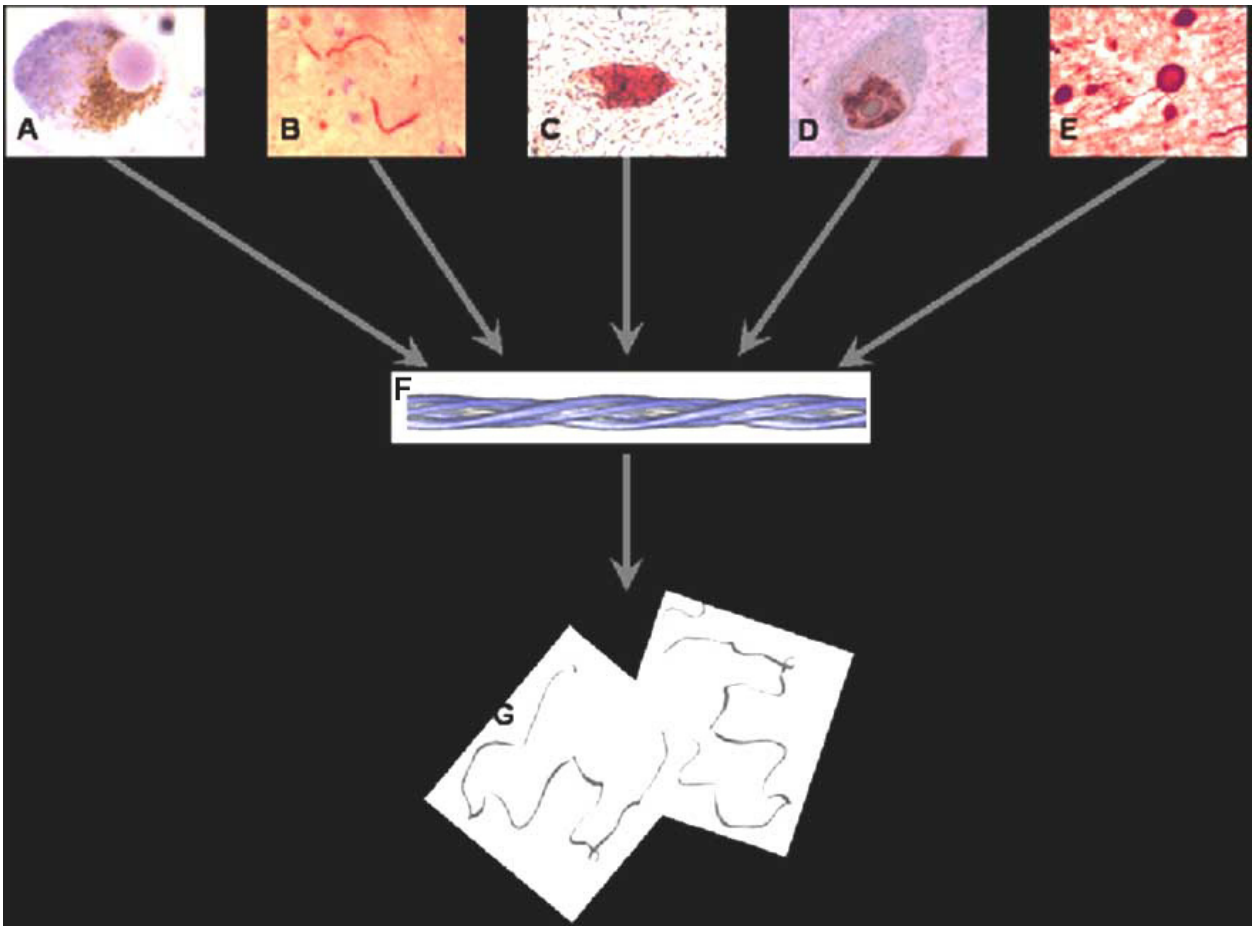


Fig. (2). A model 2 linking disease-specific deposits (LB (**A**), LNs (**B**), GCI (**C**), NCI (**D**) and axonal spheroids (**E**)) with amyloid-like fibrils (**F**) which are formed from the natively unfolded α-synuclein (**G**).

different neurodegenerative disorders which are believed to be associated with α-synuclein deposition. Some of these disorders are discussed below to illustrate a wide range of pathological manifestations in synucleinopathies.

Table 1. Human Neurodegenerative Disorders with α-Synuclein Deposits

Diseases with neuronal inclusions

- Normal aging
- Parkinson's disease
  - Idiopathic
  - Neurotoxicant-induced (incidental)
  - Familial
    - With α-synuclein point mutations
    - With α-synuclein gene triplication
    - With mutations in other proteins
  - Pure autonomic failure
  - Lewy body dysphagia
- Parkinsonism plus syndromes
  - Sporadic
    - Progressive supranuclear palsy
    - Olivoponto cerebrellar atrophy (Shy-Dragger syndrome)
    - Cortical-basal ganglionic degeneration
    - Sporadic pallidal degeneration
    - Bilateral striatopallido dentate calcinosis
    - Parkinsonism with neuroacanthocytosis
- Familial
    - Familial diffuse Lewy body disease
    - Familial dementia with swollen achromatic neurons and corticobasal inclusion bodies
    - Frontotemporal dementia with parkinsonism linked to chromosome 17
    - Associated with psychiatric disturbances
    - Associated with respiratory disturbances
    - Associated with dystonia
    - Associated with myoclonus and seizures
    - Familial progressive supranuclear palsy
- Alzheimer's disease
  - Sporadic
  - Familial with APP mutation
  - Familial with PS-1 mutation
  - Familial with other mutations
  - Familial British dementia
- Lewy body diseases
  - Dementia with Lewy bodies
    - Pure form - transitional/limbic
    - Pure form - neocortical
  - Diffuse Lewy body disease
    - Common form
    - Pure form
- Lewy body variant of Alzheimer's disease
  - Incidental Lewy body disease
  - Lewy body dementia
  - Senile dementia of Lewy body type
  - Dementia associated with cortical Lewy bodies
- Down's syndrome
- Amyotrophic lateral sclerosis-parkinsonsim/dementia complex of Guam
- Neuroaxonal dystrophies
  - Neurodegeneration with brain iron accumulation, type I (Hallervorden-Spatz syndrome or adult neuroaxonal dystrophy)
  - Motor neuron disease
- Amyotrophic lateral sclerosis
  - Familial
  - Sporadic
- Tauopathies
  - Frontotemporal degeneration/dementia
  - Pick's disease
  - Post-encephalitic parkinsonism
  - Dementia pugilistica
  - Argyrophilic grain disease
  - Corticobasal degeneration
- Prion diseases
  - Transmissible spongiform encephalopathies
    - Sporadic
      - Creutzfeldt-Jakob disease
    - Familial
      - Familial Creutzfeldt-Jakob disease
      - Gertsmann-Straussler-Scheinker syndrome
    - Infectious
      - Iatrogenic Creutzfeldt-Jakob disease
      - Variant Creutzfeldt-Jakob disease
      - Kuru
  - Fatal familial insomnia
- Ataxia telangiectatica
- Meige's syndrome

Diseases with neuronaland glial inclusions

- Multiple system atrophy
  - Shy-Drager syndrome
  - Striatonigral degeneration (MSA-P)
  - Olivopontocerebellar atrophy (MSA-C)

α-Synuclein and PD

Officially clinical symptoms of PD were first described less than 200 years ago [32]. Unofficially, there were reports of possible Parkinsonian syndromes dating back thousands of years [33, 34]. PD is the most common ageing-related movement disorder and second most common neurodegenerative disorder after AD. It is estimated that ~1.5 million Americans are affected by PD. Since only a small percentage of patients are diagnosed before age 50, PD is generally considered as an aging-related disease, and approximately one of every 100 persons over the age of 55 in the US suffers from this disorder [35]. PD is a slowly progressive disease that affects neurons of the *substantia nigra*, a small area of cells in the mid-brain. Gradual degeneration of the dopaminergic neurons causes a reduction in the dopamine content. This, in turn, can produce one or more of the classic signs of Parkinson's disease: resting tremor on one (or both) side(s) of the body; generalized slowness of movement (bradykinesia); stiffness of limbs (rigidity); and gait or balance problems (postural dysfunction). Usually, ~80-85% of Parkinsonism patients are found to have these typical features of idiopathic PD (IDP), whereas the remaining 15-20% patients belong to the other category of neurodegenerative disorders [29]. Some patients who are clinically diagnosed as having IPD with additional or atypical clinical features are currently classified as having Parkinsonism plus syndromes (PPS) [36]. It has been emphasized that several differences exist between typical IPD and PPS in the pattern of extrapyramidal features, pattern of onset of illness, and therapeutic responses [37].

The precise mechanisms of neuronal death are unknown as of yet. Some surviving nigral dopaminergic neurons contain cytosolic filamentous inclusions known as Lewy bodies (LBs) when found in the neuronal cell body, or Lewy neurites (LNs) when found in axons [38, 39].

Several observations implicate α-synuclein in the pathogenesis of PD. For example, a direct role for α-synuclein in the neurodegenerative processes in PD is demonstrated by genetic evidence. Autosomal dominant early-onset Parkinson's disease was shown to be induced in a small number of kindreds as a result of three different missense mutations in the α-synuclein gene, corresponding to A30P, E46K, and A53T substitutions in α-synuclein [2, 40, 41] or as a result of the hyper-expression of the wild type α-synuclein protein due to gene triplication [42-44]. Antibodies to α-synuclein detect this protein in LBs and LNs, the hallmark lesions of PD, a substantial portion of fibrillar material in these specific inclusions was shown to be comprised of α-synuclein, and insoluble α-synuclein filaments were recovered from purified LBs [3, 21]. The production of wild type α-synuclein in transgenic mice [45] or of WT, A30P, and A53T in transgenic flies [46], leads to motor deficits and neuronal inclusions reminiscent of PD. Under the particular conditions, cells transfected with α-synuclein might develop LB-like inclusions. Other important observations correlating α-synuclein and PD pathogenesis, being reviewed in more detail elsewhere [7, 23, 27, 30, 31, 47], are briefly outlined below. By numerous studies from different laboratories it has been established that the recombinant α-synuclein easily assembles into amyloid-like fibrils *in vitro* and this process is modulated by familial point mutations. α-Synuclein is abnormally phosphorylated, ubiquitinated, and nitrated in pathology-related inclusions. Co-expression of chaperones or β-synuclein with α-synuclein in transgenic animals was shown to suppress the neurodegeneration. α-Synuclein-positive proteinaceous deposits were shown to accumulate in several animal models where Parkinsonism was induced by exposure to different neurotoxicants.

α-Synuclein in DLB and Other Lewy Body Disorders

Dementia with Lewy bodies (DLB), being the second most frequent neurodegenerative dementing disorder after AD, is a common form of late-onset dementia that exists in a pure form or overlaps with the neuropathological features of AD. This disease is characterized clinically by neuropsychiatric changes often with marked fluctuations in cognition and attention, hallucinations, and parkinsonism [48]. Similar to PD, neurophathological hallmarks of DLB are numerous LBs and LNs in the *substantia nigra*, which are strongly immunoreactive for α-synuclein [3]. However, unlike PD, DLB is characterized by large numbers of LBs and LNs in cortical brain areas [49]. It has been noted that filaments from LBs in DLB are decorated by α-synuclein antibodies [9, 21, 50], and that their morphology closely resembles that of filaments extracted from the *substantia nigra* of PD brains [9, 21]. DLB and PD with dementia, being different in the temporal course of the disease, share most of the same clinical and neuropathologic features and are often considered as belonging to a spectrum of the same disease [51-53].

In most of DLB cases, LBs are rarely the only lesions in the brain of patients, being frequently associated with typical AD-associated lesions, neurofibrillary tangles and amyloid plaques [54, 55]. This co-appearance of the PD/DLB and AD neurophathological hallmarks leads to confusing classifications such as Lewy body variant of AD [56, 57], common and pure forms of diffuse Lewy body disease [58], Lewy body dementia [59], senile dementia of Lewy body type [60], or dementia associated with cortical Lewy bodies [61]. Furthermore, it is well recognized now that the incidence of dementia in PD is higher than expected from aging alone [48], as dementia affects about 40% of PD patients [62], and the incidence of dementia in PD patients is up to six times greater than observed in normal aged matched control subjects [63]. These observations show that the LB diseases are characterized by a clinical continuum [64] starting from pure PD cases characterized by motor dysfunction, and ending with DLB cases that combines movement dysfunction with dementia. Between these two extreme are numerous PD patients diagnosed clinically as having PD dementia (PDD), who eventually develop significant dementia. The classification of these disorders was shown to be somewhat artificial, applying what has been referred to in the literature as the 1-year rule: Those with dementia during the first year are considered to have DLB, and if the dementia occurs later in the disease course, the diagnosis of PDD is given [48].

Amyotrophic lateral sclerosis-parkinsonsim/dementia complex of Guam (Guam disease) represents another interesting example of junction between PD and dementia. Guam disease is a neurodengenerative disorder with unusually high incidence among the Chamorro people of Guam [65-67]. Originally, it was believed that this disease is an autosomal dominant disorder with complete penetrance in males and ~50% penetrance in females [65]. However, as no marker system associated with this disorder was found, it has been concluded that "local environmental factors are most likely involved in pathogenesis" [68, 69]. This hypothesis was supported by the fact that the high incidence of ALS/PD in Guam disappeared when isolation was disrupted and travel and economic changes brought food and water sources from the outside. Furthermore, it has been shown that the neurotoxic plant *Cycas circinalis*, a traditional source of food and medicine used by the Chamorro people, plays a role in the development of Guam ALS-parkinsonism-dementia [67]. Intriguingly, recent studies revealed that in general three neurodegenerative disorders, ALS, dementia, and PD, co-occur within families more often than expected by chance, suggesting that there may be a shared genetic susceptibility to these disorders [70].

It has been established that several peripheral and central areas of the nervous system can be affected by the LB deposition. Besides already discussed *substantia nigra*, this includes *hypothalamic nuclei*, *nucleus basalis of Meynert*, *dorsal raphe*, *locus ceruleus*, *dorsal vagus nucleus*, and *intermediolateral nucleus* [71]. A 'neuritic' form of Lewy body was also described in the *dorsal vagus nucleus*, *sympathetic ganglia*, and in *intramural autonomic ganglia* of the gastrointestinal tract, as well cases were demonstrated with extensive cortical and basal ganglia involvement [49, 72]. This broad spectrum of the nervous system regions potentially

affected by LB formation produces great variability in the disease manifestation and Lewy body pathology is also a characteristic feature of several rarer diseases, such as pure autonomic failure, Lewy body dysphagia, incidental Lewy body disease [23]. *Pure autonomic failure* (also known as Bradbury-Eggleston syndrome) [73] and *Lewy body dysphagia* [74] are the results of the predominant involvement of the peripheral nervous system with minimal central nervous system involvement. In *incidental Lewy body disease*, it has been shown that ~5%-10% of asymptomatic individuals have insignificant numbers of LBs bodies, usually located in *substantia nigra* [75]. In fact, the analysis of a group of the geriatric hospital patients without any neurologic or psychiatric disorders revealed that 2.3% of them had one or more Lewy bodies in brain stem locations [76]. In all cases studied, the presence of LBs was associated with at least some degree of neuronal degeneration in *substantia nigra*. Based on these facts it has been concluded that this population of patients actually represents preclinical, idiopathic Parkinson's disease and, if given enough time, will eventually develop characteristic parkinsonian symptoms [76].

α-Synuclein Deposits in AD

Alzheimer's disease (AD) was described for the first time described in 1907 by a German physician Alois Alzheimer [77]. AD is the most common aging-related neurological disorder, which constitutes about two thirds of cases of dementia overall [78, 79] and is characterized by slow, progressive memory loss and dementia due to a gradual neurodegeneration particularly in the cortex and hippocampus [80]. The clinical hallmarks are progressive impairment in memory, judgment, decision making, orientation to physical surroundings, and language [81]. From the initial symptoms, disease progression can last up to 25 years, although typically the duration ranges from 8 to 10 years. The pathological hallmarks of the AD are neuronal loss, extracellular senile plaques containing the peptide Aβ, and neurofibrillary tangles (NFTs) composed of a hyperphosphorylated form of the microtubular protein tau [82]. Detailed analysis of the α-synuclein immunoreactivity in the brains from the patients with sporadic AD revealed the presence of α-synuclein-positive inclusions resembling LBs and LNs in ~50% cases studied [83]. α-Synuclein-positive LB-like intra-cytoplasmic inclusions were found in the amygdale, the temporal cortex, the parahippocampal gyrus, and in the parietal cortex, whereas LN-like inclusions were abundant in the amygdala, the CA2/3 region of hippocampus formation, parahippocampal gyrus, the temporal cortex, substantia nigra, locus ceruleus, the frontal cortex, and in the parietal cortex [83].

Down's syndrome is a genetic disorder characterized by an extra chromosome 21 (trisomy 21, i.e., instead of having the normal 2 copies of chromosome 21, the Down's syndrome patient has 3 copies of this chromosome). The person with Down's syndrome has mild mental retardation, short stature, a flattened facial profile, a risk of multiple malformations (including heart malformations; duodenal atresia, where part of the small intestines is not developed and leukemia), and susceptibility to early-onset AD. Incidence of this disorder among the newborn is estimated at 3 in 1,000, whereas in the general population it is approximately 1 in 1,000. The difference reflects the early mortality. The analysis of Down's syndrome with Alzheimer pathology revealed presence of numerous LBs and LNs in the neurons of the limbic areas, predominantly of the amygdala. Similar lesions were less common in other regions of these brains [84, 85]. Importantly, in the vast majority of cases examined no LBs and LNs were detected in the *substantia nigra* and *locus ceruleus*, and there was no significant neuronal loss in the *substantia nigra*.

α-Synuclein and MSA

Multiple system atrophy (MSA) an adult-onset progressive neurodegenerative disorder of unknown etiology which is characterized clinically by any combination of parkinsonian, autonomic, cerebellar or pyramidal symptoms and signs, and pathologically by cell loss, gliosis and glial cytoplasmic inclusions (GCIs) in several brain and spinal cord structures. Most patients affected by MSA deteriorate rapidly and survival beyond ten years after disease onset is unusual. It is believed that the motor impairment in MSA results from L-dopa-unresponsive parkinsonism, cerebellar ataxia and pyramidal signs, with 80% of MSA cases showing predominant parkinsonism (MSA-P) due to underlying striatonigral degeneration, and the remaining 20% developing predominant cerebellar ataxia (MSA-C) associated with olivopontocerebellar atrophy [86]. Autonomic dysfunction including urogenital failure and orthostatic hypotension is common in both motor presentations, MSA-P and MSA-C, reflecting degenerative lesions of central autonomic pathways [87]. Distinguishing MSA-P from PD is problematic at early stages owing to PD-like features in MSA-P, including a transient L-dopa response in some patients [88]. MSA is less common than PD as epidemiological studies suggested a prevalence of 1.9–4.9 people per 100,000 and an incidence of 3 patients per 100,000 people per year [89-91]. Histologically, MSA is characterized by the variable neuron loss in the *striatum*, *substantia nigra pars compacta*, *cerebellum*, pons, inferior olives and intermediolateral column of the spinal cord [92]. The histological hallmark of MSA is the presence of argyrophilic fibrillary inclusions in the oligodendrocytes, referred to as glial cytoplasmic inclusions (GCIs), which are also known as Papp-Lantos bodies [93]. Fibrillary inclusions are also found in the neuronal somata, axons, and nucleus. Neuronal cytoplasmic inclusions are frequently found in the pontine and inferior olivary nuclei [94]. It has been established that α-synuclein is a major component of glial and neuronal inclusions in MSA [9, 94]. Although both LBs and GCIs contain α-synuclein, they are differently localized, with α-synuclein inclusions being neuronal in PD and DLB, and oligodendroglial in MSA. This suggests the existence of a unique pathogenic mechanism that ultimately lead to neuron loss via disturbance of axonal function [93]. In MSA, besides formation of GCIs α-synuclein also aggregates in the cytoplasm, axons and nuclei of neurons, and the nuclei of oligodendroglia. The relationship between GCIs and these additional α-synuclein deposition sites is not understood [93].

α-Synuclein and NBIA I

Neurodegeneration with brain iron accumulation type 1 (NBIA1) (formerly know as Hallervorden-Spatz disease

(HSD) or adult neuroaxonal dystrophy) represents a rare progressive neurodegenerative disorder that occurs in both sporadic as well as in familial forms. Clinically, NBIA 1 is characterized by rigidity, dystonia, dyskinesia, and choreoathetosis [95-98], together with dysarthria, dysphagia, ataxia, and dementia [98-100]. Symptoms usually present in late adolescence or early adult life and this disease is persistently progressive [95, 99, 100]. The histopathologic hallmarks of NBIA1 include neuronal loss, neuraxonal spheroids, and iron deposition in the *globus pallidus* and *substantia nigra pars compacta*, as well as by the presence of the LB-like and GCI-like inclusions and dystrophic neuritis [99]. NBIA1 is characterized by an association of extrapyramidal movement disorders with neuroaxonal dystrophy (NAD) and iron accumulation in the basal ganglia. It represents a pantothenate kinase-associated neurodegeneration caused by the PNAK2 gene linked to chromosome 20p12.3-13 [101]. It has been shown that the LB-like inclusions throughout the cortex and brainstem, axonal swellings, and rare GCI-like inclusions of the midbrain clearly possess α-synuclein immunoreactivity [102-104]. Importantly, axonal spheroids were also shown to contain α-synuclein [104, 105].

Morphologies and Composition of α-Synuclein Containing Inclusions in Synucelinopathies

As it follows from the discussion above, α-synuclein inclusions are present in neurons (both dopaminergic and non-dopaminargic), where they can be deposited in perikarya or in axonal processes of neurons, and in glia. There are at least five morphologically different α-synuclein containing inclusions, Lewy bodies, Lewy neurites (dystrophic neurites), glial cytoplasmic inclusions, neuronal cytoplasmic inclusions and axonal spheroids (see Fig. **2**). Some peculiarities of these inclusions are outlined below.

The classical appearance of the *Lewy body* in hematoxylin/eosin stained pigmented nigral neurons subjected to light microscope analysis is an eosinophilic spherical body with a dense core surrounded by a halo (see Fig. **2A**) [38]. Some LBs, however, especially those in the neocortex lack a halo [106] and may be unnoticeable with hematoxylin/eosin staining. The use of the immunolabeling for ubiquitin and α-synuclein, which superseded hematoxylin/eosin staining [107, 108], more readily reveal LB and pale bodies, which are diffuse inclusions that are weakly stained with eosin [109]. Recently, the analysis of the *substantia nigra* and cortical regions of patients with LBD immunolabeled for ubiquitin and α-synuclein revealed the existence of a wide spectrum of intracytoplasmic inclusions containing α-synuclein [106]. They ranged from diffuse, "cloud-like" deposits, which were typically ubiquitin-negative and not apparent with hematoxylin/eosin staining, through the pale bodies, which had variable immunolabeling for ubiquitin, to the classical LB with a halo, usually with α-synuclein labeling in the halo and ubiquitin labeling in the core [106]. Based on these observations it has been hypothesized that this set of different α-synuclein inclusions represent diverse stages in the LB development. In this model, the diffuse cloud-like α-synuclein-positive structures could be formed at the early stage. At the next stage, these diffuse inclusions evolve to more compact structures that may become tagged with ubiquitin and form pale bodies. Finally, these pale bodies become more condensed with a halo to produce the classic LB [106, 110]. Ultrastructural analysis of *substantia nigra* in PD using an electron microscopy revealed the existence of two types of LBs, the classical (and more common) LB with a central core and a halo when observed with light microscopy and the LB of uniform density as seeing with light microscopy [111, 112]. The more common type was characterized as being composed of granular and fibrillar components, with the granular material being more prominent in the core and the fibrils being radiating at the periphery. The second type of LB was composed almost entirely of circular or oval fibrillar material [111, 112]. Interestingly, in agreement with the light microscopy, the ultrastructural analysis of LBs in PD by immunoelectron microscopy revealed the existence of α-synuclein filaments in LB, pale bodies, and diffuse deposits (perikaryal threads) [113]. In LBs, α-synuclein was predominantly radially arranged in the periphery with only a few α-synuclein silver grains in the central core, which could be due to the tight packing of the core preventing proper labeling. In pale bodies, the loosely aggregated filaments were labeled for α-synuclein, whereas perikaryal threads were described as a loose mesh-work of small bundles of filaments immunoreactive for α-synuclein but not for ubiquitin [113]. Finally, the comprehensive list of molecules and organelles reported in LBs includes more than 50 different components starting from chondroitin sulfate, lipids and sphingomyelin and ending with numerous proteins (α-synucelin, ubiquitin, microtubule-associated protein 2, heat-shock proteins, DJ-1, and 14-3-3 protein, neurofilaments, protein tau, complement proteins and several kinases to name a few) and mitochondria [109].

Lewy neurites (LNs, dystrophic neurites) usually display a coarse and rounded form, but may extend to elongated club-shaped or serpentine structures (see Fig. **2B**), which easily escape detection by conventional staining techniques used for demonstration of PD-related lesions [114-116]. When sections of amygdala, hippocampus, entorhinal cortex, anterior cingulate areas, and dorsal vagal area, i.e., sites known for their LN predilection, were processed with immunoreaction for α-synuclein, in addition to the well-known LBs and voluminous LNs (which are the serpentine inclusions bodies developing, for example, in the dorsal motor nucleus of the vagal nerve) extensive fine thread-like immunopositive LNs were found. These LNs were usually straight, did not contain sharp kinks or deviations, were independent of each other, and did not form a continuous network. Furthermore, these thread-like structures displayed some enlargements which did not have regular shape and occurred at varying intervals from each other [114].

Glial cytoplasmic inclusions (GCIs) are argyrophilic, sickle-shaped, oval or conical cytoplasmic aggregates (see Fig. **2C**) that contain α-synuclein, ubiquitin, α- and β-tubulins, 14-3-3 protein, several isoforms of tau protein, microtubule-associated protein 5, Bcl-2, and several kinases among other proteins [117]. Originally, the main components of GCIs in low-power electron micrographs appeared to be randomly distributed loosely packed filaments, whereas the analysis higher power images revealed tubular profiles with a round or ovoid wall and an outer diameter of 20–30 nm, enclosing a clear center [93]. More recent ultrastructural analysis of GCIs revealed that they are composed of

amorphous material-coated filaments up to 30 nm in size, often organized in parallel bundles extending into oligodendroglial processes. Each core filament is made up of two α-synucelin containing fibrils, each made of a string of 3- to 6-nm particles [118]. Filaments with two distinct morphologies have been reported: twisted 5- to18-nm filaments with a periodicity of 70–90 nm, and straight filaments of 10 nm width; both types predominantly contain polymerized α-synucelin [119].

Neuronal cytoplasmic inclusions (NCIs) in the *pontine nucleus* have round or ovoid homogenous or skein-like structures (see Fig. **2D**), occupying the greater part of the neuronal cytoplasm, whereas in the *olivary nuclei*, they are irregular or reniform in shape or look like coarse granular inclusions [120, 121]. Ultrastructurally, NCIs are composed of randomly arranged loosely packed granule-coated fibrils approximately 30–40 nm in diameter [117]. Immunoelectron microscopy showed α-synucelin labeling of both granular and filamentous structures [121].

Axonal spheroids occurring in presynaptic terminals (Fig. **2E**) show accumulation of amorphous, granular, multilamellated and dense bodies, mitochondria, and tubulovesicular structures [26]. Axonal spheroids contain α-synuclein, neurofilament proteins, tau protein, ubiquitin, ferritin, suproxide dismutase and amyloid precursor protein [105].

STRUCTURAL AND FUNCTIONAL PROPERTIES OF α-SYNUCLEIN

In order to better understand the molecular mechanisms of α-synuclein aggregation and to understand the harm produced by this process, elucidation of the structural and functional peculiarities of this protein is crucial. Therefore, the subsequent section of this review is devoted to the brief description of structural properties and functions of α-synuclein.

Synuclein Family

Synucleins belong to a family of closely related presynaptic proteins that arise from three distinct genes, described currently only in vertebrates [122]. This family includes: α-synuclein, which also known as the non-amyloid component precursor protein, NACP, or synelfin [1, 18, 123]; β-synuclein, also referred to as phosphoneuro-protein 14 or PNP14 [123-125] and γ-synuclein, also known as breast cancer-specific gene 1 or BCSG1 and persyn [126-129].

Human β-synuclein is a 134-aa neuronal protein showing 78% identity to α-synuclein. The α- and β-synucleins share a conserved C-terminus with three identically placed tyrosine residues. However, β-synuclein is missing 11 residues within the specific NAC region [15, 130]. The activity of β-synuclein may be regulated by phosphorylation [124]. This protein, like α-synuclein, is expressed predominantly in the brain, however, in contrast to α-synuclein, β-synuclein is distributed more uniformly throughout the brain [131, 132]. Besides the central nervous system β-Synuclein was also found in Sertoli cells of the testis [133, 134], whereas α-synuclein was found in platelets [135].

The third member of the human synuclein family is the 127-aa γ-synuclein, which shares 60% similarity with α-synuclein at the amino acid sequence level [15, 130]. This protein specifically lacks the tyrosine rich C-terminal signature of α- and β-synucleins [130]. γ-Synuclein is abundant in spinal cord and sensory ganglia [128, 136]. Interestingly, this protein is more widely distributed within the neuronal cytoplasm than α-and β-synucleins, being present throughout the cell body and axons [128]. It was also found in metastatic breast cancer tissue [127] and epidermis [137].

As already discussed, deposition of α-synuclein has been implicated in the pathogenesis of several neurodegenerative disorders, known as synucleinopathies. It has recently been established that in addition to the traditional α-synuclein-containing LBs and LNs, the development of PD and DLB is accompanied by appearance of novel α-, β- and γ-synuclein-positive lesions at the axon terminals of hippocampus [138]. These pathological vesicular-like lesions located at the presynaptic axon terminals in the hippocampal dentate, hilar, and CA2/3 regions have been co-stained by antibodies to α- and β-synucleins, whereas antibodies to γ-synuclein detect previously unrecognized axonal spheroid-like inclusions in the hippocampal dentate molecular layer [138]. This broadens the concept of neurodegenerative "synucleinopathies" by implicating β- and γ-synucleins, in addition to α-synuclein, in the onset/progression of these two diseases. Additionally, abnormal expression of γ-synuclein has recently been reported in some breast tumors [127]. Using Northern blots and *in situ* hybridization it has been shown that a high percentage of malignant breast tumors, but not benign breast tumors or normal breast tissue, express γ-synuclein mRNA [127]. In addition, a direct link between γ-synuclein overexpression and increased invasiveness of breast tumor cells has been demonstrated [137].

Amino Acid Sequence of α-Synuclein

α-Synucleins from different organisms possess a high degree of sequence conservation. For example, mouse and rat α-synucleins are identical throughout the first 93 residues, whereas human and canary proteins differ from them by only two residues [130]. At least three α-synuclein isoforms are produced in humans by alternative splicing (AS) [139]. The best known isoform is α-synuclein-140 which is the whole and the major transcript of the protein (Fig. **3A1a**). Two other isoforms, α-synuclein-126 and α-synuclein-112, are the produced by AS resulting from the in-frame deletion of exons 3 and 5, respectively. Exon 3 localizes at the N-terminal of the protein and codes for amino acid residues 41–54, whereas exon 5 is located at the C-terminal domain of the protein, coding for residues 103–130 (see Figs. **3A1b** and **3A1c**). The whole transcript of human α-synuclein, a protein composed of 140 amino acid residues, can be divided into three regions (see Fig. **3A2**):

(*1*) Residues 1-60 form the N-terminal region. It includes the sites of three familial PD mutations and contains four 11-amino acid imperfect repeats with a highly conservative hexameric motif (KTKEGV). The N-terminal region is predicted to form amphipathic α-helices, typical of the lipid-binding domain of apolipoproteins [130, 140].

(*2*) Residues 61-95 constitutes the central region and comprises the highly amyloidogenic NAC sequence (NAC stays for Non-Aβ Component of Alzheimer's disease amy-

loid) [18, 19]. NAC contains three additional KTKEGV repeats and represents a second major intrinsic constituent of Alzheimer's plaques, amounting to about 10% of these inclusions [18]. An 11-amino-acid segment within the central part of the NAC domain (corresponding to residues 73 to 83 of α-synuclein) is missing in β-synuclein.

(*3*) The highly charged C-terminal region is constituted by residues 96-149. This part of α-synuclein is highly enriched in acidic residues and prolines, suggesting that it adopts a disordered conformation. Three highly conserved tyrosine residues, which are considered as a family signature of α- and β-synucleins, are located in this region. This region is mostly missing in γ-synuclein.

Intriguingly, α-synuclein exhibits a 40% homology with members of the 14-3-3 chaperone protein family [141]. The 14-3-3 proteins constitute a family of protein chaperones that are particularly abundant in the brain, like α-synuclein. The 14-3-3 family of proteins consists of five different isoforms that share extensive sequence homology, both among the different isoforms and between similar isoforms in different species [142, 143]. 14-3-3 proteins appear to be involved in diverse cellular functions mostly via the regulation of protein kinases [144, 145]. They bind to ligands at sites containing phospho-serine residues. Binding of 14-3-3 to phosphorylated Raf-1 stabilizes it in an active conformation [146]. 14-3-3 binds to a phosphorylated epitope of protein kinase Cε (PKCε) and stabilizes PKCε in an inactive conformation that is unable to translocate to the membrane [147]. 14-3-3 also binds to phosphorylated death agonist BAD, a very distant BCL2 family member and a pro-apoptotic oncogene that remains inactive when sequestered in the cytosol. The interaction of 14-3-3 with BAD was shown to stabilize maintenance of BAD in a cytoplasmic localization [148].

Using the Multalin algorithm, several regions of sequence homology between the α-synuclein and 14-3-3 family of proteins were found [141]. Particularly, two regions with 43 and 36% sequence homology were seen between amino acids 8 and 61 of α-synuclein and amino acids between 45 and 102 of 14-3-3 [141]. In 14-3-3, this region contains domains that are thought to be phosphorylated by PKC and involved in dimerization of 14-3-3 [149, 150]. In contrast, the C terminus of 14-3-3, which has been implicated in binding to phosphorylated Raf-1, shows no homology to α-synuclein [151]. This structural similarity raised an interesting hypothesis that α-synuclein might function as a molecular chaperone [141]. In agreement with this hypothesis, α-synuclein was shown to bind to 14-3-3 proteins, as well as some proteins known to associate with 14-3-3, including protein kinase C, BAD, and extracellular regulated kinase, but not Raf-1 [141].

Familial PD Point Mutations

Finding a small fraction of PD patients have a familial form of parkinsonism with an autosomal-dominant pattern of inheritance, raised considerable interest in understanding the potential role of genetic factors in the etiology of PD [152]. Large pedigrees have been identified where members in different generations suffer from PD, with incidence of PD in family members being greater than in age-matched controls [35, 153]. Subsequently, a twin study revealed no difference in concordance between monozygotic and di-zygotic twins of PD patients aged 60 years or older, but a significantly increased incidence was observed in monozygotic twins who developed PD at less than 50 years of age [154]. This suggests that genetic factors are important in young-onset patients, but are not likely to play a role in patients with sporadic PD [35]. In agreement with this conclusion, PD was linked to the q21-23 region of chromosome 4 in a large Italian-American family, known as the Contursi kindred, members of which had a relatively early age of PD onset [2]. A mutation in the gene encoding for α-synuclein was detected in this family, as well as in several apparently unrelated Greek families [2]. Sequence analysis demonstrated that the mutation consisted of a single base change from G to A at position 209 (G209A), resulting in an Ala to Thr substitution at position 53 (A53T) in the α-synuclein (see Fig. **3A5**). In the affected families, 85% of patients who expressed the mutant gene had clinical features of PD [2]. An unusual aspect of the mutation is that the amino acid is already a threonine in rodents and other species [155].

A second mutation in the α-synuclein (A30P, see Fig. **3A5**) has been described in a German family [41]. Recently, one more α-synuclein mutation E46K (Fig. **3A5**) was found in a Spanish family with autosomal dominant parkinsonism, dementia, and visual hallucinations of variable severity [40]. Furthermore, another genetic aberration, the triplication of the wild-type gene, has been reported in a large family from Iowa [42-44]. Pathology from three of these kindreds is available, and the postmortem examination showed α-synuclein positive Lewy bodies in the brainstem as well as nigral cell loss. However, α-synuclein pathology is not limited to the *substantia nigra* in many of these cases. In fact, the clinical descriptions of many of the patients with α-synuclein mutations reflect a disease with prominent dementia, presumably a reflection of the widespread cortical Lewy bodies in these cases. The Spanish E46K mutation was reported as LBD [40]. In the Iowan kindred, glial cell inclusions are found [42-44], which would otherwise be typical of MSA. Based on these observations, it has been emphasized that mutations in α-synuclein produce a fulminant disease that includes Parkinsonism but is much more widespread and may resemble DLB [156]. Furthermore, the disorder was shown to also be more progressive, tending to have an earlier onset than sporadic PD [156]. These findings strongly indicate that a single mutation in the human α-synuclein gene is sufficient to account for the PD phenotype.

Posttranslational Modifications

There are several sites in human α-synuclein susceptible for posttranslational modifications (PTMs). The peculiarities of α-synuclein PTMs and their roles in modulation functions and aggregation of this protein have been recently covered in an excellent review [139]. The overall significance of PTMs is in their ability to change the size, charge, structure and conformation of many proteins resulting in regulated alterations and modifications of enzyme activity, binding affinity, and protein hydrophobicity [157]. Both, the spectrum and the range of the PTM-induced changes are very broad, as they are produced by such diverse processes as proteolysis, phosphorylation, lipidation, S-nitrosylation, nitration, oxidation, glycosylation, methylation, adenosine diphosphate (ADP)-

ribosylation, acylation (acetylation, isoprenylation, myristoylation), ubiquitination, sumoylation, sulfation, farnesylation, and many, many others [158]. It has been pointed out that out of >300 known PTMs [157, 158], only a few were described for α-synuclein so far [139]. This includes phosphorylation, nitration, dityrosine crosslinking, methionine oxidation, glycosylation, ubiquitination, sumoylation, crosslinking by advanced glycation endproducts, and crosslinking by transglutaminase. The known sites of PTMs in α-synuclein are shown in Fig. (**3A5**) and briefly overviewed below. In human α-synuclein *in vivo*, serine 129 was established as a major phosphorylation site, with a second phosphorylation site being located at serine 87 (Fig. **3A5**, gray circles) [159]. Fig. (**3**) shows that Ser129 is absent in shortest α-synuclein-112 isoform produced by AS. Interestingly, phosphorylation of α-synuclein at Tyr125 was also reported [160]. α-Synuclein primary sequence contains four tyrosine residues, Tyr 39, Tyr125, Tyr133, and Tyr136 (Fig. **3A5**, black circles). These tyrosine residues are conserved in all α-synuclein orthologs and in β-synuclein paralogs, suggesting that these residues might play important functional roles [27]. It has been shown that all of these tyrosines are subjects for nitration [161-165] and dityrosine crosslinking [166]. There are also four methionines in α-synuclein located outside the repeat-containing region (Met1, Met5, Met116 and Met127) (Fig. **3A5**, open circles). All of them were shown to be highly susceptible for oxidative modification (methionine sulfoxide formation) *in vitro* [167-170]. Although α-synuclein contains 15 lysine residues (which are common targets for several PTMs in proteins, including acetylation, methylation, ubiquitination, and sumoylation) only three of them, Lys6, Lys10 and Lys12, were shown to be involved in α-synuclein ubiquitination *in vivo* (see Fig. **3A5**, gray boxes) [171] and only one yet unidentified lysine residue at the protein N-terminus was shown to be involved in α-synuclein by a small ubiquitin-like modifier SUMO1 (see Fig. **3A5**, black box) [172]. A covalent (both inter- and intramolecular) crosslinking between reactive lysine and glutamine residues leading to the formation of a characteristic N^{ε}-(γ-glutamyl)-lysine isodipeptide crosslink is known to be catalyzed by a tissue transglutaminase [173]. Although α-synuclein in PD nigral dopamine neurons was shown to be heavily crosslinked via the N^{ε}-(γ-glutamyl)-lysine isodipeptide, exact localization of all the modification sites in protein is not known as yet [174]. However, when the crosslinking of NAC by tTG was studied, it has been shown that Lys80 and Gln79 serve as a tTG donor and acceptor sites respectively (see Fig. **3A5**, gray ovals) [175]. Furthermore, the formation of the NAC/Aβ heterotetramers has been reported [175]. A novel α-synuclein isoform with the apparent molecular mass of 22 kDa was found to coprecipitate with parkin from the homogenates of frontal cortex [176]. This isoform was shown to be a result of the α-synuclein *O*-linked glycosylation as its co-incubation with *O*-glycosidase and sialidase A produced the unmodified α-synuclein monomer [176]. The exact localization of the glycosylation site(s) is not known. Finally, it has been shown that advanced glycation endproducts (AGEs, which are the products of the reactive carbonyl compounds interactions with a polypeptide chain) and α-synuclein were similarly distributed in very early LBs in the human brain in cases with incidental LBD and that α-synuclein was crosslinked by AGEs [177].

Functional Repertoire of α-Synuclein

Despite the facts that α-synuclein was estimated to account for as much as 1% of the total protein in soluble cytosolic brain fractions [178] and that it assumed to play a crucial role in the pathogenesis of several neurodegenerative disorders, the precise function of this protein remains mainly elusive. α-Synuclein is expressed in a number of neuronal and non-neuronal cell types including cortical neurons, dopaminergic neurons, noradrenergic neurons, endothelial cells, and platelets [135, 179-181]. Interestingly, torpedo synuclein was reported to localize within the nucleus and presynaptic nerve terminals [1], however most subsequent studies have shown α-synuclein localization only within nerve terminals in the central nervous system [122, 130, 182]. Although the precise function of α-synuclein remains unknown, this localization, in addition to the close association of this protein with vesicular structures, has led to the hypothesis that it may regulate vesicular release and/or turnover and synaptic function in the central nervous system [18, 122, 130, 182, 183]. In agreement with this hypothesis, mice lacking α-synuclein, being superficially normal, exhibited alterations in transmitter release from dopaminergic terminals in striatum following paired electrical stimulation and in locomotor responses after amphetamine administration [179]. Additional observations suggest that α-synuclein may play a role in neuronal plasticity responses, because its avian homologue synelphin is up-regulated in zebra finch brain at a critical period of song learning [140], and rat synuclein-1 is up-regulated during brain development [184, 185], and in cultured neonatal sympathetic neurons after nerve growth factor treatment [186]. α-Synuclein was shown to act as a high affinity inhibitor of phospholipase D2, which hydrolyzes phosphatidylcholine to phosphatidic acid and may be involved in vesicle trafficking in the secretory pathway [187, 188]. Overall, functions ascribed to α-synuclein include binding fatty acids and physiological regulation of certain enzymes, transporters, and neurotransmitter vesicles, as well as roles in neuronal survival [27]. Recently, it has been shown that α-synuclein can act as a molecular chaperone [189]. Furthermore, the role of α-synuclein in the control of the of the neuronal apoptotic response and in the protection of neurons from various apoptotic stimuli was demonstrated [190] (for more details see below, section "α-Synuclein, protein aggregation and cell death").

These diverse functions and disfunctions causing pathological conditions were discussed in numerous reviews. For example, PubMed search using a phrase "synuclein function AND review" produced 519 hits, with 120 such reviews published during last couple of years (e.g., see [48, 156, 191-200]). Intriguingly, in a case-by-case studies, α-synuclein was shown to interact with at least 50 ligands and other proteins [27], whereas a recent proteomic analysis using a SILAC technique (stable isotope labeling by amino acids in cell culture) identified 587 proteins involved in the formation of complexes with α-synuclein in the dopaminergic MES cells, with 141 proteins displaying significant changes in their relative abundance (increase or decrease) after the MES cell were treated with rotenone [201].

The list of the individually identified proteins involved in the interaction with α-synuclein includes but is not limited to

PLD2 [187], UCH-L1 [202], parkin [176], synphilin [203-205], 14-3-3 protein [141, 206], prolyl-isomerase Pin1 [207], α_B-crystallin [208], different PKC isozymes, BAD, ERK [141, 209], Rab5A [210], the ELK-1/ERK-2 complex [211], ERK-1/2, p38MAPK, and SAPK/JNK mitogen activated kinases [MAPKs [212]], Aβ [213-215], MAP1B [216], heterodimeric tubulin [217, 218], tau protein [219, 220], TBP-1 [221, 222], phospholipase D [223], protein phosphatase 2A [224], the DAT [225], the mitochondrial complex IV enzyme cytochrome oxidase [226], TH [227], aromatic amino acid decarboxylase [228], DJ-1 [229, 230], histones [231], and calmodulin [232, 233]. α-Synuclein also interacts with several polyvalent metal cations including Fe^{2+}, Al^{3+}, Zn^{2+}, Cu^{2+}, Mg^{2+} and Ca^{2+} [166, 234-244]. Furthermore, some key interactions were even attributed to the particular fragments of α-synuclein (see Fig. **3B**). It is of great interest to note that all these mapped interactions are potentially affected either by familial point mutations in α-synuclein or by one or several of its PTMs, or by both, mutations and PTMs (cf. Figs. **3A5** and **3B**). The crucial question then arose on how it is possible for one small protein to possess all those numerous functions, interactions and PTMs. The answer to this question lies in the structure of α-synuclein, and more precisely, in its natively unfolded nature.

α-Synuclein is a Natively Unfolded (or Intrinsically Disordered) Protein

α-Synuclein is an intrinsically unstructured, or natively unfolded, or intrinsically disordered protein, possessing little or no ordered structure under "physiological" conditions (i.e., conditions of neutral pH and low to moderate ionic strength) [245, 246]. The recently discovered class of intrinsically disordered proteins is gaining considerable attention from researchers due to unique capability of such proteins to perform numerous biological functions despite the lack of unique structure [247-264]. These proteins exist as dynamic and highly flexible ensembles, either at the secondary or at the tertiary structure level. In other words, in contrast to ordered proteins whose 3-D structure is relatively stable and Ramachandran angles vary slightly around their equilibrium positions with occasional cooperative conformational switches, intrinsically disordered proteins or regions exist as dynamic ensembles in which the atom positions and backbone Ramachandran angles vary significantly over time with no specific equilibrium values and typically undergo non-cooperative conformational changes. Both extended (random coil-like) regions with perhaps some secondary structure and collapsed (partially folded or molten globule-like) domains with poorly packed side chains are included in our view of intrinsic disorder [255]. Therefore, intrinsically unstructured proteins have dynamic structures that interconvert on a number of timescales and were shown to have many similarities to non-native states of "normal" globular proteins, which may exist in at least four different conformations: native (ordered), molten globule, pre-molten globule, and coil-like.

Intrinsically disordered proteins possess a number of crucial biological functions [247-261]. Furthermore, sites of posttranslational modifications (phosphorylation [265, 266], methylation [267], ubiquitination [268-270], acetylation, hydroxylation, etc.) and proteolytic attack are frequently associated with regions of intrinsic disorder [255]. This is because of the fact that similar sequence segments in different proteins can use this flexibility to conform to the active sites of the modifying and proteolytic enzymes. Recently, more than 150 proteins have been identified as containing functional disordered regions, or being completely disordered, yet performing vital cellular roles [253, 254]. Twenty-eight separate functions were assigned for these disordered regions, including molecular recognition via binding to other proteins, or to nucleic acids [253, 254, 260, 261]. An alternative view is that functional disorder fits into at least five broad classes based on their mode of action [247]. Therefore, intrinsically disordered regions are typically involved in regulation, recognition, signaling and control pathways in which interactions with multiple partners and high-specificity/low-affinity interactions are often requisite [260, 261]. In this way, the functional diversity provided by disordered regions complements those of ordered protein regions.

It seems reasonable that highly mobile proteins would provide a better basis for signaling and recognition than ordered ones. For example, disordered regions can bind partners with both high specificity and low affinity [271]. This means that the regulatory interactions can be specific and also can be easily dispersed. Obviously this represents a keystone of signaling – turning a signal off is as important as turning it on [256]. Another crucial property of intrinsically disordered proteins for their function in signaling networks is binding diversity; i.e., their ability to partner with many other proteins and other ligands, such as nucleic acids. This opens a unique possibility for one regulatory region or one regulatory protein to bind to many different partners [262, 272-275]. Furthermore, multiple disordered sequences can each adapt to fit one partner [276, 277]. These partnering abilities of disordered proteins suggest their importance and common usage in protein interaction and signaling networks and allow them to serve as hubs in these networks [260, 278, 279]. In agreement with this hypothesis it has been shown that proteins making multiple interactions are more likely to lead to lethality if deleted [280]. There are several other reasons of why intrinsically disordered proteins might be superior to their ordered counterparts. This includes, but is not limited to: binding commonality in which multiple, distinct sequences recognize a common binding site (with perhaps different folds in the various complexed intrinsically disordered proteins) [281]; the ability to form large interaction surfaces as the disordered region wraps-up [282] or surrounds its partner [283]; faster rates of association by reducing dependence on orientation factors and by enlarging target sizes [284]; and faster rates of dissociation by unzippering mechanisms [255]. As intrinsically disordered regions and proteins carry out a number of vital functions in the living cell, a new structure-function paradigm has been suggested that extends the well-known sequence-to-structure-to-function model by including disorder [248, 250, 255, 256]. These changes in the paradigm are reflected in the "Protein Trinity" hypothesis which suggests that functional proteins can exist in one of three conformational states: the solid-like ordered state (globular proteins), the liquid-like collapsed disordered state (molten globule) and the gas-like extended disordered state [256]. One more extended disordered conformation, the pre-molten globule state, was added to complete the "The Protein Quartet" model [250]. Function is

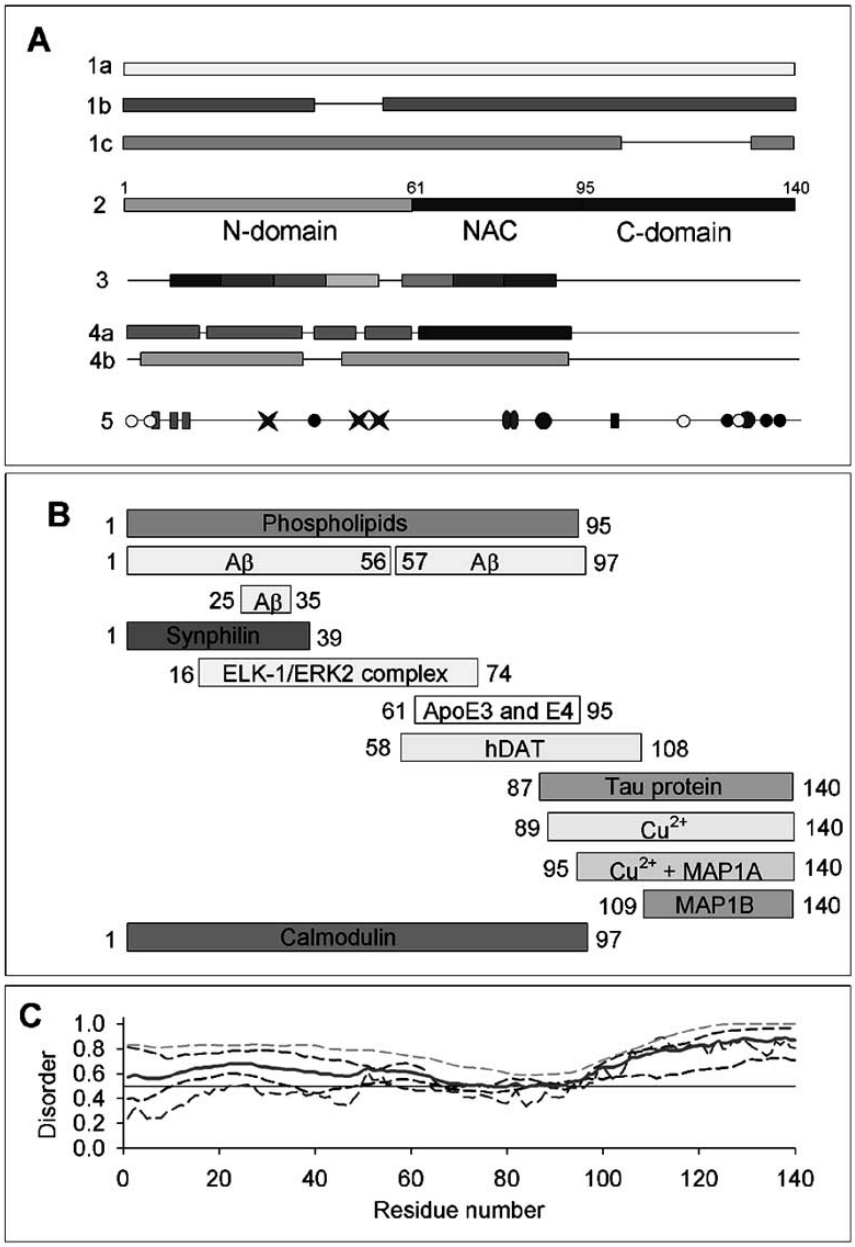


Fig. (3). Schematic representation of α-synuclein structure with the emphasis on: **A**, Peculiarities of primary structure; **B**, The putative interaction domains; **C**, Intrinsic disorder predictions.

Plot A shows three AS isoforms of α-synuclein (1a, 1b, and 1c); three formal structural domain (2); seven imperfect repeats (3); predicted (4a) and experimentally determined a-helices (4b); sites of posttranslational modifications (methionines, yellow circles; tyrosines, blue circles; phosphorylation sites (red circles); ubiquitination, green boxes; sumoylation, brown box; tTG crosslinking sites, red ovals) and PD-related mutations (three red stars) (5).

Plot B represents interaction domains responsible for binding of several ligands and proteins. The numbers on the bars correspond to the residues in α-synuclein sequence. Modified from [27].

Plot C represents results of the intrinsic disorder prediction using IUPred (pink dashed line); RONN (blue dashed line); PONDR VSL2 (red dashed line) and PONDR VL3 (cyan dashed line). The results averaged over these for predictions are shown as solid dark yellow line.

then proposed to arise from any of these three (Trinity Model) or four (Quartet Model) states, or from transitions between them.

The sequence of the typical intrinsically disordered proteins is characterized by an amino acid compositional bias and the existence of highly predictable flexibility [277]. Furthermore, the majority of the intrinsically disordered proteins, being substantially depleted in I, L, V, W, F, Y, C, and N, are enriched in E, K, R, G, Q, S, P, and A [255]. These features may account for the low hydrophobicity and high net charge of the intrinsically unstructured proteins, a property that has been pointed out for a few individual natively unfolded proteins [245, 285, 286] and that recently has been used to develop a predictor showing whether a given amino

acid sequence encodes a globular (folded) or natively unfolded protein [252, 287]. In fact, the analysis of amino acid sequences based on the normalized net charge and mean hydrophobicity performed for sets of 275 native and 91 natively unfolded proteins has established that a combination of low overall hydrophobicity and large net charge is a specific feature of intrinsically unstructured proteins. Moreover, these proteins have been shown to be specifically localized within a particular region of charge-hydrophobicity phase space, satisfying the following relationship [252, 287]:

$$< H > \leq < H >_b = \frac{< R > +1.151}{2.785},$$

where <*H*> and <*R*> are the mean hydrophobicity and the mean net charge of the given protein, respectively, whereas $<H>_b$ is the "boundary" mean hydrophobicity value, below which a polypeptide chain with a given <*R*> will be most probably unfolded. The mean hydrophobicity, <*H*>, is defined as the sum of the normalized hydrophobicities of all residues [calculated according to [288]] divided by the number of residues in the polypeptide.

Interestingly, it has been noted that α-synuclein does not fit the general trend and is located within "native" area of the charge-hydrophobicity phase space [252]. Detailed analysis of this protein amino acid sequence has established that its N- and C- terminal regions are very distinct in overall hydrophobicity (see Fig. **4**), and possess charges of opposite sign. In particular, Fig. (**4**) shows that the calculated hydrophobicity for the N-terminal fragment (dashed line) lies well above the boundary value (gray line), whereas the C-terminal hydrophobicity is located far below the boundary value. Thus, the C-terminal fragment (the last 45 residues) of human α-synuclein has parameters typical of natively unstructured proteins, whereas the parameters of the N-terminal 95 residues are typical of native folded globular proteins. It has been suggested that the disordered regions of these molecules prevent the remainder of the protein from normal folding, perhaps through extensive electrostatic attractions [252].

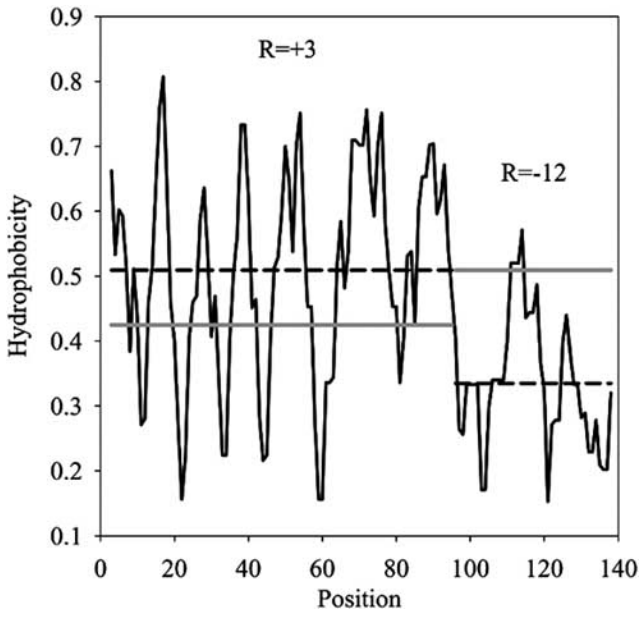


Fig. (4). Hydrophobicity distribution within the α-synuclein sequence. Gray lines represent boundary hydrophobicities for two parts of the protein (1-95 and 96-100), calculated using equation described in the text. Dashed lines represent mean hydrophobicities, calculated directly from the sequences according to [288].

More detailed analysis of the differences in amino acid compositions between ordered and intrinsically disordered proteins constituted a ground for the development of numerous algorithms aiming for the prediction of disordered proteins/regions [reviewed in [289, 290]]. Fig. (**3C**) represents the results of disorder prediction on human α-synuclein sequence using several of these predictors, PONDR® VL3 [291, 292], VSL2 [293], RONN [294] and IUPred [295]. It can be seen that α-synuclein is predicted to be almost completely disordered by all these predictors (as disorder probability scores ≥0.5 correspond to a prediction of disorder), emphasizing that its sequence is typical of the intrinsically disordered proteins.

In agreement with these predictions, α-synuclein was shown to possess little ordered structure under physiological conditions [245, 246, 249, 296]. For example, at neutral pH it is characterized by far-UV CD and FTIR spectra typical of a substantially unfolded polypeptide chain with a low content of ordered secondary structure [246]. The hydrodynamic properties of α-synuclein are in a good agreement with the results of far-UV CD and FTIR studies. In fact, it has been established that α-synuclein, being essentially expanded, does not have a tightly packed globular structure, but is slightly more compact than expected for a random coil [246, 249, 297]. It has been shown that α-synuclein sedimented more slowly than globular proteins of similar molecular weight, indicating that this protein is not compact [245]. Furthermore, based on the results of pulsed-field gradient NMR (which allows an estimation of the hydrodynamic radii), it has been concluded that α-synuclein is slightly collapsed [298]. Thus, at neutral pH, α-synuclein was shown to be essentially disordered, but slightly more compact than a random coil. In agreement with this conclusion, a high resolution NMR analysis of the protein under these conditions revealed that α-synuclein is largely unfolded in a solution, but exhibits a region between residues 6 and 37 with a preference for helical conformation [296]. Interestingly, the results of recent studies on Raman optical activity spectra were consistent with the conclusion that α-synuclein may contain some helical poly-(L-proline) II-like conformation [299].

Structural Effects of Amino Acid Substitutions

Although a genetic link between PD and α-synuclein suggests that α-synuclein might play an important role in the PD pathogenesis, the vast majority of PD cases occur in individuals carrying the wild type α-synuclein gene. It seems likely, then, that the familial early onset mutations might emphasize or enhance some property that is already inherent in wild type properties. Thus, it has been anticipated that the A30P, E46K, and A53T mutations would change the structural properties of α-synuclein, affecting their predisposition to aggregate. To check this assumption, the structural properties of WT, A30P, E49K, and A53T α-synucleins have been compared using a set of low resolution techniques, such as

circular dichroism, infrared spectroscopy, fluorescence, and several hydrodynamic approaches [300-309]. Interestingly, detailed analysis revealed that the PD-related point mutations do not affect the overall structure of human α-synuclein, which remains natively unfolded [300, 308, 309]. However, when high resolution solution NMR spectroscopy was used to analyze the residual structure in human α-synuclein and its A30P and P53T mutants, it has been shown that the A30P mutation strongly attenuates the helical propensity found in the N-terminal region of the wild type α-synuclein, whereas the A53T mutation leaves this region unperturbed and exerts a more modest and local influence on structural propensity, resulting in a slight enhancement of preference for extended conformations in a small region around the site of mutation [310]. This was an important finding which provided a basis for the better understanding of the molecular mechanism of α-synuclein aggregation and the effects of PD-related mutations. In fact, the discovered residual helical structure does not represent an exclusive property of natively unfolded α-synuclein, as it is observed in unfolded states of several other proteins. Such residual structure has been proposed to play a potential role in early intramolecular protein folding events. Based on these observations it has been concluded that this region of transient helical structure could play a role in α-synuclein aggregation, which is essentially an intermolecular folding process [310]. Obviously, the formation of a transient helical structure does not provide a clear path to the final product of the intermolecular folding/aggregation process, which is a β-sheet-rich structure [300, 308]. On the other hand, the observation that the A30P mutation eliminates this transient helicity, combined with the fact that this mutation accelerates early events in the oligomerization process, suggests that the residual helical structure may in fact retard or interfere with the initial intermolecular interactions of α-synuclein [310].

As it has been already mentioned, human β- and γ-synucleins, being 78% and 60% identical to α-synuclein, preserve some characteristic features of α-synuclein, while missing others. Therefore, they serve as good models for the analysis focused on the clarification of structural outputs of sequence variability on the structure and behavior of a natively unfolded protein. Structural properties of the members of synuclein family have been compared using several physico-chemical methods [297]. It has been established that all three proteins showed far-UV CD spectra typical of an unfolded polypeptide chain. Interestingly, α- and γ-synucleins possessed almost indistinguishable spectra, whereas the far UV-CD spectrum of β-synuclein showed a slightly increased degree of disorder. The increased unfoldedness of β-synuclein was further confirmed by hydrodynamic studies performed by size-exclusion chromatography and SAXS. In fact, size-exclusion chromatographic analysis showed that β-synuclein was slightly more extended than α- and γ-synucleins: the R_S of β-synuclein was typical of a completely unfolded polypeptide chain, while α- and γ-synucleins were more compact than expected for a random coil [297]. This emphasized the importance of the NAC region to maintain the residual partially collapsed structure in α- and γ-synucleins. SAXS analysis further confirmed this conclusion. Guinier analysis of the scattering data shows that the synucleins are characterized by rather different R_g values at neutral pH. The observed R_g value for α-synuclein at neutral pH (40±1 Å) is smaller than that estimated for a random coil conformation for a protein of this size (52 Å), indicating that the natively unfolded conformation of this protein is more compact than a random coil. On the other hand, the observed R_g value for β-synuclein (49±1 Å) matches that expected for a completely unfolded polypeptide chain of this length (51 Å), which indicates the random coil conformation for this protein. γ-Synuclein had a very large R_g (61±1 Å) under the conditions studied. This may be due to the very significant asymmetry of this protein or because of its self-association. Analysis of the SAXS forward-scattering intensity values, *I(0)* (which is proportional to the molecular weight of the molecule), confirmed that the large R_g is due to association. In fact, the *I(0)* value for γ-synuclein is more than twice that of for α- and β-synucleins [297]. Finally, the Kratky plots showed that the α- and β-synucleins do not have a well-developed globular structure, whereas γ-synuclein showed a characteristic maximum at low angles, indicating the presence of some globular structure in the oligomer [297].

α-Synuclein is a Protein-Chameleon

Important problem related to the conformational behavior of natively unfolded proteins is the understanding of the forces or factors causing their folding. Since these proteins possess low overall hydrophobicity and high net charge, it has been suggested that any changes in their environment leading to an increase in hydrophobicity and/or decrease in net charge should be accompanied by at least a partial folding. The excess net charge of these proteins at neutral pH would be neutralized at lower pH values, whereas the increase in temperature would be accompanied by the increase in strength of the hydrophobic interaction, leading to a stronger hydrophobic driving force for folding. Thus, a partial folding of α-synuclein under conditions of high temperature and/or low pH was expected [246]. In agreement with this suggestion it has been shown that α-synuclein, which was natively unfolded at neutral pH and low temperatures adopts a partially folded (pre-molten globule-like) conformation at acidic pH or at high temperatures [47, 246, 249]. This illustrates "turned out" response to the changes in the environment typical for the natively unstructured proteins, which, unlike "normal" globular proteins, gain rather than lose ordered structure at extreme pH and high temperatures [249]. The structure-forming effects of low pH were attributed to the minimization of the large net negative charge present at neutral pH, thereby decreasing intramolecular charge-charge repulsion and permitting hydrophobic-driven collapse to the partially folded conformation.

Interestingly, it has been shown that the PD-related mutations A30P and P53T do not affect the conformational behavior of α-synuclein and structural transitions induced in these three proteins by a decrease in pH or an increase in temperature or methanol concentration were shown to be indistinguishable [300, 308]. Likewise, wild type α-synuclein, and the A30P and A53T mutants may be transformed into the partially folded intermediate state by decreasing the pH or increasing the temperature [300, 308]. Importantly, the structure of this intermediate state was shown to be independent of the mutations. Thus, the mono-

meric forms of WT, A30P and A53T α-synucleins exhibit identical structural properties and conformational behavior [300, 308].

Similarly, the analysis of conformational behavior of different members of the synuclein family revealed that they possess a comparable response to the changes in their environment. In fact, although far-UV CD spectra of α-, β-, and γ-synucleins were slightly different at neutral pH, all three proteins possessed almost identical far-UV CD spectra at acidic pH suggesting that they adopt a partially folded intermediate with comparable degree of folding [297]. This hypothesis was further confirmed by the results of gel-filtration analysis, which showed that although β-synuclein was slightly more extended than α- and γ-synucleins at neutral pH, all three proteins possessed the same degree of compaction in acidic solutions [297].

An important characteristic of the α-synuclein primary structure, which is likely related to its functional activity, is seven imperfect repeats within the first 95 residues (Fig. **3A3**), resulting in a variation in hydrophobicity [18, 130, 140] with a strictly conserved periodicity of 11 [140]. Such a periodicity is characteristic of the amphipathic lipid-binding α-helical domains of apolipoproteins [130, 140], which have been extensively studied and assigned to subclasses according to their unique structural and functional properties [311, 312]. These seven imperfect 11-residue repeat sequences were predicted to form five amphipathic helices on the amino-terminal half of human α-synuclein (see Fig. **3A4a**) [183, 313, 314], with helices 1–4 predicted to associate with lipid vesicles [315, 316], whereas helix 5 being likely responsible for protein–protein interactions [183]. It has been pointed out that α-synuclein shares the defining properties of the class A2 lipid-binding helix, distinguished by clustered basic residues at the polar-apolar interface, positioned ±100° from the center of apolar face; predominance of lysines relative to arginines among these basic residues; and several glutamate residues at the polar surface [311, 312, 316]. In agreement with these structural features, α-synuclein was shown to bind specifically to synthetic vesicles containing acidic phospholipids [183, 316]. This binding was shown to be accompanied by a dramatic increase in α-helix content [183, 316] and was attributed to the formation of two curved a-helices (Val3-Val37 and Lys45-Thr92) (see Fig. **3A4b**) connected by a well ordered, extended linker [317], whereas the acidic, glutamate-rich C-terminal region (Asp98-Ala140) was shown to behave as a highly mobile tail; i.e., it remained unstructured even in the presence of membranes (see Fig. **5**) [314, 317]. Recently it has been established that C-terminal tail of the protein can gain protease-insensitive conformation when the micelle bound α-synuclein is exposed to calcium [241].

Conformational behavior of α-synuclein under a variety of environments has been extensively analyzed. This analysis has revealed that the structure of α-synuclein is extremely sensitive to the environment and can be easily modified. Intriguingly, the extended natively unfolded conformation was shown to be effectively stabilized via methionine oxidation [167, 169, 170]. It has been shown that under mild oxidative conditions (1-2% H_2O_2) all four methionines of α-synuclein, Met1, Met5, Met116, and Met127, located outside the repeat-containing region, are successfully oxidized to methionine sulfoxides [169]. The oxidized form of α-synuclein was shown to be more unfolded than non-oxidized protein as manifested by the larger contribution of unordered structure to both FTIR and far-UV CD spectra [169], and by detect-

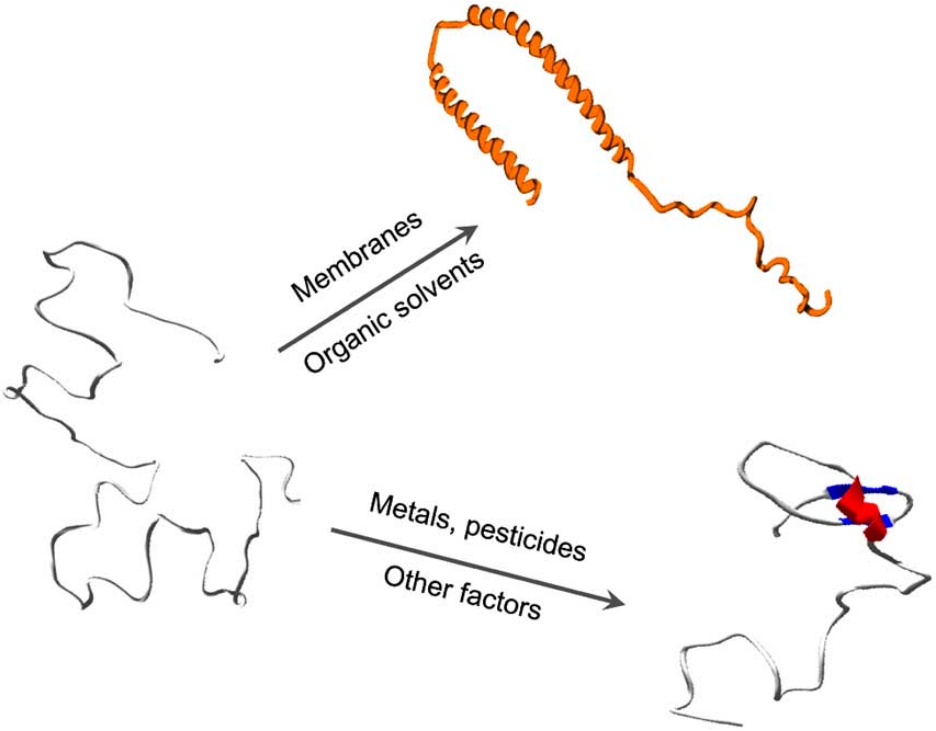


Fig. (5). A model illustrating structural changes induced in the natively unfolded α-synuclein by membrane or high concentration of organic solvents (highly α-helical conformation, 1XQ8) and by metals, pesticides or other factors stabilizing partially folded pre-molten globule-like conformation.

able decrease in the α-synuclein-MetO compactness [170]. This was attributed to the decreased hydrophobicity of oxidized methionine leading to a decrease in the overall hydrophobicity of the protein. Given the decrease in hydrophobicity, it was not a big surprise that the oxidized protein was less prone to oligomerize and aggregate, being substantially non-amyloidogenic, and even able to inhibit the fibrillation of non-modified α-synuclein [169].

It has been shown that this protein adopts a pre-molten globule-like partially folded conformation at various conditions including low pH [246], high temperature [246], the presence of low concentrations of organic solvents [318] and TMAO [319], the presence of different metal ions [238], various salts [320], several common pesticides/herbicides [321-323], heparin and other glycosoaminoglycans [324], some polycations [325], or as a result of a spontaneous oligomerization both *in vitro* and *in vivo* [326] (see Fig. **5**). Furthermore, the addition of different alcohols was shown to increase the content of ordered secondary structure in α-synuclein [318]. Interestingly, the structural transformations induced by high solvent concentrations were dependent on the type of alcohol, with simple alcohols inducing a β-sheet-enriched conformation whereas fluorinated alcohols promoting α-helix-rich species [318]. Interestingly, both α-helical and β-structural species were shown to be initially monomeric, but underwent association over longer times, and β-rich rich conformations were strongly prone to form amorphous aggregates [318]. Oligomeric α-helical globular species potentially possessing rigid tertiary structure were induced in α-synuclein by high concentrations of TMAO [319].

Besides these monomeric conformations, α-synuclein is able to form morphologically different oligomers and aggregates. For example, the prolonged incubation of this protein at different temperatures resulted in a temperature-dependent, progressive aggregation, with dimers being formed first [326]. This temperature-modulated oligomerization was shown to be accompanied by small but reproducible increase in the ordered secondary structure content. Interestingly, the trapped oligomeric conformation was structurally similar to the pre-molten globule-like partially folded monomeric confomer induced by low pH or high temperature [326]. Therefore, it has been concluded that the partially folded pre-molten globule-like conformation of α-synuclein can be stabilized as the protein undergoes a highly selective self-assembly process during prolonged incubation at elevated temperatures [326]. The formation of oxidative dimers and higher-order oligomers with dityrosine cross-links in α-synuclein under the conditions of oxidative stress was also reported [166].

In addition to covalent and non-covalent dimers, α-synuclein was shown to form a series of morphologically different soluble oligomers. There are four tyrosines, Tyr39, Tyr125, Tyr132, and Tyr135, in α-synuclein, which were shown to be easily nitrated under the appropriate conditions *in vitro* [164]. It has been established that nitrated α-synuclein remains assembled into the oligomeric spheroids even after incubation for a very prolonged time [164]. The formation of several oligomeric "protofibrilar" species with different morphologies were detected by atomic force microscopy at early fibrillation stages of α-synuclein [301, 306, 327-329]. The first-formed α-synuclein protofibrils appeared to be predominantly spherical with heights varying between 2.5 and 4.2 nm [307, 329]. Under the appropriate conditions these spherical oligomers were shown to convert into the annular structures (doughnuts) [329]. In addition to the completed rings, doughnuts, the existence of partially formed rings (crescents) has been observed [329]. The formation of both doughnuts and fibrils was shown to require initial formation of spherical, β-structure enriched, α-synuclein oligomers. However, the subsequent assembly processes seem to require different conditions. Importantly, the doughnuts were not observed once spherical oligomers have disappeared and α-synuclein was converted to fibrils [329]. Based on these observations it has been suggested that the doughnuts are not on the direct monomer-to-fibril pathway, but must "reopen" to be converted to fibrils [329]. Interestingly, the incubation of the spherical α-synuclein oligomers with brain-derived membranes was shown to produce pore-like annular protofibrils too [329]. It has been also reported that incubation of a-synuclein with different metals for one day at 4^o gave rise to three different classes of oligomers, where Cu^{2+}, Fe^{3+} and Ni^{2+} yielded 0.8-4 nm spherical particles, similar to α-synuclein incubated without metal ions, Mg^{2+}, Cd^{2+} and Zn^{2+} gave larger, 5-8 nm spherical oligomers, whereas Co^{2+} and Cd^{2+} frequent annular (doughnut-like) oligomers, 70-90 nm in diameter with Ca^{2+} and 22-30 nm in diameter with Co^{2+} [244].

Finally, α-synuclein was shown to assemble into large insoluble aggregates of two distinctive morphologies – amorphous aggregates and fibrils. The appearance of the particular type of the insoluble aggregate is determined by the environmental conditions. For example, α-synuclein precipitated from solutions containing high concentrations of simple alcohols predominantly in a form of amorphous aggregates. In many other cases, the major insoluble species were amyloid-like fibrils. In a few cases, the successful partitioning between these two pathways has been observed and α-synuclein was present in both fibrillar and amorphous forms simultaneously.

Data overviewed above indicate that α-synuclein possesses a remarkable conformational plasticity, being able to adopt structurally unrelated conformations including the substantially unfolded "basic" state, an amyloidogenic partially folded conformation, and different α-helical or β-structural species folded to a different degree, both monomeric and oligomeric [249, 297]. Furthermore, it might form several morphologically different types of aggregates, including oligomers (spheres or doughnuts), amorphous aggregates, and or amyloid-like fibrils [249, 297]. Based on this astonishing conformational behavior the concept of a protein-chameleon was proposed, according to which the structure of α-synuclein to a dramatic degree depends on the environment: the choice between all the mentioned above conformations is determined by the peculiarities of protein surroundings [249].

Fig. (**6**) provides a physical explanation for this phenomenal chameleon behavior of α-synuclein. Here the hypothetical folding-energy landscape of a typical globular protein (Fig. **6A**) is compared with that of a natively unfolded

protein (Fig. **6B**). Note both landscapes are depicted schematically in one-dimensional cross-section. Fig. (**6A**) shows that the folding landscape of a globular protein is characterized by a deep energy minimum, thus resembling a funnel [330-332]. It has been proposed that this folding landscape profile determines the ability of a globular protein to fold into a unique conformation, its native state, as a protein sequence possessing fast folding must satisfy two essential requirements: (1) thermodynamic stability meaning the existence of a deep global minimum in the energy landscape and (2) kinetic accessibility meaning the existence of a basin of attraction sloping toward that minimum [330]. Contrarily to a globular protein, the 'topology' of the landscape of a natively unfolded α-synuclein is characterized by numerous local energy minima, due to which this protein tend to behave as a highly frustrated system without any stable well-folded conformation (Fig. **6B**). This folding landscape profile determines the conformational plasticity of α-synuclein and also provides some clues on how this protein can specifically interact with so many ligands of so different nature (membrane, lipids, other proteins, metal ions, small organic molecules, etc.). If the interaction with a particular binding partner affects the α-synuclein folding landscape making some energy minima deeper and some energy barriers higher (see Figs. **6C1-3**), then this protein would fold on a template-dependent manner gaining a specific structure needed to form a given complex. This model also provides an answer to a question on how is it possible for one small protein to possess numerous functions, interactions and PTMs.

On the other hand, this conformational plasticity, strong dependence on environment and mentioned breadth of interactions, structural adjustments, and functions ascribed to α-synuclein make this protein highly vulnerable and potentially prone to misfold. In fact, mutations and/or changes in the environment may reduce the capability of α-synuclein to recognize proper binding partners thus leading to the formation of nonfunctional and deadly aggregates. Therefore, the development of different synucleinopathies may originate from the misregulation, missignaling, and misidentification of α-synuclein, accompanied by or resulted from its misfolding. The following section of review is dedicated to the consideration of the molecular mechanisms of α-synuclein aggregation and to the description of factors promoting or inhibiting the formation of these aggregates.

MOLECULAR MECHANISMS OF α-SYNUCLEIN AGGREGATION

Partial Folding is Crucial for Amyloid-Like Fibril Formation

Although α-synuclein is an intrinsically unstructured protein, it forms fibrils of highly organized secondary structure. For example, the FTIR spectrum of α-synuclein fibrils shows a major contribution from β-sheet [246, 297]. Furthermore, the X-ray diffraction analysis of α-synuclein fibrils showed the characteristic pattern of a cross β-sheet structure, in which the β-strands lie perpendicular to the long fiber axis, typical of all amyloid fibrils [333]. Thus, α-synuclein is folded within fibril.

It has been shown that early stages of fibril formation involve partial folding of α-synuclein [246]. In fact, decreasing the pH or increasing the temperature resulted in an acceleration of the α-synuclein fibrillation rates, which coincides with the pH- and temperature-driven structural transformations described above [246]. Therefore it has been concluded that the process of α-synuclein fibrillation is accelerated by the partial folding, suggesting that the pre-molten globule-like partially structured conformation is a key intermediate on the fibril-forming pathway [246]. Thus, factors that shift the equilibrium in favor of the monomeric partially folded conformation will facilitate fibril formation. In other words, the fibrillation of α-synuclein, leading to the LB formation and the development of PD and other synucleinopathies, may arise from various constituents that significantly populate the aggregation-competent species [246]. Possibilities include point mutations, non-polar molecules, such as some pesticides, that might preferentially bind to the partially-folded intermediate, and cations, that might mimic the effect of low pH (high proton concentration), as well as factors that result in an increase in the concentration of α-synuclein itself or causing modification of the protein (e.g., via oxidative damage, etc.). As it was shown above, a number of environmental factors are able to induce partial folding of α-synuclein. Thus, it was not a surprise to find that all these factors indeed resulted in the accelerated α-synuclein fibrillation (reviewed in [200, 249]).

Effect of High α-Synuclein Concentrations

As it has been pointed out that even in the absence of any endogenous factors, there is equilibrium between the natively unfolded and the partially folded conformations in α-synuclein. Obviously, high protein concentration might be predicted to increase the rate of fibrillation due to the increased total concentration of the partially folded amyloidogenic intermediate. In agreement with this assumption, it has been shown that an increase in α-synuclein concentration resulted in a decrease in the lag time and an increase in the rate constant for fibril growth [238]. Furthermore, it has been established that there is an inverse linear correlation between the logarithm of α-synuclein concentration and the duration of lag time [238]. This reflects that the formation of the aggregation-prone partially-folded conformation represents one of the rate-limiting steps in α-synuclein nucleation, and this process might be the first-order one. This would mean that the key partially folded conformation, once formed, oligomerizes very rapidly to form insoluble fibrils [238]. Similarly, a linear dependence of the first-order rate constant for fibril growth (elongation) on the protein concentration was observed [238]. The kinetics of elongation has been shown to follow first-order kinetics for several other proteins, including amyloid β-protein and insulin. The simplest explanation for this fact is that increasing protein concentration leads to increasing numbers of fibrils, due to increasing concentration of nuclei. Strong dependence of the fibrillation kinetics on α-synuclein concentration is potentially very important for the pathogenesis of PD and other synucleinopathies. In fact, it has been recently shown that a large family develops autosomal dominant PD (average age of onset, 34 years), ranging clinically from DLB to typical PD, due to the triplication of α-synuclein locus, thus, due to potentially enhanced level of α-synuclein production [42-44].

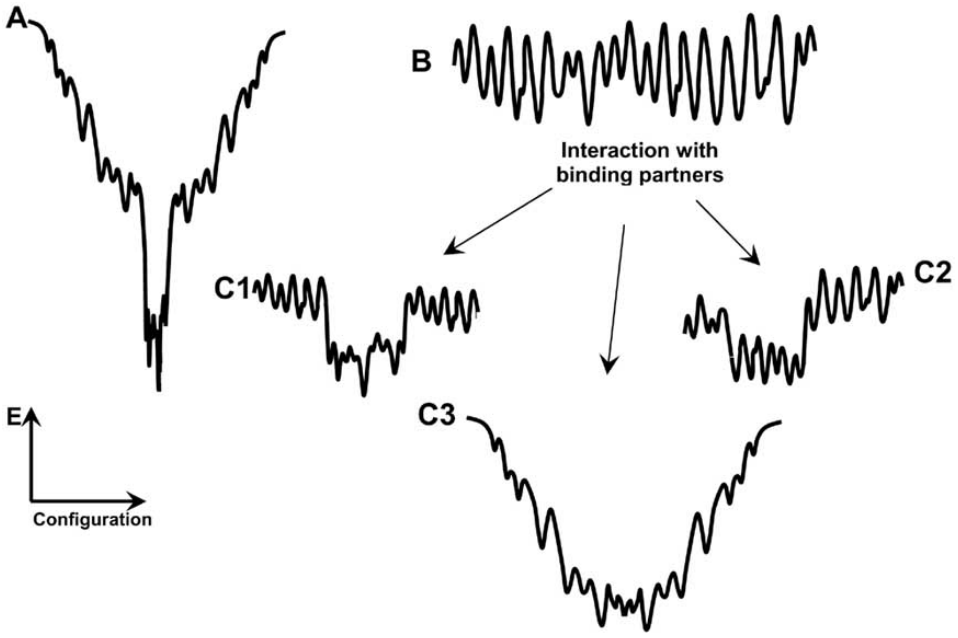


Fig. (6). A diagram showing the folding energy landscapes of a typical globular protein (**A**) [331] and of a typical natively unfolded protein in the absence (**B**) or presence of different binding partners (**C**). These landscapes are depicted schematically in one-dimensional cross-section.

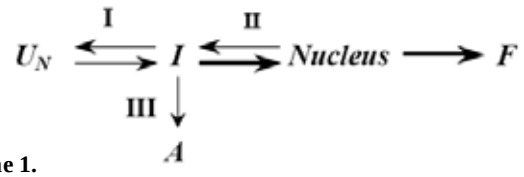
Effect of Molecular Crowding

The natural environment of any protein inside a living cell is highly crowded. The concentration of macromolecules, including proteins, nucleic acids, carbohydrates, and small solutes within a living cell can be as high as 400 g/L [334], with the intracellular solutes taking up about a half of the total cellular volume [334-337]. Obviously, the volume occupied by solutes is unavailable to other molecules, a phenomenon known as "excluded volume effects" [334, 338], which may have large effects on the stability of biological macromolecules [339-342] and on macromolecular equilibria, such as protein-protein interactions [298, 343]. It has been also suggested that volume exclusion in physiological media could modulate the rate and extent of amyloid formation *in vivo* [343]. The validity of this hypothesis has been confirmed recently for the *in vitro* fibrillation of human α-synuclein [344, 345]. High concentrations of various types of biopolymers, from neutral polyethylene glycols and polysaccharides (Ficolls, dextrans) to inert proteins (lysozyme, BSA), were shown to accelerate α-synuclein fibrillation *in vitro*. The stimulation of fibrillation increases with increasing length of polymer, as well as with increasing polymer concentration. Pesticides and metals, which are linked to increased risk of PD by epidemiological studies (see below), were shown to further accelerate α-synuclein fibrillation under conditions of molecular crowding [346].

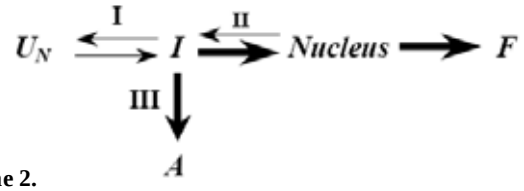
Influence of PD-Related Point Mutations

The role of familial mutations on α-synuclein aggregation deserves special discussion. This is because of the fact that the finding of several kindreds with the autosomal-dominant pattern of PD inheritance shows persuasively that a single mutation in the human α-synuclein gene is sufficient to account for the PD phenotype. Importantly, all three PD-related point mutations, A30P, E49K, and A53T, were shown to accelerate the α-synuclein aggregation (but not necessarily fibrillation) *in vitro* [300, 308]. Interestingly, detailed conformational analysis revealed that wild type and mutant α-synucleins possess similar structural properties [300-309] suggesting that the PD-related point mutations do not affect the overall structure of human α-synuclein, which remains natively unfolded [300, 308, 309]. Furthermore, similar to the wild type α-synuclein, A30P and A53T mutants were shown to adopt a partially folded conformation at low pH or high temperatures, structure of which was shown to be independent of the mutations [300, 308]. On the other hand, both mutant proteins were shown to be more prone to aggregate than wild type protein and the total aggregation (fibrillar and non-fibrillar) rates decreased in the following order A53T > A30P > WT [300, 308]. The absence of detectable structural difference between partially folded conformations of α-synucleins raises the question of how the A30P and A53T mutations affect the aggregation propensity of the protein. To explain this contradiction, the amino acid sequence of WT, A30P and A53T have been analyzed on the basis of hydrophobicity and propensities to form β-sheet or α-helix [308]. Surprisingly, both mutations have been shown to reduce hydrophobicity in the vicinity of the substitution. This was rather unexpected, because the hydrophobic interactions are assumed to be important in aggregation. At the same time, the propensity to form α-helical structure was shown to be somewhat diminished in the N-terminal region of both mutants, whereas the predisposition to form β-structure was predicted to be slightly enhanced [308]. As it has been already pointed out, high-resolution solution NMR analysis revealed that A30P mutation disrupts a region of residual helical structure that exists in the wild type protein [296], whereas the A53T mutation results in a slight enhancement of a preference for extended conformation in a

small region around the mutation site [310]. Taking these observations into account, it has been hypothesize that the increased internal susceptibility of A30P and A53T to form β-sheets may not be strong enough to alter the structure of the monomeric proteins, but may affect the aggregation behavior of the α-synuclein mutants through specific stabilization of an intermolecular β-structure [308]. This model for the effect of the familial PD mutations was illustrated by the comparison of the aggregation models for the wild type (Scheme **1**) and the mutants (Scheme **2**) [308]:



Scheme 1.



Scheme 2.

In this model ***F*** and ***A*** represent fibrils and amorphous aggregates respectively, $\boldsymbol{U_N}$ is the natively unfolded state and ***I*** represents the partially folded intermediate. The Roman numerals indicate the major stages of the aggregation process. It has been proposed that the structural properties of $\boldsymbol{U_N}$ and ***I,*** as well as the rate of their interconversion (stage I), are unaffected by the A30P and A53T mutations. However, the rates of stages II and III are facilitated by the familial Parkinson's disease point mutations (thicker arrows). This would result from the mutation-enhanced probability of forming intermolecular β-structure [308]. As a result, the mutants show a faster rate of fibrillation (A53T) or amorphous aggregation (A30P).

Interestingly, the sequence of mouse α-synuclein differs from the human sequence only at seven positions, with one of these differences being at position 53, where the mouse protein contains a threonine residue [130, 347]. Therefore, the A53T mutation in human protein is in fact a "reversion" to a residue found in a lower organism. A detailed characterization of murine protein revealed that similar to human protein, mouse α-synuclein adopts a natively unfolded conformation. At elevated concentrations mouse protein fibrillated faster than the three human synucleins. The fibrillation of mouse protein was inhibited by WT and A53T α-synuclein, leading to an accumulation of nonfibrillar oligomers [347].

Recently, the effect of E46K mutation on aggregation of α-synuclein has been analyzed [309]. It has been established that the E46K mutation is also able to increase the propensity of α-synuclein to fibrillate, but this effect was less pronounced than that of the A53T mutation [309]. Interestingly, the E46K mutation is located in the fourth KTKEGV-type repeat in the amino-terminal region of α-synuclein. It has been emphasized that a Glu residue similar to E46 is present in five of the seven degenerative repeats in α-synuclein, and the only repeat that does not have such a residue (repeat 2) has Glu residues adjacent to each side of the repeat [309]. Based on these observations it has been suggested that the N-terminal region of α-synuclein and, more specifically, Glu residues in the repeats may be important in regulating the ability of α-synuclein to polymerize into amyloid fibrils [309].

In vitro findings of the effects of the A53T and E46K mutations on α-synuclein polymerization were shown to be consistent with pathogenesis in human subjects. In fact, patients with the A53T mutation have an earlier age of disease onset (average age of onset, 45 years) than those carrying the E46K mutation (average age of onset, 60 years) [309]. These observations, together with the described above results showing that the A53T, E46K, and A30P mutations in α-synuclein lead to increased propensity to aggregate, compared with the wild-type protein, provide strong support for a direct and critical role of α-synuclein aggregation in the PD etiology.

Alterations Induced by Sequence Truncations

It has been shown that in addition to the full-length protein, LBs and LNs accumulated in sporadic PD and LBD [50], as well as GCIs of MSA [348] usually contain C-terminally truncated α-synuclein. The *in vitro* analysis revealed that the truncated α-synuclein constructs are more prone to fibrillate than the full-length protein [333, 349-351]. This suggests that the C-terminus may play a role of intramolecular chaperone preventing α-synuclein from fibrillation. Therefore, proteolytic degradation of α-synuclein may be an important factor in the assembly of α-synuclein in the various neurodegenerative diseases [349]. Furthermore, it now becomes evident that the acidic C-terminal domain of α-synuclein has several biological functions, alterations of which (induced by the proteolytic truncation) might promote development of several neurodegenerative disorders (see above). For example, it has been recently shown that human dopaminergic neuroblastoma SH-SY5Y cells expressing C-terminally truncated α-synuclein, particularly the 1–120 residue protein, were significantly more vulnerable to oxidative stress than the control cells [352]. Furthermore, the Ca^{2+}-binding site with an IC_{50} of about 2-300 μM is located within the C-terminal domain [351]. Importantly, Ca^{2+} binding affected both the aggregation and functionality of α-synuclein, modulating the efficiency of its binding to microtubule-associated protein 1A and promoting oligomer formation [351].

Furthermore, the NAC peptide was implicated in the AD pathogenesis, accumulating in the amyloid plaques isolated from the AD brains [18, 19]. *In vitro* analysis of NAC aggregation behavior revealed that this peptide readily forms amyloid-like fibrils, which was accompanied by a transition from totally random structure to predominantly β-sheet [19, 353-355]. Importantly, NAC was shown to be responsible for the α-synuclein interaction with Aβ peptides [214], which dramatically accelerated the aggregation of Aβ *in vitro* [213]. Aggregates of NAC-related peptides are toxic to cell [354, 356, 357]. All this suggests that the accumulation of NAC and C-terminally truncated α-synuclein aggregates

in the synapse may be responsible for the neurodegeneration observed in AD, PD, and other synucleinopathies. Alternatively, neurodegeneration may be caused by the loss of α-synuclein function induced by sequence truncation [19].

Aggregation Promoting Effects of Anions and Salts

Anions were shown to induce partial folding of α-synuclein at neutral pH, forcing the formation of an amyloidogenic partially folded intermediate. However, the magnitude of the fibrillation accelerating effect varied significantly between anions and generally followed the position of the anions in the Hofmeister series, indicating that the major role of anions in fibrillation is their modulation of protein-water interaction [320]. Based on these observations it has been concluded that fibrillation should be considered as a particular case of the more general phenomenon of protein salting-out. Nevertheless, the degree to which anions interact with water, stabilizing the hydrophobic interactions within the α-synuclein molecule or between protein molecules, is not the only factor determining the stability and solubility of this protein, and electrostatic effects have to be taken into account as well [320]. Therefore, the enhanced fibrillation of α-synuclein in the presence of anions is the result of the loss of the uncompensated charge, which is a factor promoting the soluble unfolded conformation, and an increase in the preferential hydration, which promotes partial folding and aggregation by strengthening hydrophobic interactions. Both key steps of fibrillation, nucleation and fibril growth, are affected by a combination of these two effects. The addition of the first small quantities of salts eliminates the strong repulsion due to the net electrical charges, giving rise to partial folding of α-synuclein, whereas the continued addition of the salt brings about dehydration that results ultimately in fibrillation of the protein [320].

α-Synuclein aggregation *in vitro* as a tool for understanding the role of environmental factors in the pathogenesis of synucleinopathies. Although aggregation of α-synuclein was implicated in a wide range of synucleinpathies (see Table **1**), the role of this process in the pathogenesis of PD continues to attract the major attention of researchers. The exact cause of PD is unknown as of yet, but considerable evidence suggests a multifactorial etiology of disease involving genetic and environmental factors. The potential role of the α-synuclein mutations and their aggregation in the development of autosomal inherited forms of PD was already discussed (see above). Importantly, PD is now considered likely to be an "environmental" disease, as several lines of evidence point to environmental exposures as potential contributing factors in the pathogenesis of this disorder [358-364], and a positive correlation between the prevalence of PD and industrialization has been recognized [358].

It has been shown that certain neurotoxins could promote and accelerate the development of PD, whereas other environmental agents (such as caffeine and nicotine may decrease the risk of PD [365-367]) may be neuroprotective. Among the PD-promoting environmental toxins are: metals [358, 368-373]; solvents [374-378]; carbon monoxide [379]; 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP) [380]; and some pesticides and herbicides [381-386]. The effect of several environmental factors as well as the influence of some cellular factors on structure and aggregation of human α-synuclein is considered below. This analysis provides basis for the understanding of the molecular mechanisms of PD pathogenesis, as it is very possible that α-synuclein plays a key role in the environmental hypothesis of PD neurodegeneration, with direct interaction between this protein and neurotoxicants directly contributing to the PD development [363].

Exposure to pesticides and herbicides. About a billion pounds of pesticides are used annually in the US. Both epidemiological and clinical observations reveal pesticides and herbicides as an important environmental PD risk factor [381-386]. Among various pesticides and herbicides, paraquat is the most often implicated as a potential neurotoxicant in PD [364, 381-387]. Multiple lines of evidence support a relationship between α-synuclein and toxicant exposure. For example, the injections of MPTP to mice enhanced α-synuclein mRNA and protein levels in midbrain extracts, and increased the number of α-synuclein-positive neurons in the *substantia nigra* [388]. Similar up-regulation of α-synuclein production was induced by the paraquat administration to mice, which also led to the accumulation of α-synuclein containing fibrilar aggregates within the neurons of *substantia nigra* [323]. Furthermore, α-synuclein-positive inclusions have been shown to accompany the neurodegeneration triggered by the rotenone infusion into rats [389, 390]. Finally, the fibrillation of purified α-synuclein *in vitro* was dramatically accelerated by several common pesticides including rotenone, paraquat, and maneb in a dose-dependent manner via the specific stabilization of the amyloidogenic partially folded conformation [321-323]. On the other hand, MPP^+ did not affect the fibrillation of the purified α-synuclein *in vitro* [322]. These facts might give a simple mechanical explanation for the presence of proteinaceous lesions in rotenone and paraquat + maneb models and the preferential lack of such deposits in MPTP model – cellular deposits develop only when toxins are directly involved in the interaction with α-synuclein and promote its aggregation (this can be easily tested in simple *in vitro* experiments). This also provides a rationale for using both *in vitro* and *in vivo* approaches for the screening of putative neurotoxicants, which, by affecting α-synuclein conformation and aggregation, may play a role in the pathogenesis of synucleinopathies [323]. Interestingly, the apparent negative association of smoking and caffeine with PD [391, 392] correlates with the observation that the presence of nicotine and caffeine had no effect on the kinetics of α-synuclein fibrillation *in vitro* [322].

α-Synuclein interaction with metal ions. The possible involvement of heavy metals in the etiology of PD follows from the results of epidemiological studies [371-373, 393, 394] and from the postmortem analysis of the brain tissues of PD patients [368, 395, 396] which revealed that LBs might contain high levels of iron and some aluminum [368]. This correlation was further proven by *in vitro* experiments, which showed that α-synuclein aggregation is facilitated in the presence of Cu^{2+} [235], and that Al^{3+} may induce structural perturbations in this protein [234]. Furthermore, a number of mono-, di-, and trivalent metal ions were shown to accelerate the process of α-synuclein fibril formation [238]. The effectiveness of metal cations to induce fibrillation was

shown to be correlated with the increasing ion charge density and with their ability to induce amyloidogenic partially folded species [238].

The mechanisms of the amyloidogenic partially folded conformation formation are different for metal cations and pesticides. The dominant effect of the metal ions on α-synuclein conformational change and fibrillation has been assumed to be due to the masking of the Coulombic charge-charge repulsion [238, 322]. For polyvalent cations, an additional important factor has been proposed, namely the potential for cross-linking, or bridging, between two or more carboxylates [238]. On the other hand, structure-forming and fibrillation-accelerating effects of hydrophobic pesticides were ascribed to their ability to bind and stabilize the partially folded conformation, which contains contiguous patches of hydrophobic clusters on its surface [321, 322].

Exposure to organic solvents. The exposure to solvents might represent another PD risk factor [374-378]. To understand the potential link between exposure to organic solvents and PD development, the structural properties and aggregation/fibrillation propensities of α-synuclein in water-organic solvent mixtures have been analyzed [318]. Low concentrations of all the solvents studied were shown to induce amyloidogenic partially folded conformation in α-synuclein favoring a very rapid formation of fibrils [318].

Effects of oxidative modifications: Methionine oxidation. The oxidation-modified proteins are known to accumulate during normal aging [397-399], and oxidative injury has been implicated in the pathogenesis of several disorders including AD [400], PD [401], dementia with LB [402], amyotrophic lateral sclerosis [403], Huntington's disease [404], atherosclerosis, inflammatory diseases [405], cronic renal failure [406], cataractogenesis [407], and brain ischemia and carcinogenesis [408]. All amino acids are susceptible to oxidation, but to a different degree [409], with methionine being one of the most readily oxidized amino acid residues. Methionine is easily oxidized to methionine sulfoxide, MetO, by a different oxidants produced in biological systems including H_2O_2, hydroxyl radicals, hypochlorite, chloramines, and peroxynitrite [410]. However, this modification can be repaired by methionine sulfoxide reductase catalyzing the thioredoxin-dependent reduction of MetO back to methionine [411, 412]. Based on these observations, it was proposed that methionines might serve as internal scavengers, as the reversible oxidation of surface-exposed methionines may protect other functionally essential residues from oxidative damage [413].

It has been shown that under the mild oxidative conditions (1-2% H_2O_2) all four methionines of α-synuclein, Met1, Met5, Met116, and Met127 were successfully oxidized to the MetOs [169]. As it has been already discussed, the oxidized form of α-synuclein was more unfolded than the non-oxidized protein [167, 169, 170], less prone to oligomerize and aggregate, and even able to inhibit the fibrillation of non-modified α-synuclein [169]. In subsequent study it has been shown that the degree of inhibition of α-synuclein fibrillation by methionine oxidation is proportional to the number of oxidized methionines [168]. It has been also shown that although the fibrillation of α-synuclein at neutral pH was completely inhibited by methionine oxidation, the presence of certain metals (Ti^{3+}, Zn^{2+}, Al^{3+} and Pb^{2+}) overcame this inhibition [170]. These observations brought about interesting hypothesis related to the biological function of α-synuclein as a natural scavenger of reactive oxygen species [167, 169]. This model was based on the following facts: methionine can react with essentially all of the known oxidants found in normal and pathological tissues; the concentration of α-synuclein may be as high as 1% of the total protein in soluble cytosolic brain fractions even under the normal conditions [178]; the concentration of α-synuclein could be further increased as a result of the neuronal response to toxic insult [323]; and MetO residues in proteins can be cycled back to their native methionines by methionine sulfoxide reductase [167, 169]. The balance between the protective anti-oxidant role of the methionine residues which is enhanced by this recycling and the protective anti-fibrillation effect of oxidized methionine residues in α-synuclein may fail under conditions of industrial pollution due to exposure of a person to lead, aluminum, zinc, titanium, and other metals. Thus, in the presence of the enhanced concentrations of such industrial pollutants, toxic insult-induced up-regulation of α-synuclein no longer plays a protective role; rather, it may represent a risk factor, leading to the effective metal-triggered fibrillation of the methionine-oxidized protein [170].

Other oxidative modifications: Tyrosine nitration. One of the most frequent oxidative modifications of tyrosines is their nitration [414, 415]. The existence of extensive and widespread accumulation of nitrated α-synuclein (i.e., protein containing the product of the tyrosine oxidation, 3-nitrotyrosine) in LBs and in the insoluble fractions of the affected brain regions with different synucleinopathies has been demonstrated [161, 401], with nitrated α-synuclein being detected in the major filamentous building blocks of these inclusions [161]. This suggests that the selective and specific nitration of α-synuclein in different neurodegenerative synucleinopaties may directly link oxidative and nitrative damage to the onset and progression of these disorders [161]. To understand the potential structure-modifying and aggregation-modulating role of α-synuclein nitration, the protein was oxidatively modified *in vitro* [164, 416]. Nitration was shown to be accompanied by the formation of a partially folded conformation and by the oligomerization. Furthermore, α-synuclein fibrillation at neutral pH was completely inhibited by nitrotyrosination due the formation of stable soluble oligomers and the addition of nitrated α-synuclein substantially inhibited the fibrillation of the non-modified protein in a concentration-dependent manner [164, 416]. The fact that the nitration-induced oligomerization inhibits fibrillation of human recombinant α-synuclein *in vitro* suggests that these oligomers must be located off the fibrillation pathway [416]. Therefore, the nitration detected in the proteinaceous deposits accumulated in the synucleinopathy-affected brain most likely occurred after, rather than before, fibril formation [416].

The role of phosphorylation. Although α-synuclein is predominantly non-phosphorylated *in vivo* under normal conditions [417], it becomes extensively phosphorylated at Ser129 in α–synucleinopathic lesions [417, 418] and in aging human brain [419]. An extensive accumulation of Ser129 phosphorylated α-synuclein has been also demonstrated in

neurons of mouse and fly PD models [420-422]. It has been shown that the specific phosphorylation of α-synuclein at Ser 129 by casein kinase 2 [159, 423] resulted in accelerated oligomerization and fibrillation [417]. This strongly suggests that excessive phosphorylation of α-synuclein in synucleinopathy brains may contribute to the development of pathological conditions [417]. In other words, disregulation of this PTM might be considered as one of the potential mechanisms in the pathogenesis of various synucleinopathies.

Interaction with membranes might promote α-synuclein aggregation. One of the α-synuclein biological functions is a transient and/or reversible interaction with synaptic vesicles [130, 183, 296, 316, 424, 425]. Membrane-bound α-synuclein isolated from rat brain represents only ~15% of total α-synuclein. However, this membrane-bound form was shown to have a high aggregation propensity and was able to seed aggregation of the cytosolic form of the protein [426]. Furthermore, studies with synthetic vesicles revealed that interactions of α-synuclein with long chain polyunsaturated fatty acids represented an important trigger factor for the rapid protein multimerization [316].

Aggregation-promoting interactions of α-synuclein with polyanions and polycations. Several glycosaminoglycans (GAGs) and proteoglycans (PGs) are known to be involved in the formation of the amyloid deposits found in a variety of human diseases [427, 428]. Agrin, which is an extracellular matrix and transmembrane heparan sulfate proteoglycan in the central neuronal system [429-431], represents an illustrative example of PG involved in neurodegeneration. In fact, recent studies have begun to investigate the role of agrin in brain, and suggest that its function likely extends beyond that of a synaptogenic protein. In fact it has been shown that agrin localizes to all lesion types in AD [432-434], and potentiates Aβ fibril formation [434]. Overall, several GAGs and PGs were shown to stimulate *in vitro* formation of amyloid fibrils from the Alzheimer Aβ protein [434-437] and from the microtubule-associated protein tau [5, 438]. Recently, it has been established that different GAGs (heparin, heparan sulfate) and other highly sulfated polymers (dextran sulfate) [324] as well as a PG agrin [439] are able bind to α-synuclein and stimulate its fibrillation *in vitro*. Furthermore, it has been demonstrated that agrin and α-synuclein co-localize to LBs and LNs in the *substantia nigra* of PD patients, indicating that agrin may contribute to the etiology of PD by modulating the aggregation state of α-synuclein in dopaminergic neurons [439].

Similar fibrillation-promoting effects have been described for such unstructured polycations as polyethileneimine, spermine, spermidine, polyLys, and polyArg [325]. In fact, interaction with polycations induced partial folding of α-synuclein and was accompanied by its oligomerization and fibrillation [231, 325]. The magnitude of the fibril-promoting effect depended on the chemical nature of the polycation, its length and concentration. Polycations were assumed to accelerate α-synuclein fibrillation by suppressing electrostatic repulsion between the negatively charged residues within the α-synuclein polypeptide chain and by the increase in local concentration of this protein [231, 325].

Protein-protein interactions: Spontaneous dimerization and oligomerization. Although the transient increase in temperature resulted in the completely reversible partial folding of human α-synuclein [246], the prolonged incubation of this protein (both in the purified form and in the crude cytosolic preparation from COS-7 cells) at different temperatures resulted in a temperature-dependent, progressive aggregation, with dimers being formed first [326]. Conformational analysis revealed that a partially folded pre-molten globule-like conformation, which is unstable in the monomeric form, can be stabilized as the protein undergoes a highly selective self-assembly process during prolonged incubation at elevated temperatures, and that these oligomers may evolve into the fibril nucleus [326]. The fact that this *in vitro* oligomerization produced the same set of oligomeric species as found in human LB preparations suggested that this *in vitro* process may in fact reflect the *in vivo* mechanism of α-synuclein aggregation in the early stage of pathogenesis [326].

Protein-protein interactions: Oxidative dimers. The formation of oxidative dimers under the conditions of oxidative stress may represent an important prerequisite for the fibrillogenesis of α-synuclein [440]. The exposure of this protein to oxidation or nitration stresses *in vitro* induces formation of dimers and higher-order oligomers with dityrosine cross-links [166]. This oxidative dimerization was shown to be accelerated in the pathogenic A30P and A53T α-synucleins, because of their greater propensity to self-interact [440]. These findings potentially link oxidation and synuclein aggregation to the same pathogenic pathway [440], providing a strong support for the hypothesis that impairments of cellular antioxidative mechanisms and/or overproduction of reactive oxidative species may provoke the initiation and progression of neurodegenerative synucleinopathies [161, 163, 401, 441-444].

Protein-protein interactions: Histones. α-Synuclein was shown to physically interact with histones *in vitro* and this interaction strongly promotes α-synuclein aggregation and fibrillation [231]. These observations brought about an interesting hypothesis that this interaction also can take place in a cell. In fact, α-synuclein was initially described as a neuron-specific protein localized to the presynaptic nerve terminals, Synapses, and the NUCLeus [1]. However, most subsequent studies have shown α-synuclein localized only within nerve terminals in the central nervous system [122, 130]. Contrary to these latter conclusions, it has been recently shown that paraquat administration triggers an upregulation of α-synuclein in the mouse brain that has been interpreted as part of the response of neurons to toxic insults [323], with this paraquat-induced injury being accompanied by the evidences of nuclear localization of α-synuclein [231]. Furthermore, α-synuclein was shown to co-localize with NeuN and histone H3, two specific nuclear markers, suggesting that, following the paraquat-induced neuronal insult, α-synuclein is translocated into the nuclei where it may interact with histones [231]. Nuclear translocation of α-synuclein and formation of histone-α-synuclein complexes was suggested to provide a mechanism by which an α-synuclein-related neuronal response may be activated or sustained. In this model, α-synuclein-histone complexes were suggested to have a regulatory role by decreasing the pool of free histones available for DNA binding. The subsequent destabilization of nucleosome and enhanced manifestation of the DNA matrix activity could lead to increased transcription, and ultimately

to the production of proteins in response to a variety of stimuli, including toxic insults [231]. Finally, it has been pointed out that the pathophysiologic implications of histone-induced α-synuclein aggregation are not very obvious, as Lewy bodies in PD are usually found in the cytosol and never in the nucleus. This might suggest that histone-α-synuclein interactions may not underlie the development of these typical inclusions. However, α-synuclein-containing aggregates have been observed in the nuclei of transgenic mice that overexpress α-synuclein [45]. This strongly suggests that the ability of histones to promote aggregation *in vitro* may contribute to pathological changes *in vivo*, at least in mice [231].

Protein-protein interactions: Transglutaminase. Transglutaminases (TGs) are a family of proteins that catalyze a calcium-dependent transamidating reaction that results in cross-linking of proteins via ε(γ-glutamyl) lysine bonds [445]. Transglutaminase 2 (tissue transglutaminase, tTG) is a unique member of this family, as in addition to the transamidating activity it functions as GTPase and ATPase [446, 447]. tTG expressed in the mammalian nervous system and human brain, localizing predominantly in neurons [448, 449]. Recent studies demonstrated that tTG catalyzes the formation of the cross-linked aggregates from neurofilament [450], amyloid precursor protein [451], tau [452], and proteins with polyglutamine repeats [449], reflecting the potential involvement of tTG in such neurodegenerative disorders as AD and HD. Furthermore, it has been established that tTG catalyzed α-synuclein cross-linking, leading to the formation of high molecular weight aggregates *in vitro* and in cellular models [453]. Furthermore, immunohistochemical studies on postmortem brain tissue confirmed the presence of transglutaminase-catalyzed ε(γ-glutamyl) lysine cross-links in the halo of LBs bodies in PD and dementia with LBs, co-localizing with α-synuclein. Based on these observations it has been suggested that tTG is able to enhance the α-synuclein aggregation, promoting the formation of LBs and, thus, contributing to neurodegeneration [453].

Protein-protein interactions: Other proteins promoting α-synuclein aggregation. Several dozens of proteins are known to interact with α-synuclein [see above and [22, 27, 454] for recent reviews]. Importantly, some of them were shown to stimulate α-synuclein aggregation *in vitro* at substoichiometric concentrations. The list of this aggregation promoters includes tau protein [455], histones [231], brain-specific protein p25α [456], tubulin [217], and agrin [439]. Furthermore, it has been shown recently that the transcriptional co-factor high mobility group protein 1 (HMGB-1) is able to bind preferentially to aggregated α-synuclein, and is present in α-synuclein filament-containing LBs isolated from brain tissue affected with LBD and PD [457]. Except for the histones, these proteins have all been identified as components of LBs and/or GCIs [217, 439, 455-457]. The mechanism by which these proteins interact with α-synuclein is unknown, but they all contain basic motifs, suggesting that the interaction with α-synuclein may be mediated through ionic interactions. It has been emphasized that the identification of proteins regulating α-synuclein aggregation *in vitro* indicates that endogenous protein factors may regulate α-synuclein aggregation *in vivo* [22]. Recently, the process of α-synuclein aggregation *in vitro* was noticeably accelerated by addition of FK506 binding proteins (FKBPs) leading to the formation of aggregates with clear fibrillar morphology [458]. FKBPs are members of the immunophilins, enzymes that bind to immunosuppressant drugs and have a peptidyl-prolyl isomerase (PPIase) or rotamase activity [459]. It has been shown that the rotamase activity of FKBP is responsible for the acceleration of the partial folding and subsequent aggregation of α-synuclein, as the addition of FK506, an inhibitor of FKBP PPIase activity, suppressed α-synuclein aggregation [458].

Proteins inhibiting α-synuclein aggregation: Chaperones. Heat shock proteins are a family of chaperones that are constitutively expressed and induced by different stresses, and that suppress protein aggregation and participate in protein refolding and/or degradation. It has been reported that torsin A, a protein with homology to yeast heat shock protein 104, co-localize with α-synuclein in LBs and is found in many peripheral tissues and brain regions [460-462]. Similar to torsin A, some other heat shock proteins have been shown to co-localize with α-synuclein in LBs [463]. Importantly, it has been demonstrated that over-expression of torsin A and heat shock proteins suppress α-synuclein aggregation in the cellular model [463].

Recently, using immunohistochemistry and confocal microscopy it has been demonstrated that αB-crystallin, a small chaperone protein that binds to unfolded proteins and inhibits its aggregation, is a prominent component of GCIs and LBs [464]. Furthermore, it has been shown that in cultured proteasome-inhibited C6 glioma cells transfected with GFP-tagged α-synuclein resulted in ubiquitin- and αB-crystallin-positive aggregates resembling GCIs in MSA brains [464]. These *in vivo* studies are supported by the detailed *in vitro* analysis, which revealed that αB-crystallin serves as a potent inhibitor of wild-type, A30P and A53T α-synucleins fibrillation [208].

It has been reported that directed expression of the molecular chaperone Hsp70 prevented dopaminergic neuronal loss associated with α-synuclein in Drosophila and that interference with endogenous chaperone activity accelerated α-synuclein toxicity [465]. Furthermore, LBs in human postmortem tissue were shown to immunostain for molecular chaperones Hsp70 and Hsp40. Similar results have been observed for specific inclusions in brain tissues from patients with DLB, LBVAD, and NBIAI [465]. These data suggest a role for chaperones in pathologies involving α-synuclein in humans, such that Hsp70 may be a critical part of the neuronal arsenal that mitigates α-synuclein toxicity [465].

Proteins inhibiting α-synuclein aggregation: β- and γ-Synucleins. Conformational analysis revealed that α-, β-, and γ-synucleins are natively unfolded under physiological conditions *in vitro*, and are able to adopt comparable partially folded conformations at acidic pH or at high temperature [297]. Although both α- and γ-synucleins were shown to form fibrils, β-synuclein did not fibrillate, being incubated under the same conditions [297]. However, even non-amyloidogenic β-synuclein can be forced to fibrillate in the presence of some metals (Zn^{2+}, Pb^{2+}, and Cu^{2+}) [466]. This metal-induced fibrillation of β-synuclein was further accelerated by the addition of GAGs and high concentrations of crowding agents. Furthermore, β-Synuclein was shown to undergo fast oligomerization and fibrillation in the presence

of pesticides, whereas the addition of low concentrations of organic solvents induced the formation of amorphous aggregates [466]. It has been shown that β-synuclein did not fibrillate in crowded environments or in the presence of glycosaminoglycans (i.e., under the conditions known to induce very fast fibrillation of α-synuclein) when metals were not present in media [466].

Intriguingly, the addition of either β- or γ-synuclein in a 1:1 molar ratio to α-synuclein solution substantially increased the duration of the lag-time and dramatically reduced the elongation rate of α-synuclein fibrillation [297]. Fibrillation was completely inhibited at a 4:1 molar excess of β- or γ-synuclein over α-synuclein [297]. β-Synuclein inhibited α-synuclein aggregation in an animal models too [467]. This suggests that β- and γ-synucleins may act as regulators of α-synuclein fibrillation *in vivo*, potentially acting as chaperones. Therefore, one possible factor in the etiology of PD would be a decrease in the levels of β- or γ-synucleins [297].

Small molecules inhibiting α-synuclein fibrillation: Dopamine and other catecholamines. The common pathway for both idiopathic and familial PD is the damage and subsequent loss of dopaminergic neurons [468], decline in the number of which below a critical threshold (~60%) produces PD symptoms as a result of the systematic reduction in the dopamine content [469]. This neurodegeneration, being accompanied by the formation of α-synuclein containing LBs and LNs in the *substantia nigra pars compacta*, obviously brings together α-synuclein, its aggregation, and dopamine. It has been shown that several catecholamines including L-dopa and dopamine are able to inhibit fibrillation of α-synuclein and Aβ and to dissolve the preformed fibrils *in vitro* [470]. Furthermore, the products of the oxidation products derived from these catecholamines were more potent Inhibitors of the α-synuclein fibrillation [470]. In a systematic analysis of the inhibitory effect of a library of 169 drug-like molecules on the α-synuclein fibrillation it has been established that all but one of 15 fibril inhibitors were catecholamines related to dopamine [471]. The inhibitory activity of dopamine was attributed to the formation of the covalent adducts between the orthoquinone derivative of dopamine and α-synuclein causing an accumulation of the covalently modified protofibrils that are unable to fibrillate [471].

Small molecules inhibiting α-synuclein fibrillation: Baicalein. A component of the traditional Chinese herbal medicine *Scutellaria baicalensis*, baicalein, is known to have multiple biological activities including antiallergic, anticarcinogenic, and anti-HIV properties [472-476]. Natural medicines containing these compounds have been reported to have beneficial effects in treating memory loss and dementia [477, 478] and it has been even reported that baicalein might protect rat cortical neurons from Aβ-induced toxicity by its inhibition of lipoxygenase [477]. Recently, the micromolar concentrations of baicalein or its oxidized forms were shown to inhibit the formation of α-synuclein fibrils, and disaggregate the preformed fibrils *in vitro* giving rise to soluble oligomers [479].

Small molecules inhibiting α-synuclein fibrillation: Rifampicin. Epidemiological studies revealed that leprosy patients have significantly lower probability of senile dementia development if they had been under antileprosy treatment with rifampicin (a semisynthetic derivative of the rifamycins, a class of antibiotics that are fermentation products of *Nocardia mediterranei* [480]) and closely related drugs for the preceding several years [481-483] suggesting that these antileprosy drugs might prevent Aβ aggregation, resulting in the absence of amyloid deposition [484]. This hypothesis has been confirmed by showing that rifampicin and its analogs, *p*-benzoquinone and hydroquinone, were able to inhibit Aβ1-40 or Aβ1-42 aggregation and neurotoxicity *in vitro* [484-486]. Similarly, rifampicin was shown to inhibit fibrillation of human islet amyloid polypeptide, amylin *in vitro* [485]. Furthermore, rifampicin inhibited the α-synuclein fibrillation *in vitro* and disaggregated preformed fibrils in a concentration-dependent manner leading to the formation of soluble oligomers comprised of partially folded α-synuclein [487].

α-SYNUCLEIN, PROTEIN AGGREGATION AND CELL DEATH

The next important question is which species present during the α-synuclein aggregation and fibrillation could be responsible for the neuronal death. Is it the natively unfolded monomer, the fibrillogenic monomeric intermediate, some oligomeric species (protofibrils), or mature fibril? It has been pointed out that synuclein toxicity may be exerted by specific populations of α-synuclein aggregates and/or mediated via various routes through proteins involved in different cellular processes [22]. The mechanisms proposed to describe the neurotoxicity of α-synuclein and its aggregates could be grouped into three major classes – mechanical distortion of cellular compartments/processes, toxic gain of function, and toxic loss of function. An analysis of the peculiarities of α-synuclein interaction with lipids revealed that some lipids can induce α-synuclein oligomerization upon binding, which in particular circumstances can disrupt membrane bilayers. Furthermore, some protofibrillar forms of α-synuclein were shown to penetrate membranes forming pore-like channels. These structures were proposed to be the neurotoxic species, which kill neurons via the abnormal increase in membrane permeability (see e.g. [488]). Alternatively, impairment of α-synuclein degradation has been proposed as a mechanism for neurotoxicity. In fact, it is known that α-synuclein is primarily degraded by proteasome. Importantly, the degradation of mutant α-synucleins is less efficient than that of wild type protein. Other modifications of α-synuclein may impair proteasome degradation as well. This could leave to the elevation of cellular concentrations of α-synuclein, aggregate formation, and neurotoxicity [489].

Toxic Loss of Function

As it has been already pointed out, one of the intriguing α-synuclein functions is its participation in the control of the neuronal apoptotic response [190]. In one study, a clonal cell line from neocortical origin (TSM1 cells) stably overexpressing wild-type α-synuclein or its PD-associated A53T mutant was utilized to examine the caspase response to various apoptotic stimuli [190]. This analysis revealed that although wild-type α-synuclein displayed antiapoptotic properties and inhibited the caspase 3 activation triggered by several apoptotic stimuli, including staurosporine, C_2-ceramide,

and etoposide, these properties were abolished by the PD-related mutation [190]. In line with an antiapoptotic physiological function of α-synuclein, it has been reported that this protein was almost always associated with normal neurons but not with those exhibiting apoptosis [490]. Subsequent analysis revealed that the antiapoptotic function of α-synuclein is mediated by the drastic lowering of p53 expression and transcriptional activity [491] and that this function can be abolished not only by a PD-related mutation, but also by 6-hydroxydopamine (6OH-DOPA) [491, 492], which is a dopaminergic derivative thought to act as a natural toxin involved in PD pathology [493]. This abrogation of the α-synuclein anti-apoptotic phenotype by both genetic and biochemical defects leading to the extensive α-synuclein aggregation suggested that anti-apoptotic activity is abolished by factors triggering protein aggregation. Furthermore, these observations could be used to explain the topological selectivity of the lesions observed in PD [492]. Thus, α-synuclein is widely distributed within the central nervous system where the protein exerts its anti-apoptotic function. However, only the dopaminergic neurons have the potential of producing the endogenous toxin able to modify α-synuclein and to modulate its biophysical characteristics. This links α-synuclein dysfunction to the anatomical selectivity of the lesions observed in PD [492].

Toxic Gain of Function

Numerous mechanisms of toxicity based on the toxic gain of function by α-synuclein have been proposed [489]. In fact, cytotoxicity could be exerted via the various forms of aggregated α-synuclein. This hypothesis is based on the evidences that α-synuclein, the known mutations, and the NAC fragment all can function as nucleation sites and promote aggregation under certain conditions. Cytotoxicity could also originate from the modifications of α-synuclein and transformation of this protein of into neurotoxic species. This could be a result of α-synuclein exposure to metals, pesticides, dopamine metabolites, oxidative stress, exogenous toxins, and mitochondrial insufficiency. The covalently crosslinked oligomers (homopolymers or heteropolymers of α-synuclein), which might be responsible for the insolubility of pathogenic inclusions such as Lewy bodies, could also be cytotoxic. On the other hand, several studies emphasize that α-synuclein-related neurotoxicity might arise from a loss of function [summarized in [489]]. Obviously, all the factors mentioned above are not necessarily mutually exclusive, but instead may be synergistic [489].

Several lines of evidence indicated that some α-synuclein oligomers might serve neuron killers. For example, it has been recently shown that oligomeric α-synuclein is deposited in detergent insoluble fractions from the brains of patients with the gene triplication mutation [494]. Furthermore, the property of the A30P mutant to dramatically accelerate the initial oligomerization of α-synuclein and to significantly retard the formation of mature fibrils [300, 307, 308] represents a key factor leading to the theory that oligomeric intermediates of α-synuclein, rather than mature fibrils, may in fact be the disease-associated species of the protein [495] and that oligomers, not fibrils, are cytotoxic [327, 328]. It has been pointed out that annular (doughnut-like) oligomers [which not only are stabilized during the *in vitro* studies (see above), but have also been isolated from human brain samples [496]] may have pore-like properties and might damage membranes [497]. Several additional facts to support the idea of oligomer toxicity were uncovered [156]:

- In cell models, toxicity is usually seen without heavily aggregated α-synuclein leading to the suggestion that soluble species mediate toxicity [206].
- Detectable aggregation of α-synuclein and deposition of this protein into insoluble fractions occurs later than cell death *in vitro* [498].
- Transgenic mice, which express human wild-type α-synuclein at a level comparable to the level of endogenous mouse protein, developed nonfibrillar intraneuronal inclusions in several brain areas, including the *substantia nigra pars compacta* [45].
- Transgenic mice expressing A53T and WT exhibited neurodegeneration outside the *substantia nigra* without fibrillar inclusions [499].
- Lentiviral-based expression of human α-synuclein in rat *substantia nigra* resulted in selective dopaminergic toxicity with nonfibrillar inclusions [500].
- The α-synuclein-containing inclusions in some animal models do not contain fibrils, and the fibril-containing inclusions of the fly can occur in the absence of neurodegeneration [465, 501].
- Although involvement of α-synuclein in PD is often associated with the fact that this protein represents a major constituent of LBs, α-synuclein aggregation has been identified in neurons in forms other than Lewy bodies. Engorged neuronal processes containing ovoid, fusiform, club-shaped, and spherical α-synuclein inclusions have been found in *substantia nigra pars compacta* and other structures in both PD and LB disease brains [502, 503]. Furthermore, threadlike aggregates of α-synuclein, which can be either coarse or fine, have also been detected in both dendrites and axons [113, 114, 504].

Based on these and several other observations it has been suggested that the LB formation does not cause but protect against neurodegeneration [505]. This strongly suggests that the death of neurons can be provoked by the formation of protofibrilar species. It has been pointed out that the process of α-synuclein fibrillation *in vitro* is frequently accompanied by the formation of protofibrils, which are usually observed by AFM and EM as a heterogeneous mixture of morphologies, including spherical, annular, pore-like, tube-like, and chain-like structures [488]. Recently, it has been demonstrated that soluble 30-50 nm-sized annular α-synuclein oligomers could be released by a mild detergent treatment from GCIs purified from multiple system atrophy brain tissue [496], suggesting that pathological synucleinopathy aggregates can be a source of annular α-synuclein species. Interestingly, the vast majority of recombinant α-synuclein was shown to yield spherical oligomers after detergent treatment. However, *in vitro* binding of Ca^{2+} to monomeric α-synuclein was shown to induce the rapid formation of annular oligomers [244]. Therefore, an intracytoplasmic Ca^{2+} concentration might affect the speciation of α-synuclein, and that soluble α-synuclein annular oligomers may be cytotoxic

species, either by interacting with cell membranes or components of the ubiquitin proteasome system [506]. Fig. (**7**) summarizes this discussion showing potentially neurotoxic and neuroprotective aggregated forms accommodated by α-synuclein either under different experimental conditions or during the aggregation/fibrillation process.

CONCLUDING REMARKS

Much has been learned in the 10 years since the identification of a specific mutation in the α-synuclein gene in familial cases of early-onset PD [2] and the demonstration that α-synuclein is highly abundant in Lewy bodies [3]. These two discoveries initiated the re-assessment of the molecular basis of PD. Data accumulated since that time has clearly shown that α-synuclein is positioned at the eye of the storm, being directly or indirectly involved in the pathology of several neurodegenerative disorders. The intrinsically disordered nature of α-synuclein, its chameleon behavior and conformational plasticity, together with the breadth of functions and interactions ascribed to this protein are central to its pathogenicity. Data accumulated to date can be used to depict a general model of α-synuclein aggregation, according to which this protein exists as a mixture of the natively unfolded and partially folded conformations prior to aggregation. Equilibrium is essentially shifted toward the unfolded conformation under normal physiological conditions. Different factors, such as an introduction of PD-related mutations, a decrease in pH, increase in temperature, the presence of amphipathic molecules (e. g. herbicides or pesticides), or presence of metal ions and other small charged molecules, interaction with charged biopolymers, other proteins, or membrane, or the effect of macromolecular crowding, and numerous other environmental factors are able to shift this equilibrium thus populating the partially folded conformation. It is important to remember that different factors may, in principle, stabilize different partially folded conformations. Under appropriate conditions, this partial folding might lead to α-synuclein self-association, which is facilitated by the formation of solvent-exposed hydrophobic clusters on the surface of a partially folded protein. The type of the end-product of the aggregation process depends on the peculiarities of the environmental conditions. Association/aggregation may result in the formation of various soluble oligomers, amorphous aggregates, or amyloid-like fibrils. Although potentially any of these three species could be neurotoxic, numerous data support the hypothesis that soluble oligomers express largest cytotoxicity. There are three major molecular mechanisms proposed to explain the neurotoxicity of α-synuclein and its aggregates – mechanical distortion of cellular compartments/processes, toxic gain of function, and toxic loss of function. Although all synucleinopathies are characterized by the accumulation of α-synuclein-containing deposits, they are clearly multifactorial diseases whose pathogeneses cannot be and should not be explained solely by the α-synuclein aggregation and various other factors have to be taken into account.

Finally, a few words on the potential implications of α-synuclein and its aggregates as potential drug targets should

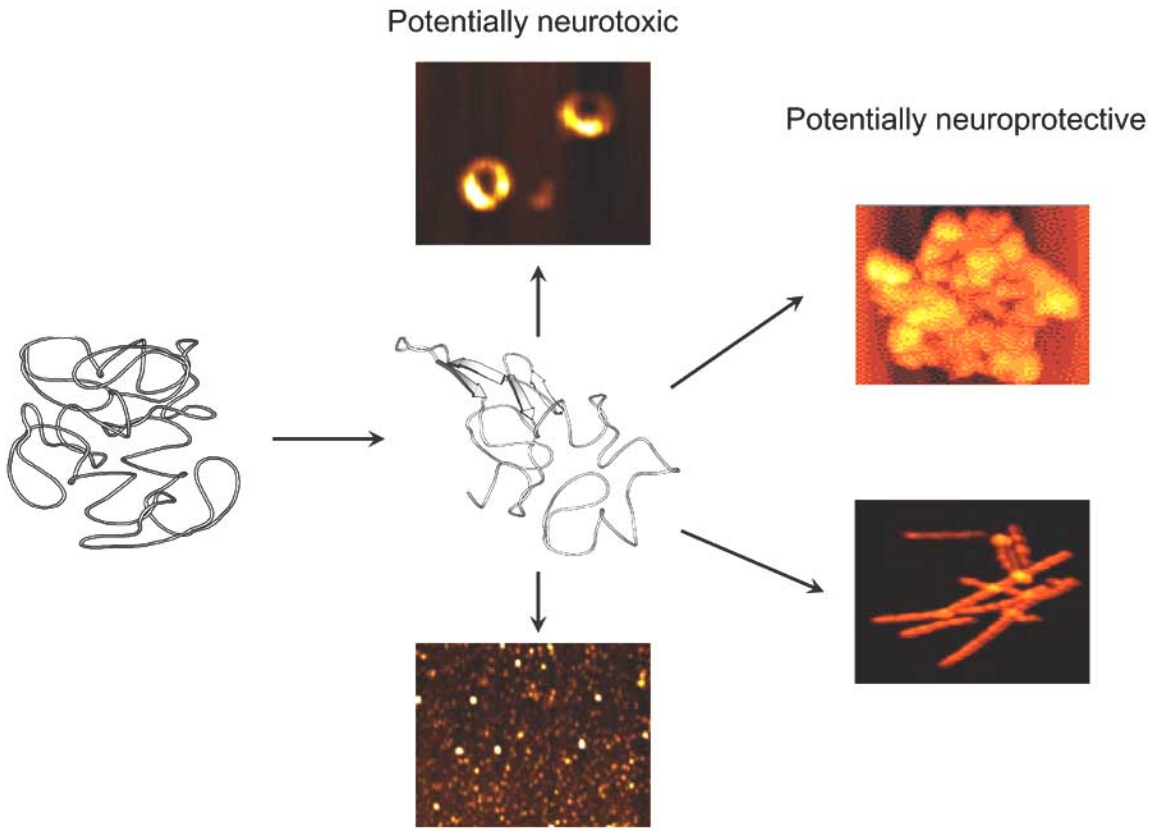


Fig. (7). Different aggregated forms achievable by the partially folded α-synuclein either under the different experimental conditions or during the fibrillation/aggregation process. Partially folded species together with soluble oligomeric forms (doughnuts and spheroids) are potentially neurotoxic, whereas insoluble aggregated forms (amorphous aggregates and fibrils) are potentially neuroprotective.

be added. Currently, there is no medication able to halt or retard neuron degeneration in various synucleinopathy and all drugs developed so far treat disease symptoms. There are several reasons for not finding means for the successful inhibition and cure of synucleopathies. A limited understanding of the key molecular events provoking neurodegeneration is first among them [507]. Furthermore, the precise knowledge on the nature of the neurotoxic species accumulated during α-synuclein misfolding and aggregation is also missing. Another complication is a very complex nature of synucleinopathies, which are known to be initiated by numerous factors, including genetic predisposition, toxic insults, failure of the chaperone system, proteosomal misfunction, rare cases of the autosomal origin, oxidative damage, etc. [489]. However, even although synucleinopathies are multifactorial disorders, some potentially effective therapeutic protocols could be developed based on the detailed analysis of α-synuclein function, misfunction, and aggregation. This is because of the fact that many of the disease-promoting factors are directly or indirectly related to the α-synuclein misfolding or abnormal processing, functioning or aggregation of this protein. This suggests that the identification of molecules that are able to inhibit α-synuclein deposition or reverse fibril/oligomer formation may be a critical step toward a better understanding of the pathophysiology of proteinaceous inclusion formation in this set of human diseases.

Obviously, a great caution should be taken in development and examining drugs that inhibit α-synuclein fibrillation or promote the disaggregation of preformed fibrils. Once again, the major pitfall here is the lack of clear understanding of the mechanisms of aggregation-related neurotoxicity. As some small α-synuclein oligomers (known as small prefibrillar species) possess high level of neurotoxicity, halting the fibrillation process at early stages that are associated with the formation of such small oligomers can create more harm than cure. Similarly, small molecules that are able to disaggregate fibrils to smaller oligomers or soluble aggregates can also promote neurodegeneration.

There are several potential solutions for these problems. One of them is a search for small molecules stabilizing the intrinsically disordered conformation of α-synuclein or completely blocking its aggregation, or resulting in the complete disaggregation of the preformed aggregates down to monomeric state. Alternatively, one can search for chemical compounds that can either clear toxic misfolded proteins or protect neurons from their impact. Finally, a very promising approach relies on compounds that promote protein aggregation, accelerate formation of large inclusions and eliminate toxic effects of misfolded protein conformations and small oligomers [508, 509]. This last approach is based on the observation that large inclusions might play neuroprotective role and therefore stimulation of fast formation of larger clumps of aggregated protein could represent a therapy for affected neurons [509].

Recent studies showing the inhibitory and disaggregating effects of some small molecules on α-synuclein fibrillation or analyzing compounds dramatically accelerating aggregation process might represent the first steps in the development of new anti-synucleinopathy drugs.

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ABBREVIATIONS USED

AD	=	Alzheimer's disease
HD	=	Huntington's disease
PD	=	Parkinson's disease
IDP	=	Idiopathic PD
PPS	=	Parkinsonism plus syndromes
DLB	=	Dementia with Lewy bodies
MSA	=	Multiple system atrophy
DLBD	=	Diffuse Lewy body disease
LBVAD	=	Lewy body variant of Alzheimer's disease
HSD	=	Hallervorden-Spatz disease
NBIA I	=	Neurodegeneration with brain iron accumulation type I
PDD	=	PD dementia
PAF	=	Pure autonomic failure
NAD	=	Neuroaxonal dystrophy
LB	=	Lewy body
LN	=	Lewy neurite
NFT	=	Neurofibrillary tangle
PTM	=	Posttranslational modification
AS	=	Alternative splicing
tTG	=	Tissue transglutaminase
NAC	=	Non-Aβ component of AD plagues
Aβ	=	Amyloid beta peptide
AGEs	=	Advanced glycation endproducts
MetO	=	Methionine sulfoxide
GAG	=	Glycosaminoglycan
PG	=	Proteoglycan

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