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Physiological and pathological properties of α-synuclein

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Abstract. α-Synuclein belongs to a small group of natively unfolded proteins that can transiently bind to lipid membranes and acquire a partial α-helical conformation. Under certain pathogenic conditions, α-synuclein aggregates to form oligomers and insoluble fibrils with increased ß-sheet configuration. Although genetic mutations and multiplications of the gene have been found in familial cases, the mechanism by which this protein aggregates in sporadic cases of Parkinson's disease, dementia with Lewy bodies and multisystem atrophy is not fully understood. Here we review the function of α-synuclein and recent insight into the mechanisms by which it aggregates.

Keywords. α-Synuclein, Parkinson's disease, α-synucleinopathies, neurodegeneration, Lewy body.

Introduction

Parkinson's disease (PD) is the most common movement disorder, clinically characterised by tremor, rigidity and bradykinesia. Neuropathologically it is defined by nerve cell loss in the substantia nigra and the presence of Lewy bodies (LBs) and Lewy neurites (LNs) [1]. LBs and LNs are also the characteristic neuropathological features of dementia with Lewy body (DLB), a common late-life dementia that exists in a pure form or overlaps with the neuropathological characteristics of Alzheimer's disease. Ultrastructurally, LBs and LNs consist of abnormal filamentous material [1].

Although LBs were first described in 1912, their composition became known only in 1997 when the discovery of a point mutation in the α-synuclein gene, in a small group of families with early-onset PD, led to identification of α-synuclein as the major component of LBs and LNs in idiopathic PD and DLB [2]. Following the original discovery of α-synuclein in LBs and LNs in PD and DLB, other diseases have also been characterised by α-synuclein positive fibrillar inclusions: these include Multiple System Atrophy (MSA), neurodegeneration with brain iron accumulation, Gerstmann-Straussler-Scheinker disease, pure autonomic failure, some cases of Parkinsonism-Dementia complex of Guam [3–9] and Alzheimer's disease, where in some brain areas they are present in approximately 60 % of both sporadic and familial cases [10].

Physiological function of α-synuclein

α-Synuclein is a 140-amino acid protein first described in *Torpedo californica* and very abundant in brain [2]. α-Synuclein belongs to the synuclein family, which includes ß- and γ-synucleins. The synucleins have a common amino-terminal sequence containing a different number of repeat regions while they differ in the carboxy-terminal part [2].

Recombinant α-synuclein in aqueous solution does not assume a uniform or consistent secondary structure; hence the protein is said to be natively unfolded [11]. However, the amino acid sequence and subcellular localisation of α-synuclein indicate that it may be capable of interacting with lipid membranes. The repeat region, which makes up a conserved apolipoprotein-like class-A2 helix, mediates reversible binding to acidic phospholipids (especially phosphatidic

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acid, PA), which in turn is associated with a large shift in protein secondary structure from around 3% to about 80% α-helical [12]. In this respect it is of interest that both α- and ß-synuclein have been identified as highly specific inhibitors of phospholipase D2 (PLD2), which produces PA by hydrolysis of phosphatidylcholine, and is localised to plasma membrane and submembranous vesicles [13]. Therefore, synuclein proteins, through their action on PLD2, may be involved in synaptic membrane biogenesis since PA metabolism has been specifically implicated in vesicle budding.

The role of α-synuclein in membrane-associated processes in the presynaptic terminal is supported by several observations: α-synuclein knockout mice have enhanced dopamine release at nigrostriatal terminals in response to paired electrical stimuli, suggesting that α-synuclein is an activity-dependent negative regulator of dopamine neurotransmission [14]. Furthermore, depletion of α-synuclein from primary hippocampal neurons with antisense oligonucleotide treatment results in a decrease in the distal pool of presynaptic vesicles as visualised by electron microscopy [15]. Finally, α-synuclein is specifically upregulated in a discrete population of presynaptic terminals of the songbird brain during a period of song acquisition, indicating a role in synaptic plasticity [16]. Interestingly, α-synuclein protects nerve terminals against injury in a pathway involving cysteine-string protein (CSP)-α and SNARE proteins on the presynaptic membrane interface [17]. In this study, transgenic expression of α-synuclein abolished the lethality and neurodegeneartion caused by deletion of CSPα, suggesting that α-synuclein acts downstream of CSPα to maintain SNARE complex assembly.

Besides its ability to bind to lipid membranes and to inhibit PLD2 activity, α-synuclein appears to interact with several other proteins [18]. By yeast two-hybrid screening, synphilin-1 was identified as a protein that binds to α-synuclein [19]. This is a 90-kDa cytoplasmic protein of largely unknown function, which might act as an adaptor molecule that anchors α-synuclein to intracellular proteins that are involved in vesicle transport and cytoskeletal function. α-Synuclein also shares physical and functional homology with 14–3-3 proteins, which are a family of ubiquitous cytoplasmic chaperones [20]. In addition, α-synuclein was found to bind to 14–3-3 proteins as well as some proteins known to associate with 14–3-3 such as protein kinase C and BAD. Based on these interactions, it was suggested that increased expression of α-synuclein could be harmful. A related observation was made in inducible neuro2a cell lines, where α-synuclein was reported to inhibit MAP kinase signalling and accelerate cell death following serum reduction [21]. However, this remains controversial since wild-type α-synuclein overexpression has also been shown to protect neuronal cells from apoptotic stimuli and to delay cell death induced by serum withdrawal [22, 23]. It has also been reported that α-synuclein protects against oxidative stress by inactivation of the c-jun N-terminal kinase, a member of the mitogen-activated protein kinase family, which plays an important role in stress response [24]. The significance of these interactions for endogenous α-synuclein and their functional consequences *in vivo* remain to be seen.

Recent data suggest that full length α-synuclein is involved in dopaminergic cell differentiation and survival in that cells from transgenic mice expressing truncated protein seem to be more sensitive to environmental conditions [25] and overexpression of α-synuclein in human neural progenitor cells appears to affect their fate and differentiation [26].

α-Synuclein carries a number of potential sites for phosphorylation. In transfected cells, α-synuclein is constitutively phosphorylated at serine residues 87 and 129, with the latter being the predominant site [27]. Residue 129 in α-synuclein lies in a consensus sequence for casein kinase 1, a sequence that is also present in ß- and γ-synuclein. Both casein kinase 1 and 2 phosphorylate this site in α-synuclein [27]. Several G-protein-coupled receptor kinases also phosphorylate α-synuclein, thus reducing its ability to interact with phospholipids and PLD2 [28]. Phosphorylation of the tyrosine residues in the carboxy-terminus of the protein by tyrosine kinase 72syk has also been reported both *in vitro* and in CHO cells [29].

Pathogenic function of α-synuclein

Effect of genetic modifications

The importance of α-synuclein in neurodegeneration is based on two seminal observations: The identification of point mutations and gene duplication and triplication in a small number of families with autosomal-dominant early-onset PD and the discovery that α-synuclein is the major component of Lewy body filaments in the sporadic cases of PD (Fig. 1) and also DLB and MSA, which are now referred to as the α-synucleinopathies [30, 31]. Polymeropoulos et al. first discovered a point mutation in a large Italian-American kindred (the Contursi family) and three smaller Greek families with levodopa-responsive parkinsonism and autopsy-confirmed LBs [32]. This mutation consists of a change of alanine residue 53 to threonine (A53T). Two other mutations, A30P and E46K, were described in unrelated families [33, 34]. These genetic modifications have been extensively investigated both *in vitro* and *in vivo* and have

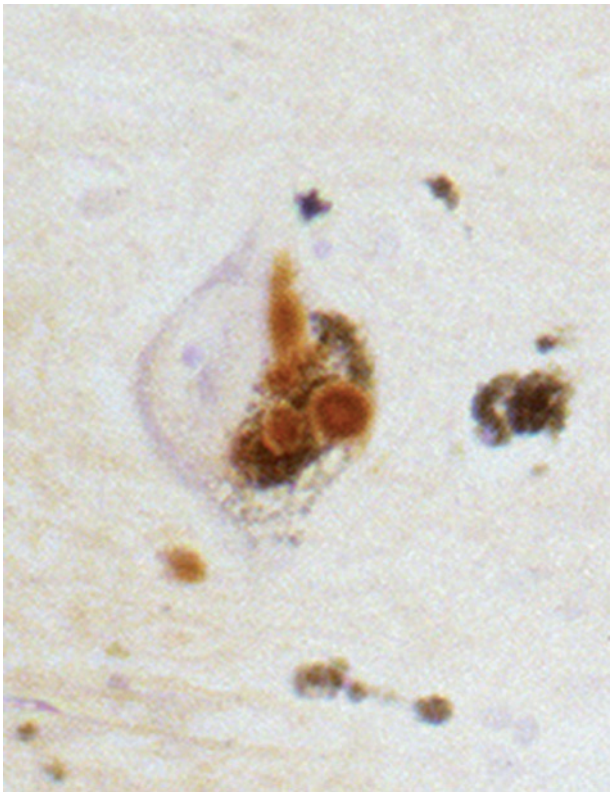

Figure 1. Lewy bodies stained with anti-α-synuclein antibodies in the substantia nigra of a sporadic Parkinson's disease patient.

provided important insights into the possible pathogenic mechanism in PD and related disorders.

Fibril formation by A53T mutation is accelerated relative to both wild-type (WT) and A30P. The effect of the A30P mutation on filament assembly is less clear since, depending on the studies, this was found to be either small [35] or absent [36]. However, under conditions that ultimately produce fibrils, the A30P monomer is consumed slightly more rapidly than the WT monomer, whereas A53T is the most rapidly consumed, suggesting that accelerated formation of pre-fibrillar α-synuclein oligomers is a shared property of both mutations [37]. On the other hand, A30P but not A53T mutation reduces the binding of α-synuclein to brain vesicles [37], thus increasing bioavailability of the protein for aberrant interactions to occur and neuropathology. The E46K mutation significantly increases binding of α-synuclein to negatively charged liposomes [38] and increases the rate of filament assembly to the same extent as the A53T mutation [38, 39]. These early studies have suggested that alteration in the propensity of α-synuclein to form fibrilar aggregates and/or its ability to bind to lipid membranes may be central events in the neurodegenerative process. More recently, duplication and triplication of the *α-synuclein* locus has been shown to cause PD [40–42], suggesting that level of α-synuclein expression/accumulation may also be pathogenic.

The deleterious effects of point mutations and the effect of high-level expression have best been investigated *in vivo* using transgenic technology and viral infection. The effect of A53T and A30P mutations and their comparison to WT protein have been investigated using both pan-neuronal (mPrP, PDGF and Thy1) as well as specific (TH) promoters. In mice where the mPrP promoter was used [43, 44], although no difference was detected in the localisation of mouse and human α-synuclein in young mice, homozygous A53T mutant α-synuclein animals between 8 and 16 months of age showed α-synuclein accumulation in cell bodies and dystrophic neurites throughout the neuraxis. Occasionally, these aggregates were made of insoluble filamentous α-synuclein. The formation of these aggregates paralleled the onset of motor impairment. However, extensive α-synuclein pathology was also detected in motor neurones and axons in the ventral root of the spinal cord, suggesting that these peripheral lesions might be the major contributors underlying the behavioural phenotype of these mice. In studies using the mPrP promoter and contrary to earlier reports, no abnormal α-synuclein accumulation or behavioural deficits were detected in mice overexpressing the WT human protein. Transgenic mice overexpressing the A30P mutant α-synuclein driven by the Thy1 promoter also develop many of the salient features of LB disease such as proteinase K-resistance, neuritic pathology and formation of some argyrophilic and thioflavin S-positive α-synuclein inclusions with increasing age [45].

It is of interest that although mPrP but not Thy1 promoter drives high expression of the transgene in neurones of the substantia nigra (SN), as revealed by *in situ* hybridisation [44], the tyrosine hydroxylase (TH)-positive neurones of the SN in the mPrP-driven transgenic mice were completely spared from α-synuclein aggregates or other deficits such as loss of striatal dopamine or dopamine transporter [43, 44]. In accordance with the latter, a study using the TH promoter to express wt or mutant α-synuclein selectively in these neurones did not result in the formation of pathogenic inclusions [46]. In this respect, adeno- or lentiviral-mediated models have been useful in studing the effect of α-synuclein in the SN neurones of adult rats [47–49]. In these studies, overexpression of either WT or mutant protein led to cellular and axonal pathology associated with loss of nigral neurones, decrease in striatal DA levels and significant motor impairment [47] but no fibrillar inclusions [49]. These *in vivo* studies contradict evidence from transgenic mouse models and support the idea that dopaminergic

neurones are vulnerable to high levels of human α-synuclein with no difference between the WT and mutant forms of the protein. This discrepancy could be related to the level of gene expression that can be achieved by either technology or the acute toxicity which is inherent to gene transfection in viral models. However, in neither transgenic mice nor viral-mediated rat models expressing full-length WT or mutated α-synuclein have fibrillar α-synuclein inclusions been reported in the SN. Therefore the mechanism by which WT human α-synuclein assembles to form LB in the SN of brains from PD patients is currently poorly understood. The only exception to the above is the presence of filamentous α-synuclein inclusions in transgenic *Drosophila*, which are associated with an age-dependent loss of dopamine cells and locomotor defects [50]. It is of interest that *Drosophila* lacks endogenous α-synuclein, and therefore they may also be deficient in the machinery that normally limits the tendency of this protein to aggregate *in vivo*. More recently, a transgenic mouse model expressing truncated α-synuclein shows filamentous aggregates in the substantia as discussed below [60].

Effect of post-translational modifications

Although study of genetic mutations in α-synuclein has been invaluable in understanding the function and pathogenic properties of α-synuclein, they only account for a very small proportion of cases of PD. More than 90% of cases are sporadic and neuropathologically characterised by insoluble fibrils of WT α-synuclein [30, 31]. Similarly, *in vitro* WT α-synuclein aggregates to form fibrils identical to those isolated from disease brains, though to a slower rate than the mutant forms [36, 51]. Therefore, a major challenge in the field of neurodegeneration is to understand what alterations occur during disease which convert normal WT α-synuclein to a toxic species.

Cellular pathways that are involved in post-translational modification of proteins might be relevant in this context. Oxidative stress appears to be one attractive candidate: under certain conditions, free-radical generators such as iron and hydrogen peroxide can stimulate the production of α-synuclein and ubiquitin-positive intracytoplasmic inclusions in cells overexpressing α-synuclein, which contain mixtures of fibrillar and amorphous material [52]. Similarly, inhibition of mitochondrial complex I in rats by chronic intravenous infusion of the pesticide rotenone induces specific neurodegeneration in the SN and formation of α-synuclein inclusions, which closely resemble LBs [53]. Reactive species may be attached to cellular proteins, thus altering their folding properties and function. Accordingly, nitrated α-synuclein species have been reported in the majority of LB pathology [54] and accelerate the fibrillation of WT protein [55]. Abnormal phosphorylation has also been implicated: serine-129 of α-synuclein has been shown to be selectively and extensively phosphorylated in α-synucleinopathy lesions, whereas phosphorylation of α-synuclein at serine-129 promoted fibril formation *in vitro* [56].

Truncation of the carboxyl-terminus is another mechanism that has been implicated in toxic gain of function of WT protein. Carboxy-teminally truncated α-synuclein forms filaments at a faster rate than the full-length protein [36, 57, 58]. Furthermore, carboxy-terminally truncated α-synuclein has been detected in LBs in human diseases and in the brains of transgenic mice expressing mutant human α-synuclein [43, 44, 59]. The significance of this modification was recently investigated by generation of transgenic mice expressing truncated human WT α-synuclein 1–120 using the TH promoter [60]. These mice developed a mixture of granular and fibrillar intracytoplasmic aggregates, progressive morphological changes in neurones of the SN and a microglial reaction similar to authentic human disease. Neurochemically, there was a decline in striatal dopamine levels with increasing age, which was paralleled by an age-related reduction in spontaneous locomotion and an increased response to amphetamine [60]. This animal model is the first to demonstrate a direct link between α-synuclein aggregates confined within brain areas primarily affected in PD, and a progressive behavioural deficit. The possibility that post-translational modifications during disease process may at least partly be secondary phenomena resulting from aggregation of α-synuclein cannot be excluded at present. This notwithstanding, it is now clear that the carboxy-terminal region of α-synuclein is a negative regulator of self-assembly. Therefore, modifications in this region, such as oxidation, nitration and phosphorylation [54, 56, 61], may influence the propensity of α-synuclein to aggregate *in vivo* in a similar way to truncation. The same is true of molecules which bind to the monomeric protein. Thus, polyamines have been shown to promote the aggregation of α-synuclein through binding to its carboxy-terminal region [62, 63]. Other positively charged molecules may act through a similar mechanism [64]. Dopamine also binds to the carboxy-terminal part of α-synuclein, and it has been suggested that this interaction could prevent α-synuclein aggregation in the SN in animal models where full-length protein is used [65].

Abnormal protein degradation is another mechanism that has been implicated in the formation of LBs but the exact mechanism is currently unclear. Evidence from polyQ proteins suggests that accumulation of misfolded proteins can overwhelm the ubiquitin-

proteasome system, leading to aberrant degradation [66, 67]. Similarly, binding of α-synuclein filaments and soluble oligomers to the proteasome results in marked inhibition of its chymotrypsin-like hydrolytic activity [68]. Monomeric WT α-synuclein in transfected cells is not a substrate for ubiquitination but instead can be directly degraded by the 20S proteasome in a ubiquitin-independent manner [69]. This process is slowed down by nitrosylation of monomeric α-synuclein [55] and under certain conditions can lead to generation of incompletely degraded, C-terminal truncated α-synuclein species [70]. In LB disease a modified form of α-synuclein of 22–24 kDa is the substrate of predominantly mono- or di-ubiquitination [59]. Taken together, these data suggest that ubiquitin-dependent degradation is unlikely to be a major physiological mechanism for α-synuclein degradation. Rather, ubiquitination of LB-associated α-synuclein most likely represents a disease-specific pathway. In this respect, ubiquitination could represent an unsuccessful 'last-ditch stand' of cells in their attempt to unfold and/or degrade misfolded proteins either through the 26S proteasome, which requires poly-ubiquitination, or the lysosome, which requires mono-ubiquitination. On the other hand, directed expression of the molecular chaperone Hsp70 prevents dopaminergic neuronal loss associated with α-synuclein toxicity in *Drosophila* [71]. Overexpression of co-chaperone carboxyl-terminus of Hsp70-interacting protein (CHIP) in cell culture inhibits α-synuclein inclusion formation and reduces protein levels via the proteasome and lysosome systems [72]. Finally, the role of lipid membranes in the conversion of WT α-synuclein to a pathogenic protein has been extensively investigated. Detergent-stable oligomers of α-synuclein have been found specifically in the brains of patients with PD and recombinant α-synuclein forms multimers *in vitro* upon exposure to vesicles containing certain polyunsaturated fatty acid (PUFA) acyl groups [73]. Furthermore, exposure of mesencephalic neurons to PUFA increases oligomerisation of the protein *in vivo* [74]. These oligomers precede the formation of insoluble fibrilar aggregates and can bind membrane bilayers via electrostatic and hydrophobic interactions and transiently permeabilize them [75, 76]. Dysregulation of dopamine homeostasis has been suggested to underlie the vulnerability of dopaminergic neurons in PD. In this respect, overexpression of both WT and mutant α-synuclein in cells isolated from transgenic mice disrupted the vesicular pH and led to a marked increase in the levels of cytosolic catechol species, which in turn can trigger oxidative damage [77]. On the other hand, dopamine stabilizes oligomeric intermediates [78, 79], which can further disrupt the integrity of synaptic vesicles, initiating a vicious circle that eventually leads to aggregation and cell death. It has been shown that overexpression of WT, A53T and A30P α-synuclein in human dopaminergic neurones but not cortical neurones led to 2–2.5-fold increase in apoptosis [80, 81]. However, more recent studies have shown that association of α-synuclein with biological membranes can also protect the protein from oxidation and nitrosylation and thus diminishes the formation of aggregates [82].

Conclusion

Over the last 10 years, since the original identification of α-synuclein as the major component of LB filaments [30], a number of studies on the recombinant WT and mutant protein and various cellular and transgenic animal models have shed light on the physiological function and pathogenic properties of this protein. The unfolded structure and conformational plasticity of α-synuclein are central to its pathogenicity. Numerous studies have indicated an important association with lipid membranes and synaptic vesicles, which taken together with histological localisation suggest that the synaptic terminal is likely to emerge as the primary anatomical substrate of neurodegeneration. Similarly, evidence from in vitro studies and novel transgenic mice [60] has identified the carboxyl-terminal region of α-synuclein as a negative regulator of aggregation and a potential molecular substrate for the toxic gain of function of this protein. The role of dopamine as a modulator of α-synuclein aggregation may help to explain the susceptibility of certain neuronal subpopulations to neurodegeneration. Finally, aberrant proteolysis by the proteasome system as a consequence of extensive modification of α-synuclein in disease may perpetuate rather than limit the pathogenic properties of the protein, and correcting this function could represent a target for therapeutic intervention.

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