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Modification of α-Synuclein by Phosphorylation: A Pivotal Event in the Cellular Pathogenesis of Parkinson’s Disease

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Abstract

Post-translational protein modifications play an important role in generating the large diversity of the proteome in comparison to the relatively small number of genes; phosphorylation being the most widespread. Phosphorylation of proteins regulates important molecular switches for several cellular events and abnormal phosphorylation events are associated in many neurodegenerative diseases. In Parkinson’s disease (PD) the main hallmark is the accumulation of cytoplasmic inclusions, Lewy bodies (LBs), consisting of α-synuclein (α-Syn) and ubiquitin. There’s another key observation which is increasingly gaining prominence is a modified-form of α-Syn; the phospho α-Syn serine129 (pSyn). The significance of pSyn has gained importance in PD because its accumulation is distinctly enhanced in the diseased condition. The revelation of the involvement of pSyn on α-Syn aggregation, LB formation and neurotoxicity is crucial to understanding the pathogenesis and progression of PD. Since some in vitro and in vivo studies have indicated that pSyn is an early event preceding apoptosis, some important questions now needs to be explored in reference to the physiological functions regulated by phosphorylation, such as dopamine synthesis, vesicle mobilization, regulation of synaptic proteins, and synaptic plasticity. An investigation of the role of enzymes on the phosphorylation and clearance of α-Syn and region-specific susceptibility is required to be determined; to identify viable targets for new therapeutics.

Keywords: α-synuclein, phospho α-synuclein serine129, PD, phosphorylation, kinases, aggregation, biomarker, neurotoxicity, Lewy bodies

1. Introduction

Parkinson’s disease (PD) is the second most common diagnosed neurodegenerative disease [1] with a prevalence of about 1% at the age of 65 and of 4–5% by the age of 85 [2]. The clinical
manifestations of classical PD are rest tremor, rigidity, bradykinesia, and postural imbalance. The loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) together with a distinct decrease in striatal dopamine, and the occurrence of cytoplasmic eosinophilic inclusions called Lewy bodies (LBs) are considered the pathological hallmarks of PD [3]. There are also reports of other neuronal cell losses in locus coeruleus and olfactory lobe during the development of the disease [4]. It has however been identified that the clinical manifestation of motor symptoms appears with the loss of DA neurons in the midbrain [5]. According to Rodriguez-Oroz et al. [6], the anatomical-functional basis of the main clinical manifestations is related to the low level of dopamine concentration in contralateral striatum and the malfunction of dopamine circuits. Current medications for PD supplement dopamine (L-DOPA), or activate DA receptors (DA-receptor agonist), or inhibit the degradation of DA (monoamine oxidase B inhibitor and catechol-O-methyltransferase inhibitor), bringing about temporary abatement of motor symptoms but failing to delay or halt disease progression. The etiology of PD is still unknown: it comprises familial (fPD) forms accounting for less than 10% of all PD cases, and the far more common sporadic (sPD) form. The striking feature in both fPD and sPD is α-synuclein (α-Syn) aggregation with ubiquitin that eventually progresses to form LBs. Three missense mutations (A53T, A30P, and E46K) [7–9] of α-Syn are known to cause autosomal dominant fPD [10], probably through a gain-of-function mechanism. Moreover, overexpression of human α-Syn in mice results in progressive loss of DA terminals in the basal ganglia and accumulation of LB-like structures in neurons [11]. Mechanisms that might control α-Syn aggregation in sPD are not clear, but may include transcription factor dysregulation [12] and the inability of normal degradation pathways to function adequately [13]. El-Agnaf et al. [14] detected α-Syn species in live-human sPD and fPD patient plasma and cerebrospinal fluid.

2. α-Syn structure and function

Syn are a vertebrate-specific family of abundant neuronal proteins. They consist of three closely related members, α-, β-, and γ-Syn, of which α-Syn has been the prime focus ever since mutations in it were recognized as a basis for fPD. Syn is a highly conserved protein with a molecular weight of approximately 14 kDa, comprising 140 amino acids [15]. This heat-resistant, soluble, acidic protein is abundant in the presynaptic terminals of central nervous system (CNS) neurons expressed pre-dominantly in the neocortex, hippocampus, SNpc, thalamus, and cerebellum [16–18]. Unlike α- and β-synuclein, γ-Syn is not concentrated in presynaptic terminals [19] and is largely found in the peripheral nervous system (PNS).

α-Syn is composed of three distinct domains: an N-terminal amphipathic repeat region that can form α-helices; a hydrophobic central segment; and a C-terminal acidic region (Figure 1). The highly conserved N-terminal domain (residues 1–65) includes 6 copies of an unusual 11 amino acid repeat that display variations of a KTKEGV consensus sequence. It is unordered in solution, but can shift to a α-helical conformation [20] comprising two distinct α-helices.
interrupted by a short break [21]. α-Syn binds strongly to negatively charged phospholipids and becomes α-helical [22, 23], suggesting that the protein may normally be associated with the membrane [24]. This N-terminal domain includes the three sites of the fPD mutations A30P, E46K, and A53T (Figure 1).

The hydrophobic central segment of α-Syn (non-amyloid component (NAC), residues 66–95) (Figure 1) [18] is the second major component of brain amyloid plaques in Alzheimer’s disease (AD) [18, 24]. This region consists of three repeats including the highly amyloidogenic part of the molecule that is responsible for α-Syn’s ability to undergo a conformational change from random coil to β-sheet structure [25] and to form Aβ-like protofibrils and fibrils [24, 25]. These properties differentiate α-Syn from β-Syn and γ-Syn, which fail to form co-polymers with α-Syn [24]. The NAC region carries a phosphorylation site on Ser87 [26].

The acidic C-terminal domain (residues 96–140) of α-Syn has a strong negative charge composed primarily of acidic amino acids [20], but has no known structural elements. It consists of an acidic domain rich in proline residues (residues 125–140) that seems critical for the chaperone-like activity of α-Syn [27], as demonstrated by deletion mutants of the C-terminal region in which the α-Syn chaperone activity is lost [27–29]. In contrast to the amphipathic N-terminal and hydrophobic NAC regions, which are highly conserved between species, the C-terminal region is variable in size and in sequence [28–31]. This region is also organized in random structure in most conditions and contains several phosphorylation sites: Ser129, Tyr125, Tyr133, and Tyr136 [32] (Figure 1).

Figure 1. Schematic representation of α-Syn amino acid sequence (1–140) with amphipathic NH2-terminal, non-amyloid component (NAC) region and acidic tail –COOH terminal. Arrows in the N-terminal, points to the three pathogenic mutations, and P in a red circle of NAC and C-terminal, points to the sites of phosphorylations.

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Although the normal functions of α-Syn are still being defined, several studies have shown that this protein has a key role to play in membrane-associated processes at the presynaptic level such as formation and maintenance of synaptic vesicle pools (Figure 2), regulation of lipid metabolism, and Ca\(^{2+}\) homeostasis [31–33]. Greten-Harrison et al. [34] using αβγ-Syn knockout mice has reported that deletion of Syns causes alterations in synaptic structure and transmission, age-dependent neuronal dysfunction, as well as diminished survival. In vivo and in vitro studies showed that abrogation of Syn expression decreased excitatory synapse size by ~30%, revealing that Syns are important determinants of presynaptic terminal size [34]. Younger synuclein-null mice show better basic transmission in comparison to older mice that showed a pronounced deterioration. Interestingly, it is further reported that the late onset phenotypes in Syn-null mice were not due to a loss of synapses or neurons but rather a reflection of specific changes in synaptic protein composition and axonal structure.

Figure 2. Schematic representation of roles of α-Syn under physiological and pathological conditions. In physiological condition, (1) α-Syn maintains synaptic functions by associating with (2) vesicle formation, (3) trafficking and (4) docking. It also associates with (5) recycling of synaptic vesicle and (6) dopamine storage. The post-translational modification of α-Syn such as (7) phosphorylation and dephosphorylation, leads to activation and deactivation of the protein. This protein undergoes (8) lysosome autophagy (9) and (10) proteosomal degradation directed by ubiquitination. However, in pathological conditions (1) due to uncertain stimulus, α-Syn undergoes (2) misfolding, (3) mutation or (4) phosphorylation leading to the (5) aggregation of the protein affecting the (6) vesicle formation, trafficking and docking, (7) impaired lysosomal autophagy, (8) and (9) ubiquitination and inhibition of proteosomal degradation. This in turn results in (10) LB formation and (11) apoptosis.
Chandra et al. [21] found selective decreases in two small synaptic signaling proteins, complexins, and 14-3-3 proteins, in α,β double-KO mice. In 2000, Abeliovich et al. [2] have shown that mice lacking α-Syn display functional deficits in the nigrostriatal dopamine system. The α-Syn−/− mice were reported to be viable and fertile, exhibited intact brain (Aβ) architecture, and possessed normal complement of DA cell bodies, fibers, and synapses; however they displayed a reduction in striatal DA and an attenuation of DA-dependent locomotor response to amphetamine [2]. Further Drolet et al.’s [35] work showed that mice lacking α-Syn have an attenuated loss of striatal dopamine following prolonged chronic 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) administration. In addition, ultrastructural analysis and imaging studies have shown reduced synaptic vesicle density at the active zone, and imaging further reveals a defect in the re-clustering of synaptic vesicles after endocytosis [36]. Increased levels of α-Syn thus produce specific, physiological defects in synaptic vesicle recycling that precedes detectable neuropathology.

α-Syn also has been suggested to function as a chaperone protein in vivo, because it appears capable of interacting with a variety of ligands and cellular proteins apart from lipids [35, 37, 38], thus modifying their activities. The N-terminal portion of α-Syn shares 40% amino acid homology with molecular chaperone 14-3-3 [36], suggesting that the two proteins could sub-serve the same function. Eliezer et al. [23] had showed that the removal of the C-terminal acidic tail of α-Syn abolished its chaperone activity. In contrast, some reports indicate that the C-terminal acidic tail is indeed necessary but not sufficient for the chaperone function of α-Syn [28, 29]. In normal physiological conditions, α-Syn exists in monomeric form and is recognized and cleared via the ubiquitin-proteasome system (UPS) and chaperone-mediated autophagy (CMA) pathways [39] (Figure 2). In the pathological state, misfolding or mutations of α-Syn (A30P/A53T) lead to the formation of pathologically modified species that bind with several cytoplasmic proteins and ultimately aggregate into LBs in the DA neuronal cells [40] (Figure 2). This aberrant level of α-Syn is also cited in idiopathic PD subjects [41].

2.1. Phosphorylation of α-synuclein at serine129

Protein phosphorylation is a reversible post-translational modification of proteins that has an important role in regulating structural and functional properties of proteins in health and disease. It is primarily associated with signaling pathways and cellular processes in all aspects of cell biology such as cell-cycle progression, differentiation, apoptosis, metabolism, transcription, cytoskeletal arrangement, intercellular communication, motility and migration [42–44]. In eukaryotes, the amino acids that are most commonly reported to be phosphorylated are serine, threonine and tyrosine [45, 46] with few reports suggesting phosphorylation at arginine, lysine and cysteine residues [45, 47, 48].

In PD too, phosphorylation appears to play an important role in fibrillogenesis, LB formation, and neurotoxicity of α-Syn in vivo [26, 49–51]. The majority of α-Syn in inclusions and LBs isolated from patients with PD and other synucleinopathies is phosphorylated at Ser129 (S129-P) [26, 49, 51]. Overexpression of wild-type or mutant α-Syn in in vivo and in vitro models showed expression of immunopositive phospho α-Syn serine129 (pSyn) in their proteinaceous inclusions [52–54]. Mass spectrometric analysis of α-Syn isolated from patients with
synucleinopathy lesions has also confirmed that the protein is phosphorylated on Ser129 [49, 50]. More than 90% of α-Syn is phosphorylated in PD patients’ brain as opposed to only 4% of phosphorylated α-Syn detected in brains of healthy subjects. Moreover, the phosphorylated α-Syn in LBs is usually ubiquitinated [50, 51, 55]. The fact that most of the α-Syn is not phosphorylated under physiological conditions in vivo suggests that α-Syn phosphorylation at serine129 contributes to the pathology of the disease [49, 56, 57].

2.2. Phosphorylation and CNS

The central processing unit of the human body is its CNS consisting of specialized cells called neurons relaying electrical and chemical signals to all parts of our body [58]. However, the most abundant cell type in the CNS is the glial cells comprising of astrocytes, oligodendrocytes and microglia [59, 60]. In addition, it is interspersed with microvasculature that provides the nutrients and support to these CNS cells. The central theme in CNS function is equilibrium among these various cell types to maintain optimal synaptic strengths, neuronal firing rates, and neurotransmitter release. The regulation of these functions can be either through inside-out or outside-in stimuli and are strongly associated with several signaling pathways within these cells. Virtually every class of neuronal protein is regulated by phosphorylation and most types of extracellular signals, including neurotransmitters, hormones, light, electrical potential, extracellular matrix, neurotrophic factors, and cytokines, can produce diverse physiological effects by regulating the phosphorylation of specific phosphor-proteins in their target cells. These extracellular signals modify the activity of protein kinases and/or phosphatases either directly (e.g. receptors with kinase activity) or via cascades of enzymatic reactions (e.g. receptor → G protein → enzyme – second messenger – protein kinase).

2.3. Kinases involved in α-synuclein phosphorylation

α-Syn phosphorylation can be induced by several kinases. Serine129 of α-Syn can be phosphorylated by G protein-coupled receptor kinases (GRK1, GRK2, GRK5, and GRK6) [61–63], casein kinases 1 and 2 (CK1 and CK2) [26, 64–69], and the polo-like kinases (PLKs) [70]. Current studies have shown that, GRKs may also phosphorylate non-receptor substrates, comprising the four members of the Syn family (α-,β-,γ-Syn, and synoretin) in addition to phosphorylating agonist-occupied G protein-coupled receptors (GPCRs) [62]. Overexpression of GRK2 or GRK5 in COS-1 cells, showed that these kinases phosphorylate α-Syn at serine129 [62]. Endogenous GRK-induced phosphorylation of α-Syn at serine129 was demonstrated in vitro in HEK293 cells, and GRK3 and GRK6 were seen to be playing the main roles in this modification [63]. Post-mortem analysis showed that GRK5 co-localized with α-Syn in the LBs of the SNCp of PD patients, but was not detected in cortical LBs of Dementia with Lewy bodies (DLB), or in the glial cytoplasmic inclusions of MSA [61]. Overexpression of α-Syn in SH-SY5Y cells and human α-Syn expressing transgenic mice also showed an increase in GRK5 protein [71]. A genetic association study revealed a haplotype association of the GRK5 gene with susceptibility to sPD in the Japanese population [61]; however, GRK5 polymorphisms in southern Italy failed to correlate with sPD [72]. The knockdown of endogenous GRK5 in SH-SY5Y cells fails to suppress the phosphorylation of α-Syn completely [71], confirming the involvement of other kinases in this phosphorylation.
The other group of kinases that phosphorylates α-Syn at serine129 is CK1 and CK2. This has been demonstrated in the yeast model [69], in mammalian cells [26, 68] and in rat primary cortical neurons [64]. It has been suggested that phosphorylation of serine129 in α-Syn by CK2 may promote \textit{in vitro} fibrillation [59] and \textit{in situ} inclusion formation [73]. Phosphorylation by CK2 and dephosphorylation by PP2C in \textit{in vitro} model indicate that these may be important enzymes that regulate the phosphorylation of α-Syn [74]. Furthermore, surplus α-Syn can form inclusions that sequester CK1, diminishing CK1 activity and aggravating synaptic defects, generating a vicious toxic cycle. CK1 has been found to co-localize with pS87 in transgenic mice and in LB-like structures in LBD/PD-diseased brains [75, 76], and phosphorylates α-Syn at serine87 as well [26]. Oxidative stress induced by iron is reported to upregulate CK2 that leads to increased phosphorylated α-Syn serine129 with an associated increase in oligomerization and inclusion formation [65]. Smith et al. [66] have shown in SH-SY5Y cells that the increase in α-Syn phosphorylation under oxidative stress is mediated by CK2 and correlates with enhancement of inclusion formation.

\textit{In vitro} studies employing kinase assays showed involvement of another family of cellular kinases in the phosphorylation of α-Syn at serine129: PLK1, PLK2, and PLK3 [70, 77]. The PLKs comprise a family of conserved Ser/Thr protein kinases that are known to play a role in cell-cycle regulation and cellular response to stress and carcinogenesis [78]. PLK2 directly phosphorylated α-Syn at serine129 in an \textit{in vitro} biochemical assay [70]. Inhibitors of PLK kinases inhibited α-Syn phosphorylation both in primary cortical cell cultures and in mouse brain \textit{in vivo}. Further, using PLK2 KO mouse, Buck et al. [79] too have shown that PLK2 plays a key role in Ser129 α-Syn phosphorylation in mouse brain. Aubele et al. [80] have shown in an \textit{in vivo} model that brain-permeable Plk-2 inhibitors reduce α-Syn phosphorylation in rat brain. In response to synaptic activation, PLK2 and PLK3 expression is reported that is associated with synaptic plasticity, remodeling, and homeostasis [81, 82], thus suggesting that these kinases could play an important role in modulating the normal physiology of α-Syn. Leucine-rich repeat kinase 2 (LRRK2) is also known to pSyn [83], but this remains debatable as there are no other studies confirming this, despite the existence of a clear interaction between the two proteins [83, 84].

\textbf{2.4. Phosphorylation at serine129 modulates α-synuclein protein-protein interaction}

The C-terminal domain of α-synuclein (residues 96–140) is an acidic tail of 43AA residues, containing 10 Glu and 5 Asp residues. C-terminal truncations of α-Syn induce aggregation, suggesting that C-terminal modifications might be involved in the pathology of α-Syn [85]. An interaction between the C-terminal domain and the NAC region of α-Syn is postulated to be responsible for the inhibition of α-Syn aggregation. Moreover, there are several studies on the interaction of α-Syn C-terminal tail with different proteins [86–91]. McFarland et al. [92] were the pioneers to address this using targeted functional proteomics [51]. The authors showed that the non-phosphorylated α-Syn peptide primarily interacts with proteins related to the mitochondrial electron transport chain (ETC) (complex I, III, and IV proteins of the ETC) [51]. It was hypothesized that changes in α-Syn phosphorylation could represent a response to biochemical events associated with PD pathogenesis. Among these, mitochondrial complex I dysfunction, oxidative stress, and proteasome dysfunction are processes that are known to...
be involved in synucleinopathies [93, 94]. The low levels of pSyn under physiological conditions as well as the absence of other phosphorylated residues such as pY39, pS87 and pY125 [26, 49, 50] suggest a faster degradation of this form under normal conditions. In fact, the phosphorylation status of α-Syn was recently correlated with clearance mechanisms [95, 96]. Another group Chau et al. [97] too reported that α-Syn phosphorylation at serine129 is toxic to DA cells and both the levels of serine129 phosphorylated protein as well as its toxicity are increased with proteosomal inhibition, emphasizing the interdependence of these pathways in PD pathogenesis.

However, the pSyn has a greater affinity for certain cytoskeletal and presynaptic proteins associated with synaptic transmission and vesicle trafficking [51]. Yin et al. [98] showed that α-Syn interacts with the switch region of Rab8a, a small guanine nucleotide-binding protein, in a serine129 phosphorylation-dependent manner; thus implicating its role in coordinating vesicle trafficking. Hara et al. [99] reported that serine129 phosphorylation of membrane-associated α-Syn modulates dopamine transporter function in a G protein-coupled receptor kinase-dependent manner. These observations suggest that pSyn could serve as a molecular switch to control α-Syn interaction with different protein partners and therefore may modulate the function of DA neurons. However, further investigations are required to assess the impact and the physiological consequences of serine129 phosphorylation on α-Syn interaction with other proteins, such as SNARE proteins [35, 100], cytoskeletal proteins (i.e. tubulin) [55, 101] and other amyloidogenic proteins (i.e. tau) [19, 102]. Jensen et al. [103] have hypothesized that an interaction between α-Syn and tau could link synaptic vesicles with microtubules. It is hypothesized that tau could bring Src PTK family members such as Fyn into closer proximity to α-Syn, thereby enhancing the activity of these kinases for α-Syn. Samuel et al. [105] showed that the membrane binding of α-Syn monomers was differentially affected by phosphorylation depending on the PD-linked mutation. WT α-Syn binding to presynaptic membranes was not affected by phosphorylation, while A30P α-Syn binding was greatly increased and A53T α-Syn was marginally lower, implicating the distal effects of the carboxyl terminal on amino-terminal membrane binding. The un-phosphorylated form of serine129 associates mainly with mitochondrial electron transport proteins, while the phosphorylated form associates with cytoskeletal, vesicular trafficking proteins and enzymes involved in protein serine phosphorylation [92]. Further work by Sugeno et al. [106] using α-Syn-over expressing cells exposed to a low dose of rotenone as an environmental toxin, showed that phosphorylation of α-Syn at serine129 promoted intracellular aggregate-formation and induced ER stress that was followed by mitochondrial damage and apoptosis.

Phosphorylation also seems to play an important role in the regulation of α-Syn axonal transport as the serine129D mutation significantly decreases its rate of transport in neurons, probably due to the modulation of α-Syn interaction with motor and/or accessory proteins involved in this process [107]. Moreover, the interplay between the different phosphorylated residues could increase the diversity in the possible protein interactors. Several differences were observed in the set of proteins that were found to interact with serine129 and Y125-phosphorylated forms of α-Syn [92]. S129 and Y125 residues both residing in the C-terminal region of α-Syn have been implicated in the majority of α-Syn interactions with proteins.
[103, 108, 109], reinforcing the significance of phosphorylation in these residues in modulating the biological role of α-Syn. All these findings together suggest that phosphorylation of α-Syn at serine129 has a widespread effect on protein-protein interaction of α-Syn.

Phosphorylation also seems to alter the subcellular localization of α-Syn. pSyn was found to be preferentially localized in the nuclei of DA neurons in rat and mouse models of synucleinopathy [67, 100]. In studies using PD rat models, the phospho-resistant S129A was found to be localized in the nucleus at higher levels than the S129D form, and was found to correlate with enhanced toxicity [110, 111]. Our group too demonstrated the nuclear localization of pSyn in SH-SY5Y cells under 6-hydroxydopamine toxicity [112]. Gonçalves and Outeiro [113] showed that S129 phosphorylation modulates the shuttling of α-Syn between nucleus and cytoplasm in human neuroglioma cells, using photo-activatable green fluorescent protein as a reporter. Moreover, the study showed that co-expression of α-Syn with different kinases altered the translocation dynamics of the protein. While G protein-coupled receptor kinase 5 (GRK5) promotes the nuclear localization of α-Syn, PLK2 and three modulate the shuttling of the protein between the nucleus and cytoplasm [114]. This difference reflects different α-Syn phosphorylation patterns in serine129 and/or other residues. Although the function of α-Syn in the nucleus is still unclear, it appears to be related to pathological insults. In particular, nuclear localization of α-Syn increases under oxidative stress conditions [112, 114, 115]. Nuclear α-Syn interacts with histones, inhibits acetylation, and promotes neurotoxicity [116, 117]. Furthermore, α-Syn may act as a transcriptional regulator, binding promoters such as PGC1-α, a master regulator of mitochondrial gene expression [106]. The significance of pSyn in regulating nuclear proteins still needs to be unraveled.

2.5. Oxidative stress and α-Syn phosphorylation

Oxidative stress can increase α-Syn phosphorylation [97]. Perfeito et al. [118] showed through an in vitro model that exposure to ferrous iron and rotenone resulted in increase in pSyn. A similar increase was reported by Ganapathy et al. [112] in the presence of the endogenous toxin 6-hydroxydopamine. Proteosomal inhibition by epoxomycin and increased oxidative stress by paraquat treatment has led to increases in pSyn [97]. This may reflect either an increased activity of the kinase responsible or a decrease of phosphatase activity. Under physiological conditions, α-Syn is degraded by chaperone-mediated autophagy [119], and studies suggest that this gets reduced upon oxidation or nitration [12, 106]. α-Syn may also be degraded by proteasomes [120]. The increase in pSyn levels in LBs suggests a change in the turnover or degradation of the phosphorylated protein (Figure 3). It is possible that at elevated levels, this phosphorylated species may be toxic.

2.6. α-Syn phosphorylation at serine129 and cellular events

The role of α-Syn phosphorylation in the cellular pathogenesis of PD remains debated [92, 110, 111, 121, 122]. This apparent controversy is due to the fact that phosphomimics (S129D/E) do not reproduce the exact properties of the endogenous authentically phosphorylated α-Syn [122–124]. The expression of a variant showing prevention of phosphorylation by site-directed mutagenesis of serine129 to alanine (S129A) caused an increase in α-Syn inclusions
On the other hand, several investigators have reported that the expression of S129A mutant protein led to fewer inclusions [66, 94, 110]. It has also been reported that the expression of phosphorylation mimicking serine129 to the aspartate (S129D) variant does not show DA deficits [92, 110, 111, 121, 123]. Hence, several groups have sought to address the issue by overexpressing α-Syn and using siRNA for its natural kinases [99, 118, 124]. As discussed in the earlier section, among the kinases primarily responsible for the α-Syn phosphorylation at serine129 are CKs, GRKs, LRRK2, and PLKs. The modulation of phosphorylation of α-Syn has also been reported by targeting the kinases in A53T mutant α-Syn expressing cells [99, 118, 124–126]. siRNA studies targeting kinases such as PLK, GRK2, and CK2 have been used to study the effect of phosphorylated α-synuclein on WT and A53T mutant α-synuclein expressing cells [99, 118, 124, 125, 127]. These studies have shown the effect of α-Syn phosphorylation with respect to ROS generation, mitochondrial alterations, proteasomal changes, and dopamine transport. The work of Perfeito et al. [118] suggest that stimuli that promote ROS formation and mitochondrial alterations highly correlate with mutant α-Syn phosphorylation at serine129, which may precede cell degeneration in PD. Similarly, we have shown in our recent work that at sub-lethal 6-hydroxydopamine concentrations, the decrease in resting vesicles (VMAT2) and vesicular dopamine release are not attributable to apoptotic cell death and occur concomitantly with the phosphorylation of α-Syn [112].

Figure 3. Schematic representation of suggested pathological role of oxidative stress in the α-Syn phosphorylation and aggregation. Normally genetic mutation, neurotoxins, dopamine auto oxidation in the presence of excess iron and the release of reactive oxygen species (ROS) leads to mitochondrial dysfunction and oxidative stress in turn leading to cell death. However, increase in the oxidative stress and direct effect of neurotoxins can lead to phosphorylation of α-Syn and their aggregation which in turn inhibits the lysosomal autophagy and impaired proteosomal degradation resulting in LB formation and cell death.
2.7. α-Syn phosphorylation at serine129 during PD pathogenesis: an early or late event?

The evidence of pSyn accumulation in the brain has been collected largely from post mortem analysis and it fails to answer if this accumulation occurs during the early or late stages of synucleinopathies. In a recent work, Walker et al. [127] investigated how pSyn levels and solubility change in cingulate and temporal cortex of DLB patients, at different stages of the disease. The authors reported a progressive accumulation of pSyn-immunoreactive species in diseased brains compared to healthy controls, as well as a positive correlation between pSyn levels and the severity of disease symptoms. A similar study using brain samples from PD patients also reported a drastic accumulation of pSyn-positive inclusions in different brain regions at the late stages of the disease [128]. Together, these results suggest that the occurrence of pSyn is linked to the severity of disease progression.

In our recent work, we demonstrated using biophysical and biochemical analysis in an in vitro model that under sub-lethal concentrations of 6-hydroxydopamine, phosphorylation of α-Syn precedes apoptosis and occurs concomitantly with the decrease in vesicular dopamine release [21]. This study provides a new perspective on the occurrence of pSyn even in early stages of the disease that may contribute to the impairment of neuronal function. Another recent work by Takahashi et al. [53] demonstrated increased accumulation of pSyn in the motor cortex of normal aging mice along with early onset motor impairment, which was further ameliorated by coenzyme Q.

2.8. pSyn in human fluids and PNS of synucleinopathies

Several studies in the field of PD and diagnosis of Parkinsonism are based on the fundamental molecular events associated with or without LBs. Total α-Syn quantification in the CSF of PD, DLB, and MSA patients in comparison to healthy controls has been proposed as a biomarker for α-Syn-related disorders [129–131]. However, an ideal biomarker for a particular disease must be easy to detect, and also reflect disease onset and progression with associated primary changes. A longitudinal study conducted by Foulds et al. [132] in the blood plasma of patients suffering from PD showed that total α-Syn in blood plasma of PD patients remained similar to that in normal individuals, but the level of α-Syn phosphorylated at serine129 was significantly higher in PD patients. Statistical analysis confirmed the usefulness of plasma levels of pSyn in discriminating patients with PD from healthy controls. In addition, pSyn inclusions were also detected in the PNS which might also serve as a useful diagnostic test for PD and related synucleinopathies. Work from two independent groups on skin biopsies showed that the majority of PD patients had accumulation of pSyn in small and large nerve fibers, while no signal was detected in healthy controls and in MSA or essential tremor control subjects [133, 134]. This cutaneous pathology was correlated with the progression of disease symptoms suggesting the use of this peripheral marker as a biomarker for the disease [135]. Presence of pSyn immunoreactivity was detected in gastric, duodenal and colonic biopsies [136, 137]. Hilton et al. [137] further reports that this accumulation of pSyn in the bowels of patients is detected in the pre-clinical phase of PD. Taken together, these reports suggest that accumulation of pSyn might provide a more reliable biomarker to detect PD at early stages and further discriminate between synucleinopathies, compared to total α-Syn.
3. Conclusion

In comparison to the relatively small number of genes, the large diversity of the proteome is achieved mainly by post-translational protein modifications, phosphorylation being the most widespread. Since the C-terminal region of α-Syn is involved in interaction with proteins [103, 104, 109] and metal ions [138–140], any phosphorylation in this site can alter its interaction capability [77]. The significance of α-Syn phosphorylation at serine129 has gained importance in PD because its accumulation is distinctly enhanced in the diseased condition. The revelation of the involvement of pSyn on α-Syn aggregation, LB formation, and neurotoxicity is crucial to understanding the pathogenesis and progression of PD and related disorders. Since some in vitro and in vivo studies have indicated that the pSyn is an early event preceding apoptosis, some important questions now need to be explored in reference to the physiological functions regulated by phosphorylation such as dopamine synthesis, vesicle mobilization, and regulation of synaptic proteins, synaptic plasticity, and its subcellular localization. A systemic investigation of the role of each kinase on the phosphorylation of α-Syn and α-Syn clearance also needs to be carried out to identify viable targets for development of new therapeutics. A further in vivo study by Hirai et al. [57] elevates the significance of pSyn as a region-specific phenomenon, and the level of pSyn in response to physiological stimuli is selectively higher in the striatum region in comparison to cortex and hippocampus [57]. The relative sensitivity of this phenomenon in the striatum in reference to PD pathology needs to be assessed to address the increased susceptibility of some brain regions to α-Syn phosphorylation and pathology. In addition, the synergistic association of PD-linked mutations and pSyn in the regulation of α-Syn toxicity needs to be evaluated. Investigation of the possible implications of phosphorylation of α-Syn on cell-to-cell transmission and its pathological propagation in PD-diseased brains is also pending. Thus, the research evidence clearly suggests that the phosphorylation of α-Syn plays a significant and possibly pivotal role both in PD pathogenesis and progression, and that answers to the questions above are crucial for the identification of novel, disease-modifying, therapeutic targets for the treatment of PD and related synucleinopathies.

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