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α -synuclein, LRRK2 and their interplay in Parkinson's disease

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Abstract

Of the various genetic factors contributing to the pathogenesis of Parkinson's disease (PD), only mutations in *α -synuclein* (*α -syn*) and *LRRK2* genes cause clinical and neuropathological phenotypes closely resembling the sporadic cases. Therefore, studying the pathophysiological functions of these two PD-related genes is particularly informative in understanding the underlying molecular pathogenic mechanism of the disease. PD-related missense and multiplication mutations in α -syn may cause both early- and late-onset PD, whereas various PD-related *LRRK2* missense mutations may contribute to the more common late-onset PD. While intensive studies have been carried out to elucidate the pathogenic properties of PD-related mutant *α -syn* and *LRRK2*, our knowledge of their normal functions and their potential genetic interplay remains rudimental. In this review, we summarize the progress made regarding the pathophysiological functions of *α -syn*, *LRRK2* and their interaction in PD, based on the available literature and our unpublished observations.

Keywords

14-3-3; α -synuclein; actin; autophagy; ER; Golgi apparatus; leucine-rich repeat kinase 2; Lewy body; microtubule; mitochondria; Parkinson's disease; proteasome

Parkinson's disease

Parkinson's disease (PD) is the most common degenerative movement disorder, which was initially described by Dr James Parkinson in the 19th century [1]. PD affects more than 1.5% of the population over 65 years old [2,3]. The clinical symptoms of PD include bradykinesia, resting tremor, rigidity and postural instability, with psychiatric and cognitive presentations in some patients. Pathologically, PD is characterized by the progressive death of midbrain dopaminergic (DA) neurons in the substantia nigra pars compacta (SNc) and the formation of abnormal intracellular protein aggregates, named Lewy bodies (LBs) and Lewy neurites (LNs), in the remaining DA neurons [2]. α -synuclein (α -syn) is enriched in LBs and LNs [4]. Interestingly, only familial cases with dominant genetic mutations in *α -syn* and *LRRK2* develop typical PD pathology, including SNc DA neuronal loss and LB/LN formation [5]. Therefore, understanding the pathophysiological functions of α -syn and *LRRK2* may

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provide critical molecular insights into the pathogenesis of PD, which is required to develop any mechanism-based therapeutic strategies to ameliorate this devastating illness.

α -synuclein

More than a decade ago, a substitution of alanine (A) at residue 53 with threonine (T) in α -syn was identified in a familial case of early onset PD, which provided the first evidence of a genetic cause of PD [6]. Following this lead, two other missense mutations in α -syn, A30P and E46K, have subsequently been linked to PD [7,8]. In addition, duplication of an α -syn gene locus region on one allele of chromosome 4, which results in three copies of the α -syn gene, was shown to cause PD with onset at late middle age [9]. Meanwhile, triplication of the α -syn gene locus at one allele or duplication of the α -syn gene loci at both alleles, which results in four copies of the α -syn gene, causes much more severe forms of disease with a much earlier onset and shorter survival time [10]. Together, these genetic studies have strongly established a prominent role of α -syn missense and multiplication mutations in causing PD.

In fact, α -syn is one of only a handful of genes consistently linked to both the etiology and pathogenesis of PD [11]. α -syn is an abundant neuronal protein present in neurons of the SNc and other brain regions, which may make up to 1% of all proteins in the cytosol. Within the neuron, α -syn is almost exclusively located in the presynaptic terminals [11], where it interacts with other presynaptic vesicle proteins, such as synphilin-1, CSP α and SNAREs, and may be involved in neurotransmitter release [12–14]. Accordingly, alterations in neurotransmitter release have been reported in α -syn-deficient and α -syn-overexpressing neurons [15,16].

At the molecular level, α -syn protein perhaps occurs in large part as an α -helically folded homo-tetramer of approximately 58 kDa under native conditions [17]. α -syn exists in either free cytosolic or membrane-bound form [18], approximately 15% is membrane-bound in neurons [19]. Both the N- and C-terminal regions of α -syn are tightly associated with membranes [18], despite the protein lacking a transmembrane domain, lipid anchor, or well-defined membrane-binding domain. α -syn preferentially binds to phospholipid liposomes that contain acidic head groups [20]. Using double labeling, Fortin and colleagues found that α -syn is specifically colocalized with the protein and lipid components of lipid rafts [21]. Lipid rafts are involved in formation of membranous organelles, for example, Golgi apparatus, endoplasmic reticulum (ER), plasma membrane, synaptic vesicles and mitochondria [22]. Overexpression of α -syn can cause Golgi fragmentation [23], mitochondrial swelling [24], lysosome enlargement [25], as well as ER stress [26]. It is possible that α -syn may bind to lipid membranes and alter the bilayer structure, which may alter the membrane curvature and destabilize membrane integrity [27,28]. In line with this notion, lipids also represent a significant portion of LBs, and they have been proposed to derive from degraded membrane organelles [29].

In addition to α -syn, two homologous genes named β - and γ -syn have also been found in mammalian cells [30]. α -syn family genes seem to have overlapping functions, only the α -, β - and γ -syn triple knockout mice display significant alterations in synaptic structure and transmission, and show age-dependent neuronal dysfunction and degeneration [31]. By contrast, α -syn single knockouts or a combination of α -syn with β - or γ -syn double knockout mice lack any obvious phenotypes, suggesting that α -syn itself may not play a crucial role in neuronal function and maintenance [15,32]. On the contrary, overexpression of α -syn produces considerable cytotoxicity and impairs synaptic transmission [16,33]. Together, these observations suggest that α -syn-induced neurodegeneration may not be due to a loss-of-function mechanism, but rather a gain of toxic properties.

LRRK2

Dominantly inherited mutations in the *LRRK2* gene are perhaps the most common cause of familial and sporadic PD, which have been identified in approximately 3–5% of familial and 1–3% of sporadic PD cases, although the frequency may vary greatly in different populations [34]. The *LRRK2* gene encodes a rather large protein of 2527 amino acids compared with α -syn. *LRRK2* contains 12 leucine-rich repeats (LRRs) in the N-terminus, a Roc domain and a MAPKKK domain in the middle, and a WD40 domain in the C-terminus [35]. The putative kinase and GTPase activities of *LRRK2* might be important in the pathogenesis of PD. *In vitro* studies indicate that several PD-specific mutations increase *LRRK2* autophosphorylation and phosphorylation of generic protein kinase substrates [36]. For example, the G2019S mutation at the kinase domain of *LRRK2* increases its autophosphorylation by twofold [37]. Meanwhile, a reciprocal regulation of its GTPase and kinase activities seems to influence the function and cytotoxicity of *LRRK2* [35]. While *LRRK2* is mainly a cytoplasmic protein, it is also partially associated with membrane organelles such as mitochondria, ER, Golgi apparatus, endosomes and synaptic vesicles [34,35]. This characteristic distribution of *LRRK2* may indicate an involvement of *LRRK2* in membrane trafficking as suggested by findings in transgenic mouse models [38].

A variety of transgenic mice have been generated to study the function of *LRRK2* [36,38]. However, none of these *LRRK2* transgenic mice have developed PD-like neuropathology, such as formation of LB-like α -syn-positive inclusions and loss of SNc DA neurons. In addition, none of the *LRRK2* knockout mice displayed obvious motor, behavioral or neuropathological phenotypes [39,40]. By contrast, *LRRK2* knockout, as well as G2019S and kinase-dead knock-in mice, developed profound pathological lesions in the periphery tissues, such as the kidney and lung of aged animals [37,41], suggesting that *LRRK2* is more critically involved in maintaining the normal functions of cells in the kidney and lung, which happen to process robust secretory pathways. A further investigation of *LRRK2*'s exact role in protein secretion and degradation in kidney and lung cells may provide significant molecular insights into the pathophysiological functions of *LRRK2* in neurons and PD. Taken collectively, these observations from *LRRK2* knockout and transgenic mice suggest that *LRRK2* might play a rather subtle or selective role in modulating the physiological function and survival of neurons. For example, studies from primary cultured neurons derived from *LRRK2* transgenic and knockout mice demonstrate an involvement of *LRRK2* in axon and den-drite outgrowth during neuronal development, likely through regulating the dynamics of actin assembly or ER/Golgi vesicle trafficking [39,42].

α -synuclein & LRRK2 interplay

Given the critical involvement of α -syn and *LRRK2* in the pathogenesis of PD, many attempts have been made to delineate a potential pathogenic interplay between the two that predisposes SNc DA neurons to be more vulnerable to various cellular stresses seen in PD. Therefore, in this section we will summarize and discuss the potential subcellular organelles and intracellular pathways in which α -syn and *LRRK2* may exert their synergistic effects during neurodegeneration (Figure 1).

Lewy bodies

One of the key pathological hallmarks in PD is the deposition of LBs of which α -syn and its phosphorylated forms are the primary structural components [40,43]. After initial identification of mutant *LRRK2* in PD, considerable efforts have been made to examine the presence of the *LRRK2* protein in LBs [44,45]. These studies estimated that *LRRK2* is present in the periphery of 10–80% of classic LBs. However, these data should be interpreted cautiously, since many of the *LRRK2* antibodies used in these early reports have

not been tested rigorously regarding their specificity and applicability for immunohistochemistry. LRRK2 was also shown to coimmunoprecipitate with α -syn from soluble protein extracts of postmortem brain tissues with apparent LB pathology [46]. While the association of LRRK2 and α -syn was later confirmed in HEK293 cells transfected with both LRRK2 and α -syn under oxidative stress [47]; a direct biochemical interaction between endogenous α -syn and LRRK2 in neurons remains to be determined.

Since the phospho-S129 of α -syn promotes formation of α -syn filaments and oligomers and is enriched in LBs [43,48], it raises the speculation that LRRK2, especially its G2019S kinase-enhancing mutant, may promote the phosphorylation of α -syn at S129. So far, there is only one published *in vitro* report showing that LRRK2 and its putative kinase domain-containing fragments have a significant capacity to phosphorylate recombinant α -syn at S129 [49]. However, other studies both in cell cultures and animal models failed to identify any increase of α -syn phosphorylation at S129 when overexpressing either wild-type or PD-related G2019S LRRK2. These findings indicate that LRRK2 may not promote the formation of α -syn aggregates by directly phosphorylating α -syn at S129.

Taken together, the present studies do not support a direct biochemical interaction between α -syn and LRRK2, or direct phosphorylation of α -syn by LRRK2, which contributes to LB formation and neuronal loss.

14-3-3 family proteins

While α -syn may not directly interact with LRRK2, they may coexist in the same protein complex through association with 14-3-3 proteins [50,51]. The 14-3-3 family of proteins consists of seven isoforms in mammalian species. They comprise 1% of total brain proteins and participate in a large variety of cellular functions by interacting with different protein partners [52].

Interestingly, 14-3-3 proteins share structural homology with α -syn [53] and also accumulate in LBs [54]. 14-3-3 proteins and α -syn can also be coimmunoprecipitated from mammalian brains [50]. Moreover, 14-3-3 proteins appear to interact specifically with the phosphorylated form of α -syn [55]. In one line of *a-syn* transgenic mice, mRNA levels of 14-3-3 isoforms θ and γ were significantly reduced [46]. Under normal physiological conditions, 14-3-3 proteins inhibit apoptosis by binding and inactivating proapoptotic proteins, including the mitochondrial Bcl-2 family member BAD and the transcription factor forkhead [56]. Increased binding to α -syn may potentially sequester 14-3-3 proteins and reduce their anti-apoptotic activity, which may increase neuronal vulnerability to reactive oxygen species generated by endogenous dopamine metabolism [57].

LRRK2 also binds to different isoforms of 14-3-3 proteins upon phosphorylation of 14-3-3 family proteins at residues S910 and S935 [51]. Mutations of these two serine residues to ala-nine prevented 14-3-3 proteins from interacting with LRRK2 [58]. Therefore, LRRK2 protein kinase activity may directly regulate the phosphorylation of 14-3-3 at the S910 and S935 sites, and hence modulate its binding with 14-3-3 isoforms [51]. Indeed, overexpression of the *LRRK2* G2019S mutation in cell lines promoted phosphorylation of 14-3-3 at S910/S935 by threefold [58,59]. However, in *LRRK2* G2019S BAC-transgenic mice, the level of phospho-S935 14-3-3 was rather decreased compared with the control mice [60]. In addition, overexpression of the other *LRRK2* pathogenic mutations, including R1441C, R1441G, R1441H, Y1699C and I2020T, in cell lines also displayed reduced phosphorylation of 14-3-3 at Ser910/Ser935 sites [58,60]. Thereby, LRRK2 may not directly phosphorylate 14-3-3 proteins at S910 or S935. Future studies may also need to further clarify the impact of 14-3-3's phosphorylation on its interaction with LRRK2. In addition, it is tempting to speculate an indirect interaction between α -syn and LRRK2 through a

common adaptor, 14-3-3. Together, the association of 14-3-3 with both α -syn and LRRK2 generates significant interest to further explore the contribution of 14-3-3 family proteins in the pathogenesis of PD.

Actin & microtubule assembly

The cytoskeleton, including actin and micro-tubule networks, is instrumental in establishing and maintaining neuronal morphology and function [61]. The impact of PD-related mutant α -syn and LRRK2 on the dynamics of actin and microtubule assembly has been summarized in a recent review article [39].

Filamentous actin (F-actin) is formed by polymerization of monomeric actin, a globular protein highly conserved across different species [62]. An ATP-dependent rapid remodeling of F-actin is critical in neurite outgrowth and synapse formation as well as plasticity [63]. Actin is found in cytoplasmic α -syn aggregates [64] and α -syn may modulate the polymerization of actin [65]. LRRK2 is also involved in regulating the dynamic arrangement of F-actin assembly. LRRK2 controls the activities of the ezrin, radixin, and moesin (ERM) family proteins via phosphorylation of ERM proteins at residue T558 [66]. The phosphorylated ERM proteins are targeted at the tip of growing neurites, where they tether the underlying F-actin with the cytoplasmic membrane and regulate the dynamics of filopodia protrusion [42]. The *LRRK2* G2019S mutation significantly enhances the phosphorylation of ERM proteins and increases the number of phospho-ERM-positive and F-actin-enriched filopodia. This may perturb the homeostasis of phospho-ERM and F-actin in sprouting neurites critical for neuronal morphogenesis. Recently, LRRK2 was also shown to interact with Rac1 and RhoA, two small GTPases that actively participate in the organization of F-actin cytoskeleton during various cellular processes [67]. However, future studies are needed to investigate whether α -syn and LRRK2 act coordinately in regulating the dynamic assembly and disassembly of F-actin in neurons.

Microtubules are built up with α - and β -tubulin heterodimers [68]. α -syn aggregates are suggested to disrupt the integrity of the microtubule network [69]. Meanwhile, LRRK2 has been shown to physically interact with both α - and β -tubulin through its GTPase domain [70,71]. Moreover, the level of free tubulin is significantly increased in the brain extract of *LRRK2* knockout mice [71], and dramatically decreased in the brain homogenate of *LRRK2* wild-type and G2019S transgenic mice [72], suggesting LRRK2 as a potential stabilizing protein for microtubule assembly. This LRRK2-mediated excessive stabilization of the microtubule network may compromise dynamic assembly and disassembly, and underlie the neurite outgrowing defects observed in neurons overexpressing *LRRK2* G2019S mutations [42].

LRRK2 may also regulate the dynamic of the microtubule network through modulating the activities of another microtubule binding protein, tau. Tau regulates the stability and dynamics of microtubule networks [73]. It has been hypothesized that phosphotau does not bind effectively to microtubules and loses the ability to stabilize them, resulting in microtubule breakdown and impairment of axonal transport [74]. Accumulation of phosphotau was found in certain lines of *LRRK2* mutant transgenic mice [75–77]. It seems that LRRK2 may play a rather complicated role in organizing the microtubule network. On the one hand, LRRK2 facilitates the polymerization of microtubules by directly binding tubulins. On the other hand, LRRK2 undermines the microtubule assembly through the phosphorylation of tau. More investigations may be required in the future to further elucidate the mechanisms of LRRK2 in regulating the dynamic organization of the microtubule network, especially in the presence of α -syn.

ER/Golgi transport

ER/Golgi-mediated secretory pathways are crucial for neuronal development, function and survival [78]. α -syn may regulate ER–Golgi transport through a small GTPase protein, Rab1 [26]. The structure and function of ER/Golgi are also dynamically regulated by the microtubule network [79]. The disruption of the microtubule lattice by aggregated α -syn may account for the fragmentation of the Golgi apparatus in cells overexpressing *α -syn* [80]. Interestingly, overexpression of *LRRK2* also caused significant fragmentation of the Golgi apparatus [72]. Moreover, coexpression of *LRRK2* and *α -syn* led to more severe and synergistic fragmentation of the Golgi apparatus, which was tightly correlated with the augmentation of α -synuclein accumulation in the soma. By contrast, inhibition of *LRRK2* expression prevented the disintegration of the Golgi apparatus in neurons overexpressing *α -syn* and suppressed the accumulation of α -syn in cell bodies. These findings indicate that overexpression of *LRRK2* may promote the improper polymerization of tubulin in neurons, which might cause the fragmentation of the Golgi apparatus and exacerbate α -syn-induced ER–Golgi trafficking defects. This process may form a vicious cycle that further exacerbates the disorganization of microtubule and Golgi networks and abnormal accumulation of α -syn in the soma, which eventually leads to the loss of neurons.

Mitochondria

It has been well known that mitochondrial complex I inhibitors, such as MPTP and rotenone, cause PD-like clinical phenotypes [81], suggesting the importance of maintaining mitochondrial energetic homeostasis in the SNc DA neurons. α -syn-positive aggregates have been shown to induce the damage of mitochondria [24]. While *LRRK2* is associated with the outer membrane of mitochondria [82], no apparent structural abnormality was observed in the mitochondria of mouse neurons overexpressing either wild-type or PD-related mutant *LRRK2* [72]. However, impaired mitochondrial function and morphology were found in *LRRK2* (G2019S) mutant patient tissue [83]. The presence of excess *LRRK2* also exacerbated α -syn-induced mitochondrial swellings and superoxide production [72]. It will be interesting to carry out more detailed studies in the future to specify the roles of α -syn and *LRRK2* in regulating the biogenesis and functions of mitochondria.

The ubiquitin–proteasome system

The ubiquitin–proteasome system (UPS) is one of the main pathways in cells responsible for the removal and recycling of undesired proteins [84]. While the stability of both α -syn and *LRRK2* is regulated by the UPS pathway [85]; α -syn aggregates may cause impairment of UPS activities [86], which may lead to the accumulation of ubiquitinated proteins in neurons. Interestingly, G2019S *LRRK2* protein is also sequestered as ubiquitin-positive clusters in neurons of aging animals [72]. The accumulation of G2019S *LRRK2* and ubiquitin-positive protein aggregates in aged mice indicate that overexpression of *LRRK2*, especially the G2019S mutation, may impair UPS activity in neurons. More interestingly, coexpression of PD-related A53T α -syn and G2019S *LRRK2* seem to further damage UPS activities and accelerate the deposition of both α -syn and *LRRK2* protein aggregates.

Autophagy

Autophagy is the other essential machinery in cells that is responsible for the removal of damaged organelles and large protein aggregates [87]. Various malfunctions of autophagy pathways have been reported in PD, in which both α -syn and *LRRK2* are involved [88,89]. The *α -syn* A53T mutation seems to interfere with the chaperone-mediated autophagy and macroautophagy pathways in cells, which impairs the clearance of α -syn aggregates [90]. *LRRK2* is also implicated in regulating autophagy induction and flux rates in cell cultures. In particular, loss of *LRRK2* leads to excessive accumulation of marker proteins involved in

the induction of autophagy in cells from the kidney and lung [37,41]. However, no overt impairment of the autophagy pathway is observed in *LRRK2*-deficient neurons. Nonetheless, future studies may focus on identifying the molecular partners of *LRRK2* in the autophagy pathway in order to fully understand the contribution of *LRRK2* in this fundamental cellular process.

Conclusion & future perspective

Since the identification of PD-related genetic mutations in α -*syn* and *LRRK2*, extensive studies have been carried out to investigate the various cellular processes affected by mutant α -*syn* and *LRRK2*, which may contribute to the preferential loss of SNc DA neurons in PD. As summarized in this review, PD-related mutant α -*syn* and *LRRK2* target many common intracellular pathways and may exert synergistic effects to exacerbate their respective cytotoxicity. Therefore, a comparative study of α -*syn* and *LRRK2*-mediated pathophysiological mechanisms in PD would be instrumental to elucidate a potential common pathogenic pathway critical for the development of PD. Of note, the majority of α -*syn* and *LRRK2*-related studies have been performed in cell lines and nonDA neurons. Considering the fact that loss of SNc DA neurons accounts for the main clinical and pathological phenotypes in PD, it may demand a more focused investigation of SNc DA neurons in response to α -*syn*- and *LRRK2*-mediated genetic insults. Although midbrain DA neurons only account for a handful of neurons in the brain, they substantially influence almost every aspect of brain activities. These neurons also possess very distinguished morphological and physiological characteristics that are different from many other types of neurons [91]. Therefore, understanding the pathogenic functions of α -*syn* and *LRRK2* and their genetic interplay in midbrain DA neurons may hold the key to finally resolving the mystery of PD and provide a cure for this devastating illness.

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Executive summary

α -synuclein

- Genetic studies have strongly established a prominent role of α -synuclein (*α -syn*) missense and multiplication mutations in causing Parkinson's disease (PD).
- In neurons, α -syn is almost exclusively located in the presynaptic terminals; alterations in neurotransmitter release have been reported in α -syn-deficient and α -syn-overexpressing neurons.
- α -syn preferentially binds to phospholipid liposomes that contain acidic head groups.

LRRK2

- *In vitro* studies indicate that several PD-specific mutations increase LRRK2 autophosphorylation and phosphorylation of generic protein kinase substrates.
- LRRK2 is mainly a cytoplasmic protein, although also partially associated with membrane organelles.
- So far, none of the *LRRK2* transgenic mice studied have developed PD-like neuropathology.

α -synuclein & LRRK2 interplay

- Lewy body (LB): phosphoserine129 of α -syn is enriched in LBs. Some studies have estimated that LRRK2 is present in the periphery of approximately 10–80% of classic LBs as well. LRRK2 may promote the phosphorylation of α -syn at S129, however, the present studies do not support this.
- 14-3-3 family proteins: the association of 14-3-3 with both α -syn and LRRK2 generates significant interest to further explore the contribution of 14-3-3 family proteins in the pathogenesis of PD.
- Actin and microtubule assembly: both mutant α -syn and LRRK2 are involved in the dynamics of actin and microtubule assembly.
- ER–Golgi transport: overexpression of LRRK2 may promote the improper polymerization of tubulin in neurons, which might cause the fragmentation of the Golgi apparatus and exacerbate α -syn-induced ER–Golgi trafficking defects.
- Mitochondria: α -syn-positive aggregates have been shown to induce damage of mitochondria; the presence of excess LRRK2 exacerbated α -syn-induced mitochondrial swellings and superoxide production.
- The ubiquitin–proteasome system (UPS): both α -syn aggregates and mutant LRRK2 may cause impairment of UPS activities. Coexpression of PD-related A53T α -syn and G2019S LRRK2 seems to further damage UPS activities and accelerate the deposition of both α -syn and LRRK2 protein aggregates.
- Autophagy: the α -syn A53T mutation seems to interfere with the chaperone-mediated autophagy and macroautophagy pathways in cells. LRRK2 is also implicated in regulating autophagy induction and flux rates in cell cultures.

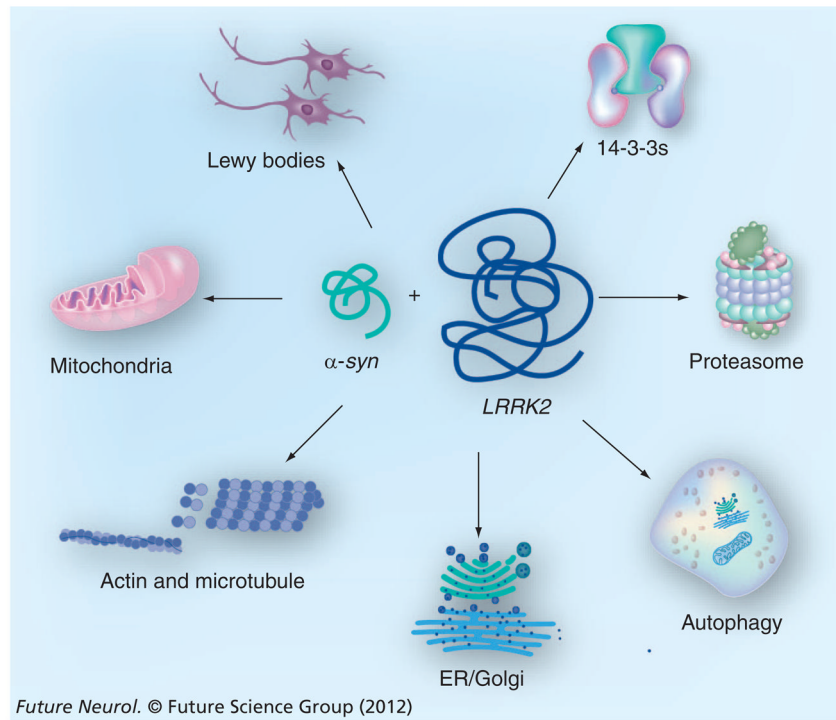


Figure 1. Intracellular pathways potentially affected by synergistic genetic interplay between α -synuclein and LRRK2
 α -syn: α -synuclein; ER: Endoplasmic reticulum.