



HHS Public Access

Author manuscript

Eur J Neurosci. Author manuscript; available in PMC 2020 February 01.

Published in final edited form as:

Eur J Neurosci. 2019 February ; 49(3): 339–363. doi:10.1111/ejn.14182.

The Unlikely Partnership Between LRRK2 and α -Synuclein in Parkinson's Disease

Noémie Cresto¹, Camille Gardier¹, Francesco Gubinelli¹, Marie-Claude Gaillard¹, Géraldine Liot¹, Andrew B. West^{2,Ⓞ}, and Emmanuel Brouillet^{1,Ⓞ}

¹Neurodegenerative Diseases Laboratory, UMR9199, CEA, CNRS, Université Paris Sud, Université Paris-Saclay, and MIRCen (Molecular Imaging Research Centre), Institut François Jacob, DRF, CEA, Fontenay-aux-Roses, France

²Center for Neurodegeneration and Experimental Therapeutics, University of Alabama at Birmingham, Birmingham, Alabama, United States 35294

Abstract

Our understanding of the mechanisms underlying Parkinson's disease, the once archetypal non-genetic neurodegenerative disorder, has dramatically increased with the identification of α -synuclein and LRRK2 pathogenic mutations. While α -synuclein protein composes the aggregates that can spread through much of the brain in disease, LRRK2 encodes a multi-domain dual-enzyme distinct from any other protein linked to neurodegeneration. In this review, we discuss emergent datasets from multiple model systems that suggests these unlikely partners do interact in important ways in disease, both within cells that express both LRRK2 and α -synuclein as well as through more indirect pathways that might involve neuroinflammation. Although the link between LRRK2 and disease can be understood in part through LRRK2 kinase activity (phospho-transferase activity), α -synuclein toxicity is multi-layered and plausibly interacts with LRRK2 kinase activity in several ways. We discuss common protein interactors like 14–3–3s that may regulate α -synuclein and LRRK2 in disease. Finally, we examine cellular pathways and outcomes common to both mutant α -synuclein expression and LRRK2 activity and points of intersection. Understanding the interplay between these two unlikely partners in disease may provide new therapeutic avenues for PD.

Graphical Abstract

Ⓞ Corresponding authors information: abwest@uab.edu, address: 1719 6th ave s., Birmingham, AL 35294 USA, Ph: 205 996 7697, emmanuel.brouillet@cea.fr, address: 18 route du panorama, 92260 Fontenay-aux-Roses, France, Ph : 33 146 549 622.

Authors' contributions

N.C. wrote the first draft of the review with E.B. with a focus on PD characteristics and pathogenesis. She also contributed to the drawing of figure 1.

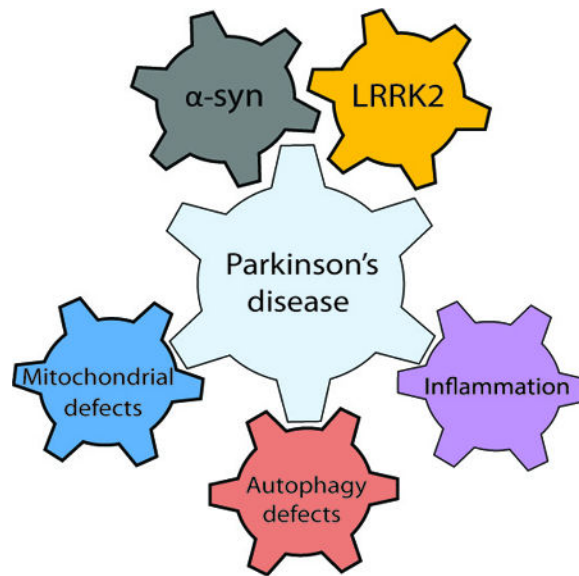
C.G. LG. and M-C G. focused on the detailed autophagy and mitochondrial mechanisms potentially involved in PD and the synergy between α -syn and LRRK2

F.G. edited the text and the figure. He took in charge the management of references to double-check for scientific relevance.

A.B.W. and E.B. supervised the writing of the manuscript and edited the text.

Conflict of interests

The authors of the present review declare to have no conflict of interest.



LRRK2 and α -synuclein probably play key roles in Parkinson's disease pathogenesis. In this review, we discuss how these unlikely partners do interact both within neurons that express both LRRK2 and α -synuclein as well as through more indirect pathways that might involve regulation of α -synuclein spreading and neuroinflammation. Understanding the interplay between α -synuclein and LRRK2 may provide new therapeutic avenues for Parkinson's disease.

Keywords

Parkinson; mitochondria; mitophagy; autophagy; neuroinflammation; Prion-like propagation; aggregation; neurodegeneration

Introduction to LRRK2 and α -Syn in PD

Parkinson's disease (PD) is a neurodegenerative disorder affecting approximately seven million people worldwide (Cacabelos, 2017). Early symptoms of disease often include REM sleep disorder, gastrointestinal disorders (e.g., constipation), and hyposmia. Later, characteristic movement-related symptoms often include shaking (resting tremor), rigidity, and slowness of movement (bradykinesia). The neuropathological hallmarks of PD are a progressive loss of dopaminergic (DA) neurons in the substantia nigra *pars compacta* (SNc) and the presence of neuronal aggregates composed mainly of α -synuclein protein (α -syn) (Lewy Bodies, LB) and dystrophic Lewy Neurites (LN) in some surviving neurons (H. Braak & Braak, 2000; Gibb, Scott, & Lees, 1991). Numerous theories exist for the causes of α -syn aggregation and preferential sensitivity of DA neurons, but without treatments that intervene in these processes, pathogenic mechanisms underlying PD remain unclear. Currently, there is no treatment capable of slowing the disease progression and although several therapies such as dopaminergic treatment and deep brain stimulation provide temporary remittance of several movement-related symptoms, the progression of the disease and therefore the aggravation of the neurodegeneration cannot be stopped.

PD is mainly a sporadic neurodegenerative disorder but a proportion of patients have demonstrated genetic origins. Rare mutations in the α -synuclein (*SNCA*) and *Leucine-Rich Repeat Kinase 2* (*LRRK2*) genes can cause autosomal dominant (AD) PD. Recently, genome-wide association studies have identified common genetic variants in both *SNCA* and *LRRK2* in susceptibility to sporadic PD, further supporting the importance of these two genes in the pathogenesis of PD (Nalls *et al.*, 2014; Satake *et al.*, 2009). The overlap between clinical phenotypes associated with mutations in *SNCA* or *LRRK2* suggest these two proteins/genes concurrently play a role in sporadic and genetic PD. A number of questions are thus raised: Does α -syn and *LRRK2* interact synergistically in disease susceptibility? Following, does *LRRK2* influence the occurrence and triggering of α -syn pathology? Through which molecular and cellular mechanisms do these two proteins interact in PD, and are they involved in both susceptibility as well as progression? Alternatively, does α -syn play a more permissive role in mutant *LRRK2* neurotoxicity?

Beyond improving our understanding of PD pathogenesis, clarifying the interplay between α -syn and *LRRK2* may help to determine whether *LRRK2* could constitute a relevant therapeutic target to slow down PD progression in patients without rare *LRRK2* mutations. Indeed, major research efforts have been conducted in the past decade to design and test novel *LRRK2* inhibitors with hopes they will benefit a large proportion of PD patients. Potentially supporting this notion, preliminary results in animal models of PD suggest that targeting *LRRK2* could be beneficial in both familial and sporadic PD. Thus, the interaction between α -syn and *LRRK2* might be central not only in terms of pathogenesis but also in understanding how to best devise effective therapeutic strategies. Here we briefly review both *in vitro* and *in vivo* studies in model systems that may shed light on the relationship between α -syn and *LRRK2* in PD.

α -Syn and prion-like propagation

In 1997, Polymeropoulos *et al.* identified the α -syn A53T missense mutation as the first genetic lesion causative for an aggressive form of familial PD (Polymeropoulos *et al.*, 1997). α -Syn is a presynaptic protein highly abundant in the brain with suspected roles in vesicle trafficking, membrane dynamics, and synaptic maintenance (reviewed in Bendor, Logan, and Edwards 2013). α -Syn has also been shown to localize to mitochondria and to be degraded in-part via chaperone-mediated autophagy (detailed in the paragraphs below on mitochondria and autophagy). The clear majority of α -syn studies focus on its dysfunction in PD pathology because of its capacity to aggregate and form LBs and LNs. Duplications, triplications and rare mutations (A53T (Polymeropoulos *et al.*, 1997); A30P (Krüger *et al.*, 1998); E46K (Zarranz *et al.*, 2004)) in the *SNCA* gene have been found in several families with dominantly inherited PD. They are associated with early-onset forms of PD with an amplification of the α -syn aggregation process (Chartier-Harlin *et al.*, 2004; Singleton *et al.*, 2003). However, while it is generally accepted that aggregation of α -syn leads to neurotoxicity, the underlying mechanisms are still debated. It is possible that α -syn assemblies (aggregates, oligomers, lewy bodies) trigger toxic mechanisms through a gain of function (e.g. novel detrimental interaction with membranes or proteins) or a toxic loss of function of α -syn as a result of the sequestration of α -syn into aggregates. Indeed, multiple studies have shown a toxic effect of α -syn knock out (Tarasova *et al.*, 2018; Benskey *et al.*,

2018; Cali *et al.*, 2012; Gorbatyuk *et al.*, 2010) while others showed that α -syn KO protected against MPTP-induced neurotoxicity (Dauer *et al.*, 2002). α -syn is a member of the synuclein protein family together with β and gamma-synuclein in mammals. The α -syn protein is composed of three regions: the first one is an amphipathic region (N-term: residues 1–60) containing apolipoprotein lipid-binding motifs and where PD mutations reside. Secondly, α -syn contains a “non- β amyloid” component region (NAC region: residues 61–95) which gives it its potential to form β -sheets inherent to protein fibrils (aggregation). The last one is a C-terminal region (residues 96–140) that harbors important post-translational modifications of α -syn including phospho-Ser129. Three post-translational modifications were identified and validated *in vivo* with phosphorylated residues detected on Ser87, Ser129 and Tyr125. In particular, post-mortem biochemical and immunohistological studies showed that in PD brains α -syn is highly phosphorylated on Ser129 in inclusions. This phosphorylation is also found in pre-LB stages suggesting that it is strongly associated to disease progression (Saito *et al.*, 2003). The pharmacological modulation of this phosphorylation may be a relevant strategy for slowing PD progression, although several studies show α -syn related toxicity is enhanced when the Ser129 residue is mutated to an unphosphorylatable alanine residue (da Silveira *et al.*, 2009; Gorbatyuk *et al.*, 2008; Khodr, Pedapati, Han, & Bohn, 2012). Some kinase interactors have been identified that can modulate Ser129 phosphorylation including polo-like kinases, casein kinases, and G-protein receptor coupled kinase (Inglis *et al.*, 2009; Ishii *et al.*, 2007; Pronin *et al.*, 2000). LRRK2, a serine/threonine protein kinase, has also been evaluated as a protein kinase for α -syn. Results suggest that LRRK2 is unlikely to directly phosphorylate Ser129 (Lin *et al.*, 2009; Herzig *et al.*, 2012). The instrumental role of Ser129 phosphorylation on α -syn is unclear and an increase of phospho-Ser129 might not be toxic *per se*. Studies using kinase interactors of α -syn to produce hyper-phosphorylation at Ser129 have shown opposite effects (accelerated neurodegeneration vs mitigated toxicity) in the rat midbrain (Sato *et al.*, 2011; Oueslati *et al.*, 2013). Also, Buck and collaborators showed that phosphorylation of α -syn at Ser129 was not enough to trigger neurodegeneration, further questioning the role of phospho-Ser129 in the pathogenesis of PD (Buck *et al.*, 2015).

The pathological mechanisms underlying α -syn mediated degeneration of DA neurons has been linked to the very high concentrations of α -syn inherent to A9 DA neurons (that can serve as substrates for α -syn inclusions). SNc DA neurons also have relatively high metabolic demands and lowered oxidative capacity and long extremely branched axonal fibers that lack myelin (Braak and Del Tredici 2004; Post, Lieberman, and Mosharov 2018). Experimental evidence also indicates central roles for mitochondrial damage and associated vulnerabilities, and deregulation of the endolysosomal and autophagic pathways. Some evidence, particularly *in vitro*, clearly demonstrates that α -syn inclusions can be transferred, through some substrate, from one cell to the next (Busquets, Espargaró, Estelrich, & Sabate, 2015; Domert *et al.*, 2016; Kordower, Chu, Hauser, Freeman, & Olanow, 2008). Kordower and collaborators suggested cell-cell transmission of α -syn in 2008. A *post mortem* observation of PD brains transplanted with fetal neurons in the striatum showed that, fourteen years after transplantation, the grafted nigral neurons presented LB-like inclusions that stained positively for α -syn and ubiquitin and had a reduced expression of dopamine transporter. Alternatively, the transplanted cells could have intrinsically formed inclusions *de*

novo in the environment of PD-affected nerve terminals in the striatum. Moreover, all healthy grafts of human midbrain transplants do not automatically show signs of α -syn pathology (reviewed in Engelender and Isacson, 2017). Experimentally, cell-to-cell transmission *in vivo* generally involves over-expression of α -syn and exposures to preformed fibrils of α -syn, brain extracts from α -syn transgenic (tg) mice, and total homogenates from post-mortem PD patients. Many experimental results are supportive of the hypothesis of a prion-like propagation of α -syn. α -Syn linked disease is different from prototypical prion disease in that there is no evidence of disease transmissibility in PD and intrinsic susceptibilities inherent to some sub-populations of neurons appears to outweigh the ability of α -syn to successfully transmit in a continuous way across circuits (Abdelmotilib *et al.*, 2017; Desplats *et al.*, 2009; Luk *et al.*, 2012; Mougenot *et al.*, 2012; Paumier *et al.*, 2015; Peelaerts *et al.*, 2015; Recasens & Dehay, 2014) (Reviewed in Surmeier, Obeso, and Halliday 2017b, 2017a).

However, even though there are many experimental studies supporting this prion-like hypothesis, it is important to note that there is a limited number of clinical evidence directly supporting it, and other experimental evidence goes against it. Indeed, Manfredsson and collaborators failed to show any significant spread of α -syn after they inoculated PFFs directly into the colon of rats and NHPs (Manfredsson *et al.*, 2018). Most of the studies supporting α -syn spreading theory use overexpression models, or brain extracts from individuals presenting synucleinopathies, and among them, not all confirm this spreading hypothesis. This contrasted point of view is very well reviewed by Engelender and Isacson, who present a “Threshold theory for PD” (Engelender and Isacson, 2017).

LRRK2 and the Kinase-Hypothesis of LRRK2-linked PD

In the mid-1990s, several families were identified that transmit autosomal dominant PD phenotypes identical to sporadic PD, a feature very uncommon for genetic forms of PD (Wszolek and Pfeiffer, 1992). In 2002, Funayama and collaborators performed a genome wide linkage analysis showing linkage to a 13.6 cM interval in 12p11.2-q13.1, dubbed the PARK8 locus. While the haplotype was shared by all the patients, it was also present in some unaffected carriers, suggesting the disease penetration to be incomplete. The authors hypothesized other parameters could affect the development of the disease such as environmental factors or other genetics factors (Funayama *et al.*, 2002).

Mutations in *LRRK2* are the most common genetic “cause” of both familial and sporadic PD, with causation understood as high-odds ratios (e.g., >20) for susceptibility to disease (Clark *et al.*, 2006; Paisán-Ruiz *et al.*, 2004; Ross *et al.*, 2011; Trabzuni *et al.*, 2013; Trinh *et al.*, 2014; Zimprich *et al.*, 2004). Kachergus and collaborators identified the G2019S mutation in 2005 (Kachergus *et al.*, 2005), known as the most prevalent pathogenic mutation in *LRRK2* that accounts for 5–6% of autosomal-dominant familial cases and 1–2% of *de novo* generic PD cases in some Western populations (Gilks *et al.*, 2005; Healy *et al.*, 2008). The prevalence can be much higher depending on the population: a recent report suggested that as many as 70% of late-onset PD patients seen at a neurology clinic in Morocco were positive for G2019S (Bouhouche *et al.*, 2017). However, in far-east Asian populations, the frequency of pathogenic *LRRK2* mutations is extremely low (Tan *et al.*, 2005; Tan *et al.*,

2007; Wu *et al.*, 2012; Zabetian *et al.*, 2009). True to the initial description of LRRK2-linked families, patients harboring the G2019S mutation are clinically indistinguishable from sporadic PD cases, including the presence of typical LBs in the majority of cases (Biskup & West, 2009; Yahalom *et al.*, 2014). The mechanisms underlying LRRK2's neurotoxicity remain unclear as it is a protein that does not itself form the proteinaceous inclusions typical of genetically linked proteins in neurodegeneration (like tau, APP, TDP43, SOD1, etc.). Rather, the *LRRK2* gene encodes a large 2527 AA multidomain protein with pathogenic mutations clustered among the central tri-domain region that forms the catalytic core (Cookson, 2010; Mata, Wedemeyer, Farrer, Taylor, & Gallo, 2006). LRRK2 is part of the ROCO protein family identified first in single-celled organisms and contains the tandem Ras-of-complex proteins (Roc) GTPase and C-terminal of Roc (COR) domains. LRRK2 may homodimerize through its COR domain and may function in a dimeric form as a GAD (G proteins activated by nucleotide-dependent dimerization) in which both ROC domains are brought close together and participate in the catalytic activity of their counterpart within the dimer (Civiero *et al.*, 2012). In addition to its GTPase domain, LRRK2 is composed of a kinase domain, with a characteristic activation loop (P-loop) with a DYG-APE motif altered to DYS-APE with the G2019S mutation. Other domains in LRRK2 include multiple protein-protein interaction regions [ankyrin (ANK), armadillo (ARM), and leucine-rich repeats (LRR) and WD40]. The number and conservation of protein-protein interaction domains suggest LRRK2 functions as a scaffolding protein contributing to the formation of multiprotein complexes that may function in trafficking and signaling.

In vitro evidence and limited *in vivo* evidence suggest pathogenic LRRK2 mutations, like G2019S, enhance kinase activities associated with autophosphorylation and Rab-substrate phosphorylation (Alessi & Sammler, 2018; West *et al.*, 2005, 2007), and LRRK2-linked neurotoxicity originates from this increased activity (Greggio *et al.*, 2006; Lee *et al.*, 2010; Smith *et al.*, 2006; West *et al.*, 2007). Based on these observations, pharmaceutical companies have developed compounds targeting the kinase activity of LRRK2 in order to block the increased kinase activity associated with LRRK2 mutations. So-called first-generation LRRK2 kinase inhibitors like staurosporine, sunitinib, CZC 54252, TAE684 and LRRK2-IN-1 have a good potential to inhibit the kinase activity of LRRK2 (Deng *et al.*, 2011). However, they are not specific for LRRK2 and inhibit other critical kinases, together with poor drug properties, making them difficult to use for modeling or functional studies. Second generation compounds included more selectivity and better *in vivo* potential, although the inhibition of LRRK2 in the brain was limited (Saez-Atienzar *et al.*, 2014; Choi *et al.*, 2012; Estrada *et al.*, 2014) (reviewed in Taymans and Greggio 2016; West 2015, 2017).

Early studies demonstrated LRRK2 is a highly phosphorylated protein in the N-terminal part between the ankyrin and the LRR domains (S910; S935; S955; S973) (West *et al.*, 2007). This cluster, more specifically Ser910 and 935, is responsible for 14-3-3 protein binding (Dzamko *et al.*, 2010; R. J. Nichols *et al.*, 2010). The 14-3-3 proteins are a highly conserved family of proteins found throughout the evolutionary scale and are implicated in many cellular functions, notably, they act to promote cell survival through inhibition of many known pro-apoptotic factors including the mitochondrial Bcl-2 family member BAD and the transcription factor Forkhead (Yuan & Yankner, 2000). The 14-3-3 proteins bind to serine/

threonine-phosphorylated residues, often functioning as direct regulators of the target proteins to which they bind (Loeffler, Klaver, Coffey, Aasly, & LeWitt, 2017). Seven isoforms are described in mammals. Studies show that 14–3–3 proteins are linked to PD pathology, as 14–3–3 also interacts with α -syn (for more details, see the common interactors paragraph). LRRK2 binding of 14–3–3 leads to a uniform LRRK2 distribution throughout the cytoplasm and may inhibit LRRK2 kinase activity (Lavalley, Slone, Ding, West, & Yacoubian, 2016). Decreased 14–3–3 binding to LRRK2 induced by PD-associated mutations leads to a re-localization of LRRK2 in cytoplasmic pools (Nichols *et al.*, 2010; Rudenko and Cookson 2010). LRRK2 may also be subject to autophosphorylation on a cluster of threonines in the Rab-like ROC GTPase domain (T1343; T1368; T1403; T1404; T1410; T1452; T1491; T1503) and on two isolated serine and threonine residues in the LRR domain (S1292) and in the kinase domain (T2031) (Kamikawaji, Ito, & Iwatsubo, 2009; Sheng *et al.*, 2012; Webber *et al.*, 2011). LRRK2 autophosphorylation appears to act as an enzymatic activator for both the GTPase and the kinase activity. In 2011, Webber and collaborators studied the effect of autophosphorylation on GTPase and on kinase activity in G2019S or in WT context. They showed that autophosphorylation on the T1503 that is localized in the GTPase domain alters both kinase and GTP binding activity, concluding that autophosphorylation in the GTPase domain induces functional and conformational changes that enhance the dimerization and leads to upregulation of kinase activity. In 2012, Sheng and collaborators validated *in vivo* S1292 as a critical autophosphorylation site (Sheng *et al.*, 2012). Familial PD mutations (N1437H, R1441G, R1441C, G2019S and I2020T) all induced significant increases in phospho-S1292 levels compared to WT-LRRK2 in cellular models, and when the S1292 residue was mutated to a non-phosphorylatable alanine residue, the toxicity associated with LRRK2 kinase activity was ablated. Taken together, these data suggest that LRRK2 kinase activity links pathogenic mutations with neurotoxicity.

Rationale for a meaningful interaction between LRRK2 and α -synuclein in PD

Evidence from humans

Clinical phenotypes—Several *LRRK2* mutations from a number of different families in different countries have been described and since they are relatively rare compared to sporadic (i.e., idiopathic or iPD) disease in most populations, comparisons that lack clinic bias are difficult to formulate. However, clinic bias tends to skew genetic forms of disease phenotypically away from sporadic disease and with LRRK2, clinical characteristics of the symptomatic carriers are very similar to one another (Adams *et al.*, 2005; Hasegawa *et al.*, 2009; Huang *et al.*, 2007; Hulihan *et al.*, 2008; Khan *et al.*, 2005; Lin *et al.*, 2008; Nichols *et al.*, 2005). Presently, there is no single clinical phenotype or scale that might predict a LRRK2 mutation carrier from typical late-onset PD. Curiously, no major difference seems to exist between homozygous patients and the ones who are heterozygous, although the number of homozygous LRRK2 mutation carriers yet described is very small, consistent with the frequency of the most common LRRK2 mutation G2019S at less than 0.1% in the Caucasian population (where it is most frequent).

LRRK2 mutations do not cause early-onset disease; the age of onset in susceptible carriers is relatively similar for iPD and LRRK2 mutation carriers (~55–65 years old). Motor symptoms of LRRK2 carriers include resting tremor, rigidity, akinesia, postural instability, and gait difficulty, which are reminiscent of those seen in iPD. Unilateral onset is often seen in LRRK2 mutation carriers as it is in iPD. As expected, LRRK2 carriers with PD are responsive to L-dopa treatment. Further, PET imaging studies of the dopaminergic system indicate alterations in symptomatic LRRK2 carriers that are typical of iPD, especially the reduction in ^{18}F -dopa accumulation in striatal DA projections, and reduced binding of ^{11}C -tetrabenazine to the vesicle dopamine transporter (vMAT) and reduced binding of the ^{11}C -methylphenidate to the dopamine transporter (DAT). The relative preservation of the DA projections in the caudate nucleus as compared to the putamen seen in iPD has been also observed in LRRK2 mutation carriers (reviewed in Biskup and West 2009).

Subtle differences have been described regarding the effect of LRRK2 mutations on the timing and progression of certain symptoms in different populations of carriers. However, a prospective longitudinal follow up of a cohort (n=122) of LRRK2 variant carriers over more than five years indicated that the onset and symptoms were not different from iPD (Oosterveld *et al.*, 2015). However, the progression of the disease after onset might be slightly accelerated in LRRK2 carriers when compared to non-carriers (Oosterveld *et al.*, 2015). Finally, some studies highlighted the possibility of differences in the prevalence and timing of onset of non-motor symptoms (REM sleep disorder, cognitive deficits), especially in PD with dementia (Goldwurm *et al.*, 2006; Sun *et al.*, 2016). In summary, the striking clinical overlap between LRRK2 mutation carriers with PD and PD without LRRK2 mutations suggests common disease mechanisms.

LRRK2 and α -syn in Lewy bodies and Lewy neurites.—Two years after the discovery of *LRRK2* as a gene responsible for autosomal dominant forms of PD, Zimprich and collaborators speculated that LRRK2 kinase activity may be responsible for the phosphorylation of α -syn and that this effect may trigger its accumulation and aggregation (Zimprich *et al.*, 2004). The authors found typical LB-dominant pathology, comparable to sporadic PD patients, in LRRK2 mutation carriers. These observations suggested that LRRK2, as a kinase, and α -syn, as a kinase substrate, could physically interact. Later, Giasson and collaborators studied via immunohistochemistry three individuals with the G2019S LRRK2 mutation and concluded LRRK2 protein was not found in LBs or in LNs, in contrast to typical α -syn pathology in these cases. However, studies including larger numbers of LRRK2 mutation carriers revealed that a proportion of cases, up to 1/3 of G2019S carriers of both non-Ashkenazi Jewish and European non-Ashkenazi Jewish descent were negative for Lewy bodies (Kalia *et al.*, 2015). Although the total number of brains evaluated remains low, the number without Lewy bodies is high compared to typical late-onset disease brains evaluated from patients clinically diagnosed with PD.

There are several possibilities to interpret G2019S-LRRK2 carrier brains clinically diagnosed with PD that lack Lewy bodies. First, in the presence of the G2019S LRRK2 mutation, LBs may be more toxic than usual and cells that harbor them may not survive through late stages of disease. Second, the G2019S LRRK2 mutation favors smaller less-phosphorylated α -syn inclusions or abnormal oligomers that are more difficult to detect than

typical eosinophilic inclusions, and this drives disease in those patients. Third, the proportion of G2019S-LRRK2 carriers negative for LBs may experience disease onset and progression without the contribution of α -syn. For example, some LRRK2 mutation carriers have been described with Tau tangle pathology. Unfortunately, there is typically insufficient clinical data associated with most tissues available to test whether disease characteristics and progression might be different in LRRK2 carriers with and with α -syn LB burden. A PET tracer that might label α -syn inclusions in life would effectively solve the riddle and such tracers are under active development. In the meantime, better clinical phenotyping within brain donation centers supported by LRRK2 mutation carriers seems vital to better understanding pathology linked to LRRK2.

SNCA genetic variants in modifying PD susceptibility in LRRK2 carriers.—

Genome-wide association (GWAS) studies indicate that variants in *SNCA* and *LRRK2*, independent from rare pathogenic missense mutations, are among the top genetic risk factors for PD susceptibility (Nalls *et al.*, 2014; Satake *et al.*, 2009; Simón-Sánchez *et al.*, 2009). Studies focused on gene-gene interactions effects of *SNCA* and *LRRK2* found no significant evidence of an interaction between the two genes, although power is still limited due to low individual effect sizes of the linked variants (Biernacka *et al.*, 2011). Botta-Orfila and collaborators genotyped the *SNCA* polymorphism rs356219 in 84 Spanish LRRK2-associated PD patients carrying the G2019S mutation (Botta-Orfila *et al.*, 2012). This single nucleotide polymorphism (SNP) is known to regulate *SNCA* expression in idiopathic PD-affected brain regions, blood, and plasma. Results showed that *LRRK2* mutation carriers carrying one or two *SNCA* rs356219-G alleles presented with an earlier PD onset than patients carrying the *SNCA* rs356219-A homozygote (55.37 \pm 1.54 vs 64.19 \pm 2.8 years respectively) (Botta-Orfila *et al.*, 2012). A similar study was performed in iPD patients, and the authors found that this SNP also influenced the age of onset (Cardo *et al.*, 2012). These initial studies suggest that *SNCA* genetic variants could be a modifier of age of onset but not necessarily disease susceptibility (Botta-Orfila *et al.*, 2012). Thus, these observations indirectly suggest that α -syn may play a permissive role in *LRRK2* mutation carriers in disease.

Evidence of α -syn and LRRK2 interaction in animal models

In 2009, Lin et al reported results from triple-transgenic mice with inducible A53T- α -syn and LRRK2 (WT or G2019S) under the *CaMKII* promoter-controlled tetracycline trans activator (tTA) (*CaMKII-tTA*), or on a LRRK2 knockout background, to examine the role of LRRK2 in the progression of neuropathological alterations and phenotypes associated with mutant α -syn. This heroic effort first determined the number of neurons that remained in the striatum of *A53T/LRRK2WT* and *A53T/G2019S* mice after months of expression; while the difference was not statistically significant in comparisons between WT-LRRK2 or G2019S-LRRK2 expression, a trend towards a decrease in the number of neurons in the A53T/G2019S striatum was reported. More convincingly, the inhibition of LRRK2 expression dramatically ameliorated α -syn-induced neuropathology almost to the level of doxycycline-induced silencing of transgenic mutant α -syn expression. Furthermore the number of neurons at 12 months of age in the striatum of mice was significantly higher in the A53T/LRRK2^{-/-} mice compared to A53T/LRRK2^{+/+} mice. Moreover, no significant increase of

astrocytosis, microgliosis, or somatic accumulation of α -syn was detected in these mice. These experiments highlighted the potential link between WT-LRRK2 expression and α -syn protein in neurodegeneration observed in PD, with a more speculative role for G2019S-LRRK2 in exacerbating α -syn neurotoxicity (Lin *et al.*, 2009).

Further testing the link between G2019S-LRRK2 and α -syn neurotoxicity, in 2012 Herzig and collaborators reported tg mice that expressed human LRRK2 (WT or G2019S) with mice that expressed human α -syn (A53T or WT) under the same neuron-specific promoter (Thy1 promoter) (A53T/G2019S; A53T/WT; WT/G2019S) (Herzig *et al.*, 2012). This strain of A53T- α -syn mice (van der Putten *et al.*, 2000) were known to develop motor coordination deficits in the rotarod test at 40 days of age and severe motor deficits, hypokinesia and weight loss at 6 months of age, although they lack the overt neurodegeneration that occurs in the Lin et al study. The G2019S-LRRK2 tg mice showed no obvious brain pathology at 19 months of age, with no further decline of motor performance in double tg mice (Herzig *et al.*, 2012). In 2017, Xiong and collaborators went back to the CamKII-induction approach to obtain a high expression level of G2019S-LRRK2 in the adult mouse forebrain, and consistent with Lin et al, these animals showed a nominal increase in α -syn aggregation, especially in the cortex (Xiong *et al.*, 2017). They obtained similar results in tg mice with a selective overexpression of G2019S-LRRK2 in the SNc, where they showed a degeneration of DA neurons and the aggregation of endogenous mouse α -syn (Xiong *et al.*, 2018). Potentially consistent, A53T- α -syn transgenic mice directed from the PrP promoter (Lee *et al.*, 2002)(see also: Giasson *et al.*, 2002), when crossed with G2019S-LRRK2 mice expressing mutant LRRK2 under the control of the CMVe-PDGFB promoter, demonstrated mild enhancement of phosphorylated inclusions but did not worsen survival rates or other phenotypes (Ramonet *et al.*, 2011; Daher *et al.*, 2012).

Outside of traditional transgenic mice, the acute induction of α -syn overexpression via viral vector or injection of preformed α -syn fibrils also suggests a role for LRRK2 in α -syn neurotoxicity. BAC-mediated expression of G2019S-LRRK2 in rats causes a very mild motor impairment with no dopaminergic cell loss even at 12 months of age (Lee and Cannon 2015; Walker *et al.*, 2014). However, injection of an AAV coding for α -syn (Daher *et al.*, 2015) in the SNc of these G2019S-LRRK2 rats induces a loss of TH-positive neurons that appears slightly more pronounced as compared to that seen in WT-LRRK2 rats. A larger effect was described in LRRK2 knockout rats that may be protected from viral-vector mediated α -syn-induced dopaminergic neurodegeneration (delivered by an AAV) (Daher *et al.*, 2014).

In summary, the effects of the G2019S LRRK2 mutation on α -syn neurotoxicity and aggregation is mixed, with either mild or no effects reported in rodent studies. In contrast, phenotypes are generally more robust in the amelioration of α -syn phenotypes in LRRK2 knockout rats and mice. For transgenic LRRK2 expression, it is important to note that except in Lin's study, the co-expression of both proteins (α -syn and LRRK2) in the same anatomical region and in the same neurons is not precisely validated thereby introducing a potential confounder to interpretation. In a recent study in primary hippocampal neurons, G2019S-LRRK2 expression mildly increases α -syn fibril induced inclusions but the effects of LRRK2 inhibition are much larger in reducing α -syn inclusion maturation (L. A.

Volpicelli-Daley *et al.*, 2016). Thus it remains possible that, at least in part, G2019S-LRRK2 expression may mediate a cell autonomous (albeit subtle) mechanism in the aggregation of α -syn in neurons. More the convincingly interactions appear from LRRK2 inhibition or knockdown which implies a general effect of LRRK2 interacting with α -syn in PD, outside of pathogenic mutations.

Molecular mechanisms to explain the interaction of LRRK2 and α -syn in PD

An important interaction between LRRK2 and α -syn may plausibly occur within cells that express both proteins at the same time (i.e., cell-autonomous), or through the interaction of cells important in disease that express one protein or the other (i.e., non-cell-autonomous). For example, in neurons, LRRK2 might modulate total α -syn levels (synthesis or degradation levels), levels of a post-translational modification in α -syn, modulate α -syn aggregation through changing interactions with α -syn interacting proteins, or change the subcellular compartments where α -syn normally resides. Within cells that express both LRRK2 and α -syn, understanding their interaction may shed light on how key organelles regulate survival, for example through mitochondria or endolysosome function. Alternatively, LRRK2 and α -syn might be functionally linked through mechanisms involving cell-cell interactions. For example, cells of the innate immune system involved in neuroinflammation that carry high levels of LRRK2 (mutated or with particular polymorphisms) could have increased sensitivity to the presence of aggregated α -syn in the brain. The complexity of the potential functional interaction between α -syn and LRRK2 may also involve different types of glial cells that act on one another in neuroinflammatory processes, namely astrocytes and microglial cells (Liddelow *et al.*, 2017).

Neuron-neuron interaction might also play a role in the apparent spread of abnormal α -syn from one susceptible brain area to the next. Whether the cellular mechanisms dictating α -syn interaction with LRRK2 are cell-autonomous or non-cell-autonomous or some combination, therapeutic targeting of LRRK2, for better or worse, is almost exclusively focused on inhibiting LRRK2 kinase activity. In the second part of this review, we shall emphasize the different directions of research that provide yet imperfect but promising answers.

LRRK2 activity in α -syn expression and aggregation

As the endogenous levels of α -syn phosphorylation are low in the unaggregated state, the phosphorylation state of α -syn, notably on Serine 129, is an excellent readout of the aggregation of α -syn. In recent studies, G2019S-LRRK2 KI mice or G2019S-LRRK2 expressing rats exhibited significantly higher levels of pSer129 α -syn in the striatum compared to WT controls (Longo *et al.*, 2017; L. A. Volpicelli-Daley *et al.*, 2016). LRRK2 could affect the subcellular localization of α -syn, notably its membrane association, promoting the aggregation by increasing the cytosolic pool of α -syn. Through G2019S-LRRK2 upregulation of total levels of α -syn, the net effect may also be an increase in the aggregation-prone cytosolic pool. In SH-SY5Y cells studied by Kondo and collaborators (Kondo, Obitsu, and Teshima 2011), the aggregation capacity of α -syn was evaluated in the presence or absence of G2019S-LRRK2. Cell viability decreases when the cells are co-

transfected with α -syn and G2019S-LRRK2 but also with WT-LRRK2 whereas viability remains unchanged in presence of each protein alone. In differentiated cells, α -syn-containing vesicles are not produced when the cells are transfected with α -syn alone. In contrast, the co-transfection of α -syn with G2019S-LRRK2 induces the formation of aggregates and vesicles. Most of the α -syn aggregates co-localize with the mitochondrial outer-membrane marker TOM20 in cells co-transfected with α -syn and G2019S-LRRK2 but not with the WT form of LRRK2. In 2014, Skibinski and collaborators demonstrated that the neurotoxicity caused by G2019S-LRRK2 could be mitigated through knockdown of α -syn (Skibinski, Nakamura, Cookson, & Finkbeiner, 2014). More recently, a study in primary cortical neurons from homozygous G2019S-LRRK2 KI mice shows that expression of G2019S-LRRK2 induced the accumulation of endogenous, detergent-insoluble α -syn, and that this effect can be reversed through pharmacological inhibition of LRRK2 kinase activity (Schapansky *et al.*, 2018). Altogether, these observations indicate that LRRK2 can accelerate aggregation of α -syn, and possibly the localization of the aggregates, but the precise mechanisms have yet to be fully elucidated.

LRRK2 mediation of α -syn cell-to-cell propagation

It is now generally established that α -syn can propagate via the synapse in model systems *in vitro*, particularly those that use triple-chamber systems. In 2011, Volpicelli-Daley and colleagues observed this cell-to-cell transmission in chambered cultured neurons with bath-application of pre-formed α -syn fibrils (Volpicelli-Daley *et al.*, 2011). Kondo and collaborators also studied the cell-to-cell transmission of α -syn through evaluating exocytosis of α -syn in the media and the internalization in neighboring neuroblastoma cells (Kondo *et al.*, 2011). In this system, G2019S-LRRK2 significantly enhances α -syn release into the conditioned media. Interestingly the phosphorylation state of α -syn is not different in the cells co-transfected compared to cells transfected with α -syn alone, suggesting that LRRK2 can influence the propagation mechanism but not the phosphorylation state of α -syn (Kondo *et al.*, 2011). These preliminary observations warrant further investigations into LRRK2 mediation of cell-to-cell transmission of abnormal α -syn.

Impact of LRRK2 on α -syn-induced neuroinflammation

Evidence of on-going neuroinflammation in affected brain regions in PD first come from analysis of brain tissue for reactive microglia and analyses of pro-inflammatory cytokines like Interferon gamma, (IFN- γ), TNF- α , Interleukin-6 (IL-6), and Interleukin-1 β , (IL-1 β) (Hunot *et al.*, 1999; Mogi *et al.*, 1994; McGeer, Itagaki, and McGeer 1988; Mogi *et al.*, 1996). In addition, GWAS studies point to polymorphisms in loci with genes related to immunity (e.g. MHC-II genes). Neuroinflammation can be seen as a defense reaction that involves both the adaptative and innate immune systems, occurring in the central nervous system (CNS) after an event that perturbs “normal” homeostasis (reviewed in Gelders *et al.*, 2018; Xanthos and Sandkühler, 2014). It is widely accepted that neuroinflammation occurs in many different acute and chronic neurological disorders. Whether it is only a consequence of neurodegeneration or is also actually a key active player favoring or opposing neurodegeneration is unknown. Presenting extensively the *pros* and *cons* of these different hypotheses on the role of neuroinflammation in degenerative diseases is out of the scoop of the present review. Comprehensive overview of this aspect of PD pathogenesis can

intraperitoneal injection of LPS is able to trigger a long-term inflammation associated with neurodegeneration of DA neurons in the SNpc, which is not the case in mice overexpressing the human wild type LRRK2 or wild type mice (Kozina *et al.*, 2018). This change produced by mutant LRRK2 is not directly linked to activation of innate brain cells but may be linked to a type II interferon immune response in peripheral cells. However, this does not rule out the possible role of LRRK2 in the regulation of brain immune cells, in particular microglia.

Microglial cells are resident innate immune cells and professional phagocytes of the brain, as well as the first line of defense against pathogens. In neurodegenerative disorders there are roles in clearing aggregated proteins and the clearance of dead cells and other debris. Microglia can switch from a resting state (ramified phenotype) into an activated state (amoeboid phenotype) through signaling molecules that mediate activation (e.g., MAPK subtypes p38, JNK and ERK). A failure in the appropriate and proportional activation process of microglia can have dramatic consequences. Excessive activation can lead to an abundant secretion of inflammatory mediators and reactive oxygen species that are cytotoxic to neurons. LPS (lipopolysaccharide) treatment, *in vitro* or *in vivo*, is known to induce a TLR4-mediated inflammatory response and microglial activation. In primary microglial cells, LPS exposure can induce the switch between the resting phenotype to the activated phenotype with an extension of processes into the environment and eventually a retraction into an amoeboid morphology. In cultured microglial cells isolated from tg mice overexpressing R1441G-LRRK2 or WT littermates, LPS treatment induces an increase in LRRK2 protein expression that is not reflected at the mRNA level, suggesting that LPS-treatment can impact the turn-over of LRRK2 (Gillardon, Schmid, & Draheim, 2012; Moehle *et al.*, 2012). In addition, R1441G-LRRK2 expression increases expression and secretion of proinflammatory cytokines (TNF) compared to WT microglia in culture (Gillardon *et al.*, 2012). LRRK2 inhibitors, LRRK2-IN-1 and sunitinib, were found to attenuate the release and transcriptional induction of TNF and reduce phospho-p38 protein levels. Similarly, knockdown of LRRK2 by a lentivirus coding for an shRNA targeting LRRK2 has the same effect, except for the phosphorylation of p38 that remains unchanged by LRRK2 shRNA. More interestingly, in the presence of LRRK2 inhibitors or LRRK2 shRNA, the LPS treatment fails to induce the switch between the resting phenotype to the activated phenotype (Moehle *et al.*, 2012). The knockdown of LRRK2 in the microglial cell line BV-2 by lentiviral vectors encoding a shRNA significantly reduces the secretion of TNF α and IL-6 with an increase of NO production as compared to control conditions. Treatment with LPS in these knockdown cells induces a decrease in the level of the phosphorylated form of p38 and induces a significant reduction of nuclear factor kappa B (NF- κ B) transcriptional activity while JNK and ERK are unaffected (B. Kim *et al.*, 2012). LRRK2 enhances the association of NFAT1 with proteins that seem to block the NFAT transport to the nucleus and thus inhibits the transcription of immune response genes (Liu *et al.*, 2011). Microglia isolated from LRRK2 KO mice have an increased α -syn uptake and clearance when treated with recombinant α -syn, compared to WT mice microglia (Maekawa *et al.*, 2016). In these studies, primary microglia and cell lines in culture adopt a macrophage-like phenotype on a transcriptional and functional level, necessitating studies *in vivo* with a preserved dynamic innate immune system (Butovsky *et al.*, 2014).

In LRRK2 knockout rats as well as LRRK2 knockout mice, LPS and Tat-induced neuroinflammation (respectively) are dramatically dulled compared to WT rodents (Daher *et al.*, 2014; Puccini *et al.*, 2015). The absence of LRRK2 protects from neurodegeneration induced by an AAV coding for α -syn (Daher *et al.*, 2014). Like LPS, injections of viral vectors coding α -syn are known to induce microglial activation. In this study, the number of CD68-positive cells recruited in response to α -syn expression is reduced in LRRK2 KO rats compared to WT rats. The morphology of IBA1-positive cells shift to the amoeboid (activated) phenotype in WT rats injected with α -syn that was not observed in LRRK2 KO rats also injected with α -syn. In addition, treatment with PF-06447475 attenuates the neuroinflammation in G2019S-LRRK2-BAC Tg rats when compared to untreated animals and to non-Tg rats expressing WT rat LRRK2 after the injection of AAV- α -syn (Daher *et al.*, 2015). Overall it seems likely that LRRK2 modulates the inflammatory response induced by α -syn. It could be imagined that G2019S-LRRK2 could increase the neuroinflammation induced by α -syn, therefore aggravating the deleterious effects of this neuroinflammation. Selective LRRK2 mutant expression or knockdown within cells of the immune system has not yet been described, limiting interpretations of mechanisms.

Many studies have correlated α -syn aggregates with activated microglia, MHC-II expression, and neuroinflammation. Studies in human post-mortem PD brain show that MHC-II detection of microglia performs as well as phosphorylated α -syn in staging brains in PD pathology (Imamura *et al.*, 2003). α -Syn fibrils, perhaps all fibrillized amyloid proteins, are substrates of TLR2 expressed in cells of the innate immune system, and can activate MyD88 pathways similar to TLR4 agonists (e.g., LPS). α -Syn fibrils and aggregates may bind to and activate both TLR2 and TLR4 (Codolo *et al.*, 2013; Daniele *et al.*, 2015; Gustot *et al.*, 2015; C. Kim *et al.*, 2013). The implication is that abnormal α -syn activates an immune response that may be potentiated by mutant LRRK2 expression. Alternatively, LRRK2 activity may initiate an abnormal immune response that causes α -syn to aggregate in neurons, with the immune response further potentiated in perpetuity with α -syn aggregates interacting with toll-like receptors. Future studies can dissect these possibilities.

LRRK2, α -syn, and 14–3-3s

Among all the pathways plausibly affected by α -syn and LRRK2, one possibility is that a common protein interactor could weave together the two proteins in a cell in response to specific stimuli. The 14–3-3 chaperone-like protein family is known to interact robustly with both α -syn and LRRK2, as well as becomes entrapped in LBs in PD (Berg, Holzmann, and Riess 2003; Kawamoto *et al.*, 2002; Nichols *et al.*, 2010). In 2002, Xu and collaborators showed that 14–3-3 proteins interact with α -syn in a 54–83-kD complex (Xu *et al.*, 2002). 14–3-3 sequestration in LBs, plausibly mediated by an interaction with α -syn, may cause loss of 14–3-3 function and lowered anti-apoptotic activity and lead to toxicity and cell death. Moreover, expression of 14–3-3 theta is significantly decreased in tg mice overexpressing human WT- α -syn under the platelet-derived growth factor beta promoter (Yacoubian *et al.*, 2008). In dopaminergic cells, the level of tyrosine hydroxylase can be modulated both with α -syn and 14–3-3 expression. 14–3-3 proteins are known to interact with phosphorylated TH to enhance its activity and to promote dopamine synthesis. α -Syn in turn may bind to unphosphorylated TH to reduce its activity (for more details see Review

from Berg, Holzmann, and Riess 2003). This is a compelling example of how 14–3–3 and α -syn binding to each other may manipulate cell phenotypes associated with a rate-limited enzyme (TH) critical for neuronal phenotypes.

As with α -syn and LRRK2, 14–3–3 proteins themselves are subject to phosphorylation. In 2015, Slone and collaborators found that modulation of 14–3–3 phosphorylation reduced their neuroprotective potential (Slone, Lavalley, McFerrin, Wang, & Yacoubian, 2015). 14–3–3 binding to LRRK2 is mediated by the LRRK2 Ser910 and 935 phosphorylation sites (Gloeckner *et al.*, 2010; Nichols *et al.*, 2010; West *et al.*, 2007). Oxidative stress may promote dephosphorylation of LRRK2 causing a loss of 14–3–3 binding to LRRK2 (Mamais *et al.*, 2014). Disruption of 14–3–3 binding to LRRK2 alters LRRK2 localization in the cell and potentially affects LRRK2 kinase activity and membrane-related functions, such as exosome release (Dzamko *et al.*, 2010; Mamais *et al.*, 2014). 14–3–3 binding to LRRK2 could prevent the dephosphorylation of LRRK2 and may stabilize LRRK2 structure. In 2017, Civiero and collaborators showed *in vitro* that the serine/threonine kinase PAK6 bound and phosphorylated 14–3–3 γ at its Ser59 residue, thus promoting 14–3–3 γ /LRRK2 complex dissociation. In neurons, the activation of this kinase rescues G2019S-LRRK2 neurotoxicity (Civiero *et al.*, 2017). Other recent work concluded that 14–3–3 binding to LRRK2 could control LRRK2 kinase activity (Lavalley *et al.*, 2016). 14–3–3 binding to LRRK2 prevents LRRK2 kinase activity and autophosphorylation, whereas a loss of 14–3–3 expression and binding result in hyper-activated LRRK2 and exacerbate LRRK2 neurotoxicity. In a cell autonomous manner, α -syn-mediated sequestration of 14–3–3 proteins may hyper-activate LRRK2 kinase activity, encourage pro-apoptotic pathways, and reduce levels of dopamine synthesis needed for proper basal ganglia function.

α -syn, LRRK2, and Mitochondria

Mitochondria have been a favorite organelle for study in PD-related research stemming back to post-mortem descriptions of a complex I deficiency in the striatum (Mizuno *et al.*, 1989) and in the SNc (Schapira *et al.*, 1990) of PD patients. Mitochondrial complex I inhibitors (for example rotenone or MPTP) can also induce parkinsonism in exposed people as well as in rodents (Langston and Ballard 1983; Schapira 2008). Mutations in the genes encoding PARKIN, DJ-1 and PINK1 cause autosomal recessive forms of PD, and these proteins are all involved in mitochondrial quality control (reviewed in Dawson, Ko, and Dawson 2010). In rodents, endogenous as well as exogenously administered α -syn (WT or A53T) localizes in-part to mitochondria (Chinta, Mallajosyula, Rane, & Andersen, 2010; Li *et al.*, 2007; Martin *et al.*, 2006; Nakamura *et al.*, 2008; Subramaniam, Vergnes, Franich, Reue, & Chesselet, 2014; Ling Zhang *et al.*, 2008). α -Syn may directly interact with TOM20 (translocase of the outer mitochondrial membrane) and inhibit mitochondrial protein import (Di Maio *et al.*, 2016). Moreover, partial homology to mitochondrial targeting sequence was found in the N-term portion of α -syn. α -Syn binding to mitochondrial membranes appears particularly high in the striatum, in the SNc and in the cortex of PD brains, regions highly affected in PD pathology (Devi, Raghavendran, Prabhu, Avadhani, & Anandatheerthavarada, 2008). α -Syn import to mitochondria may depend on intracellular pH (Cole, DiEuliis, Leo, Mitchell, & Nussbaum, 2008) and on mitochondrial membrane potential and the ATP pool (Devi *et al.*, 2008). *In vitro*, about 10% of total LRRK2 protein localizes to a mitochondrial enriched

protein fraction (West *et al.*, 2005), although a lower percentage is probably associated with the outer mitochondrial membrane without significant levels detected inside mitochondria (Biskup *et al.*, 2006).

Studies in model systems with increased or abnormal α -syn expression, or mutant LRRK2, have demonstrated compromised mitochondrial function such as decreased mitochondrial membrane potential and ATP production and increased oxidative stress (Butler *et al.*, 2012; Mortiboys *et al.*, 2015; Papkovskaia *et al.*, 2012; Parihar *et al.*, 2008; Su and Qi 2013; Wang *et al.*, 2012; Melo *et al.*, 2016; Erustes *et al.*, 2017; Martinez *et al.*, 2018; Martin *et al.*, 2006; Mortiboys *et al.*, 2010; Sarafian *et al.*, 2013). While α -syn null mice are protected against MPTP toxicity, and tg mice overexpressing human α -syn may be more sensitive to MPTP-induced toxicity (Dauer *et al.*, 2002; Klivenyi *et al.*, 2006; Song, Shults, Sisk, Rockenstein, & Masliah, 2004), LRRK2 knockout mice do not appear protected from MPTP (Andres-Mateos *et al.*, 2009). However, mice over-expressing G2019S-LRRK2 appear more sensitive to MPTP (Karuppagounder *et al.*, 2015). Thus, at least through MPTP, both α -syn and LRRK2 appear to have a functional role in mitochondria-dysfunction that can cause neurodegeneration.

While the mechanism of how LRRK2 interacts with mitochondria is speculative at this time, several studies may shed light on α -syn interaction with mitochondria. α -Syn KO mice present with reductions of mitochondria respiration and linked CI/III activity (Ellis *et al.*, 2005), and tg mice expressing α -syn A53T in DA neurons present an age-related mitochondrial CI inhibition (Chinta *et al.*, 2010). Devi and collaborators have identified in 2008 a large decrease in CI activity associated with abnormal α -syn, both in human fetal primary cortical neuronal cultures overexpressing α -syn and in mitochondria isolated from the ST and SN of autopsy brain hemispheres from PD subjects (Devi *et al.*, 2008) and these results have been reproduced in other *in vitro* models (Loeb, Yakunin, Saada, & Sharon, 2010; Reeve *et al.*, 2015). Mitochondria isolated from Wistar rats exposed to different concentrations of recombinant human α -syn also show a dose-dependent decrease in CI activity (Liu *et al.*, 2009).

Potentially corroborating these results, Tg mice expressing A53T- α -syn display dysmorphic mitochondria (Martin *et al.*, 2006). Double tg mice α -syn/parkin KO have marked alterations of mitochondrial architecture, especially in the SN and cortex (Stichel *et al.*, 2007). Similarly, tg A53T mice show changes in mitochondrial morphology and decreased mitochondrial interconnectivity (Xie & Chung, 2012) and even fragmented mitochondria that appear before DA neuron loss (Chen, Xie, Turkson, & Zhuang, 2015). *In vitro*, α -syn knock down increases the number of cells presenting elongated mitochondria (Kamp *et al.*, 2010) and overexpression of both WT and A53T- α -syn induced the fragmentation of mitochondria (Butler *et al.*, 2012; Devoto *et al.*, 2017; Guardia-Laguarta *et al.*, 2014; Martinez *et al.*, 2018) that precedes any disturbance in mitochondrial function and cell death (Nakamura *et al.*, 2011). This effect of α -syn on mitochondrial fragmentation was also shown in DA neurons derived from patients with the A53T mutation (Ryan *et al.*, 2018). Moreover, this α -syn-dependent mitochondrial fragmentation requires a direct interaction with the outer mitochondrial membrane in iPS overexpressing α -syn (Devoto *et al.*, 2017). In primary mouse cortical cultures, astrocytes internalize α -syn oligomers causing

mitochondrial fragmentation after a 24h exposure (Lindström *et al.*, 2017). In particular, Grassi and collaborators recently identified a conformationally distinct phosphorylated α -syn species (resulting from the incomplete degradation of fibrillar α -syn) that associates with mitochondria to induce mitochondrial dysfunction (Grassi *et al.*, 2018). More precisely, significant reductions of Mnf1 and 2 proteins were observed in tg A53T mice with no changes in Drp1 or OPA1 (Xie and Chung 2012). Drp1 mostly locates in the cytoplasm, but is stimulated after fission stimuli to migrate to the mitochondria. In Drp1 KO mice, the overexpression of WT- α -syn still induces significant fragmentation of mitochondria, even though it is still less important than in the WT mice, implying that α -syn-induced mitochondrial fragmentation might go through an effect on Drp1, but that it would not be the only mechanism (Nakamura *et al.*, 2011). Thus, these findings suggest that the fragmentation of mitochondria caused by α -syn aggregation and expression decreases mitochondrial fusion, rather than an increase in fission.

LRRK2 may interact with Drp1 to enhance Drp1 mitochondrial translocation, and the blockage of Drp1 inhibits mitochondrial fragmentation and dysfunction (Niu, Yu, Wang, & Xu, 2012; Su & Qi, 2013; X. Wang *et al.*, 2012). G2019S-LRRK2 might phosphorylate Drp1 leading to mitochondrial fragmentation (Su & Qi, 2013). Through this pathway, LRRK2 kinase activity could activate Drp1 and enhance its localization to mitochondria, therefore tipping the balance in favor of mitochondrial fission. Supporting this hypothesis, LRRK2 (and not its dead kinase mutant) enhances ROS production, which is known to lead to increased fission (Niu *et al.*, 2012). However, Saez-Atienzar and collaborators observed the opposite effects: in their *in vitro* study, it was the pharmacological inhibition of LRRK2 that led to a Drp-1-dependent increase in mitochondrial fission (Saez-Atienzar *et al.*, 2014). Similarly, there is no conclusion as to the effect of LRRK2 expression or activity on mitochondrial morphology. In a tg fly model, the expression of G2019S-LRRK2 is associated with mitochondrial deformity (Mortiboys *et al.*, 2015). *In vitro*, in cell lines, the expression of WT-LRRK2, and even more that of G2019S or R1441C-LRRK2 induces mitochondrial fragmentation (Su & Qi, 2013; X. Wang *et al.*, 2012). Interestingly, this effect seems to be kinase-dependent, which is consistent with the fact that the G2019S mutant has more pronounced effects on mitochondria (Su & Qi, 2013; X. Wang *et al.*, 2012). However, two other studies demonstrated the opposite results in fibroblasts derived from PD patients carrying the G2019S mutation, one observing an increased mitochondrial fragmentation, pointing towards a misbalance in favor of mitochondrial fission (Niu *et al.*, 2012), and the other enhanced mitochondrial elongation and interconnectivity, pointing towards a misbalance in favor of mitochondrial fusion (Mortiboys *et al.*, 2010). Further, the direct effect of LRRK2 on mitochondrial respiration is not clear. In HEK cells, the expression of WT-LRRK2, and to a further degree G2019S, decrease both CI and CIV activities (Su & Qi, 2013). In other model systems, tg flies expressing G2019S, G2385R, or I2020T-LRRK2 are more sensitive to rotenone-induced toxicity (Ng *et al.*, 2009; Venderova *et al.*, 2009), and in *C. elegans*, WT-LRRK2 but not G2019S-LRRK2 expression protects against rotenone-induced toxicity (Saha *et al.*, 2009).

Taken together, these studies may imply that LRRK2 and α -syn can have a direct effect on the mitochondrial respiratory chain, leading to mitochondrial dysfunction and altered energy production. However, second hypothesis that links LRRK2 and α -syn with the disruption of

the equilibrium between mitochondrial fission and fusion also seems plausible. All the data showing an effect of α -syn and LRRK2 on the toxicity of complex I inhibitors point towards a potential effect of α -syn and LRRK2 on mitochondrial respiratory chain, more precisely on CI activity. These complex I defects can lead to oxidative stress, a decrease in ATP production or in mitochondrial respiration (Devi *et al.*, 2008; Loeb *et al.*, 2010; Reeve *et al.*, 2015). All of these events ending up in mitochondrial dysfunction could therefore be a relevant explanation for the mitochondrial deficits induced by α -syn and LRRK2. As mitochondrial homeostasis is a finely regulated process with a precise equilibrium between fission (involving the proteins Drp1 (Dynamin-related protein 1), Fis1 and GDAP1) and fusion (involving the proteins Mfn 1 and 2 and OPA1), mitochondrial fragmentation can reflect an increase in mitochondrial fission or a decrease in mitochondrial fusion. Overall, these results suggest that α -syn and LRRK2 have the potential for a common effect on two major aspects of mitochondrial homeostasis: the energy production and the dynamics of mitochondria. However, there is a tight relationship between the structure and the energy function of the mitochondrial network, and an alteration of one aspect has repercussions on the other. It is therefore difficult to know which effect comes first, but newer model systems with conditional manipulation of LRRK2 and α -syn may shed light onto the relationship between these proteins, mitochondrial respiratory chain function and fusion and fission events.

α -syn, LRRK2 and Autophagy

Proteins can be removed by the ubiquitin-proteasome system (UPS) as well as the autophagy-lysosome pathway (ALP), and both have been extensively evaluated with respect to LRRK2 and α -syn in PD models. α -Syn degradation via the UPS was observed and UPS dysfunction was predicted to lead to α -syn accumulation in early studies (Bennett *et al.*, 1999; McNaught *et al.*, 2002; Tofaris, Layfield, & Spillantini, 2001; Webb, Ravikumar, Atkins, Skepper, & Rubinsztein, 2003). However, other groups failed to show an involvement of the proteasome machinery in this process (Ancolio, Alves Da Costa, Uéda, & Checler, 2000; Rideout, Larsen, Sulzer, & Stefanis, 2001; Rideout & Stefanis, 2002; Vogiatzi, Xilouri, Vekrellis, & Stefanis, 2008) suggesting another pathway to α -syn degradation such as ALP (Petroi *et al.*, 2012). Given the close overlap between LRRK2 and α -syn in other pathways we describe here, LRRK2 function may shed light on this controversy.

Autophagy clears cytoplasmic components through three distinct pathways depending of the way substrates reach the lysosomal lumen and the types of substrates supported: chaperone-mediated autophagy (CMA), macroautophagy, and microautophagy (Ana Maria Cuervo, 2004; Levine & Klionsky, 2004). In contrast, the UPS mainly drives short-lived protein degradation, typically via polyubiquitin chains, whereas autophagy is responsible for long-lived proteins, larger protein aggregates, and cytoplasmic organelles (Zhang *et al.*, 2012). CMA involves chaperone proteins (especially hsc70) which bind target proteins containing a KFERQ peptide motif. These chaperones address the proteins to lysosomes where they interact with the lysosomal receptor, LAMP2a, the rate-limiting step in CMA (A. M. Cuervo & Dice, 1996; Majeski & Fred Dice, 2004; A. Massey, Kiffin, & Cuervo, 2004). At the membrane, LAMP2a multimerizes to allow substrate translocation inside the lysosome with

the help of lys-hsc70, an intraluminal chaperone (Agarraberes, Terlecky, and Dice 1997; Cuervo, Dice, and Knecht 1997). Macroautophagy involves the creation of a double membrane structure, named phagophore, engulfing proteins and organelles. Closure completion of the phagophore forms the autophagosome which will fuse with lysosomes to generate the autolysosome, where substrates are degraded. Mitophagy is a subtype of macroautophagy which leads to dysfunctional mitochondria degradation. (Shintani & Klionsky, 2004). The molecular machinery participating in macroautophagy is composed of different type of proteins, including Atg proteins, and Beclin. Microautophagy involves the engulfment and internalization (pinocytosis) of small quantities of cytosol components directly by lysosomes (A. Massey *et al.*, 2004; Müller *et al.*, 2000). In PD brains, autophagic characteristics were shown through immunohistochemistry (Anglade, Vyas, Hirsch, & Agid, 1997) and impairment of autophagy (defective clearance of autophagosomes and lysosomal depletion) was observed in PD patient brains (Dehay *et al.*, 2010). Additionally, LAMP2a and HSC70 protein levels in the SNc of PD patient brains were decreased (Alvarez-Erviti *et al.*, 2010).

The potential degradation of α -syn by CMA is suggested since the protein contains a CMA recognition motif (95-VKKDQ-99) (Cuervo *et al.*, 2004; Dice 1990). First evidence confirming this hypothesis was depicted incubating α -syn with isolated liver lysosomes showing its degradation in a CMA-dependent manner (Cuervo *et al.*, 2004). Evidence of α -syn degradation by CMA is also demonstrated in cell lines and neurons using lysosomal inhibitors which lead to α -syn accumulation (Lee *et al.*, 2004; Paxinou *et al.*, 2001; Webb *et al.*, 2003). In 2004, Cuervo and collaborators also demonstrated that the mutant forms of α -syn (A53T and A30P) can bind to lysosome membranes but can not be engulfed and degraded efficiently by lysosomes. In fact, mutant α -syn interacts more strongly with LAMP2a, blocking CMA (Cuervo *et al.*, 2004). In different cell lines and in primary neuronal cells, mutant α -syn interaction with LAMP2a and impairment of CMA is also demonstrated (Vogiatzi *et al.*, 2008). More recently, decreasing LAMP2a expression through a strategy of shRNA-AAV injections in rat brain results in intracellular accumulation of α -syn and autophagic vacuoles (Xilouri *et al.*, 2016). Additionally, when the recognition motif 95-VDKKQ-99 is mutated, CMA is dysfunctional and compensatory mechanisms occur with the activation of macroautophagy (Vogiatzi *et al.*, 2008; Xilouri, Vogiatzi, Vekrellis, Park, & Stefanis, 2009). In cell culture, LAMP2a silencing blocks CMA but upregulates macroautophagy and sensitizes cells to oxidative stress suggesting also a compensatory mechanism (Massey *et al.*, 2006). α -Syn overexpression inhibits macroautophagy through blockade of autophagosome formation. However, cells expressing α -syn in contact with preformed α -syn aggregates leads to the formation of autophagosomes but their clearance is blocked (Tanik, Schultheiss, Volpicelli-Daley, Brunden, & Lee, 2013). In Atg7 knock out mice, macroautophagy impairment leads to ubiquitinated proteins, α -syn and p62 increased (Ahmed *et al.*, 2012). In another model of Atg7 knock out targeting TH positive cells, accumulation of α -syn in dopaminergic neurons, especially midbrain dopaminergic dendrites which exhibit numerous varicosities containing massive ubiquitinated inclusions, was identified (Friedman *et al.*, 2012). Mechanistically, α -syn compromises autophagy via Rab1a inhibition and causes mislocalization of the autophagy protein Atg9. Atg9 is implicated in the initiation of autophagosome formation (Winslow *et al.*, 2010). Additional

experiments are needed in order to decipher the molecular mechanisms implicated in macroautophagy in α -syn toxicity.

While fewer studies have explored LRRK2 function in the UPS and ALP, LRRK2 contains several CMA targeting motifs suggesting its possible degradation by CMA. The role of LRRK2 in CMA has been partially elucidated in a study from Orenstein in 2013. In this study, LRRK2 is degraded by CMA as knock down of LAMP2a increases intracellular levels of LRRK2. Degradation of LRRK2 reaches 50% of normal levels in LAMP2a knock downs whereas G2019S-LRRK2 is limited suggesting impairment of CMA through mutant LRRK2 activity (Orenstein *et al.*, 2013). Degradation of LRRK2 through macroautophagy is supported through experiments in Atg5 KO and Atg7 KO mice showing accumulation of LRRK2 in cells and tissue (Friedman *et al.*, 2012). Moreover, a fraction of LRRK2 is localized to membranes (Berger, Smith, & Lavoie, 2010; Biskup *et al.*, 2006) and more specifically to autophagic vacuoles, amphisomes, and autolysosomes and lysosomes (Alegre-Abarrategui *et al.*, 2009; Mark W. Dodson, Zhang, Jiang, Chen, & Guo, 2012; Higashi *et al.*, 2009; Orenstein *et al.*, 2013). However, contradictory results were obtained on the role of LRRK2 in macroautophagy. On the one hand, several groups observe a positive regulation of autophagy by LRRK2. Saha and collaborators demonstrate that expression of human LRRK2 or dead kinase LRRK2 in *C. elegans* increases autophagy (Saha *et al.*, 2015). In macrophages and microglial cells, LRRK2 knockdown significantly decreases the autophagic capacity (reduction of LC3 conversion), and LRRK2 inhibitors (GSK2578215A or LRRK2-IN-1) block protein aggregate clearance (Schapansky, Nardozi, Felizia, & LaVoie, 2014). In 2010, Tong and collaborators showed that LRRK2 KO mice present accumulation of lipofuscin granules and p62 protein and a decrease of LC3-II conversion in renal tubule cells of aged mice (Tong *et al.*, 2010), suggesting slowdown of autophagy in those cells. In flies, knockout of the LRRK2 ortholog dLRRK leads to an impairment of autophagy with enlarged early endosomes, accumulation of autophagosomes which involves a modulation of Rab9 activity (Dodson *et al.*, 2014). On the other hand, knock down of LRRK2 by siRNA produces an induction of autophagy suggesting LRRK2 act as a negative regulator of autophagy (Alegre-Abarrategui *et al.*, 2009). In astrocytes, inhibition of LRRK2 stimulates macroautophagy in an mTOR independent manner that requires Beclin (Manzoni *et al.*, 2016). Bravo-San Pedro et collaborators observed an increase of autophagy induction (increase of LC3, LAMP2, Beclin1 and decrease p62) in G2019S-LRRK2 skin fibroblasts from PD patients via a MAPK/ERK dependent pathway resulting in a highest sensitization to cell death (Bravo-San Pedro *et al.*, 2013). As usual, the apparent discrepancies between these different studies may originate from different experimental conditions and cell types used, but as yet there is no clear consensus of LRRK2 effect on ALP.

To go deeper in our understanding of the possible link between α -syn, LRRK2 and ALP, an interesting point is the role of Rab proteins and particularly Rab7. In fact, as mentioned above, Rab7 interacts with LRRK2 and plays a role in autophagosome and lysosome trafficking, positioning, and fusion events. Rab7 promotes clearance of α -syn aggregates (Dinter *et al.*, 2016). Rab7 is also a key regulator of late endosome maturation and autophagosome-lysosome fusion (Jager, 2004). Therefore, it is conceivable that the link between LRRK2 and α -syn in producing autophagy defects would be through Rab-dependent action, for example Rab7, at the membrane of autophagosomes. This modulation

could interfere with trafficking of autophagosomes, which need to reach the soma to fuse with lysosomes to have an efficient autophagy.

Conclusion and future directions

A compelling and growing body of evidence points towards a functional interaction between α -syn and LRRK2. Intriguingly, in many studies, larger effects are observed by knocking down LRRK2 expression or activity compared to over-expressing mutated LRRK2, giving hope that LRRK2-targeting therapeutics may offer benefit to all PD patients rather than just those that have LRRK2 mutations. Yet, the underlying mechanisms that dictate LRRK2 and α -syn interaction in PD are not clear. Based on our current understanding of both proteins in disease, cell autonomous and non-cell autonomous pathways are likely relevant in linking the proteins together in pathogenesis and disease progression. When expressed in the same cell, in disease, α -syn and LRRK2 likely interact in response to mitochondria disruption, in autophagy pathways in the endolysosomal system, as well as in normal interactions with common protein partners like 14-3-3 that maintain homeostasis of a number of systems. While α -syn expression in the brain is highly restricted to neurons, LRRK2 is also expressed in the immune system, particularly in cells known to respond to amyloid forms of α -syn, and LRRK2 appears to exacerbate damaging pro-inflammatory responses caused by α -syn. Considering current knowledge, none of the mechanisms we discussed in this review appear to be more prominent than the others. These interactions will all take time and more advanced model systems and experiments to fully resolve. In the meantime, therapeutic targeting of both LRRK2 and α -syn, if our understanding of these proteins in disease is at least partially correct, should provide the best opportunities to intervene in the disease process.

Acknowledgements:

NC is a recipient of a PhD fellowship from Association France Parkinson (2016). CG is a recipient of the PhD fellowship from the French Ministry of Research. FG is funded by the European Union Horizon 2020 Programme (H2020-MSCA-ITN-2015) under the Marie Skłodowska-Curie Innovative Training Network and Grant Agreement No. 676408. EB is supported by “Fondation de France, Parkinson committee” grants (AAP 2010, Engt 00016819 and AAP 2014, Engt 2014–0052580) and the national “Translational Research Infrastructure for Biotherapies in Neurosciences” (NeurATRIS, “Investissement d’Avenir”, ANR-11-INBS-0011). ABW is supported by NIH grants R01 NS064934, U01 NS097028, and R33 NS097643.

List of Abreviation

AAV	Adeno-associated virus
ADPD	Autosomal dominant Parkinson’s disease
ALP	Autophagy-lysosome pathway
ANK	Ankyrin
APP	Amyloid precursor protein
ARM	Armadillo
Atg	Autophagy related protein

ATP	Adenosine Triphosphate
BAC	Bacterial artificial chromosome
BAD	Bcl-2-associated death promoter
Bcl-2	B-cells lymphoma 2
CaMKII	Calcium/calmodulin-dependent protein kinase II
CI – CIV	Complex I – Complex IV of mitochondrial respiratory chain
CMA	Chaperone-mediated autophagy
CNS	Central nervous system
COR	C-terminal of Roc
DA	Dopamine
DAT	Dopamine transporter
Drp1	Dynamid related protein 1
ERK	Extracellular signal-regulated-kinase
Fis1	Mitochondrial fission protein 1
GAD	G proteins activated by nucleotide-dependent dimerization
GDAP1	Ganglioside-induced differentiation-associated protein 1
GLP1R	Glucagon-like peptide-1 receptor
GWAS	Genome-wide association
HEK	Human embryonic kidney (cell line)
HSC70	Heath shock cognate protein
IBA1	Ionized calcium-binding adapter molecule 1
IBD	Inflammatory bowel disease
IFN-γ	Interferon gamma
IL-1β	Interleukin-1 beta
IL-6	Interleukin 6
iNOS	Inducible nitrite oxide synthase
iPD	Idiopathic Parkinson's disease
iPS	Induced pluripotent stem cells

JNK	c-Jun N-terminal kinase
kD	kilo-dalton
KI	Knock-in
KO	Knock out
L-dopa	Levodopa
LAMP2	Lysosome-associated membrane protein 2
LB	Lewy bodies
LC3-II	LC3-phosphatidylethanolamine conjugate
LN	Lewy neurites
LPS	Lipopolysaccharides
LRR	Leucine-rich repeats
LRRK2	Leucine-rich repeat kinase 2
MAPK	Mitogen-activated protein kinase
MHC-II	Major histocompatibility complex II
Mnf1 – Mnf2	Mitochondrial nucleoid factor 1 – 2
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger RiboNucleic Acid
mTOR	Mammalian target of rapamycin
MyD88	Myeloid differentiation primary response 88
NF-kB	Nuclear factor kappa B
NFAT	Nuclear factor of activated T-cells
NHPs	Non-human primates
NO	Nitrite oxide
OPA1	mitochondrial dynamin like GTPase
PARK8	Old name for LRRK2
PD	Parkinson's disease
PET	Positron emission tomography
PFFs	Pre-formed fibrils
PrP	Prion protein

pSer	phosphorylated Serine
Roc	Ras-of-complex
Ser	Serine
SH-SY5Y	Homo sapiens bone marrow neuroblast cell line
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SNe	Substantia nigra pars compacta
SNCA	alpha synuclein (gene)
SNP	Single nucleotide polymorphism
SOD1	Superoxide dismutase 1
TDP43	TAR DNA-binding protein 43
Tg	Transgenic
TH	tyrosine hydroxylase
TLR4	Tool-like-receptor 4
TNF-α	Tumor necrosis factor alpha
TOM20	Translocase Of Outer Mitochondrial Membrane 20
tTa	Tetracycline trans activator
Tyr	Tyrosine
UPS	Ubiquitin-proteasome system
vMAT	Vesicle dopamine transporter
WD40	Beta transducing repeat
WT	Wild type
α-syn	α -synuclein

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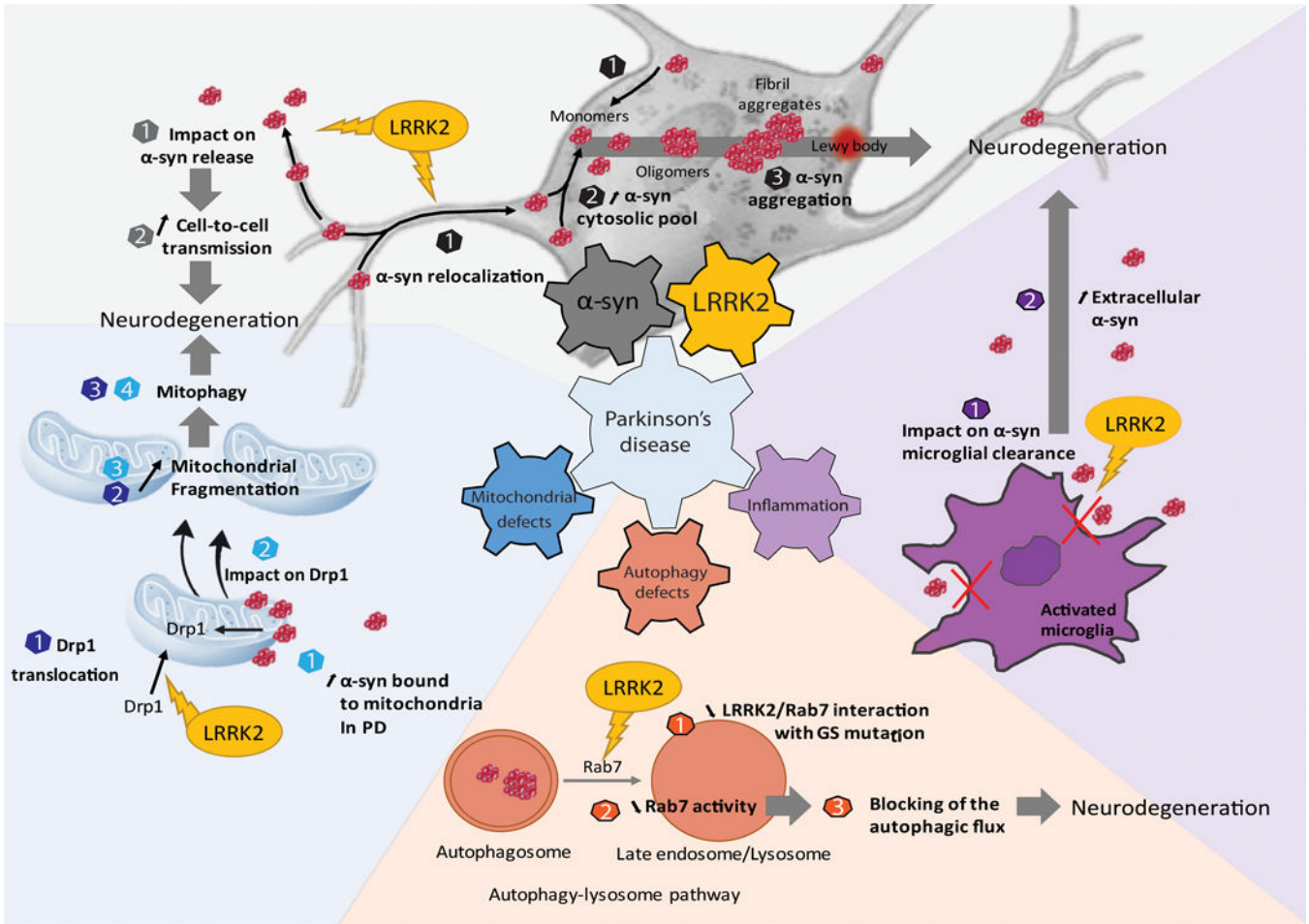


Figure 1: Cellular dysfunction and neurodegeneration through several distinct but non-mutually exclusive mechanisms.

LRRK2 could have an impact on α -syn release (grey numbers) as well as on its relocalization (black numbers) leading to a cell-cell transmission of the pathology and to an increase of the aggregation through an excess of α -syn in the cytosolic pool (grey part). Three mechanisms seem to be impacted: the mitochondrial biology (blue part), the autophagic system (orange part) and the inflammation (violet part). Both LRRK2 and α -syn are known to play a role in mitochondrial biology through Drp1 (blue number) in favor of a mitochondrial fragmentation leading to mitophagy and cell death. Inside the cell, α -syn aggregates are mainly degraded by the autophagic-lysosomal pathway, which is one of the major protein breakdown systems (orange part). LRRK2 was found to decrease the activity of Rab7, which is implicated in the maturation of the autophagosome and thus blocking the degradation of the α -syn aggregates. Outside the cell, α -syn aggregates are captured by microglia to be degraded. One hypothesis is that LRRK2 could alter the machinery of phagocytosis leading to an accumulation of extracellular aggregates.