The role of LRRK2 in Parkinson's disease

Essay
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1. Abstract

Parkinson's disease (PD) is the second most prevalent neurodegenerative disease just behind Alzheimer. It is a progressive disorder of the nervous system that affects movement.

Although it was believed to be a sporadic pathology triggered by environmental factors, nowadays a lot of PD-related genes have been identified, including LRRK2.

Mutations in LRRK2 are a major genetic risk factor for both familial and sporadic PD, being present in 4% cases of familial and 1% cases of sporadic disease. However, the pathway by which LRRK2 drives to PD development is still unknown.

Multiple pathways have been linked with LRRK2 such as autophagy, inflammation, synapsis, apoptosis, cytoskeleton interaction and mitochondria homeostasis. In this essay, these pathways and the evidences found to date of LRRK2 playing a key role in them are described.

In brief, this protein could have multiple roles because it is a big protein with two enzymatic domains and multiple protein-protein interaction domains. However, due to the strong association found between LRRK2, tau and α –synuclein and the location of LRRK2 in microtubules and membranes, I consider that the hyperphosphorylation of tau within the microtubules is the hypothesis that better brings together all the findings discovered to date and, therefore, the study of this interaction should be the future direction of research.

2. Table of contents

3. List of abreviations

- ANK: Ankirin
- AV: Autophagic vacuole
- BBB : Blood-brain barrier
- COR: C-terminal of ROC
- CNS: Central nervous system
- DD: Death domain
- EM: Electron microscopy
- GWAS: Genome-wide association
- IP: Immunoprecipitation
- K.O: Knocked-out
- LB: Lewy bodies
- LN: Lewy neurite
- LRR: Leucine-rich repeats
- NSF: N-ethylmaleimide sensitive factor
- PD: Parkinson's disease
- ROC: Ras of complex proteins
- ROS: Reactive oxygen species
- SN: Substantia nigra
- SNpc: Substantia nigra pars compacta
- SV: Synaptic vesicles

4. Introduction

4.1 Parkinson's disease

The first description of Parkinson's disease (PD) dates from 1817, when James Parkinson published an essay entitled "An Essay on the shaking palsy". In this work, he described the symptoms of six individuals and termed their disease as "paralysis agitans"(1).

Parkinson's disease is the second most prevalent neurodegenerative disease just behind Alzheimer. (1). It has a prevalence of approximately 1% at age 65, which rises to 5% by age 85 (2).

The main symptoms of PD are resting tremor, rigidity, bradkinesia and postural instability. All these motor-affecting manifestations are termed Parkinsonism and William Gowers already described them in 1886 in his work "A manual of diseases of the nervous system" (*Figure 1*) (3). Besides, it appears a variety of poorly treatable no-motor symptoms ranging from autonomic concerns to neuropsychiatric complications (1).

Figure 1. Illustration of Parkinson's disease by William Richard Gowers, which was first published in *A Manual of Diseases of the Nervous System* (1886)

Pathologically, PD is caused by degeneration in the neurons from the substantia nigra pars compacta (SNpc). Neurons in this area are dopaminergic, in charge of controlling movement. Patients with PD suffer from neurone demyelination and therefore, paralysis.

The symptoms only appear when 70% of the neurons are lost, astrocytes are dead and microglia is activated. In 90% of the cases, it is possible to find Lewy bodies (LB) or Lewy neurite (LN), intracellular inclusions largely composed of the protein α-synuclein (1).

In the last decade, the knowledge of PD pathogenesis has deeply changed. PD has historically been considered a sporadic disease triggered by environmental factors. However, recent studies in families, and genome-wide association (GWAS) studies have shown that genetics has an important role, not only in inherited but also in sporadic PD (7). Furthermore, these mutations affect to the same genes in both types of disease, suggesting that, inherited and sporadic Parkinson's disease can have common pathological mechanisms (4).

LRRK2 gene appeared to be the most common genetic cause of PD, being present in 4% cases of familial and 1% of sporadic PD.

4.2 LRRK2 gene. Structure

LRRK2 (PARK8) is a large gene located on12p11.2-q13.1 (5) whose transcript encodes a 2527 amino acid protein comprised of 51 exons called dardarin (6).

LRKK2 is mainly expressed in kidney, lungs and brains (5). Besides, it is also expressed in some cells from the immune system as monocytes, macrophages, dendritic cells and B cells (7). Even if LRRK2 is expressed in so many tissues, its activity is

much higher in the brain compared with other organs.

LRRK2 protein is mainly present in the cytoplasm, soluble or associated to membranes. Most of the time it forms dimers although it can also be found in monomeric form in the cytoplasm or forming oligomers in membranes and microtubules (8).

LRRK2 is a protein that contains multiple domains, including two enzymatic domains (kinase and GTPase) and several protein-protein interaction domains (6) (*Figure 2*). LRRK2 is considered a member of a G-superfamily of GTPase known as ROCO proteins (9), a family of proteins that presents a conserved core, consisting of a Ras-like GTPase called ROC (Ras of complex proteins) and a COR (C-terminal of ROC) domain with unknown function (10). The ROC domain can bind GTP and hydrolyse it to GDP

Furthermore, LRRK2 kinase domain phosphorylates serine/threonine residues and, due to its structure, it belongs to RIP kinase family. This family of S/T kinases collaborate with death receptors and proteins to regulate cell death. They can trigger apoptosis or necroptosis interacting with factors such as FADD, caspase 8 or NF-kB (11). It is well known that GTPase and kinase activities are strongly linked, therefore, it has been proposed that LRRK2 kinase activity could function as a down-stream effector of the GTPase activity or, contrary, that LRRK2 autophosphorylation may serve to regulate the rate of GTP hydrolysis (9).

As it was mentioned above, LRRK2 contains several protein-protein interaction domains. The most important ones are ankirin (ANK) domain, important for attachment with integral membrane proteins and cytoskeleton; leucine-rich repeat (LRR) domain, structure involved in signalling processes; and WD40 domain, present in other members of ROCO family and involved in cytoskeletal assembly and vesicle trafficking (6).

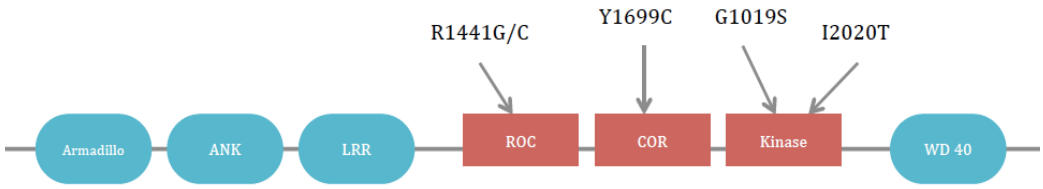

Figure 2. Schematic of LRRK2 domain organization and most important mutations for Parkinson disease (PD). Red boxes represent the catalytic core of the protein while blue boxes represent the protein-protein interaction domains. From N terminal to C terminal: Armadillo domain, Ankirin (ANK) domain, Lecuine-rich repeat (LRR) domain, Ras of complex proteins (ROC) GTPase domain, C terminal of ROC (COR) domain, Kinase domain and WD40 domain.

4.3 LRRK2 PD mutations

LRRK2 mutations are the most common genetic cause of PD, being present in 4% cases of familial and 1% of sporadic PD (5). Even if more than 40 mutations have been found (6), there are just 4 very frequent and, therefore, are the ones better studied (*Figure 2*). R1441C/G mutation affects the ROC domain while Y1699C affects the COR domain. Kinase domain is affected by G2019S and I2020T mutations (9).

The ROC domain of LRRK2 binds both GTP and GDP with similar affinity even when these domains present mutations. Nevertheless, hydrolysis of GTP to GDP is

reduced in both ROC (R1441C or R1441G) and COR (Y1699C) domain mutants whereas the GTPase activity remains unchanged when mutations affect the kinase domain (G2019S and I2020T) (9). The kinase activity is only modified under mutations in the G2019S residue, which shows increased intrinsic kinase activity (9).

4.4 Aim

Currently, the physiological function of LRRK2 is unknown. However, the presence of both protein interaction domains and enzymatic domains suggests that this protein may serve as a scaffold for proteins and act as a central integrator of multiple signalling pathways (6). LRRK2 has been suggested to play a role in the control and maintenance of neurite length, vesicle trafficking at the presynaptic site, activator of apoptosis and regulation of autophagy pathways (12). In this essay, the evidence of LRRK2 playing a role in these pathways is discussed based on the most recent literature. The main goal of this review is clarifying which are the most likely pathways by which LRRK2 promotes the development of PD.

5. Cellular roles of LRRK2

5.1 Autophagy abnormalities

5.1.1 The role of autophagy in Parkinson disease

Parkinson's disease (PD) is originated by the exposure to certain environmental toxins, age and genetic predisposition. These factors lead to block or modify cellular processes, particularly the ones related to defence against oxidative stress, mitochondrial function and protein degradation. (13).

This chapter is focused in one of the protein degradation mechanisms, autophagy, which is the catabolism of cytoplasmic molecules and organelles following sequestration in autopaghic vacuoles (AV) (14). In physiological conditions, autophagy is an important mechanism for the maintenance of neuronal cells, however, when this process is disrupted, it leads to neuron degeneration (15).

Patients with PD present an increase in neurodegenerative processes and, therefore, an accumulation of autophagic vacuoles in neurons. There are many well-known factors that inhibit autophagy (PI3K, NFkB, Bcl2, mTOR) and, on the other hand, activate it (Beclin-1, ROS, ERK/MEK) (13). Currently, scientists are studying the possible role of LRRK2 within this process.

5.1.2 LRRK2 implication in autophagy

The first indication that LRRK2 could be an important regulator of autophagy was the discovery that LRRK2 is, not only soluble in the cytosol but also bound to membranes (15). LRRK2 is considered essential for maintaining the equilibrium between cellular degradation and synthesis, and therefore, deregulation of autophagy has been associated with mutations of the LRRK2 proteins, especially the ones affecting its kinase activity (13).

The way by which LRRK2 affects autophagy processes is not yet known. Currently, there are different approaches to clarify this mechanism and it includes studies about G-protein related signalling, LRRK2 association with endolyssosomes and interaction and disruption of microtubules (13).

Plowey et al. (14) demonstrated in SH-SY5Y cell line that the number of AV increases in neurons with G2019S mutation when compared to control LRRK2 by measuring the brightness signal from GFP-LC3 (autophagy marker protein) with transmission electron microscopy (EM). Furthermore, neurite outgrowth was disrupted in the mutant strain resulting in neurite shortening.

By the combination of LRRK2-YPet strains, fluorescence microscopy and immunofluorescence techniques, *Alegre-Abarrategui et al*. (16) could demonstrate that LRRK2 is located in membrane microdomains and multivesicular bodies in VERO and HEK293 cell lines. LRRK2 is mainly located in the neck of caveolae, microvilli and intraluminal vesicles of multivesicular bodies. This suggests the important role of LRRK2 in cytoskeleton reorganization and curvature dynamics.

Other study (15) suggests that the role of LRRK2 in autophagy is Ca^{2+} dependent. They demonstrated in HEK293T cell line that LRRK2 activates NADDP-R and it controls autophagy via Ca2+/CAMKK/AMP signalling. Furthermore, they demonstrated that LRRK2 could activate a Ca2+ efflux from acidic stores causing partial alkalinisation. However, they did not find a significant difference in the signalling activity between physiological LRRK2 and its mutant G2019S, suggesting that, although this signalling pathway is not important for the pathogenesis of PD, could be important in the physiological function of this protein.

A recent study (13) has described the implication of LRRK2 within the ERK/MEK signalling pathway. The study was driven by analysing the phosphorylation levels of ERM with western blots of human dermal fibroblast from patients. They discovered that ERM phosphorylation was higher in patients with the G2019S mutation. In addition, they proved with LC3P-GFP-mCherry constructs, that fibroblasts with the G2019S LRRK2 mutation exhibit higher autophagic activity than control fibroblasts. And this activation decreased significantly when treated with the ERK/MEK inhibitor, BafA1 (*Figure 3*). Lastly, they noticed that LRRK2 knocked-out (K.O) strain also induced autophagy and cell death and, for reverting normal cell sensitivity, it is necessary to block the aberrant autophagy but maintain physiological LRRK2 functions. This is an important observation for further developing of drug targets.

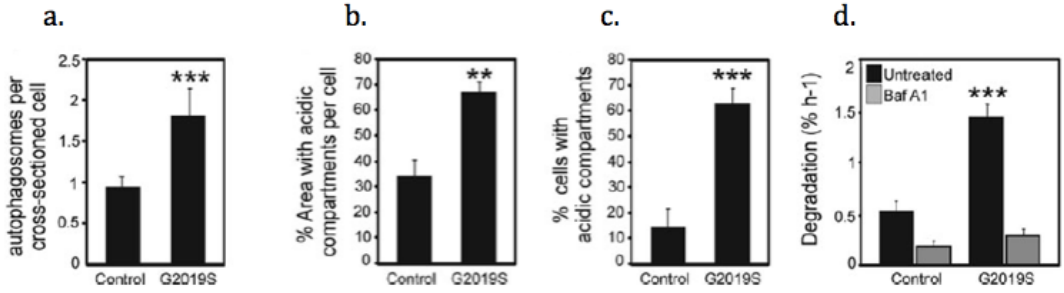

Figure 3. Detection of autophagy in the control and G2019S fibroblast groups. Results taken from (11). In these graphs is shown that fibroblasts with the G2019S LRRK2 mutation exhibit higher autophagic activity levels than control fibroblasts. In addition, this activation decreases significantly when cells are treated with the ERK/MEK inhibitor, BafA1

Altogether these data show that there is a strong link between autophagy abnormalities and the development of PD. This situation in the cell drives to accumulation of vacuoles within the neurons, which is demonstrated to occur in the SNpc of patients with PD (17), but also it drives to a stress situation, LB and LN formation and, finally, apoptosis.

Although the link between LRRK2 and autophagy is clear, the role that LRRK2 could play in this mechanism is still not well known. *In vivo* experiments performed with human tissue from patients, brings strong evidence of the implication of LRRK2 in the ERK/MEK signalling pathway. However, the implication of the $Ca2^{+}$-dependent pathways are not so clear, since there was was no difference in the activation patterns between G2019S and control LRRK2. Importantly, this over-activation of autophagic pathways could be just a side effect of the major role of LRRK2. It is not demonstrated if autophagic activation leads to protein aggregates or if it is the other way around, the accumulation of protein aggregates induce the overexpression of acidic compartments in order to control cell stress. Therefore, more study is needed in order to know if LRRK2 directly activates autophagy, or if it provokes α-synuclein and tau aggregation, which would lead to overexpression of AV.

5.2 Inflammation

5.2.1 The role of inflammation in Parkinson disease

Inflammation is a complex processes triggered under many different pathological conditions and involved in many diseases, for example in the case of patients suffering PD, microglia is strongly activated (18). Therefore, it is clear that neuroinflammation plays a key role in the progress of this disease, although it is still controversial if inflammation is the prior cause or just a secondary effect.

Some authors are sceptic about the role of inflammation as prior cause of PD (7) because it shows some weak points. First of all, it is not clear how the increased peripheral pro-inflammatory cytokines can cross the BBB (blood-brain barrier) and enter the brain or, on the contrary, they are just released *in situ.* Besides, they are doubtful about the direct link between inflammation processes and degeneration of dopaminergic neurons in the SNpc. Therefore, they maintain that neuroinflammation is just a secondary effect of a more specific process (7).

In the contrary, authors who support the important role of inflammation in PD (19) state that dopaminergic neurons are really sensitive to oxidative insults because the SNpc has lower levels of glutathione and 4,5 times more microglia when compared to other brain regions (12). Furthermore, two recent GWAS studies have found common LRRK2 mutants associated with Crohn's disease, inflammatory bowel disease, leprosy and Parkinson (12). The clearest feature from all this pathologies is the strong presence of immune response. Other fact that supports the role of LRRK2 in inflammation is that LRRK2 is highly expressed in monocytes, macrophages, dendritic cells and B cells (12).

5.2.3 LRRK2 implication in inflammation processes

Liu et al. (20) published a study in which they explained that LRRK2 interacts with NFAT modulating its retention in the cytoplasm. NFAT proteins are integrators of Ca^{2+} signalling with many other signalling pathways in T cell. They participate in a wide range of inflammatory immune responses and they act in the nucleus of the cell (21). Analysing NFAT in HEK293T cell line with confocal microscopy, brought that NFAT cannot travel to the nucleus for exert its function as transcriptional factor under the overexpression of LRRK2. However, when LRRK2 is mutated to induce Crohn's disease and inflammatory bowel disease, this protein is not able to bind NFAT anymore and the inflammation process is triggered. Nevertheless, they did not find any interaction with NF-kB.

Other study (7) proved that LRRK2 is an IFN-γ target gene. Analysing the mRNA levels of LRRK2 from human intestine cells, they discovered that they increase in presence of IFN- γ. Besides, they found a highly conserved binding site for IFN- γ after sequencing LRRK2. Moreover they support that LRRK2 enhances NF-kB dependent transcription when it is activated by IFN-γ

To sum up, inflammation is important in PD (18), even if the role of LRRK2 is still controversial.

LRRK2 presents in its sequence a highly conserved binding site for IFN- γ, an important factor in inflammation initiation (7). It was also demonstrated that LRRK2 interacts with IFN- γ (7) and NFAT (20) when studied in human cell lines.

However, even if the experiments were performed in human cell lines, these studies were focused on the understanding of Chron's disease and bowel inflammation disease; thus, the pathogenic role of LRRK2 in PD could be different. For instance, the kinase domain of LRRK2 has no apparent effect of NFAT regulation (20), however, the most frequent mutation in PD, G2019S, leads to an increase in kinase activity (9). Moreover, the experiments performed to demonstrate the link between LRRK2 and NF-kB are contradictory (7, 20) and could be an artefact of overexpressing LRRK2.

In conclusion, there is a lot of research to be done about neuroinflammation and its link with PD. Not only about the specific role of LRRK2 in this process, but also about the importance of this process in the progress of the disease. It should be clarify how a defect the immune system could lead to such a specific pathology in the SNpc.

5.3 Synapsis imbalances

Imbalances in synaptic transmission are implicated in Parkinson's disease (22). Perturbations of LRRK2 expression have been shown to influence neurite extension and vesicle endocytosis (23) and, for this reason, it has been suggested that LRRK2 could participate in the biogenesis of synaptic vesicles (SV) (9) (*Figure 4*).

First of all, LRRK2 has been localized in various membrane-containing structures in neurons, including a partial localization in SV (9); this has led to the identification of a functional role of LRRK2 in vesicular trafficking and biogenesis.

Secondly, recent GWAS studies have detected a consistent association between α-synuclein and LRRK2 (6). α-synuclein is the major component of LB and LN. Within cells, α-synuclein normally adopts an α-helical conformation. However, under certain circumstances, the protein can undergo a profound conformational transition to a beta-sheet rich structure that polymerizes to form toxic oligomers and amyloid plaques (24). These α-synuclein aggregates can disrupt proteases; sensitize neurons to stress and act at synapses to impair neuronal function (25). The big impact of α-synuclein in synapsis and the GWAS association with LRRK2, have lead to study the role of LRRK2 in SV.

It has been demonstrated the interaction of LRRK2 with many proteins involved in the synthesis of synapsis vesicles like EndoA (22) or NSF (23).

EndoA is a conserved protein critically involved in SV endocytosis, which drives vesicle formation by sensing or inducing membrane curvature (22). Its phosphorylation in Ser75 decreases its membrane affinity to liposomes (22). Using radioactive ^{33}P-ATP in a *Drosophila* model, it has been identified EndoA as a substrate of LRRK2, specifically, in Ser75 residue (22).

N-ethylmaleimide sensitive factor (NSF) is one of the major presynaptic protein, a vesicle-fusing ATPase and a key player in vesicular endocytosis (23). Western blot analysis showed that LRRK2 levels were positively associated with synapsis maturation (23). Therefore, an immunoprecipitation (IP) was performed with lysate from adult mouse brain. NSF specifically coprecipitated

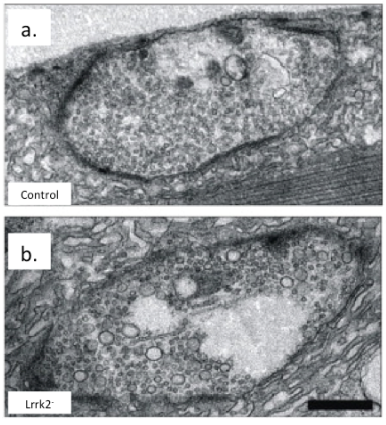

Figure 4. Loss of LRRK2 in *Drosophila* Causes Mild Defects in Synaptic Vesicle Endocytosis. Electron micrographs of a. control and b. LrrkP1 mutant (21).

together with endogenous LRRK2 suggesting that, LRRK2 has an important role in synapsis. They also proposed that the lack of this protein affects SV, meaning that the pathological mechanism would not be an increase in LRRK2 kinase activity but a defect in its protein-protein interaction activity (23).

All in all, synapsis imbalances are characteristic from PD (18). Some factors that lead to this pathology have already been identified, like α-synuclein (24). The strong association and the synergic effect between α-synuclein and LRRK2, suggests the importance of LRRK2 in this pathway. This *in vivo* experiments with *Drosophila* and mouse, shows a kinase dependent interaction with EndoA (22) and a protein-protein interaction with NSF (23) that could explain this pathway.

However, there is not an agreement in which step of the pathway LRRK2 plays an important role: vesicle trafficking, membrane curvature, or interaction with presynaptic proteins. Even more, the way in which LRRK2 interacts with this compounds is not clarify yet, if its kinase dependent or protein binding dependent.

5.4 Apoptosis

The accumulation of misfolded proteins and the presence of oxidative stress are two of the multiple cellular insults that can trigger cell death (26). The core of apoptotic pathways is composed by proteolytic caspases that, when activated, lead to a highly regulated process of cell death. Caspase activation can occur locally within neuronal processes, leading to process degeneration (26).

LRRK2 is a member from the RIP kinase family. This family collaborate with death receptor proteins to regulate cell death. They are S/T kinases that can trigger apoptosis interacting with molecules such as FADD and caspase-8 (11).

LRRK2 has been localized in various membrane-containing structures and microtubules (9). The association to these structures increase LRRK2 concentration and facilitates its oligomerization. Even more, some well-known LRRK2 PD-mutations increase the tendency of LRRK2 to form oligomers (8).

This big protein structures could be a scaffold for recruiting apoptotic proteins and, when it is located in microtubules, it is termed death effector filaments. Death effector filaments are filaments formed by DED (death effector domain) containing proteins in living cells that recruit and activate pro-caspases for inducing apoptosis with member of the TNFR superfamily (27).

In order to clarify with which elements of the apoptotic pathway LRRK2 interacts, an *in vitr*o experiment was performed (26). They demonstrated that LRRK2 interacts *in vitro* with FADD and caspase-8. What is more, they proved that all PD mutations enhance this interaction. This group also performed an IP of LRRK2 in CAD cells and they discovered that it precipitated bound with proteins containing death domain (DD) like FADD and TRADD. These findings leaded them to conclude that LRRK2 is directly linked with cell death signalling pathway and that all its PD-linked mutations enhance the activation of apoptosis.

In brief, owing to the fact that LRRK2 is a member of the RIP kinase family, it is obvious to think about its role in apoptosis. Some recent studies have shown that PD molecular pathology gets more severe if LRRK2 forms oligomers (9). The formation of scaffolds to recruit caspases would explain this observation and will also explain why

the interaction of LRRK2 with membrane and microtubules is so important for its function.

However, the experiments performed to date are *in vitro* experiments in which LRRK2 was overexpressed and, therefore, the *in vivo* affinity of LRRK2 with these factors could not be so strong. This hypothesis explains neither the strong correlation that was found with tau and α-synuclein (6). Therefore, more *in vivo* experiments should be performed in order to clarify if apoptosis is the major role for LRRK2.

5.5 Cytoskeleton disruption

Tau is a microtubule-associated protein found in the central nervous system (CNS) and highly expressed in neural axons. Its main function is promoting the assembly of microtubules for allowing neurite outgrowth, therefore, its critical for the establishment of neural cell polarity (28).

In some neurodegenerative diseases like Alzheimer disease and PD, tau is highly phosphorylated and it forms paired helical filaments that will develop until neurofibrillary lesions termed tauopathies (29). Phosphorylated tau is associated with disruption of the microtubule network (9).

The association of LRRK2, α-synuclein and tau with PD was consistent with recent genome wide association; however, the mechanism that LRRK2 contributes to α-synuclein and tau aggregation is controversial. (6).

First of all, some studies were focussed in studying the interaction of LRRK2 with the cytoskeleton. *Kett et al.* (30) studied microtubule binding of this protein with immunofluorescence, immune-EM and EM tomographic studies in HEK293T and CAD cell lines. They discovered that LRRK2 binds microtubules in a well-ordered periodic fashion, suggesting that it joins to specific binding sites or proteins. They also clarified that this bound and the filament formation requires intact kinase function and WD40 domain. What is more, they discovered that most of the major mutations, R1141C/G, Y1699C and I2020T, enhance microtubule binding, also in an ordered periodic fashion.

There are many possible explanations why LRRK2 binds so specifically to microtubules, such as its importance for microtubule dynamics and transport, formation of dead effector filaments (*See chapter 8.1*) or specifically interaction with tau.

Secondly, more research was performed in order to clarify the strong association between tau and LRRK2 (6). An IP essay in SH-SY5Y cell line exhibited that tau and tubulin co-precipitated with LRRK2. Nevertheless, tau precipitated with LRRK2 only in presence of tubulin but not in absence (28). When LRRK2 and tau were exposed to ^{32}P-ATP in presence or absence of tubulin, tau was only phosphorylated by LRRK2 when it was bound to tubulin. With Western blot experiments, was possible to identify the phosphorylation site: Thr181 (28). All this results together suggested that LRRK2 regulates the association between tau and tubulin via phosphorylation. LRRK2 can only phosphorylate tau when it is bound to tubulin and this leads to its dissociation within the complex (28) (*Figure 5*). What is more, an *in vivo* essay demonstrated that phosphorylated tau is not able to associate with tubulin any more, causing a disruption in microtubule network (28).

Finally, they studied the most common LRRK2 PD-mutations, revealing that G2019S and I2020T mutations increase kinase activity, whereas R11441C mutation shows the same activity as the control one.

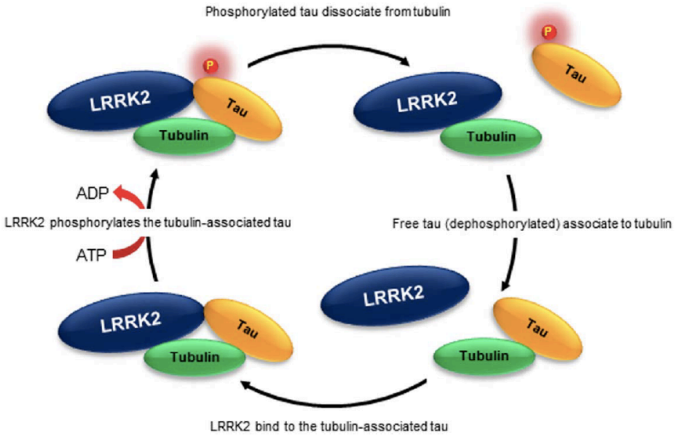

Figure 5. LRRK2 regulates the association between tau and tubulin. LRRK2 interacts with tubulin-associated tau, forming a complex. This complex induces the phosphorylation of tau by LRRK2 and sequentially induces dissociation of tau from tubulin. Tau needs to be dephosphrylated to recover its ability to bind with tubulin (28).

This hypothesis suggests why phosphorylated tau is highly present in patients with PD. Also, the disruption of microtubule network could explain other molecular pathologies already described in detail like autophagy and synaptic abnormalities. This would lead to stress within the cell, driving to α-synuclein aggregation (formation of LB and LN) and, in severe situations, to apoptosis.

In the contrary, not all the LRRK2 PD-mutations increase tau phosphorylation (28). However, those ones have an enhance microtubule binding activity (30) that could also lead to a higher tau phosphorylation.

5.6 Interaction with other pathways

5.6.1 Mitochondrial homeostasis

The dysfunction of mitochondria in PD patients can be caused by at least one of the following processes: deletion of mitochondrial DNA, accumulation of mitochondrial DNA mutations or oxidative stress from reactive oxygen species (ROS) (31). This imbalance in mitochondrial homeostasis may contribute to both familiar and sporadic neurodegenerative PD (31).

The activity of mitochondrial complex I, a major component of the mitochondrial respiratory chain, is described to be markedly decreased in substantia nigra and other tissues in PD patients (31).

After the observation that LRRK2 is present in membranes, including mitochondria, the role of LRRK2 in mitochondrial homeostasis started to be studied. There has been reported many possible interactions with LRRK2 as DRP-1 to increase mitochondrial fission (32) or Parkin with neuroprotective effects (33). The study of Lrk-1 gene, the *C.elegans* homologous of the human LRRK2, showed that, when this gene is knocked-out, mitochondrial dysfunction appears (34). Furthermore, G2019S seems to lead to a decrease in mitochondrial membrane potential and intracellular ATP and an increase in mitochondrial elongation and interconnectivity (31).

Even if it is clear that the activities of Parkin, pink1, DJ1, α-synuclein and LRRK2 impact on mitochondrial function (31), there are not direct proofs suggesting that LRRK2 plays a major role in this pathway. Currently, the evidences suggest that the imbalance of mitochondrial homeostasis is a side effect of its main role.

5.6.2 4EBP proteins

eIF4F is a complex that recruits 40S ribosomal subunit to the 5' end of mRNA to enhance translation. 4EBP is the main inhibitor of this complex (35). 4EBP binding to eIF4F is regulated by phosphorylation: the more 4EBP is phosphorylated, the weaker is the bound (35).

An *in vitro* study (36) showed that LRRK2 phosphorylates 4E-BP at T37 and T46 residues; this facilitates subsequent phosphorylation at T70 and S65 by other kinases. In this hyperphosphorylated state, 4E-BP is released and eIF4E can exert its function (*Figure 6*).

A high increase of eI4F transcription activity could reduce oxidative stress resistance and lead to dopaminergic neurodegeneration (37).

Further experiments were performed in order to discover the kinetics of this phosphorylation reaction. A study of ^{33}P incorporation over time showed that Km was really low and that ^{33}P retention in LRRK2 was higher than its transference to 4EBP (38).

In conclusion, this phosphorylation only occurs *in vitro*. This activity *in vivo* is weak when compared to autophosphorylation and, therefore, 4EBP is not a major substrate.

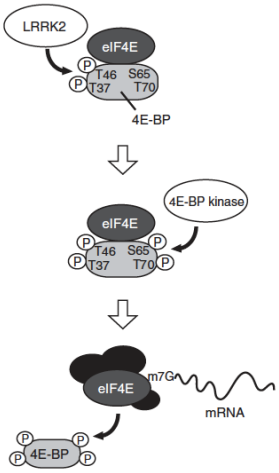

Figure 6. LRRK2 and 4EBP interaction. Phosphorylation of 4E-BP by LRRK2 facilitates subsequent 4E-BP hyperphosphorylation. In this state, it is released and eIF4E, can exert its activity (36).

5.6.3 14.3.3 proteins

14.3.3 proteins are phosphoserine-binding proteins, a family of conserved regulatory molecules expressed in all eukaryotic cells. They can bind to a multitude of functionally diverse signalling proteins (39). 14.3.3 proteins are abundant in the brain, comprising approximately 1% of its total soluble protein (39). They also have structural homology to α-synuclein (40) and co-aggregate in LB.

An IP essay showed that LRRK2 binds to member of 14.3.3 family, specifically to γ and η members(41). Nevertheless, LRRK2-PD mutations impair phosphorylation in 14.3.3 binding sites; the affinity is strongly decreased in R1441G and Y1699C mutations but not so much in G2019S mutation (41).

Owing to the properties of both factors, it remains not clear if LRRK2 modulates 14.3.3 or if it is in the other way around. A recent study (42) showed that 14.3.3 plays an essential role in LRRK2 secretion in exosomes. Exosomes could be recruited through plasma membrane-derived vesicles or through direct uptake of late endosomal vesicles. In this moment, 14.3.3 proteins may bind to soluble LRRK2 and both are packaged into the exosome. Cells can then release LRRK2-containing exosomes in either, a tightly controller or a constitutive manner (*Figure 7*).

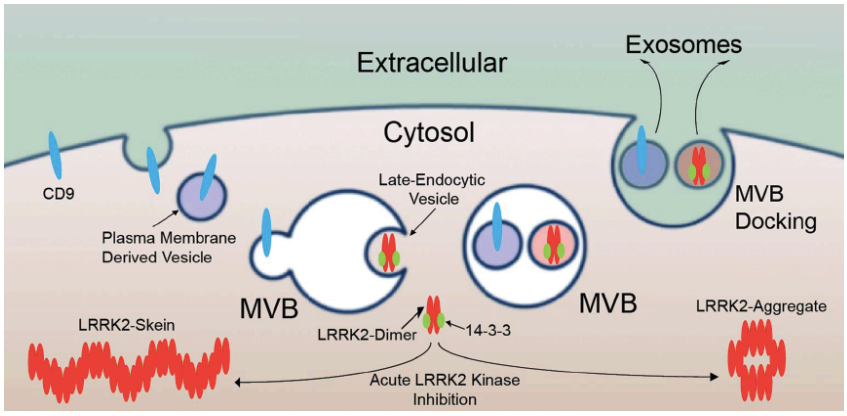

Figure 7. Proposal model for LRRK2 exosomal release (42)

These exosomes are released in urine and also appear in cerebral spinal fluid. Thus, LRRK2 was studied as a possible biomarker for PD. Unfortunately; LRRK2 expression in urinary exosomes is really variable in clinical populations, making it difficult to differentiate between PD cases and controls. Further studies are needed, but it seems unlikely that LRRK2 measurements in urine would provide a diagnostic tool (42).

Currently, it is quite clear that there is an interaction between 14.3.3 and LRRK2. *In vivo* experiments have demonstrated that both factors can regulate each other. The LRRK2 exosomal release is really interesting in order to develop diagnostic tools but it does not explain its pathological role in PD disease. What is more, even if some mutations modify LRRK2 affinity to 14.3.3, G2019S, the most common mutation, does not seem to present differences when compared to control LRRK2. Therefore, even if 14.3.3 interaction is really useful for a complete understood of PD, it does not explain LRRK2 pathology.

6. Discussion

Parkinson's disease (PD) is a progressive disorder of the nervous system that affects movement. It is the second most prevalent neurodegenerative disease just behind Alzheimer (1).

One of most important PD-related genes is LRRK2, a large gene whose transcript encodes a 2527 amino acid protein called dardarin (6). LRKK2 is expressed in many different tissues; however, its activity is much higher in the brain when compared with other organs (5).

The physiological function of LRRK2 remains unknown, thus, all the suggested roles that LRRK2 could play are being studied. In this essay these different roles are analysed for identifying the most likely pathway by which LRRK2 contributes to the development of PD. The most important and promising pathways in which LRRK2 could play a major role are autophagy, inflammation, synapsis, apoptosis, cytoskeleton dynamics and mitochondrial homeostasis (*Figure 8*).

It is important to mention that one of the reasons why the main role of LRRK2 it is not so clear is because it is a big protein with two enzymatic domains (serine/threonine kinase and GTPase) and several protein-protein interaction domains (6). It has affinity to multiple proteins and signalling factors and, therefore, it is really hard to clarify to which pathway contributes with more affinity (6).

For studying the implication of this protein in the just mentioned activities, both *in vivo* and *in vitro* essays have been performed. Furthermore, the aim of these studies were, not only discover the pathological mechanism by which LRRK2 leads to PD, but also to clarify which is the physiological role of this protein in healthy brain tissue. Both aspects are really important and, the discovery of one of them, would lead to an easier understanding of the other one and to understand both healthy and PD brain behaviour.

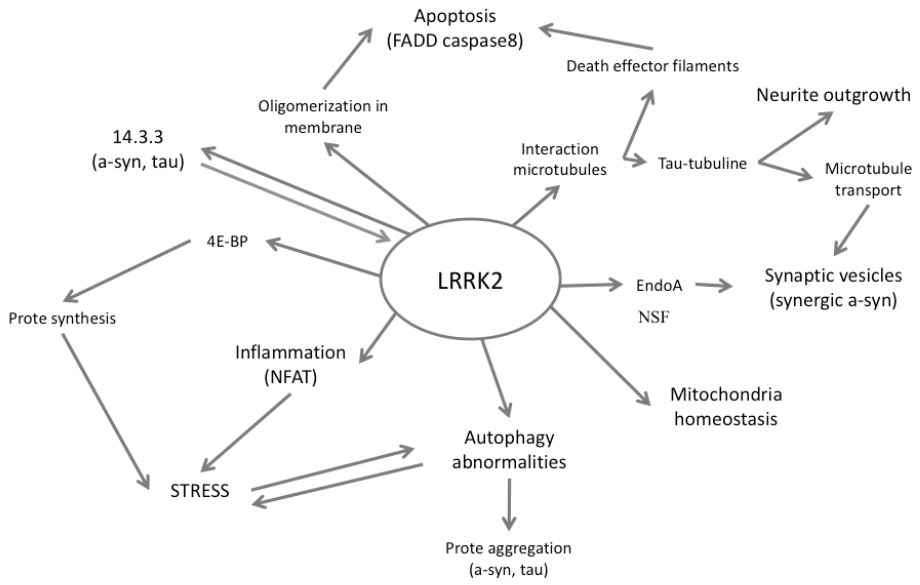

Figure 8. Suggested pathways in which LRRK2 plays it major role.

However, most of the experiments are based on overexpression studies. When LRRK2 is accumulated in high concentrations it can bind to many proteins with different affinity than in physiological concentrations. An example of this is the specific interaction that was found *in vitro* with 4EBP proteins (*see chapter 10.2*) but, after further studies, turned out that this interaction was weak when occurred *in vivo*. Therefore, studying the interaction activities under physiological conditions is really important to understand which is the real pathway in which LRRK2 plays a major role.

One of the most interesting facts of patients suffering LRRK2 mutations is that they always present either α-synuclein aggregations or tauopathies or both (9). What is more, GWAS studies showed that the three genes coding for these proteins: LRRK2, SNCA (coding for α-synuclein) and MAPT (coding for tau), are strongly associated (6). Therefore, it is clear that, whatever is the pathway in which LRRK2 is implicated, should lead to tau and α-synuclein aggregation.

In every chapter, the pros and cons of each proposed pathway have been already explained. Some of the interactions are easier to identify as a side effect of LRRK2 role than others. That is, for instance, the role of LRRK2 in mitochondrial homeostasis (*See chapter 10.1*). One of the most clear and always-present alteration in SNpc neurons of patients suffering PD is mitochondrial abnormality. It is well known that a lot of factors can drive to an imbalance in mitochondrial homeostasis (31). Thus, even if there is no direct link between LRRK2 and mitochondria, the stress that the major LRRK2 function provokes within the cell, could lead to this imbalance.

We suggest that the pathway in which LRRK2 is involved should strongly link this factor with α-synuclein and tau. Consequently, some of the possible roles that we described in this work do not fit with this premise.

Even if the idea of LRRK2 as an important mediator of apoptosis is attractive (*See chapter 8*), it does not explain the formation of LN, LB or tauopathies. Thus, we suggest that all the evidence found about LRRK2 forming structures in microtubules, is not aiming to form death effector filaments but it is important for other major function.

One of the clearest evidence found about LRRK2 protein is that it interacts with microtubules. However, the activity that it performs from this location is still unknown. From this location it could regulate many diverse activities such as autophagy, synapsis, neurite outgrowth or cytoskeleton dynamics.

First of all, if LRRK2 mutations leaded to autophagy abnormalities in PD (*See chapter 5*), it would alter protein degradation system and, therefore, it would enhance protein aggregation, such as α-synuclein or tau. The only flaw of this hypothesis is that tau only aggregates when it is hyper-phosphorylated and, thus, the over-activation of a kinase is needed to form aggregates.

Secondly, the role of LRRK2 in synaptic vesicles transport (*See chapter 7*) would explain why, when LRRK2 is mutated, it has a synergic effect with anomalous α-synuclein. Nevertheless, the flaw of this hypothesis is the same than the previous one; it does not explain hyperphosphorylated-tau aggregation.

Personally, the most convincing hypothesis that explains SNCA, LRRK2 and MAPT association and shows the other hypothesis as LRRK2 side effects, explaining as well why studies shows positive interactions with these factors, is the direct interaction between LRRK2 and microtubules, specifically with tau associated to tubulin (*See chapter 9*).

Most of the PD-related mutations (R1441C/G, Y1699C and G2019S) present a higher kinase or lower GTPase activities (9); therefore, pathological LRRK2 mechanism should be kinase dependent.

Phosphorylation of tau means its detach from tubulin network followed by microtubule network disruption. A severe disruption in microtubule network could easily impair synaptic vesicles trafficking, as well as alter autophagy system. This situation would lead to stress within the cell that would lead to mitochondrial homeostasis imbalance and protein aggregation, for instance, α-synuclein aggregation. What is more, all the hyper-phosphorylated tau protein that is not bound to tubulin any more could also aggregate forming tauopathies.

After reading the most recent articles and reviews about his topic, I consider that the interaction between LRRK2 with tau in microtubules is the hypothesis that better brings together all the findings discovered to date. It gives a nice relationship between tau, α-synuclein and LRRK2. Furthermore, all the other pathways suggested as major pathways for LRRK2 can be explained as a side effect.

In conclusion, I recommend investing in the study of the interaction of LRRK2 with tau to clarify its role in the development of PD without closing the field to new hypothesis to come out.

7. List of references

1. **'TollesoN, C., and J. 'Fang.** 2013. Advances in the mechanisms of Parkinson's disease.. Discov Med. **15:**61-66.

2. **'Shulman, J., P. 'DeJager, and M. 'Feany.** 2011. Parkinson's Disease: Genetics and Pathogenesis. Annu Rev Pathol. **6:**193-222.

3. **Goetz, C. G.** 2011. The history of Parkinson's disease: early clinical descriptions and neurological therapies. Cold Spring Harb Perspect. Med. **1:**a008862. doi: 10.1101/cshperspect.a008862; 10.1101/cshperspect.a008862.

4. **Cookson, M.** 2010. The role of leucine-rich repeat kinase 2 (LRRK2) in Parkinson's disease. Nat Rev Neurosci. **11:**791-797.

5. **Kett, L. R., and W. T. Dauer.** 2012. Leucine-rich repeat kinase 2 for beginners: six key questions. Cold Spring Harb Perspect. Med. **2:**a009407. doi: 10.1101/cshperspect.a009407; 10.1101/cshperspect.a009407.

6. **Ray, S., and M. Liu.** 2012. Current understanding of LRRK2 in Parkinson's disease: biochemical and structural features and inhibitor design. Future Med. Chem. **4:**1701-1713. doi: 10.4155/fmc.12.110; 10.4155/fmc.12.110.

7. **Gardet, A., Y. Benita, C. Li, B. E. Sands, I. Ballester, C. Stevens, J. R. Korzenik, J. D. Rioux, M. J. Daly, R. J. Xavier, and D. K. Podolsky.** 2010. LRRK2 is involved in the IFN-gamma response and host response to pathogens. J. Immunol. **185:**5577-5585. doi: 10.4049/jimmunol.1000548; 10.4049/jimmunol.1000548.

8. **James, N. G., M. A. Digman, E. Gratton, B. Barylko, X. Ding, J. P. Albanesi, M. S. Goldberg, and D. M. Jameson.** 2012. Number and brightness analysis of LRRK2 oligomerization in live cells. Biophys. J. **102:**L41-3. doi: 10.1016/j.bpj.2012.04.046; 10.1016/j.bpj.2012.04.046.

9. **Rideout, H. J., and L. Stefanis.** 2013. The Neurobiology of LRRK2 and its Role in the Pathogenesis of Parkinson's Disease. Neurochem. Res. . doi: 10.1007/s11064-013-1073-5.

10. **Lewis, P. A.** 2009. The function of ROCO proteins in health and disease. Biol. Cell. **101:**183-191. doi: 10.1042/BC20080053; 10.1042/BC20080053.

11. **Declercq, W., T. Vanden Berghe, and P. Vandenabeele.** 2009. RIP kinases at the crossroads of cell death and survival. Cell. **138:**229-232. doi: 10.1016/j.cell.2009.07.006; 10.1016/j.cell.2009.07.006.

12. **Greggio, E., L. Civiero, M. Bisaglia, and L. Bubacco.** 2012. Parkinson's disease and immune system: is the culprit LRRKing in the periphery? J. Neuroinflammation. **9:**94-2094-9-94. doi: 10.1186/1742-2094-9-94; 10.1186/1742-2094-9-94.

13. **Bravo-San Pedro, J. M., M. Niso-Santano, R. Gomez-Sanchez, E. Pizarro-Estrella, A. Aiastui-Pujana, A. Gorostidi, V. Climent, R. Lopez de Maturana, R. Sanchez-Pernaute, A. Lopez de Munain, J. M. Fuentes, and R. A. Gonzalez-Polo.** 2013. The LRRK2 G2019S mutant exacerbates basal autophagy through activation of the MEK/ERK pathway. Cell Mol. Life Sci. **70:**121-136. doi: 10.1007/s00018-012-1061-y; 10.1007/s00018-012-1061-y.

14. **Plowey, E. D., S. J. Cherra 3rd, Y. J. Liu, and C. T. Chu.** 2008. Role of autophagy in G2019S-LRRK2-associated neurite shortening in differentiated SH-SY5Y cells. J. Neurochem. **105:**1048-1056. doi: 10.1111/j.1471-4159.2008.05217.x; 10.1111/j.1471-4159.2008.05217.x.

15. **Gómez-Suaga, P., B. Luzón-Toro, D. Churamani, L. Zhang, D. Bloor-Young, S. Patel, P. Woodman, G. Churchill, and S. Hilfiker.** 2012. Leucine-rich repeat kinase 2 regulates autophagy through a calcium-dependent pathway involving NAADP. Hum Mol Genet. **21:**511-525.

16. **Alegre-Abarrategui, J., H. Christian, M. Lufino, R. Mutihac, L. Venda, O. Ansorge, and R. Wade-Martins.** 2009. LRRK2 regulates autophagic activity and localizes to specific membrane microdomains in a novel human genomic reporter cellular model**.**. Hum Mol Genet. **18:**4022-4034.

17. **Wang, Q. J., Y. Ding, D. S. Kohtz, N. Mizushima, I. M. Cristea, M. P. Rout, B. T. Chait, Y. Zhong, N. Heintz, and Z. Yue.** 2006. Induction of autophagy in axonal dystrophy and degeneration. J. Neurosci. **26:**8057-8068. doi: 10.1523/JNEUROSCI.2261-06.2006.

18. **Imamura, K., N. Hishikawa, M. Sawada, T. Nagatsu, M. Yoshida, and Y. Hashizume.** 2003. Distribution of major histocompatibility complex class II-positive microglia and cytokine profile of Parkinson's disease brains. Acta Neuropathol. **106:**518-526. doi: 10.1007/s00401-003-0766-2.

19. **Whitton, P. S.** 2007. Inflammation as a causative factor in the aetiology of Parkinson's disease. Br. J. Pharmacol. **150:**963-976. doi: 10.1038/sj.bjp.0707167.

20. **Liu, Z., J. Lee, S. Krummey, W. Lu, H. Cai, and M. J. Lenardo.** 2011. The kinase LRRK2 is a regulator of the transcription factor NFAT that modulates the severity of inflammatory bowel disease. Nat. Immunol. **12:**1063-1070. doi: 10.1038/ni.2113; 10.1038/ni.2113.

21. **Macian, F.** 2005. NFAT proteins: key regulators of T-cell development and function. Nat. Rev. Immunol. **5:**472-484. doi: 10.1038/nri1632.

22. **Matta, S., K. Van Kolen, R. da Cunha, G. van den Bogaart, W. Mandemakers, K. Miskiewicz, P. J. De Bock, V. A. Morais, S. Vilain, D. Haddad, L. Delbroek, J. Swerts, L. Chavez-Gutierrez, G. Esposito, G. Daneels, E. Karran, M. Holt, K. Gevaert, D. W. Moechars, B. De Strooper, and P. Verstreken.** 2012. LRRK2 controls an EndoA phosphorylation cycle in synaptic endocytosis. Neuron. **75:**1008-1021. doi: 10.1016/j.neuron.2012.08.022; 10.1016/j.neuron.2012.08.022.

23. **Piccoli, G., S. B. Condliffe, M. Bauer, F. Giesert, K. Boldt, S. De Astis, A. Meixner, H. Sarioglu, D. M. Vogt-Weisenhorn, W. Wurst, C. J. Gloeckner, M. Matteoli, C. Sala, and M. Ueffing.** 2011. LRRK2 controls synaptic vesicle storage and mobilization within the recycling pool. J. Neurosci. **31:**2225-2237. doi: 10.1523/JNEUROSCI.3730-10.2011; 10.1523/JNEUROSCI.3730-10.2011.

24. **Olanow, C., and P. Brundi.** 2013. Parkinson's disease and alpha synuclein: is Parkinson's disease a prion-like disorder?. Mov Disord. **28:**31-40.

25. **Kalia, L. V., S. K. Kalia, P. J. McLean, A. M. Lozano, and A. E. Lang.** 2013. alpha-Synuclein oligomers and clinical implications for Parkinson disease. Ann. Neurol. **73:**155-169. doi: 10.1002/ana.23746; 10.1002/ana.23746.

26. **Ho, C., H. Rideout, E. Ribe, C. Troy, and W. Dauer.** 2009. The Parkinson disease protein leucine-rich repeat kinase 2 transduces death signals via Fas-associated protein with death domain and caspase-8 in a cellular model of neurodegeneration.. J Neurosci. **29:**1011-1016.

27. **Siegel, R. M., D. A. Martin, L. Zheng, S. Y. Ng, J. Bertin, J. Cohen, and M. J. Lenardo.** 1998. Death-effector filaments: novel cytoplasmic structures that recruit caspases and trigger apoptosis. J. Cell Biol. **141:**1243-1253.

28. **Kawakami, F., T. Yabata, E. Ohta, T. Maekawa, N. Shimada, M. Suzuki, H. Maruyama, T. Ichikawa, and F. Obata.** 2012. LRRK2 phosphorylates tubulin-associated tau but not the free molecule: LRRK2-mediated regulation of the tau-tubulin association and neurite outgrowth.. PLoS One. **7:**.

29. **Spillantini, M. G., and M. Goedert.** 2013. Tau pathology and neurodegeneration. Lancet Neurol. **12:**609-622. doi: 10.1016/S1474-4422(13)70090-5; 10.1016/S1474-4422(13)70090-5.

30. **Kett, L. R., D. Boassa, C. C. Ho, H. J. Rideout, J. Hu, M. Terada, M. Ellisman, and W. T. Dauer.** 2012. LRRK2 Parkinson disease mutations enhance its microtubule association. Hum. Mol. Genet. **21:**890-899. doi: 10.1093/hmg/ddr526; 10.1093/hmg/ddr526.

31. **Sai, Y., Z. Zou, K. Peng, and Z. Dong.** 2012. The Parkinson's disease-related genes act in mitochondrial homeostasis. Neurosci. Biobehav. Rev. **36:**2034-2043. doi: 10.1016/j.neubiorev.2012.06.007; 10.1016/j.neubiorev.2012.06.007.

32. **Wang, X., M. H. Yan, H. Fujioka, J. Liu, A. Wilson-Delfosse, S. G. Chen, G. Perry, G. Casadesus, and X. Zhu.** 2012. LRRK2 regulates mitochondrial dynamics and function through direct interaction with DLP1. Hum. Mol. Genet. **21:**1931-1944. doi: 10.1093/hmg/dds003; 10.1093/hmg/dds003.

33. **Ng, C. H., S. Z. Mok, C. Koh, X. Ouyang, M. L. Fivaz, E. K. Tan, V. L. Dawson, T. M. Dawson, F. Yu, and K. L. Lim.** 2009. Parkin protects against LRRK2 G2019S mutant-induced dopaminergic neurodegeneration in Drosophila. J. Neurosci. **29:**11257-11262. doi: 10.1523/JNEUROSCI.2375-09.2009; 10.1523/JNEUROSCI.2375-09.2009.

34. **Yao, C., R. ElKhoury, W. Wang, T. Byrd, E. Pehek, C. Thacker, X. Zhu, M. Smith, A. Wilson-Delfosse, and S. Chen.** 2010. LRRK2-mediated neurodegeneration and dysfunction of dopaminergic neurons in a Caenorhabditis elegansmodel of Parkinson's disease.. Neurobiol Dis. **40:**73-81.

35. **Gingras, A., S. Gygi, B. Raught, R. Polakiewicz, R. Abraham, M. Hoekstra, R. Aebersold, and N. Sonenberg.** 1999. Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism.. Genes Dev. **13:**1422-1437.

36. **Imai, Y., S. Gehrke, H. Q. Wang, R. Takahashi, K. Hasegawa, E. Oota, and B. Lu.** 2008. Phosphorylation of 4E-BP by LRRK2 affects the maintenance of dopaminergic neurons in Drosophila. EMBO J. **27:**2432-2443. doi: 10.1038/emboj.2008.163; 10.1038/emboj.2008.163.

37. **Singleton, A. B., M. J. Farrer, and V. Bonifati.** 2013. The genetics of Parkinson's disease: progress and therapeutic implications. Mov. Disord. **28:**14-23. doi: 10.1002/mds.25249; 10.1002/mds.25249.

38. **Kumar, A., E. Greggio, A. Beilina, A. Kaganovich, D. Chan, J. M. Taymans, B. Wolozin, and M. R. Cookson.** 2010. The Parkinson's disease associated LRRK2 exhibits weaker in vitro phosphorylation of 4E-BP compared to autophosphorylation. PLoS One. **5:**e8730. doi: 10.1371/journal.pone.0008730; 10.1371/journal.pone.0008730.

39. **Fu, H., R. Subramanian, and S. Masters.** 2000. 14-3-3 proteins: structure, function, and regulation.. Annu Rev Pharmacol Toxicol. **40:**617-647.

40. **Yacoubian, T. A., S. R. Slone, A. J. Harrington, S. Hamamichi, J. M. Schieltz, K. A. Caldwell, G. A. Caldwell, and D. G. Standaert.** 2010. Differential neuroprotective effects of 14-3-3 proteins in models of Parkinson's disease. Cell. Death Dis. **1:**e2. doi: 10.1038/cddis.2009.4; 10.1038/cddis.2009.4.

41. **Li, X., Q. J. Wang, N. Pan, S. Lee, Y. Zhao, B. T. Chait, and Z. Yue.** 2011. Phosphorylation-dependent 14-3-3 binding to LRRK2 is impaired by common mutations of familial Parkinson's disease. PLoS One. **6:**e17153. doi: 10.1371/journal.pone.0017153; 10.1371/journal.pone.0017153.

42. **Fraser, K. B., M. S. Moehle, J. P. Daher, P. J. Webber, J. Y. Williams, C. A. Stewart, T. A. Yacoubian, R. M. Cowell, T. Dokland, T. Ye, D. Chen, G. P. Siegal, R. A. Galemmo, E. Tsika, D. J. Moore, D. G. Standaert, K. Kojima, J. A. Mobley, and A. B. West.** 2013. LRRK2 secretion in exosomes is regulated by 14-3-3. Hum. Mol. Genet. . doi: 10.1093/hmg/ddt346.