Published in final edited form as:
Exp Neurol. 2014 November ; 0: 206–216. doi:10.1016/j.expneurol.2014.05.025.

LRRK2, a puzzling protein: insights into Parkinson's disease pathogenesis

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Abstract

Leucine-rich repeat kinase 2 (LRRK2) is a large, ubiquitous protein of unknown function. Mutations in the gene encoding LRRK2 have been linked to familial and sporadic Parkinson disease (PD) cases. The LRRK2 protein is a single polypeptide that displays GTPase and kinase activity. Kinase and GTPase domains are involved in different cellular signalling pathways. Despite several experimental studies associating LRRK2 protein with various intracellular membranes and vesicular structures such as endosomal/lysosomal compartments, the mitochondrial outer membrane, lipid rafts, microtubule-associated vesicles, the golgi complex, and the endoplasmic reticulum its broader physiologic function(s) remain unidentified. Additionally, the cellular distribution of LRRK2 may indicate its role in several different pathways, such as the ubiquitin-proteasome system, the autophagic-lysosomal pathway, intracellular trafficking, and mitochondrial dysfunction. This review discusses potential mechanisms through which LRRK2 may mediate neurodegeneration and cause PD.

Keywords

LRRK2; Parkinson's disease; intracellular traffic; quality control mechanisms; mitochondria

1. Gene and protein organization

The leucine-rich repeat kinase 2 (LRRK2) gene on chromosome 12 spans a genomic distance of 144 kb and contains 51 exons. LRRK2 is a large cytosolic multidomain protein consisting of 2527 amino acids and a molecular weight of approximately 285 kDa. LRRK2 has multiple functional domains. There is a Ras-of-Complex (ROC) GTPase domain adjacent to a C-terminal-of-ROC (COR) linker region. A serine/threonine protein kinase

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domain is also present. There are putative protein-protein interaction domains that flank the central ROC-COR-kinase catalytic region like an ankyrin domain. The N-terminal region contains leucine-rich repeat (LRR) motifs, and WD40 repeats near the C-terminus of the protein probably form a beta-propeller structure (Figure 1) (Greggio and Cookson, 2009). The overall structure suggests LRRK2 may act as a scaffold for other proteins, some of which could themselves regulate LRRK2 or else undergo LRRK2-mediated modifications. LRRK2, therefore, may have the ability to integrate and modify multiple signalling pathways.

1.1 GTPase and kinase functions

LRRK2 belongs to the ROCO family. Roco family members contain both small GTPase and kinase domains, as well as a COR region. Most ROCO proteins also have either an LRR domain, a WD40 domain, or both (Bosgraaf and Van Haastert, 2003). Its structure is shared by one mammalian homolog, LRRK1, which also has a kinase and GTPase domain (Greggio and Singleton, 2007).

The kinase domain of LRRK2 apparently shares homology with mixed-lineage kinase (MLKs) and receptor interacting protein kinase (Gandhi et al., 2009; West et al., 2005). MLKs are part of the mitogen activated protein kinase (MAPK) family, and act as MAPK kinase kinases (MAPKKKs) to initiate and transduce a wide range of cell death-relevant responses (Gallo and Johnson, 2002). How and whether LRRK2 acts as a MAPKKK is unclear because the mechanisms through which it is activated, as well as its downstream kinase effects, are not well-characterized (Biskup and West, 2009; Melrose, 2008).

Dimerization is a common phenomenon among protein kinases. Dimerization can help mediate auto-regulation and modulate downstream signalling molecules. LRRK2 appears to exist as a homodimeric protein. This was first suggested by co-immunoprecipitation experiments using tagged full-length protein or LRRK2 fragments (Gloeckner et al., 2009). Studies using LRRK2 fragments or using the LRRK2 prokaryotic homolog indicate that dimerization occurs in the ROC-COR region. The crystal structure of the ROC domain also shows LRRK2 exists as a dimer, and that the COR domain that follows the ROC domain may function as a hinge between the ROC and kinase domains (Deng et al., 2008; Greggio et al., 2008; Jorgensen et al., 2009). However, neither ROC deletion by itself nor N-terminal deletion (including the ANK and LRR domains) prevents dimerization. On the other hand, a WD40 truncated-LRRK2 could not be dimerized. Together, these studies suggest that while the ROC and WD40 domains mediate LRRK2 dimerization, the WD40 domain is more crucial (Lee et al., 2012).

While specific mutations can destabilize LRRK2 fragment dimers (Deng et al., 2008; Gotthardt et al., 2008), the extent to which full length LRRK2 dimerization is affected in PD is unclear. How perturbed dimerization may affect LRRK2 function is similarly uncertain, but hypotheses exist. One hypothesis is that LRRK2 kinase activity is dimerization-dependent (Greggio et al., 2006; Sen et al., 2009; Smith et al., 2006). In support of this, Berger and colleagues found that LRRK2 dimers, which are enriched in cell membranes, have more kinase activity than monomeric LRRK2 (Berger et al., 2010; Greggio and Cookson, 2009).

Less is known about LRRK2's GTPase capacity. LRRK2 was first identified as an authentic GTPase in 2007. GTPase activity appears to be mediated by the ROC domain, which binds and hydrolyses GTP in a manner similar to that of the Ras-related small GTPase, Rac1. The ROC domain, therefore, may function in a manner typical of Ras-related small GTPases (Guo et al., 2007).

It was initially suspected that the LRRK2 GTPase domain might regulate LRRK2 kinase activity (Bosgraaf and Van Haastert, 2003). In support of this, ROC domain mutations that prevent GTP binding also inactivate kinase function (Cookson, 2010). With GTP bound, the switch region on the outside of the domain assumes an active state, which increases kinase activity. With GDP bound, the tertiary structure of the switch domain assumes an inactive state, which decreases kinase activity. Biosa and co-workers further demonstrated that normal kinase activity requires guanine nucleotide binding and, to a lesser extent, GTP hydrolysis. Interestingly, guanine nucleotide binding but not GTP hydrolysis appears to regulate LRRK2 dimerization, structure, and stability (Biosa et al., 2013). Two independent groups, however, reported neither GTP nor GDP binding affect recombinant LRRK2 kinase activity (Liu et al., 2010; Taymans et al., 2011).

It is possible that GTPase activating proteins (GAPs) and GTP exchange factors may help regulate LRRK2 function. ArfGAP1 has been shown to enhance the LRRK2 GTPase in *Drosophila melanogaster* (Xiong et al., 2012). Interestingly, the same authors had previously suggested that LRRK2 toxicity in yeast can be modulated by altering GTPase activity (Xiong et al., 2010). It is also possible that the kinase domain of LRRK2 instead regulates the ROC domain, and that the LRRK2 kinase activity alters LRRK2 GTPase activity (Cookson, 2010; Greggio and Cookson, 2009; Webber et al., 2011). Relevant to this possibility are data that show the LRRK2 kinase domain phosphorylates its ROC domain at several sites (Greggio et al., 2009; Kamikawaji et al., 2009).

While the LRRK2 GTPase can function independently, kinase activity seems to require a functional GTPase domain. Moreover, it is very likely that LRRK2 GTPase has additional independent targets (Ray and Liu, 2012). GTPase activity may also be regulated by LRRK2 dimerization or through recruitment of other cellular proteins (Gotthardt et al., 2008). Overall, data suggest that the LRRK2's ROC–COR-kinase portion probably constitutes its key regulatory region.

1.2 Phosphorylation and autophosphorylation

LRRK2 kinase activity was first measured by quantitating LRRK2-mediated transphosphorylation of myelin basic protein (MBP). However, this assay was not optimal given the LRRK2's low catalytic activity, and also because MBP is a substrate for other serine/threonine kinases (Zhao et al., 2012). More optimal substrates include moesin (also called LRRKtide), which LRRK2 phosphorylates at its Thr558 site (Jaleel et al., 2007). Moesin belongs to a group of proteins collectively called the ERM (ezrin/radixin/moesin) proteins. These proteins influence cytoskeletal dynamics by anchoring the cytoskeleton to the plasma membrane (Mangeat et al., 1999). A protein that binds eukaryotic initiation factor 4E (eIF4E), eIF4E binding protein (4E-BP) is also phosphorylated by LRRK2 (Imai

et al., 2008). The 4E-BP-eIIF4E complex promotes translation through its binding to capped mRNA species.

The search for LRRK2 kinase substrates facilitated identification of LRRK2-IN-1, the first selective LRRK2 inhibitor (Deng et al., 2011). LRRK2-IN-1, interestingly, eliminates LRRK2 phosphorylation at two sites, Ser910 and Ser935, which do not appear to arise as a consequence of autophosphorylation but appear instead to be phosphorylated by PKA suggesting that PKA is a potential upstream regulatory kinase (Li et al., 2011). Ser910/Ser935 phosphorylation mediates LRRK2's interaction with another protein, 14-3-3, so the loss of this phosphorylation disrupts 14-3-3 and LRRK2 binding. This, in turn, causes LRRK2 to accumulate within cytoplasmic pools, as opposed to adopting a more even localization (Nichols et al., 2010). Therefore, LRRK2 Ser910/Ser935 phosphorylation, LRRK2 binding to 14-3-3, and the LRRK2 cytoplasmic distribution pattern can be used to monitor LRRK2 activity (Figure 1) (Dzamko et al., 2010; Doggett et al., 2012). Other LRRK2 inhibitors, such as the small molecule inhibitor, GNE-7915, have been identified. GNE-7915 can cross the blood brain barrier, which makes it advantageous for animal-based studies (Estrada et al., 2012).These molecules that inhibit LRRK2 kinase activity were shown to arrest neurodegeneration using different LRRK2 PD models which makes them valuable to clarify the pathways in which LRRK2 play a role. Indeed in various neuronal cell culture systems kinase activity inhibition through site-directed mutagenesis or pharmacologically was shown to attenuate neurotoxicity (Liu et al., 2011a; Luerman et al., 2013; Yao et al., 2013).

While Ser910/Ser935 is not a consequence of autophosphorylation, LRRK2 autophosphorylation sites do exist (Gloeckner et al., 2010). In the presence of ATP and Mg^{2+}, efficient autophosphorylation occurs (Greggio, 2012). Autophosphorylation, therefore, can be used as a surrogate measure of kinase activity (Smith et al., 1993). Autophosphorylation sites localize primarily to the GTPase domain, which suggests some degree of kinase-GTPase inter-regulation occurs (Greggio et al., 2009).

Kamikawaji and co-workers identified Ser1403, Thr1404, Thr1410, and Thr1491 in the ROC domain, as well as Thr1967 and Thr1969 in the kinase domain, are functionally relevant autophosphorylation sites (Figure 1). Thr1410 autophoshorylation increases kinase activity, while Thr1491 autophosphorylation reduces kinase activity (Table I). Alanine substitution at Thr1967 decreases kinase activity, whereas alanine substitution at Thr1491 increases kinase activity (Kamikawaji et al., 2009). Three other putative autophosphorylation sites (Thr2031, Ser2032, and Thr2035) have also been identified within the activation segment of the LRRK2 kinase domain (Figure 1) (Li et al., 2010a).

Removal of a conserved autophosphorylation site at Thr1503 decreases both GTP-binding and kinase activity, which suggests autophosphorylation at this amino acid potentiates LRRK2 kinase activity (Table I and Figure 1). However, Thr1503 phosphorylation was not observed in transgenic mouse and cell lines with increased kinase function (Webber et al., 2011). More recently it was demonstrated that LRRK2 autophosphorylation at Ser1292 occurs *in vivo* and that this is enhanced by several familial PD mutations including R1441G, R1441C, G2019S, and I2020T (Figure 1). Converting Ser1292 to alanine (phospho-deficient

mutant) mitigates the effects of LRRK2 PD mutations on neurite outgrowth in cultured rat embryonic primary neurons. This suggests that Ser1292 autophosphorylation may reflect LRRK2 kinase activity *in vivo*, and that Ser1292 phosphorylation may mediate the effects of some PD mutations (Table I) (Sheng et al., 2012). Finally, the LRRK2 G2019S mutation associates with both increased autophosphorylation and substrate phosphorylation, which may at least partly explain this mutation's pathogenecity (Luzon-Toro et al., 2007).

Many questions relevant to LRRK2 remain. For example, to date no LRRK2 phosphatases have been identified. Further insight into LRRK2 phosphorylation and autophosphorylation will no doubt help us better understand LRRK2 function and dysfunction, and help development more accurate and sensitive LRRK2 kinase assays.

1.3 Mutations

The LRRK2 coding sequence includes 7500 nucleotides. There are a number of known variants, many of which are not linked currently to any disease and are probably phenotypically inconsequential (Paisan-Ruiz et al., 2008). Other polymorphisms, though, may act as PD risk factors. Indeed, two genome-wide association studies reported certain LRRK2 polymorphisms associate with an increased risk of sporadic PD (Satake et al., 2009; Simon-Sanchez et al., 2009). LRRK2, therefore, seems to act as a "deterministic" gene that causes autosomal dominant PD in the presence of certain mutations, and as a susceptibility gene for sporadic PD (Mata et al., 2012). Even with deterministic mutations, though, penetrance is still incomplete (Hulihan et al., 2008; Latourelle et al., 2008), individuals have survived into their 80s without developing clinical parkinsonism (Kay et al., 2005).

LRRK2 mutations typically cause a PD-typical phenotype that is characterized by tremor, rigidity, bradykinesia, and postural instability (Haugarvoll and Wszolek, 2009). Autopsies of such patients show prominent loss of melanised dopamine neurons in the substantia nigra pars compacta. The age onset is variable, but symptoms generally develop in the sixth or seventh decade.

LRRK2 mutations, and especially the G2019S mutation, are observed in both autosomal dominant and apparent sporadic PD patients. Associated histologic changes can include either Lewy Bodies (LBs) and tau inclusions. G2019S mutation carriers typically show LB pathology, whereas R1441C, Y1699C, and I2020T mutation carriers do not (Poulopoulos et al., 2012).

Six missense mutations in different domains clearly segregate with disease (Table II and Figure 1). A group of LRRK2 mutations are found within the ROC-COR domains. The R1441 position in the ROC/GTPase, when mutated to cysteine, glycine or histidine (R1441C/G/H) causes familial PD, as does the Y1699C mutation in the adjacent COR domain. The most common mutation, G2019S, is found in the kinase domain, and the original Japanese family has an I2020T mutation located in the kinase domain. These LRRK2 PD pathogenic mutations disrupt the modification of LRRK2 cellular phosphorylation sites. Ser910, Ser935, Ser955 and Ser973 are fully phosphorylated in the presence of G2019S, while these sites are hypophosphorylated in the presence of R1441G/C, Y1699C, and I2020T mutations (Zhao et al., 2012).

The G2019S mutation substitutes a serine for a highly conserved glycine located in subdomain VII of the kinase domain. This mutation increases kinase activity by increasing the catalytic rate of the enzyme; it does not enhance substrate affinity (West et al., 2005). The G2019S mutation facilitates substrate access, thereby leading to a toxic increase in kinase activity. G2019S is the only mutation that consistently shows increased kinase activity (Greggio and Cookson, 2009). Other mutations, including the R1441C mutation in the ROC domain as well as the Y1699C and I2020T mutations, have only variably been found to increase kinase activity. In these studies, discrepancies may have arisen due to the use of different substrates in the kinase assays, or because of differences in the way the enzyme was collected (Jaleel et al., 2007; Seol, 2010; West et al., 2007).Overall, though, in experimental models, changes in LRRK2 kinase activity appear to be toxic, and seem to induce degeneration of dopamine neurons (Greggio et al., 2006; Iaccarino et al., 2007; Jorgensen et al., 2009; Smith et al., 2006; West et al., 2007).

Mutations located in or next to the GTPase domain, including R1441C, R1441G, and Y1699C increase the steady-state levels of GTP-bound LRRK2. This probably results as a consequence of reduced GTP hydrolysis (Guo et al., 2007; Kumar and Cookson, 2011; Li et al., 2007; West et al., 2007).

A C-terminal WD40 domain variation that substitutes arginine for glycine (G2385R) increases PD risk and reduces kinase activity. Another mutation, the N1437H mutation that causes PD in a large Norwegian kindred, seems to increase GTP binding, which could suggest an overall reduction in GTPase activity (Table II and Figure 1) (Rudenko et al., 2012).

It is still unclear whether a common pathogenic mechanism for all PD-inducing mutations or PD-associated polymorphisms exists (Tsika and Moore, 2012) and if so what that mechanism might be. Many investigators have thus far focused on kinase function, although changes in GTP binding, constitutive phosphorylation, impaired dimerization, or excess protein degradation may all play a role (Lee et al., 2012; Rudenko et al., 2012). Interestingly, a recent study shed light to a new hypothesis for LRRK2 toxicity. In this study the authors observed that blocking LRRK2 kinase activity reduced mutant LRRK2 toxicity and this was correlated with a decrease in LRRK2 levels indicating that LRRK2 levels are more important than kinase activity per se in predicting toxicity (Skibinski et al., 2014). Given the complexity of the LRRK2 protein however, several aspects of LRRK2 function might be targeted, such as, inhibition of kinase activity and GTP binding, preservation of constitutive phosphorylation of LRRK2, disruption of LRRK2 dimerization or LRRK2 degradation (Lee et al., 2012; Rudenko et al., 2012).

2. Role in cell function

LRRK2 is expressed in most organs including brain, heart and liver. Particularly high levels are observed in kidney and lung (Paisan-Ruiz et al., 2004; Zimprich et al., 2004). It is also expressed in some immune cells (Kubo et al., 2010; Maekawa et al., 2010). In the brain LRRK2 is observed primarily in neurons of the olfactory bulb, striatum, cortex, hippocampus, midbrain, brainstem, and cerebellum (Biskup et al., 2006; Higashi et al.,

2007a). Levels are relatively low in substantia nigra and ventral tegmental area dopaminergic neurons, as compared to the higher levels that are seen in the striatum, cerebral cortex, cerebellum, and hippocampus (Han et al., 2008; Higashi et al., 2007b). Additionally, expression can be experimentally altered; MPTP, a mitochondrial toxin that destroys dopamine neurons, induces an acute increase in LRRK mRNA (Hurley et al., 2007).

Within cells, LRRK2 associates with various intracellular membranes and vesicular structures including endosomes, lysosomes, multivesicular bodies, the mitochondrial outer membrane, lipid rafts, microtubule-associated vesicles, the golgi complex, and the endoplasmic reticulum (Cookson, 2010; Tan and Jankovic, 2006). This distribution could reflect a functional role in multiple pathways, including regulation of autophagy, microtubule dynamics, and mitochondrial function. To date, investigators have largely attempted to define LRRK2-related physiology by studying the consequences of increased and decreased LRRK2 expression.

2.1 Dopamine homeostasis and vesicle trafficking

The different LRRK2 transgenic mouse models generated thus far do not faithfully recapitulate a PD phenotype. While some transgenic models exhibit DA neuron death, neurodegeneration is limited and not obviously progressive. Transgenic mice develop only mild motor deficits (Rudenko and Cookson, 2010). Moreover, LRRK2 knockout mice have an intact dopaminergic system (Andres-Mateos et al., 2009; Hinkle et al., 2012).

LRRK2 localizes to vesicles, where it interacts with vesicular proteins (Biskup et al., 2006). In presynaptic vesicles, LRRK2 silencing leads to a redistribution of vesicles, alters recycling dynamics, and increases vesicle kinetics. This suggests LRRK2 may play a role in synaptic vesicle trafficking (Piccoli et al., 2011). Shin and colleagues further report that LRRK2, in conjunction with Rab5b, contributes to synaptic function by modulating synaptic vesicle endocytosis (Shin et al., 2008). Moreover, overexpression or knockdown of endogenous LRRK2 in primary neuronal cells significantly impairs synaptic vesicle endocytosis, a phenomenon that can be reversed with co-expression of Rab5b (Heo et al., 2010a).

LRRK2 phosphorylates EndoA, an evolutionary conserved protein that is critically involved in synaptic vesicle endocytosis. LRRK2-mediated EndoA phosphorylation profoundly affects membrane tubulation and membrane association, which suggests that in synapses LRRK2, through its role as a kinase, facilitates efficient vesicle formation (Matta et al., 2012).

In regard to dopamine signaling, LRRK2 mutations have been shown to affect activity-dependent DA neurotransmission and catecholamine release (Tong et al., 2009). This manifests as reduced levels of extracellular striatal dopamine and reduced levels of dopamine metabolites. Motor deficits in these mice are minor, but these deficits nevertheless respond to L-dopa (Chen et al., 2012; Li et al., 2010b; Li et al., 2009; Maekawa et al., 2012; Melrose et al., 2010). Expression of G2019S LRRK2 induces an age-dependent loss of nigrostriatal dopamine neurons. The brains of aged G2019S mice also show perturbed

autophagy and abnormal mitochondria. Cultured dopamine neurons from these mice display markedly reduced neurite complexity (Ramonet et al., 2011). Reduced striatal DAT levels in LRRK2 transgenic mice have also been reported, which could reflect dysfunction or degeneration of dopamine neuron terminals (Chen et al., 2012). Impaired dopamine re-uptake can occur (Zhou et al., 2011); this change, along with reduced DAT levels, suggests impaired dopamine re-uptake could contribute to altered dopamine levels - in LRRK2 transgenic mice.

2.2 Relationship to α-synuclein

Potential interactions between α-synuclein (SNCA) and LRRK2 have been considered and their functions may have some degree of functional overlap. For example, they both play a role in microtubule assembly and microtubule dynamics (Parisiadou and Cai, 2010).

LRRK2 localizes to a subset of brainstem-type LBs, but not to cortical-type LBs, NFTs, other tau inclusions, or TDP-43-positive inclusions. Approximately 20–100% (mean=60%) of SNCA-positive LBs contain LRRK2 (Perry et al., 2008). It is reported that 10–80% of classic LBs are rimmed by LRRK2 (Alegre-Abarrategui et al., 2008; Vitte et al., 2010). In post-mortem brains with LB pathology, LRRK2 co-immunoprecipitates with SNCA (Yacoubian et al., 2010). In cultured cells transfected to overexpress both LRRK2 and SNCA, under conditions that promote oxidative stress LRRK2 and SNCA physically associate with each other (Qing et al., 2009b). Increasing striatal SNCA expression also concomitantly raises LRRK2 mRNA, which suggests LRRK2 and SNCA levels may be co-regulated (Westerlund et al., 2008).

The question of whether LRRK2 might phosphorylate SNCA has been considered. Experimental data does suggest LRRK2 and its putative kinase domain-containing fragments can phosphorylate recombinant SNCA at S129. This modification is believed to promote the formation of SNCA filaments and oligomers, and is enriched in LBs. Accordingly, G2019S LRRK2 phosphorylates SNCA at S129 more avidly than WT LRRK2 (Qing et al., 2009a).

It was demonstrated that in PD affected brain regions such as the amygdala and anterior cingulate cortex, but not in the unaffected visual cortex, LRRK2 levels positively correlate with SNCA phosphorylation and aggregation (Guerreiro et al., 2012). In cell culture models of SNCA over-expression, concomitant overexpression of LRRK2 facilitates SNCA release and its subsequent uptake by neighboring cells (Kondo et al., 2011). Overexpression of WT, G2019S, and R1441C LRRK2 increases SNCA expression through an extracellular signalregulated kinase pathway (ERK)-mediated pathway (Carballo-Carbajal et al., 2010).

Expressing LRRK2 in the A53T SNCA transgenic mouse PD model accelerates neurodegeneration and enhances SNCA aggregation. This is believed to arise through effects on microtubule dynamics, Golgi bodies, and the ubiquitin-proteasome pathway (UPS) (Lin et al., 2009). Loss of LRRK2, on the other hand, seems to cause both accumulation and aggregation of SNCA, as well as other ubiquitinated proteins, presumably as a consequence of impaired autophagy-lysosome pathway function (Tong et al., 2010).

In contrast to these mouse studies, basal ganglia and limbic cortex SNCA aggregation was found to be lower in LRRK2 G2019S carriers than it was in persons with idiopathic PD (Mamais et al., 2013). Furthermore, it was recently shown that by removing endogenous SNCA, LRRK2 dependent neurodegeneration and the levels of diffuse mutant LRRK2 are dramatically reduced (Skibinski et al., 2014). Overall, studies such as these suggest LRRK2 and SNCA can interact, although the true nature of this putative relationship remains unclear.

2.3 Relationship to mitochondria

LRRK2 is present in mitochondria (Biskup et al., 2006). In *C. Elegans*, over-expressing LRRK2 enhanced survival following exposure to two different mitochondrial toxins, whereas knockdown of lrk-1 (the endogenous *C. elegans* ortholog of LRRK2) reduced survival (Saha et al., 2009). Similarly, Drosophila that express mutant LRRK2 display increased sensitivity to rotenone-mediated complex I inhibition (Ng et al., 2009). WT LRRK2, but not the mutants forms, attenuate H_2O_2-induced oxidative stress, thereby suggesting a protective role for LRRK2 (Liou et al., 2008).

No structural mitochondria abnormality is observed in mouse neurons that overexpress either WT or mutant LRRK2 (Lin et al., 2009). In skin biopsies from human LRRK2 G2019S carriers, however, mitochondrial function and morphology are perturbed characterized by reduced mitochondrial membrane potential, reduced intracellular ATP levels, mitochondrial elongation, and increased mitochondrial interconnectivity (Mortiboys et al., 2010). In contrast to this, in cortical neurons LRRK2 G2019S overexpression increases DLP-1 activity and promotes mitochondrial fission (Niu et al., 2012; Wang et al., 2012).

In fibroblasts and neuroblastoma cells, the LRRK2 G2019S mutation associates with a decreased mitochondrial membrane potential and lower cell ATP levels. These changes appear to be kinase-dependent (Papkovskaia et al., 2012). SN4741 dopaminergic cells overexpressing either WT or G2019S LRRK2 are more susceptible to H_2O_2-induced cell death (Heo et al., 2010b), and WT LRRK2 expression increases cell reactive oxygen species (ROS) levels (Niu et al., 2012). LRRK2 mutations reduce activity of peroxiredoxin 3, an antioxidant enzyme located within mitochondria. This effect appears to be phosphorylation-dependent (Angeles et al., 2011).

Induced pluripotent stem cells (iPSCs) derived from fibroblasts obtained from LRRK2 carriers also produce more ROS than iPSCs derived from control subject fibroblasts. Mitochondria in iPSCs prepared from LRRK2 carriers also show less mitochondrial respiration, an increased mitochondrial proton leak, and reduced mitochondrial movement (Cooper et al., 2012).

Mouse cortical neurons that express LRRK2 G2019S or R1441C mutations show impaired calcium homeostasis and mitochondrial degradation. Calcium chelators and voltage-gated L-type calcium channel inhibitors mitigate mitochondrial degradation (Cherra et al., 2013).

Taking into account that mitochondrial dysfunction is widely recognized as a trigger of parkinsonism it is interesting to note that LRRK2 affects mitochondrial dynamics and morphology, mitochondrial calcium buffering, ROS levels and mitochondrial membrane potential highlighting once again LRRK2 intrinsic role in PD.

2.4 Relationship to the cytoskeleton

Ultrastructural analyses indicate that LRRK2 interacts with microtubules in a well-ordered, periodic fashion. This suggests the presence of LRRK2-binding sites on microtubules or microtubule-bound proteins (Kett et al., 2012). To this end, LRRK2 has been shown to bind α/β-Tubulin heterodimers and interact with microtubules (Gandhi et al., 2008). Gillardon further showed that recombinant human LRRK2 phosphorylates β-tubulin purified from bovine brain, that this phosphorylation is enhanced three-fold in the presence of theG2019S mutation, and that a LRRK2-related increase in β-tubulin phosphorylation increases microtubule polymerization (Gillardon, 2009b). LRRK2 and microtubules also interact in both neuronal and non-neuronal cells (Caesar et al., 2013). The R1441G mutation appears to interfere with this interaction, possibly by affecting tubulin acetylation and microtubule stability (Law et al., 2013).

Mice that overexpress either mutant or WT LRRK2 show elevated brain α/β-tubulin polymerization. This leads to microtubule overstabilization, which in turn impairs cell function (Lin et al., 2009; Maekawa et al., 2012). Conversely, brains from Lrrk2−/− mice have increased free tubulin levels (Gillardon, 2009b). LRRK2 also plays a role in actin dynamics, as gene expression of encode cytoskeleton-related proteins are deregulated in blood mononuclear cells from patients with LRRK2 mutations (Mutez et al., 2011). Mutant LRRK2 leads to the accumulation of polymerized actin as well as phosphorylated ERM, both of which are reversed by LRRK2 knockout (Parisiadou et al., 2009). By applying QUICK (quantitative immunoprecipitation combined with knockdown) in NIH3T3 cells, Meixner and colleagues found that LRRK2 interacts with actin isoforms, and also with actin-associated proteins that contribute to filament assembly, organization, rearrangement, and maintenance (Meixner et al., 2011).

Further links between LRRK2 and microtubule polymerization are suggested by an observed interaction between LRRK2 and elongation factor 1α (EF1A) (Gillardon, 2009a). Besides its canonical role in mRNA translation, EF1A helps maintain the microtubule cytoskeleton. LRRK2 interacts with tau, another protein that contributes to microtubule stability, and may modulate microtubule dynamics through this interaction. LRRK2 can directly phosphorylate tubulin-associated tau directly (Kawakami et al., 2012), and G2019S and I2020T LRRK2 mutations increase tau-phosphorylation. This in turn reduces tau's affinity for microtubules, which promotes its aggregation. Quantitative biochemical analysis shows the presence of unique phospho-tau species in G2019S mice (Melrose et al., 2010) and tau phosphorylation is reduced in brains from LRRK2 null mice (Parisiadou et al., 2009).

PD-associated LRRK2 mutations may directly influence the affinity of LRRK2 for microtubules or microtubule-bound proteins. Some studies suggest certain mutations increase affinity (Kett et al., 2012), while others (G2019S; R1441G, R1441H) reduce LRRK2-β-tubulin interactions (Law et al., 2013). Overall, *in vivo* and *in vitro* studies

indicate tau is a substrate of LRRK2, and suggest LRRK2 may contribute to histology changes observed in tauopathy disorders (Bailey et al., 2013).

Both LRRK2 deficiency and overexpression alter neurite length and branching (Lin et al., 2010; MacLeod et al., 2006; Plowey et al., 2008). Neurons that express the G2019S mutation have shorter neurites, and shRNA-mediated LRRK2 suppression increases neurite length (MacLeod et al., 2006).

An emerging body of literature supports a role for LRRK2 function in microtubule dynamics and trafficking. LRRK2 may interact with and/or phosphorylate or regulate the phosphorylation of several structural and regulatory components of the actin cytoskeleton and microtubule network. Therefore understanding these cytoskeletal associations may provide insight into the PD pathogenesis.

2.5 Relation to protein quality control mechanisms

Protein inclusions in PD patients brains may reflect a failure in one of the two major intracellular protein breakdown pathways, the UPS and autophagy. In the UPS, E1, E2 and E3 enzymes tag proteins with ubiquitin. Ubiquination targets the substrate to the proteasome, a barrel-shaped multiprotein complex that as protease activity, and degrades targeted substrates into peptides. In autophagy, proteins and/or other cellular components are degraded by lysosomal hydrolases.

2.5.1 Lysosomal protein degradation—Changes in autophagy are consistently observed when mutant LRRK2 is overexpressed, or with knock-down of endogenous LRRK2. Nevertheless, delineating the precise mechanism(s) by which LRRK2 regulates autophagy has been difficult.

In *C. elegans* transgenic strains, with altered LRRK2 activity, bear changes autophagy-related gene expression (Ferree et al., 2012). LRRK2 interacts with CAMKK-β/AMPK, a Ca^{2+}-dependent enzyme that induces the accumulation of autophagosomes (Gomez-Suaga et al., 2012). MAPKs, which positively regulate autophagy, are LRRK2 substrates (Gloeckner et al., 2009; Hsu et al., 2010).

In cultured cells, LRRK2 puncta co-localize with autophagic vacuoles (AVs) and multivesicular bodies (MVBs) patterns similar to those seen in human brains (Alegre-Abarrategui et al., 2009). In agreement with this, in rat brains punctate LRRK2 co-localizes with lysosomal and endosomal vesicles (Biskup et al., 2006). It is possible that alterations in the endocytic pathway are responsible for the deregulation of autophagy.

Reducing LRRK2 protein levels deregulates autophagy. For example, in the kidney, this manifests as an increase in LC3II, a protein marker of autophagy, and decreased levels of p62, an autophagic substrate. In addition, SNCA and protein carbonyls (a general marker of oxidative stress) levels also decrease whereas lysosomal proteins and proteases increase (Tong et al., 2012). However, neither autophagic nor lysosome-related structures accumulate in the brains of LRRK2 knock-out mice, which suggests LRRK2 may have different roles in different tissues, or that in the absence of LRRK2, homologs such as LRRK1 may

compensate (Tong et al., 2010). Moreover the fact that LRRK2 expression levels in the central nervous system are decreased relatively to the renal tissue can signify that mutant LRRK2 or LRRK2 absence can cause subtle pathogenic effects throughout ageing which is in agreement with the late onset of the disease.

In view of that, the inhibition of LRRK2 kinase activity stimulates macroautophagy (Manzoni et al., 2013a). In HEK cells LRRK2 knockdown increased LC3II turnover and autophagy (Alegre-Abarrategui et al., 2009). The continuous induction of autophagy caused by the absence of LRRK2 *in vivo* can ultimately cause a deficiency in the clearance or recycling of autophagic components/autolysosomes. LRRK2, therefore may, in the long run, down-regulate autophagy.

Relative to cells transfected with WT LRRK2 or with the kinase-dead K1906M mutation, cells that express the G2019S LRRK2 mutation show striking increases in neuritic and somatic autophagic vacuoles, as well as decreased neurite length. (Plowey et al., 2008). Overexpression of G2019S LRRK2 causes autophagic and lysosomal structures to accumulate in primary cortical neurons and in neuronal cell lines (MacLeod et al., 2006). Similarly, in G2019S and, to a lesser degree, R1441C transgenic mice AV accumulation in the soma and processes of cortical and striatal neurons has been described (Ramonet et al., 2011). These studies suggest that in the presence of these mutations, either autophagy is induced or autophagosome-lysosomal clearance is impaired.

Human iPSC dopaminergic neurons derived from either idiopathic or LRRK2 PD keratinocytes and fibroblasts show a decreased autophagic flux. This occurs in conjunction with increases in p62 protein, autophagosome, and lipid droplets and is consistent with impaired autophagic clearance (Sanchez-Danes et al., 2012). Compared to control fibroblasts, fibroblasts that express the G2019S mutation exhibit increased autophagy (Manzoni et al., 2013b). This is increase is mediated by the MAPK1/3 (Bravo-San Pedro et al., 2013). In SH-SY5Y cells, LRRK2 also appears to activate ERK1/2 (Liou et al., 2008). More interestingly, it is hypothesized that when excessive mitochondrial damage or excessive mitophagy is induced by neurotoxins the regenerative capacity of nigral neurons can be affected promoting PD-related pathogenic mechanisms (Dagda et al., 2008)

Calcium contributes to autophagy regulation, and links exist between calcium and LRRK2. One link is likely mediated by NAADP/Ca^{2+}, a Ca^{2+} mobilizing messenger that targets acidic (lysosome-like) Ca^{2+} stores and endoplasmic reticular stores (Guse and Lee, 2008). Recently, it was reported that the Ca^{2+} chelator BAPTA prevented an LRRK2-induced increase in autophagosomes, thereby suggesting LRRK2's autophagy effects are Ca^{2+} dependent. Further, exposing cells to a cell-permeate NAADP analogue restored the number of autophagosomes, lysosomal pH, and lipid droplet content to those that were observed when LRRK2 was overexpressed (Gomez-Suaga et al., 2012). One interpretation of this study is that LRRK2 localizes to lysosomes, where it interacts with NAADP receptors such as TPC2 to cause lysosomal calcium release. Calcium from the lysosomes then induces an endoplasmic reticulum calcium release, which activates the CaMKK/AMPK pathway. This, in turn, leads to lysosomal alkalinization and increases autophagosomes.

Rab7, a small GTP binding protein involved in late endosomal transport, lysosomal biogenesis, autophagosome-lysosome fusion, and endosome-lysosome fusion interacts with LRRK2. In morphologically altered neurons from subjects with neurodegenerative diseases, and especially those that show LBs accumulation, LRRK2 localizes to the endosomallysosomal compartment and co-localizes with Rab7B (Higashi et al., 2009). The drosophila LRRK2 homologue was also shown to localize late endosome and lysosome membranes, where it physically interacts with Rab7 and reduces Rab7-dependent targeting of lysosomes to the perinuclear region (Dodson et al., 2012). This suggests LRRK2-Rab7 interactions may regulate autophagosome-lysosome and endosome-lysosome fusion, which ultimately would perturb autophagic-lysosomal clearance pathways. Whether this happens in human neurons, though, is currently unclear.

LRRK2 itself appears to be removed via chaperone-mediated autophagy (CMA). While WT LRRK2 undergoes efficient CMA-mediated lysosomal degradation, G2019S LRRK2 does not. This raises the question of whether lysosomal G2019S LRRK2 accumulation might secondarily compromise the CMA-mediated removal of SNCA from neurons (Orenstein et al., 2013).

Overall there is still some controversy regarding how LRRK2 regulates autophagy. We can speculate that different LRRK2 mutations may cause impairment in the autophagic balance due to improper autophagic-lysosomal clearance or by an increase in the autophagic flux. Both paradigms can lead to the accumulation of autophagic components and substrates.

2.5.2 Relationship to the ubiquitin-proteasome system—In addition to being degraded by CMA, LRRK2 is also removed by the UPS. LRRK2 forms a complex with heat shock protein 90 (Hsp90) *in vivo*, and preventing this association via Hsp90 inhibition induces LRRK2 proteasomal degradation. Interestingly, a reduction in axon growth that occurs when G2019S LRRK2 is over-expressed can be reversed by Hsp90 inhibition (Wang et al., 2008). Indeed, a complex formed between LRRK2, CHIP, and Hsp90 appears to regulate cell LRRK2 levels. Hsp90 overexpression reduces CHIP-mediated LRRK2 degradation and geldanamycin, an Hsp90 inhibitor, increases CHIP-mediated LRRK2 degradation (Ding and Goldberg, 2009; Ko et al., 2009). The effect of whether or not LRRK2 mutations interfere with its Hsp90/CHIP interactions, though, is unknown. LRRK2 overexpression, both *in vitro* and *in vivo*, impairs UPS function. This leads to an accumulation of diverse substrates, including SNCA and ubiquitin (Lichtenberg et al., 2011).

3. LRRK2 and other PD-linked proteins: common pathways

Several reports suggest LRRK2 interacts with Parkin, DJ-1 and PINK1 (Samann et al., 2009; Smith et al., 2005; Venderova et al., 2009). It has further been postulated that autophagy may represent a point of functional convergence between these proteins and the pathways they contribute to (Cuervo et al., 2004; Dagda et al., 2009; Geisler et al., 2010; Narendra et al., 2008; Plowey et al., 2008).

Co-expression of human parkin in LRRK2 G2019S-expressing flies protects against DA neurodegeneration that arises with advancing age or following rotenone treatment (Ng et al., 2009). DJ-1 can also rescue neurons from LRRK2 G2019S-induced toxicity (Heo et al., 2010b). Moreover, neural cells generated from iPSCs derived from PD patients fibroblasts carrying mutations in the PINK1 and LRRK2 genes share mitochondrial abnormalities providing insight into convergence of cellular disease mechanisms between different familial forms of PD (Cooper et al., 2012).

4. LRRK2 and the Immune System

LRRK2 is expressed in circulating immune cells, and this expression is enhanced in the presence of microbial structures and viral particles. During bacterial phagocytosis, LRRK2 localizes near bacterial membranes. LRRK2 also increases NF-kB-dependent transcription and represses Nuclear Factor of activated T-cells (NFAT)-dependent transcription (Gardet et al., 2010; Hakimi et al., 2011; Liu et al., 2011b).

In monocytes, interferon-γ induces LRRK2 expression. This action is mediated through a novel ERK-dependent, interferon-γ signal transduction pathway (Kuss et al., 2014). LRRK2 may also contribute to monocyte maturation (Thevenet et al., 2011).

Microglial activation is observed in post-mortem PD brain tissue (Imamura et al., 2003), and PD patients also show other evidence of increased inflammation. Other inflammation-related changes are observed in PD subject lymphocytes, monocytes, and natural killer cells. Pro-inflammatory cytokine levels are elevated in PD subject blood, cerebrospinal fluid, and brain tissue (Collins et al., 2012). Peripheral blood mononuclear cells from PD patients with LRRK2 mutations have changes similar to those seen in mononuclear cells from sporadic PD patients (Mutez et al., 2014). Consequently, a role for LRRK2 has been implicated in microglial pro-inflammatory responses in the brains of PD subjects (Moehle et al., 2012).

In dermal fibroblasts prepared from PD subjects with LRRK2 mutations, LRRK2 silencing reduces basal and provoked cyclooxygenase (COX)-2 RNA levels (Lopez de Maturana et al., 2013). Reducing LRRK2 in murine brain microglia attenuates a lipopolysaccharide (LPS)-induced increase in cytokine mRNA and protein expression (Kim et al., 2012). Compared to isolated murine microglia obtained from mice that express WT LRRKs, microglia from mice that express the LRRK2 R1441G mutation produce more pro-inflammatory cytokines. When added to neuron cultures, conditioned medium from LPS-stimulated microglia that have the LRRK2 R1414G mutation triggers cell death (Gillardon et al., 2012). Both central and peripheral LPS administration was shown to only affect dopaminergic neurons, with no damage to either GABAergic or serotoninergic neurons and this damage was permanent in tyrosine hydroxylase-immunoreactive neurons in the substantia nigra (Herrera et al., 2000; Qin et al., 2007).

Altogether these reports pinpoint that LRRK2 dysfunction in PD may also involve immune response pathways.

5. Outlook

A better understanding of LRRK2 biology will provide insight into PD neurodegeneration. Accumulated evidence suggests LRRK2's kinase activity represents a reasonable therapeutic target for both autosomal dominant PD due to LRRK2 mutations, as well as for those with sporadic PD. It must be kept in mind, however, that LRRK2 plays a role in multiple cell pathways, including cytoskeleton maintenance and on various signalling cascades. This could limit the clinical applicability of LRRK2-directed interventions. A better molecular-level understanding of how LRRK2 mutations change LRRK2 function, as well as its cellular role, may help overcome this practical limitation.

Acknowledgments

This work was supported by the project PEst-C/SAU/LA0001/2011 from Portuguese Foundation for Science and Technology (FCT-MCTES, Portugal). AR Esteves is supported by Post-Doctoral Fellowship (SFRH/BPD/75044/2010) from Portuguese Foundation for Science and Technology (FCT-MCTES, Portugal). RHS is supported by the University of Kansas Alzheimer's Center (NIH P30AG035982).

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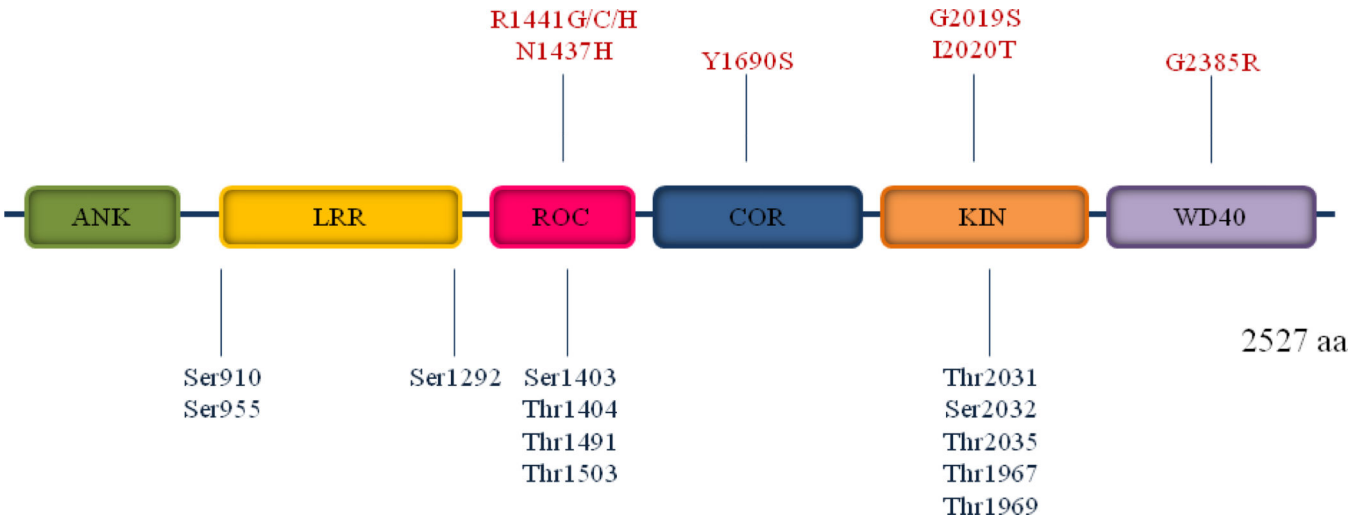

Figure 1.

Schematic representation of LRRK2. The central region of LRRK2 contains a GTPase domain also called ROC, a COR domain of unknown function and a kinase domain, flanked on either side by multiple protein–protein interaction domains: WD40 domain; ANK, ankyrin repeat domain and LRR, leucine-rich repeats. PD pathogenic mutations are depicted in red on top and phosphorylation sites are depicted in blue below. The scale of each domain or protein is not proportional to its actual size.

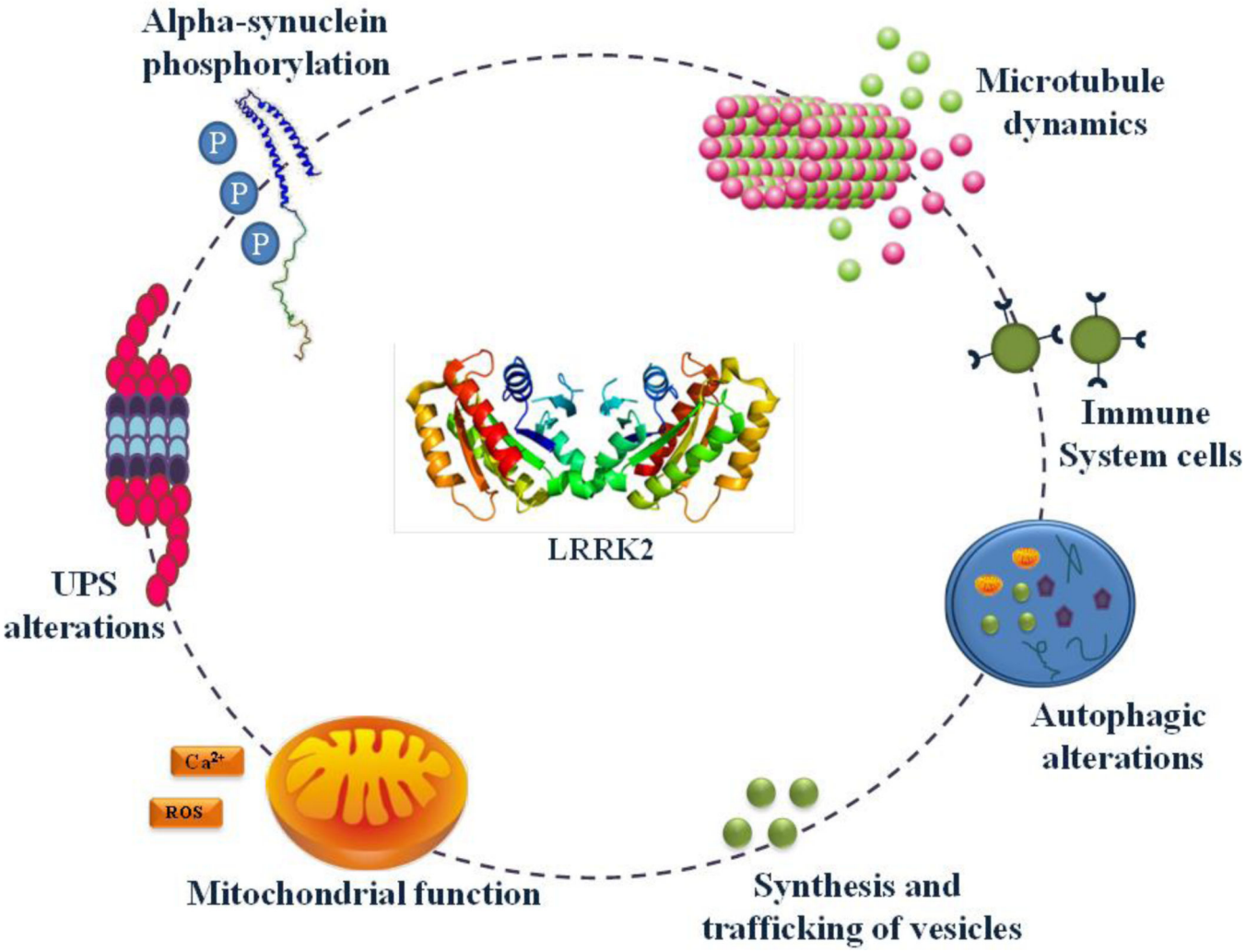

Figure 2.

LRRK2 involvement in cellular mechanisms. Several data posit that LRRK2 through its kinase and/or GTPase domain can affect mitochondrial function, ubiquitin-proteasome system, autophagy-lysosomal pathway, microtubule dynamics as well as trafficking of vesicles and proteins, alpha-synuclein phosphorylation content and immune system cells.

Table I

Overview of LRRK2 cellular phosphorylation and autophosphorylation with corresponding kinase and GTPase activity.

LRRK2 residues		Kinase Activity	References
Phosphorylation	Ser935	↑	Dzamko et al., 2010
	Ser910	↑	Dzamko et al., 2010
autophosphorylation	Ser1403 (ROC domain)	?	Kamikawaji et al., 2009
	Thr1404 (ROC domain)	?	Kamikawaji et al., 2009
	Thr1410 (ROC domain)	↑	Kamikawaji et al., 2009
	Thr1491 (ROC domain)	↓	Kamikawaji et al., 2009
	Thr1967 (kinase domain)	↓	Kamikawaji et al., 2009
	Thr1969 (kinase domain)	↑	Kamikawaji et al., 2009
	Thr2031 (kinase domain)	?	Li et al., 2010a
	Ser2032 (kinase domain)	?	Li et al., 2010a
	Thr2035 (kinase domain)	?	Li et al., 2010a
	Thr1503 (ROC domain)	↑	Webber et al., 2011
	Ser1292 (junction of the LRR and the ROC)	↑	Sheng et al., 2012

Table II

Overview of LRRK2 mutations with corresponding kinase and GTPase activity.

LRRK2 Mutations	LRRK2 Domain	Kinase Activity	GTPase Activity		References
			GTP Hydrolysis	GTP Binding	
R1441C	GTPase	?	↓	?	Kumar and Cookson, 2011; West et al., 2007
R1441G	GTPase	?	↓	=	Kumar and Cookson, 2011; West et al., 2007
R1441H	GTPase	?	?	?	Rudenko et al., 2012
Y1699C	COR	=	↓	=	Kumar and Cookson, 2011; West et al., 2007
G2019S	Kinase	↑	=	=	Greggio and Cookson, 2009
I2020T	kinase	?	?	?	Rudenko et al., 2012
I2012T	Kinase	?	?	?	Rudenko et al., 2012
N1437H	GTPase	?	?	↑	Rudenko et al., 2012
G2385R	C-terminal WD40	↓	?	?	Rudenko et al., 2012