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Exploring the contribution of LRRK2 GTPase activity to kinase activity and cellular phenotypes of Parkinson's disease-associated LRRK2

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Alice Biosa's Ph.D. thesis

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To my family and my friends

ABSTRACT

Parkinson's disease (PD) is a chronic and progressive motor neurodegenerative disorder. It is among the most common incurable diseases in the world: in the United States, at least 500,000 people suffer from PD, and about 50,000 new cases are reported annually. For these reasons, researchers are still looking for new therapies. Several genetic and environmental factors are demonstrated to be responsible for the onset of the disease.

In this work we have focused on the *LRRK2* gene in which mutations are the most common causes of familial and sporadic PD. Since, LRRK2 pathobiology is still not well understood, the aim of this study is to understand the overall contribution of the GTPase domain to the regulation of LRRK2 kinase activity and cellular phenotypes related to Parkinson's disease.

A collection of synthetic mutations analogous to well-characterized and conserved functional substitutions in members of the Ras and Raf families were developed for expression in mammalian cells. GTP binding, GTP hydrolysis, kinase activity and cellular phenotypes of FLAG-tagged LRRK2 were explored in HEK-293T cells. Since it was previously demonstrated that LRRK2 forms homodimers, the formation of dimers by FLAG-tagged wild-type (WT) LRRK2 and functional variants was verified by fast protein liquid chromatography and by co-immunoprecipitation with MYC-tagged WT LRRK2.

Finally, the effect of altered GTPase activity on neurite length was verified in cortical primary neurons obtained from P0 Sprague-Dawley rats and then transfected with WT and mutated FLAG-tagged LRRK2.

Data shows that both GTP binding and hydrolysis are independent from kinase activity and that both of them can contribute to the positive

modulation of LRRK2 kinase activity. Moreover, reduced, but not enhanced, GTPase activity critically alters neurite length in primary cortical neurons.

In summary, we generated a complete library of LRRK2 functional variants to elucidate the interplay between ROC and kinase domains. Our biochemical results seem to indicate that LRRK2 enzymatic regulation is quite complicated and it does not follow the canonical GDP/GTP cycle that is typical of the Ras/Raf/ERK pathway.

Moreover, decreased, but not increased, GTPase activity causes inhibition of axonal length. To date, we do not have any data to demonstrate that increased GTP hydrolysis can rescue GTPase-impaired neurite shortening. Therefore, new experiments are required to investigate the potential protective effects of modulating GTP hydrolysis in *in vitro* and *in vivo* model systems expressing GTPase-impaired pathological mutations.

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1. INTRODUCTION

Parkinson's disease (PD) belongs to a group of conditions called motor system disorders and it is the result of the death of dopaminergic neurons. PD is both chronic, meaning it persists over a long period of time, and progressive, because its symptoms grow worse over time. As the disease progresses, the shaking, or tremor, which affects the majority of PD patients, may begin to interfere with daily activities and sometimes these problems are accompanied by depression and other emotional changes, difficulty in swallowing, chewing, and speaking. The diagnosis of this disorder is difficult, because PD symptoms often become evident when the neuronal damage is already at an advanced stage. At present, there is no cure for this neurodegenerative disease, but there are several medications for its symptomatic treatment. Therefore, current research programs funded by the Institutes of Health of different countries are using animal models to study PD progression and to develop new drug therapies.

Among all PD causative genes, in this work we focused on *LRRK2* that largely contributes to both familial and sporadic PD cases. On the basis of these findings, LRRK2 pathobiology was hypothesized to give scientists the opportunity to better understand PD pathogenesis. Unfortunately, to date LRRK2 functions are still unclear. The aim of this work is to shed light on the enzymatic regulation of this protein, how it promotes PD onset and to hypothesize new LRRK2-related therapeutic strategies.

1.1 Parkinson's disease

Parkinson's disease (PD) is the most common neurodegenerative motor disorder and was discovered first in 1817 by James Parkinson (Parkinson, 1817). The motor abnormalities of Parkinson's disease result from the death of dopaminergic (DAergic) neurons in the *substantia nigra pars compacta*

(SNpc) and their projections to the *striatum* (Figure 1); the causes of this cell death are still unknown.

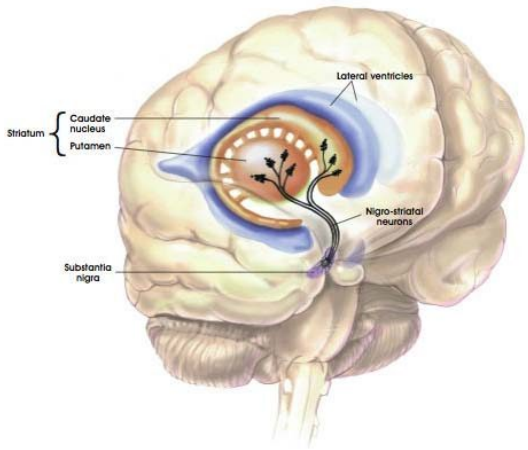


Figure 1. The nigro-striatal dopaminergic pathway implicated in Parkinson's disease.

PD affects 1% to 2% of the population over 60 years of age (Gasser, 2009), prevalently males. During the progression of the disease, the most common symptoms include resting tremor, bradykinesia, muscular rigidity and postural instability. Later, cognitive and behavioral problems may take place in the advanced stages of the disease and they can include dementia, sensory, sleep and emotional problems in ~30% of PD cases. None efficacious therapy has not yet been identified and until now PD treatment is based on L-3, 4-dihdroxyphenylalanine (levodopa, L-DOPA). This compound is the metabolic precursor of dopamine (DA) and is orally delivered; it is converted to dopamine in dopaminergic neurons after its intestinal absorption. L-DOPA is usually administered with a peripheral decarboxylase inhibitor to prevent its conversion to DA in the peripheral circulation and to increase the drug amount that reaches the central nervous system (CNS). Moreover, DA produced by decarboxylation of levodopa and present in the peripheral circulation provokes side effects including nausea and cardiovascular effects. The main limit to long-term L-DOPA treatment is the gradual decrease in its ability to rescue the

dopaminergic deficit, loading to dramatic fluctuations of striatal dopamine content in patients after each dose. Agonists of striatal dopamine receptors and inhibitors of enzymes involved in levodopa and dopamine metabolism are alternative therapies to L-DOPA.

Approximately 80% of patients with Parkinson's disease receives a probable clinical diagnosis of the pathology. The definitive diagnosis requires *post mortem* findings of neuronal loss and depigmentation of the *substantia nigra* and the presence of Lewy bodies in the brain stem (Hughes et al., 2002). Lewy bodies (intracellular inclusions, Figure 2) are the pathological hallmark of the idiopathic disorder, and their distribution throughout the parkinsonian brain varies from one individual to another. The anatomical localization of Lewy bodies is often directly related to the degree of clinical symptoms of each individual. Usually they appear first in the olfactory bulb and medulla and then take place an ascending course into the midbrain and finally into the cortex (Farrer, 2006).

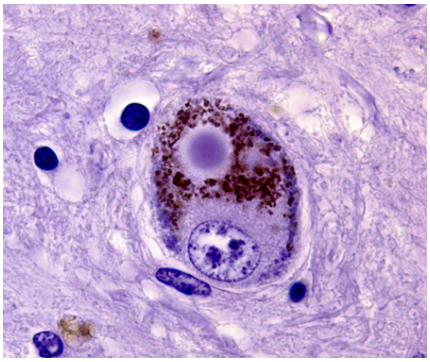


Figure 2. Lewis body in a dopaminergic neuron.

1.2 Parkinson's disease and the nigro-striatal dopaminergic pathway

PD is characterized by a deficit in the nigro-striatal extrapyramidal pathway, which is a forebrain basal ganglia circuit that connects the *substantia nigra* to the *striatum*. In the SNpc there are the neuronal cell bodies of dopaminergic neurons that extend their axons to the *striatum*. In addition to nigral afferents, projections from thalamus and from whole cortex reach the

striatum to regulate motor activities by different neurotransmitters. The nigral dopaminergic neuronal loss that occurs in Parkinson's disease is associated with a deficit of DA in the *striatum*, leading to a decrease in motor cortex excitation from the thalamus. In fact, the reduced inputs of DA in the *striatum* cause the hyper-secretion of γ-amino-butyric acid (GABA) from this basal nucleus and, consequently, the ailments of hypokinesia. Moreover, in the *striatum*, the imbalanced ratio between inhibitory DAergic and excitatory cholinergic activities stimulates the outputs from this nucleus, provoking rigidity and tremor.

DA is the main neurotransmitter of this pathway and belongs to the family of catecholamines and is involved in voluntary movement as well as in cognitive functions (O'Neill, 2005) and in the mesolimbic reward pathway (Wightman and Robinson, 2002). In dopaminergic neurons of the *substantia nigra* is expressed the tyrosine hydroxylase gene that encodes the enzyme converting the amino acid tyrosine to L-Dopa, which is transformed into DA by L-aromatic amino acid decarboxylase. At the presynaptic terminal, dopamine is concentrated into synaptic vesicles and released into the striatal synaptic cleft in response to an action potential. Then, DA binds the post-synaptic striatal receptors D1 and D2 to exert its physiological functions (Figures 3 and 6 A). The excess of dopamine in the synaptic cleft undergoes an inactivation by the catechol-O-methyl transferases (COMT) or a reuptake process for a new cycle of neurotransmitter release. The dopamine not stored in synaptic vesicles is oxidized by mitochondrial monoamine oxidases B (MAO-B).

Alterations in dopamine synthesis, exocytosis, endocytosis and catabolism in the synaptic cleft can be associated with PD; therefore different drugs might be potentially used to modulate the dopaminergic pathway (Figure 3).

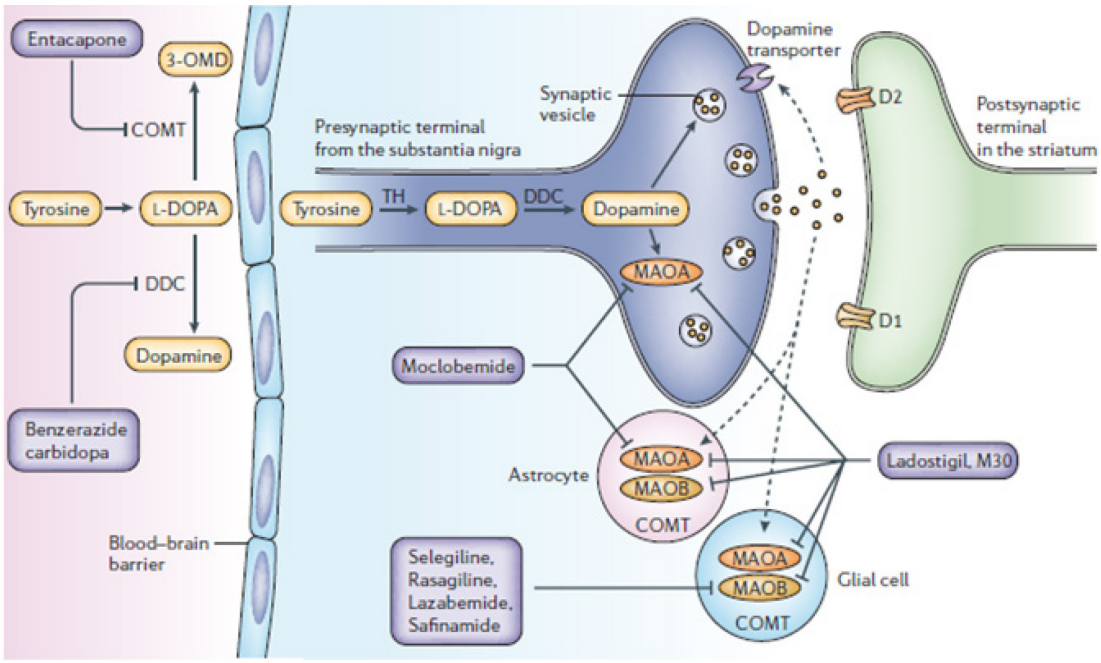


Figure 3. Dopaminergic pathway in the central nervous system and its regulation by different drugs (Youdim et al., 2006).

1.3 Parkinson's disease etiopathogenesis

At first, PD was thought to be a sporadic disease, or an environmentally-related disorder. After the discovery of *SNCA* as a causative gene of PD in 1997, there was an increasing recognition that genetic factors can be responsible for this disease, particularly in familial cases. Currently, PD is considered both sporadic and inherited disease.

Several studies in PD models suggested the existence of two main mechanisms for its pathogenesis:

- ✓ Mitochondrial dysfunction and oxidative stress;
- ✓ Protein misfolding and aggregation.

1.3.1 Environmental factors

Since many cases of Parkinson's disease occur sporadically, without any apparent cause, it has been hypothesized that the onset of PD might be the result of a complex interaction between environmental causes and genetic risk factors. The exposure to substances such as rotenone, paraquat,

transition metals, 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1 ,2,3,6-tetrahydropyridine (MPTP) (Figure 4) are among the possible causes of sporadic PD. All mentioned above compounds provoke oxidative stress and mitochondrial dysfunction.

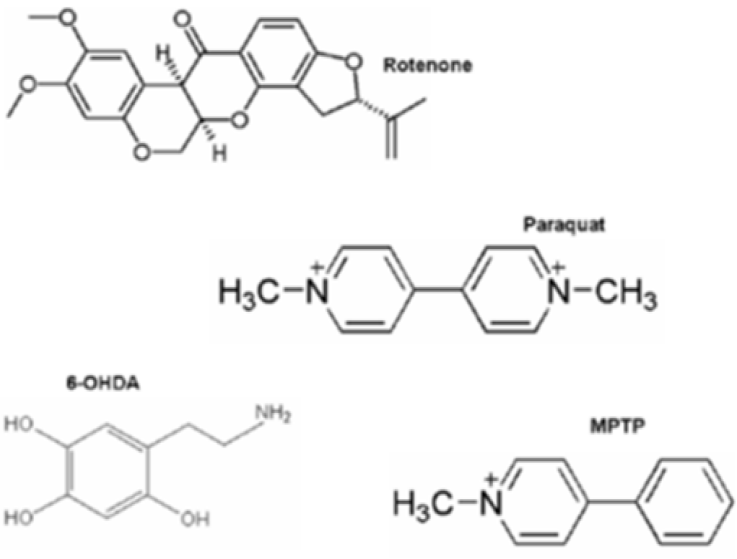


Figure 4. Main neurotoxins used in experimental models of PD.

Rotenone

Rotenone is a cytotoxic compound derived from roots extract of some tropical plants and widely used as an insecticide and pesticide (Bové et al., 2005). Rotenone is a high lipophilic molecule (Figure 4); therefore it easily crosses cell membranes and accumulates within mitochondria. Rotenone impairs mitochondrial oxidative phosphorylation by inhibiting NADH-ubiquinone reductase activity of complex I. This enzymatic down-regulation increases the production of $O_2^{\bullet-}$, which provokes the formation of hydroxyl radicals or reacts with nitric oxide to form peroxynitrite. These radicals can cause cellular damage by reacting with nucleic acids, proteins and lipids. The target of these molecules seems to be the electron transport chain (Cohen, 2000), whose inhibition leads to mitochondrial dysfunction and reactive oxygen species (ROS) production. The presence of ROS may also increase the amount of misfolded proteins. Dopaminergic neurons are

particularly sensitive to ROS production, since metabolism of dopamine produces H_2O_2 and $O_2^{\bullet-}$ and its auto-oxidation generates DA-quinone (Graham, 1978), which mostly reacts with cysteine residues of damaged proteins.

Rotenone might also inhibit microtubules formation from tubulin monomers. Since an excess of tubulin monomers may be toxic for cells, this effect could be relevant in the mechanism of degeneration of dopaminergic neurons (Bové et al., 2005).

Paraquat

The herbicide paraquat (N, N'-dimethyl-4-4'-bipiridinio), is another molecule that can induce Parkinsonism and that is considered an environmental toxin. Its toxicity is related to radical $O_2^{\bullet-}$ formation (Day et al., 1999) and so far several paraquat-related deaths have been reported. It cannot easily diffuse through the blood-brain barrier (BBB) (Figure 4).

Transition metals

As already mentioned, the neurons of the SNpc are particularly vulnerable to oxidative stress because of dopamine metabolism, even under physiological conditions. *Substantia nigra* of PD patients contains high levels of divalent iron (Fe^{2+}), which forms the highly cytotoxic hydroxyl radicals ($OH^{\bullet}$) and superoxide anions ($O_2^{\bullet-}$) by means of Fenton reactions.

In humans, high concentration of manganese (Mn^{2+}) might generate a syndrome similar to Parkinson's disease (Elbaz and Moisan, 2008). Experiments carried out on PC12 cells, a tumor line able to produce and secrete DA, showed that Mn^{2+} is able to induce apoptosis (Desole et al., 1997), moreover other *in vitro* (Migheli et al., 1999) and *in vivo* studies (Serra et al., 2000) demonstrated that subtoxic concentrations of Mn^{2+} increase

the cytotoxicity of L-DOPA. L-DOPA and DA increased auto-oxidation have been suggested as a mechanism by which Mn^{2+} causes toxicity.

6-hydroxydopamine (6-OHDA)

In 1968 Ungerstedt first demonstrated that 6-OHDA injection into the murine *substantia nigra* is associated with degeneration of dopaminergic neurons. 6-OHDA (Figure 4) is a hydroxylated analog of DA that shows a high affinity for both dopamine (DAT) and norepinephrine transporter (NET) (Luthman et al., 1989). Because of these features, the 6-OHDA can enter into dopaminergic and noradrenergic neurons causing a damage along the catecholaminergic pathway in the central and peripheral nervous system. The reason why this toxin is able to destroy catecholaminergic cells is related to a synergistic effect of both H_2O_2 and paraquinone production (Bové et al., 2005).

1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP)

In humans and monkeys, MPTP causes a severe and irreversible Parkinson-like syndrome that recapitulates PD symptoms including tremor, rigidity, slowness of movement and postural instability (Nicklas et al., 1987). In human and non-human primates exposed to MPTP it is possible to see the same benefits and complications of long-term L-DOPA administration observed in idiopathic PD patients. MPTP is a highly lipophilic molecule (Figure 4) and crosses the BBB in a few minutes after systemic administration (Markey et al., 1984). Once it reaches the brain, MPTP is oxidized to $MPDP^+$ (1-methyl-4-phenyl-2, 3-dihydropyridinium) by monoamine oxidases B (MAO-B) of glial cells and serotonergic neurons. After its spontaneous oxidation, the $MPDP^+$ is converted to MPP^+ that is released into the extracellular space. Since this is a charged molecule, MPP^+

uses its affinity to DAT to be internalized into neurons (Javitch et al., 1985; Mayer et al., 1986) (Figure 5).

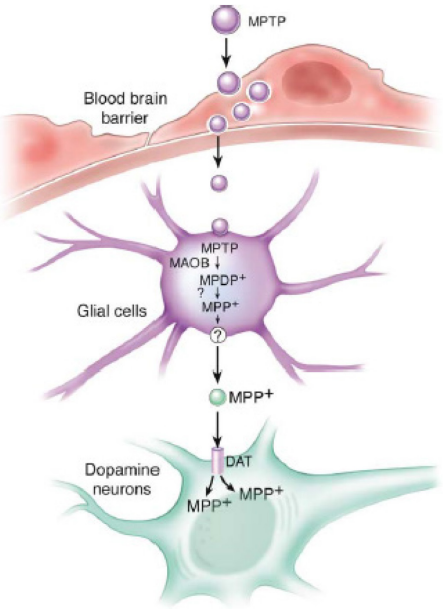


Figure 5. MPTP metabolism (Dauer and Przedborski, 2003).

Then, MPP^+ enters neuronal mitochondria and inhibits oxidative phosphorylation by down-regulating the activity of NADH-ubiquinone reductase (Nicklas et al., 1985). This inhibition leads to a drastic ATP depletion in some tissues, especially in the *striatum* and in the ventral midbrain (Chan et al., 1991; Fabre et al. 1999). Moreover, MPP^+ can stimulate ROS production and, consequently, impairs the activity of vesicular monoamine transporter 2 (VMAT2) that is no longer able to transport DA into synaptic vesicles (Johnson, 1988). These initial events are usually not directly involved in cell death, but they can trigger a cascade of reactions that cause dopaminergic neuronal death (Mandir et al., 1999; Saporito et al., 2000; Vila et al. 2001).

1.3.2 Genetic risk factors and causative genes of Parkinson's disease

By genome-wide association studies (GWAS) and linkage analyses within different populations, several susceptibility loci and causative genes were

identified (Table 1). For example, common variations in the *MAPT*, *RAB7L1, GBA, SNCA and LRRK2* genes are associated with an increased risk for developing idiopathic PD (Leroy, E. et al., 1998), although some of these genes are not exclusive to this disorder, for example, *MAPT* and *GBA*. Mutations in at least eight genes (*PINK1*, *DJ-1*, *PARK2, ATP13A2, VPS35, EIF4G1, SNCA* and *LRRK2*) are known to cause familial forms of PD (Kumari and Tan, 2009).

Locus	Gene	Chromosome	Inheritance/clinical phenotype
PARK1/PARK4	*α-synuclein*	4q21	AD and sporadic/early onset PD
PARK2	*Parkin*	6q25.2-q27	AR and sporadic/early onset PD
PARK3	*Unknown*	2p13	AD/late onset PD No causative gene identified
PARK6	*PINK1*	1p35-p36	AR and sporadic/early onset PD
PARK7	*DJ-1*	1p36	AR/early onset PD
PARK8	*LRRK2*	12p11.2-q13.1	AD and sporadic/late onset PD
PARK9	*ATP13A2*	1p36	AR/early onset PD
PARK10	*Unknown*	1p32	? AD
PARK11	*GIGYF2*	2q36-q37	AD/late onset PD Pathogenicity uncertain
PARK12	*Unknown*	Xq21-q25	Unknown No causative gene identified
PARK13	*HTRA2*	2p13	Unknown Pathogenicity uncertain
PARK14	*PLA2G6*	22q13.1	AR/L-DOPA responsive distonia-parkinsonism
PARK15	*FBX07*	22q12-q13	AR/parkinsonism-pyramidal syndrome
PARK16	*RAB7L1*	1q32	Sporadic PD
PARK17	*VPS35*	16q13-q21	AD/PD
EIF4G1	*EIF4G1*	3q27	AD/late onset PD Pathogenicity uncertain
	MAPT	17q21.3	Sporadic PD
	GBA	1p11	Sporadic PD

Table 1. Genetic risk factors and causative genes of Parkinson's disease (AD, Autosomal dominant; AR, Autosomal recessive; PD, Parkinson's disease).

Most of the PD-associated genes are directly or indirectly involved in protein misfolding and aggregation. Under physiological conditions the ubiquitin-proteasome system (UPS) is responsible for the degradation of

damaged or misfolded proteins (McNaught and Olanow, 2003). The impairment of the UPS results in protein accumulation in the cytosol and subsequent inclusion formation, leading to alterations in cellular homeostasis and integrity. Lewy bodies (LBs) are spherical aggregates of different eosinophilic cytoplasmic proteins (Spillantini et al., 1998) and are the main hallmark of PD for a definitive diagnosis (Gibb and Lees, 1988).

Microtubule-associated protein tau, RAB7L1 protein and β-glucocerebrosidase (GBA)

Common genetic variations in the *MAPT* gene (on chromosome 17) were reproducibly associated with susceptibility to PD (Rademakers et al. 2005) and are one of the major risk factors for sporadic PD.

Mutations in the *GBA* gene (on chromosome 1) occur in 7% of patients with Parkinson's disease and are a well-established risk factor for PD (Lwin et al., 2004; Lill et al., 2012; Alcalay et al., 2012).

In 2010 Tuzzi et al. identified a coding variant in PD cases within the *RAB7L1* gene (on chromosome 1, locus PARK16). Conversely, in Chinese PD patients no one *RAB7L1* mutant was found (Yan et al., 2011), raising the question about the contribution of *RAB7L1* mutant to increasing the susceptibility to PD.

ATP13A2

Mutations in the *ATP13A*2 gene (on chromosome 1, locus PARK9) cause Kufor-Rakeb syndrome (KRS), a juvenile-onset pallido-pyramidal neurodegenerative disorder. Several homozygous (F182L [Ning et al., 2008], G504R [Di Fonzo et al., 2007] and G877R [Santoro et al., 2011]) and heterozygous (T12M [Di Fonzo et al., 2007], G533R [Di Fonzo et al., 2007] and A746T [Lin et al., 2008]) mutations have recently been identified in patients with early-onset forms of familial or sporadic Parkinsonism or PD suggesting that *ATP13A2* mutations may also contribute to early-onset PD.

Vacuolar protein sorting 35 (VPS35) and eukaryotic translation initiation factor 4-gamma (EIF4G1)

Recently two groups have independently identified a single mutation in the *VPS35* gene (on chromosome 16, locus PARK17) (c.1858G>A; p.D620N) as one cause of autosomal dominant familial PD (Zimprich et al., 2011; Vilariño-Güell et al., 2011). To date, the pathogenicity of this variant requires confirmation.

Genome-wide analyses showed that a mutation in the *EIF4G1* gene (chromosome 3) segregates with late-onset Parkinson's disease in an autosomal dominant family (Chartier-Harlin et al., 2009). The encoded protein, EIF4G1, is a translation initiation factor and might be involved in the stress response. Loss of EIF4G1 leads to impaired nutrient sensing and mitochondrial bioenergetics. The same mutation was thereafter identified in several Caucasian families, but not in control individuals. These findings still need to be replicated.

PTEN-induced kinase 1 (PINK1)

Homozygous and heterozygous mutations in the *PINK1* gene (chromosome 1, locus PARK6) were identified in 1-2% of cases of early-onset recessive PD (Hatano et al., 2004). PTEN-induced kinase 1, which is the protein encoded by this gene, is ubiquitously present and includes a mitochondrial targeting motif and a protein kinase domain that is also found in the Ca^{2+}/calmodulin family of serine-threonine kinases (Valente, E. M. et al., 2004). Mutations in the *PINK1* gene provoke different effects on protein stability, localization and kinase activity (Petit et al., 2005; Beilina et al., 2005). Biological data is lacking, although wild-type (WT) PINK1 protects cultured neurons from mitochondrial dysfunction and oxidative stress-induced apoptosis (Deng et al., 2005).

DJ-1

The *DJ-1* gene is located on chromosome 1 and encodes a protein of 189 amino acids with cytoplasmic and mitochondrial localization (Lee and Liu, 2008; Shendelman et al., 2004). To date, its physiological function is unknown; however some groups have suggested a role as an antioxidant protein (Mitsumoto and Nakagawa, 2001). In this context, it seems that a Cys106 residue might have an important role, although none molecular mechanism was yet elucidated. Finally, DJ-1 may function as a molecular chaperone protein to limit α-synuclein aggregation in neurons (Shendelman et al., 2004).

Parkin

The *parkin* gene is localized on chromosome 6 (locus PARK2) with homozygous mutations causing familial recessive PD (Kitada et al., 1998). These variants are mostly found in patients with early-onset disease, about 30 years, and in patients with late-onset disease (Lincoln et al., 2003). Parkin is an E3 ubiquitin ligase (Zhang et al., 2000; Shimura et al., 2000), a component of the UPS complex that ubiquitinates unfolded or damaged proteins and targets them to proteasome for degradation (Sherman and Goldberg, 2001). Pathological mutations at the PARK2 locus impair parkin-mediated ubiquitination of its substrates leading to protein accumulation into the cytoplasm.

a-Synuclein

The *SNCA* gene is located on chromosome 4 (loci PARK1 and PARK4) and its three missense mutations A53T, A30P and E46K and gene multiplications are responsible for autosomal dominant forms of familial PD (Polymeropoulos et al., 1997; Kruger et al., 1998). Moreover, common variations in the *SNCA* gene, together with the *MAPT* gene, are two major

risk factors for the development of idiopathic PD (Pankratz et al., 2009; Satake et al., 2009; Simon-Sanchez et al., 2009; Edwards et al., 2010). α-Synuclein protein is highly expressed in the brain, mostly in cell bodies and is enriched within pre-synaptic nerve terminals (Maroteaux et al., 1988; George et al., 1995). Its biological function is not yet known, although it has been suggested to play a role in synaptic vesicle trafficking and recycling (Kahle et al., 2002). At striatal dopaminergic terminals, α-synuclein is involved in the modulation of synaptic functions probably by regulating the speed at which synaptic vesicles are recycled (Abeliovich et al., 2000). Under physiological conditions, α-synuclein is natively unfolded (Recchia et al., 2004) and mutations tend to enhance its fibrillization and the formation of amyloid fibrils (Conway et al., 1998; Giasson et al., 1999) and protofibrils (Conway et al., 1998). The evidence that PD-linked mutations promote the formation of protofibrils (Conway et al., 2000), suggested that these fibrils are responsible for α-synuclein-induced toxicity. In agreement with this hypothesis, some studies have reported that α-synuclein protofibrils might form pore-like structures at the membrane of synaptic vesicles (Volles et al., 2001; Lashuel et al., 2002), leading to the abnormal permeabilization of dopamine-containing vesicles thus enabling DA to diffuse into the cytoplasm and participate in reactions that generate oxidative stress .

Moreover, the discovery of α-synuclein in LBs suggested that its overexpression and its propensity to form fibrils could be the cause of neurotoxicity in Parkinson's disease.

Figure 6 B provides a summary of environmental and genetic factors implicated in the pathogenesis of PD.

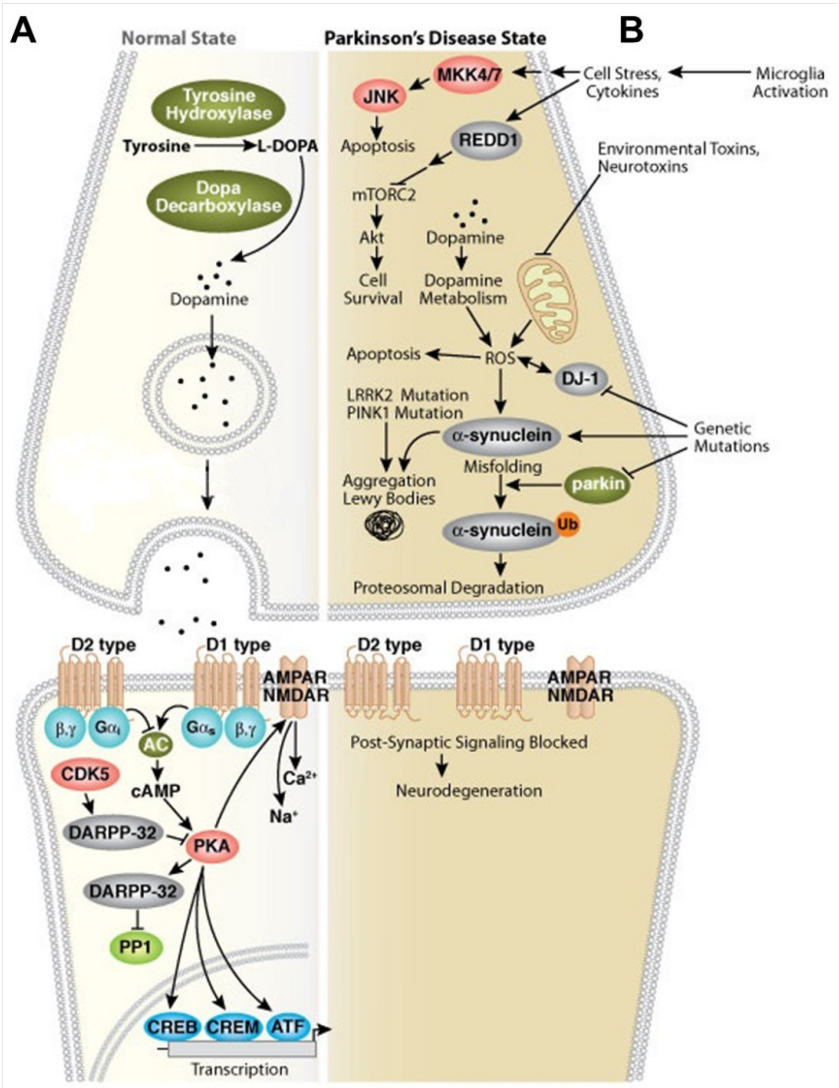


Figure 6. Dopamine signaling under physiological conditions (**A**); environmental and genetic factors associated with PD pathogenesis (**B**).

1.4 *Leucine-Rich Repeat Kinase 2* as a common genetic cause of Parkinson's disease

The discovery of the *Leucine-Rich Repeat Kinase 2* gene (LRRK2, locus PARK8, OMIM 607060) as a causative PD gene (Paisán-Ruiz et al., 2004; Zimprich et al., 2004) has significantly contributed to the understanding of PD etiopathology and to the providing some useful information for new therapies development.

Mutations in the *LRRK2* gene are the most common causes of inherited PD and genetic variations in *LRRK2* increase the risk of developing sporadic PD (Zimprich et al., 2004; Satake et al., 2009).

1.4.1 Distribution and cellular localization of LRRK2

In mammals, LRRK2 is expressed at different levels depending on tissue type and developmental stage (Biskup et al., 2007). LRRK2 expression can be detected in rat brain by embryonic day 16 to 17; the expression level increases during neuronal maturation and postnatal stages, and finally reaches a maximum level in kidney, lung and lymph nodes in adult rats. In adult mammalian brain, LRRK2 protein is particularly prominent in cerebral cortex, *striatum*, *substantia nigra*, *hippocampus* and *cerebellum* (Biskup and West, 2009; Galter et al., 2006; Higashi et al., 2007˙; Higashi et al., 2007¨; Higashi et al., 2007˙˙˙). In the nigro-striatal pathway, LRRK2 mRNA is abundantly detected in striatal medium-sized spiny output projections neurons, cholinergic and GABAergic interneurons, but at significantly lower levels in dopaminergic neurons of the *substantia nigra pars compacta* (Higashi et al., 2007˙; Higashi et al., 2007¨). Under physiological conditions, endogenous LRRK2 predominantly localizes in neurons, adopts a diffuse cytosolic distribution and is enriched upon multiple organelles and intracellular membranes, including endosomes, lysosomes, multivesicular bodies, mitochondrial outer membrane, lipid drafts, microtubule-associated

vesicles, Golgi complex and endoplasmic reticulum (Biskup et al., 2006). In primary cortical neurons LRRK2 co-localizes with lysosomal and mitochondrial markers (Biskup et al., 2006; Hatano et al., 2007) and it is enriched within synaptosomal fractions from rat brain (Biskup et al., 2006).

1.4.2 LRRK2 protein structure

ROC, COR, LRR and WD40 domains

LRRK2 is a large protein of 2527 amino acids and belongs to the ROCO protein family, including a Ras-related GTPase domain (ROC) followed by a *C*-terminal of ROC domain (COR) and a kinase domain (Bosgraaf and Van Haastert, 2003). ROC and kinase domains are also flanked by ankyrin and leucine-rich repeats at the *N*-terminus and by WD40 repeats at the *C*-terminus (Figure 7).

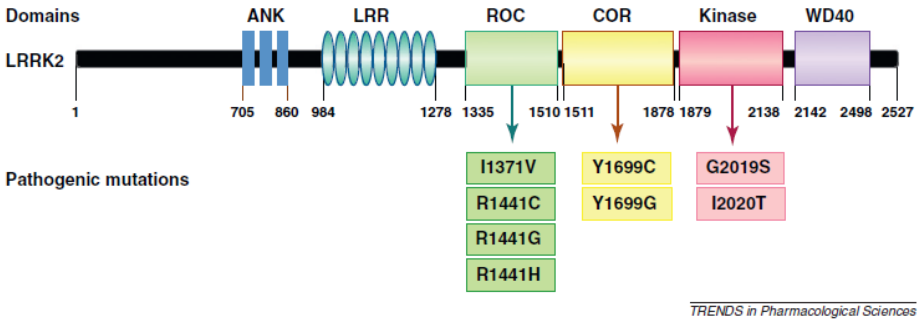


Figure 7. LRRK2 protein structure and its pathological variants (Lee et al., 2012).

The ROCO protein family consists of complex proteins, conserved among prokaryotes and eukaryotes. Three other members of human ROCO proteins were identified, including leucine-rich repeat kinase 1 (LRRK1), malignant fibrous histiocytomas-amplified sequences with leucine-rich repeats (MFHAS1) and death-associated protein kinase 1 (DAPK1). Members of the ROCO family differ from each other by the presence or absence of protein-protein interaction modules and a kinase domain.

LRRK2 is a GTPase protein, although it exhibits a weak enzymatic activity. The LRRK2 ROC domain is responsible for GTP binding and hydrolysis

and it shares homology with members of the Ras/GTPase superfamily: Ras, Rab/Ran, Rho/Rac and Arf. The GTP binding occurs through the predicted guanine nucleotide phosphate-binding loop (P-loop) which includes the conserved sequence GNTGSGKT (residues 1341-1348, Figure 8). In LRRK2 orthologous and paralogous proteins, Lys1347 and Thr1348 residues are highly conserved and they mediate the interaction between LRRK2 protein, GTP β-phosphate and magnesium (Mg^{2+}) ion. The Mg^{2+} ion interacts with $O^{\beta 3}$ atom of GTP β-phosphate, hydroxyl oxygen ($O^{\gamma 1}$) of Thr1348 and four water molecules. Moreover, $O^{\beta 1}$ and $O^{\beta 2}$ from GTP β-phosphate group and the free oxygen atom from GTP α-phosphate interact with four contiguous backbone amides from the P-loop (Gly1344, Serine1345, Gly1346 and Lys1347) through hydrogen bondings. All these water-mediated hydrogen bondings allow the Mg^{2+} ion to be linked to switch I and II regions for GTP hydrolysis (Deng et al., 2008). The motif responsible for LRRK2 GTPase activity (switch II region) includes the DFAGR sequence (residues 1394-1398, Figure 8), where Asp (D) and Gly (G) residues are highly conserved. The majority of well-known small GTPase proteins possess Gln (Q) rather than Arg (R) at 1398 position, suggesting that this difference can explain why LRRK2 exhibits such a slow rate of enzymatic activity. After GTP binding, the ROC domain changes its conformation to allow GTP hydrolysis. Different groups are studying LRRK2 GTPase activity and to date, there are contrasting results, including both negative (Ito et al., 2007) and positive (Lewis et al., 2007; Guo et al., 2007; Li et al., 2007) data, depending on experimental conditions. The principal issue is related to the slow enzymatic GTPase activity of LRRK2. In contrast, the GTP hydrolysis is much more active when the ROC domain is isolated from the full-length protein either in *E. coli* (Deng et al., 2008) or in mammalian cells (Li et al., 2007), suggesting that sequences

outside of ROC domain can directly or indirectly repress GTP hydrolysis activity.

Interestingly, some groups found that, similarly to other small GTPases, LRRK2 interacts with proteins that increase its GTPase activity (GAP proteins) (Stafa et al. 2012; Xiong et al., 2012), conversely other authors hypothesized that LRRK2 does not need a GAP protein because dimerization alone may instead be sufficient to activate GTP hydrolysis (Gasper et al., 2009).

The LRRK2 kinase domain shares closest sequence homology with a subclass of mitogen-activated protein kinase kinase kinase (MAPKKK) proteins and with the receptor-interacting protein kinases (RIPKs). In fact, LRRK2 shows all expected subdomains of a Serine/Threonine kinase. The ATP binding loop is characterized by a Glycine-rich loop (1885-1982) facilitating backbone interactions with the γ-phosphate of ATP and includes a highly conserved Lys1906 residue for ATP binding. The Asp1994 residue is part of the DLK motif within the catalytic loop: it accepts the proton from the hydroxyl group of its substrate (Ser, Tyr or Thr) and then forms the oxyanion species responsible for the further nucleophilic attack on ATP γ-phosphate. The kinase active site is located in the activation segment (2017-2043 residues, Figure 8), starting N-terminally with the tripeptide motif DFG and ending C-terminally with the APE motif (Nolen et al., 2004). The invariable Asp2017 residue is responsible for binding to a magnesium ion required for ATP phospho-transfer, while the rest of the motif contributes to hydrophobic interactions with the αC helix from the small lobe that is important for the catalytic activity (Nolen et al., 2004).

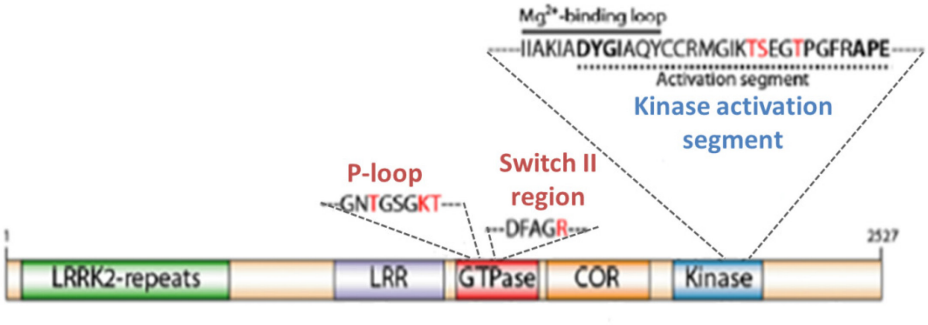


Figure 8. Highly conserved sequence in LRRK2 protein.

Protein kinases usually remain in an inactive state until a conformational changes occurs in the activation segment; often the signal is represented by autophosphorylation, phosphorylation by another kinase protein or by binding to a regulator domain. It is likely that LRRK2 kinase domain activation depends on the autophosphorylation of its activation segment, because triple mutation of Thr2031, Ser2032 and Thr2035 totally impairs the capacity of LRRK2 to phosphorylate itself. By means of *in vitro* and *in vivo* autophosphorylation assays it was demonstrated that LRRK2 is an active kinase (West et al., 2005; Li et al., 2007, Luzon-Toro et al., 2007) and that this reaction occurs in *cis* (Greggio et al., 2008). Unfortunately, this data does not prove that autophosphorylation is a relevant event under physiological conditions.

Different LRRK2 kinase substrates have been proposed: ezrin/radix/moesin (ERM) (Thr558) (Jaleel et al., 2007), β-tubulin (Thr107) (Gillardon, 2009), FoxO1 (Ser319) (Kanao et al. 2010), 4E-BP1 (Thr37/46) (Imai et al., 2008), MKK proteins (Gloeckner et al., 2009), α-synuclein (Ser129) (Qing et al., 2009), *Drosophila* Futsch (Lin et al., 2010) and ArfGAP1 (Stafa et al., 2012; Xiong et al., 2012).

The presence of so many protein-protein interaction motifs in LRRK2 (ankyrin-like, LRR and WD40 domains) might indicate that this protein is involved in different molecular pathways. For example, in other proteins, WD40 domains mediate the association with lipids, suggesting that LRRK2

could be associated with intracellular membranes. Moreover, different LRRK2 interacting proteins were identified: parkin (another protein whose variants segregate with PD), Rab5b (a small GTPase involved in endocytosis) (Shin et al., 2008), heat-shock protein 90 (a chaperone protein) (Wang et al., 2008), α- and β-tubulin (for the link to cytoskeleton) (Gandhi et al., 2008) and Fas-associated death protein (for the induction of the cell death cascade) (Ho et al., 2009).

LRRK2 protein complexes

By means of co-immunoprecipitation (Co-IP) (Gloeckner et al., 2006), fast protein liquid chromatography (FPLC) (Sen et al., 2009) and BLUE native PAGE analyses (Greggio et al., 2008; Sen et al., 2009) it was demonstrated that LRRK2 forms homodimers, similar to other ROCO kinase proteins. It was found that different points of interaction exist between two LRRK2 monomers: COR-COR (Greggio et al., 2008), ROC-COR (Deng et al., 2008; Greggio et al., 2008) and ROC-LRR (Greggio et al., 2008), suggesting that the association of two LRRK2 monomers is relatively strong. Interestingly, without the ROC-COR interaction LRRK2 dimer formation is not compromised, therefore it is possible that this interaction stabilizes the dimer but is not essential for dimer formation.

For different kinase proteins, autophosphorylation of well-conserved residues in the activation loop leads to conformational changes in this region and, consequently, to kinase activation and protein dimerization. It was hypothesized that LRRK2 kinase activity was modulated in a similar way. For this purpose, the capacity of dimeric and monomeric states of LRRK2 to phosphorylate itself and a generic substrate was investigated *in vitro*. It was demonstrated that only LRRK2 dimeric complexes show kinase activity (Sen et al., 2009). To date, it is not yet clear whether LRRK2 dimerization depends on kinase domain activation or *vice versa*. Moreover, it

was shown that phosphorylation of the kinase activation segment is not necessary for dimerization (Greggio et al., 2008), suggesting that the regulation of LRRK2 kinase activity differs from most other kinase proteins. It is worth considering that different groups have found that all LRRK2 kinase-dead mutants are unable to form dimers by Co-IP assay (Greggio et al., 2008) and BLUE native PAGE technique (Sen et al., 2009). Surprisingly, the elution profile of kinase-dead mutants corresponds to a high molecular weight (≈1.3MDa) complex by FPLC analysis (possibly due to aberrant interactions of LRRK2 monomers with other proteins) (Greggio et al., 2008 and Sen et al., 2009). All these pieces of data demonstrate the relationship between kinase activity and dimerization but leave unsolved the question of whether LRRK2 dimer formation depends on kinase domain activation or *vice versa*.

It was also shown that hydrolysable GTP, its non-hydrolysable analog GTPγS and GDP does not influence the capacity of LRRK2 to form dimers, suggesting that LRRK2 dimerization is not dependent on GTP binding and hydrolysis (Klein et al., 2009; Taymans et al., 2011).

Interestingly, James et al. demonstrated that LRRK2 localized diffusely throughout the cytosol predominantly exists as monomers, while membrane-associated LRRK2 forms dimers or tetramers (James et al., 2012). This data suggests the existence of a 'LRRK2 protein cycle': this protein remains in an inactive state in the cytosol until, in response to a specific stimulus, it is recruited to cellular membranes where it becomes active upon dimer formation.

1.4.3 LRRK2 pathological mutations

Mutations in the *LRRK2* gene are responsible for late-onset, autosomal dominant familial PD (Paisán-Ruiz et al., 2004; Zimprich et al., 2004) with clinical and neurochemical phenotypes mostly indistinguishable from the

idiopathic disease (Haugarvoll et al., 2006; Paisán-Ruiz et al., 2004; Zimprich et al., 2004).

To date, six missense mutations were identified in PD (figure 9): four within the ROC/GTPase domain (N1437H, R1441C/G/H), one within the COR domain (Y1699C) and two within the MAPKKK domain (G2019S and I2020T) (Brice 2005). Additional polymorphic variants were found: one within the COR domain (R1628P) (Tan et al., 2008; Ross et al., 2008) and one within the WD40 domain (G2385R) (Mata et al., 2005) (Figure 9).

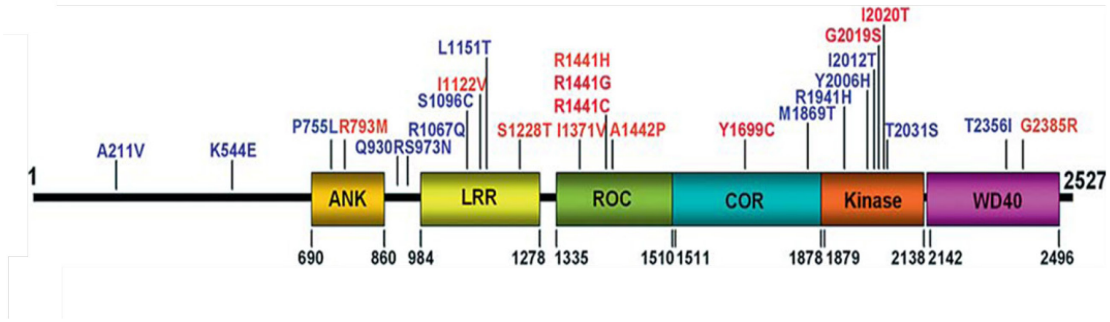


Figure 9. LRRK2 pathological mutants (red), polymorphisms considered as genetic risk factors (orange) and variants with uncertain pathogenicity (blue) (Giasson and Deerlin, 2008).

Among all LRRK2 pathological variants, G2019S is the most common and it is found in 3-41% of familial PD patients and 1-2% of sporadic cases with a high prevalence in North African families with a frequency of 41% (Lesage et al., 2005) and a lower prevalence in European populations with a frequency of 3-6% among familial cases (Goldwurm et al., 2005; Kachergus et al., 2005). Gly2019 is part of the highly conserved DF/Y**G** motif and this residue is localized at the N-terminus of the activation loop, the flexible region in the kinase domain responsible for controlling kinase activity. In most kinase proteins, the phosphorylation of specific amino acids within this loop causes a conformational change that allows access to the substrate. It is possible to hypothesize that the G2019→S substitution may allow the kinase loop to constitutively remain in an active state (Greggio

and Cookson, 2009). This idea is supported by the finding that analogous mutations to G2019→S in cancer are associated with hyperactive kinases and increased cell growth (Greenman et al., 2007).

I2020T, contiguous to G2019 residue, likely affects kinase activity but results are highly variable among different research groups (Gloeckner et al., 2006).

In 2004, the R1441C and R1441G mutations were first identified in several families (Paisàn-Ruiz et al., 2004; Zimprich et al., 2004). These mutations are associated with decreased GTPase activity, independently from GTP binding (Guo et al., 2007; Lewis et al., 2007; Li et al., 2007). Some groups found that Arg1441 stabilizes the ROC-COR domain interface by hydrophobic interactions, because this is the only residue in ROC domain that can form two hydrogen bondings with specific amino acids belonging to the opposite monomer (Deng et al., 2008; Gotthardt et al., 2008).

Y1699C substitution is localized in the COR domain and in *in vitro* systems is associated with reduced GTPase activity, independently from GTP binding, and alters the folding properties of LRRK2 (Deng et al., 2008; Daniëls et al., 2011). This suggests that this substitution destabilizes LRRK2 dimers and consequently impairs GTP hydrolysis.

There are also two polymorphic variants (G2385R and R1628P) found mostly in Asian populations that increase PD risk by approximately two-fold (Mata et al., 2005; Di Fonzo et al., 2006). The identification of risk variant carriers can be an opportunity to include these subjects in neuroprotective clinical trials for shedding light on LRRK2 physiological and pathological pathways.

1.4.4 LRRK2 cellular functions in physiological and pathological conditions

To date, LRRK2 has been implicated in different cellular pathways, including mitochondrial function, vesicular trafficking, cytoskeletal dynamics, protein aggregation, autophagy, neurite morphology and protein translation. It is known that microtubule (MT) dynamics and axon growth are mostly related to kinase function (Gillardon, 2009; MacLeod et al., 2006), whereas localization of LRRK2 to intracellular vesicles and membranous compartments are associated with LRRK2 kinase and GTPase activities (Conde and Caceres, 2009). Despite this information, to date, the LRRK2 molecular pathway that is critical for PD etiology or progression is still elusive.

Mitochondrial dysfunction

Mitochondria are the source of cellular ATP and they play a vital role in calcium homeostasis, ROS formation and apoptosis initiation. For this reason, mitochondrial function is critically important for cellular homeostasis. Moreover, cellular respiration has a critical role in brain function and its impairment is implicated in multiple neurodegenerative diseases, such as PD.

In brains of mice carrying human G2019S LRRK2 an abnormal accumulation of condensed and aggregated mitochondria was showed (Ramonet et al., 2011) implicating mitochondrial autophagy in LRRK2-related PD pathogenesis.

A significant reduction in ATP levels and mitochondrial membrane potential and altered mitochondrial morphology were identified in cultured patient-derived fibroblasts harboring the G2019S LRRK2 mutation, suggesting an impairment of mitochondrial function in PD cases (Mortiboys et al., 2010).

In rat primary neurons, LRRK2 overexpression correlates to mitochondrial fragmentation, ATP depletion and ROS accumulation through a Drp1-dependent mechanism (Wang et al., 2012).

Researchers hypothesized that mitochondrial impairment can be responsible for microtubule network integrity and autophagic-lysosomal pathway function in sporadic PD cases. According to this theory, bioenergetic defects derived from mitochondrial dysfunction would be responsible for microtubule network breakdown and, consequently, for impairments of vesicular trafficking, autophagosome maturation and autophagosome-lysosomal fusion (Figure 10). Unfortunately, to date, it is impossible to confirm that mitochondrial dysfunction is the primary pathogenic effect of LRRK2-related PD cases.

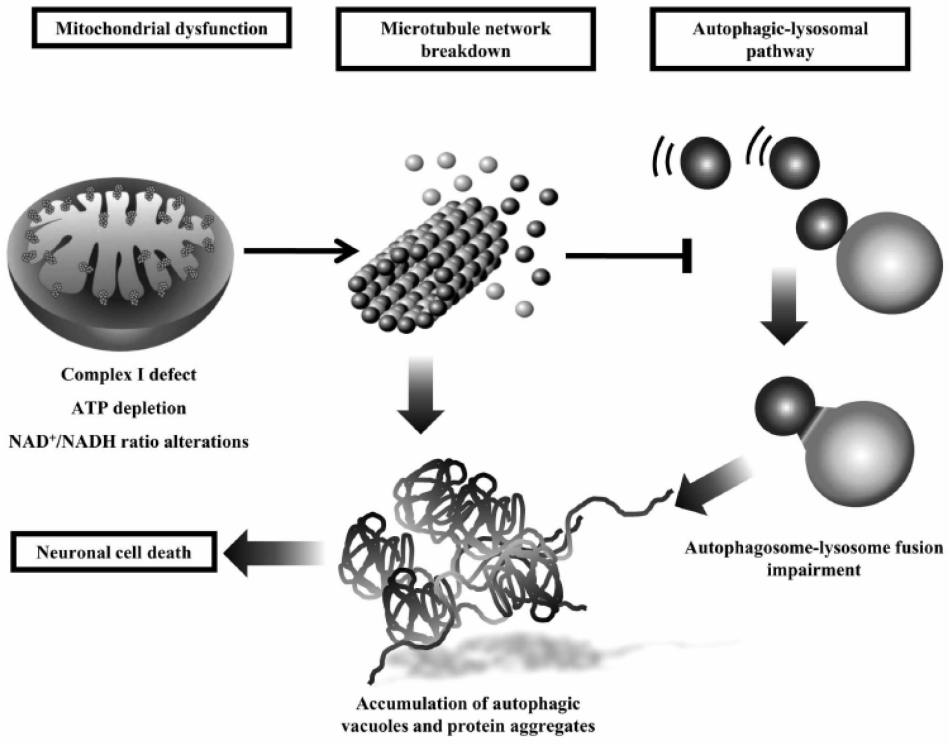


Figure 10. Relationship between mitochondrial impairment and alterations in microtubule dynamics and autophagic-lysosomal pathway in sporadic PD cases (Arduíno et al., 2010).

Synaptic vesicular trafficking

Synaptic dysfunction is one of the principal features of PD patients and severe neurotransmission defects were observed in different LRRK2 models. In fact, multiple studies showed that LRRK2 is involved in exocytosis and endocytosis, suggesting that this protein is part of the presynaptic complex (Shin et al., 2008; Xiong et al., 2010). For example, Matta et al. (2012) observed that LRRK2 affects synaptic endocytosis by phosphorylating Endophilin A (Endo A), a protein involved in the late step of this mechanism. G2019S LRRK2-mediated hyperphosphorylation of Endo A impaired synaptic endocytosis because increased Endo A phosphorylation reduces its presynaptic membrane affinity, leading to an accumulation of nascent uncoated vesicles and to an inhibition of DA reuptake. Matta et al. (2012) hypothesized the existence of a LRRK2-mediated phosphorylation-dephosphorylation Endo A cycle that modulates presynaptic endocytosis.

Moreover, given that LRRK2 can interact with different synaptic vesicle-related proteins, LRRK2 has been proposed to be involved in:

- ✓ anchoring synaptic vesicles to the cytoskeleton,
- ✓ neurotransmitter exocytosis,
- ✓ disassembling of SNARE complexes,
- ✓ endocytosis.

Moreover, several LRRK2 *in vivo* models showed defects in DAergic transmission. In fact, transgenic mice expressing LRRK2 pathological mutations present decreased DA release and re-uptake (Li et al., 2009; Li et al., 2010), impairment of D2 receptor function (Tong et al., 2009) and reduced striatal extracellular DA content compared to non-transgenic mice (Melrose et al., 2010). All these pieces of data taken together suggest a prominent role for LRRK2 in DAergic synaptic control.

Furthermore, the effects of LRRK2 on synaptic function as a primary neurodegenerative event still need to be reported. Moreover, the dysfunction of mitochondrial dynamics, vesicle trafficking or cytoskeletal dynamics could indirectly affect synaptic maintenance and functions as well.

Cytoskeletal dynamics

The cytoskeleton plays an important role in maintaining the structural polarity of neurons and, consequently, their function. Impaired cytoskeletal dynamics in dopaminergic neurons is one of the most important features of PD because a subtle interplay between MTs and MT-dependent motor proteins is necessary in these cells to transport dopamine to the synaptic bouton (Witte and Bradke, 2008; Bradke, and Dotti, 2000; McMurray, 2000).

Different studies verified that LRRK2 interacts with both α- and β-tubulin through its GTPase domain, albeit in a GTP-independent manner, and that can phosphorylate them (Gandhi et al., 2008; Gillardon 2009; MacLeod et al., 2006). LRRK2 mostly phosphorylates β-tubulin and, subsequently, regulates the MT network. In fact, *in vivo* studies showed that G2019S LRRK2 stabilizes MTs, promoting the accumulation of insoluble β-tubulin polymers (Gillardon, 2009). On the basis of this data it is possible to hypothesize that the balance in MT dynamics is critical for neuronal length, axonal trafficking and synaptic formation and that G2019S-related phosphorylation results in the deregulation of MT dynamics and, in turn, neuronal death.

Moreover, MTs and MT-axonal transport were reported to play a critical role in maintaining Golgi structure and integrity (Cole and Lippincott-Schwartz, 1995; Lane and Allan, 1998).

Protein inclusions

Brains of PD patients carrying LRRK2 pathological mutations show Lewy bodies and neurofibrillary tangles mostly in the neuronal soma (Giasson et al., 2006; Higashi et al., 2007).

In primary neurons LRRK2 promotes cytosolic protein accumulation in a kinase-dependent manner. In fact, in neurons, G2019S and I2020T mutants increase the formation of LRRK2-positive inclusion bodies containing hyperphosphorylated tau, swollen lysosomes and cytoskeletal components (MacLeod et al., 2006). Nonetheless, it is not yet understood if these inclusions are important for LRRK2-relatated neurodegeneration. Moreover, this protein does not seem to be a component of Lewy bodies or other protein inclusions (Biskup et al., 2006; Biskup et al., 2007; Higashi et al., 2007; Greggio et al., 2006; Higashi et al., 2009) but, to definitively exclude the absence of LRRK2 in Lewy bodies in brains of LRRK2-related PD patients, we must await the discovery of specific anti-LRRK2 antibodies. Given that LRRK2 pathological mutants correlate with protein aggregates, it is likely that LRRK2 could modulate protein accumulation, aggregation and degradation mechanisms, by regulating the ubiquitin-proteasome system (UPS), autophagy, chaperone proteins, endosomal and lysosomal pathways and MT-mediated transport.

Autophagy

Autophagy is the common neuronal downstream effect in some LRRK2 genetic models of PD, by participating in the clearance of protein aggregates and injured mitochondria. However, the potential consequences of autophagy dysregulation on synaptic structure and function remain unknown. Supporting this theory is the presence of components of the autophagy machinery localized to the synaptic bouton (Seichdenbecher et al., 2004).

LRRK2 is localized to autophagic vacuoles in human brains and in cultured cells and it seems to be directly or indirectly involved in autophagy regulation. In fact, G2019S LRRK2-induced neurite shortening is reverted by inhibition of autophagy components but enhanced by rapamycin, an autophagy activator (Plowey et al., 2008; artic24). Moreover, the G2019S mutation induces the accumulation of autophagic vacuoles in neuritic processes and soma of cultured neurons (Plowey and Chu, 2011). To date, it is not yet clear whether impaired autophagy is a primary neurodegenerative event in LRRK2-related PD or is a secondary consequence of other mechanisms.

Neurite morphology and branching

In neurons the cytoskeleton assumes an important role in maintaining neuronal shape, process length and function. To date, the mechanisms by which neuronal processes are extended and their length is regulated are still elusive. In fact, because of neuronal plasticity, even during the adulthood, neurons can repair cell damage, within a physiological range, by modulating cytoskeletal dynamics to re-extend injured processes and recover neuronal integrity.

To date, it was not demonstrated whether changes in axonal length occur both under physiological and pathological conditions, but it was verified that loss of dopaminergic axonal processes that extend from the SN to the striatum, precedes the loss of DAergic neuron cell bodies (Abeliovich and Beal, 2006).

This data suggests that neurite shortening or retraction might be an early feature of this neurodegenerative disease.

To verify if changes in axonal length are associated with LRRK2 expression, some groups measured axonal length using *in vitro* and *in vivo* LRRK2 models of PD. In the intact rodent CNS and neuronal cell lines,

overexpression of LRRK2 cDNA containing PD-associated mutations correlates with a phenotype of neurite injury and retraction that temporally precedes apoptotic cell death (MacLeod et al., 2006; Plowey et al., 2008; Smith et al., 2005; Smith et al., 2006). On the basis of this data, MacLeod et al. hypothesized that LRRK2 is involved in maintaining neurite length and complexity in the mammalian brain.

LRRK2-related neuronal injury is also accompanied by altered autophagy, which might contribute to neurite shortening (Plowey et al., 2008). WT LRRK2 overexpression in rat cortical neurons is unable to alter neuronal morphology and soma size. The overexpression of pathological variants (i.e. G2019S, I2020T and R1441G mutants) in cultured neurons leads to:

- ✓ a dramatic reduction in axonal length (G2019S, I2020T and R1441G mutants)
- ✓ a significant decrease in neurite branching (pathological mutations within the kinase domain only),
- ✓ tau-positive inclusions with lysosomal features (PD-related mutations within the kinase domain only)
- ✓ cell death, ~15 days after transfection (G2019S, I2020T and R1441G mutants) (MacLeod et al., 2006).

Suppression of LRRK2 overexpression by short hairpin RNA molecules (shRNAs) rescues the phenotype of reduced axonal length and branching in cultured neurons (MacLeod et al., 2006).

All these pieces of data taken together seem to show that LRRK2 protein has an important role in maintaining neuronal morphology and function.

Regulation of protein translation

It has been demonstrated that LRRK2 can control protein translation through 4E-BP1 phosphorylation (Imai et al., 2008) and interaction with the microRNA pathway in *Drosophila* (Gehrke et al., 2010).

Although the presence of all these exiting pieces of data from *in vitro* and *in vivo* LRRK2 PD models, its physiological functions in neurons are still unknown. Moreover, the molecular pathway whose impairment is the primary cause of LRRK2-induced neurodegeneration was not yet identified. Further studies are required to shed light on this question.

1.5 LRRK2: an example of kinase function autoregulated by GTPase activity?

It is known that LRRK2 GTPase and kinase domain sequences share high similarity with H-Ras and Raf-1 proteins, respectively. As kinase proteins are common effectors of Ras family members in the Ras/Raf/ERK transduction cascade, researchers hypothesized that LRRK2 might be part of a complicated molecular pathway and that its kinase activity may be a downstream effect of its GTPase activity. Scientists considered this hypothesis because:

- ✓ mutations in ROCO proteins are often associated with different diseases, suggesting that this protein family regulates multiple cellular processes;
- ✓ in *Dictyostelium*, the GTPase domain of guanosine monophosphate (cGMP)-binding protein (GbpC) autoregulates its kinase activity and this protein is involved in chemotaxis (Marín et al., 2008);
- ✓ in *D. melanogaster*, loss of the LRRK gene leads to abnormalities in locomotor activity and to a decreased number of dopaminergic neurons in the fly brain (Lee et al., 2007).

To better understand the molecular basis of LRRK2 enzymatic regulation, the H-Ras/Raf-1/ERK pathway is going to be discussed in the next section.

1.5.1 The GTP/GDP cycle in Ras proteins

Ras proteins belong to the small G-protein family and consist of three different isoforms (H-Ras, N-Ras and K-Ras) that are expressed in different cell types. These proteins contain a highly conserved P-loop for GTP binding (GAGGVGKS, 10-17 residues) and a switch II motif for guanosine nucleotide hydrolysis (DTAGQ, 57-61 residues).

The H-Ras/Raf-1/ERK pathway can be activated by cellular stress such as osmotic shock and ionizing radiation, cytokine stimulation and growth factors. Following stimulation, signals are relayed into the cell via a series of protein modifications that result in physiological cellular responses (i.e. cellular proliferation, cellular senescence, survival and differentiation) (Chung and Kondo, 2011) (Figure 11).

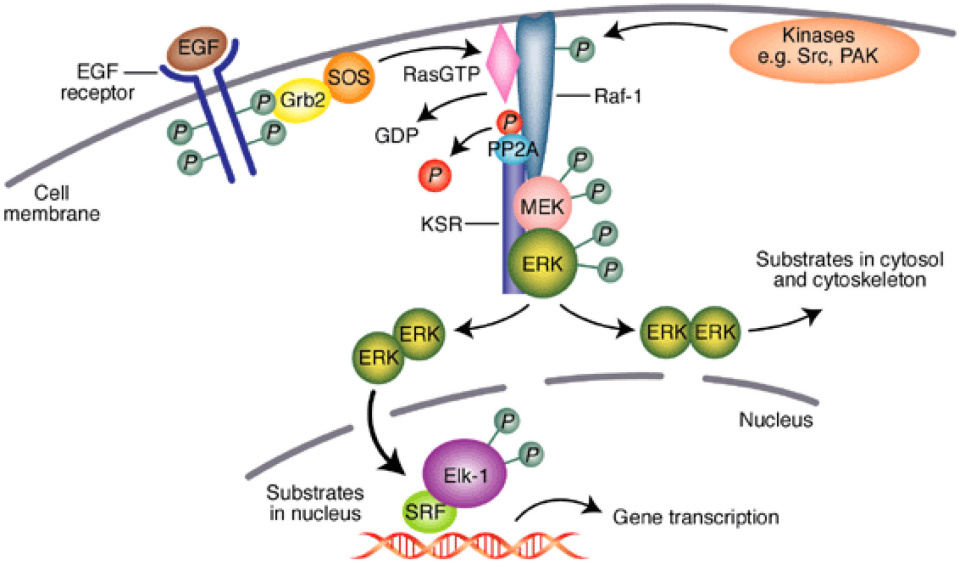


Figure 11. The Ras/Raf/ERK pathway.

The extracellular stimuli promote the dimerization of receptors tyrosine kinases (RTKs) and their kinase activity. The phosphorylation of tyrosine residues at the intracellular domain of RTKs provides docking sites for proteins containing Src homology (SH2) and phosphotyrosine binding (PTB) domains, such as the adaptor protein growth factor receptor-bound protein 2 (GRB2). Then, this adaptor protein recruits the son-of-sevenless (SOS) protein, a guanine-nucleotide-exchange factor (GEF) for the activation of the GTP/GDP cycle.

In the small G-protein Ras family, this cycle involves a GEF (guanine-nucleotide-exchange factor), a GAP (GTPase-activating-protein) and a GDI (guanine-nucleotide-dissociation inhibitor). GEFs facilitate the exchange of GDP with GTP and, consequently, activate Ras protein (GTP-

bound state). Conversely, GAPs increase the rate of Ras GTP hydrolysis and inactivate Ras protein (GDP-bound state). Finally, GDIs 'freeze' the protein in the inactive state, preventing GTP binding (Figure 12). In the GTP-bound conformation only, Ras is associated with the plasma membrane where, it can activate the extracellular-signal regulated kinase (ERK) and, consequently, the mitogen-activated protein kinases (MAPKs) cascade.

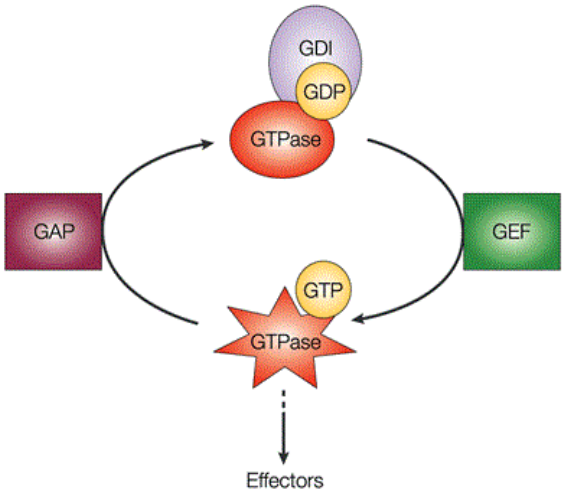


Figure 12. The GDP/GTP cycle in Ras proteins (Coleman et al., 2004).

Simultaneous to the Ras/Raf/ERK cascade and toward the same extracellular stimulus, Ras proteins may also activate the Ras/PI3K/AKT signaling pathway by the recruitment of different proteins to regulate cellular growth and survival.

1.5.2 Raf-1 kinase activity regulation

Raf-1 is the predominant effector of H-Ras proteins and is a serine/threonine kinase. In mammals there are three main isoforms of Raf protein: A-Raf, B-Raf and C-Raf (also called Raf-1), each one predominantly expressed in specific tissues. Raf-1, likely all Raf proteins, consists of a *N*-terminal regulatory domain and a *C*-terminal catalytic domain. The first one encompasses a region that binds GTP-loaded Ras (CR1) and a serine-rich region (CR2) that binds 14-3-3 when Ser259 and

Ser621 are both phosphorylated. The CR3 region of Raf-1 contains the kinase domain (Figure 13).

Similarly to other kinase proteins and within the catalytic domain, Raf-1 contains the well-conserved Lys376 for ATP binding and the sequence DFG (486-488 residues) at the *N*-terminus of the catalytic site for the phospho-transfer from ATP molecules to Ser or Thr residues of its substrate (Ito et al., 2007).

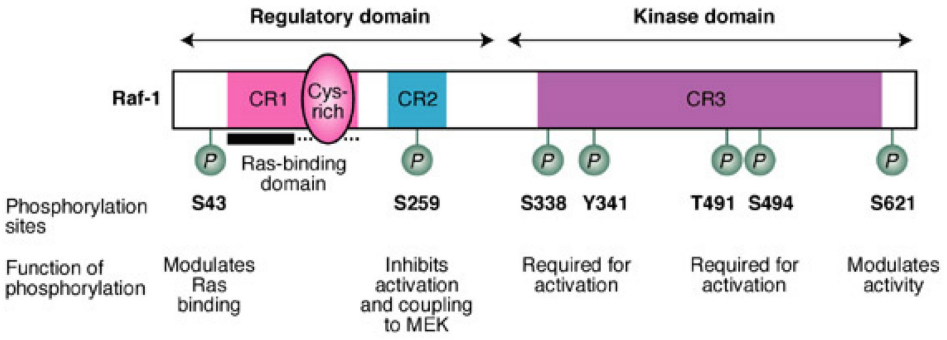


Figure 13. Raf-1 protein domains.

Raf kinase cycles between the inactive and the active state and *vice versa*. In quiescent cells, Raf-1 is blocked in the inactive conformation by 14-3-3 binding and localizes in the cytosol. In response to a specific stimulus, the interaction between GTP-bound Ras and Raf-1 is required for the displacement of 14-3-3 from the CR2 region, the translocation of Raf-1 to the plasma membrane and its dephosphorylation. Raf-1, to increase its kinase activity, forms heterodimers with B-Raf and then interacts with and phosphorylates its effector: the extracellular-signal-regulated kinase (ERK) (Figure 14). Activated ERK, in turn, phosphorylates cytosolic signaling proteins, including p90 ribosomal S6 kinase (RSK), MAPK-interacting serine/threonine kinase (MNK) and transcription factors, such as cAMP response element binding protein (CREB), c-Fos, c-Jun and mitogen-activated protein kinase/ERK kinase (MEK) to regulate cell fate, proliferation, differentiation, migration and apoptosis (Andreadi et al., 2011; Dhillon et al., 2007; Chung et al., 2011).

The temporal activation of this cascade is regulated by scaffolding proteins (Raf kinase inhibitor protein), phosphatases (PP1, PP2A and PP5) and various feedback pathways (Pearson et al., 2001; Rubinfeld et al., 2005; Jimenez et al., 2005) (Figure 14).

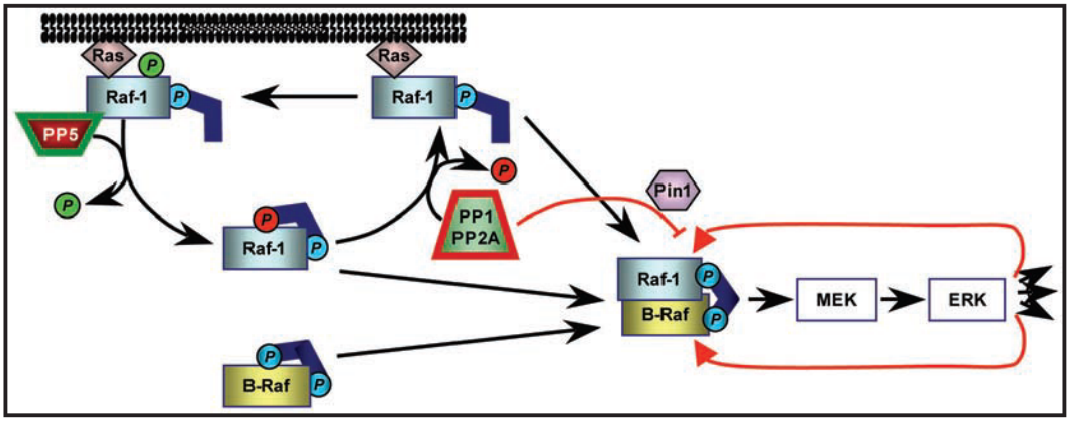


Figure 14. Model of Raf-1 regulation by phosphatases and heterodimerization (Dhillon et al., 2007).

1.5.3 Functional interplay between LRRK2 GTPase and kinase domains

LRRK2 is a dual enzymatic protein, having both GTPase and kinase activities and belonging to the ROCO family. As previously mentioned, the high degree of homology of LRRK2 with well-known and conserved proteins (H-Ras and C-Raf) raised some questions about the similarity between LRRK2 enzymatic regulation and the Ras/Raf pathway.

In this chapter, a functional comparison of LRRK2 with Ras and Raf biochemical properties is going to be discussed.

Different research groups observed that LRRK2 GTPase activity proceeds at a slower rate than in other small G-proteins (Ito et al., 2007), raising some questions about the possible causes of such a reduced enzymatic activity. The main hypothesis is that LRRK2 may possess a different GTP hydrolysis regulation compared to other small GTPases and there are several pieces of data in support of this notion.

First, it is noteworthy that there are some differences between Ras proteins and LRRK2 in the amino acid sequences. At position 1398 of the switch II region, LRRK2 possesses an arginine, critically required for GTP hydrolysis, whereas the equivalent residue in most other small GTPases is a glutamine (Ito et al., 2007). The equivalent position of R1398 is occupied by a proline in LRRK1 and by an aspartic acid in MFHAS1. Therefore it is possible that these substitutions are responsible for the decrease in the enzymatic activity of ROCO proteins even if, to date, the reasons are not well understood. Moreover, the Gln61Leu mutation decreases the GTP hydrolysis of H-Ras resulting in a constitutively active GTP-bound protein, conversely the equivalent Arg1398Leu variant increases LRRK2 GTP hydrolysis (Xiong et al., 2010; Stafa et al., 2012).

Second, the enzymatic activity of small G-proteins is regulated by GEFs and GAPs, whereas it was hypothesized that ROCO proteins do not require a GTPase domain activator since they have a high intrinsic GDP/GTP exchange rate and activate GTP hydrolysis through dimerization (Gasper et al., 2009). According to this theory LRRK2, as a putative member of GTPases activated by nucleotide-dependent dimerization (GAD), is postulated to follow a non-conventional mechanism for regulating GTP hydrolysis (Gasper et al., 2009) (Figure 15). In fact, the GTPase activity would require the contribution of specific residues in the catalytic site from each LRRK2 monomer to participate in catalysis or stabilize the dimer. According to this hypothesis, LRRK2 cycles between the inactive, monomeric GDP-bound state and the active, dimeric GTP-bound conformation, with the latter stable able to activate its kinase domain and biological functions. After GTP binding, GTP hydrolysis occurs and LRRK2 returns to an inactive form.

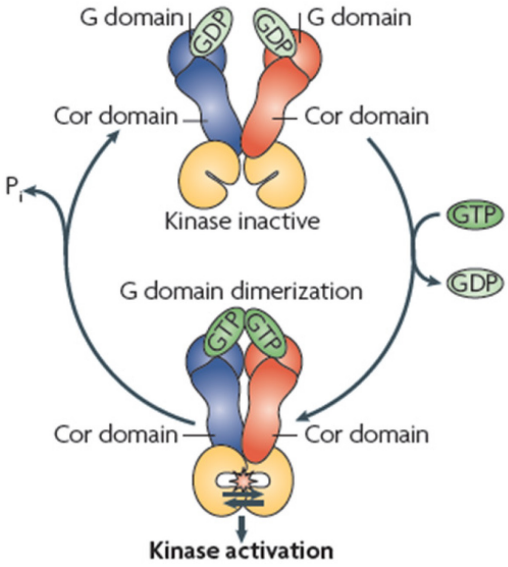


Figure 15. Model of LRRK2 enzymatic regulation according to GAD theory (Gasper et al., 2009).

This theory is supported by data coming from human LRRK2 and its homologues in *C. tepidum*. *Cis*-dimerization is crucial for GTP hydrolysis because pathological mutations that impair LRRK2 capacity to form dimers (R1441C, R1441Q, and Y1699C) are associated with reduced ROC enzymatic activity (Guo et al., 2007; Lewis et al., 2007; Li et al., 2007; Gotthardt et al., 2008; Deng et al., 2008; Daniëls et al., 2011). Moreover, mutations in *C. tepidum* of the residues that are analogous to those involved in LRRK2 dimer formation lead to impaired GTP hydrolysis, suggesting a relationship between dimeration and GTPase activity (Gotthardt et al., 2008).

Despite this, to date, there are no data demonstrating that LRRK2 is a GAD protein. In fact, this theoretical mechanism does not fit well for LRRK2 protein because it was demonstrated that there is no difference in its molecular weight in presence of GDP or GTP by Co-IP and FPLC analyses (Klein et al., 2009; Taymans et al., 2011).

Third, other research groups observed that ArfGAP1 is able to interact with LRRK2 *in vitro* and *in vivo* and increases its GTP hydrolysis (Stafa et al.,

2012; Xiong et al., 2012), suggesting a LRRK2 GTPase activity regulation that is incompatible with the GAD-like mechanism.

Like other members of the ROCO protein family, LRRK2 possesses a kinase domain that shares homology with Raf-1 (Ito et al., 2007). Since a regulatory kinase domain in ROCO proteins was not identified, they must require an alternative mechanism to modulate auto- and *trans*-phosphorylation activities. Several research groups detected LRRK2 kinase activity only in the homodimeric protein (Sen et al., 2009). This activation mechanism seems to be similar to the heterodimerization strategy used by Raf-1 to increase its kinase activity (Figure 16) (Dhillon et al., 2007). In this scenario, LRRK2 cycles between the monomeric, kinase inactive, GDP-bound state and the dimeric, kinase active, GTP-bound state in presence of specific GAPs and GEFs (Figure 16).

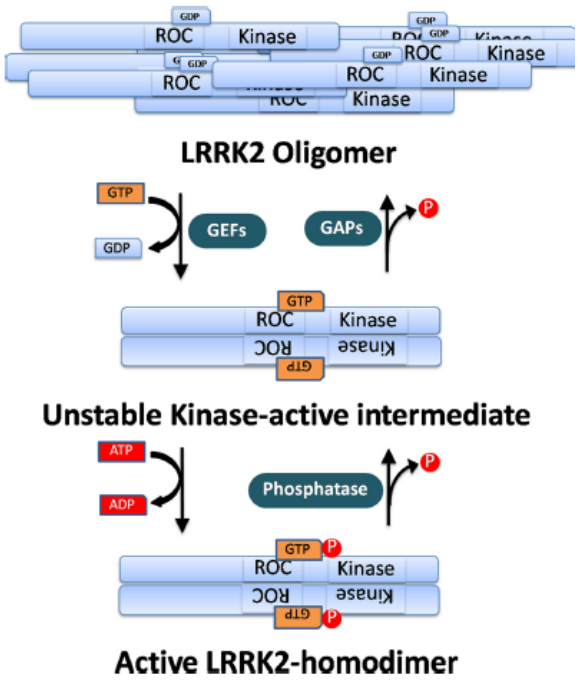


Figure 16. Model of LRRK2 kinase activation (Sen et al., 2009).

Multiple studies reported that LRRK2 kinase activity is the downstream effector of LRRK2 GTPase function, suggesting an activation model common to both the GAD mechanism and GTP/GDP Ras-like cycle. In fact, synthetic mutations in the ROC domain influence kinase activity.

First, deletion of the GTPase domain or impairment of GTP/GDP binding by Lys1347Ala and Thr1348Asn mutations impairs LRRK2 kinase activity (West et al., 2007; Ito et al., 2007).

Second, R1441C, R1441G and R1441H pathological mutations, which localize within the ROC domain and that are associated with impaired GTPase activity and normal GTP binding, present increased kinase activity (Guo et al., 2007; West et al., 2007; Webber et al., 2011). These pieces of data agree with the Ras/Raf activation pathway: oncogenic Ras, due to its reduced GTP hydrolysis, interacts longer *in trans* with Raf compared to WT Ras, causing increased kinase activity (Gasper et al., 2009).

Third, Deng et al. (2007) observed that the GTP hyperactive functional mutant R1398Q/T1343G is characterized by reduced kinase activity, suggesting that LRRK2 GDP-bound conformation is in an inactive state and does not promote LRRK2 auto- and *trans*-phosphorylation reactions.

Fourth, in a LRRK1-K745G mutant (corresponding to LRRK2-R1441G) kinase activity is reduced compared to LRRK1-WT (Korr et al., 2006), suggesting a different mechanism for regulating kinase activity by the ROC domain of LRRK1 compared to LRRK2.

Finally, LRRK2 pathological mutations in the kinase domain do not influence the GTP binding at the ROC domain (West et al., 2005), outlining the independence of GTP binding from kinase domain. In *Dictyostelium* the GTPase domain of GbpC is completely independent from other functional domains, albeit the molecular bases of the interplay between ROC and kinase domains are still unknown (Li et al., 2007).

Taymans et al. (2011) demonstrated that non-hydrolysable GTP analogs (GTPγS and GMPPCP) are able to stimulate WT-LRRK2 kinase activity in an indirect way, while GDP does not induce significant changes in kinase activity. This data suggests that LRRK2 kinase activation occurs by an

unknown activator protein when LRRK2 is in the GTP-bound conformation.

Regarding LRRK2 kinase activity, so far, no unambiguous LRRK2 substrates was identified and it is still not understood if this protein is also responsible for LRRK2 kinase activation. In this scenario, the predicted activator of LRRK2 kinase function should bind the ROC domain in a GTP-dependent manner, with a strong interaction with the GTP-bound state and a weaker interaction with the GDP-bound conformation and should be phosphorylated by LRRK2. The discovery of an authentic LRRK2 kinase substrate would be useful for understanding both LRRK2 biological function and molecular pathway.

On the other side, some researchers supposed that ROC domain activity is the downstream effector of LRRK2 kinase activity because autophosphorylation sites are localized within the ROC domain (Gloeckner et al., 2010; Greggio et al., 2009).

Taken together, these pieces of data demonstrate that LRRK2 is a complex protein characterized by an intramolecular interplay between ROC and kinase domains.To date, we are awaiting the discovery of a LRRK2 substrate to shed light on LRRK2 pathophysiological functions.

1.6 GTPase activity as therapeutic target of LRRK2-related PD

Since it was observed that a LRRK2 hyperactive kinase (i.e. G2019S) is able to generate neurotoxicity in cultured neurons (MacLeod et al., 2006), researchers hypothesized that targeting kinase activity might be a therapeutic strategy for familial LRRK2-related PD. Unfortunately, to date, no one kinase inhibitor is sufficiently selective against the LRRK2 protein: it is difficult to produce a specific kinase inhibitor because of the high similarity among kinase proteins. In fact, it was observed that LRRK2 kinase inhibitors can reduce the enzymatic activity of other kinase proteins,

leading to ambiguous results (Lee et al., 2010; Dzamko et al., 2010; Liu et al., 2011; Yun et al., 2011). It is worth noting that LRRK2 kinase-dead mutants always show both phosphorylation at Ser910 and Ser935 and 14-3-3 binding, whereas treatment with LRRK2 kinase inhibitors causes a loss of both of these biochemical properties, suggesting non-specific kinase inhibition.

Due to all these experimental issues, researchers became interested in the effects of modulating ROC domain activity and its effects on kinase function and neurotoxicity.

First, different lines of evidence showed that WT LRRK2 activates its kinase activity via the ROC domain, most likely in an indirect manner (West et al., 2005; Smith et al., 2006; Ito et al., 2007; Korr et al., 2006; Lewis et al., 2007). Moreover, Webber et al. (2011) showed that the R1441C pathological mutation has impaired GTP hydrolysis and increased kinase activity, suggesting that the LRRK2 GTP-bound conformation is associated with kinase activation.

Second, a functional relationship between GTPase activity and neurotoxicity was demonstrated. In fact, Xiong et al. (2010) observed that enhanced GTPase activity (i.e. R1398L and R1398Q/T1343G) partly reduces neuronal cell death normally induced by WT LRRK2. Moreover, Greggio et al. (2006) demonstrated that the R1441C pathological variant, which is a GTPase impaired mutant, provokes nuclei fragmentation and inclusion body formation in cultured neurons compared to WT LRRK2. All these pieces of data taken together suggest that the LRRK2 GTP-bound conformation is associated with enhanced kinase activity and, consequently, with the onset of LRRK2 PD-related phenotypes. On the basis of this experimental data, researchers anticipated that neurotoxicity associated with R1441C-LRRK2 could be rescued by enhancing GTP hydrolysis or decreasing GTP binding.

According to this hypothesis, Stafa et al. (2012) showed that ArfGAP1, as LRRK2 GAP, is able to increase WT-LRRK2 GTPase activity and autophosphorylation and that it has synergistic effect on WT LRRK2-induced neurite shortening. As demonstration of these exciting pieces of data, the knockdown of endogenous ArfGAP1 with shRNAs produced a complete rescue of WT and G2019S LRRK2-induced axonal shortening in cortical neurons. Therefore, it is likely that ArfGAP1, by enhancing LRRK2 GTPase activity increases its kinase activity and consequently causes neurotoxicity.

In contrast, other groups observed no changes in R1441C, R1441G and Y1699C kinase activity compared to WT, suggesting that the GTP-bound conformation is not associated with increased kinase activity (Kumar et al., 2010; Lewis et al., 2007; Stafa et al., 2012; MacLeod et al., 2006; Greggio et al., 2006; Greggio et al., 2007; Jaleel et al., 2007). Moreover, Xiong et al. (2012) showed that LRRK2 autophosphorylation level is reduced in presence of ArfGAP1 and that WT, R1441C and G2019S LRRK2-induced cell death can be rescued by ArfGAP1 in cortical neurons.

Because of this controversial data, the development of an efficacious and specific LRRK2 inhibitor/activator is difficult. Thus, it is necessary to obtain a better understanding of LRRK2 biochemical properties, regulatory mechanisms and dimerization process before planning a new PD therapeutic strategy. Moreover, another question is whether a LRRK2-specific drug will be efficacious for non-familial idiopathic PD cases.

2. GOALS AND OUTLINE

Although significant data supports a role for the *LRRK2* gene in Parkinson's disease, how mutations in this gene promote the initiation and progression of disease is unknown. Moreover, why LRRK2-related biological functions are so important in dopaminergic neurons is still an enigma, although recent findings demonstrated that LRRK2 is involved in synaptic vesicle endocytosis.

To date, the LRRK2 primary protein structure is known: it is a large protein composed of different domains and it shares high homology with members of Ras and Raf protein families. Since in the Ras/Raf/ERK transduction cascade kinase proteins are effectors of Ras GTP-bound proteins, it was hypothesized that LRRK2 kinase activity might be a downstream effector of its ROC domain activity.

To date, it is universally accepted that GTP binding is essential for LRRK2 kinase activation, but the role of GTP hydrolysis was not yet explored. For this purpose, a library of LRRK2 functional mutants was generated and enzymatic assays were performed to clarify the interplay between ROC and kinase domains.

Since it was observed that LRRK2 forms homodimers, our second goal was to explore the role of GTPase activity in regulating its dimerization.

Moreover, it was previously demonstrated that hyperactive kinase (i.e. G2019S pathological mutation) promotes inclusion formation, neuronal death and neurite shortening. Therefore, the third aim of this work was to study the contribution of GTPase activity to granules formation in HEK-293T cells and to axonal length in primary cortical neurons.

Only a comprehensive analysis of LRRK2 enzymatic activity and its neurotoxic effects will help us to identify new therapeutic strategies for PD.

The methods used to shed light on LRRK2 biochemical and cellular mechanisms are presented in chapter 2, whereas the results are shown in chapter 3. The final discussion is provided in chapter 4 and the conclusions are drawn in chapter 5.

3. MATERIALS AND METHODS

3.1 Animals

All animal experiments were approved by the SCAV (Service de la consommation et des affaires vétérinaires) in the Canton de Vaud and conducted in strict accordance with the European Union directive (2010/63/EU) for the care and use of laboratory animals. Animals were maintained in a pathogen-free barrier facility and exposed to a 12h light/dark cycle with food and water provided *ad libitum*. Pregnant female Sprague-Dawley rats were obtained from Charles River Laboratories (L'Arbresle Cedex, France) and resulting P0-P1 rats were used for preparation of post-natal primary cortical cultures.

3.2 Expression plasmids, proteins and antibodies

Mammalian expression plasmids containing 3xFLAG-tagged full-length human LRRK2 (WT and G2019S) in pcDNA™3.1(-) eukaryotic vector were kindly provided by Dr. Christopher Ross (Johns Hopkins University, Baltimore, USA) (Smith et al., 2005). MYC-tagged human WT LRRK2 plasmid was kindly provided by Dr. Ted M. Dawson (Johns Hopkins University) (West et al., 2005). As control plasmids, pEGFP-N1 was obtained from Clontech (Mountain View, CA, USA) and pcDNA3.1-MYC-his was obtained from Invitrogen (Basel, Switzerland). LRRKtide peptide (RLGRDKYKTLRQIRQ; 97.6% pure by HPLC analysis) was purchased from SignalChem (Richmond, Canada). GTP, GppCp, GDP and GDPβS at >95% purity by HPLC were purchased from Sigma-Aldrich (Buchs, Switzerland). 3xFLAG peptide was purchased from Sigma-Aldrich. The following antibodies were employed: mouse monoclonal anti-FLAG-(M2), anti-flag-(M2)-peroxidase and anti-β-tubulin (clone TUB 2.1), and rabbit polyclonal anti-βIII-tubulin (Sigma-Aldrich); anti-c-MYC (clone 9E10) and

anti-c-MYC-peroxidase (Roche Applied Science, Basel, Switzerland); mouse monoclonal anti-Hsp90 (BD Biosciences, Allschwil, Switzerland), peroxidase-coupled anti-mouse IgG (Jackson ImmunoResearch, Inc., West Grove, PA, USA), anti-mouse IgG coupled to AlexaFluor-488 or AlexaFluor-546 and anti-rabbit IgG coupled to AlexaFuor-633 (Invitrogen).

3.3 Oligonucleotides for mutagenesis

The mutagenic primers were designed with the following charactistics:

- ✓ length between 20 and 40 bases, melting temperature (Tm) of ≥78°C and purified by FPLC,
- ✓ minimum GC content of 40% and one or more C or G bases at the 3' terminus,
- ✓ desired mutations present in both complementary primers and in the middle (with ~10–20 bases of correct sequence on both sides).

The synthesized primers are listed in Table 2.

Mutation	Primer sequence
K1347A	Fw: 5' CACCGGCTCCGGCGCCGACCACTCTGCTCC 3' Rev: 5' GGAGCAGAGTGGTCGGCGCCGGAGCCGGTG 3'
T1348N	Fw: 5' CCGGCTCCGGCAAGAACACTCTGCTCCAGC 3' Rev: 5' GCTGGAGCAGAGTGTTCTTGCCGGAGCCGG 3'
T1343G	Fw: 5' GATGATCGTTGGCAACGGCGGCTCCGGCAAGACCAC 3' Rev: 5' GTGGTCTTGCCGGAGCCGCCGTTGCCAACGATCATC 3'
T1343V	Fw: 5' GATGATCGTTGGCAACGTCGGCTCCGGCAAGACCACAG 3' Rev: 5' CTGTGGTCTTGCCGGAGCCGACGTTGCCAACGATCATC 3'
R1398Q	Fw: 5' CTGGGATTTTGCTGGCCAGGAGGAGTTCTACAGCACGC 3' Rev: 5' GCGTGCTGTAGAACTCCTCCTGGCCAGCAAAATCCCAG 3'
R1398L	Fw: 5' CTGGGATTTTGCTGGCCTCGAGGAGTTCTACAGCAC 3' Rev: 5' GTGCTGTAGAACTCCTCGAGGCCAGCAAAATCCCAG 3'
D1994A	Fw: 5' CGCGATGATTATTTACCGCGCCCTGAAACCGCACAACGTG 3' Rev: 5' CACGTTGTGCGGTTTCAGGGCGCGGTAAATAATCATCGCG 3'
D1994N	Fw: 5' CGCGATGATTATTTACCGCAACCTGAAACCGCACAACGTG 3' Rev: 5' CACGTTGTGCGGTTTCAGGTTGCGGTAAATAATCATCGCG 3'
K1906M	Fw: 5' GGGCGAAGAAGTGGCTGTGATGATCTTTAACAAGCACAC 3' Rev: 5' GTGTGCTTGTTAAAGATCATCACAGCCACTTCTTCGCCC 3'

Table 2. Primers sequence: mutated codon is underlined, substituted nucleotides are in red.

3.4 Generation of LRRK2 functional mutants

Functional missense mutations were introduced in WT 3xFLAG-tagged LRRK2 by site-directed mutagenesis using the QuickChange II XL kit (Agilent Technologies, La Jolla, CA, USA). Mutations introduced are represented in Figure 17.

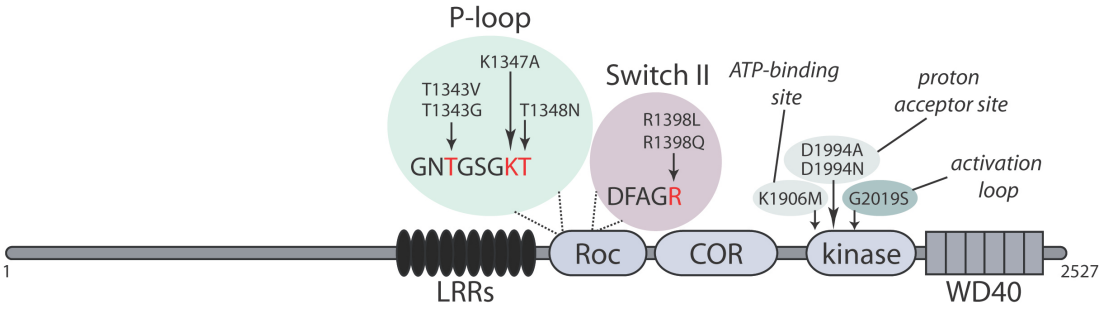


Figure 17. LRRK2 protein domains and functional mutants.

Mutagenesis reactions were prepared as indicated in Table 3 A and then subjected to the Polymerase Chain Reaction (PCR) program shown Table 3 B.

A

Volume/concentration	Product
5µl	10X reaction buffer
3µl	Quick solution reagent
20ng	WT 3xFLAG-LRRK2 plasmid
200ng	oligonucleotide forward
200ng	oligonucleotide reverse
0,5mM	dNTP mix
1µl (2,5U)	PfuUltra HF DNA polymerase
up to 50µl	ddH_2O

B

Cycle	Temperature (°C)	Time
1	92	1min
2-18	92	50sec
	60	50sec
	68	14min
19	68	7min

Table 3. Mutagenesis reaction (**A**) and PCR cycling parameters (**B**).

LRRK2 single mutants were generated using WT LRRK2 as DNA template, whereas double and triple variants were obtained from G2019S LRRK2, R1398L, R1398Q, GS2019S/R1398L and D1994A/R1398L variants.

The amplification product were subjected to *Dpn* I digestion (10U of enzyme) 1h at 37^0C, to digest parental DNA. Then, XL10-Gold® ultracompetent cells were transformed with 2µl of digested DNA following manufacturer's protocol (Agilent Technologies, La Jolla, CA, USA).
Finally, DNA was purified using NucleoSpin® Plasmid kit, according to manufacturer's protocol (MACHEREY-NAGEL, Oensingen, Switzerland) and desired mutations were confirmed by DNA sequencing.

3.5 Cell culture and transient transfection

HEK-293T cells were maintained in Dulbecco's modified Eagle's media supplemented with 10% fetal bovine serum and 1X penicillin/streptomycin at 37^0C and in a 5% CO_2 atmosphere. Cells were transfected with plasmid DNAs using X-tremeGENE HP DNA Transfection Reagent (Roche Applied Science) according to manufacturer's recommendations. Cells were harvested at 48-72h post-transfection for biochemical assays.

3.6 Co-immunoprecipitation assay and Western blotting

For co-immunoprecipitation (Co-IP) assays, HEK-293T cells were transiently transfected with 10µg of WT MYC-tagged LRRK2 and 3.3µg of flag-tagged LRRK2 in 10cm dishes and harvested after 48h in 1ml of IP buffer (10mM Tris-HCl pH 7.5, 150mM NaCl, 1% NP-40, 1X phosphatase inhibitor cocktail 2 and 3 [Sigma-Aldrich], 1X Complete Mini protease inhibitor cocktail [Roche Applied Sciences]). Cell lysates were rotated at 4°C for 1h and soluble fractions were obtained by centrifugation at 17,500g for 15min at 4°C. Soluble fractions were combined with 50µl Protein G-Dynabeads (Invitrogen) pre-incubated with anti-MYC (5µg; Roche Applied Sciences) antibody and incubated overnight at 4°C. Dynabeads complexes were washed five times with IP buffer supplemented with 450mM NaCl and twice with IP buffer. Immunoprecipitates (IPs) were eluted by heating at 70°C for 10min in Laemmli sample buffer (Bio-Rad AG, Reinach,

Switzerland) containing 5% 2-mercaptoethanol. IPs and inputs (1% total lysate) were resolved by SDS-PAGE, transferred to Protran nitrocellulose (0.2μm; Perkin Elmer, Schwerzenbach, Switzerland), and subjected to Western blot analysis with anti-flag-(M2)-peroxidase (1:5,000) and anti-MYC-peroxidase (1:2,000). Proteins were visualized by enhanced chemiluminescence (ECL; GE Healthcare, Glattbrugg, Switzerland) on a FujiFilm LAS-4000 Luminescent Image Analysis system. LabImage 1D software (Kapelan Bio-Imaging Solutions, Leipzig, Germany) was used for quantitation of protein levels by densitometry.

To assess the steady-state protein levels of LRRK2 mutants, HEK-293T cells were transfected with 3μg of flag-tagged LRRK2 (in 35mm dishes) and lysed in buffer A (1X PBS pH 7.5, 1% Triton X-100, 1X phosphatase inhibitor cocktail 2 and 3 [Sigma Aldrich], 1X Complete Mini Protease inhibitor cocktail [Roche Applied Science]) by rotating for 1h at 4°C. Clarified lysates were obtained by centrifugation at 17,500g for 15min at 4°C. The detergent-soluble supernatant fraction was quantified by BCA assay (Pierce Biotechnology, Rockford, IL, USA) and proteins (30μg) were resolved by SDS-PAGE and subjected to Western blot analysis with anti-flag-(M2)-peroxidase (1:5,000), mouse anti-β-tubulin (1:4,000) and peroxidase-coupled anti-mouse (1:40,000) antibodies.

3.7 Hsp90 binding

For assess Hsp90 binding HEK-293T cells were transiently transfected with 10μg of FLAG-tagged LRRK2 DNAs and lysated in IP buffer, as already described for the Co-IP experiments. Soluble fractions were combined with 50μl of Protein G-Dynabeads (Invitrogen) pre-incubated with mouse anti-FLAG (5μg; Sigma-Aldrich) antibody. Beads were washed three times with IP buffer supplemented with 450mM NaCl and twice with IP buffer. IPs were eluted as indicated in the previous paragraph, then IPs and lysates were resolved by SDS-PAGE and analyzed by western blot

using anti-flag-(M2)-peroxidase (1:5,000 for lysates and 1:20,000 for IPs), mouse monoclonal anti-Hsp90 (1:4,000 for lysates and 1:2,000 for IPs) and peroxidase-coupled anti-mouse (1:40,000) antibodies.

3.8 Immunocytochemistry and confocal microscopy

HEK-293T cells were seeded in 35mm dishes on glass coverslips coated with poly-D-lysine (20ng/ml; BD Biosciences) and mouse laminin (33μg/ml; Invitrogen) at a density of 80,000 cells/dish. Then, they were transfected with 2μg of FLAG-tagged LRRK2 plasmids. At 48h post-transfection, cells were fixed in 4% paraformaldehyde (PFA) and processed for immunocytochemistry with mouse anti-FLAG antibody and anti-mouse IgG-AlexaFluor-488 antibody, and stained with DAPI. Fluorescent images were acquired using a Zeiss LSM 700 inverted confocal microscope (Carl Zeiss AG, Feldbach, Switzerland) with a Plan-Apochromat 63x/1.40 oil objective in x, y and z planes. Images were subjected to deconvolution using HuygensPro software (Scientific Volume Imaging, Hilversum, Netherlands). Representative images are taken from a single z-plane at a thickness of 0.1μm.

3.9 Size-exclusion chromatography (SEC)

After transiently transfection with 10μg of FLAG-LRRK2 plasmids, HEK-293T cells were lysed in buffer B (20mM Tris-HCl pH 7.5, 150mM NaCl, 1mM EDTA, 0.5% Tween 20, 1X phosphatase inhibitor cocktail 2 and 3 [Sigma Aldrich], 1X Complete Mini Protease inhibitor cocktail [Roche Applied Science]). Cleared lysates (1ml) were incubated with anti-FLAG-M2-agarose beads by rotating overnight at 4°C. Resin complexes were washed with different buffers (twice with 20mM Tris-HCl, 500mM NaCl, 0.5% Tween 20; twice with 20mM Tris-HCl, 300mM NaCl, 0.5% Tween 20, twice with 20mM Tris-HCl, 150mM NaCl, 0.5% Tween 20, twice with 20mM Tris-HCl, 150mM NaCl, 0.1% Tween, and twice with 20mM Tris-

HCl, 150mM NaCl, 0.02% Tween 20) and LRRK2 proteins were eluted in elution buffer (20mM Tris-HCl pH 7.5, 150mM NaCl, 0.02% Tween 20 and 150μg/ml of 3xFLAG peptide) for 30min at 4°C with shaking. Eluted proteins were resolved by SDS-PAGE and stained with Coomassie G-250 to verify protein purity; LRRK2 content was calculated by densitometry using a standard curve with bovine serum albumin (BSA). Purified proteins containing equal amount of 3xFLAG-LRRK2 WT and functional mutants were injected into a AKTA Purifier FPLC system (GE Healthcare, Italy) and separated using a Superose 6 10/300 column (GE Healthcare, Italy), after equilibration with buffer C (20mM Tris-HCl pH 7.5, 150mM NaCl and 0.07% Tween 20). Pump flow rate was fixed at 0.5ml/min. Elution volumes of standards were 7.5ml for Blue Dextran (V_0), 12.37ml for thyroglobin (669 kDa), 14.21ml for ferritin (440 kDa) and 15.71ml for catalase (232 kDa). Each separated fraction obtained by FPLC analysis (1μl) was spotted on nitrocellulose membranes, incubated with anti-FLAG-peroxidase antibody (1:5,000) and subjected to detection with ECL.

3.10 GTP binding assay

HEK-293T were transiently transfected with 3μg of FLAG-tagged LRRK2 plasmids and lysed in buffer A (1X PBS pH-7.5, 1% Triton X-100, 1X phosphatase inhibitor cocktail 2 and 3 [Sigma Aldrich], 1X Complete Mini Protease inhibitor cocktail [Roche Applied Science]) by rotating for 1h at 4°C. Soluble fractions were incubated with 25μl of guanosine 5'-triphosphate-agarose (Sigma-Aldrich) by rotating for 2h at 4°C. Agarose beads were washed three times with buffer A and once with 1X PBS. GTP-bound fractions were eluted in Laemmli buffer containing 5% 2-mercaptoethanol and heating at 70°C for 10min. GTP-bound fractions and inputs lysates (1% total lysate) were resolved by SDS-PAGE and subjected to Western blot analysis with anti-FLAG-peroxidase antibody.

3.11 GTP hydrolysis assay

GTP hydrolysis assay was performed by measuring the release of free γ-phosphate (Pi) from GTP using the high sensitivity colorimetric GTPase assay kit (Innova Biosciences, Cambridge, UK). To establish time curves for LRRK2 GTPase activity, HEK-293T cells transiently expressing FLAG-tagged LRRK2 variants (WT, T1348N and R1398L) were lysed in phosphate-free lysis buffer (10mM Tris-HCl pH 7.5, 150mM NaCl, 1% NP-40, 1X Complete Mini Protease inhibitor cocktail [Roche Applied Science]) as described in the previous paragraph. Following centrifugation at 17,500g for 15min, supernatant fractions were subjected to immunoprecipitation (IP) with 2.5μg of anti-FLAG antibody pre-incubated with 25μl Protein G-Dynabeads (Invitrogen) by rotating at 4°C overnight. As control for protein contamination, mock-transfected HEK-293T cell lysates were subjected to IP with anti-FLAG antibody. Dynabeads were washed five times with lysis buffer supplemented with 450mM NaCl and once with 0.5M Tris-HCl pH 7.5, then resuspended in 100μl of 0.5M Tris-HCl pH 7.5, and finally subjected to GTP hydrolysis assays in 96-well plates in assay buffer containing 0.25mM GTP. Reactions were incubated for 30, 60, 90 and 120min at room temperature according to manufacturer's recommendations. Assay samples were measured for absorbance at 635nm and Pi concentration was determined from standard curves. The control absorbance value for the mock IP FLAG sample was deducted from the absorbance values obtained for each LRRK2 sample. FLAG IP samples (5μl total) were subjected to Western blotting with anti-FLAG-peroxidase antibody to confirm LRRK2 immunopurification. The levels of each IP LRRK2 variant was quantified by densitometry and used to normalize LRRK2-mediated Pi release in each experiment. Data was expressed as a percent of Pi release due to WT LRRK2 after 30min. Subsequent LRRK2 GTPase assays were conducted as described above with incubation for

120min at room temperature with GTPase activity (Pi release) expressed as a percent of WT LRRK2.

3.12 *In vitro* kinase assays

3.12.1 Autophosphorylation assay

For LRRK2 autophosphorylation, HEK-293T cells were plated in 10cm dishes, transfected with FLAG-tagged LRRK2 DNAs (15μg) then, lysed and subjected to IP with anti-FLAG-agarose. LRRK2 proteins were eluted with 3xFLAG peptide as described in SEC paragraph. After BSA standard curve, equal amount of LRRK2 WT and functional variants was used for kinase assay. Autophosphorylation reactions were performed in kinase buffer (25mM Tris-HCl pH 7.5, 5mM β-glycerophosphate, 2mM dithiothreitol, 0.1mM Na3VO4, 10mM MgCl2) in the presence of [^{33}P]-γ-ATP (2μCi/reaction; Perkin Elmer, MA, USA) and 5μM cold ATP (Sigma-Aldrich) at 30°C for 1h in a final volume of 25μl. The assays were terminated using 4X Laemmli buffer and by boiling at 95°C for 10 min. Autophophorylation reaction samples were resolved on 4-16% SDS-PAGE pre-cast gels (Invitrogen) and transferred to PVDF membranes. Incorporated radioactivity was detected by autoradiography and the same membranes were probed with anti-FLAG antibody (1:50,000) for 1h and 30min for protein loading control. Relative LRRK2 autophosphorylation was determined by densitometric analysis of ^{33}P autoradiograph signals for LRRK2 normalized to LRRK2 protein levels.

3.12.2 LRRKtide phosphorylation assay

To measure LRRKtide phosphorylation, HEK-293T cells were lysed and subjected to IP with anti-FLAG-agarose then, LRRK2 proteins were eluted with 3xFLAG peptide and quantified as described above. For treatment with GTP, GppCp, GDP and GDPβS, guanine nucleotides (200μM) were added to cell lysates immediately prior to incubation with anti-FLAG-

agarose and followed by rotation overnight at 4°C. For LRRKtide phosphorylation assay, LRRKtide peptide (400μM) was added to standard kinase reactions, as described above. Reaction samples were terminated by addition of 8μl 0.5M EDTA and samples were applied to P81 phosphocellulose paper (Whatman, Opfikon, Switzerland). P81 squares were dried, washed three times with 75mM phosphoric acid (Sigma-Aldrich) and incorporated ^{33}P into LRRKtide peptide was detected by scintillation counting. LRRKtide phosphorylation levels were normalized to the levels of LRRK2 protein determined by Western blotting and densitometry.

3.13 Primary neuronal cultures and neurite length assays

3.13.1 Primary neuronal cultures and transient transfection

Primary cortical neurons were prepared from Sprague-Dawley P0-P1 rats by stereoscopically isolating the cerebral cortices and dissociation by digestion in media containing papain (20U/ml, Sigma-Aldrich) and mechanical trituration. Cells were plated in 35mm dishes on glass coverslips coated with poly-D-lysine (20ng/ml; BD Biosciences) and mouse laminin (33μg/ml; Invitrogen) and cultured in Neurobasal media containing B27 supplement (2% w/v), L-glutamine (500μM) and penicillin/streptomycin (100U/ml) (Invitrogen).

To check LRRK2 expression levels in cortical neurons, cells were transfected at DIV3 with 4.5 μg of FLAG-tagged LRRK2 plasmids and lysed at DIV7, as already described in the 'cell colture and transfection' paragraph.

3.13.2 Neurite length assay

Primary cortical neurons were co-transfected at DIV3 with FLAG-tagged LRRK2 and GFP plasmids at a 10:1 DNA molar ratio (4μg total DNA per 35mm dish) using Lipofectamine 2000 reagent (Invitrogen). At DIV7,

cultures were fixed with 4% PFA and processed for immunocytochemistry using mouse anti-FLAG antibody (Sigma-Aldrich), rabbit anti-βIII-tubulin (Sigma-Aldrich), anti-mouse IgG-AlexaFluor-546 and anti-rabbit IgG-AlexaFluor-633 antibodies (Invitrogen). Fluorescent images were acquired using an EVOS inverted fluorescence digital microscope (Advanced Microscopy Group, Bothell, WA, USA) with a 10X objective. GFP images were pseudo-colored with ICA1 in NIH ImageJ software to improve the contrast of neuritic processes, and used for neurite length measurements. The length of GFP-positive axonal processes from FLAG-positive cortical neurons (β III-tubulin-positive) was measured using the line tool function of ImageJ software by an investigator blinded to each condition. Only neurons that had extended neurites were measured whereas neurons without processes were excluded from the analysis. For each experiment, axonal processes from 30 GFP-positive neurons randomly sampled across five coverslips from at least three independent experiments were measured.

3.14 Statistical analysis

Data was analyzed by one-way ANOVA with Newman-Keuls multiple comparison test or Bonferroni post-test. $P<0.05$ was considered significant. Fitting curves of SEC fractions were obtained by Gaussian regression fit analysis.

4. RESULTS

4.1 Generation of LRRK2 functional mutants

To study the interplay between LRRK2 GTPase and kinase domains, a library of 3xFLAG-LRRK2 functional mutants was created. All developed mutations are located within LRRK2 ROC and kinase domains and have been generated by the substitution of amino acids analogous to well conserved residues in members of the Ras and Raf families (Ito et al., 2007) (Figure 18). The effects of these functional mutations in Ras and Raf proteins are known, but they have not yet been completely explored in LRRK2.

In summary, the LRRK2 mutant library includes:

- ✓ P-loop variants with decreased (K1347A, T1348N, G2019S/T1348N) (West et al., 2007) or unclear (T1343G) GTP binding;
- ✓ Switch II variants with predicted altered GTP hydrolysis (R1398L, R1398L/T1343V, R1398Q/T1343G) (Ito et al., 2007; Stafa et al., 2012; Xiong et al., 2012);
- ✓ Kinase-dead mutants (K1906M, D1994A, D1994N, G2019S/K1906M, G2019S/D1994A and G2019S/D1994N) and a kinase-enhanced variant (G2019S) (Smith et al., 2006; West et al., 2007; Jaleel et al., 2007; Greggio et al., 2006);
- ✓ Double mutants including substitutions into both ROC and kinase domains (D1994A/R1398L and G2019S/R1398L) to explore the interplay between GTP hydrolysis and kinase activity;
- ✓ Triple mutants including substitutions in both ROC and kinase domains (D1994A/R1398L/T1343V and

G2019S/R1398L/T1343V) to explore the interplay between ROC and kinase domains.

Before conducting assays to measure LRRK2 GTPase and kinase activities, the steady-state level of each mutant has been evaluated in HEK-293FT cells transiently transfected with each DNA construct (Figure 18). Most of the functional mutants show equal steady-state levels compared to WT LRRK2 apart from K1347A and T1348N, which exhibit significantly lower expression levels than WT. This data suggests that impairment of GTP binding might have effects upon protein translation or stability.

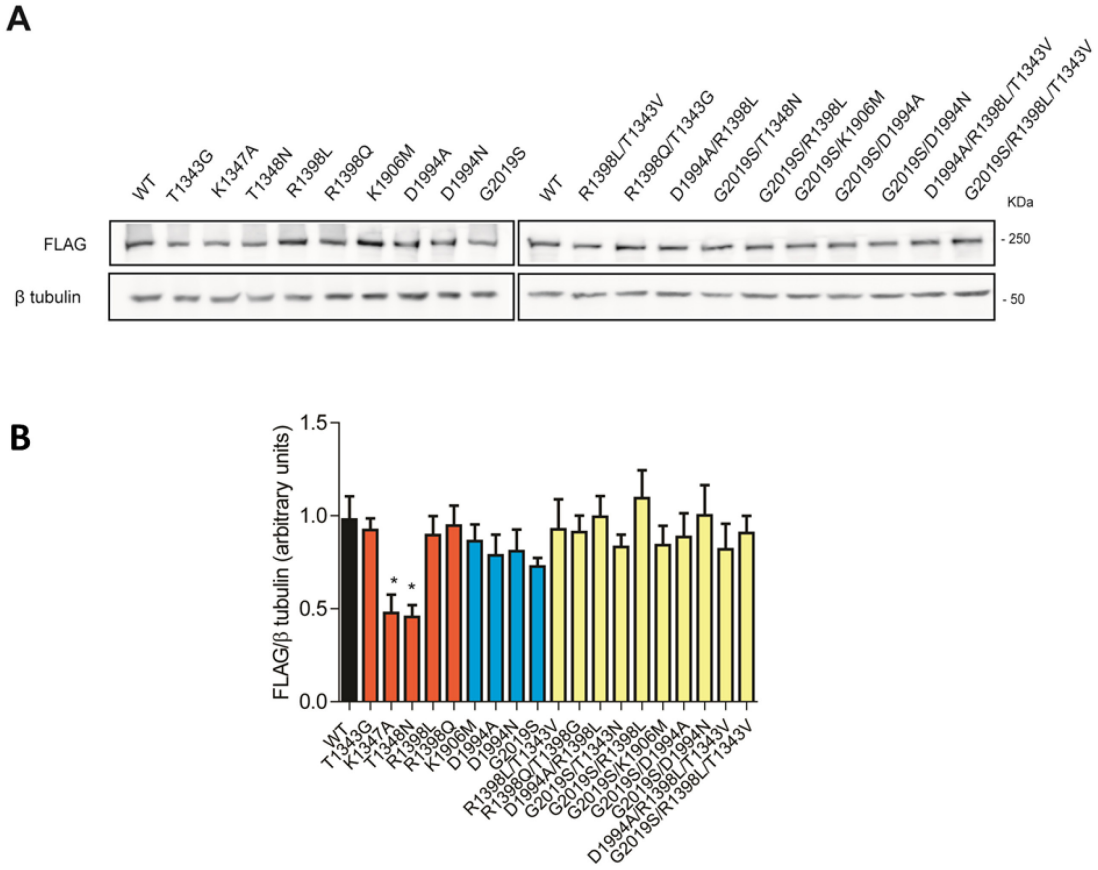


Figure 18. Western blotting showing LRRK2 functional mutants steady-state level (**A**). Quantification of LRRK2 expression levels after densitometric analysis and normalization to β tubulin signal (**B**).

4.2 LRRK2 ROC domain properties

After having verified the steady-state levels of LRRK2 variants, GTP binding and hydrolysis capacities have been assayed using well established methods (West et al., 2007; Stafa et al., 2012; Xiong et al., 2012).

GTP binding experiments were conducted in HEK-293FT cells by using a GTP-agarose resin to pull-down LRRK2 variants and to quantify the ratio between the LRRK2 GTP-bound fraction and relative input (i.e. LRRK2 expression in total lysate).

K1347A, T1348N and G2019S/T1348N P-loop mutants significantly impair GTP binding, as previously reported (West et al., 2007) (Figure 19 A and B). Single and double switch II region variants (i.e. R1398L, R1398Q, R1398L/T1343V and R1398Q/T1343G) present normal GTP binding (Figure 19). Kinase hyperactive mutants (i.e. G2019S and G2019S/R1398L) and kinase-dead variants (i.e. K1906M, D1994A and D1994N) do not show any difference in GTP binding compare to WT, suggesting that GTP binding is independent from kinase function (Figure 19). Surprisingly, kinase-dead D1994A/R1398L and D1994A/R1398L/T1343V mutants exhibit a markedly reduced GTP binding compared to WT, although single kinase-inactive variants do not have a significantly impaired GTP binding (Figure 19).

Before exploring the effects of ROC and kinase domain mutations on GTP hydrolysis, the time-enzymatic activity curve has been studied using WT, T1348N (as negative control) and R1398L (as positive control) mutants. WT and R1398L exhibit a time-dependent increase in their enzymatic activity. Among all tested time points, a period of 120 minutes allowed us to obtain the larger difference between WT- and T1348N-induced γ-phosphate (Pi) release from GTP (Figure 20 A).

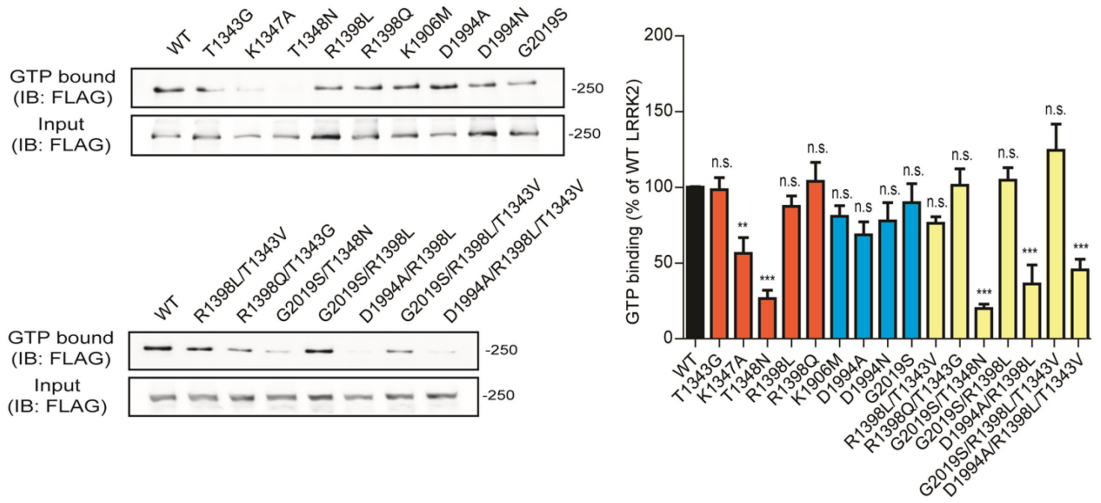


Figure 19. Western blotting showing LRRK2 GTP-bound fractions (*upper panel*) and input levels (*lower panel*). On the right, densitometric analysis of GTP binding capacity expressed as percentage of WT LRRK2.

Then, GTP hydrolysis capacity has been explored in all functional mutants. K1347A, T1348N and G2019S/T1348N show impaired GTP hydrolysis, which is consistent with their reduced GTP binding. The R1398L switch II region mutant exhibits an enhanced GTPase activity compared to WT, as reported previously (Stafa et al., 2012); while the R1398L/T1343V double mutant shows reduced GTP hydrolysis (GTP-locked variant) (Figure 20 B). Kinase function does not seem to influence GTP hydrolysis, in fact kinase-dead (i.e. K1906M, D1994A and D1994N) and kinase hyperactive (i.e. G2019S) mutants exhibit a normal GTPase function, compared to WT (Figure 20 B).

G2019S/R1398L variant exhibits the same enhanced GTP hydrolysis as the R1398L mutant alone, while G2019S/R1398L/T1343V loses R1398L/T1343V GTP locked properties (Figure 20 B). Kinase-dead D1994A/R1398L and D1994A/R1398L/T1343V mutants have a markedly reduced GTPase activity, consistent with their impaired GTP binding, making a comparison among WT, D1994A/R1398L and D1994A/R1398L/T1343V GTPase activities using this data difficult (Figure 20 B). In summary, single kinase domain mutations (i.e. K1906M,

D1994A, D1994N and G2019S) do not alter LRRK2 GTPase activity. Unexpectedly, increased kinase activity (i.e. G2019S mutation) enhances R1398L/T1343V GTP hydrolysis capacity up to the level of WT LRRK2.

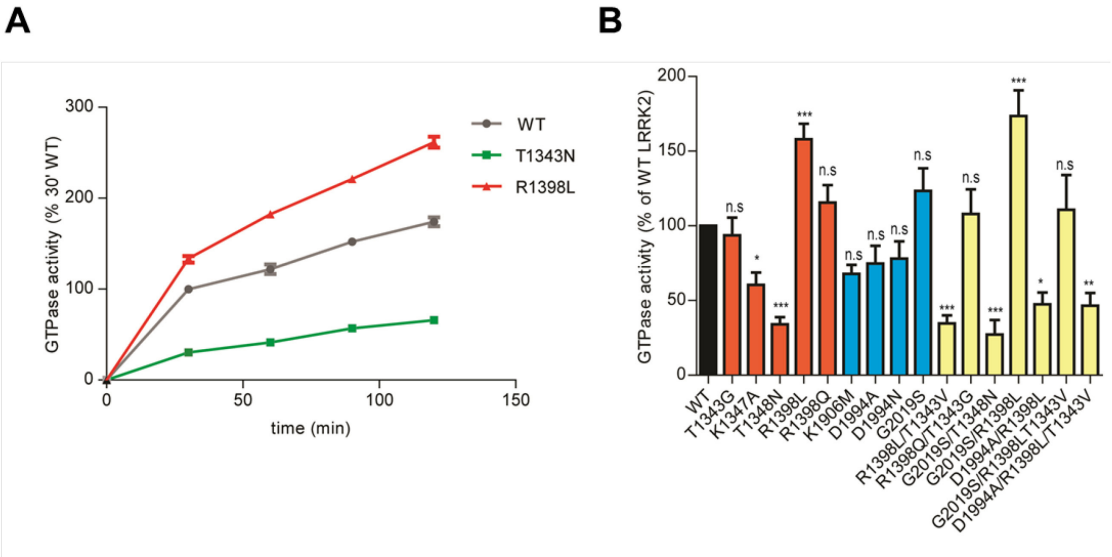


Figure 20. Time-GTPase activity curve (**A**) and LRRK2 GTP hydrolysis assay (**B**).

4.3 LRRK2 kinase activity is dependent on ROC domain

To explore the contribution of the LRRK2 ROC domain to kinase activity, *in vitro* radioactive autophosphorylation and LRRKtide-phosphorylation assays were performed on immunopurified LRRK2 protein.

Data from LRRK2 autophosphorylation assays shows that G2019S is a hyperactive kinase and that D1994A and D1994N are kinase-inactive, as previously reported in literature (Ito et al., 2007; West et al., 2007) (Figure 21 A and B). Moreover, G2019S/D1994A, G2019S/D1994N and D1994A/R1398L/T1343V mutants are kinase-dead as well, suggesting that the D1994A/N mutation markedly impairs G2019S-enhanced kinase activity (Figure 21 A and B). It is worth noting that GTP binding-deficient mutants (i.e. K1343A and T1348N) are kinase-inactive, as previously published (West et al., 2007), suggesting that the P-loop motif is critically required for kinase activity (Figure 21 A and B). R1398L (hyperactive GTPase) and R1398L/T1343V (GTP-locked variant) possess an impaired

kinase activity (Figure 21 A and B), albeit they have opposite effects on GTP hydrolysis.

G2019S/R1398L and G2019S/R1398L/T1343V mutants exhibit enhanced kinase activity, similar to G2019S alone, suggesting that addition of mutations localized in the switch II region (i.e. R1398L and R1398L/T1343V) to the LRRK2 G2019S pathological variant is not able to reduce G2019S kinase activity (Figure 21 A and B).

After having explored LRRK2 autophosphorylation capacity, LRRK2 kinase activity was studied by using the LRRK2 pseudosubstrate peptide, LRRKtide. Data obtained from LRRKtide phosphorylation assays mostly conforms to the autophosphorylation results. LRRKtide phosphorylation assays show that K1906M and G2019S/K1906M, which were not included in the previous assay, are kinase-dead mutants (Figure 21 C).

Different from the autophosphorylation data, R1398L kinase function is no longer significantly impaired. Moreover, like the autophosphorylation results, there is no difference among the kinase activity of G2019S, G2019S/R1398L and G2019S/R1398L/T1343V mutants, suggesting that addition of mutations that modulate GTPase activity is not sufficient to influence G2019S kinase activity (Figure 21 C). There are several possible reasons to explain this observation. First, it is worth noting that G2019S/R1398L/T1343V exhibits normal GTPase activity and has lost the GTP-locked properties of the R1398L/T1343V mutant which might explain why the addition of the R1398L/T1343V variant in the context of LRRK2 G2019S cannot significantly reduce G2019S kinase activity. Second, the effects of the G2019S variant upon kinase function might override the effects of switch II region mutations on kinase activity.

In summary, all these results taken together suggest that an intact P-loop motif is indispensable for kinase function and that GTP hydrolysis can modestly modulate LRRK2 kinase activity. It is important to emphasize

that the enhanced kinase function of G2019S is not influenced by GTPase activity.

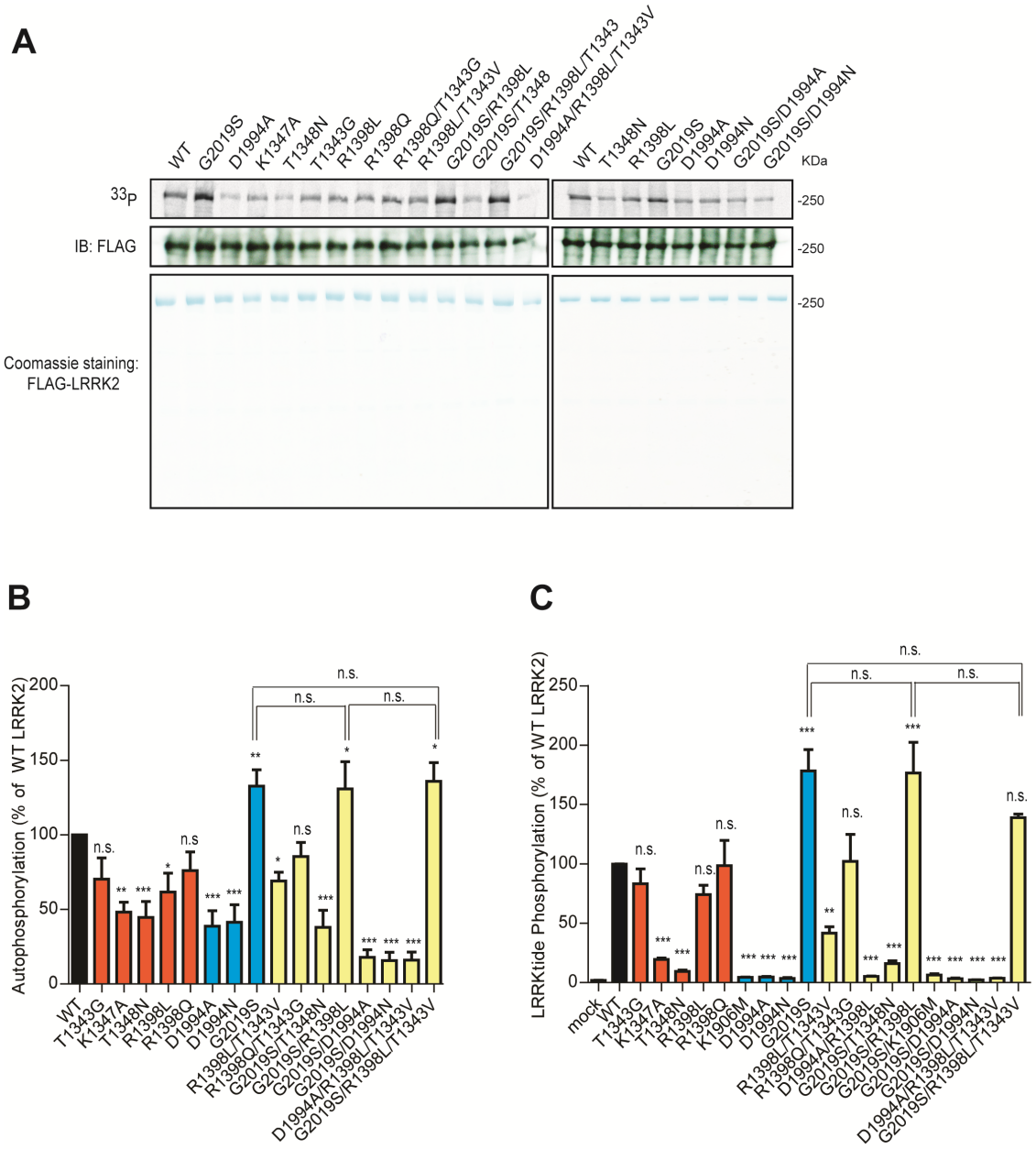


Figure 21. *In vitro* radioactive kinase autophosphorylation assay (**A**): Coomassie G-250 staining of immunopurified LRRK2 (*lower panel*), immunoblot (*middle panel*) and autoradiogram (*upper panel*) of autophosphorylated LRRK2, as analyzed by SDS-PAGE and western blotting. Graph showing radioactivity incorporated into LRRK2 protein (**B**) and into LRRKtide (**C**) after normalization to LRRK2 levels.

4.4 LRRK2 GTP-dependent kinase activation

Data from autophosphorylation and LRRKtide-phosphorylation assays suggests that:

- ✓ kinase activation is completely dependent on GTP binding capacity;
- ✓ kinase function can be modulated by GTP hydrolysis, except for the G2019S variant kinase activity.

Previous experiments (Taymans et al., 2011) have demonstrated that LRRK2 kinase activity can be increased by GTP binding, i.e. by adding non-hydrolysable guanosine nucleotides (GppCp and GTPγS) to cell extracts prior to LRRK2 immunopurification. Conversely, the contribution of GTP hydrolysis to kinase activation has not been explored until now. Therefore, to verify the contribution of GTP binding and hydrolysis to kinase activity, LRRKtide phosphorylation assays were performed with GTP, its non-hydrolysable analog (GppCp), GDP and its non-hydrolysable analog (GDPβS), which are considered to have similar GTP/GDP affinity constants (Ito et al., 2007; Liu et al., 2010; Taymans et al., 2011).

The study was based on WT and mutated LRRK2 proteins (T1348N, R1398L, R1398L/T1343V and G2019S). WT LRRK2 kinase activity is enhanced by both GTP and its non-hydrolysable analog GppCp (Figure 22); conversely, the T1348N (a GTP binding-deficient) mutant does not respond to guanosine nucleotide treatment. Moreover, GTP is able to increase LRRK2 kinase activity to a greater extent than GppCp, suggesting that also GTP hydrolysis positively modulates kinase function (Figure 22). For the R1398L/T1343V variant, GTP and GppCp induce the same increase in kinase activity, consistent with its impaired GTP hydrolysis. It was further noticed that GDP, but not GDPβS, significantly decreases WT and G2019S kinase activity (Figure 22).

Autophosphorylation and LRRKtide phosphorylation data indicates that GTP binding first is required for kinase function and that, further, GTP hydrolysis completes LRRK2 kinase activation.

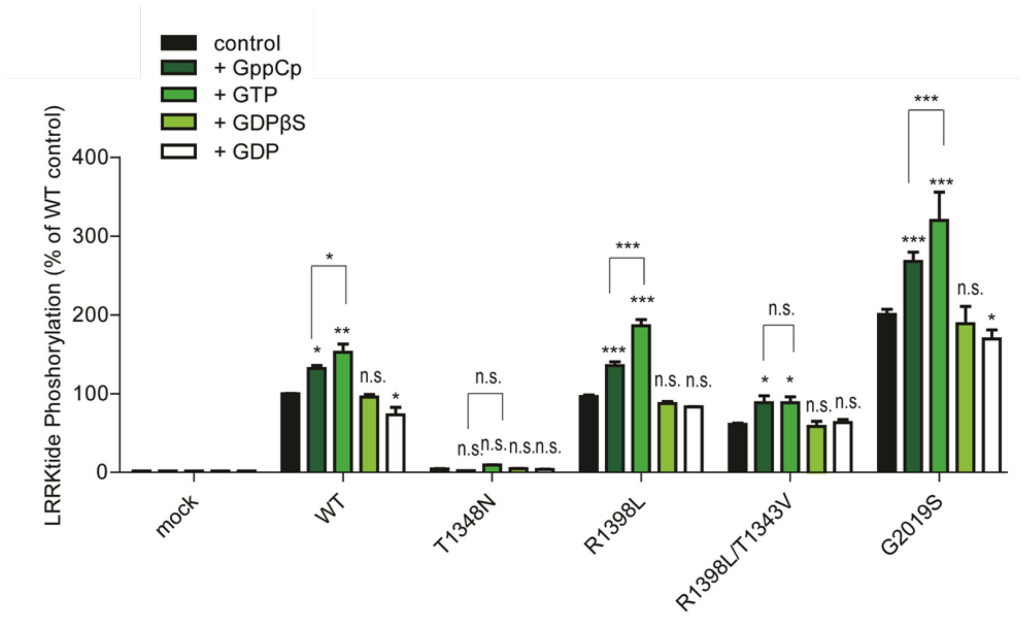


Figure 22. Effects of guanosine tri- and di-phosphate on LRRK2 kinase activity.

4.5 LRRK2 dimer formation and protein stability are not influenced by GTPase activity

It was demonstrated that LRRK2 forms homodimers and that this process is required for kinase activity, similar to other protein kinases (Berger et al., 2010; Greggio et al., 2008; Sen et al., 2009). To date, the regulation of GTPase activity by dimerization and *vice versa* have not been demonstrated.

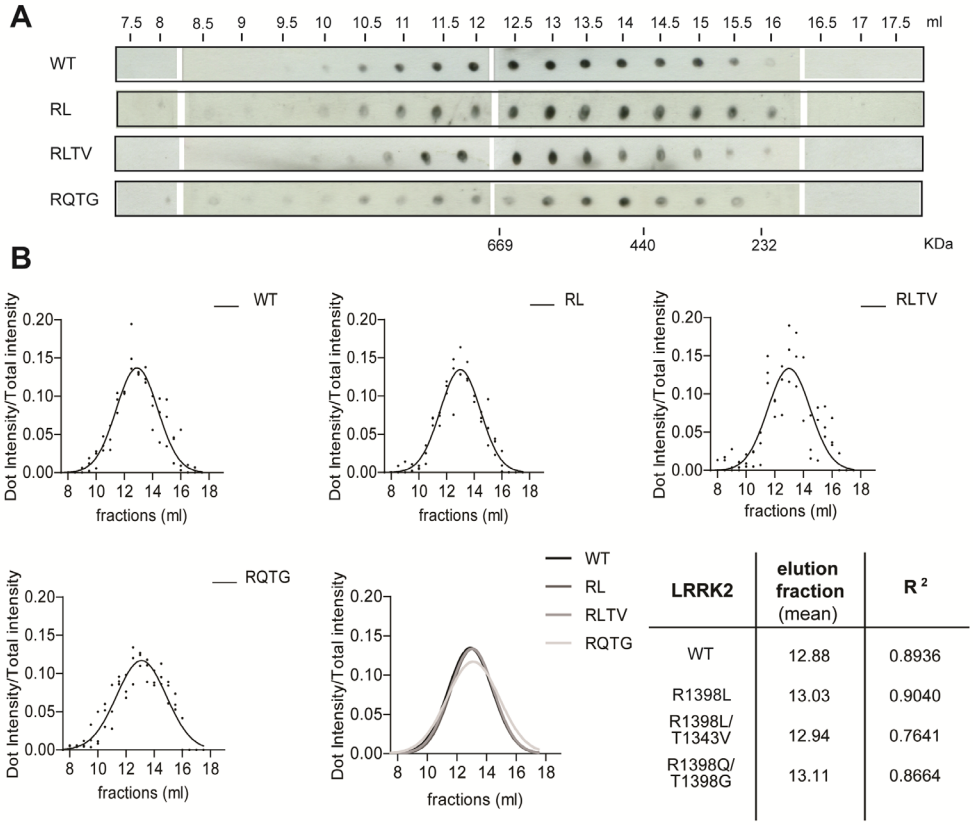
To understand whether a correlation between GTPase activity and dimerization exists, LRRK2 dimer formation of WT, R1398L, R1398L/T1343V and R1398Q/T1343G LRRK2 mutants was analyzed by FPLC and Co-IP approaches.

To study homodimers and native complex formation by mutations that alter GTPase activity, immunopurified LRRK2 proteins from HEK-293T cells were analyzed by size-exclusion chromatography. Data indicates that GTPase activity does not influence LRRK2 dimerization; in fact all tested LRRK2 variants elute within a relative large range of elution volumes, with

a peak centered at ≈13ml (Figure 23 A and B). Moreover, results show that LRRK2 native complexes possess a molecular weight within a range of 440-669 kDa, suggesting that they might include LRRK2 homodimers in addition to different LRRK2 interacting partners (i.e. Hsp90, 14-3-3, etc.).

We used a Co-IP approach to confirm that GTPase activity does not influence LRRK2 dimerization. The capacity of immunoprecipitated MYC-tagged WT LRRK2 to form heterodimers with 3xFLAG-tagged LRRK2 mutants was assessed and Co-IP data confirmed the FPLC results (Figure 24 A). Co-IP experiments also indicate that kinase-dead mutants (D1994A and G2019S/D1994A) have an impaired capacity to dimerize, as already reported (Sen et al., 2009), similar to GTP binding-deficient variants (K1347A, T1348N and G2019S/T1348N).

It was reported that Hsp90 can help to fold and stabilize LRRK2, most likely via the kinase domain, and prevents its degradation by the proteasome (Jorgensen et al., 2009). Wang et al. (2008) demonstrated that the G2385R mutation and deletion of the extreme *C*-terminus cause an increase in Hsp90 binding to LRRK2, which correlates with LRRK2 instability. Moreover, this interaction is independent from LRRK2 kinase activity because both kinase-dead (K1906M) and kinase hyperactive (G2019S) mutants are able to bind Hsp90 similar to WT protein.



LRRK2	elution fraction (mean)	R^2
WT	12.88	0.8936
R1398L	13.03	0.9040
R1398L/ T1343V	12.94	0.7641
R1398Q/ T1398G	13.11	0.8664

Figure 23. (**A**) Size-exclusion chromatography elution profiles of immunopurified FLAG-tagged LRRK2 variants derived from HEK-293T cells by Western dot-blot analysis with anti-FLAG antibody. Indicated are equivalent quantities of 0.5 ml fractions. (**B**) Densitometric analysis of LRRK2 variant elution profiles expressed as the relative signal intensity versus fraction number (ml). Table indicates the mean elution fraction for each LRRK2 variant and R^2 value for each curve fit.

To determine whether or not LRRK2 GTPase activity influences Hsp90 binding, we performed Co-IP experiments against endogenous Hsp90 using 3xFLAG-tagged LRRK2 mutants. Our data shows that altering GTPase activity does not influence Hsp90 binding (Figure 24 B). Conversely, an intact P-loop motif is critically required for the normal association with this chaperone protein (refer to K1347A, T1348N and G2019S/T1348N GTP binding-deficient mutants in Figure 24 B).

In summary, the differences in GTP hydrolysis between R1398L and R1398L/T1343V mutants do not seem to be a consequence of changes in homo- and hetero-dimerization. On the other hand, our data indicates that GTPase activity does not influence native complex formation,

heterodimerization and Hsp90 binding of LRRK2. Conversely, all tested GTP binding-deficient mutants exhibit reduced protein stability and reduced capacity to form heterodimers compared to WT. It is unclear if the impairment of GTP binding is responsible for LRRK2 protein instability and reduced dimerization or if, *vice versa*, these mutations are not well tolerated by cells and therefore provoke protein degradation, consequently resulting in reduced GTP binding and dimerization.

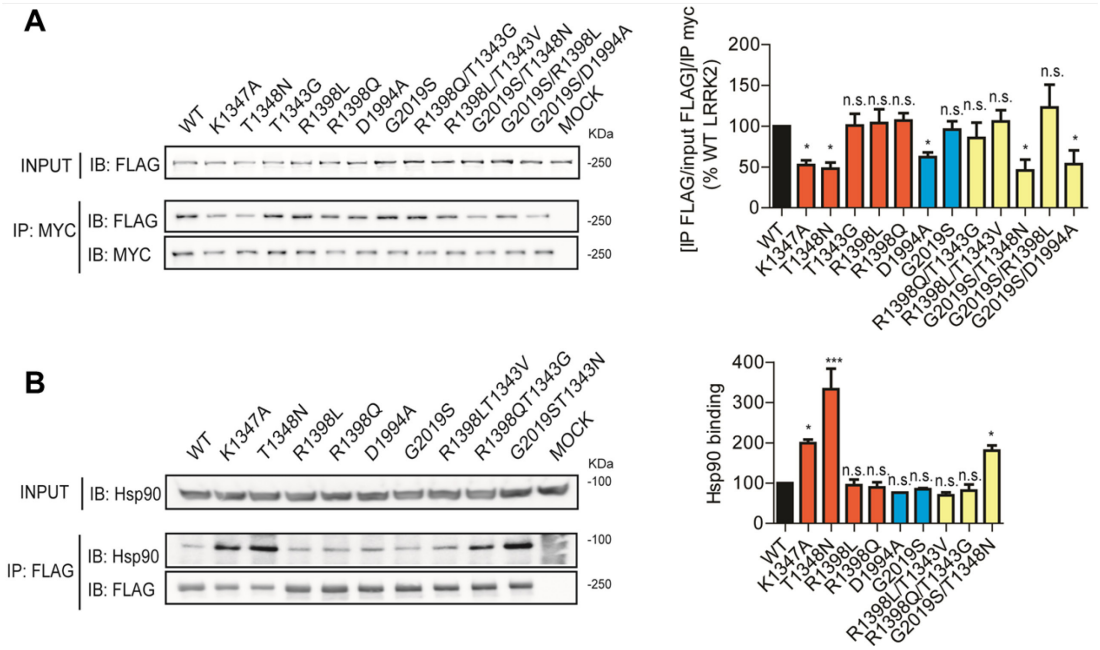


Figure 24. (**A**) Co-immunoprecipitation analysis of each FLAG-tagged LRRK2 variant with MYC-tagged WT LRRK2 from HEK-293T cells: western blotting analysis (*on the left*) and quantification (*on the right*). (**B**) Interaction of endogenous Hsp90 with FLAG-tagged LRRK2 mutants in HEK-293T cells by co-immunoprecipitation assay: western blotting analysis (*on the left*) and quantification (*on the right*).

4.6 GTPase activity does not influence LRRK2 cytosolic localization

LRRK2 is a protein that mostly adopts a diffuse cytosolic distribution in neurons and other mammalian cells (Biskup et al., 2006; Greggio et al., 2006). It was demonstrated that the G2019S mutation induces the formation of LRRK2-positive intracytoplasmic inclusions because of its hyperactive kinase function (Greggio et al., 2006). The contribution of

GTPase activity to the formation of protein inclusions has not been unexplored.

For this purpose, the cellular distribution of LRRK2 GTPase domains mutants transiently expressed in HEK-293T cells was assessed by immunocytochemistry and confocal microscopy. All examined variants adopt a diffuse cytosolic distribution similar to WT LRRK2, without strong evidence of accumulation of insoluble granules, modified membrane-association or subcellular compartmentalization (Figure 25). In summary, this data shows that LRRK2 cytosolic distribution pattern is independent of GTPase activity.

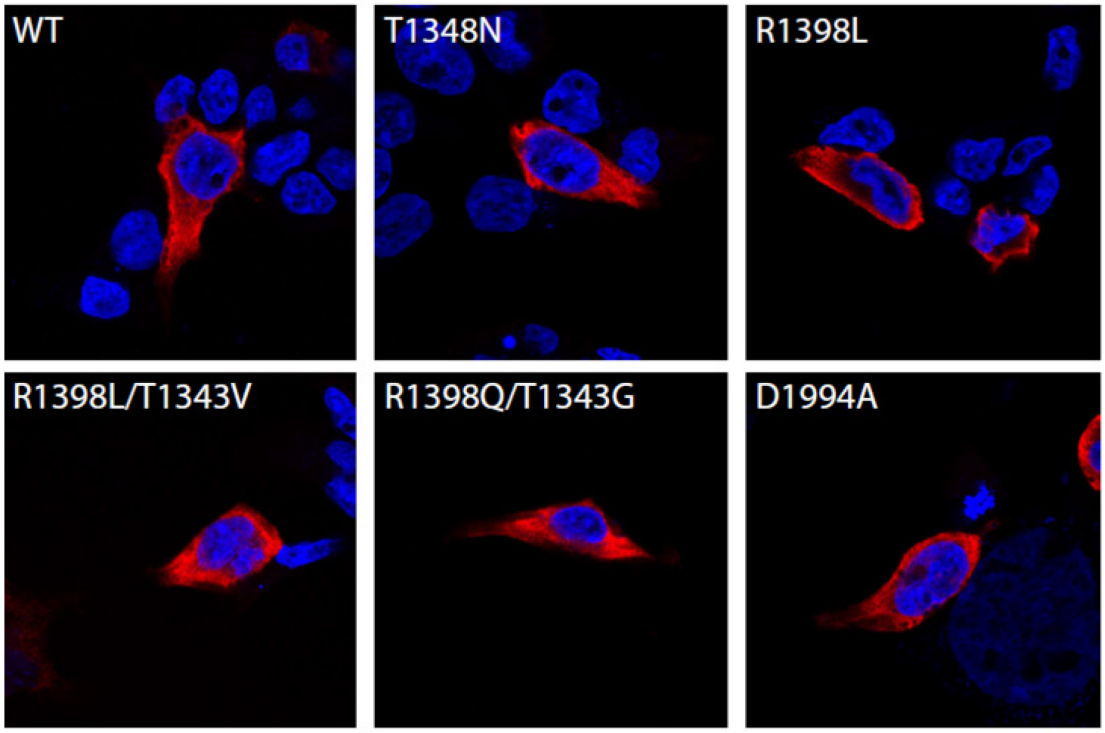


Figure 25. Confocal fluorescence microscopy reveals the diffuse cytoplasmic localization of FLAG-tagged human LRRK2 variants transiently expressed in HEK-293T cells. Staining for LRRK2 (anti-FLAG antibody, red) and nuclei (DAPI, blue) are indicated. Scale bar: 10 μm.

4.7 GTPase activity modulate neuron length

G2019S hyperactive kinase mutation was reported to be responsible for neurite shortening (i.e. inhibition of neurite outgrowth) in cortical neurons transiently transfected with LRRK2 variants and GFP (MacLeod et al.,

2006; Parisiadou et al., 2009; Stafa et al., 2012; Xiong et al., 2012; Ramsden et al., 2011).

To shed light on the role of GTPase activity on neurite outgrowth, we performed experiments to measure neurite length induced by LRRK2 GTPase domain variants. Before conducting these assays, the protein expression level of LRRK2 variants in cortical neurons was assessed (Figure 26 A). Surprisingly, all tested kinase-dead mutants (D1994A and G2019S/D1994A) exhibit significantly decreased steady-state levels and for this reason they were excluded from neurite shortening experiments. Conversely, R1398L, G2019S, R1398L/T1343V and G2019S/R1398L showed LRRK2 expression levels similar to WT, similar to their steady-state levels in HEK-293 cells. Moreover, the T1343N GTP binding-deficient variant and D1994A kinase-dead mutant, but not K1906M, are unstable in cortical neurons (Figure 26 B).

Our data from neurite shortening experiments shows that:

- ✓ LRRK2 itself promotes neurite shortening,
- ✓ G2019S hyperactive kinase mutant is neurotoxic and it provokes a ~40% of reduction in neurite length compared to WT, as previously reported.

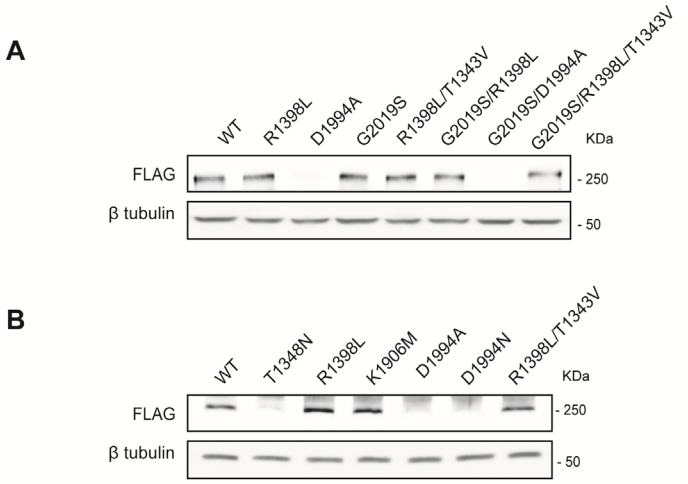


Figure 26. Western blotting showing flag-LRRK2 inputs (*upper panel*) and β tubulin signal (*lower panel*) from lysates of rat primary cortical neurons transfected at DIV 3 and lysated at DIV 6 (**A** and **B**).

Our results also indicate that impaired, but not enhanced, GTPase activity promotes neurotoxicity, to a similar extent as the G2019S pathological mutant (Figure 27 A and B). On the basis of these pieces of data it was supposed that GTPase activity might modulate neurite outgrowth. Moreover, enhanced GTPase activity is unable to prevent neurotoxicity induced by the G2019S mutant (i.e. G2019S/R1398L mutant), suggesting that G2019S hyperphosphorylation activity supersedes the effects of increased GTPase activity on neuronal length (Figure 27 A and B). Moreover, R1398L/T1343V can only modestly inhibit G2019S-induced neurite shortening (i.e. G2019S/R1398L/T1343V), albeit G2019S/R1398L/T1343V exhibits GTP hydrolysis and kinase activity similar to G2019S alone (Figures 20, 21 A, B and C). It is likely that the partial rescue in axon length is due to a non-significant reduction in kinase activity of G2019S/R1398L/T1343V compared to G2019S (refer to Figure 21 C).

In summary, impaired GTPase activity correlates with neurite shortening. Moreover, by using our genetic approach, it appears unlikely to rescue G2019S-induced axonal shortening by increasing GTP hydrolysis.

Our results are particularly interesting because most of LRRK2 pathological mutations cause impairments of GTPase activity. Therefore, in the future, it would be worth exploring the effects of modulating GTPase activity in *in vitro* and *in vivo* systems expressing additional LRRK2 pathological mutations.

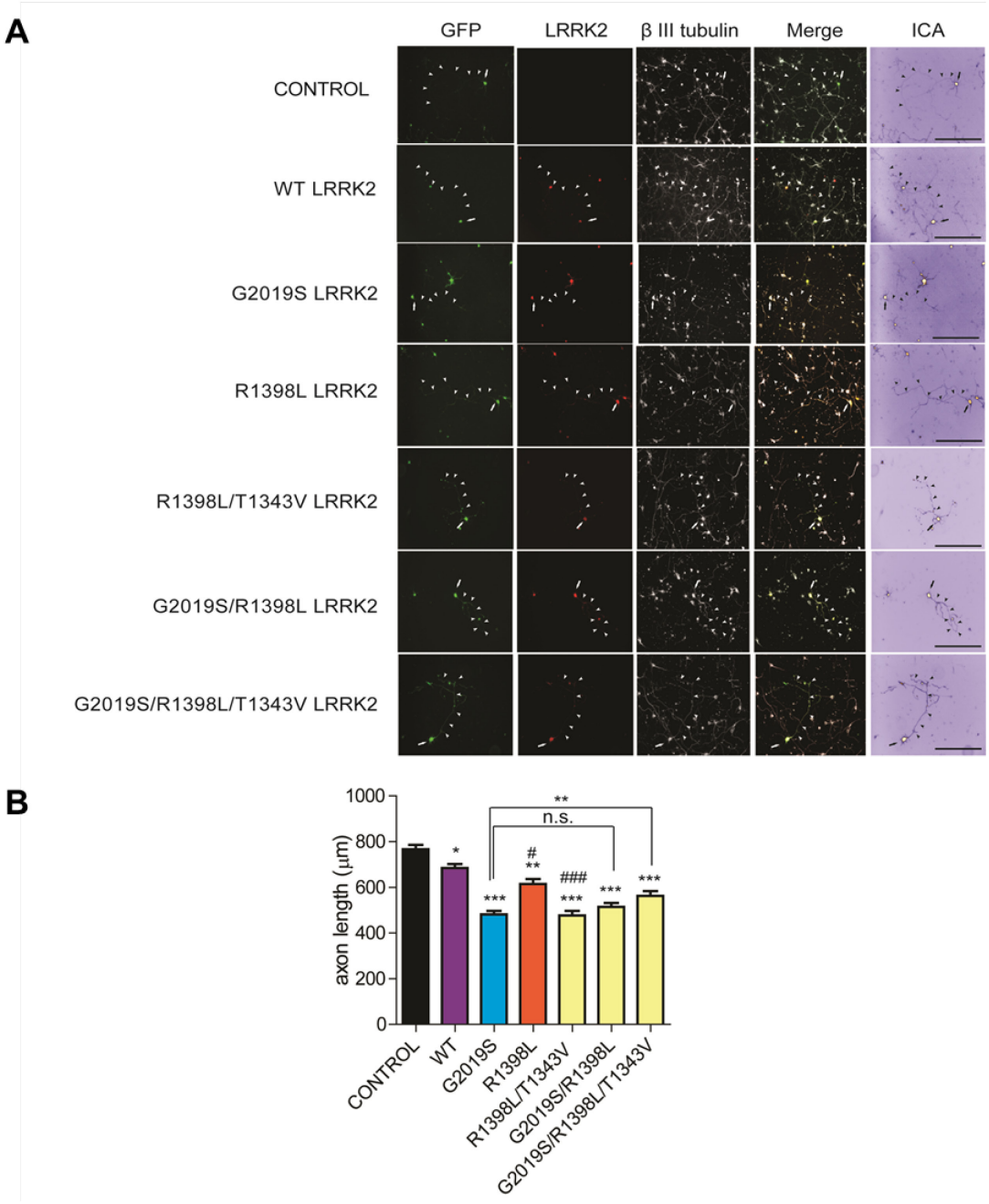


Figure 27. Staining of rat primary cortical neurons with flag-LRRK2, GFP and β III tubulin after transfection with flag-LRRK2 mutants and GFP constructs and cell fixation (**A**). *Arrows* indicate neuronal soma and *arrowheads* axonal processes. Scal bars: 400µm. Graph represents axon lengths measured on LRRK2-, GFP-, and β III tubulin-positive neurons (**B**). **P*<0.05, ***P*<0.01, ***P<0.001 comparing the axon length of each mutant to control, if not differently indicated, and $^{\#}P$<0.05 and $^{\#\#\#}P$<0.001 comparing the neurite length of each mutant to WT by one-way ANOVA with Newman-Keuls post-hoc analysis.

5. DISCUSSION

In 2004 it was first observed that several mutations localized to the *LRRK2* gene (locus PARK8) cause familial PD. To predict LRRK2 physio-pathological functions, sequence homology with well characterized proteins was explored.

LRRK2 protein belongs to the ROCO family, is a bivalent enzyme and shares high similarity with Ras and Raf protein families. On the basis of this information, researchers hypothesized that LRRK2 might take part in a signal transduction mechanism and that its kinase activity might be the downstream effect of its GTPase function. Unfortunately, LRRK2 enzymatic regulation and functions are still unclear. The contribution of LRRK2 GTP hydrolysis to kinase activation and PD-associated cellular phenotypes are not fully understood. To shed light on these processes, a complete library of functional mutations has been created on the basis of well characterized equivalent functional variants in related H-Ras GTPase and Raf-1 kinase proteins (Ito et al., 2007).

First, LRRK2 steady-state level assays were performed in HEK-293T cells. Our results, according to the literature, indicate that K1347A and T1348N are unstable compared to WT LRRK2, suggesting that single mutations in the P-loop motif that impair GTP binding are poorly tolerated in HEK-293 cells.

Then, LRRK2 GTP binding and hydrolysis were assessed. Among all the functional variants, three of them show decreased GTP binding (i.e. K1347A, T1348N and G2019S/T1348N), two GTPase-enhanced proteins (i.e. R1398L and G2019S/R1398L) and one GTPase-inactive LRRK2 version (i.e. R1398L/T1343V), independently from GTP binding affinity. Moreover, no one mutation in the kinase domain is able to modify

enzymatic activities of the ROC domain, leaving unsolved the question about the importance of autophosphorylation sites at the ROC domain.

We can summarize some important differences between LRRK2 and other small GTPases:

- ✓ LRRK2 GTP hydrolysis proceeds at lower rate than in other proteins,
- ✓ LRRK2 possess an Arg (R) residue instead of a Gln (Q) in the DFAGR sequence of the Switch II region,
- ✓ R1398L mutation in LRRK2 is associated with enhanced GTP hydrolysis, whereas the equivalent Q61L variant in H-Ras shows impaired GTPase activity (GTP-locked version),
- ✓ R1398L/T1343V mutation is the GTP-locked version of LRRK2, like the Q61L variant in H-Ras.

On the basis of this data we supposed that the regulation of LRRK2 GTP hydrolysis is distinct from most other small GTPases. Moreover, introduction of the R1398Q/T1343G double mutation in the ROC-COR-kinase fragment of LRRK2 has been associated with a GTPase-enhanced protein (Xiong et al., 2010). Conversely, LRRK2 full-length R1398Q/T1343G mutant showed GTP hydrolysis activity similar to WT. This discrepancy in GTPase activity data can be explained by postulating that sequences outside the ROC domain might negatively modulate LRRK2 GTP hydrolysis, as previously reported (Deng et al., 2008; Li et al., 2007).

Our data from autophosphorylation and LRRKtide phosphorylation assays indicates that:

- ✓ P-loop motif is critically required for LRRK2 kinase activation, in fact all GTP binding-deficient mutants are kinase-dead,
- ✓ Switch II region modulates kinase function: even in presence of an impaired GTP hydrolysis, LRRK2 can still phosphorylate itself and LRRKtide, albeit to a lesser extent.

Prior experiments based on cell treatments with non-hydrolysable GTP analogs (GTPγS and GppCp) have shown that GTP binding is responsible for kinase activation, most probably through an unidentified GTP-binding protein (Taymans et al., 2011). Our further experiments using GTP and its non-hydrolysable analog, GppCp, confirmed that both GTP binding and hydrolysis enhance kinase activity of all LRRK2 mutants except for T1348N GTP binding-deficient variant. Moreover, GTPase activity is able to further increase the GTP binding-induced kinase activation. Conversely, R1398L/T1343V (GTP-locked mutant) loses the GTP hydrolysis-related kinase activation. Therefore, on the basis of these results, we hypothesized that a GTP binding-dependent effector first activates kinase function and then, LRRK2 GTP hydrolysis allows the complete kinase domain activation cycle.

Some experiments from other groups indicated that LRRK2 cycles from a GDP-bound kinase-inactive conformation to a GTP-bound kinase-active state (West et al., 2007; Sen et al., 2009). In this scenario, our R1398L/T1343V mutant, as well as being a GTPase-inactive protein, would have stayed longer in the GTP-bound conformation and possess an increased kinase activity compare to WT. Conversely, our autophosphorylation and LRRKtide phosphorylation pieces of data were in disagreement with this hypothesis, albeit mutations that have been used are different. In support of our hypothesis are results coming from LRRK2 autophosphorylation experiments in the presence of ArfGAP1 (which increases LRRK2 GTPase activity) (Stafa et al., 2012). It has been demonstrated that ArfGAP1 promotes LRRK2 kinase activation, suggesting that either GTP binding or GTP hydrolysis contribute to LRRK2 kinase function and that LRRK2 enzymatic regulation is more complicated than that of other related G-proteins.

To explain enzymatic differences in GTP hydrolysis between R1398L and R1398L/T1343V mutants, we explored dimerization and stability properties associated with these GTPase variants. FPLC and Co-IP approaches showed that:

- ✓ LRRK2 GTPase activity does not have any effect on dimerization,
- ✓ dimer formation cannot explain the differences in GTP hydrolysis capacities between R1398L and R1398L/T1343V mutants.

Moreover, among all tested variants, K1347A, T1348N, D1994A, G2019S/T1348N and G2019S/D1994A possess reduced capacity to form heterodimers, suggesting that both an intact P-loop motif and kinase domain are necessary for dimerization, as previously demonstrated by using BLUE Native PAGE and FPLC analyses (Greggio et al., 2007; Sen et al., 2009).

Finally, FPLC results show that LRRK2 native complexes have a molecular weight (440-669 kDa) that does not correspond to a perfect LRRK2 dimer, suggesting that these complexes might include two LRRK2 monomers associated with partner proteins (i.e. Hsp90, 14-3-3, etc.).

Finally, GTP hydrolysis does not influence the association with Hsp90, suggesting that R1398L and R1398L/T1343V enzymatic activities are not a consequence of important LRRK2 conformational changes, but more likely an effect of subtle variations in the ROC domain flexibility, without impairment of LRRK2 dimerization, native complex formation and stability. To confirm this hypothesis further crystal structure analyses of GTPase mutants will be necessary. Conversely, impaired GTP binding promotes LRRK2 association with the Hsp90 chaperone protein, confirming that this enzymatic activity stabilizes LRRK2 protein structure. In the light of this data, it is important to note that both impaired GTP binding and protein instability can trigger kinase inactivity and reduced dimerization, complicating the interpretation of LRRK2 enzymatic data.

So far, two main theories have been postulated about LRRK2 enzymatic activity. The first one assumes that LRRK2 enzymatic activity regulation is based on a canonical GTP/GDP cycle, like the Ras/Raf pathway. The second hypothesis suggests that LRRK2 protein belongs to a family of GAD proteins (Gasper et al., 2009) that dimerize upon GTP binding and then stimulates its GTPase and kinase activities but in the absence of GAPs and GEFs. In summary, our kinase activity, FPLC and Co-IP pieces of data are in disagreement with both theories, in fact:

- ✓ either GTP binding or GTP hydrolysis seem be correlated with kinase activation,
- ✓ R1398L (GTPase-enhanced variant, mostly locked in the GDP-bound conformation) and R1398L/T1343V (GTPase-inactive variant, mostly locked in the GTP-bound state) mutants do not have different dimerization properties.

It is also worth noting that the LRRK2 ROC domain cannot directly stimulate kinase activity through GTP binding (Taymans et al., 2011), suggesting that LRRK2 does not follow a canonical GTP/GDP cycle. Moreover, LRRK2 GTPase and kinase activity are enhanced by ArfGAP1 (GAP protein), which is incompatible with the GAD theory (Stafa et al., 2012).

Moreover, GTP hydrolysis does not have any effect on LRRK2 cytosolic distribution in HEK-293 cells.

It was previously reported that decreased, but not enhanced, GTPase activity of LRRK2 fragments (i.e. K1343A and T1348N mutations) is correlated with toxicity in yeast and neuronal models (Xiong et al., 2010). Moreover, in cortical neurons, it was demonstrated that LRRK2 modulates axonal length and branching and that enhanced kinase activity of full-length LRRK2 is responsible for neurite shortening (Stafa et al., 2012). On the basis of this data, we assessed the contribution of GTPase activity of full-

length LRRK2 on neurite length and we found that reduced, but not enhanced, GTP hydrolysis promotes neurite shortening to a similar extent as the G2019S pathological mutant. Unfortunately, by using our genetic model, increased GTPase activity is not sufficient to rescue G2019S-induced neurite shortening.

In summary, it is possible to hypothesize the LRRK2 enzymatic regulation model that is shown in Figure 28. According to this theory, LRRK2 can be present in different conformations in cells:

- ✓ the monomeric, kinase-inactive GDP-bound state,
- ✓ the dimeric, partial kinase-active GTP-bound intermediate (after kinase activation),
- ✓ the stable homodimeric, kinase-active and physiologically-active GDP-bound state.

It is probable that following a specific stimulus and most probably in the presence of a still unidentified GEF protein, kinase-inactive LRRK2 monomers bind GTP. This GTP-bound conformation would provide docking sites for a GTP binding-dependent protein that promotes kinase activation, allowing LRRK2 to phosphorylate itself and to form an unstable dimer, but preventing this protein from efficiently phophorylating its biological substrates. For LRRK2 to complete kinase stimulation, GTP hydrolysis is also required (most probably in the presence of a GAP), with stable dimer formation. Finally, upon GTP conversion to GDP, LRRK2 becomes an efficient protein kinase that can phosphorylate both itself and its physiological substrates. After GTP hydrolysis, LRRK2 rests in a stable dimeric state, until specific phosphatases impair LRRK2 dimer stability. LRRK2 can bypass its kinase activation by GTP hydrolysis, but the GTP-bound dimeric intermediate does not exhibit all biological effects of the GDP-bound homodimeric conformation (Figure 28).

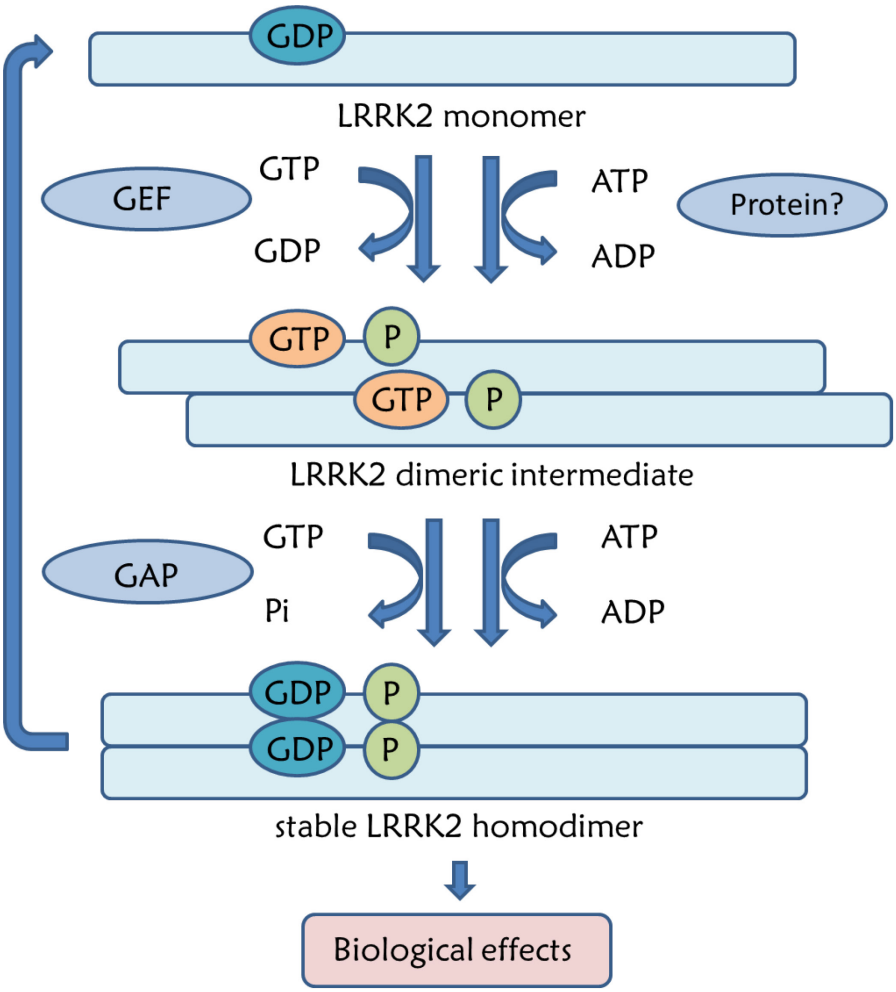


Figure 28. LRRK2 enzymatic activation model.

Our pieces of data taken together indicate that a functional relationship among ROC domain, kinase domain and dimerization exists, albeit all molecular details are not well understood. Further studies are required for clarifying the molecular pathobiology of LRRK2 protein for the purpose of finding new therapeutic strategies for PD.

6. CONCLUSIONS

We generated a complete library of LRRK2 functional mutants to explore the interplay between GTPase and kinase domains and the contribution of LRRK2 GTPase activity to cellular phenotypes.

Our biochemical data suggests that enzymatic regulation of this protein is complicated to understand and we proposed a summary model of LRRK2 activation that fits well with our findings. According to this theory, LRRK2 would require GTP binding to phosphorylate itself and dimerize, whereas GTP hydrolysis activity modulates its capacity to phosphorylate its biological substrates and, consequently, exert its biological function.

Regarding LRRK2-related neuronal phenotypes, we observed that increased GTPase activity is not sufficient to rescue G2019S LRRK2-induced neurite shortening. If neuronal length reflects cellular toxicity, this indicates that increasing GTPase activity may not provide an efficacious strategy for reverting neurotoxicity induced by the pathogenic G2019S mutation.

In the future, it will be important to confirm all biochemical and cellular findings in *in vivo* LRRK2 models and to explore the role of GTPase activity in neuronal morphology and function by using GTPase-impaired pathological LRRK2 mutations (i.e. R1441C, R1441G or Y1699C) linked to PD.

To date, we do not yet understand LRRK2-related PD and until further details of LRRK2 pathobiology have been clarified it will be difficult to develop new LRRK2-based therapies for treating PD.

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