Signal Transduction Pathways Modulated by the PD-causative Gene LRRK2

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TABLE OF CONTENTS

1 SUMMARY

Mutations in leucine-rich repeat kinase 2 (*LRRK2*) are the most frequent cause of autosomal-dominant Parkinson's disease (PD). Post-mortem studies in PD brains have shown that α-synuclein (SNCA) aggregation is a frequent neuropathological hallmark of LRRK2-associated PD, However, the pathogenic mechanisms linking LRRK2 and SNCA are largely unknown. The LRRK2 kinase domain shares homology to mitogen-activated protein kinase kinase kinases (MAPKKKs) and its kinase activity has been suggested to be a key factor for LRRK2 pathogenesis.

The objective of our study was to investigate the role of LRRK2 in PD-relevant signal transduction pathways and to identify putative downstream targets. Our results show that over-expression of wild-type LRRK2 in human embryonic kidney HEK293 cells induced the phosphorylation of extracellular signal-regulated kinases (ERK1/2) up to 4-fold without affecting basal levels of total ERK. Mutants R1441C and G2019S, but not kinase dead (KD), induced ERK activation to the same extent as wild-type LRRK2, indicating that ERK activation is dependent on LRRK2 kinase activity. Nonetheless, the time course of R1441C and G2019S induction was slower than that for wild-type LRRK2, despite similar expression levels. Furthermore, activation of the ERK pathway by LRRK2 was associated with an increase of almost 2-fold in SNCA mRNA, together with a small but significant increase in SNCA protein. Interestingly, SNCA up-regulation could be suppressed by treatment with the inhibitor U0126, which specifically blocks MEK1/2 activation, indicating that SNCA is induced by LRRK2 through ERK1/2 MAPK activation. This pathway links the two dominant PD-associated genes *LRRK2* and *SNCA,* and opens a novel venue that may be relevant to pursuit the pathophysiology in both familial and sporadic PD and to search potential therapeutic targets.

2 INTRODUCTION

2.1 Definition of PD

Parkinsonism is a neurological syndrome whose symptoms have been known for thousands of years, as collected in ancient Indian and Chinese medical texts. In 1817 James Parkinson reported the first clinical description in “An Essay on the Shaking Palsy”. The most common form of parkinsonism is PD, affecting about 6 million people worldwide at the present. The prevalence of PD is closely related to age, increasing from 1% at the age of 65 to 5% by the age of 85 (Dauer and Przedborski 2003). Indeed, the number of affected individuals is likely to double by 2050 due to the continuous increase in life expectancy in industrialized countries.

Clinically, PD is defined by the motor symptoms tremor (involuntary shaking), rigor (muscle stiffness) and bradykinesia (slow, limited movement). Pathologically, PD is characterized by the selective degeneration of dopamine-releasing neurons in the pars compacta of the substantia nigra (SNpc), together with the presence of Lewy bodies (LBs) and Lewy neurites in the surviving neurons of the brain stem and cortical areas (Goedert 2001). LBs, named after their first description by the pathologist Friedrich Lewy in 1912, are intraneuronal cytoplasmic protein inclusions found in all PD-affected brain regions. The presence of LBs is, however, not exclusive to PD as they also occur in other disease states including dementia with LBs (DLB) or Alzheimer’s disease (AD), or even in healthy elderly people. The major component of LBs is SNCA, but a variety of other aggregation-prone proteins such as parkin, ubiquitin, synphilin-1 and neurofilaments appear also in these inclusions (Dev et al. 2003). Whether LBs are toxic to neurons by interfering with normal cellular processes and/or by retaining pro-survival factors or, contrarily, they protect neurons by sequestering harmful protein aggregates, remains still controversial.

Mechanistically, the progressive and massive loss of nigral neurons causes dopaminergic depletion in the striatum, and leads to alterations in the neural circuits within the basal ganglia regulating initiation and control of movement (Fig.1). The nigrostriatal dopaminergic pathway is the most affected in PD. First symptoms including hypokinetic movement disorder accompanied by the uncontrolled movements characteristic of PD, have been reported to appear when over 60% of dopaminergic neurons in the substantia nigra have degenerated (Ottley et al. 1999).

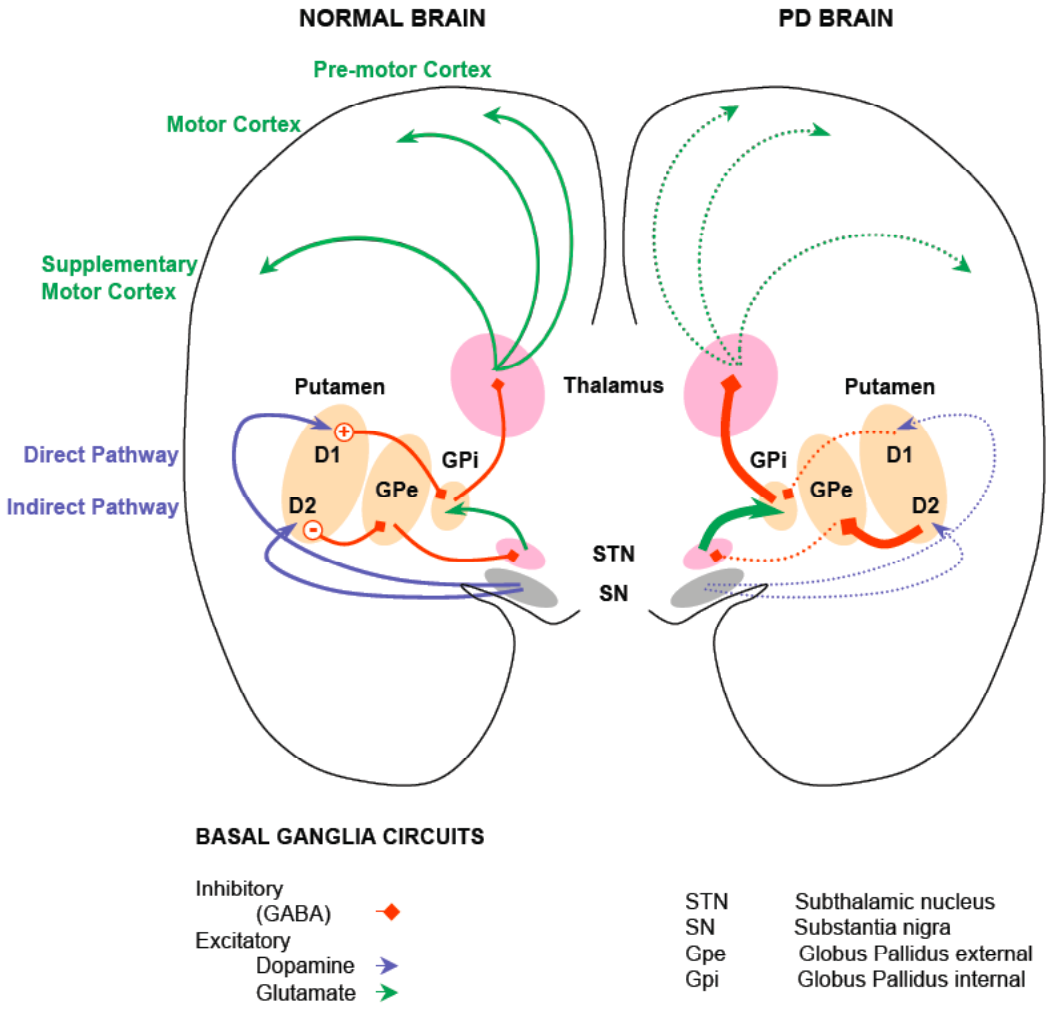


Figure 1. Basal ganglia circuits in normal (left) and parkinsonian (right) brain.

Progressive degeneration of the dopaminergic neurons in the substantia nigra pars compacta of PD patients leads to a dopamine depletion in the striatum, causing discharge abnormalities within the entire system. Thus, parkinsonism results from an excessive inhibition of components of the motor circuit in the thalamus, cortex and brainstem.

However, later disruption of the mesolimbic, mesocortical and tuberoinfundibular dopamine pathways in the brain, together with the degeneration of additional non-dopaminergic neuronal populations, likely explain much of the secondary symptoms associated with PD, including olfactory and visual abnormalities, anxiety, depression and dementia (Lim et al. 2002).

At present, there is no cure for PD patients, whose average life expectance usually does not exceed 10 years of disease duration. Standard dopaminergic substitutive therapy and, more recently, deep-brain stimulation have been shown to efficiently alleviate the symptoms in certain cases. Nonetheless, they can cause a variety of side effects and eventually become counterproductive. Thus, a better understanding of the molecular mechanisms underlying PD could provide the basis for the development of more effective therapies to cure this devastating disease.

2.2 Etiology of PD

Parkinson's disease is a complex multifactorial disease with multiple genetic and environmental factors whose synergistic interaction leads to dopaminergic neurodegeneration (Maraganore et al. 2003). To date, most of the studies aiming to identify exogenous factors causing sporadic PD have unfortunately been inconsistent. Nonetheless, several studies suggest toxins such as pesticides and herbicides as risk factors, or tobacco as protective (Tzourio et al. 1997; Smith et al. 2006). About 5-10% of the cases are familial (FPD) and follow a mendelian pattern of inheritance (Cordato and Chan 2004). Neuronal death is in both familial and sporadic forms the primary cause of the motor symptoms, suggesting putative common pathogenic mechanisms. Indeed, monogenic hereditary forms have provided valuable insights into the molecular pathogenesis of PD, including the sporadic form of the disease.

To date, 13 disease-associated loci and 5 PD-causative genes have been identified (Table 1). Mutations in *parkin*, *PINK1* and *DJ-1* typically cause autosomal-recessive early-onset PD, whereas mutations in α-synuclein (*SNCA*) and leucine-rich repeat kinase 2 (*LRRK2*) are associated to autosomal-dominant PD.

Table 1. Genes and loci associated to PD

Locus	Gene	Position	Inheritance	Onset	Putative Function
PARK1	SNCA point mutations	4q21	AD	40s	pre-synaptic protein, LBs
PARK2	Parkin	6q25	AR	20-40	ubiquitin E3 ligase
PARK3	SPR?	2p13	AD	60s	TH-biosynthesis
PARK4	SNCA genomic multiplications	4q21	AD	30s	
PARK5	UCHL1	4p14	AD	~50	ubiquitin C-terminal hydrolase
PARK6	PINK1	1p35-p37	AR	30-40	mitochondrial kinase
PARK7	DJ-1	1p38	AR	30-40	chaperone, antioxidant
PARK8	LRRK2	12q12	AD	~60	MAPKKK kinase
PARK9	ATP13A2	1p36	AR	20-40	lysosomal ATPase
PARK10	Unknown	1p32	AD?	50-60	Unknown
PARK11	GIGYF2?	2p34	AD?	late	insulin signaling (?)
PARK12	Unknown	Xq31	X-linked	late	Unknown
PARK13	Omi/HtrA2	2p12	AD?	late	mitochondrial serine protease

2.2.1 PARK1, PARK4: α-synuclein (*SNCA*)

SNCA (4q21) was the first PD-causative gene identified (Polymeropoulos et al. 1997) and codes for a small pre-synaptic phosphoprotein of 14 kDa consisting of 140 amino acids. SNCA is ubiquitously expressed in all brain regions, most abundantly in pre-synaptic terminals from neurons, a small fraction also associated with vesicular membranes (Maroteaux and Scheller 1991; Irizarry et al. 1996; Kahle et al. 2000).

Despite PD-causative *SNCA* mutations have been only found in a small number of kindreds afflicted with familial PD, the intrinsic propensity of SNCA to aggregate (Lee and Trojanowski 2006) has been shown to be crucial in the formation of LBs in both familial and sporadic PD (Spillantini et al. 1997). The three described SNCA pathogenic point mutations A53T, A30P and E46K, increase SNCA fibril formation in vitro in a seeding-dependent manner (Narhi et al. 1999; Greenbaum et al. 2005). Thus, fibrillogenesis occurs beyond a critical threshold (Wood et al. 1999) that can be reached in patients by genomic multiplications (Singleton et al. 2003) or promoter polymorphisms up-regulating SNCA expression (Chiba-Falek and Nussbaum 2001; Maraganore et al. 2006). These results suggest that transcriptional regulation of the gene and mRNA stability play a role in SNCA-associated PD. Nevertheless, it is still unclear if the aggregation is the primary insult that initiates PD or whether it is a secondary event that maintains the progression of the disease.

Although the biological and pathogenic function of SNCA remains largely unknown, there is increasing evidence of a role of SNCA in synaptic plasticity, regulation of dopamine neurotransmission and vesicle dynamics (Hardy et al. 2006). Furthermore, transgenic mice over-expressing human A53T SNCA develop mitochondrial pathology, probably because SNCA is a modulator of oxidative damage (Martin et al. 2006; Stichel et al. 2007). Indeed, mice lacking SNCA are resistant to mitochondrial toxins compared to human SNCA transgenic mice (Klivenyi et al. 2006). Biochemical abnormalities in SNCA have also been shown to activate stress-signaling protein kinases (Klegeris et al. 2008) and to impair microtubule-dependent trafficking (Lee et al. 2006). These pathophysiological findings are detrimental to normal functioning of dopaminergic neurons, providing evidence of SNCA implication in PD pathogenesis.

2.2.2 PARK2: Parkin (*PRKN*)

Parkin (6q25) was the first identified gene associated with autosomal recessive early-onset PD (Kitada et al. 1998). Up to date, more than 100 exonic variations including point mutations, insertions, and deletions, have been described in *parkin*, being one of the most common genetic causes of early-onset PD (18-49%). The parkin protein, predominantly cytoplasmic, is an ubiquitin E3 ligase that targets specific substrates for degradation by the ubiquitin-proteasome system (UPS) (Shimura et al. 2000).

Animal models have provided important insights into the parkin-associated PD pathogenesis. The parkin loss-of-function mutant in *D. melanogaster* exhibits degeneration of a subset of dopaminergic neurons, together with dramatic mitochondrial defects including swollen, fragmented mitochondria in several high energy-demanding tissues, such as the germ line in males or the flight wing muscles (Wang et al. 2007). In parkin knockout mice no severe defects in mitochondrial morphology are observed. Nonetheless, these animals displayed reduced mitochondrial respiratory activity, that is associated with signs of oxidative damage in the brain (Palacino et al. 2004). Thus, parkin is believed to play a crucial and evolutionarily conserved role of parkin in mitochondrial function that might be of relevance in the pathogenesis of PD.

2.2.3 PARK6: PTEN-induced putative kinase 1 (*PINK1*)

PINK1 (1p35-p37) was first identified as the cause of autosomal-recessive early-onset parkinsonism in three European PD kindreds (Valente et al. 2004). Mutations in the *PINK1* gene are uncommon, accounting for only 1-4% of early-onset cases. PINK1 protein contains a mitochondrial targeting motif and a serine/threonine kinase domain, where the majority of the PD relevant PINK1 mutations lie, suggesting that the most likely pathogenic mechanism is the loss of function of the kinase activity.

Cell culture studies indicate that PINK1 exerts a protective effect on the cells that is mitigated by the mutations, which increase cell susceptibility to oxidative stress (Valente et al. 2004). *Pink1* null mutants of *D. melanogaster* showed a similar phenotype as parkin knockouts, also displaying aberrant mitochondrial morphology in dopaminergic neurons, together with a modest loss of aging dopaminergic neurons. Indeed, recent *in vivo* evidence suggests that the PINK1 kinase is in a linear pathway upstream of parkin and affects mitochondrial function (Clark et al. 2006; Park et al. 2006). Supporting this idea, double knockout of *pink1* and *parkin* lead to identical phenotype and not to a stronger one (Dodson and Guo 2007). Interestingly, defects in the mitochondrial respiratory chain have also been detected in peripheral tissues from human PD patients with mutations in *PINK1* or *parkin*, but also in those with SPD, suggesting that such defects occur together with dopaminergic neurodegeneration as part of a general pathology in PD. Altogether, these data indicate that both *PINK1* and *parkin* genes act in a common genetic pathway.

2.2.4 PARK7: *DJ-1*

The gene *DJ-1* (1p38) was originally identified in a Dutch PD family as a rare cause of autosomal recessive parkinsonism (van Duijn et al. 2001), accounting for only 1% of recessive FPD (da Costa 2007). Endogenous DJ-1 is predominantly cytoplasmic but also localizes to the inner membrane space and matrix of neuronal mitochondria, and can be translocated to the outer mitochondrial membrane upon oxidative stress conditions (Canet-Aviles et al. 2004). Although the physiological function of DJ-1 and its role in dopaminergic neurodegeneration remains unknown, it has been shown to regulate oxidative stress, and to affect several major systems involved in PD pathogenesis including the ubiquitin-proteasomal and the dopaminergic systems.

Indeed, DJ-1-deficient cell lines and animal models have shown increased sensitivity to oxidative stress-induced cell death, whereas over-expression showed a protective effect (Abeliovich and Beal 2006). It still remains unknown the mechanisms by which DJ-1 might be exerting its protective effects in the cell. Possible mechanisms of action include functions as transcriptional co-activator or molecular chaperone.

2.2.5 PARK8: Leucine-rich repeat kinase 2 (*LRRK2*)

The *PARK8* locus was first mapped in a large Japanese family with autosomal-dominant late-onset parkinsonism (Funayama et al. 2002). The association was replicated in two additional kindreds and the chromosomal region further refined (Zimprich et al. 2004a) until the gene *LRRK2* (12q12) was finally identified as the disease-causative gene associated to *PARK8* (Paisan-Ruiz et al. 2004; Zimprich et al. 2004b).

Mutations in *LRRK2* have been found to be the most important genetic cause of autosomal-dominant PD. To date, over 50 variants have been identified throughout the different LRRK2 domains in PD patients. Nonetheless, only mutations R1441C/G, Y1699C, G2019S and I2020T have been found to segregate with the disease and be clearly pathogenic. Indeed, mutation G2019S has been regarded as the most common cause of dominant familial, as well as sporadic, PD with an incidence of 1-2%. This mutation itself explains 20% of Ashkanezi Jewish PD cases (Ozelius et al. 2006) and 40% in North African Arabs (Healy et al. 2008). The penetrance of G2019S, that increases with age, is reduced, suggesting the involvement of additional genetic susceptibility factors (Goldwurm et al. 2007).

LRRK2-associated PD is clinically homogenous and indistinguishable from idiopathic PD, showing late onset and the cardinal symptoms characteristic for PD. While all LRRK2-associated PD cases show dopaminergic neurodegeneration in the

SNpc, initial brain pathological analyses suggested that mutations in LRRK2 caused pleomorphic pathology with aggregation of proteins such as tau, ubiquitin and SNCA. Interestingly, diverse pathology occurred within carriers of the same pathogenic mutation or even within the same family (Zimprich et al. 2004b). Now, there is increasing evidence that the major pathology associated to mutations in *LRRK2*, in particular to G2019S, is SNCA aggregation with classical LBs. The question whether LRRK2 localizes to LBs has been addressed in a large number of studies. Nonetheless, the result seem to largely depend on the antibody used and this question needs to be further investigated.

2.2.6 Other PD-associated genes

Besides the PD-causative genes previously described, other genes have been also proposed to cause PD, but their role has not been totally clarified, mainly due to identification in single patients/families or lack of replication.

The PD candidate gene *UCHL1* (PARK5, 4p) encodes the neuronal protein ubiquitin carboxy-terminal hydrolase L1, also found in LBs. A single heterozygous missense mutation (I93M) has been found in two PD-affected siblings of German ancestry with typical PD (Leroy et al. 1998). No other pathogenic mutation in *UCHL1* has been identified up to date in families. Thus, although UCHL-1 loss-of-function mutant mice show sings of neurodegeneration (Saigoh et al. 1999), the actual role of this gene in familial or sporadic PD remains unclear.

Various loss-of-function mutations associated to PD have been described so far in the *ATP13A2* gene (PARK9, 1p36). This gene was originally identified in two kindreds presenting the autosomal-dominant, early-onset multisystemic neurodegenerative disease Kufor-Rakeb syndrome, characterized by levodopa responsiveness and a complex phenotype involving spasticity and dementia.

The gene encodes a large transmembrane protein belonging to the ATPase superfamily that is highly expressed in all brain regions, including SNpc, and that is up-regulated in SPD affected brains. In-vitro models have shown that, whereas wild-type protein localizes to lysosomes, the mutant protein is retained in the endoplasmic reticulum and subsequently degraded by the proteasome (Ramirez et al. 2006). Thus, ATP13A2-linked neurodegeneration is suggested to occur as a consequence of proteasomal dysfunction induced by protein overload or, alternatively to lysosomal dysfunction due to mutations in *ATP13A2*.

The *Omi/HtrA2* gene (PARK14, 2p12) encodes a serine protease localized to the mitochondrial inner membrane space and that is released into the cytosol in response to apoptotic stimuli. A missense variation, G399S, was found in four patients with SPD, while a polymorphism, A141S, has been suggested as a risk factor in the German population (Strauss et al. 2005). Genetic proof of pathogenicity is lacking, yet functional studies support a possible pathogenic effect of these variants, since Omi KO mice display a neurodegenerative phenotype with motor neuron dysfunction and striatal damage (Martins et al. 2004). Still, the role of the Omi/HTRA2 gene in PD remains to be further clarified.

2.3 Pathogenesis of PD

In the past two decades great efforts have been made in order to identify the primary cause of cell death in PD and to find specific therapeutic targets that might modify disease progression. Despite of the continuous progress, the precise pathogenic mechanism involved in PD is still unknown. Current knowledge of the disease suggests a complex interaction between environmental and genetic factors that act in a synergistic manner to promote cell death through different pathogenic mechanisms.

None of these mechanisms alone has been proven to be responsible for the development of PD, but observations from familial and sporadic forms of the disease, as well as from toxicity PD models, point towards three major mechanisms that are recurrently affected and may underlie dopaminergic neurodegeneration in PD: mitochondrial dysfunction/oxidative stress, proteotoxic stress, and aberrant kinase activity. (Fig. 2).

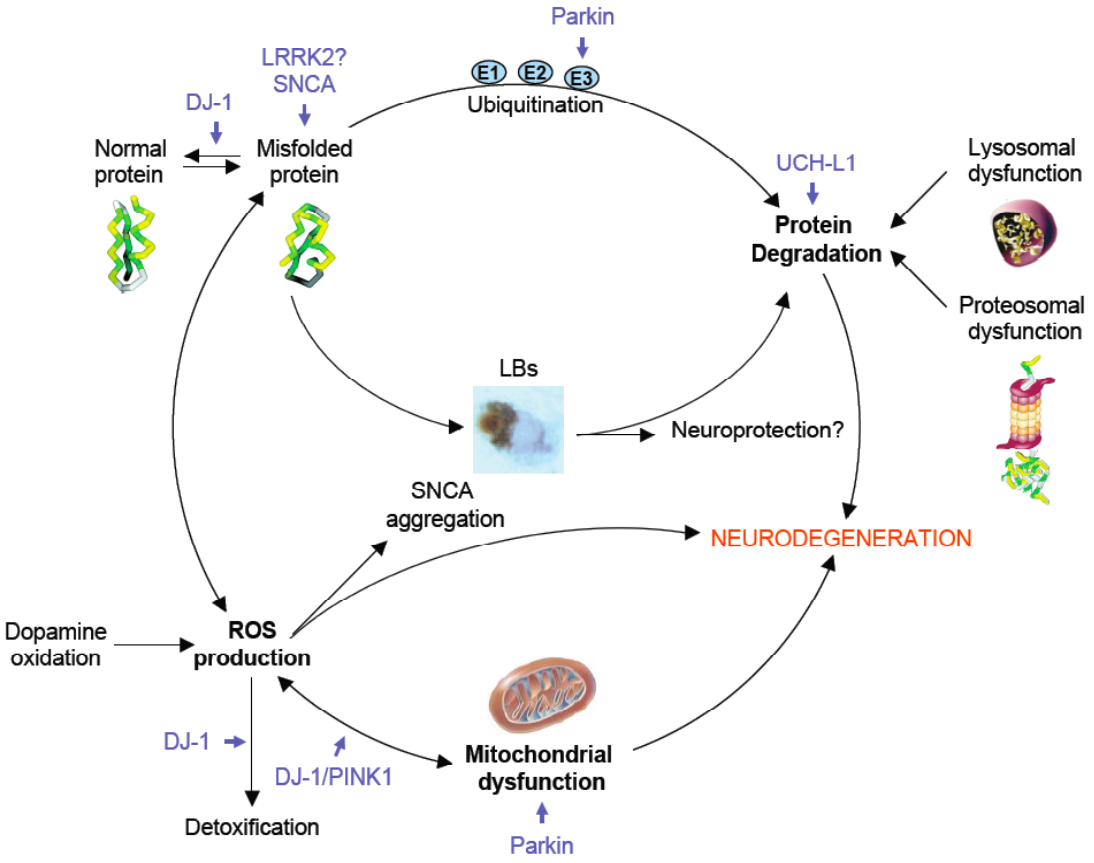


Figure 2. Role of PD-associated genes in the pathogenesis of PD.

Mutations in PD-associated genes or altered expression of the encoded proteins may contribute to PD pathogenesis through several common mechanisms including protein degradation, mitochondrial dysfunction or oxidative stress. *Parkin* and *UCHL1* are involved in protein degradation through the ubiquitin-proteasome system. *Parkin*, together with *DJ1* and *PINK1*, participates as well in maintaining mitochondrial integrity and mutations in these genes lead to mitochondrial dysfunction and oxidative stress. *SNCA* encodes an aggregation-prone protein which constitutes the main component of LBs, and that is reported to impair protein degradation and to contribute to oxidative stress.

2.3.1 Mitochondrial dysfunction/oxidative stress

Oxidative stress was the first pathogenic factor suggested to contribute to dopaminergic neurodegeneration. It results from the imbalance between the production of reactive oxygen species (ROS) and the antioxidative mechanisms, that causes the uncontrolled oxidation of biomolecules such as phospholipids, proteins, and nucleic acids, ultimately leading to cell death (Jenner 1998). First link of oxidative stress with PD was based on the observation that neurons generate H_2O_2 as a byproduct from the dopamine metabolic breakdown by the enzyme monoamine oxidase (MAO) or by auto-oxidation of dopamine. Under normal physiological conditions, H_2O_2 in the brain is cleared by the enzyme glutathione (GSH). However, when levels of H_2O_2 and GSH are deregulated, H_2O_2 reacts with redox-reactive ferrous iron (Fe^{2+}) generating highly reactive hydroxyl radicals (•OH) (Jellinger 1999). Interestingly, oxidative damage to lipids, proteins and nucleic acids (Dexter et al. 1989; Zhang et al. 1999), as well as a decrease in the levels of GSH have been detected in brain tissue from PD patients (Dexter et al. 1989; Zhang et al. 1999).

Oxidative damage is normally accompanied by decreased activity of the mitochondrial electron transport, responsible of the production of ATP by oxidative phosphorylation and main source of superoxide radicals, particularly in tissues with high-energy demand like the brain. Thus, defects in the mitochondrial respiratory chain not only compromise energy production, but also create a positive feed-back loop that increases oxidative damage and sensitization towards external toxins with a risk of excitotoxic damage (Jellinger 1999). Mitochondrial impairment and oxidative damage have been consistently detected in pathological analyses of PD brains (Jenner and Olanow 1998; Beal 2003), but not in other related diseases involving dopaminergic neurodegeneration. These observation indicate that oxidative stress may be causative, rather than a secondary event, of nigral neurodegeneration in PD.

Furthermore, inhibitors of mitochondrial complex I such as 1-methyl 4-phenyl 1,2,3,6 tetrahydropyridine (MPTP) reproduce the clinical phenotype of PD patients in animal models, as well as in humans (Schapira 2007). Reduced levels of complex I can also cause cell death and excitotoxicity due to impaired proton pumping, reduced mitochondrial membrane potential, and increased mitochondrial membrane permeability, leading to the release of apoptosis-initiating factors (Tatton and Olanow 1999). The identification of mutations in the genes *DJ-1*, *PINK-1* and *Parkin* also point to a role of oxidative stress in PD, since these three genes are involved in assessing neuronal viability through the regulation of mitochondrial integrity and oxidative stress (Abeliovich and Beal 2006).

Alternatively, the essential trace metal iron can be a source of oxygen free radical formation. In the SN, large amounts of iron are bound to neuromelanin, a molecule with redox properties and the capacity to chelate large amounts of metals, in particular iron. In fact, neuromelanin content has been suggested as an explanation for the selective vulnerability o dopaminergic neurons to oxidative stress since, under conditions of Fe^{3+} overload, neuromelanin can lead to ROS formation (Kastner et al. 1992). In addition, complex I inhibition due to GSH depletion has been reported to occur via a peroxynitrite mediated event (Chinta and Andersen 2006), suggesting that peroxynitrite-mediated oxidative damage might also play an important role in PD pathology.

2.3.2 Proteotoxic stress

The ubiquitin-proteasomal system (UPS) is responsible of degrading most of misfolded proteins and regulating protein turnover in cells. Thus, deficiencies in the UPS together with the age-related tendency to accumulate damaged proteins may lead to a pathogenic accumulation of misfolded and toxic proteins in the cell.

Accumulation of SNCA in dopaminergic neurons has been observed in sporadic PD patients (Baba et al. 1998). Furthermore, aggregated SNCA inhibits ubiquitin-dependent proteasomal function by direct binding (Snyder et al. 2003), and sensitizes cells to toxicity induced by proteasomal inhibitors (Tanaka et al. 2001). Most recent evidence indicates that the oligomeric forms of SNCA are the toxic species, rather than fibrils. This would imply that deposition of SNCA oligomers in aggregates such as LBs might contribute to detoxify the soluble oligomeric forms of SNCA. Thus, the formation of LBs may be a protective event, since most of the surviving neurons at a final stage of PD contain LBs. Interestingly, dopamine tends to stabilize the oligomeric form and prevents the aggregation of SNCA (Conway et al. 2001). This may explain the selective vulnerability of dopaminergic neurons.

A novel therapeutic approach for PD involves the use of chaperones that facilitate correct protein folding. The presence of chaperones immunostaining in LBs from human PD brains and the rescue of the pathological phenotype in transgenic flies expressing normal and mutant SNCA by over-expression of chaperones supports this approach (Auluck et al. 2002). The study of the PD-associated gene DJ-1 has been shown to inhibit SNCA aggregate formation by interacting with early-unfolding intermediates via its redox-sensitive chaperone function (Shendelman et al. 2004; Haywood and Staveley 2006).

The PD-associated genes *parkin* and ubiquitin C-terminal hydrolase-L1 (*UCH-L1*) have been also linked to UPS dysfunction in familial PD. Most *parkin* PD-associated mutations affect E3 ubiquitin protein ligase activity, suggesting that disruption of E3 ligase activity is a prominent cause of autosomal recessive PD. Interestingly, co-expression of parkin mitigates the effect of dopaminergic neuronal death in a mutant SNCA *D. melanogaster* model. Thus, parkin would participate in the detoxification of misfolded or aggregated proteins by rescuing proteasomal function (Haywood and Staveley 2006).

2.3.3 Aberrant kinase activity

The involvement of abnormal phosphorylation in neurodegenerative diseases and particularly in PD was first supported by the identification of the genes *PINK1* and *LRRK2*, both encoding protein kinases.

PINK1 encodes a serine/threonine protein-kinase with homology to the calcium-calmodulin dependent protein kinase family. *PINK1* is named after the tumor suppressor gene *PTEN*, that initially was shown to up-regulate *PINK1*, suggesting a role of PINK1 in cell cycle regulation (Unoki and Nakamura 2001).

The gene *LRRK2* encodes a multidomain protein with a serine/threonine kinase domain that shares homology with mitogen-activated protein kinase kinase kinases (MAPKKKs). A large number of mutations within the kinase domain of LRRK2, but also within other domains, appear to increase kinase activity, inducing a progressive reduction in neurite length and branching in both primary neuronal cultures and intact rodent CNS (MacLeod et al. 2006). Furthermore, mutations in LRRK2 increasing kinase activity have been shown to induce neurodegeneration whereas, accordingly, a reduction in LRRK2 kinase activity lead to reduced neuronal toxicity (Smith et al. 2006). The potential role of MAPKs in the pathogenesis of PD is supported by additional empirical evidence including the activation of JNK in several PD animal models, as well as the neuroprotection conferred by upstream inhibition of the pathway (Saporito et al. 1999). Thus, protein kinases are interesting tools to investigate PD molecular pathogenesis, and could potentially surrogate the identification of novel therapeutic targets.

2.4 *PARK8: leucine-rich repeat kinase 2 (LRRK2)*

2.4.1 Structure of LRRK2

The *LRRK2* gene spans 51 exons and encodes a large multi-domain protein (Fig. 3) of 2527 amino acids and 286 kDa. LRRK2 belongs to the ROCO protein family, whose members present a Ras-of-Complex (Roc) domain with high similarity to small guanosine triphosphatases (GTPases), and a C-terminal-of-Roc domain that is always associated to the Roc domain (Bosgraaf and Van Haastert 2003).

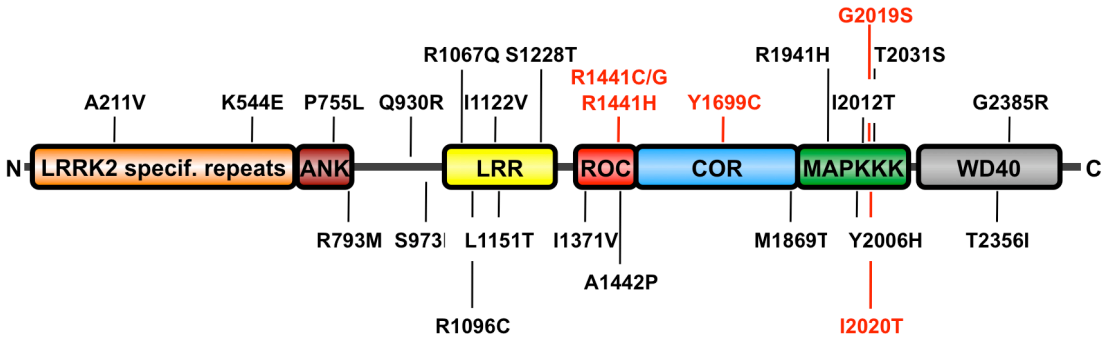


Figure 3. Predicted structure of *LRRK2* and variants identified in families.

Homology analyses of *LRRK2* structure have predicted several domains including a WD40 domain, a MAPKKK domain, a Ras of complex (ROC) and a C-terminal of Roc (ROC) domains, a Leucine-rich repeat domain, an ankyrin-like (ANK) domain and a number of repeats at the N-terminal that are specific for LRRK2. Many variants have been identified in LRRK2 spread throughout the different domains, but only five of them (highlighted in red) are proven to be pathogenic, whereas the others are either risk factors, or still lack definite confirmation of its pathogenicity.

Additionally, LRRK2 contains several other predicted domains encompassing repetitive sequences involved in protein-protein interaction including a one-thousand amino acid N-terminus sequence unique to LRRK2, four ankyrin-like repeats, fourteen leucine-rich repeats (LRR) and seven WD40 repeats. LRRK2 contains a second catalytic domain, a serine/threonine kinase with sequence homology to mitogen-activated protein kinase kinase kinases (MAPKKKs). This domain is separated from the Roc/COR domains by a 30 amino acids linker.

LRRK2 variants are spread throughout the different functional domains (Fig. 3), suggesting a relevant role for LRRK2 in integrating multiple signaling pathways. The presence of both enzymatic domains Roc and MAPKKK, as well as protein interaction domains, also indicates that LRRK2 might serve as a scaffold for multiprotein signaling complexes (Gandhi et al. 2008a).

LRRK2 has only one paralog gene, *LRRK1* (15q26.3), which shares identical domain architecture, only lacking the N-terminal *LRRK2*-specific repeats. For this reason it has been proposed that both genes may have diverged from a gene duplication of a common ancestral *LRRK2* ortholog (Marin 2006) and might functionally compensate for each other. *LRRK1* has been suggested as a candidate gene for PD, and gene variants have also been identified in this gene. However, no segregation of *LRRK1* variants with PD has been observed. Furthermore, pathogenic mutations in *LRRK2* seem to be more toxic than equivalent mutations in the paralog gene (Greggio et al. 2007). Therefore, it is likely that, if *LRRK1* participates in PD pathogenesis at all, it is more as a modifier rather than as a causative gene.

2.4.2 Expression and localization of LRRK2

To study the expression pattern of a protein is a crucial aspect to elucidate its patho/physiological role in the cell. LRRK2 is ubiquitously expressed throughout the human body, with the highest levels of expression in peripheral tissues such as lung, liver, heart, kidney and leucocytes (Zimprich et al. 2004b; Westerlund et al. 2008a). In the brain, several studies in humans and rodents have identified *LRRK2* mRNA and protein in the nigrostriatal dopaminergic pathway, with particularly high levels in dopamine-innervated areas including striatum, cortex and olfactory bulb. In contrast, modest levels have been found in dopaminergic neurons of the SNpc. In addition, LRRK2 protein has also been detected in non-dopaminergic areas such as septal

nucleus, hippocampus, and thalamus (Galter et al. 2006; Melrose et al. 2006; Simon-Sanchez et al. 2006; Taymans et al. 2006; Higashi et al. 2007). Expression of LRRK1 is similar to that of LRRK2, being widely expressed in brain, especially in the frontal cortex and hippocampus. In-situ hybridization shows a complementary pattern of *Lrrk1* and *Lrrk2* mRNA expression in rodents. Whereas *Lrrk1* mRNA reaches the highest levels in the striatum in pre-natal rodents, there is a simultaneous increase of *Lrrk2* and a decrease of *Lrrk1* during postnatal development (Westerlund et al. 2008a). This temporal pattern suggest a relevant role of *LRRK1* in early development.

Intracellularly, both LRRK1 and LRRK2 proteins are expressed diffusely in the cytoplasm. Even tough the protein does not contain any transmembrane domain itself (Biskup et al. 2006), LRRK2 associates to vesicular and membranous structures, being found in lipid rafts, Golgi, plasma membrane, synaptic vesicles, mitochondria and endoplasmic reticulum (Hatano et al. 2007). Altogether, these data suggests a prominent role of LRRK2 in the regulation of synaptic function, probably through vesicle synthesis and transport or through regulation of membranous structures or protein turnover.

2.4.3 Functional activity of LRRK2

The presence of both a kinase domain and a GTPase domain is unusual in the proteome and suggests a functional link between both activities that might be of relevance for the pathogenesis of PD. Most common LRRK2 PD-associated mutations occur within these domains, underlining the potential importance of LRRK2 enzymatic output in the pathogenesis of the disease. For this reason, the vast majority of the studies that try to elucidate LRRK2 physiological and pathogenic function are based on the function of these two predicted domains.

2.4.3.1 Kinase activity

Initial *in silico* analyses of LRRK2 kinase domain found the closest homology to mixed-lineage kinases (MLK), a subfamily of MAPKKKs that phosphorylates serine/threonine residues. Accordingly, LRRK2 protein exhibits detectable kinase activity *in vitro* in assays for both autophosphorylation and phosphorylation of the generic substrate myelin-basic protein (MBP) (West et al. 2005; Gloeckner et al. 2006; Smith et al. 2006). However, LRRK2 deviates from the critical consensus residues that define the MLKs subfamily, conferring unique characteristics to LRRK2 (West et al. 2007). Inactivating a number of functional residues in the kinase domain of LRRK2 such as the ATP-binding site (Lys1906), the proton acceptor site (Asp1994) and the Mg^{2+} binding loop in the activation segment (Asp2017/Tyr2018), has been described to impair LRRK2 kinase activity (Moore 2008). Besides those critical catalytic residues, kinase activity for many protein kinases is also regulated by phosphorylation of the activation segment. Accordingly, LRRK2 kinase activity seems to be regulated by phosphorylation on residues Thr2031, Ser2032 and Thr2035 within the activation segment (Ito et al. 2007; Luzon-Toro et al. 2007; West et al. 2007).

The common mutation G2019S, that changes the highly conserved motif DYG within the activation segment of LRRK2 kinase domain, has been shown to significantly increase *in vitro* autophosphorylation and MBP phosphorylation by 3-fold compared to wild-type LRRK2 (West et al. 2005; Gloeckner et al. 2006; Greggio et al. 2006). This effect is consistent with the dominant mode of inheritance and suggests a toxic gain-of-function mechanism probably involving misregulation of LRRK2 kinase activity. The effects of other LRRK2 mutations on kinase activity are, however, more controversial. The R1441C mutation in the Roc domain has been shown to increase LRRK2 kinase activity in some studies, but no significant change in others.

Similarly, the I2020T mutation has been shown to either slightly increase or even decrease kinase activity (Gloeckner et al. 2006; Greggio et al. 2006; Jaleel et al. 2007; West et al. 2007). These conflicting results are probably due to methodological differences among the groups, and the actual effect of these mutations has to be further clarified.

2.4.3.2 GTPase activity

LRRK2 Roc domain shares sequence homology to small GTPases of the Ras superfamily. Multiple studies have revealed that LRRK2 can bind GTP *in vitro* through the predicted guanine nucleotide phosphate-binding loop (P-loop) (Smith et al. 2006; Ito et al. 2007; Lewis et al. 2007; Li et al. 2007; West et al. 2007). Disruption of the critical residues Lys1347 and Thr1348 in this region impair GTP binding, that can be also displaced *in vitro* by competition with free GDP, GTP or the non-hydrolyzable analog GTPγS (Moore 2008). *In vitro* GTP hydrolysis activity of LRRK2 has been repeatedly reported to be very low (Ito et al. 2007; Lewis et al. 2007; Li et al. 2007). However, LRRK2 purified from transgenic mice brain shows high levels of GTPase activity (Lewis et al. 2007). These apparently inconsistent results might be explained by the lack of the specific guanine nucleotide exchange factors (GEFs) or guanine activating proteins (GAPs) in the *in vitro* experiments. It is of interest that, whereas kinase activity does not seem to influence GTP binding or hydrolysis, GTP binding to the Roc domain is required for and regulates LRRK2 kinase activity (Guo et al. 2007; Ito et al. 2007). Mutations close to the GTPase domain such as R1441C and R1441G, reduce GTP-hydrolysis activity and prolong the GTP-bound state that, in turn, enhances LRRK2 kinase activity (Guo et al. 2007; Lewis et al. 2007; Li et al. 2007). Kinase activity can be therefore substantially stimulated by non-hydrolysable GTP analogs, whereas treatment with GDP significantly represses it.

The structure of the Roc domain reveals a dimeric GTPase and, most probably, impairment of GTPase activity by mutations in this domain occurs by destabilization of the GTPase dimer (Deng et al. 2008). Altogether, these studies suggest that LRRK2 domains act as an intramolecular signaling cascade whose downstream targets are still unknown.

2.4.4 Biological effects of LRRK2

Great efforts have been done in order to understand the biological and pathogenic function of LRRK2, but only very limited information has been collected to date. While several groups, including ours, could not detect obvious signs of toxicity, some laboratories have reported toxicity of mutant LRRK2 in neuronal cell cultures (Smith et al., 2005; Greggio et al., 2006; MacLeod et al., 2006; Smith et al., 2006; West et al., 2007). However, all these models rely on acute over-expression of LRRK2 that could be exposed to additional stressors, which can enhance LRRK2 toxicity (West et al., 2007), so the toxic effect of LRRK2 should be further investigated in different cell culture systems as well as *in vivo*. So far, most robust piece of evidence supports a role for LRRK2 in neurite outgrowth, since expression of G2019S mutant in primary cortical neurons leads to a dramatic reduction in neurite length and branching morphology compared to the wild-type (MacLeod et al. 2006; Plowey et al. 2008).

Additionally, a number of *in vivo* models in *C.elegans*, *D. melanogaster* and mice have been developed to study the patho/physiological function of LRRK2. The three published *D. melanogaster* models provide controversial information. One model over-expressing the G2019S mutant and a loss-of-function model of *dLrrk*, the *LRRK2* paralog in *D. melanogaster*, report retinal degeneration, selective loss of dopaminergic neurons, motor impairment and reduced lifespam (Lee et al. 2007; Liou et al. 2008).

In contrast, a loss-of-function model lacking the dLrrk kinase domain resulted in increased oxidative stress sensitivity to H_2O_2 with no sings of neurodegeneration (Wang et al. 2007; Liou et al. 2008). *C. elegans* over-expression models point towards a role of LRRK2 in regulating stress response and neurite outgrowth (Wolozin et al. 2008; Samann et al. 2009), whereas the paralog gene, *lrk-1*, seems to be involved in regulating synaptic vesicle proteins polarity in a knockout model (Sakaguchi-Nakashima et al. 2007). Recent phylogenetic analyses of the COR, Roc and kinase domains of LRRK2 demonstrate that LRRK2 emerged from a duplication that was prior to the acquisition of the specific LRRK2-repeats at the N-terminal of the protein. Paralogs of *LRRK2* such as *LRRK1* or protostome *LRRK* encoded proteins lack this repeat, suggesting that *D. melanogaster* or *C. elegans* may not be good models to understand human LRRK2 function. Unfortunately, none of the rodent models developed so far (knock in, knockout and transgenic for LRRK2) show any clear LRRK2-associated phenotype (Li et al. 2007; Melrose et al. 2007; Melrose 2008; Wang et al. 2008).

Despite very limited *in vivo* information about LRRK2 function is available, strong evidence suggests a crucial role of LRRK2 kinase activity in the pathogenesis of the disease, probably by regulating cytoskeletal signaling pathways and neurite outgrowth.

2.4.5 LRRK2 substrates and interactors

Since kinase activity seems to be a key effector in the normal and pathogenic function of LRRK2, the identification and characterization of LRRK2 interactors and substrates of its kinase activity would provide the best way to understand the molecular pathogenesis of LRRK2-associated PD. Interestingly, most of the LRRK2-interacting proteins identified till date are cytoskeleton and trafficking proteins.

LRRK2 has been reported to interact *in vitro* with α/β-tubulin, vimentin, clathrin (Dachsel et al. 2007; Gandhi et al. 2008b), and with Rab5a, a protein that regulates synaptic vesicle endocytosis (Shin et al. 2008). LRRK2 has been also demonstrated to *in vitro* phosphorylate moesin, a cytoskeletal protein belonging to the erzin-radixin-moesin family, at the Thr558 residue (Jaleel et al. 2007). However, *in vivo* validation of this substrate is still required.

Interaction with the chaperone protein Hsp90, together with its co-chaperone Cdc37, was identified in yeast by tandem affinity purification (TAP) tag technique (Gloeckner et al. 2006). Hsp90 interacts with and maintains LRRK2 stability, since Hsp90 inhibitors can destabilize transfected and endogenous LRRK2 (Wang et al. 2008). LRRK2 has been also shown to phosphorylate in human and *D. melanogaster* the eukaryotic initiation factor 4E (eIF4E)-binding protein (4E-BP), a key mediator of stress response. LRRK2 stimulation of eIF4E-mediated protein translation, seems to attenuate resistance to oxidative stress and survival of dopaminergic neurons in *D. melanogaster* (Imai et al. 2008). However, since 4E-BP is a frequently detected substrate upon protein over-expression, these results have to be cautiously interpreted.

Finally, endogenous human LRRK2 seems to strongly interact with itself through the Roc domain forming dimmers. Indeed, LRRK2 has been shown to appear predominantly as a dimer *in vivo,* being also able to form higher molecular weight complexes (Gloeckner et al. 2006; Luzon-Toro et al. 2007; Deng et al. 2008; Greggio et al. 2008). Although dimerization is commonly required in many protein kinases to became active, the functional relationship between LRRK2 dimerization and kinase activity, as well as a precise characterization of the mechanisms leading to LRRK2 dimerization remain unknown.

2.4.6 Mitogen-activated protein kinases (MAPKs)

The structural homology between LRRK2 kinase domain and MAPKKKs suggests some degree of functional homology between both. MAPKs are a class of highly conserved signal transduction enzymes that connect extracellular stimuli with critical regulatory targets within the cell, mediating cellular adaptation. MAPKs respond to physical and chemical stress and regulate a vast number of cellular functions including proliferation, differentiation and survival, and have been shown to play a role in regulating neural functions (Sweatt 2004; Miloso et al. 2008). The activity of MAPKs is regulated through consecutive phosphorylation of the three elements of the cascade, MAPKKK, MAPKK, and MAPK (Fig. 4). Since MAPKKKs are not directly associated to membrane receptors, they require activation (phosphorylation) through intermediate components, such as GTP-binding proteins. In the case of LRRK2, GTPase and kinase are both present in the same molecule, suggesting an intramolecular activation that may favor a tighter control of the signaling cascade.

MAPKs downstream targets can be either in the cytoplasm or in the nucleus, when the substrates are factors or transcriptional co-regulators. Mammals express, at least, three differentially regulated groups of MAPKs: extracellular signal-regulated kinases (ERK MAPK), $p38^{MAPK}$ and c-Jun N-terminal kinases (JNK) (Fig. 4). Because of the described homology of LRRK2 kinase domain to MAPKKKs, many groups have investigated the effect of LRRK2 on MAPK signaling cascades. Some have reported activation of the ERK signaling pathway, but the molecular mechanisms of action have not been precisely characterized. One study demonstrates mediation of the ERK pathway in neurite shortening and autophagy in G2019 differentiated SH-SY5Y cells (Plowey et al. 2008). Over-expression of LRRK2 in HEK293 and SH-SY5Y cell lines has been reported to confer increased tolerance against H_2O_2-induced oxidative stress mediated through activation of the ERK MAPK pathway.

In this model, LRRK2 mutants were unable to appropriately activate the pathway, leading to cell death (Liou et al. 2008). LRRK2 expression also led to increases in total and phosphorylated c-Jun independently of kinase activity, as well as phosphorylated ERK1/2 and ERK5 in a manner independent of GTP binding (West et al. 2007). Hence, LRRK2 has not been yet consistently assigned to a specific MAPK signaling pathway and its putative function as a MAPKKK remains to be elucidated.

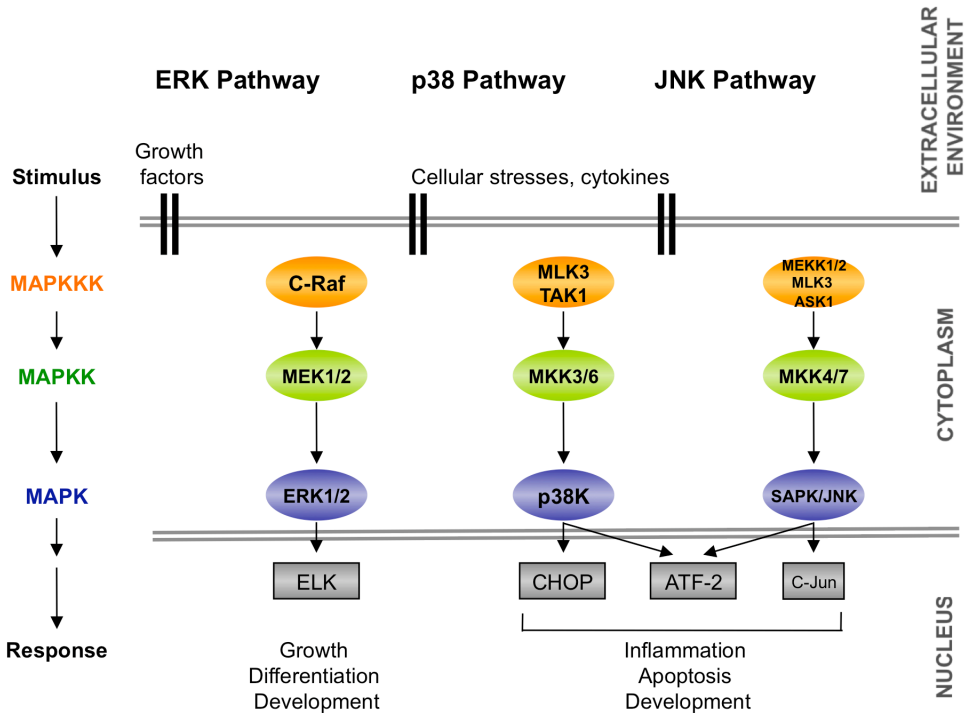


Figure 4. Mitogen-activated protein kinases (MAPKs) cascades.

MAPKs participate in critical steps in the cell response to extracellular changes. Whereas activation of the ERK MAPK cascade promotes cell proliferation, differentiation and development, activation of JNK and $p38^{MAPK}$ is mainly involved in development, inflammation and apoptosis.
Upon binding of certain stimuli to specific membrane receptors, the different components within the cascade are consecutively phosphorylated. Finally, the phosphorylated MAPK acts on its target directly in the cytoplasm or translocates to the nucleus where it phosphorylates transcription factors, typical targets of MAPKs signaling cascades.

2.5 Specific aims

The objective of this project was to investigate the implication of LRRK2 in signal transduction pathways that may be relevant to the pathogenesis of PD, as well as to identify and validate potential downstream targets of LRRK2 signaling. The specific aims of this project were:

a) to investigate the role of LRRK2 as a MAPK kinase kinase in the activation of MAPK signaling pathways.
b) to identify putative targets downstream the activated MAPK cascade.
c) to characterized the effect of LRRK2 pathogenic mutations in the identified signaling pathways.

3 RESULTS

3.1 Primary fibroblast cell cultures from PD patients to study the effect of PD-associated mutations

12 primary fibroblast lines from FPD donors were successfully established (Table 2). Outgrowth of fibroblasts started 4 weeks after skin biopsies were seeded onto 25 cm^2 flasks and reached confluent layers at about 6 weeks. However, human fibroblasts show an inverse relationship between replicative capability and donor age (Martin et al., 1970). Thus, those lines generated from younger patients, most of them carrying mutations in recessive genes leading to early-onset PD, had higher replicative capability than those lines generated from older patients with mutations in dominant genes and late-onset PD.

Table 2. Primary fibroblast cultures established from PD patients (highlighted in red appear patients with pathogenic LRRK2 mutations).

Patient ID	Birthday	Affected gene	Mutation
Tü#11	1947	LRRK2	S1228T
Tü#12	1943	LRRK2	S1228T
Tü#13	1940	LRRK2	R793M
Tü#14	1945	LRRK2	I2020T
Tü#15b	1925	LRRK2	R1441C
Tü#16	1961	LRRK2	I2020T
Tü#18	1948	PINK1	Q126P
Tü#19	1940	PINK1	Q126P
Tü#20	1935	Parkin	Del Ex3+4
Tü#21	1934	Parkin	Del Ex3+4
Tü#22	1962	DJ-1	E64D
Tü#23	1955	DJ-1	E64D

Primary fibroblast cell lines generated from *PINK1*-associated PD patients with the mutation Q126P were used to analyze changes in mitochondrial morphology. Both cell lines showed a trend towards abnormal mitochondrial morphology as compared to related control cell lines. This observation was in agreement with the results obtained after RNAi mediated downregulation of *PINK1* in HeLa cells (Exner et al. 2007). Thus, these cell lines were demonstrated to be suitable models to study basic mechanisms of PD pathogenesis.

3.2 Identification of putative phosphorylation sites in the kinase domain of LRRK2 and generation of a phospho-specific LRRK2 antibody

For many kinases, activation requires phosphorylation of the activation segment, which is defined as the sequence region between and including the two conserved tripeptide motifs DFG and APE. Within the activation segment, the activation loop is the site of regulatory phosphorylation and interaction with activity modulators. In order to identify putative phosphorylation sites in the kinase domain of LRRK2 the activation sequences from worm, fly and mouse were aligned with human *LRRK2* and its paralog gene *LRRK1*. Homology analysis revealed three phylogenetically conserved residues at the activation loop of LRRK2 that represent putative phosphorylation sites: Thr2031, Ser2032 and Thr2035 (Fig. 5A).

By alignment with several MAPKKK family members, it was found that the residue Thr2035 was particularly conserved (Fig. 5B). Indeed, this residue has been reported to be phosphorylated and essential to the activity in several MAPKKK family members including MAPKKKs 1 (MEKK1), 3 (ASK1), 4 (SSK2) and 7 (TAK1). Thus, Thr2035 was chosen to generate a LRRK2 phospho-specific antibody .

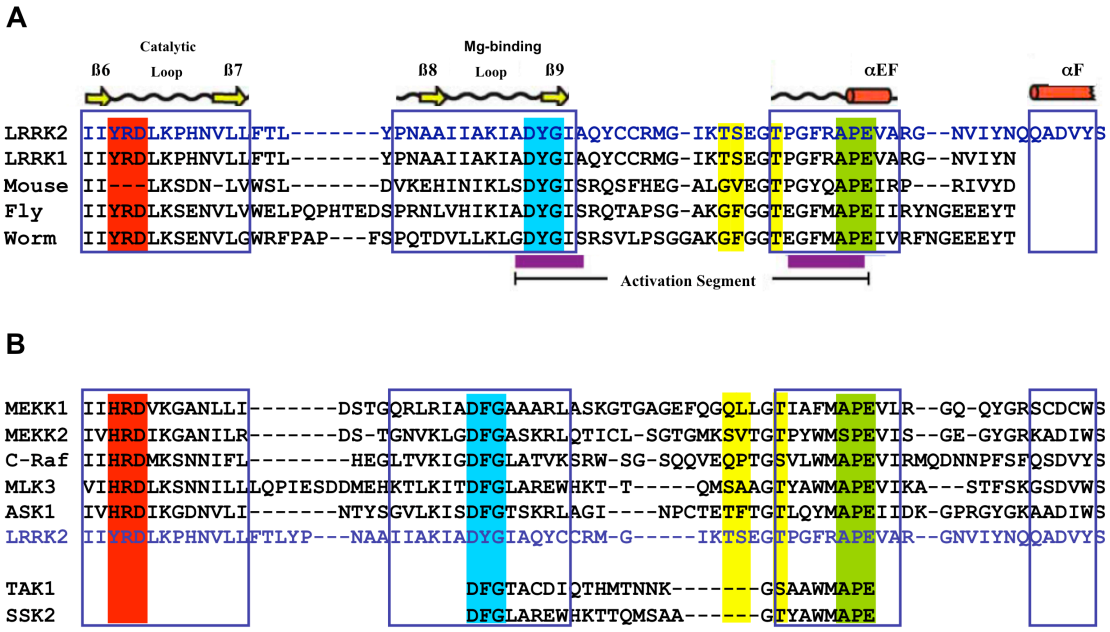


Figure 5. Identification of putative phosphorylation sites in the kinase domain of LRRK2.

For many kinases, activation requires phosphorylation of the activation segment, which is the region between and including the two conserved tripeptide motifs DFG (highlighted in blue) and APE (in green). A) Sequence alignment of the kinase activation segment of several *LRRK2* ortholog genes from human, mouse, fly and worm shows three putative phosphorylation sites within the activation segment, Thr2031, Ser2032 and Thr2035 (highlighted in yellow). Among those, the residue Thr2035 is particularly conserved in all the species.
B) Alignment of the activation segment of LRRK2 with several MAPKKKs identifies the same conserved Thr2035, a residue that has been reported to be phosphorylated and essential to the activity of some MAPKKK proteins such as MEKK1, ASK1, and SSK2. Furthermore, when this conserved threonine is substituted by any other amino acid in the proteins aligned like c-Raf and TAK1, it is exchanged by a serine, that can also be phosphorylated. This suggest a crucial role of the residue Thr2035 in the kinase activity of LRRK2.

To generate a phospho-specific LRRK2 antibody, two rabbits were immunized with the synthetic peptide KTSEGTPGFRAP, containing the putative phosphorylation site Thr2035. Sera were tested after each bleeding in western blots on whole cell lysates from HEK293 cells transiently transfected with the empty vector, [wt]LRRK2 and the synthetic mutants T2035A and T2035E. Whereas replacement of serine or threonine with alanine blocks phosphorylation on a specific site, glutamic acid substitution mimics the structure of a phosphorylated serine or threonine residue allowing the detection of increased or decrease phospho-protein levels respectively.

Although the phospho-LRRK2 specific antibody detected a faint band at the expected size of LRRK2 in HEK293 cells, no significant difference could be observed between transfection with [wt]LRRK2 and the mutants T2035A and T2035E (Fig. 6A).

Immunoprecipitation of transfected [wt]LRRK2 with anti-HA agarose beads followed by immunoblot with the phospho-specific LRRK2 antibody detected a faint band at the expected size of LRRK2 but, given the high background, further validation of the antibody is required (Fig. 6B).

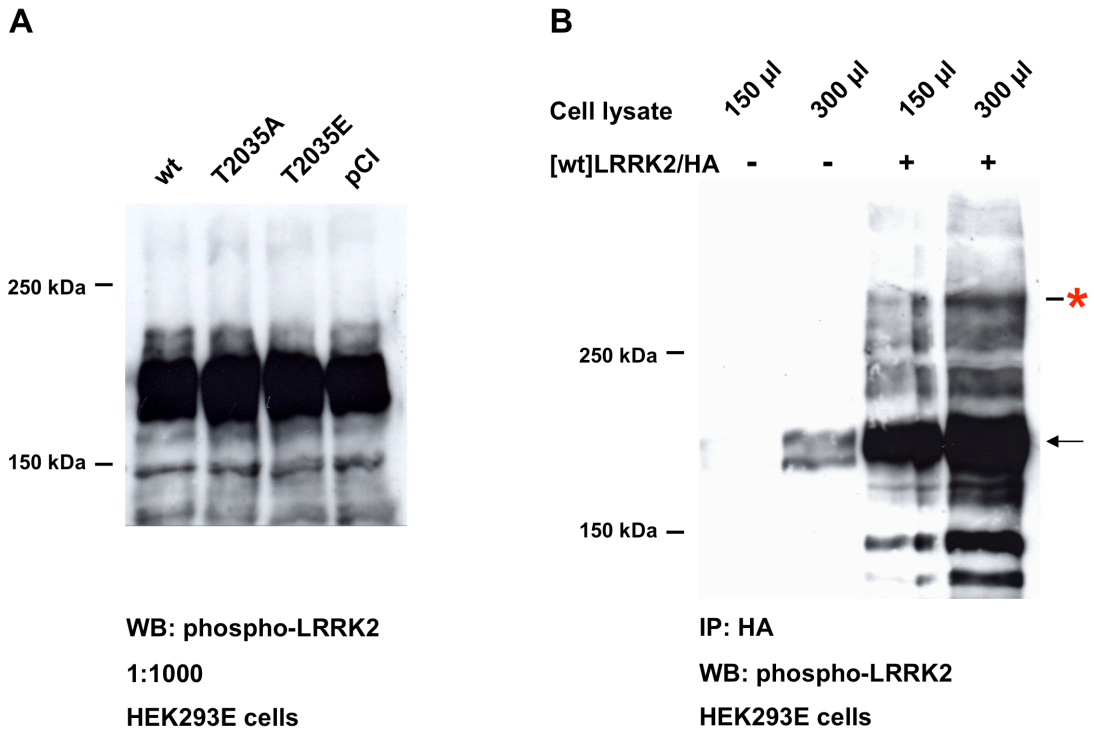


Figure 6. Characterization of the phospho-specific LRRK2 antibody in HEK293 cells.

A) HEK293 cells were transfected with [wt]LRRK2 or with the mutants T2035A, that blocks phosphorylation, and T2035E, that mimics phosphorylation. Immunoblot with the phospho-specific LRRK2 antibody did not show any difference between both mutants or with [wt]LRRK2 transfected cells. B) HEK293 cells transfected with [wt]LRRK2 were subjected to immunoprecipitation with anti-HA agarose beads followed by immunoblot with the phospho-specific LRRK2 antibody. A faint band was detected at the expected size (red asterisk) but, given the high background, further validation of the antibody is required.

3.3 Expression of LRRK2

LRRK2 expression was assessed by immunoblot 48h after transfection of HEK293 cells with [wt]LRRK2/HA. An “in house” rabbit polyclonal antibody directed against the middle (MID) region located between the ankyrin-like repeat and the leucine-rich repeat (LRR) domains of human LRRK2 (C.L.Klein, G.Rovelli, W.Springer, C.Schall, T.Gasser, and P.J.Kahle, manuscript submitted) detected a strong band at approximately 280kDa in transfected cells that was confirmed by re-probing the membrane with the antibody against the HA-tag (Fig. 7). We also detected a faint band in mock-transfected cells likely corresponding to endogenous LRRK2 (Fig. 1B). No signs of LRRK2 aggregation or degradation were observed.

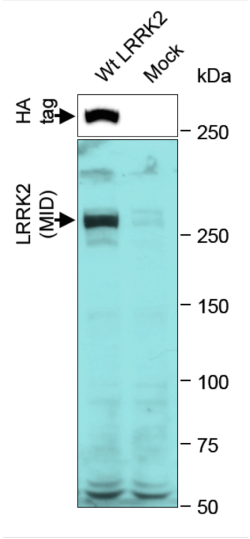


Figure 7. Expression of LRRK2 in HEK293 cells.
Extracts of HEK293 cells untransfected or transiently transfected for 48 h with [wt]LRRK2-HA were run on a 6% acrylamide gel and transferred into a PVDF membrane. LRRK2 protein was detected at the expected size of approximately 280 kDa by both HA-specific and LRRK2-specific antibodies. The MID-LRRK2 antibody detected also a faint band at this size in lysates from untransfected HEK293 cells, probably corresponding to endogenous LRRK2.

3.4 LRRK2 selectively activates the ERK pathway in a kinase-dependent manner

Because the kinase domain of LRRK2 shares homology to MAPKKK and these enzymes usually stimulate at least one of the three classical MAPK cascades, ERK1/2, $p38^{MAPK}$ and JNK, we investigated the basal phosphorylation state of these

pathways in response to LRRK2 over-expression 48h after transient transfection of HEK293 cells with [wt]LRRK2, the most common PD-associated mutant [G2019S]LRRK2 and the kinase-dead mutant [K1906N]LRRK2. Cell lysates were analyzed by immunoblot with antibodies recognizing only the activated dually phosphorylated form of the specific MAPK from each pathway. Over-expression of LRRK2 led to increased ERK1/2 phosphorylation, whereas no significant change was observed in the basal levels of phospho-p38^{MAPK} and phospho-JNK (Fig. 8). Total protein levels of ERK1/2, p38^{MAPK} and JNK remained unaltered.

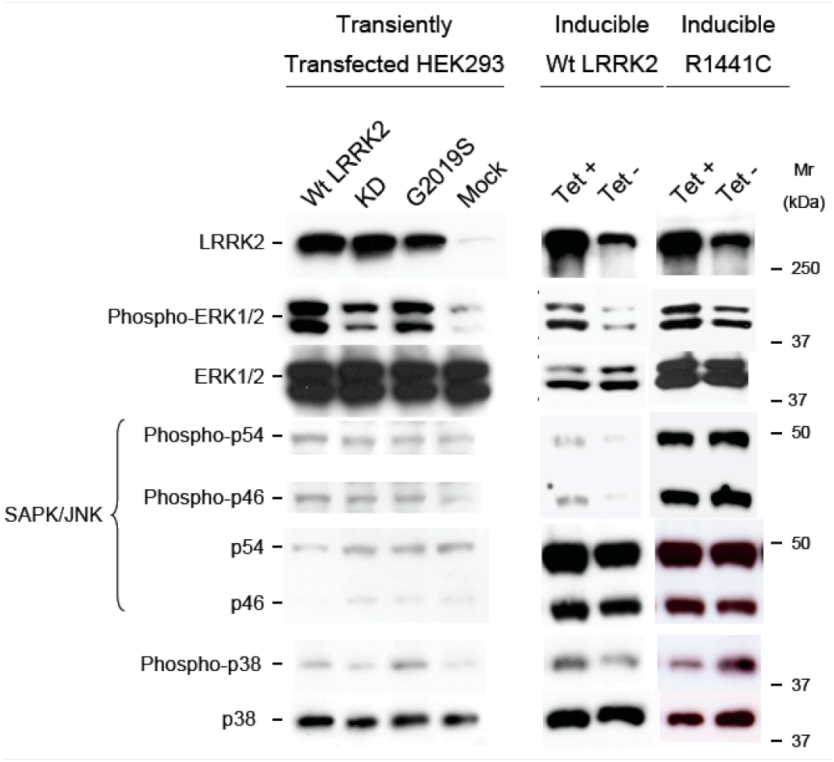


Figure 8. Activation of ERK1/2 in response to LRRK2 expression.

Equal amounts of protein cell lysates from LRRK2-inducible or transiently transfected HEK293 cells were loaded on 7.5 % acrylamide gels and subjected to immunoblot analysis using specific antibodies for activated dually phosphorylated ERK1/2, JNK and p38MAPK. Membranes were stripped and reprobed with antibodies against total MAPK proteins. Comparable LRRK2 expression levels were assessed with the specific LRRK2 antibody. LRRK2 induction was achieved by treatment with 1 µg/ml tetracycline for 48h. Immunoblots are representative from 3 independent experiments.

Moreover, ERK stimulation was dependent on LRRK2 kinase activity, since transfection with the kinase-dead LRRK2 mutant resulted in decreased phospho-ERK1/2 signal, comparable to mock transfected cells (Fig. 8). The mutant G2019S did not further enhance ERK1/2 phosphorylation in comparison to [wt]LRRK2 (Fig. 8).

Stimulation of the ERK pathway by LRRK2 was further validated in two LRRK2-inducible Tet-On HEK293 cell lines expressing [wt]LRRK2 and the PD-associated R1441C mutant. Again, tetracycline-induced expression of LRRK2 in cells led to increased levels of phospho-ERK1/2 compared to the basal levels in non-treated cells. Although some mild differences were observed in phospho-$p38^{MAPK}$ or phospho-JNK between induced and non-induced [wt]LRRK2 cells, they were neither as strong as in phospho-ERK nor consistent in both cell systems. No significant differences were observed in the total protein levels, apart from inducible R1441C cells that showed increased levels of total and phospho-JNK independently of LRRK2 expression, since they are also high in non-induced cells (Fig. 8).

3.5 PD-associated LRRK2 mutations delay ERK activation

The duration and type of stimulus that activate the ERK cascade can lead to different activation kinetics and subcellular localization of ERK, generating branch points in the pathway that can mediate different functions (Pouyssegur et al. 2002). To investigate the kinetics of ERK activation upon LRRK2 expression we treated the [wt]LRRK2-inducible cell line with 1µg/ml tetracycline for 0 to 48h and cell lysates were subjected to immunoblot and probed against phospho-ERK1/2. Phosphorylation of ERK1/2 peaked at 12h after induction, interestingly corresponding with first detectable LRRK2 expression, and remained highly phosphorylated over time (Fig. 9A).

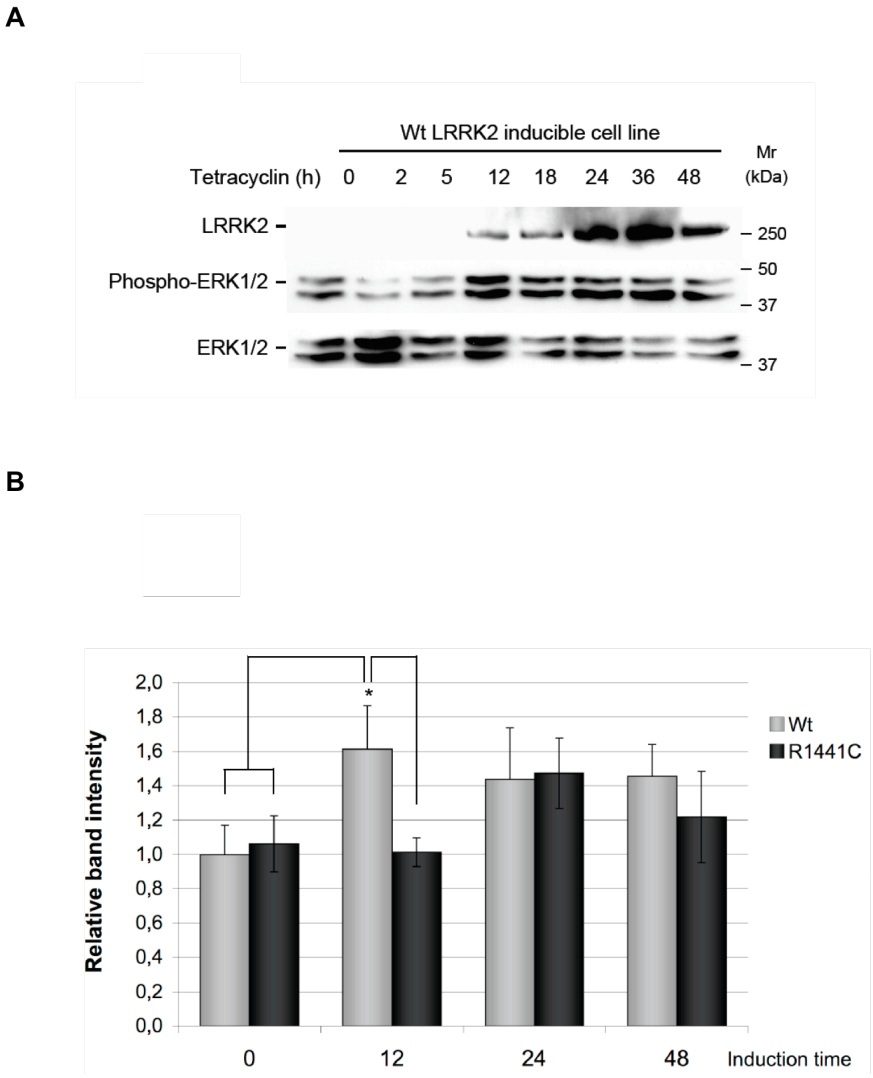


Figure 9. Time course of ERK1/2 activation by LRRK2.

A) LRRK2 inducible HEK293 cells were treated with tetracycline for the indicated times and subjected to immunoblot analysis. The samples were probed for activated phospho-ERK1/2 and total ERK1/2, showing an ERK1/2 activation peak at 12h.
B) Relative densitometry intensities for phospho-ERK1/2 immunoreactivity. [wt]LRRK2 and [R1441C]LRRK2 inducible cell lines were exposed to tetracycline for the indicated times and whole cell lysates were subjected to immunoblot and probed for phospho- and total ERK1/2. Overexpression of the PD-causative mutation R1441C led to a delayed activation of ERK1/2 compared to [wt]LRRK2. Each value is averaged from 4 independent experiments. Mock=transfection with the empty vector pCI/HA.

We next sought to determine whether LRRK2 mutant R1441C influenced the temporal activation profile of ERK1/2. R1441C inducible cells were treated with tetracycline for 0, 12, 24 and 48h, the cell lysates immunoblotted and probed against phospho-ERK1/2 and analyzed by band densitometry.

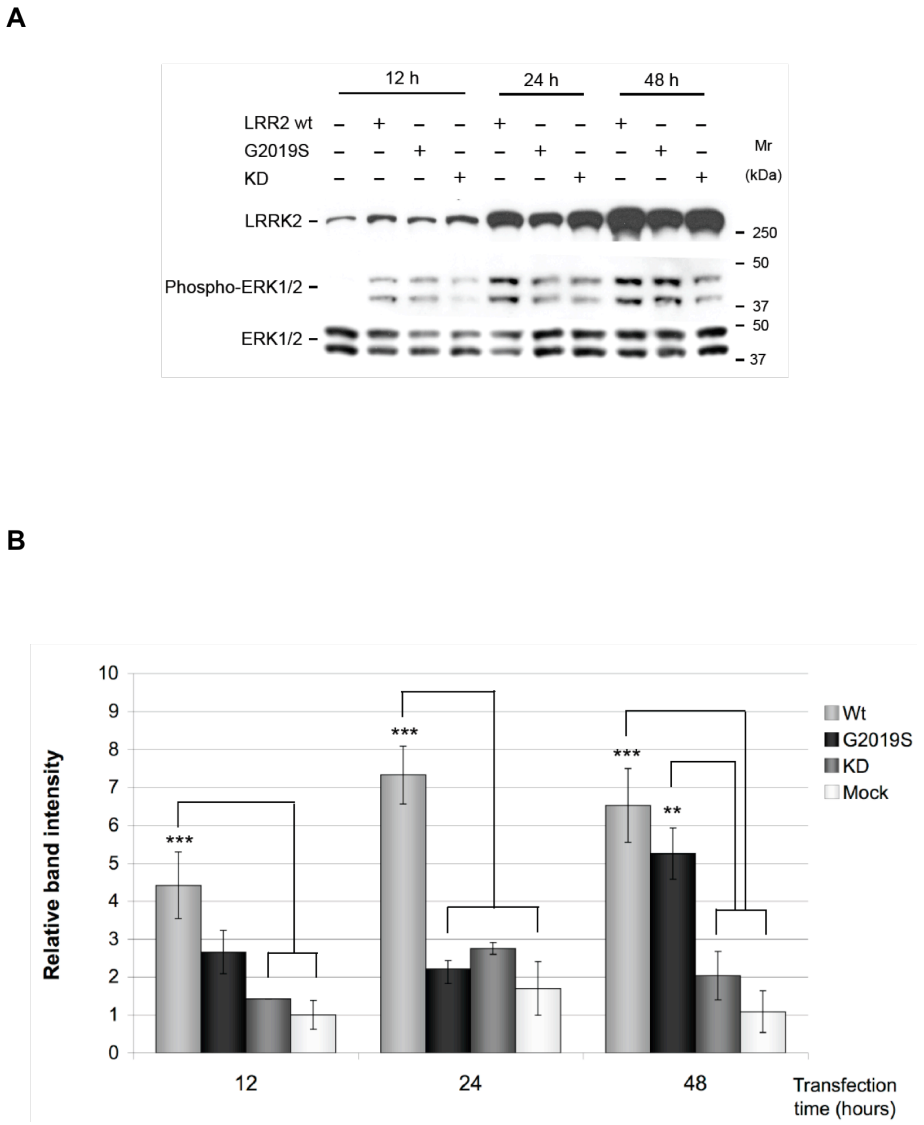


Figure 10. Delayed activation of ERK1/2 by LRRK2 mutant G2019S.

A) HEK293 cells were transfected with wild-type and G2019S mutant LRRK2 for 12, 24 and 48h and subjected to immunoblot analysis. The samples were probed for total and phospho-ERK1/2
B) Relative band intensity analysis intensities for phospho-ERK1/2 immunoreactivity in wild-type, G2019S and kinase dead LRRK2 at the indicated time points after transfection. Each value is averaged from 4 independent experiments. Mock=cells transfected with the empty vector pCI/HA.

Compared to [wt]LRRK2, over-expressed R1441C LRRK2 exhibited a significant delay of ERK1/2 phosphorylation (Fig. 9B). Thus, levels of active phospho-ERK1/2 after 12h induction are about 60% higher in [wt]LRRK2-inducible cells than in R1441C mutants that were able to phosphorylate ERK1/2 only after 48h induction

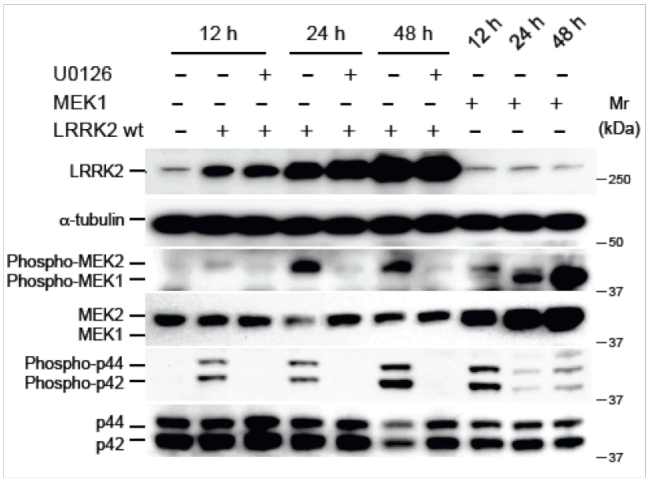
(relative intensity [RI]; wt_12h 1.611±0.254, n=6, vs wt_0h 0.996±0.171, n=5; p=0.0476; wt_12h 1.611±0.254, n=6, vs R1441C_12h 1.011±0.084, n=7; p=0.0355).

To determine whether the kinase mutant G2019S also led to a slower time course of ERK1/2 phosphorylation we transiently transfected G2019S LRRK2 in HEK293 cells. As in R1441C cell lines, phosphorylation of ERK1/2 by G2019S LRRK2 was also delayed to 48h (Fig. 10A). Band densitometry analysis of phospho-ERK1/2 showed a 4.5-fold difference between [wt]LRRK2 and mock transfected cells at the early time point of 12h (relative intensity [RI]; wt 4.417±0.876, n=4, vs Mock 1.000±0.379, n=4; p=0.0002). Active phospho-ERK1/2 in [wt]LRRK2-transfected cells increased over time compared to the basal levels with a maximum peak of about 4-fold at 24h (relative intensity [RI]; wt 7.324±0.763, n=4, vs Mock 1.698±0.706, n=4; p=0.0001). In contrast, transfection of HEK293 cells with the mutant G2019S only showed a significant increase in ERK1/2 phosphorylation after 48h (relative intensity [RI]; G2019S 5.252±0.671, n=3, vs Mock 1.088±0.551, n=3; p=0.0086), suggesting an impaired ability of this mutant to elicit a fast activation of the ERK cascade (Fig. 10B). Transfection with the kinase dead version of LRRK2 lead to levels of phospho-ERK1/2 comparable to mock transfection at all the different time points (Fig. 10B).

3.6 PD-linked mutations also delay phosphorylation of MEK, upstream activator of ERK

We next sought to determine whether LRRK2 was also able to induce phosphorylation of the upstream MAPK/ERK kinases (MEK). HEK293 cells were transiently transfected with [wt]LRRK2 for 12, 24 and 48h, immunoblotted and probed against phospho-MEK1/2 and phospho-ERK1/2.

A



B

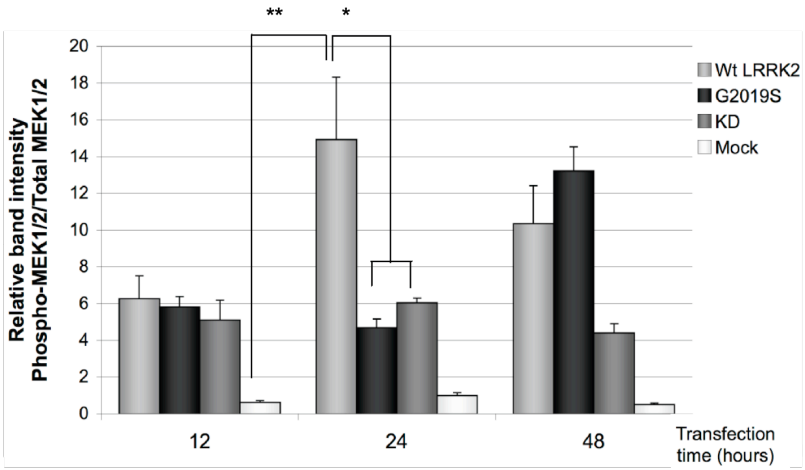


Figure 11. Delayed activation of the specific ERK1/2 upstream kinase MEK2.

A) HEK293 cells were transfected for 12, 24 and 48 h with the indicated LRRK2/HA constructs or with MEK1, non-treated or treated with the pharmacological inhibitor of the MEK/ERK kinase pathway U0126. Cell lysates were immunoblotted and probed for phospho- and total-MEK1/2 and for phospho- and total ERK1/2. B) Relative band densitometry for phospho-MEK1/2 in transiently transfected HEK293 cells. Image is representative from 3 independent blots. Mock=cells transfected with the empty vector pCI/HA.

The temporal pattern of MEK phosphorylation by [wt]LRRK2 exactly corresponded to the pattern of activated ERK1/2 shown before, with a maximum peak at 24h (Fig. 11A).

Transfection with MEK1 was used as a positive control for activation, showing that LRRK2 capability to activate the ERK pathway was comparable to that of the specific ERK1/2 activator MEK1 (Fig. 11A). Phospho-MEK1 could only be observed after transfection with the MEK1 construct because endogenous MEK1 protein was barely expressed in our cell system. However, phosphorylation of endogenous MEK2 was easily detected after LRRK2 over-expression (Fig. 11A). Treatment with the selective MEK1/2 inhibitor U0126 at the concentration of 10 μM for 12h prior to harvesting efficiently prevented phosphorylation of both MEK and ERK (Fig. 11A) and was therefore used in subsequent experiments.

Band densitometry analysis of at least 3 independent experiments allowed us to quantify MEK1 phosphorylation in cells transfected with the indicated LRRK2 constructs (Fig. 11B), showing a 4-fold increase in phospho-MEK1 24h after transfection with [wt]LRRK2 compared to basal MEK1/2 levels or to cells transfected with the mutant G2019S or with the kinase dead constructs (Fig. 11B) (relative intensity [RI]; wt 14.915±3.388, n=3, vs G2019S 4.677±1.689, n=3; p=0.0282. wt vs Mock 1.00±0.600, n=4; p=0.0051). The activation of MEK1 elicited by the mutant G2019S was delayed to 48h (Fig 11B), consistent with the activation observed in the downstream MAPK ERK1/2.

3.7 LRRK2 over-expression stimulates *SNCA* transcription in a kinase dependent manner

To test the hypothesis of a putative transcriptional regulation of SNCA by LRRK2 we quantified endogenous levels of SNCA mRNA by real time RT-PCR. Total mRNA was extracted 48h after transfection of HEK293 cells with [wt]LRRK2, G2019S or the kinase-dead mutant K1906N.

Over-expression of [wt]LRRK2 significantly increased the levels of endogenous *SNCA* mRNA approximately 75% compared to mock transfected cells (relative quantity [RQ]; wt 1.756±0.136, n=23, vs mock 1.000±0.2849, n=12; p=0.0006) (Fig. 12). The kinase dead mutant K1906N was not able to stimulate *SNCA* transcription over the basal levels, indicating that *SNCA* induction was dependent on LRRK2 kinase activity (Fig. 12).

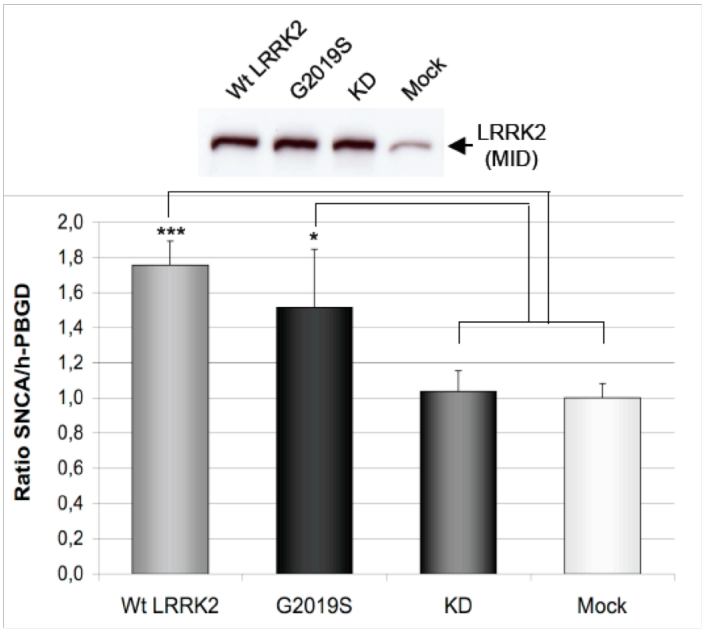


Figure 12. Upregulation of *SNCA* mRNA by LRRK2.

Endogenous mRNA levels of SNCA in HEK293 cells 48 h after transfection with the indicated LRRK2/HA constructs were determined by real time qRT-PCR. Total RNA was purified on spin columns from cell lysates and quantified in the LightCycler with specific hybridization probes for SNCA. Results are given as the ratio between SNCA and the housekeeping gene hPBGD. Comparable expression of the LRRK/HA variants is demonstrated by immunoblot against LRRK2 in 6% acrylamide gel.

Transfection with the mutant G2019S up-regulated SNCA to approximately the same extent as [wt]LRRK2, but no further induction was reached, in agreement with our observations in the activation of the ERK module. The kinase dead mutant K1906N was not able to stimulate *SNCA* transcription over the basal levels, indicating that *SNCA* induction was dependent on LRRK2 kinase activity (Fig. 12).

Transfection with the mutant G2019S up-regulated SNCA to approximately the same extent as [wt]LRRK2, but no further induction was reached, in agreement with our observations in the activation of the ERK module.

Up-regulation of *SNCA* by LRRK2 was also consistently observed at the protein level by enzyme-linked immunosorbent assay (ELISA). Relative SNCA protein levels in cells transfected with [wt]LRRK2 are mildly but highly significantly increased compared to mock transfected cells (relative quantity [RQ]; wt 109.127±3.151, n=11, vs Mock 100.000±1.994, n=10; p=0.0273) (Table 3). Neither mutant G2019S nor kinase dead LRRK2 led to any significant induction of SNCA at the protein level. No significant differences in total protein were detected.

Table 3. SNCA protein upregulation by LRRK2.

Expression of SNCA protein after 48 h transfection with wild-type, G2019S and kinase dead LRRK2 constructs and with the empty vector (mock) was determined by a specific ELISA. Results are given as the ratio between SNCA and total protein.

Construct	Ratio SNCA/total protein	SD	SEM	n	p-value
[Wt]LRRK2	109,1273	10,4509	3,1510	11	0,0273*
[G2019S]LRRK2	101,2743	17,5976	6,2217	8	0,8334
[K1906N]LRRK2	105,9705	23,1845	8,1970	8	0,4443
Mock (empty vector)	100,0000	6,3068	1,9943	10	

3.8 ERK activation mediates LRRK2-induced transcriptional up-regulation of *SNCA*

We showed that over-expressed LRRK2 was involved in transcriptional regulation of *SNCA* and, in parallel, was also able to stimulate the ERK pathway in HEK293 cells. It has been previously described that *SNCA* mRNA and protein levels are induced in PC12 after prolonged treatment with growth factors, and this signaling

has been demonstrated to be dependent in part on activation of the ERK pathway (Clough and Stefanis, 2007). Therefore we studied whether ERK signaling was also mediating transcriptional regulation of *SNCA* by LRRK2. Total mRNA was extracted from HEK293 cells transfected with [wt]LRRK2 and G2019S LRRK2 and treated or not with the inhibitor U0126 for 12h. Endogenous *SNCA*-specific mRNA was quantified by real time RT-PCR. *SNCA* mRNA levels peaked simultaneously to MEK/ERK activation, showing a relative increase of 40% compared to mock transfected cells (Fig. 13). Furthermore, LRRK2-induced up-regulation of *SNCA* could be efficiently repressed by treatment with the pharmacological inhibitor of the ERK pathway U0126, suggesting that in our system *SNCA* transcriptional regulation elicited by LRRK2 is also mediated by activation of the ERK/MAPK pathway.

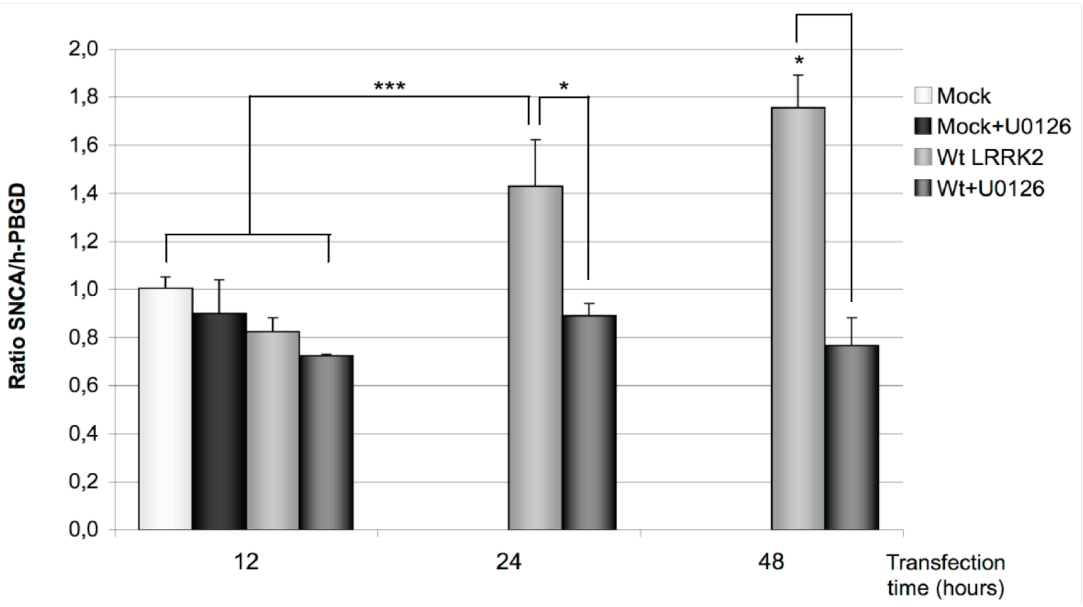


Figure 13. The MEK inhibitor U0126 represses lRRK2-mediated induction of SNCA.

HEK293 cells transiently transfected with different LRRK2 constructs were treated with 10 µg/ml U0126 or vehicle (DMSO) for 12 h prior to harvesting. Total mRNA was extracted and SNCA specific mRNA quantified by real time RT-PCR to measure the induction levels of SNCA. Similar results were achieved in 3 independent experiments.

4 DISCUSSION

The study of monogenic forms of PD has uncovered a variety of pathways and mechanisms underlying dopaminergic cell death whose convergence at one or more levels has been largely discussed. The actual observation that mutations in the gene *LRRK2* can lead to a wide range of pathologies reinforced this hypothesis by indicating a potential upstream role of the gene in protein aggregation. SNCA deposition is the neuropathological hallmark of PD, but no study up to date has been able to provide experimental evidence how LRRK2 could affect this pathological process. The present study reveals a novel pathway that integrates the two dominant PD-causative genes *LRRK2* and *SNCA*. Here we show for the first time that LRRK2 is able to up-regulate *SNCA* transcription in HEK293 cells in a kinase-dependent manner via specific activation of the MAPK/ERK cascade. Furthermore, expression of the pathogenic LRRK2 mutants R1441C and G2019S led to delayed activation and propagation of the signal. *LRRK2* is a common cause of dominant parkinsonism, whereas *SNCA*, regarded as a rare cause of disease, plays a central role in both familial and sporadic cases as major component of LBs. Hence, deciphering the pathway linking both genes could be highly valuable to understand the pathogenesis of PD and to identify new potential therapeutic targets.

4.1 Functional link between the dominant PD-associated genes *LRRK2* and *SNCA*

LRRK2 has been recently reported to co-localize with early stages of aggregating SNCA in lower brainstem of PD and DLB patients (Alegre-Abarrategui et al. 2008), suggesting that LRRK2 dysfunction might contribute to the early formation of LBs. The mechanisms involved in this process are, nevertheless, completely unknown.

According to our results, these mechanisms might imply transcriptional regulation of *SNCA* by LRRK2, since over-expressed wild-type and G2019S LRRK2 stimulate by almost 2-fold endogenous *SNCA* transcription in transiently transfected HEK293 cells. These results are consistent with a previous report showing co-regulation of *LRRK2* and *SNCA* mRNAs in rodent striatum (Westerlund et al. 2008b), indicating the existence *in vivo* of common regulatory mechanisms for both genes.

Transcriptional stimulation of *SNCA* by LRRK2 was also translated into a mild but significant increase in SNCA protein expression. The inability of LRRK2 dead kinase to elicit *SNCA* induction in our cell system shows that the effect is kinase-dependent. Since genetic regulation of SNCA expression levels is linked to the development of neuropathology, induction of SNCA by LRRK2 points towards a potential pathogenic mechanism in patients with LRRK2-associated PD, involving SNCA up-regulation. For example, multiplications of the *SNCA* locus have been shown to cause familial PD (Singleton et al. 2003) and SNCA over-expression has been extensively demonstrated to contribute to disease. Thus, modest but chronic up-regulation of *SNCA* due to mutations in *LRRK2* within or outside the kinase domain, might regulate SNCA protein levels, leading to differences in expression or local concentration that could modulate disease course or pathology, as it has been postulated to explain the genetic association of the variability in the promoter region of *SNCA* and PD.

4.2 MAPKs-mediated signaling from LRRK2 to SNCA

Tracking down signaling from LRRK2 to SNCA might be crucial in order to understand *LRRK2*-associated pathogenesis and to further characterize this pathway. Since the kinase domain of LRRK2 shares structural homology with MAPKKKs we investigated whether any of the classical MAPK cascades ERK, p38 or

JNK were activated by LRRK2. These pathways play a critical role in cell death and survival and have been repeatedly reported to be involved in different neurodegenerative diseases, including PD (Da Silva and Frossard 2005). Here, we demonstrated that LRRK2 is a functional MAPKKK that initiates the ERK signaling cascade leading to the phosphorylation of both ERK1/2 and its upstream MEK.

Despite potential cross-talk between LRRK2 and the ERK pathway in neuronal models had been previously suggested (Liou et al. 2008) (Plowey et al. 2008), a precise characterization of the effect of LRRK2 on the cascade was still lacking. Of note, in our study the mutant G2019S did not cause any further activation of ERK in comparison to wild-type LRRK2 as expected from an over-active kinase, but in agreement with our observation of similar *SNCA* induction levels by [wt]LRRK2 and [G2019S]LRRK2 expression. The mechanisms that could lead from increased kinase activity to activation delay are an interesting topic to speculate about. Many evidences in the literature show that, besides of enzymatic activation, there are additional factors required for successful transmission of a specific signal in a MAPK cascade (Shaul and Seger 2007). One of these mechanisms is the interaction with scaffold proteins that facilitate appropriate targeting of substrates to achieve a faster activation of a specific cascade (Schaeffer and Weber 1999). Further studies with regard to the more than 50 scaffolding and anchoring proteins that have been described for the ERK cascade might help deciphering the actual role of this mechanism in the delayed activation of ERK by LRRK2 mutants.

A second mechanism that has been shown to regulate signal propagation involves potential cross-talk among MAPK pathways, that is extensive since many signaling cascades can act in parallel in propagating signals from the cell surface to the nucleus. Thus, activation of the ERK pathway upon LRRK2 expression in our system does not exclude LRRK2-induced activation of additional MAPK cascades under different circumstances.

4.3 Kinetics of ERK activation may modulate LRRK2 biological response: the potential role of LRRK2 in neurite outgrowth

Correlation between the duration of ERK activation and its downstream effects has been observed in several systems, like PC12 cells, in which stimulation of the ERK pathway with different growth factors leads to different activation kinetics and downstream outputs. Whereas transient activation of the ERK cascade upon EGF stimulation causes proliferation, sustained activation caused by stimulation with NGF leads to differentiation and neurite formation (Nguyen et al. 1993). LRRK2 has been suggested to participate in neuritogenesis, and we show that *LRRK2* PD-associated mutations R1441C and specially G2019S delayed ERK activation compared to the wild-type. Thus, if LRRK2 is involved in neuritogenesis *in vivo*, delayed and/or shorter activation of ERK due to mutations in LRRK2 could interfere in the differentiation process, altering neuron morphology. Interestingly, changes in neurite length and branching due to LRRK2 mutations have been observed (MacLeod et al. 2006).

Additionally, participation of activated MEK/ERK kinases in cytoskeletal signaling and axon growth has been also extensively described (Ray and Sturgill 1987; Atwal et al. 2000; Atwal et al. 2003; Goold and Gordon-Weeks 2005). LRRK2 has been shown to phosphorylate moesin (Jaleel et al. 2007), a protein that binds to actin filaments, suggesting a role of LRRK2 in promoting actin filaments polymerization. Since the cytoskeleton is generally believed to be a convergence point of extracellular signals and intracellular machinery for the regulation of axon outgrowth, all these evidences taken together strongly support a putative involvement of LRRK2 in ERK-mediated neuritogenesis. Indeed, treatment of differentiated SH-SY5Y cells with the MEK inhibitor U0126 has been reported to reduce LRRK2-neuritic shortening caused by expression of the LRRK2 mutant G2019S, implicating ERK/MAPK-related signaling in LRRK2-regulated neurite outgrowth.

4.4 Conclusion

In conclusion, our results provide for the first time a functional link between *LRRK2* and *SNCA*, contributing to clarify the means by which LRRK2 can specifically modify SNCA biology via activation of the ERK/MAPK cascade ultimately leading to *SNCA* transcriptional up-regulation (Fig. 14).

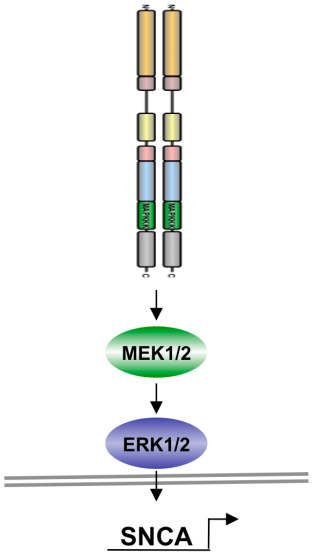


Figure 14. Proposed model of functional interaction between LRRK2 and SNCA.

LRRK2 expression activates the ERK MAPK pathway through sequential phosphorylation of the kinases MEK and ERK, which, in turn, act on the promoter region of SNCA to stimulate the transcription of SNCA. In this hypothetical model, modulation of the ERK pathway may serve to prevent excessive expression of SNCA, which is associated to the pathogenesis of PD.

The ERK pathway had been previously reported to participate in *SNCA* transcriptional regulation in a SH-SY5Y cellular model via a regulatory region lying within intron 1 of the *SNCA* gene (Clough and Stefanis 2007), but no other PD-causative gene had been ever shown to participate in this regulation. We show that LRRK2 effect on the ERK pathway and on *SNCA* regulation are related events and that activation of the ERK pathway mediates the induction of *SNCA* by LRRK2, since treatment with the specific inhibitor of the ERK pathway U0126 completely suppresses the transcriptional up-regulation of *SNCA* elicited by LRRK2. However, the relevance of this pathway in neurons and *in vivo* models will require further study.

Moreover, despite the ERK pathway is classically associated to survival, increasing data suggest that activation of the ERK MAPK pathway may play a detrimental role in certain conditions, especially following an oxidative insult (Subramaniam and Unsicker 2006). Hence, modulation of the ERK pathway may serve to prevent excessive SNCA expression associated to PD pathogenesis.

Mutations in LRRK2 alter the kinetics of ERK activation, but the effect of these changes on cell death and survival has to be further investigated in order to provide precise information about the way this signaling cascade could be involved in PD pathogenesis. This novel pathway opens, nevertheless, new chances for the development of future therapeutic treatments for PD.

5 EXPERIMENTAL PROCEDURES

5.1 Microbiology techniques

General procedures were used for handling, cultivation and storage of *E.coli* according to manufacturer's instructions. Preparation and transformation of chemo- or electro-competent *E.coli* cells, was done following standard procedures.

5.2 DNA techniques

5.2.1 Vectors and constructs

All LRRK2/HA constructs used in this study are cloned in the mammals expression vector pCI and were done by Novartis Pharma Ltd. (Basel, Switzerland) on collaborative bases.

5.2.2 Sequencing

Sequencing of DNA was performed following standard procedures in a sequencer ABI PRISM® 3100 Genetic Analyzer (Roche Applied Biosystems). DNA and RNA concentrations were measured by photometry. Agarose-gel electrophoresis was performed using standard methods. All constructs used in this study are listed in table 5 (see Materials section). All primers used in this study are listed in Table 6 (see Materials section). Sequencing primers were designed with the software Primer3 (http://ihg.gsf.de/ihg/ExonPrimer.html). PCR products were analyzed in 1x agarose gels to confirm size and integrity. LRRK2 reference sequence was obtained from the mRNA DKFZp434H211 published in the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/).

5.2.3 DNA preparation and purification

Extraction and purification of DNA fragments from agarose-gels or enzymatic reactions were done using extraction spin-columns following instruction of the manufacturer (Qiagen). Plasmid purification was done using DNA prep-columns according to the manufacturer's instructions (Qiagen).

5.3 RNA techniques

5.3.1 Preparation of RNA from cell cultures

Cells plated in 6-well plates were transferred into a 1.5 ml Eppendorf tube and washed twice with ice-cold PBS and lysed in 350 µl of RTL buffer (RNeasy Mini Kit, Quiagen). The lysate was frozen at -20°C for 24 hours for homogenization and then mixed with an equal volume of 70% DEPC-ethanol. RNA was purified with the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen).

5.3.2 Real Time RT-PCR

Total mRNA was extracted with the RNeasy Kit (Qiagen), followed by reverse transcription with the Transcriptor First Strand cDNA Kit (Roche). The sequence between the exons 2 to 5 was amplified by qRT-PCR and the specific product quantified using the LightCycler 480 System (Roche). Detection of the product was done with highly specific fluorescent hybridization primers. The crossing-point (Cp) values were calculated by the second derivate method. A low copy number constitutive gene, the porphobilinogen deaminase (h-PBGD), was used as external standard and quantified with the h-PBGD Housekeeping Gene Set (Roche). Relative transcript levels were calculated as the ratio SNCA/PBGD, normalized to the expression level in the cells transfected with the empty vector.

5.4 Cell culture

HEK293E cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum (PAA Laboratories). LRRK2-inducible cells, provided by Pro. Benjamin Wolozin (Pharmacology Department, Boston University School of Medicine), additionally with penicillin/streptomycin, 2x non-essential amino acids (Gibco), 100 µg/ml hygromycin B and 15 µg/ml blasticidin.

Confluent cells were seeded in 6-well plates and transiently transfected with 1 µg of DNA per well using the lipofection reagent FuGene6 (Roche). LRRK2 expression in stable cell lines was induced by treatment with 1 µg/ml tetracycline.

5.4.1 Primary fibroblast culture

Skin biopsies of approximately 25 mm^2 were taken from patients and unrelated sex/aged-matched controls provided by the Department of Dermatology (Molecular Oncology and Aging, Eberhard Karls University, Tuebingen), with approval of the local ethic commission and after written informed consent. After a wash in PBS, the tissue was cut in small pieces and cultured onto 25 mm^2 flasks with maintenance medium (RPMI supplemented with 10% FCS and penicillin/streptomycin). Cultures were kept at 37 °C and 5 % CO2, replacing medium every other day until primary fibroblasts proceeded out of the samples onto the culture flasks. Before total confluence, fibroblasts were trypsinized and propagated in 75 cm^2 flasks. Harvested fibroblasts were aliquoted and frozen for storage between passages 2 and 10.

5.5 Protein techniques

All antibodies used in this study are listed in Table 4 (see Materials section). Protein concentrations were determined by the BCA assay (Pierce). Proteins were separated by polyacrylamid-gel electrophoresis and western blotting was performed according to standard procedures, using PVDF membranes.

5.5.1 Protein extraction from cell culture

Lysates were prepared by resuspending PBS-washed cells from 6-well plates in 150 µl of lysis buffer (50 mM Tris-HCl pH=7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) with the protease inhibitor cocktail Complete Mini (Roche) and phosphatase inhibitors (NaF and Na_3VO_4), followed by 30 minutes incubation on ice. Lysates were spinned down and the cleared lysate loaded in the gel with the appropriate amount of 6xLB.

5.5.2 Production of antiserum

To generate LRRK2 phospho-specific antibodies, two rabbits were immunized (immunization DE07225, Eurogentec) with a synthetic peptide comprising the amino acid sequence 2031-2041 of LRRK2 (antigen code EP071133). Sera were tested after each bleeding in western blots on whole cell lysates from HEK293E cells. After conditions optimization, the antibody was used in a concentration 1:1000.

5.5.3 Inhibitors

To block the ERK-MAPK pathway, the specific phospho-MEK1/2 inhibitor U0126 (Cell Signaling) was used for 12h before lysis in a concentration of 10 μM. The inhibitor was added fresh to the medium immediately prior to use.

5.5.4 Western blotting

25 μg of lysate with the appropriate amount of 6x Laemmli buffer were loaded in 7.5 % polyacrylamid gels. Separation of the proteins through the gel was done at 100 V (stacking gel) and 140 V (resolving gel). Proteins were transferred into PVDV membranes at 100 V for 2,5 h on a wet blot chamber and membrane was blocked for 1 hour in 5 % milk powder in TBS-T (50 mM Tries pH=7.4, 150 mM NaCl, Tween-20 0.1 %). After washing three times with TBS-T for 5 min each, membranes were incubated overnight at 4°C with primary antibodies (diluted in 1% bovine serum albumin in TBS-T), followed by 3 times 10 min washing. Incubation with secondary antibodies (diluted in 2% milk powder in TBS-T) was done for 1 h at RT. Stripping of the membranes was done by incubation in stripping buffer for 30 min at 56ºC, followed by washing in TBS-T. Signal was detected using the chemiluminescent substrate HRP Immobilon Western (Millipore).

5.5.5 ELISA assays

Quantification of endogenous SNCA protein was done using the Immunoassay Kit for human α-synuclein (Biosource), following manufacturer's instructions. Cells were lysed for 30 minutes in specific lysis buffer (10 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 0,1% SDS, 0.5 % deoxicholate, 1 mM PMSF).

5.5.6 Immunoprecipitation

For the immunoprecipitation, cell lysate amounts of 250 µg from LRRK2 transfected cells were incubated in a total volume of 500 µl of lysis buffer with 50 µl of HA agarose beads (Sigma) according to manufacturer instructions at 4ºC overnight. After three washing steps in 500 µl of lysis buffer, beads were eluted with 50 µl of 2x Laemmli buffer for 5 min at 95ºC, followed by immunoblot with the phospho-specific LRRK2 antibody.

5.6 Multiple sequence alignment

Full-length protein sequences for the known LRRK2 homologs and MAPKKK family members were retrieve from the ExPASy database (www.expasy.ch). Alignment of the activation segments of LRRK2 homolog proteins and MAPKKKs family members was done using the T-Coffee package (www.tcoffee.org).

6 MATERIALS

If not stated otherwise, chemicals and reagents (analytical grade) were pursached from Merk, Sigma, Roth, Calbiochem and Biorad. Reagents for molecular biology, restriction enzymes and other enzymes were pursached from MBI Fermentas, New England Biolabs (NEB), QIAGEN, Promega, and Roche. Media for cultivation of cells was obtained from Gibco.

6.1 Antibodies

Table 4. Antibodies

Antibody	Source	Dilution	Manufacturer
anti-HA (Y-11)	rabbit polyclonal	1:1000	Santa Cruz Biotech
anti-HA Agarose Conjugate	mouse monoclonal	1:1	Sigma
anti-phospho-LRRK2 (T2035)	rabbit polyclonal	1:1000	In-house
anti-MID (LRRK2)	rabbit polyclonal	1:15000	Klein et al.
anti-phospho-ERK	rabbit polyclonal	1.2000	Cell Signaling
anti-ERK	rabbit polyclonal	1.2000	Cell Signaling
anti-phospho-MEK1/2	rabbit polyclonal	1:2000	Cell Signaling
anti-MEK1/2	rabbit polyclonal	1.2000	Cell Signaling
anti-phospho-p38^{MAPK}	Rabbit monoclonal	1:2000	Cell Signaling
anti-p38^{MAPK}	rabbit polyclonal	1:2000	Cell Signaling
anti-phospho-SAPK/JNK	rabbit polyclonal	1:2000	Cell Signaling
anti-SAPK/JNK	rabbit polyclonal	1:2000	Cell Signaling
Anti-α-tubulin	mouse monoclonal	1:15000	Sigma

6.2 Vectors and Constructs

All LRRK2 constructs used in this thesis were obtained from Dr. Giorgio Rovelli (Novartis Pharma Ltd., Basel).

Table 5. Vectors and Constructs

Name	Description	Reference
Da1397	pCI-HA tag C-term, wt LRRK2 full length	Novartis
Da1403	as Da1397 with G2019S mutation	Novartis
TF42	as Da1397, kinase dead mutant [K1906N]	Novartis
TF105	as Da1397, activation loop inactive [T2035] C-term HA tag	Novartis
TF112	As Da1397, superactive mutant [T2035] C-term HA tag	Novartis
Da73	pCI empty vector HA tag	Promega

6.3 Oligonucleotides and hybridization probes

Oligonucleotides and hybridization probes for real-time RT-PCR were pursached HPLC-purified from Metabion GmbH

Table 6. Oligonucleotides and hybridization probes

Name	Sequence 5' → 3'
SNCAx2-3F	AAGAGGGTGTTCTCTATGTAGGC
SNCAx5-6R	TGATACCCTTCCTCAGAAGGC
SNCAx3_LC	LCRed-640-TCACCACTGCTCCTCCAACATTTGTC-Pho
SNCAx3_FL	TCTGGGCTACTGCTGTCACACCC-Fluo

7 APPENDIX

7.1 List of Figures

7.2 List of Tables

7.3 Abbreviations

4E-BP	eukaryotic translation initiation factor 4E (eIF4E)-binding protein
°C	degree Celsius
µ	micro
aa	amino acid
AD	Alzheimer's disease
ANK	ankyrin
ARM	armadillo
ASK1	apoptosis signal-regulating kinase 1
ATP	adenosine triphosphate
BCA	Bicinchoninic Acid Assay
BSA	bovine serum albumin
C. elegans	*Caenorhabditis elegans*
CNS	central nervous system
COR	C-terminal of ROC
D. melanogaster	*Drosophila melanogaster*
DA	dopamine
DMEM	Dulbecco's Minimal Essential Medium
DLB	dementia with Lewy bodies
E. coli	*Escherichia coli*
ERK	extracellular signal regulated kinase
ERM	ezrin/radixin/moesin
FPD	familial Parkinson's disease
g	gram
GDP	guanosine diphosphate
DTP	guanosine triphosphate
h	hour
HEK	human embryonic kidney
HRP	*horseradish*-peroxidase
HSP	heat shock protein
IP	immunoprecipitation
JNK	c-Jun amino-terminal kinase
kDa	kilo Dalton
KO	knock out
l	liter
LB	Luria-Bertani
LB	Lewy Body
LRR	leucine-rich repeat
LRRK1	leucine-rich repeat kinase 1
LRRK2	leucine-rich repeat kinase 2
m	mill
M	molar
MAO	monoamine oxidase
MAPKKK	mitogen activated kinase kinase kinase
MBP	maltose binding protein
MEK	MAP/ERK kinase
min	minute
MLK	mixed-lineage kinase
MPTP	1-methyl-4-phenyl, 1, 2, 3, 6-tetrahydropyridine
mRNA	messenger ribonucleic acid
n	nano

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	Parkinson's disease
PINK1	phosphatase and tensin [PTEN] homolog-induced putative kinase 1
ROC	Ras of complex proteins
ROS	reactive oxygen species
RT	room temperature
SDS	sodium dodecyl sulfate
SNpc	substantia nigra pars compacta
SNCA	alpha-synuclein
SPD	sporadic Parkinson's disease
TAK1	transforming growth factor-beta-activated kinase 1
TAP	tandem affinity purification
TH	tyrosine-hydroxilase
UPS	ubiquitin-proteasome-system

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