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The role of LRRK2 in Parkinson's disease: from function to dysfunction

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Doutoramento em Ciências Biomédicas

Neurociências

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Abstract

Parkinson's disease (PD) belongs to the group of neurodegenerative disorders and it is currently considered the most common progressive movement disorder. Neurodegenerative disorders, such as Alzheimer's, Huntington's, fronto-temporal dementia and amyotrophic lateral sclerosis, share several dysfunctional molecular pathways and impairments in basic cell mechanisms. Despite intense efforts to understand to decipherthe triggers underlying these disorders, to date, there is no effective cure. This results in a growing number of cases and, consequently, in a complex social and economic problem. Therefore, it is of extreme importance to understand the common biological mechanisms involved in the pathogenesis of this devastating group of diseases, in order to develop effective therapies. The majority of the PD cases are sporadic, however, in the last decades, it has been recognized that rare genetic mutations are patholgical for PD in a number of inherited cases. Futhermore, these mutations can be as well a risk factor for sporadic PD, supporting the idea that familial and sporadic PD can share common pathlogical mechanisms.

This study focused on a key player protein in PD, Leucine-rich repeat kinase 2 (LRRK2). Mutations in *LRRK2* gene are the most frequent cause of autosomal dominant forms of PD and they are also consider a risck factor for sporadic cases. A central catalytic GTPase and kinase core, flanked by protein interaction domains, composes this large and complex multi-domain protein. The most frequent *LRRK2* PD-related mutation occurs at the animoacid 2019, a glycine subtitution for a serine (G2019S), precisely on the kinase domain of the protein resulting in its toxic gain of function. LRRK2 is known to play a role in distinct cellular mechanisms such as vesicular trafficking, microtubule network regulation and mitochondrial morphology. However, the function of LRRK2 in these important mechanisms and their related pathways is not fully understood, which is crucial for developing new therapeutic targets. Here, we investigated LRRK2 function by characterizing/identifying its protein interactors and, in particular, by exploring its relationship with two central proteins in neurodegenerative disorders, α-synuclein and Tau. In PD brain samples, we show that levels of LRRK2 are positively correlated to an increase in α-synuclein phosphorylation and aggregation in affected brain regions, where both proteins co-localize in neurons and Lewy body

inclusions. In a cell line model, this co-localization also occurs in α-synuclein inclusions and knocking down LRRK2 promotes formation of smaller inclusions. Moreover, we show an interaction between α-synuclein and LRRK2 under endogenous and over-expression conditions. These results shed light on the complex interaction of these two central PD proteins and, in particular, on underlying molecular mechanisms involved in a disease scenario. Furthermore, we demonstrate that LRRK2 also interacts with Tau protein in a cell line model, in which co-expression of both proteins promotes accumulation of Tau protein. This accumulation occurs independently of LRRK2 kinase activity and it gives rise to formation of high molecular weight Tau species and increased levels of Tau secretion. Moreover, we suggest that these effects are a consequence of an impairment of proteasomal Tau degradation and that this impairment is promoted by LRRK2. Consistently, a LRRK2-knockout mouse displayed lower levels of Tau in the brain, when compared with transgenic animals expressing human wild-type LRRK2. Our results highlight the compromised status of cellular and molecular neurodegenerative mechanisms. The identification of LRRK2 interactors is crucial to placing the protein in known biochemical pathways. To that end, we performed a screen to identify LRRK2-interacting proteins. The results obtained confirmed that this is a multifaceted protein, involved in a variety of molecular functions and biochemical pathways. α-synuclein and Tau are two proteins present in the list of interactors, which validates previously reported results. The role of LRRK2 on the cytoskeleton is also highlighted by the presence of several protein interactors linked to microtubule dynamics, which lead us to explore the effect of LRRK2 on mechanical properties of the cell. Applying a combined microscopy tecniques in cell indentation experiments, we confirmed that different distribution patterns of LRRK2 result in differential states of cell stiffness. We found that the stiffest cells exhibit a diffuse pattern of LRRK2 distribution, such that LRRK2 is dispersed throughout the entire cell, interacting with microtubule-related proteins and compromising cytoskeletal dynamics. The identification of novel interactos resulted in a better understanding of LRRK2 patho-physiological role.

Taken together, our results presented in this thesis provide novel insight into the function of LRRK2 and its particular role in neurodegenerative diseases. Ultimately, this knowledge is essential for the understanding of the molecular underpinnings of PD and for the development of novel therapeutics.

Key Words: LRRK2, Parkinson's disease, α-synuclein, Tau, protein interaction.

Resumo

A doença de Parkinson (DP) pertence ao grupo das doenças neurodegenerativas, sendo atualmente considerada a doença neurodegenerativas motora progressiva mais comum. As doenças neurodegenerativas, como a doença de Alzheimer, a demência frontotemporal ou a esclerose lateral amiotrófica, partilham várias disfuncionalidades em importantes vias de sinalização molecular e mecanismos celulares. Apesar dos esforços desenvolvidos para compreender os factores que estão na origem e na progressão destas doenças, presentemente ainda não foi encontrada uma cura eficaz. O resultante crescente número de casos destas doenças, consequentemente contribui para um complexo problema socioeconómico. É assim de extrema importância identificar os mecanismos biológicos envolvidos na patogénese deste devastador grupo de doenças, a fim de desenvolver terapias eficazes para o combate das mesmas. A maioria dos casos de DP são esporádicos, no entanto nas últimas décadas têm sido identificadas várias mutações genéticas ligadas a casos hereditários. Estas mutações podem ainda ser consideradas um factor de risco para o desenvolvimento de casos esporádicos da DP, o que suporta a ideia que os casos hereditários e esporádicos partilham os mesmos mecanismos patológicos.

Este estudo foca-se numa proteína chave na DP, *Leucine-rich repeat kinase 2* (LRRK2). Mutações na proteína LRRK2 são consideradas a causa mais frequente em casos autossómicos dominantes da doença, ocorrendo também em casos esporádicos. Esta grande e complexa proteína com múltiplos domínios, é composta por um núcleo catalítico central de GTPase e quinase, flanqueado por vários domínios de interação proteica. A mutação mais frequente em LRRK2 é a substituição de uma glicina por uma serina, que ocorre no aminoácido 2019 (G2019S). Esta mutação localiza-se precisamente no domínio da quinase da proteína, promovendo um tóxico ganho de função da mesma. É conhecido o envolvimento de LRRK2 em distintos mecanismos celulares como o tráfego vesicular, regulação da rede de microtúbulos e morfologia mitocondrial. No entanto, não é completamente conhecido o papel de LRRK2 nestes importantes mecanismos e suas vias de sinalização, o que é crucial para o desenvolvimento de novos alvos terapêuticos. Neste trabalho investigamos a função de LRRK2 através da caracterização/identificação de proteínas interatuantes, em particular

explorando a sua relação com duas proteínas centrais em doenças neurodegenerativas, alpha-sinucleína (α-sinucleína) e Tau. Em amostras de cérebro de pacientes com DP, mostramos que os níveis de LRRK2 são positivamente corelacionados com um aumento de fosforilaçao e agregação de α-sinucleína fosforilada e agregada, nas regiões do cérebro mais afectadas. Também nas regiões de cérebro mais afectadas, se verifica uma co-localização destas duas proteínas em neurónios e em inclusões de corpos de *Lewy*. Num modelo de linha celular, esta co-localização também ocorre em inclusões de α-sinucleína, onde o *knockdown* de LRRK2 promove a formação de inclusões mais pequenas. A interação entre α-sinucleína e LRRK2 é ainda confirmada em condições endógenas e de sobre-expressão. Estes resultados contribuem para uma melhor compreensão sobre a complexa interação destas duas proteínas centrais na DP, em particular sobre os mecanismos moleculares subjacentes, envolvidos num cenário de doença. Em seguida e usando um modelo celular, demostramos que a LRRK2 interatua com a Tau, sendo que a co-expressão destas proteínas promove uma acumulação de Tau. Esta acumulação ocorre independentemente da atividade de quinase da LRRK2, e promove a formação de espécies de Tau com elevado peso molecular, bem como um aumento de secreção de Tau. Estes efeitos serão a consequência de uma falha ao nível da degradação de Tau pelo proteassoma, que por sua vez será promovida pela LRRK2. Em cérebros de ratinhos *knockout* para LRRK2, verifica-se um decréscimo dos níveis de Tau, quando comparado com animais transgénicos para LRRK2 humana. Estes resultados realçam a disfunção de mecanismos celulares e moleculares, envolvidos nas doenças neurodegenerativas. A identificação de proteínas interatuantes com LRRK2 é crucial para posicionar esta proteína nas conhecidas vias de sinalização bioquímica. Com este objectivo, desenvolvemos um *screen* para identificar novas proteínas interatuantes com LRRK2. Os resultados obtidos confirmam que esta é uma proteína multifacetada, envolvida em várias funções moleculares e vias de sinalização bioquímicas. A presença de α-sinucleína e Tau nesta lista de proteínas interatuantes, vem validar os resultados acima descritos. Também a presença de várias proteínas relacionadas com a dinâmica de microtúbulos, vem realçar o papel de LRRK2 ao nível do citoesqueleto celular, o que nos levou a explorar o efeito de LRRK2 nas propriedades mecânicas das células. Aplicando uma técnica combinada em

microscopia celular, confirmámos que diferentes padrões de distribuição de LRRK2, resultam em diferentes estados de rigidez celular. Descobrimos que as células com maior rigidez são as que exibem um padrão difuso de distribuição de LRRK2, onde a proteína está dispersa por toda a célula, interagindo com proteínas relacionadas com os microtúbulos, comprometendo assim a dinâmica do citoesqueleto. A identificação de novas proteínas interatuantes resulta num melhor conhecimento da função pato-fisiológica de LRRK2. Em resumo, os resultados apresentados nesta tese, fornecem novos conhecimentos sobre as funções da LRRK2 e o seu particular papel nas doenças neurodegenerativas. Por fim, estes conhecimentos são essenciais para a compreensão das bases moleculares da DP e consequentemente para o desenvolvimento de novas terapêuticas.

Palavras-chave: LRRK2, Doença de Parkinson, α-sinucleína, Tau, interação proteica.

List of abbreviations

AD - Alzheimer's disease
AFM - atomic force microscopy
ALP- autophagy-lysosome pathway
ALS- amyotrophic lateral sclerosis
BiFC- bimolecular fluorescence complementation (assay)
CMA - chaperone mediated autophagy
DA - dopamine
FTD- fronto-temporal dementia
GO - gene ontology
GWAS- genome wide association studies
HD- Huntington's disease
HMW - high molecular weight
L-dopa - L-3,4-dihydroxyphenylalanine
LBs - Lewy bodies
LNs - Lewy neurites
LRRK2 - leucine-rich repeat kinase 2
MAPT- microtubule-associated protein tau
MPTP- 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA - messenger RNA
MS - mass spectometry
NFTs- neurofibrillary tangles
PD - Parkinson's disease
SEC- size exclusion chromatography
shRNA - small hairpin RNA
SNCA - alpha-synuclein gene
SNpc - substantia nigra pars compacts
TIRFM- Total Internal Reflection Fluorescence Microscope
UPS- ubiquitin proteasome system
WT- wild type

Chapter 1.

General Introduction

Chapter 1. General Introduction

1. The neurodegenerative disorder of Parkinson's disease

The earliest records about Parkinson's disease date back to 1817 when a British surgeon, James Parkinson, described in the monograph "An Essay on the Shaking Palsy", the first observations of patients affected by a disease he called "*paralysis agitans*". Years later, the terminology was updated by a French neurologist, Jean-Martin Charcot, that in memory of James Parkinson, named this disorder Parkinson's disease (PD).

PD is the second most common progressive neurodegenerative disorder, affecting 1-2% of people over 65 years old and 4-5% above the age of 85. The onset of PD is intimately related with age, which increases the expected number of cases due to an increased lifespan of the population in developed countries [1,2].

Almost two centuries after the first descriptions of PD, and despite intense research efforts in the field, there is still no effective cure and our knowledge about the etiology and the development of this disease is still incomplete.

The pathogenic mechanisms of PD, do not seem exclusive of this disease but actually share several common features with other neurodegenerative diseases like Alzheimer's (AD), Huntington's, fronto-temporal dementia and amyotrophic lateral sclerosis. Although these diseases present different features and hallmarks, they share several dysfunctional molecular pathways and impairments in basic cell mechanisms. Thus, it is very important to deeply investigate these common molecular pathways to determine the function of the key proteins. This cross knowledge is crucial to discover the basis of neurodegenerative mechanisms common to several dramatic diseases for targeting efficient therapies.

1.1 Clinical symptoms and pathogenesis of PD

PD is typically known for its characteristic motor symptoms such as resting tremor, bradykinesia, muscle rigidity and postural instability. Before the appearance of the first motor symptoms and the final diagnosis of the disease, there is already cognitive deterioration accompanied by pre-motor symptoms like

mood disturbances, rapid-eye-movement, sleep disorder, loss of smell (hyposmia) and depression [3,4].

The described clinical symptoms result from an increasing and selective loss of dopaminergic neurons from a particular midbrain region, the *substantia nigra pars compacta*, and consequently a massive depletion of striatal dopamine, an extremely important neurotransmitter (Figure 1A and 1B). Another typical pathological hallmark of PD, described in the early nineties by Friederich H. Lewy, is the presence of cytoplasmic protein-containing inclusions in the surviving dopaminergic neurons called Lewy bodies (LBs) (Figure 1C). LBs are agglomerates of several proteins, with α-synuclein being one of the major components and mostly present in the phosphorylated and fibrillar forms [3,5,6]. The presence of LBs is not only restricted to the dopaminergic neurons, they appear in other areas of the brain, such as spinal cord. Although considered a hallmark of PD, the LBs are also found in other neurodegenerative diseases like AD and Lewy body disease or even in healthy aged brains [7-9]. Curiously, patients with autosomal recessive forms of PD, in particular with mutations in the *PARKIN* gene, do not present LBs inclusions [10]. Despite the extensive research in the field, the question whether LBs confer toxicity or on the other hand are protective to the cells, by sequestering potential toxic species, is a hot topic for debate without a consensual answer [8,11,12].

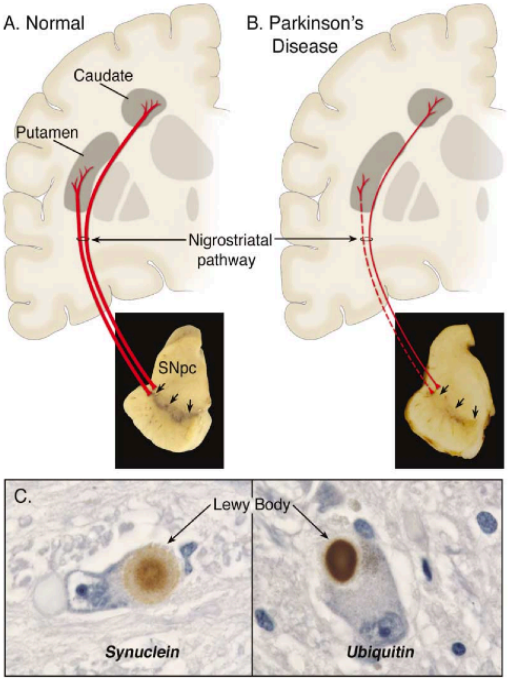

Figure 1. Neuropathology of PD.
Representation of a healthy brain **(A)** versus a PD brain **(B).** Highlighted in red are the compromised nigrostrital pathways and the depigmentation (three arrows), due to the loss of melanine present in the dying dopaminergic neurons, that projects to the striatum (putamen and caudate nucleus). **(C)** Detail of a Lewy body inclusion in dopaminergic neurons, showing the Immunoreactivity against α-synuclein and ubiquitin [6].

To date, there is no effective treatment to cure PD or stop the progressive degeneration of the dopaminergic neurons. While gene and stem cell therapies are being heavily studied and not yet available, the gold standart for the treatment of PD continues to be the pharmacological approached introduced in the eighties, aimed at amelioreating the motos symptoms of the disease. This therapy consists in the replacement of the depleted striatal dopamine by the administration of a dopamine percursor, L-3,4-dihydroxyphenylalamine, usually known as levodopa (L-dopa). Although it is considered an effective drug therapy for the motor symptoms, especialy when administered in early stages of the disease, L-dopa is not suitable for all PD cases and long-term treatment can promote severe side effects [13-15].

Currently, PD is no longer exclusively considered a disorder of the dopaminergic neurons of the substantia nigra. Although its onset and progression are still unclear, the disease is thought to happen in different brain regions and even outside the nervous system [7,15,16]. The progressive and cumulative symptoms confirm that this is a multisystem disorder that needs to be approached from a broad perspective.

1.2 Etiology of PD

PD is a typical late onset disorder and most cases (90-95%) occur sporadically, without a defined cause or relation with patient's life style, characterizing it as an idiopathic disease. It is consensual that the major risk factor for PD is ageing. However, initial finding linked the disease with environmental factors, such as the chronic exposure to several neurotoxic pesticides. Later on, in the late twenties, the discovery of rare familial genetic mutation linked with PD, brought a new perspective for the research of this disease. These findings leaded to the development of a variety of animal models, in attempt to elucidate the molecular pathogenesis of the disease [6,17]. Despite the increasing knowledge about the etiology and the development steps of PD, resulting from the intensive research in the field, the underlying molecular mechanisms of the disease are still not completely understood.

Nowadays, there is solid evidence that a synergistic combination of environmental and genetic factors is determinant to the onset and development of the disease.

1.2.1 Environmental factors

The first identified environmental factors for the development of PD was the continuous exposure to neurotoxins and chemical substances present in several pesticides. 6-hydroxydopamine was one of the first neurotoxins identified. It promotes a selective degeneration of the catecholaminergic neurons due to an increase in reactive oxygen species [18]. Another neurotoxin, MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), was identified in a synthetic drug consumed by a group of drug addicts who started to develop typical parkinsonism symptoms. This neurotoxin crosses the blood brain barrier and is converted into MPP+ in glial cells, which then displays selective toxicity in dopaminergic neurons [19]. Also, the chronic exposure to pesticides widely used in agriculture, like rotenone and paraquat, were found to be a potential risk factor for PD. Rotenone inhibits the mitochondrial complex I promoting degeneration of nigral-striatal neurons. Paraquat directly crosses the blood brain barrier, contributes for an increase of reactive oxygen species, which lead to degeneration in dopaminergic neurons [20, 21]. The knowledge about these neurotoxic chemicals was used to develop the first models of PD, which continue to be a valuable tool available for mimicking parkinsonism symptoms and consequently to test new therapeutics for impact on the disease [18].

Interestingly, there are some studies suggest that nicotine and caffeine contribute for a decrease in the incidence of PD [22], but these will not be explored further in this document, as it would be out of the scope.

1.2.2 Genetic factors

In the last two decades, genome-wide studies in several populations identified a growing number of genes associated with familial PD, reinforcing the importance of genetics as a risk factor for the disease [23-25]. The regions of the genome to which these genes map are known as the *PARK* loci. In addition, several genes associated with non-familial forms of PD were identified (Table 1). The *PARK* loci correspond to genes that might be divided into two large groups: genes responsible for autosomal dominant or autosomal recessive forms of the disease [26,27].

Table 1. Genes and loci associated PD associated genes.
AD: autosomal dominant; AR: autosomal recessive; LBs: Lewy bodies; JO: juvenile onset (age< 20); EO: early onset (age 20-40); Classic PD has a late onset (age >40). Adapted from [26] and [28].

Name	Locus	Gene	Status of inheritance	Clinical phenotype
PARK1	4q21-q23	*SNCA*	AD	Classic PD; with LBs
PARK2	6q25-q27	*PARKIN*	AR	EO; without LBs
PARK3	2p13	*unknown*	AD	Classic PD; with LBs
PARK4	4q21	*SNCA* (triplication)	AD	EO; with LBs
PARK5	4p14	*UCHL-1*	AD	Classic PD
PARK6	1p36.12	*PINK1*	AR	EO; with LBs
PARK7	1p36.13	*DJ1*	AR	EO; slow progression
PARK8	12q12	*LRRK2*	AD	Classic PD; heterogeneous pathology
PARK9	1p36.13	*ATP13A2*	AR	JO; Atypical-Kufor-Rakeb syndrome
PARK10	1p32	*unknown*	unclear	Classic PD
PARK11	2q37.1	*GIGYF2*	AD	Classic PD
PARK12	Xq21-q25	*unknown*	unclear	Classic PD
PARK13	2p13.1	*HTRA2*	AD	Classic PD
PARK14	22q13.1	*PLA2G6*	AR	EO; Parkinsonism-dystonia
PARK15	22q12.3	*FBXO7*	AR	JO; Parkinsonian pallidal syndrome
PARK16	1q32	*Rab7L1*	unclear	Classic PD
PARK17	16q11.2	*VPS35*	AD	Classic PD
PARK18	3q27.1	*EIF4G1*	AD	Classic PD
PARK19	1p31.3	*DNAJC6*	AR	JO parkinsonism
PARK20	21q22.11	*SYNJ1*	AR	EO
--	17q21.1	*MAPT*	--	Fronto-temporal dementia
--	1q21	*GBA*	--	Parkinsonism with LBs
--	5q23.1-q23.3	*Synphilin-1*	--	Classic PD
--	2q22-q23	*NR4A2/Nurr1*	--	Classic PD

SNCA was the first gene associated with familial forms of PD. Although rare, missense mutations in *SNCA* such as A53T, A30P and E46K as well as duplications and triplications, are found in familial cases of PD [29-31]. New studies are constantly updating the *SNCA* mutations related with PD, and the most

recent ones described are: A53E, H50Q and G51D [32-35]. Also several polymorphisms in the *SNCA* gene can constitute risk factors for the development of sporadic PD [36,37].

Mutations in the *LRRK2* gene are the most frequent cause of familial PD, although it is also associated with sporadic cases [38,39]. Several point mutations were identified within the multiple domains of the protein, however the G2019S substitution, in the catalytic core of the protein, is by far the most frequently identified [38].

PARKIN, PINK1, DJ-1 and *ATP13A2* are associated with autosomal recessive forms of PD, usually linked with juvenile and early-onset cases of the disease [27,40,41]. *PARKIN* mutations are responsible for the majority of the autosomal recessive PD cases, which interestingly are characterized by the absence of LBs [10].

Although familial mutations are rare, present in only up to 10% of the cases, it is known that both familial and sporadic forms of PD share common pathogenic mechanisms, which are still not clear. Studying these mutated genes and their respective encoded proteins, to know more about their physiological functions and their dysfunction in PD, is crucial to identify molecular pathways that might be used as targets for therapeutic intervention.

1.3 α-synuclein and PD

The α-synuclein protein is the main component of LBs and LNs, present in different synucleinopathies. Importantly, mutations and multiplications in the *SNCA* gene (which encodes for α-synuclein) were the first known genetic causes associated with familial forms of PD [42, 43]. These facts make this protein a central player in PD and one of the most studied proteins in the field.

α-synuclein is an abundant protein in the brain and it is particularly enriched in pre-synaptic terminals. Although its function is not completely understood, it appears to be involved in several cellular processes like synaptic activity, vesicle recycling and, as a chaperone it is involved in the formation of SNARE complexes [44, 45]. This small (140 amino acids), thermostable and natively unfolded protein is structurally composed of three different domains. The N-terminal region is usually unstructured in solution, forming amphipathic α-helices when it interacts with phospholipid membranes. The central non-amyloid-β component (NAC) is a

highly hydrophobic region, responsible for the amyloidogenic properties of the protein and the formation of β-sheet structures, which consequently potentiate aggregation. The C-terminus is a highly acidic region, and possibly responsible for the chaperone activity of the protein [46-48].

The first identified familial *SNCA* mutation was the A53T substitution, followed by two other point mutations A30P and E46K, all promoting early onset of the disease with an extremely aggressive progression [29-31]. While these mutations are considered rare, the duplications and triplications of wild-type *SNCA* are more prevalent and directly toxic via higher expression of the protein [36,37]. More studies are required to better characterize the recently described *SNCA* mutations (A53E, H50Q and G51D), which interestingly seem to be related with the aggregation capacity of the protein [32-35].

Familial mutations, multiplications, polymorphisms and post-translational modifications (particularly phosphorylation) of the protein are considered key factors contributing to α-synuclein accumulation and subsequent aggregation. As α-synuclein is such a predominant component of LBs, the mechanism through which this protein leads to aggregation and confers effects on onset and development of neurodegeneration, has been extensively studied [49-53]. Though α-synuclein is a monomeric and unfolded protein, its central hydrophobic region (NAC) has a tendency to oligomerize [54]. Briefly, the α-synuclein aggregation processes initiates with the formation of dimers that, due to a continuous oligomerization propensity, evolve into bigger oligomers, followed by protofibrils and amyloid fibrils, which ultimately are deposited in LBs. This aggregation process occurs together with neuronal dysfunction. However, there is an intense debate regarding which are the most toxic species formed along this pathway that contributes to an increase in cell toxicity, culminating in neuronal death [52-55]. To better understand this toxic oligomerization and aggregation process occurring in PD, it is also important to consider the relevance of several α-synuclein-interacting proteins. Synphilin-1 is a protein that co-localizes with α-synuclein in the LBs and is described to contribute to α-synuclein aggregation [56-58]. Some isoforms from the 14-3-3 chaperone-like protein family interact with genetic PD-associated proteins, including α-synuclein, and are present in the LBs from human PD patients [58-60].

Although several models were proposed to explain α-synuclein accumulation

and aggregation and its role in PD, this is not entirely known. This will be crucial to identifying therapeutic targets in an early phase, avoiding a massive neuronal death and disease progression.

1.4 Association of Tau with PD

The Tau protein is encoded by the microtubule-associated protein tau (*MAPT*) gene. There are six isoforms of the protein generated by alternative splicing [61]. This predominantly neuronal protein is highly expressed in the adult central nervous system, where it binds to and stabilizes microtubules. Tau interaction/stabilization with microtubules occurs through the C-terminal of the protein and is regulated by phosphorylation of specific epitopes, some of which have been described as pathogenic in some neurodegenerative disorders like AD and PD [62-64].

The Tau protein was initially related to AD, being one of the main components of neurofibrillary tangles, a defined pathological hallmark of the disease, together with extracellular plaques of amyloid-β peptides [65]. Frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) was the first neurodegenerative disorder associated with Tau mutations, therefore belonging to the group of tauopathies [66]. Common to tauopathies, is the presence of hyperphosphorylated and insoluble aggregated forms of Tau, observed inside neuronal cells in different brain regions [67,68].

Several *MAPT* mutations were described to affect the ability of Tau to bind to microtubules and to increase its aggregation propensity. Moreover these mutations were recently confirmed by GWAS as a risk factor for PD [66, 69]. Further insight into the role of Tau as a key player in PD, can be gained from understanding how this protein interacts with the other two central PD proteins: α-synuclein and LRRK2.

The link between Tau and α-synuclein was first highlighted through the co-occurrence of these proteins in insoluble protein deposits in PD and AD brains [70-73]. In addition, it was shown that the majority of AD cases display some α-synuclein enriched LBs and Tau tangles can, as well, be identified in PD brains [74-77]. *In vivo* studies confirmed the presence of hyper-phosphorylated Tau species in a mouse model of over-expressed α-synuclein [78]. In a Drosophila model of PD, the interaction between these two proteins resulted in the disruption

of cytoskeletal organization and increased neurotoxicity [79]. Moreover, *in vitro* studies show that α-synuclein and Tau are able to influence each other's polymerization. In cell models, both proteins interact through GSK-3beta and Tau enhances α-synuclein aggregation and toxicity [80-82].

The link between Tau and LRRK2 is another interesting point that correlates Tau with PD. Being the most frequent cause of the autosomal dominant form of PD, LRRK2 cases are characterized by a pleomorphic pathology including LBs, LNs and Tau tangles [83,84]. Initial studies in a mutated LRRK2 mouse, showed an extensive Tau tangle pathology, and Tau positive axonal swellings were observed in rat neuronal cultures overexpressing a fragment of LRRK2 [85]. Years later, LRRK2 as a kinase, was suggested to phosphorylate Tau in a tubulin-dependent manner [86]. Also in a mouse model of tauopathy, LRRK2 expression results in increased Tau aggregation and phosphorylation of different residues [87]. More recently *in vitro* studies demonstrate that this phosphorylation happens in the presence of tubulin and indirectly via GSK-3β [88].

1.5 Leucine-Rich Repeat Kinase 2: a key player in PD

The human *LRRK2* gene is located on chromosome 12 and encodes for a large protein named Leucine-rich repeat kinase (LRRK2). LRRK2 is also known as Dardarin, from the Basque word *dardara* (tremor), although this term is less used. The first *LRRK2* mutations associated with PD were reported in 2004 and are currently considered the most common genetic cause of PD, being responsible for a high number (5-15%) of all familial cases [83,84]. Only a few mutations, concentrated at the enzymatic domains of the protein, segregate with familial disease. Importantly, there are several LRRK2 variants reported in all other domains of the protein, which are a risk factor for sporadic PD [89-90]. The G2019S mutation is the most frequently found in PD patients, and results in a kinase gain of function of the protein [91-93].

Clinically, the symptomatology of LRRK2-related PD cases is indistinguishable from sporadic cases, presenting an average late onset with a slower progression and not frequently associated with dementia. The LRRK2-PD cases are characterized by a pleomorphic neuropathology with the presence of pure classical nigral neuronal degeneration or LBs, LNs and positive ubiquitin and Tau phosphorylated inclusions [94-95].

The clinical and pathological similarity of LRRK2-related familial cases to sporadic cases, together with the identification of *LRRK2* mutations as a common risk factor for sporadic PD cases, qualifies this protein as a candidate in bridging the gap between inherited and sporadic PD. Therefore, LRRK2 represents an extremely important target for research, with the potential to uncover the common mechanisms of familial and sporadic PD.

1.5.1 Functional structure of LRRK2 protein

LRRK2 is a multi-domain and large (2527 amino-acid) protein of approximately 285 kDa, expressed in various tissues including the brain. This multifunctional protein belongs to the Roco protein family, which is characterized by having a conserved domain containing a Ras-like GTPase domain, called ROC, and a characteristic COR domain (C-terminal of ROC). Neighbouring this GTPase domain is a serine/threonine kinase domain (Kinase), and together these two domains compose the central enzymatic core of LRRK2. Flanking these central core, the protein has additional protein-protein interaction domains such as ankirin-like repeats (ANK), leucine-rich repeats (LRRs) at N-terminal and a β-propeller-like domain (WD40) at C-terminal (Figure 2) [38, 96].

As a GTPase, LRRK2 binds GTP through its ROC domain, leading to a change of conformation and facilitating GTP hydrolysis. However, this GTPase activity is weaker when compared to other members of the Ras related GTPase family [97]. Several pathogenic mutations were found in the GTPase domain, located within the ROC (R1441G/C/H) and the COR (Y1699C) subdomains, which were associated with decreased GTPase enzymatic activity of the protein [98,99].

The kinase domain of LRRK2 shares a high similarity with mixed-lineage kinases (MLKs) and receptor-interacting protein kinases (RIPKs) [100]. Some of the studies to evaluate the kinase activity of LRRK2 are based on its capacity to phosphorylate myelin basic proteins (MBPs) like moesin and pseudo-substrate (single peptides or proteins) [101]. However, the most widely used assays to evaluate LRRK2 activity rely on its autophosphorylation capacity [102,103]. The most frequently reported mutation, G2019S, occurs precisely in the activation segment of the kinase domain and is responsible for a 2-3 fold gain-of-function of the protein. This point mutation is thought to interfere with the activated “ON-OFF” state of the kinase, due to the negative charge of the serine residue which

compromises the structural flexibility, prolonging the activated state of the kinase [104-105].

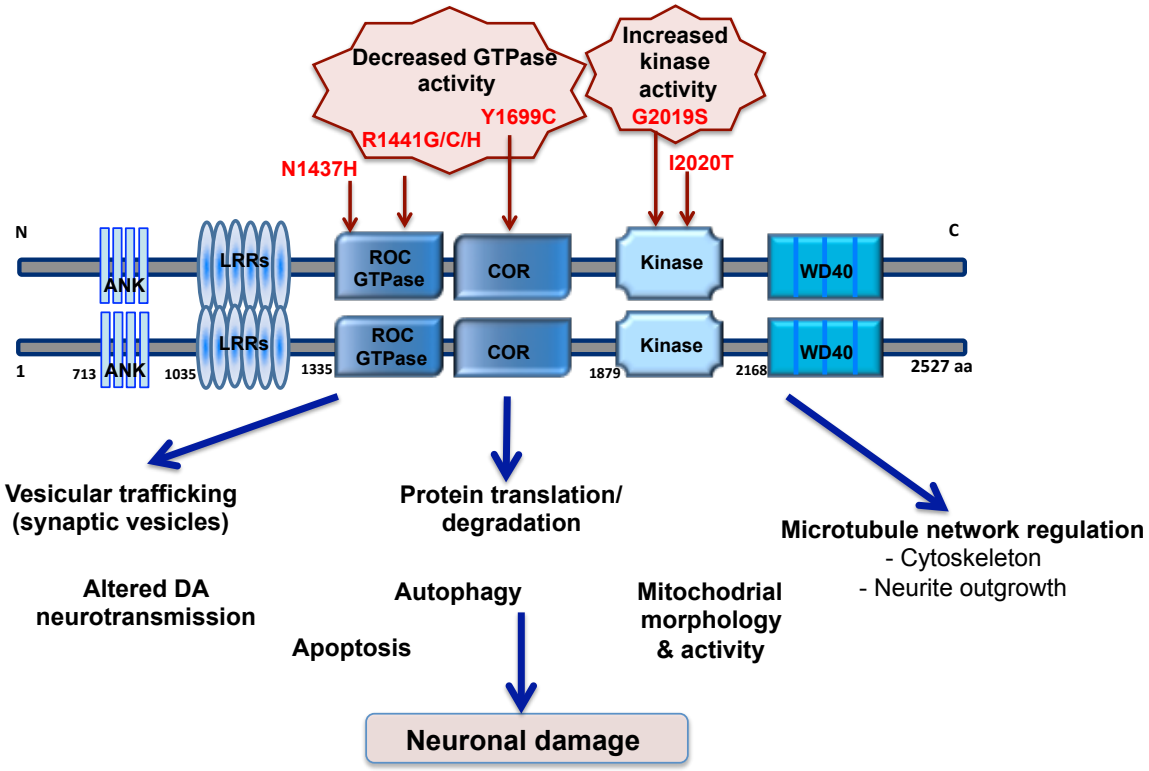

Figure 2. Domain organization of LRRK2 and cellular pathways associated with its function.
Schematic representation of LRRK2 structural domains, highlighting the functional dimeric conformation of the protein. The protein has a central enzymatic core composed by a GTPase domain (ROC) and its C-terminal (COR), together with a serine/threonine kinase domain (Kinase). Flanking these central core, the protein has several protein-protein interaction domains; at N-terminal an ankirin-like repeats (ANK) and leucin-rich repeats (LRRs) and at C-terminal a β-propeller-like domain (WD40). In the central core of the protein are placed the most frequent and pathogenic mutations thought to be responsible for a decrease in GTPase activity and an increase of kinase activity. The study of LRRK2 mutations has shed light on the protein function and on the cellular mechanisms where it could be involved in a PD scenario, ultimately contributing to neuronal damage. Adapted from [96].

1.5.2 LRRK2 mutations and PD

Since the discovery of first LRRK2 mutations related with the autosomal dominant forms of PD, several other mutations have been identified within the multiple domains of the protein. Thus far, more than 40 LRRK2 variants have been reported, however only seven mutations are considered pathogenic: N1437H, R1441C/G/H, Y1669C, G2019S and I2020T [89,88].

Among the *LRRK2* mutations identified, G2019S is located in the kinase domain and promotes its gain of function, confirmed by increased phosphorylation of LRRK2 and known generic substrates [102]. G2019S is the most frequent mutation found in PD patients, being responsible for up to 7% of the familial cases and also 1-3% of sporadic cases worldwide [107]. The frequency of the G2019S mutation varies among different populations across the globe, being particularly high in genetically isolated populations where it can account for up to 40% of total PD cases [108]. A study with data collected from over 133 families, reported a higher occurrence of the G2019S mutation in southern than northern European countries [89]. The age-dependent penetrance of the G2019S mutation, results in a probability of disease onset of 28% at age 59, rising up to 74% at age 79 [107, 109, 110]. Overall, the high but incomplete penetrance of G2019S mutation results in the existence of some carriers of the mutation, who do not develop PD in their lifetime [111, 112].

Neighbouring the G2019S, is the I2020T mutation, which seems to have a very modest effects on kinase activity of the protein, so the relationship of this mutation with the LRRK2 biochemical activities is unclear [113].

The three mutations, reported in the “hotspot” R1441 (R1414G/H/C), make this the second most common site of pathogenic LRRK2 substitutions. The R1441C mutation was initially founded in two autosomal dominant PD families. Although R1441C is found in different populations, R1414G is particularly common in the Basque region of Spain and R14141H was only found in four individual from diverse ethnicities [114]. These three mutations in the GTPase domain of LRRK2 are generally associated with a decreased GTPase activity [115, 116]. In the COR domain, a tyrosine to cysteine or guanidine mutation (Y1699C) was reported in one family from UK and other family with German heritage [99]. These mutations present a highly variable penetrance and clinical features resemble the idiopathic PD cases, with a late onset of the disease.

The N1437H mutation, in the COR domain of LRRK2, is the most recently identified mutation, in a large Norwegian family, and curiously it presents a very young age of onset (approximately 48 year old) [117]. Interestingly other two LRRK2 polymorphisms (G2385R and R1628P), which are almost absent in Caucasians, represent almost 10% risk for sporadic PD in Asian populations [118, 119].

1.5.3 Neuropathology of LRRK2 mutation in PD

The different LRRK2 mutations promote a wide spectrum of neuropathology features, resumed in table 2 [107]. The post-mortem analysis of LRRK2-associated PD patients, revealed a pleomorphic neuropathology associated with different LRRK2 mutations and even within the same mutation. Indeed, the analysis of LRRK2-associated PD brains revealed a variety of pathological features consisting in a pure nigral neuronal degeneration, similar to classical PD cases, together with typical LBs and LNs [83,120,121]. There are also some cases were LBs and LNs are absent, but instead is displayed hyperphosphorylated or ubiquitinated single proteins, in particular Tau, indicative of frontotemporal dementia [122].

The presence of LBs is the most typical and widely spread pathological feature in LRRK2-associated PD cases, occurring in the brainstem, cortex and limbic system. Indeed, LRRK2 patients who bear the most frequent mutation (G2019S) mainly exhibit α-synuclein positive LBs pathology, which is a typical feature of idiopathic PD [107].

Table 2. Pathological features of LRRK2 mutations.
Table representing the pleomorphic neuropathological features associated to the most frequent PD-LRRK2 mutations. Adapted from Lie J., et al, 2014 [107].

Mutation	Protein domain	Risk Ethnicity	Neuropathology
R1441C	ROC	Middle European	LBs; NFTs SN neuronal loss Ubiquitin staining
R1441G	ROC	Caucasian (Basque country)	SN neuronal loss Ubiquitin staining
R1441H	ROC	ND	Unknown
Y1699C	COR	ND	LBs; NFTs SN neuronal loss
G2019S	Kinase	All population	LBs; NFTs SN neuronal loss Ubiquitin staining
I2020T	Kinase	ND	LBs SN neuronal loss

ND- not determined; *LBs*- Lewy bodies; *LFTs*- neurofibrillary tangles; *SN*- substantia nigra

The wide variety of pathological features associated with different LRRK2 mutations, demonstrate that this protein influences different mechanisms of cell viability. The effect of *LRRK2* mutations may still be conditioned by genetic variations of other loci such as *MAPT* or *SNCA*, which encodes for Tau and α-synuclein, two other central proteins in neurodegeneration. The fact that LBs inclusions are a pathological feature common to LRRK2-related familial PD cases and sporadic cases, also highlight the importance of LRRK2 as a crucial protein to bridge the common features between idiopathic and familial PD pathology [112].

However, the characterization of LRRK2-associated neuropathology is still dependent on the systematic evaluation of large number of LRRK2 PD patients and respective families, in different populations.

1.5.4 Interplay between GTPase and Kinase domains

The central catalytic unit of LRRK2 confers to the protein a particular dual enzymatic activity: GTPase and kinase. Several lines of evidence suggest a potential intrinsic regulatory mechanism between these two domains [123-125]. Interestingly, it is also in this ROC-COR-kinase catalytic core, where the most pathogenic and frequently observed LRRK2 mutations are found. In particular, the G2019S mutation at the kinase domain of LRRK2 is the most frequent mutation related with autosomal dominant forms of PD. The biological consequence of this glycine substitution for a serine has been extensively investigated, and the consensus is that it promotes an exacerbated increase in the kinase activity of the protein, resulting in neurological toxicity [126, 127]. Still, in the same kinase domain, the effect of the I2020T mutation is not well established. Contradictory results showing that this mutation promotes an increase, decrease or even a null effect on the kinase activity of the protein have been reported [128, 129]. The ROC domain of LRRK2, houses three different substitutions in the Arg1441 residue, R1441C/G/H, which appear to affect GTPase efficiency, leading to a decreased ability of LRRK2 to hydrolyze GTP [130-132]. These mutations are thought to alter the folding properties of the protein and, consequently, interfere with the known ability of LRRK2 to dimerise. This occurs due to the particular location of the R1441 residue, which might be responsible for disrupting the hydrogen bond between two GTPase domains [133, 134]. More recently, the Y699C mutation at the COR domain of LRRK2 has also been shown to affect the GTPase activity of

the protein [99]. This mutation causes a decrease in GTP hydrolysis by strengthening the interaction between the ROC-COR domains, which leads to a weaker bond between LRRK2 monomers. The described alterations on the GTPase activity of LRRK2 will also subsequently modulate the downstream kinase activity of the protein, in particular, by stimulating LRRK2 autophosphorylation [131,133,134]. Interestingly two mutations in the ROC domain of the protein, K1347A and T1348N, were described to totally abolish its kinase activity [134]. Moreover, the N and C-terminal of LRRK2 were suggested to act as modulators of its kinase activity. Thus, the N-terminus is suggested to have an inhibitory effect on kinase activity, while the C-terminal tail is required for full kinase activity [129, 135]. Summing up, there are several LRRK2 mutations located within the GTPase and kinase domains of the protein, which reciprocally condition the double enzymatic activity of the protein and consequently, the downstream events. Therefore, the modulation of LRRK2 GTPase and kinase enzymatic activity is an appealing candidate for therapeutic intervention, aiming at preventing LRRK2-dependent neuronal toxicity and progression of the disease [136-138].

1.5.5 LRRK2 interacting proteins and putative function

The complex multi-domain structure of LRRK2 encompasses a central GTPase and kinase core, flanked by additional putative protein interacting domains. These last domains suggest that LRRK2 may act as a scaffold protein involved in several signalling pathways and protein complexes.

A number of *in vitro* screens revealed several potential LRRK2 substrates and/or interactor proteins, however their validation and biological meaning in *in vivo* models is not frequently achieved. To date, there are some identified and confirmed LRRK2 interactors that help to build a picture about the putative role of this complex multifunctional protein in several pathways and cellular mechanisms [139-142].

The potential role of LRRK2 in the cytoskeleton architecture and microtubule network dynamics was initially reported by the identification of ezrin/radixin/moesin, and β-tubulin, as LRRK2 substrates [101,143]. More recently, LRRK2 was reported to phosphorylate tubulin-associated Tau at the Thyrosine 181 residue, which may regulate neurite outgrowth, by promoting neurite retraction [86]. In the present thesis, we explored the biological

consequences of this interaction and proposed a mechanism through which LRRK2 regulates intracellular levels and Tau biochemical species by compromising Tau-proteasomal degradation (fully explored in Chapter 4).

The structural similarity of LRRK2 with MLKs as well as the *in vitro* results showing its capacity to phosphorylate MKK3/6 and 4/7, suggest that this protein is upstream of the MAP kinase pathways. This idea is strongly supported by the *in vivo* results obtained in LRRK2 transgenic mice, where hyper-phosphorylation of MKK4 by the mutant G2019S, activates the MKK4-JNK-c-Jun pathway, leading to degeneration of dopaminergic neurons [144]. Studies in a *Drosophila melanogaster* revealed that LRRK2 phosphorylates the transcription factor FoxO1 at Ser319. It is known that this phosphorylation promotes an enhancement of transcriptional activity, triggering a cascade of mechanisms, like oxidative stress and programed cell death. However, other direct downstream targets are poorly identified [145].

The ADP-ribosylation factor GTPase-activating protein 1 (ArfGAP1) was also identified as a robust substrate of LRRK2. The two proteins interact *in vivo* in the brain and co-localize at Golgi membranes. ArfGAP1 regulates LRRK2 GTPase activity and thereby modulate its kinase activity. Moreover, in primary cortical neurons, silencing of ArfGAP1 expression rescues the neurite shortening phenotype induced by LRRK2-G2019S, and neurite shortening induced by ArfGAP1 overexpression is also attenuated by silencing of LRRK2 [146].

The role of LRRK2 in the synaptic environment was highlighted by the discovery of EndophilinA (EndoA) as a LRRK2 substrate. EndoA is a crucial protein involved in the vesicle formation during the endocytosis process [147]. When LRRK2 is phosphorylated at serine 75, it inhibits the role of EndoA on membrane tubulation, increasing its affinity to bind to membranes and so compromising synaptic vesicle endocytosis [148]. α-synuclein is another synaptic protein that was initially reported to be a LRRK2 substrate [149]. Although this idea was very appealing, the biological meaning of this interaction was not further explored. More recently, we reported that α-synuclein interacts LRRK2 [150]. In Chapter 3, we fully explore how LRRK2 models α-synuclein aggregation pattern *in vitro*, showing they co-localize in neurons and LBs.

1.6 Cellular Quality Control Systems

The misfolding and accumulation of certain proteins is a pathology hallmark common to several neurodegenerative disorders, where it could act as an underlying cause of the disease mechanism [151]. In fact, the accumulation of misfolded proteins leads to the formation of intermediate oligomeric species and, ultimately, protein aggregates. Whether these protein aggregates promote a toxic or a defensive effect on the cells remains a topic of intense debate in the field [152]. Neurons, as post-mitotic cells with a high metabolic activity, are extremely sensitive to protein accumulation. Thus, the *post-mortem* confirmation of the presence of protein aggregates in the brain of patients with neurodegenerative diseases emphasises the importance of protein turnover in neuronal homeostasis.

To avoid the deleterious process of protein accumulation, cells have specific quality control mechanisms wish include molecular chaperons, the ubiquitin-proteasome system (UPS) and autophagy. While chaperons help other proteins in the folding process, the UPS and autophagy are responsible for the targeting and degradation of unfolded or mutated proteins. The degradation of proteins is critical to clear the cytosolic space, from proteins that might be harmfull for essential cellular processes, and also to recycle amino acids. Impairment in the cell quality control mechanisms is usually related with neurodegenerative diseases and therefore the focus of intensive research in the field [153].

1.6.1 The ubiquitin-proteasome system (UPS)

The UPS is responsible for the degradation of misfolded, mutated and excess cytoplasmic short-lived proteins. Protein substrates are tagged with a poly-ubiquitin chain and targeted for proteasomal degradation (Figure 3). This complex mechanism depends on a cascade of enzymatic events involving specific proteins for the degradation of a substrate. This mechanism requires the involvement of ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and protein-ubiquitin ligase (E3). These enzymes specifically recognize the substrates, covalently attach multiple ubiquitin molecules, which will make the protein recognized and degraded by the 26S subunit of the proteasome [151, 155].

The *PARKIN* and *UCH-L1* genes encode two proteins associated with familial forms of PD (Table 1). These two proteins play roles in the UPS. Parkin is a typical ubiquitin ligase enzyme (E3) and UCHL-1 is an ubiquitin carboxyl-

terminal esterase, involved in deubiquitylation and the recycling of ubiquitin [156, 157]. Mutations in these PD-related genes, as well as exposure to stressful environmental conditions impair the UPS, leading to the accumulation of protein aggregates and intermediary protein species, detrimental for neuronal survival [151-155]. By itself, proteolytic stress caused by large amounts of non-degraded proteins also inhibits the regular function of the UPS. This creates a vicious cycle that alters the regular mechanism of protein degradation, leading to continuous accumulations and consequent aggregations of non-degraded proteins.

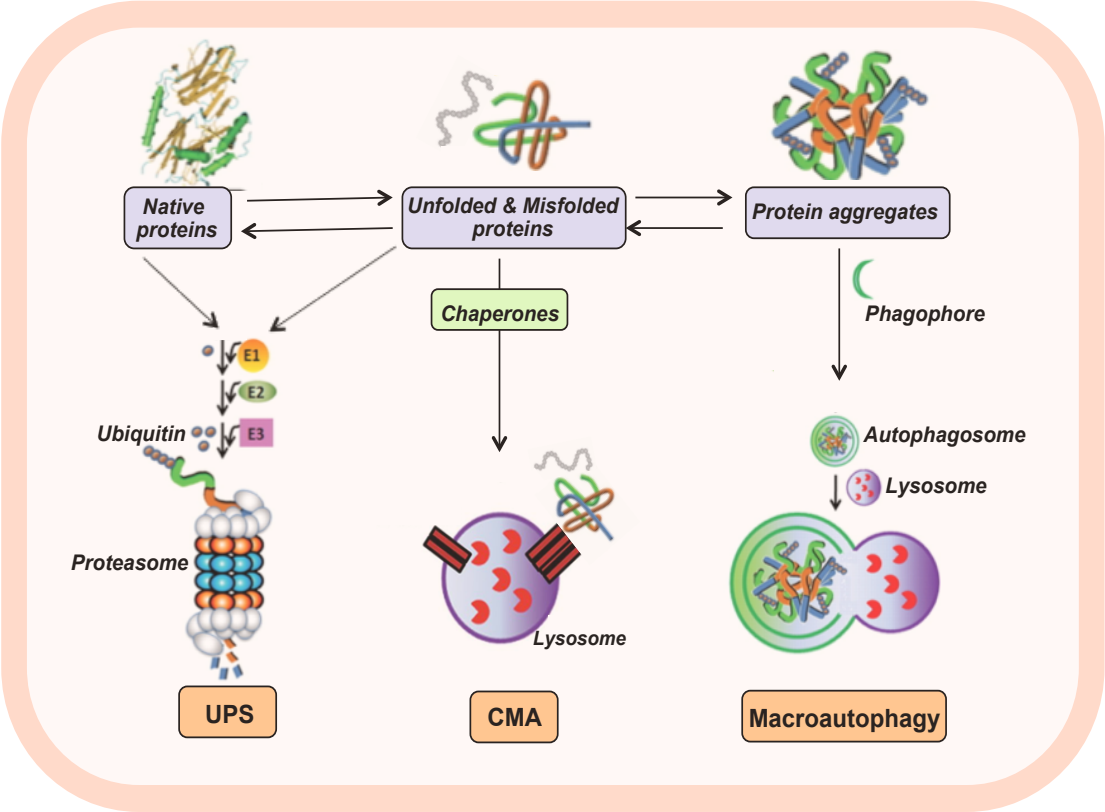

Figure 3. Cellular Quality Control Systems.
Schematic representation of the ubiquitin proteasome system (UPS), chaperone mediated autophagy (CMA) and macroautophagy. Native and misfolded protein can be targeted with a poly-ubiquitin chain to be degraded by the proteasome (UPS) or even a chaperon protein that lead them to be degraded in the lysosome (CMA). Protein inclusions and bigger aggregates are engulfed in a phagophore that after merged with a lysosome, results in an autophagosome, characterizing the macroautophagy process.

The UPS activity decreased in aged neurons. Accordingly, age-related impairment of the proteasomal activity is implicated in several neurodegenerative diseases [153]. Moreover, the presence of highly ubiquitylated protein inclusions in

different neurodegenerative diseases, suggests that impairment of protein degradation might be a common feature of these disorders [151,158].

Thus, being the UPS an essential protein quality control mechanism, disturbances on its function might lead to pathological conditions, such as those occurring in neurodegenerative disorders as PD and AD. However the exact mechanisms underlying proteasome impairment in neurodegeneration are still elusive.

1.6.2 Autophagy

Autophagy is another cellular quality control mechanism, responsible for the clearance of cytosolic components, in lysosomes. Its importance in the central nervous system has been emphasized in recent years [159]. Macroautophagy, microautophagy, and chaperone mediated autophagy (CMA) are the three types of autophagy co-existing in animal cells, which differ depending on the size of the cargo delivered to the lysosomes. Both macroautophagy and microautophagy involve the direct sequestration of the cytosolic cargo. In macroauthophagy, this sequestration happens by a vacuole that seals to form a double-membraned vesicle (autophagosome). In microauthophagy, the cargo sequestration happens by invaginations at the lysosomal membrane. The CMA does not involve sequestration of cytosolic cargo, which instead is selectively recognised by a complex of chaperones that mediates its delivery to a receptor at the lysosomal membrane (Figure 3) [159,160]. This translocation process is limited to soluble proteins that are able to be completely unfolded. Nowadays, the important role of autophagy in neural cells is well accepted and there is evidence confirming the altered autophagy in major neurodegenerative disorders. This is especially due to accumulation of autophagic vesicles in multiple diseased-brains and particularly in SNpc neurons from PD patients [161].

There are also genes related to familial forms of the disease, which are directly involved in autophagy, such as the ATP13A2, that encodes for a transmembrane lysosomal protein [162].

As already mentioned, age is the most important risk factors for neurodegenerative disorders. Interestingly, with ageing there is an impairment of the protein degradation mechanisms, leading to protein accumulation that gradually contributes to an imbalance in protein homeostasis. Consequently, this

results in inadequate response to stress, increased toxicity and overall reduced cell lifespan, which comprise the basis of neurodegenerative diseases [153,163].

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Chapter 2.

Aims of the project

Aims of the project

Parkinson's disease is a disorder, with causes and mechanisms still not completely known and, consequently, without a definitive cure. However, the study of PD-related gene mutations became a major focus to understand the underlying mechanisms of the disease in order to develop efficient therapeutics.

To date, mutations in the *LRRK2* gene are considered to be the most common genetic cause of PD. Several studies have unveiled the high degree of complexity of this multi-tasking protein. Despite the different clues about the cellular mechanisms in which LRRK2 might be involved, the function of this protein and its role in PD-associated neurodegeneration remains unclear.

In order to gain insight into both the normal function of LRRK2 and also into its role in PD, our goals were to:

1. Explore the relation between LRRK2 with another central PD-related protein, α-synuclein (Chapter 3)
   1.1 Investigate the interaction of LRRK2 with α-synuclein using cell models and also in PD brain samples.
   1.2 Evaluate the effect of LRRK2 on α-synuclein aggregation using a cell model of α-synuclein aggregation.

2. Understand the effect of LRRK2 and its kinase activity on Tau protein (Chapter 4)
   2.1 Evaluate the effect of LRRK2 on Tau intra- and extra-cellular levels
   2.2 Clarify important cellular mechanisms where LRRK2 could be involved in compromising Tau accumulations and the cellular consequences of this effect.

3. Identify novel LRRK2-interacting proteins and the molecular pathways where it could be involved (Chapter 5)

Chapter 3.

LRRK2 interactions with α-synuclein in Parkinson's disease brains and in cell models

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LRRK2 interactions with α-synuclein in Parkinson's disease brains and in cell models

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Chapter 3. LRRK2 interactions with α-synuclein in Parkinson's disease brains and in cell models

3.1 Introduction and main goals

Parkinson's disease (PD) is the most common neurodegenerative movement disorder resulting from the loss of dopamine neurons in the substantia nigra and the abnormal deposition of cytoplasmic inclusions known as Lewy bodies (LBs) and Lewy neuritis (LNs) in widespread regions of the brain [1]. The aetiology of PD is multifactorial, with a growing number of genetic abnormalities identified [2]. The first PD causative gene was α-synuclein (*SNCA*), which encodes for the presynaptic protein α-synuclein [3]. Rare patients have missense mutations (A53T, A30P, and E46K) or multiplications of *SNCA* [2], but all PD patients accumulate phosphorylated α-synuclein in the form Lewy pathologies [4,5]. Leucine-rich repeat kinase 2 (LRRK2) has been identified as the second and more common gene responsible for autosomal-dominant PD [6-9]. The function of the large LRRK2 protein remains unclear, although its serinethreonine/tyrosine kinase function is considered most important for PD etiology due to the occurrence of the most common *LRRK2* mutation (G2019S) in this domain [2]. As phosphorylation of α-synuclein is central to PD and the most common autosomal-dominant mutation occurs in a kinase, there has been intense debate about whether α-synuclein physically interacts with LRRK2 and whether it might be one of its substrates [10]. However, to date, only one report has shown that α-synuclein interacts with, and is phosphorylated by, LRRK2 and only under pathological and non-physiological oxidative stress conditions [11]. Co-immunoprecipitation is the gold standard for assessing direct protein interactions but relies on antibody specificity, a previous problem for LRRK2 antibodies that has been recently solved with the aid of resources from the Michael J. Fox Foundation (MJFF). It is now possible to revisit the question of a LRRK2 and α-synuclein interaction using these new and well-characterized LRRK2 antibodies. The aim of the present study was to establish whether LRRK2 and α-synuclein interact in human brain samples and to investigate the significance of the interaction in cell models. We report a molecular interaction between LRRK2 and α-synuclein under endogenous and over-expression conditions. We show in affected PD brain regions that the amount of LRKK2 protein is increased in association with increasing levels of phosphorylated

α-synuclein. At the neuronal level, we confirm co-localization of LRRK2 and α-synuclein in LBs in PD patients and show co-localization in a cell model of α-synuclein inclusion formation. In addition, knockdown of *LRRK2* in this cell model increases the number but reduces the size of α-synuclein inclusions. Altogether, our data provide strong evidence for an interaction between LRRK2 and α-synuclein in PD and opens novel avenues for the investigation of the interplay between different PD genes and their exploitation as targets for therapeutic intervention.

3.2 Materials and methods

Human and mouse brain samples

Human brain tissue was obtained from the Sydney Brain Bank and the NSW Tissue Resource Centre as part of the Australian Brain Bank Network funded by the National Health and Medical Research Council of Australia (NHMRC) with appropriate institutional ethics approvals. Frozen brain tissue samples and formalin-fixed paraffin-embedded tissue sections from different brain regions considered to be progressively affected by α-synuclein deposition in PD [12] were received from ten sporadic PD cases and ten matched controls (Appendix Table 3.1). The regions were the amygdala (affected pre-clinically in PD), the midbrain and anterior cingulate cortex (affected when symptoms are apparent) and the visual cortex (remains free of α-synuclein pathology even at end stage disease). Crude soluble human brain proteins were extracted from the frozen tissue as previously described [13]. Briefly, tissue was homogenized with a pre-chilled dounce homogenizer using ice-cold tris-buffered saline (TBS, pH7.4) lyses buffer (LB) containing protease and phosphatase inhibitor cocktails (Roche, Dee Why, Australia and Thermo Fisher Scientific, Waltham, MA, USA). The TBS-soluble supernatant fraction was collected after centrifugation at 16,000×g for 25 min at 4 °C, and the pellets were solubilized in LB containing 5 % SDS (SDS-soluble fraction). Protein concentration was measured using a Nanodrop1000 (Thermo scientific) for all samples. Ethics approval for the human tissue studies was from the University of New South Wales Human Research Ethics Committee.

Frozen mouse brain samples from LRRK2 knockout C57BL/6J adult mice and age-matched controls were kindly provided by Dr. Mark Cookson and Dr. Iakov Rudenko (NIH (NIA), Bethesda, MD, USA). Mouse brain tissue was lysed in

RIPA buffer (25 mM Tris–HCl pH7.6; 150 mM NaCl; 0.1 % SDS; 1 % NP40) supplemented with protease and phosphatase inhibitor cocktails (Roche diagnostics, Mannheim, Germany) using a mechanic homogenizer. Lysates were incubated in a rotor for 1 h at 4 °C and then sonicated. Following centrifuge separation (at 10,000×g for 10 min at 4 °C), the supernatants were kept and total protein concentration quantified using BCA assay (Thermo Fisher Scientific, Rockford, IL, USA).

Immunoprecipitation and western blot analyses

Immunoprecipitation experiments were performed using 1 mg (cells) or 6 mg (brain) of total protein. Lysates were pre-cleared by incubation with 20 µl of protein G beads (Invitrogen, Barcelona, Spain) for 30 min at 4 °C in rotation. Supernatants were recovered and incubated with 2 µg of the corresponding immunoprecipitation antibody: anti-α-synuclein (C-20, Santa Cruz Biotechnologies, Santa Cruz, CA, USA), anti-Myc (Cell Signaling, Danvers, MA, USA) or anti-LRRK2 (c41-2 MJFF), followed by overnight rotation at 4 °C. The next day, 40 µl of protein G beads were added for 3 h in a rotator at 4 °C. Beads were washed 5× with immunoprecipitation buffer, then re-suspended in 20 µl of protein sample buffer (50 mM Tris–HCl pH6.8; 2 % SDS; 10 % glycerol; 1 % β-mercaptoethanol; 0.02 % bromophenol blue) and boiled at 95 °C for 5 min. Supernatants were resolved on 12 % SDS-PAGE gels. Proteins were transferred overnight to nitrocellulose membranes and blocked in 5 % non-fat dry milk in TBS-Tween for 1 h. The membranes were incubated overnight at 4 °C with the primary antibodies using the following dilutions: anti-α-synuclein (syn-1, BD Biosciences, San Jose, CA, USA, 1:1,000), anti-Myc (Cell Signaling, Danvers, MA, USA, 1:4,000) and anti-LRRK2 (c41-2 MJFF, 1:1,000). Immunoblots were washed with TBS-Tween and incubated for 1 h at room temperature with the corresponding HRP-labelled secondary antibody (GE Healthcare, Bucks, UK, 1:10,000). Immunoreactivity was visualised by chemiluminescence using an ECL detection system (Millipore, Billerica, MA, USA) and subsequent exposure to autoradiographic film. Standard Western blotting was used to assess the relative amounts of LRRK2 [14] and α-synuclein [15] in 25 µg of the TBS-soluble protein from human brain samples compared with β-actin (loading control). The following primary antibodies were used: LRRK2 (c41-2, MJFF, 1:1,000), anti-α-synuclein (syn-1, BD Biosciences, San

Jose, CA, USA, 1:2,000), anti-S129 phosphorylated α-synuclein (Elan Pharmaceuticals Inc. [16], 1:10,000 or WAKO, Richmond, VA, USA, 1:10,000) and anti-mouse β-actin (Sapphire Biosciences, Waterloo, Australia, 1:10,000). LRRK2 protein was separated on precast NuPAGE 3–8 % gradient Tris–acetate gels (Invitrogen, Carlsbad, CA, USA) with constant voltage of 150 V for 50 min, and transferred onto polyvinylidene difluoride membranes (BioRad Laboratories, Hercules, CA, USA) at 30 V constant for 2 hours. α-synuclein and S129 phosphorylated α-synuclein was separated on 12 % SDS- PAGE gels with constant voltage of 100 V for 90 min, and transferred onto 0.22-μm nitrocellulose membranes (Bio- Rad Laboratories, Hercules, CA, USA) at 75 V constant for 45 min. To normalise the data between the different gels, the same control sample was loaded on all gels and probed for β-actin in addition to the proteins of interest. Immunoreactivity was visualised by chemiluminescence using an ECL detection system (GE Healthcare Biosciences, Pittsburgh, PA, USA) and the intensity of each band quantified using ImageJ software (http://rsbweb.nih.gov/ij/) with the relative expression normalised to the β-actin of the internal standard. Multivariate linear regression analysis (SPSS IBM, New York, NY, USA) was used to identify any differences in protein levels between groups and regions, and linear regression modelling (SPSS IBM, New York, NY, USA) was used to determine if LRRK2 and α-synuclein protein levels were related to each other and could predict group status. Age and post-mortem interval were cofactored into all analyses. The mean difference and standard error are given for all values.

Routine cell culture, plasmids and transfections

Both wild-type (WT) and G2019S forms of LRRK2 plasmids (pCMV-Tag3B-2xMyc-LRRK2, a kind gift from Dr. Mark Cookson, NIH (NIA), Bethesda, MD, USA) and WT pSI-α-synuclein plasmids (a kind gift from Dr. Bradley Hyman, Massachusetts General Hospital, USA) were used to over-express proteins for co-immunoprecipitation experiments.

Human embryonic kidney 293 cells (HEK-293) were cultured in DMEM media (Invitrogen, Barcelona, Spain) supplemented with 10 % fetal bovine serum and 1 % penicillin-streptomycin in 5 % CO_2 at 37 °C. One day before transfection 1.5×10^6 cells were seeded in 10 cm plates. Cells were transiently transfected

using a total of 6 µg of plasmid DNA using FuGENE®6 (Roche diagnostics, Mannheim, Germany). Forty-eight hours later, cells were washed with PBS, harvested in immunoprecipitation buffer supplemented with protease and phosphatase inhibitors and sonicated. Lysates were centrifuged at 10,000×g for 10 min at 4 °C. Pellets were discarded and the total protein concentration of the supernatants quantified using BCA assay (Pierce).

α-Synuclein aggregation model in H4 cells

A gene construct encoding for tagged version of α-synuclein (SynT, a kind gift from Dr. Bradley Hyman, Massachusetts General Hospital, US) was co-transfected with the synphilin-1 into H4 cells to recreate Lewy body-like inclusions, as previously described [17]. Briefly, H4 cells were cultured in OPTIMEM media (Gibco, Invitrogen, Barcelona, Spain) supplemented with 10 % of fetal bovine serum and 1 % of penicillin-streptomycin in an atmosphere of 37 °C and 5 % CO_2. Twenty-four hours before the transfection, 2.0×10^5cells were seeded in a 35 cm dish (Ibidi, Munich, Germany). Cells were transfected with 2 µg of each synphilin-1 and SynT plasmids using FuGENE®6 (Roche diagnostics, Mannheim, Germany). After transfection, cells were maintained for 48 h prior to further manipulations. H4 cells with reduced LRRK2 expression were created using lentiviral particles encoding *LRRK2* shRNAs or a control scramble shRNA sequence (Appendix Table 3.2) and the model for α-synuclein inclusions (described above) recreated in H4-LRRK2 knockdown cell lines.

Confocal microscopy in cells and tissue sections

LRRK2 and α-synuclein localization was performed in human tissue sections of the midbrain and anterior cingulate cortex and in transfected H4 cells with and without shRNA knockdown of LRRK2 expression. In the human tissue sections, both routine peroxidase immunohistochemistry and double immunofluorescence were performed. In H4 cells, single and double immunofluorescence was performed.

Adjacent human sections were pre-treated with 99 % formic acid for 3 min and citrate buffer (pH6.0) for 3 min, then incubated sequentially with anti-LRRK2 (MJFF c41-2, 1:200 and L955 Abgent, 1:500), anti-α-synuclein (BD Biosciences,

San Jose, CA, USA, 1:200) and anti-S129 phosphorylated α-synuclein (Elan Pharmaceuticals Inc. [16], 1:10,000) antibodies, biotinylated secondary antibodies (anti-mouse IgG for α-synuclein and anti-rabbit IgG for LRRK2; Vector, Burlingame, CA, USA), and then the avidin-biotin complex (Vectastain Elite ABC Kit, Vector, Burlingame, CA, USA) prior to visualization with DAB substrate (Sigma, St. Louis, MO, USA) in 0.1 % H_2O_2. Sections were counterstained with cresyl violet. LRRK2 and α-synuclein were co-localized in human sections and the H4 cell α-synuclein aggregation model using double immunofluorescence. Briefly, cells were washed, fixed with 4 % PFA, permeabilized with 0.5 % Triton, blocked with 1.5 % normal goat serum, then incubated in anti-α-synuclein (BD Biosciences, San Jose, CA, USA, 1:1,000) and anti-LRRK2-2 (MJFF c41-2, 1:50) antibodies, while human sections were pre-treated as above and incubated with anti-α-synuclein (BD Biosciences, San Jose, CA, USA, 1:200) and anti-LRRK2 (L955 Abgent, 1:500) antibodies or with anti-S129 phosphorylated α-synuclein (Elan, 1:10,000) and anti-LRRK2 (L955 Abgent, 1:500) antibodies. Then, a cocktail of secondary antibodies was used: for α-synuclein and S129 phosphorylated α-synuclein anti-mouse IgG conjugated with Alexa Fluor 488 (Molecular probes, Eugene, OR, USA, 1:500) and for LRRK2 anti-rabbit IgG conjugated with Alexa Fluor 568 (Molecular Probes, 1:250). Fluorescent images were captured either using a Nikon Microscope ECLIPSE 90i confocal microscope (for human tissue sections) or using a Leica Microsystems confocal microscope (for H4 cells). The proportion of neurons in the human brain sections that co-localized LRRK2 and α-synuclein was quantified in each section (total number of LRRK2 positive neurons/the total number of α-synuclein positive neurons]) and double labeling of LBs assessed (average number sampled/ section varied from 1 to 63, depending on the region assessed). Pearson correlation coefficients were used to determine whether there was any relationship between the numbers of LBs containing α-synuclein and those also containing LRRK2 in the PD cases examined. Quantification of the aggregation pattern of α-synuclein inclusions in H4 cells was performed. Briefly, for each condition (control and LRRK2-KD), a total of 40–60 cells containing α-synuclein inclusions were analyzed, and a total of three independent experiments were performed. Cells were classified into two groups: cells with <5 inclusions and cells with ≥5 inclusions, and the results were expressed as a percentage of the total number of cells with inclusions. The

average size of inclusions per cell was also quantified using the ImageJ software (http://rsbweb.nih.gov/ij/).

3.3 Results

Disclaimer: The presented results with human brain samples were performed by Yue Huang from Neuroscience Research Australia and the University of New South Wales.

3.3.1 LRRK2 co-immunoprecipitates with α-synuclein

In order to investigate the interaction between LRRK2 and α-synuclein, we used mouse brain samples from WT and *LRRK2* knockout animals. The immunoprecipitation of α-synuclein from mouse brain lysates pulled down LRRK2 in WT samples, but not in knockout samples (Figure 1A). We also verified the interaction between LRRK2 and α-synuclein when the immunoprecipitations were performed in human brain lysates.

In order to investigate whether *LRRK2* mutations alter the interaction with α-synuclein, we over-expressed WT or G2019S mutant LRRK2 together with α-synuclein in HEK-293 cells. Immunoprecipitation of LRRK2 from cells over-expressing Myc-LRRK2 (WT or G2019S mutant) together with α-synuclein pulled down α-synuclein (Figure 1B).

Consistently, when α-synuclein was immunoprecipitated, the LRRK2 proteins (WT and G2019S) were also co-immunoprecipitated (Figure 1C). We did not find significant alterations in the pattern of co-immunoprecipitation between WT and G2019S mutant, indicating that the interaction between the two proteins is not disturbed by this mutation.

In order to map the interaction between LRRK2 and α-synuclein, we express several Flag-LRRK2 constructs together with α-synuclein. After an immunoprecipitation of α-synuclein we could verify that the co-immunoprecipitation with LRRK2 only occurs in the first two constructs (Figure 1D). This result demonstrates that the interaction of these two proteins requires the N terminal of LRRK2.

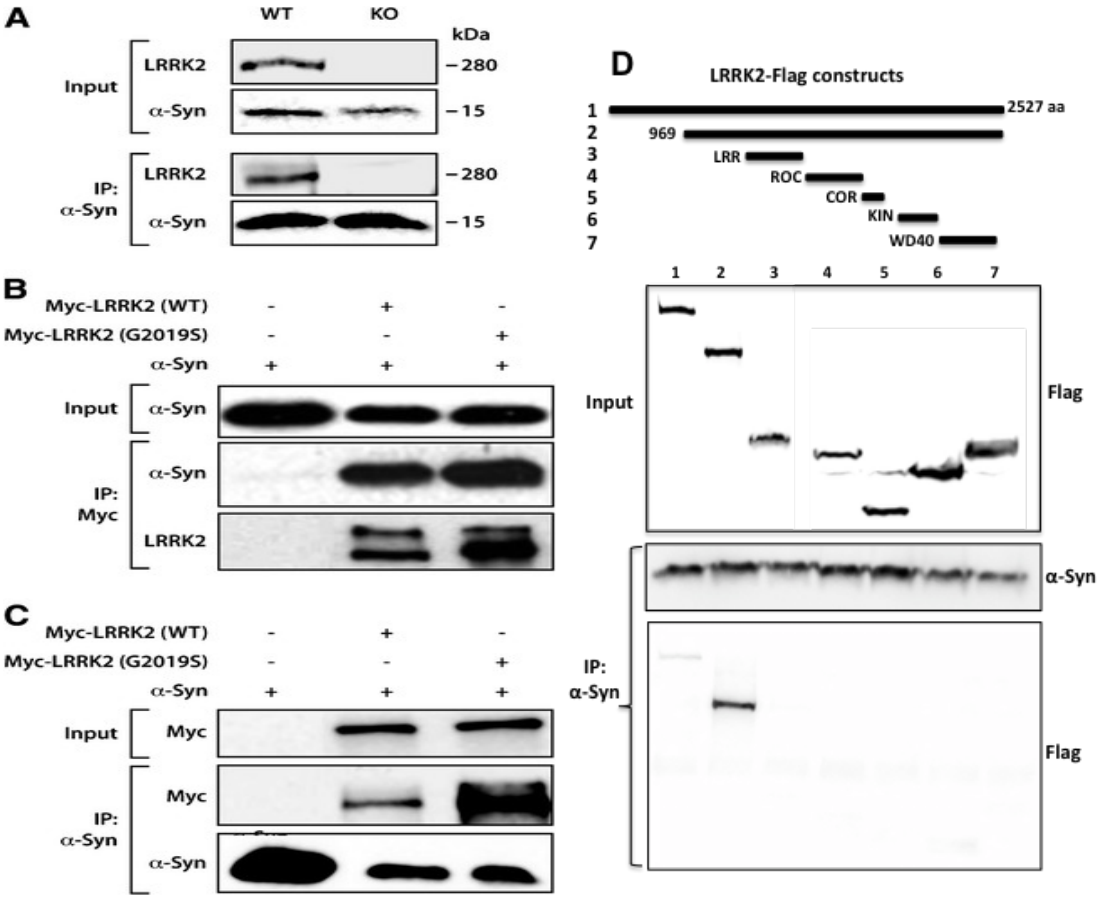

Figure 1. Co-immunoprecipitation of LRRK2 and α-synuclein. A Western blots showing the immunoprecipitation of endogenous α-synuclein in lysates from WT and LRRK2 knockout mouse brains. The co-immunoprecipitation with endogenous LRRK2 occurs in WT but not in the LRRK2 knockout brain sample. **B, C** Over-expression of Myc-LRRK2 (WT or G2019S) together with α-synuclein in HEK-293 cells showed the co-immunoprecipitation of LRRK2 (WT or G2019S) with α-synuclein. Using anti-Myc as the capture antibody and anti-α- synuclein and anti-LRRK2 antibodies for Western blotting (B) or using anti-α-synuclein as the capture antibody and anti-α-synuclein and anti-Myc antibodies for Western blotting (C). **D** Over-expression of Flag-tagged LRRK2 constructs together with α-synuclein in HEK-293 cells. Immunoprecipitating α-synuclein with a specific antibody, is showed the co-immunoprecipitation with LRRK2 constructs number 1 and number 2, using a anti-Flag antibody.

3.3.2 LRRK2 co-localizes with α-synuclein in PD brain and cell model

PD brain samples were examined to determine whether LRRK2 and α-synuclein or phosphorylated α-synuclein were co-localized. We found that LRRK2 increases along with α-synuclein in neurons prior to LB formation (Figure 2A) as well as depositing in some but not all of the hallmark inclusions (Figure 2B-D).

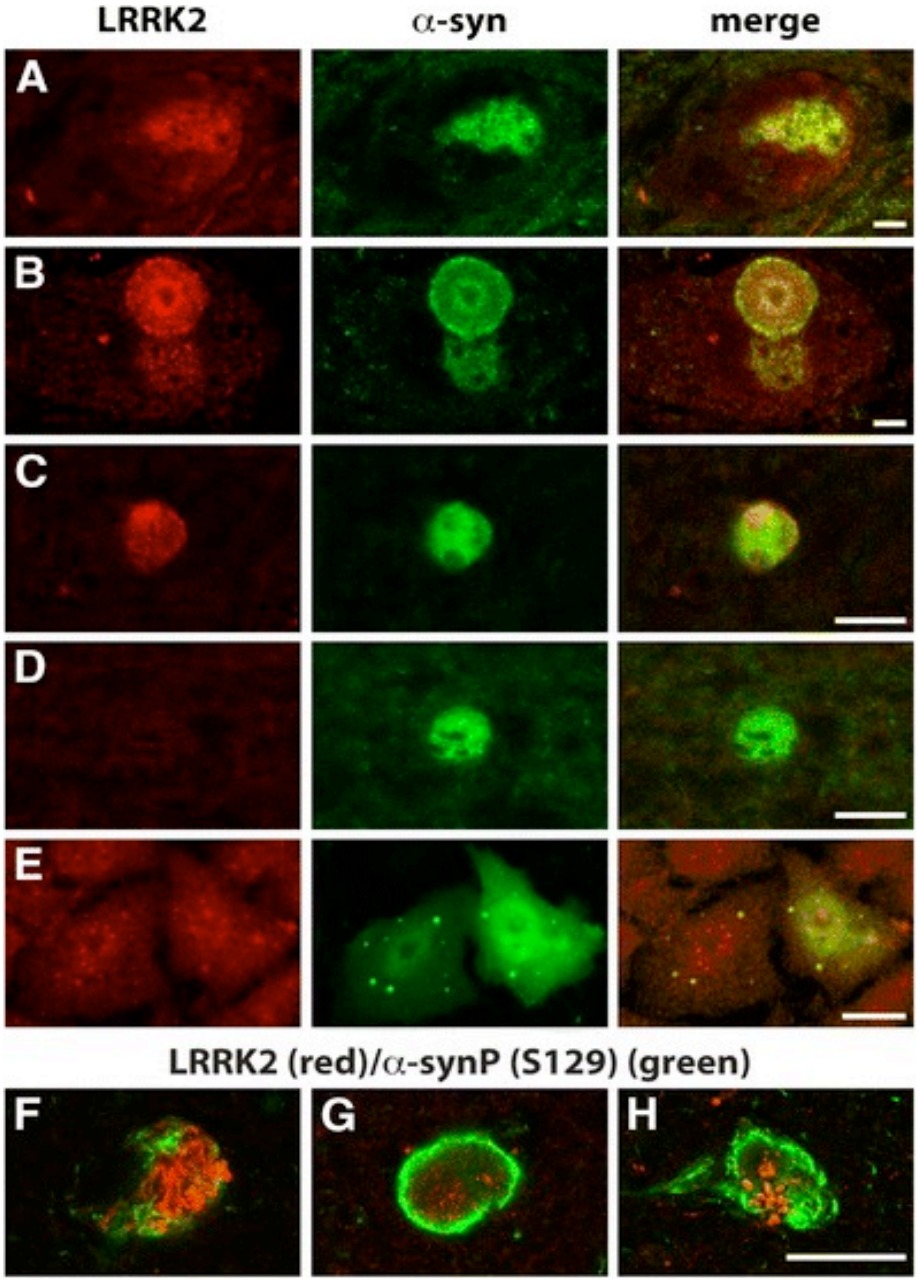

Figure 2. Co-localization of LRRK2 and α-synuclein in PD brain and cell models. In PD brains **(A-D, F-G)**, merged images clearly outline single neurons in the substantia nigra **(A, B)** and LBs **(B-D, F-G)** using double labelling immunofluorescence. There is an increase of LRRK2 and α-synuclein immunoreactivity in brainstem neurons without Lewy body formation **(A)**, with LRRK2 co-localizing with α-synuclein in LBs (donut inclusion in **B**) in these neurons. The co-localization of LRRK2 and α-synuclein was also observed in cortical LBs **(C)**. Cortical LBs without LRRK2 immunoreactivity were also observed (**D**). S129 phosphorylated α-synuclein antibody also confirmed co-localisation of LRRK2 with phosphorylated α-synuclein, with LRRK2 often centralized to a radiating pattern of phosphorylated α-synuclein fibrils **(F-H)**. In the H4 cell model, double-labelling immunofluorescence for α-synuclein inclusion formation shows that endogenous LRRK2 co-localizes with α-synuclein inclusions **(E)**. Scales in all panels are equivalent to 10 μm.

LRRK2 was also observed in phosphorylated α-synuclein-immunoreactive inclusions, often centralized to a radiating pattern of phosphorylated α-synuclein fibrils (Figure 2F,H). Quantitation of the numbers of α-synuclein inclusions immunopositive for LRRK2 in ten PD cases indicates that 60 % of cingulate LBs and 43 % of nigral LBs contained both proteins (Figure 2B-C). The specificity of the co-localization can be taken as genuine, as no 280kDa LRRK2 band was detected on Western blot and no immunoreactivity in tissue sections in peptide pre-absorption experiments. There was no correlation between the number of α-synuclein-positive LBs and those also containing LRRK2 across the cases examined. We also interrogated an in vitro model that reproduces the formation of α-synuclein inclusions in H4 cells [17]. Using this model, we observed co-localization of endogenous LRRK2 with the α-synuclein-positive inclusions (Figure 2E).

3.3.3 Knocking down LRRK2 expression reduces α-synuclein aggregation

To further investigate the effect of LRRK2 on α-synuclein aggregation, LRRK2 expression was knocked down using shRNAs in the H4 cell model. Knocking down LRRK2 expression did not produce significant changes in endogenous α-synuclein or phosphorylated α-synuclein levels (Figure 3A). Transiently transfecting these LRRK2-deficient cells with SynT and synphilin-1 expression plasmids showed that LRRK2 silencing significantly increased the number and decreased the size of α-synuclein inclusions resulting in a greater number of cells bearing smaller α-synuclein inclusions (Figure 3B-D).

3.3.4 Increasing levels of LRRK2 correlates with α-synuclein in PD

To explore the relationship between protein levels of LRRK2 and α-synuclein in PD, 20 cases (controls and Braak PD stages IV and V, Appendix Table 3.1) were analyzed. Multivariate analysis factoring in age and post-mortem delay showed that the levels of total and phosphorylated α-synuclein were significantly increased over control levels only in PD brain regions with LBs ($p < 0.001$). In the cases examined, all stage IV cases had high LB densities in the amygdala (Figure 4A), while significant densities of cingulate LBs were observed in all stage V cases (Figure 4B). No LBs were seen in the visual cortex of any case, although very small-phosphorylated deposits were observed in stage V

cases (Figure 4C).

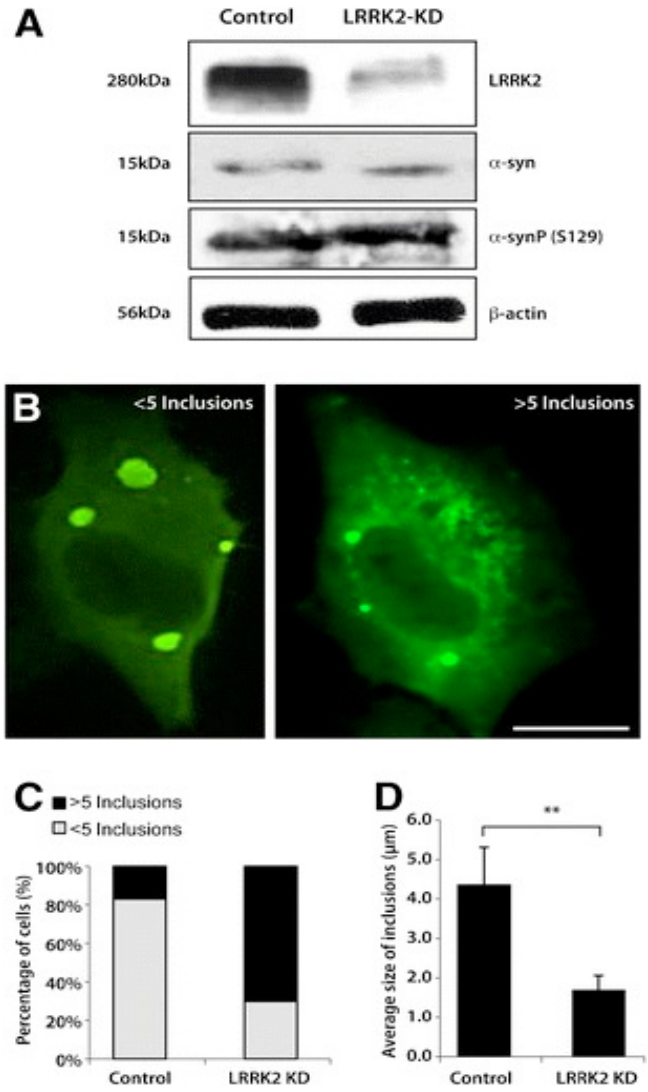

Figure 3. Knockdown of LRRK2 expression alters the size and number of α-synuclein inclusions. (**A)** Western blots showing that H4 cells infected with LRRK2-shRNA have the expected knockdown of LRRK2 protein (LRRK2-KD) compared with the scramble shRNA control, but have no significant change on the level of endogenous α-synuclein or phosphorylated α-synuclein at S129. (**B)** The model for α-synuclein inclusions was reproduced in a LRRK2 knockdown cell line and in parental control cells. Cells were classified into two groups according to the number of α-synuclein-immunoreactive inclusions observed: cells with five or more inclusions and cells with less than five inclusions. Scale bar=10μm. (**C)** Data from three independent experiments shows a greater proportion of cells containing five or more inclusions in the LRRK2 knockdown cells compared with controls. (**D)** LRRK2 silencing (LRRK2-KD) promotes a significant reduction in the average size of the inclusions, resulting in a more punctate aggregation pattern in the cells. Student's test (n=3; **p<0.01). Error bars=SEM.

In PD, there was a substantial 220±20 % increase over controls in α-synuclein protein levels in the amygdala and a less substantial 48±6 % increase in the cingulate cortex, with no change in the visual cortex (Figure 4D, E). This pattern of regional increase in α-synuclein levels was even more striking when assessing phosphorylated α-synuclein protein levels (p=0.01), as very low levels of phosphorylated α-synuclein were observed across all regions in controls compared to PD (Figure 4E).

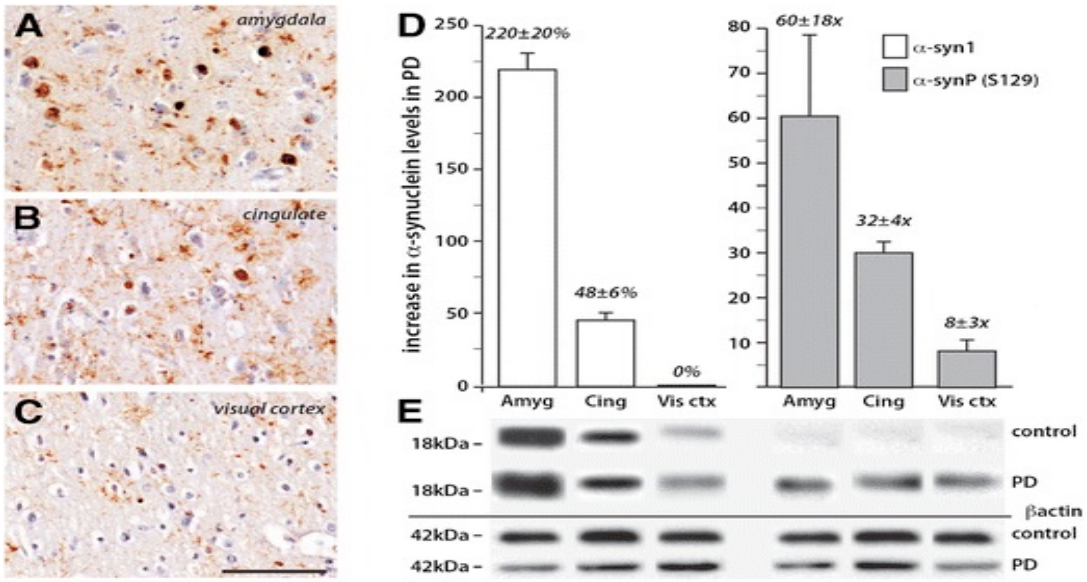

Figure 4. Increased levels of total and S129 phosphorylated α-synuclein in PD brain. **A-C** Peroxidase immunohistochemistry of brain sections from the same PD case showing the regional density of Lewy pathology as revealed by immunohistochemistry using phosphorylated α-synuclein antibody and counterstained with cresyl violet. Scale in **C**= 100 μm and is equivalent for **A** and **B**. Severe pathology is observed in the amygdala (**A**) with moderate pathology in the anterior cingulate cortex (**B**). Neuronal inclusions are not observed in the visual cortex (**C**). **D** and **E** Quantitation (**D**) of Western blots (**E**) in the same three brain regions in the PD cases (represented as an increase over control levels) confirmed the regional changes noted histologically in PD and showed considerably more phosphorylated α-synuclein compared with total α-synuclein in each regions (note the percentage at left versus fold change at right in **D**). Error bars=SEM.

There was a very large 60±18 fold increase in phosphorylated α-synuclein protein levels in the PD amygdala, a 32±4 fold increase in the PD cingulate cortex and an 8±3 fold change in the PD visual cortex relative to controls (Figure 4D,E).

The expression of LRRK2 was analyzed using the same methods in the same brain extracts (Figure 5). Multivariate analysis co-varying for age or post-mortem delay showed that the levels of LRRK2 were increased in PD compared with controls in regions containing LBs ($p<0.04$), with no difference between the LRRK2 levels in these Lewy body-containing regions ($p=0.6$). Within these regions, there was a small but significant 23±6 % increase over controls in full-length LRRK2 levels (Figure 5A,B).

To determine the relationship between LRRK2 and α-synuclein levels and PD, linear regression modeling was used assessing the protein levels obtained in the amygdala and cingulate cortex.

This analysis revealed that increasing levels of LRRK2 and total and phosphorylated α-synuclein correlated with each other in PD but not controls (Figure 5C, $p<0.001$, β coefficients=0.27, 0.33 and 0.37, respectively).

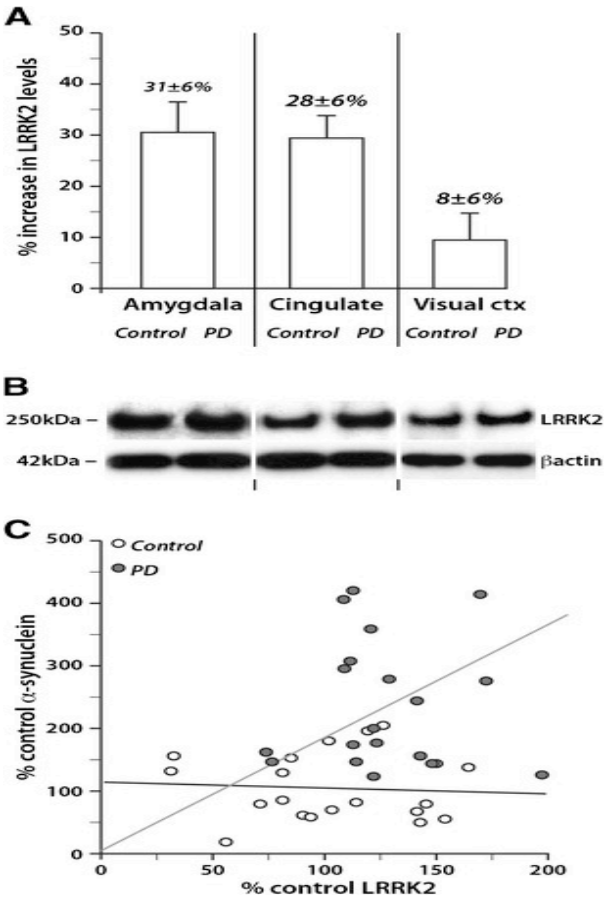

Figure 5. LRRK2 levels correlated with α-synuclein levels in PD brain. Quantitation (**A**) of LRRK2 Western blots (**B**) in the same brain regions in the PD cases (represented as an increase over control levels) and correlations with α-synuclein levels (**C**). The protein levels of LRRK2 were increased in the disease-affected areas (amygdala and cingulate) compared to the non-affected area (visual cortex) (**A**). Error bars=SEM. Multivariate analysis revealed a significant correlation between the increasing levels of α-synuclein and LRRK2 only in PD but not controls (**C**).

3.4 Discussion

Mutations in LRRK2 and α-synuclein proteins are known to be responsible for autosomal dominant forms of PD [2]. Due to the growing interest in the potential interaction of these proteins in the pathogenesis of PD [10,18], we investigated such an interaction using a variety of techniques. Co-immunoprecipitation showed that endogenous LRRK2 and α-synuclein interact in cells, mouse and human brain tissue. We also confirmed this interaction in over-

expression studies in HEK-293 cells, where the use of several LRRK2 construct revealed that the N terminal of LRRK2 is requires for the interaction with α-synuclein.

In this model, we found that the G2019S mutation did not alter the ability of LRRK2 to interact with α-synuclein. Nevertheless, we cannot exclude that the interaction with G2019S is not potentiated due to the over-expression of the protein in HEK cells. The G2019S mutation is located in the kinase domain of LRRK2, and shows an enhanced kinase activity compared to WT LRRK2 [19]. Our data indicate that the kinase domain and therefore the phosphorylation capacity of LRRK2 do not play a large role in its interaction with α-synuclein. This is consistent with recent evidence showing that the levels of rather than mutations in LRRK2 are related to the deposition of neuropathology [20]. Overall, these results unequivocally demonstrate, for the first time, a definite interaction between endogenous LRRK2 and α-synuclein, a finding that had only been detected under pathological and oxidative stress conditions [11].

In human PD brains, we show co-localization of LRRK2 and α-synuclein as well as S129 phosphorylated α-synuclein in LBs and also co-localization in neurons that have not formed LBs in LB producing regions. We have also replicated this co-localization of LRRK2 and α-synuclein in an established cell model for α-synuclein inclusion formation. Of interest, our quantitation in the PD cases showed that LRRK2 co-localized in more cortical compared with brainstem LBs. According to Braak PD staging [12], cortical LBs develop later in PD, further suggesting an early association between LRRK2 and α-synuclein in LB formation. In these neurons, S129 phosphorylated α-synuclein fibrils often appeared to radiate from more centralized LRRK2 within LBs. Overall, our results suggest that the interaction between LRRK2 and α-synuclein or S129 phosphorylated α-synuclein is enhanced prior to and during the formation of α-synuclein aggregation and fibrilization. These data are also consistent with other studies in brain tissue showing the co-localization of LRRK2 in α-synuclein-immunoreactive LBs [21–23], although questions regarding the specificity of the different LRRK2 antibodies used have been raised [24, 25]. While variance in the numbers of LBs co-localizing LRRK2 may be due to the age of the LBs assessed (see above) and account for some of the differences described in the literature, we are certain of the specificity of the antibodies used in the current study, as a number of

specificity experiments confirmed that the protein we localized to early forming LBs was LRRK2.

To test this association further, we knocked down LRRK2 in a cell model of α-synuclein inclusion formation and found that reduced LRRK2 expression altered α-synuclein inclusions, resulting in an increased number of smaller inclusions per cell. In this model, α-synuclein is co-expressed and co-aggregates with synphilin-1, as observed in LBs [26, 27]. Synphilin-1 recruits and binds α-synuclein leading to inclusion formation [26], and synphilin-1 and 14-3-3 proteins accumulate with mutant α-synuclein in A53T transgenic mice [28]. While there is little indication that synphilin-1 closely associates with LRRK2, 14-3-3 proteins are not only known to interact with and stabilize phosphorylated LRRK2 [29] but also have a preference for binding S129 phosphorylated α-synuclein [30] and accumulate in LBs [31]. 14-3-3 proteins and α-synuclein have opposing effects on regulating the activity of many enzymes [31], and such regulation may occur within a complex associated with LRRK2. The microtubule binding protein Tau phosphorylation complex has been shown to require both 14-3-3 and α-synuclein [32, 33], and it is of interest that over-expression of Tau has a similar effect on the formation of α-synuclein inclusions in this cellular model [34] to the knockdown of LRRK2 (present study), as well as sequestering phosphorylated Tau into the inclusions [34]. Either reducing the amount of unbound α-synuclein by enhanced recruitment into the tau phosphorylation complex and/or increased tau partnering of 14-3-3 to shift its binding from and decrease the phosphorylation and activity of LRRK2, would seem to produce similar effects on the formation of α-synuclein inclusions in this cellular model of inclusion formation. In mouse models, LRRK2 over-expression enhances the progression of α-synuclein-mediated neuropathological changes, and LRRK2 deletion delays the progression of pathology [20]. All of these data are consistent with an interaction between LRRK2 and α-synuclein in patients with PD.

This is the first study showing correlations between the relative protein levels of LRRK2 and phosphorylated and total α-synuclein in PD human brain tissue extracts, but not in controls. A small increase in the levels of LRRK2 in the brain tissue from PD patients directly correlated with much larger regional increases in α-synuclein levels, and more strikingly with a widespread α-synuclein S129 phosphorylation. In cell models, an increase in LRRK2 expression significantly increases α-synuclein mRNA [35], and elevated α-synuclein mRNA

levels are co-regulated with increased LRRK2 transcription [36]. The positive feedback in turn activates the ERK signaling pathway leading to phosphorylation of α-synuclein [35]. It is of interest that in long duration PD cases, the gene expression levels of both LRRK2 [37] and α-synuclein [38] are decreased in multiple brain regions forming LBs. This is possibly as a self-protective mechanism to the high levels of these proteins that accumulate within neurons in these regions, and suggests that deficits in protein degradation mechanisms play a significant role in the progression of pathology overtime. Beyond the endogenous interaction of LRRK2 and α-synuclein that we have shown in this study, we are still not able to determine whether the nature of LRRK2 interaction with α-synuclein is a direct protein-protein binding or an indirect binding within a protein complex. As it has been extensively suggested, LRRK2 interacts with other proteins also implicated in PD to form protein complexes [10, 18, 20, 29, 37, 39]. While a fine analysis of the molecular determinants of the interaction between LRRK2 and α-synuclein is still required, our study has unequivocally established that there is an interaction between LRRK2 and α-synuclein, and that this interaction appears to be enhanced in patients with PD and in cell models of α-synuclein inclusion formation. Importantly, we also provide evidence showing that the levels of LRRK2 impact on α-synuclein pathology, consistent with studies in animal models of PD [20]. Ultimately, our work paves the way for the understanding of the molecular interplay between two central players in PD.

3.5 References

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Chapter 4.

LRRK2 promotes Tau accumulation, aggregation and release

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LRRK2 Promotes Tau Accumulation, Aggregation and Release

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Chapter 4. LRRK2 promotes Tau accumulation, aggregation and release

4.1 Introduction and main goals

Mutations in the leucine-rich repeat kinase 2 (*LRRK2*) gene are known as the most frequent cause of autosomal dominant Parkinson's disease (PD). The importance of LRRK2 in PD was further substantiated by the discovery that several mutations linked to familial forms of the disease can also be associated with sporadic cases [1,2,3,4]. LRRK2 is a large protein of 2527 amino acids, composed of several defined domains. The two enzymatic domains, the GTPase (ROC/COR) and the kinase (MAPKKK), lie at the centre of the protein, but the latter is responsible for the classification of LRRK2 as a protein kinase [5,6]. Although PD-associated mutations are found dispersed throughout the protein, the most frequent G2019S mutation is present in the kinase domain. This mutation has been consistently associated with a pathological gain-of-function [7,8].

LRRK2-associated familial PD cases are indistinguishable from sporadic cases, with late onset of the disease and the presence of widespread protein aggregates, enriched in proteins like α-synuclein or Tau, in the remaining neurons, particularly [9]. LRRK2 function has been implicated in a number of cellular processes, including synaptic vesicle recycling, neurite branching, mitochondrial homeostasis, protein synthesis and protein clearance/degradation. However, the precise physiological role of LRRK2 in regulating these processes is still unclear [5,10,11].

Tau is predominantly an axonal protein, encoded by the *MAPT* (microtubule-associated protein tau) gene. Through alternative splicing, six isoforms of the protein can be originated from the *MAPT* gene (ranging from 352 to 441 amino acids). Tau is highly expressed in the adult central nervous system [12] and its main function is the stabilization of the microtubule network through the binding of the microtubule-binding domains in the C-terminal half of Tau to tubulin [13]. The interaction of Tau with microtubules is modulated by phosphorylation of specific epitopes, some of which have been described as pathogenic in neurodegenerative diseases such as Alzheimer's disease or PD [14,15]. Both the autophagy-lysosome pathway (ALP) and the ubiquitin-proteasome system (UPS) are important for Tau degradation [16,17]. Consistently, it was shown that dysfunction

in either of these two major protein clearance systems might initiate or facilitate the process of Tau accumulation and aggregation, typical in neurodegenerative processes [18,19,20].

Recent in vitro studies demonstrated that LRRK2 phosphorylates tubulin-associated, but not free Tau protein [21]. A separate study showed that LRRK2 promotes Tau phosphorylation indirectly via GSK-3β [22]. In mice, expression of transgenic LRRK2 in a model of tauopathy increases the aggregation of insoluble Tau and its phosphorylation in different residues (T149, T153, T205) [23]. Thus, our goal was to further detail the interaction between LRRK2 and Tau to provide novel insight into the role of these proteins in PD.

Here, we show an interaction between LRRK2 and Tau. We found that LRRK2 expression resulted in increased intracellular Tau levels, promoted the accumulation of oligomeric Tau, and enhanced Tau secretion. These effects appeared independently from LRRK2 kinase activity. Moreover, we show that Tau accumulation occurs due to LRRK2-mediated impairment of the proteasome, but not of the autophagy pathway. In total, our data suggest that LRRK2 increases the levels of Tau protein via proteasomal inhibition.

4.2 Materials and methods

Plasmids

Wild type human LRRK2 (LRRK2 WT) was cloned into a pLenti6/V5-Dest vector by gateway recombination. LRRK2 triple kinase-dead (K1906A, D1994N, D2017A) and G2019S mutations were introduced by site-direct mutagenesis (Agilent Technology, CA, USA). The cDNA according to the longest human Tau isoform (4R2N) was cloned into the pcDNA3.1 vector (Invitrogen, Darmstadt, Germany). For the constructs used in BiFC interactions, Venus cDNA was divided asymmetrically into a larger 5'-fragment, corresponding to amino acids 1-158, and a smaller 3'-fragment, corresponding to amino acids 159 - 239. LRRK2 cDNA was cloned to the 3'-end of the Venus 5'-fragment (VN-LRRK2) and Tau cDNA was cloned upstream of the Venus C-terminal fragment (Tau-VC) or downstream of Venus N-terminal fragment (VN-TAU). The GFP-Rab6b plasmid was a kind gift from Dr. Mika Simons, Department of Neurology, UMG, Göttingen.

Cell culture, transfections and inhibitors

Human Embryonic Kidney 293 cells (HEK-293) were grown in Dulbecco's modified Eagle's medium (PAN, Aidenbach, Germany), supplemented with 10% fetal calf serum and 1% penicillin/streptomycin, at 37ºC in 5%CO_2. The cells were transfected 24 hours after plating using Metafectene (Biontex Laboratories GmbH, Martinsried, Germany), according to the manufacturer's instructions. 48 hours post-transfection, the cells were harvested or treated for additional 24 hours with 5μM Z-Leu-Leu-Leu-al (MG132) (Sigma, Hamburg, Germany), 20mM ammonium chloride and 100μM leupeptin (NL) (Merck, Darmstadt, Germany), and 50nM bafilomycin A1 (Sigma, Hamburg, Germany).

SDS-PAGE and Western blotting

Cells were collected in lysis buffer (25mM Tris–HCl pH 7.6; 150mM NaCl; 0.1% SDS; 1% NP40) supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany), sonicated, and cleared by centrifugation for 10 minutes at 10,000xg. Total protein concentration was determined using the Bradford assay (BioRad Laboratories, CA, USA). The lysates were boiled at 95ºC for 5 minutes in protein sample buffer (PSB, 50mM Tris–HCl pH 6.8; 2% SDS; 10% glycerol; 1% β-mercaptoethanol; 0.02% bromophenol blue), and resolved by SDS-PAGE in a 10% Tris-glycine gel. Proteins were transferred for 1-2 hours at 250mA to nitrocellulose or PVDF membranes (Immobilon-FL-Membrane, Millipore, Billerica, MA, USA) and blocked for 1 hour in TBS-Tween with 3% non-fat milk or BSA, respectively. The membranes were incubated overnight at 4°C with primary antibodies using the following dilutions: LRRK2 (c41-2 MJFF, Epitomics, Burlingame, CA, USA) 1:1000, Tau (HT7, Thermo Scientific) 1:3000, GFP (C-2, Santa Cruz Biotechnology, USA) 1:3000, LC3 (5F10, Nanotools, Teningen, Germany) 1:500, p62 (SQSTM1, Sigma, Hamburg, German) 1:1000, β-actin (Sigma, Hamburg, German) 1:3000. Membranes were washed with TBS-Tween and incubated for 1 hour at room temperature with the corresponding HRP-labelled secondary antibodies (GE Healthcare, UK, 1:10,000). Immunoreactivity was visualised by chemiluminescence using an ECL detection system (Millipore, Billerica, MA, USA).

Mouse brain samples

Brains from adult LRRK2 knockout (LRRK2 KO), transgenic LRRK2 Thy-1 LRRK2-WT (LRRK2 Tg) and wild type (WT) mice were lysed in RIPA buffer (25mM Tris-HCl pH 7.6; 150mM NaCl; 0.1% SDS; 1% NP40), supplemented with protease inhibitor cocktail (Roche diagnostics, Mannheim, Germany) using a mechanic homogenizer (Precellys24, Peqlab, Erlangen, Germany). Lysates were cleared by centrifugation at 10,000*g* for 10 min at 4°C. Samples were resolved by SDS-PAGE and analysed as described above.

Co-Immunoprecipitation (co-IP)

For the co-IP assay, cells were collected in IP buffer (50mM Tris-HCl pH7,5; 0.5mM EDTA; 150mM NaCl; 0.05% NP40) supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany) and sonicated. In each IP sample, approximately 1mg of total protein lysate was pre-cleared by incubation with 20µl of protein G beads (Invitrogen, Barcelona, Spain) for 30 minutes at 4°C in rotation. Pre-cleared samples were incubated with 2µg of the IP antibody, LRRK2 (c41-2 MJFF), overnight at 4°C in rotation, mixed with 40µl of protein G beads, and incubated for additional 3 hours. Beads were washed 5 times with IP buffer, re-suspended in 20µl of PSB and boiled at 95ºC for 5 minutes. The supernatants were loaded onto a 10% SDS gel, and the Western Blotting was performed as described above.

Size-exclusion chromatography (SEC), dot blot and filter trap assays

HEK-293 cells were collected 48 hours after transfection in a phosphate buffer (1X PBS with 0,5% TritonX-100) freshly supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany) and centrifuged for 10 minutes at 10,000xg. 2-3mg of total protein in a maximum volume of 500ul was filtered using a 0,45µm Spin-X centrifuge filter (Sigma, Hamburg, Germany) before loading onto a Superose 6 (Superose 6 10/300GL. GE Healthcare Life Science, Sweden) column and subsequent high-performance liquid chromatography (HPLC) (Äkta Purifier 10, GE Healthcare Life Science, Sweden) in 50mM ammonium acetate pH 7.4 buffer with a flow rate of 0.5 ml/min. The collected HPLC fractions of 500ul were boiled at 95ºC for 10 minutes and centrifuged at 10,000xg for 5 minutes. For the dot blot assay, 150ul of the supernatant were loaded on a nitrocellulose

membrane, and for the filter trap assay, 350µl were loaded on a cellulose acetate membrane, previously soaked in PBS buffer, using a dot blot vacuum system. Membranes were further processed as described under Western blotting except that all steps were performed in PBS instead of TBS.

Measurement of 26S proteasome catalytic activity

The chymotrypsin-like activity of the 26S proteasome was determined using N-Succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Suc-LLVY-AMC, Enzo, Life-Sciences) as substrate. 48 hours after transfection, cells were collected in lysis buffer (50mM Tris, pH 7.5, 250mM Sucrose, 5mM MgCl2, 1mMDTT, 0.5mM EDTA, 0.025% Digitonin, protease inhibitors, 2mM ATP) and centrifuged for 15 minutes at 20,000g for removal of cell debris. Each reaction was performed with 12µg of total protein lysates in a final volume of 100ul in a 96-well plate and initiated by the addition of reaction buffer (50mM Tris (pH 7.5), 40mM KCl_2, 5mM MgCl2, 1mM DTT, 0.5mM ATP, 100µM Suc-LLVY-AMC). The fluorescence of AMC (380nm excitation and 460nm emission) was monitored in a microplate fluorometer (Infinite M1000, Tecan) at 37ºC for 1 hour, and values were calibrated to standard curves of AMC (0 - 5µM). Inhibition of the proteasome was controlled by the incubation of samples with 20µM MG132 (Sigma, Hamburg, Germany) prior to the measurements.

Secretion Assays

24 hours after transfection, HEK-293 cells were washed with PBS, and the culture medium was replaced by serum-free medium. 48 hours later, 2ml of the serum-free medium were collected and cleared by sequential centrifugation for 5 minutes at 300g and 10 minutes at 10,000g. The samples were boiled for 10 minutes at 95 °C and loaded on a nitrocellulose membrane using a dot blot vacuum system. Membranes were further processed as described under Western blotting using PBS instead of TBS in all steps. 100µl of cleared medium were used to determine the cytotoxicity with the Lactate Dehydrogenase (LDH) assay (Cytotoxicity Detection Kit, Roche, Mannheim, Germany), according to the manufacturer's instructions.

Immunohistochemistry and microscopy

48 hours after transfection, cells were washed with PBS and fixed with PBS/4% paraformaldehyde. The cells were permeabilized with PBS/0.1% Triton X-100, blocked for 1 hour with 1.5% BSA and incubated overnight with the primary antibody for LRRK2 (c41-2 MJFF) 1:1000. After 3 washes with PBS, the secondary antibody anti-rabbit IgG conjugated to Alexa Fluor 568 (Molecular Probes, Oregon, USA) 1:1000 was added for 1 hour at room temperature. Cell nuclei were stained with Hoechst dye (Hoechst 33258, Molecular Probes, OR, USA). Fluorescent images were captured either using a Leica Microsystems microscope (Leica DMI 6000B, Wetzlar, Germany) or an automatic microscope Olympus (IX81-ZDC, Hamburg, Germany). Venus fluorescence was quantified from 16 fields of cell images automatically collected with the Olympus microscope and analysed by the ScanR software.

4.3 Results

4.3.1 Tau levels are decreased in LRRK2-KO mice

To investigate the interplay between LRRK2 and Tau, we first compared the levels of endogenous Tau protein in transgenic mice expressing human LRRK2 under the Thy1 promoter (LRRK2-Tg), LRRK2 knockout mice (LRRK2-KO) and wild type littermate control animals (WT). Interestingly, we found that while the levels of Tau were identical in wild type and LRRK2 transgenic animals, they were drastically reduced in the LRRK2-KO mice (Figure 1).

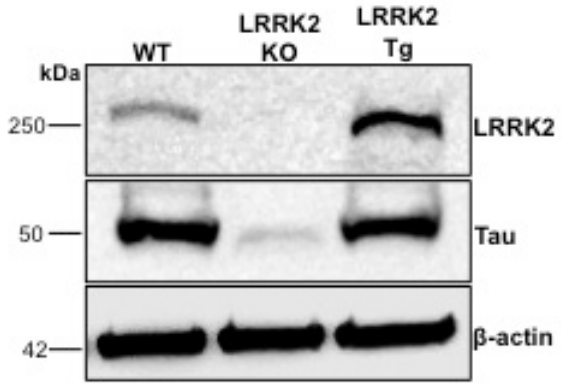

Figure 1. Tau levels are decreased in LRRK2-KO mice.
Immunoblot analysis of total brain lysates from LRRK2-KO mice, LRRK2 WT transgenic mice and littermate control mice, showing that the knockout of LRRK2 is accompanied by a decrease of the endogenous levels of Tau.

4.3.2 LRRK2 physically interacts with Tau

To investigate the interaction between Tau and LRRK2, we performed co-immunoprecipitation (co-IP) assays. We co-expressed LRRK2 and Tau in HEK-293 cells, and then immunoprecipitated LRRK2 with a specific LRRK2 antibody. The co- IP of Tau was verified with a specific human Tau antibody (Figure 2A).

Next, to investigate the subcellular localization of the interaction between the two proteins, we took advantage of the bimolecular fluorescence complementation (BiFC) assay, which enables the direct visualization of protein-protein interactions in living cells [24]. We designed BIFC constructs, combining an N-terminal fragment of Venus protein fused to the N-terminus of LRRK2 (VN-LRRK2) and the C-terminal fragment of Venus fused to the C-terminus of Tau (Tau-VC). Upon co-transfection of the two constructs in HEK-293 cells, we observed widespread fluorescence signal inside the cell, confirming the interaction between LRRK2 and Tau, as expected, in the cytoplasm, and no fluorescence in the isolated constructs (Figure 2B). The expression of LRRK2 and Tau was confirmed by immunoblot analysis with specific antibodies for each protein (Figure 2C).

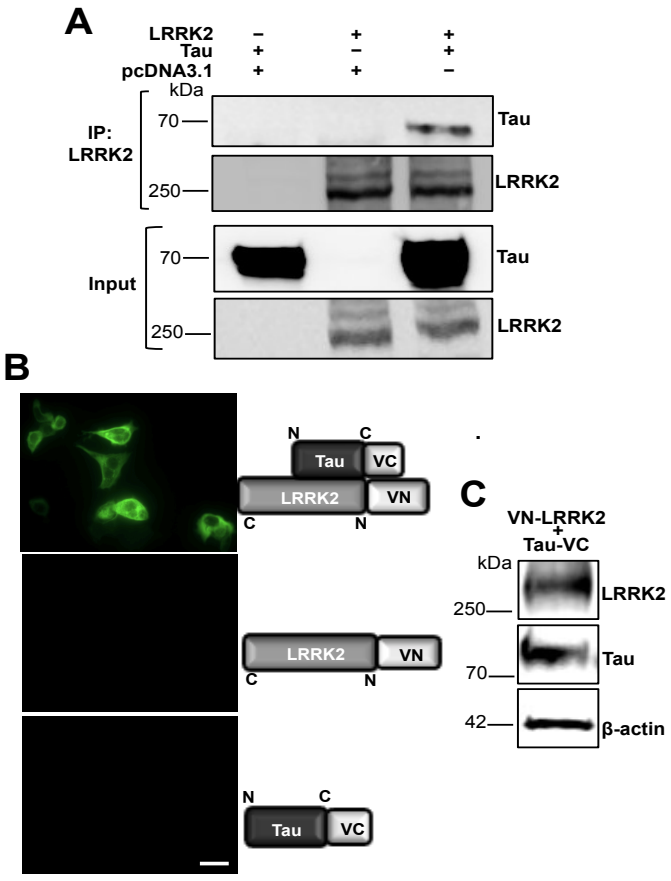

Figure 2. LRRK2 physically interacts with Tau.
(A) Human LRRK2 and human Tau were expressed in HEK-293 cells. Following the immunoprecipitation (IP) of LRRK2, the co-IP with Tau is demonstrated with a Tau-specific antibody. **(B)** Schematic representation of the BiFC constructs, VN-LRRK2 and Tau-VC. **(C)** and **(D)** The co-expression of BiFC constructs in HEK-293 cells results in the formation of reconstituted Venus fluorescent protein in the cells indicating that LRRK2 and Tau can interact. The immunoblots and fluorescence images are representative of more than 3 independent experiments. Scale bar 20μm.

4.3.3 Increased levels of Tau depend on LRRK2 expression but not on its kinase activity

Next, we investigated whether LRRK2 affected the levels of Tau in human cells. Thus, we expressed Tau in HEK-293 cells alone or together with LRRK2, and the levels of both proteins were assessed at three different time points after transfection. Expression of Tau and LRRK2 was detected already 12 hours after transfection, and the levels of both proteins continually increased over time up to 48 hours. At 24 hours and 48 hours post-transfection Tau levels were significantly increased when co- expressed with LRRK2 (Figure 3A).

We next asked whether this effect could be due to the kinase activity of LRRK2. Thus, we compared the levels of Tau when co-expressed with three different forms of LRRK2: wild type (WT), kinase-dead mutant (KD) or G2019S mutant (GS). We found that the Tau levels equally increased in the presence of any of the three forms of LRRK2 (Figure 3B). To verify whether the effect of LRRK2 on Tau levels was specific, or whether it was simply due to the clogging of degradation pathways due to the expression of a large protein such as LRRK2, we co-expressed Tau with ATP13A2, another large protein (1180 amino acids) associated with PD [25]. In this case, no increase in Tau levels was observed (Figure 3C).

In order to determine whether LRRK2 also affected the levels of oligomeric Tau, we used the BiFC assay, in living cells. The Tau BiFC constructs (VN-Tau and Tau-VC) were expressed together with the three forms of LRRK2 (WT, KD, and GS) or with a control plasmid in HEK-293 cells. The various forms of LRRK2 promoted an identical increase in Tau BiFC signal when controlled for comparable levels of LRRK2 protein expression (Fig. 3D,E), confirming that the kinase activity of LRRK2 is not required for the increase of monomeric and oligomeric Tau levels.

Next, to assess whether the elevated levels of Tau protein in the presence of LRRK2 were due to increased tau gene transcription or tau mRNA stabilization, we quantified tau mRNA levels by real-time PCR. Tau was expressed in HEK-293 cells, together with each of the three forms of LRRK2 (WT, KD, and GS), or with

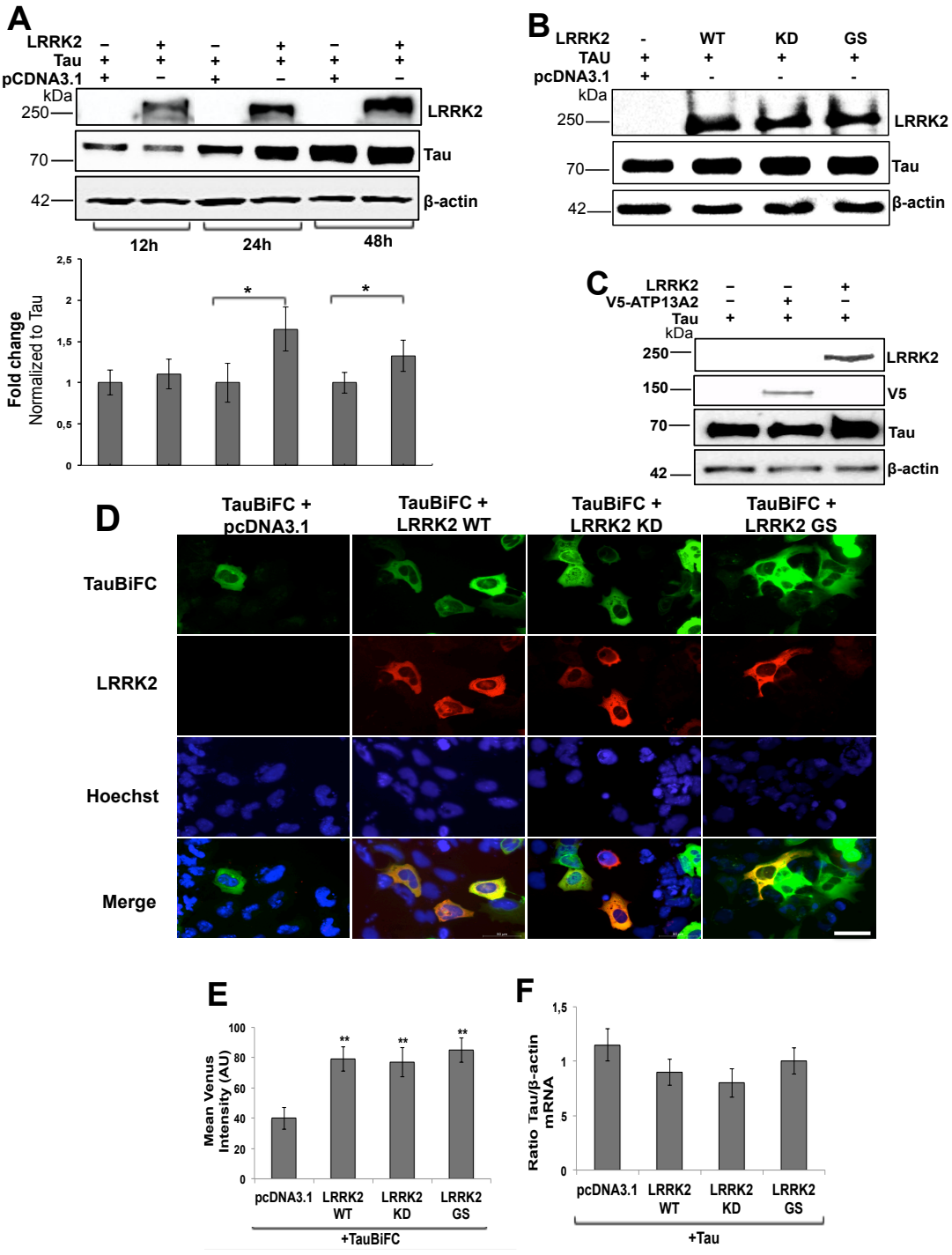

Figure 3. Increased levels of Tau depend on LRRK2 expression but not on its kinase activity.

(A) The expression of Tau±LRRK2 in HEK-293 cells over time shows significant increasing amounts of Tau protein were further augmented in the presence of LRRK2 after 24 hours. *p≤0.05. **(B)** The expression of Tau alone or together with the three forms of LRRK2, wild type (WT], kinase-dead (KD) and G2019S (GS) for 48 hours clearly shows that LRRK2 promoted an increase in the levels of Tau independent from its kinase activity (n=3]. **(C)** The expression of Tau alone and together with LRRK2 or ATP13A2 shows an increased Tau accumulation in the presence of LRRK2, but not of ATP13A2. **(D)** Tau BiFC constructs (VN-Tau and Tau-VC) were expressed in HEK-293 cells together with an empty plasmid (pcDNA3.1) or with LRRK2 wild type (WT], kinase-dead (KD) or G2019S (GS]. Fluorescence microscopy confirms the formation of increased levels of Tau oligomers in the presence of LRRK2 protein. Scale bar 20µm. **(E)** The graph shows the

quantification of the mean fluorescence intensity (±SD) in each group from three independent transfections, **p≤0.01. **(F)** Real time PCR with samples from the same transfections as in (B), demonstrating that Tau mRNA levels (±SD) are not altered in the presence of any of the three LRRK2 forms (N≥3].

the respective control. After normalization to β-actin mRNA, we found no significant differences in the levels of tau mRNA in the presence of any of the LRRK2 forms (Figure 3F). Taken together, our data demonstrate that LRRK2 promotes an increase in Tau protein levels without affecting Tau mRNA levels.

4.3.4 LRRK2 promotes the accumulation of high-molecular weight Tau species

Tau oligomerization and aggregation, with the formation of high-molecular weight species, is strongly associated with AD and PD [16-18].

To further explore whether the LRRK2-mediated increase in Tau levels affected the biochemical state of Tau, we performed size-exclusion chromatography (SEC) and analysed the various fractions using a dot blot assay. First, GFP-tagged Tau was co-expressed with LRRK2 in HEK-293 cells, and the lysates were separated by SEC. The chromatograms show that, in the presence of LRRK2, additional GFP signal peaks appeared in fractions corresponding to Tau species with higher molecular weight. The shift in Tau signal, towards high-molecular weight species, was confirmed in a dot blot assay, upon immunoblotting with a Tau-specific antibody (Fig.4).

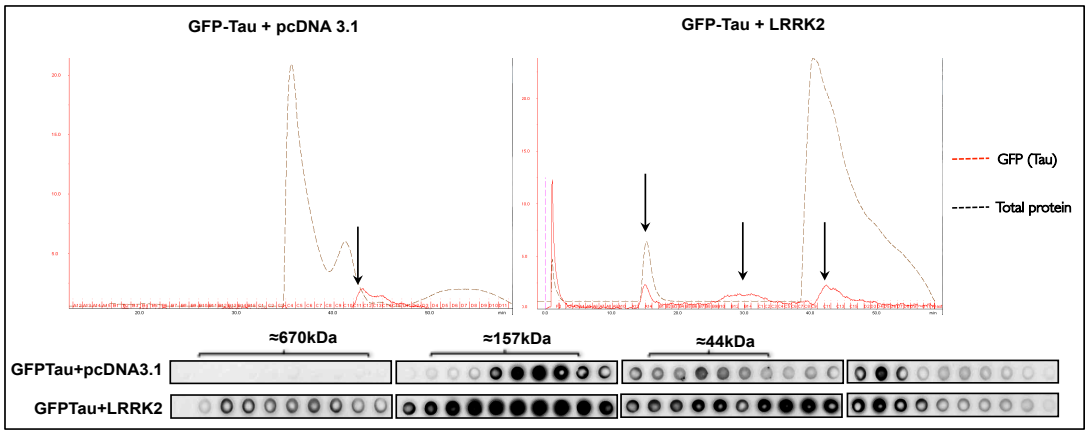

Figure 4. LRRK2 promotes the accumulation of high-molecular weight species of GFP-Tau.
Lysates from HEK-293 cells expressing GFP-Tau, or GFP-Tau together with LRRK2 were separated by SEC/ HPLC. The chromatograms show the signal of total protein (black

trace) and the signal of GFP (red trace) correspondent to GFP-Tau protein. The presence of LRRK2 promotes additional GFP signal peaks in fractions corresponding to higher molecular weight species. This shift was confirmed in a dot blot with the collected SEC-HPLC fractions onto a nitrocellulose membrane followed by staining with a Tau-specific antibody.

To ensure the effects observed were not due to the GFP tag, we also used untagged Tau.

Likewise, to rule out that the biochemical differences were simply explained due to the increased Tau levels in the presence of LRRK2, we performed an additional experiment where we transfected cells with twice the amount of Tau cDNA. Indeed, Western blot analysis of the samples with a Tau-specific antibody confirmed that the Tau levels were increased in the presence of LRRK2, and that the levels were even higher upon transfection with twice the amount of Tau plasmid DNA (Figure 5A). Analysis of these samples by SEC, and subsequent dot blot assays, revealed that, although the double amount of DNA during transfection lead to an increase in total Tau protein levels, the presence of LRRK2 promoted a shift of the Tau signal to higher molecular weight fractions (Figure 5B).

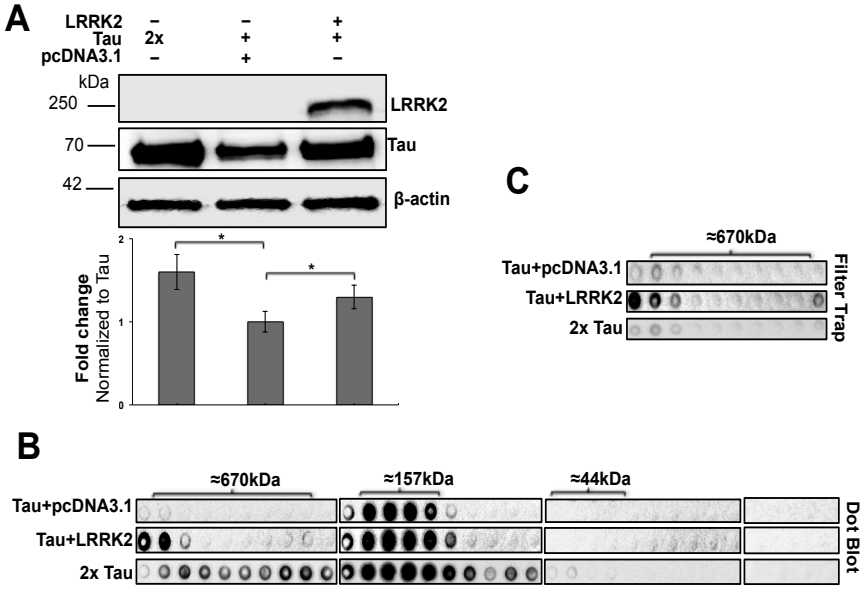

Figure 5. LRRK2 promotes the accumulation of high-molecular weight Tau species
(A) Lysates from HEK-293 cells expressing Tau, twice the amount of Tau (2X) or Tau together with LRRK2 were analysed by immunobloting using the respective specific antibodies highlights the significant effect of LRRK2 in increasing the levels of Tau is comparable with the expression of twice the amount of Tau. *p≤0.05. **(B)** The same samples were separated by SEC/HPLC and the distribution of total Tau in the collected fractions was determined by dot blot with a Tau-specific antibody. The presence of LRRK2 clearly induces a shift of Tau signal to the three first fractions from the left, corresponding to higher molecular weight species. **(C)** The same fractions were separated by filter trap using a cellulose acetate membrane and detected with a Tau-specific antibody. In the

presence of LRRK2, larger Tau species were trapped and detected in the fractions corresponding to high-molecular weight species, which was not observed by simple overexpression of Tau in the control sample (2x Tau) indicating that LRRK2 promotes the formation of larger Tau species or Tau aggregates. The Western blots and dot blots are representative of N≥3.

We also performed filter trap assays, using a low-protein binding cellulose acetate membrane, in order to confirm the accumulation of large Tau species. As expected, Tau reactivity was detected for the first three fractions corresponding to the higher molecular weight species, further confirming the accumulation of aggregated Tau in the presence of LRRK2 (Figure 5C).

4.3.5 LRRK2 impairs proteasomal protein degradation independently of its kinase activity

LRRK2 plays a role in proteostasis through modulation of protein quality control systems, such as the ubiquitin-proteasome system (UPS) and the autophagy-lysosome pathway (ALP) [11, 25, 26]. Importantly, these quality control mechanisms are indispensable for the clearance and degradation of Tau protein, thereby avoiding the accumulation of Tau and subsequent formation of high-molecular weight aggregated species that are characteristic in neurodegenerative processes [16-20]. In order to confirm the effect of LRRK2 on the UPS, we co-expressed the different LRRK2 forms (WT, KD, and GS) together with the UPS reporter GFPu, an unstable version of GFP that is degraded via the proteasome [27]. The presence of any of the three forms of LRRK2 clearly promoted an increase in the GFPu levels, visible by microscopic analysis (Figure 6A) and by immunoblot analysis (Figure 6B). The increased levels of GFPu reflect the accumulation of this protein possibly due to an impairment of proteasomal degradation promoted by LRRK2, in a kinase-independent manner, since no differences were observed in the presence of either KD or GS mutants.

We also compared the effect of LRRK2 with that of MG132, a known proteasome inhibitor and found that, although both caused an increase in GFPu accumulation, the inhibitor promoted a strong effect (~4.5 fold over the control) that was not further potentiated in the presence of LRRK2 (Figure 6C).

Next, we investigated whether LRRK2 impairs the UPS, by affecting the proteolytic activity of the proteasome. The chymotrypsin-like activity of eukaryotic

proteasomes is often considered the most important in protein degradation [28]. Thus, we performed an *in vitro* assay to measure the chymotrypsin-like catalytic activity of the 26S proteasome, in lysates from cells expressing LRRK2, using Suc-LLVY-AMC as substrate. Interestingly, we did not observe differences in the chymotrypsin-like catalytic activity of the proteasome in the presence of LRRK2 (Figure 6D).

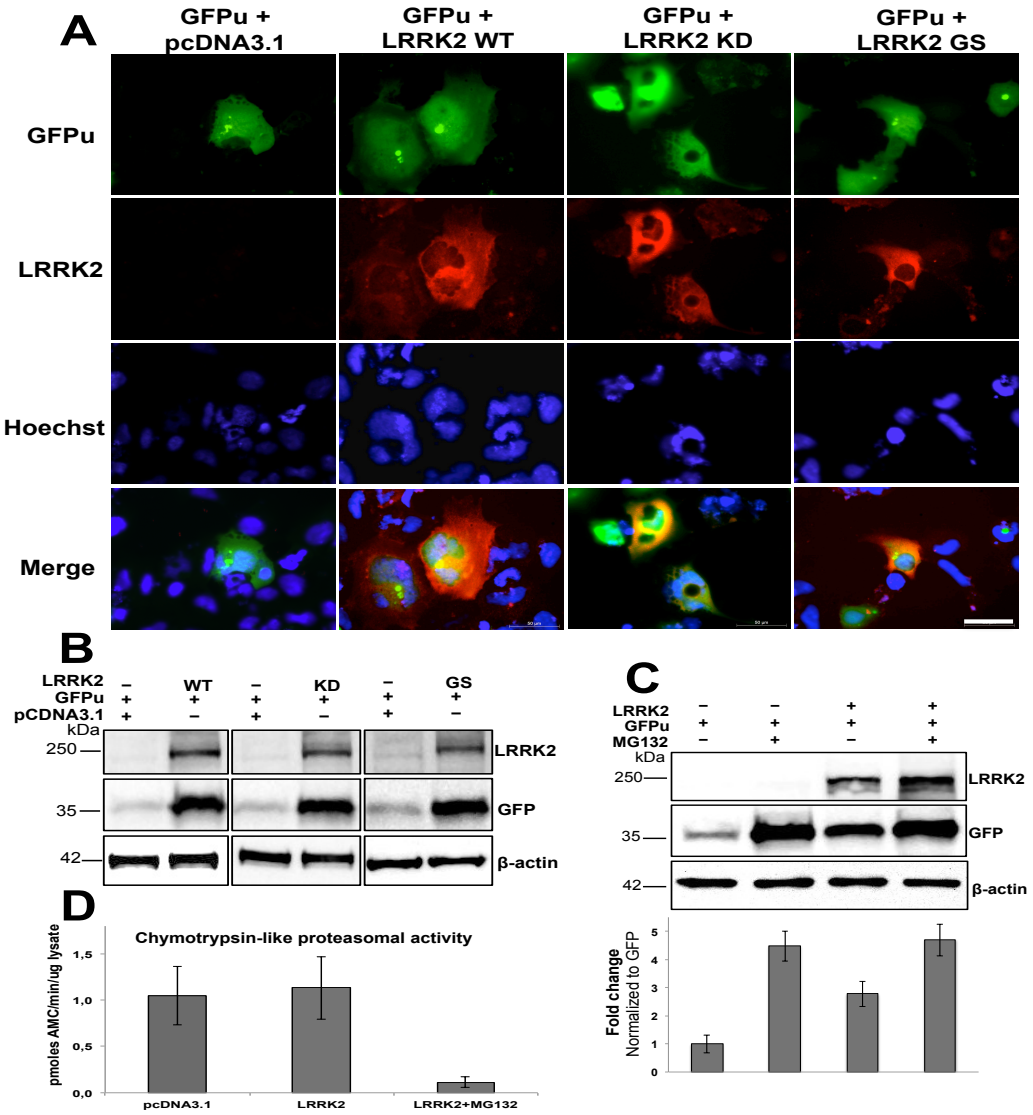

Figure 6. LRRK2 impairs proteasomal protein degradation independently of its kinase activity.
(**A**)The ubiquitin-proteasome reporter GFPu and LRRK2 wild type (WT), kinase dead (KD)or G2019S (GS) were expressed in HEK-293 cells. Fluorescence microscopy reveals the expression of GFPu and enhanced levels of GFPu in the presence of LRRK2 protein. Scale bar 20μm. **(B)** Western Blot performed with the same transfections demonstrating the accumulation of GFPu in the presence of the three forms of LRRK2 using LRRK2 and GFP-specific antibodies. **(C)** Proteasomal inhibition of GFPu-expressing HEK-293 cells using 5μM MG132 promotes a more pronounced accumulation of GFPu than the co-expression of LRRK2. Both together, LRRK2 co-expression and treatment with 5 μM MG132, do not induce further accumulation of GFPu. **(D)** HEK-293 cells expressing

pcDNA3.1 or LRRK2 were subjected to an *in vitro* assay to measure the catalytic activity of the chymotrypsin-like proteasome active site. The fluorescence (380nm excitation and 460nm emission) of the samples (±SD) calibrated to a standard curve with AMC concentrations from 0 to 5 μM shows, that LRRK2 does not alter the activity of this active site. Incubation with the MG132 proteasome inhibitor was used as control and significantly decreased the proteasomal activity. **p≤0.01. The Western blots, fluorescence images and *in vitro* assays are representative of N≥3 experiments.

4.3.6 LRRK2 impairs the proteasomal degradation of Tau but does not interfere with the autophagy pathway

In order to investigate the effect of LRRK2 on Tau clearance in the cell, we started by using a similar approach to that described above for GFPu.

We observed that blockade of the proteasome with MG132 caused a strong accumulation (2,5-fold over the control) of Tau protein. LRRK2 also promoted a pronounced increase in Tau levels, although to a smaller extent (~1,5-fold over the control). Our results confirm that the UPS is an important mechanism for Tau clearance, and that LRRK2 interferes with Tau proteasomal degradation, promoting its accumulation (Figure 7A). Next, we asked whether the effect of LRRK2 on Tau levels was solely due to proteasomal inhibition or whether it was also due to an interference with autophagy. To tackle this, we used different autophagy inhibitors, such as ammonium chloride/leupeptin or bafilomycin, to treat cells expressing Tau alone or in combination with LRRK2. Autophagy inhibition was confirmed by the accumulation of the autophagy markers LC3 and p62 (Figure 7B). The accumulation of Tau, when co-expressed with LRRK2, was not further potentiated by simultaneous treatment with the autophagy inhibitors, suggesting that the increase in Tau levels was not mediated through LRRK2 interference with the autophagy-lysosome pathway (Figure 7B). To further confirm that LRRK2 had no impact on autophagy, we assessed the levels of GFP-Rab6b, a protein known to be preferentially degraded by autophagy, in the presence of LRRK2. As predicted, we observed no differences in the levels of GFP-Rab6b, confirming that LRRK2 does not interfere with the autophagy-lysosome system (Figure 7C).

4.3.7 LRRK2 promotes the cellular release of Tau

In addition to the intracellular accumulation of aggregated Tau, the release/secretion of the protein to the extracellular space and subsequent uptake

by neighbouring cells is thought to contribute to the neurodegenerative process [29]. In order to determine if LRRK2 affected Tau release from cells, we analysed the levels of Tau in the cell culture medium of HEK-293 cells co-expressing Tau and LRRK2.

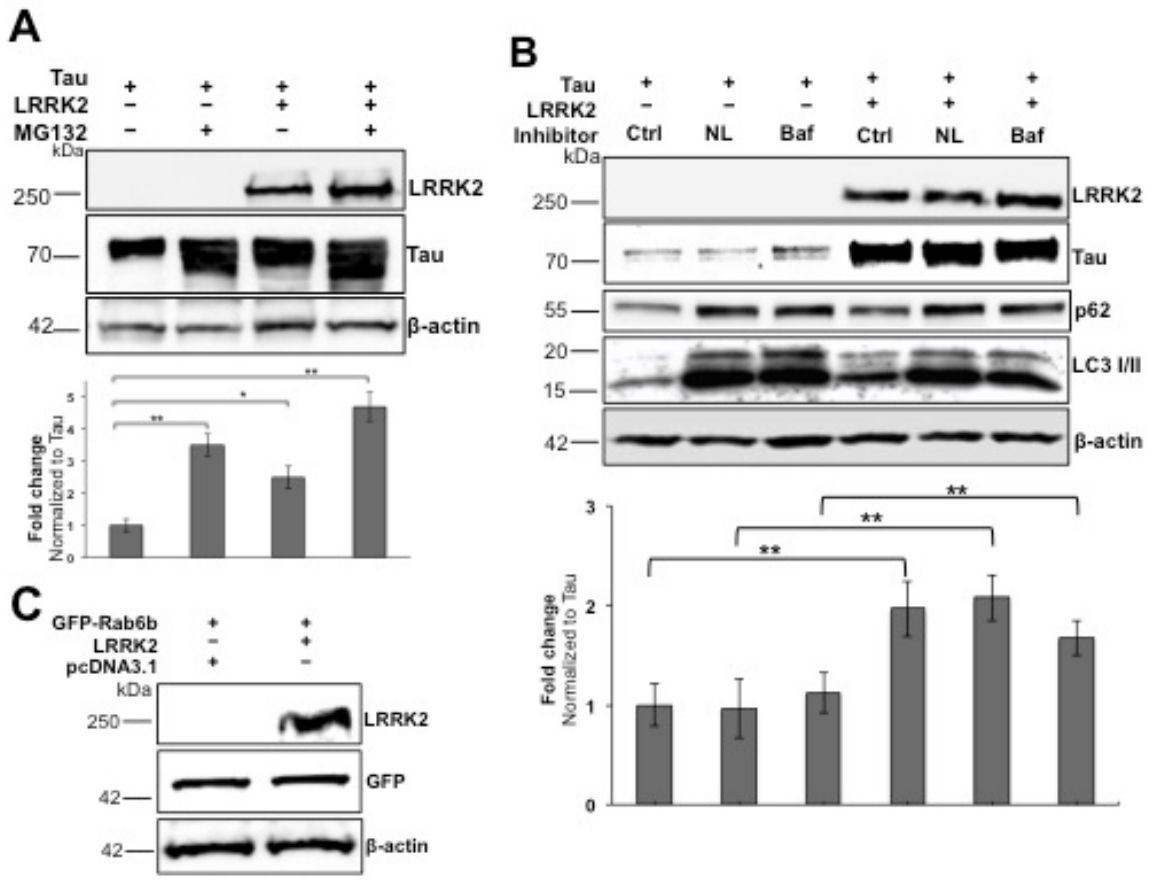

Figure 7. LRRK2 impairs proteasomal degradation of Tau but does not interfere with the autophagy pathway.
(A) Immunoblots showing that Tau levels were significantly increased in HEK-293 cells expressing the protein and being treated with 5μM of MG132 (**p≤0.01) and also when co-expressed with LRRK2 (*p≤0.059). **(B)** HEK-293 cells expressing Tau±LRRK2 remained untreated (Ctrl) or were treated with the autophagy inhibitors 20mM of ammonium chloride / 100μM leupeptin (NL) or 50nM of bafilomycin (Baf). Immunoblot showing the increased levels of LC3I/II and p62 proteins verified the efficient inhibition of autophagy. The significant (**p≤0.01) accumulation of Tau when co-expressed with LRRK2, was not further potentiated by simultaneous treatment with the autophagy inhibitors. **(C)** Lysates from HEK-293 cells expressing LRRK2 together with GFP-Rab6b, a specific target of autosomal degradation, show that LRRK2 did not alter the levels of Rab6b. The Western blots are representative of N=3.

To detect the levels of Tau, the medium was loaded onto a nitrocellulose membrane using a dot blot system, and Tau was detected after immunoblotting with an anti-Tau antibody. We found that, in the presence of LRRK2, the levels of Tau in the medium increased ~1.6-fold over the control (Figure 8A). The expression of LRRK2 and Tau in HEK-293 cells was confirmed by SDS-PAGE and immunoblot analyses with specific antibodies for each protein (Figure 8B). The enhanced release of Tau could be a consequence of increased intracellular Tau levels due to co-expression of LRRK2, a LRRK2-mediated increase in Tau secretion or simple to increased toxicity and cell death in the presence of LRRK2.

Thus, to assess whether the release of Tau was due to either the presence of LRRK2 or simply due to increased cytotoxicity and consequent membrane leakage, we performed LDH assays to measure the activity of LDH in the medium. We observed that the co-expression of Tau and LRRK2 did not increase the basal levels of toxicity induced by Tau alone (Figure 8C). This confirmed that the increased release of Tau protein was not simply due to LRRK2-mediated cytotoxicity.

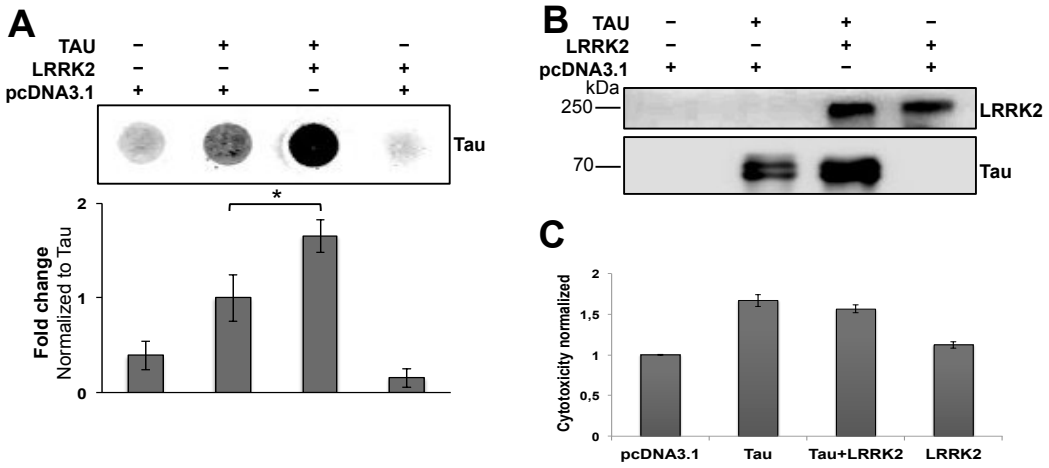

Figure 8. LRRK2 promotes the cellular release of Tau.
(A) 24 hours after transfection, HEK-293 cells expressing Tau and LRRK2 were cultured in serum-free media for additional 48 hours. Cell culture media were cleared by sequential centrifugation at 300g and 10,000g, probed on a dot blot nitrocellulose membrane and stained with specific antibodies. In the presence of LRRK2, the amount of Tau secreted into the medium was significantly enhanced (*p≤0.05). **(B)** The expression of Tau and LRRK2 was confirmed by SDS-PAGE using the cell lysates. **(C)** The graph represents the toxicity levels in the respective samples quantified by the Lactate Dehydrogenase (LDH) assay showing that enhanced release of Tau into the medium is not due to enhanced cell death. The results are representative of N=3.

4.4 Discussion

LRRK2 mutations are associated with both, familial and sporadic cases of PD and display clinical features undistinguishable from those in idiopathic PD. This suggests a high degree of similarity in the underlying molecular mechanisms and pathways in genetic and sporadic forms of the disease. Thus, investigating the interplay between LRRK2 and other PD-associated proteins is essential for our understanding of the molecular basis of PD [30-33]. Although the interplay between LRRK2 and Tau is attractive given the relevance of both proteins in PD, it is presently unclear whether this has implications in the pathophysiology of disease. Contradictory results about the role of LRRK2 in phosphorylating Tau [34, 35] suggest the interplay between the two proteins is complex. One hypothesis is that LRRK2 may influence cytoskeleton dynamics, through direct or indirect interactions with microtubule-related proteins like α-β-tubulin, ezrin/radixin/moesin or Tau [21, 22, 35-40].

The interaction of LRRK2 with Tau was previously observed using recombinant proteins in an *in vitro* assay [21]. Here we demonstrate that this interaction occurs lso in the contex og a living cellular environment. Moreover, we found that increased LRRK2 expression is paralleled by an increase in total Tau levels. This effect is also observed in the presence of a kinase-dead mutant or of the over-active mutant G2019S, suggesting the effect is independent of the kinase activity of LRRK2 on Tau. Interestingly, we found a drastic reduction in Tau levels in LRRK2-KO mouse compared to both human WT LRRK2 transgenic mice and littermate controls. This result might reflect a functional difference between the effect of LRRK2 on human Tau when compared with mouse Tau, and should be further investigated as it may impact on findings observed in LRRK2 and Tau animal models. The intracellular accumulation of Tau results in increased phosphorylation and aggregation of the protein in the brains of AD and PD patients [29, 41-43]. This abnormal Tau accumulation was speculated to occur due to impairment or age-dependent decline in the activity of the two major protein clearance systems - autophagy and the proteasome [16, 17, 44, 45]. Again, our data point to a kinase-independent role of LRRK2 on Tau accumulation by specifically impairing the proteasome degradation of Tau, promoting its cytoplasmic oligomerization and the accumulation of HMW-Tau species. The

relation between LRRK2 and the protein quality control mechanisms has been explored in previous studies. Fibroblasts from patients with different LRRK2 mutations were found to exhibit alterations in autophagy/lysosome markers [46]. In addition, over-expression of the G2019S LRRK2 mutant, but not WT LRRK2, results in increased number of autophagic vacuoles in SH-SY5Y cells [47]. LRRK2 has also been reported to impair the proteasome, resulting in accumulation of several substrate proteins like ubiquitin and α-synuclein [11]. Thus, our data are consistent with these findings, and specifically establish a connection between LRRK2-mediated impairment of the proteasome and Tau accumulation. Future studies will be required to explore the specific nature of the Tau accumulations, their phosphorylation status, and the occurrence of additional post-translational modifications such as ubiquitination.

Previously, the G2019S LRRK2 mutant was also described to enhance the aggregate formation and phosphorylation of α-synuclein, and to contribute to α-synuclein release into the extracellular medium [48]. Tau release via membrane vesicles was demonstrated upon its over-expression in HEK-293 cells [49]. Now, we report for the first time that Tau release is increased upon co-expression together with LRRK2, conceivably through LRRK2-mediated mechanisms or simply promoted by increased intracellular Tau levels, and that this is not due to increased toxicity. These novel findings shed a new light into the molecular mechanisms underlying the propagation of Tau between cells [50]. We speculate that the HMW species formed in the presence of elevated levels of LRRK2 could be released by cells and later on be uptaken by neighbouring cells, spreading Tau pathology (Figure 9). Given that LRRK2 can also be secreted, since it was found in neural exosomes where it interacts with several proteins [51], is possible to speculate that Tau release could occur in a complex together with LRRK2 and, eventually, with other proteins.

Fibrillar Tau species are thought to be responsible for the impairment of the proteasome degradation capacity, which occurs in the brains of AD patients [16, 41]. Therefore, our findings implicate LRRK2 in the proteasomal degradation of Tau in two manners: (i) it may prevent Tau-proteasomal degradation by forming a complex with LRRK2, Tau and eventually other proteins, or (ii) it may cause an indirect effect on the proteasome by promoting the accumulation of HMW-Tau species, which by themselves compromise proteasomal function, resulting in a

positive feedback loop of proteasome impairment that will be detrimental for cells (Figure 9). A direct impairment of the proteasome does not necessarily imply reduced proteolytic activity but may involve physical occupation of the proteasome by a large protein such as LRRK2 [52]. Although we speculate that LRRK2 causes the accumulation of HMW-Tau species by proteasomal inhibition, it is conceivable that LRRK2 directly interacts with Tau and thereby prevents its degradation.

Altogether, our data demonstrate an interaction between LRRK2 and Tau, and indicate that LRRK2 increases the intracellular levels of Tau probably by impairing its proteasomal degradation, one of the most important systems for Tau clearance in the cell. This accumulation might lead to formation of HMW Tau species and also influence the levels of Tau secretion. These results place LRRK2 as a central player involved in the early steps of Tau accumulation and spreading, suggesting it may constitute an important target for the design of novel therapeutic approaches in AD and PD.

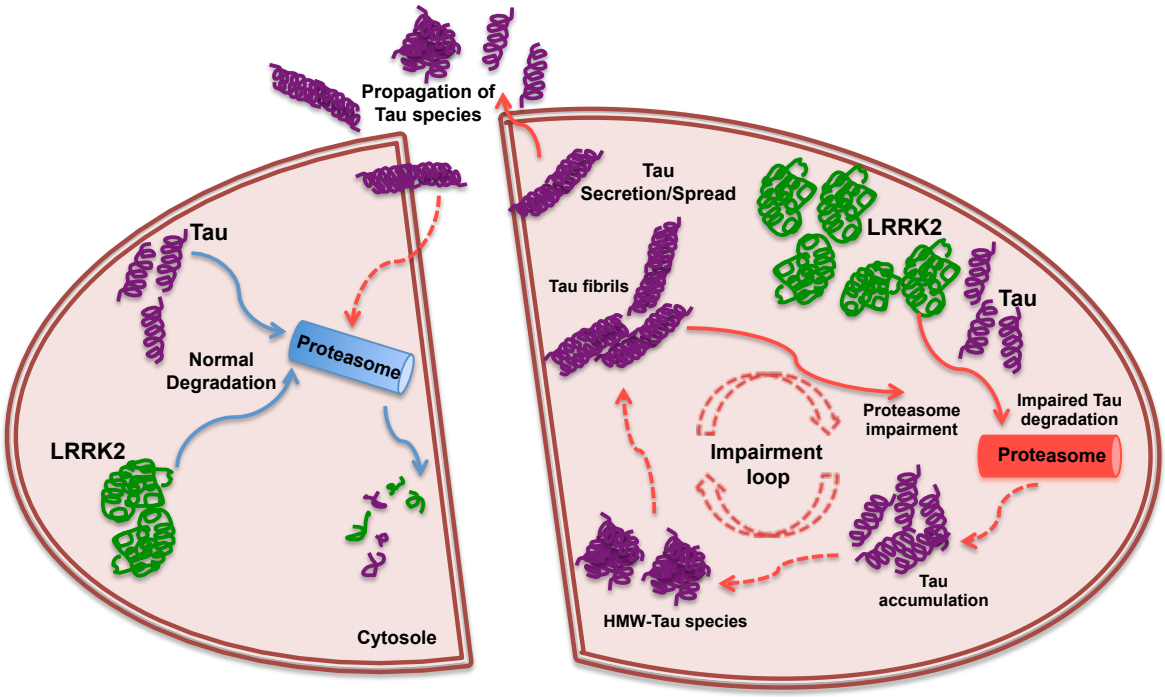

Figure 9. Proposed model for the effect of LRRK2 on Tau accumulation and release. LRRK2 and Tau are two proteins usually degraded by the proteasome. The increase of LRRK2 intracellular levels impairs Tau's proteasomal degradation and leading to its accumulation. The intracellular accumulated Tau leads to the formation of high-molecular weight Tau species and consequently to Tau fibrils, that by themselves impair the normal proteasome function. Tau is released to the intercellular medium, possibly in different molecular weight species, and in particular the fibrils might be uptaken by the neighbor cells, where they can promote a cascade impairment effect on the proteasome.

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Chapter 5.

Identification of LRRK2-interacting proteins and exploration of the molecular pathways involved

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Chapter 5. LRRK2 interactors and their biological significance

5.1 Introduction and main goals

Mutations in LRRK2 contribute to the pathogenesis of PD and the most frequent cause of inherited forms of the disease. Importantly, these mutations are also a risk factor for sporadic cases [1,2].

One of the greatest challenges in the LRRK2 field is to better understand the function of this complex protein and its dysfunction in the context of disease, thus, the identification of LRRK2-interacting proteins is critical.

Several LRRK2 interactors have been identified using different models and different methodological approaches. For instance, LRRK2 interaction with Hsp90, CHIP and DVL1/2/3 proteins was confirmed in cell lines and mouse brain, by Yeast Two-Hybrid (YTH) and Co-Immunoprecipitation (Co-IP) assays [3-6]. The interaction with α-tubulin was confirmed by a Co-IP and actin cytoskeleton-related proteins were found to interact with LRKK2 by a QUICK screen and Co-IP assays in NIH3T3 cells [7, 8].

Here, we applied a combined method of LRRK2 immunoprecipitation from HEK cells, followed by a pull down assay from mouse brain lysates, with the goal of identifying new LRRK2-interacting proteins (Figure 1). The co-immunoprecipitated proteins, potential LRRK2 interactors, were analysed by Mass Spectrometry, followed by bioinformatics analyses with specific software.

5.2 Material and Methods

Immunoprecipitation of LRRK2 and pull-down assay from all mouse brain lysate

Human embryonic kidney 293 cells (HEK-293) were cultured in DMEM medium (Invitrogen, Barcelona, Spain) supplemented with 10 % fetal bovine serum and 1% penicillin–streptomycin in 5% CO_2 at 37°C. Cells were transfected with Myc-LRRK2-WT plasmid using FuGENE®6 (Roche diagnostics, Mannheim, Germany), according to manufacturer instructions. After 48h of transfection, cells were collected with IP buffer (50mM Tris-HCl pH7,5; 0,5mM EDTA; 150mM NaCl; 0,05% NP40) supplemented with protease and phosphatase inhibitor cocktails (Roche, Mannheim, Germany) and sonicated

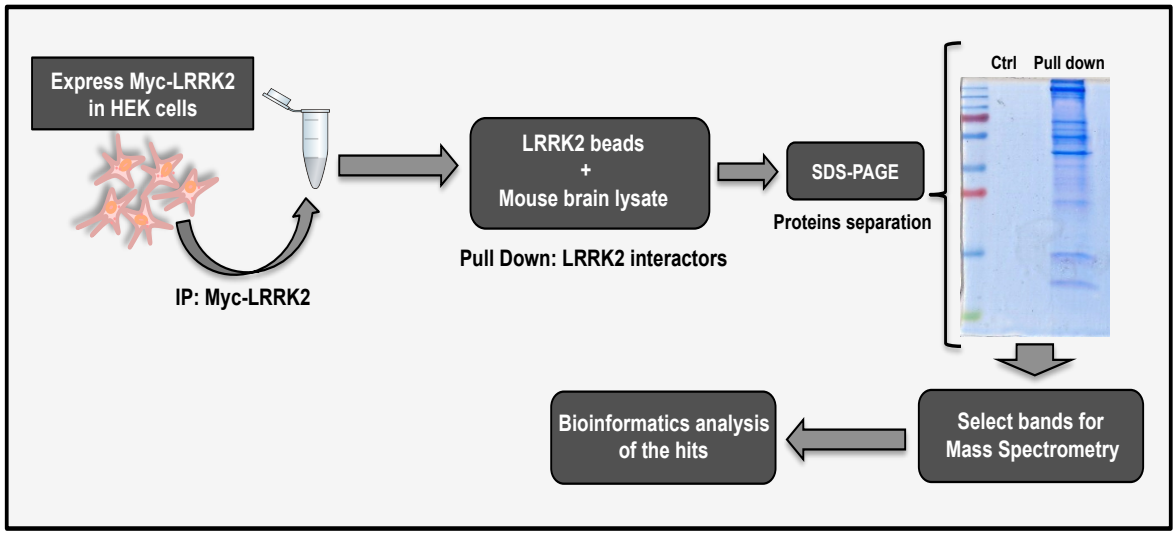

Figure 1. Experimental design for the identification of LRRK2 protein interactors.

Myc tagged LRRK2-WT was expressed in HEK cells and immunoprecipitated with an antibody against the Myc tag. Then the beads were incubated with mouse brain lysate, boiled and the supernatant was loaded onto a SDS-PAGE separation gel. After Coomassie staining, the bands were cut from the gel and the respective proteins were analysed by Mass Spectrometry. The resultant list of hits was subjected to bioinformatics analysis.

The lysate was pre-cleared by incubation with protein G beads (Invitrogen, Barcelona, Spain) for 30 minutes at 4°C with rotation. The supernatant was recovered and incubated with immunoprecipitation antibody against the Myc tag (Cell Signaling, Danvers, MA, USA), followed by overnight rotation at 4°C. The next day, protein G beads were added to the lysate-antibody complex for 3h with rotation at 4ºC. The beads were washed three times with IP buffer and then incubated with whole mouse brain (WT) lysates for an additional 4h with rotation at 4ºC. Beads were washed for the last time, boiled in protein sample buffer and the supernatant was loaded onto a SDS–PAGE (4-12% NuPAGE Bis-Tris Gel, Invitrogen), followed by Coomassie staining.

Mass Spectrometry Analysis

Disclaimer: The mass spectrometry analysis was performed in the Bioanalytic mass spectrometry facility at the Max Planck Institute for Biophysical chemistry, Goettingen.

After protein separation on a SDS–PAGE (4-12% NuPAGE Bis-Tris Gel, Invitrogen), the entire lane of the Coomassie blue-stained gel was cut into 23 slices. All slices were reduced with 10 mM DTT for 55 min at 56°C, alkylated with

55 mM IAA for 20 min at 26°C and digested with modified trypsin (Serva) overnight at 37°C. Tryptic peptides were injected into a C18 pre-column (25 mm, 360 µm o.d., 150 µm i.d., Reprosil-Pur 120 Å, 5 µm, C18-AQ, Dr Maisch GmbH) at a flow rate of 10 µl/min. Bound peptides were eluted and separated on a C18 capillary column (12 cm, 360 µm o.d., 75 µm i.d., Reprosil-Pur 120 Å, 3 µm, C18-AQ, Dr Maisch GmbH) at a flow rate of 300 nl/min, with a gradient from 5 to 36% ACN in 0.1% formic acid for 50 min using an Agilent 1100 nano-flow LC system (Agilent Technologies) coupled to a LTQ-Orbitrap Velos hybrid mass spectrometer (Thermo Fisher). The mass spectrometer was operated in the data-dependent mode to automatically switch between MS and MS/MS acquisition. Survey MS spectra were acquired in the Orbitrap (m/z 350–1600) with the resolution set to 30,000 at m/z 400 and automatic gain control target at 5 × 10E5 ions. The fifteen most intense ions were sequentially isolated for CID MS/MS fragmentation and detection in the linear ion trap. Ions with single and unrecognized charge states were excluded. Raw data was analyzed with Mascot search engine for peptide and protein identifications.

Viral transduction of H4 cells and selection

H4 cells were plated in a 12 well plate with a low confluence (approximately 20%). The day after they were transduced with LRRK2 lentivirus (LV-CMV-eGFP-LRRK2-wt), a kind gift from Dr. Veerle Baekelandt (University of Leuven). Cells were monitored daily, via fluorescence microscopy, to check for LRRK2 expression, which they start to express after three days of transduction, reaching maximum levels of expression after five days. Confluent cells were then re-plated into a 10 cm dish and after three days in culture were split. LRRK2 expressing cells were sorted by green fluorescence. Sorted LRRK2-expressing cells were kept in culture to proceed with immunostaining for phosphorylated LRRK2 (pS935, Epitomics) and with the Elastic E modulus analysis.

Microscopy and Elastic E modulus analysis

Cell probing was performed with an indention experiment. The cantilever with an attached sphere at the end pushes the cell surface until a certain force set point is reached, then the cantilever is withdrawn to the starting point. The interpretation of AFM-based indentation tests is dependent on the used theoretical model for

contact mechanics to determine Youngs modulus. It has been shown experimentally that for indentations of less than 600nm and spherical indenter geometry, the Hertz contact model is regarded as valid to derive Youngs modulus in bio material AFM experiments [36]. Youngs modulus (E) is derived from the experiments by fitting the force (f) vs. indentation (d_z) curves (Figure 2) with the Hertz model:

$$f(d_z) = \frac{4E}{3(1-\upsilon^2)} \cdot R_b^{1/2} \cdot d_z^{3/2}$$

Cell indentation is computed from the displacement of the z-piezo (z) minus the bending (b) of the cantilever (Figure 2). The sphere radius (R_b) and the Poissons ration (υ) were defined respectively for R_b=2.5µm and υ=0.4 [39].

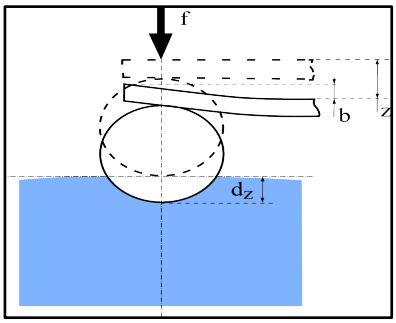

Figure 2. Schematic representation of the cantilever deformation during a force distance experiment, using a spherical cantilever probe.
The blue area represents a cell and the force is created by lowering the cantilever mounting point [39].

Distribution of the indenting load over several µm² area, averages the contribution of multiple cytoskeleton fibres and makes the contact analysis more accurate. Therefore, we used a cantilever with a borosilicate glass sphere with a radius of 2.5 µm. The nominal spring constant of this cantilever is k= 0:035N/m. To ensure indentation of the cytoskeleton, we fitted the Hertz model in the range from 100nm to 500nm indentation - the higher border is to ensure the appropriate use of the model. The associated force borders lie within the range of 150pN to 1nN, the upper border marks the set point. Indentation speed was chosen at 5 µm/s [13]. Every cell was pushed three times at three different positions in the middle, between nucleus and the outer cell peripheries. The global cell value is the mean of the three pushes (Figure 3).

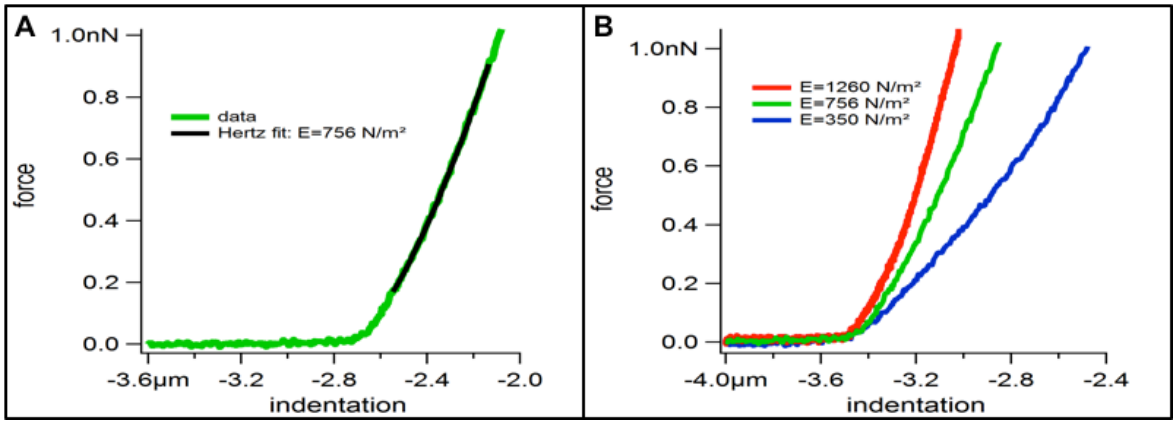

Figure 3. (A) Graphic representation for fitting a force vs. indentation f(d_z) curve with the Hertz model. **(B)** Graph from indentation experiments on the three different fluorescence cell classes. Red: diffuse cells, green: filamented cells, blue: dotted cells.

5.3 Results

5.3.1 Network of protein interactions

The Mass Spectrometry (MS) analysis was performed after the IP/Pull Down assay of LRRK2 in mouse brain lysate with the goal of identifying candidates of LRRK2 protein interactors. The resultant list was carefully examined and only hits identified with more than 2 unique peptides were considered for subsequent analysis. The final list of hits, with approximately 360 candidates, was analysed using the STRING software [9] which revealed a complex network of protein interactions, based on documented and predicted results (Figure 4). The proteins are represented by gene name and the *LRRK2* gene is located in the centre of the network, directly connected with four genes: *SNCA*, *MAPT*, *HSP90* and *CSE1L*.

5.3.2 Gene Ontology Analysis

Gene ontology (GO) is a powerful approach used to predict how gene products behave in a cellular context. The GO analysis of the LRRK2-interactors network is very useful to highlight biological processes, cellular components and molecular functions where LRRK2 and its interactors may be involved. Using STRING software, the hits were organized in three categories: cellular components, biological processes and molecular function.

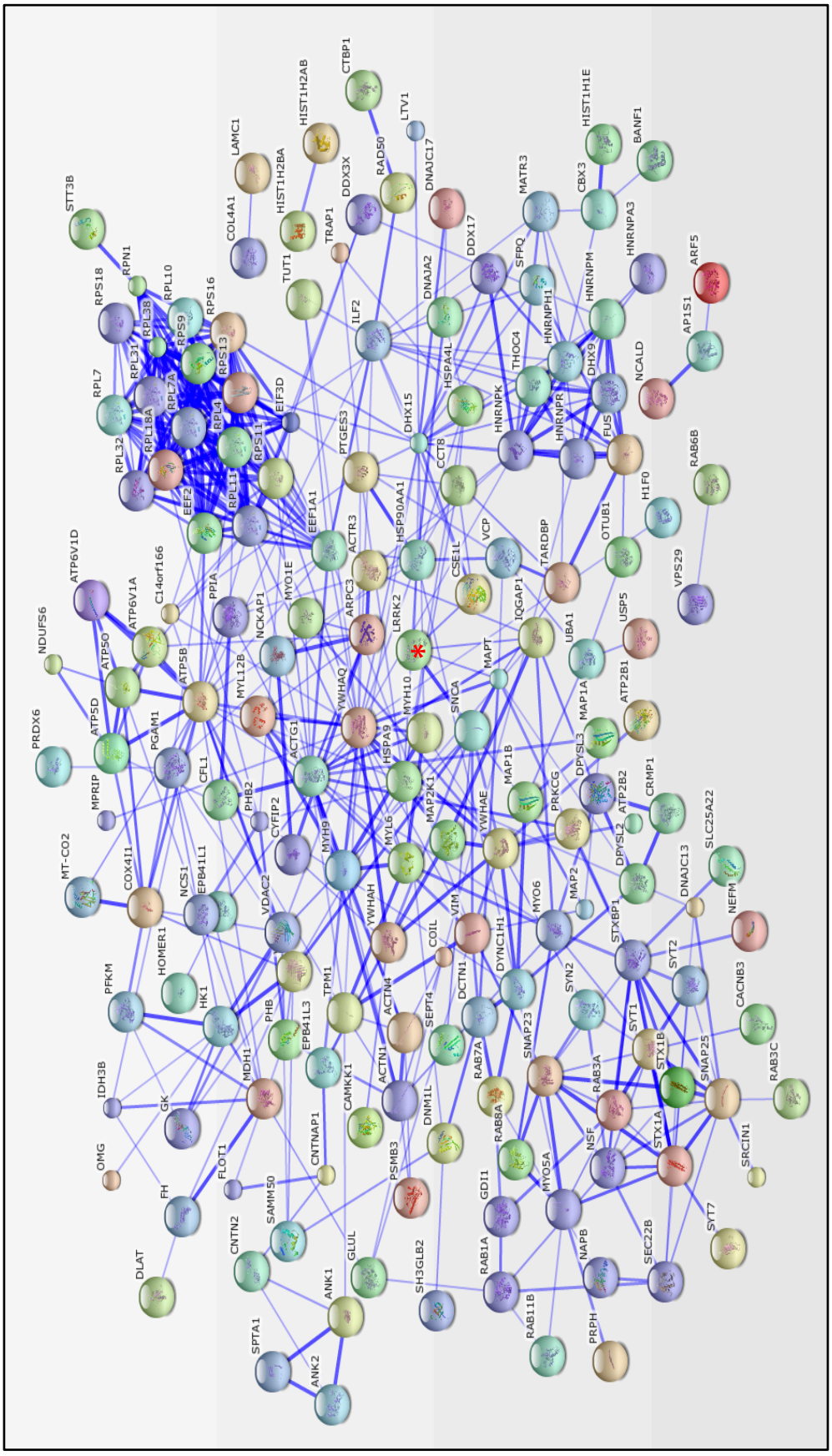

Figure 4. Network of *LRRK2* interactions using STRING software.
STRING network of direct and indirect protein interactions based on documented and predicted results, with the most probable interactions highlighted in strong blue lines. The proteins are represented by the respective gene name. *LRRK2* is centrally located in the network and marked with *.

Cellular Components

This category represents components of a cell, which may be an anatomic structure (e.g microtubule or mitochondria) or a gene product group (e.g proteasome complex or ribosome). Twenty of the most represented and statistically significant cellular components in the LRRK2-interaction network are summarized in table 1, and scored by the number of genes that correspond to each cellular component. Diverse cellular components are represented in the selected list. The most represented components are related to cytosolic structures like intracellular organelles, mitochondria, cytoskeleton and microtubule components and also to proteasomal and macromolecular complexes.

Table 1. GO - Cellular Components. Selected list of the most represented cellular components, in the LRRK2-interaction network. The list shows the number of genes belonging to each cellular component, the statistical analysis and some gene examples.

Cellular Components	*Number of Genes*	*p-value*	*Gene Examples*
Cytosol	73	8.78E-2	AP1S1; PRKCG; CAMKK1; EEF1A1
Intracellular organelle part	58	1.48E-2	ANK1; DNM1L; RPL31; DNM1L
Macromolecular complex	38	1.04E-2	DNM1L; ROL11; STXBP1; HNRPR
Intracellular non-membrane-bounded organelle	28	5.12E-2	CBX3; CCT8; DYNC1H1; VDAC2
Organelle membrane	19	7.05E-2	LRRK2; SNCA; MATR3; SYT2;PHB2
Mitochondria	17	6.38E-3	ATP50; DNM1L; SEPT4; MT-CO2
Cytoskeletal part	14	2.06E-2	CCT8; TPM1; DPYsL3; DHX9
Ribonucleoprotein complex	13	1.08E-4	RPL7A; RPS16; ILF2; ACTN4
Cell projection	12	3.84E-3	STX1A; DPYSL3; SNCA; LRRK2
Cytoplasmic vesicle	12	5.22E-3	SNAP23; STXBP1; MYO1E; SNCA
Neuron projection	11	2.2E-3	DPYSL3; SNCA; LRRK2; MYO5A
Mitocondrial envelop	10	6.3E-2	PHB2; IQGAP1; DNM1L; ATP50
Mitochondrial membrane	10	6.3E-2	LRRK2; DNM1L; PHB2; ATP5B
Ribosomal subunit	8	1.03E-4	RPL4; RPS16; RPL11; RPL38
Actin cytoskeleton	8	6.4E-3	IQGAP1; SNCA; MYO6; DPYSL3
Clathrin-coated vesicle	7	2.32E-2	RAB3A; AP1S1; SYT1
Microtubule	5	9.92E-3	CCT8; DYNC1H1; DCTN1; DNM1L
Growth cone	4	6.3E-2	SNCA; MYO5A; DPYSL3; SNAP25
Synapse part	3	7.37E-2	CNTN2; SNCA
Proteasome complex	2	2.28E-2	VCP; PSMB3

Biological Processes

The biological process category represents a collection of molecular events with a defined beginning and end. Different from the molecular function category, in general, the biological processes imply more than one distinct step.

Twenty of the most represented and statistically significant biological processes within the LRRK2-interaction network are summarized in Table 2 and scored by the number of genes corresponding to each biological process. Diverse bilogical processes are also represented in the selected list and the most represented relate to cell transport, organization and differentiation, as well as regulation of biological quality, synaptic transmission and secretion.

Table 2. GO - Biological Processes. Selected list of twenty of the most represented biological processes in the LRRK2-interaction network. The list shows the number of genes belonging to each biological process, the statistical analysis and some gene examples.

Biological Processes	*Number of Genes*	*p-value*	*Gene Examples*
Cell Transport	59	6.6E-6	RAB1A; ANK1; EPB41L3; RPL11; RPL31
Cellular localization	40	1.16E-10	ANK1; ACTN4; YWHAH; RPL11; RPL31
Cellular component organization or cell biogenesis	40	1.43E-3	MYO1E; HSPA9; DCTN1; RAB11B;VIM
Cellular nitrogen compound metabolic process	34	5.3E-3	LRRK2; VCP; MYH9; RAB7A; RAB1A
Intracellular transport	29	6.77E-9	THOC4; SNAP23; RPN1;RPL18A;ATP5O
Regulation of biological quality	28	1.23E-2	ACTN4; RAD50; YWHAH
Nervous system development	24	1.31E-4	NCKAP1; DCTN1;MAP2K1; COL4A1
Cell differentiation	21	6.73E-3	ANK1; ANK2; CRMP1
Protein transport	18	7.73E-6	RPL31; RPL4; RPL11; RPS13
Neuron differentiation	17	3.24E-5	CRMP1; ANK1; MAP2K1; COL4A1
mRNA metabolic process	17	7.29E-5	HNRNPK; FUS; DHX9; TUT1; PSMB3
Regulation of cellular component organization	17	3.01E-2	STXBP1; DNM1L; SNCA; LRRK2; PHB
Vesicle-mediated transport	16	3.87E-4	RAB7A; ACTN4; ACTN1; ANK1
Neuron development	16	3.05E-5	CFL1; CNTN2; MYL6; CACNB3; CRMP1
Synaptic transmission	13	2.73E-3	GLUL; NSF; MYO6; PRKCG; CAMKK1
Secretion	13	2.54E-5	NSF; ANK1; ACTN1; ACTN4
Protein targeting to membrane	12	2.94E-7	SAMM50; ANK1; RPL4; RPS13; RPN1
Ribonucleotide metabolic process	11	4.16E-4	LRRK2; VCP; RAN11B; ATP50; MYH9
Protein localization to endoplasmic reticulum	11	2.64E-7	ANK2; RPL31; RPL11; RPS13; RPL18
Oxidation-reduction process	11	9.33E-3	IQGAP1;STXBP1; STX1A; SNAP25

Molecular Function

Molecular function generally corresponds to activities, such as catalytic or binding activities, that occur at the molecular level. They can be the result of individual gene products, but some activities are performed by the combinations of gene products. In order avoid confusion with/between gene product name, many molecular functions are appended with the word "activity". Again, in table 3 is listed twenty of the most represented and statistically significant molecular functions in the LRRK2-interaction network, scored by number of genes corresponding to each molecular function. Also, in this category a variety of molecular functions are described. Thus, the two most represented groups are enzymatic activities (pyrophosphatases, hydrolases, ATPase, GTPase) and structural molecular activities (microfilaments activity, actin and cytoskeletal protein binding).

Table 3. GO - Molecular Function. Selected list of twenty of the most represented molecular functions in the LRRK2-interaction network. The list shows the number of genes belonging to each molecular function, the statistical analysis and some gene examples.

Molecular Function	*Number of Genes*	*p-value*	*Gene Examples*
Enzyme binding	16	5.96E-2	TUT1; YWHAH; DNM1L; ANK1; EEF1A1
Nucleoside-triphosphatase activity	14	4.76E-6	CCT8; LRRK2; RAB7A; RAB1A; MYL6
Pyrophosphatase activity	14	9.62E-6	DYNC1H1; RAB11B; LRRK2; ATP50
Hydrolase activity, acting on acid anhydrides	14	9.62E-6	RAB1A; RAB7A; MYL6; CCT8; RAD50
Structural molecule activity	13	3.25E-4	MAP1B; TPM1; ARPC3; MATR3; SPTA1
RNA binding	11	1.34E-3	TARDBP; ILF2; RSP13; RPL4; TUT1
ATPase activity	9	6.6E-5	MYH10; MYO1E; CCT8; MYL6; MYH9
Cytoskeletal protein binding	9	1.05E-2	SNCA; LRRK2; ACTN4; MYO1E; ANK1
GTPase activity	8	1.52E-4	LRRK2; RAB7A; RAB1A; SEPT4; RAB11B
Structural constituent of cytoskeleton	6	2.87E-4	VIM; NEFM; TPM1; ARPC3; ANK1
Actin binding	5	1.25E-2	MYO1E; MYO6; SPTA1; TPM1; ACTN4
Ion chanel binding	5	2.33E-2	ACTN1; LRRK2; YWHAH; ANK2; HOMER1
Microfilaments motor activity	5	6.3E-3	MYO6; MYO1E; MYH9
Protein homodimerization activity	4	2.32E-2	LRRK2; ACTN4; MYH9
Actin filament binding	4	4.23E-3	ACTN4; MYO1E; MYO6; SPTA1
ADP binding	4	2.33E-2	MYH9; MYH10; MYO6; ATP5D
Protein phosphatase binding	3	4.52E-2	IQGAP1; VCP; LRRK2
SNARE binding	2	1.3E-2	RAB11B; SYT1
Protein serine/threonine/tyrosine kinase activity	2	2.54E-2	LRRK2; MAP2K1
GDP binding	2	3.78E-2	RAB11B; RAB7A

Kyoto Encyclopedia of Genes and Genomes Pathways

The Kyoto Encyclopedia of Genes and Genomes (KEGG) is a database resource that comprises a collection of pathway maps representing knowledge of molecular interactions and reaction networks. It is essential for understanding high-level functions and utilities of a biological system, such as the cell or the organism, especially in large-scale molecular datasets, generated by high-throughput experimental approaches. The most highly represented and statistically significant KEGG pathways in the LRRK2-interaction network, were scored by the number of genes, corresponding to each pathway (Table 4). These results show that several pathways associated with neurodegenerative diseases such as PD, Huntington's and Alzheimer's, can be identified in the LRRK2-interaction network. Genes directly and indirectly linking *LRRK2* to these pathways are highlighted in the LRRK2-network, where the connection of *LRRK2* with central genes like *SNCA, MAPT* or *Rab7a* is depicted (Appendix Figure 5.1, 5.2, 5.3).

The LRRK2-interaction network also includes genes that confirm the involvement of LRRK2, with pathways related with the ribosome, oxidative phosphorylation and cytoskeleton regulation. Some of these genes, like *RPS* genes related with the cellular structure of the ribosomes and *ATP* genes, related with the supply of energy in the cell are highlighted in appendix figures 5.4 and 5.5.

Table 4. KEGG Pathways. Selected list of eleven of the most represented KEGG Pathways in the LRRK2-interaction network. The list shows the number of genes belonging to each pathway, the statistical analysis and some gene examples.

KEGG Pathways	*Number of Genes*	*p-value*	*Gene Examples*
Ribosome	14	1.33E-8	RPL32; RPL7A; RPS16, RPL11
Regulation of actin cytoskeleton	12	2.8E-2	IQGAP1; MYH19; NCKAP1; ACTN1/4
Parkinson's disease	11	2.31E-5	SNCA; LRRK2; UNA1; ATP5B
Huntington's disease	9	6.34E-3	DCTN1; COX4I1; MT-CO2; ATP5B
Oxidative phosphorylation	8	3.75E-3	COXaI1; NDUFS6; ATP5B
Alzheimer's disease	8	1.5E-2	MAPT; SNCA; NDUFS6; ATP5-D
Spliceosome	6	7.78E-2	DMX15; HNRPK; HNRPM; HNRPA3
Citrate cycle (TCA cycle)	4	2.34E-3	DLAT; IDH3B; MDH1; FM
SNARE interactions in vesicular transport	4	3.68E-3	SNAP23; SEC22B; SNAP25; STX1B
Glycolysis / Gluconeogenesis	4	2.83E-2	DLAT; PFKM; HK1; PGAM1
Adherens junction	4	5.3E-2	ACTN1; ACTN4; IQGAP1; ACTG1
Galactose metabolism	2	8.22E-2	PFKM; HK1

5.3.3 The effect of LRRK2 expression on the mechanical properties of the cell

Disclaimer: The AFM and TIRFM experiments were performed by Kai Bodensiek from Atomic Force Microscopy-biomechanics laboratory, Georg-August University, Göttingen.

The previous analysis of the LRRK2-interactors enabled us to identify proteins, and groups of proteins, that directly or indirectly interact with LRRK2. We found that several LRRK2-interactors are related to cytoskeleton structures, like actin, tubulin and myosins. The GO analysis highlighted cellular components and molecular functions such as microtubules and other parts of the cytoskeleton, proteins binding cytoskeleton, structural constituents of cytoskeleton and proteins involved in microfilaments motor activity.

The structural and mechanical properties of a cell are important parameters that influence its regular function, reflecting its health conditions. The stiffness of eukaryotic cells is a parameter conditioned by several forces from membrane tension but mainly determined by the cytoskeleton structure, that is constituted by several polymeric networks including actin, microtubules and intermediate filaments [10]. Atomic Force Microscopy (AFM) is a method widely used to study the mechanical characteristics of a cell, through the measurement of the cell's visco-elastic properties [11,12].

Given the documented effect of LRRK2 on microtubules [13, 14], we decided to explore the mechanical effect of LRRK2 on cells at the structural level. For that, we expressed LRRK2 in a human neuroglioma cell line (H4), and performed cell indentation experiments with AFM, to quantify changes in cytoplasmic crosslinking, aggregation, depolymerisation, and polymerisation. To better analyse an isolated cell in culture, we used a setup in which the AFM is combined with a Total Internal Reflection Fluorescence Microscope (TIRFM). TIRFM was used in bright field illumination to localize the different phenotypes, which could be targeted with the AFM.

5.3.4 Different LRRK2 distribution patterns result in different cell stiffness

Analysing H4 cells transduced with viruses encoding for LRRK2-GFP via fluorescence microscopy, we noticed a mixture of different distribution patterns of

the fusion protein, presenting several cytoplasmic structures. Interestingly, after immunostaining these cells with a phospho-specific LRRK2 antibody (at S935 residue), we verified that these structures, composed by LRRK2, are also enriched in phosphorylated LRRK2 (Figure 5). In order to closely analyse these different patterns, we distinguished LRRK2 expressing cells into three classes: diffuse expression, dotted expression and filamented expression (Figure 6).

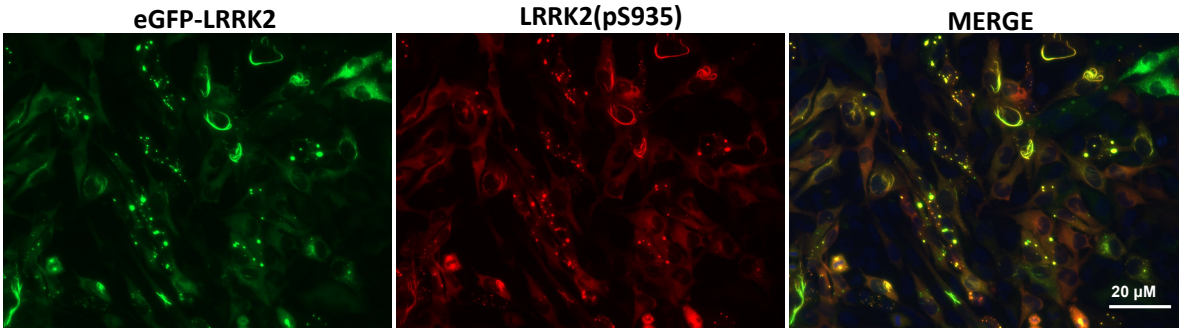

Figure 5. E**xpression of eGFP-LRRK2 in H4 cell line.**
H4 cells transduced with eGFP-LRRK2 lentivirus, stably expressing LRRK2. The fluorescent microscopic images show the different distribution patterns of LRRK2, with different shapes of cytoplasmic structures, that the immunostaining revealed to be highly phosphorylated at residue S935.

These cells were analysed by linking their viscoelastic behaviour to the grouped LRRK2-expression patterns. The chosen experimental parameters (see methods) allow for approximation of the cell as an elastic body whereby we were able to use the elastic modulus, known as Youngs modulus (E), as parameter for its characterization [15]. A detail of the different cytoplasmic structures present in the three different cell classes is shown on figure 6.

The results show that different LRRK2 distribution patterns result in differences in the elastic modulus, allowing us to characterize each cell classes according to its mechanical behaviour. Thus, cells with a diffuse pattern are the stiffest, followed by cells with filamented LRRK2 expression. Cells with a dotted pattern have lowest levels of stiffness and are similar to cells only expressing the GFP tag or the un-transduced H4 cells (Figure 7). Statistical analysis of the results shows that cells with a diffuse or filamented pattern possess a significantly higher E modulus than cells with a dotted expression. Although cells with a diffuse pattern are stiffer than cells with filamented pattern, this tendency is not statistically significant (Figure 7).

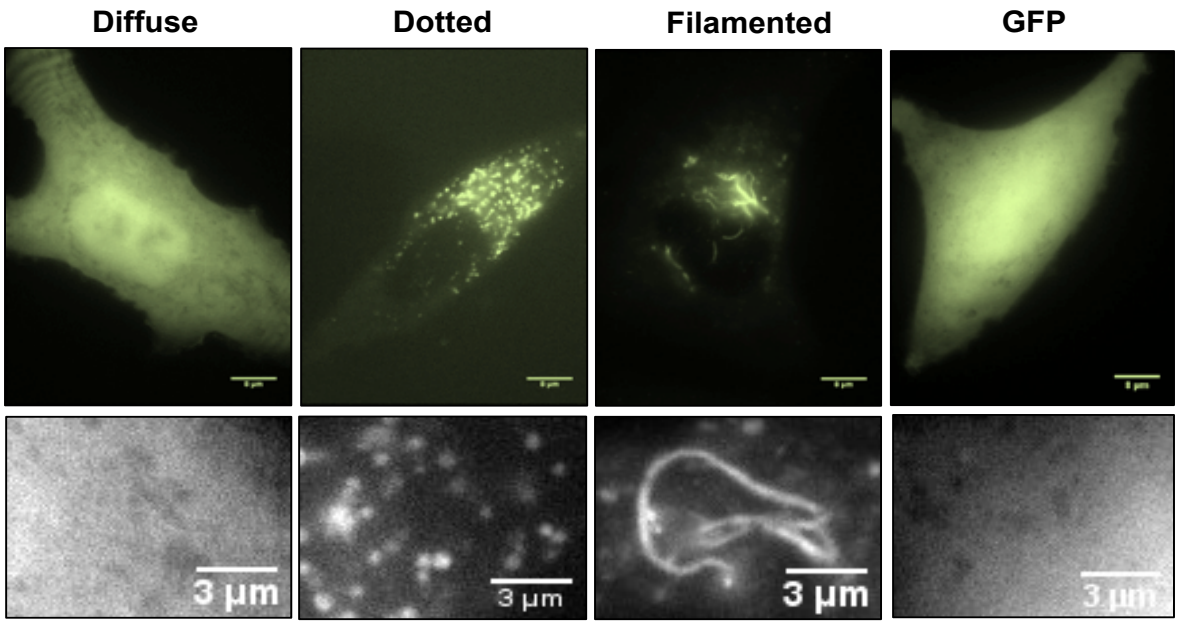

Figure 6. Different distribution patterns of LRRK2 in H4 cells. H4 cells expressing LRRK2 were classified according to their LRRK2 distribution patterns in: diffuse, dotted and filamented. In the lower row of pictures there is a detail of the cytoplasmic environment characteristic of each class. The microscope measurements of several cells per each class, like the ones exemplified in figure 5, were analysed using the Elastic E modulus.

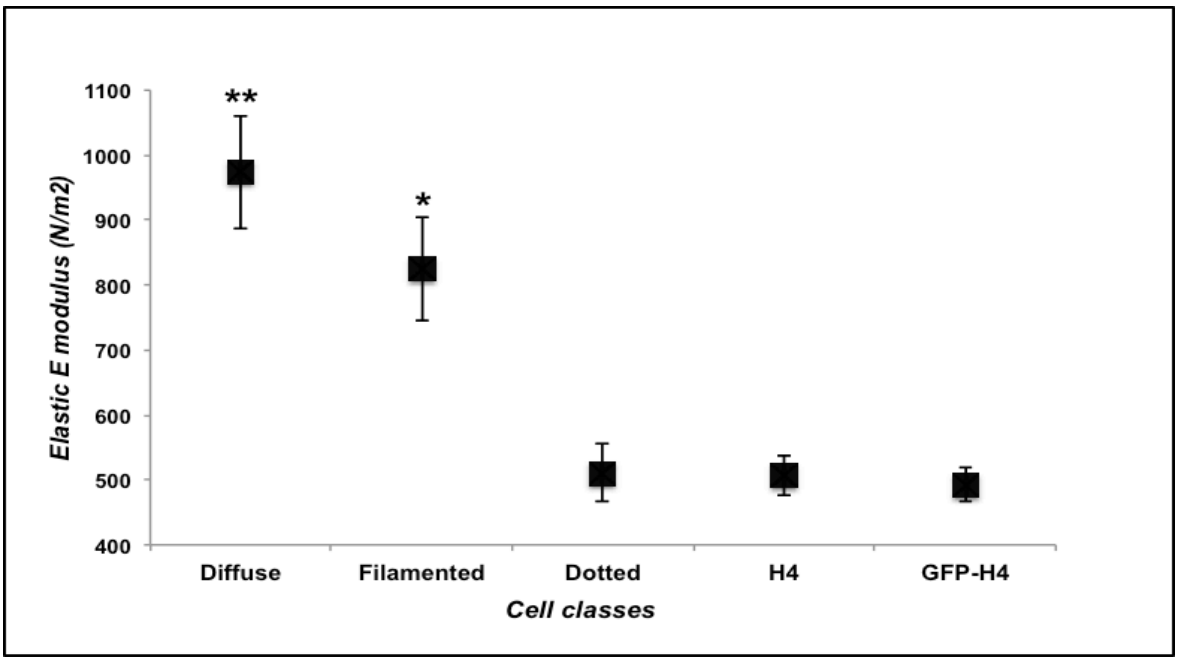

Figure 7. LRRK2 distribution patterns distinguished by Elastic E modulus analysis. Cells from the three different classes of LRRK2 distribution, and the two controls (H4 and GFP-H4), were analysed by combined AFM/TIRFM and their mechanical properties were quantified by the Elastic E modulus. The analysis distinguished cells with LRRK2 diffuse distribution as stiffer ones, followed by cells with LRRK2 filamented distribution - both differed significantly from control samples (H4 and GFP-H4). Dotted expression cells have a similar stiffness to control cells, being significantly different from diffused cells ($p<0.05$) and tendentiously different from filamented cells ($p<0.1$). Diffuse distribution (N= 13); filamented distribution (N=16); dotted distribution (N= 18); H4 cells (N=50); GFP-H4 (N=50). Significance results from T-test: **$p< 0.01$ and * $p<0.05$.

5.4 Discussion

The identification of LRRK2 interactors is crucial to place the protein in known biochemical pathways, allowing a better understanding of its physiological function and associated pathology. To fulfil this goal, a variety of LRRK2 high-throughput studies like yeast two-hybrid screens, mass spectrometry, pull-down assays and genome-wide mRNA expression screens, have been performed alone or in combination. The identified LRRK2 interactors and substrates have implicated the protein in a variety of pathways and biological processes. Some of those are cytoskeleton dynamics, protein translation, cell growth and differentiation, cell signalling, among many others.

In this study we used a sequential Co-IP followed by mass spectrometry in order to identify novel LRRK2 interactors. Within the vast list of potential LRRK2-interactors obtained, we could confirm the presence of some already described protein interactors, such as HSP90/70 [16,17], α-β-tubulin [18, 19], Rab5 [20, 21], 14-3-3 [22, 23], vimentin and clathrin [24]. This proved the robustness of the experimental approach used.

Through gene ontology analysis of the LRRK2-interactors network, we confirmed that LRRK2 is a multifaceted protein, involved in a variety of molecular functions and biochemical pathways. As a kinase, LRRK2 is naturally involved in several enzymantic reactions. LRRK2 has also a role in intracellular organelles (as mitochondria), in cytoskeleton and microtubule components, in cell transport, cell quality control and cell organization/differentiation.

As expected, LRRK2 is present in the PD pathway but, interestingly, also in other neurodegenerative diseases like Alzheimer's and Huntington's disease. This demonstrates that the important role of LRRK2 and it protein interactors is not only limited to PD.

LRRK2 is also present in pathways involving the ribosome, oxidative phosphorylation and cytoskeleton regulation. This suggests that LRRK2 plays a role in important cell pathways as protein synthesis, cellular respiration and cytoskeleton organization and rearrangement.

Within the LRRK2-interactors network, we highlight the presence of α-synuclein and Tau proteins. These two central proteins in PD and AD diseases were identified as LRRK2 interactors and potential substrates [25-28], and their

interaction with LRRK2 has been extensively explored in chapters 3 and 4 of this thesis.

The role of LRRK2 on the cytoskeleton is one of the most well accepted functions of the protein, supported by the discovery of several protein interactors linked to microtubule dynamics. LRRK2 interaction with α/β-tubulin was confirmed in different studies. It was shown that LRRK2 mutants reduce neurite outgrowth and cause an accumulation of hyperphosphorylated Tau protein, compromising the microtubule dynamics [19, 29, 30, 31]. This dynamics might also be influenced by the interaction of LRRK2 with actin and actin-related proteins [32]. The link between LRRK2 and microtubules is further supported by the interaction with proteins like EF1A, moesin (a member of ERM family) and DVL1/2/3 proteins. These proteins are respectively related to the stability of microtubules, the regulation of anchoring of the actin cytoskeleton to the plasma membrane and axon guidance and synapse formation [33-35].

Knowing that LRRK2 has an important influence on microtubules and consequently on cytoskeleton dynamics, we decided to explore how this could affect the mechanical properties of the cell. The results from cell indentation experiments, using a combined AFM/TIRFM technique, confirm that different LRRK2 distribution patterns contribute to differences in cell stiffness. Thus, the diffuse LRRK2 distribution pattern is the one presenting the stiffest cells. Curiously, the dotted pattern is the only one in which stiffness was not altered when compared to control samples. The previously described role of LRRK2 on microtubule networks, allows the speculation about these LRRK2 distribution patterns. Thus, in the diffuse pattern LRRK2 might be spread throughout the entire cell, interacting with the microtubule-related proteins and compromising cytoskeleton dynamics by increasing stiffness.

One of the pathological hallmarks of PD is the presence of inclusions composed of misfolded proteins in remaining live neurons and LRRK2 is often identified as one of the components of the inclusions [25]. The role of these inclusions has been extensively discussed and whether they are toxic to the cells or confer protection is still unclear [36, 37]. In our cell model, the LRRK2 dotted distribution pattern could be compared to the presence of these cytoplasmic protein inclusions, and we concluded that cells presenting this pattern have the same stiffness compared to the control cells. Thus, we can speculate that

cytoplasmic inclusions may be a defensive mechanism for cells to deal with a high expression of intracellular LRRK2. Therefore, the formation of these inclusions may be a way for cells to pack an excess of free cytoplasmic LRRK2, which might be interacting with microtubules and impairing the normal cytoskeleton dynamics of the cell. To confirm this theory, more experiments need to be performed, particularly to better characterize these dotted LRRK2 inclusions, evaluate the kinetics of their formation and compare the viability of cells presenting these distribution patterns. The interesting LRRK2 distribution patterns obtained in this model could still be useful to test the effect of several chemical compounds in the inclusion formation, finding an analogy with what could happen in the formation of LBs.

5.5 References

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Chapter 6.

General discussion and conclusions

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Since its discovery in 2004, LRRK2 is recognized as the most frequently mutated protein associated with familial forms of PD.

Although numerous efforts are underway in order to better understand the role of this protein in PD, the function of LRRK2 is still not fully understood. LRRK2 is a large and multi-domain protein presenting double enzymatic activity, it works as a kinase, as well as a GTPase. LRRK2 has the propensity to interact with several proteins or protein-complexes, suggesting that it is involved in several cellular mechanisms.

A solid understanding of the function(s) of LRRK2 is crucial in order to decipher the basic mechanisms compromised during the onset and development of PD. In turn, this would serve as the basis for the development of novel strategies for therapeutic intervention. Thus, this project had the goal of gaining new insight into the function and dysfunction of LRRK2 in PD. To achieve this goal, we focused on identifying new LRRK2-interacting proteins, highlighting the cellular mechanisms and pathways where it could be involved. We explored the interaction of LRRK2 with two other central players in neurodegenerative disorders: α-synuclein and Tau.

6.1 Implications of the interaction of LRRK2 with α-synuclein and Tau

α-synuclein was the first protein associated with familial forms of PD. Together with LRRK2, it is reported as being the cause of dominantly inherited forms of the disease. α-synuclein is a pre-synaptic protein highly expressed in the brain and one of the main components of the LBs, a pathological hallmark of the disease, where it appears mostly in its phosphorylated form [1,2]. As LRRK2 is a kinase and its most frequent mutation, G2019S, is responsible for a toxic gain of function, it was hypothesized that α-synuclein might be a direct LRRK2 substrate. In this context, LRRK2 would be responsible for the hyper-phosphorylation of α-synuclein in a disease context [3]. Although this idea was first put forward in 2009, the results were not conclusive, mostly due to the lack of specific and reliable LRRK2 antibodies at the time. Since then, these results were not verified in subsequent reports [4,5]. More recently, we revisited this question, taking

advantage of the new generation of LRRK2 antibodies developed by the Michael J. Fox Foundation. In chapter 3, we demonstrate the interaction between endogenous LRRK2 and α-synuclein in mouse brain samples and in a cellular model. Interestingly, we demonstrate that the hyperactive LRRK2 mutant (G2019S) does not interfere in this interaction. Moreover, in human PD brains, we showed a positive correlation between the levels of LRRK2 and α-synuclein and, even more strikingly, with widespread S129 α-synuclein phosphorylation. Still, in human brain tissue, it was possible to detect the co-localization of these two proteins in neurons, prior to the formation of LBs and also within these inclusions. The relation between LRRK2 and the formation of α-synuclein inclusions was also confirmed in a cell model where KD of LRRK2 promoted a decrease in the size of those inclusions [5]. Although we are still not able to determine whether α-synuclein is a direct interactor and/or substrate of LRRK2, the published evidences highlight that the two proteins indeed have common pathways [6-8]. LRRK2 changes the aggregation pattern of α-synuclein in a cell model and in PD brains, the levels of both proteins are directly correlated, and the two proteins co-localize in LBs, as well as in neurons without inclusions. This allows us to speculate that LRRK2 might play a role upstream of α-synuclein aggregation and phosphorylation.

Additional work is still necessary to confirm which pathways and proteins are affected by LRRK2-induced accumulation of α-synuclein, and whether this is a toxic or a survival mechanism for PD cells. It will be interesting to use models that combine LRRK2 with other proteins known to be involved in α-synuclein aggregation, for instance, synphilin-1 or a chaperone-like protein, such as 14-3-3 [9-11].

Tau is the main component of the neurofibrillary tangles characteristic of AD patients. It is a predominantly neuronal protein that binds and stabilizes the cellular microtubule network, essential in a number of cellular processes. Tau mutations have been related to several neurodegenerative disorders, known as tauopathies, characterized by the presence of hyperphosphorylated and insoluble aggregated forms of Tau in different brain regions. Several Tau mutations have also been pinpointed as risk factors for PD and, in fact, insoluble accumulation of Tau and Tau tangles can be observed in PD brains. The implication of Tau in PD is also

highlighted by its relationship with α-synuclein and LRRK2, two central proteins in PD [12-15].

In chapter 4 we prove that Tau interacts with LRRK2. Moreover, we demonstrate that increased levels of LRRK2 promote the accumulation of intracellular Tau, by interfering with its proteasomal degradation. We raised the hypothesis that this impairment may occur by a direct LRRK2 blockade of the proteasome or by an interaction with Tau, compromising its degradation. Although further experiments need to be done to clarify the mechanism behind this effect, we can speculate how LRRK2 interacts with Tau. The interaction can occur either directly or indirectly, via other common interactors, like microtubule-related proteins, influencing its intracellular accumulation and posttranslational modifications [16,17]. Accumulation of cytoplasmic Tau promotes the formation of HMW-Tau species that are considered inducers of fibrillar and toxic forms of the protein, increasing the release of Tau to the extracellular space. Along with these results, evidence for the presence of Tau tangle pathology in LRRK2 transgenic models were reported, as well as an increase of Tau aggregation and phosphorylation in a taupathy model due to LRRK2 expression [18-20].

The fact that Tau accumulation seems to occur independently of the LRKK2 kinase activity, supports the idea that Tau may not be a direct substrate of LRRK2, as it was previously suggested [19,20]. Thus, LRRK2 could act upstream of Tau phosphorylation, through the ERK pathway, for example [21]. Therefore, LRRK2 seems to play an important role in the homeostasis of Tau levels and, consequently, on Tau posttranslational modifications. This is supported by the widely accepted idea that intracellular accumulation of Tau results in increased aggregation and phosphorylation of the protein in the brains of AD and PD patients [22,23]. It can be speculated that this effect may be at the genesis of Tau cytoplasmic toxic fibrillar forms. Further, these fibrillar Tau species can be released into the intercellular media and taken up by neighbouring cells, explaining the spreading mechanism that happens in taupathies [24,25]. LRRK2 is also a protein degraded by the proteasome, thus, it will be interesting to explore the effect of Tau on LRRK2 levels. Further experiments will be required to identify which pathways and proteins are involved in this complex accumulation/release process.

Altogether, these results contribute to a better understanding of the interaction between LRRK2 and two central proteins in PD and AD, α-synuclein and Tau. In parallel, it raises new pertinent questions regarding the role of LRRK2 on the molecular mechanism behind the neurodegenerative process. Answering these questions will help to clarify the molecular mechanism behind the complex process of protein accumulation, aggregation and spreading. The increased knowledge in these processes, common among several neurodegenerative disorders, will shed light into possible targets for therapeutic intervention.

6.2 LRRK2-interacting protein network and its particular role on cytoskeleton dynamics

LRRK2 is a complex and multifunctional protein likely to be involved in a variety of cellular mechanisms; however its defined function, and particularly in a disease context, is still poorly understood. In chapter 5, we aimed to determine the cellular mechanisms and pathways where LRRK2 may be involved. For this, we applied a combined method of LRRK2 sequencial Co-IP assays, followed by Mass Spectrometry and bio-informatics analysis.

The list of candidates obtained, confirmed several LRRK2-interactors identified using other approaches. These interactors include HSP90/70 [26,27], α-β-tubulin [28,29], Rab5 [30,31] and 14-3-3 [32,33], validating the robustness of our technical approach. Moreover, the presence of α-synuclein and Tau in the list of interactors supports the claims in chapter 3 and 4 of this thesis, where we explored the interaction of LRRK2 with these two proteins.

The analysis of the protein-interacting networks identified LRRK2 as a protein that is involved in a variety of cellular biological processes, such as transport, respiration, differentiation, quality control, oxidative phosphorylation and protein synthesis. These results corroborate previous reports regarding the multifunctionality of LRRK2. This protein is involved in different cellular mechanisms and pathways, not only because of it kinase and GTPase enzymatic functions, but also resulting from the discovery of multiple key LRRK2-interactors [34, 35].

The role of LRRK2 on microtubules and the cytoskeleton is one of the most accepted functions of the protein, supported by the discovery of several protein

interactors related to microtubule dynamics [36-39]. The analysis of the obtained hits in chapter 5 corroborates this idea, in which LRRK2 is involved in several important microtubule and cytoskeleton-related cellular mechanisms. The experiments exploring the effect of LRRK2 on mechanical properties of the cell showed that different LRRK2 distribution partner contributes to differences in cell stiffness. Thus, cells with a diffuse LRRK2 pattern are the stiffest ones and cells with a dotted pattern do not experience alterations in stiffness. Therefore, the dotted pattern observed, upon an increase of LRRK2 expression, could be a common pattern that occurs with increasing levels of other proteins. This pattern could even resemble the cytoplasmic protein inclusions observed in several cellular and animal models or even the Lewy bodies present in PD brains. The formation of these dotted inclusions may represent a defensive mechanism of the cell to pack the excess LRRK2, avoiding diffuse cytoplasmic accumulation, increasing cell stiffness and resulting in an impairment of cytoskeleton dynamics. Further experiments will be needed, to understand the biological relevance of altered stiffness in different LRRK2 distribution patterns, and the implications of this in the neurodegenerative process.

6.3 Conclusions

PD is considered the second most frequent age-related neurodegenerative disorder, after Alzheimer's disease. The tendency for increasing life span of the world population potentiates the occurrence of increasing propensities of PD cases. This ultimately culminates in a complex social/economical problem that needs to be addressed from a multifaceted perspective. Despite advances obtained in minimizing symptomatology and improving life quality of the patients, to date, there is no effective treatment for PD. In this context, it is urgent to deeply understand the molecular and cellular mechanisms that are compromised during the neurodegenerative process. This will be crucial to identify effective therapeutic targets for finding a cure for PD and other similar neurodegenerative diseases.

The main goal of this thesis was to gain insight into the function of LRRK2 protein, as the most frequently mutated protein associated with dominant forms of PD.

The important role of LRRK2 in the molecular underpinnings of PD and AD was confirmed by its interaction with α-synuclein and Tau, and it influences on their biochemical characteristics and cellular distribution. Therefore, LRRK2 is an important protein for the cellular protein homeostasis, and so related with several complex biochemical pathways, wich are usually compromised in PD or AD, but also other neurodegenerative disorders. Moreover, LRRK2 reveals to be a multifunctional protein, implied in several crucial cellular mechanisms, related to the pathogenesis of PD and which should be further explored.

The results obtained in this work, provide several insights into the complex role of LRRK2 in PD-related mechanisms and also other neurodegenerative disorders. Consequently this will open new research venues, crucial for obtaining a more comprehensive level of understanding the interplay between these central neurodegenerative proteins. Ultimately, this knowledge will help to target important cellular mechanisms and pathways for therapeutic intervention.

6.4 References

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Chapter 7.

Appendix

Table 3.1. Table with details of clinical cases
PD cases fulfilled the UK PD Society Brain Bank Diagnostic Criteria, were levodopa-responsive and medicated throughout the disease course, and had no other significant neuropathological changes. Controls had no significant neuropathology and no evidence of neurological or psychiatric disease. The PD cases had a mean age of 79±9 y, mean disease duration of 15±4 y and a mean postmortem interval of 8.5±8 h. The controls were 9 years older on average (mean age of 88±6 y, ttest p=0.02) and had a similar postmortem interval (mean of 10.5±5 h, ttest p=0.5). Because of the age difference, age was co-actored into all analyses, but the use of older controls ensured that in such cases Lewy pathology in late age would not have occurred. There was no difference in the sex distribution between groups (χ2p=0.37) and both groups had similar causes of death.

Case	*Age at death (Y)*	*Gender (male/female)*	*PD duration (Y)*	*Post-mortem interval (h)*	*Braak PD stage (0-VI)*
Control 1	79	M	--	8	--
Control 2	83	F	--	7	--
Control 3	85	M	--	9	--
Control 4	85	F	--	10	--
Control 5	86	M	--	15	--
Control 6	87	F	--	5	--
Control 7	88	M	--	9	--
Control 8	92	F	--	16	--
Control 9	93	F	--	21	--
Control 10	102	F	--	5	--
PD 1	66	M	12	6	V
PD 2	69	M	17	5	V
PD 3	72	M	9	4	IV
PD 4	75	M	14	9	V
PD 5	78	M	24	6	V
PD 6	83	F	14	32	V
PD 7	83	F	14	7	V
PD 8	84	M	17	7	IV
PD 9	90	M	15	5	V
PD 10	91	F	10	4	IV

Table 3.2. Sequences of the five LRRK2 shRNA
(RNAi Consortium; Broad Institute, Ma, USA)

Clone name	*shRNA Sequence*
XM_058513.8-6782s1c1	CCGGCCACAAATTCAACGGAAAGAACTCGAGTTCTTTCCGTTGAATTTGTGGTTTTT
XM_058513.8-305s1c1	CCGGCCCAGGATGTTGGAAATGATTCTCGAGAATCATTTCCAACATCCTGGGTTTTT
XM_058513.8-7558s1c1	CCGGCGTGTGTATGAAGGAATGTTACTCGAGTAACATTCCTTCATACACACGTTTTT
XM_058513.8-5995s1c1	CCGGGCCAGAGGAAATGTCATTTATCTCGAGAGATAAATGACATTTCCTCTGGCTTTTT
NM_198578.3-7444s21c1	CCGGTCGTCGACTTATACGTGTAATCTCGAGATTACACGTATAAGTCGACGATTTTTG

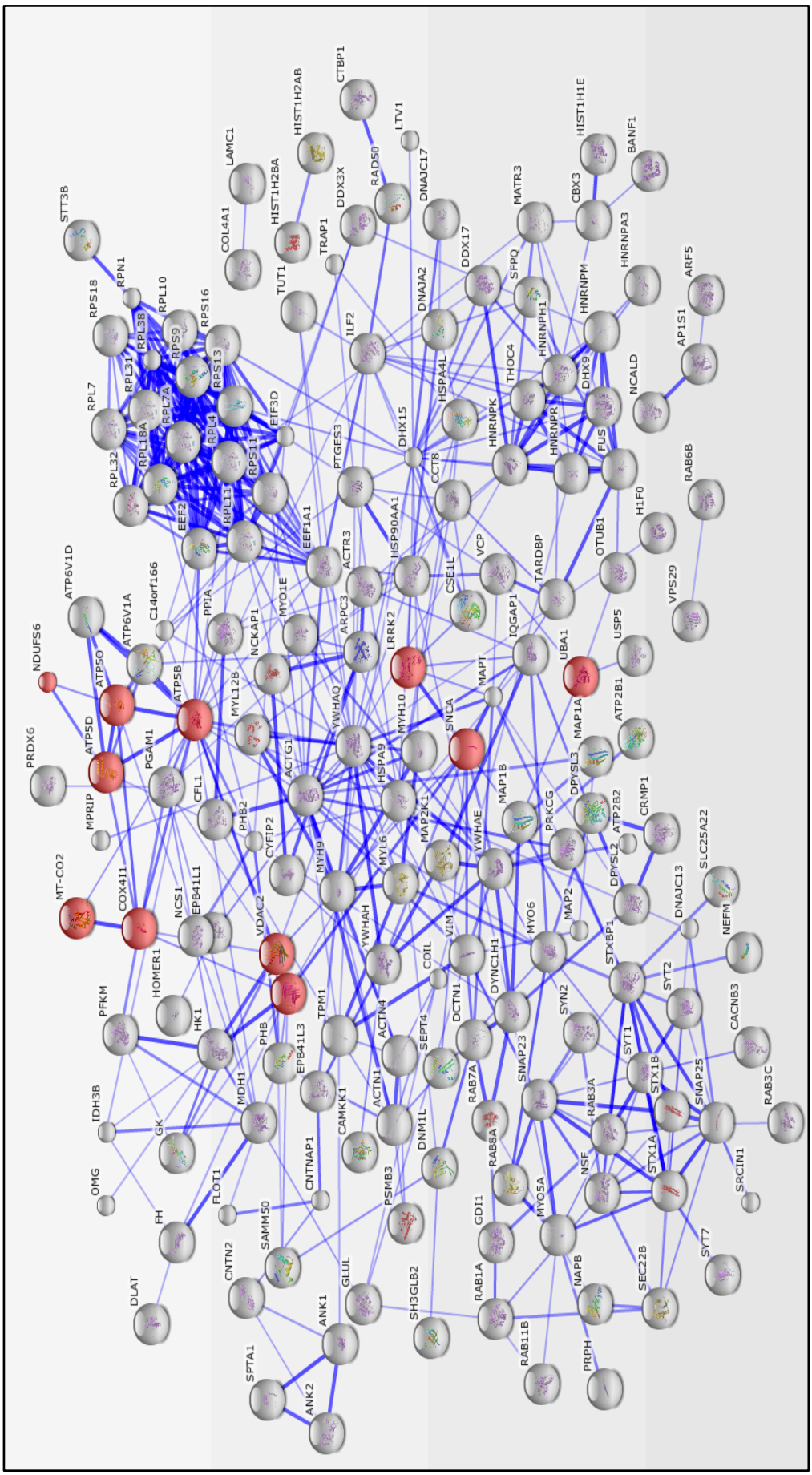

Figure 5.1. KEGG - Parkinson's Disease (PD) pathway. The KEGG pathways were analyzed within a selected list of LRRK2 interactors using STRING software. Highlighted in red and the PD-related genes, within the LRRK2 network, one of the most representative KEGG pathways.

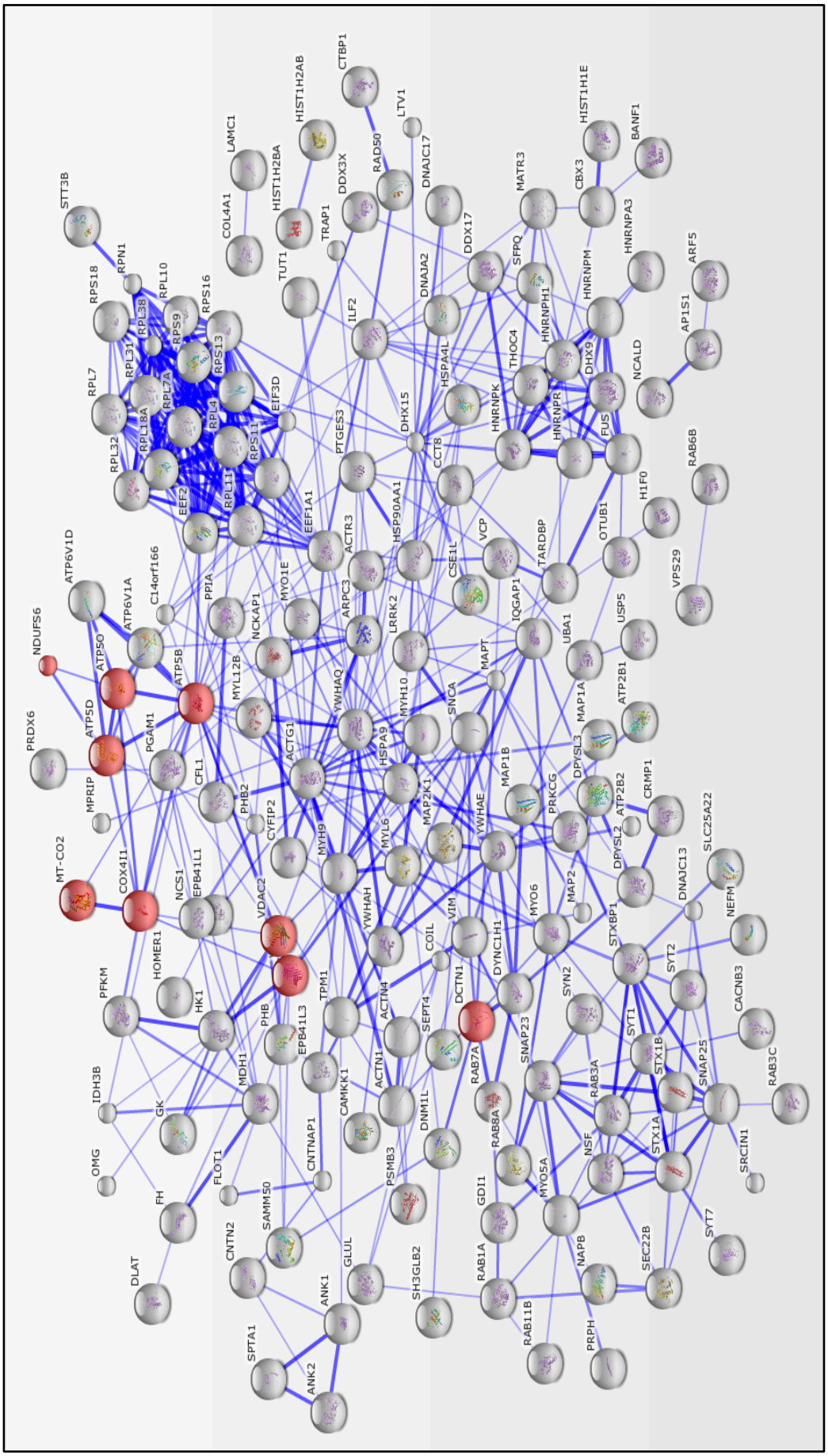

Figure 5.2. KEGG Huntington's disease (HD) pathway. The KEGG pathways were analyzed within a selected list of LRRK2 interactors using STRING software. Highlighted in red and the HD-related genes, within the LRRK2 network, one of the most representative KEGG pathways.

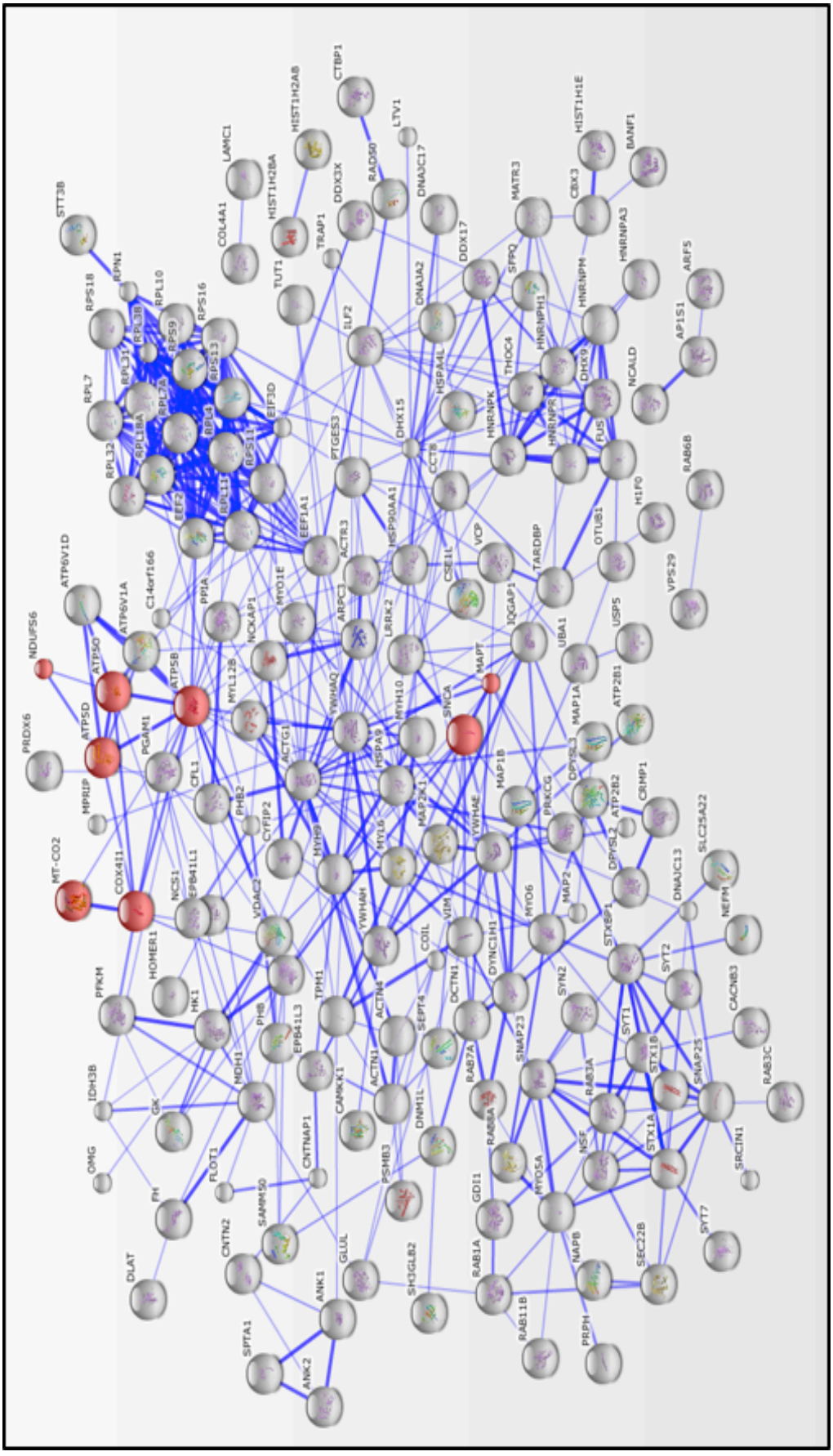

Figure 5.3. KEGG Alzheimer's disease (AD) pathway. The KEGG pathways were analyzed within a selected list of LRRK2 interactors using STRING software. Highlighted in red and the AD-related genes, within the LRRK2 network, one of the most representative KEGG pathways.

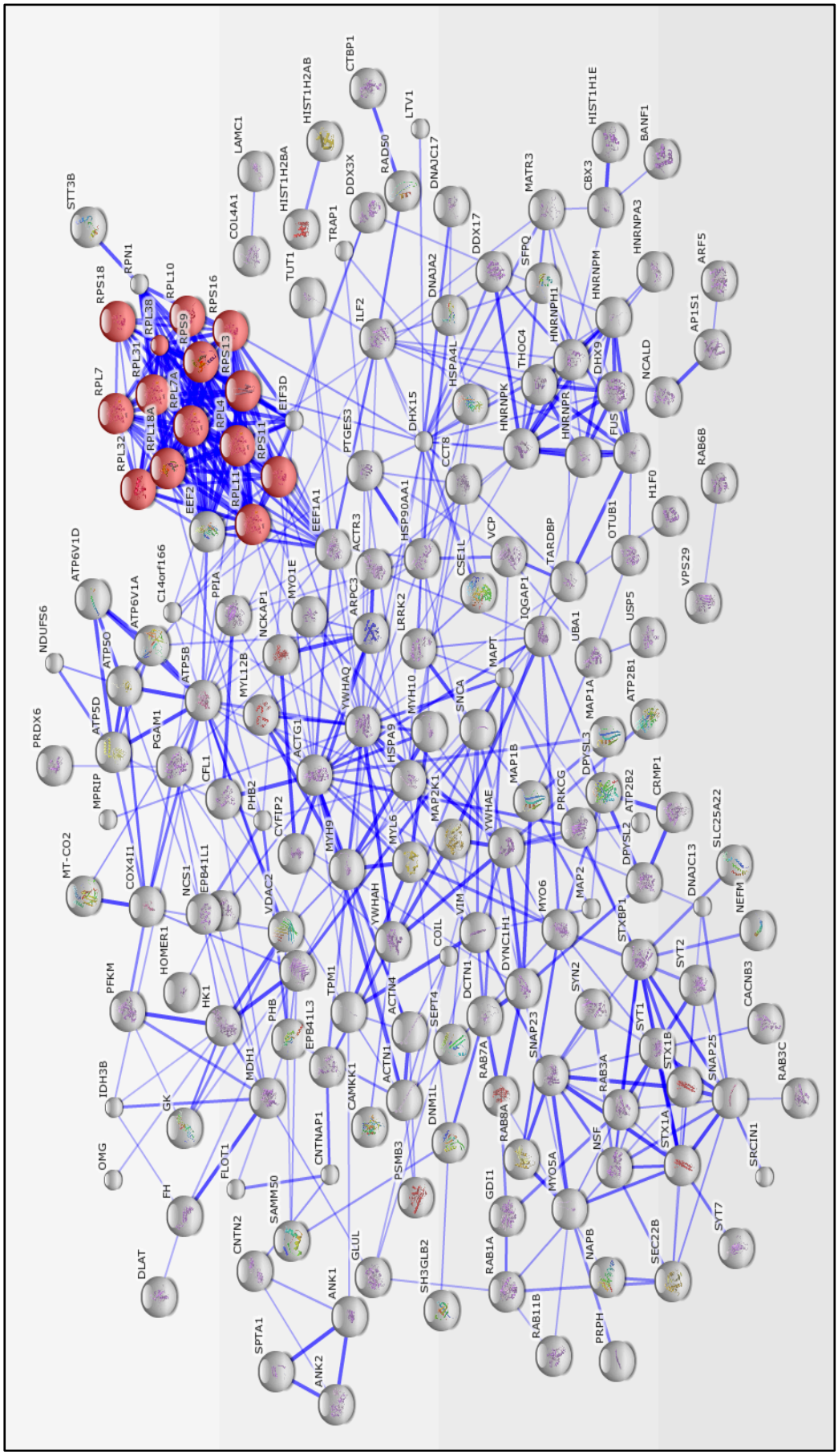

Figure 5.4. KEGG Ribosome pathway. The KEGG pathways were analyzed within a selected list of LRRK2 interactors using STRING software. Highlighted in red and the ribosome-related genes, within the LRRK2 network, one of the most representative KEGG pathways.

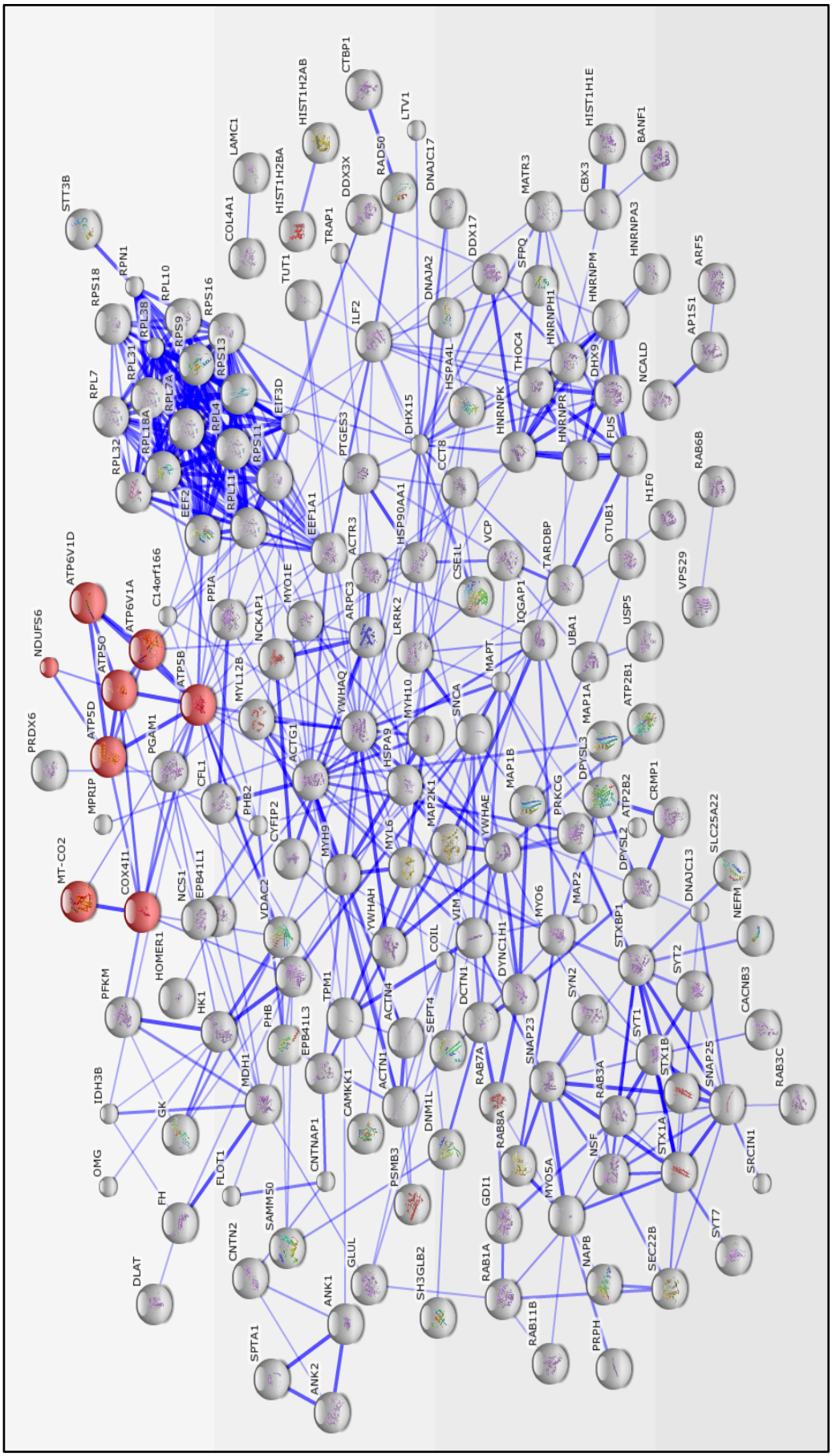

Figure 5.5. KEGG- Oxidative phosphorylation pathway. The KEGG pathways were analyzed within a selected list of LRRK2 interactors using STRING software. Highlighted in red and the oxidative phosphorylation-related genes, within the LRRK2 network, one of the most representative KEGG pathways.