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Exploring phenotypes of LRRK2 phosphorylation using a phosphosite mutation approach

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Il ne faut pas attendre d'être parfait pour commencer quelque chose de bien

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Publications and conference papers

Publications as first author:

LRRK2 Phosphorylation, More Than an Epiphenomenon. **Marchand A**, Drouyer M, Sarchione A, Chartier-Harlin M-C, Taymans J-M. Front Neurosci. 2020; 14 (June):1-21. doi:10.3389/fnins.2020.00527.

A phosphosite mutant approach on LRRK2 links phosphorylation and dephosphorylation to protective and deleterious markers, respectively. Marchand A, Sarchione A, Drouyer M, Emanuele M, Galli T, Athanasopoulos P, Kortholt A, Ho F, Greggio F, Nichols J, Chartier-Harlin M-C, Taymans J-M. Cells. 2021 ; In submission.

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Abstract

Parkinson's disease (PD) is the most common neurodegenerative motor disease. Mutations in the Leucine Rich Repeat Kinase 2 (*LRRK2*) gene are linked to autosomal dominant parkinsonism, and genomic variation at the *LRRK2* locus is associated with increased risk for sporadic PD. LRRK2 is a multi-phosphorylated protein and reduced phosphorylation is reported in PD patient brains as well as for some disease mutant forms of LRRK2. Dephosphorylation leads to alterations in LRRK2 interactions and subcellular localization. PD is characterized by impaired intracellular trafficking. However, the link between LRRK2 phosphorylation and membrane trafficking is not fully understood.

The idea behind this project is to understand the consequences of phosphorylation or dephosphorylation of LRRK2 on its cellular functions.

To this purpose, we generated LRRK2 phosphorylation site mutants and studied how these impacted LRRK2 catalytic activity, neurite growth, localization, protein binding and lysosomal physiology in cell models.

We show that phosphorylation of RAB8a and RAB10 substrates are reduced with phosphomimicking forms of LRRK2, while RAB29 induced activation of LRRK2 kinase activity is enhanced for phosphodead forms of LRRK2 (LRRK2 S860/910/935/955/973/976A). Considering the hypotheses that PD pathology is associated to increased LRRK2 kinase activity, our results suggest that for its heterologous phosphorylation sites, LRRK2 phosphorylation correlates to protective phenotypes and LRRK2 dephosphorylation correlates to deleterious phenotypes.

Abstract

La maladie de Parkinson (MP) est la maladie motrice neurodégénérative la plus fréquente. Une des causes de la MP est la présence de mutations pathogènes ou de polymorphismes associés à un risque accru de MP au sein du locus du gène *LRRK2* (Leucine Rich Repeat Kinase 2). Sa protéine LRRK2 est multiphosphorylée. Or, des défauts de sa phosphorylation sont observées dans le cerveau de tels patients. Cette déphosphorylation de LRRK2 à certains sites entraîne des modifications de ses interactions et de sa localisation cellulaire. Sachant qu'une altération du trafic intracellulaire est observée dans la MP, il mieux comprendre le lien entre la phosphorylation de LRRK2 et le trafic membranaire est nécessaire.

Cette étude a pour objectif de comprendre les conséquences de la phosphorylation ou de la déphosphorylation de LRRK2 sur ses fonctions cellulaires.

Pour cela nous avons généré des mutants des sites de phosphorylation de LRRK2 et étudié l'impact de ceux-ci sur l'activité catalytique de LRRK2, la croissance des neurites, la localisation, l'interaction protéine-protéine et la physiologie lysosomale dans des modèles cellulaires.

Nous montrons que la phosphorylation des substrats RAB8a et RAB10 est réduite avec les formes phosphomimétiques de LRRK2 (S860/910/935/955/973/976D), tandis que l'activation de l'activité kinase de LRRK2 induite par RAB29 est renforcée pour les formes déphosphomimétiques de LRRK2 (LRRK2 S860/910/935/955/973/976A). Compte tenu des hypothèses selon lesquelles la pathologie de la MP est associée à une activité kinase accrue de LRRK2, nos résultats suggèrent que pour ses sites de phosphorylation hétérologues, la phosphorylation de LRRK2 est corrélée à des phénotypes protecteurs et la déphosphorylation de LRRK2 à des phénotypes délétères.

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List of abbreviations

A2AR	Adenosine receptor 2A
AD	Alzheimer Disease
ALP	Autophagy-Lysosomal Pathway
AR	Autosomal Recessive
ATP	Adenosine Triphosphate
BBB	Blood Brain Barrier
BG	Basal Ganglia
CalA	Calyculin A
CBGTC	Cortico-Basal Ganglia-Thalamo-Cortical
CK1α	Casein Kinase 1-alpha
CMA	Chaperone-Mediated Autophagy
CNS	Central Nervous System
DBS	Deep Brain Stimulation
ENS	Enteric Nervous System
EV	Extracellular Vesicle
GBA	Glucocerebrosidase
GDNF	Glial-Derived Neurotrophic Factor
GPe	Globus Pallidus externa
GPi	Globus Pallidus interna
GWAS	Genome Wide Association Study
hiPSC	Human Induced Pluripotent Stem Cell
IL	Interleukin
LB	Lewy Bodies
LRS	Leucyl-tRNA Synthetase 1

MSNs	Medium Spiny Neurons
MT	Microtubule
MVB	Multi Vesicular Body
OMM	Outer-Mitochondrial Membrane
PD	Parkinson's Disease
PP1	Protein Phosphatase 1
SNAREs	Soluble N-ethylmaleimide-Sensitive factor Attachment protein Receptors
SNP	Single Nucleotide Polymorphism
SNpc	Substantia Nigra pars compacta
SNpr	Substantia Nigra pars reticulata
TFEB	Transcription Factor EB

Introduction

Part 1: Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disease across the world affecting 0.1 to 0.2% of people, with up to 1% of people affected over the age of 60 (Tysnes and Storstein, 2017). With the aging of the population and the lengthening of the life expectancy, the amount of people with PD will considerably increase from 2010 to 2050 and there will be close to 10 million people by 2030 (Dorsey et al., 2007; Bach et al., 2011).

1.1 Description of the pathology

The first descriptions of a state reminiscent of PD date back to 1000 BC and come from traditional Indian texts and ancient Chinese sources (Manyam, 1990). In 1817, Shaking Palsy was described by James Parkinson in *an essay on the shaking palsy* (Parkinson, 1817). James Parkinson described by visual inspection 6 patients with resting tremor, postural instability, paralysis and reduced muscle strength and an evolution of the disease over time The term PD was popularized by William Sanders and Jean-Martin Charcot who distinguished PD from multiple sclerosis and other tremor-like diseases. One century later, pathological structures, later named Lewy bodies (LB), were identified in post-mortem analysis of PD patient brains by Frederic Lewy (Lewy, 1912). Some years later, Constantin Tretiakoff identified a depigmentation of the *Substantia nigra* as another pathological hallmark of the disease (Tretiakoff, 1919).

1.2 Clinical features

The diagnosis of Parkinson's disease is most often established after the appearance of cardinal motor symptoms, a phase of the disease where up to 50% of dopaminergic cells in the nigro-striatal system are lost (Dauer and Przedborski, 2003). Symptoms include the progressive appearance of bradykinesia, associated with rigidity and resting tremor with characteristic parkinsonian asymmetry. Most patients also develop cognitive and behavioural dysfunctions in parallel with motor symptoms.

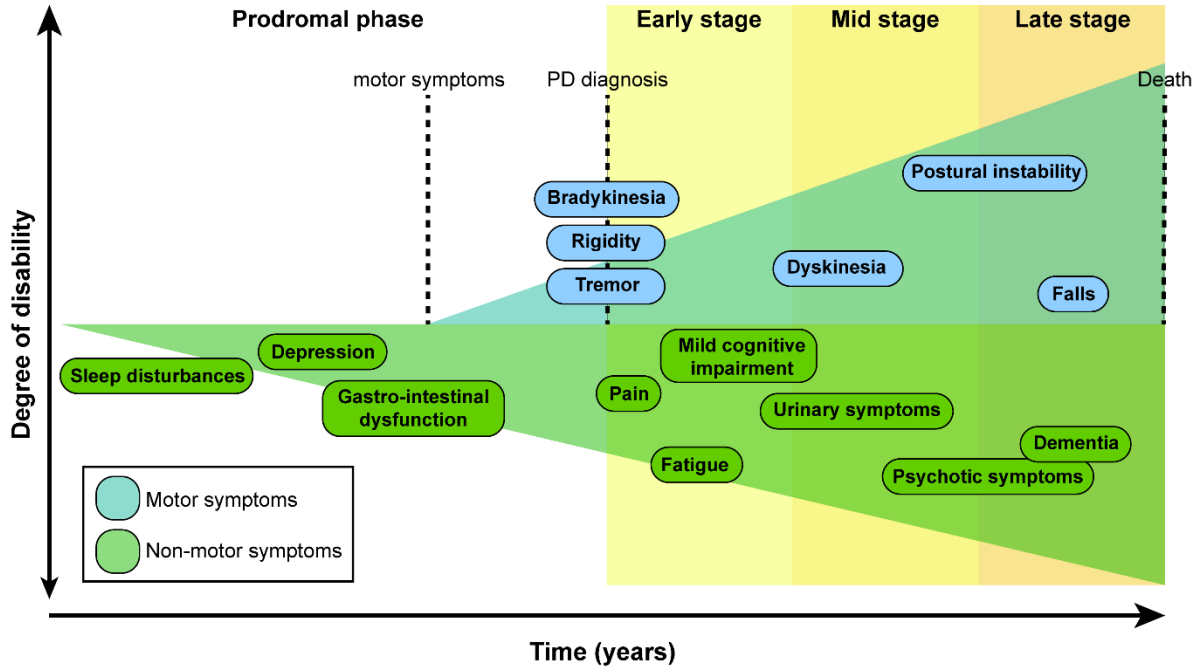


Figure 1: Evolution of PD symptoms. Non-motor symptoms that can appear before the diagnosis of PD which is usually pronounced with the appearance of motor dysfunctions. The non-motor symptoms will be more pronounced as the disease progress. The disability emerges from the combination and progression of non-motor and motor symptoms of PD. Adapted from (Poewe et al., 2017)

1.3 Symptoms

Resting tremor: It is the symptom of Parkinson's disease that is most directly associated with the pathology by the broad public. Interestingly, 20% of parkinsonian patients do not present tremors. The other 80% have limb tremor with a majority of resting pill-rolling tremor of the hand. The tremor oscillates at 4 to 6Hz at rest and disappears during initiation of movement and worsens with stress or intense concentration (Jankovic, 2008).

Bradykinesia: It is characterized by the decline of voluntary movement, and in some cases, leads to the suppression of movement (akinesia). It is identified by an inexpressive face, shuffle while walking, difficulty with repetitive tasks. It is almost always accompanied by rigidity.

Rigidity: It is a muscular hypertonia in which the muscle cannot stretch and relax normally, the patient also feels a weakness in the muscles. More than 90% of people with PD experience rigidity.

Postural instability: The postural instability appears in the early stage or later in the disease evolution. It is one of the most disabling symptoms, which can lead to falls (in 62% of patients) and serious injuries (Stolze et al., 2004).

Freezing: After several years from the appearance of PD, up to 60% of patients experience freezing of movements. Freezing varies from the inability to move for several seconds/minutes, to speak or the interruption of a movement.

Non-motor symptoms: For many years, PD was considered primarily as a motor disease. Today, important NMS are known to develop prior to motor dysfunctions (Figure 1). More than 50% of PD patients experience dysfunctions of their autonomic function daily. They are summarized in table 1.

Abnormalities of sensation
- Impairment of olfaction (90% of PD patients) (Doty et al., 1988)
- Visual acuity, contrast sensitivity, colour discrimination (Armstrong, 2011)
- Pain (76%) (Valkovic et al., 2015)
- Abnormal tactile pressure perception (Kesayan et al., 2015)
Cognitive changes
- Depression (17%) (Reijnders et al., 2008)
- Anxiety (67%) (Menza et al., 1993)
- **Apathy** (40%) (den Brok et al., 2015)
- Mild cognitive impairment (18%) (Nicoletti et al., 2019)
- Hallucinations
Autonomic dysfunction
- Orthostatic hypotension (60%) (Hellman et al., 2015)
- Gastrointestinal dysfonction (80-90%) (Fasano et al., 2015)
- Urinary symptoms (25-50%) (Winge, 2015)
- Sexual dysfunction (Bronner et al., 2014)
- Thermoregulation (Swinn et al., 2003)
Sleep disturbances review in (Schrempf et al., 2014)
- Insomnia (50%) (Lee et al., 2007)

- Excessive daytime sleepiness
- Restless legs syndrome (0-50%) (Krishnan et al., 2003)
- REM sleep behaviour disorder (30-50%) (Gagnon et al., 2002)

Other symptoms

- Excessive drooling (70%) (Barbe et al., 2017)
- Fatigue (50%) (Siciliano et al., 2018)
- Swallowing difficulties (41%) (Hartelius and Svensson, 1994)

Table 1: Non-motor symptoms of Parkinson's disease. The prevalence of symptoms for PD patient is indicated in brackets.

1.4 Anatomical pathology of Parkinson's disease

1.4.1 Organization of the basal ganglia

First mentioned by Claude Galien (129-201 AD) the basal ganglia (BG) were associated to the control of movement in the 19th century. Edouard Brissaud (1852-1909) was the first to associate the tremor in PD to the substantia nigra. The BG refers to a group of interconnected structures in the brain that control voluntary motor movement and some cognitive functions such as procedural learning, non-associative learning, behaviours and emotions. The BG can be divided into 4 structures:

- Striatum: It is the largest structure of the basal ganglia, composed of the caudate nucleus and putamen. It constitutes the principal entry of the basal ganglia and receives inputs from the cortex, substantia nigra and other brain regions. The striatum is mainly composed of GABAergic neurons called Medium Spiny Neurons (MSNs) and cholinergic interneurons.

- Pallidum: or Globus pallidus, it can be divided into two structures, globus pallidus externus (GPe) and globus pallidus internus (GPi). Mainly composed of GABAergic neurons, the GPe is the major output of the striatum and the GPi is the main output for direct and indirect pathways of the BG.

- Substantia nigra: It is composed of the Substantia Nigra Pars Compacta (SNpc) and Substantia Nigra Pars Reticulata (SNpr). They differ by the neuron composition and projection, the SNpc is composed of dopaminergic neurons projecting to the striatum. The SNpr is composed of GABAernic neurons that project to the thalamus.

- Subthalamic nucleus: the neurons of the subthalamic nucleus are glutamatergic neurons and target the GPe/GPi, SNpc/SNpr and the striatum.

These structures form a transmission loop that start in the cortex, are regulated in the basal ganglia and projected to the motor cortex. This control loop is called Cortico-basal ganglia-thalamo-cortical loop (CBGTC loop).

The cortex sends glutamatergic excitatory projections to the striatum. The neurons from the cortex are connected to the striatal GABAergic neurons (MSN). From the striatum, two pathways differ; the direct or excitatory pathway that promotes movement or the indirect pathways that inhibits movement.

When no voluntary movement is intended, the globus pallidus internus and the substantia nigra pars reticulate (GPi/SNpr) are activated, thereby inhibiting the thalamus resulting in the absence of movement. When a voluntary movement is initiated, the striatum is activated resulting in the inhibition of the GPi/SNpr, thereby disinhibiting the thalamus and allowing excitation of the motor cortex and the movement. The SNpc acts in this loop by fine tuning this circuit through its projections to the Striatum (Figure 2).

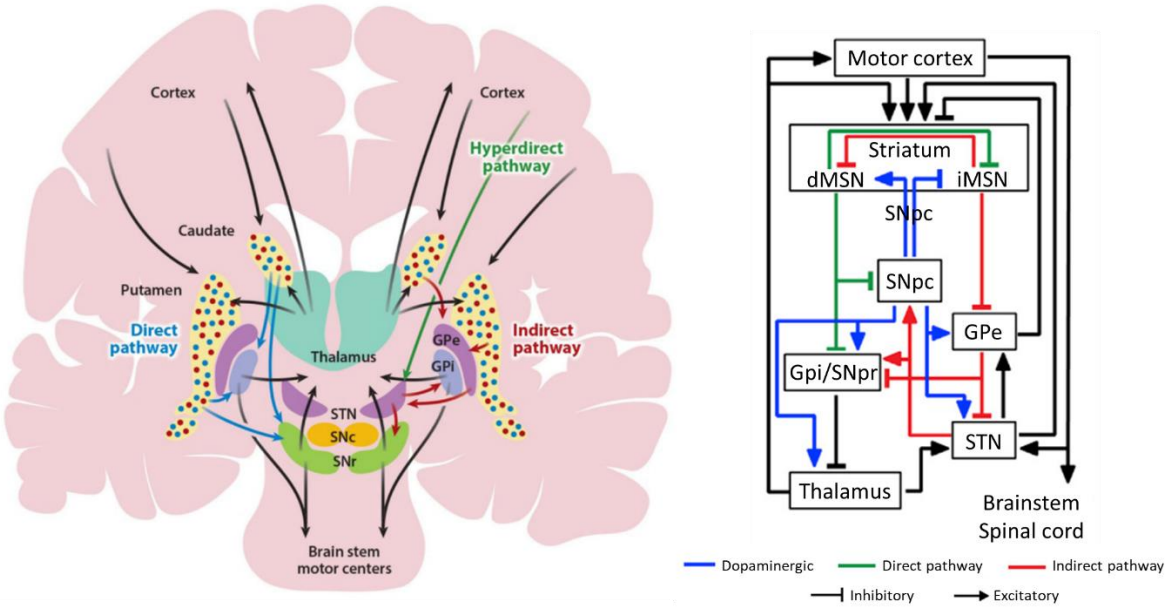


Figure 2: Basal ganglia structure and connectivity. Dopamine projections from the SNpc are represented in blue. Dopamine projections from the SNc activate the direct pathway (green) and inactivate the indirect pathway (Red). This results in higher inhibition of GPi and reduces its inhibition on the thalamus, promoting the movement. Adapted from (Nelson and Kreitzer, 2014; Mcgregor and Nelson, 2019).

1.4.2 Dopaminergic neuron loss

Since the first description of a depigmentation of the substantia nigra (SN) observed in post-mortem PD patient brains, comprehension of the mechanisms resulting in massive cell death of the dopaminergic neurons of the SN are a priority. When motor dysfunction appears in a patient, it has been estimated than more than 50% of the dopaminergic neurons are depleted. As these neurons are rich in the dark pigment neuromelanin, this explains the depigmentation of the SN (Figure 3, A) that can be observed in PD patient brains. In the surviving neurons, Dr. F.H Lewy, identified in 1912 inclusions called Lewy bodies. In the clinic it is possible to indirectly monitor the death of the dopaminergic neurons with imaging techniques such as DATscan (Cerebral Scintigraphy of striatal dopamine transporter) or [18F]-Dopa PET that measures the density of dopaminergic neurons in the brain (Figure 3, B, C).

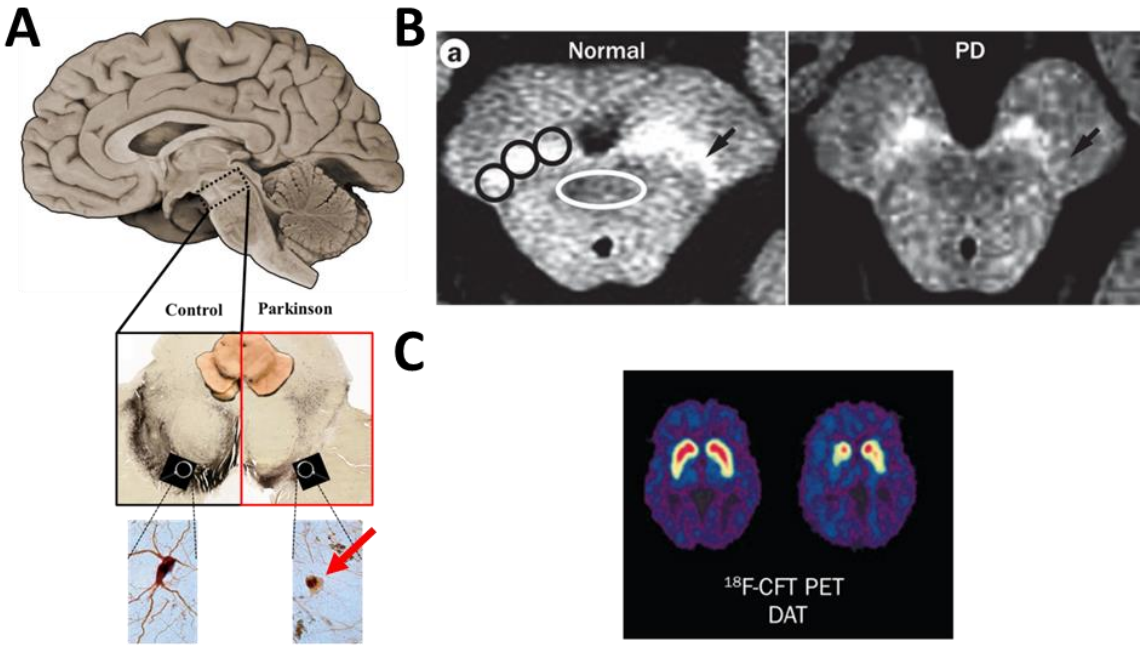


Figure 3: Evidence of PD in the human brain. (A) Brain section showing depigmentation of the substantia nigra and Lewy body inclusion from PD patient (Red arrow). (B) MRI imaging of SNpc showing reduced signal intensity. (C) DAT-scan showing reduced dopaminergic innervation in striata of a PD patient (right panel) compared to control (left panel). Adapted from (Politis, 2014).

The death of the dopaminergic neurons in the SN will reduce the fine control of the direct and indirect pathways. The inhibition induced in healthy conditions by the SNpc on the indirect pathways is weakened, resulting in a higher inhibition of the GPe, reduced inhibition of STN

and enhanced activation of the GPi/SNpr that further inhibits the thalamus and inhibits movement. In parallel, the direct pathway will no longer be activated by the SNpc lifting the inhibition of the GPi/SNpr, causing higher inhibition of the thalamus decreasing the voluntary movement (Figure 4, A, B).

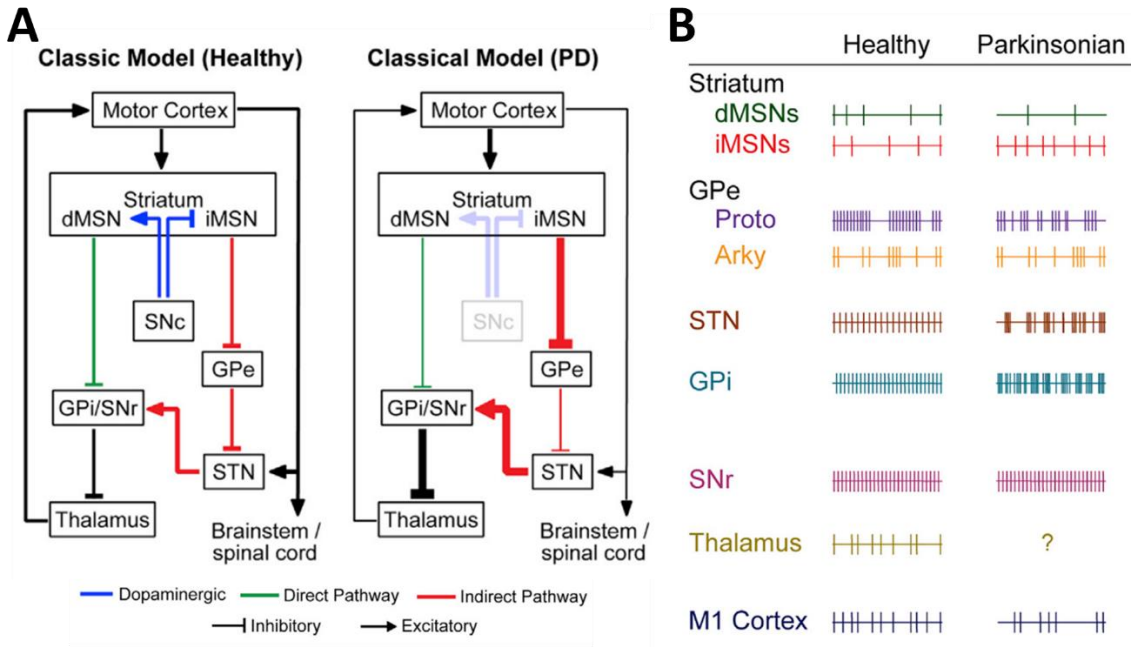


Figure 4: Neuronal circuitry causing motor dysfunctions in PD. (A) With disruption of dopaminergic neurons from the SNc, the direct pathway activity is reduced and indirect pathway is increased. The STN increases its stimulation of the GPi/SNr while inhibition from the dMSN is reduced, leading to higher inhibition of the thalamus causing inhibition of the movement. (B) Stimulation spikes differences between controls and PD patients in different structures of the basal ganglia. Adapted from (Mcgregor and Nelson, 2019).

1.4.3 Lewy bodies

In 1998, Spillantini, Goedert and their team were able to discover that alpha-synuclein was one of the major components of the LB in patients with alpha-synuclein mutation but also in sporadic patients without any alpha-synuclein mutations (Spillantini et al., 1997). Alpha-synuclein was around this period linked to hereditary PD with two missense mutations, A53T identified in an Italian family (Polymeropoulos et al., 1997) and A30P identified in a German family (Krüger et al., 1998). LBs are not specific to PD patients, it is possible to find LBs in different pathologies, such as Lewy Body Dementia, Pick's disease and others. The exact role of the LBs are still unknown; it also has been suggested that LBs could be a marker and not the cause of neurodegeneration (Corti and Brice, 2003). Recently, analysis of LBs from PD

patient brains show accumulation of organelles with lipid membranes, ubiquitin and many other components (Shahmoradian et al., 2019). They are the histopathological markers of PD (Forno, 1996).

1.4.4 Pathology outside the substantia nigra

In PD, accumulation of Lewy bodies extends beyond the CNS, affecting the enteric nervous system (ENS) (Wakabayashi et al., 1989; Braak et al., 2006). The ENS is often considered as the second brain. It contains up to 600 million neurons (200 times less than a human brain) (Furness, 2012), with similar neurotransmitters. Half of the dopamine in the body is produced by the ENS (Eisenhofer et al., 1997). The control of gastro-intestinal mobility and secretion is controlled by parasympathetic and sympathetic stimulations (Cersosimo and Benarroch, 2008). The parasympathetic stimulation is mainly ensured by the vagus nerve. Interestingly, Lewy pathology was also detected in the dorsal nucleus of the vagus nerve (Benarroch et al., 2006; Braak et al., 2006). Braak observed LB in the dorsal nucleus of the vagus nerve in all patients and he postulated that this structure was at the crossroads of the pathological process (Figure 5, A-C). An additional hypothesis suggests that PD disease starts in the gut due to disruption of intestinal permeability that allows microbial products to increase inflammation and alpha-synuclein accumulation in enteric neurons (Braak et al., 2003; Shen et al., 2021) (Figure 5, C).

Braak suggested that the distribution of alpha-synuclein inclusions can spread according to a determined anatomical path that can be divided in 6 steps depending on the progress of the pathology (Braak et al., 2003).

The process would start within the olfactory bulb and the ENS with the appearance of Lewy neurites and alpha-synuclein aggregates. It is considered to be the pre-symptomatic phase (Stage 1-2). From the brainstem, alpha-synuclein inclusions would be present in the substantia nigra and the hypothalamus and LB lesion start in the SNpc, starting to affect motor function with the appearance of motor dysfunctions (Stage 3-4). In the stages 5-6 alpha-synuclein inclusions are present in the total neocortex and cell death occurs in the SNpc (Figure 5, B) (Halliday et al., 2011).

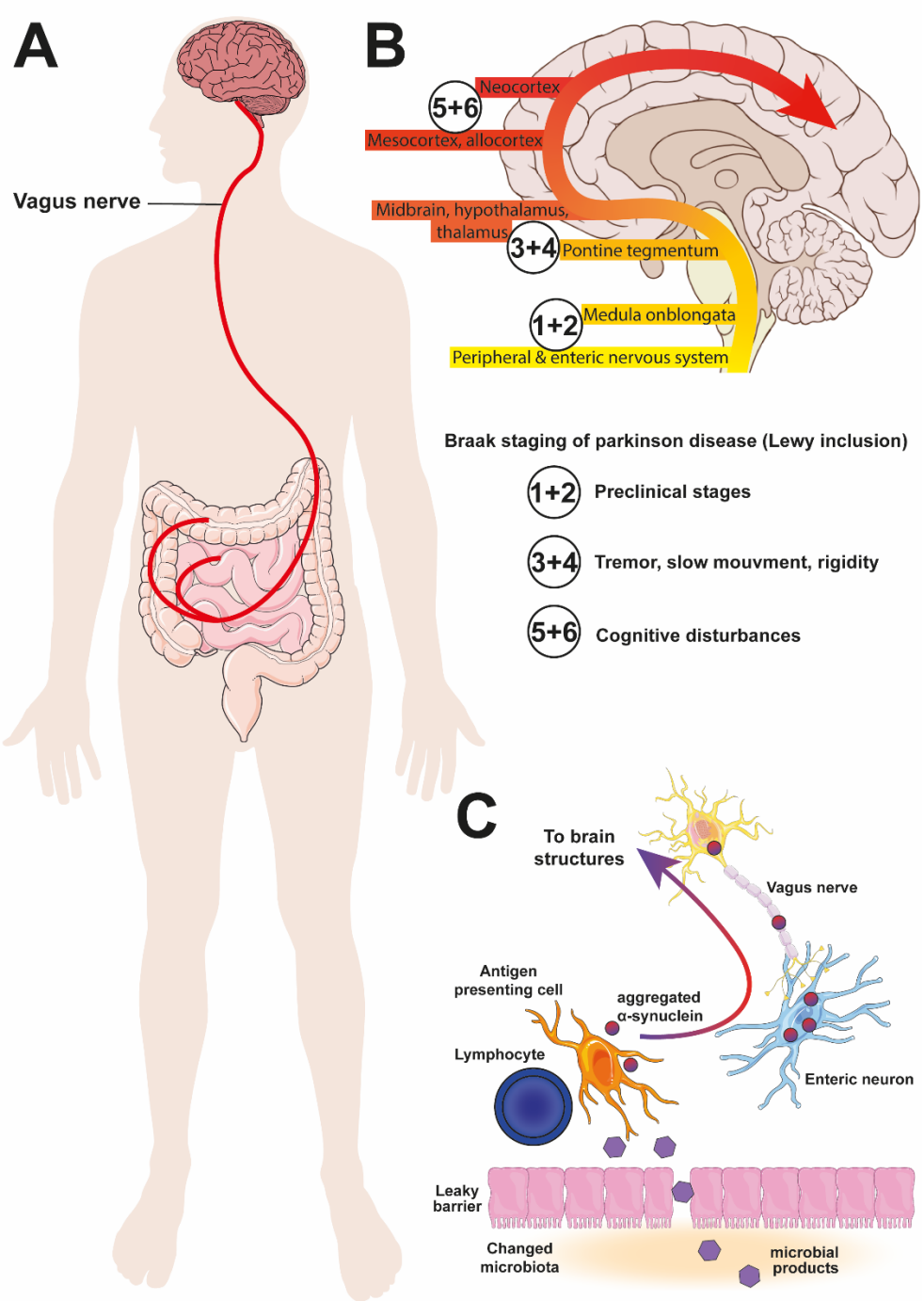


Figure 5: Representation of the PD Braak staging system. (A) Vagus nerve or pneumogastric nerve is in charge of parasympathetic stimulation controlling the digestive tract. (B) Disease progression has been observed in the brain with the progression of Lewy body pathology through the brain and can be divided in 6 stages of progression. (C) Lewy body pathology is hypothesized to start in the gut after increased alpha-synuclein aggregation resulting from disrupted function of intestinal barrier.

Not only the neurons are affected in PD. Supporting cells such as glial cells are important components of the brain, crucial for the regulation of inflammation and for the health of neurons, and are also affected (see next section).

1.4.5 Alterations in glial cells

Glial cells are non-neuronal cells present in the brain, spinal cords and the peripheral nervous system. They are associated to the neurons and represent 50% of total cells in the brain. There are 3 main types of glial cells in the brain that can all regulates cell homeostasis regulate the adverse event that happen in PD.

- **Oligodendrocytes**: Oligodendrocytes represent 5 to 8% of total cells in the adult Central Nervous System (CNS) (Levine et al., 2001). They generate myelin around axons, accelerating the electric transmission of signals. The oligodendrocytes support the integrity of axons covered by myelin by releasing glial trophic support and extracellular vesicles (EVs) (Nave, 2010; Frühbeis et al., 2020). Demyelination can lead to axonal disruption, death of oligodendrocytes and can also induce neuronal death.

- **Microglia:** Microglia are the brain macrophages and form the principal immune defence in the brain. They also contribute to the brain development by regulating the elimination of synapses. The phenomenon, called synapse pruning is controlled by environmental factors, mainly by learning in childhood (Paolicelli et al., 2011). The outgrowth of microglial processes isolates injured cells and prevent the expansion of the lesion in the brain. It helps to maintain brain homeostasis in the microenvironment around the injury (Hines et al., 2009). After injury, microglia produce an inflammatory response to prevent infections. Microglia release pro-inflammatory cytokines (TNFα, IL-6, IL-1β, IL-12, IL-23, etc). Some of the released cytokines have multiple functions: regulating neurite outgrowth (Parish et al., 1986), metabolism (Gavillet et al., 2008), ion channel activity (Viviani and Boraso, 2011). High expression levels or mutant forms of alpha-synuclein can induce activation of microgial cells (Lee et al., 2010b). iPSC-derived macrophages from PD patients with A53T mutations or alpha-synuclein triplication reduced phagocytosis capability, supporting the hypothesis of defective microglial function in PD patients (Haenseler et al., 2017).

- **Astrocytes:** Astrocytes represent the majority of the glial cells. Several roles have been attributed to astrocytes. They regulate the homeostasis of the brain microenvironment by controlling the extracellular levels of glutamate and GABA. They also regulate the brain water homeostasis though their aquaporins. Neurons depend on astrocytes for oxygen/nutrient supply. Astrocytes control the development and the survival of dopaminergic neurons by secretion of Glial-Derived Neurotrophic Factor (GDNF) (Lin et al., 1993). They also contribute to the permeability of the Blood Brain Barrier (BBB) that protects the brain from pathogenic agents, toxins, hormones. Blood vessels with oligodendrocytes, microglia and astrocytes form the neurovascular units. The end feet of the basal process completely surround the blood vessel capillaries to allow high exchanges thought the gap-junctions. In neurodegenerative diseases, such as PD or AD, the neurovascular unit is disrupted (Gray and Woulfe, 2015). The permeability of the BBB is decreased, reducing the filtering of harmful substances to the brain (Kaneko et al., 2020).

With age, many aspects of the glial homeostasis are altered (Streit, 2006). Age is one of the most important factors for PD. Many PD related genes are known to affect the functions of glial cells as presented above. Neuroprotection given by the glial cells are slowly decreased with the progress of the pathology. Development of neuroprotective compounds for the glial cells could help to prevent or slow the disease progression.

1.5 Treatments for PD

At present, PD still has no cure. The treatments currently available help to improve the patient's quality of life, with most of the treatments aimed at countering the loss of dopamine in the striatum (Figure 6).

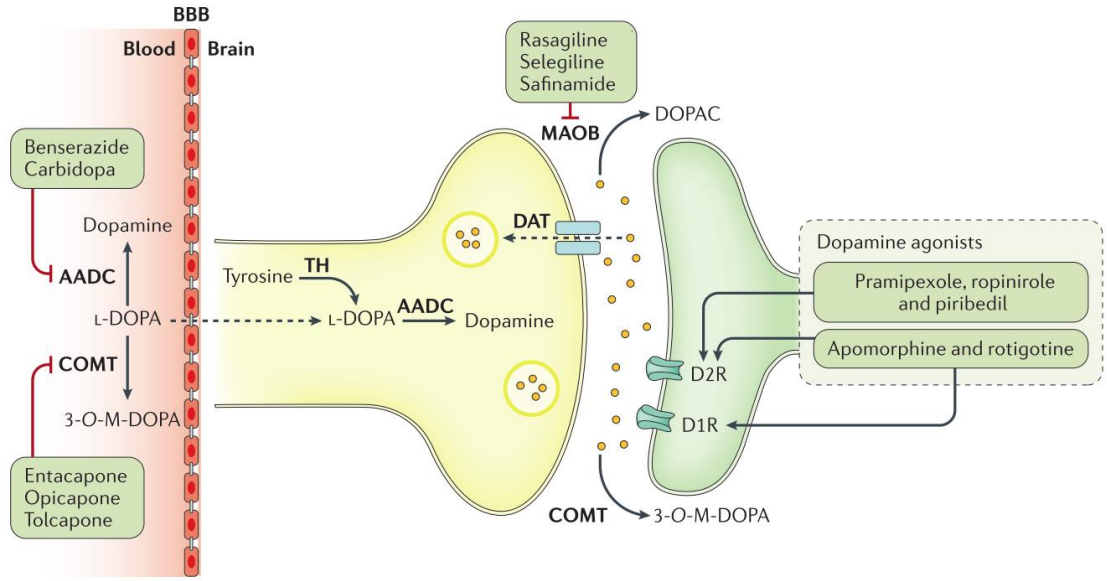


Figure 6: Drugs used in clinical practice to treat PD symptoms. Enhancing dopamine at the synapse can be achieved with catechol-O- methyltransferase (COMT) inhibitors that will reduce catecholamine degradation or Monoamine oxidase type B (MAOB) inhibitors that reduce monoamine oxidation. Treatment with L-DOPA leads to its transformation into dopamine by amino acid decarboxylase (AADC). When L-DOPA is combined with non-brain permeable AADC inhibitors to promote L-DOPA conversion in the CNS, this results in higher brain dopamine levels. From (Poewe et al., 2017).

1.5.1 L-DOPA

Levodopa was first synthetized in 1911 by Polish biochemist Casimir Funk. 50 years later, two neurologists performed a clinical trial of this compound on 20 patients presenting a parkinsonian syndrome. They were able to detect an impressive improvement of all motor symptoms (Birkmayer and Hornykiewicz, 1962). If the bradykinesia, and rigidity responds to L-DOPA, the efficacy concerning the tremor is weaker. Concerning the non-motor symptoms, the response is poor or absent. L-DOPA side effects vary, from dyskinesia (30-50% of patients) (Schrag and Quinn, 2000), to diarrhea and constipation. The first year of treatment with L-DOPA is a real relief for PD patients, usually the response to treatment is good to excellent without motor fluctuations. This period is referred as the honeymoon. With disease progression, L-DOPA response will be less and less steady with appearance of symptoms that cannot be reversed with L-DOPA treatment such as the non-motor symptoms (Poewe et al., 2017).

1.5.2 Other treatments

After the discovery of L-DOPA treatment in 1961, other compounds or surgery has been developed to increase the quality of life of PD patients (Figure 7).

Dopamine agonism

Dopamine agonists offer an alternative treatment to L-DOPA with a higher half-life that allows a longer distribution in the organism. They are less well tolerated but they delay the appearance of dyskinesias.

Non-dopaminergic treatment

- Anticholinergic drugs can be used for the treatment of tremor. In older patient, this treatment is avoided due to the side effects such as hallucination, confusions and dementia (Ffytche and Aarsland, 2017).

- Antiglutamatergic drugs are used to treat dyskinesia induced by L-DOPA treatment. It increases the release of dopamine and blocks its reabsorption. By themselves, antiglutamatergic drugs do not display significant efficacy in the treatment of PD (Crosby et al., 2003).

- Adenosine receptor antagonists are believed to be a new potential treatment for PD. In the basal ganglia, neurons express a huge proportion of A2A receptors outside of the nerve terminals. Studies in animals show an improvement of motor functions but in humans the effects on PD motor dysfunctions was less promising (Hickey and Stacy, 2012). This treatment is still in development and effects on neuroprotection and non-motor symptoms still need to be investigated.

- Surgical treatment Deep Brain Stimulation is used to re-establish an equilibrium in the basal ganglia thalamocortical circuit by placing an active electrode in the globus pallidus or subthalamic nucleus to inhibit their activity. The electrodes are connected to a stimulating impulse generator battery located in the abdomen. The surgery was approved by the US Food and Drug Administration in 2002 for the treatment of PD

tremor and advanced PD (Dostrovsky and Lozano, 2002). The surgery implicates risks, such as infection, stroke, bleeding or seizures. It is considered to be the best therapeutic advancement since the levodopa. This treatment shows a remarkable improvement in the quality of life of PD patient.

- Neuronal grafting is currently an emerging method in development. This could be used to replace neuronal loss in PD patients. It uses stem cells as a source of dopaminergic progenitor cells. Several trials are ongoing (Stoker and Barker, 2020).

- Neuroprotective compounds are in development. Currently, no drug is known to stop disease progression, however some compounds are believed to be able to slow the disease progression such as immunotherapies (to decrease the amount of alpha-synuclein aggregations in cells) (Zella et al., 2019), iron chelation (to act on ferroptosis, an iron dependent cell death) (Ward et al., 2021), LRRK2 kinase inhibition and more (Salamon et al., 2020).

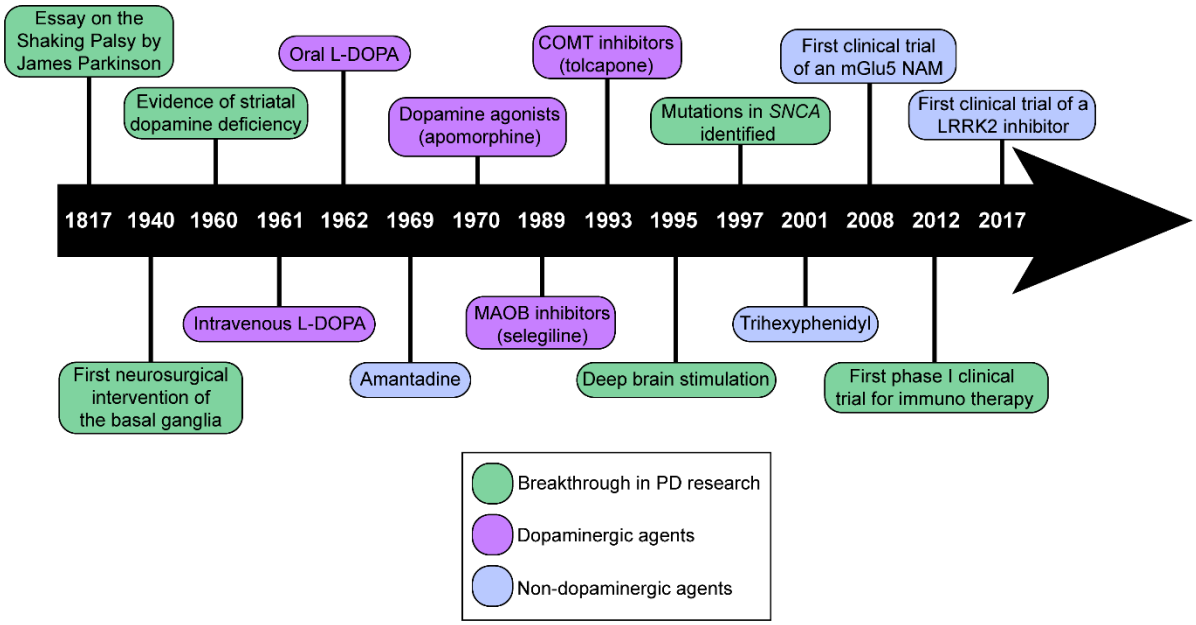


Figure 7: milestone of PD treatment. Since 19th century, diverse advances have been achieved in the research field of PD therapies. Development of compounds, neurosurgery and innovative treatment has greatly improved the quality of life of PD patients. L-DOPA, COMT (catechol-O-methyltransferase), MAOB (monoamine oxidase type B), mGlu (metabotropic glutamate receptor), NAM (negative allosteric modulator). Adapted from (Charvin et al., 2018).

1.6 Cause of pathology

The exact aetiology of PD is poorly known, although the cause of the pathology is multifactorial (Figure 8). Except few direct links between toxic molecule such as MPTP, PD etiology is always an association of different parameters; environmental factors (pollution, habits, etc) and genetic factors summarized in Table 2.

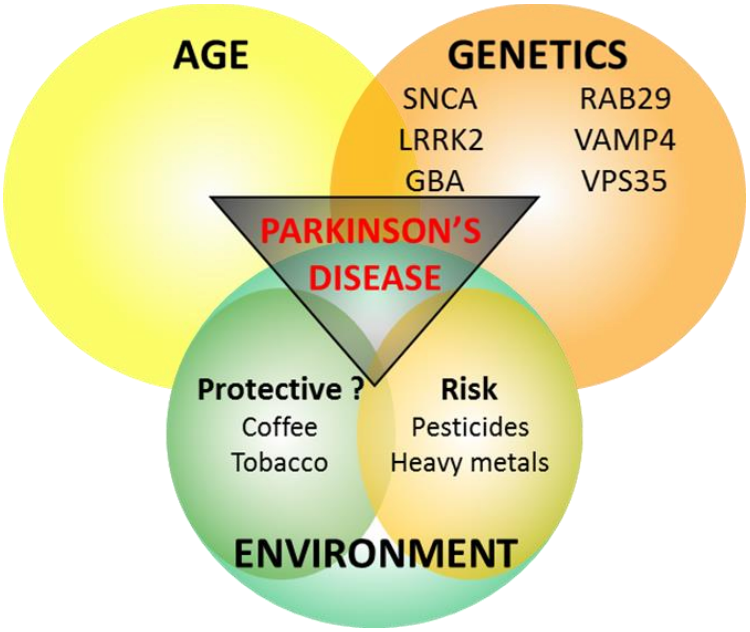


Figure 8: Component of PD factors. PD pathogenesis results from interactions between ages of patients, environmental factors (Protectives and risks factors) and genetics components. Examples of environmental factors are indicated as protective or risk factors for PD. *Loci* implicated in PD are indicated as genetics component.

1.6.1 Genetics

Before 1997, PD was not commonly considered as a non-genetic disease, heritability was ambiguous due to the lack of conclusive results in studies on twins (Tanner et al., 1999). In the last quarter century, genetic studies of disease transmission in families have identified mutations in the coding sequence or other changes such as gene multiplications of many genes as genetic causes of parkinsonism. Still, 10% of PD patient report the presence of PD in their family (Thomas et al., 2007). Many genes are identified as genetically linked for PD (Table 2).

Gene	Clinical phenotypes
Autosomal dominant inheritance	
SNCA	Missense mutations are rare cause of autosomal dominant PD. Duplication or triplication of this gene causes early-onset PD with prominent dementia, mostly for triplication.
LRRK2	Classic PD phenotype. Variations in LRRK2 include risk-conferring variants and disease-causing mutations
VPS35	Classic PD phenotype
EIF4G1	Mild to classic PD
Autosomal recessive inheritance	
Parkin	Often presents with lower limb dystonia
PINK1	Psychiatric features are common
DJ-1	Early-onset PD
DNAJC6	Onset of parkinsonism between the third and fifth decades of life
ATP13A2	Early-onset parkinsonism with a complex phenotype (for example, dystonia, supranuclear gaze palsy, pyramidal signs and cognitive dysfunction); also known as Kufor–Rakeb syndrome
PLA2G6	PLAN (or NBIA2) is characterized by a complex clinical phenotype, which does not include parkinsonism in the majority of cases
FBXO7	Early-onset parkinsonism with pyramidal signs and a variable complex phenotype (for example, supranuclear gaze palsy, early postural instability, chorea and dystonia) Juvenile-onset
DNAJC6	Juvenile-onset parkinsonism that is occasionally associated with mental retardation and seizures
SYNJ1	Patients may have seizures, cognitive decline, abnormal eye movements and dystonia
VPS13C	Young-adult-onset parkinsonism associated with progressive cognitive impairment that leads to dementia and dysautonomia

Table 2: Classification of principal hereditary parkinsonism. PD genes are associated to their clinical phenotypes. Adapted from Poewe 2017.

Thus, some genes have been reported to have very high susceptibility, such as triplication of alpha-synuclein which possesses full penetrance. Most PD patients are sporadic cases (90%), presumably caused by interactions of environmental and genetic factors with age. With the development of Genome Wide Association Studies (GWAS), identification of common genetic variations (Single Nucleotide Polymorphisms) led to the identification of 90 risk loci that taken together account for 16–36% of the heritable risk of PD (Nalls et al., 2019). The identification of genetic determinants in PD is a great help that allows the identification of molecular pathways that can be implicated in the pathophysiology of PD. Follow up analyses are fundamental to associate loci to a biological function. Research on biological functions of genetic determinants were able to uncover that *SNCA*, *RAB29*, *MAPT*, *BST1*, *GAK*, *LRRK2* and *HLA-DRB5* and others are implicated in cellular pathways such as vesicular trafficking, cytoskeleton and synaptic functions (Escott-Price et al., 2015; Nalls et al., 2019).

1.6.2 Non-genetic factors

The total contribution of the environment in the pathogenesis of PD is not fully understood and difficult to quantify. Correlation studies are often used to identify relationships between environmental factors such as exposure to a compound such as pesticides or pollutant and the development of PD. Some identified mechanisms mediated by environmental risk factors are regulation of the inflammation or oxidative stress in cells or direct action on dopamine pathways. Factors that protect against PD have also been identified such as specific diets or lifestyle, with mechanisms involving action on oxidative stress.

Risks factors

Neurotoxins: Some synthetic molecules can cause PD-like phenotypes. One such compound is MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), accidentally discovered after the production and injection of a synthetic opioid intended as a recreational drug. It specifically destroys neurons in the substantia nigra and causes permanent PD symptoms (Langston et al., 1984; Nonnekes et al., 2018).

Pesticides: Several studies pinpoint the link between PD and exposure to pesticides such as rotenone or paraquat (Tanner et al., 2011).

Methamphetamine: Methamphetamine binds to dopamine transporter resulting in a higher extracellular dopamine. It has been shown to damage dopaminergic neurons in animal models and a retrospective study in human demonstrate a 3 fold increased risk of PD in methamphetamine user (Curtin et al., 2015).

Cancer: A 44% increased risk to develop PD has been discovered in patients that had developed a melanoma (Olsen et al., 2006). Interestingly, an inverse correlation between smoker-related cancer and PD was found (Bajaj et al., 2010).

Traumatic brain injury: It results in a disruption of the blood brain barrier and accumulation of alpha-synuclein in the brain (Delic et al., 2020).

Positive risk factors

Caffein: In several studies, regular coffee consumption were associated with a lower PD risk (Hu et al., 2007; Hong et al., 2020). The lowering of the risks due to coffee is robust and seems to be linked with sex hormones, the effect is higher in men compared to women (Palacios et al., 2012). The effect is also greater in women not under postmenopausal hormones compared to women taking hormones (Ascherio et al., 2003).

Tobacco: In a prospective study, a lowering by 70% of risk to develop PD was found (Morozova et al., 2008; Li et al., 2015). In addition to these studies, results from an American cohort identify 2 polymorphisms implicated in interaction between genes and tobacco in PD risk. The SNPs are located in two genes, *RXRA* and *SLC17A6*. Interestingly, RXRA encodes the retinoic acid transport (Implicated in dopamine system) and SLC17A6 the vesicular glutamate transporter (Lee et al., 2018).

Urate: Uric acid (Urate) elevation in serum is linked to protection against dopaminergic neuron death (Guerreiro et al., 2009). Urate is known to be a strong antioxidant, so a higher concentration of urate could help preventing the increased oxidative stress in cells of PD patients. Meta-analysis suggest a decreased risk to develop PD when having high urate plasma concentration (Weisskopf et al., 2007).

Physical activity: Physical exercise is often associated with increased quality of life and lifespan. In this way, a 12.6 years follow-up of 43.000 volunteers indicates that a medium level of physical exercise lowers PD risk (Yang et al., 2015). Also, physical exercise is known to affect positively mitochondrial health (Memme et al., 2021) and the immune system (Nieman and Wentz, 2019).

1.7 Physiological dysfunction involved in Parkinson's disease

1.7.1 Mitochondrial dysfunction and oxidative stress

The mitochondrion is the powerhouse of the cell, producing adenoside triphosphate (ATP) a molecule that provides the energy necessary for the chemical reactions of the cell metabolism. Mitochondria are also regulators of cell cycle, cell death, and signalling (Horbay and Bilyy, 2016). Due to the central role of mitochondria, modification of its activity could result in modification of ATP production, increase of ROS and decrease of cell viability (Murphy, 2009). In an analysis of neurons from postmortem tissue of idiopathic PD, Complexes 1 and 2 were affected (Mizuno et al., 1989; Grünewald et al., 2016). Alpha-synuclein accumulation observed in PD results in the inhibition of mitochondrial complex I leading to an increase of ROS production (Mullin and Schapira, 2013). Another pathophysiological hallmark of PD, is the accumulation of iron in the SN of PD patients (Hirsch et al., 1991). Iron (Fe^{2+}) produces ROS through the Fenton reaction and increased ROS production also contributes to mitochondrial dysfunction (Wu et al., 2019). Many PD associated genes have been linked with mitochondrial dysfunctions, such as impairment of mitochondrial fission, biogenesis, production of ROS, mitophagy, production of ATP and calcium homeostasis (Figure 9).

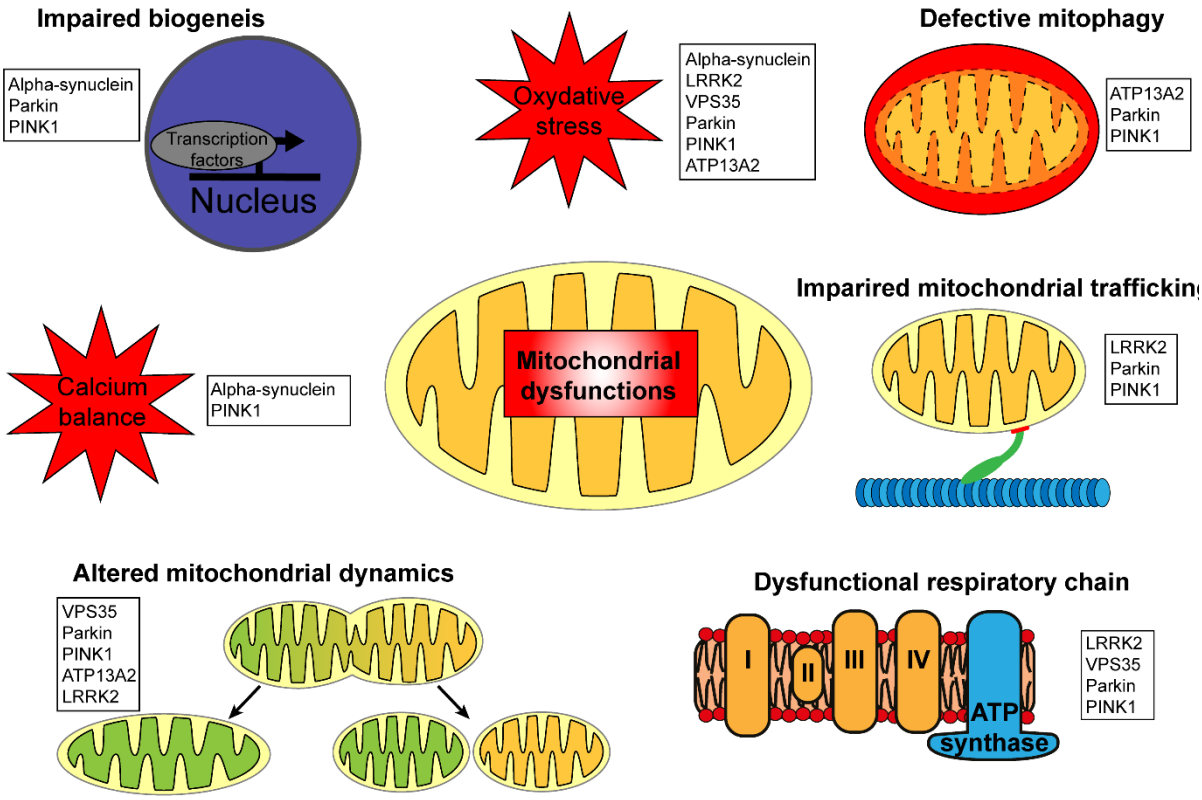


Figure 9: Mitochondrial dysfunction in PD. Different system of the mitochondria can be affected in PD. Different proteins implicated in dysregulation of mitochondrial functions are indicated in boxes. Adapted from (Park et al., 2018).

Alpha-synuclein is reported to bind to outer-mitochondrial membrane (OMM) proteins VDAC1, TOM40, TOM20. Interestingly, levels of VDAC1 are reduced in substantia nigra of PD patients (Chu et al., 2014). Overexpression of *SNCA* causes mitochondrial toxicity through increased size of mitochondria containing laminated bodies and decreased (3-(4,5-dimethythiazolyl)-2.5-diphenyl-2H-tetrazolium-bromide) MTT levels leading to oxidative stress (Hsu et al., 2000). Alpha-synuclein A53T can be found in the membrane of mitochondria and result in the inhibition of complex-1 and increase the mitophagy (Chinta et al., 2010; Risiglione et al., 2021).

1.7.2 Protein aggregation

Alpha-synuclein aggregation is a major pathological feature of PD, as these aggregates are an abundant component of Lewy bodies (Spillantini et al., 1997). As mentioned above, alpha synuclein was found to have a direct involvement in PD pathogenesis given its implication in autosomal dominant forms of PD (Polymeropoulos et al., 1997; Singleton et al., 2003; Chartier-Harlin et al., 2004; Martinez et al., 2004; Zarranz et al., 2004). Overexpression of alpha-synuclein in cell culture induces cell toxicity and neuronal death (Zhou et al., 2000) affecting cellular pathways and organelle homeostasis such as mitochondria, dopamine signalling and lysosomal degradation (Minakaki et al., 2020). Toxic species of alpha-synuclein can cause alteration of autophagy at different stages of this process (Xilouri et al., 2016). Upon aggregation, alpha-synuclein can acquire different conformations resulting in different aggregated forms (oligomers, different fibrillary strains, amorphous aggregates) (Figure 10, A).

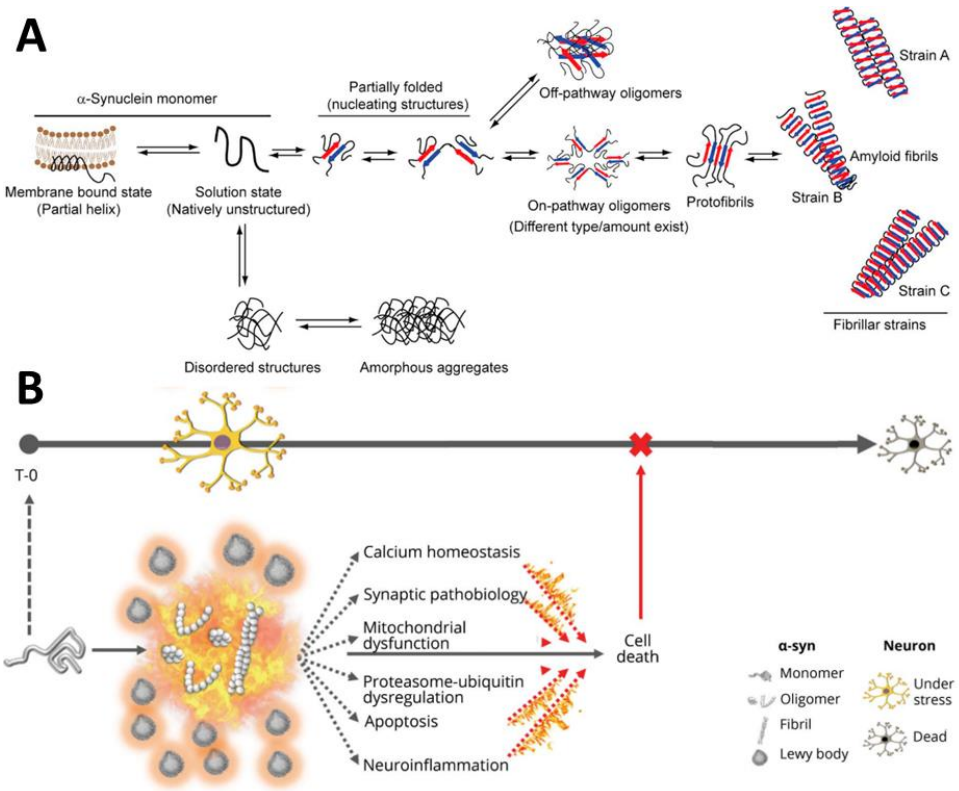


Figure 10: Representation of alpha synuclein aggregation leading to toxic species and neuronal death. (A) Alpha-synuclein monomers evolve into partially folded secondary structures, contributing to the formation of oligomers, protofibrils and finally fibrils. (B) Alternatively, or complementarily, secondary molecular changes created after protein aggregation combine with oligomers and fibrils to hasten cell death (lower panel). T-0 = time zero; α-syn = α-synuclein. Adapted from (Espay et al., 2019; Mehra et al., 2019)

When alpha-synuclein aggregates are formed, they have a negative impact on many cellular pathways such as the SNAREs complex assembly, lipid binding, dopamine synthesis and transport, vesicle synthesis and neurotransmitter release (reviewed in (Burré et al., 2018; Sarchione et al., 2021)) (Figure 10, B). Alpha-synuclein fibrils can spread from cell to cell, spreading alpha-synuclein aggregation through the brain. It was therefore described as a prion-like disease progression. Preformed fibrils can be internalize and contribute to formation of inclusions in primary cultures (Volpicelli-Daley et al., 2011).

1.7.3 Inflammation

Evidences are emerging implicating neuroinflammation in the appearance and progression of PD. Neuroimaging and analysis of post mortem brain samples show microglial activation, with increased expression of Major histocompatibility complex II (MHCII) following alpha-synuclein pathology (Tansey and Romero-Ramos, 2018). Astrocytes are also affected by alpha-synuclein aggregation. Indeed, transmission from neurons to astrocytes has been discovered through analysis of post mortem brain samples (Braak et al., 2007; Lee et al., 2010c) and alpha-synuclein has been found in lysosomal structures, possibly affecting autophagy and protein degradation in astrocytes (Loria et al., 2017). Activations of these astrocytes in PD leads to the increase of pro-inflammatory cytokines (IL1α, C1q and TNFα), adhesion molecules and increased production of ROS. Production of these molecules creates a vicious circle in the inflammatory system that will increase inflammation in the brain (Figure 11). In light of the role of neuroinflammation in PD, therapies acting on the modulation of neuroinflammation are hypothesized to have a neuroprotective role in PD (Hirsch and Hunot, 2009).

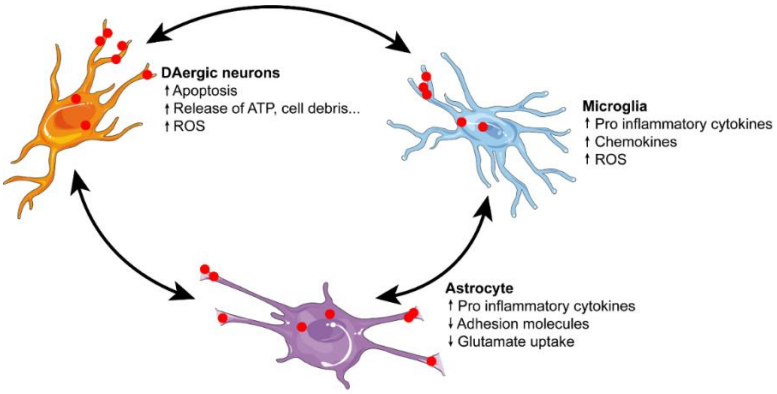


Figure 11: Neuroinflammation in PD. Illustration of the production of different neuroinflammatory compounds by astrocytes, microglia and dopaminergic neurons and indication of a crosstalk between each cell type. Adapted from (Pajares et al., 2020).

1.7.4 Cell death and stress regulation

For the moment, no therapy has been found to stop the death of neurons in PD. Research to discover compounds that target cell death is a priority therapeutic approach in the context of PD. Different cell death mechanisms have been reported in PD pathogenesis:

Apoptotic cell death: Apoptosis is a programmed cell death which is vital for numerous processes such as cell turnover, development and regulation of the immune system, embryonic development and others (Kerr et al., 1972). It is the main mechanism of cell death in PD and it is characterized by the accumulation of fragmented DNA and apoptotic chromatin that was identified in dopaminergic neurons of PD patients in post-mortem studies (Tompkins et al., 1997). Also in post-mortem studies, elevated caspase-3 activity and protein expression was shown in the SNpc (Mogi et al., 2000). Dopaminergic cell death can be prevented in *in vitro* PD models with apoptotic and caspase inhibitors (Y et al., 2002; Iaccarino et al., 2007). Diverse alterations of mitochondria lead to mitochondria-mediated apoptosis involving increased ROS production, cytochrome c release, ATP depletion, caspase-9/caspase-3 activation (Fiskum et al., 2003). Genetic determinants of PD have been linked with mitochondrial deficits including Parkin, LRRK2, PINK1, and DJ-1 (Klein and Westenberger, 2012).

Autophagy**:** Autophagy is a degradative process of the cell, through which abnormally folded proteins or defective cellular components of the cells will be sequestered in a double membranous vesicle, called autophagosome, and then degraded by the fusion with lysosomes (Klionsky, 2000). There are 3 different classes of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy (Mizushima and Komatsu, 2011). Interestingly, numerous genes linked to PD play a role in the regulation of autophagy (Figure 12). Analysis of brains from Parkinson's patients have shown reduced HSC70 (Role in protein folding and acts as a cargo protein between different organelles) et LAMP-2A (Role in chaperone-mediated autophagy and phagosome maturation) (Alvarez-Erviti et al., 2010) as well as an increased proportion of autophagosomes (Dehay et al., 2010) resulting in defective autophagy.

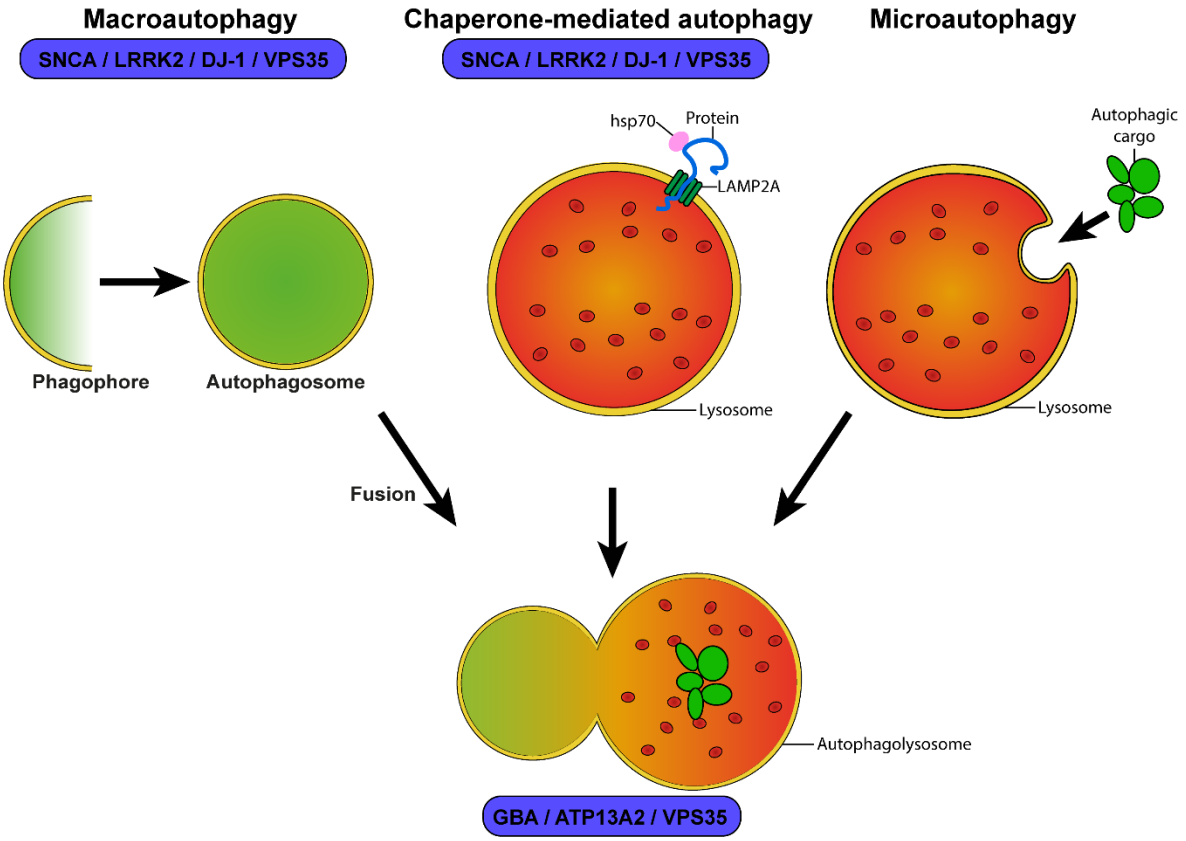


Figure 12: Implication of PD related genes on autophagy. Autophagy has been shown to be regulated by different PD genes. SNCA, LRRK2, DJ-1 and VPS35 affect the macroautophagy as well as the chaperone mediated autophagy. The fusion and homeostasis of autophagolysosome is modulated by GBA, ATP13A12 and VPS35. Adapted from (Hou et al., 2020).

Ferroptotic cell death: Ferroptosis is an iron-dependent cell death involving toxic accumulation of lipid peroxides. Iron-chelation prevents cell death induced by ferroptosis (Do Van et al., 2016). Interestingly, iron accumulation occurs in SNpc of PD patients and can be detected using magnetic resonance imaging (Lhermitte et al., 1924; Graham et al., 2000). Iron can accelerate the oxidation of dopamine and forms quinones and free radicals which are known to be involved in neurodegeneration (Chen et al., 2012). The increased production of lipid peroxides cause accumulation of alpha-synuclein (Angelova et al., 2015). Alpha-synuclein aggregation and Lewy body formation is promoted by iron accumulation, dopamine oxidation and H_2O_2 (Castellani et al., 2000).

Calcium homeostasis: Calcium is finely regulated and it is a common component of signalling pathways in neurons. It can be taken up from the extracellular space and stored mainly in the endoplasmic reticulum and in the mitochondria. Excessive calcium regulation triggers necrosis and apoptosis (Hansford, 1994; Orrenius et al., 2003). Dopaminergic neuron

health can be compromised by upregulation of calcium levels (Blandini et al., 2004; Chan et al., 2007) or downregulation of cellular calcium content (Salthun-Lassalle et al., 2004). The increased calcium concentration can trigger the activation of tyrosine hydroxylase, producing increased amount of dopamine that leads to cellular damage due to higher dopamine auto-oxidation (Michel and Hefti, 1990; Rittenhouse and Zigmond, 1999) and increased post-translational modification of alpha-synuclein (Martinez-Vicente et al., 2008). The calcium overload can activate calpains (calcium-dependent cysteine proteases). Thus, inhibition of calpains in A30P alpha-synuclein mice results in decreased alpha-synuclein aggregates (Dufty et al., 2007; Diepenbroek et al., 2014).

Unfolded protein response (UPR): To manage increases in misfolded or abnormal proteins, cells can use the Unfolded protein response or ER stress pathway. It is a complex signal transduction pathway that begins with three ER stress activation sensors, inositol-requiring protein 1 (IRE1), protein kinase RNA-like ER kinase (PERK) and activating transcription factor 6 (ATF6). Activation of UPR is needed to control ER homeostasis (Hetz, 2012). Under constant ER stress, UPR will trigger pro-apoptotic pathways resulting in death of the cells. In PD, it is hypothesized that accumulation of misfolded alpha-synuclein may trigger this UPR mechanism leading to increased dopaminergic cell death. UPR makers have been described in synucleinopathy models (Matus et al., 2011) and alpha-synuclein toxic oligomers accumulate in the ER during disease progression (Colla et al., 2012). ER-stress and activation of UPR response take place in dopaminergic neurons of PD patients, discovered in brain samples from patients (Hoozemans et al., 2007).

1.7.5 Lysosomal dysfunction

Lysosomes are membrane bound organelles whose primary role are the degradations of cellular component and endocytosed particles. They are present in all cells except red blood cells. The degradation capacity of lysosomes is enabled by 60 different enzymes acting in a luminal solution at pH 4.5-5 (Xu and Ren, 2015).

The accumulation of protein aggregates in the brain of PD patients support the idea of a defect in the degradative pathway. Indeed, reports show accumulation of autophagic vesicles with lysosomal reduction in PD patient brains (Chu et al., 2009; Crews et al., 2010; Dehay et al., 2010). The increase of ROS from mitochondria causes abnormal membrane permeabilization and disruption of its integrity (Vila et al., 2011). Autophagy use lysosomes for its degradative properties. Genetic studies also provide numerous evidences of lysosomes/autophagy disruption in PD as seen in table 3, presenting the genes linked with lysosomal/autophagy degradative pathways.

Locus	Inheritance	Protein	Involvement in ALP
SNCA	AD	Alpha-synuclein	Inhibition of CMA and TFEB sequestration
LRRK2	AD	LRRK2	Inhibition of CMA and macroautophagy
VPS35	AD	VPS35	Retromer formation
DNAJC13	AD	DNAJC13	Endosomal pathway
PARK2	AR	parkin	Mitophagy
PARK6	AR	PINK1	Mitophagy
PARK7	AR	Dj-1	Mitophagy/oxidative stress
PARK9	AR	ATP13A2	Lysosomal cation homeostasis and lipid flippase
SYNJ1	AR	SYNJ1	Endosomal pathway
ATP6AP2	X-linked	ATP6AP2	Lysosomal acidification
GBA	/	GBA	Lysosomal enzyme (ceramide metabolism)
SMPD1	/	SMPD1	Lysosomal enzyme (ceramide metabolism)
PARK16	/	RAB29	Golgi apparatus trafficking
SCARB2	/	LIMP2	Mannose-6-phosphate independent trafficking receptor for GBA
MCCC1/LAMP3	/	MCCC1 and LAMP3	LAMP3: lysosomal membrane protein MCCC1: mitochondrial protein

Table 3: Overview of PD genes connected to autophagy-lysosomal pathways. CMA: chaperone-mediated autophagy. ALP: autophagy-lysosomal pathways. TFEB: Transcription Factor EB. Adapted from (Bourdenx and Dehay, 2016).

The defective function of lysosomes contributes to PD pathogenesis. Lysosomes can degrade alpha-synuclein (Cuervo et al., 2004) and perturbations in degradative capacity of lysosomes can result in increased alpha-synuclein level in cells. Different components of the trafficking machinery are PD risks factors, leading to alterations of their pathways. PD risks factors such as GBA, VPS35, GAK, RAB29 and LRRK2 impaired the vesicular trafficking in PD leading to increased ROS production, enhancing the aggregation of alpha-synuclein, discussed in section 2.4. (Figure 13) (Ebanks et al., 2020).

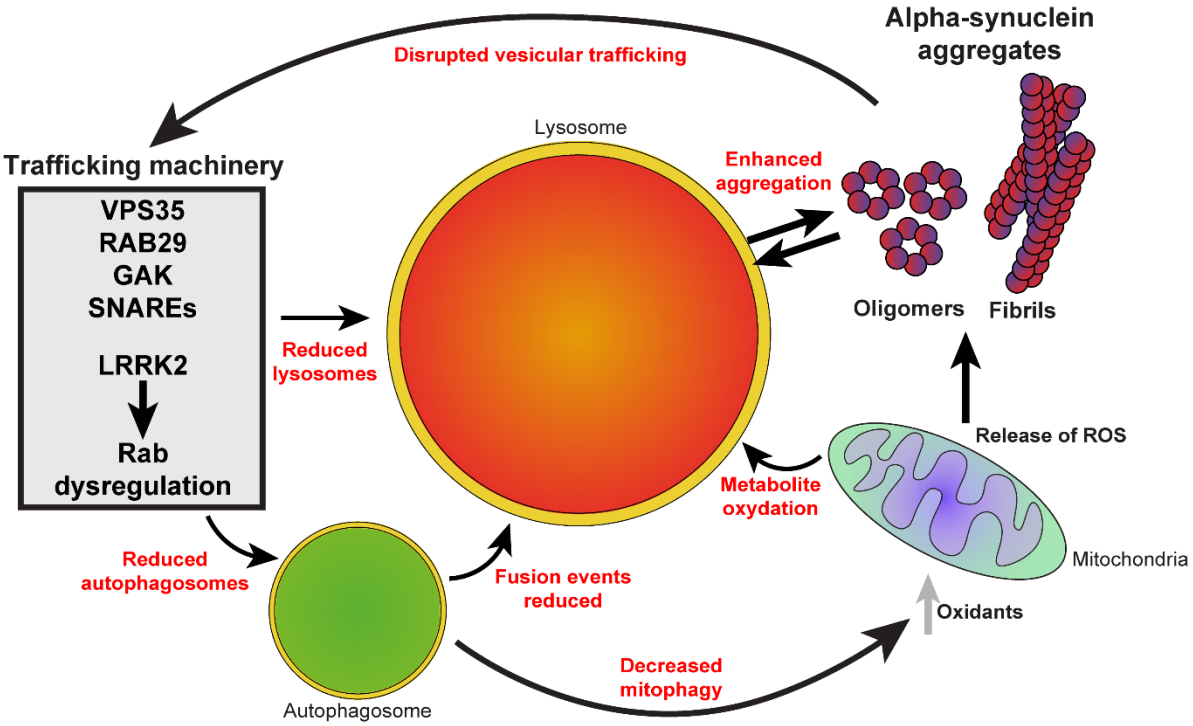


Figure 13: PD genetic risk factors regulate the trafficking machinery and increase cell toxicity. Several PD genes have been associated with dysregulation of trafficking machinery, which affect lysosomal homeostasis, decreasing its degradative properties. The impairment of mitophagy increases the release of ROS (Reactive Oxygen Species) which enhances alpha-synuclein aggregation. The alpha-synuclein aggregation promotes the disruption of vesicular trafficking as well as lysosomal degradation properties. Adapted from (Klein and Mazzulli, 2018)

Part 2: LRRK2

2.1 Discovery of LRRK2 as a genetic determinant of PD

The identification of LRRK2 started in 2002. Several families presenting a dominant PD inheritance pattern had been identified however the causative gene locus and gene was not known at that time. A genome-wide linkage analysis of a Japanese family (the so-called Sagamihara kindred) identified the PARK8 locus on chromosome 12q12 (Funayama et al., 2002). Sequencing of the locus led in 2004 to the identification by two independent groups of mutations in the gene *LRRK2* as the cause of PARK8 PD (Paisán-Ruíz et al., 2004; Zimprich et al., 2004).

2.2 LRRK2 functional domains

The *LRRK2* gene is composed of 51 exons and encodes a 2527 amino acid protein with a molecular mass of 286 kDa. LRRK2 belongs to the ROCO protein family, consisting in humans of 4 homologs (DAPK1, MASL1, LRRK1 and LRRK2). This family of proteins is defined by the presence of a ROC (Ras Of Complex proteins)/ GTPase domain followed by a COR (C-terminal of ROC) domain (Bosgraaf and Van Haastert, 2003). Another catalytic domain, KIN, is present in LRRK2 and acts as a kinase. These catalytic domains are surrounded by protein-binding domains (ARM-ANK-LRR-WD40) (Figure 14, A).

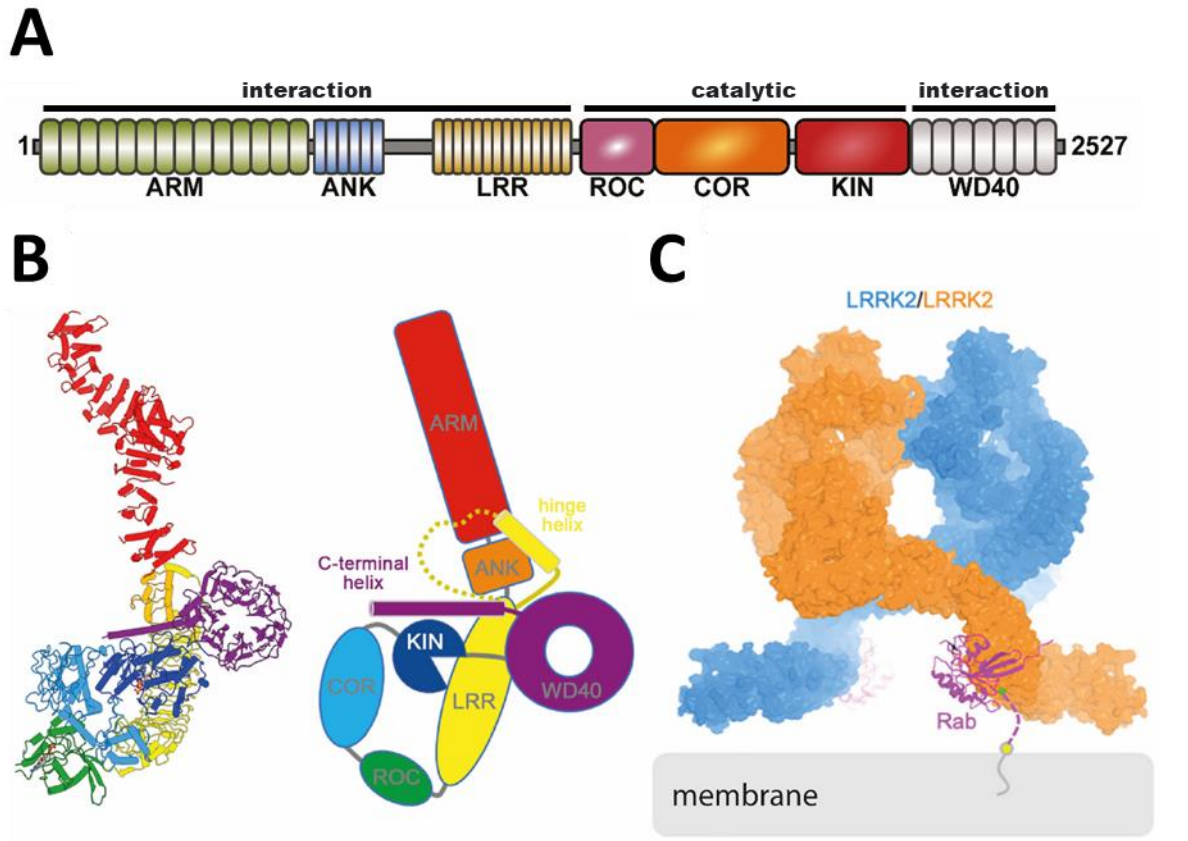


Figure 14: LRRK2 domains and structure. (A) LRRK2 is composed of 7 domains. Domains flanking the catalytic core (ROC-COR-KIN) are implicated in protein-protein interaction, Leucine-Rich repeat "LRR", Ankyrin repeat "ANK", Amardillo repeat "ARM" in the N-terminal segment of LRRK2 and the WD40 domain at the C-terminus. (B) High resolution structure of full-length LRRK2 depicting the 3D organisation of LRRK2 domains. (C) High resolution structure of homodimeric form of LRRK2 interacting with RAB partners connecting LRRK2 to membrane bound state. Adapted from (Marchand et al., 2020; Myasnikov et al., 2021).

2.2.1 Protein-Protein interaction domains

LRRK2 is known to interact with various proteins (See section 2.5) through its protein-binding domains (ARM-ANK-LRR-WD40) localized in its N and C terminal regions.

ARM Domain: This domain is found in various proteins from plants to mammals, it consists of approximately 42 repetitive amino acid modular elements. A minimum of 6 repeats is enough to form a functional structure to allow protein interaction (Hatzfeld, 1998). The motif was first discovered in the armadillo locus (homologue of mammalian β-catenin) in Drosophila melanogaster (Riggleman et al., 1989). This motif is conserved through evolution and it has been found in diverse species from yeast to human. The role of the ARM domain is to act as binding modules for numerous interacting proteins. Specifically, the ARM domain of LRRK2 is

composed of 14 putative armadillo repeats (Sejwal et al., 2017) and it is reported to interact with vesicular proteins such as the RABs at residues 386–392 (Figure 14, C) (McGrath et al., 2019; Myasnikov et al., 2021).

ANK Domain: This domain consists of a 33-residue sequence motif. It was initially discovered in Drosophila and in yeast (Breeden and Nasmyth, 1987). The ankyrin repeats locate in many proteins with different functions. The repeat is present in *Bacteria*, *Archaea* and *Eukaryotes* and in the viral genome. It serves as a scaffold for interactions and the folding of this domain play important roles such as binding to RABs and VARP protein (Mosavi et al., 2004; Barrick, 2009; Araki et al., 2018). Six putative ankyrin repeats have been discovered in LRRK2 (Sejwal et al., 2017). This domain is also an interacting domain with RAB proteins (Purlyte et al., 2018).

LRR Domain: Leucine-Rich Repeats are characterized by 20-30 amino acid residues with a rich proportion of Leucine, constituting β-sheet and α-helix folds that when repeated adopt the form of a horseshoe. 375 human proteins have been identified with LRR domains, and most of the LRR proteins remain uncharacterized. LRR motifs are implicated in protein-protein interactions (Ng and Xavier, 2011). 14 putative Leucine Rich Repeats were detected in LRRK2 (Vancraenenbroeck et al., 2012; Sejwal et al., 2017). Structural analysis of this domain reveals a modulation that stabilizes the inactive state of the KIN domain by protecting ATP entry by maintaining the ATP binding cleft open (Myasnikov et al., 2021).

WD40 Domain: WD repeats were first identified in the beta-subunit of heterotrimeric G proteins as a repetitive sequence of 43 amino acids. The length is approximately 40 amino acids and often ended by a WD dipeptide (Fong et al., 1986). The repeats form a β-sheet that fold to β-propellers. WD40 domains can be found in a broad range of species from bacteria to humans (Xu and Min, 2011). Seven putative WD40 repeats in the C-terminal domain of LRRK2 were detected. WD40, with ROC-COR domain, contributes to dimerization of LRRK2 (Jorgensen et al., 2009). The LRRK2 WD40 domain is also reported to mediate protein interaction to bind synaptic vesicles (Piccoli et al., 2014).

2.2.2 GTPase domain

The GTPase domain of LRRK2 (the ROC domain) represents a small fraction of the full-length protein (7% of total), from amino acids 1320 to 1516. Five regions can be identified in the GTPase domain of LRRK2, a P-loop domain, a switch I and Switch II region, G4 and G5 motifs. The ROC-GTPase domain of LRRK2 binds both GTP and GDP (Civiero et al., 2012; Taymans, 2012) and it is capable of hydrolysing GTP (Ito et al., 2007; Lewis et al., 2007; Daniëls et al., 2011) (Figure 15). It can be hypothesized that ROC, in analogy to other Ras family proteins, can shuttle between inactive GDP-bound state and active GTP-bound state and that ROC may be the activator of LRRK2 kinase. Interestingly, the ROC domain of LRRK2 has been found to be necessary for kinase activity of LRRK2 (Ito et al., 2007), however detailed analysis found that this is independent of its GDP or GTP bound state suggesting that the ROC GTPase cycle does not regulate LRRK2 kinase activity (Liu et al., 2010; Taymans et al., 2011).

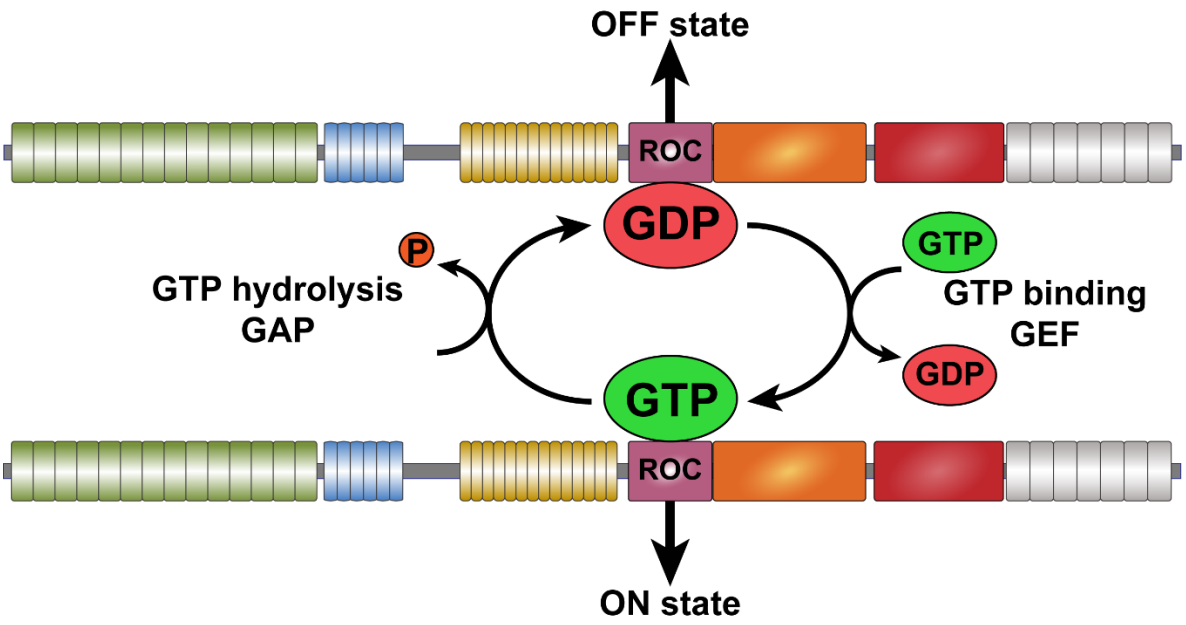


Figure 15: LRRK2 GTP binding and activation model. LRRK2 ROC domain contains a GDP/GDP binding motif. LRRK2 catalyses the hydrolysis of GTP to GDP. The GTPase function of LRRK2 is proposed to act as a molecular switch. GTP binding is associated to LRRK2 conformational changes leading to connection with effectors.

The catalytic core domains, including ROC and COR domains, are known to be a hotspot for pathogenic mutations of LRRK2 such as N1437H, R1441C/G/H, Y1699C, and R1628P reported to increase risk for PD and cause alteration of GTPase activity. The R1441C/G/H and Y1699C induce reduced GTPase activity (West et al., 2007; Daniëls et al., 2011; Liao et al., 2014). Functional mutations in the ROC domain, K1347A and T1348N, that abolish GTP binding capacity also abolish the kinase activity of LRRK2 (Ito et al., 2007; Taymans et al., 2011). The mutant R1398L increases the GTPase activity but this does not result in increased kinase activity. Structural analysis of the ROC-COR domain demonstrates that mutations are found in or near the interface between ROC and COR domain (Daniëls et al., 2011; Myasnikov et al., 2021). Mutations in the ROC/COR domain tandem can alter the affinity of GTP and disturb the GTPase activity (Ito et al., 2007; Biosa et al., 2013). Interestingly, the mutant R1398H is associated with a decreased risk for developing PD, and unlike the R1398L or Q, its GTPase activity is enhanced (Nixon-Abell et al., 2016; Gopalai et al., 2019). The R1441H mutation is reported to result in a twofold reduced GTPase activity *in vitro* (Liao et al., 2014).

2.2.3 Kinase domain

The kinase activity of LRRK2 has received comparatively more attention than LRRK2 GTPase activity. This is due to the gain of function mutations discovered in familial and sporadic PD patients, leading to increased kinase activity. LRRK2 is a serine/threonine kinase which catalyses the transfer of a phosphate from ATP to the serine or threonine in proteins. The ANK and LRR domain of LRRK2 interact with kinase domain through three sites (Myasnikov et al., 2021). Interestingly, one of these sites includes a mutation, N2081D, that increases the risk for Crohn's disease (Hui et al., 2018).

LRRK2 kinase activity is a confirmed regulator in LRRK2 in signalling pathways. Indeed, many LRRK2 substrates are found dysregulated in disease (See section 2.4). Through it kinase domain, LRRK2 also phosphorylates itself, including at a cluster of autophosphorylation sites in the ROC domain (Greggio et al., 2009; Marchand et al., 2020). Pathogenic variants have been found in the kinase domain of LRRK2, including mutations, G2019S and I2020T, N2081D (Hui et al., 2018). These variants are known to increase the kinase activity of LRRK2. It has been proposed that hyperactive kinase could cause elevated phosphorylation of LRRK2's targets and lead to cell homeostasis disruption (Greggio et al., 2006). *In vitro* studies and mice studies have linked elevated kinase activity of LRRK2 with various toxic effects such as autophagy alteration, neurite shortening, degeneration of DA neurons and organellar dysfunctions (Smith et al., 2006; West et al., 2007; Lee et al., 2010a; Madureira et al., 2020). In this way, selective kinase inhibitors were developed to reduce the toxic effect of LRRK2 (Anand et al., 2009; Deng et al., 2011; Ramsden et al., 2011). LRRK2 kinase inhibition results in a decreased kinase activity and are followed by a decreased heterologous phosphorylation (Dzamko et al., 2010). LRRK2 dephosphorylation of S910-S935-S973 has been detected in human PD brain lysates (Dzamko et al., 2017). It is also interesting to note that recently, increased kinase activity was observed in human Idiopathic PD post-mortem brain of substantia nigra, independently of LRRK2 mutations (Di Maio et al., 2018). This result reinforces the potential use of LRRK kinase inhibitors in sporadic PD. See more about Kinase inhibition in section 2.8.

LRRK2 kinase activity is regulated by LRRK2 regulators. Two PD risk factors, RAB29 and VPS35, implicated in vesicular trafficking physically interact with LRRK2 (MacLeod et al., 2013; Steger

et al., 2016). The overexpression of RAB29 induces an elevated S1292 autophosphorylation with a LRRK2 recruitment to the TGN (Purlyte et al., 2018). In a study in human PBMCs carrying the VPS35 D620N pathogenic mutation, an elevated LRRK2-mediated T73-RAB10 phosphorylation is reported as well as LRRK2 mediated-phosphorylation of RAB8a, RAB10 and RAB12 (Mir et al., 2018). Interestingly, this emerging evidence suggests a relationship between LRRK2, RAB29 and VPS35 in regulating the retromer complex and neurite growth. Expression of LRRK2 G2019S or RAB29 knock down results in neurite shortening in rat primary neurons, and this can be prevented by overexpression of VPS35. Moreover, expression of VPS35 D620N mimics the neurite shortening induced by LRRK2 G2019S (MacLeod et al., 2013).

2.3 Involvement of LRRK2 in Parkinson's disease

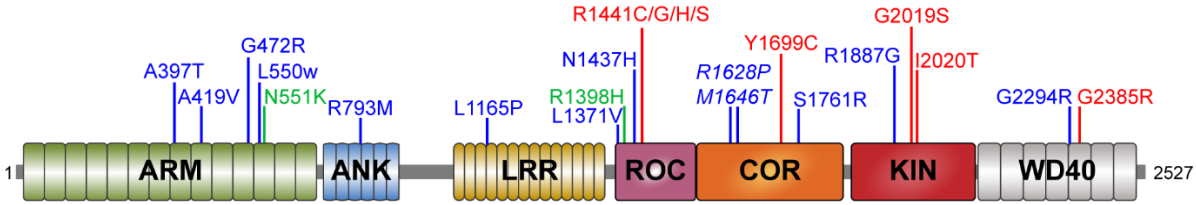


Figure 16: LRRK2's pathogenic mutations. Pathogenic variants of LRRK2 are indicated in red, rare variants pathogenic in blue and protective variants are indicated in green. Adapted from (Goveas et al., 2021)

Genetic variations at the LRRK2 locus have been reported to be implicated in PD development with missense mutations being the most common cause of familial PD. The first reported mutation was discovered in a German-Canadian and Western Nebraska family (Zimprich et al., 2004). After this discovery, other mutations have been reported in different families, from all around the globe. In 2005, a mutation in the activation loop of LRRK2 at the position 2019 was reported (Kachergus et al., 2005). This missense mutation changes a glycine residue to a serine, noted G2019S. This mutation is the most common LRRK2 mutation linked to PD. LRRK2 G2019S segregates with PD in 4% of familial cases and 1% of sporadic PD worldwide (Healy et al., 2008). Most LRRK2 PD mutations are located in the catalytic core of the protein and potentiate the kinase activity of LRRK2. The pathogenic mutants R1441C/G/H, Y1699C cause decreased GTPase activity with decreased phosphorylation at S910, S935 and increase kinase activity leading to the hypothesis of a potential interplay between the kinase activity and GTPase activity of LRRK2 (Taymans, 2012).

Apart from pathogenic mutations, protective risk factors have also been discovered in the GTPase domain (R1398H) but also in the ARM domain (N551K) of LRRK2 (Gopalai et al., 2019) (Figure 16). Interestingly, these two protective mutants lead to a reduced RAB10 phosphorylation in Peripheral Blood Mononuclear Cells (PBMC) of healthy carriers without change of S935 phosphorylation level (Wang et al., 2021). Many additional PD risk factor mutations in LRRK2 have been reported, although many remain to be confirmed and few of them are subjected to deeper investigations (Table 4).

Mutation	Catalytic activities	Hallmarks	References
ARM-ANK-LRR domain			
A397T	Unknown		(Kishore et al., 2019)
G472R	Unknown		(Kishore et al., 2019)
L550W	Unknown		(Kishore et al., 2019)
N551K	Unknown	Reduced pRAB10	(Ross et al., 2011; Wang et al., 2021)
R793M	Unknown		(Covy et al., 2009)
L1165P	Unknown		(Covy et al., 2009)
ROC domain			
R1398H	Decreased kinase activity	Reduced pRAB10 Decrease in active GTP-bound LRRK2	(Chen et al., 2011; Nixon-Abell et al., 2016; Wang et al., 2021)
N1437H	Increased kinase activity		(Puschmann et al., 2012)
R1441C	Increased kinase activity	Reduction in lysosomal GBA activity. Increased pRAB10.	(Haugarvoll et al., 2008)(Ysselstein et al., 2019)
R1441G	Increased kinase activity	Reduction in lysosomal GBA activity.	(Simón-Sánchez et al., 2006)(Ysselstein et al., 2019)
R1441H	Increased kinase activity		(Ferreira et al., 2007)
R1441S			(Mata et al., 2016)
COR domain			
R1628P	Unknown		(Oosterveld et al., 2015)
S1647T	Unknown		(Oosterveld et al., 2015)
M1646T	Unknown		(Sosero et al., 2021)
Y1699C	Increased kinase activity		(Khan et al., 2005)
S1761R			(Lorenzo-Betancor et al., 2012)
Kinase domain			
D1887G	Unknown		(Kishore et al., 2019)
G2019S	Increased kinase activity	mtDNA damage in iPSC derived NPC and neural cells. Reduction in lysosomal GBA activity. Impaired mitochondrial respiration. Enlarged lysosomes.	(Kachergus et al., 2005; Sanders et al., 2014; Hockey et al., 2015; Schwab et al., 2017; Ysselstein et al., 2019)
I2020T	Increased kinase activity		(Funayama et al., 2005)
WD40 domain			
G2294R	Unknown	Decreased RAB8a and RAB10 levels in macrophages	(Ogata et al., 2021)
G2385R	Decreased kinase activity	Alteration of synaptic vesicle trafficking.	(Di Fonzo et al., 2006; Carrion et al., 2017; Rudenko et al., 2017)

Table 4: LRRK2 mutations found in patients. LRRK2 mutations reported in PD patients are classified according to their domain location. Changes in catalytic activities are indicated and the hallmarks of the mutations are described if observed in patients' cells.

2.4 LRRK2 functions

Improving our comprehension of the precise role of LRRK2 is crucial for the development of new therapies. As seen before, LRRK2 is subjected to modifications, such as its phosphorylation rate, in sporadic PD. Moreover, LRRK2 mutations represent a non-negligeable proportion of PD patients. By studying the protein-protein interaction network of LRRK2 as well as exploring the phenotypes of different LRRK2 mutations, it is possible to deduce the involvement of LRRK2 in diverse pathways resumed in Figure 17.

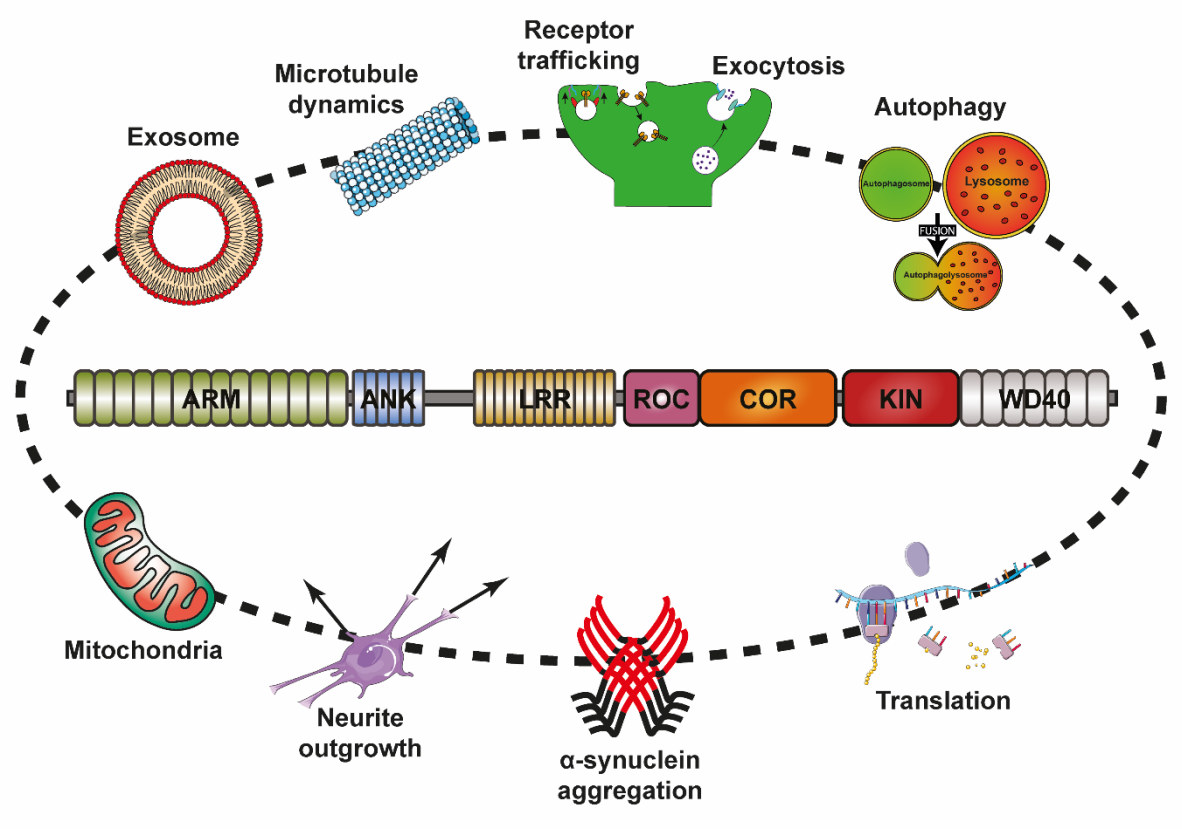


Figure 17: Cellular Functions of LRRK2. This scheme compiles different cellular events that have been reported to be influenced by LRRK2 in physiology or in disease. This representation associate finding from different research groups and from different cellular/animal models. It is not yet determined if all of these events are all disrupted by LRRK2 at the same time in the same cell model.

It is possible to identify a pathway involving LRRK2 by looking at the pathways of its cellular partners. In Table 5, a non-comprehensive list of LRRK2's cellular partners are given. Partners are grouped based on their implication in specific pathways.

Protein	Relation to LRRK2	Potential role	reference
ArfGAP1	GAP-like protein for LRRK2. LRRK2 genetic modifier LRRK2. Kinase substrate.	Golgi to ER retrograde vesicular sorting. GTPase activating protein (GAP) for LRRK2.	(Xiong et al., 2010, 2012; Stafa et al., 2012)
AP3B1	LRRK2-interacting protein. LRRK2 and AP3B1 complex together to recycle lysosomal membrane proteins LAMP1 and LAMP2.	AP3 protein complex component. Localized to endosomal membranes. Regulates endosome maturation and vesicle exocytosis, and recycling of lysosomal membrane proteins. Generation of new synaptic vesicles.	(Cheung and Cousin, 2012; Park and Guo, 2014; Kuwahara et al., 2016)
ATP13A2	Increased protein expression in G2019S. LRRK2 brain tissue.	P5-type ATPase localized to late endosomal and lysosomal membranes.	(Henry et al., 2015)
Auxilin	LRRK2 kinase substrate. LRRK2-interacting protein.	Synaptic Vesicle Endocytosis.	(Nguyen and Krainc, 2018)
GAK	LRRK2-interacting protein	Clathrin uncoating. Facilitates clathrin receptor binding at Golgi and plasma membrane.	(Lee et al., 2005; Beilina et al., 2014)
Dynamin 1, 2, 3	LRRK2-interacting proteins.	Membrane scission in clathrin-mediated endocytosis.	(Stafa et al., 2014)
GBA	LRRK2 familial mutants decrease lysosomal Glucocerebrosidase activity.	Lysosomal hydrolase.	(Nguyen and Krainc, 2018; Ysselstein et al., 2019)
LAMP2a	LRRK2-binding partner.	Lysosomal membrane protein. Chaperone-mediated autophagy receptor.	(Orenstein et al., 2013)
NSF	LRRK2 kinase substrate.	Golgi disassembly. SNARE complex dissociation synaptic vesicle endocytosis	(Piccoli et al., 2011; Belluzzi et al., 2016; Lanning et al., 2018)
SEC16A	LRRK2-interacting protein.	ER to Golgi anterograde transport.	(Cho et al., 2014)
Syntaxin-6	LRRK2-interacting protein.	SNARE protein at TGN.	(Beilina et al., 2020)
VPS35	LRRK2-interacting protein. D620N mutation enhances phosphorylation of LRRK2 kinase substrates.	Retromer complex subunit.	(MacLeod et al., 2013; Mir et al., 2018; Nguyen et al., 2020)
VPS52	LRRK2-interacting protein.	Golgi-associated retrograde protein (GARP) complex subunit.	(Beilina et al., 2020)
Endophilin A	LRRK2 kinase substrate.	Synaptic Vesicle Endocytosis. Autophagosome formation at neuromuscular junction synapses.	(Arranz et al., 2014; Soukup et al., 2016; Pan et al., 2017)
P62/SQSTM1	LRRK2 kinase substrate.	Autophagy.	(Kalogeropulou et al., 2018)
RAB1a/b/c	LRRK2 kinase substrate.	Endoplasmic reticulum - Golgi trafficking.	(Steger et al., 2016)

RAB3a/b/c/d	LRRK2 kinase substrate.	Exocytosis, neurotransmitter release	(Schlüter et al., 2004; Steger et al., 2017)
RAB5a/b/c	LRRK2-binding partner. Putative LRRK2 kinase substrate.	Endocytic vesicle fusion to early endosomes. Synaptic Vesicle Endocytosis.	(Shin et al., 2008; Dodson et al., 2012; Yun et al., 2015)
RAB7a	LRRK2-interacting protein.	Late endosome and lysosome membrane protein. Endocytic trafficking and lysosome biogenesis.	(Dodson et al., 2012)
RAB8a/b	LRRK2 kinase substrate.	Trafficking and recycling of epidermal growth factor (EGF) and EGF receptors from cell surface membrane. Lysosomal overloading response. Endocytosis and secretion. Post-Golgi trafficking, ciliogenesis.	(Healy et al., 2008; Steger et al., 2017; Eguchi et al., 2018; Rivero-Ríos et al., 2019)
RAB9	LRRK2-interacting protein.	Retrograde trafficking.	(Dodson et al., 2014)
RAB10	LRRK2 kinase substrate.	Exocytosis, trans-Golgi/recycling endosome trafficking to plasma membrane. Lysosomal overloading response.	(Steger et al., 2017; Eguchi et al., 2018)
RAB12		Recycling of endosomes and lysosomes, ciliogenesis	
RAB29	LRRK2 kinase substrate. Recruits LRRK2 to the TGN and to overloaded lysosomes. Activates LRRK2.	Endo-lysosomal sorting/degradation. TGN maintenance, retrograde trafficking from late and recycling endosomes to TGN. Lysosomal overloading response.	(Wang et al., 2014; Eguchi et al., 2018; Fujimoto et al., 2018; Liu et al., 2018)
RAB32		Retrograde trafficking.	(Waschbüsch et al., 2014, 2019)
RAB35	LRRK2 kinase substrate. Recruited to LRRK2-positive overloaded lysosomes.	Recycling endosomal trafficking, exosome secretion. Endosome to plasma membrane recycling. Plasma membrane receptor recycling.	(Chua et al., 2010; Eguchi et al., 2018; Jeong et al., 2018)
RAB43	Putative LRRK2 kinase substrate.	anterograde ER-Golgi trafficking	(Li et al., 2017; Steger et al., 2017)
Snapin	LRRK2 phosphorylate snapin.	Adaptor protein interacting with the SNARE protein SNAP-25 Synaptic vesicle trafficking.	(Yun et al., 2013)
Synaptojanin-1	LRRK2 kinase substrate.	Synaptic vesicle endocytosis. Clathrin uncoating, down-regulation of actin polymerization, modulation of dynamin activity.	(Islam et al., 2016; Pan et al., 2017)
FoxO1	LRRK2 kinase substrate	Transcriptional regulation of pro-apoptotic genes.	(Kanao et al., 2010)
4E-BP-1	LRRK2 kinase substrate	Cap-dependent protein translation. Survival under starvation, oxidative and unfolded protein stress.	(Imai et al., 2008)
RPS15	LRRK2 kinase substrate	Bulk protein translation.	(Martin et al., 2014)
eIF2C1	LRRK2 interactor	miRNA machinery	(Dächsel et al., 2007)
eIF2C2	LRRK2 kinase substrate	miRNA machinery	(Dächsel et al., 2007)
eIF4B	LRRK2 kinase substrate	binding of mRNA to ribosomes	(Krumova et al., 2015)
eIF3C	LRRK2 kinase substrate	Translation initiation	(Krumova et al., 2015)
eEF1D	LRRK2 kinase substrate	enzymatic delivery of aminoacyl tRNAs to the ribosome	(Reyniers et al., 2014)
RPS11	LRRK2 kinase substrate	40S ribosomal subunit	(Martin et al., 2014)
RPS15	LRRK2 kinase substrate	40S ribosomal subunit	(Martin et al., 2014)
RPS20	LRRK2 kinase substrate	40S ribosomal subunit	(Martin et al., 2014)
RPS27	LRRK2 kinase substrate	40S ribosomal subunit	(Martin et al., 2014)

β-tubulin	**LRRK2 kinase substrate**	A component of microtubule (MT) Neurite outgrowth	(Gillardon, 2009)
Futsch	LRRK2 kinase substrate	Microtubule-association protein, regulation of MT dynamics Negative regulator of synaptic functions	(Lee et al., 2010d)
MARK1	LRRK2 kinase substrate	Regulation of MT stability through phosphorylation of MAPs.	(Krumova et al., 2015)
Moesin/Ezrin/ Radixin	LRRK2 kinase substrate	Actin cytoskeleton rearrangement, neurite outgrowth, neuronal morphogenesis	(Jaleel et al., 2007)
Tau	LRRK2 kinase substrate	Modulation of microtubule dynamics Neurite outgrowth	(Kawakami et al., 2012)
ARHGEF7	LRRK2 kinase substrate	guanine nucleotide exchange factor for LRRK2. Neurites outgrowth.	(Chia et al., 2014)(Häbig et al., 2013)
DLP-1	LRRK2-interacting protein.	Mitochondrial dynamics	(Wang et al., 2012)
RGS2	Interacting partner of LRRK2	GTPase Activating Protein for LRRK2	(Dusonchet et al., 2014)
14-3-3	LRRK2-interacting protein	Diverse roles	(Nichols et al., 2010)
CK1-alpha	Phosphorylate LRRK2	Cell cycle regulation	(Chia et al., 2014)
IKB	Phosphorylate LRRK2	Immune response	(Dzamko et al., 2012)
PP1 PP2A	Phosphatase of LRRK2	Regulate LRRK2 phosphorylation state	(Lobbestael et al., 2013; Drouyer et al., 2021)

Table 5: Identified cellular partners of LRRK2. From (Erb and Moore, 2020; Jeong and Lee, 2020). In RED are indicated the proteins involved in vesicular trafficking. In ORANGE the proteins linked with protein translation. In BLUE proteins related to microtubules. In GREEN proteins linked with mitochondria. In GRAY proteins linked with LRRK2 regulation.

From this list, it is possible to identify pathways in which LRRK2 regulates a large number of factors. By performing an Interaction Network Analysis, Tomkins and colleagues were able to link LRRK2 to functions in intracellular organization, transport and protein metabolism (Tomkins et al., 2018). Other reports show implications in the regulations of autophagy or Golgi-related functions (Beilina et al., 2014). We will discuss in this section the different pathways where LRRK2 has been linked.

Vesicular trafficking:

From synthesis of proteins to their release, the intracellular membrane system communicates via vesicles, which is a structure enclosed by a lipid bilayer, similar to the plasma membrane. A vesicle can fuse to the plasma membrane to release its content in the extracellular space (exocytosis) or fuse with intracellular organelles. A vesicle can also be released out of the cell, for examples via specialized endosomes (Multi-Vesicular Bodies), containing intraluminal vesicles. The MVBs can be degraded via fusion with the lysosomes or released by fusing with the plasma membrane, this fusion will lead to the release of extracellular vesicles (Hessvik and Llorente, 2018). A vesicle can be formed from the invagination of the plasma membrane and

form intracellular vesicles (Endocytosis). These mechanisms are regulated by many different protein classes such as adaptor proteins, tethering proteins and coat proteins, Soluble N-ethylmaleimide-Sensitive factor Attachment protein Receptors (SNAREs) and RAB-GTPases, to name a few.

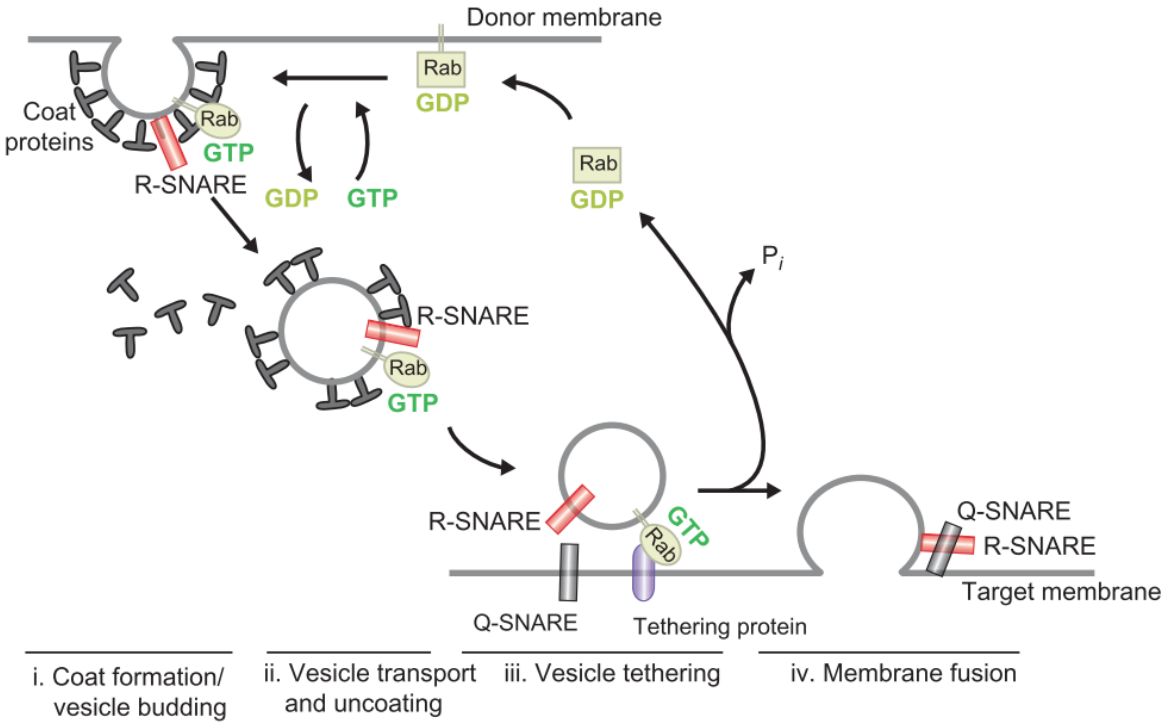


Figure 18: Basic principles of membrane trafficking. Intracellular trafficking involved different coat proteins and interaction with SNARE protein and RABs. Relationship between these proteins allow docking and fusion event on plasma membrane. From (Søreng et al., 2018).

To fuse two membranes, Soluble N-ethylmaleimide-Sensitive factor Attachment protein Receptors (SNAREs) are involved. SNARE proteins can form trans complexes and create marked curvature of membranes. They are located on vesicle membranes (v-SNARE) and on targeted membrane (t-SNARE) (Ungermann and Langosch, 2005) (Figure 18). The curvature of membrane will increase the tension on both membranes leading to hemifusion and yielding to a fusion pore. The fusion pore expands until the complete release of tension and complete fusion (Chen and Scheller, 2001).

The RAB-GTPase proteins localize to diverse compartments in cells in a close relationship to membranes. They regulate membrane trafficking events such as vesicle formation, vesicle transport and membrane fusion (Søreng et al., 2018). RAB proteins localize at specific

organelles, regulating their transport or processes such as autophagy (Ao et al., 2014). LRRK2 is found at SNARE complexes and interacts with different RAB proteins that regulates membrane trafficking in the cells (Figure 19).

LRRK2 is found in a homogenous distribution throughout the cytoplasm and associated with organellar membranes and the microtubule network (Biskup et al., 2006). LRRK2 can localize to the Trans-Golgi network (TGN) where one of its interactors, RAB29, regulates TGN structure. The overexpression of RAB29 recruits LRRK2 to the TGN and induces it fragmentation and causes centrosomal cohesion deficits by accumulation of phosphorylated RAB8a (Fujimoto et al., 2018; Madero-Pérez et al., 2018; Purlyte et al., 2018).

In addition to the many vesicular traffic interactors with which LRRK2 interacts, the role of LRRK2 in the regulation of vesicular trafficking has been confirmed in animal models. LRRK2 KO mice display increased lysosomal markers such as cathepsin D, lipofuscin and p62 in the kidney but not in the brain (Herzig et al., 2011; Tong et al., 2012; Baptista et al., 2013; Wallings et al., 2019). Overexpression of LRRK2 G2019S leads to a reduction in RAB7 activity and delays early endosomal trafficking (Gómez-Suaga et al., 2014). G2019S also results in the accumulation of multi vesicular bodies (MVB) and autophagic vacuoles (Plowey et al., 2008a; Alegre-Abarrategui et al., 2009; Gómez-Suaga et al., 2012). Similar results are obtained in the cerebral cortex of G2019S mice and in iPSC derived dopaminergic neurons from G2019S carriers (Ramonet et al., 2011; Sánchez-Danés et al., 2012; Reinhardt et al., 2013). The observations made with pathogenic mutations of LRRK2 emphasize its role in regulation of vesicle trafficking.

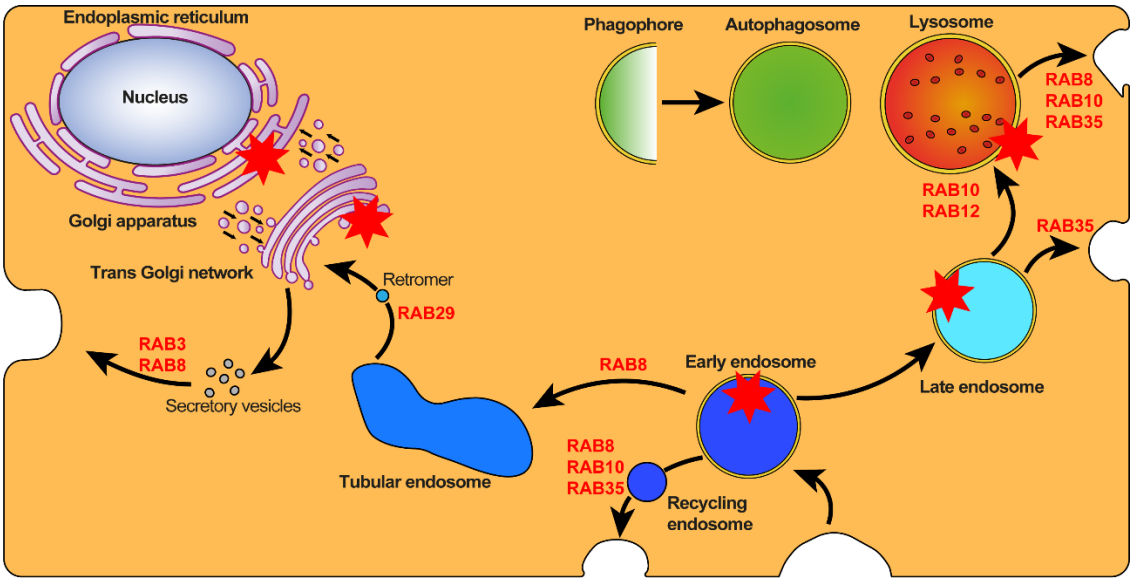


Figure 19: LRRK2 RAB substrates are implicated in the regulation of vesicular trafficking. At the Golgi, RAB1, 29 and 43 regulate the trafficking between the Golgi, TGN and ER. The secretion is regulated by RAB3a, 8, 10 35. The endosomal system transport and maturation is regulated by RAB8a, 10, 12, 35. Adapted From (Bae and Lee, 2020).

Autophagy:

Dysregulation of autophagic flux in neurons, in a context of proteinopathies such as PD, leads to the alteration of the degradation system and accumulation of toxic species such as alpha synuclein. LRRK2 have been linked with different autophagy step. It is possible to divide autophagy in different steps, phagophore formation, maturation with completion of the autophagosome, fusion with lysosome, and at the final step with the degradation of autolysosomes content (Figure 20). LRRK2 has been found to act on all of these steps.

To initiate autophagy, phosphorylated p62 binds to LC3 and ubiquitinated proteins and therefore are associated to the nascent phagophore membrane. LRRK2 phosphorylates p62 and expression of pathogenic mutants (N1437H, R1441C/G/H, Y1699C, and G2019S) result in an increased p62 phosphorylation. Overexpression of p62 increased LRRK2 degradation through autophagic pathways (Park et al., 2016). LRRK2 is able to phosphorylate Leucyl-tRNA synthetase 1 (LRS), which is responsible of mTOR activation (Bonfils et al., 2012; Ho et al., 2018b). Expression of LRS phosphomutant T293D (a phosphomimicking variant at the LRRK2 phosphosite) in cells increased the expression of LC3B and p62 and result in accumulation of alpha-synuclein (Ho et al., 2018b). The same observation was established in brain lysate from G2019S mice (Ho et al., 2018b). Expression of WT-LRRK2 and G2019S-LRRK2 in rat primary

cortical neuron culture induced decreases of LC3 puncta compared to non-transgenic cultures after induction of lysosomal biogenesis, indicating that LRRK2 expression as well as G2019S inhibits the production of autophagosomes. Authors where able to block this effect with LRRK2 kinase inhibition (Su and Qi, 2013; Manzoni et al., 2016; Wallings et al., 2019).

To form autolysosomes, the fusion between a lysosome and autophagosomes is needed. In iPSC-derived LRRK2-G2019S astrocytes, colocalization between markers of autophagosomes (LC3) and lysosomes (LAMP1) is decreased, suggesting a reduced fusion event (di Domenico et al., 2019). The same results were described in SH-SY5Y expressing G2019S mutant (Obergasteiger et al., 2020). After LRRK2 kinase inhibition, the number of autolysosomes in these cells was lowered, due to alteration of autophagosomes/lysosome fusion (Saez-Atienzar et al., 2014).

Maintenance of lysosomal pH (around 4.5-5) is a crucial step for the activity of enzymes and degradation of lysosomal content (Hu et al., 2015). Interestingly, LRRK2 interacts with the ATP6V0A1 proton pump, a risk factor for PD (Chang et al., 2017). ATP6V0A1 is involved in the acidification of various organelles (Chang et al., 2017; Wallings et al., 2019). Interestingly, in cultured primary cortical neurons, the interaction between LRRK2-R1441C and ATPV60A1 is reduced, with reduced acidification of lysosomes followed by a reduction in lysosomal degradation (Wallings et al., 2019). In LRRK2-G2019S derived fibroblasts as well as *post-mortem* tissue, enlarged lysosomes were observed (Henry et al., 2015). In HEK293T cells, lysosomal enzymes Cathepsin B and L activities was lowered with G2019S expression (Henry et al., 2015). These two enzymes, when inhibited, abolish the degradation of alpha-synuclein and promote its aggregation (McGlinchey and Lee, 2015).

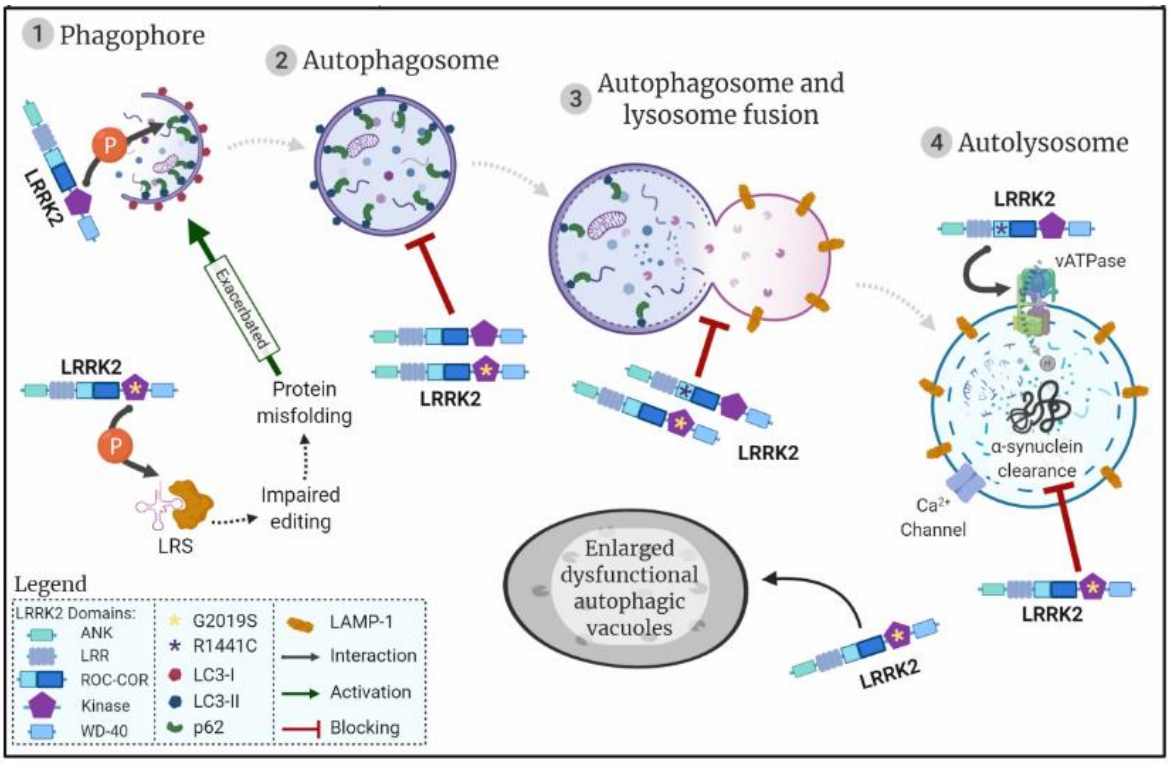


Figure 20: LRRK2 and the autophagy. At different steps indicated in brackets, LRRK2 has been linked with autophagy. (1) Once the cargo is fully encapsulated by a bi-layered membrane the autophagosome (2) fuses with the lysosome (3) to produce the autolysosome (4). Proteins/organelles are degraded via lysosomal enzymes. The diagram shows how LRRK2 wildtype and the two most common LRRK2 mutations are likely to impact autophagic flow and lysosomal protein degradation. LRRK2 is represented by its respective domains (ANK, LRR, ROC, COR, Kinase, and WD-40). LRRK2 mutations are represented by asterisks in the respective domains where they are located. G2019S, yellow asterisk in kinase domain; R1441C, purple asterisk in ROC domain; interactions represented by arrows; flow of autophagic phases represented by dashed arrows. From (Madureira et al., 2020).

Synaptic function:

BAC transgenic mice expressing LRRK2 G2019S are reported to present age dependent decreases in release and uptake of dopamine without loss of dopaminergic neurons in SNpc at 12 months of age (Li et al., 2010b). LRRK2 G2019S was responsible of degeneration of dopaminergic terminals as well as downregulation of dopaminergic homeostasis (Thomas et al., 2007). LRRK2 participates in different protein pathways regulating synaptic vesicle trafficking at the presynaptic and postsynaptic terminals.

LRRK2, by its interaction can modulate exocytosis events:

RAB3a, 3b, 3c and 3d regulate exocytosis events and lysosome exocytosis (Lledo et al., 1994; Vieira, 2018). RAB10 phosphorylation by LRRK2 induces lysosomal release (Vieira, 2018; Kuwahara et al., 2020). LRRK2 can also interact with AP3B1 which is a component of the AP3 complex to regulate synaptic vesicle generation from endosomes (Cheung and Cousin, 2012). LRRK2 modulates the SNARE complex during synaptic transmission by a negative regulation of snapin and SNAP-25 (Yun et al., 2013).

Endocytosis events can also be regulated by LRRK2 interactors:

RAB5 is an important player in the regulation of clathrin-mediated endocytosis (He et al., 2017). RAB5 is also a regulator of the readily releasable pool, a pool of vesicles at the pre-synaptic bouton that can be easily deployed compared to other vesicles (Hoopmann et al., 2010). Finally, RAB5 controls AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor internalization in a clathrin-dependent manner (Brown et al., 2005). RAB8a modules EGFR trafficking and EGFR (Epidermal Growth Factor Receptor) internalization is disrupted by LRRK2 G2019S, and the deficits of EGFR trafficking induced by LRRK2 G2019S can be rescued by RAB7 overexpression (Gómez-Suaga et al., 2014; Rivero-Ríos et al., 2019).

Due to the hyperactivity of LRRK2, increased phosphorylation of auxilin (DNAJ6) was reported in patient-derived iPSC dopaminergic neurons carrying G2019S, R1441C/G (Nguyen and Krainc, 2018). This led to an impairment of clathrin binding leading to disrupted synaptic vesicle endocytosis. LRRK2 is able to interact with and phosphorylate NSF (N-ethylmaleimide Sensitive Fusion), this interaction increases LRRK2 kinase activity and SNARE complex dissociation, leading to alteration of synaptic vesicle recycling (Belluzzi et al., 2016). LRRK2 directly phosphorylates synaptojanin-1 that leads to the disruption of the endophilin-synaptojanin1 interaction leading to a decreased endocytosis (Pan et al., 2017). Synaptojanin-1 is a crucial protein for coating of endocytosed vesicles (Cremona et al., 1999). LRRK2 is reported to phosphorylate a partner of synaptojanin-1, endophilin A, which is a protein hub for clathrin-independent endocytosis (Matta et al., 2012; Arranz et al., 2014; E et al., 2015; Renard et al., 2015).

Translation:

Dysregulation of eIF2 signaling and eIF2 phosphorylation has been demonstrated in sporadic and familial PD patients (Mutez et al., 2014). It has been suggested that key PD protein could interact with the translation machinery, resulting in its deregulation (Taymans et al., 2015). LRRK2 is reported to interact with a subset of translation initiation factors (eIF2C1, eIF2C2, eIF4B, and eIF3J) and ribosomal proteins (S15, S20 and SA) and elongation factor eEF1D (Dorval and Hébert, 2012; Martin et al., 2014; Reyniers et al., 2014) (Figure 21). Drosophila models harboring LRRK2 pathogenic mutants presented alteration in cap-dependent mRNA translation. LRRK2 is able to interact and phosphorylate 4E-BP1 leading to the release of eiF4E from the complex. eiF4E binds to the cap-structure of mRNA and allows the translation, while excess phosphorylation of 4E-BP will increase cap-dependent protein translation and dysregulation of gene expression such as Furin-1, required for synaptic function (Dorval and Hébert, 2012; Penney et al., 2016). G2019S is responsible, in drosophila, of increased phosphorylation of Ribosomal Protein S15, resulting in upregulation of protein synthesis and dopaminergic neurodegeneration in aged drosophila (Martin et al., 2014). LRRK2 also interacts with argonaute proteins that are essential for the regulation of translation/degradation of mRNA (Gehrke et al., 2010). In fibroblasts from PD patients (G2019S and sporadic cases), protein synthesis is reduced by 40%, a phenomenon that can be reverted with LRRK2 kinase inhibition (Katsuda et al., 2020).

Ribosome profiling of G2019S Human Induced Pluripotent Stem Cell (hiPSC)-derived dopaminergic neurons presented alteration of genes involved in calcium homeostasis, which could lead to disruption of mitochondrial respiration, increased ROS production (Nicholls, 2008; Surmeier et al., 2017; Kim et al., 2020).

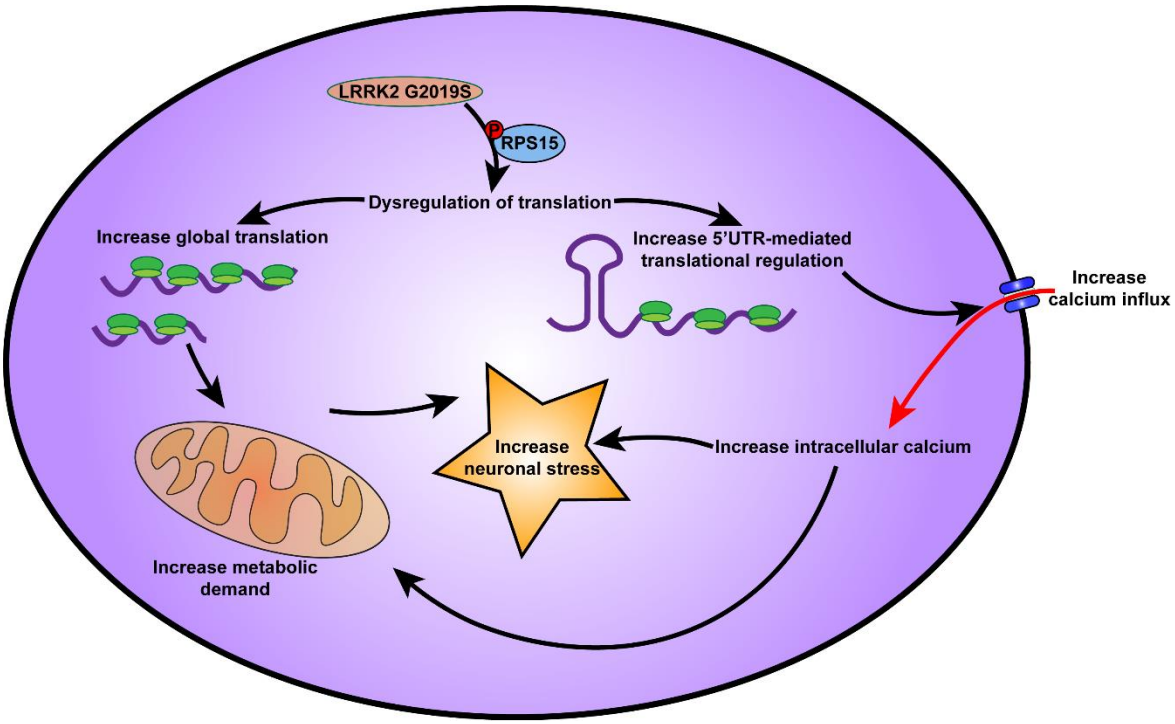


Figure 21: LRRK2 impact on protein translation and calcium homeostasis. LRRK2 pathogenic mutations have been shown to regulate proteins from the translation mechanism. LRRK2 is reported to impact the global translation, increasing the metabolic demand of the cell leading to an increased cellular stress. LRRK2 has been shown to specifically increase the 5'UTR translation, increasing the calcium influx in the cell. Increased calcium content led to impairment of mitochondrial functions and is associated to neuronal stress. Adapted from (Kim et al., 2020).

Mitochondria:

Mitochondrial dysfunction have been observed in PD patients including PD patients carrying LRRK2 mutations (Bose and Beal, 2016; Hsieh et al., 2016). LRRK2 is present on the outer membrane of mitochondria (Biskup et al., 2006). iPSC-derived neural cells from LRRK2 G2019S carriers presented damaged mitochondrial DNA (Sanders et al., 2014; Howlett et al., 2017). This damaged DNA can result from higher ROS production. Some reports suggest a small ROS production with LRRK2 pathogenic mutations (Heo et al., 2010; Angeles et al., 2011), but mutants increased the susceptibility to oxidative stress (Cooper et al., 2012).

Mitochondria fuse and divide constantly and the balance between fusion and fission constitutes one part of mitochondrial health regulation. Maintenance of the mitochondrial dynamics is crucial for ATP production, Ca^{2+} homeostasis, ROS production and apoptosis (Detmer and Chan, 2007). LRRK2 has been shown to regulate fusion/fission event. For instance, LRRK2 G2019S promotes mitochondrial fission and elongated mitochondria

(Mortiboys et al., 2010; Ho et al., 2018a, 2019). In primary cortical neurons, LRRK2 G2019S induces mitochondrial fragmentation through interaction with dynamin like protein 1 (DLP1), a mitochondrial fission protein (Niu et al., 2012; Su and Qi, 2013), which is an important factor for normal morphology of mitochondria (Pitts et al., 1999).

LRRK2 has been shown to regulate mitophagy in iPSC-derived cells. LRRK2 G2019S slows down the transport of the damaged mitochondria on the cytoskeleton by impairing the kinesin motor complex, reducing the autophagosome engulfment and its degradation (Hsieh et al., 2016). LRRK2 mutants G2019S and R1441C have been shown to regulate the PINK1/parkin-dependent mitophagy (Wauters et al., 2020). LRRK2 G2019S and R1441C increased RAB10 phosphorylation, impairing the binding with optineurin that binds to depolarized mitochondria and promotes their degradation (Lazarou et al., 2015). These effects can be reversed with LRRK2 kinase inhibition (Wauters et al., 2020).

Microtubule dynamics and neurite outgrowth:

LRRK2 G2019S and I2020T overexpression in cultured cells or *in vivo* cause neurite shortening and reduced process branching (MacLeod et al., 2006). LRRK2 pathogenic mutants are able to co-localize with microtubules in an organized and repetitive fashion (Kett et al., 2012). LRRK2 can interact with and phosphorylate beta-tubulin and activate tubulin polymerization in presence of Microtubule Associated Proteins (MAPs) (Gillardon, 2009). In Drosophila, expression of LRRK2 G2019S enhances Tau phosphorylation and mislocated Tau, resulting in dendrite degeneration (Lin et al., 2010). Expression of LRRK2 G2019S in differentiated SH-SY5Y induced neurite shortening and generated an increase accumulation of autophagic vacuoles in the soma as well as in the neurites (Plowey et al., 2008b). Cultured neurons from G2019S mice reveal alterations of neurite complexity and autophagic vacuole accumulation (Ramonet et al., 2011). When a crucial gene for the autophagosome formation (ATG7) is repressed in mice, accumulation of alpha-synuclein and LRRK2 is found in the presynaptic space (Friedman et al., 2012). This supports the idea of a disrupted transport and autophagy regulation that will lead to alpha-synuclein accumulation and disruption of the neurite's complexity.

Alpha-synuclein aggregation:

LRRK2 is reported to accelerate the propagation of alpha-synuclein aggregates in multiple models. In the alpha-synuclein A53T mouse model, the overexpression of LRRK2 increases the rate of alpha-synuclein accumulation (Lin et al., 2009) and KO of LRRK2 in mice overexpressing alpha-synuclein reduces the synuclein-induced dopaminergic neurodegeneration (Daher et al., 2014). In LRRK2 KO mice, at 20 months of age, accumulation and aggregation of alpha-synuclein is increased in the kidney due to autophagy impairment (Tong et al., 2012). Ablation of the LRRK2 kinase activity with pharmacological treatment enhance the proportion of alpha-synuclein directed to the lysosomes of alpha-synuclein transgenic mice (Daher et al., 2015; Bae et al., 2018). A mechanism due to another RAB substrate of LRRK2, RAB35 is known to regulates exosome secretion but its precise role in the protein degradation needs to be defined (Hsu et al., 2010; Bae and Lee, 2020).

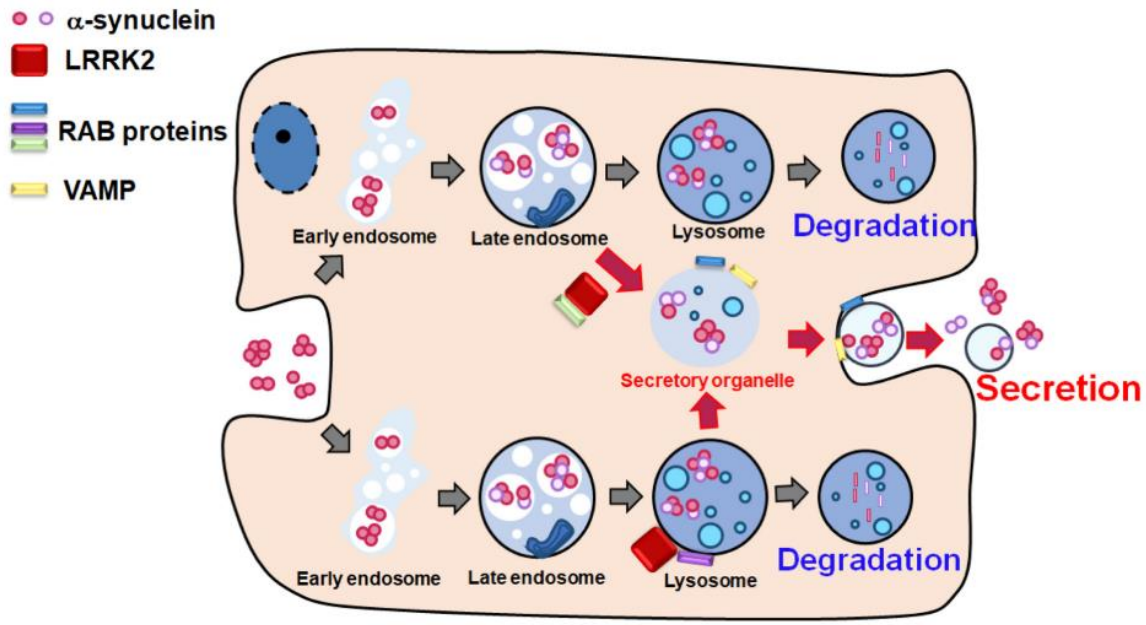


Figure 22: LRRK2-RAB mediated alpha-synuclein propagation. Secretion of alpha-synuclein is possible through secretory organelles derived from late endosomes or directly from the lysosomes. From (Bae and Lee, 2020).

2.5 LRRK2 phosphorylation

LRRK2 is a highly phosphorylatable protein (Figure 23). First, LRRK2 exists as a phosphorylated protein in mammalian cells under basal conditions as observed after metabolic labelling of LRRK2-expressing cells with radioactive phosphate or by detection of LRRK2 in phosphoprotein isolates from cell culture (Greggio et al., 2007; Lobbestael et al., 2013; Reyniers et al., 2014). Second, additional phosphorylation potential above the cellular phosphorylation of LRRK2 can be observed when purified LRRK2 is submitted to *in vitro* autophosphorylation. Third, in a similar fashion, *in vitro* incubation of LRRK2 with a separate kinase, such as protein kinase A (PKA), can also lead to additional phosphorylation of LRRK2 (Muda et al., 2014). The notion that LRRK2 is a highly phosphorylated protein is confirmed by phosphosite mapping studies via mass spectrometry, showing at least 74 phosphorylation sites on isolated LRRK2 protein, corresponding to almost 3% of all amino acid residues of the protein (Greggio et al., 2009; Kamikawaji et al., 2009; Gloeckner et al., 2010; Pungaliya et al., 2010). Phosphorylation sites include a majority of serines (59%), followed by 37% threonines and some tyrosines (4%). Further compilation of the reported LRRK2 phosphosites indicates that 37 are reported by two or more separate studies, meaning that half of the reported LRRK2 phosphorylation sites still await independent confirmation.

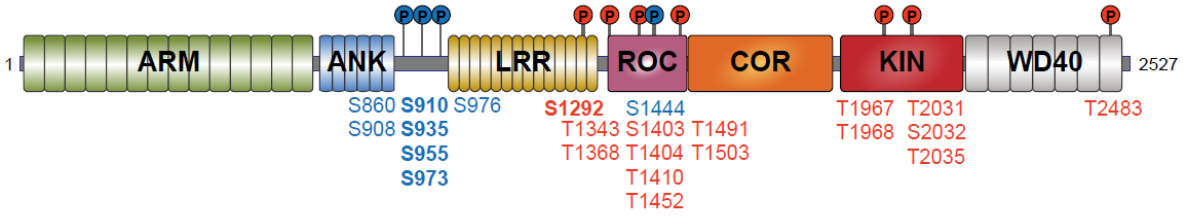


Figure 23: Schematic representation of LRRK2 domains and phosphorylation sites. Parts of known phosphorylation site are indicated. Heterologous phosphorylation sites are indicated in blue, autophosphorylation site are indicated in red. The most studied sites are indicated in bold. Adapted from (Marchand et al., 2020).

As is the case for many kinases, LRRK2 can autophosphorylate, and as for any phosphorylated kinase, it is therefore possible to divide the phosphorylated sites into two groups, the heterologous phosphorylation sites and the autophosphorylation sites. In addition, one can distinguish phosphosites that are observed from LRRK2 directly isolated from cells or tissues without any further manipulations (cellular phosphorylation sites) from sites that are

submitted to additional *in vitro* phosphorylation. Sites that are qualified as autophosphorylation sites are confirmed when their phosphorylation rates increase *after in vitro* phosphorylation, while this is not the case for heterologous phosphorylation sites. Using these criteria, 60% of the identified LRRK2 phosphorylation sites are autophosphorylation sites and 36% are heterologous, while the remaining 4% of sites have been identified as both autophosphorylation and PKA phosphorylation sites (threonine 833, serine 1443, and serine 1444) (Gloeckner et al., 2010; Pungaliya et al., 2010; Muda et al., 2014).

Looking at the distribution of the phosphorylated residues across the LRRK2 protein, one prominent phosphorylation cluster is located between the ANK and the LRR domain at serines S860, S910, S935, S955, S973, and S976 for the most studied sites. The importance of the heterologous phosphorylation sites for LRRK2 function has been supported by the findings that 14-3-3 binding to LRRK2 is dependent on S910 and S935 phosphorylation and that LRRK2 phosphorylation levels at heterologous phosphorylation sites affect subcellular distribution of LRRK2.

The LRRK2 autophosphorylation occurs on at least 20 different serine or threonine residues located in and around the ROC domain and some in the kinase domain. While *in vitro* phosphorylation has revealed a large number of autophosphorylation sites, it remains unclear which proportion of these exist under physiological conditions. One example of an autophosphorylation site identified in cells and *in vivo* is the S1292 site that is positively modulated in LRRK2 mutants (N1437H, R1441C/G/H, Y1699C, G2019S, and I2020T) (Reynolds et al., 2014; Steger et al., 2016).

2.5.1 Regulators of LRRK2 phosphorylation: Kinases

The first kinase reported as a candidate to regulate LRRK2 phosphorylation was PKA in 2007 (Ito et al., 2007). The authors of the study were able to identify PKA as an upstream kinase responsible for the phosphorylation of LRRK2 in HEK293 cells using two different potent inhibitors of PKA. They also showed that PKA efficiently phosphorylates LRRK2 K1906M kinase-inactive mutant. Several years later, two independent groups confirmed PKA as a kinase acting on the S910 and S935 sites (Li et al., 2011; Muda et al., 2014) and also on the S1444 site both *in vitro* and *in cellulo* (Muda et al., 2014). Treatment with PKA activator forskolin increased phosphorylation at S910 as well as at S1444. The S1444 phosphorylation site was proposed as a new alternate 14-3-3 binding phosphosite. However, another study showed an opposite effect of PKA activation on LRRK2, with a decrease of phosphorylation at S910, S935, S955, and S973 and reduced 14-3-3 binding on LRRK2 overexpressed in HEK293 T-Rex cells and endogenous LRRK2 in A549 lung derived cell lines (Reynolds et al., 2014). Finally, PKA activation or inhibition had no effect on the level of phosphorylation at pS935 (Hermanson et al., 2012). Further work will be needed to better decipher the role of PKA in the regulation of LRRK2 phosphorylation. Interestingly, the recent literature supports the notion of a functional cross-regulation between LRRK2 and PKA that might be cell type specific (Parisiadou et al., 2014; Russo et al., 2018).

Dzamko et al. showed that the inhibitor of Ikappa B kinases (IKKα and β) phosphorylates the S910 and S935 sites in macrophages derived from bone marrow during stimulation of Toll-like receptor signalling (Dzamko et al., 2012). Further data indicate that IKKβ is also a potential kinase regulating LRRK2 phosphorylation in SH-SY5Y and HEK293 cells (Hermanson et al., 2012). Intriguingly, LRRK2 dephosphorylation induced by kinase inhibition with LRRK2-IN1 and CZC25146 was completely prevented by LPS stimulation (Dzamko et al., 2012). Finally, Chia et al. provided the evidence that Casein Kinase 1-alpha (CK1α) is a physiologically upstream kinase regulator of LRRK2 at the constitutive phosphorylation sites using an unbiased siRNA kinome screen in HEK-293T cells as well as in the mouse brain with *ex vivo* experiment (Chia et al., 2014). In addition, the repression of the expression or inhibition of CK1α led to a decrease in phosphorylation at S910 and S935 as well as an increase in the association of ARHGEF7 with LRRK2, which decreased GTP binding. Treatment with siRNAs targeting CK1α

also reduced RAB29-dependent Golgi fragmentation caused by LRRK2, indicating that phosphorylation of heterologous LRRK2 sites modulates recruitment of LRRK2 within the TGN (Chia et al., 2014). A number of additional upstream kinases have been proposed (Lobbestael et al., 2012).

2.5.2 Regulators of LRRK2 phosphorylation: Phosphatases

The phosphoregulation of LRRK2 is a complex mechanism (Figure 24). The rapid induction of LRRK2 dephosphorylation after LRRK2 kinase inhibition suggests the involvement of protein phosphatases. Moreover, cAMP stimulation downregulated LRRK2 phosphorylation that suggests that a phosphatase may be activated in HEK293 but also in A549 cells (Hermanson et al., 2012).

The search of phosphatases related to LRRK2 pathophysiology has seen some advances in recent years. Regarding phosphatases regulating heterologous phosphorylation sites, only the alpha catalytic subunit of Protein Phosphatase 1 (PPP1CA) has been demonstrated to regulate phosphorylation of LRRK2 at S910, S935, S955, and S973 (Lobbestael et al., 2013). Indeed, pharmacological inhibition of Protein Phosphatase 1 (PP1) with Calyculin A (CalA) prevented the dephosphorylation of LRRK2 induced by LRRK2 kinase inhibitors. Interestingly, the effects of PPP1CA on LRRK2 phosphorylation were confirmed in several cell types HEK-293T, SH-SY5Y, NIH 3T3, A549, and U-2 OS but also in mouse primary cortical neurons. This shows that PPP1CA is active as an LRRK2 phosphatase independent of the cell type tested. Moreover, under LRRK2 dephosphorylation conditions, the association between PP1 and LRRK2 is increased, for example: during treatment with LRRK2 kinase inhibitors or in the presence of LRRK2 mutants with low level of phosphorylation (Lobbestael et al., 2013). Furthermore, a study on LRRK2 and oxidative stress (Mamais et al., 2014) also highlighted the importance of the physiological role of PP1 in the dephosphorylation of LRRK2. Arsenite-mediated stress leads to a reduction in the phosphorylation of LRRK2 at S910 and S935 in cell culture, and this reduction is reversed by CalA treatment. In addition, CalA counteracted arsenite and H2O2- induced S935 dephosphorylation, but only arsenite induced an increase association of PPP1CA with LRRK2 (Mamais et al., 2014). PP1 target specificity is driven by the association of regulatory subunits (Bollen et al., 2010). We do not yet know which regulatory subunits form the active PP1 holoenzyme responsible for catalyzing LRRK2 dephosphorylation. Therefore, a key issue to

understand how LRRK2 dephosphorylation is regulated is to identify the composition of the PP1 holoenzyme by identifying the LRRK2-specific subunits that form the active PP1 holoenzyme that acts on LRRK2.

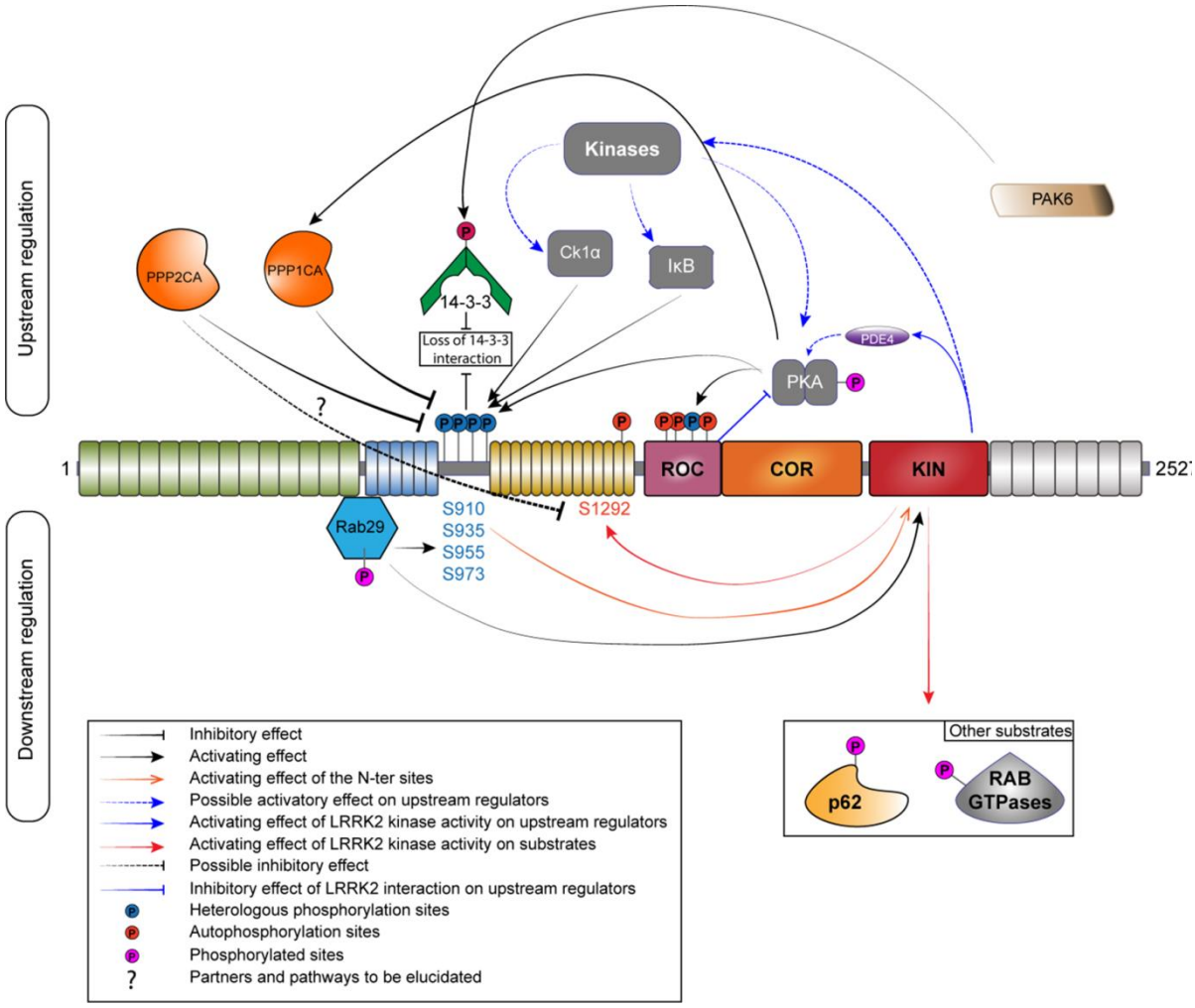


Figure 24: Phosphoregulation of LRRK2. Phosphoregulation of LRRK2 protein put together a lot of different partners, and some of those partners can also be regulated by LRRK2 itself. On the upstream regulation, the inhibitory phosphatases are localized on the left and the activating kinases are localized on the right. Kinases and phosphatases are implicated in the regulation of the N-ter phosphorylation sites (S910/935/955/973). N-ter sites and S1444 are phosphorylated by PKA while LRRK2 is also able to regulate the activity of PKA by a direct interaction with its ROC domain or by an indirect manner, by acting on the phosphodiesterase 4 (PDE4). PPP1CA has been confirmed to act on LRRK2. The holoenzyme PP2A could regulate the phosphorylation at S1292. The phosphorylation of the N-ter sites allows the interaction with 14-3-3. If phosphorylated by PAK6, the binding to LRRK2 is abolished. RAB29 interacts with LRRK2 in the Trans-Golgi network; this interaction leads to an increased phosphorylation of the N-ter and the kinase activity of LRRK2. LRRK2 can phosphorylate RAB29 and avoid LRRK2 activation, creating an inactivation loop. From (Marchand et al., 2020)

It is unclear which phosphatases are regulating LRRK2 phosphosites outside the ANK-LRR interdomain region. However, PP2A has been identified as a partner interacting with LRRK2 (Athanasopoulos et al., 2016). This study reports that LRRK2 interacts with all three subunits of PP2A and that this is mediated by the ROC domain in cultured cells. This is consistent with the recent report by Sim and colleagues who identified in a Drosophila model the three components of PP2A that are required to form a functional holoenzyme, i.e., scaffolding, regulatory, and catalytic subunits, as a modulator of LRRK2 function. Although PP2A has been found to be responsible LRRK2 dephosphorylation (Athanasopoulos et al., 2016; Drouyer et al., 2021). In addition, silencing of the catalytic subunit of PP2A by shRNA aggravated cell degeneration in SH-SY5Y cells expressing the LRRK2 R1441C variant as well as in cultured cortical neurons derived from G2019S overexpressing transgenic mice. Interestingly, relevance of PP2A as an LRRK2 phosphatase for the regulation of S1292 phosphorylation site could be demonstrated by pharmacological and genetic approaches in mutant LRRK2 flies (Sim et al., 2019). Pharmacological activation with either ceramide or fingolimod (FTY720) ameliorates their disease-associated phenotypes. In addition, under conditions of PP2A subunit overexpression, LRRK2 phosphorylation at S1292 was found reduced. This is consistent with a report demonstrating that S1292 dephosphorylation is mediated by phosphatases that are sensitive to CalA and okadaic acid (Reynolds et al., 2014).

2.5.3 Phosphorylation phenotypes of LRRK2

While the global picture of how LRRK2 phosphorylation levels at its various phosphorylation sites influence LRRK2 function is still incomplete, several studies have shown that changes in LRRK2 phosphorylation influences LRRK2 biochemical or cellular properties. Links between LRRK2 phosphorylation and disease or pathological mechanisms are being established in different ways: by monitoring LRRK2 phosphorylation in patient-derived samples, disease models, and study of phosphomutant forms of LRRK2 and how these affect cellular phenotypes. To investigate the links between LRRK2 phosphorylation and its kinase activity, phosphomutants are used. When testing for autophosphorylation activity of the S910A/S935A mutant, no change in S1292 autophosphorylation was observed in cells compared to WT (Reynolds et al., 2014). Other phosphorylation site mutants or combinations of phosphorylation site mutants from the S935 cluster on LRRK2 kinase activity remain to be tested. Mutant S2032A, T2035A, and S2032A/T2035A showed a reduced autophosphorylation activity, assessed by *in vitro* autophosphorylation with 32P-labeled ATP (Li et al., 2010a). The overall conclusion here is that specific LRRK2 phosphorylation sites may affect LRRK2 kinase activity. Conversely, there is not a uniform correlation between LRRK2 phosphorylation and its kinase activity. Besides kinase activity, GTP-binding and GTPase activity may also be influenced by LRRK2 phosphorylation levels. Of particular interest are the autophosphorylation sites that are clustered in and around the ROC GTPase domain and several sites map to G-box motifs that mediate GTP binding, which point to the possibility that autophosphorylation may affect GTPase functions (Webber et al., 2011; Taymans, 2012). In particular, some phosphomimetic mutants such as T1491D and T1503D showed impaired GTP binding, although GTP binding is unchanged for another phosphomimetic LRRK2 mutant, T1410D (Kamikawaji et al., 2009; Webber et al., 2011). By extension, a potential role of heterologous phosphorylation sites of LRRK2 on its GTP-related functions cannot be excluded and has been studied in the present work.

2.6 LRRK2's interactors

The phosphorylation at S910 and S935 sites, as well as the S1444 site, have been shown to be responsible for the interaction of LRRK2 with 14-3-3 proteins (Dzamko et al., 2010; Nichols et al., 2010; Li et al., 2011; Muda et al., 2014). Indeed, phosphodead mutations (substitution of the serine residue for alanine) at S910 and S935, but not at S955 and S973, lead to a strong reduction of 14-3-3 binding (Doggett et al., 2012). Moreover, if 14-3-3 binding is blocked using difopein (dimeric fourteen-three-three peptide inhibitor), LRRK2 appears to be dephosphorylated at S910 and S935 (Fraser et al., 2013; Zhao et al., 2015). Therefore, it has been suggested that 14-3-3 interaction could protect against dephosphorylation at these two phosphorylation sites and influence the subcellular localization of LRRK2 in the cell (Nichols et al., 2010; Li et al., 2011). The absence of 14-3-3 binding to LRRK2 when S910 and S935 sites are dephosphorylated induces accumulations of LRRK2 in the cytoplasm of cells. Accumulation types include filamentous "skein-like" structures (Dzamko et al., 2010; Nichols et al., 2010; Reyniers et al., 2014) and/or punctate accumulations (Chia et al., 2014). Likewise, pathogenic mutants that exhibit a reduction in phosphorylation at S910 and S935 sites (N1437H, R1441C/G/H, Y1699C, I2020T, and the risk factor G2385R, but not the G2019S variant) display a similar loss of 14-3-3 binding and relocalization of LRRK2 to cytoplasmic accumulations pools and filamentous skein-like structures (Dzamko et al., 2010; Nichols et al., 2010; Deng et al., 2011; Doggett et al., 2012). The brain is the tissue with the highest 14-3-3 concentration (Boston et al., 1982). The role of 14-3-3 proteins in neurodegeneration has been reviewed in (Shimada et al., 2013) and is known to affect protein localization and activity through its binding to targeted substrates. Interestingly, there is an additional layer of regulation of 14-3-3 proteins that affects LRRK2 phosphorylation. Indeed, 14-3-3γ is phosphorylated by PAK6 (kinase 6 activated by p21), a serine/threonine kinase (Civiero et al., 2017). Phosphorylated 14-3-3γ is no longer able to bind S935 site, thus causing its dephosphorylation.

Several teams have demonstrated an interaction of LRRK2 with RAB29 (MacLeod et al., 2013; Beilina et al., 2014; Liu et al., 2018; Purlyte et al., 2018). This interaction takes place in the ANK domain of LRRK2 and regulates the heterologous phosphorylation sites of the S935 cluster (Purlyte et al., 2018). Purlyte et al. discovered that all RAB29 binding-deficient ankyrin domain LRRK2 variants are also dephosphorylated on these heterologous phosphorylation sites. In

addition, the loss of endogenous RAB29 in A549 cells moderately reduces the phosphorylation of these sites. However, these data do not exclude the possibility that another Golgi resident, a protein kinase or phosphatase, regulates the phosphorylation of these sites. The LRRK2 kinase activity seems also to be regulated by RAB29 through the phosphorylation of the S935 cluster. In fact, the kinase activity of LRRK2 is reduced when a phosphomimetic mutant of RAB29 is expressed but no change is found with the dephosphomimetic form of RAB29. In particular, RAB29 is itself phosphorylated by LRRK2, suggesting that RAB29 binding to LRRK2 may mediate a potential positive feedback loop between LRRK2 phosphorylation at the S935 cluster and LRRK2 kinase activity, although further work would be required to confirm this (Purlyte et al., 2018). LRRK2 has other RABs as substrate but none of these have yet been reported to increase LRRK2's kinase activity.

2.7 Involvement of LRRK2 in other pathologies

2.7.1 Cancer

As seen in section2.3-translation, LRRK2 is able to regulate translation through regulation of eIF4E-binding protein is known to be a specific target of the Drosophila homologue of mTOR (Imai et al., 2008). The deregulation of mTOR by LRRK2 might occur in some cancers (Looyenga et al., 2011). In type 1 papillary renal cell carcinoma, LRRK2 was amplified and overexpressed (mRNA, DNA and protein level) (Gera et al., 2004). Another evidence of a possible implication of LRRK2 in cancer is the number of mutations identified in the catalogue of Somatic mutations In cancer database (human cancer mutation database) founded in a large number of tumour types.

2.7.2 Leprosy

Leprosy is a chronic disease caused by bacteria, *Mycobacterium leprae*. The disease progression is known to be dependent of the host genetic background (Fava et al., 2019) and patients are subjected to Type 1 reaction leading peripheral nerve damage. LRRK2 has been identified by GWAS to be associated to leprosy (Zhang et al., 2009). The LRRK2 variant, R1628P, was recurrent in patients without Type 1 and seems to confer a protection against Type 1 reaction in leprosy.

2.7.3 Crohn's disease

LRRK2 N551K and R1398H are protective risk factors for Crohn's disease (Hui et al., 2018) as well as PD. Hui and Collaborators also identified the risk factor N2081D, that increases the kinase activity of LRRK2 and reduced acetylation of alpha-tubulin and decrease lysosomal pH whereas the pH was increased for N551K and R1398H. These results present similarities between PD and Crohn's disease with LRRK2 mutants (Hui et al., 2018).

2.8 Kinase inhibition

Many pharmaceutical groups have since developed inhibitors that target the LRRK2 kinase function converging on ATP active site and describe at type I inhibitor or more recently type II inhibitor (non-ATP-competitive inhibitor). Starting in 2006, different scaffolds against the ATP site were publish (Deng et al., 2012; Kethiri and Bakthavatchalam, 2014; Galatsis, 2017). In 2018, the first clinical trial (NCT03710707) was announced by Denali Therapeutics Inc., followed by a second clinical trial in 2019 (NCT04056689).The structures of DNL201 and DNL151 are not published yet (Konstantinidou et al., 2021).

As mentioned in the section on LRRK2 Phosphatases, pharmacological inhibition of LRRK2 kinase activity induces LRRK2 dephosphorylation. Moreover, the induction of LRRK2 ubiquitination has been observed after LRRK2 pharmacological kinase inhibition followed by decreased protein levels, due to proteasomal degradation (Zhao et al., 2015; Lobbestael et al., 2016). This suggests that one of the consequences of prolonged LRRK2 dephosphorylation at the S935 cluster may be LRRK2 degradation, although this effect may be tissue and condition specific. In rats, administration of LRRK2 kinase inhibitor PFE360 in food leads to a decrease of LRRK2 total protein level in the brain but not in lung (Kelly et al., 2018). In contrast, loss of LRRK2 protein level was not detected in mouse brain, consistent with results reported in other studies using MLi-2-treated mice (Fell et al., 2015). At the phenotypic level, LRRK2 kinase inhibitors induced abnormal cytoplasmic accumulation of secretory lysosome in the lungs but no change in the kidney in non-human primates (Fuji et al., 2015). Six hours of treatment with inhibitors of CK1α, an upstream kinase of the S935 cluster, induced dephosphorylation of S935 and protein destabilization. In fact, CK1α inhibition is able to destabilize LRRK2 mutant R1441G/I2020T and also mutant without ARM domain (De Wit et al., 2019). While these data suggest the notion that LRRK2 dephosphorylation at S935 cluster may be a priming event for LRRK2 degradation, the reality of the mechanism is likely more complex. Loss of phosphorylation does not seem to be enough to destabilize the protein; LRRK2 dephosphomutant at six heterologous sites for S908A/910A/935A/955A/973A/976A does not show reduced basal expression levels, but this mutant is still degraded after 24 h of pharmacological inhibition in cell culture (De Wit et al., 2019). Other examples of discrepancies in LRRK2 expression in different conditions include KI mice for the kinase dead

variant of LRRK2, D1994S, that display decreased protein levels. However, those observations are not replicated in cells (Herzig et al., 2011). Also, R1441G and Y1699C mutants with low GTPase activity and reduced steady-state phosphorylation at the S935 cluster have an increased basal level of ubiquitination compared to the I2020T mutant that shows normal GTPase activity and increased kinase activity (De Wit et al., 2019). Pharmacological inhibition does not affect the ubiquitination level of those mutants and no destabilization is found after 48 h of kinase inhibitor treatment by MLi-2 or PFE-475. Ubiquitination level of those mutants can be restored with the PP1 and PP2A inhibitor CalA. Intriguingly, the N-terminal LRRK2 sequence as well as the LRRK2 S935 phosphosite is involved in inhibitor-induced LRRK2 destabilization. Indeed, a truncated form of LRRK2 (170-kDa) that lacks the ARM domain is dephosphorylated on S1292 after kinase inhibition but not destabilized. Nevertheless, this version of LRRK2 does not present a phosphorylation at S935 (De Wit et al., 2019). Due to the LRRK2 protein destabilization observed in certain conditions after kinase inhibition, it should be noted that some of these phenotypes may correspond to phenotypes observed in LRRK2 KO animals. For instance, increased number and size of lysosomes in kidney proximal tubule cells and lamellar bodies in lung type II cells is found in LRRK2 KO mice (Herzig et al., 2011), while similar findings are made in LRRK2 KO rats (Baptista et al., 2013). Further research should be performed to further determine the hypothesized parallel between LRRK2 kinase inhibition and LRRK2 KO.

Part 3: Objectives

Understanding the pathophysiological mechanisms involved in Parkinson's disease is a crucial issue in the search for a cure. The LRRK2 protein is intimately linked to PD pathophysiology and constitutes an interesting therapeutic target for novel PD therapies. Indeed, expression of LRRK2 pathogenic mutations such as G2019S lead to deleterious phenotypes in cells and animal models. The phosphorylation of LRRK2 that is the focus of this PhD thesis is a key property of LRRK2 that may be central to LRRK2 pathogenic mechanisms, although some questions remain. *Post*-mortem analysis of PD patient brains revealed dephosphorylation of LRRK2, and similar observations are made after treatments with experimental therapies under clinical trials. It is not clear if phosphorylation or dephosphorylation is associated to healthy of deleterious conditions. The elucidation of the cellular phenotypes of LRRK2 phosphorylation has the potential to lead us to further understand the link between phosphorylation of LRRK2 and PD and open future venues to develop novel treatments.

We hypothesize that dysregulation of LRRK2 phosphorylation could contribute to PD pathogenesis by modification of it control of proteins in charge of maintaining cellular homeostasis, in particular processes related to vesicular physiology or lysosomal dysfunction.

The overall objective of this PhD thesis is to determine whether a specific phosphorylation profile of LRRK2 can drive LRRK2 into a toxic state.

To achieve this evaluation, we generated expression constructs of phosphomutant forms of LRRK2, i.e. LRRK2 that is mutated at its phosphorylation sites in order to mimic phosphorylation or dephosphorylation and studied how these impacted **(1)** LRRK2 biochemical properties (Catalytic activities, protein binding, protein phosphorylation), **(2)** its subcellular localization, **(3)** known cellular phenotypes of LRRK2 such as lysosomal protein degradation, neurite outgrowth, or RAB29 induced kinase activation (Figure 25).

Together, these results will help us to increase the knowledge on the precise role of LRRK2 phosphorylation and how it may be associated to deleterious phenotypes. Taken together, these results will provide new insight between LRRK2 phosphorylation and PD pathophysiology.

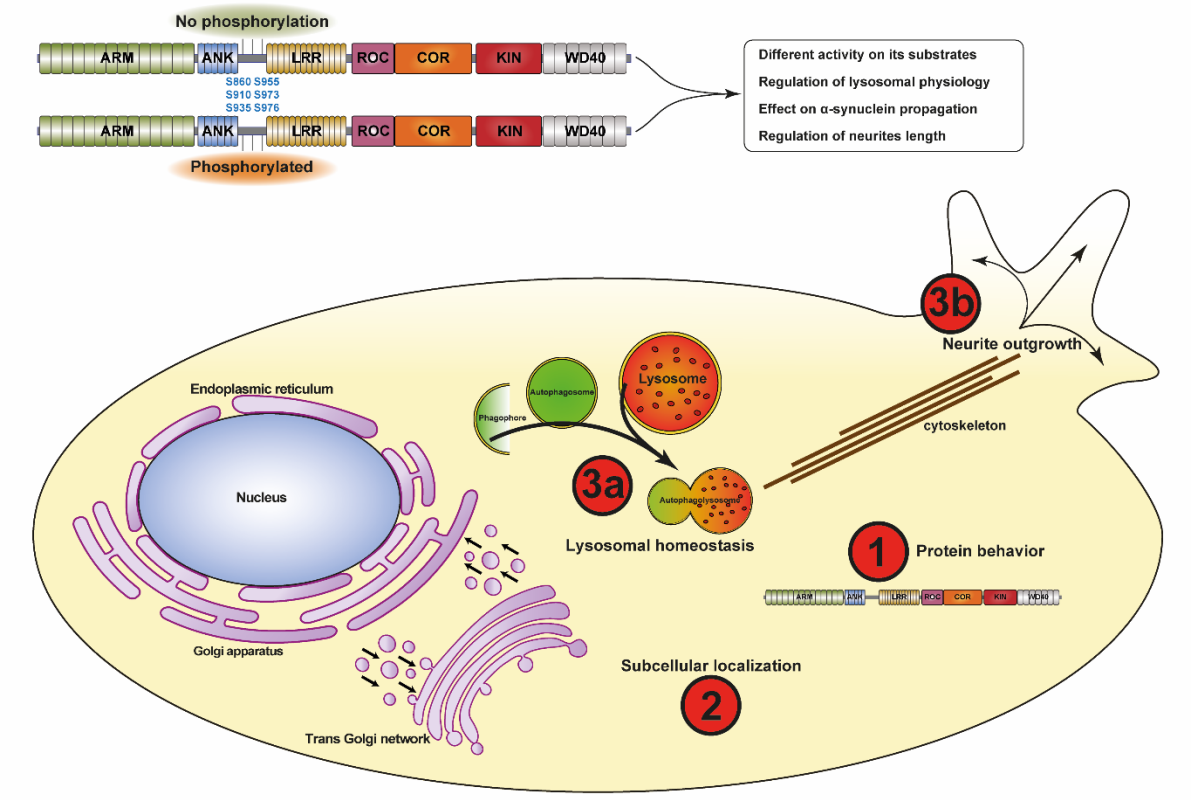


Figure 25: Objectives. Depicted is a schematic representation of the sub-aims of the project. **(1)** Could LRRK2 phosphorylation profile be in charge its biochemical properties? **(2)** Is LRRK2 phosphorylation in charge of its localization? **(3)** Is LRRK2 phosphorylation profile a regulator of neurite outgrowth, a regulating factor of the lysosomal homeostasis?

Part 4: Materials and methods

1) Models

1.1) Cellular models

HEK293T were grown in Dulbecco's modified Eagle's medium containing high glucose 4500 mg/L (ThermoFisher, USA) and 10% fetal bovine serum, HEPES 25mM (Life technologies), Penicillin-Streptomycin 20U/mL (ThermoFisher, USA) at 37°C.

SH-SY5Y were grown in 50% DMEM-F12 and 50% MEM (ThermoFisher, USA) containing 10% fetal bovine serum, non-essential amino acids (Life technologies, USA), L-Glutamine 1X (Life Technologies), Penicillin-Streptomycin 20U/mL (ThermoFisher, USA) at 37°C.

PC12 were grown in RPMI 1640 medium (ThermoFisher, USA) containing 10% fetal horse serum, 5% fetal bovine serum. Supplemented with 20U/mL Penicillin-Streptomycin (ThermoFisher, USA). Differentiated cell were grown on collagen-coated plates. Differentiation was induced with RPMI 1640 medium with 1% fetal horse serum and 0.5% fetal horse serum containing 50µg/mL of NGF, medium was changed every 48H. Differentiation was completed when neurites network is fully developed, after 10 days of differentiation.

1.2) Plasmid construction

1.2.1) List of plasmids

LRRK2 was previously cloned in or lab into the pLV.CSJ vector backbone which is itself derived from pLV-mCherry (Addgene plasmid #36084). pLV-CSJ-mCherry-LRRK2 plasmids were generated by excising the 3flag sequence (NheI and BamHI) and ligating the mCherry sequence (obtained by PCR using the pLV-mCherry and primers with sequences). All coding sequences were sequenced (Eurofins genomics) and digested with EcoRI and EcroRV enzymes (New England Biolabs) (Figure 26).

Plasmid name	Protein expressed	Plasmid size	Size coding sequence	Host sequence
pLV-CSJ-3xFLAG-LRRK2	LRRK2	14373 bp	7659 bp	*Human*
pLV-CSJ-mCherry-LRRK2	LRRK2	1517 bp	8294 bp	*Human*
Myc-RAB29	RAB29	4967 bp	744 bp	*Human*
HA-14-3-3zeta	14-3-3zeta	4852 bp	740 bp	*Human*

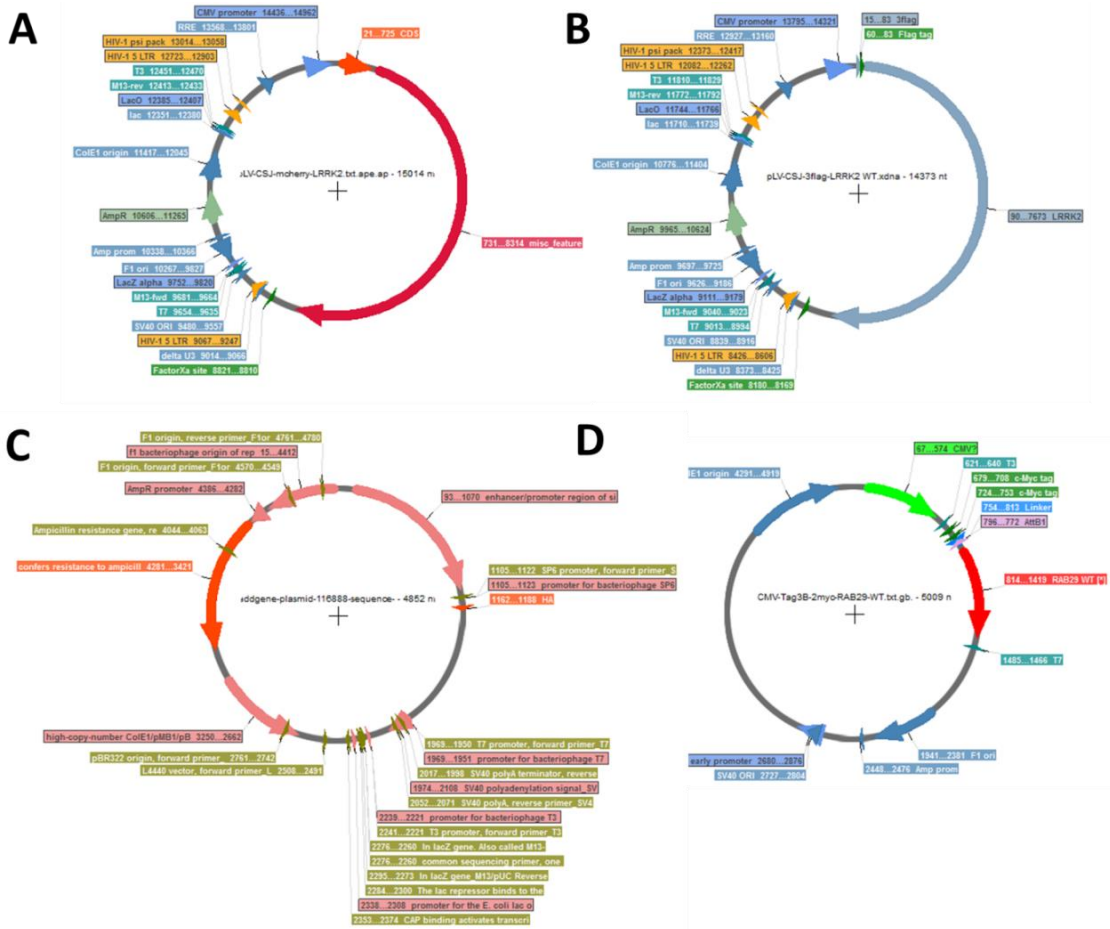
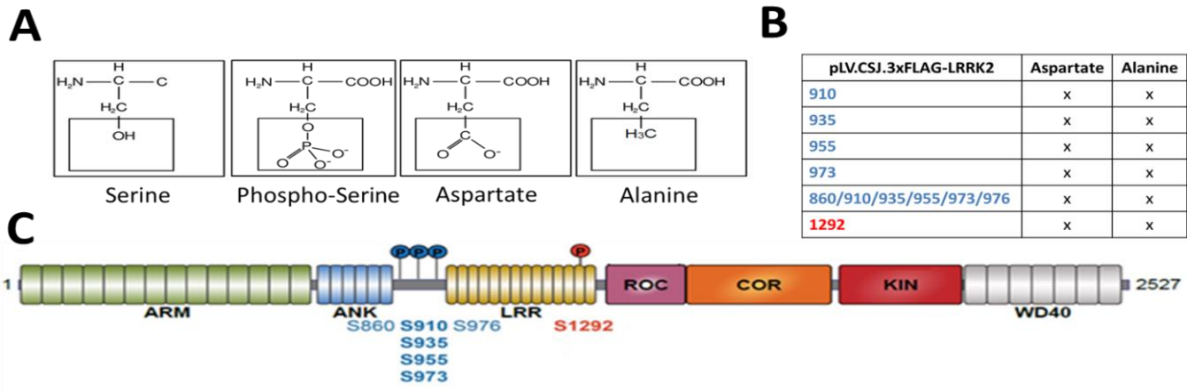


Figure 26: Plasmid map of vectors used in this study. (A) Plasmid pLV-CSJ-3xFLAG-LRRK2. (B) Plasmid pLV-CSJ-mCherry-LRRK2. (C) Plasmid Myc-RAB29. (D) Plasmid HA-14-3-3zeta.

1.2.2) Site directed mutagenesis

Mutations (S910A, S910D, S935A, S935D, S955A, S955D, S973A, S973D, S1292A, S1292D, S860/910/935/955/973/976A, S860/910/935/955/973/976D) were introduced using site directed mutagenesis or synthesized on demand at a cloning service company (eZyvec, Lille, France). Alanine (A) substitution result in complete dephosphorylation while Aspartate (D) mimic the phosphorylation of a phospho-serine (Figure 27, A). pLV.CSJ-mCherry-LRRK2 plasmids were generated by excising the 3flag sequence (restriction sites to include) and ligating the mCherry sequence (obtained by PCR using the pLV-mCherry and primers with sequences). All coding sequences were sequenced (Eurofins genomics) and digested with EcoRI and EcroRV enzymes (New England Biolabs).



pLV.CSJ.3xFLAG-LRRK2	Aspartate	Alanine
910	x	x
935	x	x
955	x	x
973	x	x
860/910/935/955/973/976	x	x
1292	x	x

Figure 27: Construction of phosphomutant of LRRK2. (A) structures of the amino acid serine, a phosphorylated serine, mimicking aspart since the phosphorylation of serine and alanine that mimick a dephosphorylated serine. (B) All phosphomutants generated by site-directed mutagenesis from the plasmid pLV.CSJ-3xFLAG-LRRK2. (C) Localization of the different positions of the generated phosphomutants, heterologous phosphorylation sites are in blue and autophosphorylation in Red.

1.3) Cell transfections

Cells were plated and at transfected with FuGENE® 6 Transfection Reagent (Promega Corporation, Madison, USA) at 30% confluency with a ratio 3:1 or with polyethylenimine (PEI) (Sigma Aldrich, Darmstadt, Germany), at 50% confluency with 1µg of DNA for 3µL of PEI (1 µg/µL). Culture medium was changed after 24H of incubation with transfection agents.

1.4) Cell transduction

To obtain stable expression in our cell models we use transduction with lentiviral particles. This expression method allows us to maintain our cell lines overtime with identical protein expression through time.

1.4.1) Lentiviral particle production

Lentiviral particles encoding 3xFLAG-LRRK2; mCherry-LRRK2 construct were prepared as described (Lobbestael et al., 2010). A mixture of 3 different plasmids is needed, packaging plasmid, envelop plasmid and the transfer plasmid encoding the sequence of interest. HEK293T are triple transfected with these plasmids at 60% confluency. Medium is refreshed at 24H post-transfection with 10mL of Optimen 0% FCS. 48H later, cell supernatant containing LV particles is filtered through 0.45µm filter and concentrated with Amicon filter unit (Merck Millipore, USA). Lentiviral particles concentration is measured by ELISA quantification of the capsid protein P24 (Anti-HIV p24, Eurobio).

1.4.2) Cell transduction and selection

Cells are plated into P24 dish, with same amount on lentiviral particles for each LRRK2 construction. Alpha-synuclein LVs co-express a resistance to blasticidin, allowing us to select the cells transduced with the lentivectors. Transduced cells were selected with 15µg/ml of blasticidin for one week. For LRRK2 constructs, we use Fluorescent Activated Cell Sorter cytometer to sort the cells positive for mCherry-LRRK2.

2) Molecular biology

2.1) Western Blot

2.1.1) Protocol

Quantity of lyzed samples is determined with Pierce BCA protein assay kit (Thermo scientific, USA). 10µg of proteins are mixed with NuPAGE LDS sample buffer 4X (Life technologies, USA) and reduced 10mn at 95°C. Proteins samples are loaded into a Novex 4-20% Tris-Glycine mini

gel (Fisher Scientific, USA) into a Tris-Glycine running buffer (Life technologies, USA) for 45mn at 225V.

The proteins are transferred on a PVDF membrane low fluorescence (Bio Rad, USA) for 16H at 5V. Membranes are blocked in milk 5% in TNT 1X (15mM Tris base, 0.14M NaCl, 0.5M tween 20, pH 8) for 1H at room temperature. The membrane and then incubated with selected antibodies in Signal Boost solution (Merck Millipore, USA) for 16H at 4°C. After 4 washes in TNT 1X membranes are incubated with secondary antibodies for 2H at RT, washed in TNT and imaged with Typhoon FLA 9500.

The quantification of the evolution of phosphorylation is done with the use of ImageQuant software (GE Healthcare Life Sciences, USA), for each phosphorylation site the levels observed for the phosphomutants are compared to the level of the wild form of the protein to allow us to evaluate the variations in the level of phosphorylation of the other phosphorylation sites.

2.1.2) List of antibodies

Name	Target	Type	Clone	Concentration for WB	Concentration for ICC
N241A/34	Total LRRK2	Monoclonal	N241A/34	1µg/mL	1µg/mL
MJFR11	pS955-LRRK2	Monoclonal	MJF-R11 (75-1)	1/1000	
MJFR12	pS973-LRRK2	Monoclonal	MJF-R12 (37-1)	1/1000	
MJFR19	pS1292-LRRK2	Monoclonal	MJFR-19-7-8	1/1000	
UDD1	pS910-LRRK2	Monoclonal	UDD1 15(3)	1/1000	
UDD2	pS935-LRRK2	Monoclonal	UDD2 10(12)	1/1000	
FLAG M2	FLAG tag	Monoclonal	M2	1/1000	1/1000
Myc	Myc tag	Monoclonal	4A6	1/2000	
GFP	GFP tag	Monoclonal		1/1000	
RAB8a	Total RAB8A	Monoclonal	D22D8	1/1000	1/200
RAB8a p T72	RAB8a-pT72	Monoclonal	MJF-R20	1/1000	
RAB10	Total RAB10	Monoclonal	D36C4	1/1000	
pRAB10 p T73	pT73-RAB10	Monoclonal	MJF-R21	1/1000	

RAB29	Total RAB29	Monoclonal	MJF-R30-104	1/1000	
RAB29 pT71	pT71-RAB29	Monoclonal	MJF-R24-17-1	1/1000	
LAMP2a	Total LAMP2	Monoclonal	EPR4207(2)		
LC3B	Total LC3B	Polyclonal			
Tubulin	Tubulin	Monoclonal	DM1A	1/500	
B-Actin	β-Actin	Monoclonal	AC-15	1/5000	
Alexa Fluor 488 anti-mouse				1/1000	1/1000
Alexa Fluor 568 anti-rabbit				1/1000	1/1000
IR Dye 680				1/1000	

Table 6: List of antibodies used in the present study.

2.1.3) LRRK2 purification

HEK293T expressing 3xFLAG-LRRK2 were lysed in IP lysis buffer (Tris 20mM pH7.4, NaCL 150mM, EDTA 1mM pH 8.8, Glycerol 10%, Triton x-100 1%, protease/phosphatase inhibitor 1X) and incubated with anti-FLAG-M2-magnetic beads (Sigma-Aldrich, USA) overnight at 4°C. Beads were washed in different buffer (4X IP lysis buffer) followed by (2X elution buffer, Tris pH 7.4 25mM, NaCl 200mM, MgCl2 5mM, DTT 1mM, Triton X-100 0.02%). LRRK2 proteins are eluted from the beads by competition with 0.1µg/µL of 3xFLAG peptide in elution buffer for 30mn at 4°C on a vertical agitator. Quantities of purified protein are quantified by silver staining using standard dilution of BSA.

2.2) LRRK2 enzymatc activity tests

2.2.1) in vitro kinase assays

-^{32}P incorporation:

The kinase activities of purified LRRK2 were measured at 30°C in Kinase assay buffer consisting of 25mM Tris (pH7.5), 15mM MgCl2, 20mM β-glycerol phosphate, 1mM sodium fluoride, 1mM EGTA, 1mM sodium orthovanadate, 2mM DTT and 0.1mg/ml BSA. 0.1µM purified LRRK2 and 75µM LRRKtide (LRRK generic substrate) were used in the assay. The reaction was initiated by addition of 25µM 32P-γ-ATP (4Ci/mmol). At different time points until 30min of reaction, the reaction mixtures were quenched in 100mM ice-cold EDTA. The quenched samples were

then spotted on P81 phosphocellulose paper discs pre-rinsed with 75mM ice-cold phosphoric acid and further washed with phosphoric acid to remove free ATP. The paper discs were then air dried before scintillation counting.

-Phosphorylation of LRRK2 substrate:

40ng of purified LRRK2 proteins are mixed with 200ng of recombinant RAB8a (Abcam) and 100µM of ATP in kinase buffer (Tris pH 7.4 25mM, MgCl2 10mM, DTT 2mM, Triton X-100 0.02%) for 1H at 37°C. Phosphorylation of RAB8a is assessed by SDS-PAGE, followed by western blotting for total and phosphorylated RAB8a.

2.2.2) GTPase assay

LRRK2 WT and mutants are produced in HEK293T cells and purified on beads as described in 2.2.1. Hydrolysis of GTP is measured at 25°C in a solution (20mM HEPES pH 7.5, 150mM NaCl, 5mM MgCl2, 5% glycerol and 1mM DTT). The resulting solution containing GTP and newly produced GDP is separated on a column (C18-reversed phase, Phenomenex, Jupiter 5µm C18 300 Å) coupled with an HPLC system (Waters). An absorbance measurement is made at 254nm, the surface of the GDP is converted into concentration with a comparison to a standard curve.

2.3) Co-localisation LRRK2/RAB8a

2.4) Immunocytochemistry

SH-SY5Y stably expressing 3xFLAG-LRRK2 WT and its mutants are splitted and transfer to P24 plate containing glass coverslips coated with Cell tak solution (292.5µL of bi-carbonate buffer, 2.5µL of NaOH 1M, 5µL of Cell Tak). Once desired confluence obtained, cells are washed one time with cold PBS and fixed for 20mn with paraformaldehyde 4%. Coverslips are washed with PBS and permeabilized for 5mn with triton 0.1% solution and cells are blocked with PBS + 0.5% BSA for 20mn. Primary antibody is incubated overnight at 4°C, cells are washed 3 times with PBS and coverslips are incubated with secondary antibody for 2H at room temperature with fluorescent antibodies (See table 6 for antibodies concentration). Coverslips are placed on glass slides with a drop of mounting medium (ProLong Diamond black Antifade Mountant with DAPI, Invitrogen). Coverslips are visualized through confocal microscope Zeiss LSM 710 and analysed with IMARIS software.

3) Lysosomes

3.1) Isolation of lysosomes from cells

HEK293T cells express 3xFLAG-LRRK2 cultured for 24H in a medium containing an iron-coupled dextran. This compound accumulates in lysosomes. After a washing time of 24H in a medium without dextran the cells are centrifuged 60g for 5mn then washed with PBS. A mechanical lysis is performed with a Dounce Tight followed by 8 passes in a 23 G needle. After a 400G centrifugation for 10 min the lysate is placed on a magnetic column (MS Coumns, Miltenyi Biotec) (Figure 28). The lysosome-enriched fraction is then collected by detaching the column from the magnetic base in PBS/0.1mM sucrose buffer. The samples are then analysed by western blot.

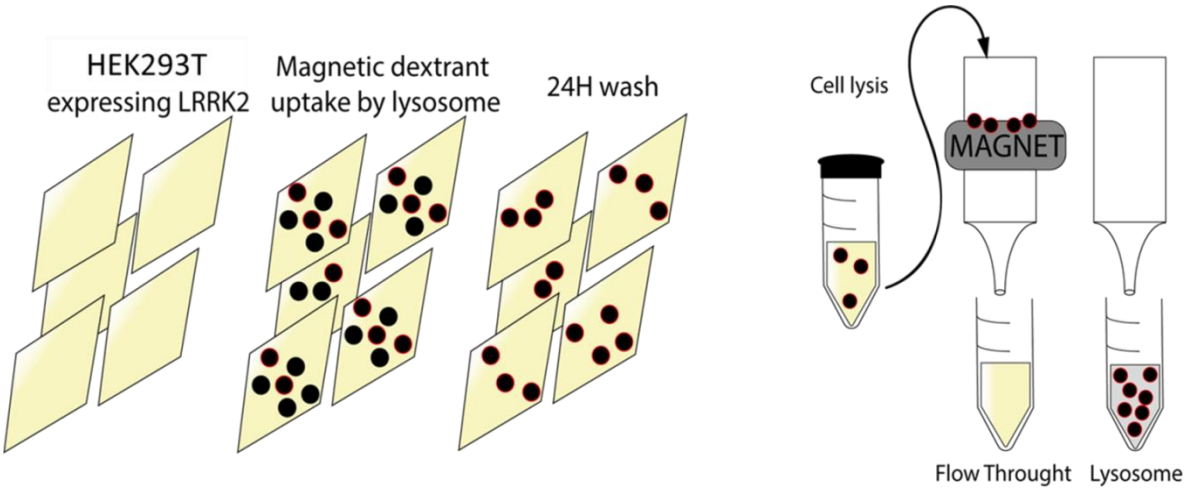


Figure 28: Lysosomes isolation with dexomag40 treatment. Briefly, cells uptake a ferromagnetic compound, dexomag40, that will be integrated into the endosomal system. After 24H of wash, lysosomes are enriched with the ferromagnetic compounds. After the lysis, magnetic lysosomes are collected on a magnetic stand for further analysis.

3.2) Image Stream analysis

Imaging of lysosomal density is measured by flow cytometry-microscopy. Image Stream MK2 instrument integrates flow cytometer coupled with fluorescence microscopy it allows the measurement at single cell cytometry data and microscopy. This allows us to measure at high throughput the quantification of lysosomes density for a representative large number of cultured cells.

3.2.1 Lysosomal density and lysosomal enzyme activity

5 million PC12 cells are centrifuged 500G, 5mn and resuspended in 1mL of medium containing 10µg of Hoechst 33258 (Invitrogen). After 30mn of incubation at 37°C cells are centrifuged 500G for 5mn and incubated in medium containing 75nM of Lysotracker Deep Red (ThermoFisher, USA) and 75µg of FDGlu (Tebu-Bio, France) for 1H at room temperature. Cells are centrifuged and resuspended in 20µL of PBS and EDTA 0.5M. Cells are analysed by ImageStream MKII at 60X magnification and spectral compensation was carried out of single stained controls. Data were analysed with IDEAS Software (EMD Millipore).

4) Incucyte analysis

To analyse the neurite complexity, we used the IncuCyte live-cell imaging system (Sartorius, UK). 4000 PC12 cells stably expressing mCherry were seeded on collagen coated Costar® 24-well Clear TC-treated Multiple Well Plates (Corning, USA). Differentiation was induced with RPMI 1640 medium containing 1% fetal horse serum and 0.5% fetal bovine serum + 50 µg/mL NGF. This medium was refreshed every 48H until full differentiation at day 10. Plates were scanned by the Incucyte system using a 20x objective and 36 images were taken per well. Assessment of neurite length and branch points was performed using the Incucyte®Neurotrack analysis software module. Average Neurite length and branch points per cell were calculated by dividing total neurite length or branch points per well by the cell confluence expressed in mm^2.

5) Statistical analysis

All data presented is this document represents mean ± SEM (Standard Error of Mean) with a minimum of three independent replicate for each experiment. Exact number of replicates are indicated in the legend of the figures. Statistical tests were performed with GraphPad Prism 7. Significance is assessed by different tests, as presented in the results section.

Part 5: Results

5.1 Characterization of LRRK2 phosphomutants

To investigate the links between LRRK2 phosphorylation and its functions, phosphomutants were used. In cells, LRRK2 is constantly phosphorylated at ANK-LRR domain (West et al., 2007).

Single change was able to modified the localization of LRRK2 within the cell by inducing accumulation of LRRK2 in the cytoplasm (S910A; S935A; S910A/S935A) (Doggett et al., 2012). Phosphorylation changes can also lead to decreased of its kinase activity or GTP binding and destabilization. Phosphorylation changes are known to modify adjacent phosphorylation sites, S910A or 935A dephosphorylation induce diminution of S973 phosphorylation (Doggett et al., 2012).

Despite the number of described LRRK2 pathogenic mutant's phosphorylation profile, there is little evidence establishing a direct link between the loss of a specific phosphorylation site and pathway dysregulation.

First, to determine if the phosphorylation motif of LRRK2 could induce new phosphorylation profile we mutated the Serine (S) residues of position 910, 935, 955, 973, and 1292 to Alanine (A) to mimic non-phosphorylated Serines and to Aspartate (D) to mimic phosphorylated Serine. A compound mutant was also generated to mimic phosphorylated/non-phosphorylated LRRK2 presenting 6 mutations of Serines to Alanines or Aspartates in the heterologous phosphorylation cluster noted 6xA or 6xD (Serines 860/910/935/955/973/976).

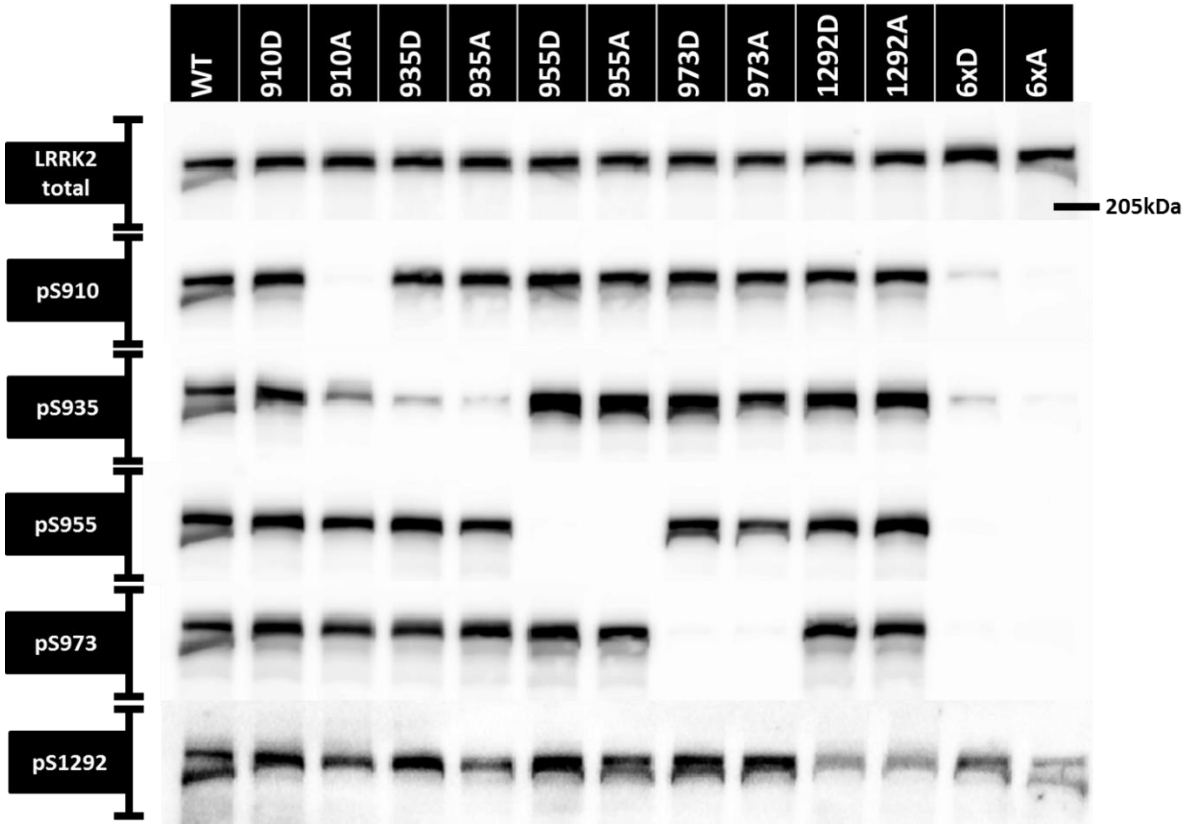


Figure 29: Phosphorylation state of LRRK2 phosphorylation mutant. HEK293T cells were trans transfected with the Wild Type human LRRK2 as well as the phosphorylation mutant of LRRK2. The phosphorylation state of LRRK2 was analysed by Western Blot. The quantification of the signals was obtained by Typhoon FLA 9500 imaging.

Expression in HEK293T was assayed and on each expressed protein, LRRK2 phospho-antibodies was applied (S910, S935, S955, S973, S1292). LRRK2 phosphorylation mutant are expressed in the same proportion, independently from their phosphorylation profile. Specific antibodies against phosphorylated serine seems unable to bind to mutated sites except for the S910D and a strong reduction in binding can be observed with mutant S935A and S935D (Figure 29).

Phosphodead mutant S910A, S935A and S955A change the phosphorylation of adjacent serine (Figure 30, A, B, D), matching previous observations (Nichols et al., 2010; Doggett et al., 2012). By comparison, phosphomimicking mutants of these same sites presents the same effect on neighbouring Serines as the dephosphorylation mutants, except S910D which does not display altered S935 phosphorylation.

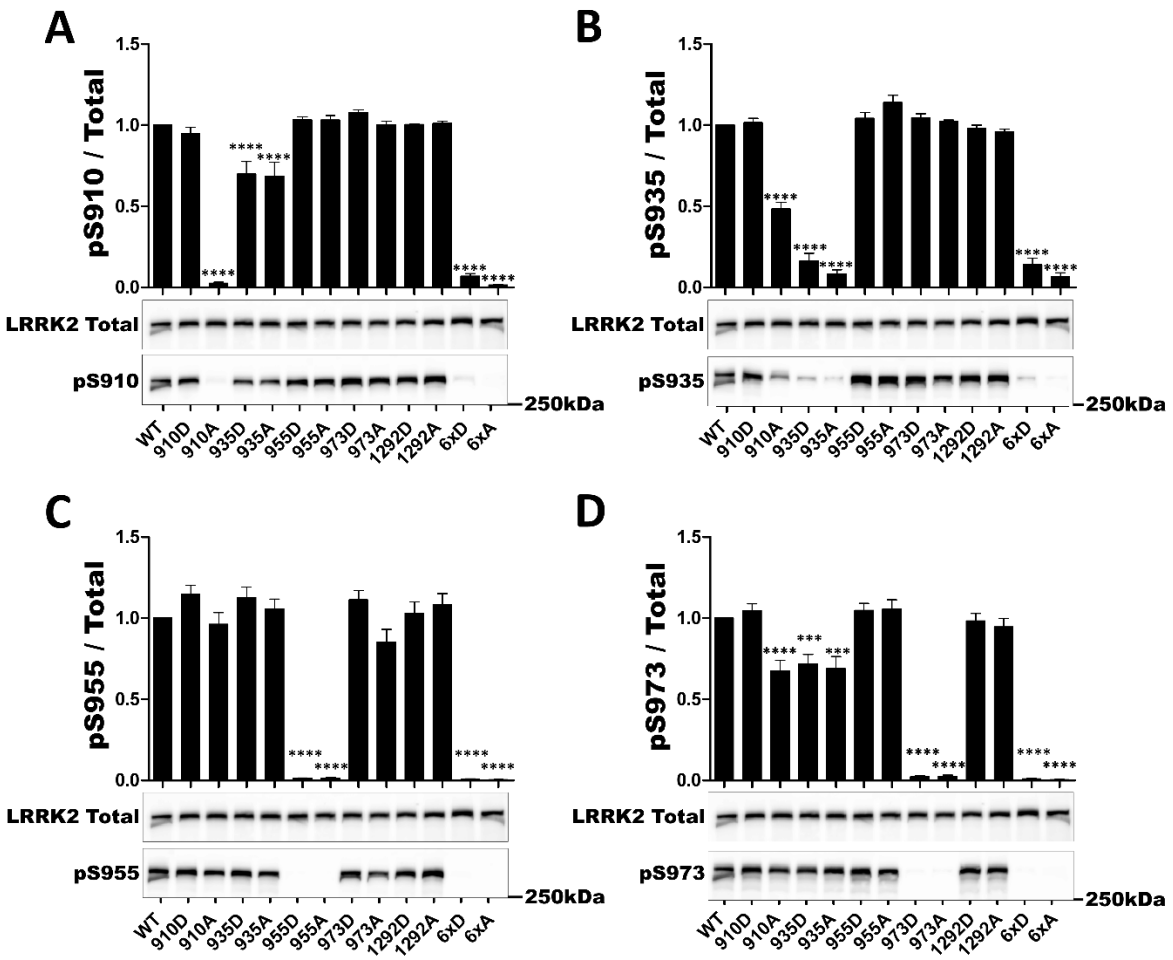


Figure 30: *In cellulo* phosphorylation profile of LRRK2 phosphorylation mutants.
Western blot analysis of HEK293T cell lysates after transfection of LRRK2 phosphomutants. The ratio of phosphorylated site over total LRRK2 signal is shown in (A) for S910, (B) for S935, in (C) for S955 and in (D) for S973. The data represents the mean ± SEM from four independent experiments. The quantification of the signals was done by using Typhoon FLA 9500 and ImageQuant software. The data was analysed by One-way-ANOVA with Dunnet's multiple comparison. (*** P <0.001), (**** P <0.0001).

We tested our phosphorylation mutants for S1292 autophosphorylation site, representative of the kinase activity of LRRK2. Individually, S910 S935, S955, and S973 had no significant effect on the phosphorylation rate of the S1292 autophosphorylation site (Figure 31). Unexpectedly, our mutants LRRK2 6xA and 6xD presented a 30% reduced phosphorylation a S1292 (Figure 31). Other dephosphorylation mutant (S910/S935/S955/S973A) induce a kinase activation (pSer1292 p = 0.066; pThr1491 p = 0.097; pThr2483 p = 0.055) (Reynolds et al., 2014).

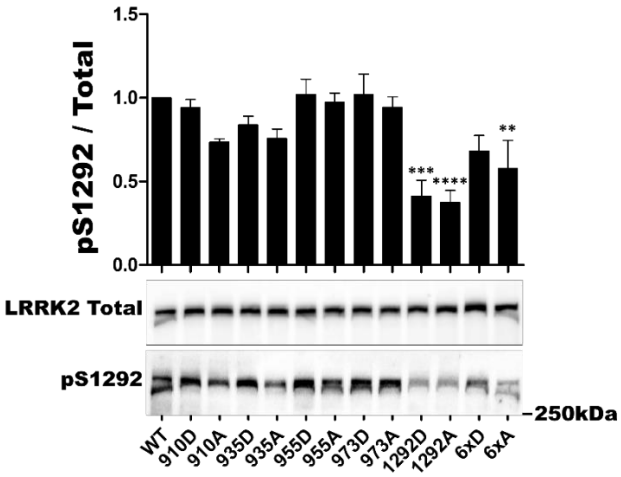


Figure 31: *In cellulo* auto-phosphorylation profile of LRRK2 phosphorylation mutants. Western blot analysis of HEK293T cell lysates after transfection of LRRK2 phosphomutants. The ratio of 1292 phosphorylated site over total LRRK2 signal is shown. The data represents the mean ± SEM from four independent experiments. The quantification of the signals was done by using Typhoon FLA 9500 and ImageQuant software. The data was analysed by One-way-ANOVA with Dunnet's multiple comparison. (*** P <0.001), (**** P <0.0001).

Interestingly, there were no detection of increased phosphorylation after induction of phosphorylation mutants (Figure 32). Phosphorylation mutants seems to only induce a reduction of phosphorylation level over the other phosphorylation sites.

	pS910	pS935	pS973	pS973	pS1292
910D	=	=	=	=	=
910A	x	↘	=	↘	=
935D	↘	x	=	↘	=
935A	↘	x	=	↘	=
955D	=	=	=	=	=
955A	=	=	x	=	=
973D	=	=	x	x	=
973A	=	=	=	x	=
1292D	=	=	=	=	x
1292A	=	=	=	=	x
6xD	x	x	=	x	↘
6xA	x	x	=	x	↘

Figure 32: Representation of phosphorylation effect in HEK293T. Data from quantified western blot are merged and modification of phosphorylation are shown in red for decreased phosphorylation, X when the phosphorylation is not possible with phospho-antibodies, = when no change is observed.

5.2 Kinase activity of LRRK2 phosphomutant

5.2.1 In whole cell lysate

To assess a potential deficit in kinase activity of these two particular mutants (6xA/6xD), we measured in total cell lysate the phosphorylation of two known substrates of LRRK2, RAB8a and RAB10. LRRK2 is able to phosphorylation of RAB8a at T72 and RAB10 at T73.

In HEK293T, we were able to detect a decreased phosphorylation for both RAB8a and RAB10 *in cellulo* for LRRK2 6xD but not for LRRK2 6xA (Figure 33, A, B).

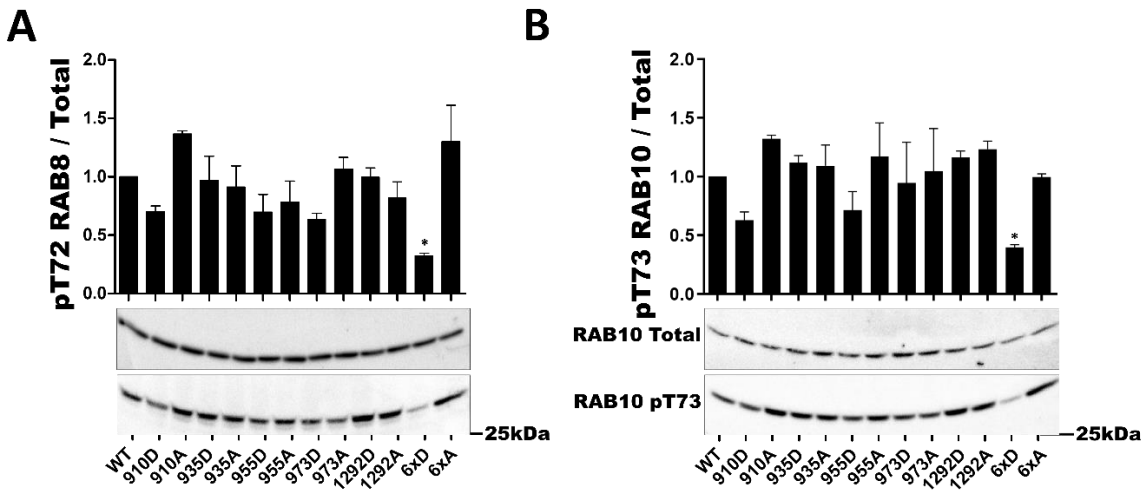


Figure 33: *In cellulo* phosphorylation profile of LRRK2 phosphorylation mutants. Western blot analysis of HEK293T cell lysates after transfection of LRRK2 phosphomutants. The ratio of phosphorylated site over total protein signal is shown, (A) Endogenous phosphorylation level of RAB8a T72 (A) and RAB10 T73 (B) were also quantified. The data represents the mean ± SEM from 3 independent experiments for (A), (B). The quantification of the signals was done by using Typhoon FLA 9500 and ImageQuant software. The data was analysed by One-way-ANOVA with Dunnet's multiple comparison. (* P <0.05).

We then tested whether or not LRRK2 6xD could trigger dephosphorylation of RAB29, a PD risk factor that has previously been reported as an interactor and substrate of LRRK2 (Beilina et al., 2014; Reyniers et al., 2014). Measurement of endogenous RAB29 phosphorylation was no completed du to performance of phosphor-RAB29 antibody (Figure 34). Expression of the LRRK2 phosphorylation mutants 6xA and 6xD did not induce changes in RAB29-T72 phosphorylation compared to LRRK2 WT (Figure 34).

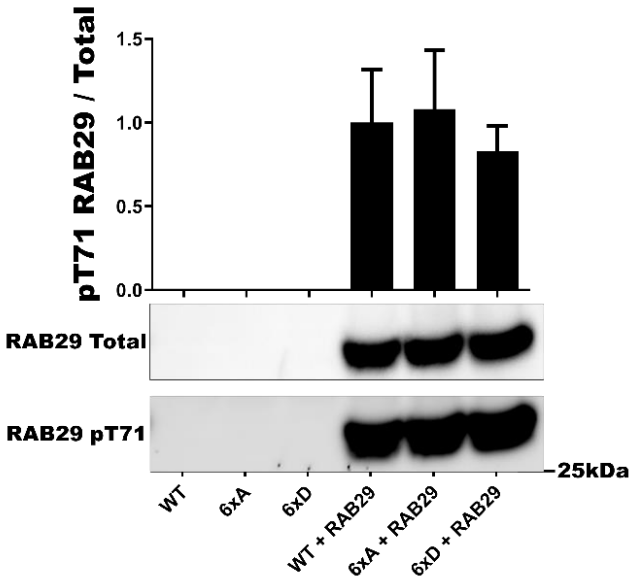


Figure 34: *In cellulo* phosphorylation of RAB29 by LRRK2. Western blot analysis of HEK293T cell lysates after transfection of LRRK2 phosphomutants and 2myc-Rab29. The ratio of phosphorylated site over total protein signal is shown. Phosphorylation level of RAB29 T72. The data represents the mean ± SEM from four independent experiments. The quantification of the signals was done by using Imager 600 and ImageQuant software.

RAB29 phosphorylation interact with LRRK2 on a different domain than RAB8a and RAB10. It has been reported that RAB29 can bind to LRRK2 in the ankyrin domain (Purlyte et al., 2018) while another RAB, RAB35, has been reported to bind to the ARM domain (Myasnikov et al., 2021).

In this experiment, we decided to also test for effects of RAB29 on LRRK2, given previous reports that RAB29 overexpression activates autophosphorylation of LRRK2 at S1292 (Purlyte et al., 2018). We found that expression of RAB29 led to an activation of LRRK2-S1292 phosphorylation of LRRK2 6xA that was 3-fold higher compared RAB29 induced activation of LRRK2-WT or LRRK2-6xD (Figure 35).

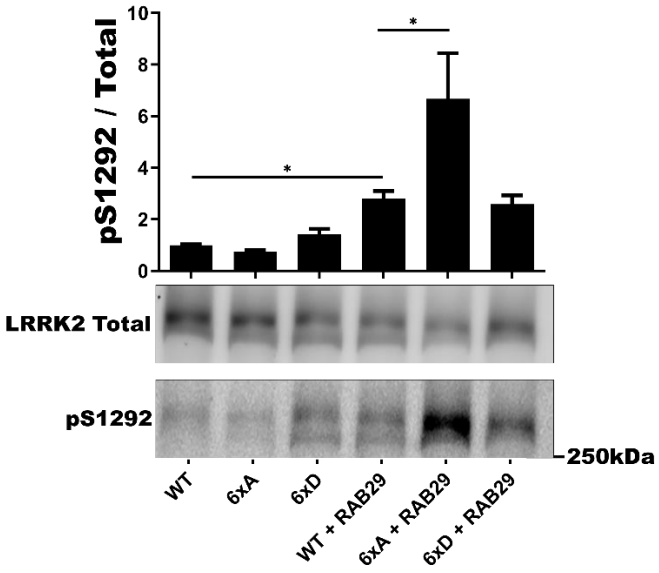


Figure 35: *In cellulo* LRRK2 activation by RAB29. Western blot analysis of HEK293T cell lysates after transfection of LRRK2 phosphomutants and 2myc-RAB29. The ratio of phosphorylated site over total protein signal is shown. LRRK2 autophosphorylation site S1292 was quantified over total LRRK2 after transfection of 2myc-RAB29. The data represents the mean ± SEM from 3 independent experiments. The quantification of the signals was done by using Imager 600 and ImageQuant software. The data was analysed by One-way-ANOVA with Dunnet's multiple comparison. (* P <0.05).

5.2.2 In vitro catalytic assay

We show that our phosphorylation mutant can induce phosphorylation changes on RABs substrates, as well as a decrease autophosphorylation at S1292. Phosphorylation mutant of autophosphorylation sites are known to decrease GTP binding (T1343A, T1348A/D, T1349D, T1357A/D, S1403A, T1404A, T1410A T1503A/D) (Greggio et al., 2009; Webber et al., 2011; Kamikawaji et al., 2013). To test whether if our phosphorylation mutants induce change of catalytic activity of LRRK2, we assay our mutants for *in vitro* kinase activity (LRRKtide peptide phosphorylation in the presence of 32P-γ-ATP) and GTPase activity (hydrolysis of 32P-γ-GTP).

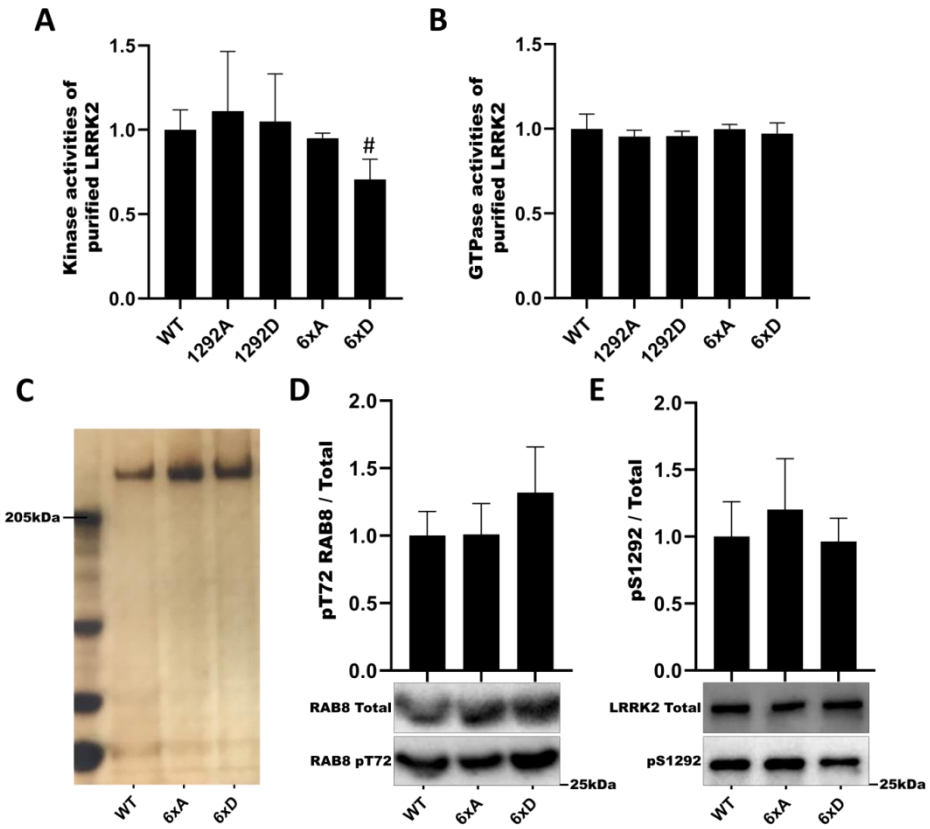


Figure 36: Catalytic activities of LRRK2 phosphorylation mutants. The kinase activities of purified LRRK2 were measured by addition of 32P-γ-ATP for 30mn (A) and 32P-γ-GTP for 180mn (B) and then quenched. Samples were assay for scintillation counting. (C) Silver staining of purified LRRK2 proteins. (D) Purified proteins are incubated with recombinant RAB8 (Life technologies) and ATP for 60mn. Measurement of LRRK2 autophosphorylation level pS1292 (D) and level of phosphorylation of RAB8a (E). The data represents the mean ± SEM from three independent experiments and analysed by ANOVA Kruskal Wallis test; comparison to WT. (# p=0.0549).

Mutation of phosphorylation sites S1292A, S1292D had no effect on the incorporation of 32P-γ-ATP and 32P-γ-GTP. However, in the multi-mutant LRRK2 6xD kinase activity trend to decrease (P = 0.0549) (Figure 36, A, B). As we identified LRRK2 6xD to present, *in cellulo*, a decreased phosphorylation at RAB8a and RAB10, we tested LRRK2 multi-mutant for *in vitro* kinase assay on recombinant RAB8a. The loss of T72 RAB8 and S1292 phosphorylation decreased obtain in cellulo were not replicated by only using the two purified partners (Figure 36, C, D, E). We hypothesized that the decreased phosphorylation of RAB8a and S1292 could only happened when LRRK2 6xD a part of a protein complex or in a close relationship with membranes.

5.3 Interaction with 14-3-3

LRRK2 phosphorylation is an important factor allowing the interaction with 14-3-3. LRRK2 S910A/S935A abolish the binding to 14-3-3 through overlay assay (Nichols et al., 2010; Doggett et al., 2012). LRRK2 kinase inhibition which result in a decreased phosphorylation of the ANK-LRR domain disrupt the interaction with 14-3-3 protein. Interaction with 14-3-3 is known to induce localization change in the cells (Dzamko et al., 2010).

We first use Micro Scale Thermophoresis (MST) that allow us a quick identification of binding between overexpress fluorescent protein and recombinant partners, here 14-3-3zeta. Binding with LRRK2-WT was confirmed (Figure 37, A, B). Surprisingly, binding with dephosphomimic mutant 6xA was confirmed, as well as 6xD mutant.

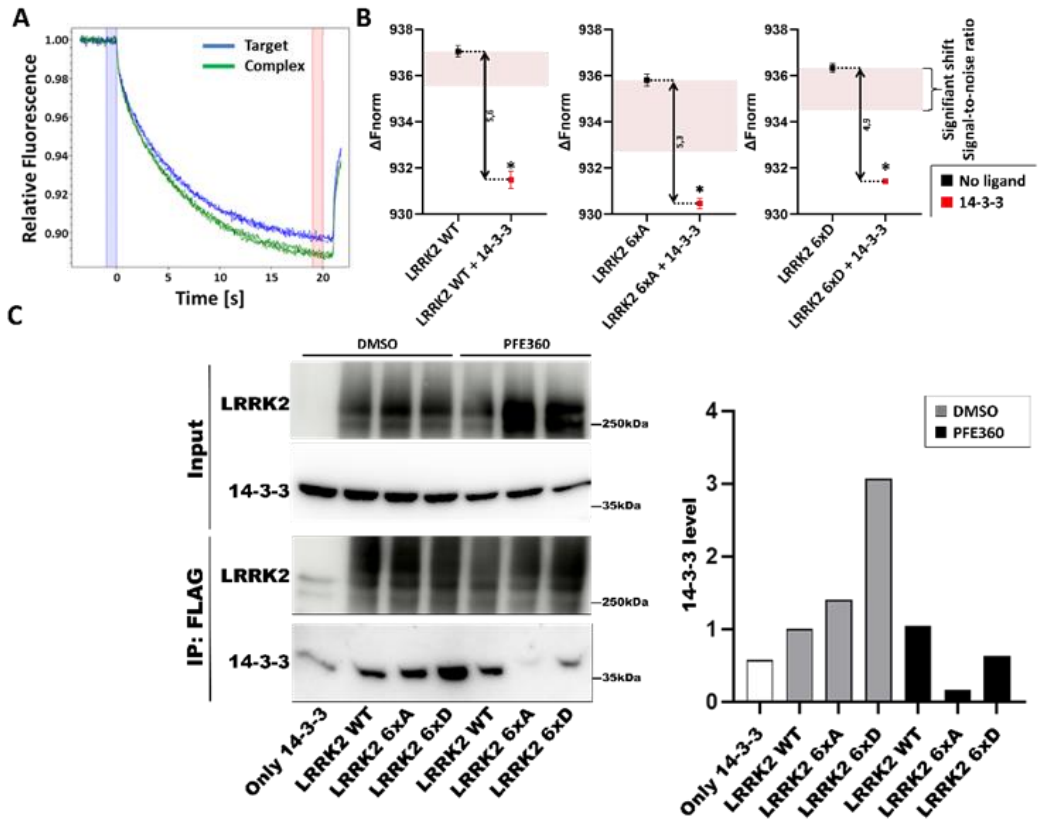


Figure 37: Assessment of interaction between LRRK2 and 14-3-3. (A) MST raw data showing shift between after incubation of mCherry-LRRK2 + 14-3-3. (B) Binding check comparison between LRRK2 WT/6xA and 6xD with and without ligand. Significant shift signal noise-to-noise ratio is calculated by Noise = 3x S/N, with S/N $= \frac{\text{Response amplitude}}{\sqrt{\frac{\sum_i (r_i - \bar{r})^2}{n-1}}}$. (C) HEK293T were transfected with 14-3-3 or FLAG-LRRK2 in combination with 14-3-3, as indicated. After coimmunoprecipitation with anti-FLAG beads, the immunoprecipitate (IP) and input samples were analysed by SDS- PAGE and western using the indicated antibodies.

To confirm the binding with LRRK2 6xA and 14-3-3 with performed Co-immunoprecipitation of 3xFLAG-LRRK2 and myc-14-3-3zeta in HEK293T.

We were able to co-immunoprecipitate LRRK2 with 14-3-3, more importantly, no change in interaction was confirmed with LRRK2 dephosphomimic 6xA and phosphomimic 6xD. Our findings suggested that phosphorylation of LRRK2 need a specific pattern to guide it interaction with 14-3-3. As previously reported, dephosphomimic mutant S910/935A mutant result in a lost binding with 14-3-3 while our multi mutant that contain the mutated site 910/935A do not replicate the previous finding.

5.4 Localization of LRRK2

5.4.1 Localisation of LRRK2 phosphomutants in cells

LRRK2 localisation in cells has repeatedly been shown to correlate with 14-3-3 binding. It is possible to change the diffuse cytoplasmic distribution of LRRK2 by kinase inhibition of LRRK2, this treatment induces disruption of both 14-3-3 binding and the diffuse cytoplasmic distribution of LRRK2 and creates filamentous LRRK2 accumulations in cells described as skein like, puncta or amorphous. The new subcellular distribution of LRRK2 in cells may be responsible for the interaction of LRRK2 to specific interactors in specific compartment.

To test if the phosphorylation of LRRK2 can trigger a specific localization of LRRK2 we expressed our LRRK2 phosphorylation mutants in HEK293T and subjects them to immunocytochemistry and immunofluorescence analysis.

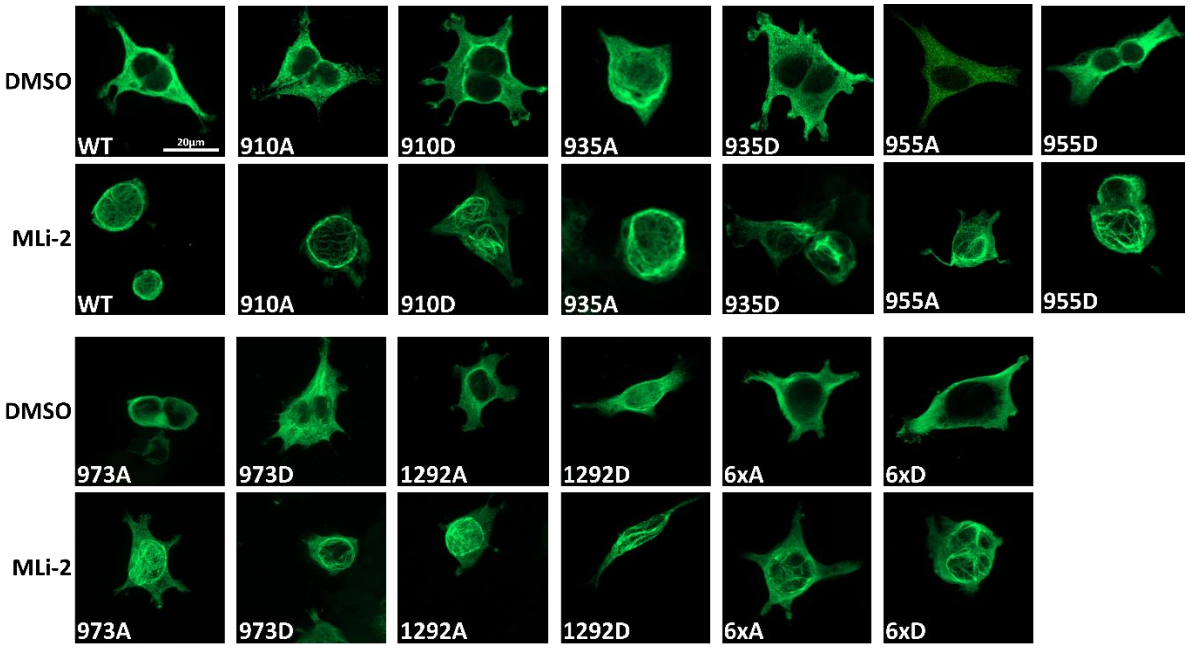


Figure 38: Localization of LRRK2 phosphorylation mutants in cells. HEK293T cells were transfected with 3xFLAG-LRRK2 construct. After 48H of transfection, cells were treated with DMSO or MLi-2 10nM for 2H followed by paraformaldehyde fixation. Representative fluorescent imaging of the indicated phosphosite mutants is shown with and without inhibitor treatment.

Localization of S973A phosphorylation mutant is in agreement with the localization already reported (Doggett et al., 2012) and same as the S973N, PD associated mutation (Nichols et al., 2010). We reported no significant effect on the cellular localization of LRRK2 mutants (Figure

38). Interestingly, the dephosphorylated mutant LRRK2 6xA does not present skein like LRRK2 positive accumulations without MLi-2 treatment.

Binding to 14-3-3 is not altered by S860A, S955A, S973A, S976A, S973A/S976A phosphorylation mutant (Nichols et al. 2010). 14-3-3 binding is dissociated after a kinase inhibition resulting of an ANK-LRR dephosphorylation. For instance, no consensus prone the direct link between phosphorylation and loss of 14-3-3 binding. Mimicking the dephosphorylation of 6 sites in the ANK-LRRK domain does not result in a loss of binding with 14-3-3 (Figure 37, C). In agreements with our findings, no change of interaction was detected between LRRK2 6xA and 14-3-3, consistent with the presence of a homogenous cytoplasmic distribution with LRRK2 6xA and 6xD.

5.4.2 Analysis of LRRK2 phosphomutants in lysosome enriched fractions in cell culture

LRRK2 is found to stabilize RAB8a and RAB10 on lysosomes depending on their phosphorylation. LRRK2 can be mobilized to enlarged lysosomes to interact and phosphorylates its substrates RAB8a and RAB10 (Eguchi et al., 2018). Repair of damaged endolysosomes is dependent of LRRK2's recruitment of RAB8a, this recruitment prevent the lysophagy of damaged endolysosome (Herbst et al., 2020). To test if the dephosphorylation of RAB8a and RAB10 was due to a miss localization of LRRK2 to a positive RAB8a/RAB10 compartment, we induce this know colocalization with chloroquine in SH-SY5Y stably expressing 3xFLAG-LRRK2. We found no changes in the subcellular distribution of LRRK2 6xA or 6xD compared to WT in any of the conditions tested (Figure 39).

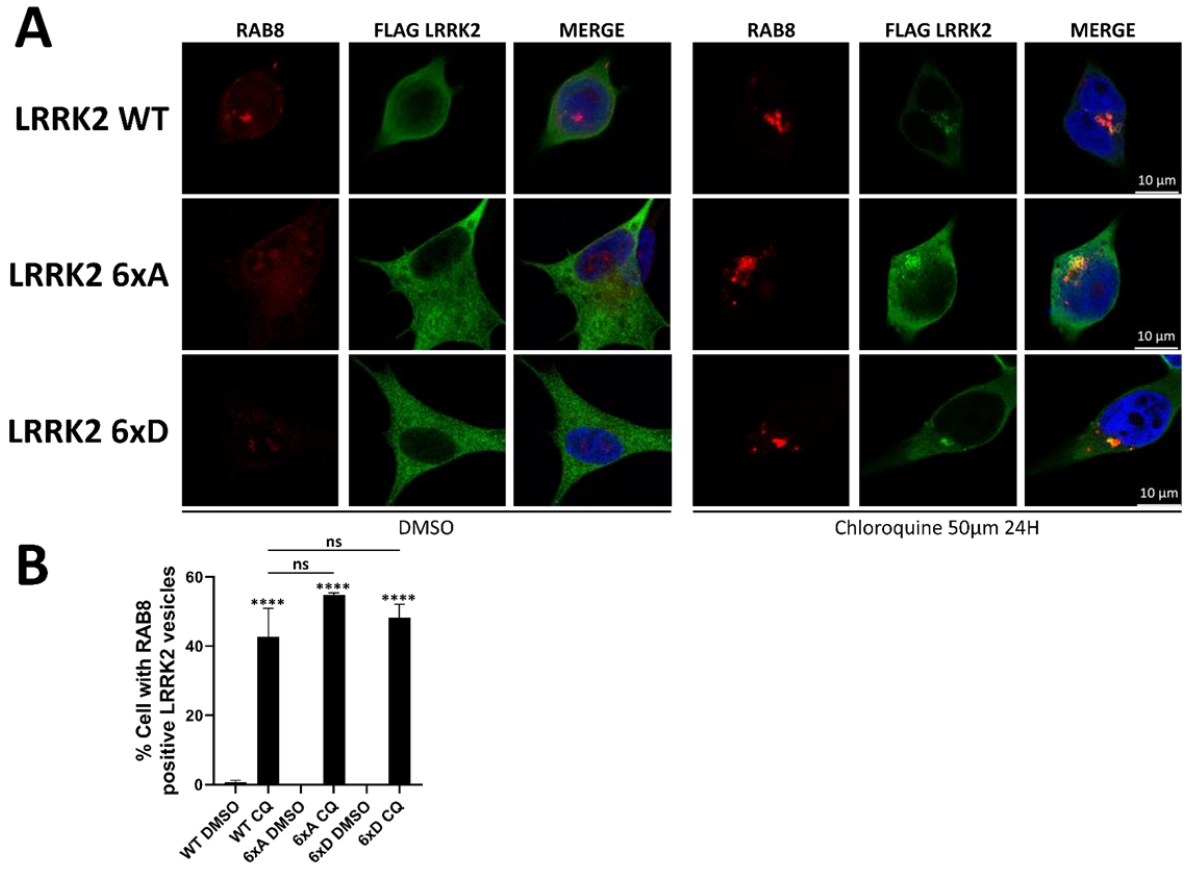


Figure 39: LRRK2 and RAB8a are localized to vesicular structures after chloroquine treatment. (A) SH-SY5Y stably expressing LRRK2 were treated with 50µM of chloroquine for 24H prior fixation. (B) Quantification of the percentage of cells presenting RAB8a positive LRRK2 vesicles. Data are mean ± SEM (n = 3 independent experiments, 100 – 130 cells were analysed per mutant in each experiment).

To further investigate LRRK2 relocalization to the lysosomes, we measure abundance of LRRK2 in purified lysosomes (Figure 40). In our experiment, we were able to identify 3xFLAG-LRRK2 and its phosphorylation mutant in the isolated lysosomes (Figure 40, B) without chloroquine treatment. Chloroquine treatment do not significatively increased the proportion of 3xFLAG-LRRK2 in the isolated lysosomes (Figure 40, B).

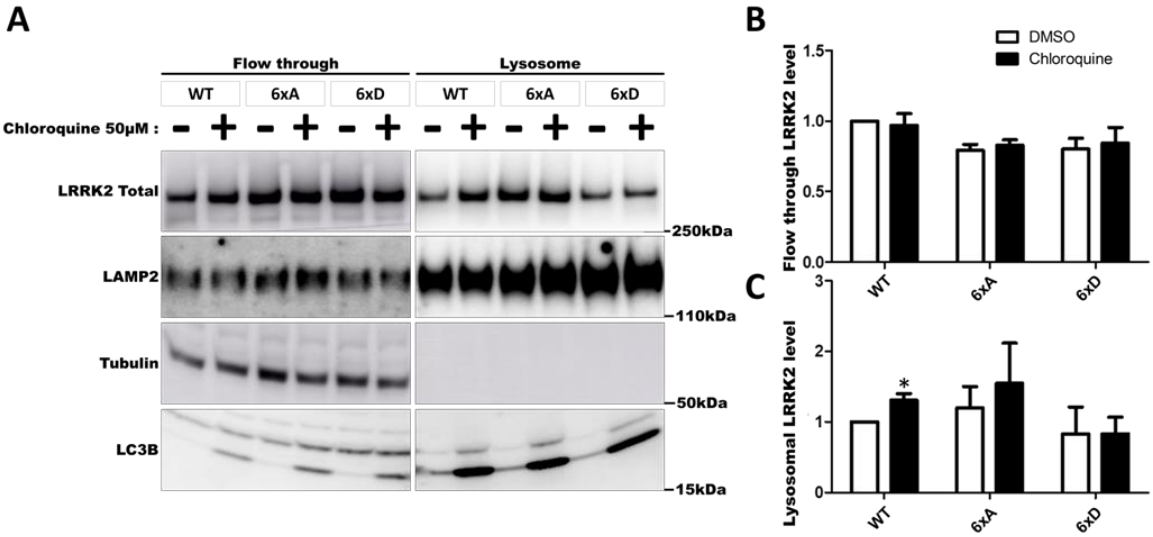


Figure 40: Isolation of lysosomes from HEK293T. Abundance of LRRK2 in purified lysosomes. Lysosomes were magnetically isolated from HEK293T cells expressing 3× FLAG-LRRK2 and phosphorylation mutants. Cells were treated with Chloroquine 50µM for 24H to induce LRRK2 enrichment to lysosomes. Levels of LRRK2 in the Flow through and in the lysosomes isolated are quantified by western blotting (A). Relative abundance of LRRK2 in Flow Through (B) and in isolated lysosomes (C) is normalized with LAMP2 levels. The data represents the mean ± SEM from three independent experiments and analyzed with one sample t test (* $p < 0.05$).

Under chloroquine treatment, LRRK2 6xD conserved a dephosphorylated RAB8a (Figure 41). The difference with the previous finding by Eguchi and colleagues could be explained by a higher expression level of LRRK2 resulting in a bigger proportion of LRRK2 in the lysosomes at the basal level.

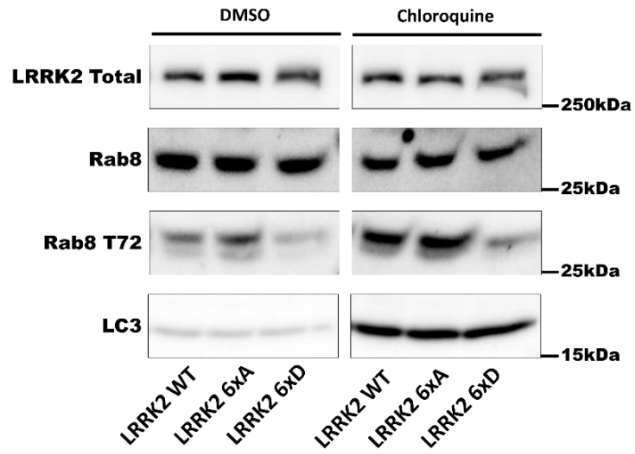


Figure 41: RAB phosphorylation under chloroquine treatment. HEK293T were transfected with 3xFLAG-LRRK2 constructs and treated with chloroquine (50µM for 24H) 48H post transfection. Lysates were analysed by western blot with the indicated antibodies.

In imaging flow cytometry, we were able to detect LRRK2 at the same position as lysotracker spots (Figure 42). To assess if LRRK2 phosphorylation influences the number of lysosomes in cells, we generated PC12 expressing LRRK2 and its 6xA/D mutants and control the number of lysosomes by imaging flow cytometry (ImageStream MKII, luminex). Chloroquine treatment did not increase the number of lysotracker spots in PC12 cells (Figure 42, B). Expression of mCherry-LRRK2-6xA or 6xD reduced the number of lysotracker spot per cell and chloroquine treatment is able to restore the loss of lysotracker spots induced by expression of LRRK2 phosphomutants.

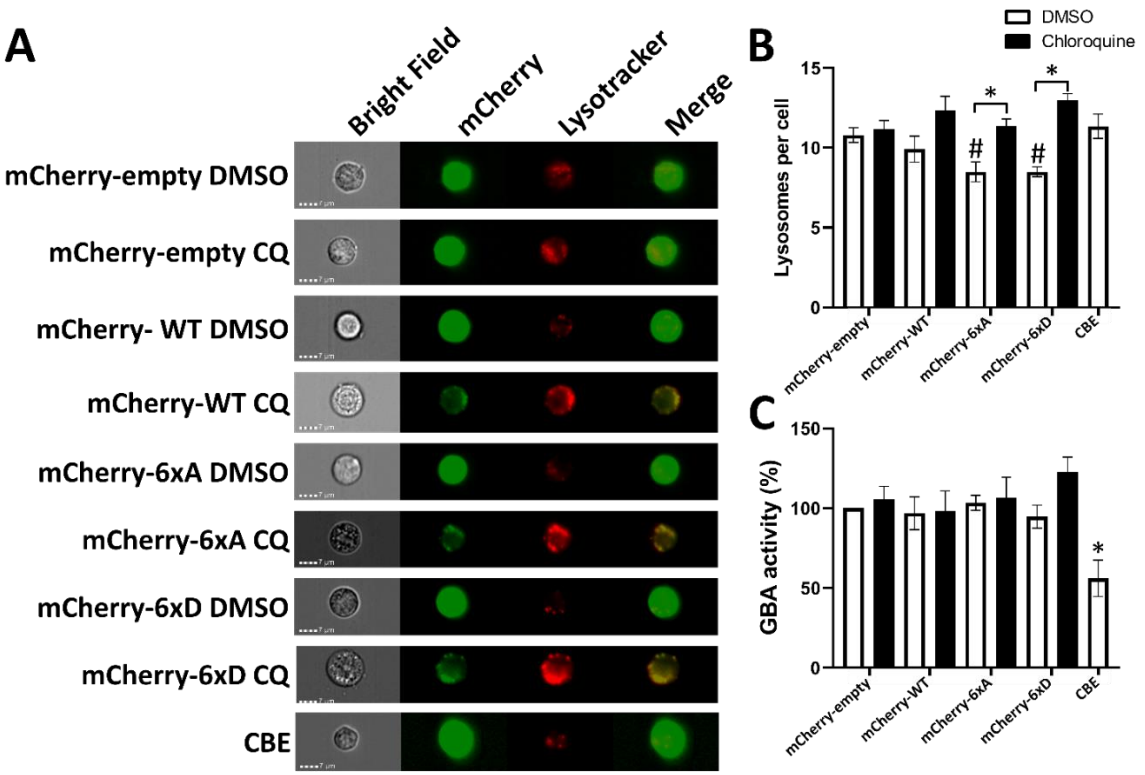


Figure 42: Lysosotracker analysis PC12 cells expressing WT and phosphomutant LRRK2 treated with and without chloroquine via imaging flow cytometry (ImageStream MKII, Amnis®). PC12 cells were incubated with lysotracker for 1H and subjected to ImageStream analysis. (A) Representative images of PC12 cells labelled with lysotracker, showing red-coloured positive spots corresponding to lysosomes. (B) Quantification of the number of spots imaged per cell. Data represent the mean spots detected from each genotype. (n= 4 independent experiments, 3000 – 5000 cells were analysed per mutant in each experiment).

Lysosomal Glucocerebrosidase (GBA) activity in live PC12 cells were measured using the quenchable substrate FDGlu that allow us to measure in real time the activity of this enzyme (Migita et al., 1995). LRRK2 G2019S and R1441C are known to decrease the activity of this enzymes in iPSC derived cells due to regulation of RAB10 (Ysselstein et al., 2019). Overexpression of RAB10 is known to increase the activity of GBA, but overexpression of phosphomimick RAB10 T72E had no effect on GBA activity (Ysselstein et al., 2019). In our experiment, LRRK2 overexpression or its phosphorylation had no effect on lysosomal GBA activity (Figure 42, C).

5.4.3 LRRK2 phosphomutants and neurite outgrowth

LRRK2 kinase activity is known to affect neurite length, mutants G2019S and R1441G cause neurites lengths reductions in primary cultured hippocampal neurons and increased RAB10 phosphorylation (Lavalley et al., 2016; Fan et al., 2021). We generated PC12 cell line stably expressing mCherry-LRRK2 to measure the neurite length of each construct, after 10 days of differentiation no change in neurites length or neurites branch points was shown between our phosphorylation mutants (Figure 43, A, B).

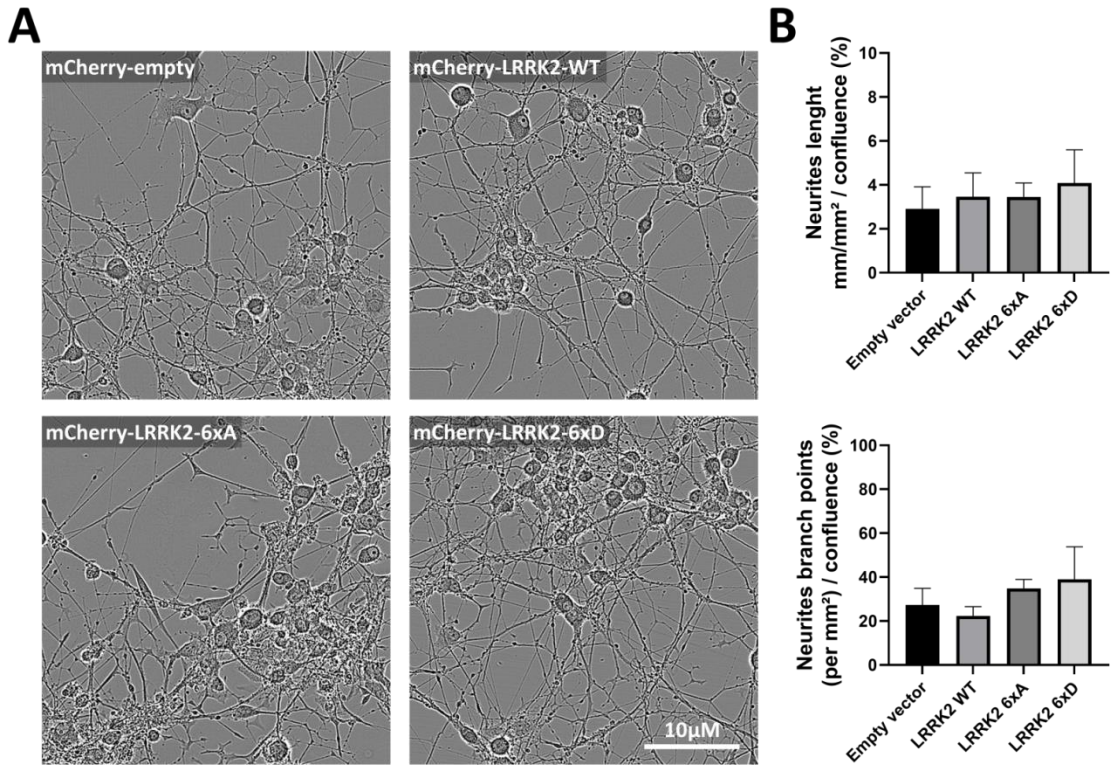


Figure 43: LRRK2 phosphorylation does not affect neurite complexity in PC12 cells. PC12 transduced wit mCherry-LRRK2 are coated on P24 plate and differentiations are followed by Incucyte and analysis of neurites complexity was performed when cells were fully differentiated, 10 days of differentiation are needed to obtain differentiated cells. (Incucyte, sartorius). Phase-contrast imaging of differentiated PC12 is show (A) and measurement of neurites length and neurites branch points was assay (B). The data represents the mean ± SEM from three independent experiments.

Parts 6: LRRK2 interaction with VAMPs

6.1 Introduction

In section 5.2.1, we presented a phosphorylation mutant, LRRK2 6xA, hyperactivated by overexpression of RAB29. LRRK2 binding to RAB29 allows recruitment of the complex to the trans Golgi network and allows LRRK2 to interact with others factors that can result in a change of trans Golgi network morphology (MacLeod et al., 2013; Beilina et al., 2014). LRRK2 was recently reported to be part of the Golgi-associated retrograde protein (GARP) complex, promoting retrograde transport through interaction with SNARE proteins, Syntaxin-6 (t-SNARE) and VAMP4 (v-SNARE) (Beilina et al., 2020). Interestingly, LRRK1, the closest homolog of LRRK2, can interact with a protein from the SNARE family VAMP7. This interaction is proposed to be at the ANK-LRR interface of LRRK1 (Figure 44) (Toyofuku et al., 2015).

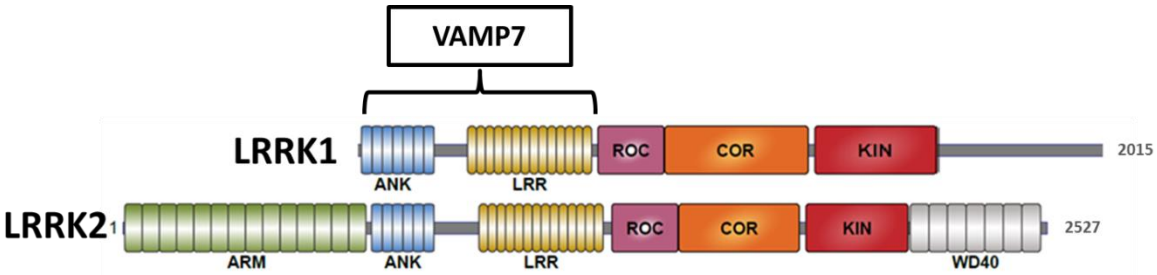


Figure 44: Interaction simulation between LRRK2 and VAMP7.

VAMP7 is expressed in all tissues and is enriched in neuronal cells including mesencephalic neurons and particularly in the somatodendritic compartment where most of the neurons of the dopaminergic pars compacta are affected in PD (Coco et al., 1999). VAMP7 has also been implicated in lysosome secretion via the LRRK1/VARP complex that are in competition (Wang et al., 2017). At present, the precise relationship between LRRK2 and VAMP7 is not known. Since the region of interaction between LRRK1 and VAMP7 is, the ANK and LRR domain is conserved in LRRK2. Thus it is in this region where the mutations of LRRK2 6xA are localized. Given this information's, we choose to evaluate LRRK2 as an interactor of VAMP7.

6.2 Hypothesis

As described in the first part of the thesis, LRRK2 is at the interface of different membrane fusion events in the cells. LRRK2 pathogenic mutations have been shown to decrease fusion events such as autophagosome/lysosome fusion. Due to the implication of LRRK2 in the SNARE complex machinery and the homology with LRRK1, we hypothesize that LRRK2 could interact with VAMP7, inducing changes on the neurite's complexity. Thus, the phosphorylation of LRRK2 in the ANK-LRR domain could be a trigger for the interaction/disruption of the interaction with VAMP7 leading to modification of trafficking event in the cells.

6.3 Aims

To evaluate the interaction between LRRK2 and VAMP7 we (**1**) performed a Co-immunoprecipitation between our LRRK2 and VAMP7 (**2**) assessed whether this interaction is could be controlled by the phosphorylation status of LRRK2 phosphorylation profile and (**3**) whether loss of VAMP expression can lead to changes in neurite complexity in differentiated PC12 cells.

6.5 Results

In order to assess whether LRRK2 can physically interact with VAMP7 we performed co-immunoprecipitation of mCherry-LRRK2 with GFP-VAMP7. We show a decreased interaction with VAMP7 with dephosphorylated LRRK2 6xA while the phosphorylation mutant 6xD conserves the interaction with VAMP7 (Figure 45).

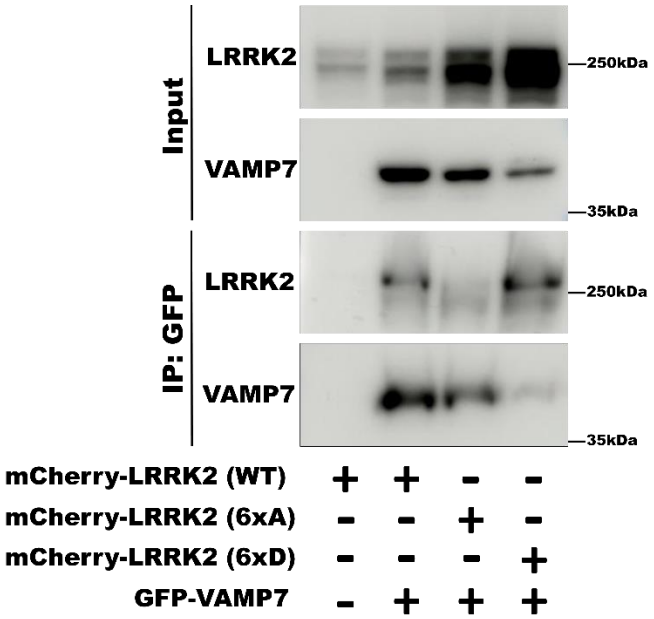


Figure 45: Co-immunoprecipitation with mCherry-LRRK2 and GFP-VAMP7. HEK293T were transfected with GFP-VAMP7 or FLAG-LRRK2 in combination with GFPVAMP7, as indicated. After coimmunoprecipitation with GFP-trap beads, the immunoprecipitate (IP) and input samples were analysed by SDS-PAGE and western blotnusing the indicated antibodies.

LRRK2 interacts with VAMP7, in a phosphorylation dependent manner. Indeed, LRRK2 phosphorylation mutant 6xA presented a decreased binding with VAMP7, an important factor for fusion event in cell (Wojnacki et al., 2020). No change was detected in size of neurites or branch points in PC12 lacking of VAMP2, VAMP4 or VAMP7. We were able to detect a trend in a deficit of neurite complexity (neurite length and neurite branch points) in PC12 VAMP7 KO models, as previously described (Figure 46) (Wojnacki et al., 2020).

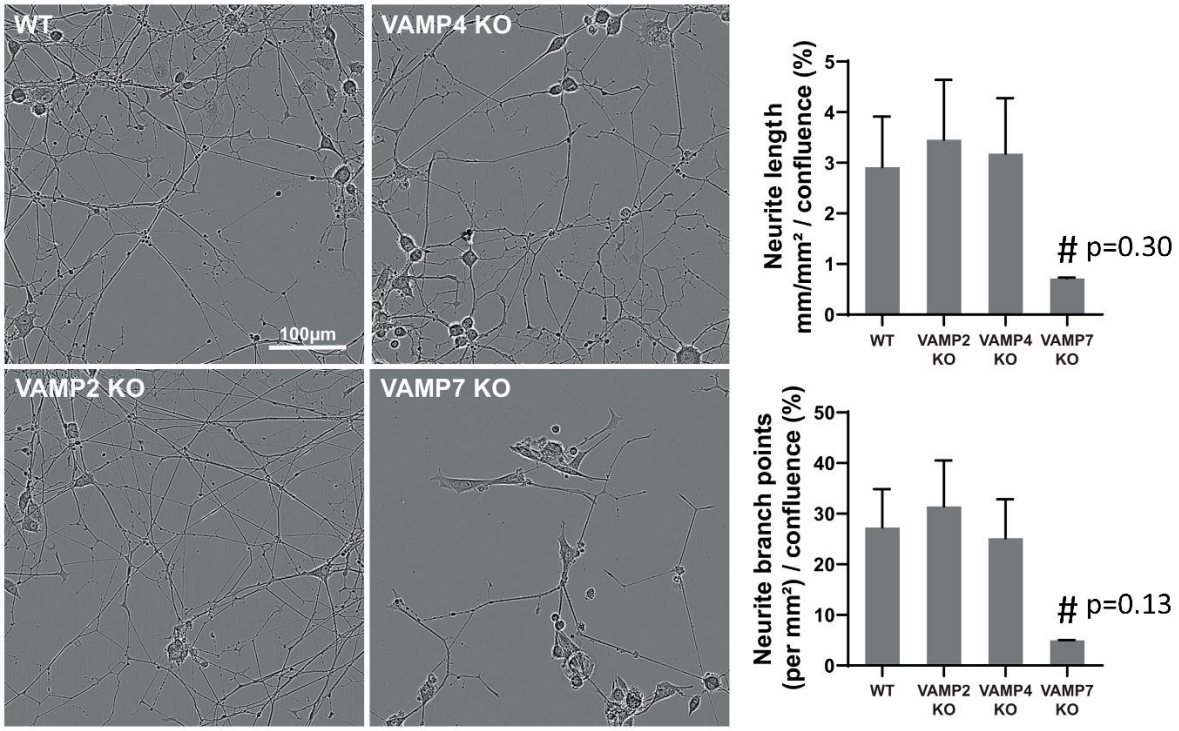


Figure 46: Neurite complexity in PC12 VAMPs KO cells. PC12 cells of different genotypes (WT, VAMP2 KO, VAMP4 KO and VAMP7 KO) expressing mCherry-empty are analyzed with Incucyte and analysis of neurites complexity was performed when cells were fully differentiated, 10 days of differentiation are needed to obtain differentiated cells (Incucyte, sartorius). Representative phase-contrast images are shown of the differentiated PC12 cells at day 10 taken by live cell imaging as described in materials and methods. Scale bar, 100 µm (B) Neurite length and neurite branch points were measured by automated quantification as described in materials and methods. The data represents the mean ± SEM from three independent experiments. The data was analysed by One-way-ANOVA with Dunnet's multiple comparison with indicated P value.

In comparison to LRRK2 WT, the phosphorylation mutant of LRRK2 6xA and 6xD doesn't seems to regulates neurites shortening or number of branch points (Figure 46, 47). We demonstrate that VAMP7 KO alone can reduce the size of neurites and the number of branch points in differentiated PC12 (Figure 47, D) (Figure 48, D). In the VAMP7 KO model, the overexpression of LRRK2, independently of its phosphorylation status, increase the neurite length and number of branch points.

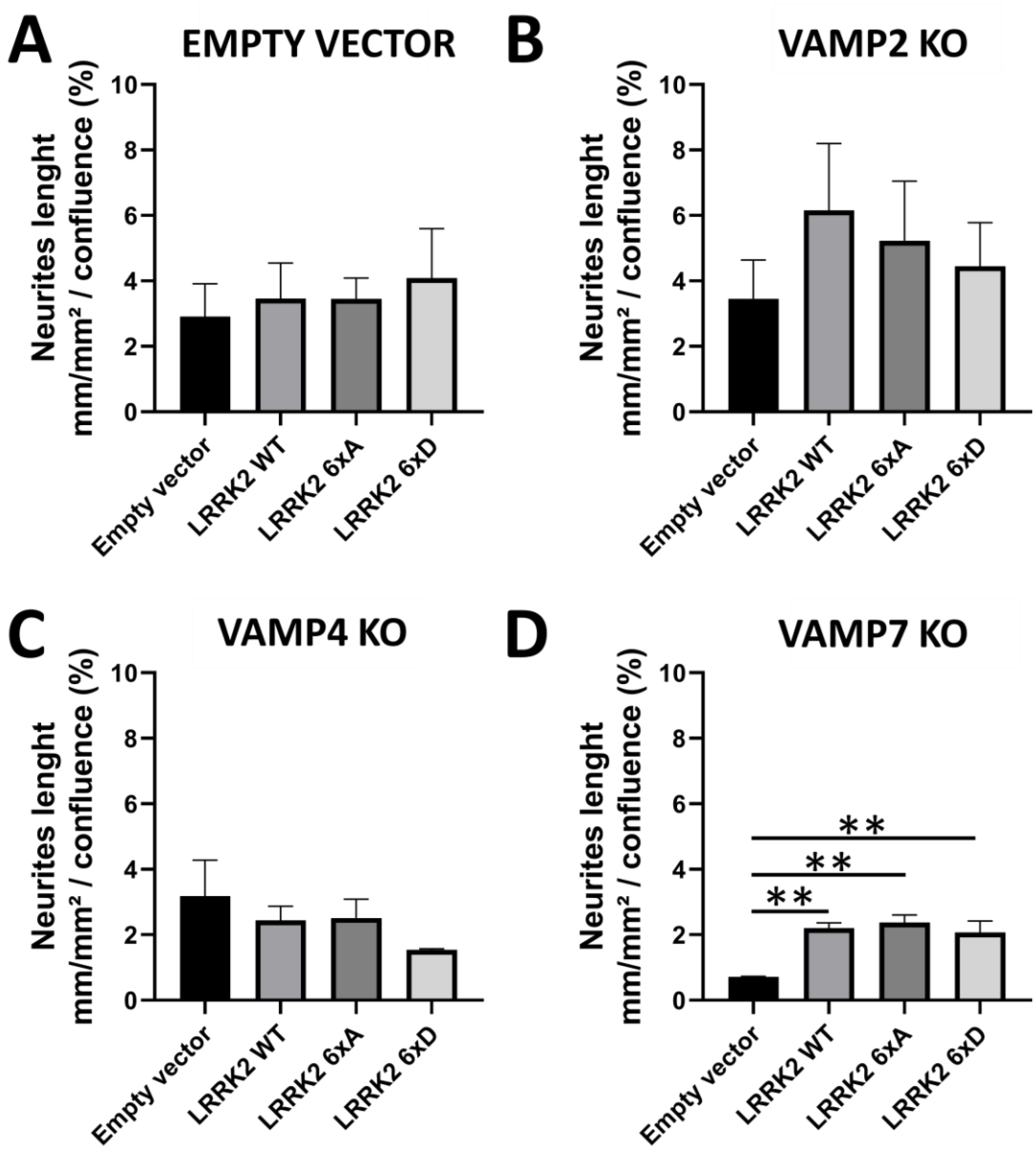


Figure 47: Neurite's lengths and VAMP KO. PC12 transduced wit mCherry-LRRK2 are coated on P24 plate and differentiations are followed by Incucyte and analysis of neurites complexity was performed when cells were fully differentiated, 10 days of differentiation are needed to obtain differentiated cells. (Incucyte, sartorius). Phase-contrast imaging of differentiated PC12 transduced with LRRK2 phosphorylation mutants are shown and measurement of neurites length is shown for PC12 navies (A), PC12 VAMP2 KO (B), PC12 VAMP4 KO (C), PC12 VAMP7KO (D). The data was analysed by One-way-ANOVA with Dunnet's multiple comparison. (** P <0.01).

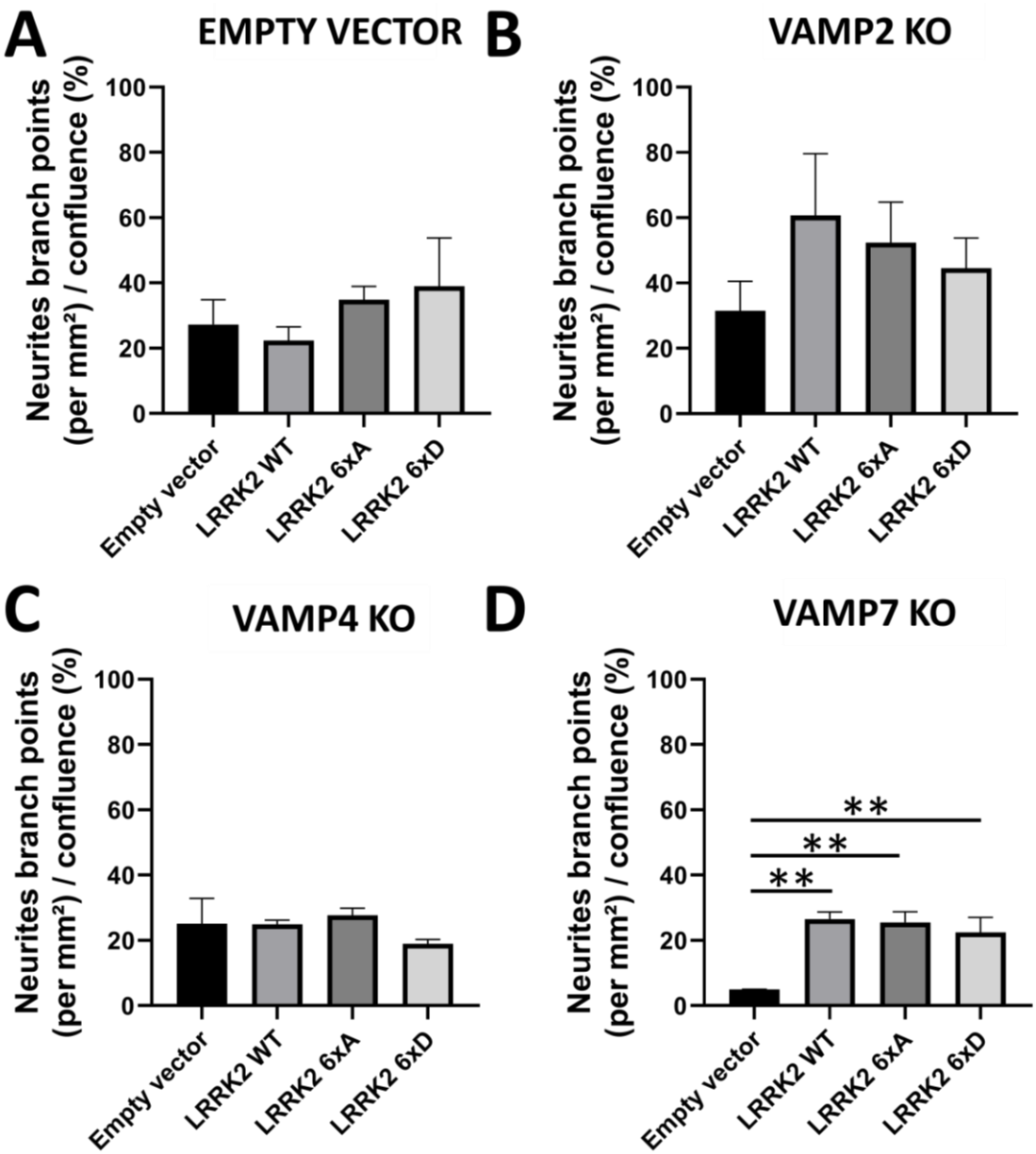


Figure 48: Neurites branch points and VAMPKO. PC12 transduced wit mCherry-LRRK2 are coated on P24 plate and differentiations are followed by Incucyte and analysis of neurites complexity was performed when cells were fully differentiated, 10 days of differentiation are needed to obtain differentiated cells. (Incucyte, sartorius). Phase-contrast imaging of differentiated PC12 transduced with LRRK2 phosphorylation mutants are shown and measurement of neurites branch points is shown for PC12 navies (A), PC12 VAMP2 KO (B), PC12 VAMP4 KO (C), PC12 VAMP7KO (D). The data was analysed by One-way-ANOVA with Dunnet's multiple comparison. (** P <0.01).

Discussion

The question of the phenotypic consequences of LRRK2 phosphorylation or dephosphorylation has remained mostly unanswered. Here, we present results that show that changes at the phosphosites in the ANK-LRR interdomain region affect LRRK2 activity in cells, including cellular kinase activity, RAB29 induced activation of LRRK2 differently following it phosphorylation profile.

Using a phosphomutant approach, we first made a survey of LRRK2 phosphomutant variants, including modifications of individual sites compared to modifications of multiple sites. While some of the phosphodead mutants [Serine (S) to Alanine (A)] have been reported before, few reports have tested phosphomimicking mutants [(Serine (S) to Aspartate (D)] of LRRK2 and our report is also the first to include side by side comparisons of phosphodead with phosphomimicking mutants. For those constructs that have been previously reported, such as the S910A or S935A mutants, our observations were similar to published observations, namely that these mutants affect the phosphorylation rate at other sites in the S910/S935 cluster, underscoring the robustness of our results. The phosphomutant approach was thus implemented to verify which known phenotypes of LRRK2 are modified by LRRK2 phosphorylation.

Previous data indicates that several grey zones persist as to whether LRRK2 dephosphorylation linked to its toxicity. LRRK2 is dephosphorylated in PD patient brains (Dzamko et al., 2017), and in cells with LRRK2 pathogenic mutations (Marchand et al., 2020). A particular case is for LRRK2-G2019S, the most common LRRK2 mutation, where heterologous phosphorylation is unchanged in cell culture but reduced in brains, lungs and kidney of G2019S knock-in mice (Ito et al., 2014). Dephosphorylation also occurs in cells/tissues treated with LRRK2 type I inhibitors, that have also shown in several studies the ability to abolish adverse phenotype induced by LRRK2 pathogenic mutations such as LRRK2 mutant induced neurite shortening (Lavalley et al., 2016). Dephosphorylation of the N-TER region does not occur with Type II LRRK2 inhibitors, stabilizing LRRK2 in an open conformation (Tasegian et al., 2021). Induction of filamentous accumulations of LRRK2, where LRRK2 binds to microtubules, is possible with LRRK2 stabilized in a closed conformation (Deniston et al., 2020; Tasegian et al., 2021). In our experiment, LRRK2 6xA and 6xD can also present filamentous cellular accumulations after treatment with MLi-2, a type I LRRK2 kinase inhibitor, suggesting that immobilization of LRRK2 in a closed conformation by MLi-2 is independent of the phosphorylation state of LRRK2 ANK-

LRR domain. Interestingly, our previous work showed that cellular treatment with type I inhibitors of LRRK2 kinase leads to rapid recruitment of phosphatases to the LRRK2 complex (subunits of both protein phosphatase 1 and 2A holoenzymes), suggestive of a conformational change in LRRK2. We postulated that such a conformational change enables LRRK2 dephosphorylation, and given our observation that LRRK2 phosphomutants remain sensitive to type I inhibitor induced filamentous accumulations, we postulate that this phenomenon is related to a conformational change in LRRK2 rather than to dephosphorylation of LRRK2 per se. A recent paper from Myasnikov presents the highest resolution of LRRK2 structure, unfortunately, the region between the ANK and LRR remains to be solved. Our mutations are localised to this unresolved region. The absence of resolution could be due the high dynamic conformational change due to the phosphorylation change.

Looking at cellular kinase activity, when we tested effects of the individual LRRK2 phosphosite mutants on phosphorylation of the LRRK2 substrates RAB8a and RAB10 in cells, we had no or low changes, while a significant decrease was observed for the 6xD mutant, consistent with a decreased kinase activity of LRRK2 in cells when it is phosphorylated. Intriguingly, when tested *in vitro* this result was not as pronounced as the *in cellulo* experiment. It is possible that, *in cellulo*¸ yet unidentified partners act as a sensor for the phosphorylation motif at the ANK-LRR. We hypothesized that the decreased dephosphorylation of RAB8a and S1292 preferentially occurs when LRRK2 6xD is found in a cellular context, perhaps via the aid of additional proteins as a part of a LRRK2 complex.

While we showed *in cellulo* that LRRK2's phosphorylation profile specifically regulates the phosphorylation of RAB8a and RAB10, we did not detect any effect on RAB29 phosphorylation. It has been reported that RAB29 binds to LRRK2 via the ankyrin domain and increased RAB29 expression is a trigger to activate LRRK2 kinase activity (Purlyte et al., 2018). While the exact mechanisms of RAB29 induced activation of LRRK2 remains to be elucidated, our observation that the phosphodead variant of LRRK2 is significantly more activated than the phosphomimicking variant of LRRK2 suggests that LRRK2 phosphorylation is a modulator of LRRK2 activation. Further work would be warranted to explore the link between RAB29 mediated activation of LRRK2 and LRRK2 phosphorylation. For instance, a recent study has modelled binding between RAB38 and ARM domain, using a high resolution structure of LRRK2 (Myasnikov et al., 2021), a similar analysis could be performed for LRRK2 and RAB29. Also,

RAB29 overexpression recruits LRRK2 to the Trans Golgi network and this is assumed to modulate kinases/phosphatases and stimulate LRRK2's activity (Purlyte et al., 2018).

Another phenotypic effect of LRRK2 mutations is its ability to affect neurite outgrowth, however the role of LRRK2 phosphorylation in this phenotype was never explored with phosphorylation mutants. LRRK2 G2019S is reported to reduce stable contact between growth-cones (Onishi et al., 2020) and G2019S impair neurite outgrowth in mice (Winner et al., 2011). LRRK2 G2019S also increases the phosphorylation of RAB8a/RAB10 that are crucial factors required in rat hippocampal neurons to control neurite outgrowth (Huber et al., 1995; Wang et al., 2011). In brief, a protein from the trans Golgi network, TRIO, regulates the axonal guidance by interacting with RABIN8, a guanine exchange factor (GEF) that activates RAB8/RAB10 (Villarroel-Campos et al., 2016). RAB8a interaction with RABIN8 is dependent of the phosphorylation of RAB8a's S111 phosphorylation site (Pourjafar-Dehkordi et al., 2019) the Rab8-RABIN8 interaction is also blocked with RAB8a T72 phosphorylation (Mamais et al., 2020) and this interaction promotes neurite outgrowth (Homma and Fukuda, 2016). Despite the reduced RAB8a and RAB10 phosphorylation in the presence of LRRK2-6xD, no significant effect on neurite complexity and length was detected in the tested conditions of NGF induced neurite outgrowth in PC12 cells. Our result suggests that other mechanisms may be at play besides RAB phosphorylation in LRRK2-mediated regulation of neurite complexity and further work is warranted such as testing different experimental conditions (such as testing other neurite forming cell types) and/or analyzing finer phenotypes such as growth-cone formation deficits.

LRRK2 kinase activity has been associated with the regulation of lysosomal properties. Enhanced kinase activity results in enlarged lysosomes and reduced GBA activity and reduced lysosomal degradative properties (Henry et al., 2015; Hockey et al., 2015; Ysselstein et al., 2019; Obergasteiger et al., 2020). Our results show that LRRK2 phosphorylation at S860/910/935/955/973/976 is not associated with modification of glucocerebrosidase activity. Interestingly, our results suggest that this cluster of Serine residues is involved in determining cellular lysosome numbers, as both the phosphodead and the phosphomimicking variants showed reduced numbers on lysosomes in PC12 cells (Figure 9, B). Further work is needed to investigate the link between phosphorylation change and lysosomal dysfunction. Our results also suggest that the localization of LRRK2 to damaged lysosomes is not dependent

on its ANK-LRR phosphorylation status. In (Eguchi et al., 2018) LRRK2, in absence of chloroquine treatment, is not detected in the lysosomal compartment. We were able to detect LRRK2 with and without chloroquine treatment in lysosomes. LRRK2 6xD shows a non-statistically significant tendency to be less abundant in the lysosomes, with or without chloroquine treatment. The difference with the previous finding by Eguchi and colleagues could be explained by a higher expression level of LRRK2 resulting in a bigger proportion of LRRK2 in the lysosomes at the basal level. It is important to correlate these results with microscopy imaging, without chloroquine, LRRK2 and RAB8a are not present in the same vesicular structures. Therefore, we conclude that LRRK2 can be recruited to lysosomes independently of its phosphorylation, on the conditions tested.

As its homologue LRRK1, we demonstrate that LRRK2 can interact with VAMP7. We show that this interaction is disrupted by LRRK2 6xA dephosphorylation mutant but not with the phosphorylation mutant 6xD. Tyrosine phosphorylation of VAMP7 increase its activity and lead to exocytosis of resting pool and neurites growths (Burgo et al., 2013). Our results show decreased neurites complexity in PC12 KO for VAMP7 as previously described (Wojnacki et al., 2020). It was possible to restore the neurites deficits induced by the KO of VAMP7 in PC12 by overexpressing LRRK2, independently of its phosphorylation profile. Further investigation is required to verify if VAMP7 is phosphorylated by LRRK2 and what the precise mechanisms are for the LRRK2:VAMP7 interaction and how this affects neurite complexity.

Reduced phosphorylation of RAB8a and RAB10 observed in the presence of LRRK2 6xD phosphomimicking could be accompanied by reduced RAB35 phosphorylation, however this would need to be verified. Recent resolution of LRRK2 structure in its inactive state indicate that RAB proteins may bind to the ARM domain of LRRK2 (Myasnikov et al., 2021). In its inactive conformation, the ARM is remotely positioned relative to the kinase domain that is also covered by the LRR domain that wraps around the ATP binding cleft and block its use. The coordination of the RAB with the ARM domain suggests that LRR and ARM would shift positions in the active state to allow access of the RAB to the kinase.

Perspectives

The new insight provided in this thesis demonstrate the importance of the phosphorylation profile of LRRK2 in the physiology of LRRK2 in PD and opens several perspectives for future research. For instance, this thesis has studied specifically the phosphorylation sites of the ANK-LRR interdomain region, that are only a fraction of the many LRRK2 phosphorylation sites (see figure 23). As we focused out work on the ANK-LRR interdomain region, it would be worth using the phosphorylation mutant approach outside of this hotspot. Assays should be performed in such a way that all LRRK2 phosphorylation sites are measured simultaneously in order to identify a subset of sites that act in parallel (for instance, a subset of sites always phosphorylated or not). Phosphorylation changes in specific functional subsets of phosphosites could be important for the regulation of specific pathways.

Autophosphorylation sites tend to be increased in the disease while heterologous sites are decreased. An interesting experiment would be to construct a phosphorylation mutant with constant dephosphorylation of autophosphorylation sites and constant phosphorylation of heterologous sites (as well as the other way around) and then study the behaviour of the protein. The results of such modification could be, as suggested by (Myasnikov et al., 2021), a modification of its structure. They described that the addition of negative changes by phosphorylation of S1292 could lead to the modification of the interface of LRR with KIN domain (Myasnikov et al., 2021). Therefore, it would be interesting to test single phosphorylation for its effect on structural properties and with modification of binding to partners (Protein, ATP, GTP/GDP), which is dependent on its 3D structure. These experiments would allow us to understand the relationship between LRRK2 structure and its phosphorylation status and point to mechanisms explaining how a toxic effect could be derived from an altered phosphorylation profile.

As such, monitoring the phosphorylation status of LRRK2 or its targets could have the potential to define a biomarker for Parkinson's disease (Taymans et al., 2017). Several studies have begun to survey the phosphorylation state of LRRK2 in different fluids of parkinsonian patients (Rideout et al., 2020). A more complete description of the relationship between LRRK2

phosphorylation and its association with a deleterious or beneficial state could open the way to new therapeutic strategies.

Today, the strategies developed focus on the kinase activity of LRRK2. Acting directly on the kinase function modifies the phosphorylation state of all LRRK2 substrates. We were able to demonstrate in our work that the phosphorylation state of LRRK2 can act on the phosphorylation of certain substrates while others substrates are unaffected. In the future, the development of compounds that can block the action of LRRK2 phosphatases or its kinases could lock LRRK2 into a certain phosphorylation profile and thus enable it to remain in a less pernicious state. To this end, a better description of LRRK2 phosphorylation regulation dynamics is also needed to identify the precise phosphatases and kinases.

Considering our results showing different phosphorylation profile between RAB8a/RAB10 and RAB29 in the presence of LRRK2 phosphomutants, it would be worth to investigate the phosphorylation of other RABs and substrates of LRRK2 with our phosphorylation mutants. Several RABs are involved in pathogenic mechanisms such as RAB35 for alpha-synuclein release, RAB11/RAB43 regulating TRAPPII complex and other substrates (Bae et al., 2018; Jenkins et al., 2020). It is known that LRRK2 can regulate alpha-synuclein secretion by RAB35 phosphorylation. A possible experiment to carry out is to test if RAB35 phosphorylation is dependent on LRRK2 phosphorylation profile and if this modification led to alteration of alpha-synuclein secretion. In our laboratory, we are currently working on this hypothesis by performing measurements of the alpha-synuclein release in cell culture in response to different experimental conditions (PhD thesis of Alessia Sarchione under the supervision of Dr Chartier-Harlin). The preliminary data she obtained in collaboration with myself and Dr Taymans suggest that LRRK2 phosphorylation is able to modulate alpha-synuclein release.

This thesis also reveals that binding of the v-SNARE VAMP7 to LRRK2 is dependent on the LRRK2 phosphorylation profile. VAMP7 is an important factor regulating fusion events in cells. This study should be extended to the whole family of VAMP proteins such as VAMP4, considered as a potential PD risk factor. Our collaborators are currently working on this hypothesis (PhD of Francesca Filippini under the supervision of Dr Thierry Galli). In our study, we were not able to detect differences in the neurite's complexity of our PC12 between 6xA and 6xD mutants. As described by (Wojnacki et al., 2020), VAMP7 is factor regulating axonal

growth and neuronal polarity. Further work is needed to identify a potential role of LRRK2 phosphorylation the membrane fusion mechanism.

This thesis work also observed a reduced number of lysosomes per cell in the cells overexpressing LRRK2 6xA and 6xD relative to WT, with no differences between phosphorylated or dephosphorylated LRRK2. We also observed that these two mutants do not reduce the activity of GBA in lysosomes. A more precise description of the structure of the lysosomes and its content (RAB phosphorylation, LRRK2 kinase or phosphates, expression of ATPases…) after expression of LRRK2 phosphomutants could pinpoint importance of LRRK2 phosphorylation on lysosomal physiology. In this study, we did not test the degradative properties of lysosomes in the presence of LRRK2 phosphomutants, this could contribute to further documenting deleterious or beneficial states of LRRK2 relative to PD pathology.

We believe that the complex regulation of LRRK2 phosphorylation is a crucial factor regulating diverse processes in cells. Developing new tools to study LRRK2 phosphorylation states and correlate these to disease related phenotypes will open new opportunities to develop new therapeutic and diagnostic approaches for PD.

Conclusions

Considering the postulates that enhanced kinase activity and increase of RABs phosphorylation are detrimental processes, our study results are consistent with the hypothesis that phosphorylation of LRRK2 at the ANK-LRR phosphosites corresponds to a protective state for LRRK2 while dephosphorylation at these sites correspond to a deleterious state. Future research should pursue the investigations on this hypothesis, notably to understand the precise role of LRRK2 phosphorylation on disease related processes such as LRRK2 effects on membrane fusion, endosomal trafficking, neurite complexity and release of alpha-synuclein.

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