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Leucine-Rich repeat kinase 2 (LRRK2) in Parkinson's Disease - a role in endocytosis of synaptic vesicles

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2012

Acknowledgments

I would like to express my gratitude for this opportunity to learn/work at the Janssen Pharmaceutica, a Johnson & Johnson company in Beerse, Belgium. During my master training I had the opportunity to interact with many excellent and dedicated researchers who shared their experience, ideas and enthusiasm for science. The collection of skills, experiences, and memories acquired while studying/working here was made so much more pleasant with the wonderful people who stayed by my side every step of the way.

I would like to specify my gratitude to my supervisors:

Dr. Diederik Moechars, for being a great mentor, for his leadership, for his excellent scientific support and for the opportunity I had to work in a very stimulating environment. Thank you for guiding me through science and for helping me to mature as a scientist;

Dr. Hamdy Shaban, for his energy at lab, patience, and commitment to research were one of the driving forces of my work. The advice and support that he gave me in difficult circumstances were invaluable to me;

Dr. Kristof Van Kolen, for thesis comments, incitement to scientific discussion and leadership in the lab. Thank you!

To my colleagues, Erik De Prins, Luc Hoskens, Marie Pollard and Mohammad Rahman for support in the electrophysiology lab, provide a great scientific environment and friendship. Thank you for endure me. I will never forget you!

To my group colleagues, Guy Daneels, Lore Delbroek, Jelle Verloes, Liesbeth Steegmans for scientific discussion and team spirit in the lab;

To Jacobine Kuijlaars and Christoph Tombeur for patience, for teaching me the cell culture procedure and to support in cell culture room;

To Prof. Dr. Bart De Strooper to allow my presence in his lab, even a few hours, and scientific discussion in LRRK2 meetings, and Dr. Wim Mandemakers to provide me assistant in cell culture in the same lab;

My friends that came to Janssen Pharmaceutica with me, Ana Farinha, Joana Ramalho and Sara Calafate for their interest in my work, for the great moments we spent together and for a lot of moral support;

To people that in one way or another helped me in any circumstances, but I do not have specified their names;

And finally, to my family:

First I want to thank my parents (Luisa Guimarães and Carlos Guimarães) for giving me the best prerequisites to develop, and for always supporting me with my studies and my work;

And most of all, thanks to my ever supportive girlfriend and best friend, Lisbeth Silva. Although not agreeing with my coming to Beerse, Belgium, she gave me the support that I needed during the internship. Thank you!

Thank you all!

Obrigado a todos!

Contents

Resumo

A doença de Parkinson (PD) é uma perturbação cerebral e prolongada que se caracteriza pelo inclusão de corpos de Lewy (LB) e pela degeneração dos neurónios dopaminérgicos da *substância nigra pars compacta* (SNpc) até ao estriado. É a segunda doença neurodegenerativa mais comum e tem como principais sintomas a dificuldade em controlar os movimentos voluntários, movimentos lentos, tremor, rigidez, instabilidade postural e demência. Actualmente, a patogênese da PD não está totalmente clarificada mas alguns estudos têm sugerido que o envelhecimento, factores ambientais e genéticos desempenham um papel preponderante para o desenvolvimento da doença.

Mais recentemente, estudos genéticos têm descrito várias mutações no gene Leucine-Rich Repeat Kinase 2 (LRRK2) que causam PD do tipo autossómico dominante com início tardio sendo clinicamente impossível diferenciar da PD idiopática. De facto, as mutações no gene LRRK2 são responsáveis por 5-10% dos casos familiares e 1-2% dos casos esporádicos da PD.

A proteína LRRK2 é uma ampla e complexa proteína com diversos domínios exibindo duas principais actividades enzimáticas, GTPase e cinase. A mutação mais conhecida, G2019S, leva ao aumento da actividade cinase, enquanto mutações no domínio GTPase, tal como R1441C/G, também afectam a actividade cinase. A função biológica/patológica da proteína LRRK2 permanece desconhecida, mas várias evidências descrevem que esta proteína desempenha papéis no encurtamento das dendrites, disfunção mitocondrial, tradução proteica, autofagia, libertação de neurotransmissores e na endocitose de vesículas sinápticas.

De facto, a proteína LRRK2 tem sido descrita como uma proteína reguladora devido às suas interacções com elementos chave para o tráfego das vesículas sinápticas dentro da célula pré-sináptica tais como a proteína Rab5b, subunidades do complexo AP-2, glicoproteína 2A da vesícula sináptica (SV2A), NEM-sensitive factor (NSF) e a clathrin coat assembly protein, AP180.

Neste projecto, culturas primárias de hipocampo de ratinhos Wild Type e LRRK2 Knockout foram estabelecidas para estudar, via whole-cell patch clamp, o papel da LRRK2 na reciclagem de vesículas sinápticas e na libertação de neurotransmissores. A ausência de LRRK2 e a inibição farmacológica da actividade cinase da proteína LRRK2 prejudica a libertação de neurotransmissores, ao nível da célula pré-sináptica, devido a perturbações na endocitose de vesículas sinápticas. Juntamente com estas observações, o nosso estudo mostra que existe uma conservação evolucionária desta função fisiológica em *Drosophila*

melanogaster e em neurónios de mamíferos. Perante isto, a LRRK2 também regula, em neurónios de mamíferos, a endocitose de vesículas sinápticas na célula pré-sináptica através de um ciclo de fosforilação da endofilina.

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Abstract

Parkinson's disease (PD), the second most common neurodegenerative disorder, is characterized by progressive degeneration of dopaminergic projections from the *substantia nigra pars compacta* (SNpc) to the striatum and presence of Lewy bodies (LBs). PD affects the control of voluntary movement leading to tremor, postural imbalance, rigidity, and slowness of movement, depression and dementia. The pathogenesis of PD is not fully understood but many studies have suggested that aging, environmental factors and genetic susceptibility play an important role.

Human genetics has defined several mutations in the Leucine-rich repeat kinase 2 (LRRK2) gene as a cause of late-onset autosomal dominant PD that is clinically indistinguishable from idiopathic PD. LRRK2 mutations account for 5-10% of familial and 1–2% of sporadic PD cases.

LRRK2 is a large, complex, multidomain protein displaying kinase and GTPase enzymatic activities and multiple protein–protein interaction domains. The best characterized mutation, G2019S, leads to increased kinase activity, while mutations in the GTPase domain, such as R1441C/G, have been reported to influence kinase activity as well. The biological/pathological function of LRRK2 remains to be established, but several lines of evidence describe a role in decreased neurite outgrowth, mitochondrial dysfunction, increased protein translation, altered synaptic vesicles endocytosis and autophagy.

LRRK2 has been suggested as a regulatory protein by its interaction with key elements of synaptic vesicle trafficking within the recycling pool such as Rab5b, AP-2 complex subunits, synaptic vesicle glycoprotein 2A (SV2A), NEM-sensitive factor (NSF) and clathrin coat assembly protein AP180.

In this project different primary neuronal cultures of Wild Type (WT) and LRRK2 Knockout (KO) mouse were established to study, via whole-cell patch clamp recordings, the role of LRRK2 in synaptic vesicle recycling and neurotransmitter release. The absence of LRRK2 and the pharmacologic inhibition of LRRK2 kinase activity impair the neurotransmission, at level of pre-synaptic cell by disrupting the endocytosis of synaptic vesicles. Together, our work shows that there is evolutionary conservation of this previously identified physiologic function of LRRK2 from fly to mammalian neurons. Also in mammalian neurons, LRRK2 regulates the endocytosis of synaptic vesicles in the pre-synaptic cell, via an endophilin phosphorylation cycle.

Abbreviations

(4E-BP) 4E-binding protein

(6-OHDA) 6-hydroxydopamine

(ADBE) activity-dependent bulk endocytosis

(AD) Autosomal dominant

(AlzD) Alzheimer 's disease

(AgCl) Silver chloride

(AMPA) α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid

(ANK) Ankyrin

(AP-2) Heterotetramer adaptor complex

(APs) Assembly proteins

(AR-JP) Autosomal recessive forms of juvenile parkinson

(ARM) Armadillo

(ATP) Adenosine triphosphate

(ATP13A2) ATPase Type 13A2

(BAC) Bacterial artificial chromosome

(BAR) Bin/Amphiphysin/Rys

(BBB) Brain blood barrier

(Ca^{2+}) Calcium

(CCVs) Clathrin-coated vesicles

(cDNA) Complementary Deoxyribonucleic Acid

(CMA) Chaperone-mediated autophagy

(CME) Clathrin-mediated endocytosis

(CHIP) C-terminal Hsp70 interacting protein

(CNS) Central Nervous System

(CO_2) Carbon dioxide

(COR) c-terminal of ROC

(CRMP-2) Collapsing response mediator protein-2

(DA) Dopamine

(DAergic) Dopaminergic

(Dapk1) Death-associated protein kinase

(DAT) Dopamine transporter

(DLB) Dementia with LB

(DNA) Deoxyribonucleic acid

(DVL1-3) Dishevelled family of phosphoproteins

(E1) Ubiquitin-activating enzyme

(E2) Ubiquitin-conjugating enzyme

(E3) Ubiquitin ligase enzyme

(EOP) Early-onset parkinsonism

(EPS15-EPS15R) 15-EPS15 related

(ER) Endoplasmatic reticulum

(ERK's) Extracellular-signal regulated kinase

(ERM) Ezrin/radixin/moesin

(FADD) Fas-associated protein with death domain

(F-actin) Filamentous actin

(FCH) Fes/CIP4 homology

(FCHO) FCH only domain

(GAK) Cyclin G-associated

(GAP) GTPase activating protein

(GDP) Guanine diphosphate

(GEF) Guanine nucleotide exchange factor

(Gly) Glycine

(GTP) Guanine triphosphate

(GWAS) Genome-wide association studies

(GYKI 52466) 2,3-benzodiazepine

(h4E-BP1) human 4E-binding protein 1

(H_2O_2) Hydrogen peroxide

(HEK293T) Human embryonic kidney 293T cell line

(HSC 70) ATPase heat shock cognate

(Hsp) Heat shock protein

(iNOS) Nitric oxide syntahse induction

(JNK) c-Jun amino-terminal kinase

(KCl) Potassium chloride

(KD) Kinase-domain deletion or kinase deficient (dead) mutation

(KO) Knockout

(KRS) Kufor-Rakeb syndrome

(LB) Lewy body

(LPAAT) Lysophosphatidic acid acyl transferase

(LPS) Lipopolysaccharide

(LRR) Leucine-rich repeat

(LRRK1) Leucine-rich repeat kinase 1

(LRRK2) Leucine-rich repeat kinase 2

(LRRK2-IN-1) 5,11-Dihydro-2-[[2-methoxy-4-[[4-(4-methyl-1-piperazinyl)-1-piperidinyl] carbonyl]phenyl]amino]-5,11-dimethyl-6H-pyrimido[4,5-b][1,4]benzodiazepin-6-one

(LC) Locus coeruleus

(L-DOPA) Levodopa

(LN) Lewy neurite

(MAO-B) Monoamine oxidase-B

(MAPK) Mitogen-activated protein kinase

(MAPKKK) Mitogen- activated protein kinase kinase kinase

(MAPs) Microtubule-associated proteins

x

(Masl1) Histiocytoma-amplified sequence with leucine-rich tandem repeats-1

(MEFs) Mouse embryonic fibroblasts

(mEPSP) Miniature Post Synaptic potential

(MLRs) Mixed lineage kinase (MLRs)

(MT) Microtubules

(MPP$^+$) 1-methyl-4-phenylpyridinium

(MPPP) 1-methyl-4-phenyl-4-propionoxypiperidine

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

(MSA) Multiple-system atrophy

(Na$^+$) Sodium

(NAC) Non-Aβ component

(nbM) Nucleus basalis of Meynert

(NBQX) 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione

(NMDA) N-methyl-D-aspartate

(NBIA) Neurodegeneration with brain iron accumulation

(NO) nitric oxide

(NP) nigrostriatal pathway

(PAF) Pure autonomic failure

(Paraquat) (N,N'-dimethyl-4-4'-bipiridinium

(PBMC) Human peripheral blood mononuclear cells

(PD) Parkinson's disease

(pERM) Phosphorylated ERM

(PICK1) Protein interacting with C kinase 1

(PINK1) Phosphatase and tensin (PTEN)-induced kinase 1

(PIP2) phosphoinositol-(4,5)-bis-phosphate

(PLD2) Phospholipase D2

(PP2A) Phosphoprotein Phosphatase 2A

(PRD) Proline-rich domain

(RIPKs) Receptor-interacting protein kinases

(ROC) Ras of complex protein

(ROS) Reactive oxygen species

(sEPSC) Spontaneous Excitatory Post Synaptic Currents

(Ser) Serine

(siRNA) Small interfering RNA

(sIPSC) Spontaneous Inhibitory Post Synaptic Currents

(SH3) SRC homology 3

(Sh-RNA) Short-harpin RNA

(SN) Substantia nigra

(SNpc) Substantia nigra pars compacta

(SNX9) Sortin nexin 9

(SV) Synaptic vesicles

(SV2) Synaptic vesicle glycoprotein

(TH) Tyrosine hydroxylase

(Thr) Threonine

(TLR4) Toll like receptors 4

(TNF-R) Tumor necrosis factor receptors

(TKL) Tyrosine kinase like

(TRADD) Tumor necrosis factor receptor type 1-associated DEATH domain protein

(tRNA) Transfer RNA

(TTX) Tetrodoxin

(Tyr) Tyrosine

(Ubl) Ubiquitin-like domain

(UCH-L1) Ubiquitin C-terminal hydrolase L1

(UPS) Ubiquitin proteasome system

(VGLUT-1) vesicular glutamate transporter-1

(VTA) Ventral tegmental area

(WT) Wild type

1. Introduction

1.1. Parkinson's Disease

Parkinson's disease (PD) is the second most frequent neurodegenerative disorder (Alzheimer's disease - AlzD is more common) [1, 2] and was described, for the first time, in 1817 by James Parkinson (1755-1824) in his publication "*An essay on the Shaking Palsy*" [2, 3]. The disease's name, "Parkinson's Disease", is a tribute of Jean Martin Charcot (1825-1893), a French Neurologist, to the British Physician, some years after the discovery [4].

PD is characterized by chronic and progressive neurodegenerative pathology of the Central Nervous System (CNS), which affects voluntary movements in approximately 1-2% of the population over the age of 65 years and 4-10% over the age of 80-85 years [5, 6]. The main symptoms are bradykinesia (slow movement), akinesia (impaired muscle movement), rigidity, rest tremor, loss of postural reflexes and flexed posture [4, 7], but the patients often also suffer from symptoms not associated with motor performance, such as depression [8], sleep disturbances, sexual dysfunction and dementia [8-12].

At the pathological level, PD is characterized by degeneration of dopaminergic (DAergic) neurons from the *substantia nigra pars compacta* (SNpc) to the striatum and emergence of cytoplasmatic inclusion bodies, Lewy bodies (LBs)/neurites (LNs) in the *substantia nigra* (SN), latin denomination for "black substance". This area is rich in DAergic neurons and plays an important role in movement (voluntary movement), in reward and addiction [12]. This "black substance", due to the presence of neuromelanin, is located in mesencephalon, also called midbrain, and is a part of the nigrostriatal pathway (NP) [5]. This pathway, as expected, is composed of DAergic neurons whose cell bodies are located in the SNpc and send projections to the basal ganglia and to the striatum, which is composed by the putamen and caudate nucleus (fig.1) [5, 7, 13]. In fact, the disease is hypothesized to start in striatum, because many studies show a larger degeneration of terminal nerves than cell bodies in SNpc, in early stages of PD [5, 7, 13].

The pathophysiology of PD remains elusive and for the majority of the PD cases the cause is uncertain [14]. An estimated 1 to 2% of PD patients have a clear familial etiology, exhibiting a classical recessive or dominant Mendelian mode of inheritance [7, 15] however, for the majority of PD patients the disease is probably caused by a combination of age, genetic and environmental factors [16, 17]. Both these genetic and environmental components

together can lead to pathophysiological phenomena like inflammation, mitochondrial dysfunction, oxidative stress, protein misfolding and apoptosis [16, 17].
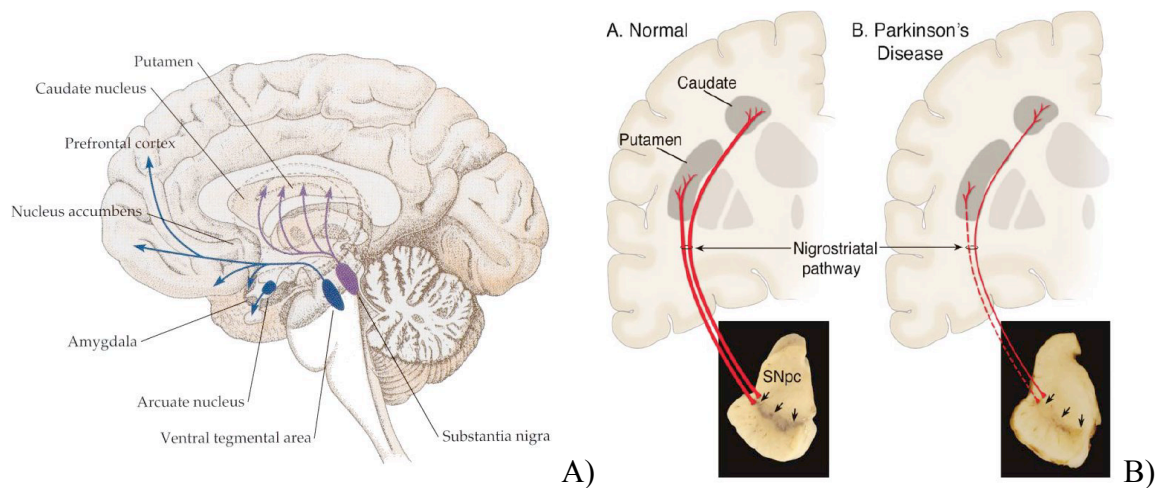

Figure 1. A) Schematic representation of NP and its constituents (purple) and reward circuit (blue). B) Schematic representation of the normal and of the diseased (in PD patients) NP (in red). It is composed of Daergic neurons whose cell bodies are located in the SNpc and their projections in the striatum (putamen and caudate nucleus). In PD, the NP degenerates and there is a marked loss of Daergic neurons that project to the putamen (dashed line) and a much more modest loss of those that project to the caudate (thin red solid line) [5].

1.1.1. Hallmarks of Parkinson's disease

As mentioned earlier, the main pathological hallmarks of PD are degeneration of DAergic neurons from the SNpc to the striatum and appearance of cytoplasmatic inclusion bodies, LB and LN [7, 13]. In fact, the initial PD symptoms are accompanied by significant neurodegeneration (some 70%) in SNpc, striatum, putamen and caudate nucleus, as above described, resulting in a strong reduction of dopamine (DA), the main neurotransmitter present in NP (fig.1) [5]. Nevertheless, the nigral damage is not uniform. The ventrolateral neurons in SNpc degenerate earlier and more severely than dorsal neurons in SNpc, which is also accompanied by extensive extranigral pathology in the dorsal motor nucleus of the glossopharyngeal, vagal nerves (i.e. dorsal IX/X motor nucleus) of the medulla oblongata and other zones like the intermediate reticular zone, locus coeruleus (LC), basal forebrain, thalamus and amygdala, affecting many other systems such as the mesocortical DAergic system, noradrenergic, serotonergic, cholinergic and limbic system [12, 18-20].

Lewy bodies (LBs) (fig.2) are abnormal aggregates of protein, including neurofilaments, α-synuclein (pre-synaptic protein, and major protein of these bodies), synphilin-1 and other components of the ubiquitin proteasome system (UPS), an important system in the clearance of proteinaceous complexes or misfolded proteins which are of high relevance for neurodegenerative diseases like Alzheimer's, Parkinson's and Huntington's disease [12, 21]. Like PD, the name LB was given to these structures to pay tribute to the

neurologist Frederic Lewy (1885-1950), the first man who described these lesions in PD patients, in 1912 [15]. LBs appear in many regions of brain such as LC, nucleus basalis of Meynert (nbM), cerebral cortex, olfactory bulb, but despite extensive investigation, mechanisms of LB formation remain unknown [12, 20].
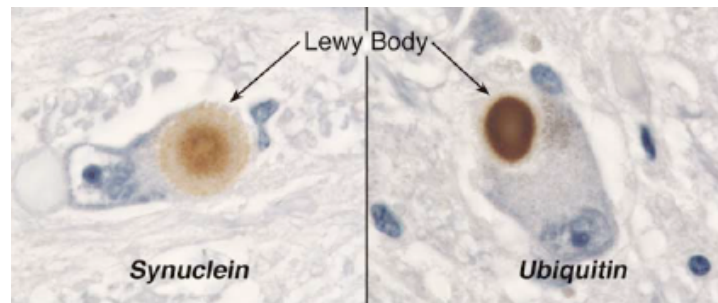
Figure 2. Lewy bodies, in a SNpc DAergic neuron. Immunostaining with antibodies against α-synuclein and ubiquitin reveal that these protein are constituents of LB's [5].

Notwithstanding the presence in many regions of brain, LBs have some different characteristics and, until today, these are not clarified completely. The LBs have, mainly, two forms: (1) classical, which are eosinophilic cytoplasmic inclusions with some fibrils [1] and (2) cortical form, where LBs are more homogeneous [12]. Therefore it is difficult to say when LB bodies arise or formed, because they can appear in different stages of PD and with different characteristics [12].

1.1.2. PD diagnosis

PD diagnosis is often difficult, especially in early stages. Early signs and symptoms of the disease may sometimes be dismissed as the effects of normal aging. In fact, the majority of clinical diagnoses about 40% of patients may not be diagnosed, ≈25% are misdiagnosed and only 75% of clinical diagnoses of PD are confirmed at autopsy [22].

To decrease these percentages, in the last ten years, many studies have been performed to explain how PD initiates and progresses, however, the neuronal damage does not develop randomly but, rather, follows a predetermined sequence marked by characteristic changes in topographical extent [12]. In 2003, a study of Braak et al., [12] was published and defines 6 stages in PD: in the first stage, there are inclusion bodies only within the spindle-shaped projection neurons of the dorsal IX/X motor nucleus and/or intermediate reticular zone and in the second stage there is some cell death and LB formation in the LC. These stages do not have, yet, any consequences in the movements and hence the diagnosis is very difficult [12].

In the third stage, the features of stage 2 become more severe, SNpc is affected such as neuronal projections in the magnocellular nuclei of the basal forebrain. Notably, there is no indication, macroscopically detectable, of the SN depigmentation. At this stage, the cortical involvement is confined to the temporal mesocortex and allocortex (CA2-plexus) while the neocortex is unaffected. It is in this stage, that diagnosis is possible. In stage 4, the odor sensory areas of the neocortex and prefrontal neocortex are affected, there is a significant loss of neurons, especially in the posterior regions of the SNpc and the presence of inclusion bodies in the interstitial nucleus of the striatal terminals is observed [12].

In stage 5, the disruption of olfactory areas is severe and LNs and LBs gradually decrease while in SN a visible loss of neurons is observed. Finally, in stage 6 there is the involvement of the entire neocortex and the premotor areas, the primary motor field, the first sensory association and primary sensory areas usually are subjected to relatively mild pathological changes the first motor symptoms [12].

1.1.3. PD treatment

Actually, there is no treatment for this complex disorder, despite the large number of studies that have been done [2, 23]. The most significant advanced treatment occurred in the 1960's by Anthony Carlsson (133 years after discovery of disease) with the discovery that the DA precursor, levodopa (L-DOPA), could replenish DA and attenuate most motor symptoms when administered orally or intravenously [2, 23]. L-DOPA is however ineffective in treating dementia and in non-motor symptoms that occur in PD [2]. When L-DOPA enters the brain, it is metabolized in DAergic neurons and leads an increase of DA levels in presynaptic cells, in synaptic vesicles (SVs) and in the synaptic cleft. These features improve DA synaptic transmission and attenuate most motor symptoms, as described above [2, 24-26]. Normally, L-DOPA is co-administered with other drugs that prevent peripheral metabolism, enhancing the function of L-DOPA. In an advanced stage, with symptoms that can no longer be treated with L-DOPA, invasive surgical interventions can be made. Pallidotomy, which destroys the overactive globus pallidus, results in attenuation of many symptoms, that however return at a later stage. Alternatively, thalamotomy - which destroys part of the thalamus - to block the abnormal brain activity, can be applied but with this approach the risk of cognitive and/or speech problems is substantial [24-26].

1.1.4. Risk Factors of Parkinson´s Disease

The exact cause of PD remains unclear. In the last century, PD was thought to result from environmental factors (e.g. toxins), and there were some clinical and epidemiological evidences that support the notion that PD occurred as a result of a neuronal infection, for example neuro-virulent strains of influenza A virus [2]. In addition to environmental influence, several studies reported genetic factors that govern PD [2]. While an exact cause of the disease is unknown, it is thought that there is a conjunction of many risk factors contributing to nigrostriatal dysfunction, such as increasing age, environmental and genetic factors [16, 17].

Actually, it is estimated that 90% of all PD patients are sporadic/idiopathic PD but it is possible that in these cases some deoxyribonucleic acid (DNA) mutations are yet to be discovered[16, 17].

1.1.4.1. Aging

As the majority of PD patients are older than 65, it is widely accepted that aging is the largest risk of PD [4]. In fact, the percentage of affected individuals ranges from 1% after 65 years and 5% from 85 years. Nevertheless, the number of young patients suffering from PD is substantial [4]. Therefore, PD is classified in juvenile PD (<21 years), early (between 21 and 50 years) or late (>50 years) onset [4, 5]. The common aging mechanisms, contributing to development of the disease are still unknown but oxidative stress, inflammation, phenomena characteristic of aging, have been suggested to play a relevant role in PD [4, 5].

1.1.4.2. Environmental factors

In many studies, PD was shown to develop due to environmental risk factors, such as viruses, toxins affecting the CNS, via the postganglionic enteric neurons, during earlier stages of PD [4, 5]. An important trigger consists in pharmacological inhibition or alteration of some cell functions including complex I of the mitochondrial respiratory chain, formation of reactive oxygen species (ROS) and displacement of DA from vesicular stores. Among these compounds that alter the functions described above, there are many toxins, neurotoxins and herbicides such as 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP), 6-hydroxydopamine (6-OHDA) rotenone and paraquat [5].

MPTP (fig.3A), a lipophilic compound, was discovered in the early 1980's within a group of heroin addicts. The toxin derived, 1-methyl-4-phenyl-4-propionoxypiperidine

(MPPP) an analog of narcotic meperidine (Demerol), crosses the brain blood barrier (BBB) and is metabolized in glial cells and serotonergic neurons by monoamine oxidase B (MAO-B) into 1-methyl-4-phenylpyridinium (MPP$^+$) [27, 28]. After cellular uptake, via dopamine transporter (DAT), MPP$^+$ accumulates, preferentially, into mitochondria of DAergic neurons and inhibits complex I of the respiratory chain. This leads to production of ROS, disruption of the NP and adenosine triphosphate (ATP) depletion, suggesting a possible mechanism for nigral cell death (fig.3B) [5, 27, 28]. In fact, MPTP in mice has been shown to result in motor deficits, like as tremor, rigidity, slowness of movement, postural instability (many symptoms of PD), caused by cell death, microtubule depolymerization and α-synuclein/ubiquitin positive inclusions in the SNpc [5, 27, 28]. Despite the use as Parkinsonism model in rodents (study the molecular mechanisms of DAergic neurons) and primates (study of novel therapeutics), the MPTP toxicity can be reverted. Cells lacking α-synuclein are insensitive to mitochondrial toxicity induced by MPP$^+$ [29] and it has been shown that α-synuclein could play an important role in mediating MPP+ toxicity, perhaps through regulation of nitric oxide (NO) signaling [30] and/or by inhibition of MPP$^+$-induced mitogen-activated protein kinase (MAPK) activation, that reduces autophagy and mitochondrial impairments/degradation [31, 32].
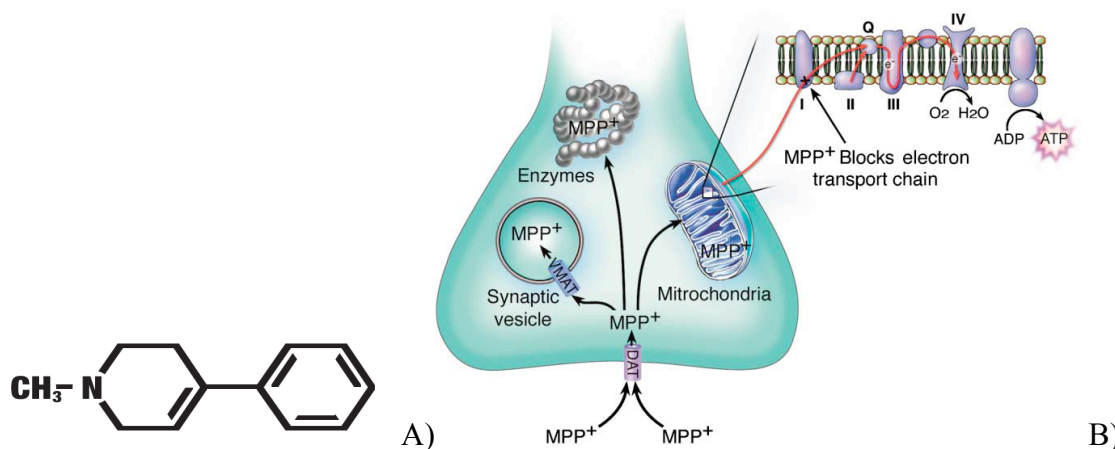
Figure 3. A) Chemical structure of MPTP. B) Schematic pathway of action of MPTP. MPTP crosses BBB and is metabolized by glial MAO-B. After MPP$^+$, into mitochondria inhibits complex I of respiratory chain [5].

6-OHDA is a neurotoxin with a similar mode of action as MPTP (i.e. decreased mitochondrial complex I function) leading to production of ROS in catecholaminergic neurons [33]. Because many studies have reported an important role for mitochondrial dysfunction in PD, actually, MPTP and 6-OHDA are used to develop cellular and animal

models of PD. Nevertheless, MPTP, and 6-OHDA are not the only compounds with these properties [33].

Paraquat, an herbicide (N,N'-dimethyl-4-4'-bipiridinium) that shows similarity to MPP$^+$ induces superoxide radical production (via mitochondrial dysfunction), which causes of DAergic neurons and locomotor dysfunction. This herbicide does not require DAT for uptake and oxidizes the cytosolic form of thioredoxin resulting in activated c-Jun N-terminal kinase (JNK) leading to caspase-3 activation and cell death [33]. The systemic administration of paraquat in mice leads to DAergic cell loss in the SNpc with LB appearance [34].

Rotenone also blocks complex I of mitochondrial chain and its low-dose intravenous administration in rodents promotes the same consequences as paraquat [35].

The relevance of PD models (cellular and animal), for understanding the impact of oxidative stress and complex I inhibition was further emphasized by the identification of point mutations in mitochondrial transfer RNA (tRNA) genes in the SN of PD cases [33]. In addition, some PARK genes are interfering with mitochondrial function, such as *P-TEN induced putative kinase 1* (PINK1), that protects against oxidative stress, and *DJ-1* that prevents protein aggregation and oxidative stress [36-38]. On the other hand all the pharmacologic interventions that can reverse MPTP- or 6-OHDA-induced neurotoxicity have failed in the clinic for efficiency reasons. Hence these animals' models are valuable symptomatic models but not PD pathogenic models [36-39].

1.1.4.3. Genes Associated with Parkinson's disease

As previously described, the majority of PD cases are believed to be idiopathic, but some cases (10%) have the influence of genetic factors, showing both autosomal dominant and recessive manners of inheritance [7, 16, 17]. During the last 20 years, many studies have showed that a substantial amount of PD cases are linked to specific gene mutations (see table 1) [7, 16, 17]. These discoveries provide opportunities to investigate the functions of the proteins that play an important role in neurodegenerative cascade and consequently, allow studying the pathways involved in the PD pathogenesis such as protein aggregation, mitochondrial dysfunction and secretory pathway dysfunction [17].

In fact, chromosomal regions (loci) have been mapped in familial PD (autosomal dominant/recessive manner) referred to as PARK1-16 (See table 1) [7, 40]. The main PD-linked loci include two autosomal dominant genes, *α-synuclein* and *Leucine-rich repeat kinase 2* (LRRK2), and three autosomal recessive genes, *Parkin*, *DJ-1*, and *Phosphatase and*

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tensin (PTEN)-induced kinase 1 (PINK1). A mutation in a sixth gene, *Ubiquitin C-terminal hydrolase L1* (UCH-L1) has only been found in one family and the importance of this gene in familial PD is still uncertain. The identification of mutations in PARK genes in families with hereditary forms of the disease has revolutionized the study of PD [7].

1.1.4.3.1. Autosomal dominant genes
PARK1/4 (*α-synuclein*)

At the end of the 20th century, the PARK1 locus was mapped for the first time in an Italian family on chromosome 4q21 [41]. It was identified as the first locus linked to autosomal dominant form of PD and contains the α-synuclein gene, where some missense mutations A53T, A30P, E46K and G46L, that modified the properties of the α-synuclein protein, were identified [41, 42].

The gene that encodes α-synuclein protein spans 117 kb, contains 6 exons [41] and has been a major focus of PD genetic research because α-synuclein was found not only to govern rare forms of familial PD but also to be relevant for the pathology in sporadic PD [17].

Locus	Chromosome	Gene	Mode of inheritance	Phenotype
PARK1/4	4q21-q23	α-synuclein	AD	Classic PD and dementia
PARK2	6q25.2-27	Parkin	AR-JP	Slow progression and no LB
PARK3	2p13.3-2p13.1	Unknown	AD	Classic PD
PARK5	4q14	UCH-L1	AD	Classic PD
PARK6	1q35-p36	PINK1	AR-JP	Parkinsonism
PARK7	1p36	DJ-1	AR-JP	Slow progression and no LB
PARK8	12p11.2-q13.1	LRRK2	AD	Classic PD
PARK9	1p36	ATP13A2	AR	KRS and dementia
PARK10	1p32	Unknown	Trans. unknown	Classic PD
PARK11	2q36-q37	GIGYF2	AD	Classic PD
PARK12	Xq21-q25	Unknown	Trans. unknown	Classic PD
PARK13	2p12	HTRA2/OMI	Trans. unknown	Classic PD
PARK14	22q13.1	PLAG26	AR	Parkinsonism + LB
PARK15	22q12-q13	FBXO7	AR	Parkinsonism
PARK16	1q32	Unknown	Trans. unknown	Classic PD
-	17q21.1	MAPT	Trans. unknown	Parkinsonism
-	1q21	Glucocerebrosidase	AD	Parkinsonism + LB
-	5q23.1-q23.3	Synphilin-1	Trans. unknown	Classic PD
-	2q22-q23	NR4A2/Nurr1	Trans. unknown	Classic PD

Table 1. PARK loci and the genes implicated in Parkinsonism and PD. This table describes the familial PARK loci (1-16) and genes shown to be associated to PD in non-familial forms. AD: autosomal dominant, AR: autosomal recessive, AR-JP: autosomal recessive-juvenile Parkinsonism, LB: Lewy body. Classic PD refers to the late- onset. KRS: Kufor-Rakeb syndrome Adapted from Lees et al., 2009 [15].

α-Synuclein is a 19kDa, 140 amino acid protein and is the major structural component of LB, providing compelling evidence that it plays a major role in the pathogenesis of sporadic and familial PD [43, 44]. This unfolded protein contains three domains, the acidic region (carboxyl terminus), non-Aβ component (NAC), which confers the β-sheet potential domain and the N-terminal alpha-helical lipid binding domain, which contains 7 eleven residue repeats and are predicted to form amphiphilic helices conferring the propensity to form α-helical structures upon membrane binding. These repeats form an amphipathic helix upon membrane binding that allows binding to synaptic vesicles. This property suggests that α-synuclein is required for the formation and/or maintenance of a reserve pool of presynaptic vesicles [43, 44]. This binding is altered by A30P and A30T mutations that reduce this ability in a step in exocytosis before calcium (Ca^{2+})-induced fusion [17, 45-49].

Predominantly, this protein, a member of the synuclein family proteins, which also include β- and γ-synuclein, is localized in presynaptic terminal of cholinergic and DAergic neurons, comprises 1% of total cytosolic protein but is also found, for unclear reasons, in erythrocytes and platelets. Originally identified as a result of its association with synaptic vesicles, α-synuclein lacks a transmembrane domain or lipid anchor and has been considered a peripheral membrane protein [50]. Indeed, α-synuclein binds to artificial membranes *in vitro* by adopting an α-helical conformation [50]. α-Synuclein also associates with axonal transport vesicles, lipid droplets, and yeast membranes [51]. However, α-synuclein behaves almost entirely as a soluble protein in brain extracts [17, 42, 45-49].

α-Synuclein also plays a role in synaptic plasticity, chaperone mechanisms, storage, exocytosis and endocytosis/vesicle recycling and serves as a potential negative regulator of DA neurotransmission via interaction with the SNARE complex (mediates vesicle fusion/release) [52, 53]. In PD, this protein is phosphorylated at Ser129 residue (and S87 residue) which leads to structure and form modifications suppressing tyrosine-hydroxylase (TH) activity [52, 53] improving DA storage into vesicles. This reduces the activity of DAT and has been shown to inhibit the exocytosis of vesicular glutamate transporter-1 (VGLUT-1) in glutamatergic vesicles and in neurons [6, 17, 43, 54]. Indeed, overexpression of Wild Type (WT) α-synuclein, and presence of A30P and A53T α-synuclein mutations cause an increase in levels of protein leading to aggregation and impairments in normal role of α-synuclein. A30P α-synuclein mutation, which abolishes the protein ability to bind to small phospholipids vesicles and A53T α-synuclein mutation, which impairs its association with planar lipid membranes bind to small phospholipids vesicles and A53T α-synuclein mutation, which

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impairs its association with planar lipid membranes, inhibit exocytosis, endocytosis, synaptic transmission, leading to few symptoms relevant to PD [44, 49, 52, 53, 55-58].

Nevertheless, it was also demonstrated, that knockout (KO) of α-synuclein causes impairments in neurotransmission by increasing of DA contents in the presynaptic cell and the in synaptic cleft [49, 52, 53, 55-57]. Therefore, loss of normal function of α-synuclein, as well a toxic effect of altered forms of mutant proteins impair the neurotransmission. Moreover, α-synuclein can also leads to neuronal cell death by blockade of ER-Golgi trafficking, sequestering vesicle-trafficking proteins, such as Ypt1p, and interfering with its function. Conversely, overexpression of these proteins, in particular Ypt1p and its ortholog Rab1, can attenuate α-synuclein-induced toxicity [44, 59].

A possible strategy to reduce the phosphorylation of α-synuclein at serine (Ser) 129 and perhaps attenuate the PD symptoms is to stimulate the Phosphoprotein Phosphatase 2 A (PP2A), the primary Ser/threonine (Thr) phosphatase in the brain, protecting against α-synuclein neurotoxicity [42, 60].

Endocytosis, as described below, is considered to be particularly important in neurons and synaptic transmission, and a study of Kuwahara et al., (2008) [49] described that overexpression of WT α-synuclein and mutants A30T and A53T inhibits AP-2 function [49]. AP-2 is involved in endocytosis, because this heterotetramer, composed of two large subunits, α and β2, and two small subunits μ2 and σ2, recruits clathrin and cargo receptors to the endocytic pits, which in turn are progressively invaginated and internalized into the cytosol by forming vesicles [49, 61]. The AP-2/endocytosis inhibition by α-synuclein is potentiated by A30P and A53T mutations, suggesting a link between AP-2/endocytosis function and α-synuclein neurotoxicity. These findings raise the possibility that accumulation of WT α-synuclein or mutated α-synuclein perturbs the endocytic pathway (fig.4A) [49, 61]. Furthermore, overexpression of WT α-synuclein or A30P and A53T mutants also inhibits activity of phospholipase D2 (PLD2) *in vitro* and a similar action *in vivo* can result in a reduction of the number of vesicles available for DA storage and increase in the oxidative stress (fig.4B) [47]. PLD2 is a membrane-bound enzyme located in plasma and endosomal membranes, hydrolyzes phosphatidylcholine into lysophosphatidylcholine and phosphatidic acid in response to external stimuli. PLD2-derived phosphatidic acid then, recruits AP-2 and triggers the budding of vesicles from donor membranes. Thus, it is also possible that α-synuclein inhibits endocytosis by negatively regulating PLD2 activity [49] which is accompanied by the genomic multiplication of the complete α-synuclein gene, found on

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PARK4 in other families around the world (French, Spanish, American, Japanese families). This gene multiplication was linked to familial PD and, actually, there is a direct relationship between gene dosage (protein synthesis) and disease age at onset, disease progression and phenotypic severity [62, 63]. As described, α-synuclein pathology in PD is not confined to the cell soma, but is also prominent in neuritic processes, it is widespread in various regions of brain in PD and it is present in a number of other synucleinopathies such as multiple-system atrophy (MSA), dementia with LB (DLBs), many cases of AlzD (the so-called LB variant of AlzD), neurodegeneration with brain iron accumulation (NBIA) type 1, pure autonomic failure (PAF) and even a subtype of essential tremor [62, 63].
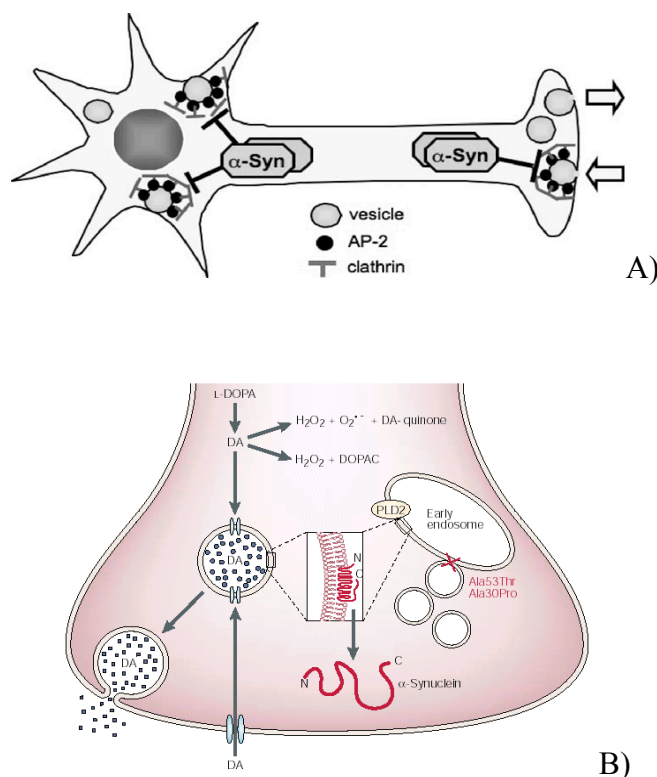
Figure 4. Possible models of toxic effect of α-synuclein. **A)** α-Synuclein may inhibit endocytosis at the presynaptic terminals or cell bodies [49]. **B)** DA is synthesized in the cytoplasm and immediately sequestered into synaptic vesicles. If unstored, dopamine can auto-oxidize to dopamine–quinone or 3,4-dihydroxyphenylacetic acid (DOPAC). α-Synuclein is highly enriched in presynaptic terminals. α-Synuclein might be to regulate the formation of synaptic vesicles from early endosomes through interactions with PLD2. Mutations in α-synuclein might result in a reduced number of vesicles being available for dopamine storage, leading to an accumulation of dopamine in the cytoplasm and increased levels of oxidative stress [47].

PARK5 (*Ubiquitin C-terminal hydrolase L1*)

The PARK5 locus contains a gene for an autosomal dominant form of PD and was mapped on chromosome 4p14 [64]. This gene encodes the enzyme Ubiquitin C-terminal hydrolase L1 (UCH-L1) that has a function on UPS (hydrolyses polymeric ubiquitin chains into monomers) and plays an important role in the regulation of synaptic function and

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plasticity [64]. Moreover, this protein is suggested as Ubiquitin ligase enzyme (E3) ligase like Parkin protein (described below) that may control the ubiquitin ligase activity dimerization-dependent and may maintain the ubiquitin homeostasis through ubiquitin monomer stability [64]. In 1998, a single missense mutation in the UCH-L1 gene was identified to cause autosomal-dominant PD that causes the UCH-L1 aggregation in LB [65, 66].

PARK8 (*Leucine-rich repeat kinase 2 or dardarin - LRRK2*)

PARK8 locus was originally identified early of XXI century in a Japanese family with autosomal dominant PD, the Sagamihara kindred, and was the second causal gene linked to autosomal dominant inherited PD [1, 67-69].

The PARK8 locus is located on chromosome 12p11.2-q13.1 (chromosome 15 in mouse) [70] and was also identified in European/American families, therefore seems be relatively common throughout the world [71, 72]. Strikingly, neuropathological analysis of Japanese patients showed loss of SNpc neurons and no LB, so called "pure nigral degeneration or no dementia", whereas the European families presented signs of dementia and motor-neuron degeneration [2, 68, 69, 72].

The gene responsible for PARK8 linked PD was identified as Leucine-rich repeat kinase 2 (LRRK2). It has 144 kb and contains 51 exons, which encodes a protein with 2527 amino acids (286 kDa) [1, 2, 6, 7, 71-74].

Missense mutations, such as Y1654C and R1396G, were identified in LRRK2 gene in British and Spanish families, respectively [75]. These and additional mutations such as G2019S, I2020T, R1441C/G/H, Y1699C, G2385R and I1122V mutations show clear segregation with PD [1, 2, 7, 17, 39, 76, 77]. The most prevalent mutation, G2019S (28% at 59 years to 74% at 79 years) [78], is identified in Caucasian populations of North America and Western Europe. Probably, this mutation derived from Arabic and/or Jewish family and was spread via Northern Africa or Arabic and/or Jewish Diasporas, because the populations with more prevalence of G2019S LRRK2 mutation are Arab Berbers (North Africa) with 39% of idiopathic PD and 36% of familial PD; and Ashkenazi Jews (Jews) with 10% of idiopathic PD and 28% of familial PD [79, 80]. In Europe, the frequency is higher in southern European countries than in northern European countries and it is rarely seen in Asians (Chinese, Korean, and Indian) countries [79]. The prevalence of this mutation does not differ significantly by gender [73, 79].

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At the molecular level, the G2019S mutation increases kinase activity, suggesting that LRRK2-driven PD is due to deregulated enzyme function (kinase) [73, 79]. At the pathological level, this mutation leads an increase of cell toxicity, including frontotemporal lobar degeneration, corticobasal syndrome, nigral neuronal degeneration and gliosis but with variable intraneuronal protein inclusions and in some cases leading to AlzD [31, 81]. These inclusions may contain α-synuclein-positive LB and LN tau-positive neurofibrillary tangles, ubiquitin-positive intranuclear and cytoplasmic inclusions [77].

The PARK8 locus and mainly LRRK2 protein has been associated with both idiopathic and familial PD [1]. The interaction of LRRK2 with other PARK loci (dominant and/or recessive), and its implication in both synucleinopathies and tauopathies, suggests that it might play an important role in various pathways. Therefore, an examination of LRRK2 biochemical properties is important for understanding the mechanisms underlying the disorder. In addition, kinase inhibition is an attractive strategy that has the potential to lead to therapeutics [73, 82].

Other loci have been linked to autosomal-dominant PD such as the PARK3 locus, present on chromosome 2p13, where the responsible gene leading to an autosomal dominant form of PD with a late onset and formation of LB was not yet identified [83] and PARK11 locus, present on chromosome 2q36-q37 that contains GIGYF2 gene and where the mutation N457T seems to be related with PD appearance [84].

1.1.4.3.2. Autosomal recessive PD genes

PARK2 (*Parkin*)

Another gene linked to PD is PARK2 (*Parkin*) [64]. In 1997/1998, this locus was described/mapped on chromosome 6q25.2-27 in several Japanese families with autosomal recessive juvenile Parkinsonism (AR-JP) [17, 85, 86]. Patients that carry mutations in this gene have manifested PD symptoms at 20 years of age, becoming mutations in this gene the most common cause of early-onset Parkinsonism [85, 87]. In fact meta-analysis of parkin mutation carriers suggests that pathogenic alterations to parkin gene account for up to 50% of AR-JP in some populations [88].

The *Parkin* gene contains 12 exons encompassing 1,38Mb. This gene encodes the Parkin protein with 465 amino acids [85] that contains ubiquitin-like (Ubl) domain in its NH$_2$-terminus and a COOH-terminal cysteine-rich region that included a motif similar to a RING. Later, it was established that the COOH-terminal region contains two RING fingers,

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characterized by the signature C3HC4 (that is, three conserved cysteine residues followed by a conserved histidine and then four additional conserved cysteines) plus an intermediate, cysteine-rich region (characterized by a C6HC pattern), that was called IBR or DRIL domain [86]. Actually, this characteristic protein structure is called as RBR domain [85, 87].

Parkin protein is expressed in multiple tissues and particularly in different regions of the brain, including SN. These findings suggest that parkin function is normally required for long-term survival of DAergic neurons although it is likely to have functions in other tissues as well. This protein is responsible for transferring activated ubiquitin molecules to substrates targeted for degradation by the UPS, i.e., recognizes the specific protein to be ubiquitinated and catalyzes the transfer of ubiquitin from E2 (ubiquitin-conjugating enzyme) to this target protein [85, 87]. These specific proteins (targets) such as α-synuclein, synphilin-1, cyclin E, synaptotagmin and parkin itself are degraded in the proteasome, a component of the UPS that cleaves proteins in amino acids in 3 steps, as shown in figure 5 [85, 87].
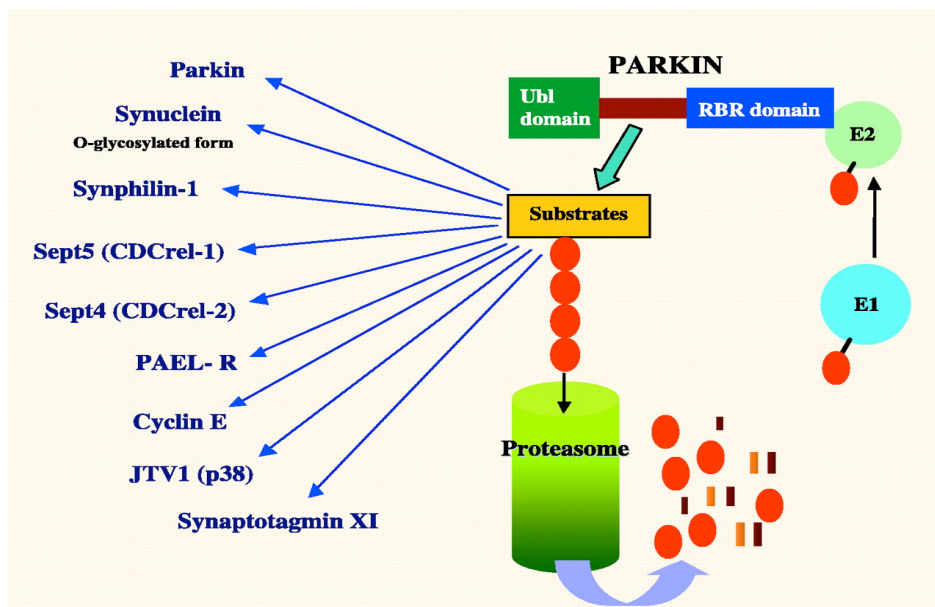
Figure 5. Parkin substrates and the ubiquitin-proteasome system. Ubiquitin (red) is first activated by the E1 (ubiquitin-activating) enzyme. Then it is transferred to the ubiquitin conjugating enzyme (E2). From E2, ubiquitin is finally added to the substrate of a ubiquitin protein ligase (E3), in this case Parkin. Polyubiquitinated substrates bind to the proteasome and are degraded, liberating short peptides and free ubiquitin, ready to be used again [86].

In the cell, the Parkin protein appears in the cytoplasm membranous organelles, like endoplasmatic reticulum (ER) and Golgi apparatus, vesicular structures of dendrites and nerve terminals suggesting that this protein modulates trafficking of synaptic proteins such as endophilin-A1, an endocytic protein with a N-terminal BAR domain that interacts with

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amphiphysin to regulate lipid membrane curvature [87]. Therefore, some modifications in this protein lead to alterations in synaptic function [17].

After mutations in LRRK2, the second most common genetic cause of Parkinsonism and PD are found throughout the Parkin protein [79]. Some of these mutations are C289G, R275W, R256C, R334C, C431F in RING domain and R33Q, R42P, V56E and N52MfsX29 in the Ubl domain [87]. These mutations confer either a decreased ubiquitin ligase activity or disrupt its localization, solubility or interactions with substrates such as loss of the C-terminus, a common truncation of Parkin. This loss reduces its localization to the post-synaptic cell, reduces the scaffold and reduces ubiquitination of Parkin post-synaptic substrates [17, 87]. Thus target proteins and organelles cannot be degraded and are accumulated in the cell ultimately leading to PD features. Although synucleinopathy is not a typical feature of AR-JP in humans [89], LB has been observed in some cases [17, 86, 89, 90].

The Parkin protein is also responsible for mitochondrial maintenance and might induce subsequent autophagy of dysfunctional mitochondria [17, 91]. Several mice models demonstrated nigrostriatal synaptic deficits, mitochondrial dysfunction and deficits in learning in Parkin mutants. However these phenomena are not associated with nigral DAergic cell loss [85, 89, 92].

In addition, Parkin has been suggested to bind DNA and represses the tumor suppressor protein p53 on a transcriptional level [86], and seems to be important to control calcium (Ca^{2+}) levels in glutamatergic neurons, by regulating protein interacting with C kinase 1 (PICK1), a protein that is implicated in internalization activity-dependent and retention of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor GluA2 (GluR2) subunit. Together, Parkin and LRRK2 act as a part of a complex to regulate glutamatergic synapse formation/elimination and/or excitatory synapse protein trafficking and degradation [17, 93].

Furthermore, in the post-synaptic compartment, Parkin has been reported to function as a PDZ-binding protein via its C-terminus and its association with CASK, a post-synaptic multidomain scaffolding protein [17], suggesting that Parkin and CASK interaction have a relevance for neurodegenerative disorder and protein degradation [17, 94].

PARK6 (Phosphatase and tensin (PTEN)-induced kinase 1)

In 2004, a second autosomal-recessive gene inside the PARK6 locus was identified in three families with early-onset autosomal-recessive PD. The P-TEN induced putative kinase 1

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(PINK1) localized on chromosome 1p35-p36 [6, 36, 95, 96] encodes a protein that plays an important role in regulation of mitochondrial morphology (fission and fusion) and functionality by control Ca^{2+} efflux [36, 37].

PINK1 is a 581 amino acid protein with a catalytic Ser/Thr kinase domain. PINK1 protein exhibits kinase activity *in vitro* and it has been hypothesized that PINK1 plays a role in signaling cascades between the mitochondria (maintenance of mitochondrial function) and the nucleus by potentiating the expression of essential proteins with protective properties [97]. PINK1 protein regulates mitochondrial trafficking, reduces ROS formation and facilitates neuroprotection, regulates mitochondrial respiration efficacy, the opening of the mitochondrial permeability transition pore (MpTP), interacts with cell death inhibitors and chaperones [36, 37, 98]. Two candidate substrates were described for direct or downstream phosphorylation by PINK1 in mitochondria. These putative substrates are the heat shock protein (Hsp) 75 chaperone TRAP1, a ubiquitously expressed protein with significant sequence homology to the HSP90AA1 family of molecular chaperones, and the protease HtrA2, well known for its involvement in apoptosis - release from mitochondria to promote cell death [98].

PINK1 is ubiquitously expressed and is localized into mitochondria (inner mitochondrial membrane) and two homozygous PINK1 mutations were identified: a truncating nonsense mutation (W437X) and a G309D missense mutation. Its functional loss can be substituted by Parkin protein, nevertheless, studies with mouse embryonic fibroblasts (MEFs) from PINK1 KO mice have shown that Parkin is unable to localize to the mitochondria, suggesting that the mitochondrial function is dependent of PINK1 presence [37, 98]. In flies, PINK1 loss-of-function leads to muscle and DAergic degeneration due to mitochondrial dysfunction, a phenotype rescued by overexpression of Parkin. Thus, PINK1 and Parkin could act in a common biochemical pathway. Furthermore, studies with small interfering RNA (siRNA)-mediated depletion of PINK1 increase the susceptibility to apoptotic cell death [36, 37, 98].

PARK7 (DJ-1)

The third most relevant autosomal recessive PD gene was discovered in 2003 and is localized in locus PARK7 on chromosome 1p36 [76]. The gene was called *DJ-1* and encodes a protein called DJ-1 protein [38].

DJ-1 gene spans 24kb and contains 8 exons while DJ-1 protein is a homodimer constituted by 189 amino acids. This protein is localized to both neuronal and glial cells in the brain and was found to be hydrogen peroxide (H_2O_2)-responsive, suggesting that DJ-1 protein represents a sensor for oxidative stress, for example, DA toxicity. It acts as an antioxidant (direct scavenger of ROS) and protects cell against neuronal cell death, therefore, a complete loss of DJ-1 function may lead to mitochondrial dysfunction and PD [99, 100]. More recently it was hypothesized that the DJ-1 protein is part of a novel E3 ubiquitin ligase complex together with parkin and PINK1. Conversely, DJ-1 protein is not a component of LB like PD autosomal recessive genes mentioned above [6, 38, 99, 100].

Other loci have been linked to autosomal-recessive PD: these include PARK9, 14 and 15. PARK9 locus, present on chromosome 1p3 is associated with rare form of autosomal-recessive PD and is linked to Kufor-Rakeb syndrome (KRS). KRS is a rare hereditary disease with juvenile onset. In addition to typical signs of Parkinson disease, affected individuals show symptoms of more widespread neurodegeneration, including dementia [101, 102]. This locus contains the ATPase Type 13A2 (ATP13A2) gene. Mutations in this gene have been shown to cause AR-JP and early-onset Parkinsonism (EOP) associated with atypical features including dementia, pyramidal degeneration. Aggregation of this protein in endoplasmic reticulum causes proteasomal or lysosomal dysfunction [101]. PARK14 and PARK15 loci are present on chromosome 22q13.1 and 22q12-q13 respectively [103].

1.1.4.3.3. Loci with hereditary transmission unknown

Until now, there are still some loci and genes that is unclear if they are autosomal dominant or recessive, but mutations in these loci have been linked to PD. Some mutations in the CDCP2 gene on PARK10 locus, presents on chromosome 1p32, lead to classic PD without LB [104]; PARK12 locus presents on chromosome Xq21-q25 shows classic PD [105] and variants of the HTRA2/OMI gene on PARK13 locus, present on chromosome 2p12, lead to classic PD with LB [106].

Additionally, three other susceptibility genes, Nurr1 (NR4A2) on chromosome 2q22-q23, Synphilin-1 on chromosome 5q23.1-q23.3 and tau (MAPT), which show no linkage to previously described PARK loci, have been linked to families with PD [40, 106].

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1.2 LRRK2 Protein

The majority of PD patients are a result of complex interactions between genetic and environmental toxins that lead to disease, but, as previously described, a gene that seems to play a role in both idiopathic/sporadic PD patients and familial PD patients is *LRRK2* [1].

The *LRRK2* gene, contains 51 exons and a 9kb mRNA transcript that is predicted to encode a 286kDa (2527-amino acids) multi-domain protein, LRRK2 [1, 2, 6, 7, 71-74].

The LRRK2 protein is a ROCO superfamily member and there are at least 40 members in this superfamily. These proteins are found in a variety of species including prokaryotes, *dictyostelium*, plants, *metazoa* and *mamals* but not in *plasmodium* and yeast. Three other proteins belonging to this family have been identified in humans; leucine-rich repeat kinase 1 (LRRK1), death-associated protein kinase (Dapk1) and malignant fibrous histiocytoma-amplified sequence with leucine-rich tandem repeats-1 (Masl1) [107].

LRRK2 is moderately expressed in many tissues [93], such as bone marrow, lung and kidney, having an important role during aging [39, 108, 109]. In fact, LRRK2 mRNA and protein was found in circulating in peripheral blood mononuclear cells (PBMCs) and a deletion of LRRK2 in mice induces cytopathological abnormalities in kidney, lung tissues [78, 110]. In the brain, LRRK2 mRNA and protein are expressed in neurons, astrocytes, and microglia. LRRK2 expression is first detected in the rodent brain by embryonic day 16 to 17 with increasing expression during neuronal maturation and postnatal stages [78]. However, *in situ hybridization* studies indicated sites of mRNA and protein expression throughout the mammalian brain with highest levels of expression detected in forebrain regions, including the cerebral cortex and striatum, intermediate levels observed in the hippocampus, olfactory tubercle and cerebellum, and low levels in the thalamus, hypothalamus and SN [39, 77, 111]. Despite the low expression of LRRK2 in SN, the presence of LRRK2 mRNA in the SNpc of PD brains is dramatically reduced compared to that observed in normal control brains, indicating that LRRK2 is essential to DA neurons. A possible explanation is that LRRK2 regulates proteins involved in the synthesis or release of neurotrophins which are then transported, via retrograde transport, from the striatum to the SNpc [111, 112]. The apparent absence of LRRK2 mRNA in the SNpc, despite the detection of low protein levels in this region may be caused by LRRK2 mRNA and protein characteristics. LRRK2 mRNA may have a short half-life or be transported to distal sites in nigral DAergic neurons or the LRRK2 protein may have a particularly long half-life in these neurons so only small number of mRNA copies are required to maintain protein levels [112, 113].

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At the subcellular level, many reports have shown that LRRK2 (monomer) is found in cytoplasm (mainly in soluble state) and cytoskeleton-associated (α-tubulin; β-tubulin; microtubules - MTs) [114].

LRRK2 is also able to form a dimer (~600 kDa) and is found with membrane-bound organelles of the secretory pathway, like ER, Golgi apparatus; structures of the endocytic pathway (lipid rafts, clathrin-coated endosomes and multi-vesicular endosomes) and outer mitochondrial membrane within the rat brain, suggesting a potential role in the formation and/ or regulation of vesicular structures [39, 77]. LRRK2 dimer is substantially enriched at the membrane, which coincides with elevated *in vitro* kinase activity of the membrane-associated pool of LRRK2 compared to cytosolic LRRK2 [39, 77]. In addition to its increased kinase activity, an increased level of GTP binding, with decreased level of phosphorylation (decreased GTPase activity), was observed [115]. The decreased phosphorylation status of the membrane-associated LRRK2 compared to cytosolic LRRK2, suggests that phosphorylation at certain sites may inhibit LRRK2 activity, dimer formation, trafficking to the membrane or a combination of these processes [115]. The discrete localization of LRRK2 to membranous and vesicular structures broadly points to a role for this protein in the biogenesis, regulation and/or trafficking of such lipid based structures or their associated protein components [77]. An unpublished observation from the De Strooper lab has demonstrated presence of LRRK2 in synaptosomal preparations from mouse brain (Wim Mandemakers, unpublished observation).

1.2.1. LRRK2 protein structure

As mentioned earlier, LRRK2 protein contains 2 catalytic domains: a ras of complex proteins (Roc) GTPase domain and a kinase domain like mitogen-activated protein kinase kinase kinase (MAPKKK). These domains are separated by a C-terminal of Ras (COR) domain. Roc and COR domains are always expressed together in the same molecule suggesting a combined function of these domains. The kinase domain of LRRK2 is situated C-terminal of the COR domain, a sequence with identity to receptor-interacting protein kinases (RIPKs), a crucial regulator of cell survival and death, and to mixed-lineage kinases (MLKs), a subclass of the MAPKKK family [2]. Because LRRK2 has multiple protein-protein interaction regions, such as a C-terminal WD40 motif, an N-terminal leucine rich repeats (LRR) domain, an armadillo (ARM) domain and an ankyrin (ANK)-like domain, a

role as a scaffolding protein contributing to the formation of multi-protein signaling complex has been suggested (fig. 6A) [1, 6, 73, 107].

According to its complex structure, LRRK2 seems to function as a cell signaling enzyme, as a scaffolding protein and modulator of other proteins via direct interaction (fig. 6B) [1].
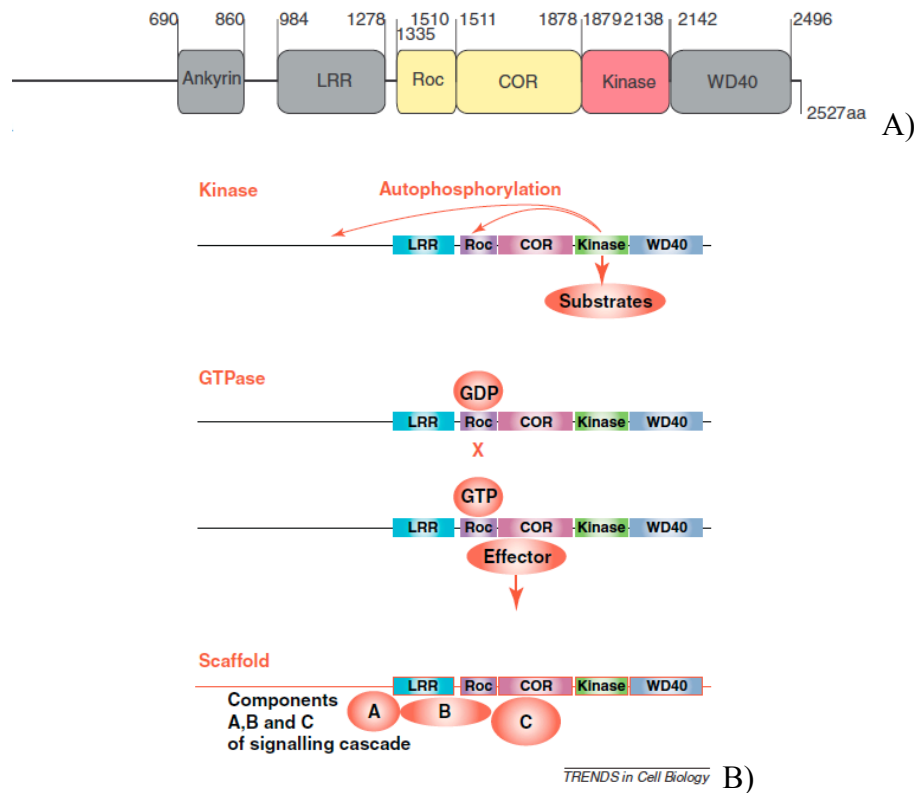

Figure 6. **A)** Schematic structure of LRRK2 protein and their functional domains **B)** Proposed models of LRRK2-mediated signaling based on structure/function relationship. LRRK2 acts as a kinase, small GTPase or scaffolding protein [1].

1.2.2. Ras of complex protein (Roc) domain

As stated above, the Roc domain is one of the ROCO family members, a group of the Ras superfamily of small GTPases. It is constituted by 200-250 amino acids and plays diverse cellular functions. Indeed, it appears to be a GTP-binding protein with functional GTPase activity [73, 85][65, 77]. GTPases act as molecular/regulatory switches in a cycle between guanine triphosphate (GTP) and guanine diphosphate (GDP)-bound conformations, regulated by guanine-exchange factors (GEFs) such as the guanine nucleotide exchange factor ARHGEF7 and the small GTPase CDC42 and GTPase-activating proteins (GAP) such as ArfGAP1. In fact, ARHGEF7 binds, as guanine nucleotide exchange factor, on dimeric GDP bound LRRK2. Subsequently the GDP-GTP exchange leads to activation of intrinsic GTPase activity of LRRK2, which induces autophosphorylation and promotes the activation/

phosphorylation of the kinase domain (fig.7) [116]. In human embryonic kidney 293T cell line (HEK293T), it was observed that overexpression of R1441C mutated LRRK2 increases the interaction between ARHGEF7 and GTP binding of the protein LRRK2 in comparison to WT, but shows a decrease in GTPase activity due impairments in cycle between GTP and GDP-bound conformations, nevertheless LRRK2 kinase activity is stimulated upon binding of GTP to the Roc domain [116]. In fact, initial studies demonstrated that GTP binding enhanced the kinase activity of LRRK2, whereas abolishing GTP binding via P-loop null mutations (i.e. T1348N) critically impaired kinase activity [78].
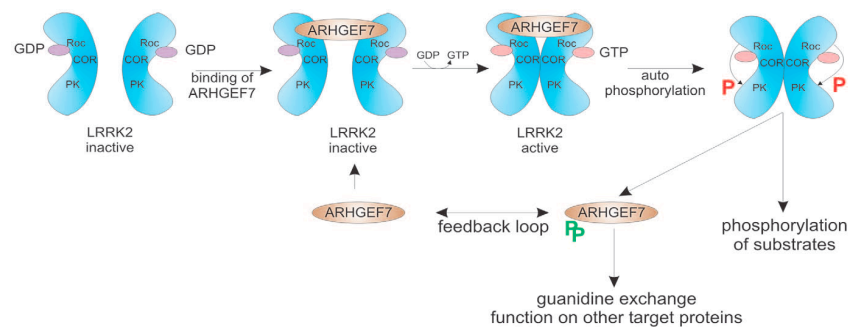
Figure 7. Suggested model of the intermolecular regulation of ARHGEF7 and LRRK2. ARHGEF7 binds as guanine nucleotide exchange factor on dimeric GDP bound LRRK2. Subsequently the GDP-GTP exchange leads to activation of intrinsic GTPase activity of LRRK2, which induce autophosphorylation followed by kinase activation of LRRK2. LRRK2 kinase domain active recognizes different substrates among them ARHGEF7 [116].

Within Ras-related small GTPase superfamily there are five subfamilies that play important roles in signaling pathways: Ras (regulates gene expression), Rho (controls cytoskeletal organization and gene expression), Rab, Sar/Arf (regulates vesicular trafficking) and Ran (regulates microtubule organization) [39]. The Roc domain of LRRK2 protein shows high sequence homology with the Rab subfamily of Ras-related GTPases. There are at least 60 Rab genes in the human genome and a number of Rab GTPases are conserved from yeast to humans. Members of this family have been implicated in subcellular targeting, mainly on vesicular trafficking [39]. In general, Rab GTPases differ most in their carboxyl termini and share a structure that is similar to all small GTPases of Ras superfamily, consisting of six stranded β sheet surrounded by α-helices that form 5 loops that connect the α-helices and β-strands, and that harbour the elements responsible for guanine nucleotide, Mg^{2+} and GTP hydrolysis. Four of these 5 loops are conserved in Roc domains and . some studies describe that like other Ras-related GTPases, Rab proteins are activated by GEFs and adopts two distinct conformations, GDP- and GTP-bound states [109, 116, 117].

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These GTPases are located at the cytosolic side of membrane, where they are implicated in regulation of membrane traffic pathways by facilitating vesicular trafficking and transport processes. In the ROC domain some mutations have been identified, such as R1441 (R1441C/G/H), Y1699C and I1371V, that alter GTP hydrolysis (fig.8) [81, 118]. However, these mutations show ambiguous and sometimes conflicting results, which many times is due to the use of different substrates in kinase assays or may be due to different enzyme preparations [82].

R1441C mutation is the second most recurrent mutation after G2019S that occurs in kinase domain. R1441C was first found in of North America and in a small Caucasian family. The R1441G mutation was first found in Basques families and it has been found to be prevalent in Northern Spain while R1441H mutation has never been found to co-segregate with PD in a large family but in small families or in sporadic PD patients of diverse ethnicities including Portuguese and Australian people [81]. These mutations decrease GTPase activity of LRRK2, affecting its dimerization and some studies suggest an increase in kinase activity but there is some controversy in that observation. These mutations also lead to impairments in nigrostriatal DAergic innervation (degeneration of the nigrostriatal projections) [73, 118], suggesting that the poor GTPase activity contributes to LRRK2 toxicity [39]. More recently, an *in vitro* study using a recombinant Roc domain fragment of LRRK2 demonstrated that the R1441C mutation destabilizes the LRRK2 dimer, implying a potential role for altered dimerization of LRRK2 in PD pathogenesis [39, 70, 73]. As the ROC domain regulates LRRK2 kinase activity, mutations in this domain are expected to alter downstream signaling properties of LRRK2. On top of this, these mutations might affect dimerization, possibly via the COR domain that acts as a molecular hinge [73, 119]. The kinase domain remains inactive until a change in the conformation of the activation segment within the large C-terminal lobe is induced by phosphorylation. In addition to the kinase domain, the ROC domain is, without any doubt, an important domain of LRRK2 function, specially, via the strict relation to kinase activity and via its direct interaction with potential signaling partners [73, 119].

1.2.3. C-terminal of Roc (COR) domain

Little is known about this domain, but today it is described that the COR domain is always found in combination with the Roc domain and thought to regulate the activity of this domain. The COR domain of ROCO proteins is 300-400 amino acids and does not show

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significant sequence homology to any domain or protein described today [109] indicating that more studies are necessary to obtain a better knowledge about the function of the COR domain. Nevertheless, in this domain a mutation, Y1699C, was one of the first mutations to be identified in LRRK2 and has been shown to co-segregate with the disorder in two large kindreds with autosomal dominant PD [81]. This mutation is positioned at the intra-molecular Roc:COR interface and the Y1699C substitution strengthens the intra-molecular Roc:COR interaction, thereby locally weakening the dimerization of LRRK2 at the Roc-COR tandem domain, resulting in decreased GTPase activity and changes in kinase activity (fig.8) [120, 121]. Another COR domain-associated mutation is the R1628P mutation, a mutation that was brought in connection with AlzD in one study [121]. The R1628 amino acid is evolutionarily conserved across species. Substitution of a highly basic polar arginine with proline is postulated to result in a conformational change in LRRK2's secondary structure which could disrupt the function of the COR domain. The R1628P variant could also affect the GTP binding capacity, the kinase activity either directly or via its interaction with the different functional domains of LRRK2 or other interacting protein [81].

1.2.4. MAPKKK domain (kinase domain)

This catalytic domain belongs to the tyrosine kinase-like (TKL) subfamily of human protein kinases, whose members show sequence similarity to Ser/Thr and tyrosine (Tyr) kinases [73, 122]. Nevertheless, LRRK2 has the Thr as the main phosphoacceptor, when compared to Ser and Tyr, although peptides with Ser as the phosphoacceptor were also identified[123].

In conditions of cellular stress, the MAPKKK domain of LRRK2 activates all major MAPK pathways that have been linked to PD - extracellular-signal regulated kinase (ERK's), mainly ERK1/2 and ERK5; p38 $\alpha/\beta/\gamma/\delta/$ MAPKs and JNKs (MKK3⁄6 and MKK4⁄7 phosphorylation). LRRK2 also plays an important role in alternative signaling pathways like TNF-α/FasL pathways and Wnt signaling pathways. In fact, post-mortem studies showed that ERK1/2, p38 MAPK and JNK were activated in PD brains [1, 67, 118].

This domain itself has a very low activity but, together with other domains, mainly with WD40 and GTPase domain, it is able to mediate *in vitro* autophosphorylation and phosphorylation of the artificial substrate myelin basic protein [2]. As in other functional domains, PD-associated LRRK2 mutations have been described to be present in this domain.

Some mutations, such as G2019S and I2020T, are localized at the N-terminal border of the activation segment and increase kinase activity (fig.10) [39, 77].

As described above, the high prevalence of the G2019S mutation, reported in Ashkenazi Jews and North African Arabs, has led to the hypothesis that the mutation originated in the Middle East about 2000 years ago. The G2019S mutation, the most prevalent mutation, occurs in exon 41 of LRRK2 and substitutes glycine (Gly) to Ser [124]. It occurs within the DYG hinge of the Mg^{2+}-binding⁄activation region and is associated to symptoms like tremor, bradykinesia and rigidity. Furthermore, the increase of LRRK2 kinase activity (gain-of-function effect of mutant LRRK2) was determined with use of LRRKtide (H-Arg-Leu-Gly-Arg-Asp-Lys-Tyr-Lys-Thr-Leu-Arg-Gln-Ile-Arg-Gln-OH) in substrate phosphorylation or autophosphorylation experiments [73, 123]. Based on structural modeling, it has been postulated that the Gly to Ser substitution forces LRRK2 to remain in a constitutively active state, keeping the catalytic site open, which leads to increased kinase activity. The DFG⁄DYG conserved region in the active site loop region tends to create a flexible conformation as a result of the presence of a small Gly residue [73, 118].

The I2020T mutation was initially found in the Sagamihara family, the original large Japanese family which had been used to map the LRRK2 locus [81]. However, the functional outcome of this mutation remains controversial. Some authors defend that I2020T mutation increases kinase activity, while others claim a decreased kinase activity [67, 73]. Nevertheless, modeling analysis of a homologous MAPKKK indicates that the isoleucine to the Thr substitution is not predicted to result in a conformational change in the active site of the enzyme but increases the ATP binding affinity, despite its lower catalytic activity compared with wild-type LRRK2 protein [67, 73]. This mutation, as well R1441C mutation, is also linked to patients that have phenotypes which are similar to idiopathic PD [81].
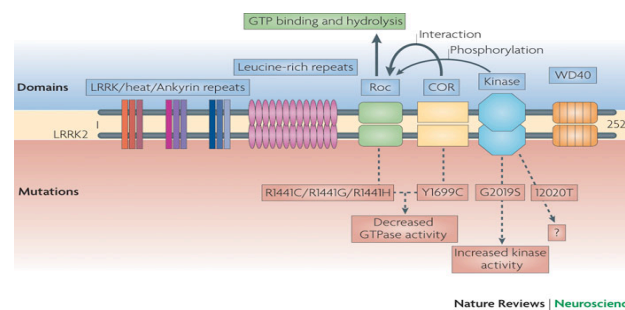
Figure 8. Summary about main LRRK2 pathogenic mutations and its implications. LRRK2 is a large multidomain and contains a Roc domain, a COR domain, a kinase domain and main protein–protein interaction regions, the LRR and WD40 domains. Both R1441 and Y1699 mutations decrease the GTPase activity of LRRK2, whereas G2019S increases kinase activity. These two activities may be related because kinase domain autophosphorylates the Roc domain at several sites [118].

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1.2.5. Main interaction domains: WD40 domain, Leucine-rich repeat (LRR) domain, armadillo (ARM) domain and ankyrin (ANK)-like domain

As previously described, LRRK2 contains 7 WD40 repeats and 13 LRRs forming the two interaction domains and two other domains, ARM and ANK domains, until today unknown functions [39, 122]. The WD repeat contains a GH dipeptide (11-24 residues) from its N-terminus and a WD dipeptide at the C-terminus separated by a conserved core sequence. LRR are 20-30 residues that contain a conserved segment, generally with 11-13 residues. Both WD40 domain and LRR domain adopt circular/arch-like structure, respectively. Each repeat of WD40 domain contains a four-stranded, anti-parallel β-pleated sheet that potentiates formation of a circular form and LRR are tandem sequences that potentiate arch structure formation in these domains [39, 122].

These protein domains participate in many key biochemical and biological processes such as hormone-receptor interactions, enzyme inhibition, cell adhesion and cellular trafficking, early mammalian development, neural development, cell polarization, regulation of gene expression, apoptosis and regulation of cytoskeletal dynamics [39, 77]. The functional role of these domains is underscored by fact that mutations in these domains lead to altered protein-protein interactions that in turn alter LRRK2 function with pathological and biological consequences. Indeed mutations like T2358I, G2385R, R1067Q, S1096C and S1228T; all localized in the WD40 domain may alter protein interactions, the dimerization and kinase activity [39, 78, 122]. Within these mutations, the G2385R mutation is associated with an elevated rate of apoptosis and cell death under conditions of oxidative stress [81].

1.2.6. Cellular functions of LRRK2

Despite the strong genetic interaction between LRRK2 and PD, the biological/ biochemical function of LRRK2 needs to be identified. It has been shown that membrane targeting and dimerization of WT LRRK2 are critical biochemical mechanisms governing LRRK2 kinase activity. Downstream LRRK2 candidate substrates and their biological relevance in neuronal survival, cytoskeletal rearrangement, mitochondrial dysfunction, protein homeostasis, autophagy, apoptosis, neurotransmission (neurotransmitter release) and vesicle endocytosis is becoming identified [1, 17, 31, 39, 78, 82, 115, 125, 126]. LRRK2 has been shown to phosphorylate moesin at Thr-558 (Thr ^{558}) *in vitro*, suggesting that this residue is critical to phosphorylation by LRRK2 protein (kinase domain) *in vivo*. This protein is an actin-binding ERM (ezrin, radixin and moesin) protein that has been implicated in neurite

outgrowth and anchors the actin cytoskeleton to the plasma membrane. Two peptides derived from LRRK2, LRRKtide and Nictide [78] that are phosphorylated in the equivalent Thr residue present in ERM protein, and some substrates like as 4E-binding protein (4E-BP) 1 (Thr37/46), β-tubulin (Thr107), FoxO1 (Ser319), collapsin response mediator protein-2 (CRMP-2) and creatine kinase have been described as LRRK2 candidate substrates [2, 39, 73, 123, 127].

1.2.6.1. Neuronal survival, cytoskeletal rearrangement

Many studies have suggested that cytoskeleton disruption may contribute to PD pathogenesis [39, 82, 115, 128]. MT cytoskeleton is critical for the generation and maintenance of neuronal axons and dendrites (neuronal survival), transport of synaptic vesicles and organelles along the synaptic processes, and the initiation and maintenance of synaptic transmission, while ERM proteins link the actin cytoskeleton with membrane proteins and play prominent roles in the determination of cell shape, growth, and motility [39, 82, 115, 128, 129]. The activity of an ERM protein is regulated by the intramolecular interaction between the N- and C-terminal regions. This interaction leads to an "inactive" conformation and prevents the ERM protein from associating with other proteins, including filamentous actin (F-actin). The phosphorylation of a conserved Thr residue in the C-terminal domain of ERM proteins blocks the intramolecular association and induces a conformational change to an "active" state, which allows their association with F-actin and other proteins [128].

LRRK2 is physiologically involved in cytoskeletal rearrangement because many studies have shown that LRRK2 associates with various cytoskeleton proteins including α/β-tubulin via Roc domain (GTPase domain) and in a guanine nucleotide independent manner (*in vivo* studies) [78, 114], F-actin, ERM family members, and the dishevelled family proteins (DVL1-3), supporting that LRRK2 plays a critical role in the regulation of microtubule and actin dynamics, and in morphogenesis of developing neurons [39, 128]. Indeed, several studies using overexpression, and knockdown in cell lines; studies on KO and transgenic in *Drosophila melanogaster*, *Caenorhabditis elegans* and mouse models have implicated LRRK2 in decreased neuronal cell viability [39, 82, 115], disruption of neurite outgrowth and synaptic morphogenesis (through distinct substrate proteins at the presynaptic and post-synaptic compartments) [39, 82, 115].

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In the presynaptic side, LRRK2 forms a complex with tubulin and the *Drosophila melanogaster* variant of vertebrate MAP1B, the MT-binding protein Futsch. This protein, which is required for axonal and dendritic growth during embryogenesis and for synaptic morphogenesis, is phosphorylated by LRRK2, which negatively regulates the presynaptic function of Futsch in controlling MT dynamics [39, 128]. At the post-synaptic side, LRRK2 has been suggested to interact with 4E-BP1 affecting protein synthesis, nevertheless without conclusive results [130]. It is thought that when LRRK2 is mutated, these interactions cause defects in presynaptic MT cytoskeleton dynamics and post-synaptic protein synthesis [73, 82, 126, 130]. LRRK2 also controls the formation and stability of MTs in the presence or absence of microtubule-associated proteins (MAPs), respectively [39, 128, 129]. If MAPs are present, LRRK2 phosphorylates MAPs promoting microtubule stabilization, but in the absence of MAPs, LRRK2 phosphorylates β-tubulin promoting microtubule formation. In LRRK2 G2019S neurons, the latter phosphorylations are enhanced, and lead to defects in neurite outgrowth [39, 128, 129]. Indeed, overexpression of WT LRRK2 and G2019S LRRK2 mutant has been shown to reduce the neurite length and branching in primary neuronal cell cultures of rat cortical neurons, whereas LRRK2 deficiency [with use of short-harpin RNA (sh-RNA)] results in increased neurite length and branching [125].

More recently a paper of Kawakami et al., (2012) [131] describes that LRRK2 phosphorylates tubulin-associated tau and reduces its tubulin-binding ability, whereas LRRK2 does not phosphorylate the free tau molecule. In fact, tau has not been reportedly detected as the LRRK2 binding protein in other studies involving proteomic analysis, except in neurons of G2019S LRRK2-transgenic flies that exhibited hyperphosphorylation of tau at Thr212, which was ascribed to phosphorylation by the activated GSK-3β homologue, and not to direct phosphorylation by LRRK2 [78, 131]. Nevertheless, in the G2019S bacterial artificial chromosome (BAC) transgenic mice, an increased of Tau phosphorylation has been observed [131].

Other reports have shown that LRRK2 and its mutants phosphorylate ERM family members [128, 132]. In fact, LRRK2 modulates positively and negatively ERM but in LRRK2 G2019S neurons the numbers of phosphorylated ERM (pERM) and F-actin enriched filopodia were significantly increased, which correlates with the retardation of neurite outgrowth in these neurons. Conversely, the levels of pERM and F-actin within the filopodia of LRRK2 KO neurons were significantly decreased and neurite outgrowth was promoted

[39, 128, 132]. These findings suggest that LRRK2 serves as a regulator of cytoskeleton dynamics [39, 128, 131, 132].

1.2.6.2. Mitochondrial dysfunction

Mitochondria are important cellular organelles, because they do not only have a key role in electron transport and oxidative phosphorylation, but are also the main cellular storage of free radicals, Ca^{2+} homeostasis and involved in cell-death pathways [133-135]. Therefore, impaired mitochondrial function is likely to increase oxidative stress and might render cells more vulnerable to this and other related processes, including excitotoxicity and apoptosis [133-135]. Indeed, mitochondrial dysfunction is implicated in pathophysiology of PD and there is evidence that Parkin, α-synuclein, DJ-1 and PINK-1 associate with mitochondrial function and/or dysfunction. In 2005 a study reported that cytosolic LRRK2 interacted with Parkin protein, but not with α-synuclein or DJ-1 [93]. Therefore, LRRK2 was suggested to be implicated in mitochondrial dysfunction when it is mutated [134]. LRRK2 association with the mitochondrial outer membrane in rodent brain is supportive evidence in this matter [133, 136].

Mitochondria are dynamic organelles and undergo frequent fission (cell death induction) and fusion (cell protection). The process of fission and fusion is regulated by molecular machinery that includes dynamin-related GTPases and WD40 repeat–containing proteins. Because LRRK2 combines both GTPase and WD40 domains within the same protein, it could potentially serve as a scaffold during mitochondrial fission and fusion events [133, 136]. Furthermore, WT LRRK2 but not Y1699C, G2019S or R1441C mutants attenuated H_2O_2-induced cell death in HEK293 cells and in SH-SY5Y cells. Further mechanistic research indicated that mutations such as Y1699C, G2019S or R1441C compromise the inherent protective capacity of WT LRRK2 against oxidative stress via its inability to activate the ERK1/2 pathway [134].

1.2.6.3. Protein homeostasis, autophagy and apoptosis

Protein aggregation, such as LB formation, is thought to play an important role in neurodegeneration and PD pathogenesis [108]. As described above, in the post-synaptic side, LRRK2 is capable to interact with and phosphorylate 4E-BP1, a negative regulator of protein translation [39, 73, 126, 130]. Phosphorylation of 4E-BP1 causes its release from the eIF4E and relieves its inhibitory effect on translation. At least six phosphorylation sites have been

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identified in human 4E-BP1 (h4E-BP1), including T37, T46, S65, T70, S83 and S112. A sequential phosphorylation of 4EBP1 in the order of T37/T46>T70>S65 has been proposed [130]. Although the regulatory mechanisms involved in 4E-BP phosphorylation are not fully understood, it appears that a combination of perhaps all phosphorylation events is required to dissociate 4E-BP1 from eIF4E [39, 73, 126, 130]. Although LRRK2 is not the only kinase that phosphorylates 4E-BP T37/T46 sites, the phosphorylation of 4E-BP1 at T37/T46 sites by LRRK2 prevents its inhibitory binding to eIF4E and, thus, stimulates eIF4E-mediated protein translation and increases oxidative stress-induced neurotoxicity in DA neurons [82, 126, 130]. LRRK2 also phosphorylates forkhead transcription factor FoxO1 at Ser 319 residue, which controls various cellular processes involved in cell cycle, cell death, metabolism and oxidative stress and regulates 4E-BP1 protein transcription [39].

In a study published in 2009 [108], absence of LRRK2 in kidneys was shown to lead to impairments on autophagy-lysosomal pathways, potentiating protein aggregation, inflammatory responses, oxidative damage and apoptotic cell death in aged mice [108]. This important observation demonstrates an essential cellular function of LRRK2 during aging in the maintenance of protein homeostasis and, in particular, α-synuclein (main component of LB) through the regulation of protein degradation pathways [108]. In fact, impairment in neurite outgrowth induced by G2019S LRRK2 is prevented by genetic inhibition of autophagy components but exacerbated by rapamycin, an activator of autophagy. G2019S LRRK2 also promoted the accumulation of autophagic vacuoles in neuritic and somatic compartments of cultured neurons although it is not known whether these observations result from enhanced autophagy, impaired autophagic flux, or impaired fusion of autophagosomes with lysosomes [78]. Nevertheless, in primary neuronal cultures it was also shown that LRRK2, in response to cellular stress such as an accumulation of misfolded proteins, or oxidative stress (both of which have been implicated in PD), has an important in apoptosis [39, 137]. In fact, LRRK2 interacts with the C-terminal R2 RING-finger domain of Parkin and in turn, Parkin interacts with the COR domain of LRRK2, enhancing the ubiquitination, decreasing the misfolded protein aggregation [78, 93].

The two main pathways that can trigger programmed cell death/apoptosis are (1) the intrinsic pathway, which is controlled by factors that are released by mitochondria (cytochrome C, for example) and activate caspase-9 and (2) the extrinsic pathway, which is typically initiated by cell surface "death receptors" such as tumor necrosis factor receptors (TNF-R) and Fas that lead to caspase-8 activation via the death adaptor Fas-associated protein

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with death domain (FADD) [137]. LRRK2 has been shown to interact with FADD and with tumor necrosis factor receptor type 1-associated death domain protein (TRADD), death adaptor proteins important for apoptosis activation [39, 137]. The interaction between LRRK2 and FADD is enhanced by R1441C, G2019S, Y1699C, I2020T mutations and leads to the recruitment and activation of caspase-8 [137]. This observation suggests that LRRK2, FADD, and caspase-8 are components of a multi-protein complex. In fact, blocking LRRK2 kinase function eliminates the FADD binding, decreasing apoptosis activation and provides a potential mechanism to prevent LRRK2-mediated neuronal death [137].

The chaperone machinery is important to chaperone-mediated autophagy (CMA) and it has been described that LRRK2 interacts with many elements that are involved in CMA. Overexpression of WT LRRK2 and pathological mutants allows showing that the molecular chaperone, Hsp90 (and its co-chaperone, p50^{cdc37}) interacts with LRRK2 [39, 67]. This chaperone protein may help to maintain the proper folding of LRRK2. The HSP90/p50^{cdc37} chaperone complex binds to LRRK2 and may assist with the activation of other protein kinases. Inhibition of Hsp90 disrupts the LRRK2 and Hsp90 interaction leading to LRRK2 degradation via UPS. Therefore, Hsp90 inhibitors are potential therapeutics against mutant LRRK2-induced toxicity [39]. Furthermore, LRRK2 can interact with other molecular chaperones like HSp60, Hsp70, and the c-terminal Hsp70 interacting protein (CHIP). The last one binds, ubiquitinates and promotes degradation of LRRK2 via UPS and thereby reduces mutant LRRK2-induced toxicity [39]. Overexpression of CHIP protects against mutant LRRK2-induced toxicity and enhances ubiquitination of α-synuclein in cells [39, 138] whereas knockdown of CHIP exacerbates toxicity mediate by mutant LRRK2 via reducing degradation of LRRK2 proteins [39].

1.2.6.4. LRRK2 in Immune System and Inflammation

LRRK2 has been suggested, through genome-wide association studies (GWAS), as one of a few genes where common genetic variability underlies susceptibility to the chronic autoimmune Crohn's disease and Mycobacterium leprae infection (leprosy), raising the possibility that variations in LRRK2 may modify immunogenic responses in PD [110, 139, 140]. As described above, LRRK2 mRNA and protein was found in circulating in PBMCs such as CD19$^+$ B cells and in CD14$^+$ monocytes, whereas CD4$^+$ and CD8$^+$ T cells were devoid of LRRK2 mRNA. Within CD14$^+$ the CD14$^+$CD16$^+$ sub-population of monocytes exhibited high levels of LRRK2 protein in contrast to CD14$^+$CD16$^-$ cells. However both

populations expressed LRRK2 mRNA. In fact, in two recent studies, the IFN-γ increased LRRK2 mRNA and protein levels in monocytes concomitant with a shift of CD14$^+$CD16$^-$ cells towards CD14$^+$CD16$^+$ but, with LRRK2 inhibitor IN-1 (LRRK2-IN-1), this shift towards CD14$^+$CD16$^+$ was attenuated after IFN-γ stimulation [110, 141]. In fact, LRRK2-IN-1, has been used to inhibit LRRK2 because it induces dephosphorylation of Ser 910 and Ser 935 in kinase domain and accumulation of LRRK2 within aggregate structures. LRRK2-IN-1 inhibits both WT and G2019S mutant LRRK2 kinase activity with IC50 (concentration required to inhibit 50% of enzyme activity) values of 13 nM and 6 nM, respectively, and serves as a versatile tool to pharmacologically interrogate LRRK2 biology and study its role in PD [142].

In another study of Hakimi et al., (2011) [139] demonstrated that LRRK2 expression in cultured bone marrow-derived macrophages from mice is upregulated in response to lipopolysaccharide (LPS), a potent agonist of toll-like receptor 4 (TLR4) [139]. Therefore, LRRK2 might have a role in immune cells, monocyte maturation and regulation at the transcriptional and translational level [110, 139, 140]. In the mouse brain, LRRK2 is highly expressed in peripheral monocytes and macrophages, and a study of Moehle et al., (2012) [140] described that LRRK2 is expressed in activated microglia, after LPS induction, and that LRRK2 modulates pro-inflammatory responses in these cells, like as is the case in PD. Interestingly, the accumulation of LRRK2 protein, which occurs during inflammatory signaling in primary microglia, is not accompanied by significant changes in mRNA levels, suggesting important post-transcriptional regulation [140]. In these mice, they also found LRRK2-positive small cells in the corpus callosum of mice after an intrastriatal LPS injection and that LRRK2 inhibition either by RNAi knockdown or small-molecule kinase inhibitors like LRRK2-IN-1 and Sunitinib, attenuates pro-inflammatory signaling in response to TLR4 activation, by attenuation of TNFα secretion and nitric oxide synthase induction (iNOS [140].

Thus, alterations in LRRK2 function may modify inflammatory responses in neurodegenerative and infectious diseases, potentially leading to disease initiation or modification of progression. Hypothetically, LRRK2 G2019S missense mutation in the kinase loop, may serve to exaggerate neuroinflammatory responses that predispose to neurodegeneration susceptibility in PD. Indeed, LRRK2 may function as a stress response kinase during a neuroinflammatory stimulus in the brain by facilitating signal transduction pathways in affected cells [110, 139-141].

1.2.6.5. Neurotransmission and Synaptic Vesicle Endocytosis

In the CNS, neurons are communication specialists that convert electrical into chemical signals at special junctions called synapses, a term introduced by Charles Sherrington. There are two types the synapses: electrical and chemical. In the electrical synapse the gap between pre- and post-synaptic is greatly reduced (3.5 nm) and allows, bidirectional, the flow of ions through junction channels [61, 143]. In chemical synapses (20-40 nm), an action potential induces depolarization and Ca^{2+} (50-100 μM) influx; the synaptic vesicles fuse with the plasma membrane at the active zone and release their content (neurotransmitters – chemical substance), a process called exocytosis, to the synaptic cleft. Then these unidirectional diffuse across the synaptic cleft and bind to specific receptors on the post-synaptic cell membrane and trigger an electrical signal [61, 144, 145]. The strength of both electrical and chemical synapses can be increased or diminished by cellular activity. However, on high-frequency stimulation, the amplitude of these responses decays rapidly, a conclusion that Birks and MacIntosh, primarily, and Elmqvist Quastel, subsequently obtained [146]. The decay depends of stimulation but also of the SV number, therefore, they proposed that there are two distinct presynaptic stores of transmitter: a "readily releasable" fraction, which is rapidly depleted at high frequencies of stimulation and a "non-readily releasable" fraction [146]. This idea gained strength and actually there are three different vesicle pools considered: the readily releasable pool (RRP) (1-2%), the recycling pool (5-20%) and the reserve pool (80-90%) (fig.9) [146, 147]. The RRP localizes in the active zone and is immediately available upon stimulation and depleted in a few milliseconds of depolarization, defined by a rapid and instantaneous kinetic [146, 147]. The more scattered localized recycling pool is responsible for maintaining the synaptic communication in physiological stimulations and is depleted in a few seconds. The last pool, the reserve pool is released upon high frequency stimulation and is depleted in 30-60s [146, 147].
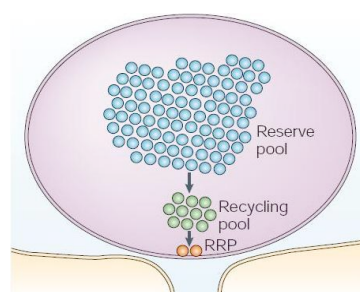
Figure 9. Classic model with three pools. The reserve pool constitutes ~80–90% of the total pool, and the recycling pool is significantly smaller (~5–20%). The readily releasable pool (RRP) consists of a few vesicles (~1-2%) that seem to be docked and primed for release [146].

Even though the release of synaptic transmitters appears to be smoothly graded, it is actually released in discrete packages called *quanta*. Each quantum of transmitter produces a post-synaptic potential of fixed size, called the *quantal synaptic potential* or miniature excitatory post-synaptic potential (mEPSP). Normally, these small events or small potential differences are recording in present of of Tetrodoxin (TTX). TTX is a potent neurotoxin, frequently used to suppress neuronal activity in cell culture by blockade of action potentials. This neurotoxin binds to the voltage-gated sodium (Na^+) channels and fast Na^+ channels in presynaptic cell, preventing the uptake of Na^+ and, as consequence, the entrance of Ca^{2+} in nerve terminal. The blockade of action potential propagation impairs all the synaptic vesicle cycle, by decreasing the ratio of recycling SV and the fusion of synaptic vesicles derived from recycling and reserve pool. Nonetheless. this release is linked to spontaneous excitatory post synaptic currents (sEPSCs), a mixture of action potential evoked (increase of Ca^{2+} concentration) and mEPSC. These sEPSCs depend of vesicle number (N), probability of release (Pr) (can depend on [Ca^{2+}] influx, sensitivity or vesicle priming), amount of neurotransmitter in a vesicle and post-synaptic response to a vesicle (Q=quantal size) (Response = NPrQ) and each sEPSC measured is the result of AMPA- and *N-Methyl-D-aspartate* (NMDA)-mediated currents (Glutamatergic-mediated responses) recording in post-synaptic cell. These sEPSC can be suppressed by using AMPA antagonists like 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) (at 10-30 µM) and 2,3-benzodiazepine **(**GYKI 52466) (at 20-50 µM) allowing thus the study of inhibitory post-synaptic currents (sIPSC) when cells are clamped at -60 mV [148].

These sEPSCs are commonly studied to gain insight into release probability, instantaneous frequency (Hz), believed to be as results of presynaptic mechanisms [149], since the velocity of synaptic vesicle and neurotransmitter release is an indication of how the synaptic vesicle cycle machinery response to boosting stimulus [149]. Other important parameter is peak amplitude, to gain insight in changes of receptors. The peak amplitude measures how the post-synaptic cell works by uptake of neurotransmitters [150, 151].

In fact, via whole-cell patch clamp is possible measure these events and study the currents through multiple channels. This can be achieved by selectively choosing the ionic composition of the pipette and/or the bath solution; by controlling the holding potential of the cell membrane; or by using different pharmacological agents that selectively block or activate specific channels or channel groups [152]. To measure the currents of different channels, the electrode, a glass micropipette that has an open tip (diameter 1µm) and resistance around 3-5

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MΩ, is filled with a solution (internal solution) matching the ionic composition of the bath solution. When the electrode is in the bath solution, current level is set to zero allowing a constant voltage while observing changes in current (voltage clamp), or keep a constant current and observing changes in voltage (current clamp). The electrode is placed on the cell membrane and at this moment a small suction is applied to form a GΩ between the glass and the cell membrane. The cell membrane inside the electrode tip is broken by an electrical pulse (-600 mV, 0.100 ms) allowing access to the intracellular space of the cell [153]. The electrical circuit is completed with silver chloride (AgCl) wire placed in contact with the bath solution. The events are recorded by the amplifier through specific software.

After exocytosis, SV membrane must be retrieved from the presynaptic plasma membrane and recycled back to the active zone via endocytosis. This process is essential to preserve synaptic transmission as well as the general organization of the synapse [61, 154]. The first study about SV endocytosis was done by Heuser and Reese [155] and these two scientists found that after depolarization, using a fluid-phase cytosolic marker the terminals were able to label some structures, later known clathrin-coated structures [155, 156]. They proposed that SV fuse completely with the plasma membrane of pre synaptic cell and then is retrieved by clathrin-coated invaginations at an area outside the active zone [155, 156]. During the subsequent years, the hypothesis proposed by Heuser and Reese, was strengthened by a wide variety of other studies demonstrating for example that clathrin-coated vesicles isolated from brain contain synaptic vesicle proteins and that most components of the clathrin endocytic machinery are enriched in brain [155].

Actually, it is known that endocytosis is involved in all of the physiologic functions associated with the plasma membrane because it controls the protein and lipid composition at the surface, regulates signaling pathways, modulates the cell surface area, regulates the surface expression of proteins, brings nutrient into cells, retrieves proteins deposited after vesicle fusion and turns over membrane components by sending these components for degradation in lysosomes [61, 144]. In fact, there are two major types of endocytosis, pinocytosis (uptake of fluid and solutes) that occurs in all cell types and phagocytosis (uptake of large particles) that occurs in cells of immune system [61, 144].

Pinocytosis of synaptic vesicles is thought to be clathrin-mediated endocytosis (CME); the best studied type of endocytosis [61, 144]. This process occurs constitutively in most mammalian cells and is important for the internalization of receptors and extracellular ligands, recycling of plasma membrane components, and retrieval of surface proteins destined

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for degradation [61, 144]. CME is critical in many aspects of development as well as in synaptic transmission, where CME provides the recycling of synaptic vesicle proteins and maintains the plasma membrane architecture at the synapse [144, 145, 157]. However, there are other possible pathways of endocytosis both clathrin-independent and clathrin-dependent such as: Kiss-and-run; Bulk retrieval; CLIC/GEEC endocytic pathway; arf6-dependent endocytosis, flotillin-dependent endocytosis, macropinocytosis, circular doral ruffles, phagocytosis, and trans-endocytosis (clathrin-independent modes of retrieval); collapse of one and retrieval of another one from the RRP, collapse of a vesicle followed by brief dispersal into a few patches and collapse followed by complete dispersal of vesicle molecules that occur in different times, depending of stimulation and number of SV released (fig 10) [156].
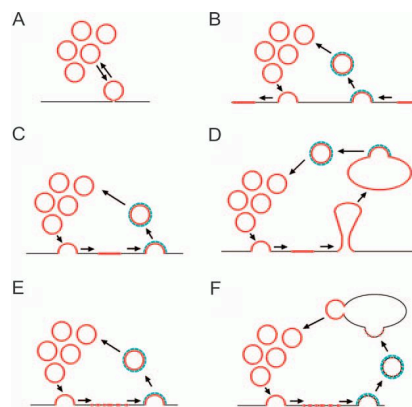
Figure 10. Possible pathways of synaptic vesicle recycling. The speed and fidelity of the recycling process tend to decrease from A to F. A) Kiss-and-run. B) Collapse of one vesicle and retrieval of another one from the 'readily retrievable' pool. C) Collapse and (classical) CME. D) Bulk retrieval: strong stimulation followed by formation of infoldings, which are broken up into vesicles by CME. E) Collapse of a vesicle followed by brief dispersal into a few patches, which are recovered by interaction with the CME machinery. F) Collapse, followed by complete dispersal of vesicle molecules. They are retrieved by CME but need endosomal sorting to be made into new vesicles [156].

Despite the existence of different pathways to retrieve the SV, clathrin was identified as being the major protein making the lattice-like coat around vesicles, which were described as "vesicles in a basket" [154, 158]. However, this protein does not work single-handed. There are a large number of proteins involved in the endocytosis process in different stages with different functions [136, 140]. In attachment, the table summarizes the proteins that have a role in CME according to function: core components, cargo-specific adaptors, inositol-5-phosphatases, kinases, actin nucleation at clathrin-coated vesicles and other proteins with different functions or not clear function in CME [154].

Through the use of FM1-43 dye and synaptoPHlorin, a pH-sensitive fluorescent protein, it was possible determine that CME is a slow process (1 min) and occurs in majority

of synapses together with the "kiss-and-run" endocytosis model (less than 2 s) in separate molecular mechanisms [61, 144]. The "Kiss-and-run" model allows a rapid neurotransmitter release through a small pore without collapsing into the plasma membrane and unlike CME does not allow control plasmamembrane area and retrieval of SV proteins [61, 144].

However, when neuronal activity increases, CME lacks the capacity to deal with the additional retrieval demands placed on the nerve terminal, and activity-dependent bulk endocytosis (ADBE) is triggered [159]. ADBE is commonly described by large invaginations of plasma membrane which then fission to form endosomal-like compartments [159]. SVs can then bud from these bulk endosomes to the active zone and release their content [159].

CME forms coated vesicles, referred to as clathrin-coated vesicles (CCVs) and to their formation, addition to clathrin, a number of other proteins have been identified for assembling and/or uncoating of CCVs [136]. Thus, it was proposed, in a review of Jung and Haucke (2007) [143] that CME can be divided in four steps: adaptor recruitment, clathrin assembly, fission and uncoating [143]. Notwithstanding, in 2011, a review of McMahon and Boucrot described the same process in 5 states by division of the first step: Initiation/Nucleation, cargo selection, coat assembly, scission and uncoating [154]. In both suggestions the four major components of CME are proposed: clathrin, heterotetramer adaptor complexes (AP-2), transmembrane cargo receptors, and dynamin (fig.11) [61, 144, 154, 159].

In the first stage (Initiation/Nucleation) of CME, there is the membrane invagination called a pit [154]. This clathrin-coated pit initiation was thought to be triggered by the recruitment of several putative nucleation proteins such as Fes/CIP4 homology (FCH) (a short conserved region of around 60 amino acids first described as a region of homology between FER and CIP4 proteins), domain only (FCHO) 1,2 proteins, EGFR pathway substrate 15– EPS15related (EPS15–EPS15R), intersectins, epsin and endophilin [154]. This putative nucleation module is thought to assemble only at the plasma membrane because its preference for phosphoinositol-(4,5)-bis-phosphate (PIP2). The F-BAR domain of these proteins can bind to very low curvatures and its membrane-binding activity is required for progression of the clathrin-coated pits, suggesting a need for membrane curvature generation even before clathrin recruitment [154].

In the second stage (Cargo Selection) there is a recruitment of coat/endocytic proteins such as the clathrin adaptor/assembly proteins (APs) [(AP-2, dynamin, amphiphysin, and AP180)] by intervention of PIP2, endophilin and FCHO 1,2 proteins [61, 144, 154]. Both AP-2, a heterotetramer complex that mediates the recruitment of clathrin to the membrane,

and clathrin coat assembly protein AP180, a component of adaptor complex that links clathrin to coated vesicles. AP2 binds both cargo and PIP2 to induce the nucleation module at PIP2 enriched areas, to next promote internalization. AP-2 interacts with synaptotagmin protein (Ca^{2+} sensor) and synaptic vesicle glycoprotein 2 (SV2) and AP180 interacts with synaptobrevin (important protein to SNARE complex formation) potentiating the internalization. In this step, as described above, amphiphysin is also recruited to assemble dynamin, a GTPase protein that will have a role downstream in the CME process [61, 144, 154].

In the third stage, clathrin coat assembly occurs. The clathrin *triskelia* are recruited directly from the cytosol to sites of adaptor concentration on the membrane to help organize the coated vesicle formation. AP-2 (mainly) and AP180 with help of regulatory proteins, such as Eps15, epsin and endophilin (BAR domain and SH3 domain) assemble individual clathrin molecules into regular cage (polymerization) in a process that induces invagination and stabilization of the budding coated pit [136]. Indeed, endophilin, described below, induces by lipid-modifying activity - lysophosphatidic acid acyl transferase (LPAAT) activity, inward curvature of the plasma membrane. Any perturbation in this step stops these mechanisms, impairing and blocking CME and subsequently the neurotransmitter release [61, 144, 154].

In the fourth step, vesicle fission is occurring. Clathrin-coated vesicle budding depends, mainly, on the mechanochemical GTPase, dynamin, but other proteins seem to be involved such as again endophilin, sorting nexin 9 (SNX9) and amphiphysin which have SRC homology 3 (SH3) domains that bind the Pro-rich domain of dynamin [154]. These proteins cause impairments in neck invagination, essential for dissociation of CCV. Despite endophilin and amphiphysin actions, downstream and upstream of vesicle fission, these proteins have been suggested to help the large GTPase dynamin to form a helical collar around the neck of an invaginating CCV, where it may regulate, pinch or pop the vesicle from the parent membrane [61, 144, 154]. It is shown that GTP hydrolysis is coupled to vesicle scission and that on GTP hydrolysis dynamin spirals undergo length-wise extension, which drives the vesicle away from the membrane causing lipid fission [154]. In fact, inhibition of dynamin reveals a approach to study the endocytosis. An inhibitor available is the dynasore. Dynasore interferes *in vitro* with the GTPase activity of dynamin1, dynamin2, and Drp1, the mitochondrial dynamin, but not of other small GTPases. Dynasore acts as a potent inhibitor of endocytic pathways known to depend on dynamin by rapidly blocking coated vesicle formation within seconds of dynasore addition. Two types of coated pit intermediates

accumulate during dynasore treatment, U-shaped, half formed pits and O-shaped, fully formed pits, captured while pinching off [160, 161].

In the fifth and final step, CCVs must be uncoated, in a process called Uncoating and Clathrin component recycling [154]. The clathrin coat is disassembled from its lattice arrangement back to triskelia by the ATPase heat shock cognate (HSC70), the clathrin-associated protein auxilin or cyclin G-associated kinase (GAK) [154]. Auxilin or GAK is recruited after clathrin-coated vesicle budding by binding to the terminal domains and "ankles" of clathrin triskel*ia*. There auxilin/GAK recruits HSC70 to initiate the uncoating reaction. To facilitate and accelerate, endophilin and AP2 are detached from plasma membrane and help on recruitment of synaptojanin and auxilin, respectively [154]. Therefore, the disruption of HSC70, synaptojanin, auxilin interaction and recruitment of endophilin and AP2 leads to impairments in neurotransmission by decreased SV and increased CCVs [61, 144, 154].
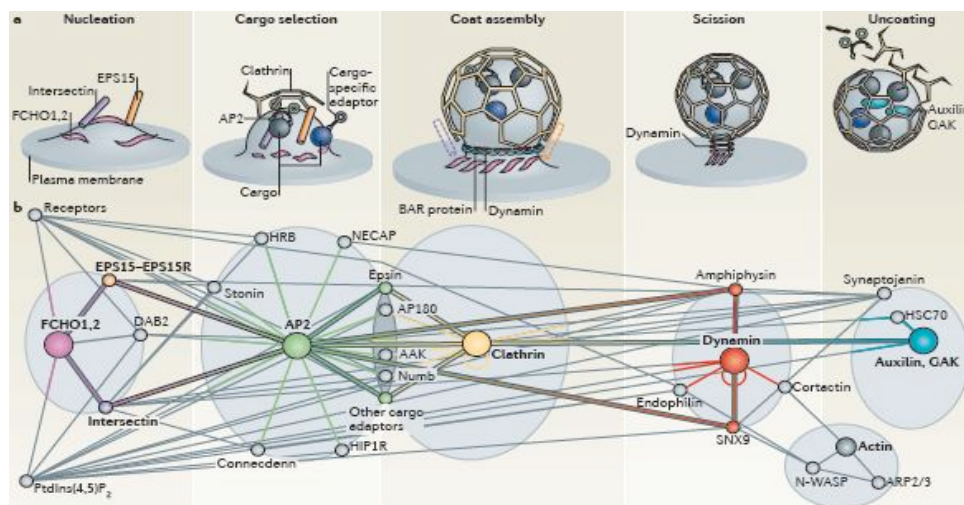
Figure 11. A) Clathrin-coated vesicle formation. Nucleation: FCHO proteins bind PIP2-rich zones of the plasma membrane and recruit EPS15–EPS15R and others proteins to initiate clathrin-coated pit formation by recruiting AP2. Cargo selection: AP2 recruits several classes of receptors directly through its μ subunit and σ subunit. Cargo-specific adaptors (for example, stonin, HRB and Numb) bind to AP2 appendage domains and recruit specific receptors to the AP2 hub. Coat assembly: clathrin triskelia are recruited by the AP2 hub and polymerize in hexagons and pentagons to form the clathrin coat around the nascent pit. Scission: the GTPase dynamin is recruited at the neck of the forming vesicle by BAR domain-containing proteins, where it self-polymerizes and, upon GTP hydrolysis, induces membrane scission. Uncoating: auxilin or GAK recruits the ATPase HSC70 to disassemble the clathrin coat and produce an endocytic vesicle containing the cargo molecules. Synaptojanin probably facilitates this by releasing adaptor proteins from the vesicle membrane through its PtdIns lipid phosphatase activity. The components of the clathrin machinery are then free for another round of clathrin-coated vesicle formation. B) The clathrin network. The protein–protein interactions underlying the different stages of vesicle progression are shown. Major hubs are obvious because of their central location in the network and the large number of interacting molecules. They are essential for pathway progression and are denoted by the central colored circles. Possible pathways of progression between hubs are shown with thicker lines [154].

Subsequently, the SVs fuse with the early endosomes to generate new vesicles or are conducted to active zone (recycling vesicles), for uptake of neurotransmitters by neurotransmitter transporter with help of vacuolar ATPase control present in SV membranes. The fusion with early endosomes is mediated by ras-like small G-proteins, such as Rab5b protein, that control trafficking, exocytosis, endocytosis and endosome fusion [39, 61, 80, 144].

In cell, the active traffic of proteins, as in CME, is very common and the Bin/Amphiphysin/Rvs (BAR) domain proteins constitute an important heterogeneous superfamily of cytoplasmic proteins with a strong commitment to membrane traffic [144, 162]. The best characterized BAR domain proteins are endophilins (fig 12) that are involved in SV retrieval, mitochondrial network dynamics, receptor Tyr kinase signaling, apoptosis and autophagy. There are five endophilin protein types, endophilin A1 (**SH3GL2**), A2 (SH3GL1), A3 (SH3GL3), B1 (SH3GLB1) and B2(SH3GLB2). Endophilin A1 is localized in the brain (pre-synaptic terminals) while endophilin A2, B1 and B2 are expressed in all tissues and the endophilin A3 is expressed in testes and in brain [144, 162].
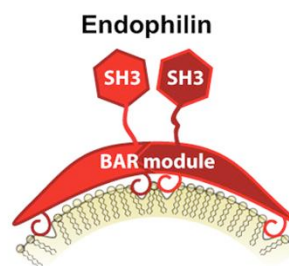
Figure 12. Diagram of endophilin binding to membranes. The BAR domain is responsible for membrane binding dimerization and curvature sensing. An amphipathic helix and the N terminus inserts into the membrane like a wedge helping to drive positive membrane curvature [163].

All these endophilins contain, in addition to N-terminal BAR domain, a C-terminal SH3 domain. BAR domains are involved with dimerization domains and are able to induce and stabilize membrane curvature by formation a crescent-shaped dimer, with each monomer made up three kinked, antiparallel, α-helices. SH3 domains are protein-recognition modules that adopt the β-barrel core typical of SH3 domains with a hydrophobic groove accommodating proline-rich peptide sequences present in binding partners [162]. Indeed, the SH3 domain binds proline-rich domain (PRD)-containing proteins such as synaptojanin, dynamin, and ataxin-2, important proteins to CME[162]. Recently, a study of Milosevic et al., (2011) [164] proves that endophilin, a membrane-binding protein with curvature-generating and -sensing properties also binds the GTPase dynamin and the phosphoinositide phosphatase

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synaptojanin and is thought to coordinate constriction of coated pits with membrane fission (via dynamin) and subsequent uncoating (via synaptojanin). They used the knockout of the 3 mouse endophilins (endophilin A1, A2, A3) to demonstrate that this lack results in the accumulation of clathrin-coated vesicles, but not of clathrin-coated pits at synapses. This can be explain by absence of synaptojanin, an important protein to promote the uncoating [164]. In fact, the absence of endophilin impairs but does not abolish synaptic transmission and results in perinatal lethality, whereas partial endophilin absence causes severe neurological defects, including epilepsy and neurodegeneration. This paper suggests a model in which endophilin As have two main roles: membrane curvature and uncoating CCVs [164, 165].

As described above, PD-linked mutations in Parkin protein can disrupt SV endocytosis [79]. Indeed the same happens with LRRK2 [13, 80, 166]. LRRK2 and its mutants interact with domains of endocytic proteins modulating the SV endocytosis process [13, 80, 166]. In 2008 and 2011, studies showed that alteration of LRRK2 levels in the presynaptic terminal impairs the normal synaptic activity by reducing endocytosis [13, 80, 166]. In Shin et al., (2008) a reduced rate of vesicle recycling upon LRRK2 overexpression was found and this was shown to be mediated by interaction with Rab5b protein, a ras-like small G-protein that regulates endocytic vesicular transport from plasma membrane to early endosomes [80]. In Piccoli et al., (2011) study, LRRK2 is implicated in the control of vesicle dynamics [13]. LRRK2-silenced neurons have unusual high numbers of active synapses under resting conditions and when cells are depolarized with potassium chloride (KCl) which increases vesicle release of the recycling pool, no additional increase in synaptotagmin staining was found in these cells, suggesting that LRRK2 is necessary to control endocytosis [13]. The same observation in RRP was obtained with hypertonic solution, sucrose, solution that is thought to stimulate the release of the entire RRP in neuron in culture, thereby allowing the estimation of the RRP itself [13].

In addition to these studies, LRRK2 also has been associated to other crucial proteins/ complex for SV endocytosis, such as α-actin, a component of cytoskeleton, AP-1, AP-2, AP180 protein, SV2, vacuolar ATPase, dynamin, endophilin and clathrin, suggesting that LRRK2 may influence endocytosis of SV and neurotransmission by interaction with these or other proteins [13].

In fact, a recent manuscript under review (Neuron) from the host lab describes that endophilin A is a direct substrate of LRRK2. In this study, we found that loss of *Drosophila melanogaster* endophilin A rescues synaptic endocytosis deficits in LRRK mutants,

suggesting that Endophilin A dominantly impairs vesicle recycling in the absence of LRRK, the LRRK2 variant in *Drosophila melanogaster*. They also found that the LRRK phosphorylates Endophilin A at Ser 75 in the BAR domain, a domain important in the initiation of the membrane curvature in the first stage of CME. These results prove that LRRK2 mediated endophilin A phosphorylation is required to the normal function of endocytosis. But, when the phosphorylation is too strong (LRRK clinical mutant) there is a impairment on synaptic vesicles endocytosis as well, suggesting that LRRK is part of an endophilin A phosphorylation cycle that regulates synaptic vesicle formation and predicts that both increased and decreased LRRK/LRRK2 kinase activity may have a deleterious effect in synaptic vesicle endocytosis. Therefore it is thought that this (de)phosphorylation alters the Endophilin A function (Matta et al., 2012-manuscript accepted).

They further showed that when Endophilin A is phosphorylated, it is removed from the plasma membrane, blocking the recruitment of some core components, essentials to membrane curvature initiation, stopping the CME in the initial stage. In fact in flies, mutant G2019S LRRK2 exhibits selective loss of DA neurons, locomotor dysfunction and early mortality which is in according with WT LRRK2 (Bacterial artificial chromosome -BAC) transgenic mice, where striatal DA transmission and motor performance is enhanced, while similar expression of G2019S mutants decreased striatal DA content as its release and uptake [82, 167]. Furthermore, the R1441 (R1441C/G/H) mice show abnormal activity-dependent DA neurotransmission, including impairment in stimulation-induced locomotor activity and catecholamine release, as well as dopamine D2 receptor-mediated functions, leading to degeneration of nigrostriatal projections [82, 167].

In flies, absence of LRRK, or in the presence of LRRK kinase inhibitors also cause an impairment in synaptic vesicles endocytosis because it is thought that the endophilin A phosphorylation is required to facilitate recruitment of HSC70, auxilin and synaptojanin, important proteins to uncoating reaction. These findings suggested that LRRK2 is essential to proposed Endophilin cycle.

In figure 13 is represented the possible normal/pathogenic of LRRK2 functions.
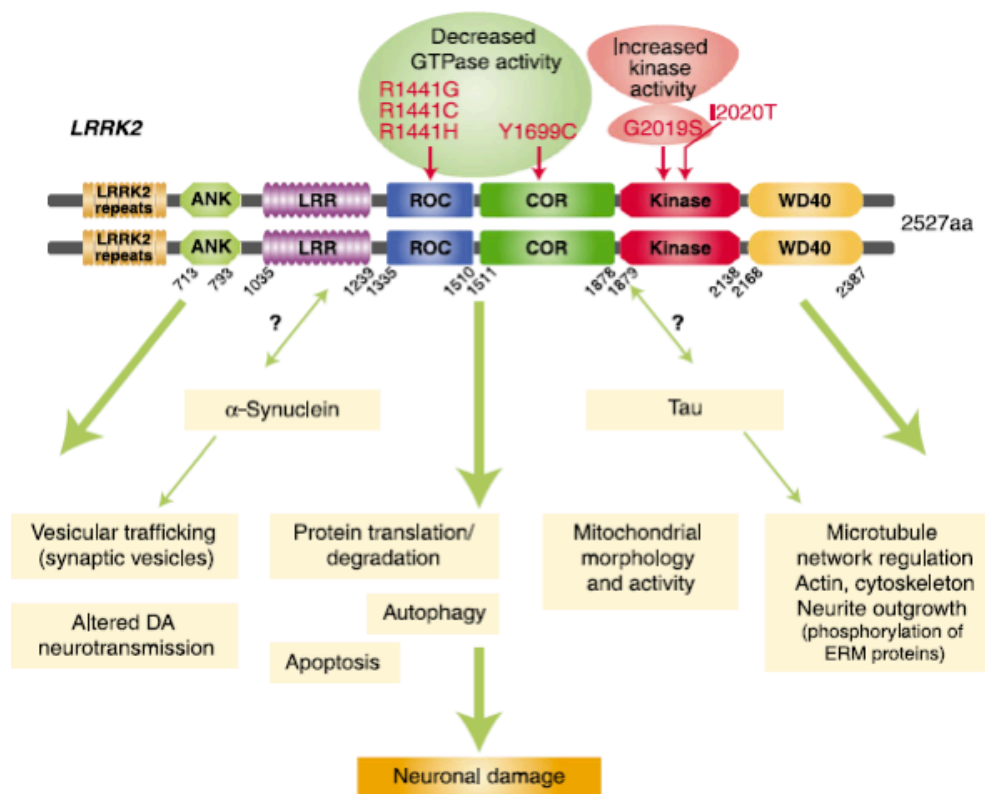
Figure13. Normal and pathogenic functions of LRRK2. LRRK2 has been implicated in regulating the microtubule network, actin cytoskeleton, neurite outgrowth, autophagy-lysosomal pathway, apoptosis, mitochondrial morphology and activity, vesicular trafficking (ie, synaptic vesicle exocytosis/endocytosis), dopaminergic (DA) neurotransmission, protein translation, and degradation pathways, which could potentially underlie neuronal damage. The potential functional interaction of LRRK2 with α-synuclein and tau as well as the molecular mechanisms through which disease-associated mutations lead to neurodegeneration.

1.2.7. LRRK2 Rodent Models

1.2.7.1. LRRK2 knockout (KO) mice

The first LRRK2 KO mice was published in 2007, by Biskup and his coworkers [124, 157]. LRRK2 KO mice are viable and have no major neuropathological abnormalities or motor dysfunction. No loss of DAergic neurons was observed even at 24 months of age suggesting that expression of LRRK2 for neuronal survival and to early embryonic development is not essential [39, 157]. These features were confirmed by a study that shows no significant difference in the susceptibility of LRRK2 KO and WT mice to MPTP. This fact could be explained by compensation mechanisms by LRRK1 since LRRK1 shares high homology with LRRK2 and is expressed in the brains (fig.14). In addition, this observation is supportive with the gain-of-function hypothesis of LRRK2 mutants. Nevertheless, the LRRK2 KO mice have some complications in the kidneys [39, 157]. The kidneys of these mice accumulate and aggregate α-synuclein, suggest some defects in autophagy, apoptotic cell death, oxidative damage and inflammation response [39, 157].

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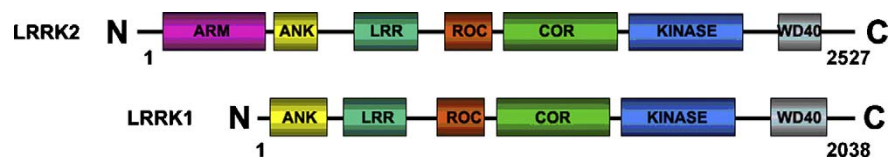
Figure 14. Schematic of LRRK1 and LRRK2 domain structures: ARM = armadillo, ANK = ankyrin, LRR = leucine-rich repeat, ROC = Ras of complex, COR = C terminus of ROC [97].

1.2.7.2. Bacterial artificial chromosome (BAC) transgenic mice of LRRK2

The use of BAC transgenic mice was initially described in 1997 by Antoch and his coworkers [159, 162]. It is a milestone in the past decade because this methodology potentiates gene functions studies *in vivo*, mainly in CNS. The application of BAC transgenics is advantageous over conventional transgenics for studying LRRK2. The application of BAC transgenic is advantageous over conventional transgenics for studying LRRK2. The main reasons are: generation of LRRK2 BAC transgenic mice does not involve the synthesis of full-length LRRK2 complementary DNA (cDNA), which is a > 7 kb nucleotide and technically difficult to manipulate as a result of the large size; the entire genomic sequence of mouse or human LRRK2 is approximately 180 kb, which is the average length of BAC clones that are readily available in public domains; and LRRK2 BAC transgenes with introduced PD mutations are suitable for modeling the LRRK2-mediated pathological process as a result of the dominant disease transmission for LRRK2 mutations [124, 157].

BAC transgenic mice have been developed for LRRK2 WT, LRRK2 R1441C/G, LRRK2 G2019S and display some hallmarks of neurodegeneration, demonstrated by measuring the dopamine content after pharmacologically blocking the dopamine uptake [39, 157, 162]. The LRRK2 protein expressed in transgenic mice, normally, shares 86% homology with the human protein and this expression is broadly distributed in many regions, including cortical cortex, hippocampus, striatum, amygdala, cerebellum and OB, as well as in ventral tegmental area (VTA) and SN [39, 157].

The BAC transgenic mice overexpressing the human LRRK2 R1441C/G mutant develop typical motor phenotype, beginning with reduced mobility that was reminiscent of hypokinesia in PD [39, 157, 162]. By 10–12 months of age, the hypokinesia in most LRRK2^{R1441G} mice had progressed to a visually apparent immobility, reminiscent of akinesia in late PD [162]. These symptoms are reversed by use of L-DOPA and DA receptor agonists, recapitulating the progressive motor deficits and responsiveness to L-DOPA that are characteristic of human PD [162]. However, immunohistochemistry for TH in 9–10-month-

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old LRRK2^{R1441G} mice revealed that DAergic neurons in the SNpc and VTA were normal in number and anatomical organization but in striatum and piriform cortex, two areas that were enriched in DAergic projections, TH–positive axons appeared to be beaded and fragmented and exhibited spheroids and dystrophic neurites [162].

LRRK2^{R1441C} BAC mice and also LRRK2^{G2019S} BAC mice show reduced DA release in striatal system in the absence of neuropathology, suggesting that LRRK2 mutants have a pathogenic role in this system [39, 157, 162]. In fact, impairment of endocytosis can be involved in this reduced DA release as this would compromise the "reconstruction" of SV, recycling of plasma membrane proteins of SV and dynamics of plasma membrane [13, 80].

1.2.7.3. Double transgenic mice

There is clear evidence that LRRK2 and α-synuclein are associated in PD pathophysiology [166]. Therefore a double transgenic mice was generated in 2009 expressing an inducible PD-related A53T α-synuclein mutant combined with the G2019S LRRK2 mutant. Interestingly synergistic toxicity to neurons was observed with exacerbated progression of α-synuclein-mediated neuropathology [166].

2. Main objectives of this study

As described above, PD is the second most common disease, and despite nearly 200 years since the first description of this disease, a definitive understanding of disease pathogenesis and a preventative therapeutic approach is still missing. So gaining insight into the pathogenesis and development of an effective disease modifying therapy are two of the major objectives of the host lab. So far, it is known that PD is a neurogenerative disorder characterized by DAergic neuron loss and presence of LBs. Nevertheless, the mechanisms and pathways that lead to these specific loss and presence of LB remain unknown [2, 23].

The discovery of gene-linked PD has provided an opportunity to investigate the PD pathogenesis. One of this genes/protein, called LRRK2, is a cause of autosomal dominant PD and involved in sporadic PD and hence is a good target for investigation. The multi-domain structure of LRRK2, with both enzymatic (kinase and GTPase) and interaction domains, makes that there are many layers of regulation controlling the functional outputs of this protein. In fact, LRRK2 has been implicated in a wide variety of physiologic function such as neuronal survival and cytoskeleton rearrangement, mitochondrial dysfunction, protein homeostasis, autophagy, apoptosis, in immune system and in neurotransmission and synaptic vesicle endocytosis [78]. An involvement of LRRK2 in the pathogenesis of PD, notably in synaptic vesicle endocytosis was discovered in the host lab just before the beginning of this study (Matta et al., 2012 - manuscript accepted). These initial findings were however made in *Drosophila melanogaster*. To study the functional conservation of the role of LRRK2 in synaptic vesicle endocytosis in the mammalian neuronal system, to evaluate the LRRK2 kinase dependency of this phenomenon and to confirm the functional conservation of mammalian endophilin in this pathway the study presented in this thesis was performed.

The working hypothesis is that in analogy with *Drosophila melanogaster* LRRK, mammalian LRRK2 regulates synaptic vesicle endocytosis by acting as a switch via phosphorylation of a variant of endophilin. This phosphorylation regulates the subcellular localization of endophilin affecting the SV endocytosis with non-phosphorylated and phosphorylated endophilin respectively competent and incompetent to interact with the membrane of the synaptic vesicle

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3. Material and Methods

3.1. Mediums and Solutions

Cell Culture

- Hank's balanced salt sodium solution[1] (HBSS) [without Ca^{2+} and Mg^{2+}] and HEPES[2] solution (HBSS/HEPES) – 3.5 ml of HEPES 1M, 5 ml of Penicillin-Streptomycin[3], fill up to 500 ml with HBSS filter through a 0.22μm filter[4] and stored at 4°C;

- MEM-Horse medium *(plating medium)* - 50 ml Horse serum (heat-inactivated)[5], 15 ml of 20% glucose[6] (glucose was dissolved in milli-Q water, filter through a 0.22μm filter store at 4°C), fill up to 500 ml with MEM 1x [+ Earle's, + L-Glutamine][7], filter through a 0.22μm filter and stored at 4°C;

- B27 L-glutamine *(sustaining medium)* - 485 ml Neurobasal medium, 10 ml B27 supplement[8], 5 ml L-glutamine[9], filter through a 0.22μm filter and stored at 4°C.

Electrophysiological solutions

- Internal solution (mM): K-gluconate[10] 146; HEPES 17.80; Mg-ATP[11] 4.00; Na_2-ATP[12] 0.30; EGTA[13] 1.00 and Phosphocreatine[14] 12.00, pH 7.3 adjusted with KOH (2M) and osmolarity 300 mOSmol kg^{-1}, adapted from Rost et al., 2010 [168].

- Bath Solution - Extracellular saline solution (mM): NaCl[15] 140.00; KCl[16] 2.40; HEPES[17] 10.00; D-Glucose-H_2O[18] 10.00; $CaCl_2$[19] 2.50 and $MgCl_2$[20] 1.30, pH 7.3 adjusted with KOH (2M) and osmolarity 300 mOsmol kg^{-1}, adapted from Rost et al., 2010 [168].

- Sucrose solution contained the same composition as bath solution. Sucrose[21] was added to a final concentration of 50 mM (350 mOsmol kg^{-1}, pH 7.3).

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[1] Product number: 14175 – Company: Invitrogen/Gibco
[2] Product number: 15630-122 (20 x 100 ml) – Company: Invitrogen/Gibco
[3] Product number : 15140 – Company: Invitrogen/Gibco
[4] Product number Stericup & Steritop system Express PLUS (0.22μm) Cat. N° SCGPU05RE – Company: Millipore.
[5] Product number: 26050088 – Company: Invitrogen – Quantity: 500 ml
[6] Product number: 104074.1000 – Company: Merck – Quantity: 1kg
[7] Product number: 31095-052 (10 x 500 ml) – Company: Invitrogen/Gibco
[8] Product number: 17504-044 (10ml) – Company: Invitrogen/Gibco
[9] Product number: 25030 – Company: Invitrogen/Gibco
[10] Product number: D-Gluconic Acid (2,3,4,5, 6-Pentahydroxycaproic Acid) Potassium Salt G4500 - Company: Sigma
[11] Product number: Adenosine 5-Triphosphate magnesium salt, from bacterial source A9187-500MG - Company: Sigma
[12] Product number: Adenosine 5-Triphosphate A7699 - Company: Sigma
[13] Product number: Ethylene glycol-bis (2-aminoethyl-ether) - N,N,N',N'-tetracetic acid E4378-100G - Company: Sigma
[14] Product number: Phosphocreatine disodium salt hydarte enzymatic, approx. 98% P7936-5G - Company: Simga
[15] Product number: 27810295 - Company: BDH Prolabo/VWR - Quantity: 1kg
[16] Product number: 1049361000 - Company: Merck - Quantity: 1kg
[17] Product number: H75223-250G - Company Sigma
[18] Product number: D(+)-Glucose monohydrate 1083421000 - Company: Merck - Quantity: 1kg
[19] Product number: 21107-1L – Company: Sigma
[20] Product number: M8266-1KG – Company: Sigma
[21] Product number: S0389-500G – Company: Sigma

3.2. Animals

All experiments were performed with embryos WT (B6.Cg-Tg(Lrrk2)6Yue/J), KO LRRK2 (B6.129X1(FVB)-*Lrrk2^tm1.1Cai*/J) C57BL/6J mice 17-19 days (E17-E9 days). The animals were housed in an animal facility that is fully compliant with the European policy on the use of Laboratory Animals. Experimental protocol was approved by the Institutional Review Committee of Janssen Pharmaceutica (Beerse, Belgium) and meet the European and Belgian guidelines on animal experimentation.

The animals (26, 15 WT and 11 LRRK2 KO mice) were maintained on a 12 hours light/dark cycle and were provided food with and water *ad libitum*. Care was taken to reduce the number of used animals.

3.3. Preparation of Petri dishes/cover glasses

The coating process takes 2 days. To prepare the Petri dishes/cover glasses coating solution, 5 ml of milli-Q water was added to 5 mg poly-D-lysine [22] (PDL) to obtain a final concentration of 1 mg/ml (stock solution, stored at –20 ºC). The PDL solution was mixed and 2 ml aliquots with a concentration of 0.1 mg/ml (work solution) were stored at 4 ºC.

Cover glasses[23] were cleaned for 3 hours with nitric acid (HNO_3) and washed 3 times with milli-Q water (30 min) before the coating process. Cover glasses were pre-sterilized in 70% ethanol for 30 minutes and sterilized by heat (180 ºC, 4h).

The coating process was done by addition of 200 μl of work PDL solution in the center of 3.5 cm Petri dishes[24] or 100 μl on cover glasses. Petri dishes/cover glasses were incubated at room temperature overnight and next day the solution was aspirated. Petri dishes/cover glasses were washed 2 times (20 min) with milli-Q water and left in a laminar flow hood to dry.

3.4. Cell culture

One day before dissection, the plating and sustaining medium were added to tissue culture flasks[25] (T250) and placed in a humidified incubator at 37 ºC and 5% CO_2.

On the day of dissection the HBSS/HEPES solution was pre-warmed to 37 ºC on a heating plate. 10 cm Petri dishes[26] (10 ml) and in 3.5 cm Petri dishes[27] (1ml) were used to

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[22] Product number: P6407-5MG - Company: Sigma
[23] Product number: A10143263NR1 – Company: Thermo Scientific
[24] Product number: 150318 - Company: DishesNuclon™/Thermo Scientific
[25] Product number: Falcon 353003 tissue culture dishes, 100 x 20 mm – Company: Becton Dickinson Labware
[26] Product number: Falcon 353003 tissue culture dishes, 100 x 20 mm – Company: Becton Dickinson Labware
[27] Product number: Falcon 353001 tissue culture dishes, 35 x 10 mm – Company: Becton Dickinson Labware

collect the embryo's heads and embryonic brains, respectively. One of the 3.5 cm Petri dishes was filled with 2.5 ml HBSS/HEPES to collect hippocampi. A 15 ml centrifuge tube[28] with 4.5 ml HBSS/HEPES was placed in a warm water bath (37 °C). The trypsin[29] solution 0.5% (10X) [with EDTA] was retrieved from the fridge and left in laminar flow hood.

Large forceps and scissors were sterilized with 70% ethanol. The pregnant mice (day E17-E19) were sacrificed by cervical dislocation. A large incision was made in the skin of abdomen on the midline starting from the pelvis up to the thorax. The skin was separated from muscle tissue, exposing the surface of the abdomen. A large area was opened to reach the embryos. The uterus was placed in a 10 cm Petri dish and covered with a lid.

In laminar flow hood, the embryos were removed from the uterus and decapitated. The heads were transferred to the 10 cm Petri dish containing pre-warmed HBSS/HEPES. The forehead was perforated with curved forceps to keep the head stable. The skull was removed by perforation above the eyes, using a lateral movement, one hemisphere at time. The brains were removed out the skull and placed with the ventral side facing up into 3.5 cm dishes containing HBSS/HEPES. When all brains were collected, the hippocampi were isolated under a dissection microscope.

The 2 hemispheres were removed from the diencephalon and the brain stem. With the internal side hemispheres facing up, the meninges was removed. Then the hippocampus was collected and transferred into to 3.5 cm dish with 2.5 ml pre-warmed HBSS/HEPES.

After hippocampi were dissected, they were transferred into a 15 ml centrifuge tube. The HBSS/HEPES solution was removed and 500 μl of trypsin 0.5% (10 xs) solution was added to the 15 ml centrifuge tube with 4.5 ml HBSS/HEPES. This solution was added to hippocampi and incubated for 10-15 minutes in the warm water bath. The solution was removed and hippocampi were washed 2 times with 3-5 ml plating medium.

After enzymatic dissociation, tissue was resuspended in 3 ml of plating medium. The mechanical dissociation was done by first pipetting 30 times using a sterile glass Pasteur pipette[30] with standard tip diameter and then by pipetting 30 times using a Pasteur pipette with reduced fire-polished tip diameter. The cells were centrifuged at 1000 rpm for 5 minutes, resuspended in 2 ml of plating medium and then counted using the Vi-Cell automatic counter.

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[28] Product number: Falcon Blue Max Jr. 15 ml polypropylene conical tube, 17 x 120 mm 352097 – Company: Becton Dickinson Labware
[29] Product number: 15400 – Company: Invitrogen/Gibco (without phenol red)
[30] Product number: Disposable glass Pasteur pipettes, 230 mm, pre-plugged D812 – Company: Volac

The cells were plated at a density 50.000 cells/ml in Petri dishes/cover glasses coated with PDL. Cells were left overnight in the incubator at 37 $^{\circ}$C and 5% CO_2. Then, plating medium was removed and replaced with sustaining medium.

Cells were used for electrophysiological experiments 7-12 days after plating.

3.5. Electrophysiology

Petri dishes/cover glasses containing the neuronal network were placed under a Carl Zeiss Axioskop 2FS upright microscope (Jena, Germany). Sustaining medium was changed to bath solution and all experiments were performed at room temperature (18-22 ºC)

Pyramidal neurons were voltage clamped at a holding potential of -70 mV with pipettes for patch-clamp recording (electrodes) under HEKA software. Recordings were digitized and stored on the computer hard drive (sample frequency 10 kHz, filter frequency 5 kHz). Electrodes[31] (thick-walled borosilicate glass capillaries with outer diameter 1.5 mm and inner diameter 0.86 mm) were pulled with a P97 Flaming/Brown Micropipette Puller (Sutter Instruments). The micropipette tip was heated with a micro forge to produce a smooth surface. The diameter and the resistance of these polished tips were ≈ 1μm and 3-5 MΩ, respectively. The electrodes were filled with internal solution.

Signals/events were recorded during 10 seconds with an EPC10 USB Patch Clamp Amplifier (HEKA) and analyzed using a PC and Igor Pro 6.2.2.2 software (WaveMetrics, Inc.). The GABA-mediated responses were avoided by voltage clamping neurons at -70 mV. The use of 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX)[32], an AMPA receptor antagonist, at 10 μM showed no sEPSC in control neither in sucrose, which prove that we were recording the Glutamatergic-mediated responses.

After recording 10s in bath solution, this solution was replaced to a hypertonic sucrose solution (50 mM sucrose, 350 mOsmol kg^{-1}) by perfusion. After 2 min perfusion, a new recording was made and same parameters were measured.

3.6. Drugs

Dynasore[33] was stored frozen at a concentration of 20 mM (in DMSO[34]) in 200 μl aliquots and diluted to a final concentration of 40 μM (0.2% DMSO) in bath solution and sucrose solution. After placing the dish under the microscope the sustaining medium was

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[31] Product number: 300057 - Company: Harvard Apparatus
[32] Product number: N171-5MG – Company: Sigma
[33] Product number: 324410 – Company: Calbiochem
[34] Product number: 1.02952.1000 – Company: Merck

changed, to bath solution containing the compound. After 5 min, a cell was selected, patched and control recording was made. By perfusion the solution was replaced to sucrose solution plus compound in the same concentration for 2.5 min and the recording was repeated.

LRRK2-IN-1[35] was stored at -4 ºC at a concentration of 10 mM (in DMSO) and diluted to a final concentration of 0.1 μM, 0.3 μM or 1 μM (0.2% DMSO). Cells were pre-treatment for 1 hour with compound. After placing the dish under the microscope the medium with the compound was changed, to bath solution containing the compound in the same concentration. A cell was selected, patched and control recording was made. By perfusion the solution was replaced to sucrose solution plus compound in the same concentration for 2.5 min and recording was repeated.

To control, the cells were pre-treatment for 1 hour with DMSO 0.2% dissolved in sustaining medium.

3.7. Data Analysis

Using Igor Pro 6.2.2.2 software, raw data traces obtained with HEKA software were transferred to an Excel template, which detects the sEPSC events based on time and threshold of the event. Events that exceed 5 ms in duration and have amplitude less than -10 pA were not considered as a sEPSC.

Graphs are generated with GraphPad Prism 4.02 and values are represented as mean ± Standard Error of the Mean (SEM) from *n* cells/experiments. Statistical significance was assessed by a two-tailed Student's *t* test for the experimental versus control condition, one-way ANOVA followed by Dunnett's Multiple Comparison Test or two-way ANOVA. A *p* value of 0.05 or less was considered for significant differences.

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[35]   Product number: 438193-5MG: 5,11-Dihydro-2-[[2-methoxy-4-[[4-(4-methyl-1-piperazinyl)-1-piperidinyl]carbonyl]phenyl]amino]-5,11-dimethyl-6H-pyrimido[4,5 b][1,4]benzodiazepin-6-one, Company: Merck Millipore Home

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4. Results

LRRK2 is widely expressed in neurons throughout the CNS, including cortex, hippocampus, striatum, and other brain areas [78, 111, 113].

Recent publications and findings in the host lab propose an involvement of LRRK2 in neurotransmitter release [70] and a function in the pre-synaptic protein network, coordinating both the storage and the mobilization of SVs [13] by interaction with Rab5 [80] and endophilin A1 (Matta *et.* al.*,* 2012-manuscript accepted). In this study, we studied the functional conservation of the role of LRRK2 in synaptic vesicle endocytosis in the mammalian neuronal system and the LRRK2 kinase dependency of this phenomenon (Matta *et.* al., 2012-manuscript accepted).

LRRK2 absence causes impairments in pre-synaptic function

To confirm a role of LRRK2 in modulation of synaptic function in mammalian neurons, we measured the number, frequency and amplitude of sEPSCs, via whole-cell patch clamp in hippocampal neurons derived from WT and LRRK2 KO mice. As LRRK2 expression is only detected in the rodent brain from embryonic day 16 to 17 (E16-E17) [78], WT and LRRK2 KO mice were used between embryonic day 17 to 19 (E17-E19 days) between 7-12 days in culture (fig. 15).
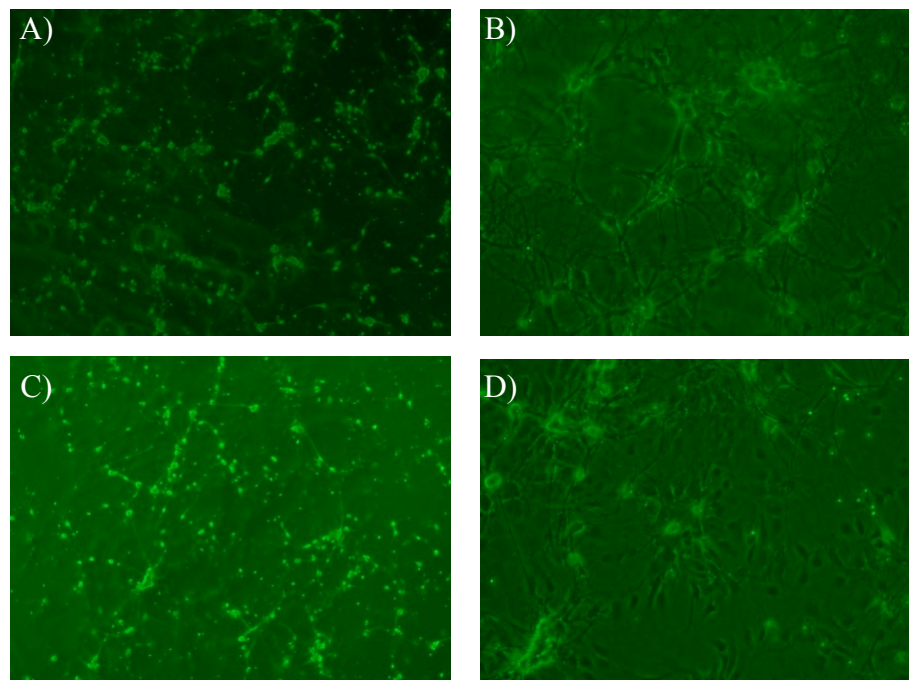

Figure 15. Hippocampal cells in 7 days culture. A, B; WT hippocampal cells, 7 days in culture, with magnifications of 40x and 100x, respectively. C, D; LRRK2 KO hippocampal cells, 7 days in culture, with magnifications of 40x and 100x, respectively.

To make sure that we were recording the sEPSC and not sIPSC, during the experiments, we performed some recordings in WT and LRRK2 KO hippocampal cells in control and sucrose conditions (n=7) in the presence of NBQX, an AMPA receptor antagonist at 10 μM and compared those to the currents measured in the absence of NBQX. NBQX impaired the AMPA- and NMDA-mediated currents (Glutamatatergic-mediated responses) recording in post-synaptic cell by blocking the AMPA receptor. This indicates that sEPSC are the result of binding of glutamate to AMPA receptors that are essential to neurotransmission (fig. 16)
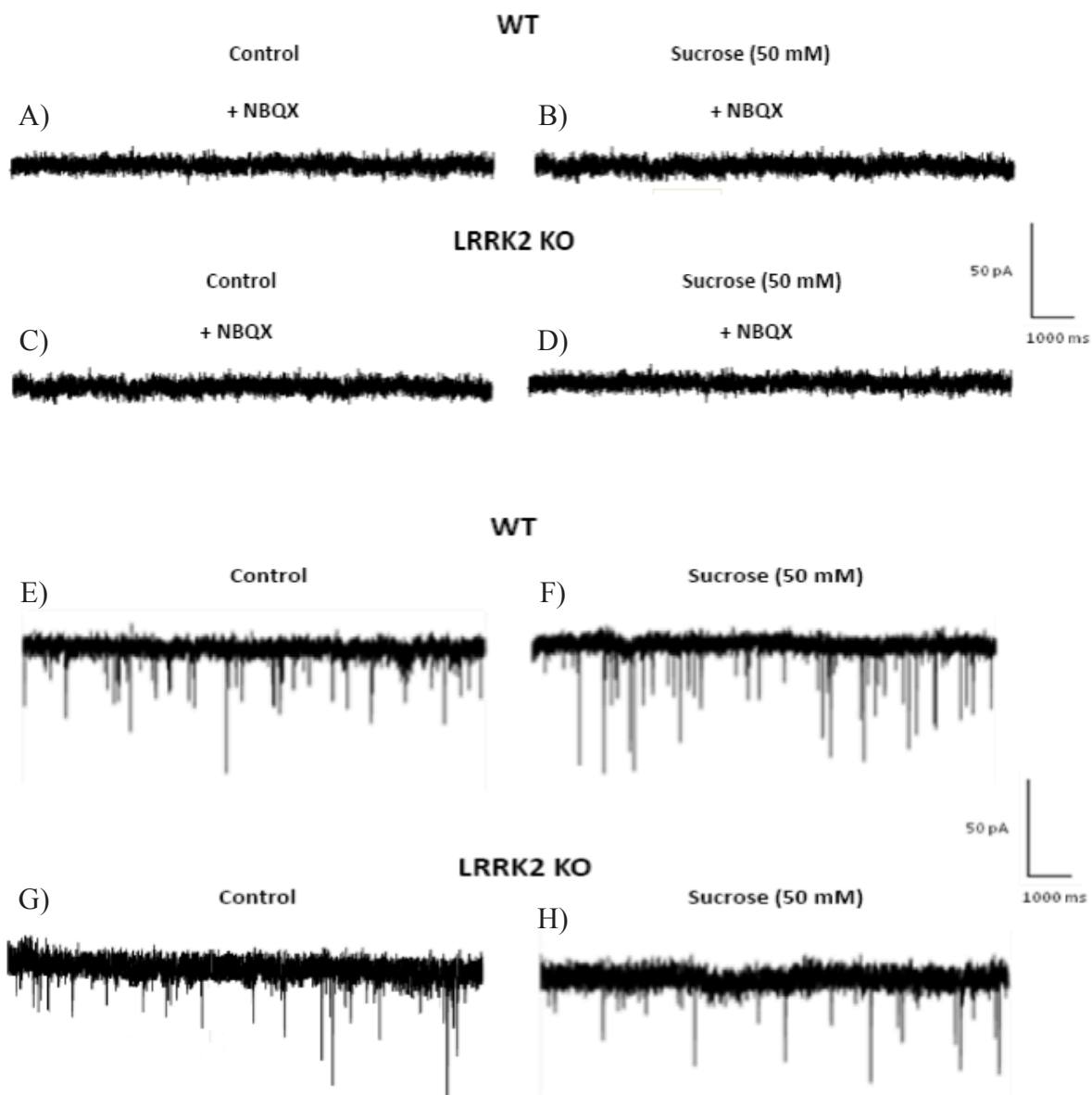
Figure 16. AMPA receptor antagonist (NBQX) blocks the sEPSC. Representative recordings of WT hippocampal cells (9 days in culture) in presence of NBQX in control (A) and sucrose (B), and in absence of NBQX in control (E) and sucrose (F). Representative recordings of LRRK2 KO hippocampal cells (9 days in culture) in presence of NBQX, control (C) and sucrose (D), and in absence of NBQX in control (G) and sucrose (H).

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The sEPSCs, as described above, are the result of the different number of release points (synapses) that each cell can form, number of receptors available in post synaptic cells and the network of the cell culture. Under basal conditions, the currents measured are similar in the two genotypes. In presence of sucrose (50 mM), the number of EPSCs in WT hippocampal cells was drastically increased in the presence of sucrose (n=19; control, 47.74 ± 14.03; sucrose, 100.84 ± 19.62) (fig.17A,B and 17E; ρ<0.05, two-tailed Student's t test). Conversely, in LRRK2 KO hippocampal cells (n=22; control, 32.05 ± 8.07; sucrose, 43.14 ± 9.76) sucrose treatment failed to induce an increase in the number of sEPSC (fig.17C, D, E). Sucrose is thought to stimulate the release of the entire RRP (depletion of total RRP) in the neuronal culture, by mechanical stress (independently of Ca^{2+}), producing a parallel depletion and refilling of the pool used by action potential-evoked release (recycling pool and reserve pool) [169]. Determination of the sucrose/bath ratio showed that the application of sucrose promotes a fold-change of 4.07 ± 1.38 in WT (n=19) and 1.69 ± 0.37 in LRRK2 KO hippocampal cells (n=22) (fig. 17F, ρ<0.05, two-tailed Student's t test).

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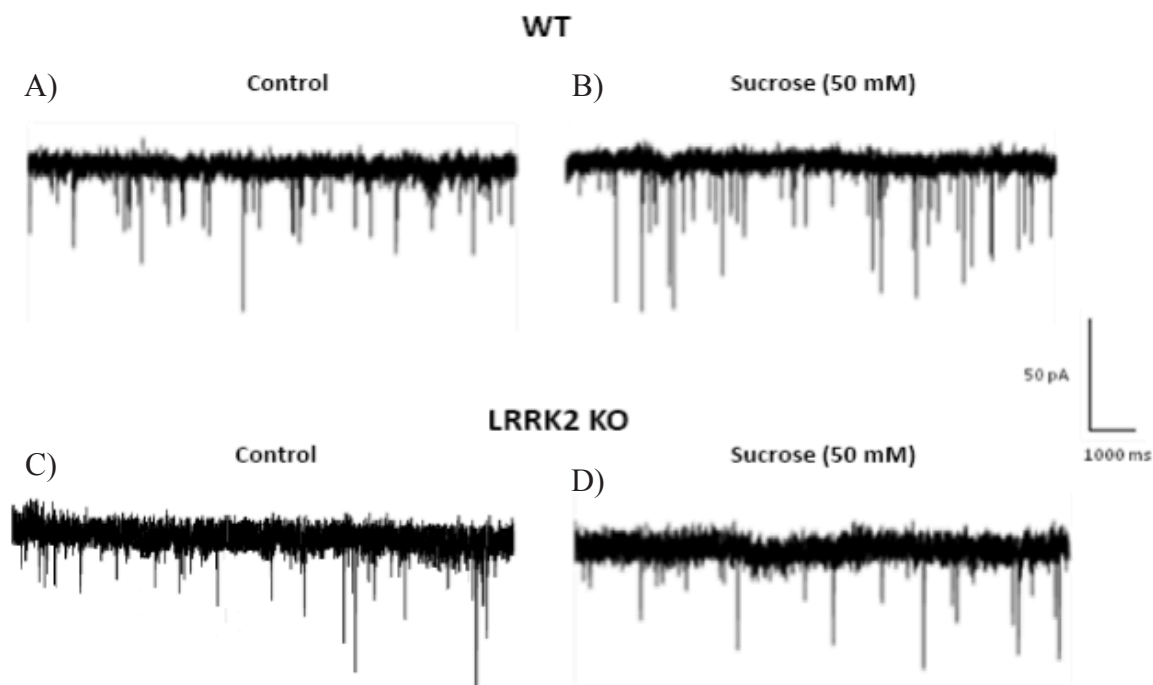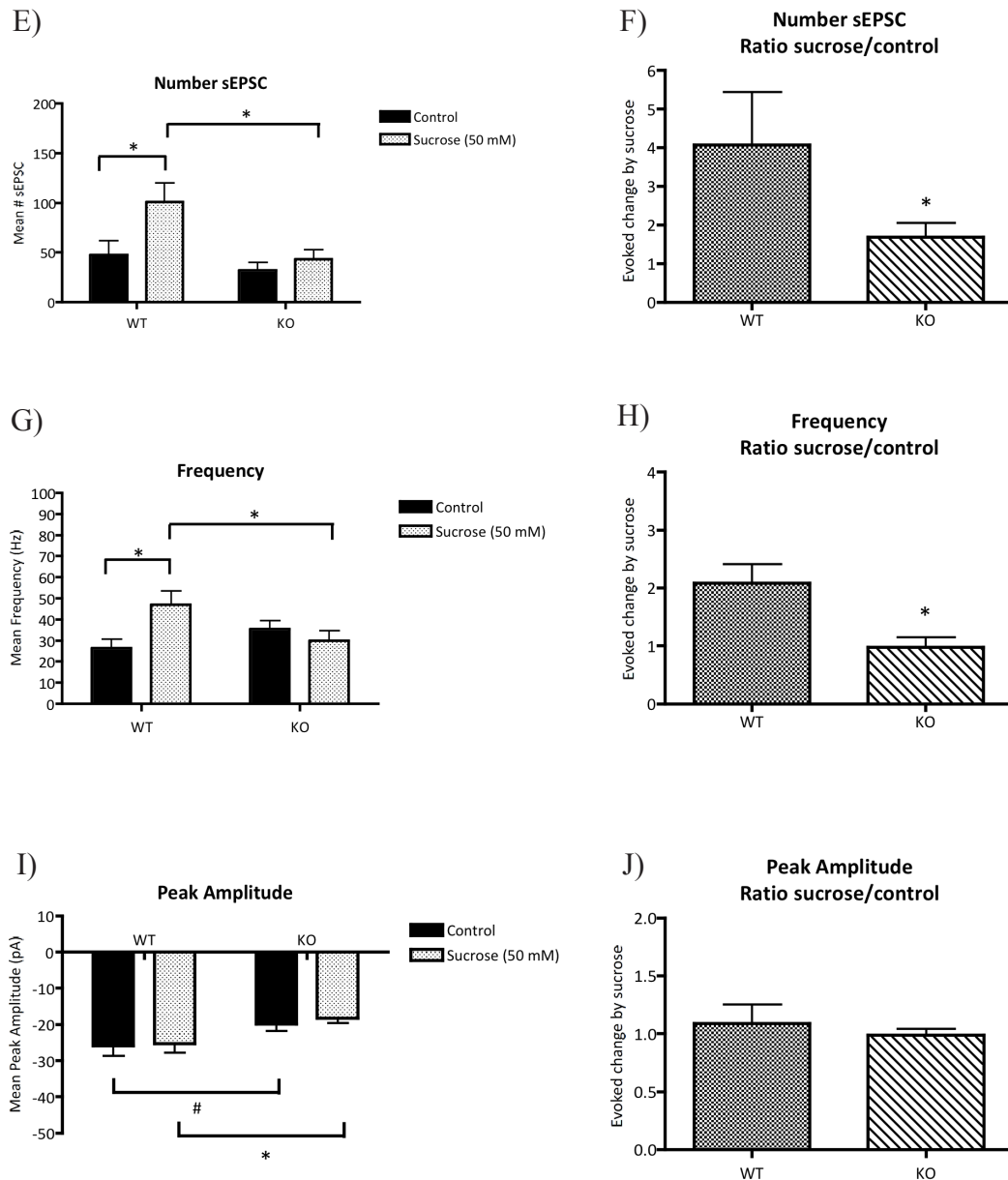
Figure 17. LRRK2 KO causes impairment in neurotransmitter release. Single recordings were performed in post-synaptic WT or LRRK2 KO cells that receive inputs from the neuronal network. Representative whole-cell patch clamp recordings from WT hippocampal cells in control **(A)** and sucrose (50 mM) **(B)**; Representative whole-cell patch clamp recordings from LRRK2 KO hippocampal cells in control **(C)** and sucrose (50 mM) **(D)**. **(E)** Sucrose significantly increased the number of sEPSC in WT hippocampal cells (n=19; control, 47.74 ± 14.03; sucrose, 100.84 ± 19.62) but not in LRRK2 KO hippocampal cells (n=22; control, 32.05 ± 8.07; sucrose, 43.14 ± 9.76). **(F)** Sucrose (50 mM) induces a fold change on number of sEPSC in WT and LRRK2 KO hippocampal cells of 4.07 ± 1.38 and 1.69 ± 0.37, respectively. **(G)** Sucrose significantly increased the frequency (Hz) in WT hippocampal cells (n=19; control, 26.32 ± 4.27; sucrose, 46.64 ± 6.48) but not in LRRK2 KO hippocampal cells (n=22; control, 35.44 ± 4.01; sucrose, 30.00 ± 4.67). **(H)** Sucrose (50 mM) induces a fold change on frequency (Hz) in WT and LRRK2 KO hippocampal cells of 2.08 ± 0.33 and 0.97 ± 0.18, respectively; **(I)** Sucrose did not affect the peak amplitude in both genotypes (WT: n=19; control, -25.90 ± 2.84; sucrose, -25.27 ± 2.47); (KO: n=22; control, -19.44 ± 1.97; sucrose, -18.26 ± 1.37) and **(J)** the fold change does not show a significant difference (WT; n=19; 1.09 ± 0.17) and (KO; n=22; 0.99 ± 0.06). Data are expressed as mean ± SEM. # p=0.08, *p<0.05, two-tailed Student's t test.

As the number of sEPSC and the efficiency of synaptic transmission depend on the availability of post-synaptic receptor and not only the pre-synaptic probability of release, we asked whether LRRK2 might be involved in presynaptic and/or post-synaptic cell. We thus looked whether presence and absence of LRRK2 might affect presynaptic mechanisms including recycling of the synaptic vesicles. To study this, we analyzed the frequency (Hz) of sEPSC (basal and sucrose-stimulated) in neurons from WT and LRRK2 KO mice. As expected, the presence of sucrose stimulates the neurotransmitter release and induces a significant increase in frequency in WT hippocampal cells (n=19; control, 26.32 ± 4.27; sucrose, 46.64 ± 6.48). Nevertheless, in LRRK2 KO hippocampal cells (n=22; control, 35.44 ± 4.01; sucrose, 30.00 ± 4.67) the stimulating effect is absent (fig 17G, *ρ<0.05, two-tailed Student's t test). Data in figure 17G show that in control, the LRRK2 KO neurons shows a slightly (not significant) increase in frequency when compared to control in WT hippocampal cells. The increase in frequency is dependent on presynaptic machinery proteins and showed a normal increase in WT hippocampal cells (2.08 ± 0.33, n=19) but in LRRK2 KO hippocampal cells the frequency remains the same (0.97 ± 0.18, n=22) (fig. 17H, *ρ<0.05, two-tailed Student's t test). As frequency of sEPSC is a valid parameter [149] of pre-synaptic functions, these results suggest that presence of LRRK2 is essential in pre-synaptic mechanisms to replenish the synaptic vesicles pool to govern high frequency neurotransmission.

As mentioned above, neuronal communication is dependent on both the pre-synaptic cell and post-synaptic functions in the neurons. While both pre- and post-synaptic mechanisms can determine the peak sEPSC amplitude, conventionally sEPSC amplitude changes are thought to reflect changes in the response of post-synaptic receptors [170, 171]. Thus, to evaluate whether LRRK2 affects the post-synaptic mechanism, we evaluate the peak amplitude in the same recordings. For both WT and LRRK2 KO hippocampal cells, there were no differences observed between control and sucrose conditions (WT: n=19; control, -25.90 ± 2.84; sucrose, -25.27 ± 2.47); (KO: n=22; control, -19.44 ± 1.97; sucrose, -18.26 ± 1.37) (fig 17I, #ρ=0.08, *ρ<0.05, two-tailed Student's t test). These results were also further quantified by looking at the ratio sucrose/control, where it was confirmed that the peak amplitude remain unchanged (WT; n=19; 1.09 ± 0.17; KO; n=22; 0.99 ± 0.06) (fig. 17J). Although, the sucrose solution did not affect the peak amplitude in the two genotypes, we can observe that peak amplitude is lower in LRRK2 KO hippocampal cells than in WT hippocampal. The ρ value is < 0.05 under sucrose-stimulated conditions but in control the ρ value is 0.08, which can suggest that in both conditions, LRRK2 KO hippocampal have

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smaller peak amplitude than WT hippocampal cells. This evidence might be attributed to the fact that in LRRK2 KO cells there are less synaptic vesicles to release, even under hypertonic solution. These results again suggest a role of LRRK2 in modulating presynaptic vesicle release.

Blockade GTPase Dynamin by dynasore mimics the effect of LRRK2 KO

Synaptic transmission between neurons requires the coordinated activity of numerous cellular elements to achieve the sustained communication necessary for normal nervous system function. Given the electrophysiological changes showed in previous results, we asked if LRRK2 is involved in endocytosis or exocytosis. We thus treated the cells with dynasore (40 µM, 5 min), a cell-permeable, small molecule and noncompetitive inhibitor of dynamin 1, 2, often used to study the endocytosis (fig.18A-H).
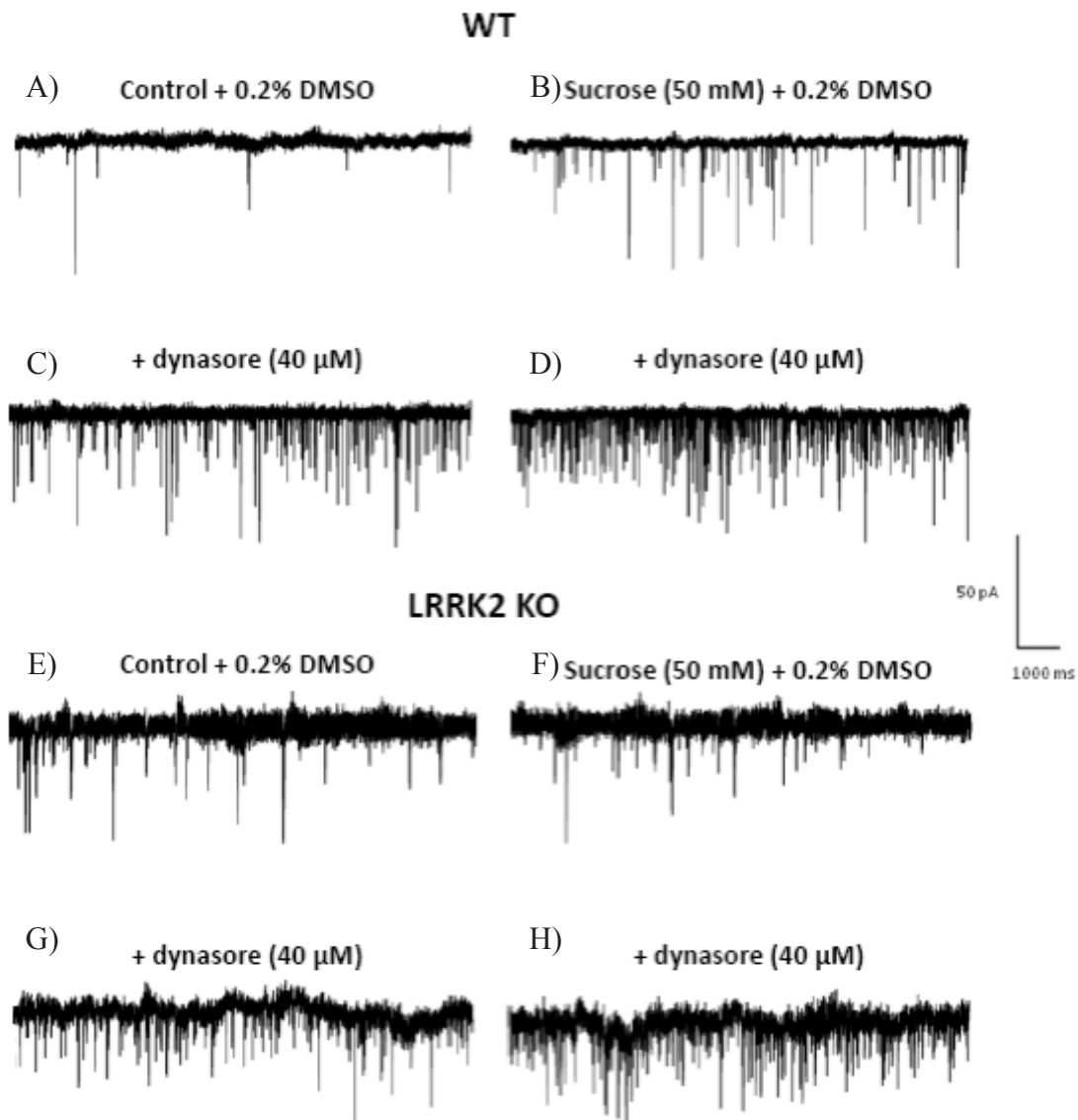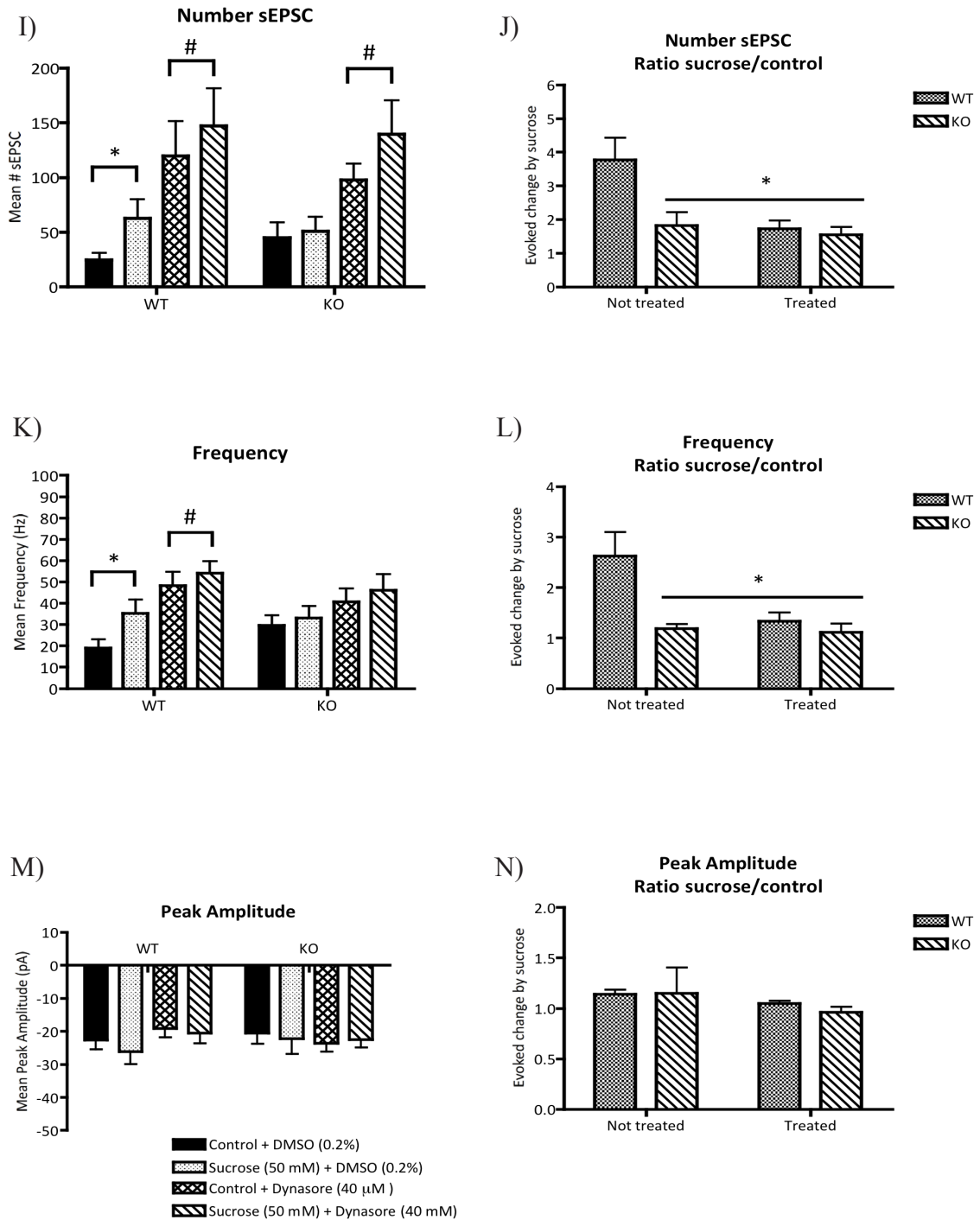
Figure 18. Blockade of GTPase dynamin by dynasore mimics the effect of absence of LRRK2. Single recordings were performed in post-synaptic WT or LRRK2 KO hippocampal cells that receive inputs from the neuronal network. Representative whole-cell patch clamp recordings from WT hippocampal cells in control **(A)** treated with dynasore (40 µM) **(C)** and in sucrose (50 mM) **(B)** treated with dynasore (40 µM) **(D)**. Representative whole-cell patch clamp recordings from LRRK2 KO hippocampal cells in control **(E)** treated with dynasore (40 µM) **(G)** and in sucrose (50 mM) **(F)** treated with dynasore (40 µM) **(H)**. **(I)** Sucrose (50 mM) significantly increased the number of sEPSC in WT hippocampal cells not treated with dynasore (40 µM) (n=15; control, 24.80 ± 6.35; sucrose, 62.73 ± 17.21) but not in LRRK2 KO hippocampal cells (n=12; control, 45.09 ± 14.00; sucrose, 51.09 ± 12.89). The dynasore (40 µM) increases the number of sEPSC in both genotypes but abolish the effect of sucrose in WT hippocampal cells (WT; n=14; control, 119.57 ± 31.76; sucrose, 147.36 ± 34.02) (KO; n=14; control, 97.56 ± 15.00; sucrose, 139.78 ± 30.78). **(J)** Sucrose (50 mM) induces a fold change on number of sEPSC in WT and LRRK2 KO hippocampal cells no treated with dynasore (40 µM) of 3.77 ± 0.66 and 1.82 ± 0.40, respectively. The fold change on number of sEPSC in cells treated with dynasore (40 µM) is: WT; n=14: 1.72 ± 0.25 and KO; n=14: 1.55 ± 0.23. **(K)** In hippocampal cells not treated with dynasore (40 µM), sucrose significantly increased the frequency (Hz) in WT hippocampal cells (n=15; control, 18.98 ± 4.05;

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sucrose, 35.37 ± 6.43) but not in LRRK2 KO hippocampal cells (n=12; control, 29.66 ± 4.74; sucrose, 33.10 ± 5.70). In hippocampal cells treated with dynasore (40 μM), WT hippocampal cells (n=14; control, 48.30 ± 6.52; sucrose, 54.17 ± 5.61) show larger frequencies in two conditions when compared with cells not treated but the effect is abolish (KO: n=14; control, 40.60 ± 6.38; sucrose, 46.15 ± 7.66). **(L)** Sucrose (50 mM) induces a fold change on frequency in WT and LRRK2 KO hippocampal cells no treated with dynasore (40 μM) of 2.63 ± 0.47 and 1.18 ± 0.10, respectively. The fold change on frequency in cells treated with dynasore (40 μM) is: WT; n=14: 1.33 ± 0.18 and KO; n=14: 1.11 ± 0.17. **(M)** Sucrose (50 mM) did not affect the peak amplitude in both genotypes not treated with dynasore (40 μM) (WT: n=15; control, -22.69 ± 2.76; sucrose, -26.18 ± 3.68); (KO: n=12; control, -20.56 ± 3.24; sucrose, -22.27 ± 4.50) and treated with dynasore (40 μM) (WT: n=14; control, -19.13 ± 2.63; sucrose, -20.48 ± 3.19); (KO: n=14; control, -23.62 ± 2.55; sucrose, -22.42 ± 2.50) and **(N)** the fold change does not show a significant difference (WT not treated; n=15; 1.14 ± 0.05; WT treated; n=14; 1.05 ± 0.03) and (KO not treated; n=12; 1.15 ± 0.25; KO treated; n=14; 0.96 ± 0.05). Data are expressed as mean ± SEM. *p<0.05 versus WT untreated, two-tailed Student's t test, #p<0.05 versus WT/KO untreated, two-tailed Student's t test.

The number of sEPSC in WT hippocampal cells was increased in presence of sucrose when the cells were exposed to the DMSO (vehicle) (n=15; control, 24.80 ± 6.35; sucrose, 62.73 ± 17.21) but the cells exposed to dynasore (40 μM) were unable to induce a significant increase (n=14; control, 119.57 ± 31.76; sucrose, 147.36 ± 34.02). As seen before, in LRRK2 KO hippocampal cells, the sucrose was unable to increase the number of sEPSC in both cells treated with DMSO (n=12; control, 45.09 ± 14.00; sucrose, 51.09 ± 12.89 and dynasore (40 μM) (n=14; control, 97.56 ± 15.00; sucrose, 139.78 ± 30.78) (fig. 18I, #ρ<0.05 versus WT or KO not treated, *ρ<0.05, two-tailed Student's *t* test). In addition, WT hippocampal cells treated with dynasore (WT not treated: n=15; 3.77 ± 0.66; WT treated: n=14: 1.72 ± 0.25) show the same ratio that is observed in LRRK2 KO hippocampal cells treated (n=14: 1.55 ± 0.23) or not treated (n=14: 1.82 ± 0.40) with dynasore (fig 18J, *ρ<0.05, two-tailed Student's *t* test).

The frequency of the events in WT hippocampal cells was increased by sucrose (50 mM) (n=15; control, 18.98 ± 4.05; sucrose, 35.37 ± 6.43) but, as we expected in LRRK2 KO hippocampal cells, this effect is abolished (n=12; control, 29.66 ± 4.74; sucrose, 33.10 ± 5.70). When the cells are treated with dynasore (40 μM), the stimulating effect of sucrose is neutralized and the frequency is not significantly different in both genotypes (WT: n=14; control, 48.30 ± 6.52; sucrose, 54.17 ± 5.61) (KO: n=14; control, 40.60 ± 6.38; sucrose, 46.15 ± 7.66) (fig. 18K, #ρ<0.05 versus WT or KO not treated, *ρ<0.05, two-tailed Student's *t* test). From quantification of sucrose/control ratio in the presence and absence of dynasore, it could be determined that the frequency in untreated WT hippocampal cells (n=15; 2.63 ± 0.47) was increased while in WT hippocampal cells treated (n=14; 1.33 ± 0.18) with dynasore (40 μM) this was not the case. Both LRRK2 KO hippocampal cells treated (n=14: 1.11 ± 0.17) and not treated (n=12; 1.18 ± 0.10) with dynasore (40 μM) do not exhibit differences in ratio (fig. 18L, *ρ<0.05, two-tailed Student's *t* test).

A remarkable observation is the fact that treatment with dynasore increased the number of sEPSC detected in both genotypes and the frequency of WT hippocampal cells in control and sucrose. It is not clear if these effects are caused by non-specific effects of dynasore or if dynamin inhibition leads an adaption of synaptic cycle recruiting, but some studies have described a significant increase in spontaneous release and frequency at excitatory synapses between cultured cortical neurons [161, 172, 173].

The last parameter evaluated was the peak amplitude. The peak amplitude in WT and LRRK2 KO hippocampal cells in both control and sucrose was not altered with the treatment with vehicle (WT: n=15; control, -22.69 ± 2.76; sucrose, -26.18 ± 3.68); (KO: n=12; control, -20.56 ± 3.24; sucrose, -22.27 ± 4.50) and dynasore (40 μM) (WT: n=14; control, -19.13 ± 2.63; sucrose, -20.48 ± 3.19); (KO: n=14; control, -23.62 ± 2.55; sucrose, -22.42 ± 2.50) (fig. 18M). Also no alteration was observed in terms of ratio (WT not treated; n=15; 1.14 ± 0.05; WT treated; n=14; 1.05 ± 0.03) and (KO not treated; n=12; 1.15 ± 0.25; KO treated; n=14; 0.96 ± 0.05) (fig.18N).

Collectively, the experiments shown in figure 18 show that treatment of neurons with dynasore mimicked the effect of LRRK2 absence, suggesting that the observed impairment in synaptic function in LRRK2 KO neurons has a strong endocytic component.

LRRK2 kinase activity is essential to endocytosis of synaptic vesicles

In accordance with the proposed hypothesis, LRRK2 is part of a complex of presynaptic proteins that modulates synaptic vesicle endocytosis. A recently identified mechanism in *Drosophila melanogaster* involves the vesicle binding of Endophilin A1, which is regulated by phosphorylation at residue 75 in BAR-domain of endophilin (Matta et al., 2012 – manuscript accepted). If conserved in mammalian neurons, this would mean that the effect of LRRK2 on endocytosis depends on kinase activity. To confirm this with functional data, we asked if the effects observed in LRRK2 KO neurons can be mimicked in WT neurons treated with a LRRK2 kinase inhibitor. Therefore, we did recordings (basal and sucrose-stimulated) in the presence of LRRK2-IN-1 (0.2% DMSO) at 0 µM (n=15), 0.1 µM (n=10), 0.3 µM (n=10) and 1 µM (n=10) in WT neurons (fig. 19A-H).
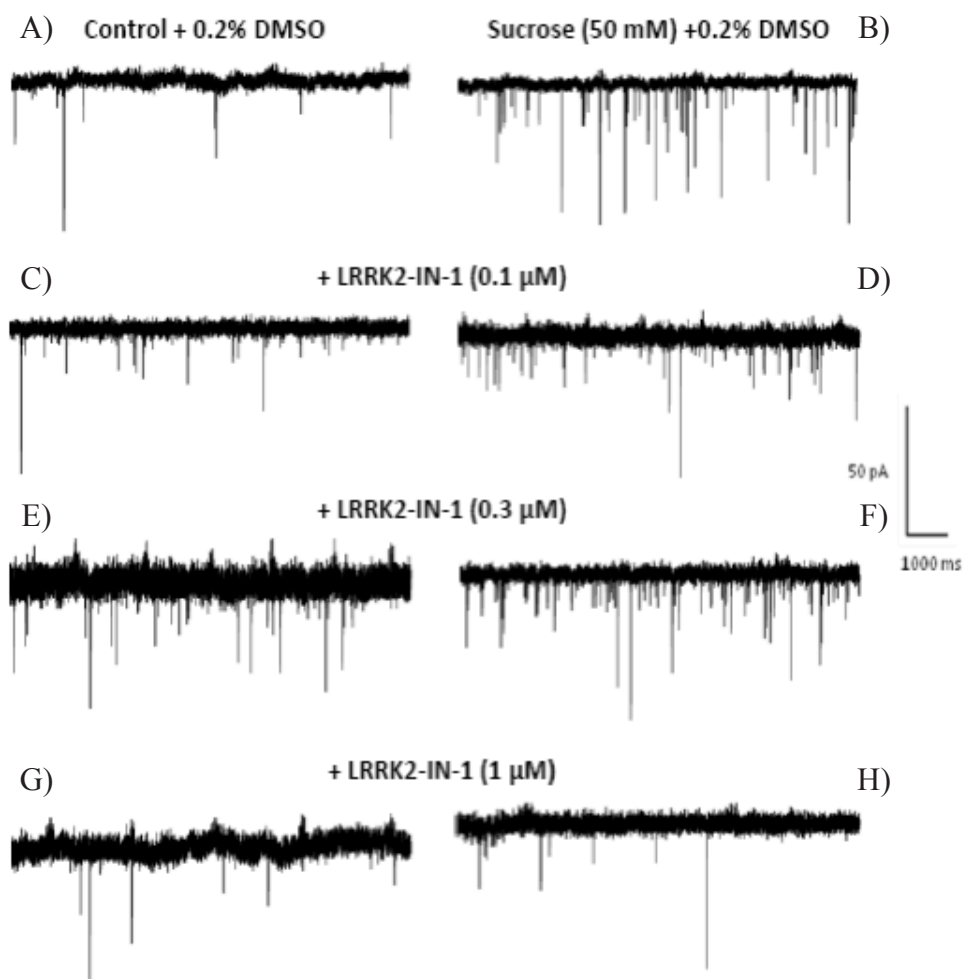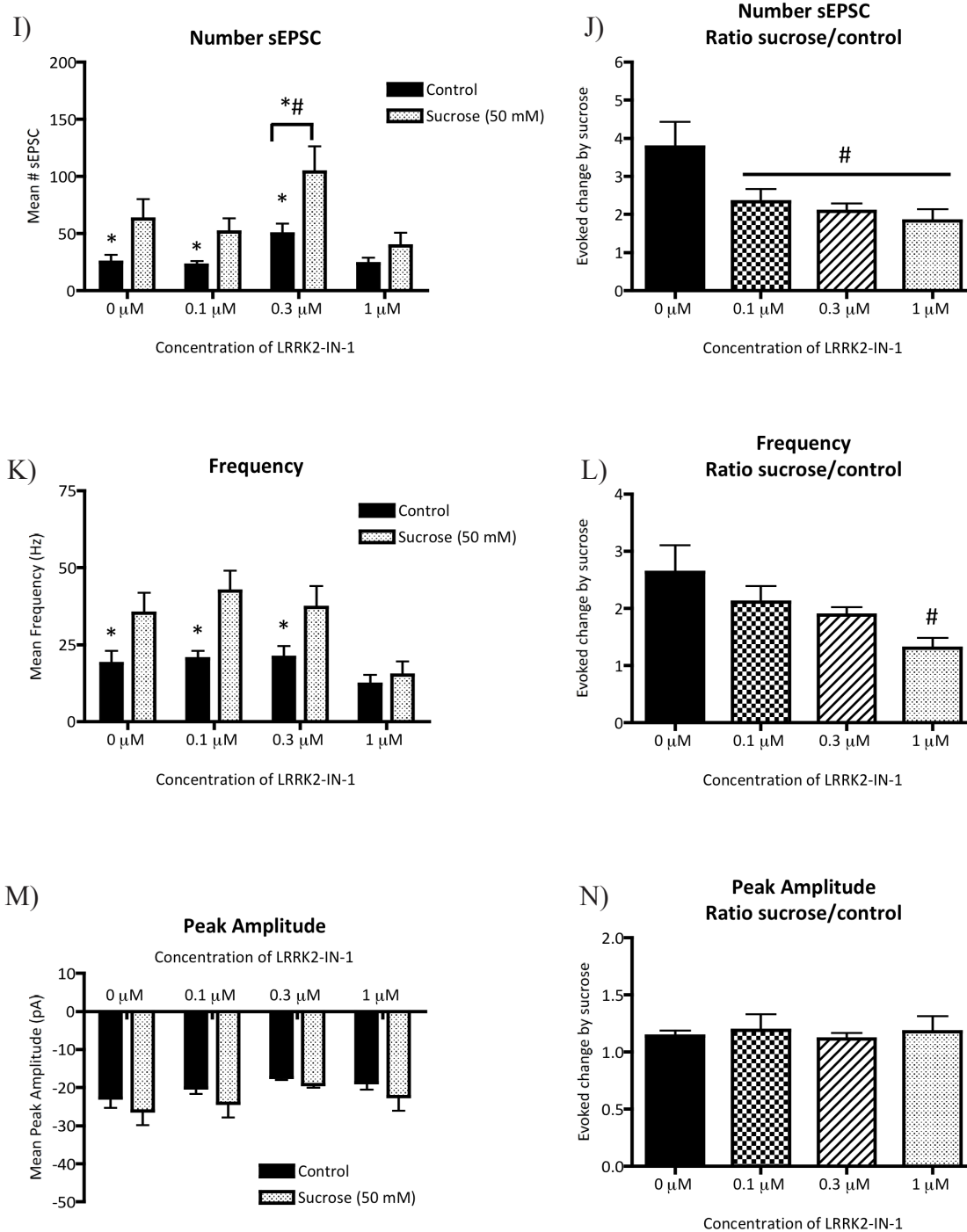
Figure 19. LRRK2 kinase domain is essential to endocytosis of synaptic vesicles. Single recordings were performed in pre-treated (1h) WT hippocampal cells that receive inputs from the neuronal network in presence of LRRK2-IN-1 (0.2% DMSO) at 0 μM, 0.1 μM, 0.3 μM and 1 μM. Representative whole-cell patch clamp recordings from WT hippocampal cells (LRRK2-IN-1, 0 μM) in control **(A)** and in sucrose (50 mM) **(B)**. Representative whole-cell patch clamp recordings from WT hippocampal cells (LRRK2-IN-1, 0.1 μM) in control **(C)** and in sucrose (50 mM) **(D)**. Representative whole-cell patch clamp recordings from WT hippocampal cells (LRRK2-IN-1, 0.3 μM) in control **(E)** and in sucrose (50 mM) **(F)**. Representative whole-cell patch clamp recordings from WT hippocampal cells (LRRK2-IN-1, 1 μM) in control **(G)** and in sucrose (50 mM) **(H)**. **(I)** Sucrose (50 mM) significantly increased the number of sEPSC in WT hippocampal cells treated with LRRK2-IN-1 at 0 μM (n=15; control, 24.80 ± 6.35; sucrose, 62.73 ± 17.21), 0.1 μM (n=10; control, 22.50 ± 3.11; sucrose, 51.60 ± 11.48), 0.3 μM (n=10; control, 49.60 ± 9.00; sucrose, 104.10 ± 22.00), but not at 1 μM (n=10; control, 23.60 ± 4.93; sucrose, 39.2 ± 11.42). **(J)** Sucrose (50 mM) induces a fold change on number of sEPSC in WT hippocampal cells (n=15) no treated with LRRK2-IN-1 of 3.77 ± 0.66. The fold change on number of sEPSC in cells treated with LRRK2-IN-1 at 0.1 μM (n=10; 2.34 ± 0.32), 0.3 μM (n=10; 2.08 ± 0.20) and 1 μM (n=10; 1.84 ± 0.30) are significantly decreased. **(K)** In WT hippocampal cells treated with LRRK2-

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IN-1 at 0 µM (n=15; control, 18.98 ± 4.05; sucrose, 35.37 ± 6.43), 0.1 µM (n=10; control, 20.54 ± 2.48; sucrose, 42.47 ± 6.53) and 0.3 µM (n=10; control, 20.90 ± 3.65; sucrose, 37.24 ± 6.74) sucrose significantly increased the frequency (Hz) but not at 1 µM (n=10; control, 12.19 ± 3.02; sucrose, 15.21 ± 4.31). **(L)** The effect of sucrose (50 mM) in presence of LRRK2-IN-1 at 1 µM (n=10; 1.31 ± 0.17) is significant decreased but in LRRK2-IN-1 at 0 µM (n=15; 2.63 ± 0.47), at 0.1 µM (n=15; 2.11 ± 0.27) and 0.3 µM (n=10; 1.89 ± 0.13) the effect remains. **(M)** Sucrose (50 mM) and LRRK2-IN-1 at different concentrations did not affect the peak amplitude (0 µM: n=15; control, -22.69 ± 2.76; sucrose, -26.18 ± 3.68); (0.1 µM: n=10; control, -20.10 ± 1.61; sucrose, -24.10 ± 3.78); (0.3 µM: n=10; control, -17.27 ± 0.77; sucrose, -19.16 ± 0.99); (1 µM: n=10; control, -18.66 ± 1.93; sucrose, -22.27 ± 3.90) and **(N)** the fold change does not show differences in presence of LRRK2-IN-1 at 0 µM; (n=15; 1.14 ± 0.05), 0.1 µM (n=10; 1.19 ± 0.14), 0.3 µM (n=10; 1.12 ± 0.05) and 1 µM (n=10; 1.18 ± 0.13). Data are expressed as mean ± SEM. *p<0.05 versus WT untreated, Student's t test, *#p<0.05 versus WT/KO untreated, Student's t test, #p<0.05 versus WT untreated, One-way ANOVA followed by Dunnett's Multiple Comparison Test, Two-way ANOVA for dose-dependent in frequency

From the data shown in figure 19 (A, B and I) it is clear that the number of sEPSC is increased after perfusion with sucrose in presence LRRK2-IN-1 at 0 µM (n=15; control, 24.80 ± 6.35; sucrose, 62.73 ± 17.21), LRRK2-IN-1 at 0.1 µM (n=10; control, 22.50 ± 3.11; sucrose, 51.60 ± 11.48) and LRRK2-IN-1 at 0.3 µM (n=10; control, 49.60 ± 9.00; sucrose, 104.10 ± 22.00) but in LRRK2-IN-1 at 1 µM (n=10; control, 23.60 ± 4.93; sucrose, 39.2 ± 11.42) there is no significant increase (fig.19I, *ρ<0.05, two-tailed Student's t test). Also here this effect was quantified and in the absence of LRRK2-IN-1 (0 µM) sucrose can trigger a larger increase (n=15; 3.77 ± 0.66) when compared with cells treated with compound at different concentrations (LRRK2-IN-1 at 0.1 µM (n=10; 2.34 ± 0.32), 0.3 µM (n=10; 2.08 ± 0.20) and 1 µM (n=10; 1.84 ± 0.30) (fig.19J, #ρ<0.05, one-way ANOVA followed by Dunnett's Multiple Comparison Test). In figure 19I, it is possible see that in LRRK2-IN-1 0.3 µM there is a significant difference before and after perfusion with sucrose compared with cells no treated with LRRK2-IN-1. Although there is no straight forward explanation for this observation, these measurements in hippocampal cells were done with 2 days longer in culture which may explain this result. On the other hand, when the number of sEPSCs is expressed as sucrose/control ratio, the effect observed in cells treated with 0.3 µM LRRK2-IN-1 is situated in between 0.1 and 1 µM of the compound (fig. 19J).

Together with number of sEPSC, we evaluated the frequency of these events and cells treated with LRRK2-IN-1 at 0 µM (n=15; control, 18.98 ± 4.05; sucrose, 35.37 ± 6.43), LRRK2-IN-1 at 0.1 µM (n=10; control, 20.54 ± 2.48; sucrose, 42.47 ± 6.53) and LRRK2-IN-1 at 0.3 µM (n=10; control, 20.90 ± 3.65; sucrose, 37.24 ± 6.74) demonstrated an increase in the frequency after perfusion with sucrose. In cells treated with LRRK2-IN-1 at concentration of 1 µM (n=10; control, 12.19 ± 3.02; sucrose, 15.21 ± 4.31), sucrose was unable to promote a significant increase in frequency (fig.19K, *ρ<0.05, two-tailed Student's t test). The analysis of the ratio of frequency at different LRRK2-IN-1 concentrations (0 µM (n=15; 2.63 ± 0.47), at 0.1 µM (n=15; 2.11 ± 0.27), 0.3 µM (n=10; 1.89 ± 0.13 and at 1 µM

(n=10; 1.31 ± 0.17)) (fig.19L, #ρ<0.05, one-way ANOVA followed by Dunnett's Multiple Comparison Test) shows that only in cells treated with 1 µM LRRK2-IN-1 there is a significant different when compared with cells no treated with LRRK2-IN-1. Nevertheless, with two-away ANOVA statistics test is possible to see that there is a trend towards a concentration-dependent effect.

Lastly, we measured the peak amplitude of all events to evaluate if the compound has an effect in the post-synaptic cell. As, we predicted the LRRK2 kinase inhibition with LRRK2-IN-1 (0 µM: n=15; control, -22.69 ± 2.76; sucrose, -26.18 ± 3.68); (0.1 µM: n=10; control, -20.10 ± 1.61; sucrose, -24.10 ± 3.78); (0.3 µM: n=10; control, -17.27 ± 0.77; sucrose, -19.16 ± 0.99); (1 µM: n=10; control, -18.66 ± 1.93; sucrose, -22.27 ± 3.90) did not have an effect in peak amplitude at any concentration in control or in sucrose conditions (fig. 19M). That was quantified by the ratio sucrose/control and it is possible that the variation is about 1 (0 µM; (n=15; 1.14 ± 0.05), 0.1 µM (n=10; 1.19 ± 0.14), 0.3 µM (n=10; 1.12 ± 0.05) and 1 µM (n=10; 1.18 ± 0.13) for all concentrations, which means that there is no change in post-synaptic cells (fig. 19N).

These results suggest that LRRK2 kinase activity is involved in the regulation of endocytosis of synaptic vesicles and subsequent neurotransmission.

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5. Discussion

In light of the presented data, we suggest that LRRK2 kinase activity is important in the regulation of endocytosis of synaptic vesicles.

LRRK2 had been suggested as an important protein in pre-synaptic vesicular trafficking [13, 80] and in neurotransmitter release [70] but the molecular mechanism involved was unclear. The host lab in collaboration with Prof. Bart De Strooper's and Prof. Patrik Verstreken's group found that LRRK2 phosphorylates endophilin A1 at S75 residue, a residue located in the helix1 appendage of the BAR-domain, as described in the hypothesis earlier. This domain is crescent-shaped, binds lipid membranes to remodel their structure and coordinates CCP neck constriction (to mediate CCP fission from the plasma membrane, crucial features of endocytosis. The absence of this phosphorylation (by absence or inhibition of LRRK2) promotes the attachment of endophilin to the membrane, blocking SH3-driven recruitment, of dynamin and synaptojanin (both needed for in uncoating), leading to impairments in endocytosis (Matta et 2012 – manuscript accepted). This lack of phosphorylation together with lack on interactions with AP-2 complex subunits, NSF and Rab5, by absence of LRRK2, may explain the results obtained in figure 17 [13]. The hypertonic sucrose was unable to increase the number of sEPSC and frequency in LRRK2 KO cells while this increase could be observed in WT cells (see ratio figure 17). In fact the differences were only observed in presence of hypertonic sucrose, showing that hypertonic sucrose was a good approach to study endocytosis dependence of synaptic vesicle release [13, 174]. This is the reason why we did not use TTX. TTX is a potent neurotoxin, frequently used to suppress neuronal activity in cell culture by blockade of action potentials. This neurotoxin binds to the voltage-gated Na^+ channels and fast Na^+ channels in presynaptic cell, preventing the uptake of Na^+ and, as consequence, the entrance of Ca^{2+} in nerve terminal. The blockade of action potential propagation impairs all the synaptic vesicle cycle, by decreasing the ratio of recycling SV and the fusion of synaptic vesicles derived from recycling and reserve pool [13].

In control condition, RRP is the major pool involved in the basal activity, conferring the same results in both genotypes being released by hypertonic sucrose stimulation. The small increases observed in control in number of sEPSC and frequency in LRRK2 KO cells may be attributable to an adaptive activity in docking or priming, conferring a higher

probability to contact the membrane and fuse [175, 176] or a higher expression of a homologous LRRK2, LRRK1 leading to compensatory mechanisms.

Despite the small increase (not significant) in frequency in LRRK2 KO neurons (fig. 17C, G) and decrease shown by LRRK2 KO neurons in peak amplitude in both conditions (fig. 17 I,J), these results suggest that LRRK2 acts in presynaptic cell by controlling a pool of SV larger than the RRP [13]. The latter observation is supportive for the hypothesis that LRRK2 acts presynaptic. One possible explanation for the overall decrease in amplitude in LRRK2 KO neurons is that there are less SV available because the recycling machinery is impaired. The small amount of SV is not enough to maintain the same response, decreasing the peak amplitude in both conditions (fig. 17I ; #ρ=0.08 in control and *ρ<0.05 in sucrose) [13].

As LRRK2 has been suggested as a protein involved in presynaptic mechanisms, the previous results can be explained by impairments in exocytosis. In fact, together with proteins involved in endocytosis, LRRK2 has been described to interact with proteins involved in exocytosis like SNAP-25, syntaxin 1, proteins of SNARE complex [13, 78]. Given these evidences, we asked whether interference with dynamin1 function could mimic the effect observed in LRRK2 KO neurons. Dynasore is often used to study the endocytosis by blocking the GTPase activity of dynamin. Cells treated with vehicle (DMSO, 0.2%) (fig. 18) show the same result presented in figure 17. This shows that DMSO, a lipophilic solvent, at 0.2%, does not have an effect in the number of neurotransmitter release, frequency or peak amplitude in control or sucrose. However, during the experiments, the percentage of patched cells was 25% lower which might be explained by lipophilicity of DMSO that disturbs the cell membrane, and consequently the stability of gigaseal. In cells treated with dynasore, this compound did not alter the peak amplitude but increased the number of sEPSC in WT and LRRK2 KO cells in control and sucrose such as frequency in WT in both conditions (fig 18 C, D, G, H, I, K). Despite that, in presence of dynasore, sucrose was unable to induce the same effect showed by WT cells treated with vehicle (fig. 18K, L). If we consider, that the block of dynamin, prevents the recovery of synaptic vesicle membrane after transmitter release, this results in an accumulation of SV membrane in cell membrane, increasing the capacitance [$C_{in}=C_m(4\pi a^2)$] [36] of the cell. In other words, the cell size and nerve terminal increase, and possibly more vesicular Ca^{2+} channels are in cell membrane, facilitate the formation of new Ca^{2+} channels

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[36] C_m = specific capacitance per unit are of all biological membranes, has the same value, approximately 1 μF cm^{-2} of membrane. C_{in}= total input capacitance of a spherical cell. a= area (cm^2)

clusters. This induces a flow of current to the cell to produce the same change in membrane potential [161, 172, 173]. These vesicular Ca^{2+} channels [177, 178] allow the entrance of Ca^{2+} increasing the SV synthesis and increasing spontaneous release even in the absence of nerve stimulation. Other possibility, that might contribute to dynasore effects on transmitter release, is calcium-independent mechanism, but without conclusive results. The theory is based in that the dynamin and its blockade alter the many protein–protein interactions and it has an impact in exocytosis. In high-frequency, the interaction of syndapins, which is important in actin cytoskeletal alterations, and in the synaptic vesicle cycle, with dynamin I [179] can be altered by dynasore, since dynasore interferes with the stability of actin [180], altering the neurotransmitter release. These observations indicate that the time of exposition to dynasore did probably not allow a good evaluation of dynamin1 inhibition. In a next experiment, increase in time of exposition, around 15 min or 30 min, should avoid the observed increase in number of sEPSC in both genotypes and in frequency of WT hippocampal cells. In terms of concentration, we think that this was well chosen, since that at this concentration, dynasore blocks 80% of dynamin and LRRK2 does not block the endocytosis completely [172].

Regarding the effects of dynasore in endocytosis, these observations suggest that the impairment caused by absence of LRRK2 is mimicked by blockade of dynamin with dynasore at 40 µM, which indicates that the impairment caused by absence of LRRK2 is at the endocytosis level. This idea is supported by role of endophilin (endophilin cycle). This protein has been suggested to play a role in two main roles in endocytosis: formation of pit (membrane invagination) and recruitment of adaptors proteins like AP-2, dynamin, amphiphysin, and AP180 to promote the membrane curvature in the 1st step of endocytosis (described above); and in recruitment of dynamin (5th step), amphiphysin, auxilin SNX9 and synaptojanin, to facilitate pinching off (scission) and the uncoating of CCV. Blocking the dynamin with dynasore, would arrest the cycle in the 5th step (dynamin is the main protein to execute this step, which is important for construction of the vesicle neck) impairing the endocytosis in U-shaped pit and in the stage where the neck needs to be "broken", impairing the endocytosis in O-shaped-pit [181]. This causes that CCVs not to be formed completely or remains attached to the cell, leading to less synaptic vesicles available to release, which leads a less number of sEPSC detected and a decrease in the frequency (increasing the time to complete the synaptic vesicle cycle). Although in LRRK2 KO hippocampal cells the effect in frequency is not exacerbating by dynasore exposure, it can be attributable to the fact there is impairment in recycling machinery [161].

Finally, we also tested in rodent brain if the phosphorylation described in hypothesis is dependent of LRRK2 kinase activity. Inhibition of LRRK2 using LRRK2-IN-1 [142] results in pre-synaptic but not post-synaptic defects, similar to those found in LRRK2 KO cells with and without dynasore. The changes in peak amplitude are not significant but it is possible to observe that since LRRK2-IN-1 at 0.1 μM, there is a decrease in evoked change by sucrose in number of sEPSC when compared with cells not treated. But in terms of frequency, the analysis with two-away ANOVA statistics test shows that this inhibition by LRRK2-IN-1 displays a dose dependent effect. Thus, and in our understanding, inhibition of LRRK2 kinase activity results in defects on sEPSC frequency due to synaptic vesicle recycling defects. This effect is likely due to reduced phosphorylation of the LRRK2 substrate Endophilin A. In fact, in this host lab, we found that the treatment with LRRK2-IN-1 at 1 μm switches the sub cellular localization of endophilin from to cytosol to cell membrane by reducing the phosphorylation of endophilin A as was hypothesized. This evidence supports the data shown in this work and that LRRK2 acts at endocytosis of synaptic vesicles (unpublished data). Nevertheless, it remains to be clarified whether more proteins phosphorylate endophilin A and if these proteins are inhibited by LRRK2-IN-1. Although LRRK2-IN-1 is claimed to be selective for LRRK2 [142] our inhibition experiment needs to be done in LRRK2 KO neurons as a negative control to LRRK2-IN-1. Furthermore, further studies with a selective GTPase inhibitor for LRRK2 would be useful to elucidate if endophilin phosphorylation is only kinase dependent since the two enzymatic activities of LRRK2 regulate its own activity and impairments in each enzymatic activity causes variations in all LRRK2 activity [13, 78, 118].

In fact, using of GTPase inhibitor may also reveal a good therapeutic target since mutations in Roc domain also cause impairments in diverse pathways. In neurotransmission, the knock in of R1441C mutation in LRRK2, shows defects in DAergic neurotransmission in brain slices from *substantia nigra* [70].

More recently a study has been described that LRRK2 interacts with tau protein [131]. As Tau protein, a risk factor for PD as well, is involvement in neurite outgrowth, this interaction suggests that LRRK2 affects the spines formation, via Tau interaction, and together, the two proteins may have a role in synaptic transmission [131]. In fact, in *Drosophila melanogaster*, LRRK2 has been described as an important protein for synaptic morphogenesis and neurotransmission, via interaction with microtubules where tau has an important in maintenance of spines and neurites, transport of synaptic vesicles and maintenance of neurotransmission. Despite that, the regulation of these two synaptic

phenomena involves different mechanisms and pathways. It is also possible that LRRK2 may have an effect in post-synaptic cell since it has been described an interaction with 4E-BP at the post-synapse, but only in studies with LRRK2 mutants because the studies through WT vs LRRK2 KO comparison, like this presented work is not possible to see any observable phenotype [126, 130].

The fact that the main mutations that have been shown to segregate with PD are located in the enzymatic domains of LRRK2 (G2019S (kinase domain) and R1441C/H/G (Roc domain)), suggest an altered enzymatic activity drives LRRK2-dependent pathology. Given the correlation described in this study and manuscript accepted from Matta et al., 2012, these results will stimulate further studies mainly in determination whether perturbed endocytosis may contribute to PD.

6. Conclusions

The results presented in this work reveal that use of sucrose was a good strategy to study the role of LRRK2 in endocytosis. In presence of this sugar at 50 mM was possible observe that LRRK2 KO impairs the neurotransmission, by disrupt the endocytosis. Taking in account, the papers of Macia et al., 2006 [160], Douhitt et al., 2011 [161] and Chung et al., 2010 [173] that describe the use of dynasore as a good approach to study endocytosis, the time of exposition (5 min) was not the most correct in this study. Further experiments need to be done with more time of treatment (30 min) and that possible, would decrease the misleading in total values.

Regarding the experiment with LRRK2-IN-1 it is possible to conclude that only at 1 μM (treatment for 1h), LRRK2 kinase activity is full inhibited and that kinase activity is essential to endocytosis. At this concentration there was a significant decrease in number of sEPSC and frequency, mimicking the effect of LRRK2 absence.

Towards this work, the major conclusion is that the LRRK2 protein, via the kinase activity, plays an essential role in neurotransmission, at the level of endocytosis of synaptic vesicles. These findings need to be complemented with further studies in cultured primary neurons (hippocampal and striatal neurons) and animal models to advance our understanding in the physiological and pathophysiological role of LRRK2. The whole-cell patch clamp is a good technique to study the exocytosis and endocytosis but this approach need to be complement with techniques such as fluorescence imaging (with FM1-43 dye), optical imaging of vesicle dynamics (overexpression of synaptopHluorin or vGLUT1-pHluorin) electron microscopy. In fact, using autaptic cultures, cells that are pre- and post-synaptic cells itself, allow, via whole-cell patch clamp a better control in evoked release, in frequency of stimulation, evaluate the kinetics of the different pools of vesicles and a large understanding on role of LRRK2 at endocytosis of synaptic vesicles. Disease-associated mutations or LRRK2 KO alter enzymatic activity *in vitro* but also induce phenotypes in cultured neurons in a kinase-dependent and occasionally a GTPase dependent manner. It will be important to clarify whether kinase inhibition represents a feasible strategy for attenuating LRRK2-dependent dopaminergic neurodegeneration (by impairments in neurotransmission-endocytosis), or it is necessary include GTPase inhibitors as strategy to PD.

In fact, a complete understanding of LRRK2 function and the pathogenic mechanisms of familial mutations will offer a number of opportunities for the identification of novel

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molecular targets which might prove useful for attenuating LRRK2-dependent neurodegeneration in PD.

7. References

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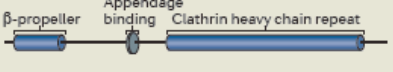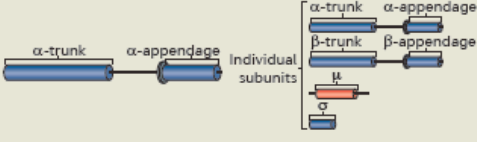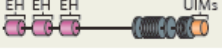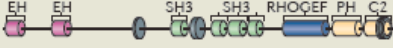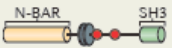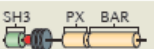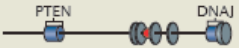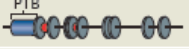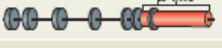
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8. Attachments

Protein	Human genes	Function	Domain architecture*
Core components			
Clathrin	CLTA‡, CLTB, CLTC	Self-polymerizing protein composed of three heavy and three light chains that form the clathrin triskelion, which can polymerize into flat lattices or cages	
FCHO	FCHO1, FCHO2‡	F-BAR domain-containing proteins that nucleate clathrin-coated pits and generate the initial membrane curvature	
AP2	AP2A1‡, AP2A2, AP2B1, AP2M1, AP2S1	A heterotetrameric adaptor complex (α-, β2, μ2 and σ2 subunits) that links membrane cargo to clathrin and accessory proteins	
EPS15–EPS15R	EPS15‡, EPS15R	AP2 clustering and scaffolding proteins	
Intersectin	ITSN1‡, ITSN2	Scafolding protein linking various components of the clathrin machinery	
AP180, CALM§	SNAP91‡, PICALM	ANTH domain-containing PtdIns(4,5)P$_2$-binding protein that binds AP2 and clathrin and is thought to regulate vesicle size	
Epsin	EPN1‡, EPN2	ENTH domain-containing membrane-bending protein that is a cargo-specific adaptor for monoubiquitylated receptors	
Amphiphysin	AMPH1‡, BIN1	N-BAR domain-containing protein that bends the membrane and recruits dynamin to clathrin-coated pits	
SNX9	SNX9	BAR domain-containing protein that binds AP2 and dynamin	
Dynamin	DNM1‡, DNM2, DNM3	Self-polymerizing mechanoenzyme that triggers vesicle scission upon GTP hydrolysis	
Auxilin, GAK‖	DNAJC6‡, GAK	J domain-containing protein that recruits HSC70 to clathrin cages for uncoating	
HSC70	HSPA8	ATPase triggering uncoating of clathrin cages	
Cargo-specific adaptors			
ARH	LDLRAP1	Recruits LDLR to AP2	
DAB2	DAB2	Recruits megalin and LDLR to AP2	
Stonin	STON1, STON2‡	Recruits synaptotagmin to AP2	
HRB	AGFG1	Recruits the SNARE protein VAMP7 to AP2	
NECAP	NECAP1‡, NECAP2	Potential cargo-specific adaptor	
Numb	NUMB	Recruits Notch to AP2	
β-arrestin	ARRB1, ARRB2‡	Recruits GPCRs to AP2 and clathrin	

Protein	Human genes	Function	Domain architecture*
Inositol 5-phosphatases			
Synaptojanin	SYNJ1‡, SYNJ2	Lipid phosphatase recruited to clathrin-coated pits by AP2 and endophilin	SAC1 5′-phosphatase
SHIP2	INPP1L	Lipid phosphatase recruited to clathrin-coated pits by intersectins	SH2 5′-phosphatase SAM
OCRL	OCRL1	Lipid phosphatase recruited to mature clathrin-coated pits by AP2 and clathrin	PH 5′-phosphatase RHOGAP ASH
Kinases			
AAK1	AAK1	Phosphorylates the μ-chain of AP2	Kinase
CVAK104	SCYL2	Phosphorylates the β2 subunit of AP2	Kinase
DYRK1A	DYRK1A	Phosphorylates several proteins involved in clathrin-mediated endocytosis	Kinase
Actin nucleation at clathrin-coated vesicles			
HIP1–HIP1R	HIP1‡, HIP1R	ANTH domain-containing proteins that bind clathrin, actin, AP2 (HIP1 only) and cortactin (HIR1R only)	ANTH
Cortactin	CTTN	Recruits actin polymerization machinery to budding clathrin-coated vesicle through dynamin and HIP1R	SH3
Other proteins potentially involved in clathrin-mediated endocytosis			
Endophilin	SH3GL1‡, SH3GL2, SH3GL3	N-BAR domain-containing protein that bends the membrane and recruits dynamin and synaptojanin	N-BAR SH3
SGIP1	SGIP1	Membrane-tubulating protein containing a μ-homology domain	?¶ μ-like
TTP	SH3BP4	Controls TfR recruitment to clathrin-coated pits	SH3 SH3

Table: Summary of all proteins that have a role in CME according to function: core components, cargo-specific adaptors, inositol-5-phosphatases, kinases, actin nucleation at clathrin-coated vesicles and other proteins with different functions or not clear function in CME. AAK1, AP2‑associated kinase 1; AGFG1, ARFGAP with FG repeats 1; ANTH, AP180 amino-terminal homology domain; AP2, adaptor protein 2; ARH, autosomal recessive hypercholesterolaemia; ASH, ASPM1–SPD2–hydin; CALM, clathrin assembly lymphoid myeloid leukaemia; CLT, clathrin light chain; CVAK104, coated vesicle associated kinase of 104 kDa; DAB2, Disabled homologue 2; DYRK1A, dual-specificity Tyr phosphorylation-regulated kinase; EH, EPS15 homology; ENTH, epsin N-terminal homology domain; EPS15, EGFR pathway substrate 15; EPS15R, EPS15‑related; FCHO, FCH domain only; GAK, cyclin G-associated kinase; GAP, GTPase-activating protein; GED, GTPase effector domain; GEF, guanine nucleotide exchange factor; GPCR, G protein-coupled receptor; HIP1, huntingtin-interacting protein 1; HIP1R, HIP1‑related; HSC70, heat shock cognate 70; LDLR, low-density lipoprotein receptor; NECAP, adaptin ear-binding coat-associated protein; OCRL, oculocerebrorenal syndrome of Lowe; PH, pleckstrin homology; PICALM, phosphatidylinositol-binding CALM; PRD, Pro-rich domain; PTB, phosphotyrosine binding; PTEN, phosphatase and tensin homologue; PX, phox homology; SAC1, suppressor of actin; SAM, sterile α-motif; SGIP1, SH3‑containing GRB2-like 3‑interacting protein 1; SH, SRC homology; SHIP2, SH2 domain-containing inositol phosphatase 2; SNAP91, synaptosomal-associated protein 91 kDa homologue; SNX9, sorting nexin 9; TfR, transferrin receptor; TTP, TfR trafficking protein; UIMs, ubiquitin-interacting motifs; VAMP7, vesicle-associated membrane protein 7. *Clathrin-binding motifs are denoted by a red dot. ‡Denotes the proteins for which the structure is depicted in the domain architecture column. §AP180 is the brain-specific protein, CALM is the ubiquitous one. ‖Auxilin is the brain-specific protein, GAK is the ubiquitous one. Lipid binding module of undefined character. Table removed from McMahon and Boucrot -(2011) [154]

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