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Unraveling the role of LRRK2 at the postsynaptic site in health and Parkinson's disease

Coordinator: Prof. Ildikò Szabò

Supervisor: Prof. Elisa Greggio

Ph.D. student : Giulia Tombesi

"Like the entomologist in search of colorful butterflies,
my attention has chased in the gardens of the grey matter cells
with delicate and elegant shapes, the mysterious butterflies of the
soul, whose beating of wings may one day
reveal to us the secrets of the mind."

Santiago Ramon y Cajal

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Abbreviations

4E-BP 4E binding protein

ABPs Actin-binding proteins

Acb Accumbes nucleus

AD Alzheimer disease

ADF Actin-depolymerizing factor

AMPA α-amino-3-hydroxy-5methyl-4-isoxazolepropionic acid

ANK Ankyrin

AP-3 Adaptor protein complex 3

ARM Armadillo

ATP Adenosine triphosphate

AVs Autophagic vesicles

BDNF Brain Derive Neurotrophic Factor

BG Basal ganglia

CaM kinase Calmodulin dependent protein kinase

CASK Calcium/calmodulin-dependent serine protein kinase

CD Crohn's disease

CK1α Casein kinase 1

CN Caudate nucleus

CNS Central nervous system

COR C-terminal of Roc

DA Dopaminergic

DAG Diacylglycerol

DANs Dopaminergic neurons

DAPK1 Death-associated kinase 1

DAT Dopamine transporter

DBL Dementia with Lewy bodies

DBS Deep brain stimulation

DOPAC 3,4-dihydroxyphenylacetic acid

DOPET 3,4-dihydroxyphenylethanol

DRD1 Dopamine receptor subtype 1

DRD2 Dopamine receptor subtype 2

ER Endoplasmic reticulum

F-actin Filamentous actin

GDI GDP dissociation inhibitor

GDP Guanosine diphosphate

GEF Guanine exchange factor

GKAP Guanylate kinase-associated protein

GPe Globus pallidus external

GPi Globus pallidus internal

GRIP Glutamate-receptor-interacting protein

GWAS Genome-wide association studies

HD Huntington disease

HLA Human leukocyte antigen

IFN-γ Interferon

IL Interleukin

IP3 Inositol trisphosphate

IP3R Inositol trisphosphate receptors

JNK c-Jun N-terminal kinase

KIN Kinase

KO Knockout

L-DOPA Levodopa

LBs Lewy bodies

LMPs Lysosomal membrane proteins

LNs Lewy neurites

LRR Leucine-rich repeat

Lrrk1 Mouse leucine-rich repeat kinase 1

Lrrk2 Mouse leucine-rich repeat kinase 2

LRRK2 Human leucine-rich repeat kinase 2

LTD Long term depression

LTP Long term potentiation

MAGUK Membrane-associated guanylate kinases

MAO Monoamine oxidase

MAPK Mitogen-activated protein kinase

MAPKKK Mitogen-activated protein kinase kinase kinases

MAPs Microtubule-associated proteins

MASL1 Malignant fibrous histiocytoma amplified sequences with leucine-rich tandem repeats 1

mGluR Metabotropic glutamate receptor

MNTB Medial nucleus of the trapezoid body

MPTP 1-methyl-4phenyl1,2,3,6-tetrahydropyridine

MSNs Medium spiny neurons

MTs Microtubules

NFAT Nuclear factor of activated T cells

NGF Nerve Grow Factor

NMDA N-methyl-D-aspartate

NMDAR N-methyl-D-aspartate receptor

NSF N-ethylmaleimide sensitive factor

NT-3 Neurotrophin-3

NT-4 Neurotrophin-4

NTRs Neurotransmitter receptors

NTs Neurotransmitters

PAK p21-activated kinase

PAK6 p21-activated kinase 6

PD Parkinson's disease

PI3K Phosphatidylinositol 3-kinase

PKA Protein kinase A

PKC Protein kinase C

PNS Peripheral nervous system

PP1 Protein phosphatase 1

PP2A Protein phosphatase 2

proBDNF Pro brain-derived neurotrophin factor

PSD Postsynaptic density

PTMs Post-translational modifications

Put Putamen

RhoA Ras homolog gene family member A

ROC Ras of complex

ROS Oxygen radical species

RP Reserve pool

RRP Readily releasable pool

RyR Ryanodine receptors

SER Smooth endoplasmic reticulum

SNARE Soluble NSF Attachment Protein Receptor

SNpc Substantia nigra pars compacta

SNpr Substantia nigra pars reticulata

SPAR Spine-associated RapGAP

STN Subthalamic nucleus

SVs Synaptic vesicles

synj1 Synaptojanin1

TGN Trans-Golgi network

TH Tyrosine hydroxylase

TNF-α Tumor necrosis factor

Trk Tropomyosin-related kinase

TrKB Tropomyosin receptor kinase B

VAMP2 Vesicle associated membrane protein 2

VGCC Voltage-gated calcium channels

VPS35 Vacuolar protein sorting 3

Wnt Wingless signaling pathway

WT Wildtype

α-Syn Alpha-synuclein

Abstract

Synapses constitute the fundamental elements of the nervous system: they form an extremely dense and complicated map of connections that make neuronal cells capable of exchanging an impressive amount of information. Synapses and their highly specialized structures named *dendritic spines*, are very plastic elements that continually reshape in response to environmental cues, a crucial process that occurs throughout the entire lifespan. Thus, neuronal communication requires functional and plastic neuronal circuits and any impairment in this system results in information loss. A growing body of evidence indicates that synaptic vulnerability is a common feature in multiple neurodegenerative disorders, including in Parkinson's disease (PD). PD is the second most common neurodegenerative disorder affecting over 230.000 Italians and more than 1 million people in Europe and, although its etiology is still uncertain, in approximately 10% of patients it manifests as a monogenic disease. One of the main hallmarks of PD is the degeneration of dopaminergic neurons (DANs) in the *substantia nigra pars compacta* (SNpc) projecting to the striatum. However, the loss of striatal dopaminergic (DA) fibers precedes the degeneration of DANs cell bodies in SNpc (axonal dying back hypothesis) and synaptic failure may be an early igniter of axonal degeneration.

Among all genetic contributors of PD, mutations in Leucine-rich repeat kinase 2 (LRRK2), represent the most common cause of autosomal dominant late onset familial PD and variations around LRRK2 locus increase lifetime risk for PD. LRRK2-PD is almost indistinguishable from sporadic PD in terms of clinical and pathological phenotype, suggesting that understanding LRRK2 pathobiology will inform on general mechanisms of PD. Pathogenic mutations are clustered into the enzymatic core of LRRK2 protein, composed by a Roc/GTPase and kinase domains, bridged by a COR scaffold. The most frequent mutation (G2019S) located in the kinase domain results in a protein with a gain of kinase activity, associated with increased cellular toxicity.

To date, the current understanding of the role covered by LRRK2 in the physiology of the neuron is still limited. LRRK2 has been linked to membrane remodeling and trafficking events, all key processes at the synaptic compartments. Consistently, several laboratories including our group provided evidence that LRRK2 regulates synaptic vesicle cycling through interaction and phosphorylation of a panel of synaptic

proteins. In contrast, the involvement of LRRK2 at the postsynaptic element has been only marginally explored. Of note, LRRK2 is highly expressed in MSNs of the dorsal striatum, rich in dendritic spines and representing the postsynaptic element of the nigrostriatal pathway. Dendritic spines are the sites in which excitatory synaptic contacts occur and their architecture is shaped by the actin cytoskeleton, whose remodeling is at the base of synaptic plasticity. Interestingly, in the past few years our group as well as other laboratories provided exciting evidence that LRRK2 is physically and functionally associated with cytoskeletal-related components.

In this scenario, this PhD project aimed to investigate the physiological role of LRRK2 in orchestrating dendritic spines structural plasticity and the pathological implications that increased kinase activity associated with the common G2019S mutation may have in this compartment.

Specifically, we confirmed that LRRK2 is highly expressed in the *striatum*, whose predominant neuronal type is represented by medium spiny neurons (MSNs). Moreover, by performing affinity purification coupled with mass spectrometry analysis, we found that LRRK2 mainly interacts with actin- and myosin- related proteins. Based on these findings and on previous literature suggesting a role for LRRK2 at the postsynapse, we hypothesized that LRRK2 may orchestrate postsynaptic dynamics by regulating the actin cytoskeleton. To this end, we analyzed striatal synapses carrying out a longitudinal study on developing, mature and aged brains from Lrrk2 wildtype (WT) and knockout (KO) mice. We obtained compelling evidence that LRRK2 influences striatal synaptic contacts number and dendritic spines maturation during the first post-natal stages. We then went a step further and investigated the involvement of LRRK2 in development of new spines, exploiting brain derived neurotrophic factor (BDNF) as a model to study this process *in vitro,* given its well-established role in dendritic spines plasticity and long term potentiation. After observing that LRRK2 promotes dendritic spines formation upon BDNF treatment, we investigated how its interactome is reshaped in response to stimulation. Affinity purification coupled with mass spectrometry in *SH-SY5Y* cell lines stably expressing GPF-LRRK2 uncovered that BDNF increases the interaction of LRRK2 with proteins involved in cytoskeletal dynamics and remodeling. We further pursued the functional validation of LRRK2 interaction with drebrin, an actin binding protein highly enriched

in dendritic spines. In parallel, we observed that LRRK2 phosphorylation at Ser935 is increased upon BDNF treatment, which also stimulated its relocalization with the BDNF receptor TrKB within punctate structures. Moreover, in LRRK2 KO *SH-SY5Y* cells the receptor accumulates in vesicular-like compartments. These lines of evidence suggest that LRRK2 responds to BDNF stimulation to regulate actin-cytoskeleton dynamics and TrKB trafficking.

In the second part of this PhD project we evaluated whether the common LRRK2 pathological mutation G2019S affects the structural plasticity of the postsynaptic compartment. We observed an increased number of branched spines in the striata of 18 month-old Lrrk2 G2019S mice (knockin and BAC overexpressors), while the number of synapses is reduced. These results further corroborate the hypothesis that LRRK2 activity is relevant at the postsynaptic compartment, impacting on dendritic spines dynamics.

Taken together, our data reveal that LRRK2 is an important regulator of dendritic spines structural plasticity. We propose that LRRK2 acts as a hub for actin cytoskeleton remodeling *via* interaction with actin-related proteins and receptor trafficking modulation, in agreement with its established role at the interface between vesicular and cytoskeletal pathways. Unraveling how LRRK2 functions at the synaptic compartment could be very helpful in light of the design of effective therapeutic and preventive PD strategies, as they allow to focus on dysfunctional mechanisms that are prodromal to the irreversible neuronal loss.

Chapter 1

Introduction

The Synapse

1. The Synapse

The human brain has an extraordinary but largely unresolved complexity, which makes it one of the most mysterious structures in the universe. The nervous system works to ensure that the diverse body functions are fully operational and coordinated, as well as to execute higher associative functions such as cognition and memory, behavior and personality. All information are processed and stored inside the neuron, an electrically excitable cell representing the key element of the nervous system. It is estimated that the human brain hosts ~ 83 x 10^9 neurons (Herculano-Houzel, 2009), which form an extremely dense and complicated map of connections, the synapses. Synapses represent the principal structure involved in the transfer of messages from one neuron to another. Synapses are very plastic elements that continually reshape through a process named synaptic plasticity. This term refers to any change in the efficacy of synaptic transmission at preexisting synapses in response to environmental cues. These modifications, that could be of different nature (morphological, biochemical molecular and genetic), impact on synapses number, size and strength. Synaptic plasticity is crucial during the development and maturation of the organism, but also in the process of learning and memory and in the recovery after brain damage.

Considering these two important aspects, synapses are essential for the proper functioning of brain circuits. Their damage and/or loss can harm the correct information transfer and the capacity of the nervous system to modify its own organization, leading to pathology.

1.1. Synaptic physiology

In 1890 Santiago Ramón y Cajal, exploiting an improved Golgi impregnation method to visualize neuronal cells, provided for the first time clear proofs that the nervous elements are in contiguity and not in continuity. From this observation it arose the "*Neuron Doctrine*", according to which the nerve structures are constituted by independent cells communicating with each other *via* "*intracellular articulations*", that we now know as synapses (Fig.1) (Sotelo, 2020).

The word "*synapse*" was coined by the neurophysiologist Charles Sherrington in 1897 from the Greek term synaptein, composed of syn-, meaning "together", and haptein, meaning "to clasp". This word implies the physical discontinuity of the two

synaptic elements and encompasses the idea, sustained by Sherrington, that the nervous impulse has to change its nature as it passes from one cell to other. Thus, the beauty of this term lies in the fact that it combines both the anatomical and physiological concepts of the neuron theory (Todman, 2009).

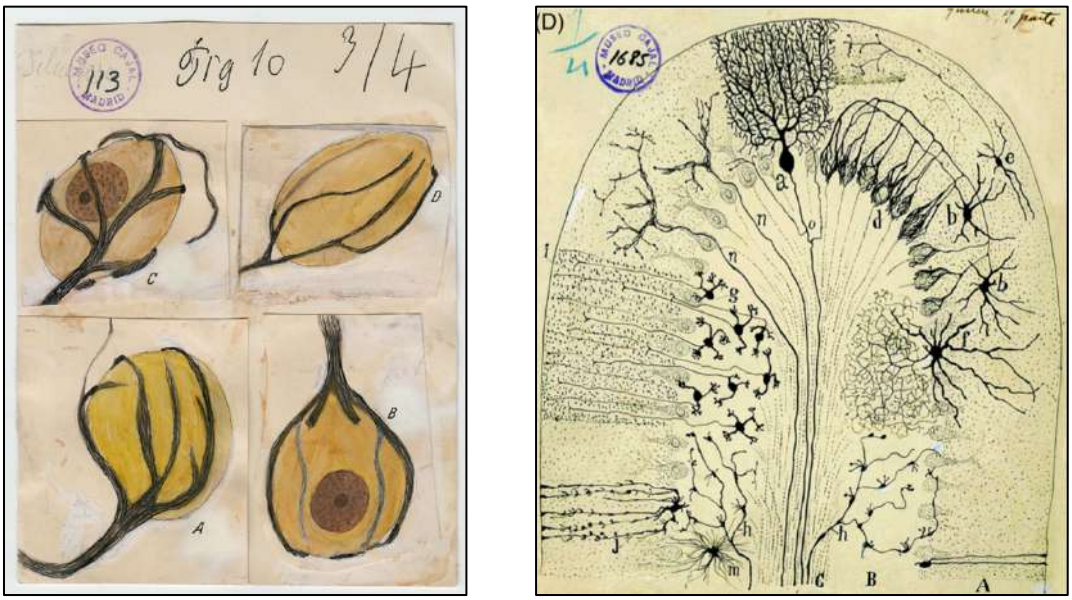


Fig.1: Cajal drawing. (A) *Calyces of Held. They constitute the largest type of synapse in the mammalian brain, with important role in the auditory system. This synapse type consist in a giant axon terminal with the cell body in the cochlear nucleus contacting the principal neurons in the medial nucleus of the trapezoid body (MNTB). In the drawing the giant axons appear as black lines enfolded around MNTB principal neurons (yellow cells), resembling the calyces of flowers that cover the base of petals. This synapse structure was exploited by Ramón y Cajal to sustain his Neuron Doctrine of separate neurons.* ***(B)*** *Schematic Cerebellar Cortical Circuit. Adapted from (Sotelo, 2020).*

The nature of synaptic communication has been a subject of debate for many years, known as "*The War of the Soup*". What is clear now is that the functional interaction among nerve cells can take place through electrical or chemical synaptic transmission (Pereda, 2014). While at the electrical synapses the information pass from one cell to another directly across the cytoplasm via clusters of intercellular channels called gap junctions or connexons, chemical synapses utilize neurotransmitters (NTs) to propagate and elaborate signals between neighbor cells. The two types of synapses coexist in most organisms and brain region but chemical synapses represent the most prominent type.

Chemical synapses, hereafter referred as synapses, consist of a *presynaptic element* (for example an axon terminal) separated from a *postsynaptic element* (for

example a dendritic spine) by a tiny space named *synaptic cleft*. Together, these three elements constitute a synaptic complex, the *non-reducible basic unit* of a chemical synapse (Hammond & Esclapez, 2015): the electrical signal in the form of depolarizing currents reaches the presynaptic area and is turned into a chemical message in form of discrete packets of NTs termed "*quanta*" released in the synaptic cleft; the binding of NTs to specific receptors localized on the postsynaptic membrane reconverts the chemical message into an electric signal whose properties depend on the nature and number of postsynaptic receptors.

A synaptic connection can be established between two neurons or between a neuron and a non-neuronal cell. In the inter-neuronal synapses the *presynaptic element* is seldom represented by a dendrite (dendro-dendritic synapse) or soma (soma-somatic or soma-dendritic synapses), instead it is more frequently formed by the axon, either as terminal axonal branches (*terminal boutons*) or as swelling along the axon (*boutons en passant*). Axonal terminals originate different types of synapses (Fig.2) making connections with a *postsynaptic element* of various nature: dendrite (axo-dendritic synapse), soma (axo-somatic synapse) or axon (axo-axonic synapse) (Hammond & Esclapez, 2015).

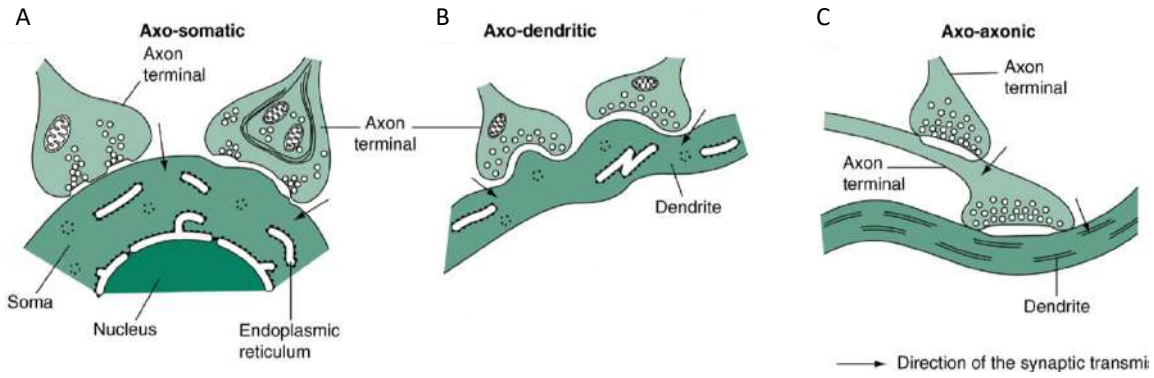


***Fig.2: Inter-neuronal synapses** : **(A)** Axo-somatic synapses: synapses between axon terminals and soma. **(B)** Axo-dendritic synapses: synapses between axon terminals and dendrite. **(C)** Axo-axonic synapses: synapses between two axons. Adapted from (Hammond & Esclapez, 2015).*

A variety of ultrastructural specializations occur at the synapse enabling unambiguous identification of pre- and post-synaptic partners (Fig.3).

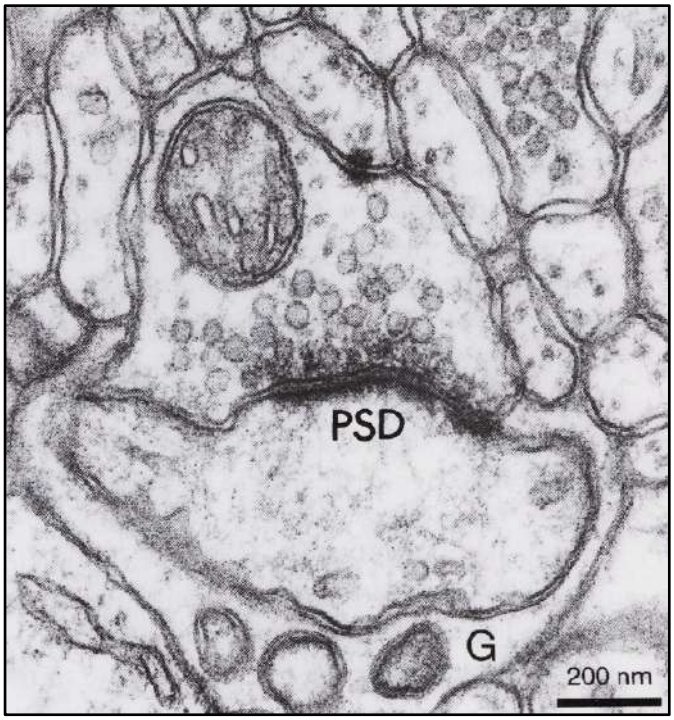


Fig.3: Ultrastructure of the synapse *: Electron microscopy image of a synapse between an axon terminal and a dendritic spine. In the presynaptic element a group of synaptic vesicles is visible; in the postsynaptic element it can be appreciated an electron-dense membrane thickening right in front of the presynaptic active zone called postsynaptic density (PSD). Of note, a glial cell (G) surrounds the dendritic spine. Adapted from (De Camilli et al., 2001)*

1.1.1. Presynaptic element

The presynaptic element contains many organelles that cooperate to allow the efficient release of the chemical message. In this section I will provide a rapid description of the main components of the presynaptic site (Fig.4).

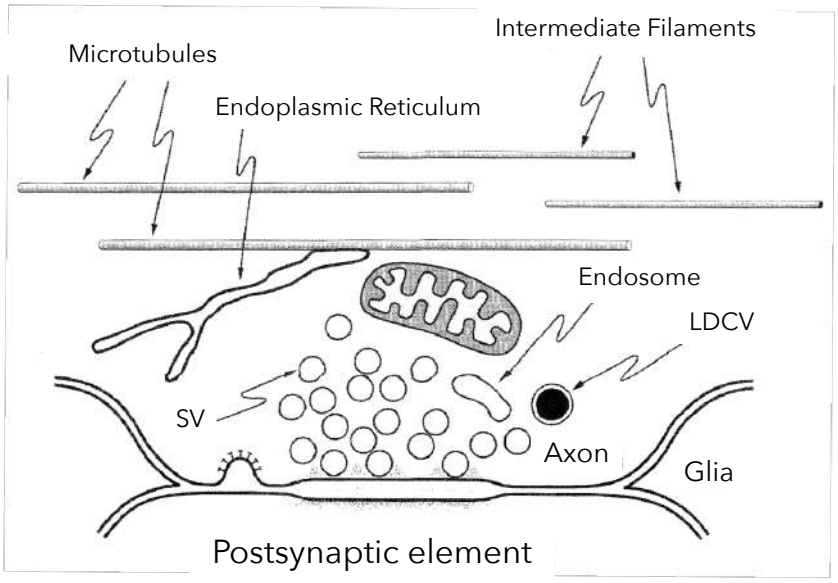


Fig.4: Principal components of the presynaptic element: *Image illustrates the principal components of the presynaptic site. Adapted from (De Camilli et al., 2001).*

Due to the distinctive morphology of neuronal cells, the distance between the cell body and the nerve terminals is particularly pronounced, placing a neuronal cell to face the problem of delivering new synthesized components to the periphery in a relatively short amount of time. The simple diffusion process is not sufficiently fast and specific to handle this task, whilst the anterograde and retrograde transport takes advantage of motors proteins that exploit cytoskeletal filaments as tracks to move different types of cargos with high specificity. Nevertheless, also the fastest axonal transport may take hours to supply cargos, such as membranous organelles important for the release of NTs, to the synaptic terminal. Considering that the translation from electrical to chemical messages must be very fast, nerve terminals compensate the slow restock of membranous organelles using a very specialized secretory apparatus: *synaptic vesicles* (SVs). These organelles can be recycled and refilled with NTs locally, without the involvement of the cell body protein-synthesizing machinery. Representing by far the most remarkable hallmark of chemical synapses (Takamori, 2009), SVs consist of small vesicles clustered at the nerve endings that possess a clear and electron-lucent center with a size range of 35-50 nm that store quanta of nonpeptidic NTs such as glutamate, GABA, glycine and acetylcholine (De Camilli et al., 2001). They are reported to be homogeneous in size but their diameter and shape appear to differ according to the NTs content (GRAY, 1959): most excitatory synapses contain spherical vesicles, while inhibitory synapses display ovoid or flat structures owing to an artifact linked to the osmolarity of the fixative. A subclass of SVs is represented by dense-core vesicles containing amine NTs (e.g. catecholamines), responsible for the electron-dense centers.

The process of NTs release takes place in multiple steps (Fig.5), collectively known as SVs cycle. The first stage (step1) relies in the NTs uptake within SVs: after vesicles reformation, newly formed SVs are restocked with NTs actively transferred into SVs by the action of two transport proteins. The vacuolar proton ATPase pumps protons into the lumen of SVs thanks to an integral Vo domain, which builds up a ring structure in the membrane and mediates proton translocation, and a peripheral V1 domain which catalyzes ATP hydrolysis. The subsequent electrochemical gradient generated across the membrane is utilized by vesicular transporters to mediate specific NTs uptake into the vesicle. In the central nervous system (CNS) there are four uptake systems: VGLUT for glutamate, VIAAT for GABA and glycine, VMAT1/2 for monoamines and VAChT for

acetylcholine (Chanaday et al., 2019; Takamori, 2009). In their resting state, SVs (either those clustered in the functional vesicles pools or those newly formed via the recycling process) are restricted in their movement by synapsins, which tether them to filamentous actin (F-actin) cytoskeleton. In the presence of an action potential-mediated membrane depolarization, the influx of calcium through voltage-gated calcium channels (VGCC) triggers the phosphorylation of synapsin, which loses affinity for SVs, freeing them from the cytoskeleton. SVs are thus allowed to reach a restricted area of the presynaptic plasma membrane called "active zone" and to come in physical contact with it. This docking phase (step2) is initiated by a group of cytomatrix proteins such as piccolo, bassoon, munc13, which bring the SVs closer to the RIM proteins, orchestrating SVs positioning at the active zone. Docked vesicles are then converted in fusion-competent SVs by priming process (step3). As the membrane fusion process has a huge energy barrier, spontaneous membrane fusion is too slow to occur in a biological relevant timeframe, thus necessitating the action of the so-called Soluble NSF Attachment Protein Receptor (SNARE) proteins as catalysts. More in detail, the two plasma membrane SNARE proteins Syntaxin and SNAP-25 interact with the vesicular SNARE protein Synaptobrevin (also called VAMP2) to form partially coiled *trans*-SNARE complexes (Rizzoli, 2014), bringing the vesicle and plasma membranes into close proximity and providing the energy required for fusion (Rizo & Rosenmund, 2008). SNAREs are stalled in this conformation by a clamping complex formed by proteins such as synaptotagmins and complexins (Yoon & Munson, 2018; Zhou et al., 2017), until the neuronal activity triggers calcium entry via VGCC located in the active zone. Synaptotagmin, a vesicle membrane protein, represents the primary calcium sensor exploited by neurons to unlock the clamping complex allowing the full zippering of the SNARE complex (Meriney & Fanselow, 2019): once calcium is bound, it promotes conformational changes in the clamping complex engaging simultaneous interactions with SNAREs and the plasma membrane (Rizzoli, 2014). This event brings to the complete fusion (step4) of the two membranes with the formation of a pore and the exit of NTs. After exocytosis, SVs components integrated into the plasma membrane are recovered to form a new SVs by endocytosis (step5). The most studied modes of synaptic vesicle retrieval differ in molecular mechanisms and speed. Slow endocytosis (10-30 s) is orchestrated by a protein called clathrin that forms a coat on the presynaptic surface and a subsequent invagination that detaches through the action of

dynamin. Fast endocytosis (<1-2 s) is called “kiss and run” since the fusion pore closes rapidly without collapse of the vesicle. In both of this two mechanisms a GTP hydrolysis by the GTPase protein dynamin is fundamental for the fission of the invaginated membranes of newly formed SVs. A third form of endocytosis is the activity-dependent bulk endocytosis that happens in response to a very strong exocytic burst and consists in the generation of endocytic vacuoles from the excess plasma membrane derived from the fusion of a large number of synaptic vesicles with the plasma membrane within a short time. To date, the mechanism underlies the conversion of vacuoles in SVs remains elusive. Reformed SVs through one of these processes are subsequently moved through the early endosomal intermediates or immediately recycled back (step6) and refilled with NTs (step1) (Takamori, 2009). Interestingly, based on the phase of release in which they intervene, SVs are organized in three functional pools. The docked and primed vesicles are located near the active zone and represent the readily releasable pool (RRP), immediately available for fusion upon the arrival of action potentials. In conditions of moderate physiological stimulation, the fraction of SVs that undergoes exocytosis is replenished by vesicles constituting the so-called recycling pool, that represent the 10-20% of all vesicles. Finally, the reserve pool (RP) is composed by vesicles that are recruited only upon high-frequency stimulation and after depletion of the recycling pool, as they are reluctant to be mobilized. They represent the majority of vesicles in most presynaptic terminals (Rizzoli & Betz, 2005). Overall, the amount and the mobility of these vesicle pools affect post-synaptic stimulation and, consequently, neuronal plasticity (Takamori, 2009).

Nerve terminals also secrete peptide neurotransmitters (or neuropeptides) through large dense-core vesicles, synthesized in the cell body and transported at the periphery once they are mature. These secretory granules do not accumulate in the proximity of the plasma membrane and their exocytosis is mostly para-synaptic, occurring in structurally non specialized sites, at a distance from the active zones. The release of the content of these organelles is caused by a trains of action potentials and it is thought to modulate the synaptic transmission: neuropeptides bind slow-acting receptors that are located far away from the release site.

An important role in the functionality of the nerve terminals is played by the endoplasmic reticulum (ER), a network of tubular membranes that extends from

dendrites to the periphery. It is involved in calcium homeostasis, acting as a store for intracellular Ca^{2+} that is released in an highly regulated fashion.

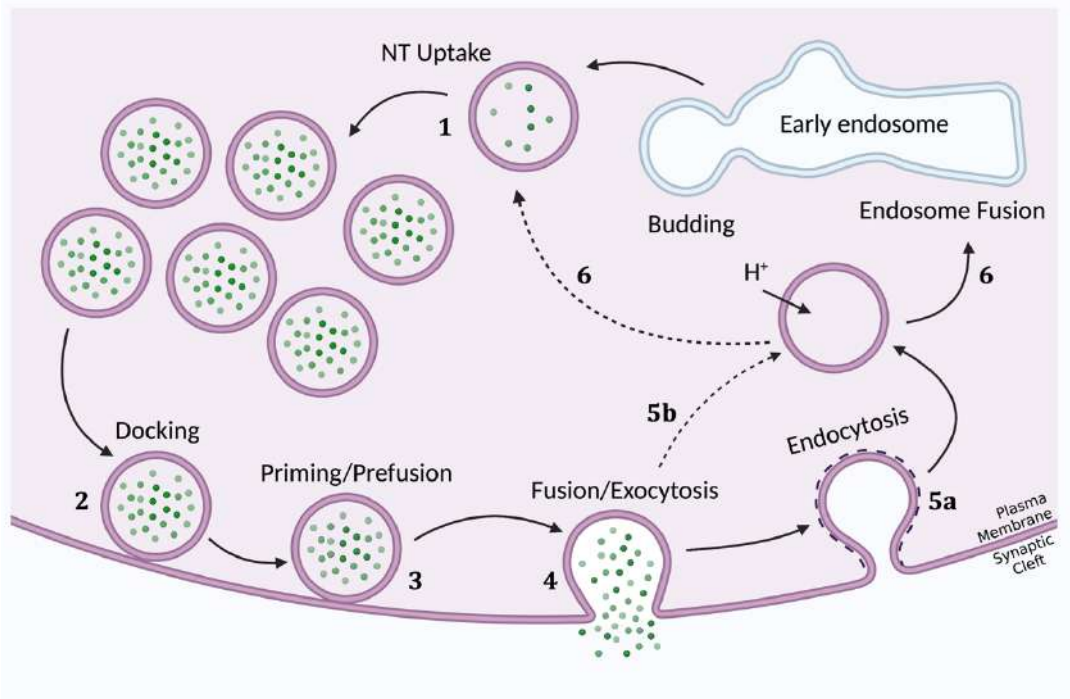


Fig.5: The synaptic vesicle cycle: *Image illustrates the major steps of the SVs trafficking:* ***(1)*** *SVs are filled with NTs;* ***(2)*** *SVs are translocated to the presynaptic plasma membrane active zone where they attach in a process named docking;* ***(3)*** *SVs are subjected to a prefusion reaction that primes them for calcium-triggered NTs release (priming);* ***(4)*** *Upon calcium influx the fusion pore expand and NTs are released in the synaptic cleft (Fusion/Exocytosis);* ***(5)*** *Empty SVs undergo endocytosis budding from the plasma membrane (a, clathrin-mediated endocytosis) or rapidly closing the fusion pore (b, "kiss and run") endocytosis;* ***(6)*** *SVs are acidified via proton pump and, after fusion with early endosomes, are regenerated and refilled with NTs. Alternatively, after acidification, some SVs can directly go to the step 1.*

Also mitochondria are frequently observed in presynaptic terminals where they are involved in the regulation of energy metabolism. Indeed, they ensure the supply of ATP required for SVs cycle. Indeed, ATP is needed across different steps of SV cycle, including vesicles priming, disassembly of the SNARE complex, endocytosis and uptake of NTs.

It is well established that the presynaptic site is enriched in actin cytoskeleton (Fifkovà & Delay, 1982): ultrastructural studies revealed the presence of a meshwork of actin filaments associated with SVs (Gotow et al., 1991; Hirokawa et al., 1989; Landis et al., 1988). Thus, it has been thought that actin may modulate NTs release operating in the maintenance and regulation of vesicle pools. It can act both as a scaffold to restrict the mobility of SVs or as a track to transport them toward the different pools.

At the active zone, actin has been suggested to play either a positive role in facilitating the docking of SVs or a negative role by forming a barrier for the priming process, preventing SVs fusion. The state of synaptic activity seems to impact on the role that actin assumes (Dillon & Goda, 2005). Moreover it has been reported that actin filaments intervene in the endocytosis of SVs providing mechanical force to form endocytic pits (X. S. Wu et al., 2016). By contrast, the presence and functional role of microtubules (MTs) at the presynaptic site are still controversial. Some electron microscopy studies reported the presence of MTs at presynaptic terminals, suggesting a model whereby a sub-group of MTs interact with SVs to reach the active zone, whereas another set forms marginal filaments in the terminal that are associated with mitochondria. Thus MTs may work as tracks to allow SVs and mitochondria trafficking at the pre-synapse (Bodaleo & Gonzalez-Billault, 2016; Parato & Bartolini, 2021).

Of note, the area of presynaptic plasma membrane destined to synaptic transmission is in close apposition to the postsynaptic plasma membrane. It can also been identified by the presence of an hexagonal grid of dense projections, cone-shaped structures of a size around 50nm, localized in between vesicles. Different studies support the hypothesis that these presynaptic densities are important in linking SVs, Ca^{2+} channels and the fusion machinery at the plasma membrane (Südhof, 2012).

1.1.2. Synaptic cleft

The synaptic cleft is defined as the space separating the plasma membranes of two communicating neurons. Although the intracellular space among central neurons is usually very small, about 10nm, it becomes larger in the proximity of synapses, where its average thickness is around 20nm. This widening is not strictly a real space: indeed, in central synapses, it is filled by undefine dense material (GRAY, 1959; Lučić et al., 2005) probably composed by standard extracellular matrix proteins and specialized synaptic proteins that connect the two synaptic sides in mechanical and functional terms. Indeed, not only does the synaptic cleft stabilize pre- and postsynaptic plasma membranes in a parallel orientation, but it also mediates the *trans-synaptic* signaling between the pre- and the post-synaptic element, providing the space for NTs diffusion. Possible candidates for the execution of this role are cell adhesion molecules such as the well-known cadherins and integrins, as well as neuron-specific neuroligins and neurexins. Of note, within the synaptic cleft glial cells help to remove NTs from inter-

neuronal space and, occasionally, could participate to synaptic communication (Burns & Augustine, 1995).

1.1.2. Postsynaptic element

While the presynaptic element is involved in the synthesis, storage and release of NTs, the postsynaptic plasma membrane is specialized to receive and elaborate the chemical signal - in the form of NT - and in the transmission of this information to the rest of the neuron. The postsynaptic cell possesses a characteristic equipment of structural specializations to achieve this function. The cardinal functional component is represented by neurotransmitter receptor (NTRs) proteins, localized in front of the presynaptic plasma membrane. Their amount and classification depend on the type of synapse. NTRs can be conventionally categorized in two classes. The first is composed of *ligand-gated ion channels* (or *ionotropic receptors*) that form a pore for the passage of specific ions and also bind NTs, which modulate receptor opening. The second class is represented by *G protein-coupled receptors* (or *metabotropic receptors*) consisting of a single protein passing through the cell membrane seven times and activating, upon NTs binding, a signaling cascade culminating in opening or closure of ion channels. The change in membrane potential triggered by *ionotropic receptors* occurs rapidly and is proportional to the amount of NT bound, whereases *metabotropic receptors* mediate slow neurotransmission with a change in membrane potential being more finely tuned due to their dependence on signal transduction cascade. NTRs are embedded in a dense and rich protein system composed by signal transduction proteins coupling NTRs activation to second messenger pathway, signaling enzymes modulating NTRs activity, and also cytoskeletal proteins and scaffolding proteins that are important for NTRs clustering.

Of note, the molecular organization and the morphology of the postsynaptic specializations differ between inhibitory and excitatory synapses (De Camilli et al., 2001; M. Sheng & Kim, 2011). Excitatory synapses are made on small protrusions of distal dendritic branches called dendritic spines, whereas inhibitory synapses are typically formed on the dendritic shaft or on cell bodies and axon initial segments. An interesting aspect is the thickness that characterized these synaptic contacts. Excitatory synapses present a remarkable accumulation of electron dense material on the post-synaptic side, placed just opposite to the active zone and named postsynaptic

density (PSD). This structure provides to the synapse a "asymmetrical" appearance (or type I) (Fig. 6 A, B, E), according to Gray classification (GRAY, 1959). Instead, inhibitory synapses show no marked differences among per- and postsynaptic membranes, with only a slight electron-dense thickening associated with the latter. For this reason inhibitory synapses were described as symmetric (or type II) synapses (Fig. 6 C, D, E) (GRAY, 1959).

Over the years many efforts have been made to better understand the molecular organization of excitatory synapses, whilst much remains to be solved about the inhibitory synapses.

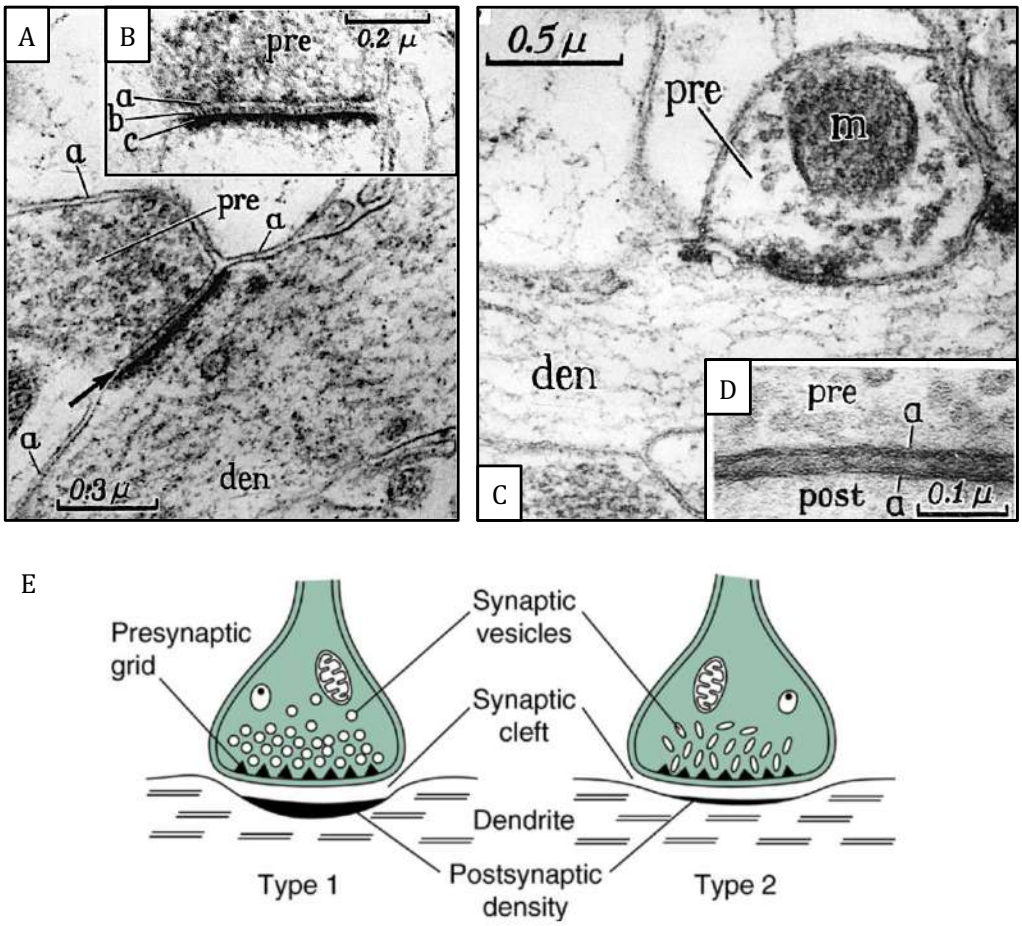


Fig.6: Images of asymmetric and symmetric synapses: (A) *Electron Microscopy image of an asymmetric or Type I synapse on a dendrite. Non-thickened regions of the membranes are indicated by [a], while the thickened region is denoted by arrow.* ***(B)*** *Magnification of the asymmetric synaptic contact among the pre- [a] and the post- [c] synaptic membranes separated by synaptic cleft [c] and characterized by thick PSD.* ***(C)*** *Electron Microscopy image of an symmetric or Type II synapse on a dendrite.* ***(D)*** *Details of the symmetric synaptic contact among the pre- and the post- synaptic membranes, characterized by the relatively thin postsynaptic specialization.* ***(E)*** *Schematic representation of the two synaptic type. Images adapted from (GRAY, 1959) and from (Hammond & Esclapez, 2015).*

1.1.2.1. Excitatory post-synaptic compartment: dendritic spines

In the great majority of neurons the total area of the dendritic tree is increased by the presence of small, thin, and specialized postsynaptic protrusions known as *dendritic spines*. As mentioned above, dendritic spines represent the principal postsynaptic element of most excitatory synapses and a mature spine contains one single synaptic contact on its head (Kristen M. Harris, 1999; Kristen M. Harris & Kater, 1994; Hering & Sheng, 2001). In mammalian brains, for instance, the majority of excitatory neurons bear dendritic spines: these elements are abundant in pyramidal neurons of the neocortex, in medium spiny neurons of the striatum and in Purkinje cells of the cerebellum (Hering & Sheng, 2001). The distribution of these structures varies among different brain regions and cell types, making it difficult to have an accurate quantification. As a general indication, mature neurons show a density of 1-10 spines per μm of dendritic length (Sorra & Harris, 2000). However, there are some aspiny neurons, represented mostly by the local interneurons, that lack dendritic spines along dendrites and have large swelling in the neurites: in this case, both inhibitory and excitatory synapses occur within the dendritic shaft (Kristen M. Harris & Kater, 1994). Notable, it is quite rare to find spiny neurons in lower organisms such as *Drosophila* and *Caenorhabditis elegans* suggesting that dendritic spines evolved to cope with the complex information transfer and processing distinctive of more advanced nervous systems (Hering & Sheng, 2001).

1.1.2.1.1. Dendritic spines structure

The existence of dendritic spines has been documented for over a century by Santiago Ramon y Cajal (Ramón y Cajal, 1888; Yuste, 2015) (Fig.7A), when he was exploring the architecture of the avian cerebellar cortex. At that time he made the following observation:

" the surface of the Purkinje cells' dendrites appear ruffled with thorns or short spines, which on the terminal dendrites look like light protrusions "

Later, he noted that dendritic spines are also present in numerous classes of neuronal cells and brain areas, as well as in various animal species (Ramón y Cajal, 1899). So, he

put forward the hypothesis that dendritic spines serve to increase the surface area of dendrites to accommodate the vast complexity and number of neural connections in the brain (Ramón y Cajal, 1899). With the progress made in the microscopy field, the structure and the ultrastructure of these protrusions started to be explored more in detail.

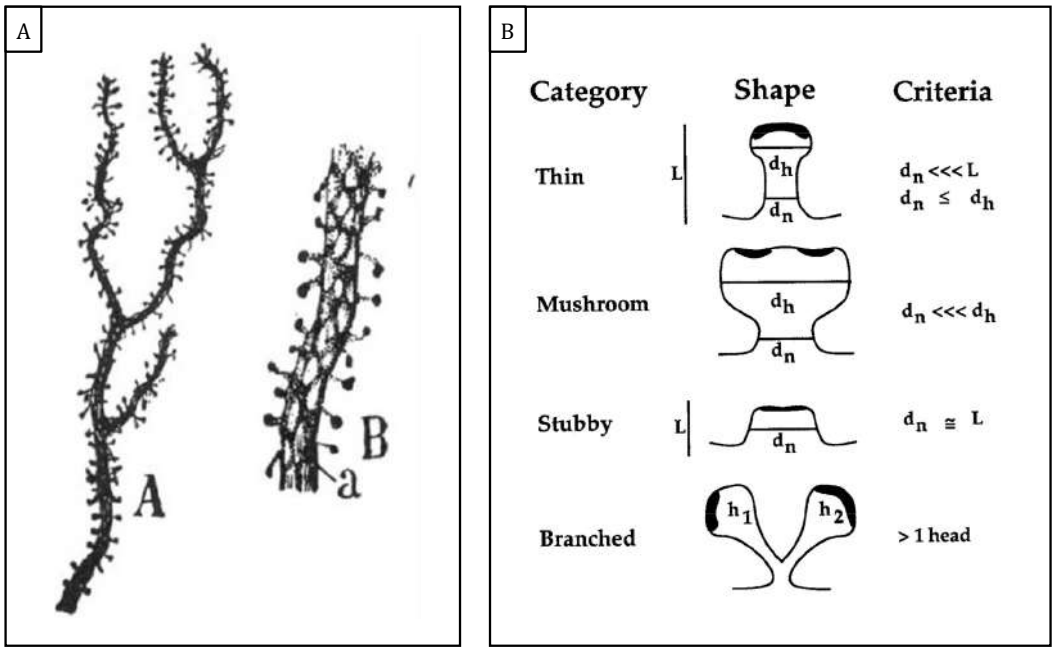


Fig.7: Dendritic spines: (A) *Illustration from Ramón y Cajal of cerebellar Purkinje cell dendritic spines, visualized via Golgi impregnation. Adapted from (Yuste, 2015)* ***(B)*** *Schematic representation of the criteria for dendritic spines morphological classification. Thin: the neck length is greater than its diameter and the diameters of the head and the neck are similar; Mushroom: the diameter of the head is much bigger than the neck diameter; Stubby: the neck diameter is comparable to the total length of the spine; Branched: the spine possess more than one head. L= length, d_n= diameter of spine neck; d_h= diameter of spine head. Adapted from (K. M. Harris et al., 1992).*

Dendritic spines typically possess bulbous heads attached to the dendritic shaft by narrow necks (Nimchinsky et al., 2002). They are found in a large variety of shapes (K. M. Harris et al., 1992; Jones & Powell, 1969; Peters & Kaiserman-Abramof, 1970) and sizes, ranging in neck length from 0,5 μm to 2 μm and in head diameter from 0,2 μm to 1,5 μm. Moreover, the total volume oscillates from less 0,01 μm^3 to 0,8 μm^3 (Kristen M. Harris, 1999; Kristen M. Harris & Kater, 1994). According to detailed anatomical studies, dendritic spines have been classified in four main morphological classes (K. M. Harris et al., 1992; Peters & Kaiserman-Abramof, 1970) (Fig.7B): “thin” spines possess a small head and a narrow neck and are also the less stable spines type; “stubby” or sessile spines are characterized by the lack of a clear constriction between the head

and the attachment to the shaft and probably represent an intermediate stage between excitatory shaft synapses and spine with a neck; “mushroom” spines consist of a narrow neck with a large head and constitute the more stable class of spines; “branched” or cup-shaped spines present two heads attached to a single narrow neck.

Another kind of dendritic protrusion is represented by the so called “filopodia”: long, very motile protuberances devoid of any expansion of the head. They were mainly observed during the early phases of synaptic development, when they constitute about the 20% of all protrusions, and then their amount reduces in mature brain tissue in favor of more mature spines. Due to this observation and also to the fact that they can protrude and retract from dendrites within minutes, filopodia are extensively believed to be the precursor of dendritic spines (Ziv & Smith, 1996): they are considered as indicators of synaptic contacts along dendrites that give way to the development of shaft synapses from which mature spines subsequently emerge (Kristen M. Harris, 1999).

The dendritic spines shape classification underestimates the huge variety of existing dendritic spines morphology and conveys a fallacious static concept of dendritic spines structure. Actually dendritic spines are highly dynamic and, in an extremely regulated way, they can change their shape and size across the different categories, allowing the remodeling of spine volume, size and thus structure. It is now well recognized as the different dendritic spines classes correspond to different phases of spine life and how this wide heterogeneity in spine configuration reflects the great variability that physiologically characterizes synaptic efficacy of excitatory synapses (Kristen M. Harris & Kater, 1994). Precisely, there is a general consensus among the experts that spines with larger spine head originate stronger synaptic contacts and, accordingly, they are defined “mature”. Indeed, the head size is proportional to the area of PSD, to the number of postsynaptic NTRs and also the amount of presynaptic docked vesicles (Hering & Sheng, 2001).

Thanks to the development of high resolution microscopy techniques such as confocal microscopy, two-dimensional transmission electron microscopy, super-resolution microscopy and three-dimensional reconstruction, dendritic spines ultrastructure has been extensively investigated, revealing a complex structure with several different elements (Fig.8). The most evident component is the PSD, that correlates perfectly with spine head volume and marks the synaptic contacts. The

external side of PSD is full of adhesion molecules that extend transapically and are inserted in postsynaptic plasma membrane. Moreover, it is rich in receptors that recognize glutamate, the major neurotransmitter of fast excitatory synapses in the brain. In particular, while metabotropic glutamate receptors are situated just outside the edges of PSD, the ionotropic glutamate receptors are distributed within this area: α-amino-3-hydroxy-5methyl-4-isoxazolepropionic acid (AMPA) receptors stand more centrally compare to N-methyl-D-aspartate (NMDA) receptors, that are found at the ends of PSD (Kharazia & Weinberg, 1997). It is also possible to find other membrane-bound receptors inside PSD, such as Tropomyosin receptor kinase B (TrKB), that binds BDNF. Interestingly, the surface of dendritic spines, that looks regular in small synapses, becomes more complex and discontinues, with the presence of interruptions devoid of PSD material, while the size of the spine increases. It is believed that these perforated PSDs represent the increased AMPA receptors insertion in the plasma membrane occurring during synaptic potentiation (Lüscher et al., 2000).

PSD also contains numerous classes of scaffold proteins differently involved in synaptic function and constituting the structural core of PSD. The best known group is a subfamily of the membrane-associated guanylate kinases (MAGUK proteins) including PSD-95, PSD-93, SAP102, SAP97, uniformly distributed along the synaptic membrane. In particular, PSD-95 was thought to play a key role in anchoring NMDA and AMPA receptors to the synaptic surface. Moreover, some of these PSD scaffold proteins are implicated in AMPA receptors trafficking, such as glutamate-receptor-interacting protein (GRIP), ABP and PICK1 that occupy a intermediate position in the cytoplasmatic area of the PSD. MAGUK proteins can be bound by another class of scaffold proteins called guanylate kinase-associated protein (GKAP) that, in turn, are coupled with the Shank family proteins, located in PSD cytoplasmic margin. This last protein type could interact with Homer proteins family, also positioned in cytoplasmic side of PSD (X. Chen et al., 2008).

Several others proteins in the PSD are involved in downstream signaling. Among them it is worth citing the small GTPases of the Rho family (such as RhoA, Rnd1, Rac1, Cdc42) and the Ras family, both implicated in the regulation of spine morphogenesis and synaptic plasticity.

Besides PSD, many other intracellular organelles are found within dendritic spines. Of particular importance is the presence of smooth endoplasmic reticulum (SER), a network of interconnected membranes that are spread throughout the entire neuron (Spacek & Harris, 1997). The extent of spines carrying SER differs according to the brain region: in the cerebellum almost all spines possess this organelle, while SER is present in less than 15% of hippocampal dendritic spines. Within spines, SER operates as an intracellular calcium store that can release calcium via activation of inositol trisphosphate receptors (IP3R) and the ryanodine receptors (RyR) in response to a long term potentiation (LTP) inducing stimulus. More interesting is the fact that, within larger spines, SER acquires the appearance of laminae separated by dense bars full of the actin binging protein synaptopodyn. This structure is known as "spine apparatus". While its precise role is unknown, some hypotheses have been formulated, including a role in calcium homeostasis (i.e. it acts as an efficient calcium buffer upon the ion influx triggered by pre-synaptic stimulus), in membrane protein trafficking, in protein synthesis and in posttranslational modification (Kristen M. Harris & Weinberg, 2012).

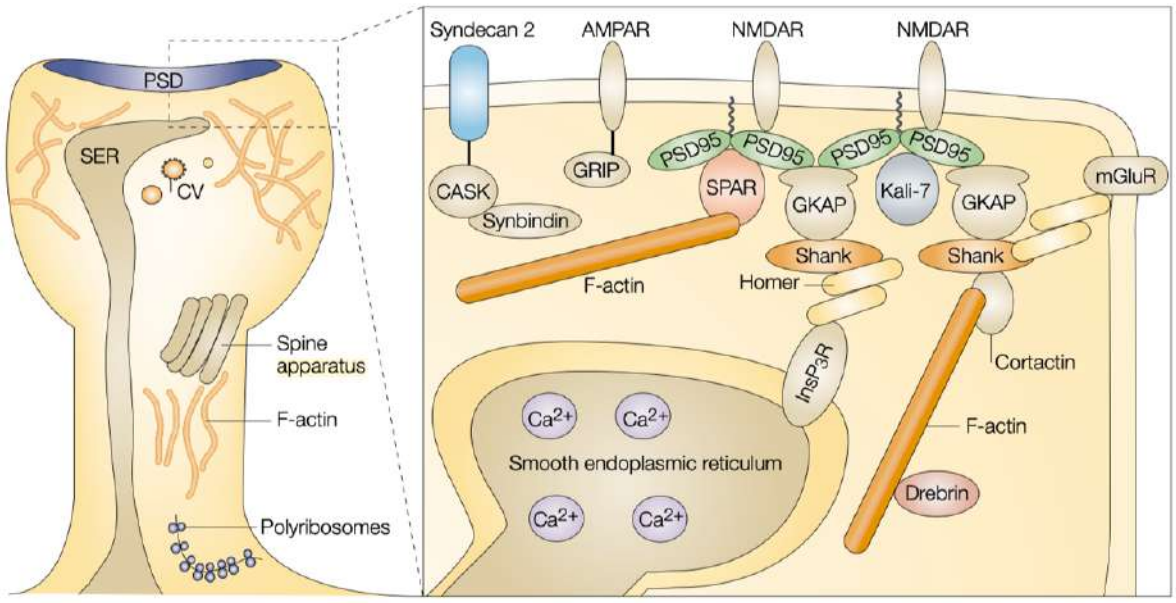


Fig.8: Dendritic spine structure: *Illustration of the princ$_{ip}$al components of dendritic spines:* ***(A)*** *Organelles located within mature dendritic spines: SER and spine apparatus, polyribosomes, clathrin-coated vescicles and actin cytoskeleton;* ***(B)*** *Representation of proteins and protein-protein interactions within PSD (see the text for more details). NB: GRIP (glutamate-receptor-interacting protein), and CASK (calcium/calmodulin-dependent serine protein kinase) and synbindin, scaffold proteins that bind to AMPA receptors and syndecan 2, respectively.* ***Legend:*** *AMPAR, AMPA receptor; F-actin, filamentous actin; GKAP, guanylate-kinase-associated protein; Kali-7, Kalirin-7; mGluR, metabotropic glutamate receptor; NMDAR, N-methyl-D-aspartate receptor; SPAR, spine-associated RapGAP. Adapted from (Hering & Sheng, 2001).*

It has been reported that local protein translation occur within dendritic spines. Accordingly, ribosomes can be found in dendritic spines, identified as clusters of three or more organelles called polyribosomes. Ribosomes are often found at the base of dendritic spines. They can appear as free polyribosomes synthesizing cytoplasmic proteins or they can be associated to ER synthesizing integral membrane proteins such as receptors. The distribution of ribosomes is not uniform in dendritic spines, reflecting different degrees of local protein synthesis and, thus, diverse levels of synaptic growth and plasticity (Kristen M. Harris & Weinberg, 2012).

Dendritic spines may also contain a set of endosomal structures such as clathrin-coated vesicles and pits, large uncoated vesicles, tubular compartments and multivesicular bodies. The presence of these structures suggests the existence of a local membrane trafficking process occurring in dendritic spines.

An important component of dendritic spines is the cytoskeleton, mainly constituted of F-actin that plays a crucial role in spine formation, plasticity and dynamics (Hotulainen & Hoogenraad, 2010) (Fig.9A). On the contrary, dendritic spines are commonly devoid of MTs, which instead represent the major component of dendritic cytoskeleton. However, it has been documented how a small portion of dendritic spines can be transiently entered by the growing EB3 positive MT plus ends (Jaworski et al., 2009), that via the interaction with F-actin binging protein cortactin, modulate actin dynamics in dendritic spines. This MTs invasion is dependent on neuronal activity as increasing neuronal activity enhances both the number of spines invaded by MTs and the duration of this invasions (Hu et al., 2008).

1.1.2.1.2. Dendritic spine dynamics and actin cytoskeleton

Dendritic spines exhibit the ability to change their morphology, number, density and motility over periods ranging from minutes to days and months, representing highly dynamic and plastic elements. Different forms of dendritic spine dynamics were observed, according to the age-related stage of the nervous system. During development, the dendritic shaft is very dynamic, with a rate of extension and retraction of dendritic protrusions that is quite high (Lendvai et al., 2000), allowing proper and accurate synapse formation and neural circuits assembly. Critical periods such as adolescence and early adulthood are characterized by refinement and maturation of neuronal circuits and a consequent decrease in dendrites structural

plasticity, whereas both the degree of dendritic spines motility among the morphological classes and the level of dendritic spines turnover appear to be still pronounced and profoundly sensitive to sensory and motor experience. By the adulthood, dendritic spines motility and rate of dendritic spines formation and pruning decrease, reaching a relatively stable number of protrusions (Koleske, 2013). However, it is important to highlight that, even if at low level, also in the adult mature brain dendritic spines dynamics remain active, especially in response to experience, such as learning processes (Grutzendler et al., 2002; Trachtenberg et al., 2002).

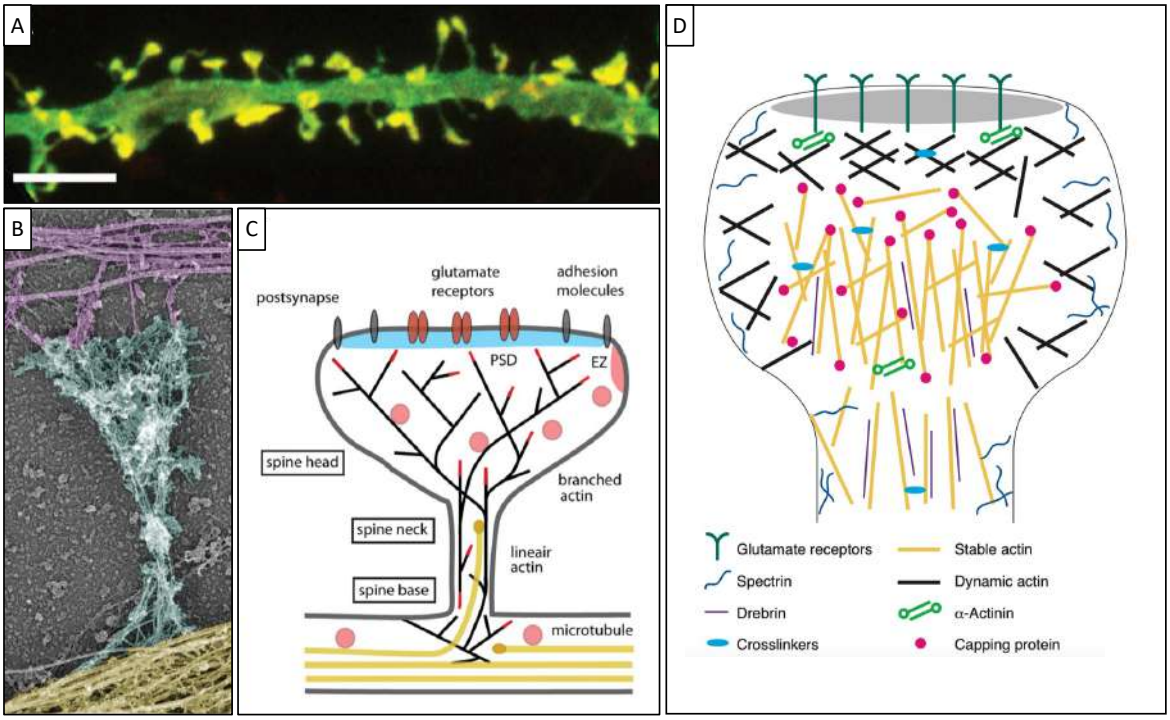


Fig.9: Actin in dendritic spine: (A) *Hippocampal neuron with actin highly localized in dendritic spines, as it can be appreciated by the yellow staining.* ***(B)*** *Actin cytoskeleton organization in mature dendritic spine of a hippocampal neuron visualized by platinum replica electron microscopy. The axonal cytoskeleton is visualized in purple, the dendritic shaft in yellow and the dendritic spine in cyan.* ***(c)*** *Representation of cytoskeleton in a mature mushroom-shaped spine: actin constitutes a continuous network of both straight and branched filaments (black lines), with barbed ends indicated as red lines; MTs (yellow lines) are the main component of the dendritic shaft cytoskeleton and some of them are dynamic and able to exit from the dendritic shaft and transiently enter dendritic spines. A-B-C adapted from (Korobova & Svitkina, 2010)* ***(D)*** *Representation of stable and dynamic actin pools distribution within dendritic spines. In spine head a set of filaments forms a stable core of F-actin in the central region of the spine (yellow), whereas a pool of dynamic filaments exist towards the periphery (black). Other cytoskeletal elements of dendritic spines include α-actinin (green), drebrin (purple), myosin and gelsolin; spectrins and the PSD (gray). Adapted from (Halpain, 2000).*

The fate of a dendritic spine depends on the nature of the stimulus (e.g. weak or strong) (Chidambaram et al., 2019). Formation, development, maturation and stabilization are all processes induced by high-frequency stimulation that promotes LTP, meaning synaptic potentiation. In this case, around 30 minutes of LTP leads to the so called short-term plasticity concerning the establishment and the expansion of new spines. While a sustained LTP brings to long-term plasticity lasting from several hours to days and consisting in the maturation and stabilization of newly formed spines, a low frequency stimulation generates long term depression (LTD) and a consequent synaptic de-potentiation. Indeed, LTD comes with reduction in dendritic spines size, shrinkage of spine head and pruning of few spines and/or filopodia. The events at the base of LTP and LTD start with the change in intracellular calcium levels through NMDA receptors and the subsequent activation of different kinases and GTPases, followed by a rearrangement of actin cytoskeleton and a repositioning of NTRs (AMPA and NMDA). In addition, transcription factors are activated, promoting gene expression, which leads to structural reorganization (Gipson & Olive, 2017).

Ultimately, the formation and loss of dendritic spines as well as their morphological plasticity is driven by actin cytoskeleton rearrangements (Matus, 2000). Actin is able to rapidly polymerize and depolymerize, constituting a very efficient tool for coupling synaptic activity to dendritic spines structural adaptation. Actin cytoskeleton is mainly composed of small globular (G) actin that assembles in paired and twisted filaments originating filamentous (F) actin. F-actin filaments differently combined together give rise to a mix of linear and branched actin networks, which extend across all the entire spine up to the postsynaptic density (Fig.9B-C). In the spine neck the mixed actin filaments are loosely arranged in a longitudinal orientation, and in the spine base the branched filaments frequently reside directly on the microtubule network in the dendritic shaft. To note, actin grid undergoes extensive branching at the neck–head junction and stays highly branched in the spine head (Korobova & Svitkina, 2010). Also, it was discovered the exitance of a periodic F-actin mesh that extends from dendrites in the spine neck and gradually vanishes in spine head (Bär et al., 2016). Periodic F-actin is composed by membrane-associated structures characterized by a remarkable stability and resistance to depolymerization.

In this picture actin appears to be at the base of two apparently contradictory effects on dendritic spines: on one hand it is associated with cell motility allowing the

dendritic spines remodeling, on the other hand it is crucial for the maintenance and stabilization of these structures when they mature. The explanation lies in the fact that spines hold a heterogenous population of stable and dynamic actin filaments (Halpain, 2000) (Fig.9D). Stable actin filaments possess capped ends that make the removal and the addition of actin monomer a very slow process. They form a core of filaments that provides the structural bases of the spine and allows the persistence of individual spines over hours to days. A second population of actin filaments constitutes the dynamic pool, located at the tip of the spines and holding free barbed ends that display a very high turnover of actin monomer. Under the proper stimulus, fast actin polymerization brings to the rapid length excursions of individual filaments towards the plasma membrane, generating the propulsive force for spine head expansion. Thus, dynamic actin pool possesses huge extensive force that appears to be determinant for spine head volume oscillations.

The stable and the dynamic nature of the two pool of actin filaments is influenced by a set of actin-binding and actin-regulatory molecules (Sekino et al., 2007). The main regulators of actin are the Rho small GTPases (particularly RhoA, Rnd1, Rac1 and Cdc42) that receives both extracellular and intracellular signals related to actin dynamics. The switch between the active GTP-bound and inactive GDP-bound states is able to change the activity of specific actin-binding proteins (ABPs) which, in turn, promote or suppress the polymerization of actin filament**.** There are ABPs that influence the structure and organization of actin cytoskeleton: capping proteins (e.g. Actin-CP and Eps8) bind to filaments ends affecting filaments turnover and length; crosslinking proteins (e.g. α-actinin, calponin, CaMKIIb, NeurabinI, Drebrin) are able to arrange and stabilize F-actin into different networks as actin bundles or actin mesh. Others ABPs promote F-actin nucleation, severing and depolymerization. The main player belonging to the first category is the actin-related protein (Arp2/3) complex that enhances actin polymerization and branching and is activated by the interaction with other proteins such as cortactin, WAVE1 and N-WASP. Also profilin, a multifunctional G-actin-binding protein, induces F-actin polymerization increasing the exchange of ADP for ATP bound to actin. Gelsolin instead possesses a strong actin-severing activity: in a Ca^{2+} dependent manner it promotes the shortening of F-actin filaments. The primary responsible for actin depolymerization is the ADF (actin-depolymerizing factor)/cofilin family protein. ADF/cofilin cuts F-actin increasing the

dissociation rate of its pointed ends and sequesters G-actin in pH dependent manner. As mention above, ABPs are the target of a wide range of signal transduction cascades that comprise the action of Rho GTPases and also serine/threonine kinases to finally respond to the cellular needs.

1.1.2.1.3. Dendritic spine function

The function of dendritic spines has always aroused great interest from the scientific community and different hypotheses have been formulated about that. The oldest one dates back to the time of dendritic spines discovery and was made by Ramon y Cajal (Kristen M. Harris & Kater, 1994; Ramón y Cajal, 1899). He postulated that dendritic spines serve to increase the surface area of dendrites available for the formation of new synapses. Spiny dendrites tightly condensate numerous synaptic connections into a limited brain volume, increasing this way their capacity to receive synaptic inputs. This hypothesis remains current and valid nowadays, even if over the years it has been enriched with new aspects. Specifically, it became clear soon that each dendritic spine builds a single synaptic contact suggesting that the meaning of spines might be the formation of a local synapse-specific compartment, rather than the mere expansion of postsynaptic surface area (Kristen M. Harris & Kater, 1994; Shepherd, 1996). It has been proposed that this integrative unit can electrically separate the synapse from the dendritic shaft, but this is true only to some degree (Koch & Zador, 1993; Shepherd, 1996). Today, the dominant view is that the main function of spine is to isolate the synapse chemically (Koch & Zador, 1993). The spine head acts as a microcompartment that localizes postsynaptic biochemical response, whereas the spine neck operates as a diffusional barrier for organelles and signaling ion and molecules, maintaining them at high concentration in dendritic spines and preventing their diffusion to neighboring synapses along the parent dendrite. For example dendritic spines are able to compartmentalize calcium (Majewska et al., 2000; Sabatini et al., 2001) bringing interesting advantages. First of all, being the volume of dendritic spines head quite small the opening of few channels causes large changes in intra-spine calcium levels. Moreover, the presence of the spine neck allows the shortening of calcium response latency and the slowdown of the calcium response kinetics decay. This effect is influenced by dendritic spines morphology (Noguchi et al., 2005; Volfovsky et al.,

1999), indeed it is stronger in long neck dendritic spines than in dendritic spines with short neck. It should be stressed that, despite the broadly acceptance that spines can segregate and integrate synaptic signals, the physiological significance of spines for brain function is still not clear. An hypothesis can be made based on the well-recognized role of dendritic spines as major sites of synaptic structural plasticity (Bosch & Hayashi, 2012). Francis Crick in 1982 speculated that dendritic spines 'twitch' and proposed that this rapid twitching may perhaps subtend short-term information storage (Halpain, 2000). This was the first of many theories about a role of dendritic spines in learning and memory process. Dendritic spines actually "dance" or twitch and a great open question remains to understand when and why they shows this particular behavior and if it is linked with the higher functions of the brain.

1.2. Synaptic pathology

Aging makes neuronal degeneration an inevitable event, although this process can be anticipated by a number of causes, of environmental nature, genetic origin or both. A growing body of evidence arising from human studies and animal models suggests that synaptic vulnerability is a common feature in multiple neurodegenerative disorders, such as Alzheimer (AD) and Huntington (HD) diseases. Precisely, synaptic impairment is thought to be an early pathological event that precede disease clinical manifestation. In AD, synaptic connections are lost in disease mouse models before any signs of cognitive decline start (Hong et al., 2016) and synapse loss is also observed in pre-symptomatic AD patients (Scheff et al., 2006). Early synaptic pathophysiology is also a hallmark of HD, with mouse and human studies indicating that reduced synaptic connectivity starts long before the onset of classical disease signs (Milnerwood & Raymond, 2010). Finally, it is important to note that early synaptic decay was highlighted also in Parkinson's disease (PD). It was found that at the onset of the pathology the synaptic terminals of DA neurons are more degenerated than the DA cell bodies. Moreover, synaptic dysfunction has been documented in PD animal model, with both pre and postsynaptic alterations reported. Therefore, an interesting hypothesis is that in PD there is a connection between disruption of synapse homeostasis and pathogenic mechanisms underlying the disorder (Picconi et al., 2012).

S. No	Neurological/neurodegenerative condition	Dendritic spine pathology
1.	Alzheimer's disease	Marked spine loss, increase in premature dendritic spines and varicosities formation
2.	Parkinson's disease	Decrease in dendritic length, dendritic spine loss and varicosities formation
3.	Huntington's disease	In mild cases, significant increase in dendritic branching, spine density and spine size are seen. In advanced cases, decrease in dendritic arborization, focal swellings on dendrites and decrease in spine density are reported.
4.	Intellectual disability or mental retardation	Both changes in dendritic spine density and/or spine shape
5.	Autism spectrum disorder	Higher dendritic spine density
6.	Fragile X syndrome	Increase in dendritic spine density and many dendritic spines show immature morphology like long, thin, torturous nature
7.	Rett syndrome	Size of neurons gets reduced along with increase in neuronal cell density; lower dendritic spine density especially mushroom-type spines; and impaired dendritic arborization
8.	Down's syndrome	Dendritic spines show larger spine heads along with distinct reduction in DS number and altered spine morphology
9.	Schizophrenia	Marked reduction in spine number
10.	Depression	Spine dysfunction, impaired signal transmission across synapse and dendritic spine loss
11.	Epilepsy	Frequent loss of dendritic spines, varicose swelling of dendrites and dendritic abnormalities such as changes in dendritic length, shape and branching patterns
12.	Stroke	Spine loss and reduction in spine number
13.	Prion disease	Significant decrease in spine density and dendritic spine loss
14.	Sleep disorders	Harmful effects on signaling molecules, polymerization of F-actin and dynamics of dendritic spines

Table1: List of neurological disorders presenting dendritic spines pathology*. Adapted from (Chidambaram et al., 2019).*

To note, studies conducted on transgenic animal models and post-mortem brains revealed a strong association between pathology affecting dendritic spines and both psychiatric disorders and neurodegenerative diseases (Fiala et al., 2002). Dendritic spines pathology can be due to an alteration in the spines distribution along the dendrites affecting number, shape, and loci of spine origin on the neuron, or to an impairment of spine ultrastructure, involving aberrant distribution of subcellular organelles inside spine. Dendritic spines pathology has been documented in diseases characterized by altered mental status and impaired cognitive skills such as intellectual disability or mental retardation, autism spectrum disorder, Fragile X syndrome, Rett syndrome, Down's syndrome, schizophrenia, major depression, epilepsy, stroke, prion disease, sleep disorders (Table1). As already mentioned, dendritic spines alterations are documented also in the most common neurodegenerative disorders. AD shows a massive loss of dendritic spines in different brain regions (e.g. neocortex and hippocampus) and near amyloid plaques regions (Knobloch & Mansuy, 2008; Serrano-Pozo et al., 2011; Spires-Jones et al., 2007), plus aberrant signaling pathways and postsynaptic proteins organization. In mild cases of HD it has been reported a marked increase in dendritic branching, spine density and size in the striatum, but with the progression of the disorder a parallel loss of dendritic spines and the appearance of dendritic anomalies was documented (Ferrante et al., 1991; Graveland et al., 1985).

Finally, also PD associates with an evident striatal spine loss, that appears to be correlated with the degree of dopamine denervation and that is prevalently due to glutamate excitotoxicity in striatal neurons (Chidambaram et al., 2019).

Accumulating evidence indicate that more than half of causative genes and risk factors for Parkinson's disease work at the synapse. Among these genetic players, the PD kinase LRRK2 plays an important role at this compartment, in both pre and post-synaptic sites. In the following sections I will provide an overview of the literature about PD and LRRK2, with a focus on the studies supporting a link with the synapse.

Chapter 2

Introduction

Parkinson's Disease

2. Parkinson's Disease

In "*An Essay on the Shaking Palsy*" (Fig.10A), published in 1817 by James Parkinson, symptoms and clinical features of a painful disorder were described in detail for the first time (Parkinson, 2002). With regard to the observations made on six individuals, Parkinson wrote:

"[they exhibit] involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forward, and to pass from a walking to a running pace: the senses and intellect being uninjured"

In the same dissertation the British physician termed this illness *paralysis agitans* (shaking palsy), sustaining the pressing need to bring it to the attention of scientific and medical community.

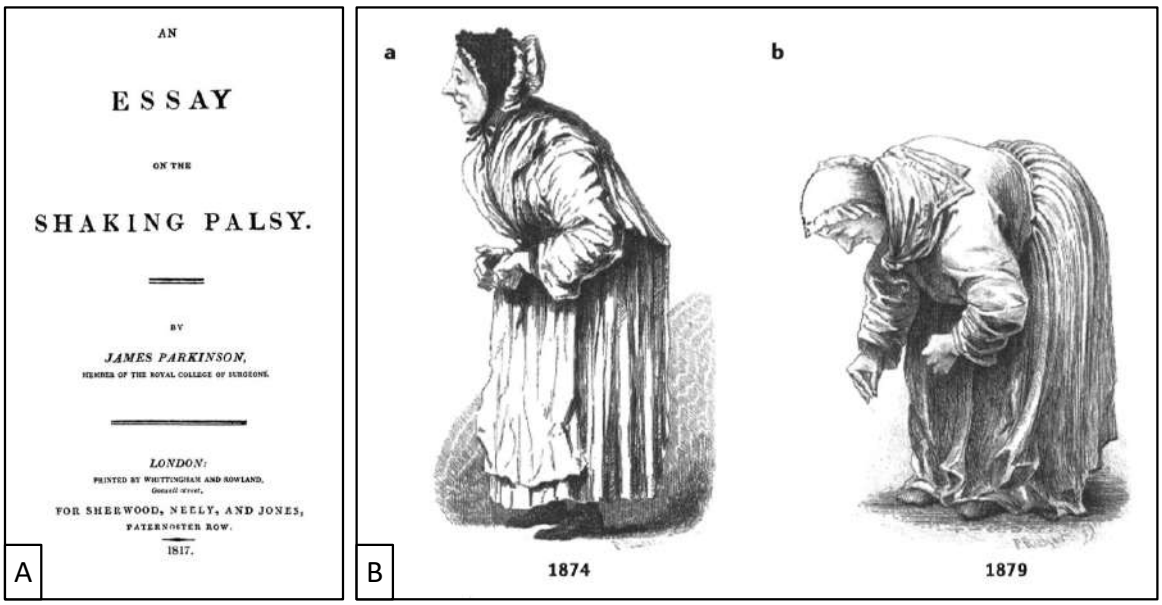


Fig.10: (A) Title page of An Essay on the Shaking Palsy. *The essay was published by James Parkinson in 1817* ***(B) A woman with paralysie agitante.*** *Drawings by Paul Richer of "Anne-Marie Gavr…" in 1874 (part a) and 1879 (part b), highlighting the progression of the motor symptoms (Goedert & Compston, 2018).*

Over 50 years later, French neurologist Jean-Martin Charcot emphasized the importance of slowness of movement (bradykinesia) rather than paralysis and the presence of muscle rigidity, highlighting the existence of the tremorous and the rigid/akinetic form of the disease (Fig.10B). In the English transcription (Charcot, 1877) of one of Charcot's lectures about diseases of the nervous system (*Leçons sur les maladies du système nerveux*), the following sentence can be read:

*"This man, aged 50 years, was attacked by '**Parkinson's disease**' in consequence of a strong emotion occasioned by the attempts of the Federalists, during the time of the Commune, to incorporate him into their battalions"*

Thus, thanks to Charcot's words, from that moment on, the disease was denoted with the name of who has discovered it.

Nowadays, PD is recognized as the second most common progressive neurodegenerative disorder after AD, with an incidence ranges from 5 to >35 new cases per 100000 individual per year, probably reflecting the differences in the population demographics (Fig.11A) (Poewe et al., 2017). The disorder affects up to 0,3% of the entire population, a prevalence that increases from 1% to 2% for people over the age of 60 and from 4% to 5% for people over the age of 85 (de Lau & Breteler, 2006; H. Deng et al., 2018) (Fig.11B). Of note, in most populations PD is more common in men than women, with a possible explanation linked to the neuroprotective effects of estrogens. Besides some cross-cultural variations in the incidence and in the prevalence of PD, potentially due to differences in environmental exposures or distribution of genetic risk factors, it is extremely clear that PD is an age-related disease. To this regard, it is important to mention the existence of early-onset parkinsonism, that includes all the cases with an onset occurring before the age of 40 (Ascherio & Schwarzschild, 2016). Within this category, cases with disease onset before age of 21 can be distinguished and are classified as juvenile forms of parkinsonism.

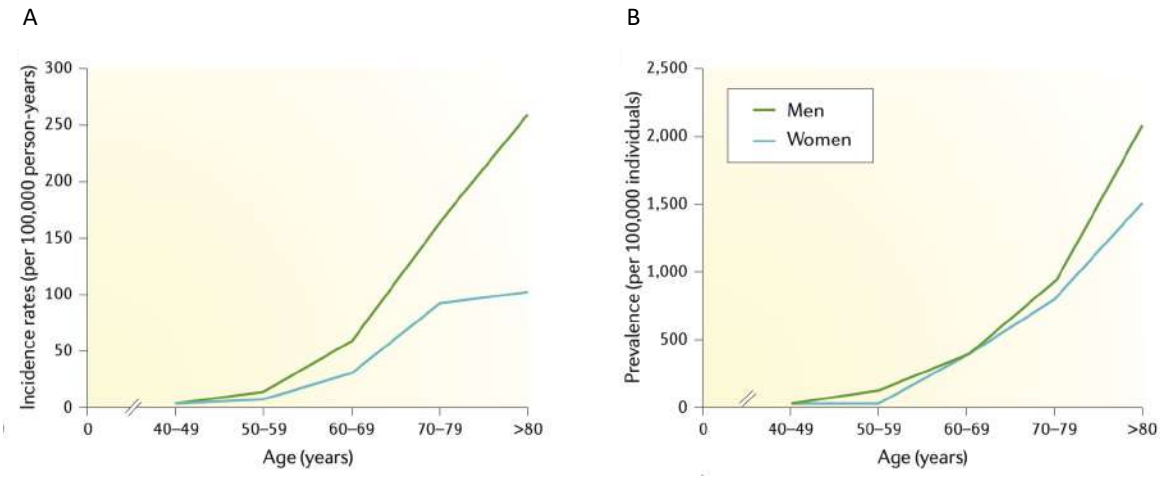


Fig.11: Epidemiology of Parkinson disease (A) *Prevalence of Parkinson disease in men and women per 100,000 individuals.* ***(B)*** *Incidence rate of Parkinson disease per 100,000 person-years. Adapted from (Poewe et al., 2017).*

The progressive and pronounced global population aging is associated with increasing prevalence of PD over the years, and the amount of affected people is predicted to double between 2005 and 2030. This points out the extreme need of treatments to prevent the onset of the disease, avoiding also the progressive increase in the social and economic burden associated with this disorder (Poewe et al., 2017).

2.1. Clinical manifestation, diagnosis and treatment

PD is characterized by a heterogeneous symptomatology (Fig.12), with both motor and non-motor features, a slow progression through aging after the onset, and the involvement of multiple neuroanatomical areas (Kalia & Lang, 2015). Cardinal motor symptoms of PD are grouped under the acronym of TRAP: Tremor at rest, Rigidity, Akinesia (or bradykinesia) and Postural instability. In addition, some others secondary motor symptoms have been described, comprising hypomimia, dysphagia, festination, freezing, dystonia, flexed posture (Jankovic, 2008). The existence of such a great range of symptoms has led to the identification of three major PD subtypes: tremor-dominant PD, which does not present other motor symptoms; non-tremor dominant PD, defined by postural and gait instability and akinetic-rigid movements; and a further subgroup of patients with intermediate phenotypes (Kalia & Lang, 2015). Non-motor symptoms, including sensory defects (e.g. olfactory dysfunction, pain), cognitive impairment and neurobehavioral abnormalities (e.g. depression, anxiety, apathy, dementia, hallucinations), sleep disorders (e.g. sleep-wake cycle regulation, excessive sleepiness or insomnia, rapid eye movement sleep behavior disorder), intestinal dysfunction (e.g. constipation), autonomic dysfunction and fatigue, are distinctive of the prodromal phase of PD, that precedes the onset of the typical motor symptoms (Postuma et al., 2012).

The complexity of the symptomatologic picture generates clinical challenges such as the difficulty to make a definitive diagnosis of PD in the early stages. Despite the standard criterion for diagnosis consists in the pathological assessment of the occurrence of neuronal loss and Lewy bodies in the *substantia nigra pars compacta* (SNpc), in clinical practice the diagnosis is based on a mixture of cardinal motor features, secondary motor symptoms, and response to levodopa (Jankovic, 2008). This guidelines have been defined by the UK Parkinson's Disease Society Brain Bank and the National Institute of Neurological Disorders and Stroke (Gelb et al., 1999) even if there

is still a bit of uncertainty regarding the reliability and validity of these criteria. Recently, the development of strategies to define biomarkers for PD has started to emerge as a very promising diagnostic alternative, with the aim of intervening early in the disease course, ideally before the onset of motor symptoms.

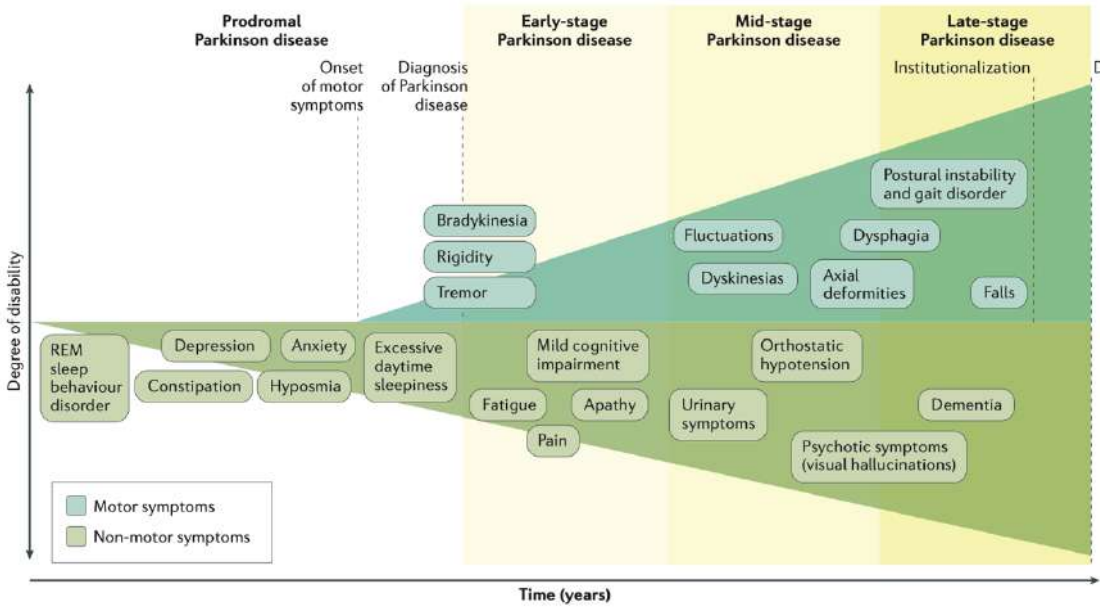


Fig.12: Symptomatology progression in Parkinson disease: *Scheme of the progressive appearance of clinical features during the disease develop (Poewe et al., 2017).*

It is believe that, during the premotor phase, the pathologic mechanisms implied in the disease's onset are taking place, offering a temporal window to intervene with disease-modifying therapies. Unfortunately, to date the available therapies are only symptomatic and do not slow down or halt disease progression (Kalia & Lang, 2015). They are focused on restoring the dopaminergic signaling with the administration of the dopamine precursor levodopa (L-DOPA) or dopamine receptors agonists. The big problem is the appearance of adverse reactions also associated with the patient medical history, so the choice of the right treatment needs to be carefully considered. Currently, the L-DOPA treatment is the most efficient in alleviating the motor symptoms, even if the long-term use is associated with motor fluctuations and dyskinesia. An alternative approach, typically reserved for patients that suffer L-DOPA side effects, is the deep brain stimulation (DBS) of either the subthalamic nucleus or globus pallidus internus, that has been proved to be effective in improving motor-symptoms and some non-motor features in moderate and severe PD (D. Lee et al., 2018). However, the mechanism of DBS is not completely understood and comes with

some side effects, probably resulting from the fact that DBS interferes not only with pathological neuronal circuitry but also it partially impacts on physiological connectivity. In the last years, new strategies are being considered as immunotherapy, gene therapy and transplantation, but they resulted to be effective only in terms of safety and not from a clinical point of view. Thus, it appears clear that further studies are urgently needed to elucidate the mechanisms underly PD pathology in order to identify more efficient treatments through the development of molecular or gene target therapies.

2.2. Pathology

From a pathological point of view, the motor symptoms manifestation is due to the loss of dopamine in the striatum resulting from the degeneration of DA neurons of the SNpc (Dickson et al., 2009) (Fig.13A). The most affected region in the SNpc is the ventrolateral area which contains DA neurons with unmyelinated axons projecting via medial forebrain bundle to the dorsal putamen of the *striatum* (nigrostriatal neurons), where they make contacts with GABAergic MSNs. In early-stage disease, loss of DA neurons is limited to this area, while as disease progresses towards the advanced stages it becomes more spread.

In addition to neuronal degeneration, surviving neurons are characterized by Lewy pathology, consisting in the formation of proteinaceous cytoplasmic inclusions that can be found within the neurons cell body (Lewy bodies, LBs) and processes (Lewy neurites, LNs) (Goedert et al., 2013), according to the description made by the German neurologist Dr. Friedrich Lewy in 1912 (Lewy, 1912). In 1997, Spillantini and colleagues identified alpha-synuclein (α-Syn) as the main component of LBs (Fig.13B-C) (Spillantini et al., 1997): when in misfolded state, it becomes insoluble and aggregates generating intracellular inclusions. Immunohistochemical studies showed that, besides α-Syn, more than 90 molecules can be found inside these intraneuronal structures, including structural elements of the LBs, α-Syn -binding proteins and ubiquitin (Wakabayashi et al., 2013). These aggregates are present not only in the brain, but also in the spinal cord and in the peripheral nervous system, suggesting a systemic nature of the disease (Dickson et al., 2009). Back in 2003, LBs were proposed by Braak and colleagues to spread in the nervous system with a temporal and spatial progression that support the PD clinical course. According to Braak's model, aggregates

reach the CNS via nasal or gastric route, transmitting from neuron to neuron across synapses (Braak et al., 2003). These observations laid the bases for considering PD as a prion-like pathology (Visanji et al., 2014). Even if the presence of LBs and LNs is by now well documented in PD patients, the nature of their actual function remains an open question: indeed, it has been postulated that oligomers and protofibrils of α-Syn could be toxic for neurons, while fibrillar aggregates of α-Syn appear to play a neuroprotective role (Wakabayashi et al., 2013).

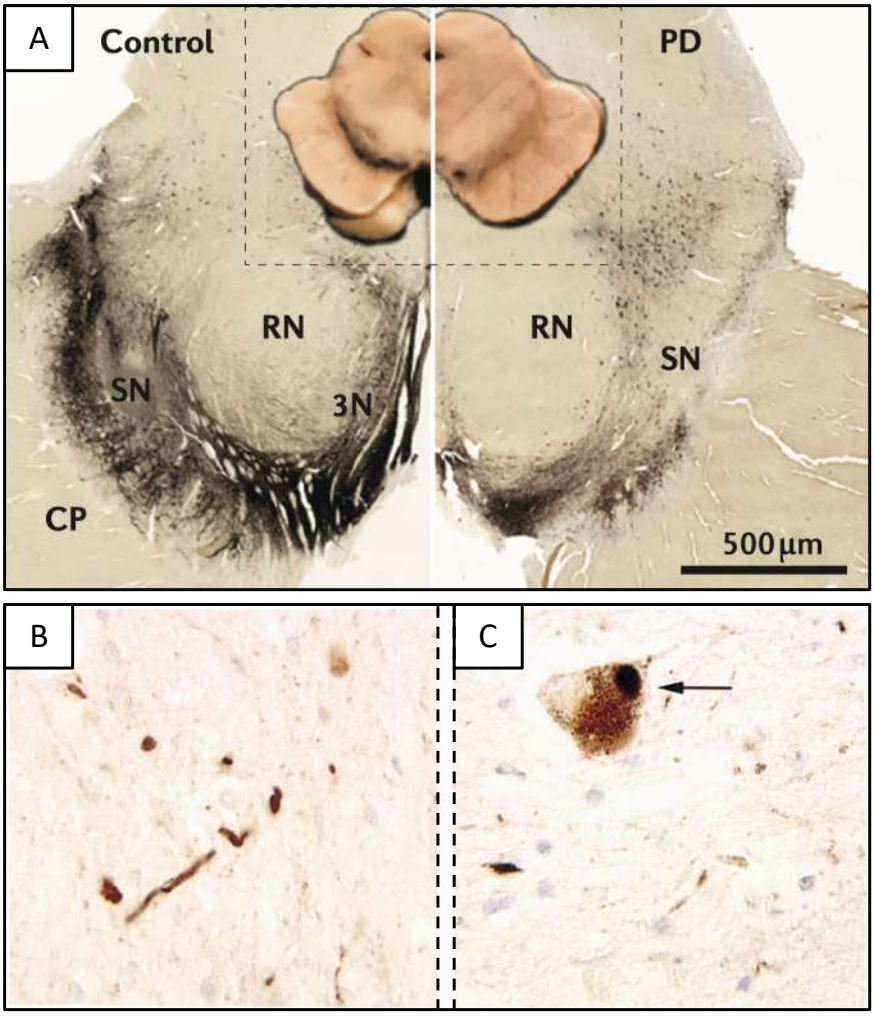


Fig.13: Neuropathological hallmarks of PD (A) *Degeneration of nigrostriatal neurons: compare to the control (left panel), the affected midbrain (right panel) presents the PD typical depigmentation resulting from the loss of DA neurons in the SNpc. 3N, 3rd nerve fibres; CP, cerebral peduncle; RN, red nucleus. Adapted from (Poewe et al., 2017).* ***(B)*** *Lewy neurites pathology: a-Synuclein-positive Lewy neurites in the substantia nigra* ***(C)*** *Lewy bodies pathology: a-Synuclein-positive Lewy body (arrow) in pigmented nerve cell of the substantia nigra. B and C adapted from (Spillantini et al., 1997).*

2.3. Pathways deregulation

The term basal ganglia (BG) refers to a group of subcortical nuclei at the base of the forebrain that exerts, as a primary function, the control of voluntary actions. The damage of this system contributes to the development of movement disorders such as PD and HD (Lanciego et al., 2012).

From an anatomical point of view, BG comprises the *striatum,* the external and internal segments of the globus pallidus (GPe and GPi), the subthalamic nucleus (STN), the SNpc and the *substantia nigra pars reticulata* (SNpr) (Fig.14).

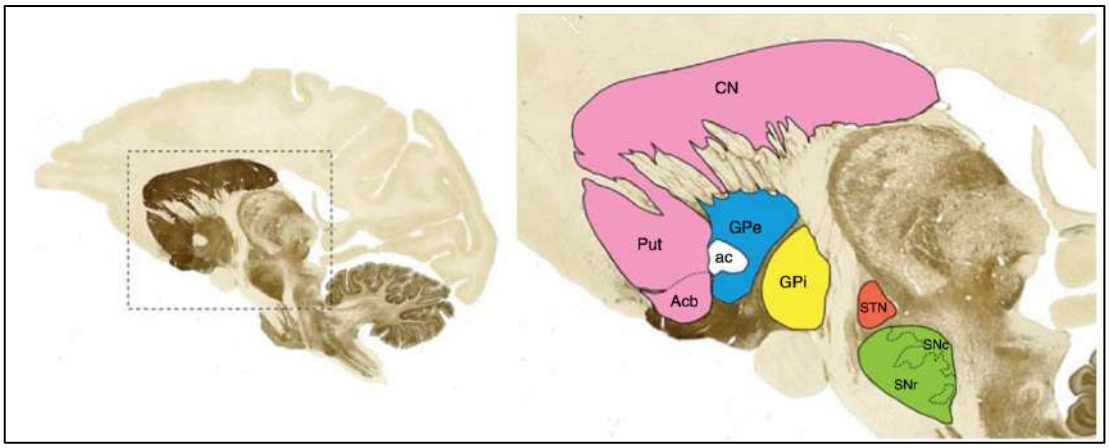


Fig.14: Basal ganglia nuclei. *Sagittal section of the monkey brain in which the components of the basal ganglia are shown. CN= Caudate Nucleus; Put= Putamen; Acb= Accumbes Nucleus, (Lanciego et al., 2012).*

The *striatum* is the biggest structure in the subcortical region and is composed by the caudate nucleus (CN), the putamen (Put) and the accumbes nucleus (Acb). The striatal complex receives glutamatergic excitatory projections from several cortical and subcortical areas and dopaminergic projection from the SNpc. Also the STN collects the external signals, especially from the cortical afferents. Altogether *striatum* and STN represent the input elements of the BS. The information are then processed and sent to the thalamus *via* the output nuclei, involving the GPi and the SNpr. Eventually, the thalamus projects back to the cerebral cortex controlling this way the movement execution. The correct functioning of this system relies in the appropriate DA release: an affected DA tone in the striatum results in alteration of the information transfer activity.

To note, the *striatum* is mainly composed by MSNs, that represent approximately the 90% of striatal neurons. The remining 10% of neuronal cells are interneurons, that

can be cholinergic or GABAergic. MSNs can give rise to two distinct and complementary pathways. According to the classical model of BG functionality, two neuronal subcategories can be identified based on their target and the receptors that they possess (Wichmann & DeLong, 2008) (Fig.15A). MSNs originating the "direct" monosynaptic pathway express the dopamine receptor subtype 1 (DRD1) and project directly to the GPi and the SNpr inhibiting their activity and thereby disinhibiting the thalamocortical projection. The "indirect" polysynaptic pathway is composed by MSNs that express the DA receptor subtype 2 (DRD2) and contact the basal ganglia output (GPi/SNr) indirectly through GPe and STN. As a result, GPi is stimulated and inhibits the thalamocortical projection. Thus, ultimately, the direct pathway is associated with an excitatory function that promotes movement, whereas the indirect pathway is postulated to have an inhibitory activity that hinders movement. Even if the two distinct pathways compete functionally, under normal circumstances they are finely balanced, allowing to process signals through the BG (McGregor & Nelson, 2019).

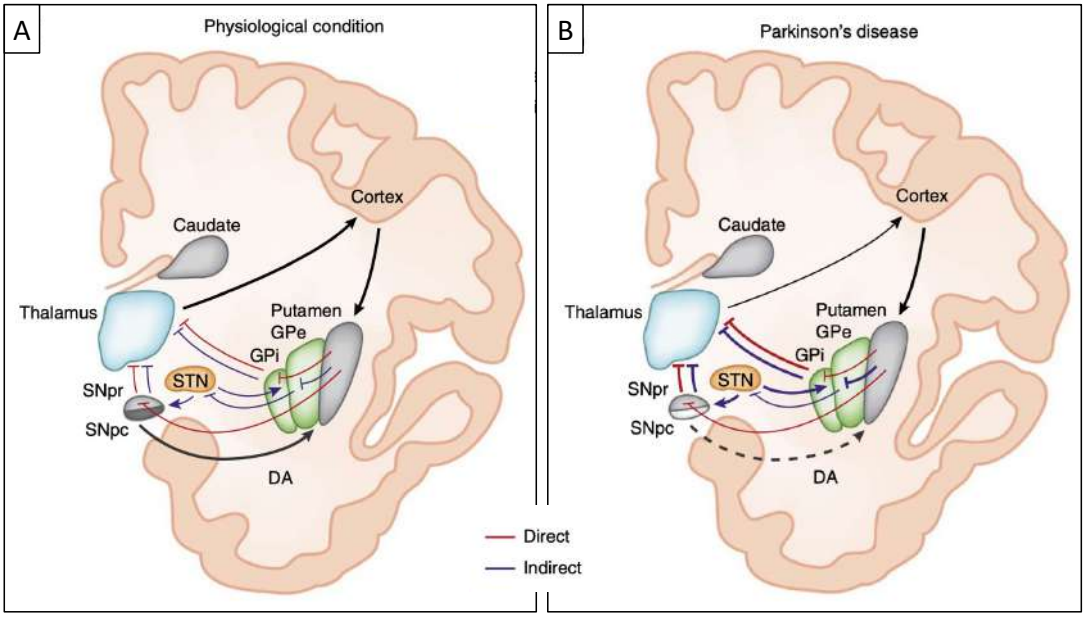


Fig.15: Schematic representation of the direct and indirect pathway in the physiological condition and in Parkinson's disease: (A) *in physiological condition, SNpc DA neurons project to the striatum balancing the activity of the two different pathways.* ***(B)*** *in Parkinson disease, DA tone decreases do to the loss of DA neurons triggering an unbalance in the activity of the two pathways. CN= Caudate Nucleus; Put= Putamen; Acb= Accumbes Nucleus, (Lanciego et al., 2012).*

The activity of these two pathways is modulated by DA released from the DA nigrostriatal projections arising from the SNpc, that directly make contacts on the neck of striatal MSNs dendritic spines. Any fluctuation, also physiological, in DA tone

modifies the existing balance between the activity of the two pathways. Importantly, the decrease in DA transmission characterizing PD affects the BG circuits, acting in an opposite fashion on the two pathways (Fig.15B). Reduced facilitation of the direct pathway and increased promotion of the indirect pathway finally result in GABAergic inhibition of thalamocortical projection with consequent impairment in movement initiation and execution (Wichmann & DeLong, 2008).

2.4. Etiology

Despite the precise cause of PD remains unknown, over the recent years it became clear that the disease results from a complicated interplay between environmental and genetic factors in which aging represents the main risk factor for the development of the pathology (Kalia & Lang, 2015).

Whilst the majority of PD cases are sporadic with unknown etiology, about 15% of patients show a family history and some polymorphisms within certain genes have been found to influence the risk of developing PD (H. Deng et al., 2018).

The discovery of monogenetic forms of PD, representing about 5%-10% of all cases, has been key to dissect the molecular pathways that lead to this pathology (H. Deng et al., 2018). This finding is an unprecedented opportunity to model the pathogenic mechanisms of the disease using appropriate cellular and animal models. Genetic investigations are key to develop new therapies, since the treatments currently available are only symptomatic and do not halt or slow the neurodegeneration process.

2.4.1. Sporadic Parkinson's Disease

As mention before, the majority of PD cases have an unclear etiology, although multiple lines of evidence indicate that a number of factors can predispose to disease (Ascherio & Schwarzschild, 2016). The most established risk factor is aging. Another one is the biological sex: the chance to develop PD is two-fold higher in men than in women (Baldereschi et al., 2000), possibly depending a protective effect exerted by female hormones (estrogens) (Thadathil et al., 2021). Also ethnicity is counted among risks factors. Indeed different PD incidence exists among different populations: idiopathic PD is less common among Asian and African people than Europeans, North Americans and South Americans (Sauerbier et al., 2018; Van Den Eeden et al., 2003).

Other risk factors are linked with environmental exposure and life style. In particular, epidemiological studies have found that pesticides, herbicides and heavy metals can increase the risk of PD (Nandipati & Litvan, 2016). This correlation emerged for the first time when the neurotoxic metabolite of 1-methyl-4phenyl1,2,3,6-tetrahydropyridine (MPTP) was identified in synthetic heroin assumed by drug abusers that manifested rapid-onset PD. MPTP is classified as a mitochondrial poison inhibiting the complex I of the electron transport chain causing the specific degeneration of DA neurons because MPTP is up-taken by dopamine transporter (DAT) (William Langston et al., 1983). Other compounds with a similar mechanism of MPTP are the herbicide paraquat and the pesticide rotenone (Nandipati & Litvan, 2016). These compounds are exploited in laboratory as useful tools to generate animal models that mimic PD symptomatology and pathology. Numerous studies have also revealed an important association of long-term exposure to heavy metals (coming from diet, environmental pollution and occupational exposure) on the risk to developing PD (Bjorklund et al., 2018). In particular, iron and manganese increase the risk of PD by two folds (Bjorklund et al., 2018; Nandipati & Litvan, 2016). Furthermore, certain life style habits were found to reduce the odds of developing PD, with a quite strong correlation for tobacco smoking and coffee intake (Wirdefeldt et al., 2011).

Environmental and epigenetic factors are thought to act in combination with genetic predisposition in modulating PD risk. To date, genome-wide association studies (GWAS) have identified more than 90 risk variants explaining 16-36% of all PD cases depending on the population (Gasser, 2015; Nalls et al., 2019). Among all identified risk factors, *SNCA*, *LRRK2*, *MAPT, GBA* show the most robust and reproducible associations (Gasser, 2015; Kalinderi et al., 2016). *SNCA* and *LRRK2* genes are pleomorphic risk loci, being both mutated in monogenetic forms of PD and containing risk variants influencing the risk of sporadic disease (Cookson, 2015). *MAPT* gene encodes the microtubule-associated protein tau which promotes the assembly and the maintenance of microtubular network allowing the correct neuronal axonal transport. Pathological accumulations of tau protein are found in several neurodegenerative disorders such as AD. Moreover, variants in this gene lead to increased risk of PD (Pascale et al., 2016). However, the greatest genetic risk factor for PD is represented by mutations in *GBA1* gene, encoding the beta-glucocerebrosidase lysosomal enzyme. *GBA1* mutations cause an autosomal recessive lysosomal storage disorder named

Gaucher's disease while heterozygous *GBA* variants increase the risk of PD by ~ 5 times (Riboldi & Di Fonzo, 2019; Sidransky & Lopez, 2012; Thaler et al., 2017).

2.4.2. Familiar Parkinson's Disease

During the past 25 years, large efforts have been made at defining the genetic bases of PD. Different genes have been linked to monogenetic forms of the disorder, caused by single mutations inherited in a dominant or recessive fashion. At present, 23 chromosomal loci, called PARK and numbered chronologically, have been linked to PD and in 19 of them the causative gene has been identified (Table 2) (H. Deng et al., 2018). However, only six of these genes (*SNCA, LRRK2, VPS35, Parkin, PINK and DJ1*) carry mutations that have been definitively shown to segregate with PD following Mendelian inheritance.

The very first gene that has been associated with PD was *SNCA,* located in the *PARK1* locus on chromosome 4 and originally found to carry the missense mutation A53T (Polymeropoulos et al., 1996, 1997). Later on, additional missense mutations were identified within the same gene together with gene multiplications, all associated with early-onset autosomal dominant familial PD (Chartier-Harlin et al., 2004; Singleton & Gwinn-Hardy, 2004). The consequent aminoacidic substitution or increased protein expression make α-Syn prone to aggregate and accumulate in LBs (Burré et al., 2015). In 2004, gain of function mutations in the *LRRK2* gene were discovered to cause *PARK8*-linked PD (Paisán-Ruíz et al., 2004; Zimprich et al., 2004). It is now clear that mutations in *LRRK2* represent the most common cause of late-onset autosomal dominant PD and also account for a percentage of sporadic PD cases. Even if 80 variants have been identified, only seven have been proven pathogenic (Corti et al., 2011; Iannotta & Greggio, 2021). Among these, the G2019S variant is the most common and studied mutation, while the others are less frequent worldwide. More detailed discussion about *LRRK2* and its relevance in PD will be provided in the next section.

The *VPS35* gene has been associated with late-onset dominantly inherited PD (Vilariño-Güell et al., 2011; Zimprich et al., 2011). It maps within the *PARK17* locus on chromosome 17 and encodes vacuolar protein sorting 35 (VPS35), the main component of the retromer complex that plays a central role in endosomal-lysosomal trafficking and in retrograde transport of membrane-associated proteins from endosomes to trans-Golgi network or plasma membrane (Williams et al., 2017). To

date, the D620N substitution constitutes the only certain pathogenic *VPS35* mutation, although additional variants have been described (Williams et al., 2017). Clinically, the symptoms shown by patients carrying mutated VPS35 are comparable to those of idiopathic cases while the neuropathological bases are still unknown due to the lack of post-mortem studies. Some cellular activities are found to be affected by the expression of D620N mutation, including neurotransmission, autophagy, lysosome and mitochondrial function. Intriguingly, a connection among *VPS35* and others PD-associated genes (e.g. *Parkin, LRRK2, SNCA*) has been proposed, suggesting an important role for *VPS35* in the pathogenesis of PD (Sassone et al., 2021).

LOCUS	GENE NAME	PROTEIN PRODUCT	DISEASE MANIFESTATION
Autosomal domaniant PD			
PARK1/4	SNCA	α-syn	Early Onset PD
PARK8	LRRK2	LRRK2	Late Onset PD
PARK17	VPS35	VPS35	Late Onset PD
Autosomal recessive PD			
PARK2	PRKN	Parkin	Early Onset PD
PARK6	PINK1	PINK1	Early Onset PD
PARK7	DJ-1	DJ1	Early Onset PD
Unconfirmed autosomal dominant PD			
PARK3	PARK3	Unknown	Late Onset PD
PARK5	UCHL1	Ubiquitin C-terminal hydrolase L1	Late Onset PD
PARK11	GIGYF2	GRB10 interacting GYF protein 2	Late Onset PD
PARK13	HTRA2	HtrA serine peptidase 2	Late Onset PD
PARK18	EIF4G1	Eukaryotic translation initiation factor 4 gamma 1	Late Onset PD
PARK21	TMEM230	Transmembrane protein 230	Late Onset PD
PARK22	CHCHD2	Coiled-coil-helix-coiled-coil-helix domain containing 2	Late Onset PD
Complex syndromes with parkinsonism			
PARK9	ATP13A2	ATPase 13A2	Parkinsonism
PARK14	PLA2G6	Phospholipase A2 group VI	Parkinsonism
PARK15	FBXO7	F-box protein 7	Parkinsonism
PARK19	DNAJC6	DnaJ heat shock protein family (Hsp40) member C6	Parkinsonism
PARK20	SYNJ1	Synaptojanin 1	Parkinsonism
PARK23	VPS13C	Vacuolar protein sorting-associated 13 homolog C	Parkinsonism

Table2: Table showing the PD-related genes. *For any genes the name, the protein product, the mode of inheritance and the clinical manifestation are reported.*

Autosomal recessive PD is linked to mutations in *Parkin, PINK* and *DJ-1* and is manly characterized by early onset. *Parkin,* the second identified causative gene of monogenetic PD, sits in the *PARK2* locus on chromosome 6 (Matsumine et al., 1997). It represents the most common affected gene associated to autosomal recessively inherited PD, with more than 100 different mutations identified, including point mutations, deletions, insertions and multiplications. They account for about 50% of familial cases and for 15% of sporadic cases in patient with early-onset PD (Lücking et

al., 2000). In 2004, *PINK1* was discovered to be located in the *PARK6* locus mapped to chromosome 1 (Eriza Maria Valente et al., 2004) with two homozygous mutations in the kinase domain identified (Enza Maria Valente et al., 2004). Afterward, other studies found *PINK1* mutations in European, American and Asian populations, with a frequency around 4-7% in sporadic cases (C. Y. Kim & Alcalay, 2017). Proteins encoded by *Parkin* and *PINK1* are both involved in mitochondrial quality control and have been proposed to act in concert to eliminate damaged mitochondria via a process named mitophagy (van der Merwe et al., 2015). Recently, it has been proposed that in depolarized damage mitochondria PINK1, a serine-threonine kinase active even in its unphosphorylation state, dimerizes and, consequently, undergoes trans-autophosphorylation at the level of Ser228. Phosphorylation causes conformational changes in the N-lobe of the protein that destabilize the dimers allowing phosphorylated PINK1 to act as a monomer and to become an active ubiquitin kinase (Gan et al., 2021). In this state PINK1 is able to phosphorylate parkin, an E3 ubiquitin ligase protein, recruiting it into mitochondria and triggering its ability to transfer ubiquitin to specific target proteins, ultimately promoting the removal of injured organelles.

Finally, *DJ-1* was identified in the *PARK7* locus within chromosome 1 (Bonifati et al., 2003). PD mutations in this gene are very rare, representing the 1-2% of early onset PD cases. DJ-1 is thought to function as a sensor of oxidative stress and could protect neurons and mitochondria itself, possibly interacting with PINK1 and parkin (Kalinderi et al., 2016).

In addition to the above mentioned genes, there is another set of PARK loci (e.g. *PARK3, PARK5-UCHL1, PARK11, PARK13-HTRA2, PARK18, PARK21, PARK22*), that have been linked with familial PD, even though this association remains to be further confirmed. Mutations in some of the genes located in PARK loci cause atypical form of PD (*PARK9-ATP13A2, PARK14-PLA2G6, PARK15-FBX07, PARK19-DNAJC6, PARK20-SYNJ1, PARK23-VPS13C*) (H. Deng et al., 2018; Kalinderi et al., 2016; Klein & Westenberger, 2012).

2.5. Molecular mechanisms

Some of the genes responsible for inheritable forms of PD were found mutated also in sporadic forms of the disorder with the associated neuropathology being similar or

even indistinguishable between the two conditions (Poewe et al., 2017). This observation suggested the possibility that the cellular mechanisms altered in genetic PD may be similar to those occurring during sporadic PD. Thus, the inheritable forms of PD, even if they represent a small percentage of total cases, constitute a strategic and wildly accepted platform to investigate and shed light into the molecular mechanisms underlying the development of PD pathology. α-Syn proteostasis, mitochondrial function, oxidative stress, calcium homeostasis, axonal transport and neuroinflammation are some of the pathways perturbed in PD (Poewe et al., 2017).

α-Syn proteostasis – α-Syn, encoded by the *SNCA* gene, is a small acidic protein made of 140 residues, with expression in the brain, where it can be found ubiquitously (Jakes et al., 1994). Within neurons, it is highly enriched at the pre-synaptic terminals, while its localization in the cell body and dendrites is relatively low (Maroteaux et al., 1988). Within the cell, α-Syn is in equilibrium between a soluble state and a membrane-bound state that is thought to be essential for its physiological function. The precise role of this protein is, however, still controversial, although many lines of evidence propose its involvement in regulating synaptic activity (e.g. synaptic plasticity, neurotransmitter release and vesicle trafficking,), lipid metabolism, membrane biogenesis and dopamine metabolism (Burré, 2015). Moreover, α-Syn has been found associated with mitochondria, Golgi apparatus and endoplasmic reticulum. In PD brains, a large amount of α-Syn aggregates, especially fibrils, is observed within neurons. This accumulation is triggered by mutations causing an overproduction of the protein or by an increased protein misfolding probability. Although it is still debated whether large fibrillar α-Syn aggregates are the actual toxic products or whether smaller oligomers are the detrimental component, α-Syn misfolding and aggregation is thought to affect several neuronal functions, including proteostasis, which in turn results in neurodegeneration. On the other side, impairment in the mechanisms responsible for cellular proteostasis (e.g. ubiquitin-proteasome system and lysosomal autophagy system) may contribute to α-Syn accumulation (Burré, 2015). α-Syn aggregates have been proposed to propagate in prion-like manner, spreading the pathology in multiple brain regions and contributing to the onset of motor dysfunction once they reach the SNpc (George et al., 2013).

Mitochondrial dysfunction and oxidative stress – Mitochondria are ubiquitous organelles that satisfy the cellular energetic demand providing ATP *via* oxidative phosphorylation, a process mediated by ATP synthase and other four respiratory chain complexes constituting the OXPHOS system. Besides being the main cellular energetic source, the OXPHOS system also produces superoxide radicals. Any perturbation in this machinery could therefor lead to an impaired energy metabolism and increased oxidative stress. Accumulating of evidence indicates that complex I is inhibited by different PD causing-factors, such as neurotoxins (e.g. MPTP and rotenone), mutations in *LRRK2, PINK1* and *DJ-1*, and *SNCA* triplication or aminoacidic substitutions. Subsequently, the electrons leakage from the respiratory chain increases oxygen radical species (ROS) production and oxidative stress, eventually activating the apoptotic process. PD-related mutations in *PINK1* and *Parkin* are also associated with defective removal of damaged mitochondria and imbalanced mitochondria dynamics. Defects in mitochondrial morphology, function and quality control as well as oxidative stress damage could trigger or sustain the loss of neuronal cells (Bose & Beal, 2016; Schapira, 2007). Of note, DA neurons display higher levels of mitochondria oxidative stress, either as a consequence of their autonomous peacemaking activity and/or of their sustained metabolism. The vulnerability of DA neurons to oxidative stress is particularly pronounced when this damage comes concomitantly with others alteration such as neuroinflammation and α-Syn aggregation, as I will discuss more in detail below.

Neuroinflammation – The term neuroinflammation delineates the inflammatory processes occurring in the CNS and includes the participation of the innate and the adaptive immunity. Accordingly, both activated microglial cells and T lymphocytes have been detected in the brain of PD patients with an increased expression of pro-inflammatory mediators. (Badanjak et al., 2021). Neuroinflammation is a *"double-edged sword"* because, even if it promotes the removal of detrimental factors (e.g. toxins, impaired and dysfunctional synapses) with beneficial effects, when it becomes sustained and prolonged it can lead to cytotoxicity and neurodegeneration causing significant tissue and cellular damage (Badanjak et al., 2021). Several lines of evidence pointed neuroinflammation as a characteristic feature of PD that contributes to the progression of DA neurons degeneration. Microglia cells represent the resident

macrophages of the brain are the key players in this process. In resting state, they possess a ramified morphology that serve to survey the surrounding environment and, in response to abnormal changes, they become active and respond (e.g. secreting inflammatory mediators) to eliminate the perturbations cause. The levels of some pro-inflammatory cytokines (e.g. tumor necrosis factor (TNF)-α, interferon (IFN)- γ , interleukin (IL)-1 β , and IL-6) was found to be high in striatum, SNpc and cerebrospinal fluid of experimental animal models and PD patients (Russo et al., 2014) and they are proportional to the severity of PD in peripheral tissues (Badanjak et al., 2021). An excessive microglia reactivity in the midbrain favors the progression of PD as it positively correlates with dopaminergic neuronal death and motor symptom severity (Russo et al., 2014). It has been also detected a trace of adaptative immune system in PD post-mortem brain samples consisting in the presence of lymphocyte infiltrates made of both $CD8^+$ and $CD4^+$ T cells, whereas B and natural killer cells are absent. Interestingly, interplay between resident macrophages and the infiltrated lymphocytes has been proposed: in a rat model of α-Syn PD, infiltrated T-cells induce a pro-inflammatory response of microglia that increases the expression of MHC-II, accelerating neuronal death (Badanjak et al., 2021). Finally, increased risk of PD development is associated with polymorphisms in genes encoding inflammatory cytokines like TNF-α and IL-1 β , and cell-surface human leukocyte antigen (HLA), IFN-γ (Russo et al., 2014) (Gelders et al., 2018).

Possible factors that trigger this response are environmental neurotoxins such as MPTP or mutations in α-Syn and LRRK2, which are found to affect microglia and T-cell function (Gelders et al., 2018).

2.6. Axonal decay in Parkinson Disease

Neurons are specialized cells equipped with a wide cytoplasm extending far from the cell body. Their post-mitotic nature makes neurons highly dependent on a plethora of mechanisms that ensure the maintenance of cellular homeostasis. An efficient and perduring machinery able to preserve neuronal homeostasis is key as neurons are constantly subjected to different sources of perturbation, including developmental processes, synaptic plasticity, changes in extracellular signals, and tissue damage (Harnack et al., 2015).

As extensively discussed, one of the main hallmark of PD pathology is the preferential degeneration of DA neurons of the SNpc, which, at the onset of motor symptoms, accounts for about 70% of neuronal loss. Neurodegeneration has been observed also in others brain areas, even if to a lesser extent (10-40%) (Wong et al., 2019).

Over the years, many efforts have been made to understand what renders this specific subpopulation of neurons so vulnerable in PD. To date, at least three different models have been proposed. A first model suggests that DA neurons of the SNpc are more prone to degeneration because of their unique axonal morphology: they are projecting neurons presenting long, thin, unmyelinated or poorly myelinated axons that give rise to an extensive axonal arborization including a large amount of axon terminals and a massive number of synapses in the striatum, two orders of magnitude greater than other neurons in the basal ganglia (Bolam & Pissadaki, 2012). Such large and highly branched neuronal architecture puts the cell under an impressive energy demand to ensure vital physiological functions such as transmission of action potentials, a process very expensive in terms of energy, also considering the lack of myelinization of DA neurons. In healthy conditions, DA neurons are in perfect balance between energy production and expenditure. However, this equilibrium may be perturbed by the different factors and deregulated processes linked to PD (Bolam & Pissadaki, 2012).

Another major hypothesis about SNpc DA neurons selective vulnerability in PD is based on one of their peculiar physiology: they act like autonomous pacemakers capable, without stimuli, to generate action potentials (spikes) that promote a slow rhythmic activity (Grace & Bunney, 1983, 1984). This pacemaking activity is needed to maintain tonic DA release in target regions (e.g. the striatum) and relies on the expression of different types of voltage-gated ion channels. However, unlike other pecemaking neurons, in SNpc DA neurons this activity is strongly supported by the engagement of calcium channels and the subsequent presence of calcium currents that contribute to oscillations in the intracellular calcium concentration. In particular, SNpc DA neurons exploit a L-type Ca^{2+} channels owning pore-forming subunit (Cav1.3) that opens at relatively hyperpolarized membrane potentials bringing to sustained calcium entry. The resulting calcium oscillations guide a persistent mitochondria stimulation to produce ATP via OXPHOS. This feed-forward mechanism is advantageous in a

situation of high energy demand, but when the ATP production exceeds the need, it runs the risk of generating ROS and, thus, oxidative stress (Surmeier et al., 2017). Moreover, calcium intake promotes DA synthesis ensuring a rapid replacement of NTs.

A third model that may explain SNpc DA neurons susceptibility regards dopamine itself at the level of presynaptic terminals (Mosharov et al., 2009). Dopamine is synthetized starting from tyrosine through two consequential enzymatic reactions. The rate limiting enzyme is tyrosine hydroxylase (TH), which catalyzes the conversion of tyrosine to L-DOPA. DA is characterized by a chemically unstable nature and neurotoxic metabolites. Thus, its levels in the cytoplasm are firmly regulated by an homeostatic equilibrium among biosynthesis, synaptic vesicle loading, uptake from the extracellular space by DAT, and catabolic degradation via monoamine oxidase (MAO). MAO promotes oxidative deamination of DA with the production H_2O_2 and ammonia together with DOPAL, further converted in non-toxic metabolites (3,4-dihydroxyphenylacetic acid (DOPAC) or 3,4-dihydroxyphenylethanol (DOPET)). Several events, such as dysfunction of DA storage in synaptic vesicles, can lead to increased DA and L-DOPA levels resulting in endotoxicity considered as one of the major causes of oxidative stress in PD. These two molecules are not stable and can undergo auto-oxidation with the consequent production of ROS and quinones, harmful for neurons. ROS are known to be responsible for DNA damage, protein cross-linking and lipid peroxidation, while quinones, both DA and L-DOPA-derived, have been shown to induce oxidative stress by affecting mitochondrial function (e.g. covalently modify mitochondrial proteins) and to promote the activation of necrotic and apoptotic pathways (e.g. modulating mPTP opening). Moreover, given its tendency to covalently modify lysines and cysteines residues, L-DOPA can induces enzyme inhibition and neuronal proteostasis alterations in terms of protein aggregation (e.g. α-Syn), competition with functional post-translational modifications (PTMs, i.e. ubiquitination, alteration of neuronal, SUMOylation, acetylation) and accumulation of ubiquitinated proteins (Masato et al., 2019). The ongoing mitochondria activity due to enhanced oxidative stress could lead, over time, to mitochondria dysfunction increasing mitophagy at the expense of other proteostatic tasks, such as the elimination of misfolded proteins like α-Syn.

Overall, these lines of evidence strongly support the extreme sensitivity of this class of neurons to metabolic and oxidative stress, that can worsen over the years (aging)

and/or in presence of stressful and pathological conditions such as environmental risk factors or PD mutations.

The lack of substantial progress in the development of successful neuroprotective therapies may be ascribed to a research largely focused on DA neuronal loss (Burke & O'Malley, 2013; Tagliaferro & Burke, 2016), with the aim of halting the degeneration of the neuronal soma, implying that this cell compartment is affected first or at the same time as the axon. However, recent evidence supports the idea that the degeneration of DA neurons in PD starts in axon and synaptic terminals and only later involves neuronal cell bodies (Burke & O'Malley, 2013; Tagliaferro & Burke, 2016), through retrograde degeneration described as "dying back" hypothesis. Evidence in support of this model came from the assessment of human post-mortem and functional imaging studies, but also from genetic studies in animal models, which contributed to dissect the role of PD-mutated proteins have in different neuronal compartments. Human studies highlighted that about 30% of DA neurons (Fearnley & Lees, 1991; Greffard et al., 2006; S. Y. Ma et al., 1997) and 60-80% of striatal DA terminals are lost (Beach et al., 2008; Kish et al., 1988; Kordower et al., 2013) at the onset of the motor symptoms. This implicates that the loss of DA axonal terminals in the striatum is much more pronounced than that of DA neurons in the SNpc. The critical role of axon degeneration in PD is further supported by the abundant α-Syn pathology in axons and presynaptic terminals. Of interest, brains affected by dementia with Lewy bodies (DBL), a pathology that like PD is characterized by α-Syn deposition, displays more than 90% of α-Syn aggregates at the presynaptic terminals rather than in the cell bodies (Kramer & Schulz-Schaeffer, 2007). To further supporting the dying back hypothesis, a recent work by Surmeier and collaborators showed that mitochondrial complex I disfunction brings to a metabolic switch in DA neurons underling early loss of axonal function and the consequent peripheral degeneration (González-Rodríguez et al., 2021).

Chapter 3

Introduction

Leucine Reach Repeat Kinase 2

3. Leucine-reach repeat kinase 2

Back in 2002, Funayama and collaborators described an autosomal-dominant form of PD in a large Japanese kindred, named "Sagamihara family" from the region of origin. The genetic locus associated with this form of PD was termed *PARK8* and mapped on chromosome 12 (Funayama et al., 2002). Later, in 2004 two independent studies identified *LRRK2* as the causative gene of *PARK8* associated PD (Paisán-Ruíz et al., 2004; Zimprich et al., 2004). Due to the Basque origin of the studied families and based on the tremor characterizing PD, the homonymous protein encoded by *LRRK2* gene was also named "dardarin", from the Basque word "dardara", meaning tremor (Paisán-Ruíz et al., 2004).

LRRK2 is a large multi-domain protein (280 kDa) comprising a central catalytic core surrounded by several protein-protein interaction regions (Fig.16). LRRK2 core possesses a dual enzymatic activity, including a Ras of complex domain (ROC) owing GTPase activity, a serine-threonine kinase domain (KIN) and a spacer domain in between the two catalytic activities termed C-terminal of Roc (COR), which is important form protein dimerization (Cookson, 2010; Myasnikov et al., 2021). The LRRK2 N-terminus harbors armadillo (ARM), ankyrin (ANK) and leucine-rich repeat (LRR) domains, mediating the interaction with others proteins. Instead, the WD40 domain is located at LRRK2 C-terminus and may influence protein folding and consequently LRRK2 function (Wallings et al., 2015).

Among all genetic contributors of PD, mutations in *LRRK2* gene represent the single most common cause of the disease (Paisán-Ruiz et al., 2013), that manifests with clinical and pathological phenotype almost indistinguishable from sporadic PD. Therefore, since its discovery, LRRK2 has occupied a relevant place in PD research, and several studies were conducted to shed light into its genetic, biochemistry and cellular functions.

3.1. LRRK2 genetics

Soon after its identification, several pathogenic mutations were identified in *LRRK2.* The most common mutation, the G2019S located in the kinase domain, is a relatively common cause of PD, with a frequency from 1 to 40% of all LRRK2 cases, depending on the population (Greggio & Cookson, 2009). For all the mutations described, the

phenotype and the age of onset are similar between the homozygous and the heterozygous carriers, supporting the autosomal dominant inheritance of these mutations (Cookson, 2010). Despite the many aminoacidic substitutions described, only seven of them segregate clearly with the disorder. These mutations are all located within the core of the protein, affecting its enzymatic function. Precisely, N1437H/D, R1441C/G/H, mutations sit in the ROC domain, Y1699C mutation is found in the COR domain and the kinase domain carry the G2019S and the I2020T mutations (Greggio & Cookson, 2009; Marchand et al., 2020; Wallings et al., 2015). Penetrance varies depending on the mutation type, but in general it increases with age, according to the age-dependent nature of PD, and is incomplete, meaning that not all carriers will develop pathology during their lifetime.

The most well-studied mutation is the G2019S as it represents the most common across many populations (Greggio & Cookson, 2009). It is responsible for up to 10% of sporadic PD and up to 42% of familial PD (Paisán-Ruiz et al., 2013). Indeed, its prevalence varies according to the ethnic background: it is rare in Asian people (<1%), low in Caucasian populations (5% of familial cases and around 2% of sporadic cases) and high in North African Arab and Ashkenazi Jewish populations, where it rises up to 30-40% for familial cases (Hernandez et al., 2016). The penetrance of the G2019S mutation is elevated but incomplete (Healy et al., 2008), and is reported to change from 25% to 45,5% at 80 years of age (A. J. Lee et al., 2017). The second mutation located in the kinase domain is the I2020T, which is the mutation identified in the Sagamihara family (Funayama et al., 2005).

But how do these mutations affect LRRK2 function? It is recognized that mutations clustered in the ROC domain lower GTP hydrolysis causing a consequent sustained signaling. Also the COR mutations are thought to have the same effect (Greggio & Cookson, 2009). It is supposed that the substitution of key residues in the phosphate binding P-loop region of ROC affects the dimeric-monomeric equilibrium that underlies the activation of LRRK2, locking the GTPase activity in the monomeric state and impacting on the binding kinetics of guanine nucleotide (Wauters et al., 2019; C. X. Wu et al., 2019). Regarding the kinase domain, it is well established that G2019S mutation causes a gain of phosphorylation activity, that appears to be enhanced approximately 3-4 fold *in vitro* and associated with neuronal degeneration (Greggio et al., 2006; Smith et al., 2006; West et al., 2005). The increase of *in vitro* kinase activity

associated with the G2019S mutation is predicted to have a structural explanation. The G2019S mutation is thought to impact on the conformational flexibility of the kinase activation loop. Indeed, Glycine2019 is part of the DYG motif located in the ATP binding site of the activation loop and playing a central role in switching the protein orientation allowing or restricting substrate access. Glycine2019S is replaced with a bulkier serine that probably lock the kinase in the active form (Greggio & Cookson, 2009; Wallings et al., 2015). Thus, the G2019S mutation causes a structural activation of LRRK2 whist ROC-COR mutations prolong LRRK2 binding to GTP, prolonging its active state in the cell (e.g. more time available to phosphorylate substrates).

The kinase and GTPase activities of LRRK2 are tightly linked. LRRK2 monomer, whose conformation has been recently solved together with the dimeric one, assumes a J-like shape with the C-terminus WD40 that folds over the LRR bringing the two catalytic domains close together (Myasnikov et al., 2021). However, for many years the ROC-COR pathogenic mutations were found to have an inconsistent impact on the kinase activity, as different laboratory reported contrasting results performing *in vitro* assays with recombinant proteins and autophosphorylation or peptide/substrate phosphorylation as readouts. With the development of the first antibody against the autophosphorylation site Ser1292 and the identification of a subset of RAB GTPases as a physiological LRRK2 kinase activity substrates, it was finally clear that, in cellular and tissues contests, the majority of the pathogenic mutations augment the kinase activity of the protein (Iannotta et al., 2020; Kluss et al., 2018; Z. Sheng et al., 2012; Steger et al., 2016). This highlighted the intrinsic limitations of *in vitro* kinase assays that cannot mimic the complex cellular machinery (made of cofactors, interactors, compartmentalization) required by the mechanisms underlying the effects of the different mutations.

Interestingly, besides the known photogenic mutations mentioned so far, LRRK2 carries also a number of rare coding variants, including the G2385R in the WD40 domain and the R1628P in the COR domain. They represent the most common *LRRK2* susceptibility variants for PD (Funayama et al., 2007; Ross et al., 2008). In particular, the risk of PD doubled in presence of G2385R variant (Ross et al., 2011).

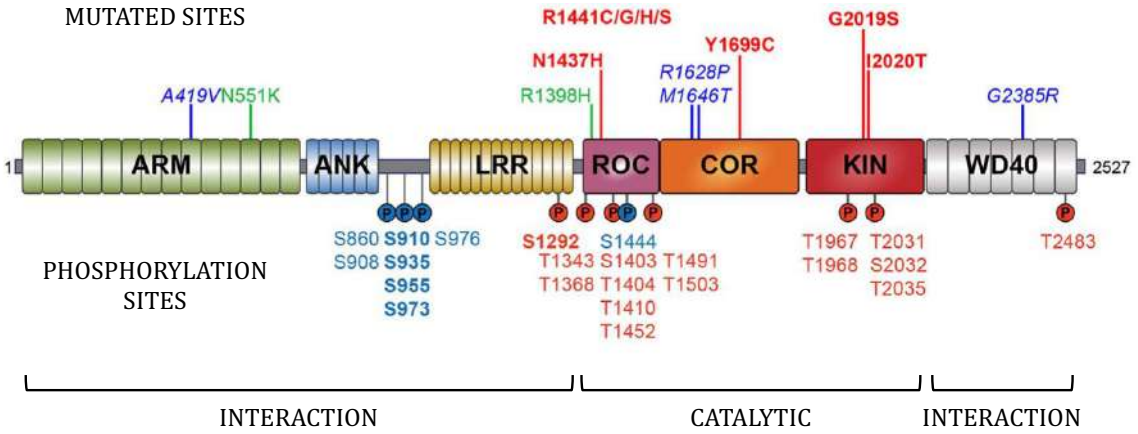


Fig.16: Schematic representation of LRRK2: *The image shows the domains topology and their main function. The upper part illustrates the mutations segregating with PD: in red are reported the pathogenic mutations, while in blue the risk variants. The lower part displays the phosphorylation sites: the sites of autophosphorylation are in red and the heterologous phosphorylation sites are indicated in blue. The most described and studied sites are in bold. Adapted from (Marchand et al., 2020).*

3.2. LRRK2 expression

LRRK2 is physiologically present in multiple tissues and organs during development and adulthood. The highest LRRK2 protein levels are found in kidney, lungs and liver (Biskup et al., 2007; Giasson et al., 2006), but it has a remarkable expression also in circulating immune cells and brain. Here, Lrrk2 mRNA and protein show a broad localization, with particularly great amount in anatomical regions of direct relevance to PD pathogenesis. More in detail, different studies have underlined that the mRNA levels are pronounced in cerebral cortex, olfactory tubercule and *striatum,* whereases they became moderate in the cerebellum and hippocampus and low in the hypothalamus and *substantia nigra* (Fig.17). Importantly, what emerges is that Lrrk2 mRNA is actually localized throughout the nigrostriatal dopaminergic pathway, displaying elevated expression in neuronal populations within the striatum, the target of DA neurons of the SNpc, that instead exhibit very low levels of LRRK2 mRNA (Gaiter et al., 2006; Higashi et al., 2007; H. Melrose et al., 2006; H. L. Melrose et al., 2007). In parallel, other lines of evidence confirmed the same expression pattern for LRRK2 protein levels (Higashi et al., 2007). More precisely, LRRK2 was detected in mouse SNpc and in human DA SNpc cells, even if its expression is reported to be relatively low

compare to the BG (Greggio et al., 2006; Iannotta et al., 2020; West et al., 2014; Westerlund et al., 2008). Furthermore LRRK2 appears to be enriched in certain *striatum* subcompartments, named striosomes (Mandemakers et al., 2012; West et al., 2014). The function of striasomal compartments is still debated, but a quite recent work has suggested that neurons of these striatal regions have specific functional features and that they represent the predominant striatal output to SNpc DA neurons (McGregor et al., 2019), suggesting that Lrrk2 could affect striosomes function and that this expression profile could be related to PD. In the mouse striatum Lrrk2 RNA and protein have been clearly detected in the majority of both direct and indirect GABAeric MSNs, constituting the 90% of striatal neurons, and Lrrk2 protein also in about 10% of cholinergic interneurons, that modulate the release of striatal dopamine (Higashi et al., 2007; Mandemakers et al., 2012; West et al., 2014). LRRK2 is also present in several non-PD related areas such as hippocampus (in particular in supremammilary nuclei and GABAergic interneurons), thalamus (where it is expressed widely), brainstem (in various nuclei and in cholinergic motor neurons) and also cerebellar cortex and the deep cerebellar nuclei.

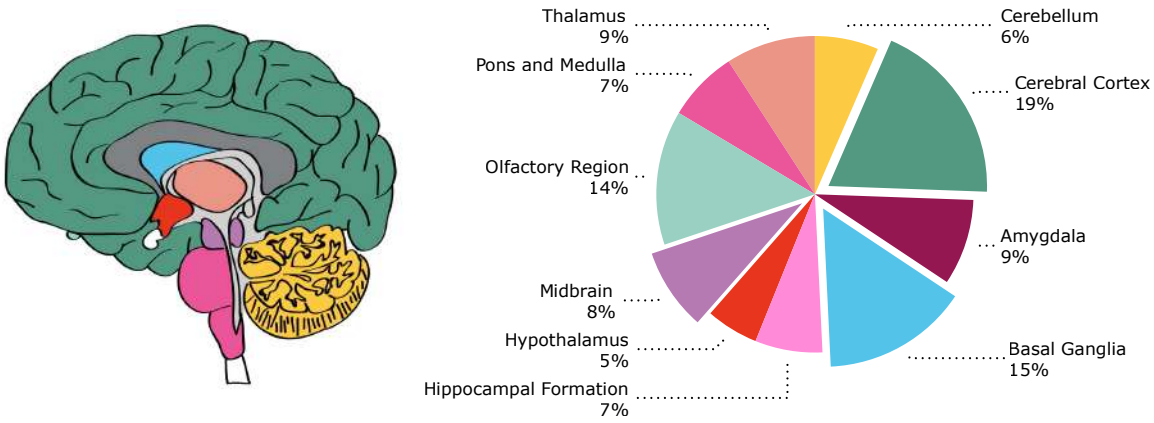


Fig.17: Lrrk2 mRNA expression levels in the brain: *On the left, a graphic illustrating the normalized mRNA expression levels of Lrrk2 in the different brain anatomical areas. On the right, a brain image illustrating the different brain anatomical areas colored as in the graph to allow their visualization. Adapted from: https://www.proteinatlas.org/ENSG00000188906-LRRK2/brain.*

Interestingly, the developmental gene expression pattern of LRRK2 appears to be complementary to the one of its paralogue LRRK1 (Biskup et al., 2007; Westerlund et al., 2008). In the brain, while Lrrk1 displays relatively high expression levels during embryogenesis, with a pick around E15.5 and a subsequent decline below Lrrk2 levels

after birth, Lrrk2 amount increases progressively from embryonic day 15 to day 17 and then stabilizes remining almost unchanged throughout the adulthood. Contrary to the brain, in peripheral tissues such as lungs, heart and skeletal muscles similar levels of Lrrk1 and Lrrk2 were detected once reached the maturation. This highlights a putative compensative function by Lrrk1 when Lrrk2 is not working properly: this Lrrk1 compensatory role may lack in the brain where its levels are low, implying a possible consequence on the develop of PD brain pathology .

At a subcellular level, in many brain area, such as cortex and striatum, Lrrk2 is mainly localized in the cytoplasm confined in the soma and proximal dendrites (Biskup et al., 2006; West et al., 2014). In this environment, it is often visualized with a punctate organization: indeed, it has been extensively demonstrated a LRRK2 association with a variety of membranous and vesicular structures, MTs and membrane bound organelles. These elements include lysosomes, transport vesicles, mitochondria and also endosomes. Thus, a possible involvement of LRRK2 in the biogenesis, trafficking and recycling of vesicular and membranous intracellular structures has been suggested.

3.3. LRRK2 biochemistry

LRRK2 is a complex multi-domain protein owing both a ROC and a COR domain. The presence of this bidomain defines Lrrk2 as a member of the so called ROCO proteins family, part of the Ras/GTPase superfamily (Bosgraaf & Van Haastert, 2003). In the ROCO proteins RAS-COR superdomain exists within a large set of others functional domains. In human, there are four genes encoding for ROCO proteins (e.g. Malignant fibrous histiocytoma amplified sequences with leucine-rich tandem repeats 1 (MASL1), Leucine-rich repeat kinase 1 (LRRK1), LRRK2 and death-associated kinase 1 (DAPK1)) differing principally in the domains that surround the ROC-COR region: among them, one does not possess the kinase domain (MASL1), whereas the other three, including LRRK2, display kinase activity. The kinase domain is a serine/threonine kinase that belongs to the family of mitogen-activated protein kinase kinase kinases (MAPKKK). Of note, LRRK1 is the closest LRRK2 paralog in mammals and encodes a protein with similar domain organization, differing mainly in the terminals regions: indeed, LRRK1 lacks the ARM domain and the binding sites for 14-3-3 proteins at the N-terminus; moreover it possesses a C-terminus with a low degree of homology with the LRRK2

WD40 domain (Civiero et al., 2012). Interestingly, no mutations in LRRK1 have been so far associated with the development of PD (Taylor et al., 2007) and when LRRK2 mutations are reproduced in LRRK1, a lower toxicity is detected *in vitro* (Greggio et al., 2007), overall suggesting a divergent functions of these two proteins.

The two enzymatic domain have been extensively investigated to shed light on how they relate to each other in modulating LRRK2 activity. As a phospho-protein, LRRK2 is predicted to phosphorylate several targets: among these, LRRK2 itself has been widely confirmed as the main one. Indeed, LRRK2 is able to auto-phosphorylates *in vitro* more that 20 serine and threonine residues, that are grouped in two main autophosphorylation clusters (Fig.16). One resides in the kinase domain, while the bigger one sits in and around the ROC-COR bidomain (Gloeckner et al., 2010; Greggio et al., 2009; Kamikawaji et al., 2009; Marchand et al., 2020). However, their existence *in vivo* is still under investigation, with the exception of Ser1292 that has been detected in brain, lung and kidney lysates from transgenic G2019S knock in (KI) mice. Among the auto-phosphorylated residues identified in the kinase domain, only T1967, S2032 and T2035 have been demonstrated to modulate the enzymatic activity of this domain (Xiaojie Li et al., 2010). Notably, many of the auto-phosphorylation sites found in ROC-COR bidomain are located specifically within the GTP binding pocket, strongly suggesting that kinase activity modulates GTP binding and hydrolysis (Gloeckner et al., 2010). Nevertheless, none of the mutations in the kinase domain were demonstrated to impact on GTPase activity (Wallings et al., 2015). Reciprocally, it was extensively proposed that the GTPase domain influences Lrrk2 kinase activity. Many evidence indicate a gain of kinase activity induced by Roc domain (Marín et al., 2008): for example, it has been shown that the binding of non-hydrolysable GTP analogues stimulates the kinase activity (Korr et al., 2006). LRRK2 ROC domain is a G-protein and works as molecular switcher cycling between an GTP-bound active state and a GDP-bound inactive state (Liao & Hoang, 2018). Until fairly recently, the accepted view was that LRRK2 presents higher kinase activity when in its GTP-bound active conformation, while the enzymatic activity decreases once GDP is bound (Greggio & Cookson, 2009). This theory has been overcome when LRRK2 kinase activity was demonstrated to depend on the presence of a functional GTPase domain, capable to bind guanosine nucleotides, regardless the guanosine nucleotides type (Taymans et al., 2011). Back in 2008, the dimeric nature of LRRK2 under native conditions has been reveled (Greggio

et al., 2008) and, later, it has been further confirmed by electron microscopy (Civiero et al., 2012). According to the model developed by Guaitoli and collaborators (Guaitoli et al., 2016), the dimerization of LRRK2 monomers is mediated by the interaction among two ROC-COR domains. Importantly, it has been also found that LRRK2 dimeric conformation makes the protein an active serine-threonine kinase. Reciprocally, both the kinase and the GTPase activity are important to stabilize dimeric LRRK2: reduced kinase activity leads to higher molecular weight oligomers unable to phosphorylate substrates and the R1441C/G and Y1699C ROC-COR mutations reduce dimerization with a subsequent lower LRRK2 GTPase activity. The latest insights into the cycle of ROCO proteins comes from a novel study in which the dimeric nature of LRRK2 is taken into account: basically the ROCO proteins are proposed to switch between dimeric and monomeric form during GTP hydrolysis (Deyaert et al., 2017).

Besides autophosphorylation sites, there is also a cluster of serine residues (S860/908/910/935/955/973/976) at the N-terminus and one (S1444) in the ROC domain which are constitutively subjected to heterologous phosphorylation, that finely impacts on LRRK2 function. Casein kinase 1 (CK1α) and the IkB family of kinases have been found to mediate the phosphorylation at the mentioned sites (Chia et al., 2014; Dzamko et al., 2012). In addition, phosphorylation is counter-regulated by cellular phosphatases, the most validated ones being protein phosphatase 1 (PP1) and protein phosphatase 2 (PP2A) (Athanasopoulos et al., 2016; Lobbestael et al., 2013). Given that S910/935 phosphorylation is decreased in pathogenic mutants or kinase-inhibited LRRK2, they are considered an indirect readout of LRRK2 activity (X. Deng et al., 2011; Vancraenenbroeck et al., 2014). Additionally, when phosphorylated, S910/935 bind 14-3-3 proteins, important effector in the regulation of LRRK2 subcellular localization, kinase activity and substrates/interactors availability (Nichols et al., 2010).

3.3.1. LRRK2 interactors and substrates

Over the past decade a notable amount of interactors and substrates have been described for Lrrk2 and the search of unique Lrrk2 function has been very challenging. An attractive explanation is that Lrrk2 behaves as a “date-hub” (Manzoni, 2017), i.e. as a very flexible protein able to form macromolecular complexes with different proteins in different locations, developmental stages and under specific stimuli. This idea

reflects the high complexity of Lrrk2 and also its expression in various tissues and cell types.

A recent study on human and mouse LRRK2 protein interactions has reported into the IntAct database the presence of approximately 2400 unique interactors (Gloeckner & Porras, 2020). Interestingly, In 2015 Manzoni and colleagues performed a *in silico* investigation of LRRK2 interactome based on the previous published literature (Manzoni et al., 2015). Among all the putative interactors reported, they obtained filtered list of hits that appear mostly associated with cellular transport and trafficking and implicated in the regulation of enzymatic events orchestrating cytoskeleton and vesicles dynamics.

Indeed many studies reported an interaction of LRRK2 with proteins involved in cytoskeletal dynamics, synaptic transmission and vesicular trafficking. Among these it is worth mentioning those related to cytoskeletal function that include microtubules and filamentous actin, together with some actin-regulatory proteins such as PAK6, ERM and WAVE2 proteins. Moreover, several of pre- and post- synaptic proteins controlling SVs trafficking have been observed to interact with LRRK2, e.g. Endophilin A (EndoA), SynapsinI, N-ethylmaleimide sensitive factor (NSF). LRRK2 also interacts with proteins involved in protein translation (e.g. 4E-BP1 and s15) and in autophagic flux (e.g. TPC2 and p62) (Cogo & Greggio, 2020). Of note, a subset of 14-3-3s proteins have been identified back in 2010 as capable of binding phosphorylated S910 and S935 in LRRK2, influencing LRRK2 localization, stability and activity (Iannotta & Greggio, 2021). Additionally, certain Rab GTPases protein, controlling vesicular trafficking, have been shown to interact with LRRK2 or with other PD-related proteins (e.g. α-synuclein, PINK1 and Parkin, VPS35 and TMEM230) (Cogo & Greggio, 2020).

Noteworthy, some of LRRK2 interactors serve also as substrates of its kinase activity that, consequently, impacts on their physiological functions. The identification of LRRK2 substrates represent a remarkable milestone in the PD research, given they can serve as readout of pathogenic LRRK2 activity in PD patients. Several *in vitro* substrates have been reported over the years (Cogo & Greggio, 2020), such as synaptic proteins (endophilin A1, NSF, WAVE2), factors involved in protein translation (4E-BP1) and microtubule-associated proteins (MAPs) (moesin, β tubulin, tau) (Gillardon, 2009; Jaleel et al., 2007; Kawakami et al., 2012). Nevertheless, an important contribution in the delineation of LRRK2 function came when a subset of Rab GTPases were validated

as LRRK2 kinase *bona fide* substrates (Steger et al., 2016), 12 years after the initial cloning of LRRK2. RAB proteins belong to the RAS superfamily of small GTPases and represent the master regulators of membrane trafficking, impacting on vesicle budding, tethering, transport and membrane fusion (Homma et al., 2021). Precisely, exploiting a combination of genetic and pharmacological approaches Rab3a, Rab8a, Rab10 and Rab12 were shown to be targets of LRRK2 phosphorylation activity. Moreover, the phosphorylation level of RABs, as well as that of Ser1292, is enhanced *in vivo* by the majority of photogenic mutations, confirming that LRRK2 mutations are gain of function. Later, the same authors systematically investigated whether additional RAB proteins are LRRK2 targets, coming up with a total of 14 proteins, ten of which (RAB3A/B/C/D, RAB8A/B, RAB10, RAB12, RAB35 and RAB43) are phosphorylated without overexpression (Steger et al., 2017).

3.4. LRRK2 functions

The understanding of LRRK2 biological function has been and remains to date an important goal in the field of LRRK2 research. Based on the intriguing idea that it may behave as a date-hub protein, LRRK2 was supposed to modulate a plethora of cellular signaling pathways (Wallings et al., 2015). This hypothesis is also supported by its structure that suggests putative regulatory and scaffolding roles *via* the two enzymatic activities and protein-protein interaction domains, respectively. Indeed, to date LRRK2 has been linked to a variety of signaling pathways. Among them, the mitogen-activated protein kinase pathways (MAPK) has been one of the first candidates to be investigated. It has been reported that LRRK2 binds and phosphorylates MAP2K 3-6 leading to the activation of p38 and MAP2K 4-7 associated with JKN, that affect both cell proliferation and differentiation, apoptosis, inflammation, immune response and the production of cytokines. Moreover, the G2019S LRRK2 has been also found to activate MAP2K1 and MAP2K2 with the consequent hyperphosphorylation of their effectors, thus impacting on autophagy (Wallings et al., 2015). The Wingless signaling pathway (Wnt) is important to induce the transcription of several genes implicated in apoptosis, cell growth, immune functions and inflammation, synaptogenesis during embryonic development and synaptic maintenance in adulthood. A strong interaction between LRRK2 and key components of this pathway has been demonstrated.

Moreover, this interaction is affected by the presence of LRRK2 pathogenic mutation (Wallings et al., 2015).

To date, a clear picture about the molecular mechanisms in which LRRK2 is involved is still missing, however the protein has been associated to several and sometimes unexpected processes and functions in the cell. I will now underlie LRRK2-mediated pathways that might be of particular relevance for PD with a brief overview about the role that LRRK2 plays in inflammation, protein translation, vesicular trafficking, endo-lysosomal, autophagic pathways and axonal transport. In the subsequent sections, I will extensively discuss the contribution of LRRK2 in cytoskeleton dynamic and analyze the implications of this interplay on the synaptic compartment.

3.4.1. LRRK2 in inflammation

The first evidence of LRRK2 linked with inflammation dates back in 2010, when in human immune cells it has been observed an enriched LRRK2 expression, that can be induced by INF-γ stimulation in intestinal tissues upon Crohn's disease (CD) inflammation (Gardet et al., 2010). Additionally, GWAS highlighted that common LRRK2 variants are associated with CD (Barrett et al., 2008).

As microglia represent the first line of defense of innate immune system within the brain, many studies have been conducted to understand a possible role of LRRK2 in this cell type. Interestingly, our group found that a protein kinase A (PKA)-mediated pathway is involved in promoting the attenuation of inflammatory processes in LRRK2 deficiency conditions (Cogo et al., 2020). In parallel, it has been shown that R1441G mutated LRRK2 might prompt the pro-inflammatory microglia phenotype with an exacerbated inflammatory state (Russo et al., 2014). Cytoskeleton reorganization is important for the function of microglia as macrophages, being fundamental for the phagocytic and migration processes. Indeed, several lines of evidence revealed that LRRK2 impacts on both these aspects affecting microglia activity, thus supporting the notion of LRRK2 interaction with cytoskeletal elements. For instance, inflammatory stimuli have been reported to fail to induce evident morphological changes and phagocytosis in inhibited LRRK2 microglia (Russo et al., 2014). Moreover, in myeloid cells, LRRK2 mediates phagocytosis regulation by modulating the actin cytoskeleton via interaction/phosphorylation of the cytoskeletal regulatory protein WAVE2 (K. S. Kim et al., 2018). LRRK2 also acts through class III PI3K/Rubicon complex to inhibit

Mycobacterium tuberculosis autophagosome maturation in macrophages (Härtlova et al., 2018).

3.4.2. LRRK2 in the regulation of protein translation

Several studies have focused in the investigation of LRRK2 as a potential player in protein synthesis. Early studies exploited *Drosophila melanogaster* as a preferential animal model. One of the first evidence that links LRRK2 with protein translation comes from the observation that the translation repressor eukaryotic initiation factor 4E binding protein (4E-BP) represents a substrate of *Drosophila Melanogaster* homologue dlrrk, as dlrrk deficiency leads to reduced phosphorylation and increased activation of 4E-BP (Imai et al., 2008). Instead, an opposite effect has been documented in presence of pathological mutations (Imai et al., 2008). Later, LRRK2/Lrrk has been found to phosphorylate the ribosomal protein S15 both in human neurons and in *Drosophila*, promoting an increase in protein synthesis that is further pushed in presence of PD-mutations (Martin et al., 2014). Another interesting finding is the existence of an interference promoted by LRRK2 on microRNAs, that are known to prevent protein translation. More precisely pathogenic LRRK2 has a negative impact on microRNA activity leading to protein expression that could be toxic for neurons (Gehrke et al., 2010). Furthermore, a genome wide study revealed that the Lrrk2-G2019S overexpression in mouse models positively regulates the transcription process (Nikonova et al., 2012).

Interestingly, several studies reported that by regulating protein translation LRRK2 could also exert a further impact on inflammatory response. Of note, LRRK2 has been described as a negative regulator of nuclear factor of activated T cells (NFAT) transcription factor, decreasing its nuclear translocation. Moreover, LRRK2 deficiency correlates with a reduction in NF-kB transcriptional activity and a subsequent diminished cytokines release (Russo et al., 2014). Intriguingly, LRRK2 is able to modulate the expression of CX3CR1, a receptor that promotes the migration of glial cells at the expense of their inflammatory activity (B. Ma et al., 2016).

3.4.3. LRRK2 and vesicular traffic

Vesicular traffic is the biological process by which different cellular components are transported across different cellular compartments or between a cell and the surrounding environment thanks to the movement of vesicles. The tight regulation of vesicle formation, function and location appear to be fundamental especially in neuronal cells due to their post-mitotic nature and extended morphology. Vesicular traffic underlies numerous important biological processes essential for cell survival and functionality such as nuclear transport, autophagy, endo-lysosomal trafficking, axonal transport, mitochondria dynamic/damage response and NTs release at the synapse (Lewis, 2021). Vesicular biology has been linked to the activity of many proteins whose coding genes have been found mutated in familial neurodegenerative disease or identified as risk loci for these disorder (Lewis, 2021).

Along this line, mutated LRRK2 is emerged in last years as a perturbating factor of intracellular trafficking of vesicles, suggesting a physiological role of the protein in the regulation of this process. Consistently, LRRK2 localizes at the level of multiple membranous compartments where Rab GTPases proteins exert their physiological role (Cookson, 2016) (Fig.18). As mentioned above, Rab GTPases regulate all the aspect of membrane traffic, impacting on the formation, maturation, transport, tethering and fusion of vesicles. Basically, when in GDP-bound state, the RAB protein inserted into a specific target membrane is activated by guanine nucleotide exchange factor (GEF) that promotes the binding of GTP. Appropriate effector proteins are recruited by GTP-bound Rab protein that, consequently, is hydrolyzed back to its inactive state and is extracted from the membrane by the action of GDP dissociation inhibitor (GDI) to start a new cycle (Hur et al., 2019). As a subset of RABs are LRRK2 substrates, it has been suggested that these proteins may control the recruitment of LRRK2 to the membranes.

Among the Rab GTPases proteins suggested to have an interaction (physical, genetic or functional) with LRRK2 we can find Rab5, Rab32 and Rab38. Rab5 proteins target clathrin-coated endocytic vesicles to endosomes, thus representing orchestrators of the SVs recycling at the presynaptic and synaptodentritic compartments. Moreover it has been proposed also a role for these protein in autophagy. Rab 32 and Rab38 have been reported to present an elevated expression in melanocytes (Cookson, 2016).

Rab7L1, also named Rab29, is particularly relevant for at least two reasons. First, it has been shown to interact genetically and biochemically with LRRK2 by different laboratories. Second, it is considered a sporadic PD risk factor due to its location in *PARK16* locus. Rab29 is involved in the retromer-associated sorting between lysosomes and the trans-Golgi network (TGN). It has been suggested that Rab29 can recruit LRRK2 to this membranous compartment increasing its kinase activity and inducing the subsequent LRRK2 mediated-phosphorylation of Rab8 and Rab10. In addition, LRRK2 can be recruited to stressed lysosomes by Rab7L1 and inducing the accumulation of other LRRK2 substrate (such as Rab3, Rab8, Rab10, and Rab35) at the level of these organelles (Bonet-Ponce & Cookson, 2021; Cookson, 2016; Hur et al., 2019).

Of note, Rab8 is implicated in tubular endosome formation in the recycling of several proteins such as receptors (M. Sharma et al., 2009). Rab10 and Rab12 are found associated to numerous membranous compartments and regulates the endo-lysosomal pathway (Bae & Lee, 2020). In addition they have been shown to intervene in the autophagic process. Importantly Rab10 is essential for the endocytic recycling compartment formation and impacts on ER dynamics and morphology (English & Voeltz, 2013). The Rab GTPases affinity for their regulatory partners GD1/2 and GEF Rabin8 decreases when LRRK2 is phosphorylated, resulting in an increased binding between Rab8a/Rab10 and RILPL1/2 that impacts on centrosome positioning and cilia formation (Iannotta & Greggio, 2021). Moreover, there are evidence linking Rab3 and Rab35 in the regulation of vesicle exocytosis (Bae & Lee, 2020) . Of interest it has been shown that Rab35 mediates the propagation of α-Syn upon LRRK2 phosphorylation: the overexpression of WT LRRK2 or G2019S mutant enhanced the propagation of α-Syn that is indeed reduced when LRRK2 kinase activity is inhibited (Iannotta & Greggio, 2021).

Steger and collaborators proposed that photogenic hyperactive mutant LRRK2 affects the cellular and membranous distribution of its substrates like Rab8, Rab10 and Rab12, hyperphosphorylating them and promoting in this way their localization to the membranes. The result of such alteration is an abnormal Rab GTPases distribution and a consequent possible acquirement of pathogenic function (Steger et al., 2016).

All together these evidence imply that LRRK2, *via* Rab GTPase interaction and phosphorylation, can intervene in in multiple critical processes of the vesicular trafficking. In the next two paragraphs I will provide a better insight in LRRK2 involvement in endo-lysosomal and autophagic pathways.

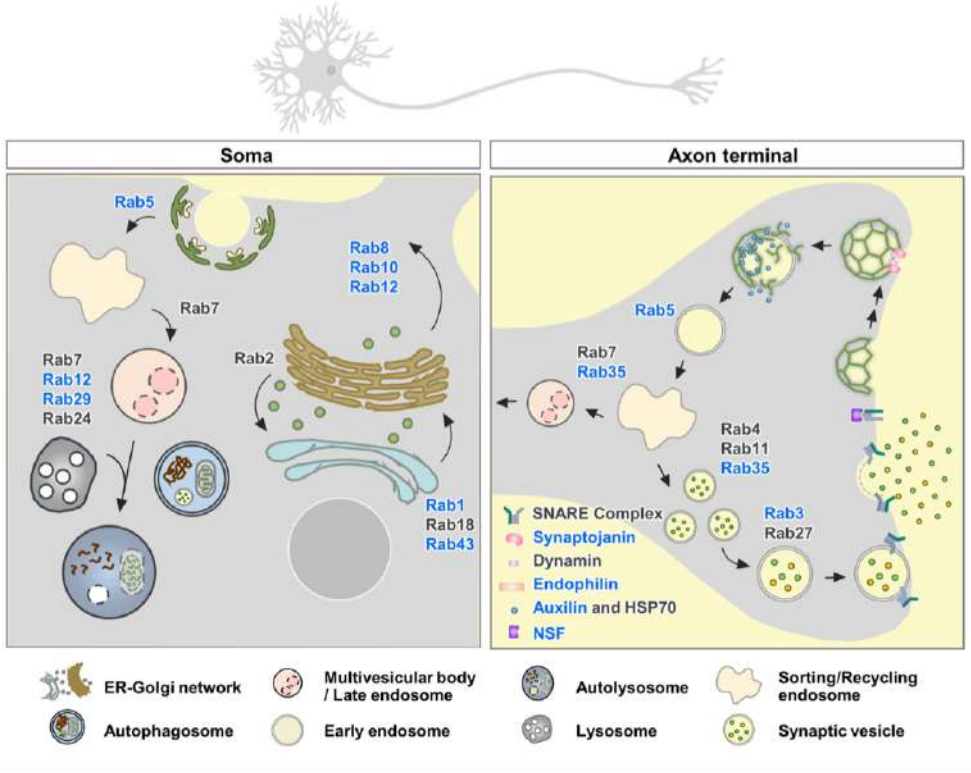


Fig.18: Cellular functions of LRRK2-interacting RAB proteins in vesicular trafficking: *Image showing the principal Rab proteins involved in vescicluar trafficking regulation. The proteins representing substrates of Lrrk2 kinase activity are reported in blue. Adapted from (Hur et al., 2019)*

3.4.3.1. The role of LRRK2 in the endo-lysosomal pathway

The endo-lysosomal system is made by a number of intracellular membranous compartments that mediate the internalization, recycling and degradation of several players involved the physiological cell function. These membranous organelles are highly dynamic as capable to interconvert early endosomes, recycling endosomes, late endosomes and lysosomes. Early endosomes represent a sorting station from where cargo can take different routes: they can return to the plasma membrane, reach the TGN or be targeted for degradation. In the latter case, early membranous organelles

mature in late endosomes that, in turn, fuse with lysosome and, consequently, cargo are subjected to proteolytic degradation.

Of note, LRRK2 has been extensively described to influence the activity of several proteins involved in this pathways, such as Rab GTPases.

Alterations in LRRK2 expression or in kinase activity can impact on endosomal trafficking and protein degradation. Moreover, sporadic PD human brain tissue show colocalization of LRRK2 with late endosome protein Rab7b and LAMP2, all accumulated in enlarged granules (Erb & Moore, 2020). LRRK2-Rab7 interaction is supported by the observation that in *Drosophila* dLRRK localizes in the late endosome inhibiting Rab7-induced lysosome clustering. Additionally, the presence of an equivalent G2019S mutation in dLRRK gene prompts the clusters formation (Dodson et al., 2012). Another interesting finding is that pathogenic LRRK2 mutant decreases endosomal budding diminishing Rab7 activity, with a consequent retardation in the trafficking of epidermal growth factor (Gómez-Suaga et al., 2014). In addition the same group reported that impaired endo-lysosomal trafficking correlated to pathogenic LRRK2 is due to an inactivation of Rab8A (Rivero-Ríos et al., 2019). Also the endosomal trafficking of lysosomal membrane proteins (LMPs) such as LAMP1 and LAMP2 has been shown to be regulated by LRRK2 activity via regulation of adaptor protein complex 3 (AP-3). In condition of lysosomal stress, LRRK2 recruits to lysosomes JNK-interacting protein 4 via Rab35 and Rab10 phosphorylation and promotes lysosomal tubulation and sorting. Finally, in stress conditions LRRK2 accumulates to lysosomes or TGN thanks to Rab7L1 activity and stimulates the elimination of dysfunctional organelles through autophagic process or lysosomal exocytosis. (Iannotta & Greggio, 2021)

3.4.3.2. The role of LRRK2 in autophagy and synaptic autophagy

Autophagy is one of the process considered to be impaired in PD. Notably, macroautophagy was proposed as one of the mediators of retrograde axonal degeneration (Cheng et al., 2011), although the precise molecular mechanisms are still unknown. A great body of evidence suggests an involvement of LRRK2 in the regulation of lysosomal-based degradative pathways (Cogo et al., 2020), including the autophagic process. For instance, it has been reported that the macroautophagy flux *in vitro* is modulated by the overexpression of WT LRRK2 and the expression of mutated LRRK2

in cell models, as well as the LRRK2 kinase activity inhibition and knock down. However, it is still unclear if the macroautophagy process is regulated positively or negatively by LRRK2 (Wallings et al., 2015). Noteworthy, from the evaluation of macroautophay markers in brain tissue, it emerged that the BG of individuals carrying G2019S mutation exhibits decreased levels of LAMP1, supporting a model of impairment of lysosomal targeting in PD (Mamais et al., 2018). Importantly, several studies show that in LRRK2 pathological models autophagic defects are found at the periphery, in the axon and neurites. The description of BAC transgenic mouse model expressing the human disease-causing LRRK2 (R1441G) mutant suggests an early pathology that affects the terminals of the DA system. Young mice display accumulation of autophagic vacuoles in "giant", polymorphic axonal spheroids in the medial forebrain bundle. They also present other axonal abnormalities, including intra-axonal autophagic vacuoles alone and intra-axonal myelin invagination. However, there are not modifications in the number and in the morphology of neuronal soma and neurites (Tagliaferro et al., 2015). The neuronal architecture become affected as animal age, with the appearance of clear signs of axonopathy: dystrophic neurite, fragmented axon and phosphorylated tau-positive axonal spheroids. These defects are accompanied by the development of progressive motor deficits (Y. Li et al., 2009). Both in the cortex and in the striatum of another transgenic mice expressing G2019S mutant LRRK2 it was observed the presence of enlarged vacuolar structures with multiples membranes resembling autophagic vacuoles, including early and late autophagosomes, within regions enriched in axon and synapses. R1441C LRRK2 mice display similar but less marked cytopathology in the cortex (Ramonet et al., 2011). A study carried out on autophagy deficient mouse model showed an early dendritic and axonal dystrophy in DA neurons of the midbrain lacking in autophagy gene Atg7. They also demonstrated for the first time that this impairment in the autophagy brings to presynaptic accumulation of LRRK2 within *cerebellum* and autophagy-deficient MEF cells. Further evidence supports a role for LRRK2 in the neurites growth and maintenance. It has been demonstrated that in primary cortical neurons following the expression of mutant forms of LRRK2 there is a degeneration of neurites length and branching (MacLeod et al., 2006). In particular, the overexpression of mutated G2019S LRRK2 in these cultured neurons induces the accumulation of swollen lysosomes and multivesicular bodies, distended vacuolated mitochondria and phosphorylated tau-

positive spheroid axonal inclusions. Moreover, in neuronally differentiated neuroblastoma cells the G2019S mutant induces shortening of neurites with a concomitant activation of autophagy process (Plowey et al., 2008). Furthermore, a recent study demonstrated that LRRK2 regulates autophagy at the level of the synapse thought EndoA phosphorylation (S. F. Soukup et al., 2016). Taken together, these data highlight a significant function for LRRK2 in the autophagic process both *in vitro* and *in vivo*.

3.4.4. The role of LRRK2 in axonal transport

Altered axonal transport is widely considered to be one of the factor contributing to axonal degeneration in PD. Aggregated α-Syn was demonstrated to be tightly associated with reductions in axonal motor proteins levels in the neurites of nigral neurons of sporadic PD cases (Chu et al., 2012). Interestingly, an interaction between endogenous LRRK2 and α-Syn has been observed in mouse and human brain tissue and further confirmed in cells in overexpressing conditions, suggesting that dysfunctional LRRK2 could affect the accumulation of α-Syn contributing to axonal degeneration (Guerreiro et al., 2013). Moreover, LRRK2 was found to be in connection with microtubule cytoskeleton. MTs form tracks made of α and β tubulin polymers along with motor proteins to move cargos (proteins and organelles), along dendrites and axons. LRRK2 interacts with tubulins through its ROC domain (Gandhi et al., 2008) and human recombinant LRRK2 is able to phosphorylate β tubulin, enhancing microtubule stability (Gillardon, 2009). Additionally, in LRRK2 KO MEF cells the level of β tubulin acetylation is increased meaning that, once bound to MTs, LRRK2 interferes with microtubule acetylation (Law et al., 2014). Notably, it was demonstrated that the rate of transport along MTs is influenced by the status of their acetylation, with increased acetylation promoting axonal transport (Reed et al., 2006). Another work reported that LRRK2 carrying R1441C and Y1699C mutations in the ROC-COR domain localizes selectively on deacetylated MTs *in vitro* and harms axonal transport in primary neurons and in *Drosophila Melanogaster*, leading to locomotor deficits *in vivo*. Thus, it seems that the inhibition of axonal transport is connected to decreased LRRK2 GTPase activity. By increasing MT acetylation the physiological phenotype is restored (Godena et al., 2014). Another interesting study reported that fibroblasts derived from patients carrying the G2019S LRRK2 mutation display huge increase in acetylated tubulin and

a destabilization of MT, together with a marked alteration of cellular morphology compared to healthy controls (Cartelli et al., 2012). Very recently, exploiting a wide variety of model system, the axonal transport of autophagic vesicles (AVs) has been found to be damaged in presence of G2019S mutation. In particular the LRRK2 increased kinase activity causes a tug of war between anterograde and retrograde transport of AVs *via* re-localization of JIP4 at the level of AVs membrane and the activation of kinesin. Moreover the same effect was observed by overexpressing Rab29, corroborating a possible involvement of the two protein in the same pathway (Boecker et al., 2021). Possibly, both LRRK2 enzymatic domains influence microtubule stability and post-transactional modification, affecting the axonal transport.

3.4.5. Lrrk2 and cytoskeleton

The maintenance of structural polarity is essential for the physiological function of neurons and it is provided by the combination between MTs and actin, the two main components of the cytoskeleton. Actin plays important role in synaptic maintenance and function and also in supporting neurite outgrowth and growth cone navigation (Cingolani & Goda, 2008). MTs cytoskeleton ensures mechanical support, influences local signaling events and provides tracks for long-distance cargos transport (Kapitein & Hoogenraad, 2015). Growing body of evidence suggest a physical and functional association of Lrrk2 to cytoskeletal-related components in neurons (Parisiadou & Cai, 2010).

From a structural point of view, MTs are polymers made by α and β tubulin subunits. As mentioned before, LRRK2 was showed to interact with tubulin *via* its ROC domain in a guanine-nucleotide independent fashion (Gandhi et al., 2008). Consistently, a colocalization between the two proteins was found both in primary neurons (Gandhi et al., 2008) and mouse brain (Lin et al., 2009). In addition, LRRK2 stabilizes MTs during neurite outgrowth phosphorylating β-tubulin, preferentially in Thr107, and this effect is enhanced in presence of G2019S mutation, possibly resulting in a deregulation of microtubule dynamics (Gillardon, 2009). LRRK2 was also reported to interfere with tubulin acetylation, as demonstrated by the increase in β-tubulin acetylation documented in LRRK2 KO models (Law et al., 2014). Of interest, it has been found that transgenic mice overexpressing Lrrk2 present an increased fragmentation of the Golgi apparatus as consequence of enhancement of tubulin stabilization: this results in an

ER-Golgi vesicular trafficking impairment that could be the cause of α-Syn accumulation in the brain of mice co-expressing A53T α-syn and LRRK2 (Lin et al., 2009). Along this line, an accumulation and hyperphosphorylation of MT-associated protein Tau has been described in several transgenic mice expressing mutant G2019S LRRK2. These studies point out an implication of Lrrk2 in promoting α-Syn and Tau mediated PD neuropathology *via* deregulation of MTs dynamics.

Back in 2006, MacLeod and colleagues realized for the first time that LRRK2 is involved in the maintenance of neuronal process length and complexity (MacLeod et al., 2006): in contrast to LRRK2 WT neurons, mutant LRRK2 G2019S neurons display reduced neurite outgrowth together with the accumulation of inclusions positive for tau. In the same period LRRK2 was demonstrated to phosphorylate the protein moesin at Thr558, regulating its activation state (Jaleel et al., 2007). Moesin, acting jointly with the others two ERM proteins ezrin and radixin, links the actin cytoskeleton to the plasma membrane. This finding is the first clear evidence of LRRK2 involvement in actin dynamics. It was also found that increased amount of pERM and F-actin enriched filopodia is associated with the impairment of neurite outgrowth reported in mutant LRRK2 G2019S (Parisiadou et al., 2009). Soon after, the analysis of LRRK2 interactome revealed that LRRK2 binds actin and is able to promote its polymerization (Meixner et al., 2011). Among the LRRK2 interactors identified over the years, the p21-activated kinase 6 (PAK6) appears particularly interesting. Its expression is almost restricted to the brain, where it is found at elevated levels, and it belongs to PAKs proteins. PAKs are a group of serine-threonine kinases regulating actin dynamics through LIM kinase-cofilin pathway (Civiero & Greggio, 2018): activated PAKs phosphorylate and trigger the activity of LIM-kinase which downregulates cofilin activity by phosphorylation, resulting in the inhibition of actin severing and in filamentous actin stabilization. The kinase activity of PAK6 promotes neurite complexity *in vivo* in the mouse striatum by binding to the GTPase/ROC domain of LRRK2, which stimulates PAK6 autophosphorylation and LIM kinase pathway activation (Civiero et al., 2015). Moreover, PAK6 negatively influences LRRK2 phosphorylation: it binds and phosphorylates 14-3-3γ inducing the loss of its affinity for Lrrk2 phospho-Ser935 and rendering this way LRRK2 an easy target of phosphatases (Civiero, Cogo, et al., 2017). Importantly, the neurite shortening phenotype showed by mutant G2019S neurons, is rescued by the expression of an hyperactive form of PAK6, *via* a mechanism dependent

on 14-3-3γ phosphorylation at Ser59. Altogether, these findings indicate that LRRK2 regulates actin-cytoskeleton dynamics by interplaying with multiple targets.

As widely emphasized in the first chapter of this dissertation, neurons are highly specialized non-mitotic cells, characterized by processes (both dendrites and axons) often extending at long distance from the soma. In this scenario, vesicle traffic along the cytoskeletal elements, that is implicated in multiple physiological processes, becomes of primary importance for the maintenance of neuronal homeostasis. The discovery of a functional link between LRRK2 and a subset of Rab GTPases has strengthened the already known role of LRRK2 in orchestrating vesicles dynamics. Based on all these considerations, an interesting hypothesis is that Lrrk2 behaves like a bridge between vesicles and cytoskeleton. In support of this, the main cellular processes attributed to LRRK2 are connected to MTs and actin dynamics: neurite outgrowth, synaptic vesicles trafficking, axonal transport, Trans-Golgi transport, macroautophagy, endocytosis and phagocytosis. In particular, at the synapse LRRK2 orchestrates the movement of molecules involved in the neurotransmission.

3.4.6. Lrrk2 at the presynaptic compartment

At the presynaptic site LRRK2 has been demonstrated to bind SVs through interaction with actin and a number of presynaptic proteins, potentially covering a key role in governing different steps of the bouton-specific SVs dynamics (Fig.19A).

Lrrk2 was shown for the first time to have a role in regulating SVs endocytosis when, in 2008, Shin and colleagues found that it interacts with Rab5b and that both endogenous LRRK2 overexpression or knockdown strongly harm the endocytic process (Shin et al., 2008). Later, LRRK2 has been also described to obstruct the clathrin-dependent endocytosis phosphorylating some of its effectors (Pischedda & Piccoli, 2021). Among them, EndoA acts in the initial phases of endocytosis promoting the curvature of plasma membrane. It has been identified as a substrate of LRRK2 in *Drosophila melanogaster* (Arranz et al., 2015) and later also in mammalian cells (Matta et al., 2012) by the same research group. They also showed that G2019S mutant increases EndoA phosphorylation altering its recruitment to clathrin-coated pit, fundamental for the uncoating of vesicles. Additionally, they found that the KO of LRRK2 causes a significant delay in clathrin-mediated endocytosis in striatal neurons along with ultrastructural abnormalities consisting in aberrant endocytic

intermediates (Matta et al., 2012). Interacting with dynamin, EndoA stimulates the separation of SVs from the plasma membrane and the following recruitment of synaptojanin1 (synj1). Synj1 enables the removal of clathrin coat facilitating the attachment of auxilin. The exacerbated kinase activity that mutant LRRK2 exerts towards dynamin (Stafa et al., 2014), synj1 (Islam et al., 2016) and auxilin (Nguyen & Krainc, 2018) negatively influences the endophilin-dependent endocytosis. Alongside, LRRK2 has been also clearly showed to promote the exocytosis process. Among the proteins involved in SVs cycle, NSF and synapsin I have been identified as LRRK2 kinase activity substrates. NSF is phosphorylated by LRRK2 at T645 with a subsequent increase of its ATPase activity that promotes its ability to dissociate the SNARE complex (Belluzzi et al., 2016). Moreover, LRRK2 kinase activity affects the phosphorylation of synapsin I, implicated in the tethering of the SVs, at T337 and T339 residues. The G2019S mutation has been reported to increase this phosphorylation hampering the interaction among SVs and actin (Marte et al., 2019). It was also shown that this pathological mutation decreases the synapsin I phosphorylation at residues that are not LRRK2 substrates, exerting the opposite effect on its association to actin and SVs (Beccano-Kelly et al., 2014). Moreover, the Ca^{2+} inward current via pre-synaptic CaV2.1 channel is enhanced in presence of G2019S LRRK2 mutant (Bedford et al., 2016). Eventually, LRRK2 is also able to phosphorylates sinapin, inhibiting its interaction with SNAP25 and prolonging this way the exocytic release with a concomitant depletion of the RRP (Yun et al., 2013). Thus, it appears clear that LRRK2 is involved in promoting the exocytosis process.

It is noteworthy that the C-terminal WD40 LRRK2 domain has been found to bind some of the mentioned SVs-associated proteins such as synapsin-I, NSF, dynamin-1, and synaptojanin but also with others like MUNC18-1, VAMP2, synuclein and syntaxin 1A (Piccoli et al., 2011, 2014).

Any damage in the correct function of both the endo- and exocytic process may cause defects in synaptic transmission in the long run. Many literature have been produced about this topic. As a first clear evidence, it was found that LRRK2 loss of function does not dramatically affect the NTs release, even if an altered SVs endocytosis was reported in some studies. Essentially, basal and evoked dopamine and glutamate release appear to be unchanged without functional LRRK2 (Pischedda & Piccoli, 2021). Interestingly, the overexpression of human WT LRRK2 causes a decrease in basal

striatal DA release in mice (Beccano-Kelly et al., 2015; H. L. Melrose et al., 2010) and in evoked DA release in rats (Sloan et al., 2016). On the contrary, overexpression of murine WT LRRK2 produces an increase in evoked DA release (Xianting Li et al., 2010), suggesting that the effect of the genetic manipulation depends on multiple factor.

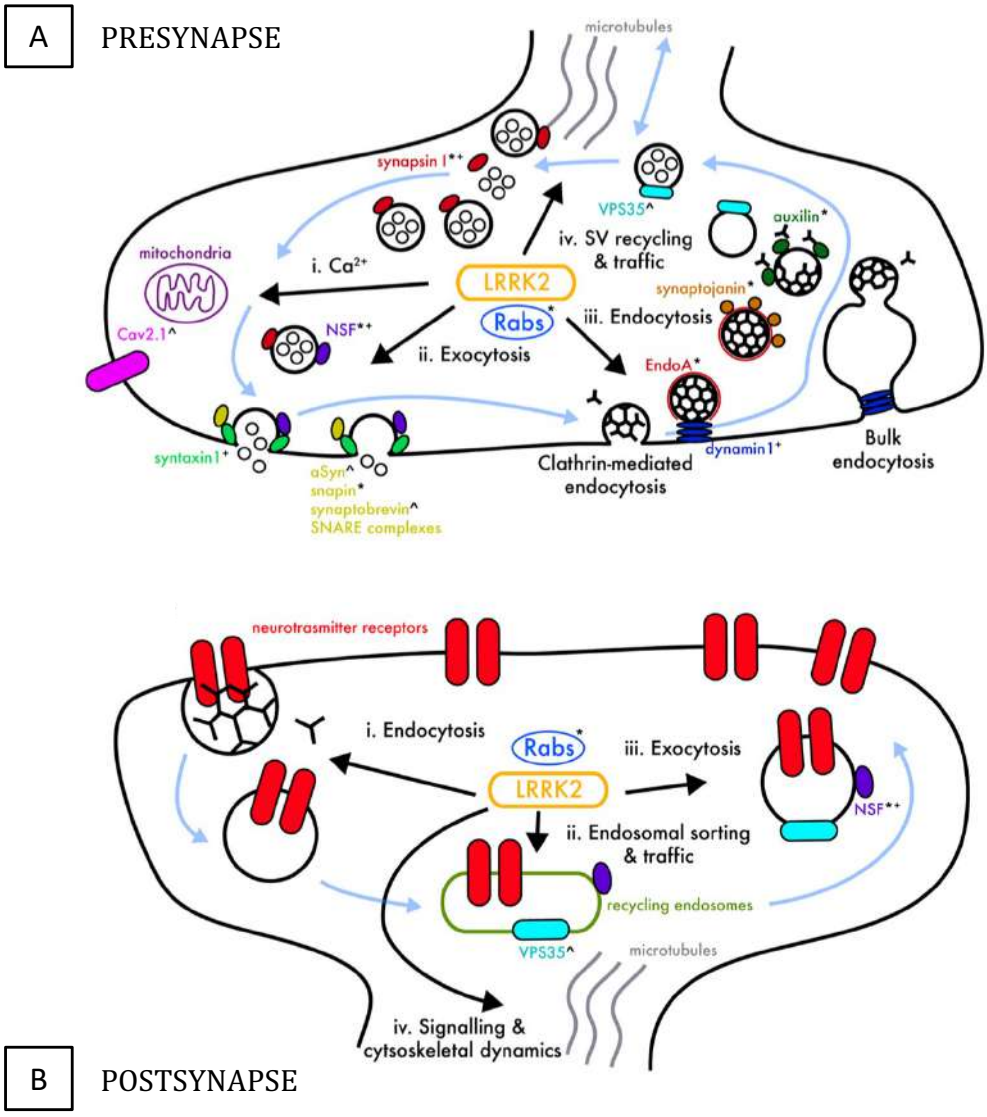


Fig.19: Lrrk2 potential involvement in pre- and post- synaptic pathways. *Adapted from (Kuhlmann & Milnerwood, 2020).* ***(A)*** *Presynaptic element: Lrrk2 is involved in SVs trafficking via interaction with several different proteins like NSF, synapsin, auxilin, synj1, EndoA, syntaxin1, VPS35 eventually affecting the endo- and exo-cytosis processes* ***(B)*** *Postsynaptic element: Lrrk2 has been associated to cytoskeletal dynamics and NTRs trafficking.*

Regarding the pathological mutations, evidence from the literature reported that the BAC model overexpressing LRRK2 G2019S mutation brings an increase in glutamatergic activity, while causes a reduction in DA release (Pischedda & Piccoli,

2021). Also the expression of the G2019S mutation at endogenous levels has a stimulation effect on glutamatergic neurons, indeed it has been reported an increased glutamate miniature event frequency in mutated cortical neurons (Beccano-Kelly et al., 2014) and also increased glutamatergic transmission in mutated striatal slices (Matikainen-Ankney et al., 2016; Volta et al., 2017). Moreover, some alterations in dopaminergic transmission were highlighted in this KI model: for example 3 months old mice present an increased DA release together with an elevated extracellular DA lifetime (Volta et al., 2017), whereases in 12 months old mice striatum there is a reduction in DA levels around the 50% (Yue et al., 2015). Thus, the majority of the literature suggest that G2019S models possesses an enhanced glutamatergic activity with a parallel decrease in DA release: it has been hypothesized that the mutation, operating from the postsynaptic glutamatergic element, could weaken dopaminergic transmission (Pischedda & Piccoli, 2021).

3.4.7. Lrrk2 at the postsynaptic compartment

The function of LRRK2 in the post-synapse has not been so investigated as its role in the presynaptic element. Nevertheless, some studies have suggested that the protein is actually able to impact also in this compartment (Fig.19B).

Interestingly, growing body of evidence bared an involvement of LRRK2 in NTRs trafficking. For instance, DRD1 have been shown to possess an altered distribution in the striatum of G2019S mice in respect to WT: precisely a significant increase in the membrane-associated receptors has been documented (Migheli et al., 2013). Later, the same group corroborated this finding, observing a compromised DRD1 internalization in presence of mutant G2019S LRRK2. In addition they found that this mutation reduces the rate of DRD2 trafficking from the Golgi to the cell membrane (Rassu et al., 2017). Recently, Tozzi and colleagues have provided evidence that a strong reduction of the glutamatergic activity of striatal neurons carrying the G2019S mutation is triggered by the activation of DRD2 receptors, with an endocannabinoid-dependent mechanism of action (Tozzi et al., 2018). Similarly to DA receptors, also the glutamatergic receptors traffic is affected by mutant LRRK2. For instance, mice carrying the G2019S mutation are deficient in calcium-permeable AMPA receptors content (Matikainen-Ankney et al., 2018); moreover in hippocampal slices from mice overexpressing the G2019S mutation it was found an altered AMPA/NMDA receptor

ratio suggesting an aberrant recycling of these receptors (Sweet et al., 2015). Different hypothesis were made regarding the molecular mechanisms by which LRRK2 influences the receptor trafficking. For example, it has been found to interact with Sec16A, important for the ER-Golgi export: in the absence of LRRK2 activity the activity-dependent transport of glutamate receptors onto cell surface is harmed (Cho et al., 2014). It is also likely that LRRK2 affects AMPA receptors trafficking *via* its phospho-targets Rab8a or NSF, both reported to mediate the insertion of AMPA receptor subunits in the postsynaptic membrane. An elegant work of Parisiadou and colleagues (Parisiadou et al., 2014) identified LRRK2 as capable to negatively modulate the activity of the regulatory subunit IIb of the cAMP-dependent protein kinase A (PKA) controlling its predominantly dendritic shaft localization. The loss of Lrrk2 triggers the PKA recruitment within the spines, where the enzyme phosphorylates both the actin related protein cofilin and the glutamate receptor GluR1, involved in synaptic formation and transmission. Cofilin phosphorylation affects cytoskeleton dynamics and is mediated by LIMK1, recently found to be linked with LRRK2 (Civiero et al., 2015). Moreover, GluR1 phosphorylated by PKA is well known to have a crucial role in spine maturation and plasticity (Esteban et al., 2003). MSNs of Lrrk2 KO pups present indeed abnormal spine morphology and transmission: spines appear less mature and less functional (decrease of mEPSCs frequency), suggesting a delay in their development (Parisiadou et al., 2014). Additionally, PKA-dependent phosphorylation of GluR1 was abnormally enhanced in both young and aged LRRK2 KO mice after treatment with a D1 agonist, suggesting that LRRK2 maintains its negative regulator activity of PKA function also after the developmental phase. Also the LRRK2 R1441C/G mutation induce increased PKA activity in MSNs. To note, another work discovered the presence of larger spines and greater postsynaptic activity in developing LRRK2 G2019S MSNs (Matikainen-Ankney et al., 2016).

Importantly, all the studies mentioned until now do not considered the presence within the striatum of two pathways with a complementary role: the direct and the indirect pathway. This could implicate important limitations in understanding the pathological impact of LRRK2 mutations. Recently, it was observed that both the R1441C and G2019S KI mice present an altered AMPA receptors organization with decrease frequency of mEPSC. Interestingly, these effects are greater for the R1441C mutation and in MSNs of the direct pathway (C. Chen et al., 2020). These evidence imply

that cellular specificity should be take into account when studying LRRK2 function in health and disease.

Chapter 4

Introduction

Brain Derived Neurotrophic Factor

4. Neurotrophins and the brain derived neurotrophic factor

Neurotrophins represent a group of growth factors located within the brain and peripheral tissues that are historically known to modulate several aspects of neuronal function, comprising neuronal growth, survival and maintenance (E. J. Huang & Reichardt, 2001). Notably, during development they regulate the apoptotic programmed cell death of neurons in order to eliminate the improper formed connections and to ensure the appropriate amount of surviving innervating neurons required by the different target organs. In the adulthood neurotrophins stop to be directly involved in the acute survival of neurons, but rather their main function relies in the preservation of neuronal phenotype and function. It has been also discovered that, during adulthood, neuronal electrical activity regulates the neurotrophins synthesis, secretion and action in the brain and that, in turn, neurotrophins are able to modify both synaptic function (transmission) and structure (connectivity) in response to stimulus-dependent activity, suggesting that they play a remarkable role in synaptic plasticity (E. J. Huang & Reichardt, 2001).

So far, four are the members that compose the mammalian neurotrophins family, being Nerve Grow Factor (NGF) the first to be identified in the 1950s by Rita Levi-Montalcini, Stanley Cohen and Viktor Hamburger as a molecule functioning in the peripheral nervous system (PNS), even if years of research clarify that it acts also in the CNS. Back in 1982, Yves-Alain Brade and collogues described a factor exerting analogous neurotrophic and neurotropic activity in CNS neurons become known as BDNF. Subsequently, two more factors were isolated based on their homology to NGF and BDNF: neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4).

All the four neurotrophins are initially synthesized as precursors or pro-neurotrophins containing at their N-terminal signal peptides followed by a pro-region. Pro-neurotrophins can be secreted or undergo proteolytic cleavage by furin or convertases to produce the mature forms, that, after the translation process, form stable, non-covalent dimers. Whilst for a long time a real biological function has been attributed only to mature neurotrophins, several roles have been discovered to be exerted also by pro-neurotrophins. Neurotrophins exert their action by binding and activating two different types of cell surface receptor: the tropomyosin-related kinase (TrK) receptor tyrosine kinases and the p75 neurotrophin receptor. More precisely, it

has been observed that mature neurotrophins preferentially stimulate Trk receptors promoting survival. NGF mainly activates TrKA receptors, BDNF and NT-4 specifically signals through TrKB receptors and NT-3 principally binds TrKC receptors. In addition they can exert their function binding with low affinity the p75 receptor. On the contrary, this type of receptor is activated in a more selectively fashion by pro-forms that, this way, induce apoptosis (Chao, 2003).

Different studies have highlighted the exitance of different neurotrophins sources. An important font of trophic factors is represented by target organs (especially in peripheral nervous system) that, this way, finally controls the terminal size of the innervating neuronal population. Second, neurotrophins can be secreted by glial cells stimulated by cytokines in response to some kind of nerve injuries and also by neurons themselves (E. J. Huang & Reichardt, 2001). Neuronal cells can release neurotrophins both pre- or post- synaptically, according to the brain area. Indeed, neurotrophins may be secreted from neuronal dendrites as a consequence of synaptic activity and function as retrograde factors on the presynaptic neuron. Otherwise, they can undergo anterograde transport from the soma to presynaptic compartment and, after being released in response to neuronal activity, they can act on the postsynaptic neuron.

Among all neurotrophins, BDNF has received great interest due to its strong involvement in neurodegenerative disorders such as HD, AD and PD. Human BDNF gene is composed by 11 exons controlled by 9 different promoters that allow alternative splicing with the consequent production of transcripts that are tissue-specific. Within the adult brain, BDNF protein is found in different areas such as cerebral cortex, hypothalamus, striatum and cerebellum. Interestingly, in contrast to high proteins levels, *bdnf* mRNA is almost absent in the striatum, suggesting that striatal BDNF comes from others brain regions: it is likely that neurons of the cerebral cortex and SNpc synthesize and anterogradely transport the neurotrophin in the striatum, where it is fundamental for the its maintenance and proper function (Baydyuk & Xu, 2014). Notably, also TrKB mRNA and protein possess a broad distribution in the nervous system, even if with a certain region and cellular specificity. For instance, in the striatum it is preferentially expressed by MSNs of the indirect pathway, those that are prevalently affected in HD (Baydyuk & Xu, 2014).

The process of BDNF production involves multiple steps and starts with the synthesis of the pre-pro BDNF in the ER. One transported in Golgi apparatus, the pre-

sequence is cleaved and the pro-neurotrophin isoform is translated (proBDNF) and accumulated in the TGN. At this point it is directly secreted as immature isoform or further cleaved to produce the mature BDNF (mBDNF) that is then released via secretory vesicles. They can be the small secretory granules of the *constitutive* pathway when pro-BDNF is cleaved immediately in the TGN, or larger vesicles of the *regulated* pathway when it is cleaved within the vesicles themselves. In neuronal cells, the second way is by far the most frequent and it is strictly activity-calcium-dependent. Once in the extracellular space mBDNF and proBDNF are able to exert their physiological function (Kowiański et al., 2018; Mercado et al., 2017). The ratio of this two isoforms changes based on brain developmental stages and regions. More in detailed, pro-BDNF with an high affinity for p75 neurotrophin receptor, is present in greater concentration in early postnatal period during which it plays a negative influence on neuronal remodeling *via* reducing dendritic complexity and promotion of cell death, guaranteeing this way the proper neuro and synaptogenesis and the elimination of the mature neurons surplus and of the anomalous connections. During the adulthood, the more prevalent isoform is mBDNF that enhances the developmental process and also synaptic transmission and plasticity. Thus, given both the two isoforms have been shown to be biologically active and given their partially opposite function, a dynamic balance between the two appears to be crucial to ensure the maintenance of physiological homeostasis (Kowiański et al., 2018; Mercado et al., 2017).

4.1. BDNF-mediated signaling pathways

BDNF is able to perform its biological functions thanks to the binding of its different isoforms to specific receptors and the subsequent activation of ad hoc pathways. Synaptically secreted mBDNF once dimerized recognizes TrKB receptor triggering its dimerization and autophosphorylation of intracellular tyrosine residues. These class of receptors are indeed compose of intracellular tyrosine kinase domains and extracellular immunoglobulin domains that bind ligand. Phosphorylated TrKB stimulates the initiation of three major intracellular signaling cascades culminating in gene expression and protein translation (Fig.20). More in detail, the MAPK/Ras signaling pathway activates ERK1/2 and CREB, being important for the early response gene expression and for protein synthesis, as those involved in cytoskeletal dynamics and dendritic growth and branching. The cytoskeleton development and the fostering

of dendritic development and ramification are promoted also by the phosphatidylinositol 3-kinase (PI3K)/Akt-related pathway that in addition enhances antiapoptotic and pro-survival effects and modulates the NMDAR-dependent synaptic plasticity. Also the PLC-γ-dependent pathway is stimulated with the production of diacylglycerol (DAG) and inositol trisphosphate (IP3), which activate protein kinase C (PKC) and increase the intracellular Ca^{2+} concentration respectively, triggering the Ca^{2+}-calmodulin dependent protein kinase (CaM kinase) activity and resulting in enhancement of synaptic plasticity. Finally, *via* binding TrKB BDNF can activate a fourth signaling cascade, that is the Rho-GTPases-mediated pathway. It arouses the synthesis of actin and microtubule resulting in growth of neuronal fibers (Kowiański et al., 2018) (Fig.20).

Alongside, pro-BDNF is capable of signaling through the preferential interaction with the p75 neurotrophin receptor through its mature domain and with the co-receptor sortilin *via* its pro-domain, forming the pro-BDNF/p75/sortilin complex. As result, three principal pathways are activated (Fig.20) : the c-Jun N-terminal kinase (JNK), Ras homolog gene family member A (RhoA) or NF-κB – related signaling cascades. They lead to expression of genes involved respectively in programmed cell death, neuronal development and survival.

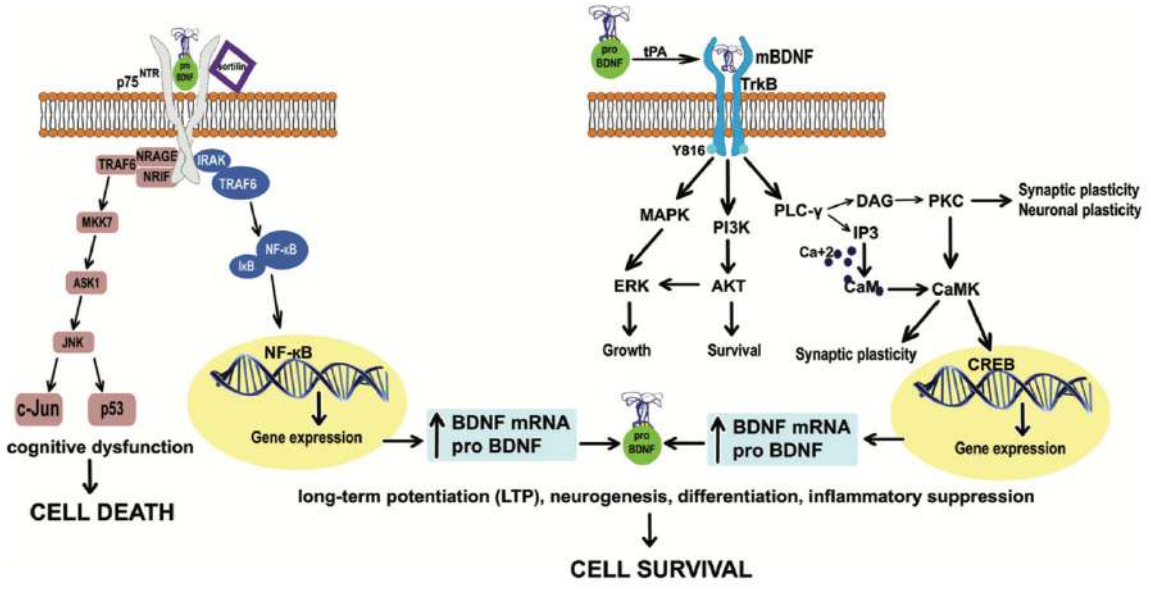


Fig.20: BDNF signaling: *Representative image of the main signaling pathways activated by BDNF. The left side of the image reports the proBDNF signaling upon the binding of p75 receptor; the right side of the image reports the mBDNF signaling upon the binding with TrKB receptor. Adapted from (Hernandez-Baltazar et al., 2019).*

Overall, what is extremely evident is that the transcription, translation, secretion and signaling of BNDF isoforms originate a variety of different outcomes on neuronal function, making this neurotrophin a pleiotropic factor. On one hand, pro-BDNF acts preferentially during the development promoting a delicate equilibrium between a negative influence on neuronal remodeling, development and LTP with apoptosis stimulation and the supporting of survived neurons. On the other side, the function of mBDNF consists in enhancing processes linked to development likewise those associated with the improving of memory and cognition in the adulthood and related to increased brain activity and synaptic transmission (Kowiański et al., 2018; Mercado et al., 2017).

4.2. BDNF synaptic plasticity and Parkinson's disease

Synaptic plasticity can be defined as experience-dependent modifications in the efficacy and strength of preexisting synapses. Lasting changes are at the base of information storage for the formation of memory and are the consequence of the promotion of late LTP, involving protein synthesis (Bramham & Messaoudi, 2005). Among all neurotrophins, BDNF emerges due to its elevate brain expression and its powerful effect on synapses. Indeed, nowadays there is a quite consensus in identifying synapse regulation as the main function of BDNF in the mature brain, with both functional and structural endings on excitatory or inhibitory synapses. In particular, BDNF/TrKB axis has been widely demonstrated to mediate synaptic consolidation participating in the induction of both early LTP and late LTP, principally in hippocampus but also in other brain regions (Leal et al., 2014). The short-lasting processes controlled by BDNF relate on the modification of components already available at the synapse: pre- and post- synaptic preexisting proteins are subjected to post-translational regulation, like phosphorylation enhancing overall the strength of the synaptic transmission (Kowiański et al., 2018; Leal et al., 2014). For instance, at the presynapse BDNF has been reported to increase the number of docked vesicles per active zone potentiating the NTs (e.g. glutamate) release; while in the postsynaptic compartment it has been showed to intensify glutamate receptors opening probability phosphorylating their subunits and also to promote the synaptic accumulation of AMPA receptors potentiating the postsynaptic response to NTs release. These transient outcomes could be followed by more prolonged synaptic changes, at the base

of long-term effects and dependent on gene expression and protein synthesis modification with a final modification in synaptic protein content (Leal et al., 2014). The set of gene induced during the establishment of LTP includes also BDNF itself: basically, the expression of BDNF rises in a dependent-stimulation fashion and, on the other hand, the increased amount of BDNF reinforces synaptic function. Interestingly, de novo gene transcription and protein synthesis associate LTP with the enlargement and the formation of dendritic spines suggesting that sustained stimulation of excitatory synapses inducing LTP is also connected to post-synaptic structural changes. In this regard, even if the underlying mechanism remains partially unknown, it has been extensively demonstrated that BDNF-TrKB signaling is an important factor involved in the regulation of dendritic spines density and shape (Mercado et al., 2017; von Bohlen und Halbach & von Bohlen und Halbach, 2018). Indeed, mice lacking TrKB receptor display a significant reduction in the dendritic spines density. Recent work showed that, besides LTP strength promotion acting on the presynaptic site, BDNF operating postsynaptically is mostly involved in LTP maintenance. In hippocampal neurons, even a spine autonomous autocrine signaling has been described: the NMDAR and CaMKII-dependent pathway activation induces the release of BDNF that subsequently activates TrKB on the same stimulated spines (von Bohlen und Halbach & von Bohlen und Halbach, 2018). Among the possible actions exerted by BDNF on dendritic spines there is the possibility that the neurotrophin increases the polymerization of spines actin mesh by regulating p21-activated kinase (PAK) and ADF/cofilin (Leal et al., 2015). The same effects on actin cytoskeleton is achieved after the activation of m-calpain via ERK-dependent phosphorylation. The actin filaments remodeling and elongation is fundamental for the maintenance of LTP *in vivo*.

4.3. BDNF and its involvement in PD

Several studies have reported an outstanding role of BDNF in promoting the survival and function of neurons belonging to two brain areas related to PD: SN and striatum (Mercado et al., 2017). The neurotrophin signal is indeed involved in the support of DA neurons located in the SN, as proved by the fact that the lack of TrKB receptor is associated with neuronal degeneration with accumulation of α-Syn in the survived neuronal cells (Bohlen und Halbach et al., 2005). Also the electrical activity of these neurons, DA turnover and motor behaviors are stimulated by BDNF. Another

fundamental role is played by BDNF in striatal compartment, both during development and adulthood. The production of BDNF in the striatum, as mentioned before, is almost absent and the protein is supplied by cortical and SNpc dopaminergic projections. During the embryogenesis the main source of BDNF has been found to be the DA neurons of the SN, given the expression of BDNF is low also in the cortex. Thus, it has been proposed that the small SNpc area is able to regulate the size of its larger target, the striatum, controlling *via* BDNF, together with other neurotrophins, the survival of immature MSNs starting from their origin. In developing brain BDNF helps the MSNs maturation and the proper establishment of striatal connection that depend on expression of appropriate neuronal markers, the complexity of dendritic arbor and the morphology of dendritic spines. Finally, in the adult brain, BDNF, derived also from cortical projections, represent a critical factor influencing dendritic spine and synapse dynamic and plasticity (Baydyuk & Xu, 2014; Mercado et al., 2017).

The critical role of BDNF-TrKB axis in the nigrostriatal system, especially its involvement in the maintenance of dendritic spine density and synapse function, suggests that any kind of abnormalities in BDNF signaling, such as alterations in anterograde transport and gene expression from brain regions providing BDNF to the striatum, might cause neuronal dysfunction significantly and negatively impacting on BG physiology. Interestingly, many lines of evidence linked BDNF to PD pathology. In particular, post mortem analysis of PD brains has highlighted a significant decrease in BDNF mRNA and proteins levels in DA neurons of the SNpc that correlates with the severity of motor symptoms (Mercado et al., 2017). It has been found that the loss of BDNF is present in some early onset form of PD associated to mutated Syn (Zuccato & Cattaneo, 2009). The reduction in BDNF production in PD midbrain could be involved in the death of nigral dopaminergic neurons, but also could contribute to disfunctions in striatal neurons that have been found to show a consistent reduction in the length of dendrites, with the remaining dendrites often showing few to no spines (Mercado et al., 2017). Consistently, when delivered in experimental animal models of PD, BDNF is able to rescue the SNpc DA neurons that are degenerating (Zuccato & Cattaneo, 2009).

Chapter 5

Aim of the project

Aim of the project

This PhD project investigated the physiological role of the PD-associated protein LRRK2 in orchestrating post-synaptic functionality *via* actin cytoskeleton remodeling, together with the potential pathological implications of the PD G2019S mutation in this compartment.

Functional synapses are essential for the proper activity of brain circuits. Among all existing categories, dendritic spines, small protrusions originating from neuronal dendrites, constitute the postsynaptic element of most excitatory synapses and are particularly abundant in some classes of neuronal cells such as striatal MSNs (Hering & Sheng, 2001). They increase synaptic efficacy by compartmentalizing the postsynaptic signaling machinery and can assume various shapes, which correlate with different levels of maturation and functionality (Chidambaram et al., 2019). Being very dynamic elements capable of changing their morphology in response to appropriate stimuli along the entire life span, dendritic spines are at the base of synaptic plasticity (Chidambaram et al., 2019).

Synapses damage or loss can harm the information transfer and the capacity of the nervous system to modify its own organization, leading to pathology. To this regard, accumulating evidence indicates that synaptic loss and vulnerability, including alterations in dendritic spines, constitute early pathological events in several neurodegenerative diseases (Fiala et al., 2002). These processes have been extensively characterized in Alzheimer disease (AD) and Huntington disease (HD), where loss of synaptic connections has been shown to precede disease clinical signs (Hong et al., 2016; Milnerwood & Raymond, 2010). PD, a progressive neurodegenerative disorder affecting 1-2% of the population over the age of 65, is pathologically characterized by the loss of SNpc DA neurons projecting to the dorsal striatum. At the time of the onset of motor symptoms, the loss of striatal DA synaptic terminals has been reported to exceed the loss of DA cell bodies in the SNpc (Tagliaferro & Burke, 2016). All these lines of evidence point to early synaptic dysfunction as an initial mechanism of PD neurodegeneration, prodromal to neuronal death and disease clinical manifestation (Picconi et al., 2012; S. Soukup et al., 2018). Nevertheless, the onset of such a process and its molecular mechanisms and triggers are still unknown.

Previous data from our team and other laboratories clearly suggest a key role for LRRK2 at the synapse. In this compartment LRRK2 physically interacts with vesicles and actin cytoskeleton, providing the scaffold for the assembly of local signaling and the achievement of the main synaptic functions (Civiero et al., 2018). The role of LRRK2 at the presynaptic site has been extensively studied: it has been shown to regulate SVs cycling through interaction and phosphorylation of a panel of pre-synaptic proteins (Pischedda & Piccoli, 2021). For example, LRRK2 binds and phosphorylates NSF at T645, enhancing its ATPase activity with consequent increased rate of SNARE complex disassembly (Belluzzi et al., 2016). LRRK2 was also found to phosphorylate other presynaptic substrates, including auxillin, Endophilin A and synapsin I, impacting on neurotransmitter release and local autophagy (Pischedda & Piccoli, 2021).

In contrast, a putative LRRK2 activity at the postsynaptic element has not yet been investigated as extensively. Of note, LRRK2 is highly expressed in MSNs of the dorsal striatum (Mandemakers et al., 2012; West et al., 2014), rich in dendritic spines and constituting the postsynaptic element of the nigrostriatal pathway. The postsynaptic compartment is mainly represented by dendritic spines, which possess a cytoskeleton made essentially by actin filaments. In neurons, besides playing a critical role in the growth of dendrites and axons in coordination with microtubules, actin dynamics is the driving force that underlies synapse formation and plasticity (Cingolani & Goda, 2008), shaping dendritic spines morphology and influencing their maturation.

The central hypothesis of this project is that LRRK2 regulates the dynamics of the actin cytoskeleton in dendritic spines, influencing their genesis and maturation.

The **first aim** was to **understand whether and how LRRK2 influences the structural dynamics of postsynaptic spines**, taking advantage of several complementary approaches, including Golgi-Cox imaging of mouse striatal neurons, immunofluorescence of mouse brain, imaging analysis of dendritic spines, affinity purification coupled with mass spectrometry, cell biology and biochemistry. In this part of the work we assessed the physiological impact of mouse Lrrk2 on dendritic spines maturation and dissected the molecular mechanisms and players behind Lrrk2-dependent spine remodelling. In particular, we will take advantage of BDNF-TrKB signalling cascade as a model to explore Lrrk2-mediated spinogenesis. BDNF is

fundamental for the formation, maintenance and adaptation of striatal synapses and its deficiency has been linked to PD development (Mercado et al., 2017). We collected and discussed compelling data pointing to a Lrrk2-dependent actin cytoskeleton remodeling with impact on BDNF-receptor TrKB traffic and actin-related protein function. A special focus was given to drebrin, a postsynaptic actin-binding protein, whose interaction with LRRK2 was influenced by neurotrophic signaling.

The **second aim** of the project was to **evaluate the pathological consequences of increased LRRK2 kinase activity caused by the common LRRK2 G2019S mutation on dendritic spine structure**. Impaired actin dynamics and the consequent alterations of the delicate cytoskeletal network in the presence of mutant LRRK2 may severely affect neuronal homeostasis, including the proper postsynaptic functionality. We provided interesting data describing the impact of Lrrk2 G2019S pathological mutation on striatal dendritic spines.

LRRK2 is a very complex protein affecting multiple cellular processes. While fifteen years of research on LRRK2 have contributed to make important steps forward in the definition of the mechanisms underlying LRRK2 physiology and pathology, there are still many biochemical and cellular aspects that remain to be explored. By exploring the role of LRRK2 in synaptic function, this PhD work contributed to make a step forward in the research field of LRRK2 pathobiology.

Chapter 6

Materials and Methods

6. Materials and Methods

6.1. Animals

C57Bl/6J Lrrk2 wild-type and knock-out mice were respectively provided by Dr. Heather Melrose and Jackson Laboratory (B6.129X1 (FVB)-Lrrk2 tm1.1Cai/J). G2019S-Lrrk2 knock-in mice were obtained from Prof. Michele Morari and Novartis Institutes for BioMedical Research, Novartis Pharma AG (Basel, Switzerland). G2019S-Lrrk2 BAC mice were obtained from Jackson Laboratory [B6.Cg-Tg(Lrrk2*G2019S)2Yue/J]. Housing and handling of mice were done in compliance with national guidelines. All animal procedures were approved by the Ethical Committee of the University of Padova and the Italian Ministry of Health (license 1041/2016-PR and 105/2019).

6.2. Constructs

GFP-LRRK2 WT cloned in pDEST53 (Life Technologies) was previously described (Greggio et al., 2006); The different LRRK2 truncation constructs containing an N-terminal GFP tag were previously described (Greggio et al., 2008).

6.3. Mammalian cells cultures

6.3.1. SH-SY5Y cell culture and differentiation

Human neuroblastoma *SH-SY5Y* cells were grown in a complete medium composed of a 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM, ThermoFisher Scientific) and Ham's F-12 Nutrient Mixture (F12, ThermoFisher Scientific), supplemented with 10% Fetal Bovine Serum (FBS, ThermoFisher Scientific) and 1% Penicillin-Streptomycin solution (PS, ThermoFisher Scientific). Cells were maintained at 37°C in a humified atmosphere with 5% CO_2.

When 80-100% confluence was reached, *SH-SY5Y* cells were washed with Dulbecco's Phosphate Buffered Saline (DPBS, ThermoFisher Scientific) to remove the cell culture medium and, after removal of DPBS, cells were incubated with trypsin 0.1X (ThermoFisher Scientific) in order to detach. Fresh medium was, then, added to inactivate the proteolytic enzyme and, once centrifuged for 5 minutes at 400g, the supernatant was discarded and the cells were resuspended and counted.

About 350.000 cells were plated in 6 well-plates (Sarstedt Limited) in 2mL of DMEM/F12 containing 1% PS and 1% FBS. 10μM all-trans-retinoic acid (RA, Sigma-Aldrich®) was added to promote N-type (neuronal-like cells) cell differentiation. Furthermore, about 50.000 cells were plated in 24-well cell culture plates (Sarstedt Limited) containing 12mm glass coverslips in 500μl of DMEM/F12 added with 1% FBS, 1% PS and 10μM RA to induce N-type cell differentiation. Coverslips were pre-treated with poly-L-Lysine 1X (Sigma-Aldrich®) in order to improve cell adhesion. Cells were, subsequently, incubated at 37°C in 5% CO_2. At regular intervals of 48 hours, RA was newly provided to the differentiating cells: on differentiation day 2, RA was added directly to the medium, while, on differentiation day 4, the differentiation media (supplemented with RA) was refreshed. Cells were incubated again at 37°C in 5% CO_2 and the differentiation process was carried on until differentiation day 6, when cells were subjected to various treatments (Paragraph 6.3.4.2.).

6.3.2. HEK293T cells

HEK293T cells (Life Technologies) were cultured in DMEM supplemented with 10% FBS and maintained at 37°C in a humified atmosphere with 5% CO_2. When at 80% confluency, cells were washed with DPBS to remove the cell culture medium and incubated with trypsin 0.1X. The enzyme was inactivated with fresh medium and, after centrifugation (5 minutes at 400g), the supernatant was discarded and the cells were resuspended and counted. About 8000000 cells were plated in 150mm cell culture dishes (Sarstedt Limited) in 12mL of supplemented DMEM. 24 hours after plating, when at 80% confluency, cells were subjected to transfection.

6.3.3. Primary cultures of cortical neurons

Primary cortical neurons were obtained from postnatal Lrrk2 WT and Lrrk2 KO C57BL/6J pups between day 0 and 1 (P0-P1) exploiting the Papain Dissociation System (Worthington Biochemical Corporation). The brain was removed and, once isolated, cortices were snipped into smaller pieces and transferred in Papain solution where they were further triturated 10 times. Then, the solution was incubated for 40 minutes at 37°C and was mixed by inversion every 10 minutes. Subsequently, cells were triturated 10 times and spun down in a swinging-bucket rotor for 5 minutes at 200g.

The supernatant was discarded and 3mL of STOP solution was added to the pellet: after triturating 3 times, the tubes were incubated for 10 minutes at room temperature (RT) to allow the bigger pieces of tissue to precipitate. Then, the supernatant was gently pipetted drop-by-drop upon 5mL of 10/10 solution and spun down for 10 minutes at 100g. After discarding the supernatant, the pellet was resuspended in about 5mL of Neurobasal plating medium and cells were diluted to 1:10 in 0.4% Trypan blue to be counted. High-density (1000-1500 cell/mm^2) and medium density (150-200 cells/mm^2) neuronal cultures were plated onto 6-well plates or on 12mm glass coverslips in 24-well plates respectively and grown in Neurobasal (Life Technologies) supplemented with 5% FBS, 2% B27 supplement (Invitrogen), 0.5mM Glutamine (Life Technologies), penicillin (100Units/ml) and streptomycin (100µg/ml) (Life Technologies), in a 5% CO_2 atmosphere at 37°C. After 7 days, 50% of the Neurobasal medium was removed and replaced with fresh one. Neurons were cultured until DIV14, then they were exposed to BDNF treatment (Section 6.3.4.2.).

6.3.4. Mammalian cells transfection and treatments

6.3.4.1. Transfection

For the purification assay and the following WB analysis HEK293T were transiently transfected with plasmid DNA using polyethylenimine (PEI, Polysciences), utilizing a DNA:PEI ratio of 1:2. A total amount of 25µg DNA was dissolved in 750µl of DMEM deprived of FBS for LRR and KIN tagged GFP constructs, whereas for all the others constructs 40µg of plasmid were employed. The respective amount of PEI was added to 750µl of DMEM deprived of FBS. After 5 minutes the two solutions were mixed together and incubated for 20 minutes to allow the formation of DNA/PEI complexes. The mix was then added to the cells that had previously been plated in 150mm dishes and the cells were lysates after 48 hours.

For the immunohistochemistry experiments, Lrrk2 WT and KO primary neurons were plated in 24-well plates and transfected with DNA 3-4 days after the seeding. Lipofectamine® 2000 (LIPO) (Invitrogen) was exploited as transfection reagent, with a DNA:LIPO ratio of 1:2. A total amount of 1µg DNA was dissolved in 250µl of OPTI-MEM (Life Technologies) and the respective amount of LIPO was added to 250µl of OPTI-MEM. The solutions were mixed together after five minutes and incubated for 20

minutes to allow the formation of DNA/LIPO complexes. The mix was then added drop by drop to neurons, whose medium was previously changed with OPTI-MEM. After 45 minutes of incubation the transfection medium were replaced with the supplemented Neurobasal and neurons were maintained in culture for additional 9-10 days.

6.3.4.2. Treatments

On differentiation day 6, the differentiation media were completely removed from each dish and replaced with DMEM/F12 added with only 1% PS: *SH-SY5Y* cells were serum-starved for 5h at 37°C in 5% CO_2 in order to prevent any possible interference of the serum with the subsequent treatment. Then, the inhibition of LRRK2 kinase activity was achieved by pre-treating cells with 0.5μM MLi-2 (abcam) for 1.5 hour. Hence, in the same serum-free medium, cells were stimulated for different time periods (5, 15 and 60 minutes) with BDNF (100ng/mL, 50240-MNAS, Sino Biological) in the presence or absence of the LRRK2 inhibitor. Since BDNF and MLi-2 were dissolved, respectively, in H_2O and dimethyl sulfoxide (DMSO, SigmaAldrich®), a negative control sample was included treating cells with Cell Culture Water Pyrogen free (Biowest®) and DMSO in equal final concentrations.

One reach the fourteenth day in vitro, Lrrk2 WT and KO mature neurons cultured in the 6-well plates for WB analysis were subjected to BDNF and Mli2 treatments for 5, 30, 60 and 180 minutes in Neurobasal completed medium. The appropriate controls were also included. The cells were then fixed in 4% PFA. Lrrk2 WT and KO primary neurons plated in 24-well plates and subjected to the transfection protocol, were treated for 24 hours with BDNF (100ng/mL) to study the spinogenesis process, or for different time periods (5 minutes, 15 minutes, 1 hours and 24 hours) to investigate the drebrin exodus. The appropriate controls were also included. The cells were then fixed in 4% PFA.

6.4. Cells and tissues lysis and western blot analysis

For Western Blot (WB) analysis mammalian cells and mouse brain tissues were harvested in appropriate volume of RIPA Lysis Buffer (20mM pH 7.5 Tris-HCl, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1%, NP40, 1% sodium deoxycholate, 2.5mM sodium pyrophosphate, 1mM β-glycerophosphate, 1mM sodium orthovanadate)

supplemented with 1% protease inhibitor cocktail (SigmaAldrich®). More precisely, mammalian cells were lysed at the end of each treatment, after the removal of culture medium and two washes with DPBS.

The appropriate volume of each sample, containing 50μg for *SH-SY5Y* cells and primary neurons and 70μg, for brain tissues was denatured by diluting 4X Sample Buffer (SB, 200mM Tris-HCl pH 6.8, 8% SDS, 400mM DTT, 40% glycerol, Bromophenol Blue, Sigma-Aldrich® , in the amount needed to reach the desired color intensity) to a final 1X concentration.

Proteins were, then, resolved on ExpressPlusTM PAGE precast 4-20% gels (GenScript Biotech Corporation, Piscataway, NJ, US) in MOPS running buffer at 150V or 8% Tris-glycine polyacrylamide gels in SDS/Tris-glycine running buffer, according to the size-resolution required. Also, Precision Plus Protein TM Standard Dual Color (Bio-Rad, Hercules, CA, US) molecular weight marker was loaded in a separate lane of each gel.

Resolved proteins were transferred to polyvinylidenedifluoride (PVDF) membranes using semidry Biorad transfer machine (Trans-Blot Turbo Transfer System, Biorad, Hercules, CA, USA). The nonspecific binding sites were subsequently blocked incubating membranes with 5% non-fat dry milk diluted in 0.1% Tween-20 Tris-buffered saline (TBS-T) for 1 hour at RT under agitation. PVDF membranes were subsequently incubated over-night (ON) at 4°C with primary antibodies in TBS-T plus 5% non-fat milk or 5% BSA. Membranes were then washed in TBS-T (3x10 minutes) at RT to remove the excess of primary antibody and subsequently incubated for 1 hour at RT with horseradish peroxidase (HRP)-conjugated α-mouse or α-rabbit IgG. After three more washes of PVDF sheets in TBS-T (3x10 min), immunoreactive proteins were visualized using chemiluminescence (Immobilon ECL western HRP substrate, Millipore, Burlington, MA, USA). Densitometric analysis was carried out using the Image J software. The antibodies used for WB are as follows: rabbit α-LRRK2 (MJFF2 c41-2, ab133474, abcam, 1:300); rabbit α-phospho-Ser935 LRRK2 (ab133450, abcam, 1:300); mouse α-phospho-Ser473-AKT (sc-293125, Santa Cruz Biotechnology, 1:500); rabbit α-AKT (9272S, Cell Signaling Technology, 1:1000); rabbit α-phospho(Thr202 /Tyr204 , Thr185 /Tyr187)-ERK1/2 (12-302, EMD Millipore, 1:1500); rabbit α-ERK1/2 (4695, Cell Signaling Technology, 1:1000); mouse α-GAPDH (CSB-MA000195, 1:5000); mouse α-βIII tubulin (T8578, Sigma-Aldrich, 1:40000); mouse α-DREBRIN

(MA1-20377, Thermo-Fisher, 1:500); α-Flag® M2-HRP (A8592, Sigma-Aldrich, 1:5000); α-mouse IgG-HRP (A9044, Sigma-Aldrich, 1:80000); α-rabbit IgG-HRP (A9169, Sigma-Aldrich, 1:16000).

6.5. Staining on brain tissues and mammalian cells

6.5.1. Immunocytochemistry

For the immunocytochemistry (ICC) analysis, *SH-SY5Y* cells were cultured onto 12mm glass coverslips in 24well plastic tissue culture plates that had previously been coated with poly-L-lysine (Sigma-Aldrich). After the described treatment (paragraph 6.3.4.2.), cells were washed with DPBS and fixed with 4% paraformaldehyde (PFA, pH 7.4) for 20 minutes at RT. Subsequently, cells were washed thrice with DPBS and subjected to staining protocols. After the last washing step, the basal fluorescence of the cells was quenched incubating the coverslips in 50mM NH_4Cl (in 1X PBS) for 10 minutes at RT in agitation. Cells were washed another time with DPBS and subsequently permeabilized in ice-cold methanol for 10 minutes at -20°C. This passage in methanol allowed us to optimize the staining for LRRK2. Then, cells were washed once with DPBS and incubated with blocking solution (5% FBS in 1X PBS) for, at least, 2 hours at RT in agitation. After the saturation, cells were incubated ON at 4°C with primary antibodies diluted in the blocking solution. The day after, coverslips were washed with DPBS (5 minutes x 3) and then incubated with secondary antibodies diluted in blocking solution for 1 hour at RT in the dark. After rinsed with DPBS (5 minutes x 3), cells were stained with Hoechst 33258 (DAPI, ThermoFisher ScientificTM) diluted 1:10000 in DPBS for 5 minutes at RT in agitation. Lastly, coverslips were washed again with DBPS, cleaned in ddH_2O and mounted on microscope slides (ThermoFisher ScientificTM). As mounting medium Mowiol (Calbiochem) was used. Images were collected with Zeiss LSM700 confocal microscope.

For the ICC analysis, primary cortical neurons derived from WT C57BL/6 and LRRK2 KO mouse pups (P0) were cultured in 12 mm glass coverslips in 24well plastic tissue culture plates that had previously been coated with poly-L-lysine. 14 days after the seeding, when synapses reach the maturation, neurons were treated as previously described (paragraph 6.3.4.2.). After the treatment, neurons were fixed with 4% PFA for 20 minutes at RT and subsequently they were washed thrice with DPBS and

subjected to staining protocols. Neurons were permeabilized with 0,3% Triton® X-100 in DPBS for 5 minutes at RT and then incubated with blocking solution (1% BSA, 50mM glycine, 2% goat normal serum, 0,1% Triton® X-100 in PBS) for 1 hour at RT in agitation. After the saturation neurons were incubated with primary antibodies diluted in working solution (20% blocking solution in PBS) for 1 hour at RT. After Once rinsed in working solution (5 minutes x 3) in agitation, coverslips were incubated with 1 hour at RT in the dark with the appropriate secondary antibody diluted in working solution. After additional washes in working solution (5 minutes x 3), neurons were stained with Hoechst 33258 diluted 1:10000 in DPBS for 5 minutes at RT in agitation and then rinsed in DPBS and cleaned in ddH_2O. Finally, coverslips were mounted on microscope slides with Mowiol mounting medium. Images were collected with Zeiss LSM700 confocal microscope.

The antibodies used for ICC are the following: mouse α-PSD95 (ab2723, abcam, 1:200), rabbit α-MAP2 (sc-20172, Santa Cruz, 1:200); mouse α-DREBRIN (MA1-20377, Thermo-Fisher, 1:400), mouse α-TrkB (sc-377218, Santa Cruz Biotechnology, 1:75); Anti-Mouse AlexaFluor® 568-conjugated, 647-conjugated (Invitrogen, Thermo Fisher Scientific); Anti-Rabbit AlexaFluor® 488-conjugated, 647-conjugated and 405-conjugated (Invitrogen, Thermo Fisher Scientific).

6.5.2. Immunofluorescence

Animals were terminally anesthetized with xylazine (Rompun®) and ketamine (Zoletil®) and transcardially perfused with 0.9% saline followed by ice cold 4% PFA. Brain were dissected and post-fixed in 4% PFA at 4°ON, then transferred to a sucrose gradient in DPBS (20% and 30%) at 4°C for cryopreservation. Once saturated in sucrose, 40 µm thick coronal slices were obtained sectioning the brains with a vibratome. Sections were rinsed three times with DPBS and then the sample autofluorescence is quenched in 50mM NH_4Cl in DPBS. After three more washings, the slices were permeabilized and saturated for 2h in blocking solution (15% vol/vol goat serum, 2% 2% wt/vol BSA, 0.25% wt/vol gelatin, 0.2% wt/vol glycine in DPBS) containing 0.5% Triton X-100. Incubation with the primary antibodies was carried out ON at 4°C in blocking solution Samples were extensively washed three times with DPBS and then sections were incubated with appropriate secondary antibodies diluted

1:200 in blocking solution. Nuclei were stained with Hoechst 33258, diluted 1:10000 in DPBS for 5 minutes at RT in agitation. Sections were rinsed with DPBS (5 minutes X 3) and mounted on microscope slides with Mowiol mounting medium. Images were acquired with Zeiss LSM700 confocal microscope, using 63X/1,40 Oil DIC M27 objective.

The antibodies used for IF are the following: mouse α-βIII tubulin (T8578, Sigma-Aldrich, 1:1000); rabbit α-total LRRK2 [MJFF2 (c41-2)] (ab133474, abcam, 1:200); rabbit α-phospho-S935 LRRK2 [UDD2 10(12)] (ab172382, abcam, 1:200), guinea pig α-GLT1 (AB1783, EMD Millipore, 1:400), rabbit α-GFAP (Z0334, Dako-Agilent, 1:400); α-Rat CD11b [M1/70] (14-0112-82, eBioscience™ from Thermo Fisher Scientific, 1:200); mouse α-PSD95 (ab2723, abcam, 1:200); rabbit α-VAMP2 (homemade gently provided by Ornella Rossetto, 1:200); α-mouse AlexaFluor® 568-conjugated (Invitrogen, Thermo Fisher Scientific); α-rabbit AlexaFluor® 488-conjugated (Invitrogen, Thermo Fisher Scientific); α-rat AlexaFluor® 647-conjugated (Invitrogen, Thermo Fisher Scientific); α-guineapig AlexaFluor® 488-conjugated (Invitrogen, Thermo Fisher Scientific).

6.5.3. Golgi-cox staining

Animals were terminally anesthetized with xylazine (Rompun®) and ketamine (Zoletil®) cocktail and transcardially perfused with 0.9% saline. Brains were dissected, washed with dd-H_2O and cut into two halves to allow better impregnation. One half was put into Golgi-Cox solution (made by Potassium dichromate, Mercuric chloride Potassium chromate prepared according to (Zaqout & Kaindl, 2016) and stored in the dark at RT. After three days, the Golgi-Cox solution was refreshed. The tissues were kept at RT in the dark for 14 days. Following this period brains were transferred into a 30% sucrose solution in DPBS to give malleability to the sample. This is fundamental to avoid the cracking of the slices during the cutting. The brains were embedded in 1,5% agarose (Low Melting Point, Promega) and then mounted onto a vibratome stage with glue. The vibratome reservoir was filled with 30% sucrose solution in DPBS. The section thickness is optimally 100 μm for dendritic spines studies. While cutting, sections were collected with a thick brush and transferred onto SuperFrost Ultra Plus® Adhesion Slides (Thermo Scientific). As soon as all sections

have been loaded on slides, the sucrose solution in excess around the sections was cleaned off with absorbent paper. The sections were then blotted by pressing an absorbent paper moistened with sucrose solution onto the slides. The slides with sections were kept for drying for 7-10 minutes.

The samples were then subjected to colour development procedure. The mounted sections were washed (5 minutes x 2) with ddH_2O to remove trace of impregnation solution and then were placed in 50% ethanol for 5 min. After that, slides were kept for in 20% ammonium hydroxide for 10 minutes. Sections were washed in ddH_2O for 5 minutes and immersed in 5% sodium thiosulfate for 8 minutes at RT in the dark. After additional washings (1 minutes x 2) in ddH_2O, the slides were passed through ascending grades of ethanol (70%, 95% and 100%) in order to dehydrate the tissue. Finally, they were placed in xylene (6 minutes x 2) and then covered applying coverslips and Eukitt® mounting medium. Images were acquired with Zeiss LSM700 confocal microscope, using 100X/1,40 Oil DIC M27 objective with phase contrast acquisition mode.

6.6. Protein purification from mammalian cells

For the purification protocol, *SH-SY5Y* OE-GFP and *SH-SY5Y* hLRRK2-GFP were plated onto 100mm dishes (Sarstedt Limited) and differentiated into neuronal-like cells for 6 days (paragraph 6.3.1.). On differentiation day 6, *SH-SY5Y* OE-hLRRK2-GFP cells were treated with recombinant BDNF (100ng/mL) or with an equal volume of Cell Culture Water Pyrogen free for 15 minutes, while *SH-SY5Y* OE-GFP cells were left untreated. Once the treatment medium was removed, cells were washed with DPBS and, then, solubilized in 500µL of Lysis Buffer (20mM Tris-HCl pH 7.5, 150mM NaCl, 1mM EDTA, 1% Tween® 20, 2.5mM sodium pyrophosphate, 1mM β-glycerophosphate, 1mM sodium orthovanadate) containing 1% protease inhibitor cocktail. For the purification of LRRK2 deletion constructs, HEK293T cells were plated onto 150mm dishes, transfected with the appropriate amount of DNA and solubilized after 48 hours in 1ml of the same Lysis Buffer. In both of the cases, after clearing of the lysates containing GPFtagged protein by centrifugation at 20000×g for 30 minutes at 4°C, the supernatants were incubated ON with an appropriate volume of GFP-Trap ® Agarose resin (ChromoTek, Planegg-Martinsried, Germany) overnight at 4°C in rotation. Resins

were subsequently washed twice with each of the following Washing Buffers (WBu) supplemented with Tween® 20:

- WB1: 20mM Tris-HCl pH 7.5, 500mM NaCl, 1% Tween® 20
- WB2: 20mM Tris-HCl pH 7.5, 300mM NaCl, 0.5% Tween® 20
- WB3: 20mM Tris-HCl pH 7.5, 150mM NaCl, 0.5% Tween® 20
- WB4: 20mM Tris-HCl pH 7.5, 150mM NaCl, 0.1% Tween® 20
- WB5: 20mM Tris-HCl pH 7.5, 150mM NaCl, 0.02% Tween® 20

Centrifugation steps (7500g x 1 minute) were performed between washes to pellet the resin. Proteins were eluted in 2X SB and then resolved in SDS-PAGE. Acrylamide gels containing samples from SH-SY5Y OE-GFP and SH-SY5Y hLRRK2-GFP cells were stained with Colloidal Coomassie Brilliant Blue (0.25% Brilliant Blue R-250, Sigma-Aldrich® , 40% ethanol, 10% acetic acid in milli-Q water) for at least 1 hour and then destained with a destaining solution (10% isopropanol, 10% acetic acid in milli-Q water). Eventually, the gel was preserved in 10% acetic acid in agitation to remove the background color and visualize proteins. Finally, gel band was excised and assessed by mass spec. The samples from HEK293T cells transfected with LRRK2 truncated constructs were processed for WB analysis.

6.7. Mass spectrometry analysis

Gel slices were cut into small pieces and subjected to reduction with dithiothreitol (DTT 10 mM in 50 mM NH_4HCO_3, for 1 h at 56 °C), alkylation with iodoacetamide (55 mM in 50 mM NH_4HCO_3, for 45 min at RT and in the dark), and finally in-gel digestion with sequencing grade modified trypsin (12.5 ng/µL in 50 mM NH_4HCO_3, Promega). Samples were analyzed using a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) coupled to a HPLC UltiMate 3000 (Dionex – Thermo Fisher Scientific) through a nanospray (NSI). Peptides were separated at a flow rate of 250 nL/min using an 11-cm-long capillary column (PicoFrit, 75-µm ID, 15-µm tip, New Objective) packed in house with C18 material (Aeris Peptide 3.6 µm XB C18; Phenomenex). A linear gradient of acetonitrile/0.1% formic acid from 3 to 40% was used for peptide separation and the instrument operated in a data dependent acquisition mode with a Top10 method (one full MS scan at 60,000 resolution in the Orbitrap, followed by the

acquisition in the linear ion trap of the MS/MS spectra of the four most intense ions). Raw data files were analyzed using MaxQuant v.1.5.1.2. (Cox and Mann, 2008) connected to Andromeda server (Cox et al., 2011) and searched against the human section of the UniProt database (version September 2020, 75093 entries) using the following parameters: trypsin was selected as digesting enzyme with up to two missed cleavage allowed, precursor and fragment tolerance was set to 10 ppm and 0.6 Da respectively, carbamidomethylation of cysteine residues was set as a fixed modification and methionine oxidation as a variable modification. The precursor area ion detector node of Proteome Discoverer was used to integrate the area of precursor ions. A search against a randomized database and the algorithm Percolator were used to assess the false discovery rate (FDR), and data were filtered to keep into account only proteins identified with at least two peptides and a FDR = 0.01 both at peptide and protein levels. Proteins were grouped into protein families according to the principle of maximum parsimony.

6.8. Generation of LRRK2 KO SH-SY5Y CRISPR/Cas9 edited monoclonal cell line

The KO of LRRK2 was performed using CRISPR/Cas9-mediated genome editing technology following the protocol by Sharma et al., 2018. Two sgRNAs were selected among those designed by the laboratory of Zhang F. and double-checked using the online platform Benchling (www.benchling.com). Benchling was also exploited to design an additional sgRNA and to score all the three sgRNAs. The oligos encoding the 20-nt guide sequence were cloned into the pSpCas9(BB)-2A-Puro (PX459) V2.0 vector (Addgene, Watertown, MA, US) and amplified in chemically competent E. coli StbI3 cells (ThermoFisher ScientificTM). *SH-SY5Y* cells were transfected and subjected to puromycin selection, as the plasmid contains the antibiotic resistance. Then the selected cells were diluted to the limit in order to obtain monoclonal cell lines. Approximately one week after plating, the colonies were inspected for a clonal appearance and, when sufficiently grown, they were moved to progressively larger wells and flasks in order to increase the population size. Finally, the deletion of LRRK2 was verified in multiple lines by WB with LRRK2 specific antibody. We were able to get two fully KO lines and some lines in which the expression of LRRK2 is downregulated.

6.9. Data analysis and software

The relative protein amount detected in WB was measured calculating relative band intensities with the freeware ImageJ software. Total protein levels were normalized over the loading control whereas the phospho/total ratios were estimated as the relative abundance of phospho- over total protein, after being normalized to their respective loading controls. GraphPad Prism 9 was exploited to perform statistical analysis and generate Graphs. The Golgi-Cox images were analyzed with the software Reconstruct (http://synapses.clm.utexas.edu).

Chapter 7

Results

LRRK2 promotes dendritic spines remodelling through the regulation of actin cytoskeleton

7. Introduction

As discussed in the introduction of this dissertation, dendritic spines are small protrusions originating from neuronal dendrites and characterized by highly dynamic nature, meaning that they can change their shape within relatively short timeframes in response to appropriate stimuli, e.g. LTP or LTD. The shape of dendritic spines determines their classification into different morphological classes, representing different levels of maturation (Kristen M. Harris, 1999). Mature spines present larger heads, that correlate with an higher density of receptors and other specialized post-synaptic proteins that encode and elaborate a post-synaptic response (Hering & Sheng, 2001). The activity-controlled dendritic spines strengthening and weakening is strongly influenced by the remodeling of their cytoskeleton, mainly composed by actin filaments (Matus, 2000). Thus, the reorganization of the actin cytoskeleton plays a major role in dendritic spine morphology and function.

LRRK2 is a multidomain enzyme with a double enzymatic activity, playing multiple functions in different cell types and tissues (Wallings et al., 2015). Accumulating evidence indicates that LRRK2 sits at the crossroad between cytoskeletal dynamics and vesicular trafficking through interaction with a host of partners and phosphorylation of a subsets of targets such as Rab GTPases. LRRK2 binds actin cytoskeleton and orchestrates its dynamics by interacting with a subset of actin-regulatory partners such as PAK6, ERM and WAVE2 proteins (Civiero et al., 2018).

Based on vast literature evidence as well as on our findings that LRRK2 interacts with a number of cytoskeletal-related proteins highly represented in dendritic spines (Kneussel & Wagner, 2013; Lei et al., 2016), in this chapter I present results supporting a role for LRRK2 in orchestrating dendritic spines structural plasticity. In detail, I (i) explored the consequences of loss of LRRK2 function on postsynaptic morphology, maturation and synaptic contacts formation and (ii) investigated the mechanisms whereby LRRK2 may regulate dendritic spines formation and maturation by exploiting BDNF as a model to study spinogenesis in vitro.

7.1. Lrrk2 is highly expressed in the *striatum*

As discussed in the introduction, murine Lrrk2 is expressed in different brain areas, with reported high levels in the cortex and striatum (West et al., 2014). Consistently, we confirmed Lrrk2 localization in mouse in three brain regions relevant for PD: cortex, midbrain and striatum. Our analysis revealed that the steady state levels of the protein are higher in the cortex and striatum compared to midbrain (Fig.21).

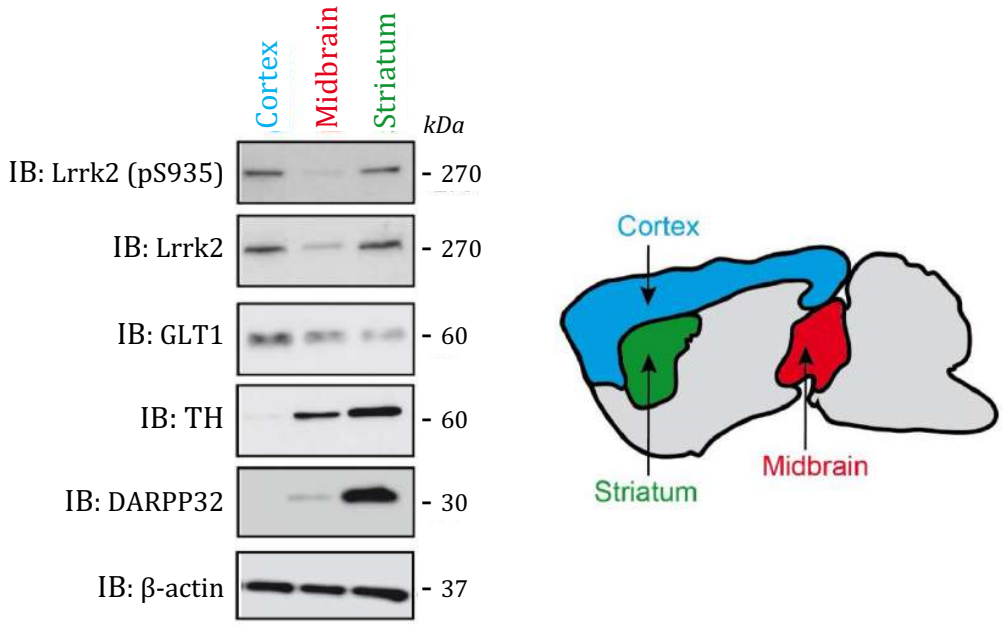


Fig.21: Lrrk2 steady state levels in PD relevant disease areas. *On the right, a schematic representation of cortex (blue), midbrain (red) and striatum (green) localization in mouse brain is reported. On the left, a representative western blot analysis shows Lrrk2 steady state levels and phosphorylated Ser935 levels in these PD-relevant areas of 24 month-old WT mice. Also the different levels of others markers can be appreciated (GLT1 for astrocytes, TH for dopaminergic neurons and DARPP32 for striatal neurons). β-actin was used as loading control. Adapted from (Iannotta et al., 2020).*

In addition, we investigated Lrrk2 cellular localization by immunofluorescence on coronal brain slices obtained from 1 month and 12 month-old BAC-G2019S (BAC-GS) mice. In these animals, the murine Lrrk2 locus is overexpressed by ~ 6-8 folds under the control of the endogenous promoter (Xianting Li et al., 2010), simplifying the visualization of Lrrk2 by immunofluorescence, which is very challenging when the protein is expressed at physiological levels. Of note, we detected high levels of total and

phospho-Ser935 signals in the dorsal striatum, whilst Lrrk2 signals are barely present in the cortex, suggesting that the protein is more expressed in the basal ganglia compared to the nearby cortical regions (Fig.22, A,C). Importantly, we checked the specificity of anti-total and anti-phospho-Ser935 antibodies Lrrk2 in Lrrk2-KO mice, which, as expected, do not display any fluorescent signal (Fig.22, B,D).

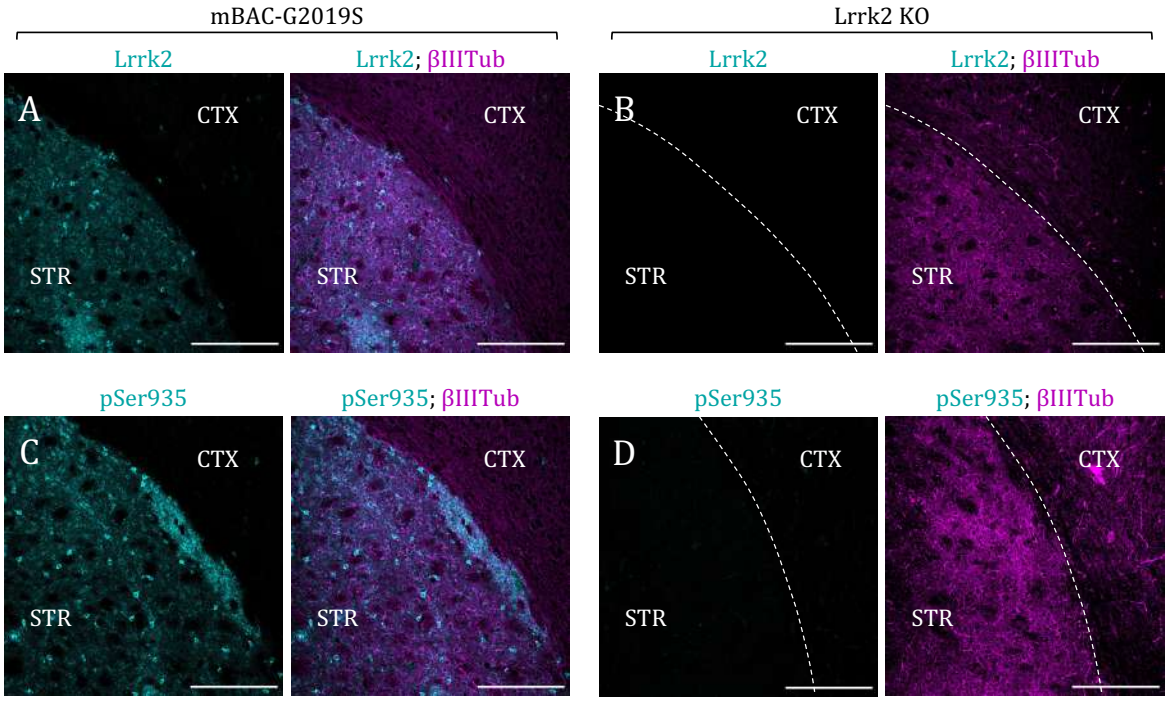


Fig.22: Immunofluorescence staining of brain slices from hemizygous transgenic mice overexpressing murine BAC-Lrrk2-G2019S and from Lrrk2-KO mice. *Coronal brain slices from BAC-Lrrk2-G2019S show higher Lrrk2 and pSer935 signals in the striatum compared to the cortex. The lack of fluorescence in Lrrk2-KO mice for both total and phospho-Lrrk2 confirms the specificity of the staining. Cyan: total Lrrk2 and pSer935 Lrrk2; Magenta: β-III-tubulin. Scale bar: 200 µm. Adapted from (Iannotta et al., 2020).*

Interestingly, in the dorsal striatum of 1 and 12 month-old BAC-GS mice we observed that Lrrk2 partially colocalizes with β-III-tubulin, a neuronal marker (Fig.23, A-B) and, to a lesser extent, with GLT-1, the major glutamate transporter mainly expressed by astrocytes (Fig.23, C-D). Instead, no co-localization with the microglial marker CD11b has been detected (Fig.23, E-F). Moreover, phospho-Ser935 Lrrk2 colocalizes with β-III-tubulin (Fig.24, A-B) but no (or very little) co-localization with GLT1 could be observed (Fig.24, C-D), suggesting that Ser935 phosphorylation is higher in neurons than in astrocytes. Also CD11b signal does not overlap with that of phospho-Ser935 Lrrk2 (Fig.24, E-F). Taken together, these results indicate that the

expression and phosphorylation of Lrrk2 in the mouse brain is high in neurons of the striatum.

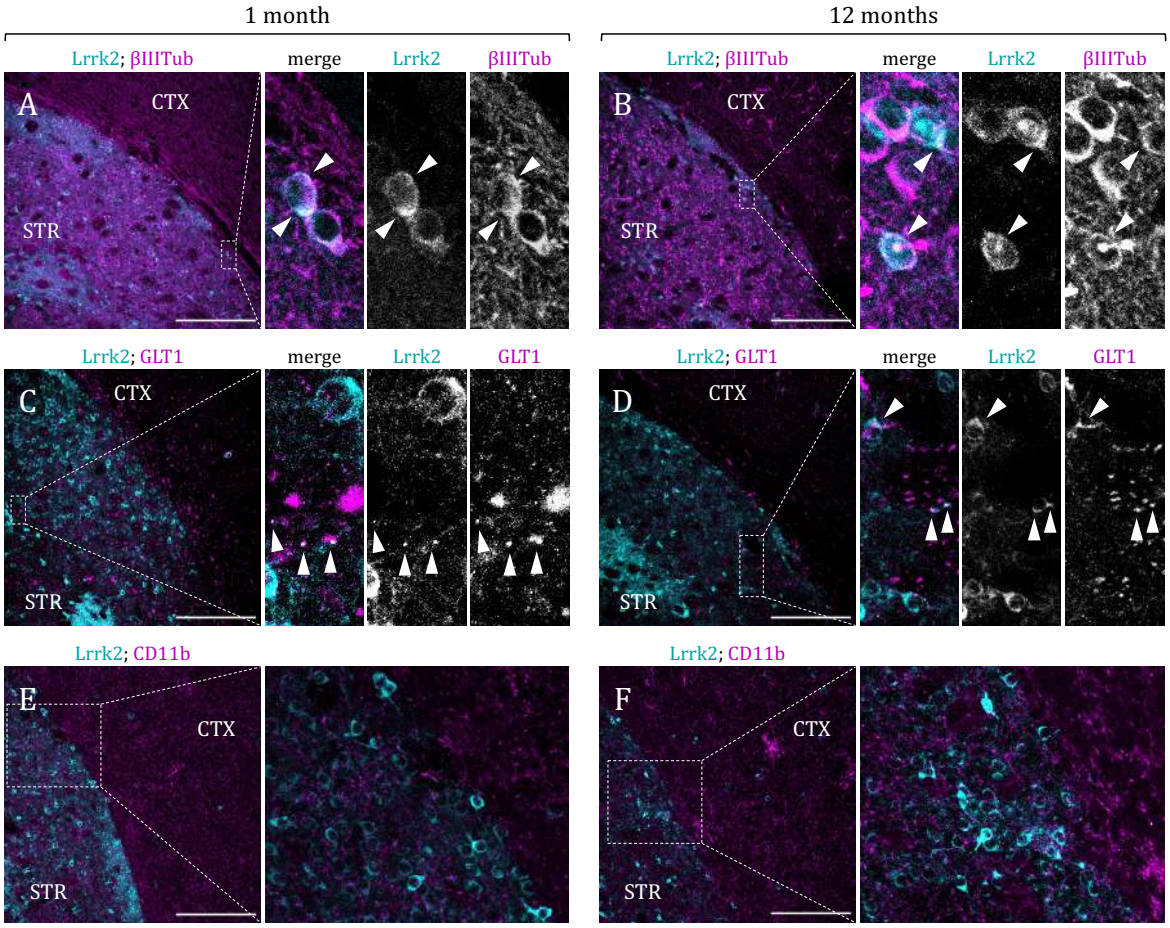


Fig.23: Immunofluorescence staining of brain slices from hemizygous transgenic mice overexpressing murine BAC-Lrrk2-G2019S showing Lrrk2 cellular localization. *Dorsal striatal coronal brain slices from 1 (A, C, E) and 12 (B, D, F) months old BAC-Lrrk2-G2019S mice show an intense Lrrk2 signal localizing in β-III-tubulin positive cells (A, B). The localization is barely visible in astrocytes positive for GLT1 (C, D) and almost absent in microglia marked with CD11b (E, F). Cyan: total Lrrk2; Magenta: β-III-tubulin (A, B), GLT1 (C, D), CD11b (E, F). Scale bar: 200 μm. Adapted from (Iannotta et al., 2020).*

The striatum is a nucleus of the basal ganglia where the large majority of the neuronal population (approximately 90-95%) is represented by GABAergic inhibitory spiny projecting neurons or medium spiny neurons (MSNs) (Lanciego et al., 2012). As suggested by their name, these neurons are characterized by an extensive dendritic arborization enriched in dendritic spines. The dorsal area of the striatum (composed by the caudate and the putamen) receives dopaminergic inputs from the DA neurons of the SNpc, forming the nigrostriatal pathway. The DA afferent terminals make

synaptic contacts onto the necks of MSNs spines, modulating the glutamatergic inputs that arrive at the same spines from the cortico-striatal projections. Thus, MSNs represent the post-synaptic cells of the nigro- and the cortical- striatal pathways and possess high levels of Lrrk2 expression. It can be hypothesized that an impaired response of the postsynaptic element associated with mutant Lrrk2 may result in a synapse that is weakened. As a consequence, the DA presynaptic neurons can sense this change in synaptic strength, a process that could contribute to their degeneration through a dying back mechanism. Based on these considerations, we decided to explored the physiological role of LRRK2 at dendritic spines.

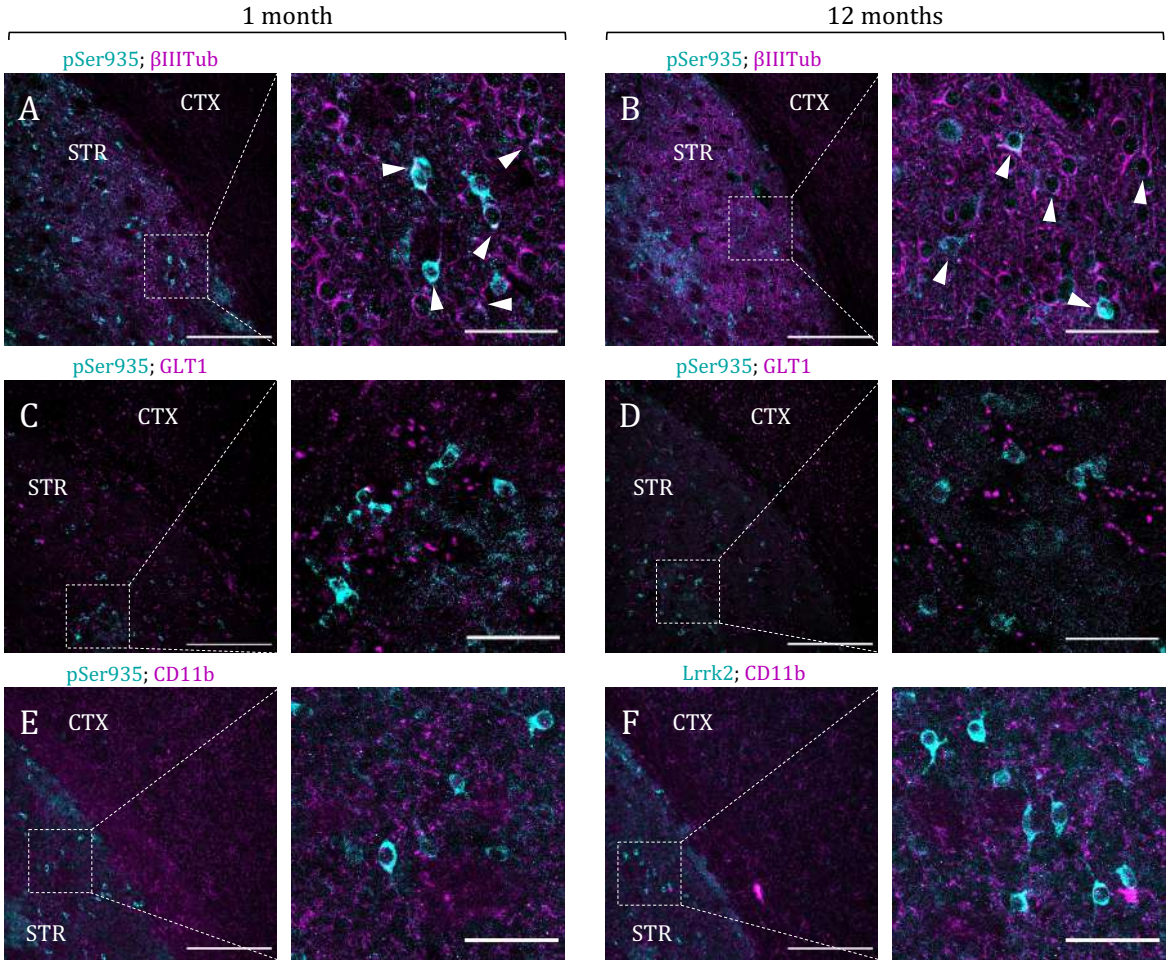


Fig.24: Immunofluorescence staining of brain slices from hemizygous transgenic mice overexpressing murine BAC-Lrrk2-G2019S showing phospho-Ser935 Lrrk2 cellular localization. *Dorsal striatal coronal brain slices from 1 (A, C, E) and 12 (B, D, F) months old BAC-Lrrk2-G2019S mice show a strong phospho-Ser935 Lrrk2 signal in neurons labeled with β-III-tubulin (A, B). On the contrary, it is not detectable in astrocyte and microglia (C, D, E, F). Cyan: phospho-Ser935 Lrrk2; Magenta: β-III-tubulin (A, B), GLT1 (C, D), CD11b (E, F). Scale bar: 200 µm. Magnifications scale bar: 50 µm. Adapted from (Iannotta et al., 2020).*

7.2. Lrrk2 interacts mostly with proteins involved in cytoskeletal dynamics

We initially set out to expand the current knowledge on LRRK2 interactome, with the aim of identifying candidate interactors that could support a role for the kinase at the postsynaptic compartment. Protein-protein interactions (PPI) can be analyzed via several strategies (Cookson, 2019). Here we chose to exploit affinity purification combined with tandem mass spectrometry analysis (AP-MS/MS) due to its high throughput and sensitivity. Basically, the target protein is isolated in conditions that maintain the binding with its interacting partners and, when purified, this protein complex is digested and submitted to MS/MS to identified its components. Notably, given a number of purified non-specific interactors is quite expected, the discrimination between contaminants and *bona fide* binders is essential. Thus, negative controls, consisting commonly in mock purifications conducting on naïve cells that do not express the protein of interest, are important to compare the abundance of purified proteins with that of the experimental condition.

To this purpose, we purified GFP-tagged LRRK2 from a stable *SH-SY5Y* neuroblastoma cell line that was previously generated in the lab (*SH-SY5Y* hLrrk2-GFP OE). To mimic the physiological environment as close as possible, cells were previously differentiated with retinoic acid (RA) for 6 days to obtain neuronal-like cells. Importantly, human-derived neuroblastoma *SH-SY5Y* cells have been found to give rise to both substrate adherent (S-type) and neuroblastic (N-type) cells. Undifferentiated *SH-SY5Y* cells, characterized by a continuous proliferation and by the lack of mature neuronal markers, resemble catecholaminergic immature neurons expressing tyrosine hydroxylase (TH) and possessing moderate levels of dopamine- β -hydroxylase (DßH) activity, specific of noradrenergic neurons (Filograna et al., 2015). Over the years, several protocols have been optimized to promote the differentiation of human-derived neuroblastoma *SH-SY5Y* cells in neuronal like cells (N-type), the most widely accepted and implemented of which is based on the treatment with RA (Kovalevich & Langford, 2013). RA is a vitamin A that inhibits cell growth and induces their transformation into a more neuronal-like population (N-type cells) that is biochemically, ultrastructurally, and electrophysiologically similar to primary neurons and, morphologically, presents long and branched processes (Encinas et al., 1999). Moreover, differentiated human-derived neuroblastoma *SH-SY5Y* cells have a

decreased proliferation rate and an increase in the expression of specific neuronal markers such as βIII-tubulin and neurofilament (Filograna et al., 2015). Differentiated *SH-SY5Y* hLrrk2-GFP OE cells were then solubilized and lysates overexpressing GFP-tagged LRRK2 were incubated with appropriate amount of GFP-trap®, a GFP Nanobody (VHH) coupled to agarose beads that allows to immunoprecipitate GFP-fusion proteins. Purified GFP-tagged LRRK2 together with its shell of co-purified interactors were subsequently analyzed *via* mass spectrometry, in collaboration with Prof Giorgio Arrigoni (Department of Biomedical Sciences). Differentiated naïve and GFP-expressing *SH-SY5Y* cells incubated with GFP-trap® served as negative controls, in order to exclude non-specific interactions (Fig. 25). While very few interactors were identified with GFP-expressing cells, more binders were detected by MS when using a mock lysate (naïve cells), suggesting that the GFP-trap resin is sticky. Thus, we used naïve cells as control to calculate the fold change (FC) of LRRK2 interactors over the background. Strikingly, besides a plethora of known LRRK2 interactors and PD related proteins, our analysis revealed that LRRK2 interactome is significantly enriched in actin-related or myosin-related proteins (Fig. 26), including cytospin-A (CYTSA), LIM domain-containing protein C (LIMCI), protein flightless-1 homolog (FLII), drebrin (DREB), myosin phosphatase target subunit 1 (MYPT1), actinin-1 (ACTN1), tropomyosin-1 (TMOD1) and many others (Table1).

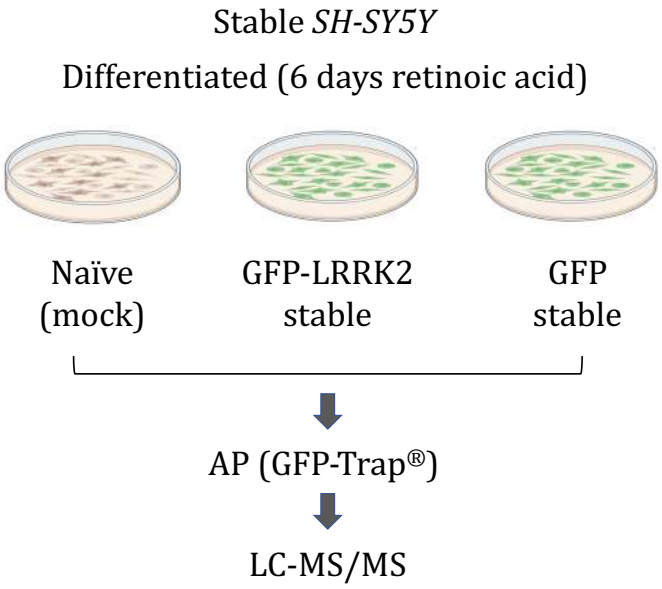


Fig.25. Schematic representation of experimental setup to evaluate LRRK2 interactome: *Differentiated SH-SY5Y cells were solubilized and subjected to affinity purification assay with GF-Trap®; samples were then analyzed with LC-MS/MS.*

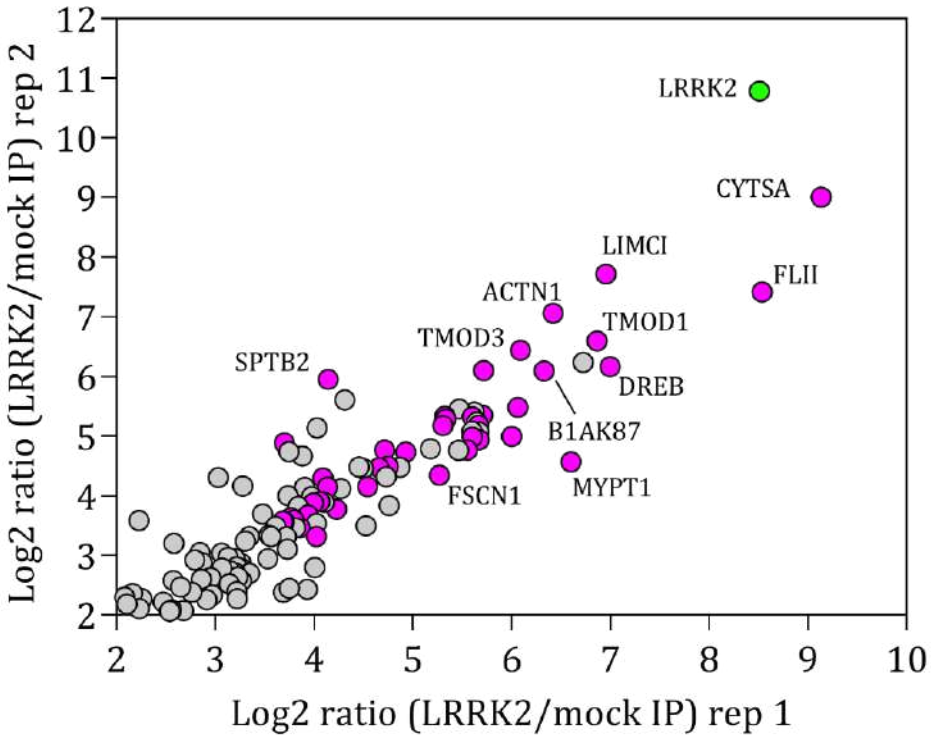


Fig.26: LRRK2 mainly interacts with actin-related and myosin-related proteins. *Scatter plot of mass spectrometry analysis showing in pink the LRRK2 interactors related to actin dynamics, mainly consisting of myosin- and actin- related proteins. Each axis reports the fold change (FC) in protein-protein interactions (LRRK2 IP / mock IP) expressed as Log_2FC, of each technical replicate.*

INTERACTOR	***PROTEIN***	***FUNCTION***
CYTSA	Cytospin-A	actin cytoskeleton organization and microtubule stabilization
FLII	Protein flightless-1 homolog	regulation of cytoskeletal rearrangements involved in cytokinesis and cell migration
LIMC1	LIMC1 protein	actin stress fibers-associated protein activating non-muscle myosin Iia
TMOD1	Tropomodulin-1	actin-related protein that blocking the elongation and depolymerization of the actin filaments at the pointed end
ACTN1	Alpha-actinin-1	F-actin cross-linking protein anchoring actin to a variety of intracellular structures
DREB	Dehydration responsive element-binding protein	acting-binding protein playing a role in dendritic spine morphogenesis and organization
TMOD3	Tropomodulin-3	actin-related protein blocking the elongation and depolymerization of the actin filaments at the pointed end
B1AK87	F-actin-capping protein subunit beta	F-actin-capping protein binding to the fast growing ends of actin filaments blocking the exchange of subunits at these ends
FSCN1	Fascin	actin-binding protein organizing filamentous actin into parallel bundles
SPTB2	Spectrin beta chain, brain 1	actin crosslinking and molecular scaffold protein linking the plasma membrane to the actin cytoskeleton
MYH9, MYH10	Myosin-9; Myosin-10	cellular myosin playing an important role in cytoskeleton reorganization

Table1: LRRK2 mainly interacts with actin-related and myosin-related proteins. *List of some of the main actin- and myosin- related LRRK2 interactors.*

The dynamics and remodeling of actin mesh at dendritic spines relies on the activity of a set of actin-related proteins exerting different functions from polymerization/ depolymerization and severing of filamentous actin (Matus, 2000). Alongside, myosin-related proteins also influence the organization of actin filaments into dendritic spines by regulating the activity of non-muscle-myosin motors, mechanoenzymes that hydrolyze ATP to move along the actin filaments different cellular components (Kneussel & Wagner, 2013).

To gain insight into common pathways shared by the most significant LRRK2 interactors, we carried out a gene ontology (GO) enrichment analysis for biological processes using the list of interactors with FC > 2 (gProfiler; https://biit.cs.ut.ee/gprofiler/gost). The analysis revealed enrichment for actin organization pathways (Fig. 27; pathways with $p < 10^{-11}$ are shown).

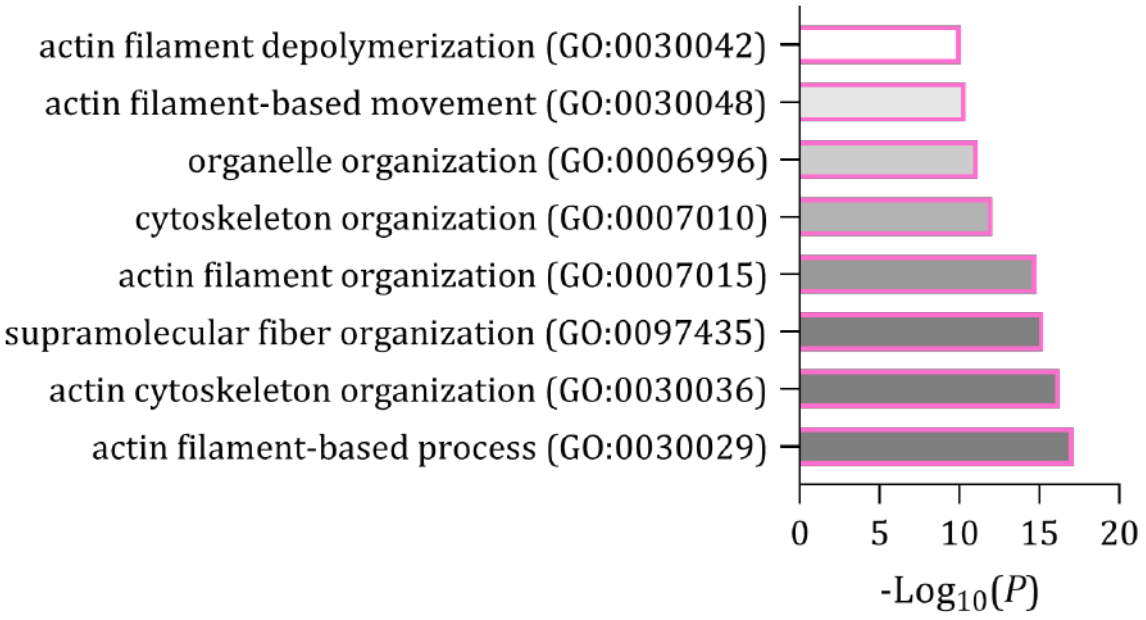


Fig.27: Lrrk2 mainly interacts with proteins involved in actin cytoskeleton dynamics. *Graphical representation of GO enrichment analysis for the biological processes conducted on the mass spectrometry hits presenting a FC > 2. We reported the categories showing an adjusted p-value > 10^{-10}. The analysis confirms that the major interactors of Lrrk2 that we found with mass spectrometry are involved in functions related to the actin cytoskeleton dynamics (n=2 technical replicates).*

7.3. Lrrk2 impacts dendritic spines maturation during the development

Previous studies from our laboratory highlighted a link between LRRK2 and actin in the process of neurite outgrowth. In particular, Civiero et al. found that LRRK2 cooperates with the kinase PAK6 (p21-activated kinase 6) to control neurite complexity via the LIM kinase-cofilin-actin pathway (Civiero et al., 2015; Civiero, Cogo, et al., 2017). Furthermore, Parisiadou and colleagues observed that Lrrk2 KO mice display defects in dendritic spines maturation at post-natal stages, pointing to altered PKA pathway dependent cofilin phosphorylation (Parisiadou et al., 2014). Based on this previous evidence together with our PPI studies on *SH-SY5Y* cells, we set out to understand whether LRRK2 physiological activity is important to shape postsynaptic spines, whose structure is heavily affected by actin-remodeling processes. To this aim we explored the consequences of Lrrk2 loss of function on striatal dendritic spine morphology, number and ultrastructure, providing an extensive characterization of synapses architecture. For the first time to our knowledge, we carried out a comprehensive longitudinal study in developing (1 month-old), mature (4 month-old), and aged (18 month-old) brains of Lrrk2 knock out (KO) mice in parallel with the wild-type (WT) littermates.

7.3.1. Lrrk2 and dendritic spines morphology

We initially analyzed the morphology and the size of dendritic spines performing Golgi Cox staining on coronal brain slices. The Golgi method, initially described in 1873, provides fine neuro-anatomical information and the advantage of staining fewer than 5% of the neuronal population, which allows the visualization of individual neuronal processes.

Here, we used a modified Golgi impregnation method named Golgi-Cox staining, in which mercury chloride is employed to foster the impregnation of neurons. Since this technique was not present in the laboratory, we set out to optimize the staining in our samples. As shown in figure 8, we were able to visualize different brain areas containing neurons with distinctive orientation and morphology. The cortical area of the coronal slices (Fig. 28, A) contains both non-pyramidal and pyramidal neurons organized in the typical layers that distinguish the cortex. The pyramidal neurons, as expected, are the most numerous and present the characteristic morphology with a

conic shaped soma, a large typical dendrite orientated toward the upper layers of the cortex and basal dendrites faced in the opposite direction. In the hippocampus both pyramidal and granule cells can be appreciated (Fig. 28, B). Moreover, the striatum appears enriched in MSNs with an extensive neurite network. These neurons exhibit ramifications of the primary dendrites that divide one or twice to form secondary and tertiary processes and of the axonal branches originating from the main axon (Fig. 28, C). To further confirm the suitability of our impregnation protocol, we used WT mouse brains to compare the number of spines per unit of length in the dorsal striatum versus the cortex: as expected, MSNs possess more spines with respect to cortical neurons (Fig. 29).

We have then compared the morphology, average length, average width and number of protrusions in MSNs of the dorsal striatum of Lrrk2 WT versus Lrrk2 KO mice. Lrrk2 KO mice were generated by deletion of exon 41 and were a kind gift of Dr. Heather Melrose (Hinkle et al., 2012). The dataset collected and analyzed includes: 1) 1 month old (n=3 WT and n=3 KO), 2) 4 month-old (n=3 WT and n=3 KO) and 3) 18 month-old (n=3 WT and n=3 KO) brains. Mice have been randomly paired per genotype and each pair (1 WT and 1 KO) were processed, stained and imaged in parallel. About 20 neurites of ~20-30 μm in length were imaged and analyzed per each mouse. The average number of each parameter (width, length, number and spine category) was calculated and the mean value from the 3 individuals was used to calculate the overall mean value ± SEM. Images have been acquired at the confocal microscopy using the phase contrast acquisition mode and the analysis has been performed with the software Reconstruct (http://synapses.clm.utexas.edu).

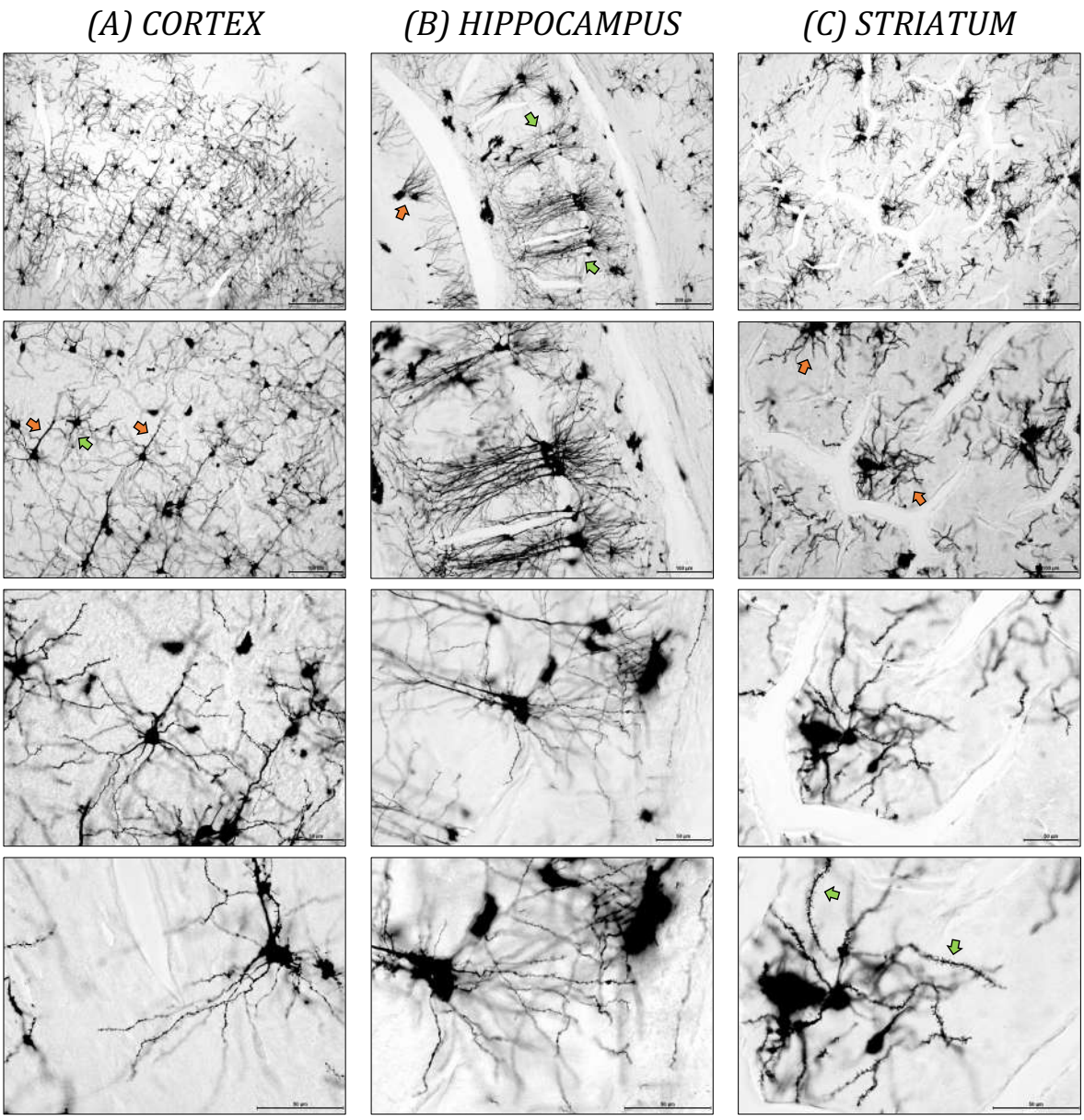


Fig.28: Representative images of Golgi-Cox impregnated slices of 3 month old WT mouse brains at different magnifications and showing neuronal cell types typical of specific brain areas. (A) *Cortex: This region presents pyramidal (orange arrow) and non-pyramidal (green arrow) neurons organized in the distinctive cortical layers;* ***(B)*** *Hippocampus: Both granular (orange arrow) and pyramidal cells (green arrow) are visible in this part of the brain;* ***(C)*** *Striatum: In this brain compartment the main cell type is represented by medium spiny neurons (orange arrow) that, notably, are particularly enriched in dendritic spines (green arrow). Scale bar: 1st line 200µm, 2nd line 100µm, 3rd and 4th line 50µm.*

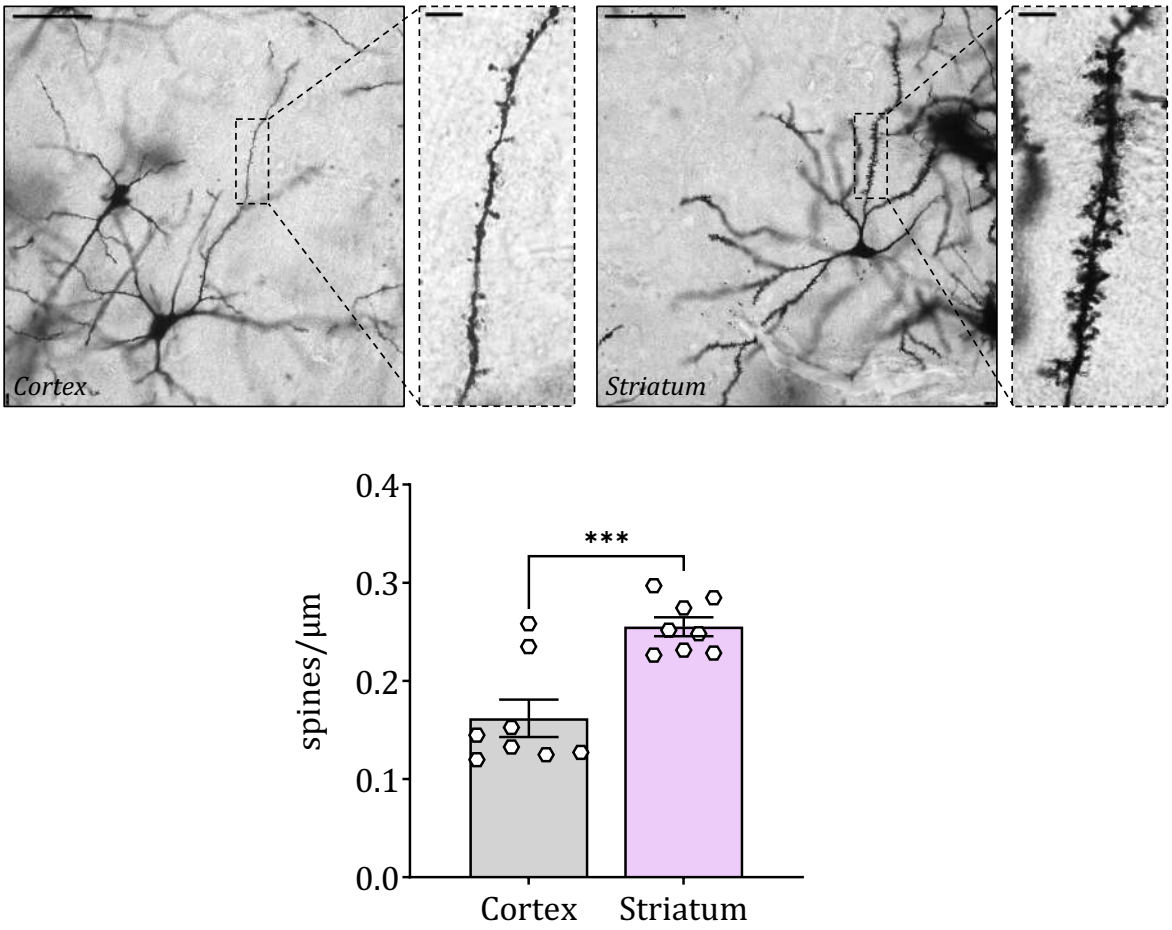


Fig.29: Analysis of dendritic spine density in cortical neurons versus MSNs: *the analysis has been performed on 8 images per brain area measuring the number of dendritic spines per unit (µm) of dendritic length. Differences between the two brain areas have been evaluated using Student's t-test (significance ***p<0.001). Scale bar: 50 µm; Magnifications scale bar: 5 µm.*

We initially measured the length of the spine neck and the width of the dendritic spine head, two parameters that allow to classify the protrusions into the different morphological classes. In particular, *filopodia* represent the precursor of dendritic spines and they are considered the less mature structures. Among dendritic spines, the so- called *thin spines* constitute the immature protrusion and are characterized by long necks and small heads. The *mushroom* and the *branched spines* consist instead in mature postsynaptic elements with large or ramified heads, respectively. Finally, *stab spines* sit in between immature and mature protrusions. As shown in figure 30, one month-old Lrrk2 KO animals present a different distribution pattern of protrusion classes as compared to Lrrk2 WT mice, whereas these differences disappear when the animals age (4 months and 18 months) (Fig. 30). Specifically, 1 month-old KO mice

exhibit a reduced number of filopodia and an increased quantity of thin protrusions, suggesting that loss of Lrrk2 promotes the transition from filopodia to thin spines, an intermediate stage between non-functional and fully functional post-synaptic structures. Instead, the proportion of fully mature spines (mushroom and branched) remained unaltered. Of note, a non-significant trend of increased thin spines persist at older ages. To test if this substle effect is real, a much larger number of mice should be employed. Using gPower 3.1, I estimated a sample size of 11 animals per group (with alpha 0.05 and beta 0.8) considering an effect size of 0,82 based on our current data (Fig. 30).

We next evaluated the width, the length and the number of dendritic protrusions (all classes) in the two genotypes across the three time points. As shown in figure 31, one month-old KO mice display a reduction in the average neck height of about 15% (Fig.31 A-C) and a decrease in the average head width of about 27% (Fig.31 A-B), meaning that spines are smaller in Lrrk2 KO brains compared with Lrrk2 WT mice. Of note, the total amount of protrusion does not change between the two genotype and across the different ages (Fig.31 A). Interestingly, the number of protrusions increased significantly around 4 months in both of genotypes and then decreased at 18 months, a possible consequence of physiological synapse loss (Fig.31 A).

Taken together, these results indicate that murine Lrrk2 plays an important function in the process of spine maturation during the post-natal stage, as further supported by other results (see below). Of interest, Parisiadou and collaborators observed a substantial decrease of mature spines and increase of dendritic filopodia in developing MSNs in Lrrk2 KO brains compared with WT, as well as a higher proportion of thin and 'less mushroom' spines and increased phospho-cofilin in KO neurons within the first postnatal days (Parisiadou et al., 2014). Although we did not find a reduced number of spines in 1 month-old Lrrk2 KO brains, our study similarly suggests that there are spine maturation defects in the absence of Lrrk2 and it adds the important notion that these defects occur during development and normalize upon aging.

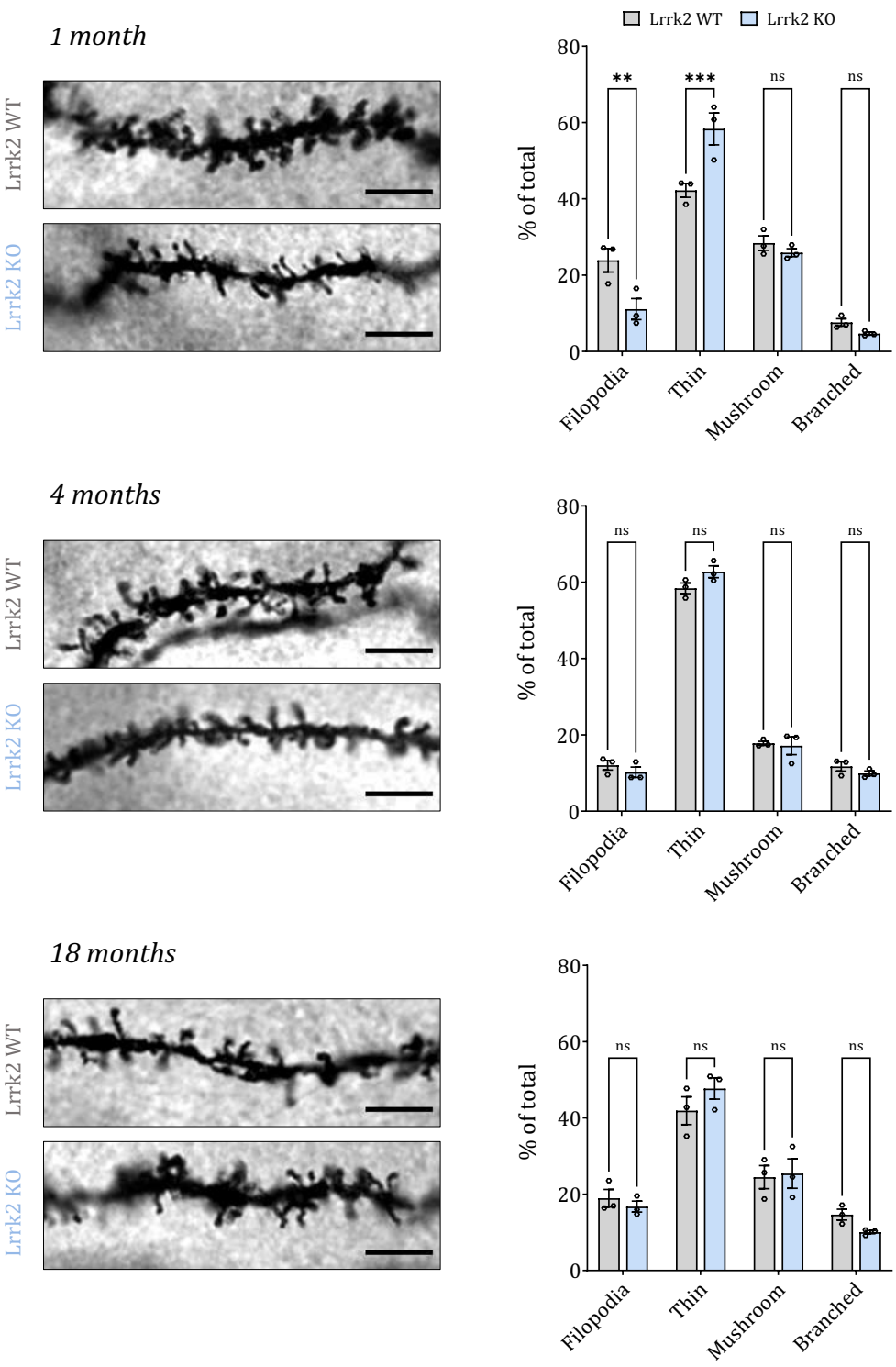


Fig.30: Lrrk2 influences striatal dendritic spines maturation in one month old mice: *Representative images of the neurite segments analyzed (about 20 segments per animal) are reported in the left side for each age. Scale bar: 3 µm. As showed in the right side, protrusion were classified in four morphological classes (filopodia, thin, mushroom, branched) and graphically represented as % of the total number. Maturation defects are observed only in Lrrk2 KO one month old mice (upper figure). Significance between genotypes was tested using 2 way ANOVA with Tukey's multiple comparison test (significance ***p<0.001; **p<0.01), n=3 animals for genotype for each age.*

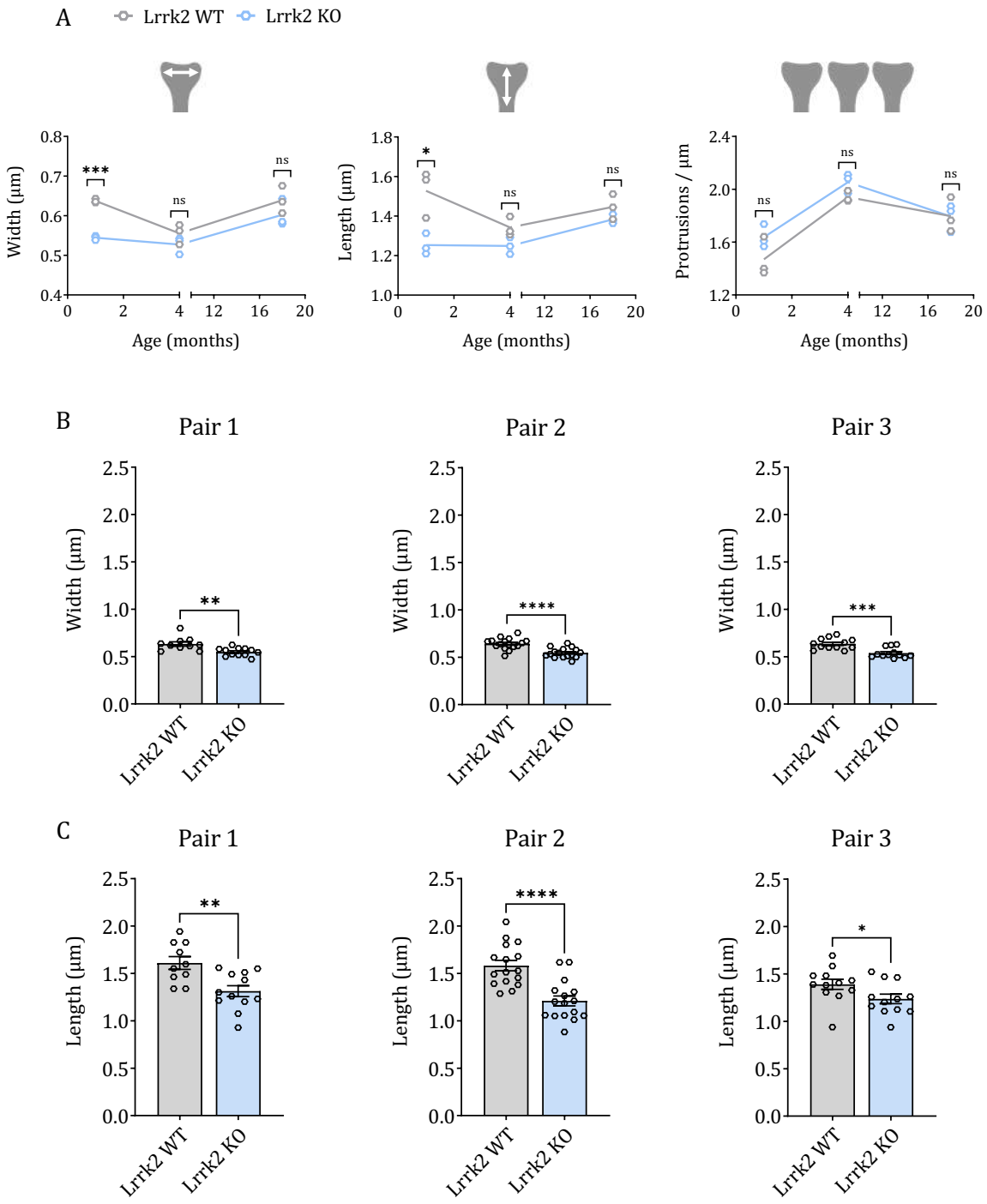


Fig.31: Lrrk2 influences the average width of dendritic spines head and the average length of dendritic spines neck in one month old mice, whereas it does not impact on the number of total protrusions. (A) *Graphical representation of average spine width (left), length (middle) and number (right) across n=3 KO brains and n=3 WT brains at different ages. Each dot represents the average value from each animal (n=3 animals). Differences between genotypes were evaluated using Student t-test (significance ****p<0.0001; ***p<0.001; **p<0.01; *p<0.5).* ***(B-C)*** *Representative analyses of individual mice (WT/KO pairs) at 1 month ages. Each dot represents the average width (B) or length (C) of the spines in each segment. Statistical significance was assessed with Student's t-test (****p<0.0001; ***p<0.001; **p<0.01; *p<0.5).*

7.3.2. Lrrk2 and synapses number

We next wondered whether the increased amount of "less mature" dendritic spines (thin protrusions) could affect the formation of synaptic contacts. To assess this aspect we have performed immunofluorescence on dorsal striatal brain slices utilizing as a specific presynaptic marker VAMP2 and as a specific postsynaptic marker PSD95. Then, exploiting the Fiji plugin ComDet v.0.4.1, we counted the number of yellow spots that represent the colocalization of the two compartments and, thus, the presence of synaptic contacts. The analysis showed a significant reduction in the number of synapses in 1 month-old Lrrk2 KO mice compared to WT littermates, whereas in 4 and 18 month-old KO mice the decrease is still present but not statistically significant (Fig. 32).

The smaller spine size in one-month old mice (Fig. 31) may underlie the reduced synapse number in 1 month-old KO mice (Fig. 32), suggesting that fewer dendritic spines are sufficiently mature to make synaptic connections.

7.3.3. Lrrk2 and the postsynaptic ultrastructure

To further explore potential differences in postsynaptic structure morphology, we used transmission electron microscopy (TEM) on striatal brain slices of 1 and 18 month-old WT vs KO mice. Specifically, we measured the postsynaptic density (PSD) length as it directly correlates with the amount of those proteins orchestrating post-synaptic signalling, such as the postsynaptic receptors, thus providing an indication on the maturation levels and the functionality of the synapse. The analysis revealed that PSD is significantly shorter in 1 month-old Lrrk2 KO animals compared to WT mice, while this difference is not observed in 18 month-old mice (Fig. 33).

Taken together, these results support the presence of subtle postsynaptic maturation defects in the striatum of post-natal Lrrk2 KO mice.

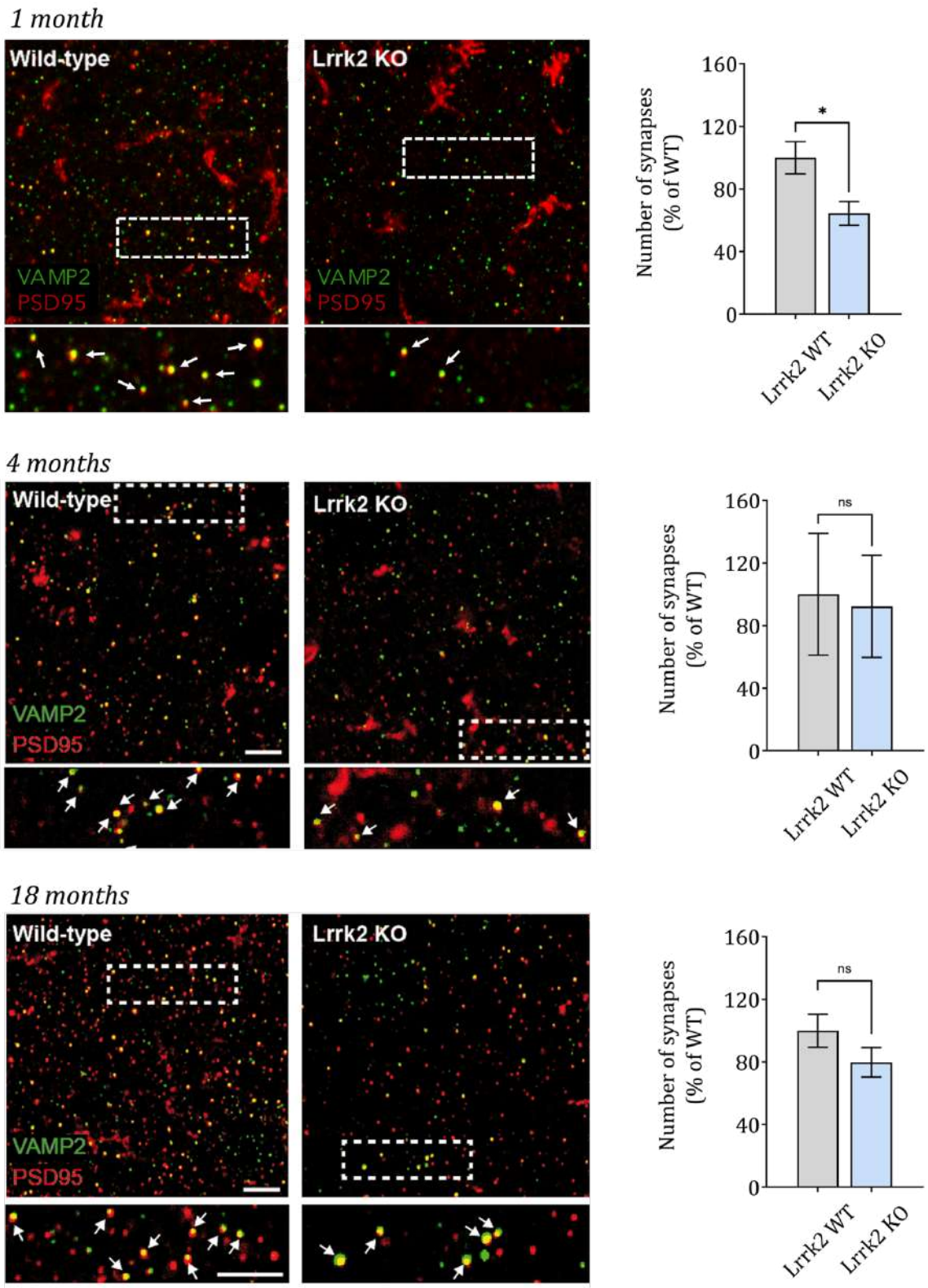


Fig. 32. Lrrk2 influences the number of striatal synapses: *On the left, representative immunofluorescence images of striatal brain slices stained with presynaptic VAMP2 (in green) and postsynaptic PSD95 (in red) markers to compare synapse number in Lrrk2 WT versus Lrrk2 KO mice at 1 month, 4 months and 18 months of age. Scale bar 5 µm. On the right, quantification for each age is reported. Significant reduction in synapses number is observed only in Lrrk2 KO one month old mice (upper figure). Statistical significance was assessed with Student's t-test (*p<0.5), n=3 animals per condition were analyzed.*

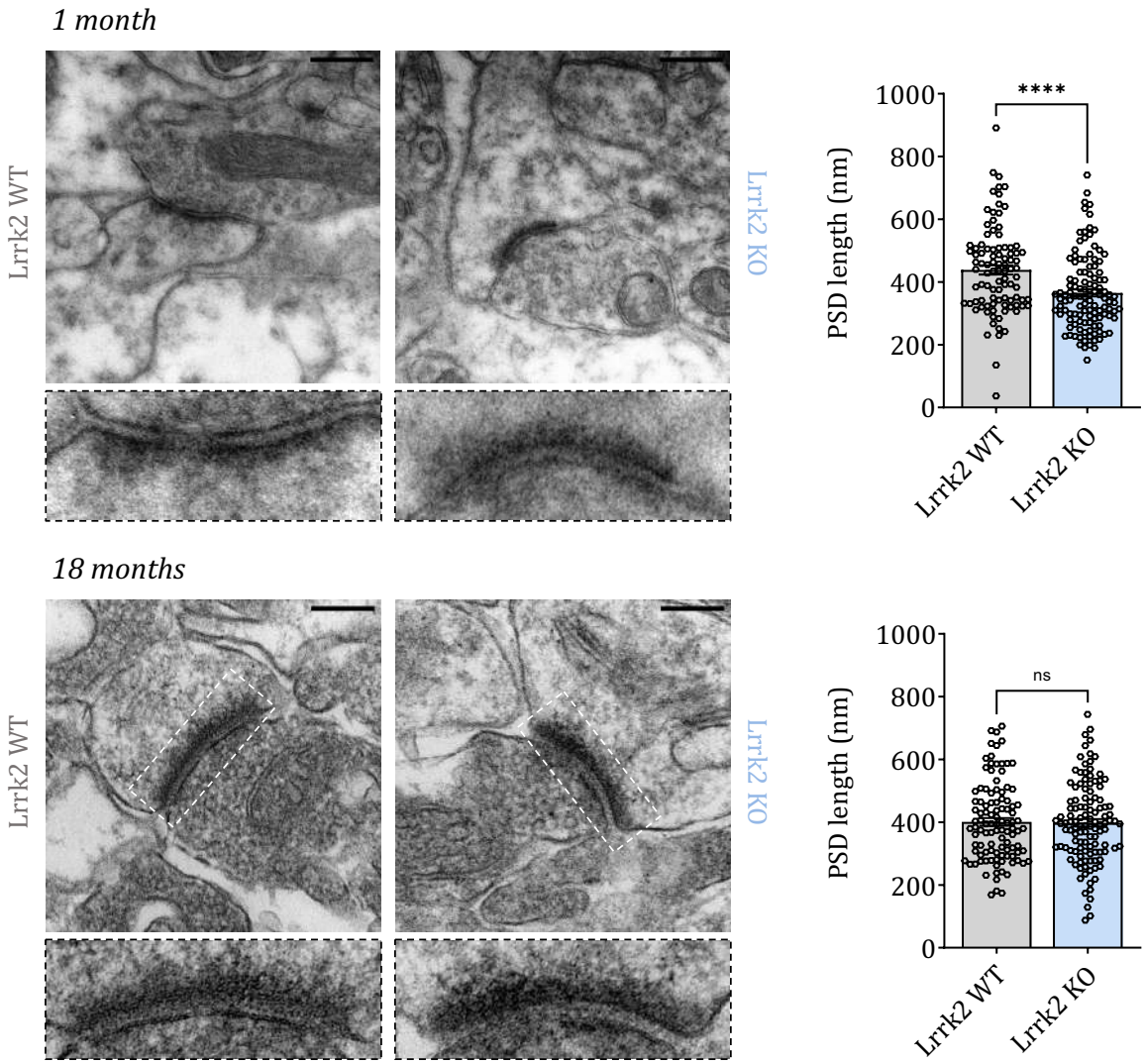


Fig.33. Lrrk2 loss of function is associated with a reduced PSD length during development: *the length of the PSD has been evaluated performing transmission electron microscopy on striatal brain slices from 1 and 18 months old mice (representative images on the left). 1 month old Lrrk2 KO mice, but not the 18 months old mice present shorter PSD compare to the control. Differences between genotypes have been evaluated using Student's t-test (significance ****p<0.0001), n=4 (80 synapses per genotype) and n=3 animals (120 synapses per genotype) per genotype were analyzed at 1 month and at 18 months of ages respectively. Scale bar: 200 nm.*

7.4. Lrrk2 is involved in spinogenesis induced by BDNF

The results we obtained from the analysis of Lrrk2 KO brains support a role for Lrrk2 in orchestrating dendritic spine formation and maturation during the first post-natal stages. To gain mechanistic insights into the involvement of LRRK2 in these processes, we set out to establish an *in vitro* model of dendritic spine formation taking advantage of the neurotrophic activity of brain derived neurotrophic factor (BDNF). In particular, BDNF is well known to positively influence dendritic spine maturation and

synapse formation in striatal MSNs (Mercado et al., 2017). In particular, BDNF is able to promote spinogenesis in primary mouse neurons (Lai et al., 2012), thus constituting a great model to study the possible link between LRRK2 and spine maturation. Based on a previously established method (Lai et al., 2012), we evaluated the formation of new spines upon BDNF treatment in Lrrk2 WT and KO primary neurons (Fig. 34). Specifically, at DIV4 (days in vitro), primary cortical neurons were transfected with GFP to fill the entire dendritic arbor, including dendritic spines. At DIV14 when synapses are fully mature, cultures were exposed to BDNF for 24 hours. After the treatment, neurons were fixed and stained with the primary antibody against PSD95, a post-synaptic marker of dendritic spines that labels the PSD, a structure that is present within dendritic spines head. The specific neuronal marker MAP2 has been also used to rule out astrocytes or microglia. We then assessed the number of spines by counting the number of PDS95-positive puncta along dendrites. While WT neuronal cultures responded to BDNF as expected, Lrrk2 KO neurons showed an impaired response to BDNF treatment, which failed to promote the formation of new PSD95-positive puncta (Fig. 35). This finding supports a mechanism whereby Lrrk2 is important to stimulate spinogenesis in response to BDNF exposure.

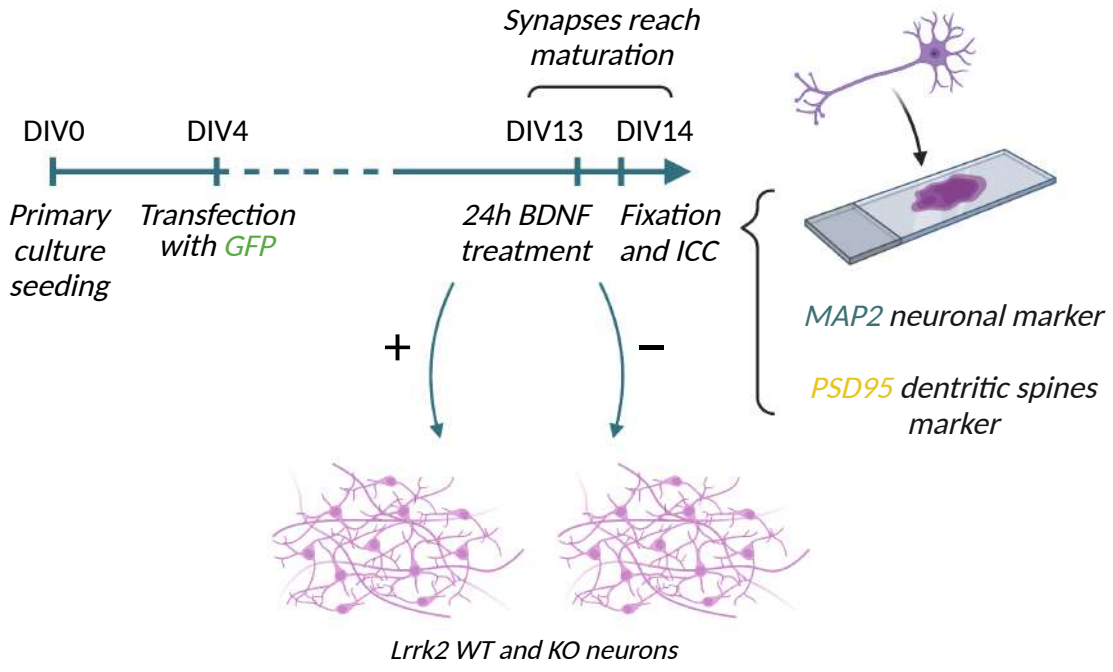


Fig.34. Schematic representation of experimental setup to evaluate spinogenesis upon BDNF treatment. *Created with BioRender.com.*

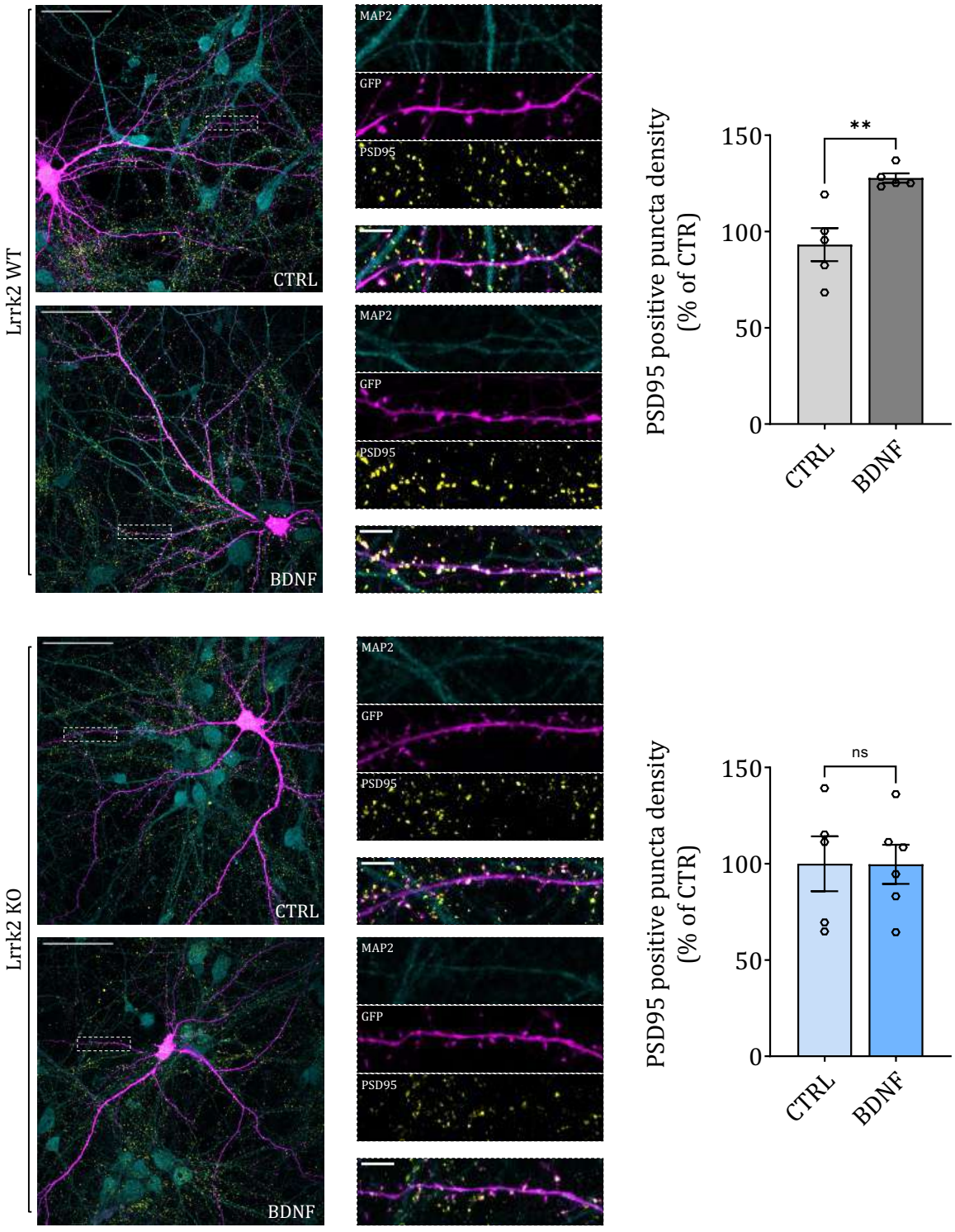


Fig.35. Primary cortical neurons with Lrrk2 KO present reduced dendritic spines formation upon BDNF stimulation: *Representative images of Lrrk2 WT and KO neurons treated for 24 hours with BDNF. GFP is colored in magenta, PSD95 in yellow and MAP2 in cyan. Scale bar: 50 µm. The density of PSD95 positive puncta along each neuronal dendrite has been assessed via Image-J plugin SynapCount. Differences between the two treatment conditions have been evaluated using Student's t-test (significance **p<0.01), n=3 biological replicates.*

Given that Lrrk2 KO neurons do not respond to 24-hour BDNF stimulation, we next wondered whether LRRK2 activity is modulated directly by BDNF signaling. LRRK2 is a protein with GTPase and a serine-threonine kinase activities and it possesses different clusters of serine residues that can be phosphorylated *via* autophosphorylation or *via* the action of heterologous kinases. While autophosphorylation at Ser1292 is considered a direct indicator of LRRK2 activity but it is of difficult detection under physiological LRRK2 expression (Iannotta et al., 2020), phosphorylation of Ser935 in the N-terminal region can be robustly detected in brain and in cells (Reynolds et al., 2014). While the exact consequence of this phosphorylation is still unclear, it is well-established to serve as a docking site (together with other neighboring serine residues) for 14-3-3s proteins, which influence LRRK2 dimerization (Civiero, Russo, et al., 2017), subcellular localization (Civiero, Russo, et al., 2017; X. Deng et al., 2011; Nichols et al., 2010) and, as a consequence, access to its cellular substrates (Lavalley et al., 2016). The phosphorylation and dephosphorylation of Ser935 is mediated by the activity of several upstream kinases and phosphatases (Iannotta & Greggio, 2021).

To evaluate the involvement of Lrrk2 in the BDNF pathway, we monitored the phosphorylation of Ser935 in primary neurons and then exposed to BDNF at different time points. As shown in figure 36, we observed a significant increase in Lrrk2 phosphorylation at Ser935 upon BDNF exposure. Specifically, the phosphorylation reached a peak after 5 minutes of treatment and rapidly decreased at later timepoints, suggesting that Lrrk2 may be quickly recruited/activated upon BDNF binding to its receptor TrkB, similar to Akt and Erk1/2, two of the three major intracellular signaling pathways activated by BDNF (Fig.37). As a control, we pharmacologically inhibited Lrrk2 activity by a 90 minute pretreatment of neurons with 0.5μM of the potent and selective inhibitor MLi-2, which is well-known to induce Ser935 dephosphorylation (Fell et al., 2015). As expected, MLi-2 leads to a dephosphorylation of Ser935 residue in control condition and, interestingly, BDNF is not able to increases Ser935 phosphorylation in presence of the inhibitor (Fig. 36). The recent structural information on LRRK2 (Deniston et al., 2020; Watanabe et al., 2020) highlighted that type I inhibitors, which includes MLi-2, promotes the transition of the kinase domain to the closed conformation, similarly to the effect produced by the majority of LRRK2

pathogenic mutations. As both LRRK2 inhibited with type I compounds (not type II) and mutant LRRK2 are dephosphorylated at Ser935, it is reasonable to conceive that the closed kinase conformation promotes the activity of phosphatases or inhibit the action of kinases (Iannotta & Greggio, 2021). Based on our results, we can infer that the activity of the upstream kinase phosphorylating LRRK2 at Ser935 upon BDNF stimulation cannot override the conformational effect of the inhibitor, suggesting that the site is inaccessible to phosphorylation.

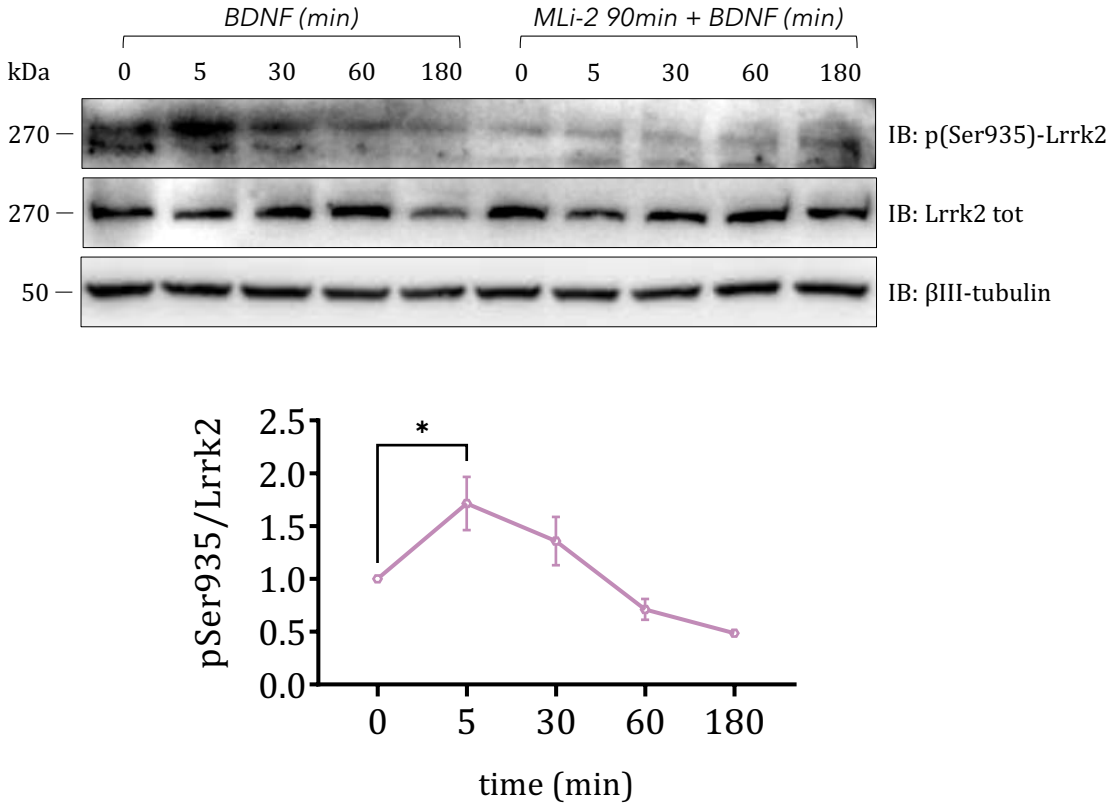


Fig.36. Lrrk2 WT primary neurons respond to BDNF treatment increasing phospho-Ser935 Lrrk2 levels: *Western blot analysis of primary neurons from Lrrk2 WT mice treated with BDNF shows that Lrrk2 rapidly responds to the stimulation by increasing its phosphorylation state. The intensity of the bands were normalized to βIII-tubulin, used as loading control. Changes in the levels of phospho-Ser935 Lrrk2 at the different time points of the treatment with respect to the basal condition have been evaluated by one-way ANOVA with Dunnett's multiple comparisons test (significance *p<0.5), n=6 biological replicates. The increase in Lrrk2 phosphorylation is significant only after 5 minutes of treatment.*

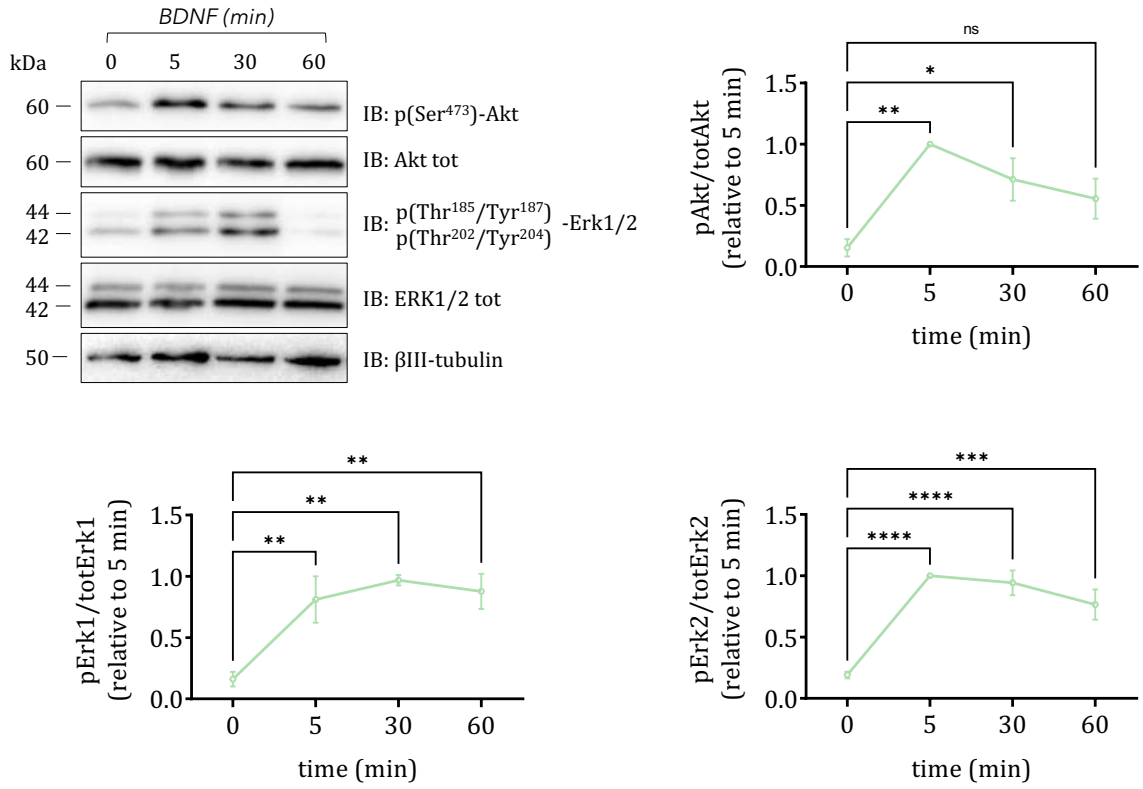


Fig.37. Primary cortical neurons respond to BDNF treatment: *Western blot analysis of primary cortical neurons treated with BDNF showing that Akt and Erk1/2 rapidly respond to the stimulation by increasing their phosphorylation state. The intensity of the bands of interest were normalized on βIII-tubulin , used as loading control. Changes in the levels of pAkt and pErk1/2 in respect to the basal condition have been evaluated performing one-way ANOVA with Dunnett's multiple comparisons test (significance ****p<0.0001; ***p<0.001; **p<0.01; *p<0.5), n=4 biological replicates.*

After having found that Lrrk2 phosphorylation state is influenced by BDNF stimulation in primary neurons, we decided to investigate the same LRRK2 activity readout in human-derived neuroblastoma *SH-SY5Y* cells, a more accessible cellular model to conduct mechanistic studies. Indeed, this cell line is advantageous for a number of reasons. First of all it possesses a neuronal origin, as it derives from a subclone of a parental *SK-N-SH* neuroblastoma cell line, established from a metastatic tumor of bone marrow (Kovalevich & Langford, 2013). Moreover, the manipulation of this cell model allows to overcame the post-mitotic nature of primary neurons, thus reaching large-scale expansion. Moreover, *SH-SY5Y* cells can be genetically engineered to overexpress or downregulated a gene of interest. Importantly, human-derived neuroblastoma *SH-SY5Y* cells can be differentiated into neuronal-like cells through

several protocols, among which the most widespread is based on the use of retinoic acid (Kovalevich & Langford, 2013). It has been also reported that RA promotes the expression of high levels of BDNF receptor, TrkB , that lacks in undifferentiated *SH-SY5Y* cells (Kaplan et al., 1993), making them able to respond to the neurotrophin stimulation.

After differentiation of *SH-SY5Y* cells with RA for 6 days, we first evaluated whether our model responded appropriately to BDNF treatment. To this end, we assessed the activation of two of the three major BDNF effectors: Akt, a serine/threonine kinase possessing a key role in the PI3K/AKT pathway, and ERK1/2, serine/threonine kinases MAPKKK in the MAPK signaling cascade. Upon treatment with BDNF, both AKT (Fig. 38, A) and ERK1/2 (Fig. 38, B) are rapidly phosphorylated. The phosphorylation reaches a peak after 5 minutes and then starts to return at the basal levels. This result indicates that differentiated *SH-SY5Y* cells are competent to sense BDNF stimulation and reply with the activation of signaling pathways that are important for synapse plasticity.

To then establish whether LRRK2 responds to BDNF stimulation, we evaluated the expression of endogenous LRRK2 in differentiated versus undifferentiated *SH-SY5Y* cells. Interestingly, the expression of LRRK2 is greatly increased after cell neuronal differentiation, suggesting that its activity is important for the maintenance of neuronal specific functions and structures (Fig. 39). At this point, we wanted to see whether BDNF stimulates LRRK2 phosphorylation also in differentiated *SH-SY5Y* cells. As shown in figure 40, upon BDNF stimulation LRRK2 phosphorylation increases following the same trend observed in primary neurons, with a maximal response at 5 minutes. Similarly, the presence of MLi-2 prevents BDNF-induced LRRK2 phosphorylation. Of note, LRRK2 phosphorylation returns to the basal level (or even lower) after 60 minutes of stimulation, whilst the phosphorylations of AKT and ERK1/2 are still above the unstimulated condition at this time point (Fig. 38).

Taken together, these results further strengthen the notion that LRRK2 is directly involved in BDNF signaling, and that differentiated *SH-SY5Y* cells represent a suitable model to study the mechanisms behind BDNF-LRRK2 signaling pathway and its role in modulating spine architecture.

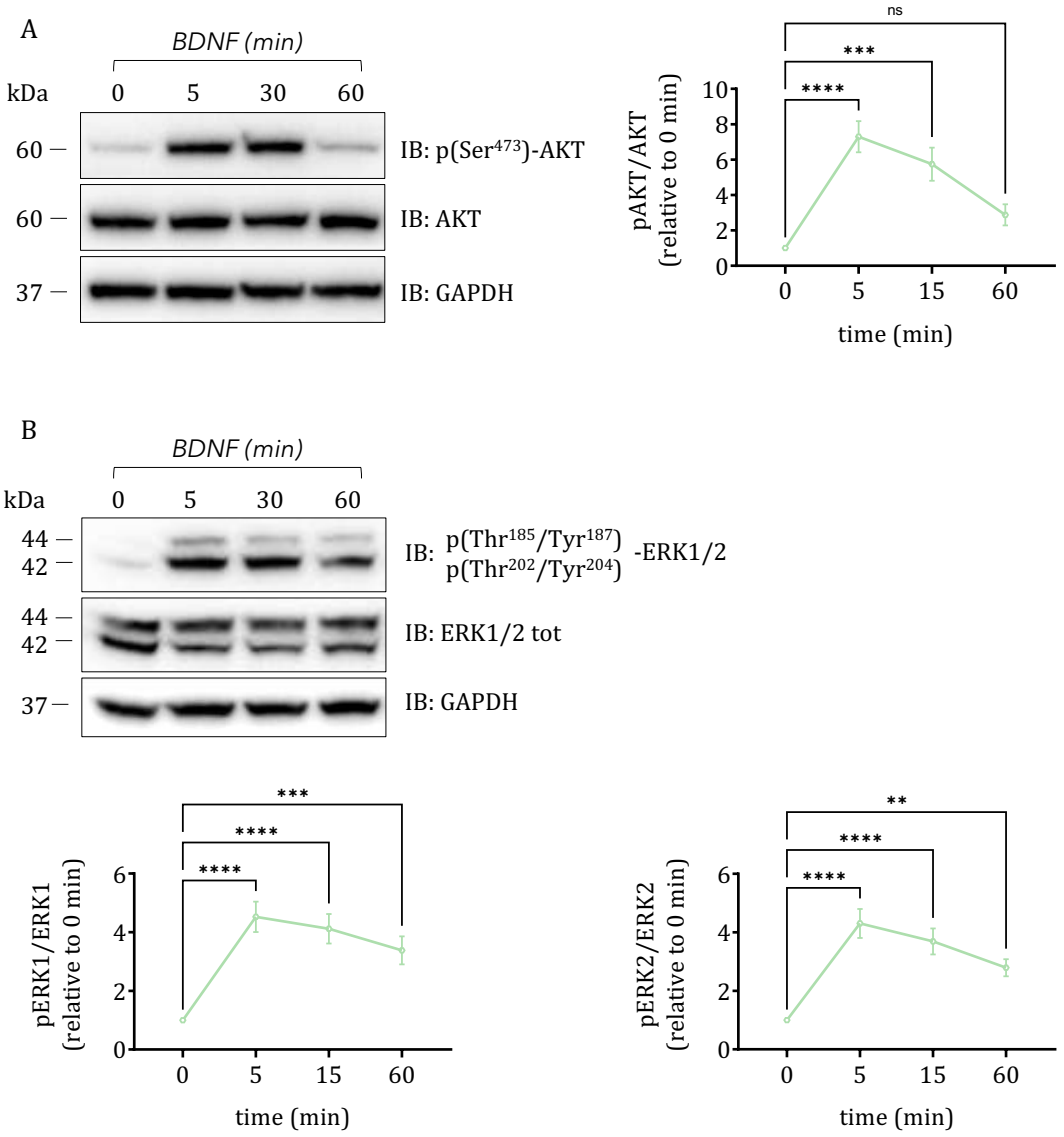


Fig.38. Differentiated human-derived neuroblastoma SH-SY5Y cells respond to BDNF treatment: *Western blot analysis of SH-SY5Y cells treated with BDNF showing that AKT* ***(A)*** *and ERK1/2* ***(B)*** *rapidly respond to the stimulation by increasing their phosphorylation state, that reaches a peak after 5minutes of BDNF exposure. The intensity of the bands of interest were normalized on GAPDH, used as loading control. Changes in the levels of pAKT and pErRK1/2 in respect to the basal condition have been evaluated performing one-way ANOVA with Dunnett's multiple comparisons test (significance ****p<0.0001; ***p<0.001; **p<0.01; *p<0.5), n=7 and n=13 biological replicates for AKT and ERK1/2 respectively.*

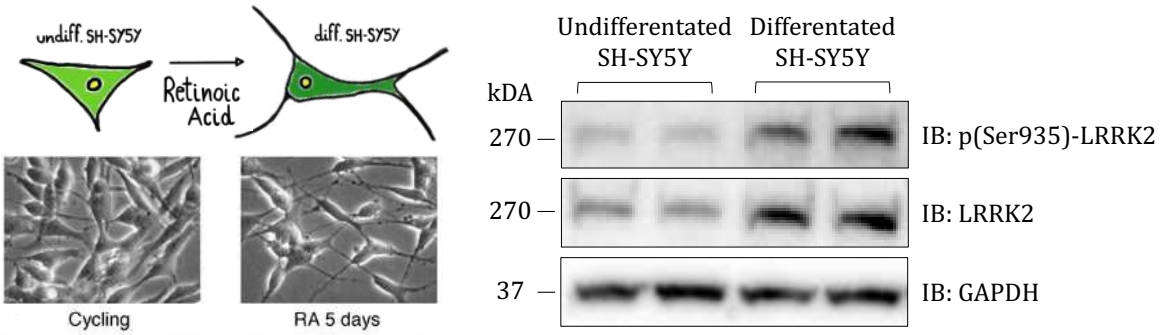


Fig.39. LRRK2 protein levels increase during the differentiation process: *Western blot analysis comparing the expression of LRRK2 in undifferentiated versus differentiated human-derived neuroblastoma SH-SY5Y cells. The amount of the protein becomes higher after the treatment with RA.*

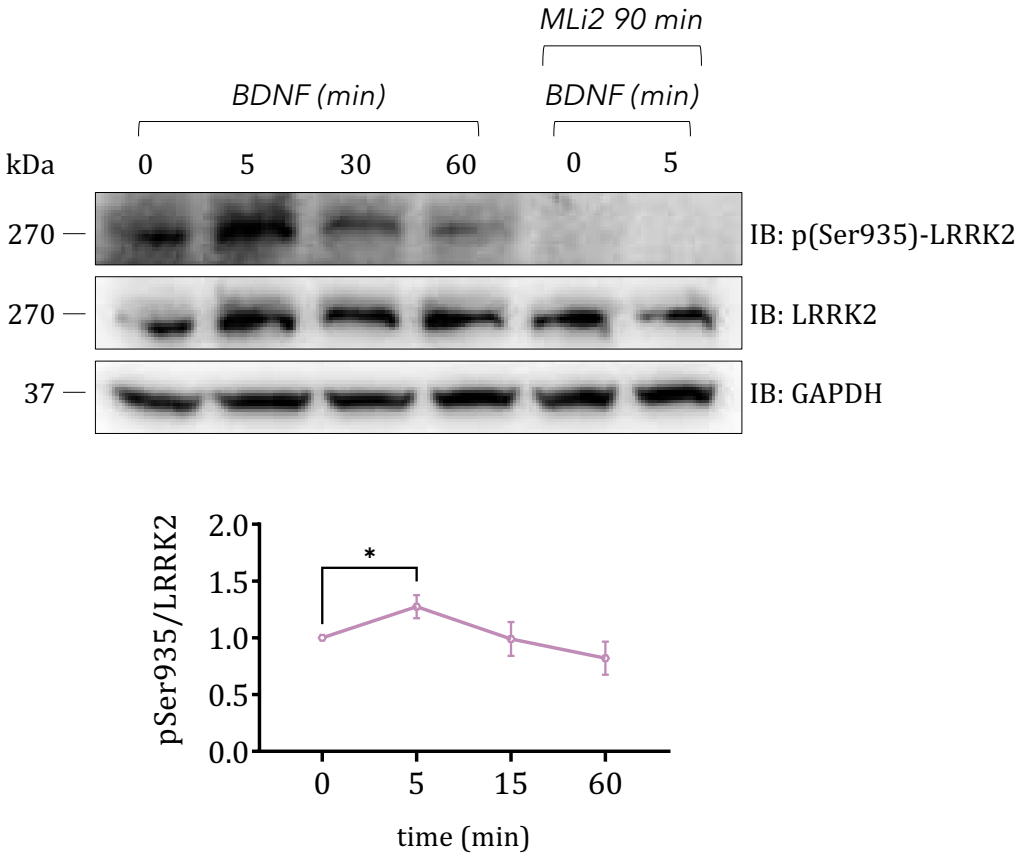


Fig.40. Differentiated human-derived neuroblastoma SH-SY5Y cells respond to BDNF treatment increasing phospho-Ser935 LRRK2 level: *Western blot analysis of human-derived differentiated SH-SY5Y treated with BDNF showing that LRRK2 rapidly responds to the stimulation by increasing its phosphorylation state. The intensity of the bands of interest were normalized to GAPDH, used as loading control. Changes in the levels of phospho-Ser935 LRRK2 at the different time points of the treatment with respect to the basal condition have been evaluated by one-way ANOVA with Dunnett's multiple comparisons test (significance *p<0.5), n=4 biological replicates. The increase in LRRK2 phosphorylation is significant after 5 minutes of treatment.*

7.4.1. Lrrk2 may regulate TrkB internalization and trafficking

The receptor tyrosine kinase TrKB possesses high affinity for BDNF, which, upon binding, triggers its dimerization and autophosphorylation (von Bohlen und Halbach & von Bohlen und Halbach, 2018). As a consequence the mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and phospholipase C (PLC-γ1) pathways are activated. These three major intracellular signaling cascades eventually modulate gene expression influencing a number of physiological processes. During brain development, BDNF modulates neuro-, glio- and synapto-genesis, cell death and elimination of improperly formed connections (Kowiański et al., 2018). In adulthood, it acts as a promoter of neuronal maturation, survival and maintenance and enhances synaptic transmission. Notably, in striatal MSNs it has been shown to influence dendritic spines and synapse dynamic (Kowiański et al., 2018). After the binding between BDNF and TrkB, occurring at the level of axon terminals and dendritic spines, the receptor is internalized via clathrin-mediated endocytosis (Eric J. Huang & Reichardt, 2003).

Several years of research on LRRK2 function highlighted that the kinase localizes in cytoskeletal and vesicular cell compartments where it is distributed across multiple cellular membranes (Cookson, 2016). Indeed, LRRK2 plays a well-recognized role in orchestrating intracellular vesicle trafficking both via phosphorylation of different substrates and interaction with membrane-associated proteins (Hur et al., 2019). This is true also in the pre- and postsynaptic compartment, where LRRK2 has been reported to influence neurotransmitter release and receptor trafficking, respectively (Cirnaru et al., 2014; Pischedda & Piccoli, 2021; Rassu et al., 2017). Given the lack of BDNF-induced spinogenesis in Lrrk2 KO primary neurons and the fast activation timeframe of Lrrk2 upon BDNF treatment we have postulated that Lrrk2 could control TrKB internalization.

To assess this possibility we stained with primary antibodies against LRRK2 and TrKB differentiated human-derived neuroblastoma *SH-SY5Y* cells unstimulated or exposed to BDNF for 5 and 30 minutes. Interestingly, these experiments revealed that, while in the control both LRRK2 and TrkB present a diffused distribution, 5 minutes of BDNF stimulation induces the clusterization of both fluorescence signals (Fig. 41). More precisely, LRRK2, that we found rapidly phosphorylated upon BDNF stimulation,

redistributes into punctate structures that colocalize with TrKB, suggesting its involvement in TrKB trafficking.

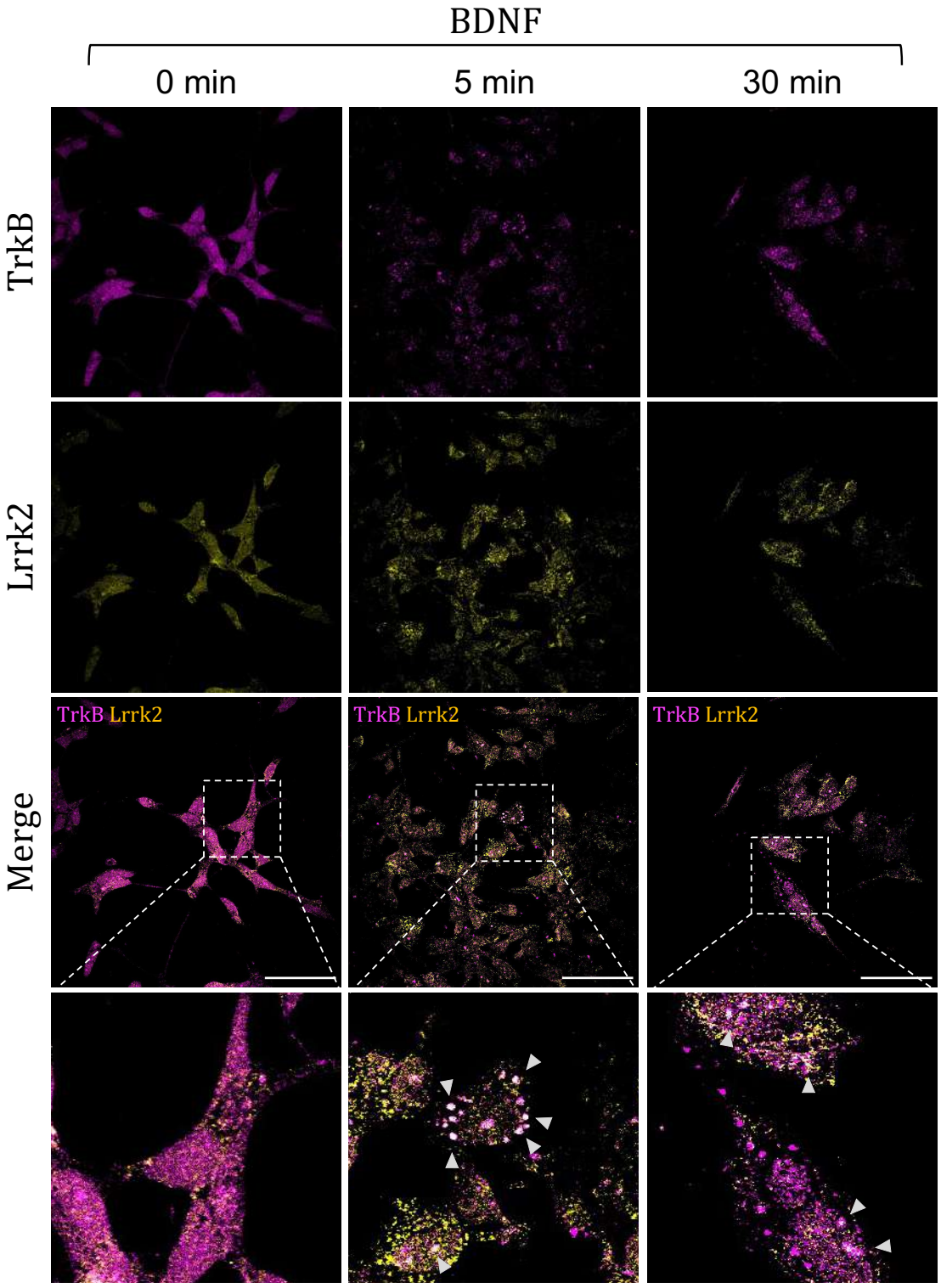


Fig.41: Upon BDNF stimulation LRRK2 redistributes in clustered structures together with TrkB. *Representative images of differentiated human-derived neuroblastoma SH-SY5Y cells showing a colocalization between LRRK2 and TrkB after BDNF treatment in punctate structures (white arrows). TrkB is coloured in magenta, Lrrk2 is coloured in yellow. Scale bar: 50µm.*

To collect more evidence of a possible involvement of LRRK2 in TrKB trafficking, we generated *SH-SY5Y* cell line KO for LRRK2 exploiting the CRISPR/Cas9 technology. To increase the chance of success we utilized three different guide RNAs (gRNAs) that we cloned in the PX459 vector and amplified by replication in bacterial cells. After having checked the absence of recombination in our plasmid by enzymatic digestion, we independently transfected *SH-SY5Y* cells with the three gRNAs. The transfected cells have been selected based on their resistance to puromycin and the successful of these two steps was controlled by assessing the presence of Cas9 via western blot analysis of the three polyclonal populations (Fig 42, A). Notably, the presence of LRRK2 in the polyclonal population relies on the fact that the nuclease was not able to knockout *LRRK2* gene in all the transfected cells. To get monoclonal LRRK2 KO colonies, transfected cells were diluted to the limit and then further expanded. Ultimately, we were able to obtain two monoclonal *SH-SY5Y* cell populations completely KO for LRRK2 (Fig.42, B) and a number of other lines knockdown for the protein. Indeed, *SH-SY5Y* have a tetraploid karyotype and, for this reason, it is likely that not all the alleles have been effectively knocked out leading to downregulation of Lrrk2 expression. Importantly, the Cas9 protein (containing a flag tag) was no longer detectable, suggesting that it was lost during the subsequent cell divisions and did not integrate into the cellular genome.

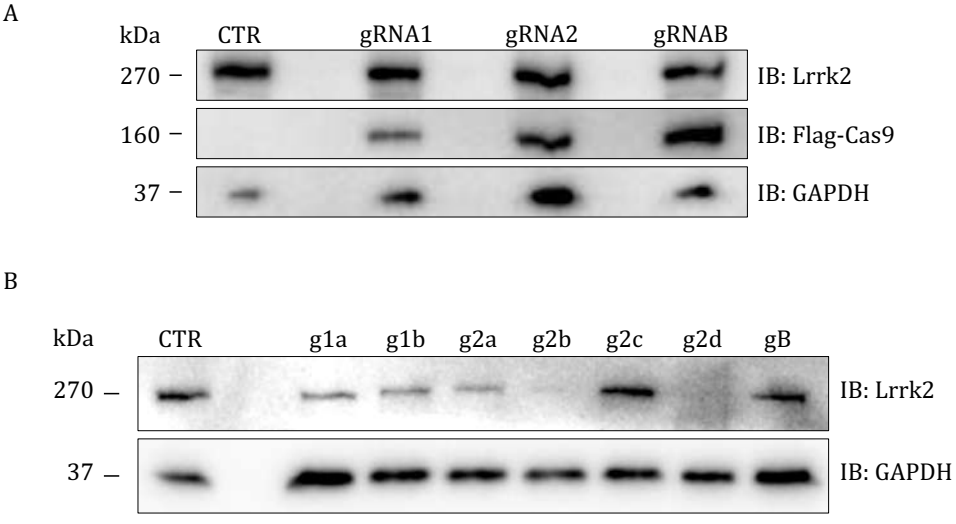


Fig.42: Western blots showing the generation of Lrrk2 KO SH-SY5Y cells. (A) *Western blot of polyclonal populations of SH-SY5Y cells transfected with the different gRNAs, attested by the presence of Cas9. LRRK2 protein expression was compared to that of a control population of naïve untransfected cells.* ***(B)*** *Western blot of different monoclonal populations of transfected SH-SY5Y cells showing that LRRK2 was successfully knocked out in two monoclonal lines (g2b and g2d). GAPDH was used as a loading control.*

We then perfomred IF experiments in differentiated *SH-SY5Y* WT versus KO cells (g2b line) to detect possible alteration in TrkB localization. As shown in figure 43, under unstimulated conditions the receptor appears diffused in WT cells, as expected from the previous experiments, whereas in the KO line it accumulates in structures that resemble tubules. Further experiments will be carried out to determine the nature of these vesicle structures by using different markers of the endolysosomal pathway (Fig. 43).

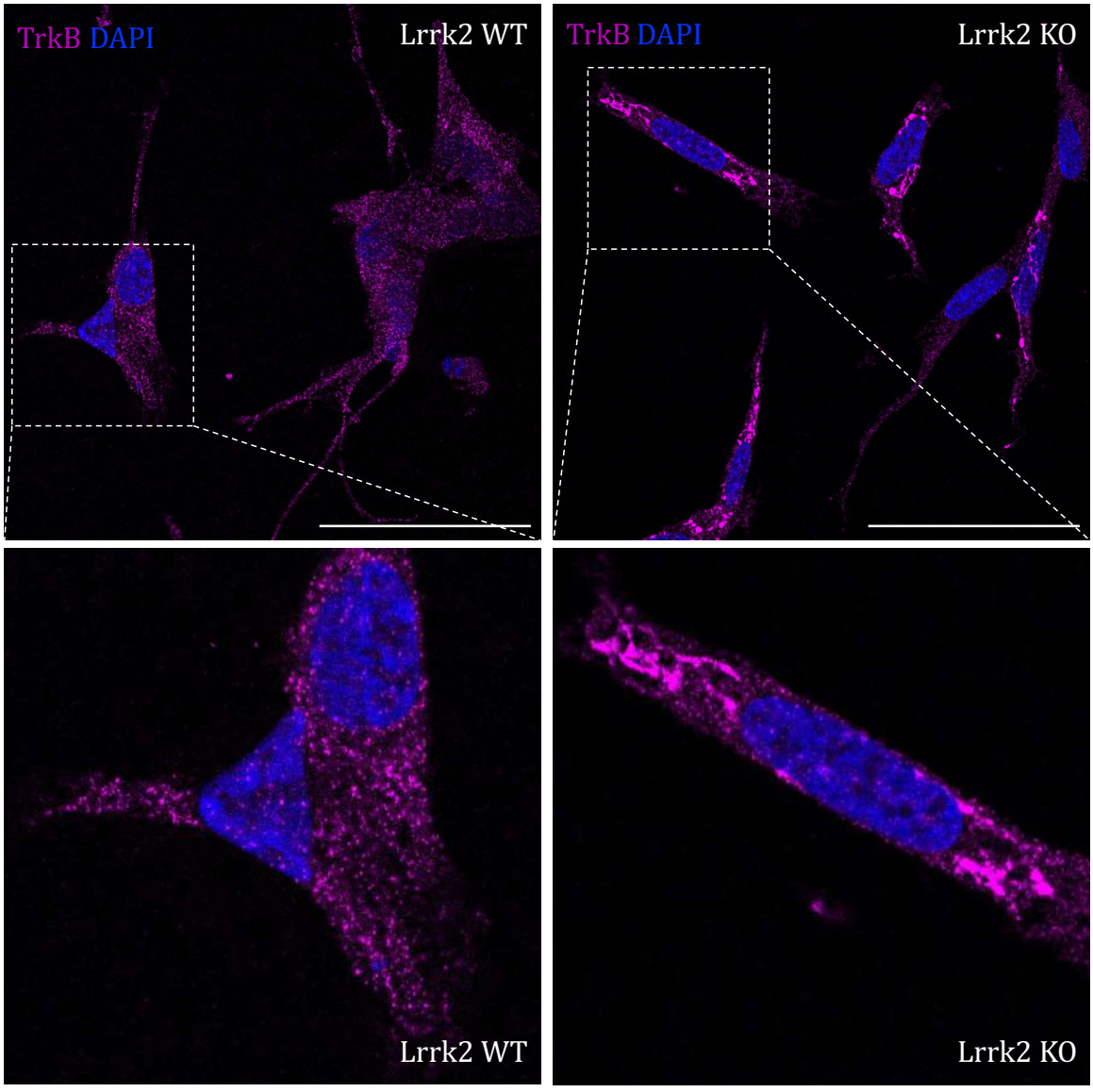


Fig.43: In differentiated LRRK2 KO SH-SY5Y cells TrkB accumulates in structures that resemble endo-lysosomal vesicles. *Immunofluorescence of differentiated Lrrk2 WT and KO SH-SY5Y cells stained for TrkB (magenta). The diffuse TrkB signal characterizing Lrrk2 WT cells, becomes strongly localized in vesicle-tubular structures when Lrrk2 is not present. Scale bar: 50µm.*

Altogether these preliminary data suggest that LRRK2 may play a role in orchestrating TrkB trafficking at basal condition and also upon BDNF treatment, opening interesting possibilities on which steps of the process LRRK2 may intervene.

7.4.2. BDNF stimulation increases LRRK2 interaction with actin-cytoskeletal proteins

In order to gain more insights into the mechanism through which LRRK2 may affect the process of dendritic spines formation, maturation and dynamics, we investigated the change in its interactome upon BDNF treatment. We used a similar approach as described above (paragraph 7.2.) but this time we purified GFP-LRRK2 under BDNF-stimulated versus unstimulated conditions. We took advantage of two *SH-SY5Y* cell lines already present in our laboratory and stably overexpressing (OE) hLRRK2 protein tagged with GFP at the N-terminus or only GFP, representing the negative control. These two lines have been differentiated for 6 days with RA to obtain neuronal-like cells that express a protein profile similar to that of neurons and then starved for 5 hours in cell culture medium deprived of serum. 100 ng/mL of BDNF or an equal volume of vehicle (H_2O) (unstimulated) has been applied to *SH-SY5Y hLrrk2-GFP OE* cells for 15 minutes, whereas *SH-SY5Y-GFP OE* cells have not been subjected to the treatment. After lysis, samples were incubated with the appropriate amount of GFP-Trap® resin overnight and eluates were loaded onto SDS-PAGE and subsequently analyzed via MS/MS (in collaboration with Prof Giorgio Arrigoni, Department of Biomedical Sciences). We performed two independent rounds of experiments to analyze by MS/MS. We first cleaned the list of interactors removing contaminants represented by common environmental keratins or undesirable sticky peptides that we found in the negative control sample. This round of MS confirmed many of the cytoskeletal interactors that we previously found (Fig. 26) but also reveal additional putative binders. In particular, LRRK2 co-precipitated proteins involved in endosome/lysosome/synaptic-vesicle trafficking (i.e. VPS13A, CTSD, SEC23A/B, DNAJA1, and known LRRK2 interactors such as Rab12) and proteins encoded by other PD-linked genes (i.e. VPS13C, VPS35, GCH1, CTSB) (Fig. 44). When calculating the fold change (FC) of LRRK2 interactors between BDNF-treated over untreated cells, we

found a striking result. Many LRRK2 interactions are re-shaped upon BDNF stimulation and hits whose binding with LRRK2 increases are mainly actin-related proteins .

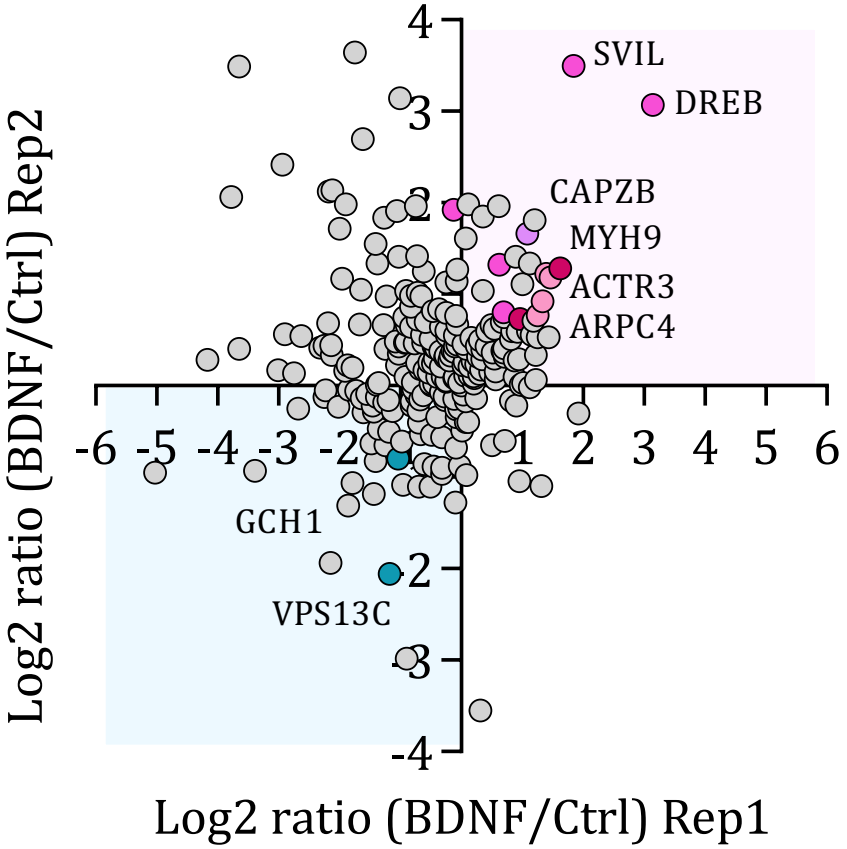


Fig.44: Upon BDNF treatment most of LRRK2 interactions are found to be reshaped. *Scatter plot of mass spectrometry analysis showing the alterations in LRRK2 interactome after 15 minutes of BDNF stimulation. Each axis reports the magnitude of the change in protein-protein interactions for one biological replicate, expressed as Log_2 FC of BDNF over the Ctrl (unstimulated). Proteins that exhibit a decreased interaction with LRRK2 in both the biological replicates are reported in the lower left quadrant. VPS13C and GCH1 are highlighted in blue, as they represent two PD-related genes. The proteins that display an increased interaction with LRRK2 in both the biological replicates are reported in upper right quadrant. We emphasize proteins involved in actin filaments capping (CAPZB) in purple, in actin polymerization/depolymerization (ACTR2, ACTR3, ARPC4, TMOD1) in magenta, and in actin stabilization (SVIL, FLNA, SPTBN1, ACTN1, DREB) in pink. Moreover some myosin-related proteins (MYH9, and MYH10) are represented in red. N=2 biological replicates.*

Next, we separated the interactors in two groups, one with $Log_2FC > 1$ and one with $Log_2FC < 1$ and performed GO enrichment analysis for biological processes with gProfiler.

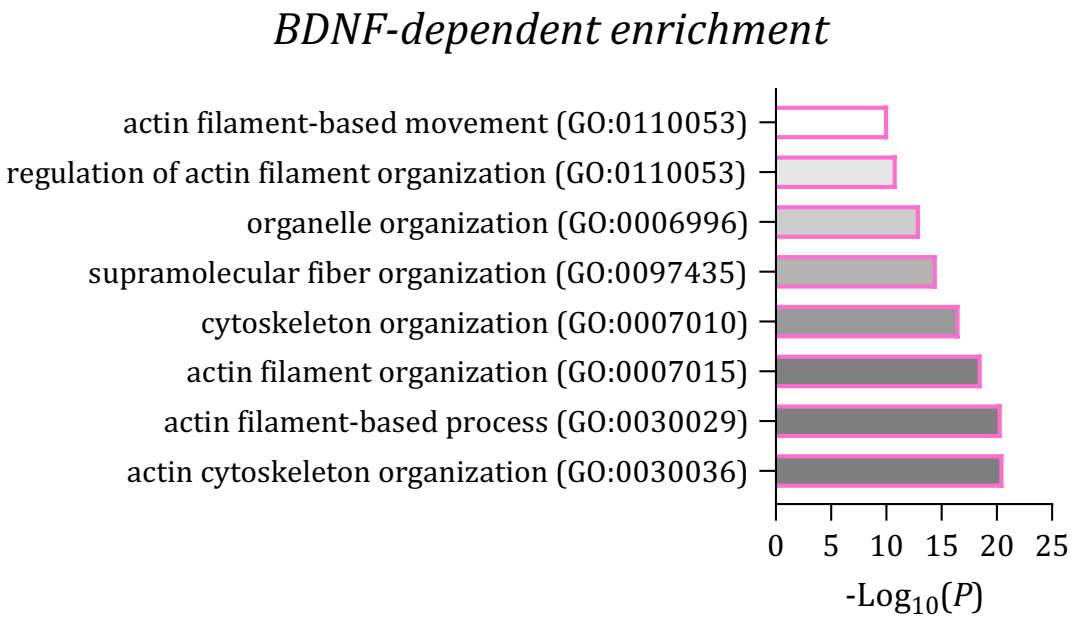


Fig.45: Most of LRRK2 partners increased upon BDNF stimulation are involved in actin cytoskeletal dynamics. *Graphical representation of GO enrichment analysis for the biological functions conducted on the mass spectrometry hits with increased interaction with Lrrk2 after BDNF treatment > 2 FC. We reported the categories showing an adjusted p-value < 10^{-10}.*

As shown in figure 45, LRRK2 interactions enriched under BDNF stimulation are mostly proteins involved in actin cytoskeleton dynamics (selected categories with p value < 10^{-10}), suggesting that BDNF treatment increases the binding of LRRK2 with actin-regulatory proteins (Fig. 45). Post-synaptic structural changes are often driven by activity-dependent and neurotrophic factor-dependent reorganization of the actin cytoskeleton (Lu et al., 2005). Among the increased LRRK2 interactors there are important players in actin filaments capping (CAPZB), in actin polymerization/depolymerization (Arp2/3 complex, TMOD1) and stabilization such as supervillin (SVIL), FLNA (filamin A), spectrin beta chain brain 1 (SPTBN1), alpha actinins (ACTN1) and drebrin (DREB). It is worth mentioning the Arp2/3 complex, which mediates the nucleation of branched F-actin networks providing the force for cell structural motility, such as dendritic spine head expansion (Lei et al., 2016). We further identified a subset of BDNF-increased LRRK2 interacting proteins forming the Arp2/3 complex: actin-related protein 3 (ACTR3), actin-related protein 2/3 complex subunit 4 (ACTR4), actin-related protein 2/3 Complex Subunit 2 (ACTR2). Moreover, this landscape of BDNF-increased interactions is enriched by at least two vertebrate non-muscle myosin, heavy chains myosin IIA (MYH9) and myosin IIb (MYH10).

Interestingly, non-muscular myosins II (NMMII) are present in neuronal spines neck and proximal spines head but also overlapping with the PSD95. NMMII were shown to impact on the morphology and dynamics of spines, especially myosin IIb (Kneussel & Wagner, 2013).

We further selected those hits whose interaction with LRRK2 increased by >1 fold change and subjected them to a more specific gene ontology analysis for synaptic proteins. To this aim we used SynGO (https://www.syngoportal.org/), an evidence-based, expert-curated resource for synapse function and gene enrichment studies (PMID: 31171447). Strikingly, we found that 18 out of 59 BDNF-enriched LRRK2 interactors with FC> 1 (~30%) were unique SynGO annotated genes and fall into post-synaptic (not presynaptic) categories (figure appendix 1). In particular, post-synaptic cellular components (CC) and biological processes (BP) mainly refer to actin cytoskeletal categories, strongly supporting a mechanism whereby BDNF induces LRRK2 interaction with postsynaptic actin cytoskeleton.

In parallel, we performed GO analysis of those hits whose interaction with LRRK2 decreased upon BDNF-treatment. As shown in figure 10, enriched categories include ER targeting and translation/transcription processes (Fig. 46). Among the decreased interactions, we identified at least two that are related to PD. GTP cyclohydrolase I, an enzyme essential for DA synthesis in nigrostriatal cells, is encoded by disease-causing gene (GCH1) for dopa-responsive dystonia (DRD), frequently found in concomitance with PD in families carrying the mutated gene (Yoshino et al., 2018). However, the most outstanding decreased interaction is that with vacuolar protein sorting-associated protein 13C (VPS13C), belonging to the VPS13 protein family. The biology of VPS13 proteins remains still unclear, even though they were suggested to be implicated in several different processes including membrane traffic among Golgi and endosomes and mitochondrial health. Interestingly, a work from De Camilli's group has proposed that VPS13C (similar to other VPS13 proteins) operates as a direct lipid exchanger between ER and endo-lysosomes (Kumar et al., 2018). Despite the details of its function are still unclear, VPS13C is associated with early-onset forms of PD, characterized by rapid and severe disease progression and early cognitive decline, with a parallel mitochondrial dysfunction (Lesage et al., 2016). Thus, LRRK2 may function together with VPS13C in regulating lipid transfer from the ER to the endolysosomes, when, for example, LRRK2 is recruited to the lysosome under lysosomal stress where it promotes

lysosomal repair (Bonet-Ponce & Cookson, 2021; Eguchi et al., 2018; Herbst et al., 2020). Another protein whose interaction with LRRK2 is reduced upon BDNF is VPS13A, encoded by a gene associated with the neurodegenerative disorder Chorea Acanthocytosis (Yeshaw et al., 2019). VPS13A sites between ER and mitochondria, where it tethers the two organelles, and also lipid droplets affecting their motility (Kumar et al., 2018). One biological replicate has shown a decreased in LRRK2 interaction with VPS35, a key component of the retromer complex involved in the retrograde transport of cargo proteins from endosomes to the trans-Golgi network and reported to modulate DA neurons survival (Williams et al., 2017). Several studies described that mutations in *VPS35* coding gene are associated to late-onset forms of PD, even if limited data are currently available regarding their neuropathology (Sassone et al., 2021). A functional interaction between VPS35 and others genes associated with familial PD including *SNCA*, *PARKIN* and also *LRRK2* have been previously observed (Williams et al., 2017).

Taken together, these data suggest that under a specific stimuli LRRK2 is recruited to a specific compartment, in this case BDNF drives LRRK2 toward the actin-cytoskeleton.

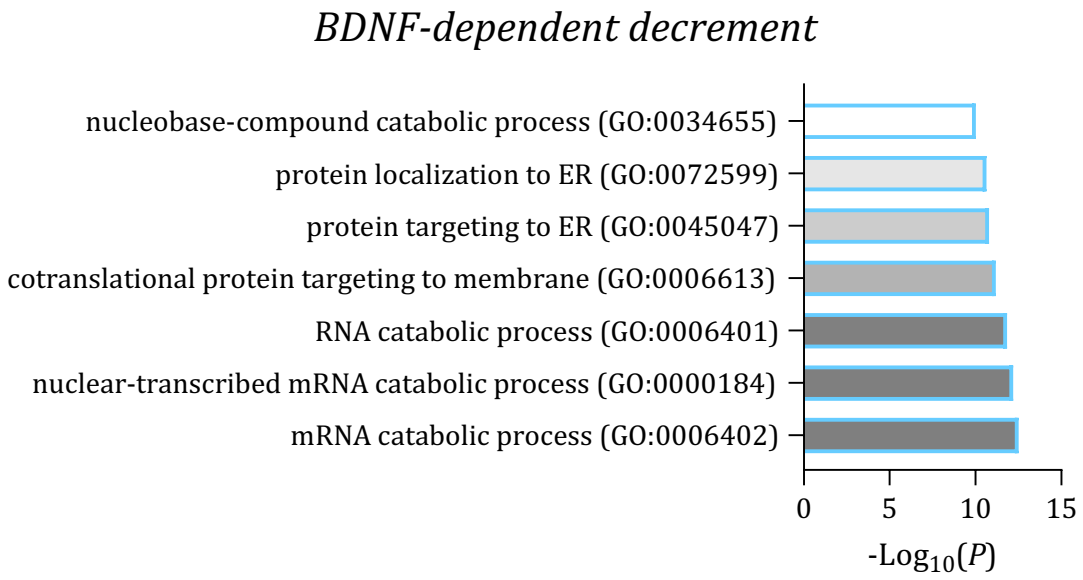


Fig.46: Most of the LRRK2 partners whose interaction decreased upon BDNF stimulation are involved in the ER targeting and in the translation/transcription processes. *Graphical representation of GO enrichment analysis for the biological functions conducted on the mass spectrometry hits presenting a decreased interaction with Lrrk2 after BDNF treatment. We reported the categories showing an adjusted p-value > 10^{-10}.*

7.4.2.1. Drebrin and LRRK2 cooperate to promote dendritic spines structural remodeling

A compelling result from the AP-MS/MS analysis is that the most significant interaction upregulated in the presence of BDNF is that with drebrin, with a 4.5 fold change increase over the untreated condition (Fig. 44). Drebrin (developmentally regulated brain protein) constitutes a major postsynaptic actin-binding protein highly accumulated in dendritic spines, whose function is to organize and stabilize F-actin to regulate spine plasticity and function (Ivanov et al., 2009). Mechanistically, when bound to F-actin drebrin forms a helix with a length pitch of 40nm, causing morphological variations that spread also in undecorated actin regions (S. Sharma et al., 2011). Frequently, drebrin-decorated F-actin acquires properties that change their responsiveness to several stimuli reaching dendritic spines (e.g. in presence of G-actin sequestering agent latrunculin A, drebrin-decorated F-actin depolymerization is inhibited) allowing a great range of outcomes within dendritic spines. More importantly drebrin slows down the F-actin treadmilling and decreases the rate of its depolymerization, bringing to a stabilization of actin cytoskeleton (Shirao et al., 2017). Based on this function, drebrin appears to be crucially involved in dendritic spine morphogenesis and remodelling in the embryonic and adult brain respectively (Koganezawa et al., 2017). During the earliest developmental stages the predominantly expressed isoform is termed Drebrin E, whereases Drebrin A represents the adult isoform, specifically expressed in neurons during the adult life (Shirao et al., 2017). Based on the substantial (4.5 FC) and reproducible (across 2 independent experiments) enrichment of LRRK2-drebrin interaction upon BDNF stimulation, we decided to investigate the functional role of this interaction in dendritic spines dynamics.

First, we validated the interaction by co-immunoprecipitation across three independent experiments. Specifically, we immunoprecipitated with GFP-trap® resin either GFP alone (control) or LRRK2-GFP form unstimulated or BDNF treated differentiated *SH-SY5Y OE* cells. Under equal amounts of purified LRRK2, we confirmed that the quantity of drebrin interacting with Lrrk2 is ~2.2 fold higher in treated conditions (Fig. 47, A). We also wanted to rule out that drebrin does not bind the resin but exclusively GFP-LRRK2 bound to anti-GFP nanobodies coupled to the resin. To this aim, GFP-LRRK2 has been purified from cell lysates containing a mix of *hLrrk2-GFP OE*

SH-SY5Y cells and naïve *SH-SY5Y* cells at different ratios: 4:0, 3:1, 2:2, 1:3, 0:4 (OE:naïve). As shown in Fig. 47, the levels of drebrin bound to GFP-LRRK2 diminish proportionally with the reduction of LRRK2 purified from the lysate, with a complete lack of signal in the eluate from the resin incubated with naïve cells only (0:4 condition), confirming the specificity of drebrin binding to GFP-LRRK2 (Fig. 47, B).

We then have checked the post-synaptic localization of drebrin performing immunofluorescence on primary cortical Lrrk2 WT neurons (Fig. 48). To this end, we transfected neurons at DIV 4 with PSD95 to visualized dendritic spines and stained them for endogenous drebrin and the neuronal marker MAP2. We confirmed that in neurons drebrin is highly enriched within dendritic spines. Interestingly, we noticed that some spines are positive only for drebrin, suggesting that it is present also in protrusions that have not yet reached the maturation.

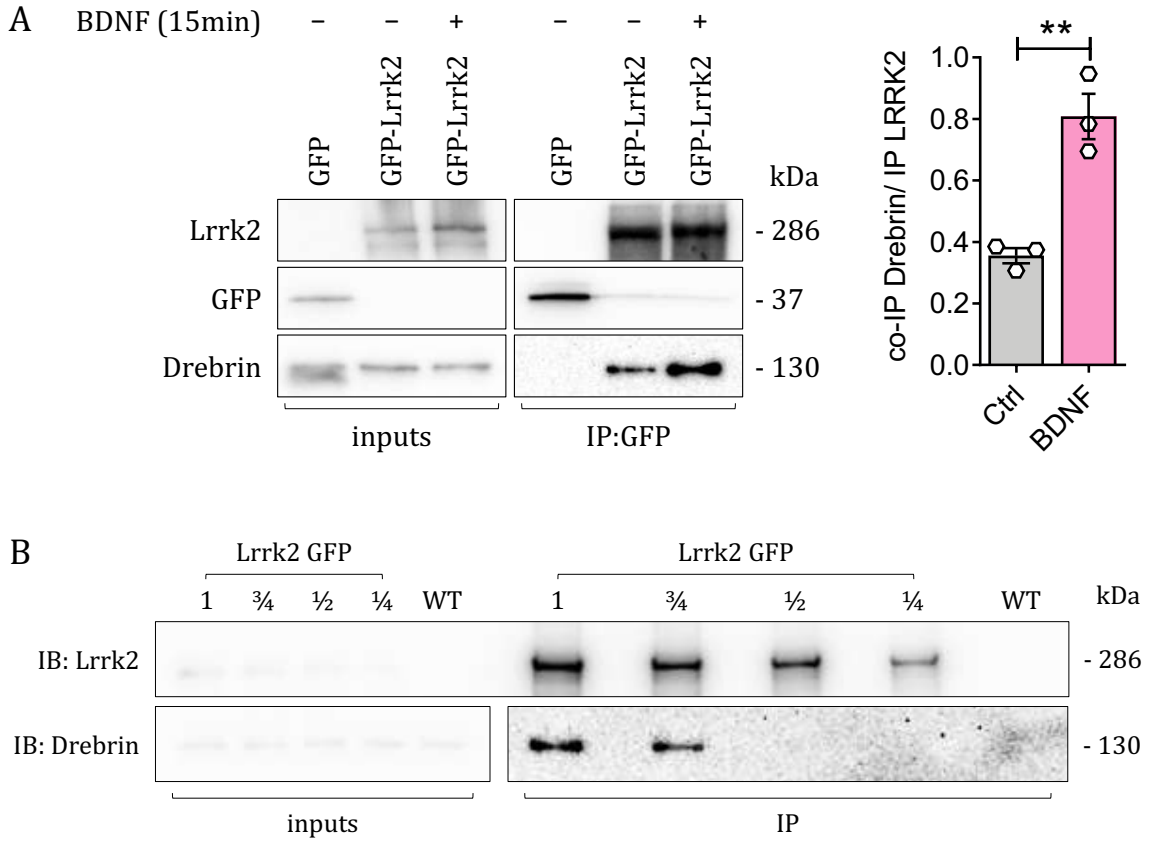


Fig.47: Western blot analysis validating the increased Lrrk2-Drebrin interaction upon BDNF treatment. (A) *Western blot analysis of purified Lrrk2-GFP and the relative quantification showing how the amount of drebrin interacting with Lrrk2 is significantly augmented upon BDNF treatment compare to control state. Differences between the two conditions have been evaluated using Student's t-test (significance **p<0.01), n=3 biological replicates.* ***(B)*** *Western blot analysis proving that drebrin binds in a specific fashion purified Lrkk2 and not the resin.*

Having validated LRRK2-drebrin interaction, we next hypotesized that loss of LRRK2 could affect the expression of drebrin within the brain. To test this possibility, we performed western blot analysis on Lrrk2 WT and Lrrk2 KO brain samples derived from the same mice where we carried out Golgi-Cox staining analysis. In line with the greater amount of immature spines observed in one month old Lrrk2 KO brains compare to the matched WT, we found that these mice also exhibit a significant decrease in drebrin quantity (Fig. 49, upper image). Both 4 month-old and 18 month-old old mice show a trend of decrease in drebrin levels, although the difference does not reach statistical significance (Fig. 49, central and lower image), in agreement with the possible compensatory effect on spine maturation that was also observed in adult and old KO mice. This result is also consistent with the well-established evidence that the spine head size is proportional to the amount of the stable F-actin pool. In other words, spines with larger head size contain a larger proportion of stable F-actin and this proportion is positively correlated with drebrin content (Kobayashi et al., 2007). In contrast, less mature spines with smaller heads contain lower stable F-actin together with decreased drebrin fraction.

Dendritic spines formation, maturation and remodeling relies on actin cytoskeleton reorganization: spine shape is regulated by the stable and dynamic configurations of F-actins, with the first forming the spine head structural foundation and the second being responsible for changes in spine head shape (Halpain, 2000). It has been proved that after stimuli inducing calcium entry and LTP, drebrin undergoes exodus from dendritic spines and consequently accumulates in dendritic shafts and cell body (Koganezawa et al., 2017). The exit of drebrin from dendritic spines head allows the reestablishment of normal helical pitch and the subsequent association of F-actin with other actin-binding proteins, together with new monomeric actin accumulation inside the dendritic spine head (Shirao et al., 2017). These events bring to an increase in the dynamic actin pool content resulting in a facilitation of F-actin polymerization and spine head remodeling Importantly, after the exodus drebrin reaccumulates inside the spine where it restores the stable F-actin pool. In light of these considerations, drebrin plays a pivotal role in the maintenance of spine size after stimuli inducing its reshaping.

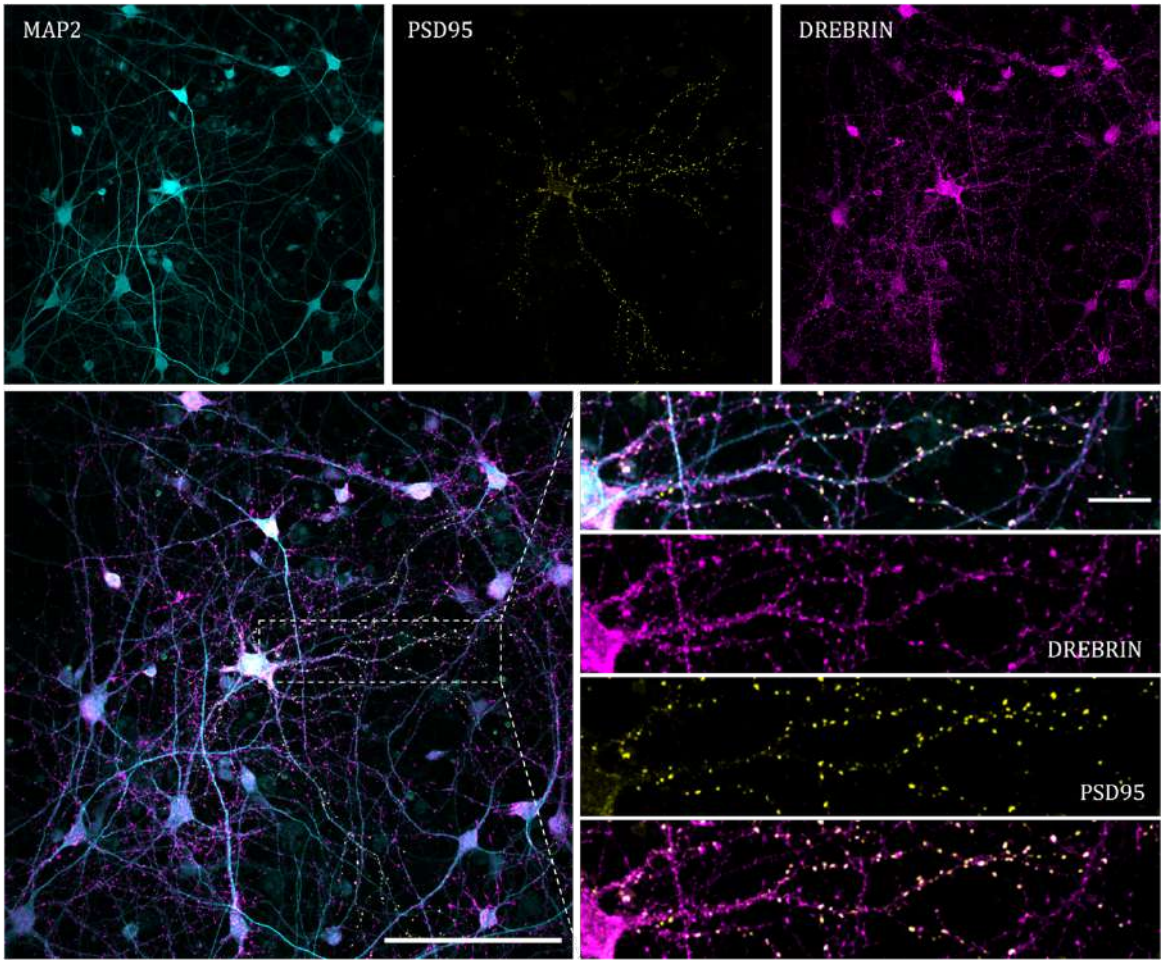


Fig.48: Immunofluorescence on WT Lrrk2 primary neurons showing the preferential localization of drebrin within dendritic spines. *Primary neurons has been stained for the specific neuronal marker MAP2 (cyan) and for endogenous drebrin (magenta). Moreover, they has been transfected with RFP-PSD95 (yellow), to visualize dendritic spines. Scale bar: 100 µm ; Magnifications scale bar: 15 µm.*

Considering this well-recognized role of drebrin and our discovery of its interaction with LRRK2, we next tested the hypothesis that loss of LRRK2 function could affect drebrin relocalization upon a stimulus prompting the maturation of dendritic spines. To this end, we treated DIV14 primary cortical neurons from Lrrk2 WT vs. KO mice with BDNF for 5 mins, 15 mins and 24 hours, and subsequently performed confocal imaging of drebrin post-synaptic localization (Fig. 50). At DIV4 neurons have been transfected with GFP, for the visualization of the entire dendritic shaft, and with PSD95, to recognized dendritic spines, whereases endogenous drebrin has been stained with a mouse anti-drebrin primary antibody. We then calculated the integrated density of the signal from endogenous drebrin overlapping the area defined by PSD95 positive puncta for each time points of BDNF treatment in Lrrk2 WT and KO neurons. In this

way, we were able track the movement of drebrin after BDNF stimulation in presence or absence of Lrrk2 activity. As shown in figure 50, Lrrk2 WT neurons show, as expected, a relocation of drebrin following BDNF stimulation. The drebrin exodus from dendritic spines starts after 5 minutes of treatment and further increases at 15 minutes. At 24 hours, drebrin co-localization with PSD95 appeared higher as compared to the unstimulated (Fig. 50), suggesting that spines underwent a remodelling process. In contrast, Lrrk2 KO neurons did not display a clear change in drebrin localization at none of the treatment times (Fig. 50). Although these data are still preliminary (n=2 independent experiments), they overall suggest that Lrrk2-drebrin interaction is important for maintaining dendritic spine architecture and promoting their remodelling via the BDNF pathway.

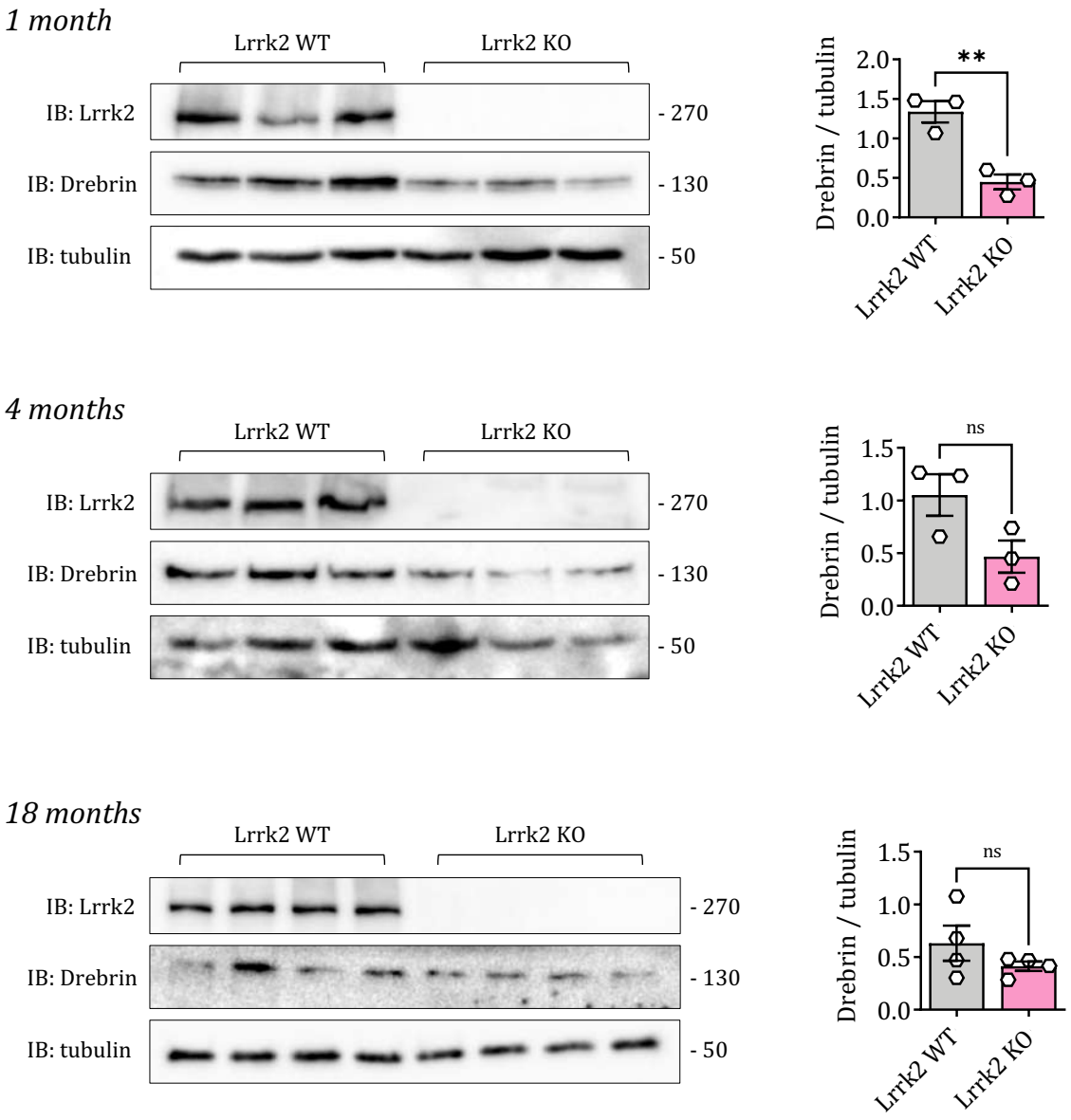


Fig.49: Drebrin levels are significantly reduced in one month old Lrrk2 KO mice compare to the matched Lrrk2 WT animals. *Western blot analysis of brain samples derived from Lrrk2 WT and KO mice where Golgi-Cox staining has been performed. The reduction in drebrin content is significant in Lrrk2 KO mice at 1 month of age. Differences between the two genotypes have been evaluated using Student's t-test (significance **p<0.01), n=3 animals for each genotype for each ages.*

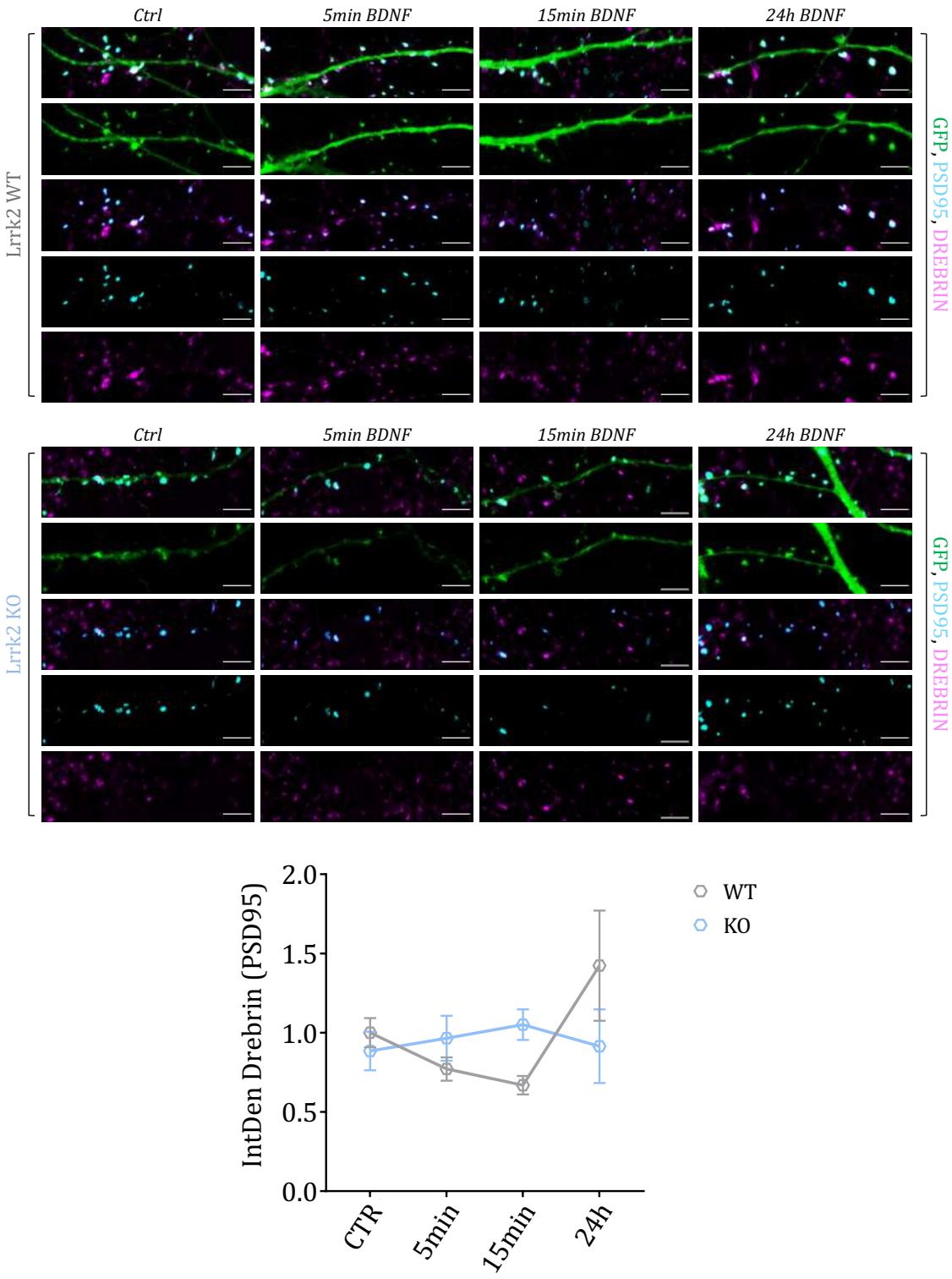


Fig.50: Lrrk2 affects drebrin exodus from dendritic spines. *After the exposure to BDNF for different times (5 minutes, 15 minutes and 24 hours), primary neurons from Lrrk2 WT and KO mice have been transfected with GFP and PSD95 and stained for endogenous drebrin. The amount of drebrin localizing in dentritic spines outlined by PSD95 has been assessed. The graph shows a pronounced decrease in drebrin content in WT neurons during the treatment and a consequent increase after 24 hours. KO neurons present only a blunt effect on drebrin localization upon the stimulation. Scale bar: 4 µm.*

LRRK2 is an multidomain protein that interacts with a plethora of partners via its PPI domains and that acts on several substrates through its GTPase and kinase activities (Iannotta & Greggio, 2021). To gain insights into the LRRK2 domains responsible for drebrin interaction, we performed co-immunoprecipitation. To this propose, different LRRK2 truncation constructs containing an N-terminal GFP tag were expressed and immunoprecipitated from HEK293FT cells. Specifically, we transfected a LRRK2 full length construct, a ΔN construct lacking the N terminus region, deletion constructs expressing single domains (LRR, ROC, COR, KIN) and a RCK construct including the entire enzymatic core composed by the ROC-COR GTPase domain and the kinase domain. We performed the transfections three independent times and evaluated the LRRK2 domain immunoprecipitation with anti GFP antibodies and co-precipitated endogenous drebrin. Indeed, we had previously noticed during a MS experiment in HEK293T cells where we immunoprecipitaed overexpressed LRRK2 that drebrin was consistently coimmunoprecipitated in this experimental conditions. Thus, we took advantage of endogenous drebrin to evaluate its interaction with LRRK2 without overexpressing it. As shown in figure 31, we were able to precipitate all the domains using GFP trap resin even though, due to a great level of variability, we were not able to obtain the same quantity of purified proteins (Fig. 51). Nevertheless, coimmunoprecipitation of drebrin is consistently detected in the presence of ROC, COR, and KIN domains. Unexpectedly, the tridomain RCK always failed to co-precipitate drebrin, possibly suggesting that this domain folds in a non-physiological conformation that hinders the interaction interfaces with drebrin. Importantly, the lack of drebrin signal in the resin incubated with naïve lysates, confirmed that drebrin binds the purified proteins and not the resin matrix. Overall, these results indicate that the interaction may require the full catalytic core of LRRK2 (ROC-COR-kinase), opening interesting possibilities about the putative interaction mechanisms that could involve the GTPase and/or the kinase activity. Due to the high variability across replicates, more experiments are required to obtain a statistically relevant result.

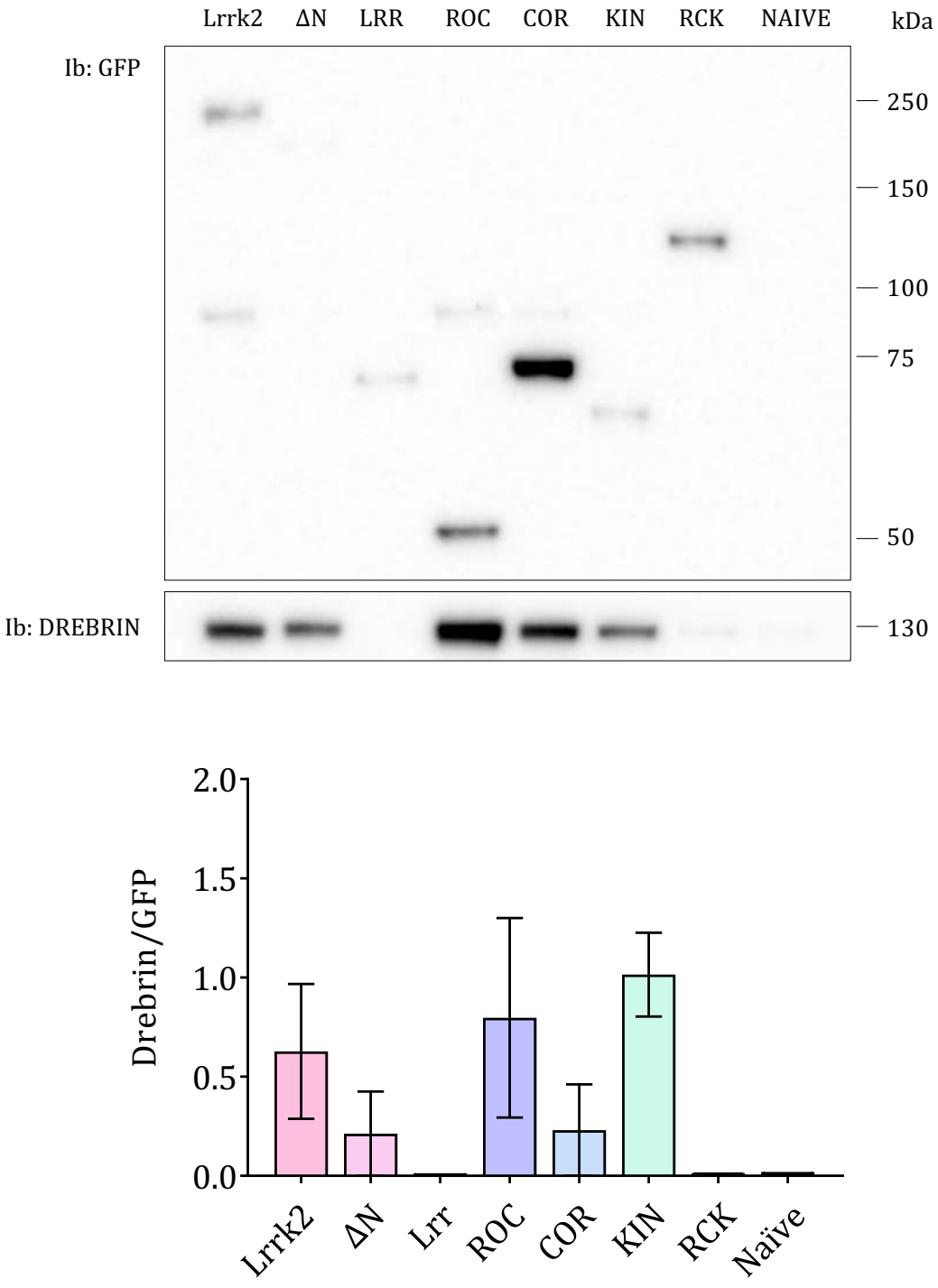


Fig.51: Drebrin interacts with the enzymatic core of Lrrk2. *The upper image reports a representative western blot analysis of purified Lrrk2 truncation constructs containing a GFP tag and drebrin coimmunoprecipitation. The lower image shows the quantification of drebrin fraction that binds the different Lrrk2 domains, expressed as the ratio between coimmunoprecipitated drebrin and purified GFP tagged constructs.*

Chapter 8

Results

Altered Lrrk2 activity impacts on dendritic spines structural plasticity

8. Introduction

LRRK2 contains several scaffolding regions implicated in protein-protein interaction, as well as serine-threonine kinase and ROC GTPase catalytic domains, all together making a large and complex signaling protein. Mutations in the *LRRK2* gene cause autosomal dominant PD, while common variants in the *LRRK2* locus increase the lifetime risk of disease (Cookson, 2015). Most pathogenic mutations sit within the enzymatic core of the protein, resulting in a decrease of GTP hydrolysis (R1441C/G/H and Y1699C) or in an increase of kinase activity (G2019S), consequently affecting the activity of downstream LRRK2 effectors. The G2019S substitution is the most recurrent LRRK2 mutation accounting for approximately 1% of sporadic PD and comprising an high proportion (approximately 4–5%) of familial PD cases, making this mutation the most common known cause of PD. Furthermore, the prevalence of G2019S is significantly enriched in some ethnic groups (e.g. 40% in North Africans and 30% in Ashkenazi Jews) and the penetrance is incomplete and increases with age (25% to 42.5% at age 80) (A. J. Lee et al., 2017). This mutation is located in the kinase domain and results from a G > A substitution at position 6055 of exon 41 of LRRK2 gene that causes the replacement of a glycine with a bulkier serine at the codon 2019 of LRRK2. It is thought that the conformational flexibility of the serine residue is so reduced, freezing the kinase in a more active conformation (Greggio & Cookson, 2009).

Increased kinase activity associated with G2019S has been link to several different outcomes, including abnormal Erk, c-Jun and Akt signaling pathways, dysfunctional autophagy, mitochondrial dysfunction, accumulation of αSyn and tau proteins, but also synaptic vesicle traffic alteration and reduce neurite growth (Ren et al., 2019). To this regard, our group obtained interesting evidence highlighting as LRRK2 G2019S i) impairs synaptic vesicle dynamics via aberrant phosphorylation of two important SV related protein, namely NSF and synapsin I (Belluzzi et al., 2016; Marte et al., 2019), and ii) affects the extension of neurites, which become shorter and decreased in number after 14 day in vitro in BAC-G2019S neurons (Civiero, Cogo, et al., 2017). Furthermore, the G2019S mutation is linked with an altered post-synaptic activity in terms of responses to glutamatergic and dopaminergic stimulations (Beccano-Kelly et al., 2015; Matikainen-Ankney et al., 2016; Pischedda & Piccoli, 2021; Plowey et al., 2014; Volta et al., 2017).

Based on these consideration and on our results indicating that LRRK2 participates in postsynaptic remodelling through interaction with actin-cytoskeletal components, in this chapter I report a number of experiments assessing the consequences of Lrrk2 gain of kinase function on dendritic spines morphology and maturation. As Lrrk2 G2019S mice do not display overt signs of neurodegeneration (Liu et al., 2015; Seegobin et al., 2020; Yue et al., 2015), they may represent pre-symptomatic models of disease. Thus, we reasoned that, if mutant Lrrk2 impairs dendritic spine dynamics, this might manifest as an early pathological sign preceding neuronal death.

8.1. Mutant LRRK2 G2019S impacts dendritic spines morphology in aged mice

With the aim of investigating whether the common, hyperactive G2019S mutation affects synaptic architecture, we analyzed the consequences of Lrrk2 gain of kinase function on striatal dendritic spine morphology, number and ultrastructure. To perform this analysis, we took advantage of two different mouse models: the knockin Lrrk2-G2019S mouse (KI-GS) (Herzig et al., 2011) and the BAC Lrrk2-G2019S mouse (BAC-GS) (Xianting Li et al., 2010). KI-GS is recognized as a "physiological model" to study the effect of this mutation being the mutation inserted in the endogenous murine gene (Herzig et al., 2011). Although some different observations were made across different laboratories, there is a general consensus of subtle dysfunctions in dopaminergic neurotransmission and hyperkinetic movements (Beccano-Kelly et al., 2015; Longo et al., 2014, 2017). Moreover, our group and collaborators reported an accumulation of phosphoS129 α-Syn in 12-months old GS-KI brains, which may represent a prodromal sign before clinical manifestation (Longo et al., 2017). The BAC mouse overexpresses murine G2019S-Lrrk2 under the control of the endogenous promoter (Xianting Li et al., 2010) and is characterized by shorter neurites in cultured neurons, increased post-synaptic efficiency and reduced striatal dopamine content and uptake (Xianting Li et al., 2010; Sepulveda et al., 2013; Sweet et al., 2015). However, these phenotypes are mild and far from fully recapitulating PD-related phenotypes like nigral loss, α-syn deposition in Lewy bodies-like inclusions, and motor impairment. The lack of clinical PD symptoms in mutant Lrrk2 mice may be reconciled with the incomplete penetrance of Lrrk2 mutation in humans, where aging acts in combination with genetics, a situation that cannot be fully reproduced in the shorter lifespan of the

mouse. Thus, GS Lrrk2 mice may constitute a valuable pre-symptomatic model of the pathology, where early sign of synapse degeneration/loss can be evaluated and studied, with BAC-GS mice representing an exacerbated model of G2019S pathological effects. Given these mice are presymptomatic models and PD is an age-related disorder we firstly have analysed the spines and synapses of 18 month-old mutant Lrrk2 mice to search for defects that may emerge from the combination between genetic and age.

8.1.1. Mutant Lrrk2 affects dendritic spines morphology

We used the Golgi-Cox staining to visualize dendritic spines morphology to perform a similar analysis as described for Lrrk2 KO mice (7.3.1.) in coronal dorsal striatal brain slices derived from KI-GS and BAC-GS mice. As controls, we used WT mice (C57BL/6J) that were used to breed both genotypes. Three mouse brains per genotype were collected and about 20 neurites of ~20-30 μm in length were imaged and analyzed per each brain. The mean value ± SEM of every parameters (width, length, number and spine category) was calculated starting from its average value in each of the three mice. Phase contrast acquisition mode was exploited to acquire images at the confocal microscope and the analysis was performed with the software Reconstruct (http://synapses.clm.utexas.edu). Unexpectedly, the Golgi-Cox staining revealed that the KI-GS and, at even higher extent, the BAC-GS, possess more branched spines and less thin protrusions compared to WT mice. Moreover, the BAC-GS animals display also a significant reduction in filopodia protrusions with respect to both the KI-GS and the WT mice. These findings suggest that the G2019S mutation promotes the formation of more mature spines and a reduction in the amount of non-mature protrusions, with a exacerbated effect in presence of overexpressed mutant Lrrk2 (Fig. 52). We have also assessed the total amount of protrusions together with the average width of dendritic spine head and the average length of dendritic spine neck. As shown in figure 53, only the length of dendritic spines neck appears to be reduced in BAC-GS mice compared to the control WT mice, while we were not able to detect any additional alterations in the others parameters, implying that BAC mice possess spines that are shorter on average compared to those of the other genotypes (Fig.53, A). To rule out whether this phenotype occurs with age or is already present at earlier stages, we also examined 4 month-old GS-mouse striata. We did not detect any significant difference, although there is a trend of increased branched spines in both KI-GS and BAC-GS mice (Fig. 52).

Only the KI-GS animals exhibit a statistically significant decrease in the number of thin spines (Fig. 52). A larger sample size is needed to prove or disprove whether these subtle differences are real. Non changes were observed in the average number of protrusions and width of the spine head, while the average length of the spine neck showed a significant decrease in GS-KI mice at 4 months of age (53, B). Instead, in 18-month old striata spine density decreased compared to 4 month-old mice with no differences across genotypes(Fig. 53, C). A similar trend was observed in the WT vs. KO analysis (Fig. 31), likely due to physiological decay during the aging process, as previously reported (Dickstein et al., 2013). Of interest, an increased frequency of occurrence of spines with enlarged heads was reported in aged cats compared with young individuals (Levine et al., 1986). In our case, we did not find significant differences in spine width between adult and aged mice but we did see an increased in branched spines, which, similarly, represent a class of spines with increased density for postsynaptic transmission.

Overall these results suggest that G2019S mutation stimulates drives a shift toward the formation of branched spines and a parallel reduction of thin spines, suggesting that synapses maybe more efficient. This is in line with a previous study where the authors reported that developing Lrrk2 G2019S MSNs possess larger spines and parallel larger postsynaptic activity in 1 month old mice (Matikainen-Ankney et al., 2016). We plan to extend these data with the analysis of 1 month-old mice as well.

8.1.2. Mutated Lrrk2 affects synapses number and postsynaptic ultrastructure

We next analyzed the number of synaptic contacts comparing WT with KI-GS and BAC-GS. Striatal brain slices obtained from 18 month-old mice were stained for the presynaptic marker VAMP2 and the postsynaptic marker PSD95. Synaptic contacts are represented by overlapping fluorescence signals, i.e. yellow puncta. We analyzed 12 random fields per animal (n=2 animals per WT and n=3 animals per KI and BAC). As shown in figure 54, the number of synapses has a trend of reduction in both the GS animal models, although on the overexpressing BAC-GS mice show a stronger effect (Fig. 54). We have to increase the WT sample size in order to perform statistical test.

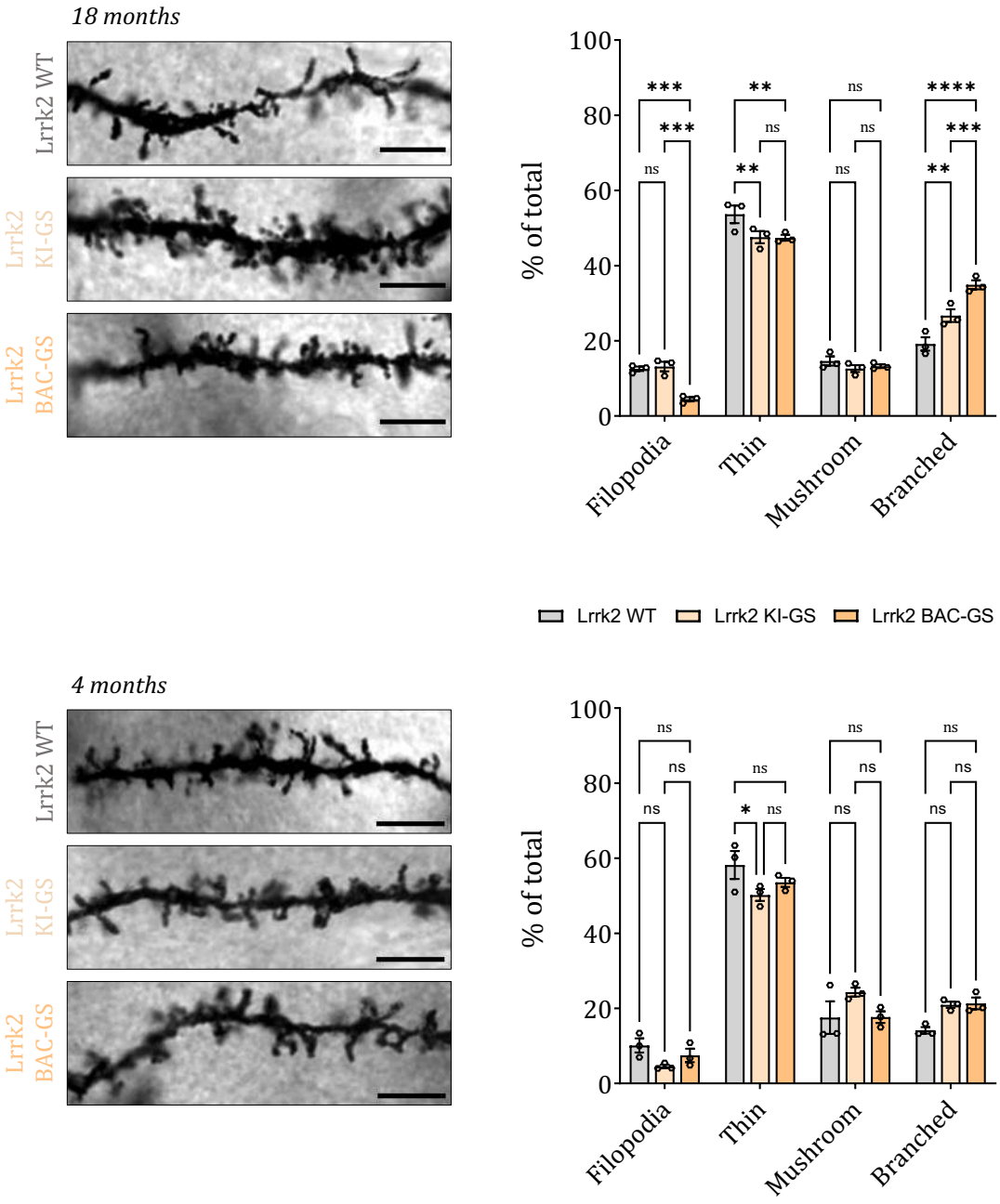


Fig. 52: G2019S Lrrk2 mutation influences striatal dendritic spines maturation in aged mice: *Representative images of the neurite segments analyzed (about 20 segments per animal were analyzed) are reported in the left side for each age. Scale bar: 5 µm. As showed in the right side, protrusion were classified in four morphological classes (filopodia, thin, mushroom, branched) and graphically represented as % of the total number. Maturation defects are observed in Lrrk2 GS eighteen months old mice (upper figure). A significant decrease in the amount of thin protrusions has been detected also in 4 months old GS-KI mice. Significance between genotypes was tested using 2 way ANOVA with Tukey's multiple comparison test (significance ***p<0.001; **p<0.01; *p<0.5), n=3 animals for genotype for each age.*

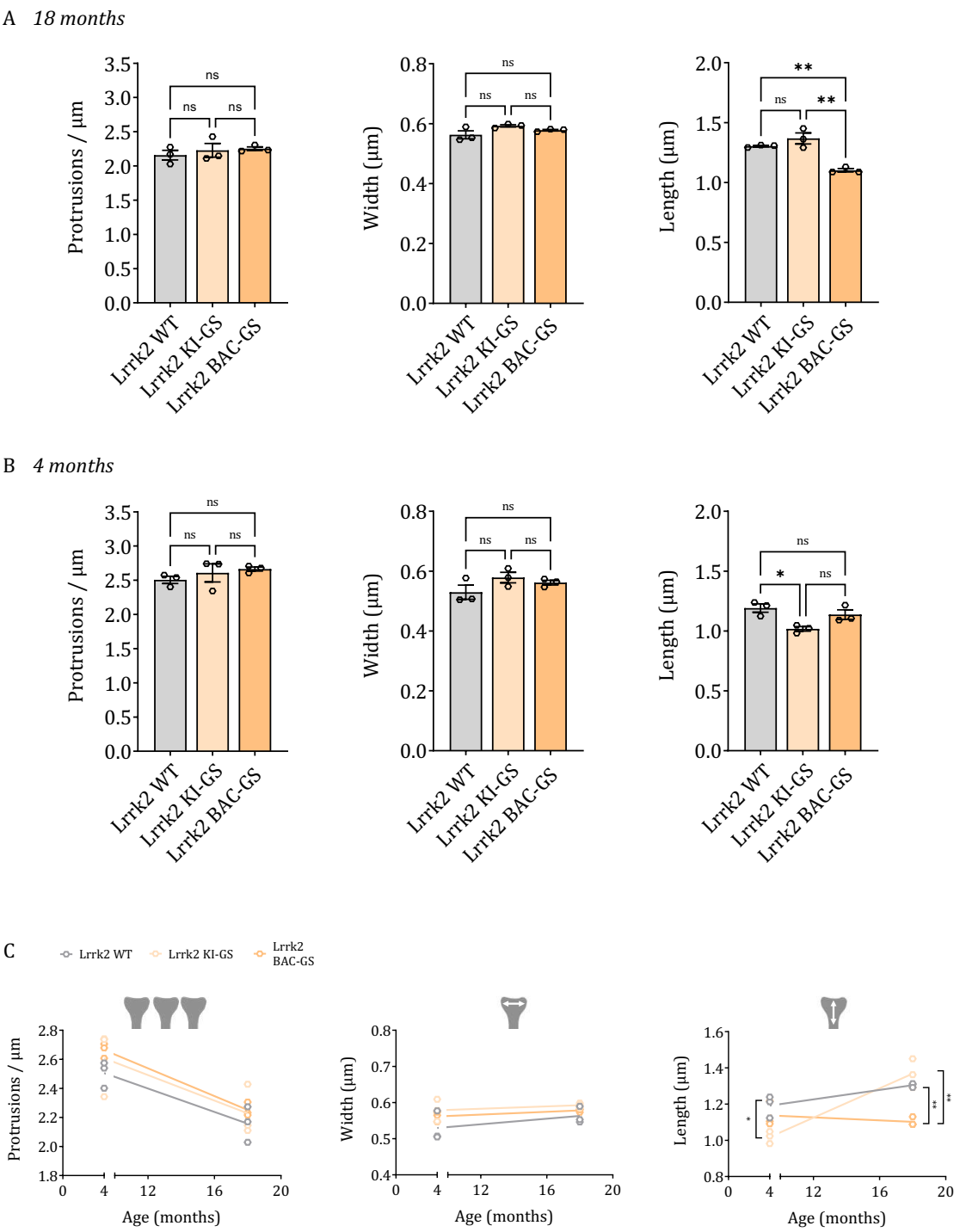


Fig. 53: G2019S Lrrk2 mutation influences the length of dendritic spines neck, whereas it does not impact on the number of total protrusions. *Analyses of protrusions number, spines width and length in 18 months* ***(A)*** *and 4 months* ***(B)*** *old GS-mice. The lower figure* ***(C)*** *reports the different parameters comparison between the two analyzed ages. Statistical significance was assessed with one-way ANOVA with Tukey's multiple comparisons test (significance **p<0.01; *p<0.5), n=3 animals for genotype for each age.*

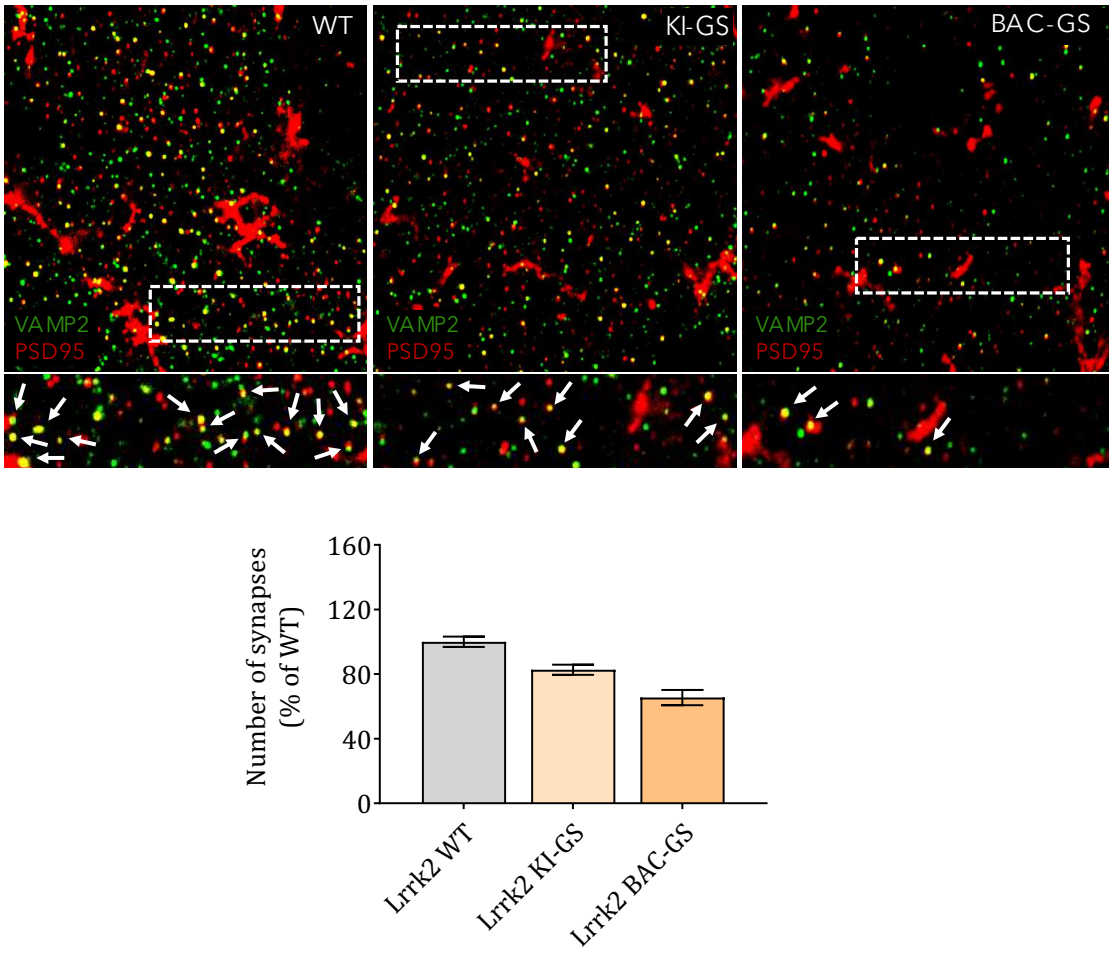


Fig. 54: G2019S Lrrk2 mutation influences the number of striatal synapses: *In the upper panels, representative immunofluorescence images of striatal brain slices stained with presynaptic VAMP2 (in green) and postsynaptic PSD95 (in red) markers to assess synapse number in Lrrk2 WT versus Lrrk2 GS mice at 18 months of age. The quantification is reported in the lower part of the figure. Even if not significant, there a trend of reduction in synapses number is observed in Lrrk2 GS mice. Statistical significance was assessed with one-way ANOVA with Tukey's multiple comparisons test, n=3 animals per condition were analyzed.*

Finally, we checked for the presence of possible ultrastructural differences in 18 months-old mice performing electron microscopy analysis of synaptic structure in the striatum. Specifically, we measured the length of the PSD to infer further information about the predicted postsynaptic efficiency, which is proportional to the length of PSD. As shown in figure 55, PSD is significantly longer in GS models compared to WT animals (Fig. 55).

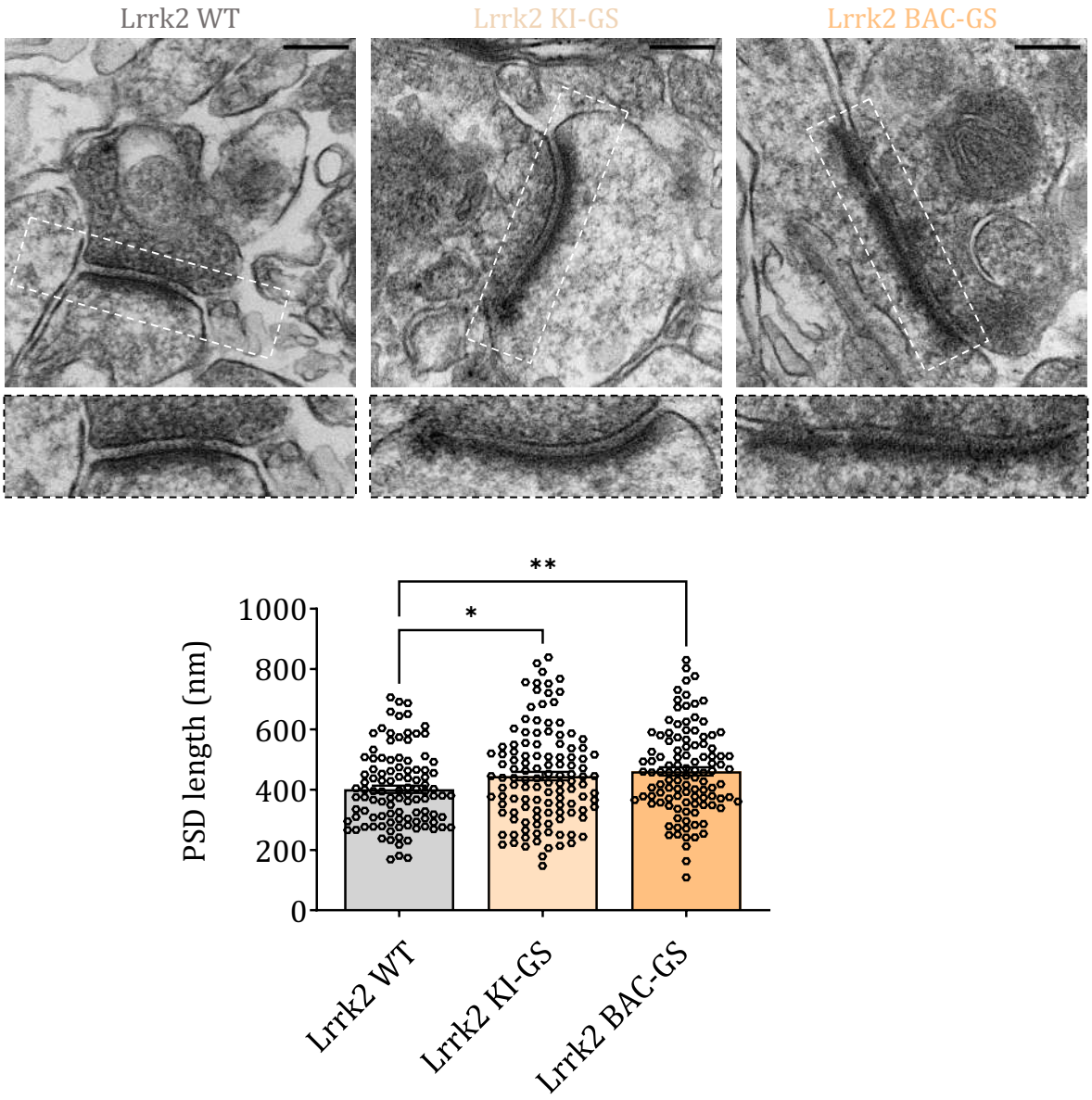


Fig. 55: G2019S Lrrk2 mutation is associated with an increased PSD length in aged mice: *the length of the PSD has been evaluated by transmission electron microscopy on striatal brain slices from Lrrk2-GS 18 months old mice (representative images in the upper part of the figure). Lrrk2-GS mice present longer PSD compared to controls. Differences between GS and WT mice have been evaluated using one-way ANOVA with Dunnett's multiple comparisons test (significance **p<0.01; *p<0.5), n=3 animals per genotype were analyzed. Scale bar: 200 nm.*

Overall, these findings indicate that aged mice carrying the pathological G2019S mutation spines are enlarged with an increased PSD, suggesting that they may be more functional. Coherently, in the GS brain in which we performed Golgi-Cox staining (Fig. 52) we observed a trend of increase in drebrin expression compare to the WT (Fig. 56). However, the overall synaptic contacts is reduced in mutant mice. The reason behind this apparently contrasting findings is unclear, however one possibility could be that a sustained synaptic activity due to the presence of larger spines could results in an overstimulation of the cortical-striatal circuits with a consequent glutamate excitotoxicity. This scenario would also fit with the increased cortico-striatal activity that has been previously documented in G2019S models (Huntley & Benson, 2020). In particular, in the dorsal striatal MSNs of KI-GS acute brain slices the frequency of spontaneous excitatory postsynaptic currents (sEPSCs) was found to increase of about 4 folds compared to the WT (Volta et al., 2017). It can be speculated that this non-physiological condition may result in increased extracellular glutamate, already observed in GS mice (Beccano-Kelly et al., 2014; Volta et al., 2017), which in turn leads to synapse removal.

Interestingly, we have also collected evidence in support of an altered brain environment in aged mice carrying the G2019S mutation. We stained coronal slices from 1 and 12 months old BAC-GS mice for GFAP, a specific astrocytic marker whose expression increases during astrogliosis. As shown in Figure 57, striatal astrocytes from 12 months-old mice display a more ramified morphology as compared to 1 month-old animals, overall suggesting the presence of enhanced gliosis in aging BAC-GS brains. Moreover, we noticed that CD11b signal, that marks microglial cells, is more intense in striatal slices obtained from 12 month-old BAC-GS mice with respect to those from 1 month-old animals (Fig. 57, C-D). In these aged mice microglia also appears more ramified.

Taking together these data point are indicative of an increased inflammatory environment in aged BAC-G2019S brains, coherently with some previous studies in GS models (Schildt et al., 2019; Xiong et al., 2018). Noteworthy, glia reactivity and neuroinflammation represent conditions frequently associated with glutamate extra-synaptic diffusion and associated toxicity (L. Iovino et al., 2020).

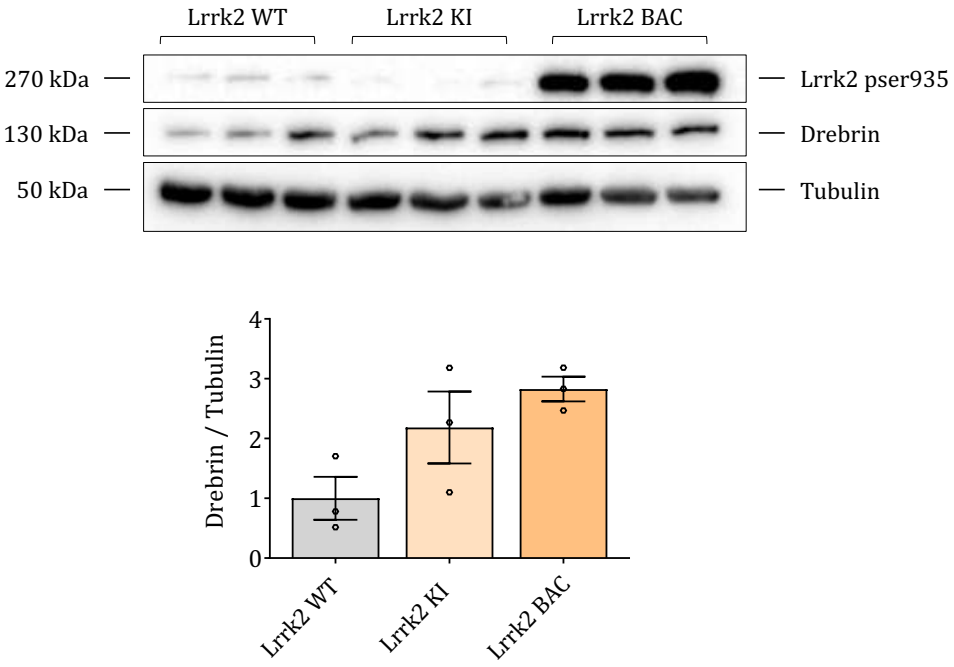


Fig. 56: Drebrin levels have a trend of increase in 18 month-old Lrrk2 GS mice compare to the matched Lrrk2 WT animals. *Western blot analysis of brain samples derived from Lrrk2 WT and GS mice where Golgi-Cox staining has been performed. The increase in drebrin content is not significant.*

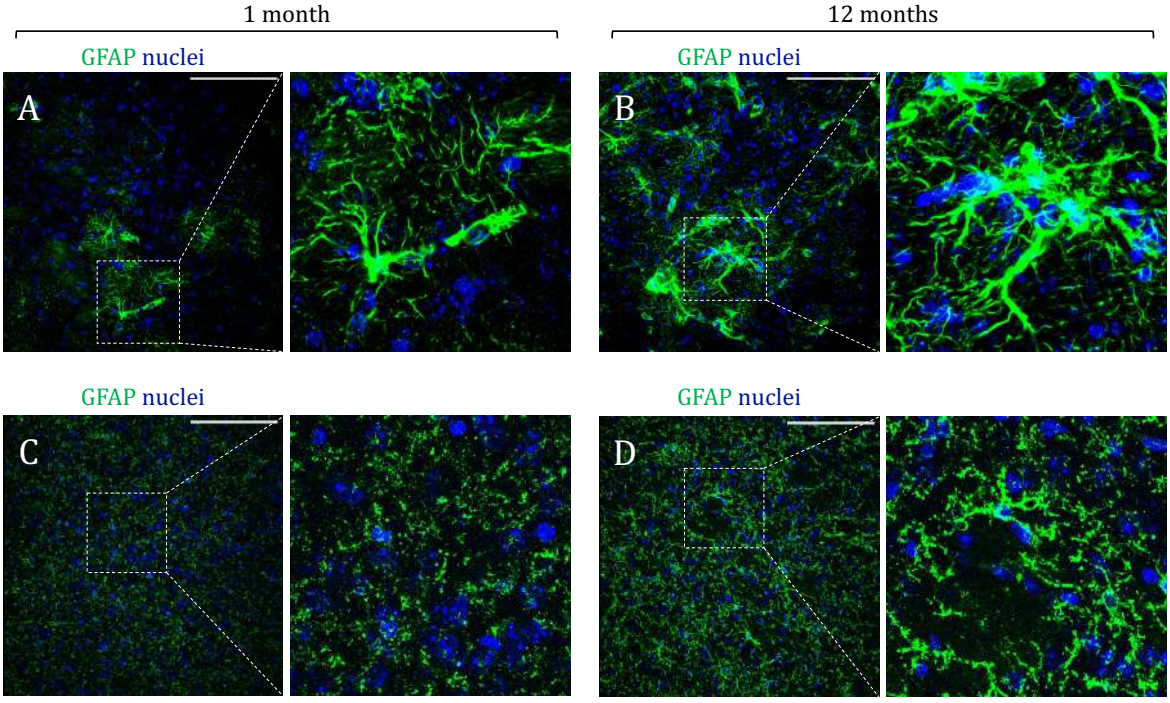


Fig.57: Immunofluorescent staining for glial cells of brain slices from hemizygous transgenic mice overexpressing murine BAC-Lrrk2-G2019S. *Representative immunofluorescent staining of GFAP (A,B) and CD11b (C,D) -positive cells highlighting the different shape and activation level of striatal astrocytes and microglia respectively, at 1 and 12 months of age. Scale bars 200 µm. Adapted from (Iannotta et al., 2020).*

Chapter 9

Discussion and conclusions

9. Discussion and conclusions

Several lines of evidence indicate that LRRK2 sits at the crossroads between vesicular traffic and cytoskeletal dynamics, through interaction with a host of cytoskeletal and vesicle-associated proteins and phosphorylation of a subset of RAB GTPases (Civiero et al., 2018; Iannotta & Greggio, 2021). This activity is particularly important in a specialized compartment such as the synapse, where LRRK2 has been shown to influence SVs cycle and consequent NTs release (Pischedda & Piccoli, 2021). The presence of a functional cytoskeleton, in particular actin filaments, is of fundamental importance at the synapse, as it provides the microarchitecture for synaptic structure maintenance and plasticity-driven reshaping. Thus, the dynamics of actin cytoskeleton are central for dendritic spines plasticity and, consequently, for brain circuits functionality (Matus, 2000). Via remodeling of actin filaments, these specialized structures respond to environmental cues and contribute to the experience-dependent refinement of neuronal circuits, learning and memory (Chidambaram et al., 2019). Robust literature points to synaptic damage and dendritic spines dysfunction as early pathological events proceeding neurodegeneration and clinical symptoms in multiple neurodegenerative disorders, such as PD (Fiala et al., 2002; Hong et al., 2016; Milnerwood & Raymond, 2010; Picconi et al., 2012; S. Soukup et al., 2018).

As mutations in LRRK2 represent the most common known cause of PD, understanding the role covered by this kinase in the physiology of the neuron is critical to gain better insight into the possible mechanisms contributing to PD neurodegeneration. The data of this doctoral thesis highlighted that LRRK2 regulates cytoskeletal dynamic impacting on the structural plasticity of dendritic spines and that the hyperactive G2019S mutation affects the processes that underlies this structural regulation.

We first assessed the expression of Lrrk2 in the mouse brain, focusing on midbrain, striatum and cortex, the three major compartments of relevance in PD. We observed that Lrrk2 is highly expressed in the mouse striatum, while the protein shows significantly lower levels in the midbrain. Performing immunofluorescence analysis on coronal brain slices from 1 month and 12 month-old BAC-GS mice, overexpressing by

~ 6-8 folds the murine *Lrrk2* locus, we further noticed that both total and phospho-Ser935 Lrrk2 signals are elevated in dorsal striatum but low in the nearby cortical region. High levels of LRRK2 in the striatum were previously reported in different studies, confirming our findings (Mandemakers et al., 2012; West et al., 2014). The dorsal striatum is the main gateway of the basal ganglia (BG), receiving afferent projections either from DA neurons of the SNpc, located in the midbrain, and from glutamatergic neurons of the cortex. Interestingly, we observed that total and pospho-Ser935 Lrrk2 signals is mainly present in cells positive for the neuronal marker β-III-tubulin, whilst it is poorly detectable in astrocytes and almost absent in microglia cells. Our data suggest that the expression and phosphorylation of this protein in the mouse brain is elevated in neurons of the striatum, according to other studies showing a specific functions of Lrrk2 in these cells (Matikainen-Ankney et al., 2016; Parisiadou et al., 2014). We performed this analysis in BAC-G2019S mice as they overexpress Lrrk2, allowing its visualization by immunofluorescence, otherwise undetectable under endogenous expression by the commercially available antibodies. While we predict that WT and G2019S have identical cell-type expression, these results warrant confirmation in the BAC mouse overexpressing WT Lrrk2. Nevertheless, it is clear that LRRK2 is enriched in the striatum, whose neuronal cells are mainly composed by MSNs, representing the post-synaptic cells of the nigro- and the cortical-striatal pathways. PD is characterized by the loss of DA neurons in the SNpc and and one hypothesis is that this degeneration starts at the periphery and only later reaches the cell body, through a dying back mechanism (Tagliaferro & Burke, 2016). Thus, we can speculate that a functional damage in post-synaptic MSNs causes an alteration in the synaptic functionality, contributing to DA neurons dysfunction and degeneration. Why DA neurons and not glutamatergic neurons are more susceptible to this dying back process may be found in the intrinsic vulnerability of these neurons. Indeed, DA neurons possess highly branched, unmyelinated axons, each providing as many as 245,000 synaptic contacts, putting them under an extraordinary energy demand, which requires a highly efficient delocalization of protein quality control systems (Bolam & Pissadaki, 2012; Mosharov et al., 2009; Surmeier et al., 2017). Interestingly, it has been shown that LRRK2 impacts the regulation of DA release: in presence of Lrrk2 BAC-GS (the same mice used in our study), DA release and uptake are reduced in the striatum (Xianting Li et al., 2010). While this points to a presynaptic impairment, we cannot rule

out that such an effect could be also an indirect consequence of an altered postsynaptic function. Moreover, the activity of cortical glutamatergic terminals is modulated by other neurotransmitters released in the local area, including DA. This reduction in the DA tone associated with mutant Lrrk2 alters DA-mediated plasticity at cortico-striatal synapses (Volta & Melrose, 2017). In addition, a recent study connects the changes in motor cortex excitability in PD with basal ganglia pathology, as a consequence of DA reduction, rather than to a loss of direct dopaminergic innervation of the motor cortex (Swanson et al., 2021). Moreover, glutamate release is regulated in a negative fashion by endocannabinoids that act *via* retrograde signaling from MSNs (Kreitzer & Regehr, 2002). Thus, mutant LRRK2 in MSNs may trigger several dysfunctional processes in this compartment contributing to the initiation of basal ganglia pathology in PD. Supporting this possibility, Chen and co-workers recently observed that Lrrk2 causes alteration in the functionality of MSNs spines in the presence of G2019S and R1441C mutations (C. Chen et al., 2020).

In parallel, by exploring LRRK2 interactome in neuronal-differentiated *SH-SY5Y* to find molecular clues about LRRK2 function at the synapse, we observed that the most significantly enriched hits are actin-related or myosin-related proteins. Actin-related proteins constitute a repertoire of players mostly specialized in regulation of actin cytoskeleton rearrangement (Pollard, 2016). Non-muscular myosins are actin-based cytoskeletal motors with a recognized role at neuronal synapses, where they drive cargo transport but also regulate actin cytoskeleton dynamics (Kneussel & Wagner, 2013). Consistently, GO enrichment analysis for biological processes revealed enrichment for actin organization pathways. Remarkably, the remodeling of actin network in postsynaptic dendritic spines heavily relies on the combined activity of actins and myosins (Kneussel & Wagner, 2013; Matus, 2000).

Several lines of evidence support a nexus between LRRK2 and cytoskeletal pathways (Civiero et al., 2018). Our group previously showed that LRRK2 Roc domain in its GTP-bound state interacts with PAK6 and that this interaction promotes neurite complexity in the striatum by modulating actin dynamics via the LIMK/cofilin pathway (Civiero et al., 2015). Moreover, it has been demonstrated that Lrrk2 controls the activity of cofilin also *via* PKA signaling pathway, impacting on dendritic spines remodeling at post-natal stages (Parisiadou et al., 2014). LRRK2 is able to

phosphorylate moesin (Jaleel et al., 2007), a member of the ERMs, proteins that are localized at the actin-rich sites in filopodia where they control neurite outgrowth by regulating filopodia architecture. Of interest, the phosphorylation state of ERM and F-actin content are increased in G2019S LRRK2 resulting in retardation of neurite outgrowth (Parisiadou et al., 2009). All these evidences and our protein-protein-interaction data point to a role of LRRK2 in neuronal actin dynamics. Based on these considerations together with the elevated LRRK2 expression in postsynaptic striatal MSNs, we set out to investigate whether LRRK2 influences the structural properties of dendritic spines. TEM and confocal imaging of Golgi-Cox stained striatal sections from 1, 4 and 18 month-old Lrrk2 WT and KO mice, showed that LRRK2 deficiency leads to changes in the maturation of striatal dendritic spines and in striatal synaptic contacts number during the developmental stages. Striatal dendritic spines of 1 month-old KO mice show a shift from *filopodia* to *thin spines* - while the amount of *mushroom* and *branched spines* remains unaltered - with a parallel reduction of PSD length, overall suggesting that loss of Lrrk2 results in subtle defects in spine maturation at young ages. Of note, Parisiadou and collaborators previously observed an higher proportion of thin and 'less mushroom' spines in LRRK2 KO pups, further supporting our findings (Parisiadou et al., 2014). Spines with small heads are not able to form strong synaptic contacts, suggesting that the reduction in the number of synapses observed in KO mice may be explained by the fact that fewer dendritic spines are sufficiently mature to make synaptic connections. PSD area correlates perfectly with spine head volume and also it is proportional to the number of postsynaptic NTRs and also to the amount of presynaptic docked vesicles, giving an indication about the level of synaptic functionality (Hering & Sheng, 2001). In 1 month-old Lrrk2 KO mice we detected a reduction in the PSD length, further indicating that dendritic spines are less mature and, probably less functional. However, 1month-old WT mice exhibit a higher proportion of filopodia (which are devoid of synaptic contacts), making the interpretation of these results complicated. One possibility is that the reduced PSD length observed in KO mice may also involve mushroom and branched spines. Indeed, accumulating evidence indicates the existence of dendritic spine shapes as a continuum rather than separated classes. To this end, it will be interesting to perform additional analyses using algorithms that rely on clusterization rather than classification of spines (Pchitskaya & Bezprozvanny, 2020). Importantly, even if a slight decrease in dendritic

spines maturation and synaptic contacts number is still still detectable in mature and aged mice, none of these alteration are statistically relevant, suggesting that Lrrk2 has an impact on neuronal development and leads to very early alterations, while differences are ironed out by some sort of compensatory mechanisms during the adulthood (Volta & Melrose, 2017), in agreement with previous studies showing that aged mice present normal electrical activity compared to young mice (Hinkle et al., 2012; Volta & Melrose, 2017). One possible explanation is that the paralog LRRK1 is able to compensate for LRRK2 deficiency. Even if no changes in the gene expression levels of Lrrk1 have been detected in BAC, KO or KI models (Volta & Melrose, 2017), it has been shown that Lrrk1 and Lrrk2 double KO mice show highly debilitating age-dependent defects, with α-Syn pathology, disruption of the autophagy-lysosomal pathway, and DA neurodegeneration in the CNS (Giaime et al., 2017). Of note, Parisiadou and collaborators found a significant decrease in the total amount of spines and in the quantity of fully-mature spines in 15 day-old pups Lrrk2 KO as compared to WT, accompanied to an increase in filopodia protrusion (Parisiadou et al., 2014). Instead, in our hands, the proportion of mature spines does not change between the two genotypes 1 month-old mice, possibly suggesting that this compensatory mechanism has already started to operate. Thus, our study adds the important notion that spine morphological defects in Lrrk2 KO normalize over time.

BDNF is a neurotrophin playing fundamental roles in the formation, maturation and plasticity of dendritic spines (Baydyuk & Xu, 2014; Kowiański et al., 2018; Leal et al., 2014). In addition, it promotes the process spinogenesis *in vitro*, possibly through a mechanism involving the regulation of actin mesh polymerization (Leal et al., 2015). Thus, BDNF represents an interesting model to study the possible link between LRRK2 and spine maturation in primary cultures. In agreement with the morphological analysis, we found that Lrrk2 promotes dendritic spines formation *in vitro*, as BDNF treatment is not able to induce spinogenesis in Lrrk2 KO cortical primary neurons. Furthermore, we found that Lrrk2 phosphorylation at Ser935 is enhanced by BDNF stimulation in primary neurons. Even if the precise role of this phosphorylation is still unclear and is likely to be cell-type specific, it has been shown to be required for the binding of 14-3-3 chaperons. LRRK2-14-3-3 interaction regulates LRRK2 dimerization (Civiero, Russo, et al., 2017), subcellular localization (Civiero, Russo, et al., 2017; X.

Deng et al., 2011; Nichols et al., 2010) and, as a consequence, access to its cellular substrates. Thus we can predict that BDNF exposure, by increasing the phosphorylation of LRRK2, stimulates its relocalization and activity. Accordingly, we observed that BDNF induces LRRK2 relocalization in TrKB-positive clusters in differentiated SH-SY5Y cells, possibly indicating LRRK2 recruitment to TrKB-endosomes during receptor internalization. Indeed, differentiation of *SH-SY5Y* into neuronal-like cells, made them competent to respond to BDNF stimulation and promoted an increase in LRRK2 expression.

After the binding between BDNF and TrKB, occurring at the level of axon terminals and dendritic spines, the receptor is internalized *via* clathrin-mediated endocytosis. Even if the precise mechanism of TrKB endocytosis is still incompletely resolved, it was shown to undertake different fates, including recycling back to the plasma membrane, trafficking to lysosomes for degradation or to the cell body via retrograde traffic within "signalling endosomes" (Cosker & Segal, 2014; S. H. Huang et al., 2013; Zahavi et al., 2021). Of note, in LRRK2 KO *SH-SY5Y* neuroblastoma cells generated with the Crispr/Cas9 technology, TrKB appears clustered into tubular structures under non-stimulated conditions, further supporting a role for LRRK2 in TrKB trafficking. This is not surprising if we think that LRRK2 has been propose to control different steps of vesicle traffic through phosphorylation of a subset of RAB GTPases and interaction with key components of the endocytic pathway. As a future step, it will be interesting to monitor TrKB-LRRK2 trafficking, using fast recycling (e.g. Rab4), slow recycling (e.g. Rab11) and degradation (e.g. LAMP1) markers to investigate in which steps of the process LRRK2 may intervene. Interestingly, we and others found that LRRK2 mutations increase autophosphorylation and Rab10 phosphorylation, although with different mechanisms and tissue specificity (Iannotta et al., 2020; Iannotta & Greggio, 2021). Recently, the Schiavo's laboratory proposed that the sorting and axonal retrograde transport of activated TrKB receptors is strongly influenced by Rab10, a mechanism that could be important to control BDNF availability at the synapse (preprint in bioRxiv, doi:10.1101/2021.04.07.438771). As LRRK2 phosphorylation of Rab10 is crucial for endo-lysosome dynamics (Kuwahara & Iwatsubo, 2020), it is tempting to speculate that TrKB sorting to Rab10-positive endosomes may depend on LRRK2 activity.

Based on our MS data indicating that LRRK2 interacts with a number of proteins involved in actin-related processes and that it intervenes in BDNF-induced spinogenesis, we reasoned that repeating the AP-MS experiments GFP-LRRK2 SH-SY5Y cells upon BDNF stimulation could have provided additional clues behind the role of LRRK2 in BDNF-dependent spinogenesis. First, this unbiased approach confirmed that LRRK2 interacts with proteins related to vesicular trafficking, cytoskeletal processes and also with proteins encoded by other PD-linked genes. Second, we observed that the LRRK2 interactome is profoundly remodeled in response to BDNF treatment. Specifically, the group of LRRK2 partners that showed reduced interaction with LRRK2 upon BDNF exposure are mostly involved in ER targeting and in translation/transcription processes, as indicated by GO enrichment analysis for biological processes. As extensively discussed, LRRK2 is well known to modulate both endosomal traffic and transcription/translation processes. For instance, LRRK2/Lrrk has been found to phosphorylate the ribosomal protein S15 both in human neurons and in *Drosophila*, promoting an increase in protein synthesis (Martin et al., 2014) and BDNF signaling is known to promote LTP inducing the expression of synaptic proteins involved in the enhancement of the synaptic strength (Leal et al., 2014). It can be speculated that, by reducing a putative inhibitory interaction between LRRK2 with translation-associated proteins, BDNF may stimulate the expression of genes involved in synaptic plasticity and function. Another interesting finding is that the LRRK2 interaction with VPS13C, a PD-linked protein, is significantly inhibited upon BDNF treatment. VPS13C is a pleomorphic risk locus, meaning that loss-of-function (LoF) variants impact both inherited and idiopathic PD ((Lesage et al., 2016)). Although the biology of VPS13C is almost unexplored, one study proposed that loss of function mutations in VPS13C cause mitochondrial dysfunction (Lesage et al., 2016). More recently, De Camilli's team described VPS13 proteins as a family of novel tethers working as channels for the direct exchange of lipids between the ER and other organelles (endo-lysosome for VPS13C and mitochondria for VPS13A) (Kumar et al., 2018). Our data indicate that LRRK2 and VPS13C interact in neuronal cells and that this interaction is released upon BDNF stimulation. This can add an interesting piece in the mechanisms proposed above. Given that i) TrKB is trafficked via Rab10 positive endosomes, ii) LRRK2 phosphorylates RAB10, iii) BDNF induces the activation of

LRRK2 and iv) decreases the affinity between LRRK2 and VPS13C, we can postulate that TrKB traffic is orchestrated by the coordinated actions of LRRK2, VPS13C and RAB10. While it would be extremely interesting to dissect the physiological meaning of this interaction and understanding why it decreases upon BDNF stimulation, we focused our attention on the hits whose interaction with LRRK2 was positively regulated. Strikingly, using SynGO we found that increased interactions upon BDNF stimulation were highly enriched in proteins involved in actin organization pathways at the postsynapse. Considering that post-synaptic structural changes are driven by activity-dependent and neurotrophic factor-dependent reorganization of the actin cytoskeleton (Lu et al., 2005), these data strengthen the hypothesis that LRRK2 regulates spine architecture influencing the cytoskeleton of actin.

Among all interactors, developmentally regulated brain protein, known as drebrin, showed a remarkable increased binding to LRRK2 over the untreated condition. Drebrin is well established to be highly enriched in neurons where it localizes within dendritic spines and to play a major role in regulating actin dynamics in this structure (Koganezawa et al., 2017). Drebrin is found as two main isoforms, named drebrin E and drebrin A, whose levels change based on the developmental stage. Indeed drebrin E is predominantly expressed during the development and localizes in migrating neurons where it accumulates in the growth cones of axons and dendrites. Here, it influences the formation of axonal filopodia and collateral branches promoting neurite outgrowth and axonal elongation (Ketschek et al., 2016). Upon neuronal maturation, there is an increase in the expression of drebrin A, which accumulates at nascent synapses until, at the end of the processes, drebrin A-decorated F-actin mainly concentrates in the central region of dendritic spines of fully mature neurons (Koganezawa et al., 2017). Back in 2011, Shamara et al. (S. Sharma et al., 2011) observed that drebrin remodels filamentous actin and, accordingly, drebrin-decorated actin filaments exhibit atypical longer helical crossover that results in two main features: (i) the exclusion of other existing actin-binding protein (e.g. tropomyosin) with the consequent acquirement of novel proprieties that change the type of responses to the different stimuli that reach dendritic spines (Koganezawa et al., 2017); (ii) the stabilization of actin filaments that present a slower depolymerizing rate (Mikati et al., 2013). Importantly, drebrin was reported to be crucially involved in dendritic spine morphogenesis and plasticity both

in developing and adult brain (Koganezawa et al., 2017). Based on the pivotal role of drebrin in regulating spine architecture via actin-remodelling, we decided to validate and explore the functional significance of drebrin-LRRK2 interaction. Besides confirming the interaction and its enhancement upon BDNF stimulation, we further found that the expression of drebrin in reduced in Lrrk2 KO brains at 1 month of age but not at later timepoints (using brain tissue from the same animals employed for the Golgi-Cox morphological analysis). Of interest, the proportion of drebrin within dendritic spine head positively correlates with the amount of stable F-actin and with the volume of the dendritic spine head (Kobayashi et al., 2007). Thus, the reduced drebrin content in KO brains is consistent with a reduction in the amount of mature spines. During development, drebrin A accumulates at postsynaptic sites via a regulatory mechanisms likely involving a neuron-specific sequence (Ins2) in the middle of the protein, and there it regulates the formation of synaptic protrusions. Drebrin A-decorated F-actin is thought to form a scaffold to coordinate the assembly of post-synaptic proteins by direct and indirect binding with drebrin. In addition, the protein has been found to accumulate in structures called "megapodia", where F-actin and PSD-95 content correlates with the amount of drebrin (Koganezawa et al., 2017). This suggests that drebrin promotes the transition to mature synapses. Dendritic spines plasticity depends on actin cytoskeleton remodeling and, in particular, on dynamic actin filaments reorganization. In response to appropriate stimuli inducing LTP and consequent synapse strengthening, drebrin was shown to transiently exit from dendritic spines to allow the remodeling of the actin dynamic pool and the enlargement of spines head (Koganezawa et al., 2017). Once the reshaping is completed, drebrin returns inside the spine head where it locks actin filaments located in the center of the spine, restoring the stable actin pool and ensuring the maintenance of the enlarged spine head (Koganezawa et al., 2017). Of note, our data show that drebrin exodus after BDNF treatment is barely detectable in Lrrk2-KO primary neurons, whilst Lrrk2-WT neurons show, as expected, a relocation of drebrin following BDNF stimulation. Interestingly, the exodus of drebrin is significant at 15 minutes of treatment, while the protein returns inside the spine head after 24 hours and its levels appear higher compared to the pre-stimulation condition, suggesting that the remodeling process has occurred. Considering that loss of Lrrk2 function affects drebrin stimulus-dependent relocalization, the LRRK2-drebrin interaction appears

relevant in promoting dendritic spines structural remodeling. This applies for BDNF stimulation, but potentially also for other stimuli inducing synaptic potentiation and strengthening, e.g. NMDA-dependent LTP. Based on literature and on our own data, we propose a possible mechanism whereby LRRK2 controls drebrin exodus. A first consideration relates to the fact that among all the increased interaction upon BDNF treatment we also found two NMMII, heavy chains myosin IIA (MYH9) and myosin IIb (MYH10). NMMII have been reported to impact on spines morphology and dynamics, as they can reversibly bind and contract actin (Kneussel & Wagner, 2013). Interestingly it has been proposed that Ca2+ influx following a stimulus inducing the activation of NMDA receptors, disinhibits myosin II ATPase activity within dendritic spine (Rex et al., 2010). When active, the enzymatic activity of myosin II ATPase severs drebrin-decorated F-actin, resulting in fragmentation of stable F-actin in shorter filaments which can move out from the dendritic spines into the dendritic shaft (Mizui et al., 2014). Thus we hypothesize that LRRK2 cooperates with myosins II in inducing drebrin exodus. Moreover our preliminary data suggest that LRRK2 interacts with drebrin via its entire catalytic core, with a prevalent binding at the level of the kinase domain, suggesting that drebrin could be a LRRK2 kinase substrate. It has been shown that drebrin phosphorylation at S142 by Cdk5 makes accessible a second actin binding site, which is masked by a C-terminal domain in the dephosphorylated form. Drebrin can then bundle F-actin or relocate inside the cells (Worth et al., 2013). Therefore LRRK2 may phosphorylate drebrin directly or via other kinase partners influencing drebrin actin-binding properties and thus its exodus. We could not collect evidence that LRRK2 is physically present in the spine head (due to the lack of sensitive antibodies to detect endogenous LRRK2) and overexpressed LRRK2 does not localizes within spines. If this holds true also for the endogenous protein, we can predict that during synaptic plasticity LRRK2 may be present in the shaft at the entrance of the spine neck and recruit drebrin outside the dendritic protrusion via the interaction with /phosphorylation of intermediate players, allowing in this way actin cytoskeleton remodelling.

The second part of this project focused on understanding the pathological consequences of mutant LRRK2 kinase activity on dendritic spines maturation and remodeling. We observed that LRRK2 gain of kinase function associated with the

G2019S mutation results in altered striatal dendritic spines maturation and striatal synaptic contacts number in aged mice. Our data indicate that 18 month-old Lrrk2 G2019S mice display an increased amount of mature spines and a parallel decrease in non-mature spines compared to controls. In particular, the proportion of *branched* spines is enhanced with a concomitant reduction in the fraction of *thin* protrusions, suggesting that the presence of mutant LRRK2 promotes the maturation of dendritic spines. Consistently, we also observed that the length of the PSD is significantly longer in Lrrk2 pathological models. Importantly, these effects are exacerbated in BAC GS mice, suggesting that the more Lrrk2 activity the more the effect is pronounced. A previous study highlighted that 1 month-old G2019S KI Lrrk2 mice possess MSNs with larger spines and parallel larger postsynaptic activity (Matikainen-Ankney et al., 2016). As alterations in striatal neurotransmission have been detected in GS-Lrrk2 at 3-4 months of age (L. Iovino et al., 2020), we subsequently analyzed younger mice. In 4 month-old Lrrk2 GS mice we only observed a slight reduction in the amount of *thin* protrusions in KI-GS animals even if there is a trend of increase in branched spines in both pathological models. A larger sample size will be required to confirm or disprove this subtle difference. We also plan to conduct the analysis in 1 month-old mice, as this represents the time window were Lrrk2 exerts it physiological function in spine maturation. Mature spines are characterized by larger head, whose formation is influenced by actin cytoskeleton remodeling. Thus, the hyperactive kinase mutation may affect Lrrk2-drebrin interaction causing an excessive enlargement of spine heads. On the other hand, we can speculate that increased Lrrk2 kinase activity alters TrKB trafficking. The downstream consequences are unknown at the moment. However, a previous study from our group showed that LRRK2 G2019S alters the trafficking of the glutamate transporter Glt-1 in astrocytes, resulting in reduced receptor availability at the membrane (preprint (Iovino et al., 2021)). Whether a similar situation occurs for TrKB and how this influences LRRK2-drebrin binding will require additional investigations. Surprisingly, we detected a reduced number of synaptic contact in 18 month-old KI-GS and BAC-GS mice, with a more pronounced effect in BAC-GS animals. One possible interpretation of this apparent inconsistency is that the presence of stronger synapses could be detrimental in the long run as sustained activity may affect synaptic function. There are a number of evidence documenting overstimulation of the cortico-striatal projections in Lrrk2 GS mice. For instance, Volta and collaborators

observed that MSNs in acute striatal slices display an augmented sEPSC frequency compared to control (Volta et al., 2017). Moreover, glutamate release is increased in Lrrk2 GS KI neurons neurons (Beccano-Kelly et al., 2014). Increased extracellular glutamate could result in a well-documented phenomenon named excitotoxicity. We can speculate that non-physiological levels of glutamate may trigger the mechanism of synaptic pruning, whereby non-functional synaptic contacts are eliminated. Supporting the presence of a synaptic pruning mechanism we observed an increase in the activation of glial cells in BAC-GS old mice. Neuroinflammation is a dominant feature of PD and relies on the activity of astrocytes and microglia. Glia reaction and neuroinflammation states represent conditions frequently associated with glutamate extra-synaptic diffusion and associated toxicity (L. Iovino et al., 2020). However, it should be pointed that all these analyses were conducted without discriminating among the different cell types constituting the striatum. Indeed, Parisiadou and collaborators (C. Chen et al., 2020) observed that LRRK2 gain-of-function pathogenic mutations cause alterations in MSNs excitatory synapses function that are more pronounced in the neurons of the direct pathway. Thus, in the future, it would be interesting to explore what type of synapses are affected in their maturation process in the absence of LRRK2.

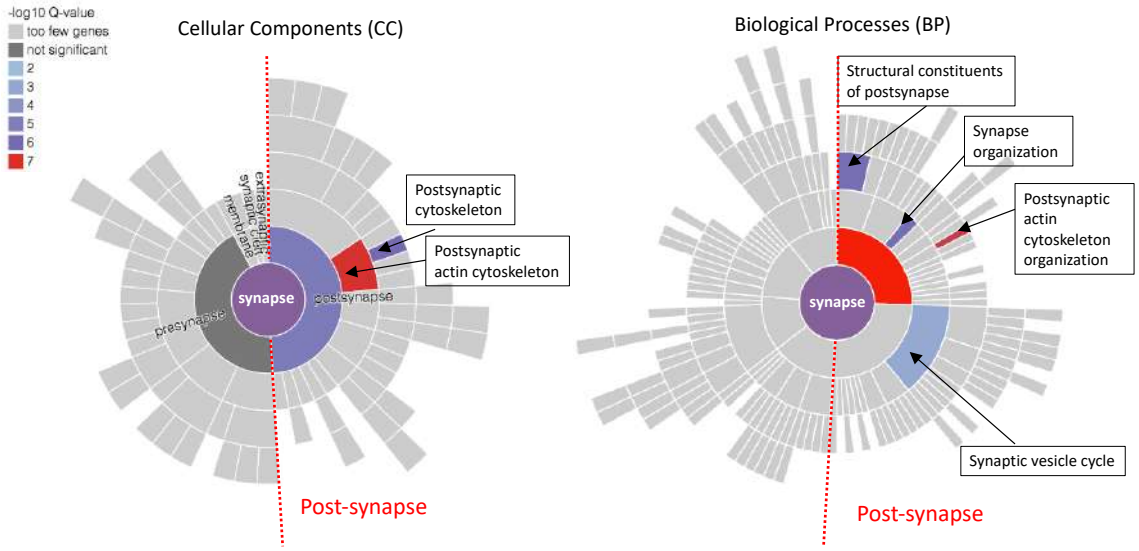
In conclusion, the results obtained during my PhD shed light on a novel function for LRRK2 in the processes underling postsynaptic physiology. Taking advantage of multiples approaches, we demonstrated that LRRK2 is involved in dendritic spine structural plasticity by orchestrating actin cytoskeletal remodeling. In particular, we propose a mechanism whereby LRRK2 recruits the actin-related protein drebrin allowing the reorganization of F-actin filaments and the reshaping of dendritic spine heads in the presence of a stimulus inducing synaptic strengthening, like BDNF. Furthermore, we collected evidence that LRRK2 influences actin dynamics modulating TrKB receptor trafficking and the consequent postsynaptic response. This may involve additional targets such as VPS13C and RAB10. Importantly, LRRK2 influences dendritic spine genesis and maturation mostly during the post-natal stage. Under pathological hyperactivation of LRRK2, we observed an increased amount of mature spines and diminished number of synaptic contacts accompanied by a parallel gliosis in aged mice. Whether these impairments depend on altered BDNF/TrKB and/or actin/drebrin

mechanisms will require further investigations. However, it is difficult to conclude that this phenotype is directly linked to LRRK2:actin-related proteins complex deregulation rather than being the consequence of other dysfunctional processes, e.g. excessive neuroinflammation resulting in synaptic dysfunction, calcium dyshomeostasis or mitochondrial impairment. Future studies investigating spine maturation and LRRK2-drebrin interaction in young G2019S mice should be performed to explore this aspect. Indeed, addressing the involvement of pathogenic LRRK2 in the postsynaptic elements may support the development of therapeutic interventions that target the early steps of the degeneration process, prior to neuronal terminal loss characterizing PD. For example, non-manifesting LRRK2 mutation carriers may benefit from the administration of LRRK2 inhibitors as a preventing therapy able to delay or block early synaptic dysfunction.

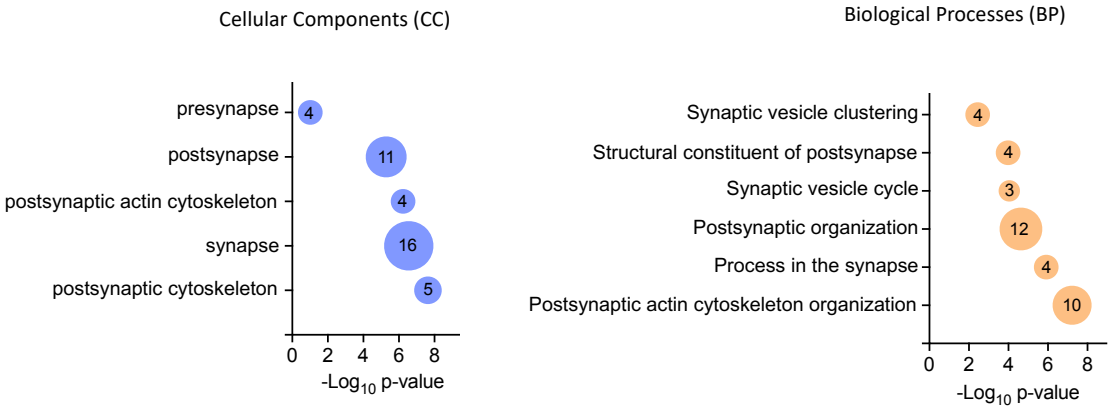
Overall this project contributed to increase our understanding of LRRK2 role at the post-synaptic site, adding another piece to the complex puzzle of LRRK2 pathobiology.

Appendix 1

a



b



Supplementary figure 1. *SynGO gene onlotogy analysis reveals enrichment of post-synaptic actin cytoskeleton categories among BDNF-enrichd LRRK2 interactors. A) SynGO CC terms and BP terms are visualized in a sunburst plot for terms with at least 5 unique annotated genes. B) Bubble plots highlighting p values of the GO categories.*

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