Review

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Neuronal microtubules and proteins linked to Parkinson's disease: a relevant interaction?

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Abstract: Neuronal microtubules are key determinants of cell morphology, differentiation, migration and polarity, and contribute to intracellular trafficking along axons and dendrites. Microtubules are strictly regulated and alterations in their dynamics can lead to catastrophic effects in the neuron. Indeed, the importance of the microtubule cytoskeleton in many human diseases is emerging. Remarkably, a growing body of evidence indicates that microtubule defects could be linked to Parkinson's disease pathogenesis. Only a few of the causes of the progressive neuronal loss underlying this disorder have been identified. They include gene mutations and toxin exposure, but the trigger leading to neurodegeneration is still unknown. In this scenario, the evidence showing that mutated proteins in Parkinson's disease are involved in the regulation of the microtubule cytoskeleton is intriguing. Here, we focus on α-Synuclein, Parkin and Leucine-rich repeat kinase 2 (LRRK2), the three main proteins linked to the familial forms of the disease. The aim is to dissect their interaction with tubulin and microtubules in both physiological and pathological conditions, in which these proteins are overexpressed, mutated or absent. We highlight the relevance of such an interaction and suggest that these proteins could trigger neurodegeneration via defective regulation of the microtubule cytoskeleton.

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Introduction

Microtubules are ubiquitous structures playing many roles in all eukaryotic cell types from fungi to mammals. Neurons are an impressive example of cells in which the contribution of microtubules is fundamental to achieve their sophisticated cell architecture and to sustain their functional complexity (Kelliher et al., 2019). Indeed, neuronal microtubules are essential for the health of the nervous system starting from neurodevelopment up to neuronal differentiation as well as for the maintenance of the mature neuron's phenotype: they are key determinants of cell polarity, contribute to trafficking of cargo molecules and organelles to pre-, post- or extra-synaptic domains, and regulate not only dendritic spine morphology, but also synaptic plasticity (Hoogenraad and Bradke, 2009; Kapitein and Hoogenraad, 2015). This extraordinarily wide range of properties is closely dependent on complex and multiple-level regulation (Song and Brady, 2015; Baas et al., 2016). First, the expression of different tubulin genes and the accumulation of post-translational modifications of tubulin generate the so-called "tubulin code", which modulates microtubule building and rearrangement giving rise to highly specialized microtubules (Park and Roll-Mecak, 2018). Second, the large number of microtubule-interacting proteins strongly influences many aspects of the microtubule's life, from the control of their dynamics and the stabilization of microtubule lattice to the movement of cargos along microtubules themselves (Goodson and Jonasson, 2018).

Moving from physiology to pathology, many studies indicate that defective regulation of microtubules may have a role in the pathogenesis of a broad range of nervous system disorders, including neurodevelopmental, psychiatric and neurodegenerative diseases (Penazzi et al., 2016). Remarkably, a growing body of evidence, including our work, indicates that microtubule defects are linked to Parkinson's disease (PD) pathogenesis (Cartelli and Cappelletti, 2017a; Pellegrini et al., 2017). The concept

that microtubule dysfunctions can participate in, and perhaps lead to, PD progression has been suggested by studies on toxin-based and genetic experimental models of the disease. We have shown that the PD-neurotoxin 1-methyl-4-phenylpyridinium (MPP^+) reduces microtubule polymerization and interferes with the dynamic instability of microtubules *in vitro*, acting as a destabilizing factor (Cappelletti et al., 2005), and leads to microtubule alteration and, in turn, to mitochondrial trafficking impairment in cultured neurons (Cartelli et al., 2010). In addition, we have shown that systemic injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in mice induces early microtubule dysfunction that precedes axonal transport deficit, depletion of tyrosine hydroxylase and, ultimately, dopaminergic neuron degeneration (Cartelli et al., 2013). The importance of microtubules in PD is also supported by several independent studies reporting a genome-wide significant association with PD of single nucleotide polymorphisms in the *MAPT* locus, containing the gene coding for the microtubule-associated tau protein (Nalls et al., 2011). This review assesses the emerging data that point to the link between the mutated proteins in the familial forms of PD and the microtubule system. Starting from a brief introduction on their structure, localization and known functions, we will move to the experimental, and sometime debatable, evidence of their impact on microtubules and microtubule-dependent functions in both physiological and pathological contexts. Our goal is to critically discuss the relevance and future perspectives of the interaction between PD-linked proteins and the microtubule system, thus contributing to puzzle PD pathogenesis out.

α-Synuclein

α-Synuclein is a protein deeply involved in PD, as demonstrated in 1997 by two seminal studies showing that (i) it is the main component of Lewy bodies (LB), the well-known histopathological hallmark of this neurodegenerative disorder (Spillantini et al., 1997) (Figure 1), and (ii) its gene, *SNCA*, is mutated in familial PD (Polymeropoulos et al., 1997). Starting from these two discoveries, many studies have underlined the importance of α-Synuclein in the disease (Goedert et al., 2017). Despite this, to date little is known not only about the mechanisms through which α-Synuclein acts in the disease, but also about its physiologic role. α-Synuclein, encoded by the *SNCA* gene (4q21.3–q22), is a protein of 140 amino acids (aa) that, together with β-Synuclein and γ-Synuclein, belongs to the synuclein family of proteins.

Structure, localization and function

α-Synuclein can be divided into two main regions: (i) an N-terminal region, which includes a basic N-terminal and a central hydrophobic 'non-amyloid component' (NAC) domains, and (ii) a shorter C-terminal region composed by the acidic C-terminal domain (Figure 2). There are major differences between them. First, the N-terminal region is highly conserved among species and within the synuclein family, while the C-terminus is less conserved (George, 2002). Second, along the N-terminal region are located seven 11 aa repeats containing a KTKEGV consensus

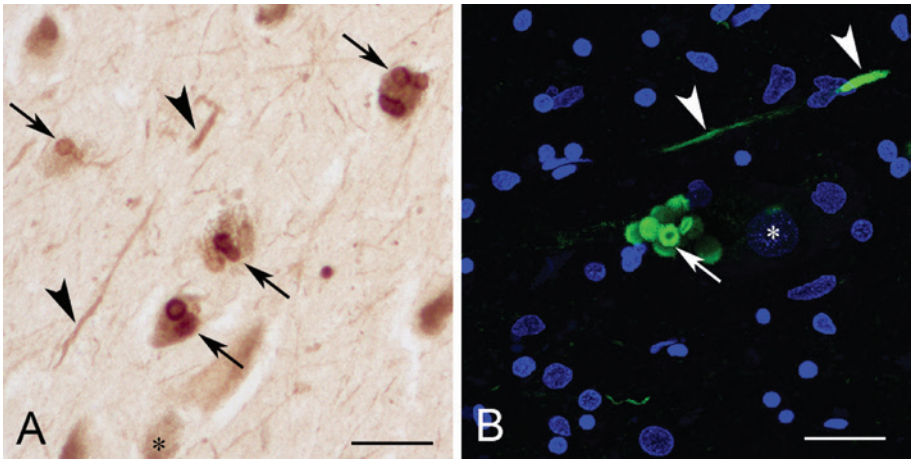

Figure 1: α-Synuclein localization in Lewy bodies and Lewy neurites in *substantia nigra*.
(A) Immunoenzymatic staining performed in *postmortem* brain from patients affected by Parkinson's disease reveals the presence of α-Synuclein in Lewy bodies (arrows) and Lewy neurites (arrowheads) inside neurons containing neuromelanin (black asterisk). (B) Confocal maximal projections showing that α-Synuclein (green) is localized in Lewy bodies (arrow) in a neuron (recognizable by the presence of an eucromatic nucleus, asterisk) and in Lewy neurites (arrowheads). Nuclei are visualized in blue. Scale bar: (A) 50 μm; (B) 17 μm.

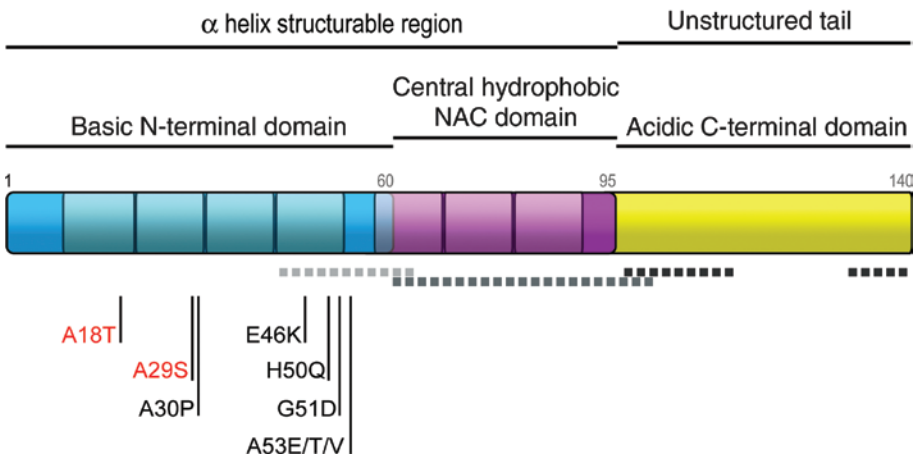

Figure 2: Domain structure of human α-Synuclein.
α-Synuclein is composed by three domains: a basic N-terminal domain, a central hydrophobic NAC domain and an acidic C-terminal domain. While the acidic C-terminal domain forms the unstructured tail of the protein, the basic and the hydrophobic NAC domains form the α-helix structurable region, as they acquire α-helix structures upon interaction with protein substrates. The seven repeats containing a KTKEGV consensus sequence are indicated in light blue and light violet. Dotted lines indicate the regions that are suggested to be involved in tubulin binding. Missense mutations found in PD patients are reported in black (familial PD) or in red (sporadic PD).

sequence (Jakes et al., 1994), completely lacking in the C-terminal domain. Third, the N-terminal region is able to acquire α-helix structures under specific conditions and the aggregation-prone NAC domain mediates the β-sheet conformation of the protein, while the C-terminal domain remains unstructured. Finally, all the known mutations are located only in the N-terminal region. The main characteristic of α-Synuclein is that it is a "natively unfolded" protein, intrinsically unstructured, with little or no ordered secondary structure under physiological conditions (Uversky, 2002). Indeed, it has been proposed that, in physiological conditions, α-Synuclein is mainly present as unfolded monomers, or as tetramers that have α-helical conformation and resist aggregation (Bartels et al., 2011). α-Synuclein is able to acquire a tertiary structure upon interaction with its partners, like lipidic membranes and synaptic vesicles [reviewed in (Auluck et al., 2010; Lassen et al., 2016)].

α-Synuclein owes its name to its localization into the nucleus and at the pre-synaptic terminals (Maroteaux et al., 1988). The latter localization has been the subject of most investigations. Indeed, α-Synuclein is mainly considered a pre-synaptic protein, although its low expression is detectable also in the soma, in dendrites and in axons (Iwai et al., 1995). α-Synuclein is expressed in both the central and the peripheral nervous system, suggesting that it has a role in synaptic transmission in the entire nervous system. α-Synuclein is enriched in presynaptic terminals, interacts with synaptic vesicles and promotes SNARE complex assembly (Burrè et al., 2010), and modulates vesicular trafficking to the membrane and vesicular endocytosis, thus playing an important role in the regulation of synaptic transmission (Benskey et al., 2016; Longhena et al., 2019). In addition to its involvement in exocytosis, endocytosis and vesicle clustering processes, other proposed roles for α-Synuclein, mainly based on its numerous identified partners, include regulation of intracellular trafficking, cell death, protein clearance and maintenance of neuronal mitochondrial homeostasis (Lassen et al., 2016; Rocha et al., 2018; Huang et al., 2019).

α-Synuclein in Parkinson's disease

α-Synuclein aggregates are found not only in the brain but also in the peripheral nervous system of PD patients (Braak et al., 2006; Beach et al., 2010). Although the mechanisms leading to their formation have been supposed to be "cell-autonomous" for a long time, emerging and debatable data support spreading and a prion-like behavior for α-Synuclein in PD patients (Brundin and Melki, 2017). Familial forms of PD can be linked to *SNCA* duplication and triplication (Singleton et al., 2003; Chartier-Harlin et al., 2004; Farrer et al., 2004; Ibáñez et al., 2004), as well as to point mutations. To date, seven α-Synuclein mutants have been identified in familial PD: A53T (Polymeropoulos et al., 1997), A30P (Kruger et al., 1998), E46K (Zarranz et al., 2004), H50Q (Appel-Cresswell et al., 2013), G51D (Lesage et al., 2013), A53E (Pasanen et al., 2014), and, more recently, A53V (Yoshino et al., 2017) (Figure 2, in black). Additionally, Hoffman-Zacharska and colleagues discovered other two variants, A18T and A29S, in two Polish patients (Hoffman-Zacharska et al., 2013) (Figure 2, in red).

As no family history of neurodegenerative disorders has been reported for them, these two mutations are actually associated with sporadic PD. Considering that none of their parents were available for genetic sequencing, it is intriguing to speculate that they could be carriers of two *de novo* mutations, an hypothesis that would justify the absence of familial positivity to neurodegenerative disorders.

Patients carrying different α-Synuclein alterations actually show different phenotypes and age at onset. Briefly, patients with A18T, A29S, A30P and H50Q mutated α-Synuclein experience late onset of PD, while E46K, G51D, A53E and A53T mutations cause earlier onset. Finally, patients with a duplication of the *SNCA* locus and patients with idiopathic PD experience onset of the disease at similar ages, while triplication of the *SNCA* locus in early onset of the disease.

Considering the multiple partners and the involvement in a large number of processes, α-Synuclein alterations in PD leads to many dysfunctions, mainly at the synapse but also in the axon, where causes impairment of axonal transports (Longhena et al., 2019). However, its misfolding and aggregation into insoluble deposits is considered the main event in the pathogenesis of PD, even if how the accumulation of α-Synuclein leads to neurodegeneration is still unclear (Spillantini and Goedert, 2018; Mehra et al., 2019).

α-Synuclein, tubulin and microtubule cytoskeleton

The link between α-Synuclein, tubulin and microtubules has been under investigation for about two decades, nevertheless this remains a controversial issue. The first question is whether α-Synuclein directly interacts with tubulin. Originally, Payton and colleagues found that in zebra finch and mouse brains α-Synuclein co-immunoprecipitates with several proteins, including α- and β-tubulin (Payton et al., 2001). The ability of α-Synuclein to be co-purified with both α- and β-tubulins was confirmed also in rat brain (Alim et al., 2002) and its interaction was reported not only with free tubulin, but also with assembled tubulin (Alim et al., 2004). More recently, we found that α-Synuclein interacts with tubulin tetramers, rather than with dimers, and acquires α-helical structures upon incubation with purified tubulins, thus supporting the hypothesis that tubulin is one of its physiological partners (Cartelli et al., 2016). Interestingly, α-Synuclein co-localizes with microtubules in HeLa cells (Zhou et al., 2010) and preferentially with tyrosinated tubulin in human mesencephalic neurons (Cartelli et al., 2016). Although these data indicate that α-Synuclein directly interacts with tubulin, the binding region has not been identified so far. Unluckily, crystal structures of α-Synuclein in complex with tubulin are not available as well as, as to date the region responsible for this interaction has not been clearly identified. A first study shows that aa 96–102 and/or 131–140 are important for microtubule assembly, suggesting that they mediate binding to tubulin and that the microtubule binding domain is located in the C-terminal region of α-Synuclein (Alim et al., 2004). Later, Zhou and colleagues proposed a central region (spanning from aa 60 to aa 100) as the one responsible for interaction with tubulin (Zhou et al., 2010). More recently, based on sequence similarity to spastin, we suggested that the region responsible for α-Synuclein/tubulin interaction could be located just before this sequence (i.e. aa 43–63) (Cartelli et al., 2016) (Figure 2, dotted gray lines). The reported hypothetic binding sequences could indicate that the microtubule binding domain of α-Synuclein is spread along the entire protein sequence, and that all regions are necessary to elicit α-synuclein functions on tubulin and microtubules. Although further work is needed to clearly identify the amino-acidic residues involved in this binding, α-Synuclein interaction with tubulin seems to be at this point more than just a hypothesis and its impact on tubulin behavior is an intriguing aspect to be investigated.

The effects of α-Synuclein on tubulin polymerization and dynamics have been poorly investigated and the results are still somewhat controversial. Some authors have reported that monomeric α-Synuclein does not influence tubulin polymerization *in vitro* (Chen et al., 2007), whereas others that it inhibits (Zhou et al., 2010) or promotes (Alim et al., 2002, 2004) microtubules assembly, suggesting a regulatory role of the protein on microtubule formation. More recently, we suggested that α-Synuclein is not just a microtubule associated protein, but rather a dynamase, i.e. a protein able to regulate both microtubule nucleation and catastrophe (Cartelli et al., 2016). In addition, α-Synuclein influences microtubule polymerization and dynamics in cultured cells (Zhou et al., 2010; Cartelli et al., 2016) and regulates the partitioning between tubulin dimers and microtubules at the neuronal growth cone (Cartelli and Cappelletti, 2017b). Finally, looking at the impact of α-Synuclein on microtubule-dependent functions, recent papers show that α-Synuclein promotes the formation of short transportable microtubules that play an important role in axonal transport (Toba et al., 2017), and that WT α-Synuclein induces vesicle endocytosis impairments via microtubule overassembly (Eguchi et al., 2017).

In pathological conditions, α-Synuclein interactions with tubulin and microtubules are altered. First of

all, microtubule assembly kinetics are lower with A30P and A53T mutants than with WT α-Synuclein (Alim et al., 2004). As A30P, E46K and A53T mutants partially lose the ability to acquire α-helical secondary structures upon incubation with tubulin (Cartelli et al., 2016), this could explain why these mutations could compromise α-Synuclein binding to tubulin and, consequently, induce polymerization defects. In cells, the expression of A30P and A53T mutants affects microtubule assembly at the neuronal growth cone (Cartelli and Cappelletti, 2017b). α-Synuclein overexpression correlates with disruption of the microtubule network, impairment of microtubule dependent trafficking and neurite degeneration (Lee et al., 2006).

Interestingly, the discovery that tubulin co-localizes with α-Synuclein in LB and other α-Synuclein-positive pathological structures, including pale bodies and Lewy neurites, strongly suggests that the microtubule cytoskeleton could be directly involved in triggering α-Synuclein aggregation (Alim et al., 2002). In support of this fascinating hypothesis, the formation of α-Synuclein inclusions might be attenuated in cell and *Drosophila melanogaster* models following the increase of tubulin acetylation by pharmacological inhibition or gene silencing of the enzyme SIRT2, which deacetylates tubulin (Ouitero et al., 2007). In addition, it has been reported that β3-tubulin binds α-Synuclein and promotes its aggregation in cultured cells and in a mouse model of Multiple System Atrophy, a neurodegenerative disease characterized by accumulation of α-Synuclein in oligodendrocytes (Nakayama et al., 2012). All together, these data clearly support the concept that a direct link between tubulin, the microtubule cytoskeleton and α-Synuclein exists in both physiological and pathological contexts. However, this is a two-faced relationship: on the one hand, α-Synuclein overexpression leads to defects in the microtubule cytoskeleton and to neurodegeneration, whereas, on the other hand, microtubule modulation affects the formation and accumulation of α-Synuclein aggregates. Notably, as recently reviewed, α-Synuclein effects on microtubule stability can be both direct and indirect as it could involve Tau and GSK-3β pathways. This indicates that the alteration of α-Synuclein (overexpression or missense mutations) could impact the microtubule system at different levels (Carnwath et al., 2018).

Parkin

The E3 ligase Parkin is a protein of 465 aa encoded by *PRKN* (*PARK2*) gene, located on chromosome 6 in humans (6q25-q27), firstly identified in patients affected by autosomal recessive juvenile Parkinsonism, from which its name originated (Kitada et al., 1998).

Structure, localization and function

Parkin is characterized by the presence of the following domains from the N-terminus to the C-terminus: an Ubiquitin-like domain (Ubl), two consecutive RING (Real Interesting New Gene) finger domains (RING0 and RING1), an In-Between-Ring domain (IBR), a Repressor domain (REP) and, finally, a last RING domain (RING2) (Figure 3) (Zhang et al., 2016). RING1 is required for E2-conjugating enzyme recruitment, while RING2 is the catalytic domain containing the cysteine needed for ubiquitin transfer. Three-dimensional structures of Parkin show that the enzyme exists in a closed, inactivated state under normal conditions. In this auto-inhibited state RING0, also called Unique Parkin Domain (UPD), closes the active site on RING2, while REP and Ubl domains prevent access to the putative E2-binding site on RING1 (Riley et al., 2013; Trempe et al., 2013; Wauer and Komander, 2013). Parkin activation requires the kinase PINK1 and consists of phosphorylation at S65 (Ubl) and binding of a phosphorylated ubiquitin to H302 (RING1), causing structural rearrangements that open protein structure and allow enzymatic activity (Gladkova et al., 2018).

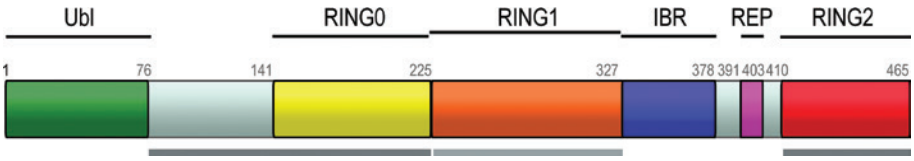

Figure 3: Domain structure of human Parkin.
Parkin is composed of six domains: a Ubiquitin-like domain (Ubl), two consecutive Real Interesting New Gene domains (RING0, RING1), an In-Between-Ring domain (IBR), a Repressive Element of Parkin (REP) and a RING domain (RING2). The three independent microtubule binding domains are indicated with gray lines. Due to the large number of pathological mutations, they were not indicated in this scheme.

Parkin is expressed in many human tissues including the brain, where its expression is high in several regions (Kitada et al., 1998). Parkin is considered to be a cytosolic protein that localizes in neuronal axons, soma and dendrites. Due to its localization at both pre- and post-synaptic terminals, it has been proposed that Parkin elicits its function by also regulating synaptic functions (Sassone et al., 2017). Being an E3 ligase, Parkin acts as an E2-dependent ubiquitin ligase associated with the ubiquitin-proteasome system (Imai et al., 2000; Shimura et al., 2000; Zhang et al., 2000). Furthermore, Parkin is also involved in mitophagy and in autophagy-associated protein quality control, making it clear that Parkin is a key regulator of protein and mitochondrial homeostasis, as recently reviewed (Zhang et al., 2016). In addition to these E3 ligase-dependent roles, the involvement of Parkin in regulation of gene expression, thanks to its emerging activity as transcription factor, has been pointed out very recently (Alves da Costa et al., 2019).

Parkin in Parkinson's disease

Parkin is historically correlated to PD, as it was identified for the first time in families affected by autosomal juvenile PD (Kitada et al., 1998). Actually, more than 100 pathogenic alterations have been found on the *PRKN* gene in PD patients (Corti et al., 2011). These consist of exon rearrangements (deletions and multiplication) and point mutations (missense, nonsense and frameshift mutations) that can affect the entire coding sequence. Furthermore, *PRKN* mutations, as well as Parkin inactivation, can also be found in sporadic PD (Dawson and Dawson, 2014). Due to the great number of mutations, it is difficult to study the effect of each of them, notwithstanding the general idea is that they lead to a loss of function impairs the role of Parkin as an E3 enzyme, and, consequently, causes the toxic accumulation of (one or several of) its substrates, thereby triggering neurodegeneration. Otherwise, different mutations might elicit different effects: C289G and C418R missense mutations, but not K161N and R256C, are characterized by subcellular re-localization and rapid sequestration into insoluble aggregates (Gu et al., 2003). Furthermore, analysis of another six mutations revealed that point mutations located in the RING1 domain (i.e. R256C and R275W) show an unusual distribution of the protein as they are concentrated in large inclusions called aggresomes (Cookson et al., 2003). Then, Hampe and colleagues investigated some of these mutants together with others and found that only a part of them show altered solubility, binding to some substrates, ligase activity or attitude to aggregation (Hampe et al., 2006), thus supporting the diversity of Parkin mutation effects. More recently, the role of Parkin expression has been investigated in human midbrain dopaminergic neuron induced iPSCs derived from two PD patients with exon deletions in the *PRKN* gene, which cause the loss of Parkin expression. Using this experimental model, Jiang and coworkers showed that Parkin absence leads to many dysfunctions, including abnormalities in dopamine release and uptake (Jiang et al., 2012). To date, many additional Parkin-related defects have been disclosed in PD models ranging from the higher susceptibility of neurons from Parkin null mice to rotenone, a PD-causing toxin (Casarejos et al., 2006), to alterations in mitochondria homeostasis and mitophagy, autophagy, and synapse functions (Zhang et al., 2016; Sassone et al., 2017). Considering the central role played by Parkin in regulating mitochondria biology and the importance of mitochondrial dysfunction in PD, the main pathological mechanism in PD associated with Parkin is linked to changes in mitochondrial homeostasis and mitophagy (Pickrell and Youle, 2015).

Parkin, tubulin and microtubule cytoskeleton

Among the others, α- and β-tubulin are substrates of Parkin, which ubiquitinates them and accelerates their degradation by the ubiquitin-proteasome system (Ren et al., 2003), indicating that this enzyme is required for tubulin turnover. Moreover, Parkin binds to microtubules and localizes along them with a punctate staining (Ren et al., 2003; Yang et al., 2005). It has been suggested that three separate domains of Parkin (i.e. a linker region containing the RING0 domain, RING1 and RING2 domains) bind independently tubulin and microtubules with high affinity (Figure 3, gray lines). Notably, this binding stabilizes the microtubule network against microtubule depolymerizing agents (Yang et al., 2005). The localization of Parkin on microtubules and the attachment of endoplasmic reticulum (ER) to microtubules, together with its involvement in the degradation pathways of misfolded membrane proteins, suggest that Parkin might efficiently ubiquitinate misfolded transmembrane protein substrates, which have to be retrotranslocated to the cytosol for ubiquitination and degradation. In addition, Parkin might also ubiquitinate misfolded proteins transported along microtubules to the aggresome (Yang et al., 2005; Feng, 2006). Although the tridimensional structure of Parkin is well studied, to date the conformation adopted in its tubulin-bound state is unknown, due to the difficulty in obtaining a crystal structure of the complex.

Indeed, we do not know whether the binding to tubulin and/or microtubules can in some way limit the ability of Parkin to interact with their substrates or partners, or whether this interaction leads to activation/inactivation of the protein, thus acting as a regulator of Parkin activity, or whether Parkin binds to them preferentially in its active/inactive state.

Parkin interaction with the microtubule cytoskeleton has also been investigated in pathological conditions. Some of its pathogenic mutants (K161N, T240R, C431F) show defects in their ability to ubiquitinate α- and β-tubulin and, therefore, to accelerate their degradation (Ren et al., 2003). Most likely, this is not due to their defective ability to bind tubulin (Yang et al., 2005). On the other hand, experiments performed in both murine and human midbrain dopaminergic neurons showed that mutations or exons' deletion in *PRKN* can lead to microtubule destabilization, abolishing the stabilizer effect of Parkin against microtubule destabilizing toxins (Ren et al., 2009, 2015). These observations are in agreement with data obtained in skin fibroblasts: patients with *PRKN* mutations (point mutations and/or exon deletions) display reduced microtubule mass and higher microtubule destabilization following pharmacological treatments, while over-expression of WT Parkin restores control phenotype (Cartelli et al., 2012). In addition, Ren and co-workers studied the cellular morphology of midbrain dopaminergic neurons obtained from PD patients with *PRKN* mutations (exon 5 and/or 3 deletions) and found that Parkin absence, caused by these mutations, reduces the length and complexity of neuronal processes, microtubule stability and, after treatments with microtubule destabilizer (colchicine), makes microtubules even less stable (Ren et al., 2015). All these defects are rescued by overexpression of WT Parkin, but not T240R mutant, indicating that Parkin is involved in the regulation of cytoskeleton and that, for this, the ubiquitin ligase activity is necessary. The fact that Parkin is required for proper microtubule cytoskeleton dynamics has been confirmed by a recent study performed on *PRKN* KO mouse (Cartelli et al., 2018). Analyzing brain tissues of mice at different ages, an overall misregulation of the microtubule cytoskeleton emerged: Parkin absence leads to the imbalance of tubulin post-translational modifications in both corpus striatum and substantia nigra. In particular, dopaminergic neurons of *PRKN* KO mice are characterized by an early accumulation of tyrosinated tubulin and a decrease in acetylated tubulin in young mice, while at the later ages the situation is overturned. Next, we demonstrated that Parkin downregulation leads to defects in microtubule dynamics and in microtubule-dependent mitochondria transport in the neuronal cell line and changes in acetylated microtubules are evident in iPSC-derived dopaminergic neurons obtained from PD patients (carrying exons' deletions or point mutation).

All together these data clearly support the pivotal role of Parkin in microtubule rearrangement and functions and suggest that some of the pathogenic mutations of *PRKN* can contribute to PD pathogenesis, at least in part, by compromising this regulatory task.

LRRK2

Leucine-rich repeat kinase 2 (LRRK2) is a protein encoded by the *LRRK2* gene (12p11.2-q13.1), which was identified as causative gene in familial PD in 2004 (Paisán-Ruíz et al., 2004; Zimprich et al., 2004). Mutations in *LRRK2* not only are the most frequent cause of familial PD, but this gene is also a risk factor for idiopathic PD (Cookson, 2015).

Structure, localization and function

LRRK2 is a large protein (2527 aa) composed of several domains distributed all along the entire sequence: Armadillo (ARM), Ankyrin (ANK) and Leucine-Rich Repeat (LRR) domains are followed by the catalytic core, composed of Ras of complex protein (ROC), C-terminal of ROC (COR) and a kinase (KIN) domains. Finally, WD40 domain is located in the C-terminus (Figure 4). Thanks to its catalytic core, LRRK2 carries out two enzymatic functions: it is both a GTPase (ROC and COR domains) and a serine/threonine kinase. The other remaining domains mediate protein-protein interactions and are often characterized by repeats: ANK domain contains seven ankyrin-type repeats, LRR domain contains 13 leucine-rich repeats, WD40 domain consists of C-terminal repeats. The LRRK2 activation mechanism is complex, involving both auto- and hetero-phosphorylation of different sites and upstream kinases and GTPases. Its catalytic activity is also regulated by its dimerization and subcellular localization as well as by interactions with its substrates and effectors, such as various members of the Rab family (Pfeffer, 2018; Harvey and Outeiro, 2019; Wauters et al., 2019). Notably, differently from the other Rab family members, Rab29 is a key upstream activator of LRRK2 (Purlyte et al., 2018).

LRRK2 is expressed in many organs, including the brain, where it is found expressed at different levels in almost all regions (Miklossy et al., 2006; Vitte et al., 2010). LRRK2 is a cytoplasmic protein that can be also found associated with membranes and vesicles. Indeed,

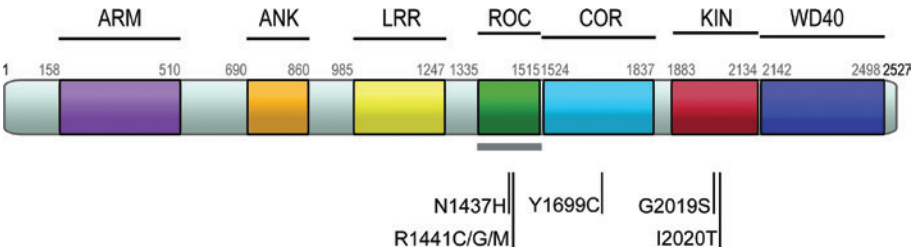

Figure 4: Domain structure of human LRRK2.
LRRK2 protein is characterized by several domains: Armadillo (ARM), Ankhirine (ANK), LRR repeats (LRR), Ras of Complex (ROC), C-terminal of the ROC (COR), Kinase (KIN) and WD40 domains. Tubulin binding region is indicated by a gray line. Pathological mutations found in familial PD are reported.

in brain it is mainly associated with the ER (Vitte et al., 2010). Notably, LRKK2 kinase activity has been reported to be higher when the protein is localized to the membrane. This finding supports the idea that LRRK2 is regulated also by its subcellular localization. Although the physiological functions of LRRK2 are largely unknown, recent studies indicate its involvement in a broad range of cellular functions, including neurite outgrowth, cytoskeletal maintenance, vesicle trafficking, autophagic protein degradation, inflammation, immune response, as well as in various signaling pathways as reviewed in (Rideout and Stefanis, 2014; Wallings et al., 2015; Civiero et al., 2018; Harvey and Outeiro, 2019)

LRRK2 in Parkinson's disease

Mutations in *LRRK2* were firstly identified in families with autosomal-dominant, late onset Parkinsonism and are the most common cause of familial PD (Paisán-Ruíz et al., 2004; Zimprich et al., 2004). In addition, *LRRK2* mutations have also been found in patients affected by idiopathic PD and this is the reason why they are considered also a risk factor for the disease (Cookson, 2015). Starting from 2004, numerous mutations in *LRRK2* have been identified along the entire sequence, although the dominant, pathogenic mutations are all located in the catalytic core: N1437H and R1441C/G/H in ROC domain, Y1699C in COR domain while G2019S and I2020T in KIN domain (Corti et al., 2011). Due to their localizations, these PD-associated mutations can alter both LRRK2 enzymatic activities. While pathogenic mutations in ROC and COR domains impair GTPase activity (Tsika and Moore, 2013), G2019S, the most prevalent point mutation in PD patients, leads to hyper-activation of kinase, increasing auto-phosphorylation and phosphorylation of its substrates (West et al., 2005). Similar effects have been reported for I2020T *LRKK2* mutant (Gloeckner et al., 2006). Interestingly, higher LRRK2 activity has been found not only in familial, but also in idiopathic PD patients, with possible consequences in vesicular trafficking and inflammation (Kluss et al., 2019).

The clinical phenotype associated with *LRRK2* mutants is late onset PD, very similar to idiopathic PD, with bradykinesia, rigidity, tremor and good L-dopa response. *LRRK2* mutations are associated with heterogeneous neuropathology, which can include or not include LB, neurofibrillary tau tangles and ubiquitin-positive inclusions (Li et al., 2014). This variability in neuropathology is further proof of the fact that *LRRK2* is involved in several signaling pathways (Esteves et al., 2014). Furthermore, *LRRK2* has been detected inside the LB and Lewy neurites in both idiopathic PD and in patients carrying G2019S mutated *LRRK2* (Vitte et al., 2010). Interestingly, the link between LRRK2 and α-Synuclein is supported by *in vitro* experiments showing that α-Synuclein can be phosphorylated by LRRK2 and that this effect is enhanced by G2019S mutation (Qing et al., 2009). Given the complexity of the LRRK2 protein, its ability to act as an enzyme and as a scaffold protein, and its involvement in numerous signaling pathways, it is very difficult to clear its function up in neurodegeneration. Nevertheless, its main pathogenic role in PD seems to be linked to its increased enzymatic activity, which leads to altered signaling pathways and, ultimately, to autophagy and lysosomal impairment and protein aggregation (Obergasteiger et al., 2018). To note, LRRK2 could be important for both α-Synuclein and Tau aggregation processes, even if the mechanism through which it happens are still unclear (Outeiro et al., 2019).

LRRK2, tubulin and microtubule cytoskeleton

LRRK2 interaction with microtubule cytoskeleton was first supported by confocal analysis performed in both cell lines and rat hippocampal neurons (Gandhi et al., 2008; Godena et al., 2014). *In vitro* experiments showed that the

ROC domain is sufficient to interact with tubulin heterodimers, being able to pull-down both α- and β-tubulin from cell lysates (Gandhi et al., 2008). The ability of LRRK2 to interact with tubulin heterodimer was confirmed by the co-precipitation of the protein with β-tubulin from mouse brain (Gillardon, 2009). Later, it has been shown that LRRK2 directly interacts with three β-tubulin isoforms (TUBB, TUBB4 and TUBB6). This finding suggests that the distribution of LRRK2 along microtubules is β-tubulin isoform dependent (Law et al., 2014). Performing molecular modeling experiments, these authors established that the location of LRRK2 binding region of β-tubulin is at the luminal face of microtubules. In detail, this region is located near Lys40 on α-tubulin, which is the acetylatable site, and near the binding site for the stabilizing drug taxol on β-tubulin, clearly relating LRRK2 to microtubule stability. Based on this, LRRK2 binding to β-tubulin could physically block taxol access, thus interfering with microtubule stabilization, or impede α-tubulin acetylation, thus interfering with microtubule aging and mechanical resistance (Portran et al., 2017; Xu et al., 2017). This is in accordance with LRRK2 localization only on de-acetylated microtubules observed in cells (Godena et al., 2014) and could explain why microtubule acetylation is increased in LRRK2 knock out MEF cells (Law et al., 2014). In addition, as the luminal face of microtubules is most likely not easily accessible to such a large protein as LRRK2, particularly in mature long-lived microtubules, LRRK2-tubulin interaction is more likely to occur with dynamic pools of microtubules. This is in accordance with LRRK2 localization on growth cones in cultured cells (Sancho et al., 2009). Within this context, LRRK2 interaction with microtubules may play a role in regulation of neurite outgrowth, as suggested by alterations in neurite length found in several experimental models [see (Civiero et al., 2018)]. On the other hand, it is important to remember that LRRK2 is a kinase and that recombinant LRRK2 can phosphorylate β-tubulin *in vitro*, enhancing microtubule stabilization (Gillardon, 2009). Therefore, in physiological conditions, on the one hand, LRRK2 may prevent (hyper)acetylation of microtubules, on the other hand, it may regulate microtubule stabilization through its kinase activity on β-tubulin. In addition to these direct effects, it should be borne in mind that LRRK2 interacts and phosphorylates Tau bound to microtubules (Kawakami et al., 2012), as well as it is able to interact, phosphorylate and negatively regulate the microtubule binding protein Futsch in *Drosophila melanogaster* (Lee et al., 2010) and its human homolog MAP1B *in vitro* (Chan et al., 2014), suggesting that LRRK2 impact on microtubules dynamics could be also indirect, namely mediated by other proteins (e.g. Tau or MAP1B).

LRRK2 interaction with tubulin can be maintained or lost depending on the different pathogenic mutations. R1441C, Y1699C and G2019S mutants preserve the ability to co-precipitate with α- and β-tubulin (Gandhi et al., 2008; Law et al., 2014). In addition, a quantitative two hybrid yeast assay showed that R1441G and R1441H mutations reduce LRRK2 interaction with TUBB and TUBB4 at different levels, while R1441C increases this interaction (Law et al., 2014), confirming that each mutation can lead to a specific alteration. Among the pathological LRRK2 mutants, G2019S is probably the most investigated. Even if this mutation does not affect the ability to co-precipitate with tubulin, dopaminergic neurons obtained from primary culture of G2019S-*LRRK2* transgenic mice are characterized by a marked reduction in neurite complexity (Ramonet et al., 2011). Furthermore, dopaminergic neurons obtained from iPSCs derived from patients carrying the G2019S *LRRK2* mutation show early neuritic branching defects in addition to defects in neurite length and complexity (Borgs et al., 2016). These changes in cellular morphology are in accordance with data obtained from fibroblasts of patients carrying the G2019S *LRRK2* mutation, in which cellular morphology is deeply altered compared to healthy controls, acetylated tubulin is drastically increased, and microtubules are destabilized (Cartelli et al., 2012). These observations also indicate that LRRK2 interaction with microtubule cytoskeleton could play a relevant role in PD.

Conclusion

Undeniably, the large number of studies summarized in this review converges on the concept that α-Synuclein, Parkin and LRRK2 interact with and strongly influence the dynamics and functions of neuronal microtubules (Table 1). Of note, further proteins linked to familial PD seems to be involved with tubulin/microtubules, including DJ-1 (Sheng et al., 2013) and UCH-L1 (Kabuta et al., 2008).

Taking into account that microtubules are integral to neuronal function from neurodevelopment to neuronal differentiation and maintenance of the mature neuron's phenotype, the evidence gathered in these studies provides novel insights into the physiological roles of these proteins and suggests the relevance of their interplay with microtubules for a healthy nervous system. Likewise, the consequences of the PD-linked mutations on microtubule "homeostasis" may play an important role and be crucial in the pathogenic events. As reported above, the pathogenic forms of proteins linked to PD may exert their pathological

Table 1: Impact of PD-linked proteins on a microtubules system.[a]

Protein	Microtubule stability/dynamics	Tubulin acetylation	References
α-Synuclein	– Effect on microtubule polymerization *in vitro*[1] – Regulation of microtubule dynamics *in vitro* and in cultured cells[2]		[1]Alim et al., 2002, 2004; Zhou et al., 2010 [2]Cartelli et al., 2016; Cartelli and Cappelletti, 2017b; Eguchi et al., 2017; Toba et al., 2017
	– Reduction of microtubule assembly *in vitro* by A30P, E46K and A53T variants when compared to WT protein[3] – Inhibition of microtubule assembly in cells by A30P and A53T variants when compared to WT protein[4]		[3]Alim et al., 2004 [4]Cartelli and Cappelletti, 2017b
Parkin	– Regulation of tubulin ubiquitination and turnover[5] – Binding to and stabilization of microtubules[6]		[5]Ren et al., 2003 [6]Yang et al., 2005
	– Defective α-and β-tubulin ubiquitination induced by K161N, T240R and C431F mutations[7] – Destabilization of microtubules by *PRKN* mutations or exons' deletion in murine and human dopaminergic neurons[8] – Destabilization of microtubules in skin fibroblasts obtained from PD patients harboring *PRKN* mutation[9] – Alterations of microtubules dynamics in *PRKN*-silenced neuronal cells[10]	– Alterations of acetylated tubulin in *PRKN* KO mouse model and in human dopaminergic neurons from patients carrying *PRKN* mutation[10]	[7]Ren et al., 2003 [8]Ren et al., 2009–2015 [9]Cartelli et al., 2012 [10]Cartelli et al., 2018
LRRK2	– Increase of microtubules stability *in vitro*[11]	– Maintenance of low levels of acetylated tubulin in cells[12]	[11]Gillardon, 2009 [12]Godena et al., 2014; Law et al., 2014
	– Destabilization of microtubules in primary cultures obtained from G2019S *LRRK2* transgenic mice, in human dopaminergic neurons and skin fibroblasts from patients carrying G2019S *LRRK2* mutation[13]	– Increase in the levels of acetylated tubulin in skin fibroblasts obtained from PD patients harbouring *LRKK2* mutation (G2019S)[14]	[13]Ramonet et al., 2011; Cartelli et al., 2012; Borg et al., 2016 [14]Cartelli et al., 2012

[a]The effects of WT proteins and of pathogenic variants are summarized in green and red boxes, respectively.

effects through microtubule regulation at multiple levels, from the alteration of microtubule stability and dynamics, to the unbalancing of post-translationally modified tubulin and the impairment of microtubule-based intracellular transport. Importantly, as mentioned in the introduction, the association between PD and single nucleotide polymorphisms in the *MAPT* locus, containing the gene coding for the microtubule-associated tau protein (Nalls et al., 2011) indicated that Tau could play a relevant role in PD in addition to the tauopathies including Alzheimer's disease. To date, the evidence of Tau pathology in PD is striking, ranging from pathological Tau aggregation to the interaction of Tau with α-Synuclein, and contributes to neuronal dysfunction, impairment of axonal transport and cell death underlying PD (Zhang et al., 2018).

Given the relevance of the interplay between PD-linked protein and microtubules for neuronal functions and dysfunctions, a microtubule-directed therapeutic intervention is not merely an hypothesis and, interestingly, many preclinical studies providing supportive evidences are emerging (Cappelletti et al., 2017). We are convinced that a multi-level and mechanism-based strategy is feasible, based on microtubule stabilization (Brunden et al., 2017), on modulation of the enzymes that modify tubulin, including tubulin deacetylases (Outiero et al., 2007; Godena et al., 2014; Esteves et al, 2018), and/or on targeting microtubule-interacting proteins, which include the PD-linked proteins α-Synuclein, Parkin and LRRK2. The strategy based on targeting PD-linked proteins implies increasing understanding of their interplay with microtubules, the ultimate goal being the design of selective drugs or peptides that could modulate this interaction and, hopefully, achieve neuroprotection.

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