LRRK2 function on actin and microtubule dynamics in Parkinson disease

Loukia Parisiadou and Huaibin Cai

Unit of Transgenesis; Laboratory of Neurogenetics; National Institute on Aging; National Institutes of Health; Bethesda, MD USA

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Cytoskeleton and Parkinson Disease

Although MTs play an important role in establishing structural support of a cell, they also take part in other processes as well. MTs have been implicated in vital cellular functions such as motility, division, organelle transport, cell shape, organization and vesicle transport.8,9 Structurally, MTs are polymers produced by the interaction of α and β subunits.8 The polymerization which is directed by polarity can be reversed depending on cellular demands, and it is this dynamic property of MTs that makes them critical for specific biological functions such as spatial and temporal flexibility inside neurons and cellular mobility.10 Due to the important roles MTs play within the cell, it is clear that a disturbance of their molecular dynamics could potentially lead to devastating effects and possible onset of disease.

Several studies have shown a connection between tubulin and MTs with PD pathology.12-13 One study in particular demonstrated that depolymerization of MTs underlies the selective vulnerability of dopaminergic (DA) neurons by PD toxins such as rotenone.13 The DA neurons send very long axons to the striatum for dopamine release. Because of the axonal length of DA neurons, a high degree of cooperation between MTs and MT-dependent motor proteins is required to ensure dopamine is transported successfully through vesicle transport. Depolymerization of MTs causes an impairment of dopamine transport leading to an abnormal accumulation of dopamine in the soma. It is then eventually oxidized producing an increased amount reactive oxygen species that lead to cell death.13 Moreover, Alim et al. reported an interaction of PD-related α-synuclein with tubulin as well as the colocalization of these proteins in Lewy bodies and other intraneuronal structures. They also showed that tubulin initiated and promoted α-synuclein fibril formation in vitro.15 Parkin, a gene implicated in recessive forms of PD, has also been shown to bind to tubulin and to stabilize MTs at least in HEK293 cells.16 Lee et al. further demonstrated that α-synuclein aggregation leads to disorganization of the MT network and MT-associated trafficking resulting in Golgi fragmentation and neuritic degeneration. This strongly suggests a close association between α-synuclein aggregation, MT dysfunction and Golgi disruption.17

Accumulating data also suggest a relationship between PD and actin dynamics.18-22 Actin is a globular protein that is highly conserved among species. Monomeric or G-actin is readily hydrolyzed to form F-actin with the concomitant hydrolysis of

Introduction

The cytoskeleton plays an important role in maintaining structural polarity of neurons which is important for their physiological function. The development and maintenance of the nervous system appears to require a delicate interplay between two key components of the cytoskeleton, microtubules (MTs) and actin, and the assembly of abnormal cytoskeletal elements is thought to be one major characteristic of neurodegenerative diseases.1-3 One example of this phenomenon is the accumulation of neurofibrillary tangles, a pathological marker of Alzheimer disease, which consists of paired helical filaments of the MT-stabilizing protein tau.4 Another such example is seen in Parkinson disease (PD) where the formation of Lewy bodies, a pathophysiological hallmark of the disease are shown to contain tubulin, MT-associated proteins (MAP) and neurofilaments.5 Furthermore, an increasing amount of evidence links these cytoskeletal components to neurotransmission impairment leading to neuronal degeneration.6,7 Here, this paper aims to discuss the relationship of PD and the cytoskeleton, and more specifically, the emerging role of LRRK2 protein on cytoskeletal balance and how this interplay relates to the cellular events that underlie the disease pathology.

The mutations in the LRRK2 gene cause clinically typical, late-onset Parkinson disease, strengthening the idea that the familial forms of the disease represent an important tool for the study of the idiopathic forms. Despite the great effort to describe and functionally characterize the LRRK2 gene product, its physiological role remains elusive. In this article, we will discuss along with other references, our recent findings that assigned a critical role of LRRK2 protein on cytoskeletal dynamics and how this direction could provide a valuable platform to further appreciate the mechanism underlying LRRK2-mediated pathophysiology of the disease.

Correspondence to: Loukia Parisiadou and Huaibin Cai; Email: parisiadoul@mail.nih.gov and caih@mail.nih.gov
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ATP. Rapid remodeling of actin cytoskeleton has been implicated in several fundamental functions. In the nervous system, the neurite outgrowth and the synapse formation and maintenance are associated with actin dynamics.\textsuperscript{23,24} Studies have shown that actin is found in cytoplasmic α-synuclein aggregates,\textsuperscript{19} and that these two proteins were shown at least partially to colocalize in neuronal cell lines.\textsuperscript{25} Moreover, a recent study revealed structural and functional regulation of actin dynamics by α-synuclein. More specifically, it was demonstrated that wt α-synuclein binds to actin and modulates its polymerization and this ability is profoundly changed by the A30P mutation.\textsuperscript{36} Parkin was also implicated with actin remodeling through phosphorylation of the depolymerizing molecule cofilin.\textsuperscript{21} Recently, PINK1 protein function was connected with modulation of actin dynamics, possibly through the same pathway.\textsuperscript{20} The loss of PINK1 function causes a autosomal recessive form of familial PD.\textsuperscript{26} Thus, the genes that are related to familial forms of the disease are dynamically involved in the regulation of cytoskeleton.

### LRRK2 Protein and Cytoskeleton

**LRRK2 and actin regulation.** Mutations in *LRRK2* gene lead to PD phenotypes with strong overlap to typical late onset disease that neuropathologically is characterized by the presence of α-synuclein-positive Lewy bodies.\textsuperscript{27} Therefore, the understanding of LRRK2-related pathology may unravel the cellular events underlying PD. LRRK2 protein is a large protein of 2,527 amino acids and consists of several domains including a N-terminal leucine rich repeat region, ankyrin and C-terminal WD40 domain that possibly mediates protein-protein interaction.\textsuperscript{28} Interestingly, it combines a functional GTPase domain called Roc domain (Ras of complex protein) and kinase domains making up one molecule.\textsuperscript{29}

Mutations associated with PD have been described throughout all LRRK2 domains. The main genetic cause of PD found thus far is the G2019S substitution that lies in the activation segment of the kinase domain.\textsuperscript{30} In the brain, LRRK2 is expressed in neurons, astrocytes and microglia. Recent studies have detected LRRK2 in specific brain regions including the cortex, striatum, hippocampus, cerebellum and as well as in dopaminergic neurons of the substantia nigra.\textsuperscript{31-35} In the subcellular level, it was found mainly in the cytoplasm, lipid rafts, lysosomes, endosomes, mitochondria and in association with Golgi transport vesicles.\textsuperscript{34} Since the pathophysiology of PD has been closely associated with dysfunction or deregulation of cytoskeletal dynamics, this paper will discuss recent findings that assign a crucial emerging role of LRRK2 on the regulation of microtubule and actin dynamics and how these events are related to the neuropathological phenotypes of PD.

Soon after the cloning of LRRK2 and in the absence of a physiological protein substrate, a number of in vitro studies attempted to evaluate the kinase activity of LRRK2 and to examine how the PD-related mutations modulate its activity.\textsuperscript{35,36} The first description of a potential functional role of LRRK2 is shown in the work of McLeod and colleagues where they associated LRRK2 kinase activity with the maintenance of neuronal process.\textsuperscript{37} They demonstrated by using both neuronal cultures and by delivering the wild-type (wt) and G2019S kinase domain into rat brains that the neurons with G2019S mutation but not wtLRRK2 had shorter neurites. Suppression of LRRK2 expression by shRNAs led to an increase in neurite length. Furthermore, they established a connection between LRRK2 and the MTs-associated protein tau by showing that the expression of G2019S mutation led to tau-positive inclusions and also co-localized with tau in these inclusions.\textsuperscript{37}

In 2007, an elegant biochemical work reported the protein moesin as the first substrate for LRRK2 kinase activity in vitro. Kinase substrate tracking and elucidation screening (KESTREL) was used in rat brain extracts in order to identify proteins that are phosphorylated by G2019S. They showed that LRRK2 phosphorylated moesin at Thr558, a highly conserved phosphorylated site. Moesin, together with ezrin and radixin are known as ERM proteins as they link the actin cytoskeleton to the plasma membrane.\textsuperscript{38} This study represents the first strong indication that LRRK2 is implicated in actin dynamics. Phosphorylation of ERM’s critically regulates their activity.\textsuperscript{38,39} An intramolecular interaction between the N- and C-terminal domains of the proteins is characteristic of their “inactive” state and prevents their interaction with F-actin and other proteins.\textsuperscript{40} Upon phosphorylation of the C-terminal threonine residue, their intramolecular association is abolished and the proteins are converted into an “active” state. At the subcellular level, they are localized to the actin-rich sites in filopodia and are shown to play a key role in neurite outgrowth by regulating filopodia restructuring.\textsuperscript{41}

In a recent study we established a critical connection between LRRK2 and actin dynamics through phosphorylation of ERM proteins.\textsuperscript{20} Phosphorylated ERM (pERM) proteins link F-actin to the plasma membrane by binding their N-terminal to the plasma membrane while the C-terminal interacts directly with F-actin.\textsuperscript{42} It was shown that ERM proteins are genuine downstream targets of LRRK2 in vivo and that the function of LRRK2 on neurite outgrowth during development is achieved through the regulation of ERM proteins and actin dynamics. More specifically, in developing LRRK2 G2019S neurons, the numbers of pERM and F-actin enriched filopodia were significantly increased, which correlates with the retardation of neurite outgrowth in these neurons. Conversely, the levels of pERM and F-actin content in the filopodia of LRRK2 knockout neurons were significantly decreased and neurite outgrowth was promoted. These observations establish a physiological link between LRRK2 and pERM in neuron development. To directly determine whether the aberrant phosphorylation of ERM proteins is involved in the G2019S-mediated neurite outgrowth defects, we decided to examine the effect of inhibiting ERM phosphorylation on neurite outgrowth. We found that the pERM levels in the filopodia of G2019S neurons were modulated by the introduction of an exogenous polypeptide containing the phosphorylation site of ERM proteins.\textsuperscript{43} Interestingly, the application of the peptide competed with the phosphorylation of the endogenous ERM proteins and rescued the neurite growth defects.\textsuperscript{22}

Moreover, we tested whether increased F-actin content also contributed to delayed outgrowth of G2019S neurons by treating them with the actin depolymerizing agent latrunculin. Indeed,
disruption of F-actin led to normal outgrowth of neurites. Proper neurite outgrowth requires a dynamic interplay between F-actin and microtubules.\(^2\) F-actin restricts additional protrusion of underlying microtubules at the very early stages of development, however, at some point the F-actin becomes less stable allowing the protrusion of the underlying microtubules and the formation of the axons.\(^{23}\) The significantly elevated F-actin content in G2019S neurons may act as a barrier that prevents the extension of microtubules, leading to the inhibition of neurites' outgrowth. That is why, when the F-actin is disrupted, a rescue of the outgrowth defect is achieved.

We also attempted to elucidate the intracellular signaling pathways by which G2019S regulates neuronal morphogenesis. The application of forskolin (FSK), the activator of adenyl-cyclase reversed the neurite outgrowth defects in G2019S neurons. It remains to be seen how G2019S mutation is associated with the degeneration of the midbrain dopaminergic neurons in PD. Given the phosphorylation of ERM proteins is also implicated in the neuronal regeneration,\(^{44}\) the G2019S mutation of LRRK2 may potentially hinder the sprouting of neurites in the PD brain as it did during the development, resulting in potentially accelerated neuron degeneration. In a recent study, gene expression patterns in peripheral blood mononuclear cells from elderly, healthy controls and from Parkinson disease (PD) patients carrying the G2019S mutation were compared. Results from these experiments showed deregulation and alterations in genes associated with actin cytoskeleton maintenance.\(^{45}\)

**LRRK2 and microtubules.** There is growing evidence linking LRRK2 with microtubules. Colocalization of GFP-tagged LRRK2 with β-tubulin in HEK293 cells was initially described.\(^{46}\) A later study reported the colocalization of endogenous LRRK2 with tubulin in primary hippocampal neurons.\(^{57}\) Additionally, the interaction of LRRK2 and tubulin through LRRK2 Roc domain was also identified. This specific interaction takes place in a guanine-nucleotide independent manner, indicating tubulin is not a downstream effector of LRRK2 GTase activity. α/β tubulin heterodimers interacted similarly both with wild-type and R1441C mutant LRRK2, suggesting that the abolishment of this interaction is not the mechanism underlying PD pathogenicity.\(^{57}\) Consistently, Lin et al. found that wt and G2019S LRRK2 coexpressed with BIII tubulin in mouse brains.\(^{48}\)

The work of Gillardon further confirmed the LRRK2-tubulin endogenous interaction in mouse brain by showing tubulin association with overexpressed LRRK2 in HEK293 cells using coimmunoprecipitation experiments. Notably, recombinant human LRRK2 appeared to preferentially phosphorylate β-tubulin in Thr107 in mouse brain, and the phosphorylation was significantly enhanced by G2019S mutation. Tubulin phosphorylation during neurite outgrowth results in stabilization of microtubule cytoskeleton.\(^{59}\) In accordance, in vitro biochemical assays showed that tubulin phosphorylation by LRRK2 enhanced microtubule stability in the presence of microtubule associated proteins and this finding may represent an additional potential function of LRRK2 in neurons.\(^{50}\) The maintenance of microtubule dynamics balance is critical for neuronal development, axonal trafficking and synaptic formation and maintenance; the G2019S-enhanced tubulin phosphorylation may thus result in microtubule dynamics deregulation that may in turn interfere with proper neuronal function.\(^{50}\)

Towards this direction, a more detailed investigation in mouse brains overexpressing wtLRRK2 and G2019S mutation also showed that LRRK2 leads to perturbation of microtubule dynamics, revealed by the comparison of RAB (Reassembly High-Salt Buffer)—soluble and insoluble fractions of mouse brains. RAB buffer was used to extract intracellular free tubulin.\(^{51}\) The levels of β-tubulin in (RAB) soluble fraction was dramatically decreased in the presence of LRRK2, reflecting a depletion of free tubulin with a concomitant increase in RAB insoluble fraction indicating an enhancement of tubulin polymerization.\(^{48}\) In accordance, the free tubulin levels were significantly increased in the brains of LRRK2KO mice.\(^{59}\) Furthermore, Lin et al. identified a pathophysiological interplay between LRRK2 and α-synuclein by demonstrating that LRRK2 accelerates the progression of α-synuclein-dependent neuropathological features using a series of compound transgenic mice. The mechanism by which LRRK2 affects α-synuclein neurodegeneration was extensively described; the enhanced tubulin polymerization in the presence of LRRK2 was found to characterize the sequence of events that lead to α-synuclein-dependent neuropathology. This then leads to the question, how does perturbation of microtubule dynamics by LRRK2 leads to neuronal degeneration?

Microtubules and microtubule-axonal transport has been reported to possess a critical role in maintaining Golgi structure and integrity.\(^{52,53}\) The Golgi apparatus consists of cisternal stacks whose main function is to sort proteins from the endoplasmic reticulum (ER) to lysosomes and plasma membrane.\(^{54}\) The stacks are found around the centrosome, the major organizing center for cytoplasmic microtubules. Microtubules are described as tracks along which vesicles with the help of dynein and kinesin move to Golgi. In addition, the Golgi elements move along microtubules to concentrate towards their minus end.\(^{55}\) Increased fragmentation of the Golgi apparatus was reported in transgenic mice overexpressing LRRK2, and this strongly suggests that the enhancement of tubulin polymerization affects the organization of microtubule in neurons leading to Golgi disruption.\(^{58}\) In accordance, previous studies reported that treatment with the microtubule stabilizer taxol resulted in Golgi fragmentation.\(^{56}\) The disruption of the Golgi is not toxic to the neurons but does lead to impaired coordination of ER-Golgi vesicular trafficking which may explain the abnormal somatic accumulation of α-synuclein in the brains coexpressing A53T α-synuclein and LRRK2 compared to α-synuclein alone expressing neurons. α-synuclein accumulation in turn may trigger pathogenic events leading to degeneration of the neurons. The above study revealed a function of LRRK2 in accelerating the progression of α-synuclein-mediated neuropathological features through deregulation of microtubule assembly, providing the functional link between cytoskeleton and PD-related pathophysiology.

**Concluding Remarks**

The fact that LRRK2 mutations lead to typical PD and represent a very common cause of both familial and sporadic PD has
prompted researchers to functionally characterize the protein in order to understand the cellular and molecular mechanisms of PD. From the study of Mac Leod that first assigned a role for LRRK2 in neurite outgrowth, to the most recent paper of Lin et al. that proposed a critical role of LRRK2 in α-synuclein dependent neurodegeneration, a common parameter exists: the strong relationship of LRRK2 to the regulation of cytoskeletal dynamics. Towards this direction, the broad expression of LRRK2 in multiple tissues throughout development indicates a more general function, not exclusively restricted to midbrain dopaminergic neurons. The exact reason why the specific neuronal population is affected in PD by cytoskeletal deregulation is still unknown.

The close association of LRRK2 function with the cytoskeleton provides a solid foundation to concentrate our tasks.

LRRK2 through microtubule deregulation seems to be the regulator of α-synuclein neurodegeneration and therefore its inhibition may ameliorate the underlying neuropathology. LRRK2 inhibition as a potential therapeutic target is favored by the fact that LRRK2 knockout mice were shown to be viable, fertile and absent of any obvious motor phenotypes. Although a significant amount of progress has been made, extensive research is still needed in order to shed more light on the mechanistic events that underlie LRRK2 function on actin and microtubule dynamics.

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