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LRRK2 guides the actin cytoskeleton at growth cones together with ARHGEF7 and Tropomyosin 4

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ABSTRACT

Mutations in the *leucine-rich repeat kinase 2* (*LRRK2*) gene represent the most common genetic cause of Parkinson's disease (PD). However, LRRK2 function and molecular mechanisms causing the parkinsonian phenotype remain widely unknown. Most of LRRK2 knockdown and overexpression models strengthen the relevance of LRRK2 in regulating neurite outgrowth. We have recently identified ARHGEF7 as the first guanine nucleotide exchange factor (GEF) of LRRK2. This GEF is influencing neurite outgrowth through regulation of actin polymerization. Here, we examined the expression profile of neuroblastoma cells with reduced LRRK2 and ARHGEF7 levels to identify additional partners of LRRK2 in this process. Tropomyosins (TPMs), and in particular TPM4, were the most interesting candidates next to other actin cytoskeleton regulating transcripts in this dataset. Subsequently, enhanced neurite branching was shown using primary hippocampal neurons of LRRK2 knockdown animals. Furthermore, we observed an enhanced number of growth cones per neuron and a mislocalization and dysregulation of ARHGEF7 and TPM4 in these neuronal compartments. Our results reveal a fascinating connection between the neurite outgrowth phenotype of LRRK2 models and the regulation of actin polymerization directing further investigations of LRRK2-related pathogenesis.

1. Introduction

Understanding the molecular mechanisms underlying the pathogenesis of Parkinson's disease (PD) is of major interest in current neurodegenerative disease research. Especially in Leucine-rich repeat kinase 2 (LRRK2) associated PD, this goal is far from being reached. Up until now, more than 50 variants in this 286 kDa protein have been described [1]. Multiplying the complexity of the research projects, the proven pathogenic mutations cover all enzymatic domains of this protein. Among those are N1437S/H and R1441C/R/H mutations in the GTPase domain (Roc) of LRRK2 [2–5]. The COR-domain harbours the Y1699C mutation and in the kinase domain the I2020T and the most common G2019S mutation have been found [2,3,6–8]. Additionally, it is not yet clear whether the pathogenicity is being induced by a differentially active GTPase activity or kinase activity of LRRK2, or both.

Research in LRRK2 invertebrate models, cell culture and mouse models clearly indicates an influence of LRRK2 on neurite outgrowth. In *Caenorhabditis elegans* the LRRK2 homologue lrk-1 plays a role in the specification of axons and dendrites, their outgrowth ability and capacity in pathfinding [9,10]. This LRRK2 associated phenotype is supported by analyzing primary murine neurons, in which an enhanced kinase activity of LRRK2 results in reduced neurite growth whereas reduced kinase activity has the opposite consequence of increased neurite outgrowth [11–14].

The molecular basis for this phenotype is far from being fully determined. Investigations on LRRK2 interacting proteins hint at underlying signalling cascades. It has been shown that LRRK2 directly interacts and phosphorylates tubulin leading to more stable yet less dynamic microtubules [15–17]. The interaction of LRRK2 with the elongation factor

Abbreviations: F-actin, filamentous actin; G-actin, globular actin; LIMK, LIM domain kinase; LRRK2, leucine-rich repeat kinase 2; PAK, p21 protein (Cdc42/Rac)-activated kinase; qRT-PCR, quantitative reverse transcription polymerase chain reaction; GEF, guanine nucleotide exchange factor; shRNA, short hairpin RNA; siRNA, small interfering RNA; TPM, tropomyosin

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1α (EF1A), known for maintenance of microtubule stability, further supports a function of LRRK2 in this pathway [18]. Axonal guidance, neuronal maintenance and microtubule stabilization are also influenced by the dishevelled protein family (DVL) and the Wnt signalling pathway. Not only the interaction of LRRK2 with DVL1/2/3 was recently identified, but also the expression profile of SH-SY5Y cells with reduced LRRK2 levels and the expression profile of mononuclear cells of PD patients with the G2019S mutation points to a dysregulation of the Wnt signalling pathway [19–21].

In addition to microtubule cytoskeleton structures that are influenced by LRRK2, the impact of LRRK2 on actin cytoskeleton regulation is being heavily investigated. Recently, the interaction of LRRK2 with the Rho GTPases Rac1 and CDC42, that have critical roles in actin cytoskeleton remodelling, was shown by us and others [5,22]. In these studies, the authors identified that LRRK2 attenuates Rac1 activation which in turn causes neurite retraction through disassembly of actin filaments [22]. The influence of LRRK2 on the ratio of filamentous actin (F-actin) to monomeric actin (G-actin) was also shown by the group of M. Ueffing [23]. They identified that LRRK2 is interacting with actin isoforms as well as with proteins that regulate actin stability and maintenance like capping proteins, subunits of the Arp2/3 complex, tropomyosins and actin motor proteins [23]. Additionally, LRRK2 is interacting and phosphorylating ezrin, radixin and moesin that are crucial for the fixation of actin filaments on the cell membrane [16,24].

Together, these hints as well as the expression profile of SH-SY5Y cells after knockdown of LRRK2, clearly point to LRRK2 as a central mediator of the cellular actin network [19]. The detailed analysis of this expression profile highlights a dysregulation of actin cytoskeleton pathways, with ARHGEF7 being one of the most profoundly up-regulated cytoskeleton-associated transcripts. In subsequent studies, ARHGEF7 was identified as the first guanine nucleotide exchange factor (GEF) for the GTPase activity of LRRK2 [5]. This GEF is known to regulate the maintenance of the actin cytoskeleton through (i) regulating the activity of CDC42 and, additionally, (ii) by interacting with PAKs that are important in activating LIM-kinases [25,26]. In this study, we further analyzed the interplay of LRRK2 and ARHGEF7 with respect to downstream partners for the actin cytoskeleton signalling. Given that the upregulation of ARHGEF7 leads to enhanced neurite growth [27,28] and the downregulation of LRRK2 results in upregulation of ARHGEF7 [19], the enhanced neurite growth in LRRK2 knockdown models [12,14,29] could be in connection to ARHGEF7 and additional until now not elucidated actin cytoskeleton associated partners.

For that purpose we used the joint downregulation of ARHGEF7 and LRRK2 in SH-SY5Y cells, followed by transcription profiling. By comparing the expression values of the single LRRK2 and the joint knockdown (lower may resemble a rescue effect) we identified four actin associated transcripts that could be relevant for the phenotype. Finally, the results were transferred into *ex vivo* studies to validate their relevance in regulating neurite outgrowth and growth cone morphology.

2. Material and methods

2.1. Antibodies and other reagents

The following antibodies were used in this study: rabbit monoclonal anti-LRRK2 antibody MJFF2 (c41-2) and MJFF3 (c69-6) (Epitomics), rabbit polyclonal anti-SH3 ß-Pix antibody 07-1450 (Millipore), rabbit polyclonal TPM4 antibody ab77683 (Abcam) for detection of mouse TPM4, and mouse monoclonal TPM4 antibody 4E4-102 (Abnova) for detection of human TPM4. Additionally, we used the mouse monoclonal Beta-Actin antibody clone Ac15 (Sigma), the p97 ATPase (VCP) antibody #65278 (Progen) and two different Map2 antibodies (sc20172 Santa Cruz, M1406 Sigma). For the immunofluorescence staining we applied 4′,6-diamidino-2-phenylindol (Sigma) to detect nuclei and Alexa Fluor 488 phalloidin (Invitrogen) to visualize the actin cytoskeleton. The coating was done with poly-DL-ornithine hydrobromide (P8638, Sigma) for neurons and with poly-L-lysine (P8920, Sigma) for SH-SY5Y or NIH3T3 cells.

2.2. Cell cultures and mouse model

SH-SY5Y cells (ACC 209) and NIH3T3 (ACC 59) were purchased from DSMZ. The shRNA based LRRK2 knockdown mouse model was published previously [30,31] and kindly provided by the group of W. Wurst (Munich, Germany).

2.3. RNA interference

RNA interference experiments were performed in SH-SY5Y cells according to the previously published protocol [19]. In addition to the published siRNA against LRRK2 (siLRRK2-1) and the control siRNA, we used the siRNA against ARHGEF7 with the target sequence CAAGCGCA AACCTGAACGGAA. Forty eight hours after transfection, the cells were analyzed further.

2.4. RNA isolation, cDNA synthesis and qRT-PCR

RNA was isolated using an RNeasy Mini Kit (Qiagen) according to the protocol. A QuantiTect Reverse Transcription Kit (Qiagen) was used for cDNA synthesis. The primer design for exon–exon boundary-spanning oligonucleotides was done with Primer3 software (http://frodo.wi.mit.edu/primer3/). The used oligonucleotides for the measurement of the human genes are listed in Table 1.

The qRT-PCR was done using a QuantiTect SYBR Green PCR Kit (Qiagen) on the LightCycler 480 (Roche) in a 348-well plate and 10 µl volume. The PCR settings were applied according to the manufacturer's protocol. The assay specificity was analyzed by melting curves, and standard curves were measured to obtain primer-specific PCR efficiency.

Table 1
Oligonucleotides for qRT-PCR measurement, reference genes are displayed in grey.

Gene symbol	Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
PDHB	Pyruvate dehydrogenase beta	ggtttcccattcaagacctg	tggtttccatgtccattggt
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	agccacatcgctcagacac	gcccaatacgaccaaatcc
SDHA	Succinate dehydrogenase complex, subunit A	agaagccctttgaggagca	cgattacgggtctatattccaga
LRRK2	Leucine-rich repeat kinase 2	atgatgacagcacagctagga	aaacggtcaagcaagattgta
ARHGEF7	Rho guanine nucleotide exchange factor (GEF) 7	ccagcaaatgctcgtacagt	tcactgcagaagggtgattg
ARHGEF9	Cdc42 guanine nucleotide exchange factor (GEF) 9	actcagcagatcaacgaacg	ccgagctcctgtctaggatg
CDC42	Cell division cycle 42	ccagagactgctgaaaagctg	gcacttccttttgggttgag
MGMT	O-6-methylguanine-DNA methyltransferase	ctcttcaccatcccgttttc	agggctgctaattgctggta
NDN	Necdin homolog	tcactgaggagttcgtccaa	ccatgatttgcatcttggtg
SCG5	Secretogranin V (7B2 protein)	tgaagggaggagagagacga	gacagacttctttgcaacaaca
SEMA4F	Semaphorin 4F	cagtctgtgcctggagctt	tccaggctctttaggacacaa
SYT1	Synaptotagmin I	tgcaaagtgctgagaaggaa	tgcctccagaatgacaacag
TPM4	tropomyosin 4	tgaaaaggaggacaaatatgaaga	ctttggcctgggcaagtt
ZMAT3	zinc finger, matrin-type	aatcctcagagctgggtcaa	gagagcggggattgaagtaa