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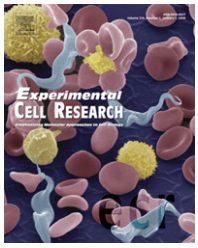

Research Article

LRRK2 regulates synaptic vesicle endocytosis

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ABSTRACT

The leucine-rich repeat kinase 2 (LRRK2) has been identified as the defective gene at the PARK8 locus causing the autosomal dominant form of Parkinson's disease (PD). Although several LRRK2 mutations were found in familial as well as sporadic PD patients, its physiological functions are not clearly defined. In this study, using yeast two-hybrid screening, we report the identification of Rab5b as an LRRK2-interacting protein. Indeed, our GST pull down and co-immunoprecipitation assays showed that it specifically interacts with LRRK2. In addition, subcellular fractionation and immunocytochemical analyses confirmed that a fraction of both proteins co-localize in synaptic vesicles. Interestingly, we found that alteration of LRRK2 expression by either overexpression or knockdown of endogenous LRRK2 in primary neuronal cells significantly impairs synaptic vesicle endocytosis. Furthermore, this endocytosis defect was rescued by co-expression of functional Rab5b protein, but not by its inactive form. Taken together, we propose that LRRK2, in conjunction with its interaction with Rab5b, plays an important role in synaptic function by modulating the endocytosis of synaptic vesicles.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease and affects more than 1% of the population over 65 years old [1,2]. Major PD symptoms are bradykinesia, resting tremor, rigidity and postural instability. PD is pathologically characterized by progressive death of midbrain dopaminergic neurons in the substantia nigra and formation of Lewy bodies [1,2]. Recently, leucine-rich repeat kinase 2 (*LRRK2*/*dardarin*) has been identified as a gene responsible for PARK8-linked autosomal dominant inherited familial PD [3,4]. LRRK2 mutations were found in approximately 3 to 5% of familial and 1 to 3% of sporadic PD cases [5–10], with the highest prevalence (up to 40%) in North Africans and Ashkenazi Jews [11]. LRRK2 is predicted to be a 285 kDa multidomain protein coded by 51 exons. LRRK2 belongs to the Roco protein family [12] and contains 12 leucine-rich repeats (LRRs), a GTPase domain of Ras of complex

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proteins (Roc), a C-terminal of Roc (COR) domain, a mitogen-activated protein kinase kinase kinase (MAPKKK) domain and WD40 repeats [3,4]. Among PD-specific LRRK2 mutations, R1441C is located in the GTPase domain, Y1699C in the COR domain, and I2012T, G2019S and I2020T in the kinase domain, implying that these domains' malfunctions lead to PD. Recent studies demonstrated that LRRK2's autophosphorylation activity is enhanced by PD-specific mutations [13,14]. Overexpression of LRRK2 wild type (WT) and pathogenic mutants in various cell lines led to formation of cytoplasmic aggregates whose numbers increased with the expression of various PD-specific mutants and decreased with the expression of kinase-defective mutants [15]. Importantly, expression of PD-specific mutants caused neuronal degeneration in both dopaminergic SH-SY5Y cells and primary neuronal cultures [15,16] and induced a progressive neurite loss in primary cortical neuronal cultures [17]. In addition, it was reported that the GTPase-defective mutant of LRRK2 shows reduced LRRK2's kinase activity and its expression is associated with decreased neuronal death compared to expression of PD-specific mutant(s) [15,18].

Rab proteins are small GTPases which localize to various cellular vesicle compartments and function as regulators of membrane trafficking [19,20]. Rab5 is a key regulator of endocytic vesicular transport from plasma membrane to early endosomes [21]. In hippocampal neurons, Rab5 was detected in endosomes as well as in the membrane of synaptic vesicles at dendrites and axons, suggesting that axonal endosomes may function in biogenesis of synaptic vesicles [22,23]. In *Drosophila*, Rab5 functional defects have been reported to affect endocytosis rates [24].

To investigate the potential biological function of LRRK2, we sought to identify its interacting protein(s). Using yeast two-hybrid screening, we identified Rab5b as an LRRK2-interacting protein. We characterized the LRRK2–Rab5b interaction by biochemical and immunostaining assays. Furthermore, in primary neuronal cell cultures, we found that alterations of LRRK2 expression level by either overexpression or knockdown caused the endocytosis defects which were rescued by co-expression of Rab5b WT or the constitutive active mutant (Q79L), but not by that of the dominant negative mutant (N133I) protein. Our overall data strongly suggest that LRRK2 regulates synaptic function by modulating endocytosis of synaptic vesicles.

Materials and methods

Plasmid and oligomers

The full-length human LRRK2 cDNA was generated by PCR amplification of short cDNA fragments using a human cerebral cortex cDNA pool as the template and gene-specific primers based on the human LRRK2 sequences from the NCBI database. The resulting cDNA was sequenced and the sequence was compared to that of the LRRK2 full-length clone (NCBI AY792511). Our clone contained three nonsynonymous substitutions (635C>T, 4939T>A, 7190T>C) and six synonymous substitutions (457C>T, 2196A>G, 2857C>T, 4872C>A, 4911A>G and 6324G>A; the numbers indicated are from the LRRK2 clone AY792511). The three nonsynonymous substitutions are due to LRRK2 polymorphism since two of them (4939T>A, 7190T>C) were already reported as polymorphisms [25] and the 635C>T was also found in other independent human EST clone in the NCBI databases (BC117180). All synonymous substitutions except 2196 A>G were also reported [25]. To generate myc-tagged vector, full-length human LRRK2 cDNA was subcloned into pCDNA3.1–myc/his-A (Invitrogen), resulting in pCDNA3.1–myc/his-hLRRK2 which was named as Myc–LRRK2 and contains the myc/his tag at the C-terminus. Two more copies of the myc tag were inserted into Myc–LRRK2 to make Myc3–LRRK2. The Rab5b clone was synthesized by PCR from human brain cDNA library as templates and cloned into the pcDNA3.1 plasmid as a Flag-tagged form, resulting in the pcDNA3-Flag–Rab5b plasmid. Both full-length LRRK2 and Rab5b genes were cloned into the pGEX4T-1 plasmid to express GST fusion proteins.

To make LRRK2 G2019S, R1441C and K1906M and Rab5b Q79L and N133I mutations, site-directed mutagenesis was carried out with a pair of oligomers containing the corresponding mutated bases (G2019S-F: 5′ GATTGCTGACTACAGCATTGCTCAGTAC; G2019S-R: 5′ GTACTGAGCAATGCTGTAGTCAGCAATC; R1441C-F: 5′ CTTCAATATAAAGGCTTGCGCTTCTTCTTC; R1441C-R 5′ GAAGAAGAAGCGCAAGCCTTTATATTGAAG; K1906M-F: 5′ GAAGAAGTGGCTGTGATGATTTTTAATAAACATAC; K1906M-R 5′ GTATGTTTATTAAAAATCATCACAGCCACTTCTTC; Q79L-F: 5′ CTGGGACACAGCTGGGCTGGAGCGATATCACAGC; Q79L-R: 5′ GCTGTGATATCGCTCCAGCCCAGCTGTGTCCCAG; N133I-F: 5′ ATTGCCCTGGCAGGGATCAAAGCTGACCTGGCCAAC; N133I-R: 5′ GTTGGCCAGGTCAGCTTTGATCCCTGCCAGGGCAAT) and pcDNA3–Myc–LRRK2 and pcDNA3-Flag–Rab5b as template, respectively, using *in vitro* site-directed mutagenesis kit (Stratagene, CA, USA). The specific mutations were confirmed by sequencing of the resulting clones. LRRK2-specific siRNAs (siRNA-1: 5′ GGAUCUUCCUUUGGAUGAACUG; SiRNA-2 5′ AGCCUUGGCUCUUCAAUAUAAA) were synthesized.

Cell culture and transfection

HEK293T cells were cultured in DMEM with 10% FBS at 37 °C in CO_2 incubator. Transfections were done using Lipofectamine2000 (Invitrogen) with indicated plasmids according to the manufacturer's direction. E-18 primary rat hippocampal neurons were prepared as described [26]. Neurons were transfected at DIV 10 for endocytosis assay using the calcium-phosphate method with synaptopHluorin (spH) alone or with indicated LRRK2 plasmids in the ratio of 1:2 [26] and then used 5 days after transfection. The amount of spH construct transfected was fixed for constant fluorescent signal.

Yeast two-hybrid screening

Yeast two-hybrid screening was carried out using Matchmaker Two-hybrid system 3 (Clontech, CA, USA). LRRK2 was divided into LRR (amino acids 967–1360), GTPase-MAPKKK (amino acids 1334–2128), and WD40 (amino acids 2168–2510) domains, fused to GAL4 DNA binding domain (DBD) of pGBKT7 and each fusion protein expression was determined by Western analysis using GAL4 antibody. Each of three plasmids was used as bait for the screening against human brain cDNA library (Clontech). Several positive clones were

identified and tested for interaction with bait expressing the full-length LRRK2.

GST pull down assay

Specific GST fusion proteins were expressed in *Escherichia coli* BL21 strain and isolated as previously described [27]. Mouse brain lysates or HEK293T cell lysates expressing Myc-tagged LRRK2 proteins were dialyzed in the binding buffer (20 mM HEPES [pH7.4], 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT) and incubated with purified GST fusion proteins for at least 2 h. To preload GST–Rab5b with GTPγS, the experiments were done as previously described [28]. Bound proteins were eluted by incubation with elution buffer (20 mM reduced glutathione, 150 mM NaCl, 50 mM Tris [pH 8.0], 1 mM DTT) and detected by specific antibodies.

Co-immunoprecipitation and immunocytochemistry

For co-immunoprecipitations, 293T cells were harvested 3 days after transfection with Myc3–LRRK2 and lysed with lysis buffer I (20 mM TrisCl [pH 7.5], 50 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 5% glycerol, 1 mM DTT, 1 mM Na-orthovanadate, 1 mM PMSF, 1× protease inhibitor cocktail [Calbiochem], 0.1 mM ATP). Cell lysates were immunoprecipitated with anti-Rab5b antibodies (Santa Cruz, SC-598, CA, USA) or normal rabbit IgG (Pierce) at 4 °C overnight. Protein A-agarose (Pierce) was added to each sample and incubated at 4 °C for 2 h. Co-immunoprecipitated proteins were eluted by protein gel loading buffer, loaded in a 4–20% gradient gel (Bio-Rad) and detected using anti-Myc (Sigma, M5546) or anti-Rab5b antibody by Western analysis.

Immunocytochemistry was performed with HEK 293T expressing myc–LRRK2 or hippocampal neuronal cells as previously described [13,29]. Primary LRRK2 antibody used was the one previously reported [30].

Subcellular fractionation

To prepare the synaptosome fraction, rat cortex homogenates were fractionated as described [31,32]. Rat tissues were homogenized in sucrose buffer (320 mM sucrose, 4 mM HEPES [pH 7.3]) and fractionated by differential centrifugation. Total proteins were divided into P1 (nucleus and intact cells) and S1 (soluble) fractions by centrifugation at 500 ×*g* for 10 min. The S1 fractions were further fractionated into S2 and P2 by centrifugation at 10,500 ×*g* for 15 min. The P2 fraction contains synaptosomes as well as membranous organelles such as Golgi, endoplasmic reticulum and plasma membrane [32]. The

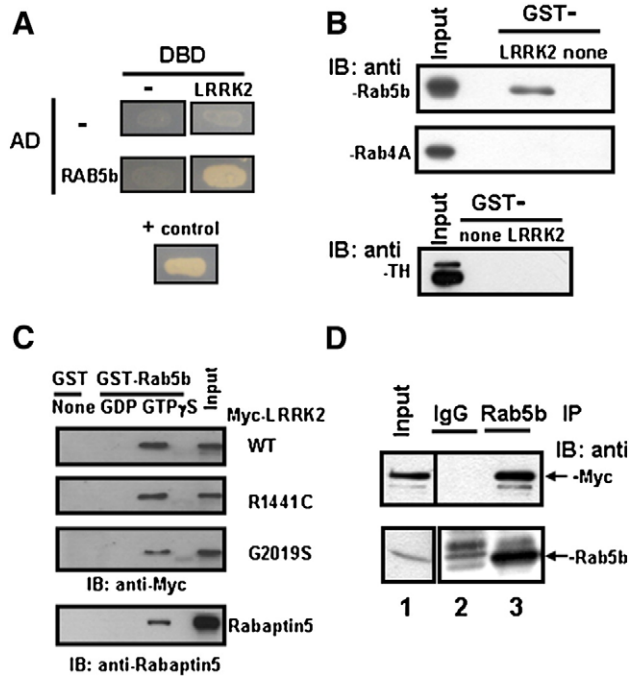

Fig. 1 – LRRK2 interacts with Rab5b. (A) The LRRK2 fusion of GAL4 DNA binding domain (DBD) interacted with Rab5b fused to GAL4 activation domain (AD) in the yeast two-hybrid system (MATCHMAKER, Clontech, CA, USA). Yeast transformants containing one of the DBD plasmids and one of the AD plasmids as indicated were grown on selective plates lacking leucine, adenine, tryptophan and histidine. The specific interaction of LRRK2 with Rab5b was determined by growth on the selective medium. The full-length LRRK2–DBD plasmid and the Rab5b-AD plasmid isolated from the original yeast two-hybrid screening were used in this interaction assay. The yeast transformants containing GAL4DBD-T antigen and GAL4AD-p53 fusion plasmids (Clontech) are shown as a positive control. (B) GST–LRRK2 interacts with Rab5b. Both GST alone and GST fused to full-length LRRK2 were expressed in *E. coli* and purified by glutathione-S-sepharose. Mouse whole brain lysates were dialyzed against GST interaction buffer and subsequently incubated with purified GST proteins. Bound proteins were washed, eluted with 20 mM glutathione and subjected to Western analysis using anti-Rab5b, anti-tyrosine hydroxylase (TH) or anti-Rab4A antibody (Santa Cruz, SC-598, SC-25269 or SC-312, respectively). (C) GST–Rab5b interacts with LRRK2 in the presence of GTP. Both GST alone and GST–Rab5b were prepared as in B and GST–Rab5b was preloaded with 10 μM GDP or GTPγS. Lysates of HEK 293T cells expressing indicating myc-tagged LRRK2 wild type (WT), G2019S or R1441C proteins were dialyzed and incubated with GST proteins with 1 mM GDP or GTPγS. Bound proteins were washed, eluted and subjected to Western analysis by anti-myc antibody (Sigma, M5546). Endogenous Rabaptin5, a strong Rab5 interactor, was also detected as a positive control by anti-Rabaptin5 antibody (BD Transduction Laboratory, R57420). (D) LRRK2 and Rab5b were co-immunoprecipitated. LRRK2 was exogenously overexpressed in 293T cells by transient transfection with Myc3–LRRK2 plasmid followed by incubation for 3 days. After cell lysis, the endogenous Rab5b proteins were immunoprecipitated by anti-Rab5b antibody (lane 3) and normal IgG as a negative control (lane 2). Bound proteins were eluted and analyzed by Western blotting. An upper part of the membrane was analyzed using anti-myc antibody to detect LRRK2 and the lower part was analyzed using anti-Rab5b antibodies. A fraction of total proteins (5%) was loaded (input). Lane 1 in the lower panel was exposed longer as the exposure time similar to those used in lanes 2–3 failed to detect any Rab5b protein. The arrows indicate the position of corresponding proteins.

P2 fraction was centrifuged at 25,000 ×*g* for 20 min, generating LP1 and LS1. LS1 was further centrifuged at 165,000 ×*g* for 2h to yield LS2 and LP2. The LP1, LS2 and LP2 fractions are corresponding to enriched synaptosomal membrane, soluble synaptosomal proteins and synaptic vesicles, respectively [32]. Equal amounts of each fraction were loaded to protein gels and LRRK2, Rab5b and other synaptic vesicle markers were detected by Western blot analysis.

SynaptopHluorin (spH) endocytosis assay

Hippocampal neurons at DIV 10 were co-transfected with synaptopHluorin (spH) and the indicated plasmid or LRRK2-specific siRNAs. The neurons transfected with spH alone were used as control. Transfected neurons were assayed for synaptopHluorin endocytosis and exocytosis as described [29,33]. Quantitative measurements of the fluorescence intensity at individual boutons were obtained by averaging a selected area of pixel intensities using MetaMorph software. Net fluorescence changes were obtained by subtracting average of the intensities of the first 4 frames (F_0) from the intensity of each frame (F_t) for individual boutons. Then, they were normalized to the maximum fluorescence intensity ($F_{max}-F_0$) and averaged. The decay of fluorescence was fitted with a single exponential. Data are presented as mean ± SEM. After the endocytosis assays, expression of each construct was confirmed by retrospective immunostaining using specific antibodies (anti-myc, anti-flag and/or anti-GFP depending on samples) and only immunopositive neurons were included in the analysis. The sypHy assay was carried out identically except that sypHy was co-transfected instead of spH.

Results

LRRK2 interacts with Rab5b

While overexpression of pathogenic LRRK2 has been reported to induce protein aggregation, defect of neurite extension and neuronal cell death [15–17], its cellular functions in normal and diseased brains are largely unknown. To elucidate LRRK2's functional role(s), we sought to identify LRRK2-interacting proteins. Using yeast two-hybrid screening, we isolated Rab5b as a LRRK2-interactor with its second codon as the fusion site. Thus, the isolated clone contained the full-length coding sequence of Rab5b minus the start codon. The Rab5b clone was originally isolated to interact with the LRR domain of LRRK2 but neither with the GTPase-MAPKKK nor with WD40 domain (data not shown) and, later, confirmed to interact with the full-length of LRRK2 (Fig. 1A). To confirm LRRK2's interaction with Rab5b, we carried out GST pull down assay. As shown in Fig. 1B, LRRK2 was found to interact with Rab5b but not with Rab4A, another Rab protein functioning in endosome trafficking [34] or tyrosine hydroxylase (TH), a dopaminergic neuron marker (Fig. 1B). We further showed that Rab5b interacted not only with LRRK2 wild type but also with two pathogenic mutants, R1441C and G2019S, and their interactions were GTP dependent (Fig. 1C) similar to that of the Rabaptin5, an effector of Rab5b [35], which was used as a positive control. The interaction of LRRK2 with Rab5b was isoform-specific since neither Rab5a nor Rab5c interacted with LRRK2 (Supplementary Fig. 1). Interaction of LRRK2 and Rab5b was further confirmed by co-immunoprecipitation assay. Myc-tagged LRRK2 was co-immunoprecipitated with anti-Rab5b antibody, but not with normal IgG (Fig. 1D). Together, these data suggest that LRRK2 and Rab5b specifically interact with each other and form a complex inside cells.

LRRK2 partially co-localizes with Rab5b

We performed subcellular protein fractionation of rat cortex to address whether LRRK2 co-localizes with Rab5b. We have enriched synaptosomal membranes, soluble proteins, and synaptic vesicles [31,32] and used the LRRK2 antibody to detect the LRRK2 expression in each fraction. The LRRK2 antibody used in current study is known to specifically detect the over-expressed LRRK2 as well as the endogenous LRRK2 in brain lysates at approximately 280 kDa [30]. We found that LRRK2 was enriched in the soluble synaptosome fractions (Fig. 2, LS1 and LS2 of LRRK2) although it was also present in the cytosol and other membrane fractions (data not shown) as previously reported [13,14,16,30,36]. Rab5 is known to associate with the membranes of synaptic vesicles as well as early endosomes [22]. Consistent with this, considerable amount of Rab5b was

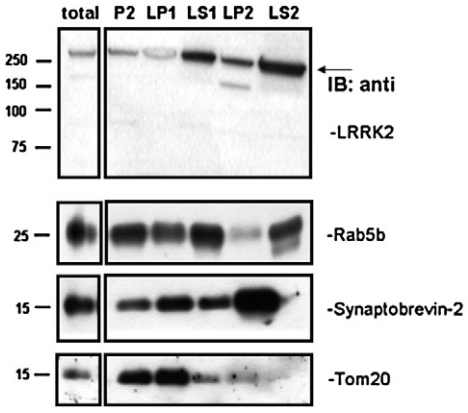

Fig. 2 – LRRK2 and Rab5b co-localize in the synaptosome fraction. Rat cortex was homogenized and subjected to differential centrifugation according to Huttner et al. [31]. The P2 fraction contained synaptosomes and membranous organelles such as mitochondria and endoplasmic reticulum. The P2 fraction was lysed and centrifuged to yield LP1 and LS1 fractions. The LP1 fraction was enriched with synaptosomal membranes and other membranous organelles such as mitochondria. The LS1 fraction was further centrifuged to yield the LP2 and LS2 fractions enriched with synaptic vesicles and soluble synaptosomes, respectively [31,32]. Each lane was loaded with the same amount of proteins, 120 μg for LRRK2 in a 6% gel and 40 μg for other proteins in a 15% gel. LRRK2, Rab5b and other synaptic marker proteins were visualized with specific antibodies (LRRK2: [30]; Rab5b: Santa Cruz, SC-598; Synaptobrevin-2: Stressgen, VAS-SV006; Tom20: Santa Cruz, SC-11415). The arrow indicates the intact LRRK2 band and numbers in the left indicate sizes of the molecular weight markers in kilodaltons.

present in the soluble synaptosomal (LS2) and synaptosomal membrane (LP1) fractions. (Fig. 2, Rab5b). A synaptosome membrane marker, synaptobrevin-2, and a mitochondrial marker, Tom20, were not detected in the LS2 fraction, confirming that the LS2 fraction was not contaminated by the other membrane proteins (Fig. 2, LS2 of synaptobrevin-2 and Tom20). These results suggest that a fraction of LRRK2 and Rab5b co-localize at synaptic vesicles.

We next examined co-localization of LRRK2 and Rab5b in 293T cells and rat hippocampal neurons by immunocytochemistry. Anti-myc and anti-Rab5b antibodies stained 293T cells exogenously expressing myc–LRRK2 WT, R1441C or G2019S. Endogenous Rab5b proteins were detected as punctate forms as well as perinuclear aggregates (Fig. 3A). LRRK2 WT and mutants were cytoplasmic proteins, but some of them also formed aggregates, as previously described [15,16]. Most of these LRRK2 aggregates co-localized with Rab5b aggregates (closed arrowheads in Fig. 3A). The Rab5b aggregates were also observed in untransfected cells (open arrowhead in Fig. 3A, R1441C) suggesting that exogenous overexpression of LRRK2 is

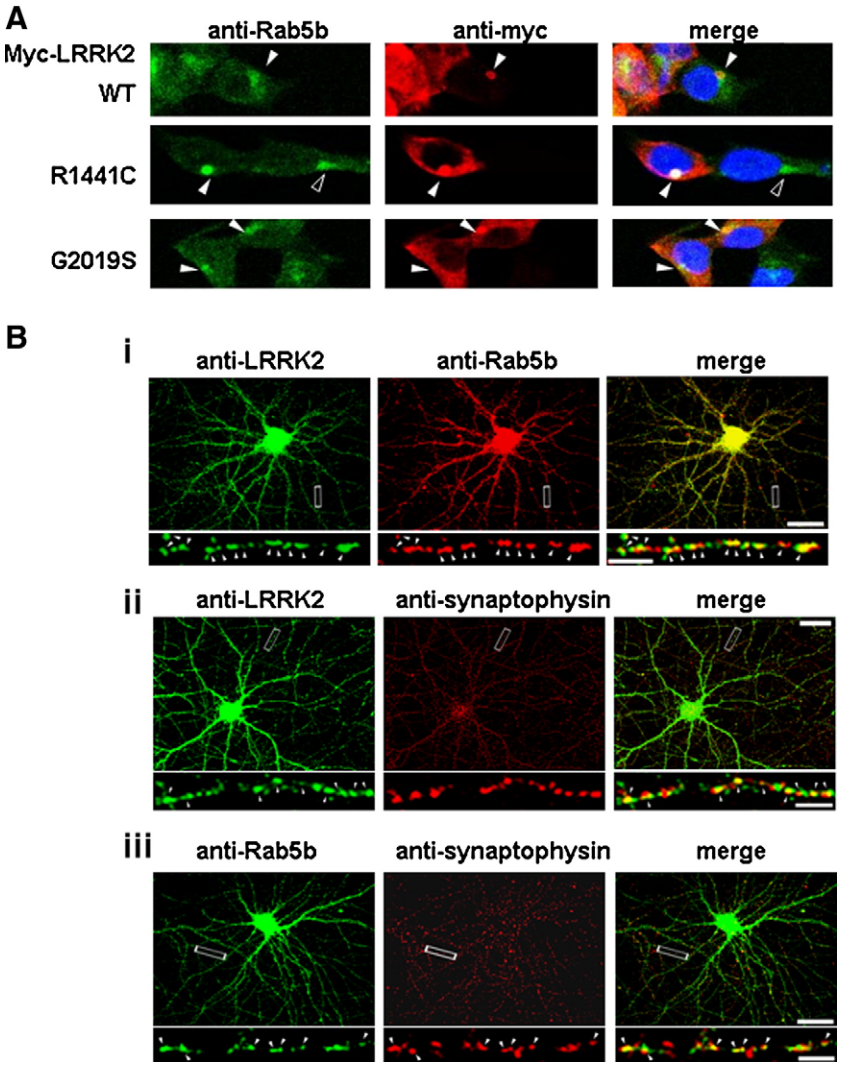

Fig. 3 – LRRK2 co-localizes with Rab5b. (A) Myc-tagged LRRK2 WT, R1441C or G2019S plasmid was exogenously expressed by transient transfection of HEK 293T cells cultured on a coverglass. Cells were incubated for 3 days, fixed and immunostained by anti-Rab5b (green) and anti-myc (red) antibodies and observed by a confocal microscope. Rab5b partially formed perinuclear aggregates in which LRRK2 was partially co-localized. Merged immunostaining images are also shown. Nuclei stained blue. Closed arrowheads indicate regions where co-localization of LRRK2 and Rab5b are readily seen. An open arrowhead indicates that Rab5b aggregates without LRRK2 overexpression. (B) LRRK2 and Rab5b co-localized at the presynaptic terminals. Cultured hippocampal neurons at DIV 14 were immunostained with anti-LRRK2, anti-Rab5b and anti-synaptophysin antibodies and observed by a fluorescent microscope. LRRK2 (i—green) co-localized with Rab5b (i—red) and LRRK2 (ii—green) and Rab5b (iii—green) co-localized with synaptophysin (ii and iii—red), a presynaptic marker, suggesting co-localization of LRRK2 and Rab5b at presynaptic terminals. Merged images are shown in the last columns. Low panels are high magnification view of the region enclosed by the rectangles. Arrowheads indicate the regions where co-localizations are readily seen. Scale bars: 20 μm at low magnification, 5 μm at high magnification.

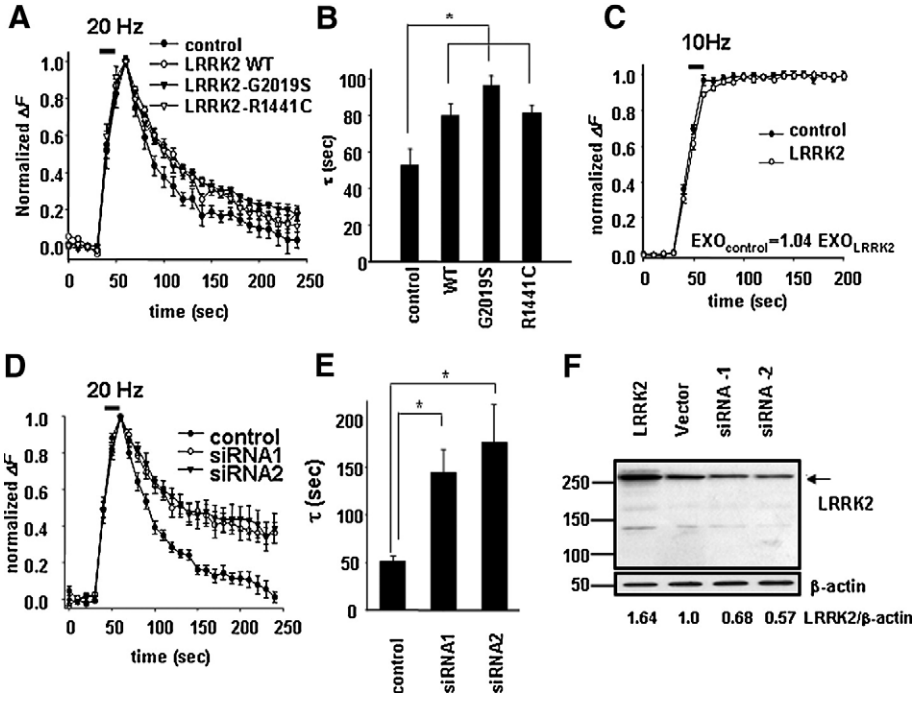

Fig. 4 – LRRK2 expression impairs synaptic vesicle endocytosis. (A, B) Overexpression of LRRK2 WT or PD-specific mutants caused endocytosis defect. Rat primary hippocampal neurons were transfected with spH alone (control) or co-transfected with spH and myc–LRRK2 WT-, G2019S- or R1441C-expressing plasmid (WT, G2019S, R1441C). Following the onset of stimulus (600 action potentials at 20 Hz, dark bar), exocytosis of spH causes a rapid increase in fluorescence, followed by exponential decay after the cessation of stimulation. The average fluorescence intensity profiles were plotted as F/F_0 against time. The decay of fluorescence was fitted with a single exponential with $\tau = 52.9 \pm 8.8$ ($n = 4$, bouton = 154) for control, 80.0 ± 6.3 ($n = 6$, bouton = 506) for LRRK2 WT, 96.2 ± 5.4 ($n = 4$, bouton = 170) for G2019S, or 81.0 ± 4.4 ($n = 5$, bouton = 164) for R1441C. (C) Exocytosis was not affected by LRRK2 WT expression. Neurons expressing spH (control) or myc–LRRK2 WT with spH (LRRK2) were preincubated with bafilomycin A1 (0. 5 μM) for 60 s, and stimulated for 30 s with 10 Hz (dark bar) in the presence of bafilomycin A1 to exclusively measure exocytosis [29]. The fluorescence values were normalized to the peak fluorescence change for each experimental condition. The rate of exocytosis at each condition was obtained from linear fits to the data during the stimulation. The slopes were 0.025 for control ($n = 6$, bouton = 149) and 0.024 for LRRK2 ($n = 6$, bouton = 146) and the ratio of the slope values (EXO_{LRRK2} vs. $EXO_{control}$) was 1.04 as indicated ($p > 0.8$, Student's t-test). (D, E) Knockdown of endogenous LRRK2 by transfection with siRNAs resulted in slower endocytosis kinetics. Hippocampal neurons were transfected with spH alone or spH and LRRK2-specific siRNA-1 or -2, and endocytosis kinetics was measured after 4 days incubation. Expression of either siRNA significantly slowed the kinetics of endocytosis with $\tau = 51.5 \pm 5.7$ ($n = 5$, bouton = 149) for control, $\tau = 144.9 \pm 23.6$ ($n = 6$, bouton = 166) for LRRK2–siRNA-1 and $\tau = 176.5 \pm 39.4$ ($n = 6$, bouton = 183) for LRRK2–siRNA-2. (F) LRRK2 specific siRNAs decreased endogenous LRRK2 expression. Human LRRK2 specific siRNAs at 200 pmol were transfected using Oligofectamine (Invitrogen) into PC12 cells seeded at a 6 well plate. After 3 days of incubation, only transfected cells sorted by MACS (magnetic cell sorting, Miltenyi Biotec.) were collected. For comparison, cell lysates transfected with LRRK2 expressing plasmids (LRRK2) or empty vector (Vector) were also prepared. The same amount of each cell lysates were subjected to Western analysis using anti-LRRK2 (Novus) and anti-β-actin (Sigma) antibodies. Relative expression level of each sample was shown. For all experiments, spH and LRRK2 plasmids were co-transfected at 1:2 ratio. *Significantly different from the control value with $p < 0.05$, ANOVA or Student's t-test.

not required for Rab5b aggregate formation. In rat hippocampal primary neurons, LRRK2 and Rab5b were present in the cell body as well as along the neurites where they mostly co-localized with the presynaptic marker synaptophysin (Fig. 3Bii and iii) and with each other (Fig. 3Bi). Their localization at the presynaptic terminals was further confirmed by immunostaining with another presynaptic vesicle marker, synaptobrevin-2 (Supplementary Fig. 2). Taken together, our biochemical and immunocytochemical data indicate that LRRK2 and Rab5b are co-localized in neurons, primarily at the presynaptic terminals, suggesting that the interaction of LRRK2 with Rab5b may have a role in synaptic vesicle trafficking.

Alteration of LRRK2 expression impairs endocytosis of synaptic vesicles

The above findings prompted us to hypothesize that the LRRK2 may have important regulatory roles in synaptic functions. In particular, we speculated that LRRK2 might modulate the synaptic vesicle endocytosis based on the established role of

Rab5 in endocytosis. To address this possibility, we carried out the assay to measure synaptic vesicle endocytosis in cultured hippocampal neurons using synaptopHluorin (spH). The spH is a pH-sensitive green fluorescence protein (GFP) fused to the synaptic vesicle membrane protein, synaptobrevin-2 [29,33]. The fluorescence of the spH is quenched by the acidic conditions inside synaptic vesicles. The relatively basic pH of the extravesicular space allows the spH to fluoresce. Thus, the spH has been utilized as a reliable tool to measure synaptic vesicle recycling in real time [29,33]. To measure kinetics of synaptic vesicle endocytosis, spH with the indicated plasmids or siRNAs were co-transfected into hippocampal neurons. Then, endocytosis kinetics was assessed by fitting fluorescence decay with a single exponential time constant (τ). Overexpression of LRRK2, regardless of WT or the pathogenic mutants, slowed down the synaptic vesicle endocytosis (τ=52.9±8.8 for control; τ=80.0±6.3 for LRRK2 WT; τ=96.2±5.4 for LRRK2 G2019S; τ=81.0±4.4 for LRRK2 R1441C; Figs. 4A and B). Expression of LRRK2 WT or the pathogenic mutants had no effect in exocytosis kinetics of synaptic vesicle (Figs. 4C, 5C and D). We further tested whether LRRK2 WT or the pathogenic mutant also affects the rate of endocytosis during the stimulation but found that neither affects the endocytosis during a train of action potentials (Supplementary Table 1).

To investigate the effect of endogenous LRRK2 on the endocytosis kinetics, we synthesized two LRRK2-specific siRNAs and confirmed their activity to reduce endogenous LRRK2 expression (Fig. 4F). Transfection with siRNA1 or siRNA2 markedly slowed down the synaptic vesicle endocytosis compared to the control (τ=51.5±5.7 for control; τ=144.9±23.6 for LRRK2–siRNA1; τ=176.5±39.4 for LRRK2–siRNA2; Figs. 4D and E).

To further confirm the effect of LRRK2 expression on endocytosis rate, we carried out the alternative assay using sypHy, another pH-sensitive GFP fused to synaptophysin [37]. Overexpression of LRRK2 WT with sypHy slowed down endocytosis kinetics (τ=57.8±7.8 for control; τ=112.8±18.37 for LRRK2; Figs. 5A and B), confirming that endocytosis proceeds more slowly when LRRK2 is overexpressed.

To rule out the possibility that LRRK2 affects the acidification rate of synaptic vesicles rather than endocytosis rate in the spHluorin assay, we employed FM4-64, a fluorescent lipophilic dye which has been widely used to measure the kinetics of synaptic vesicle recycling [38,39]. We used the following protocol. After the first loading and unloading of FM 4-64, the second loading was applied 20 s after the onset of electrical stimulation. In that 20 s some vesicles undergo endocytosis and escape being labeled, *i.e.*, the slower the endocytosis, the higher the intensity of FM 4-64 staining [38]. The results

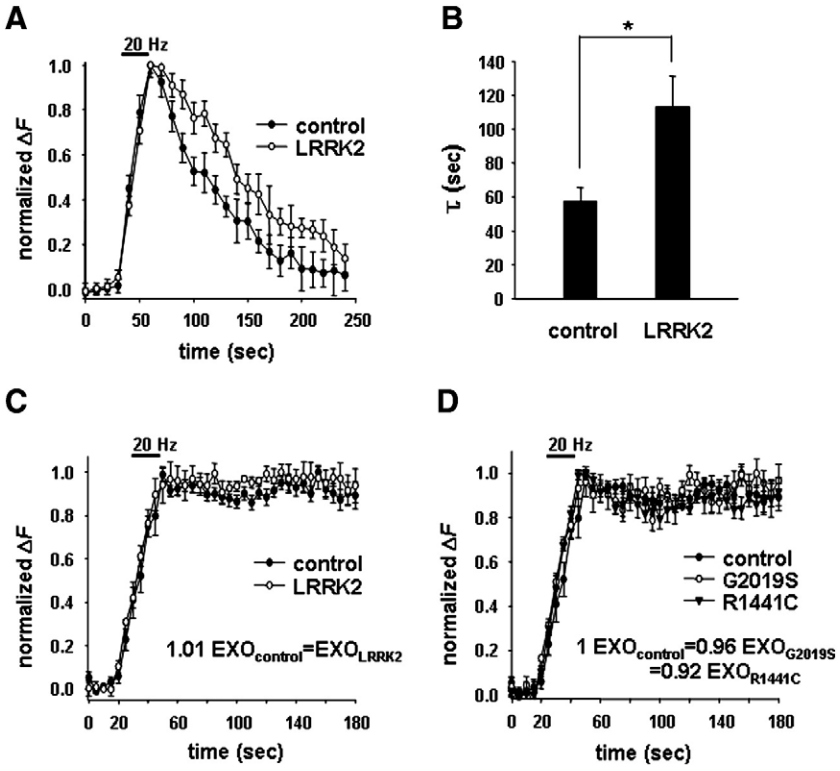

Fig. 5 – LRRK2 expression impairs endocytosis, but not exocytosis, in sypHy assay. Both endocytosis and exocytosis assays were carried out essentially as described in Fig. 4 except that sypHy instead of spH was used. (A, B) Endocytosis rate was measured after transfection of sypHy with or without LRRK2 WT plasmids into rat hippocampal neurons (τ=57.8±7.8, n=4, bouton=130 for control; τ=112.8±18.37, n=4, bouton=116 for LRRK2 WT). (C) The exocytosis rate was measured after co-transfection of sypHy with or without LRRK2 WT plasmids in the presence of bafilomycin A (n=5, bouton=175 for control; n=4, bouton=156 for LRRK2). (D) The exocytosis rates of PD-specific LRRK2 mutants were compared to that of the control. (n=5, bouton=175 for control: n=4, bouton=140 for G2019 S; n=4, bouton=111 for R1441C). *Significantly different from the control value with p<0.05, ANOVA or Student's t-test.

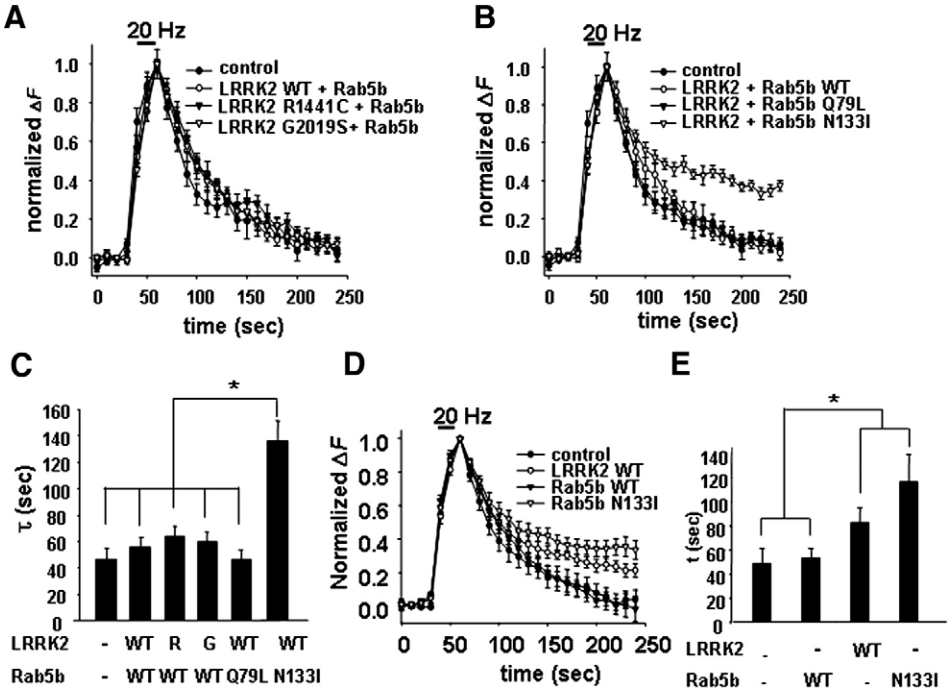

Fig. 6 – Overexpression of Rab5b rescues endocytosis defect caused by LRRK2 overexpression (A, B, C). Overexpression of Rab5b WT rescued endocytosis defect caused by overexpression of either LRRK2 WT or PD-specific mutants (A). Overexpression of the Rab5b WT and the constitutively active mutant, Q79L, but not of the dominant negative mutant N133I, rescued the endocytosis defects caused by LRRK2 overexpression (B). The spH, LRRK2 and Rab5b plasmids were triply transfected at the 1:2:3 with total DNA amount at 24 μg in 60 mm dish. The bar graph of time constant (τ) was shown in (C). R and G in (C) indicate LRRK2 R1441C and G2019S, respectively. Time constant values are $\tau = 48.9 \pm 8.2$ ($n = 6$, bouton = 143) for control, $\tau = 55.3 \pm 7.3$ ($n = 7$, bouton = 146) for LRRK2 WT and Rab5b WT, $\tau = 64.4 \pm 7.8$ ($n = 5$, bouton = 154) for LRRK2 R1441C and Rab5b WT, $\tau = 60.5 \pm 7.9$ ($n = 6$, bouton = 152) for LRRK2 G2019S and Rab5b WT, $\tau = 46.4 \pm 7.1$ ($n = 5$, bouton = 147) for LRRK2 WT and Rab5b Q79L, $\tau = 136.2 \pm 15.3$ ($n = 5$, bouton = 191) for LRRK2 WT and Rab5b N133I. (D, E) Overexpression of the Rab5b dominant negative mutant, N133I, but not of the WT, caused the endocytosis defect similar to that of the LRRK2 overexpression. The τ values were $\tau = 48.4 \pm 13.1$ ($n = 5$, bouton = 145) for control, $\tau = 83.1 \pm 12.0$ ($n = 5$, bouton = 146) for LRRK2 WT, $\tau = 53.4 \pm 8.0$ ($n = 6$, bouton = 166) for Rab5b WT, $\tau = 116.3 \pm 23$ ($n = 5$, bouton = 205) for Rab5b N133I. The spH and LRRK2 or Rab5b were transfected at 1:3 with total DNA amount at 20 μg in 60 mm dish. All the experiments were done as described in the legend of Fig. 4. *Significantly different from the control value with $p < 0.05$, ANOVA or Student's t-test.

showed that the intensity of FM 4-64 staining of LRRK2 WT or siRNAs-transfected synaptic boutons was invariably higher that that of the control boutons, indicating that the endocytosis defect by LRRK2 or LRRK2–siRNAs were not due to artifact in the acidification step of the spHluorin assay (Supplementary Fig. 3).

Since Rab5 is involved in endocytic trafficking of synaptic vesicle [22,23] and we showed that Rab5b specifically interacts with LRRK2, the endocytic defects caused by LRRK2 overexpression or knockdown could be due to interaction with Rab5b. To test this possibility, first we overexpressed Rab5b WT either with LRRK2 WT or with mutants in hippocampal neurons and measured endocytosis rate. Overexpression of Rab5b with LRRK2, regardless whether LRRK2 is WT or the PD-specific mutant, restored the endocytosis defect caused by LRRK2 overexpression (Figs. 6A and C). Next, we co-expressed LRRK2 WT with either Rab5b WT or one of two well-studied mutants (N133I and Q79L) and measured endocytosis rate. The N133I is inactive since it is unable to bind to GTP, and the Q79L is constitutively active since it is unable to hydrolyze the bound GTP [40,41]. The overexpression of Rab5b Q79L, but not of N133I, rescued the endocytosis defect caused by overexpression of LRRK2 (Figs. 6B and C), suggesting that the interaction of LRRK2 with GTP-bound form of Rab5b is primarily responsible for the effects on endocytosis. Rab5b alone had no effect on endocytosis while Rab5b N133I caused endocytic defect as previously reported (Figs. 6D and E; [42]).

Discussion

LRRK2 is the recently identified pathogenic gene involved in PD [3,4] and its variants occur most frequently in both familial and sporadic cases [5–10]. Therefore, characterization of LRRK2 and its pathogenic forms' biological functions are of paramount importance to understand underlying mechanisms of PD pathogenesis. Since LRRK2 contains several functional domains, we hypothesized that identification of its interacting proteins may provide important clues about its functional role(s).

Recent studies using subcellular fractionation, immunocytochemistry and immunogold electron microscopic analyses

of rat brain tissue or cultured cell lysates indicated that LRRK2 is a cytoplasmic protein associated with membrane organelles such as mitochondria, endoplasmic reticulum, endosomes and synaptic vesicles [13,14,30,36]. In agreement with these studies, our results using subcellular fractionation of rat cortex lysates (Fig. 2) and immunocytochemistry of rat hippocampal primary neuronal cells (Fig. 3B) indicate that LRRK2 is present in synaptosomes of neurites. These findings indicate that LRRK2's physiological functions may be related to vesicular structures and/or synaptic functions.

Using the yeast two-hybrid screening, we isolated an almost full-length cDNA encoding Rab5b. We also confirmed that LRRK2 and Rab5b interact with each other by GST pull down and co-immunoprecipitation assays. In addition, immunocytochemical and subcellular fractionation analysis indicated that they co-localize at the presynaptic regions (Figs. 2 and 3). Since Rab5 is well known to regulate endocytic vesicular transport [21], our findings prompted us to hypothesize that LRRK2 may modulate synaptic function by regulating clathrin-mediated endocytosis of synaptic vesicles. Indeed, in hippocampal neuronal cultures, we found that alterations of LRRK2 expression levels either by overexpression or knockdown significantly impaired endocytosis of synaptic vesicles. An interesting feature is that either overexpressed or reduced expression of LRRK2 similarly resulted in reduced rates of endocytosis. Such phenomena were frequently observed in similar experiments with regulators functioning specifically in endocytosis such as N-WASP, SPIN90 and SNX9 [29,43,44]. During endocytosis or exocytosis, numerous proteins participate as effectors and/or regulators via protein–protein interactions. For example, hundreds of proteins are believed to participate in exocytosis [45,46]. Physiologically abnormal high concentration of a regulator protein might perturb homeostasis of these protein and work as a dominant negative mutant. Too low concentration of the protein is not enough to carry out its physiological functions. Both situations will make networks to impair the cascade.

Previous studies showed that toxic effect of LRRK2 upon overexpression in neurons [15,16]. Thus, the endocytic defects shown here may come from the toxic effect of LRRK2. This is, however, highly unlikely since we observed defect only in the endocytosis after stimulation (post-endocytosis), but not in the endocytosis during the stimulation or in the exocytosis after overexpression of LRRK2. Moreover, the similar endocytic defect occurred under conditions reduced expression of LRRK2 by siRNAs (Fig. 4). We further confirm this point by immunostaining the nuclei of hippocampal neurons transfected with LRRK2–GFP to assess whether pyknosis occurred. The result showed that the nuclei of transfected neurons were intact and the neurons have long extended neurites (Supplementary Fig. 4). The difference may result from a different amount of DNA used for transfection, or a different culture system used. Indeed, we observed neuronal death when we used high level of LRRK2 expression (data not shown).

Notably, the defect of synaptic vesicle endocytosis by altered LRRK2 expression was largely rescued by co-expression of both wild type and constitutively active Rab5b proteins, but not by its dominant negative form (Figs. 6A and C), further supporting the notion that LRRK2 and Rab5b co-operatively regulate endocytosis. To our knowledge, we provide the first evidence that LRRK2 have a functional role in regulating synaptic vesicle endocytosis.

We could not observe any significant difference in endocytosis kinetics by expressing LRRK2 WT or PD-specific mutants (Fig. 4). Since LRRK2 causes late-onset PD in most cases, we speculate that pathological symptoms may be manifested only after long-term accumulation of functional difference caused by LRRK2 mutations and that their functional differences may not be detectable in our experimental settings. In line with this, LRRK2 mutation-caused PD often demonstrates clinical and neurochemical manifestations that are typically indistinguishable from late-onset cases. An alternative explanation is the possibility that the endocytosis regulation by LRRK2, albeit an important biological function, is not directly related to PD pathogenesis. If this is the case, LRRK2 may have an as-yet-unidentified additional function(s) that may underlie the pathogenic effect by these mutations. We observed that both G2019S, the pathogenic mutant whose kinase activity was enhanced, and K1906M, a kinase-defective mutant, slowed down endocytosis (Fig. 4A and Supplementary Fig. 5). This result implies that at least kinase activity of LRRK2 is not directly related to the endocytosis phenotype.

Taken together, our results suggest a new role for LRRK2 in regulating synaptic vesicle endocytosis by interacting with Rab5b. The reduced rate of endocytosis may cause the defects in synaptic transmission in the long run, especially during intense neuronal activity, where the vesicle replenishment from the endosomal compartments is crucial for effective neurotransmitter secretion. Interestingly, parkin, another PD-causing gene, has been recently reported to regulate endocytosis and trafficking of EGFR [47].

Since its cloning, several laboratories have investigated the potential functional role of LRRK2 mutations in PD pathogenesis. An emerging theme from these studies is that at least one of these mutations, G2019S, provides a gain-of-function role for PD, in particular by increased kinase activities [13,18,48]. Interestingly, the GTP binding activity of LRRK2 has been reported to regulate its kinase activity in a non-reciprocal manner [13,18,48]. Increased kinase activity in mutant forms appears to induce decrease of neurite length and branching, formation of inclusion bodies, and/or neuronal toxicity [13,15–18,48]. However, no physiological function of LRRK2 is defined yet although moesin is reported as a target protein for LRRK2 kinase activity [49]. In this regard, our present study provides new functional insights by showing its potential regulatory function of endocytosis as well as by identifying its interacting protein, Rab5b. In addition, discovery of other interactors for LRRK2 could be crucial for a better understanding of physiological functions of LRRK2 and pathogenic mechanisms of PD as well as for development of novel therapeutic treatments.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yexcr.2008.02.015.

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