

Apoptotic mechanisms in mutant LRRK2-mediated cell death

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Mutations in the gene coding for leucine-rich repeat kinase 2 (LRRK2) cause autosomal-dominant Parkinson's disease. The pathological mutations have been associated with an increase of LRRK2 kinase activity, although its physiological substrates have not been identified yet. The data we report here demonstrate that disease-associated mutant LRRK2 cell toxicity is due to mitochondria-dependent apoptosis. Transient transfection of mutant LRRK2 leads to neuronal death with clear apoptotic signs. Soluble caspase inhibitors or the genetic ablation of Apaf1 protects cells from apoptotic death. Moreover, we explored the function of two protein domains in LRRK2 (LRR and WD40) and demonstrate that the lack of these protein domains has a protective effect on mitochondria dysfunctions induced by mutant LRRK2.

INTRODUCTION

The primary neuropathological hallmarks of Parkinson's disease (PD) are the progressive degeneration of various neuronal groups and the presence of intraneuronal cytoplasmic inclusions composed of α -synuclein and other proteins (Lewy bodies and Lewy neurites) (1,2). In particular, the loss of dopaminergic neurons in the substantia nigra (SN) pars compacta is responsible for both depletion of dopamine in the caudate-putamen and motor dysfunction. Although the pathogenesis of PD remains incompletely understood, it apparently involves both genetic susceptibility and environmental factors (3).

The identification of rare familial forms of parkinsonism and the subsequent cloning of causal genetic mutations has had significant impact on our understanding of the molecular mechanisms underlying idiopathic PD. Genes whose mutations have been associated with Parkinsonism include α -synuclein, parkin, DJ-1, PINK1 and ATP13A2 (4,5). Specific mutations in leucine-rich repeat kinase (*LRRK2*, *PARK8*) gene have been identified as responsible for autosomal dominant familial PD (6,7), and most of the

LRRK2-linked families show a clinical and *in vivo* neurochemical phenotype that is indistinguishable from idiopathic PD (8). Clinicogenetic studies from several independent groups have evaluated the frequency of *LRRK2* mutations in several different populations and such mutations have been found not only in the 5–6% of familial PD but also in ~1–2% of idiopathic PD (9,10).

LRRK2 is a protein of 2527 amino acids composed of different predicted functional domains: Roc (Ras in complex proteins), COR domain (C-terminal of Roc), a leucine-rich repeat (LRR) domain, a protein kinase catalytic domain, a WD40 domain and an ankyrin domain. Mutations in *LRRK2* associated with PD are found in all different protein domains but no deletions or truncations have been reported so far. The pattern of dominant inheritance is consistent with a gain of function mechanism and most of the mutations appear to increase the kinase activity of the protein (11,12).

Controversy has surrounded a role for apoptosis in the loss of neurons in PD. Although a variety of studies have supported an apoptotic contribution neuronal loss particularly in the SN of PD patients (13,14), many others have failed to confirm such evidence (15). This discrepancy could be explained considering the

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slow progression of the disease; it is possible that in PD patients only a small percentage of dopaminergic cells may present signs of active cell death at certain given time-point. On the other hand, the presence of apoptotic markers has been well characterized in experimental mice and rats after treatment with either 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or 6-hydroxydopamine (13,16), the best-characterized toxin paradigms for PD.

In this study, we have investigated the role of apoptosis in mutant LRRK2-mediated cellular death. We found that the LRRK2-mediated cell toxicity involves cytochrome *c* release and caspase 3 activation. The genetic ablation of Apaf1 abrogates the caspase 3 activation and the neuronal death. Moreover, we explored the role of LRR and WD40 protein domains in the neuronal death using different LRRK2 deletion mutants.

RESULTS

Expression and cellular localization of LRRK2

The full-length LRRK2 cDNA was amplified by RT-PCR on total RNA extracted from human lymphoblasts. Point mutations R1441C, Y1699C and G2019S were generated by PCR. All LRRK2 cDNAs were cloned in different plasmids for eukaryotic expression with or without tag (1x-FLAG or 1x/5x-myc tag) (Fig. 1A). All constructs were transfected in SH-SY5Y cell line and the cells were analysed 24 h after transfection. The 5X-myc-LRRK2 protein expression was easily detected by western blot experiments on protein extracts from transiently transfected cells showing the expected molecular weight (Fig. 1B). No difference in protein levels was visible between WT and mutant LRRK2 in all our experiments (Fig. 1B). All LRRK2 constructs were analysed by immunofluorescence experiments. As expected (12), immunofluorescence experiments after transfection using an anti-myc antibody showed a cytoplasmic localization for both WT and mutant LRRK2 (Fig. 1C). Similar results were obtained using the other genetic construction we made even if the 1X-myc and 1X-FLAG fusion proteins were hardly detectable by western blot experiment using specific antibody for the two epitopes (data not shown).

Apoptotic pathway in neuronal death mediated by mutant LRRK2

Although a role for mutant LRRK2 in cell toxicity has already been suggested by different lines of evidence (17) (18), the molecular mechanisms underlying this event are completely unknown. To explore the role of apoptosis in LRRK2-mediated neuronal cell death, we co-transfected human neuroblastoma SH-SY5Y cells with the GFP and 5x-myc-LRRK2 constructs described in Materials and Methods and measured into the GFP-positive cells different parameters of the intrinsic apoptotic pathway (19). As evidenced in Figure 2A and B, by direct immunofluorescence analysis, the expression of mutant LRRK2^{R1441C} determines cell nuclei condensation in a high percentage of transfected cells when compared with a small percentage of apoptotic nuclei in cells transfected with wild-type LRRK2 (Fig. 2B). The mild but significant toxicity observed in cells transfected with LRRK2^{WT} construct can be explained with the

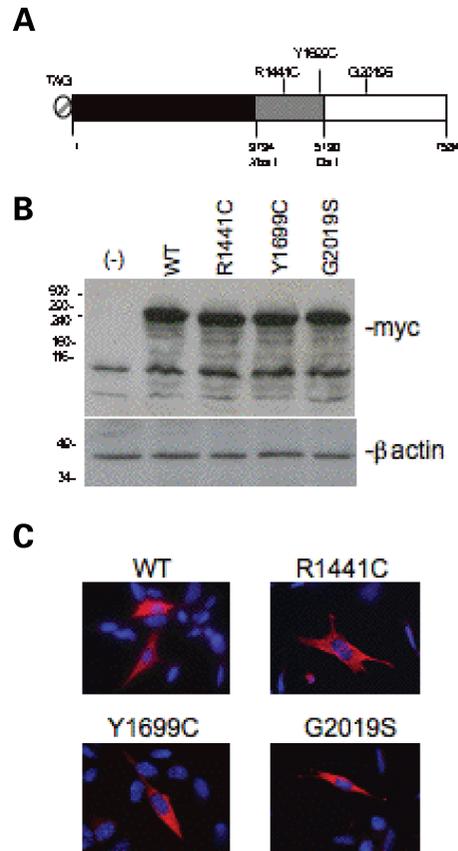


Figure 1. Expression of LRRK2 mutants in neuronal SH-SY5Y cell line. (A) Schematic representation of WT and mutant LRRK2s used in this work. (B) Western blot analysis using anti-myc and anti-β-actin antibodies on protein extracts prepared from SH-SY5Y cells transiently transfected with plasmids coding for wild-type or mutant human LRRK2. (C) Direct immunofluorescence using an anti-myc antibody showing the cytoplasmic localization of WT and mutant LRRK2 in SH-SY5Y transfected cells.

high level of protein in transiently transfected cells, a condition where the LRRK2 kinase activity could be not fully regulated.

We also evaluated the activation of caspase 3 in our *in vitro* cell system 24 h after transfection. As shown in Figure 2C, activated caspase 3 is easily detected in cells transfected with LRRK2^{R1441C}. Furthermore, treatment with the cell permeable caspase inhibitor z-VAD-fmk strongly impairs caspase 3 activation (Fig. 2C and D) and nuclei condensation (data not shown) due to the presence of mutant LRRK2. Identical results were obtained when the 1x-flag, 1x-myc and non-tagged LRRK2^{R1441C} were used (data not shown), suggesting that the presence of 5x-myc is not altering LRRK2^{R1441C} toxicity. These results strongly suggest that, in a neuronal cell line, caspase 3 activation is the main mediator of cell death due to the presence of mutant LRRK2.

Apaf1 knock-out blocks cellular death mediated by expression of mutant LRRK2

In order to gain more insights into the molecular mechanisms responsible for mutant LRRK2-induced apoptosis, we used a neuronal cell line defective for Apaf1 [ETNA^{-/-}, embryonic

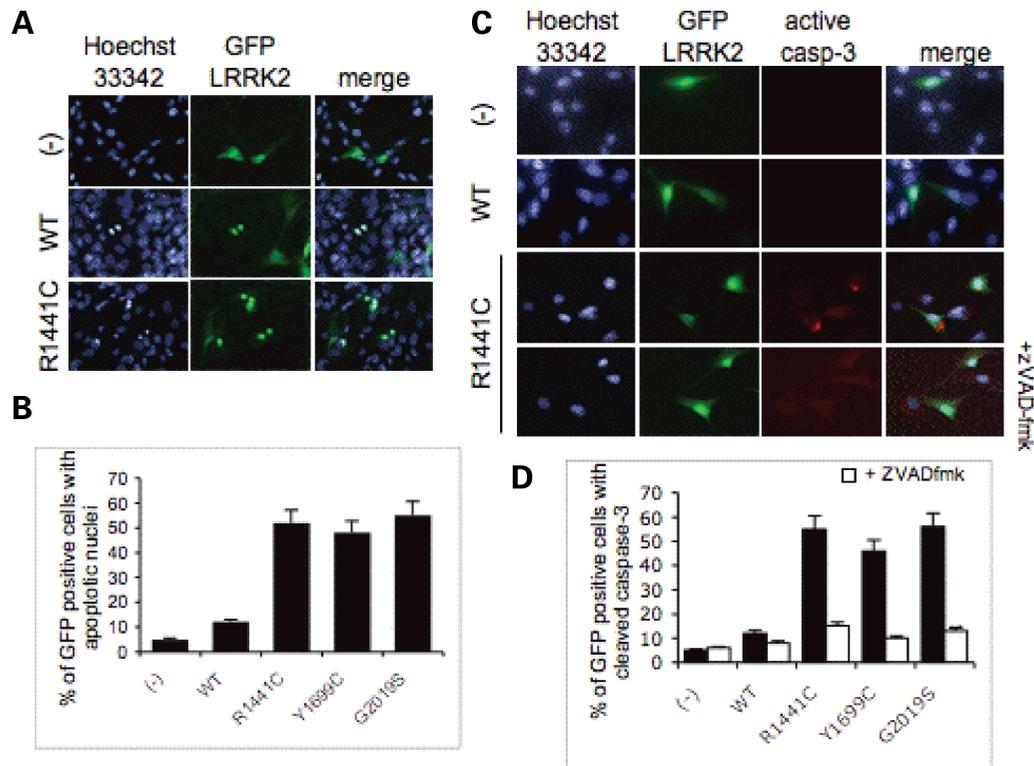


Figure 2. LRRK2 mutants induce cell death in SH-SY5Y cells. SH-SY5Y cells were transiently co-transfected with the indicated plasmid coding for wild-type or mutant LRRK2 and a pLanterm GFP in a ratio 5:1. (A) Representative direct immunofluorescence of apoptotic nuclei in co-transfected cells using a fluorescent probe Hoechst 33342. (B) Quantification of apoptotic nuclei 48 h after transfection. At least 100 GFP-positive cells from randomly chosen fields for each transfected construct were counted. (C) Direct immunofluorescence using an anti-cleaved-caspase 3 antibody in co-transfected cells 24 h after transfection. Only the mutant R1441C is shown as an example. Use of cell-permeable caspase inhibitor z-VAD-fmk to block caspase 3 activation (lower panel). (D) Quantification of cleaved caspase 3-positive cells 48 h after transfection in the presence or absence of z-VAD-fmk treatment.

telencephalic naïve Apaf1 knock-out (20)]. Apaf1 is a scaffold protein participating with pro-caspase-9 and cytochrome *c* in the formation of the apoptosome, which operates as an engine of caspase activation. This, in turn, initiates and/or executes apoptosis (19). Apaf1-deficient cells (ETNA^{-/-}) or Apaf1 normally expressing cells (ETNA^{+/+}) were exposed to WT or mutant LRRK2 and different apoptotic signals upstream and downstream to Apaf1 were analysed. As shown in Figure 3A (upper line), in ETNA^{+/+} cells, transient transfection of mutant LRRK2 leads to increased caspase-3 activation when compared with control. On the contrary, no activation of caspase-3 was observed in ETNA^{-/-} cells with all LRRK2 constructions. Using a specific antibody against Apaf1, we were able to confirm the presence of the protein only in ETNA^{+/+} but not in ETNA^{-/-} cells (Fig. 3A, bottom line). To exclude the differences in transfection level and in protein loading between the two different cell types, we performed a western blot experiment using a specific antibody against myc-tag and total caspase 3 on the same protein extracts (Fig. 3A, middle lines). We confirmed the lack of apoptosis in ETNA^{-/-} cells transfected with mutant LRRK2 by quantifying the picnotic nuclei (Fig. 3B) in cells transfected as described in Figure 2. On the contrary, in both cell types, the expression of mutant LRRK2 induces cytochrome *c* release (Fig. 3C), indicating that the apoptotic pathways are normally activated. Although cytochrome *c* is

normally released in ETNA^{-/-} cells transfected with mutant LRRK2, only a small percentage of picnotic nuclei are observed (Fig. 3B). The lack of caspase 3 activation and condensed nuclei in ETNA^{-/-} cells strongly suggests that the mutant LRRK2-mediated-death is dependent on mitochondria dysfunction and apoptosome formation. Moreover, in order to exclude that mutant LRRK2 over-expression in Apaf1-deficient cells can induce cell loss, independently from apoptosis, we evaluated the ratio between GFP-positive cells and total cell number in ETNA^{-/-} cells transfected with different genetic constructions. We did not observe any significant reduction in the percentage of GFP-positive cells between ETNA^{-/-} cells transfected with GFP alone or in combination with wild-type or mutant LRRK2 48 h after transfection (GFP: $130 \pm 9/500$; GFP and LRRK2: $123 \pm 7/500$; GFP and LRRK2^{R1441C}: $125 \pm 10/500$).

In order to demonstrate that the protection from death observed in ETNA^{-/-} cells was a monogenic phenotype, we co-transfected the mutant LRRK2^{R1441C} with a construct carrying the Apaf1 cDNA under the control of the strong constitutive promoter CAGGs. As shown in Figure 3D, the presence of Apaf1 in ETNA^{-/-} cells restores the R1441C-mediated caspase 3 activation 24 h after transfection that is not observed in the absence of Apaf1. The experiment strongly indicates that the lack of R1441C-mediated cell death in ETNA^{-/-} cells is mainly dependent on the absence of Apaf1.

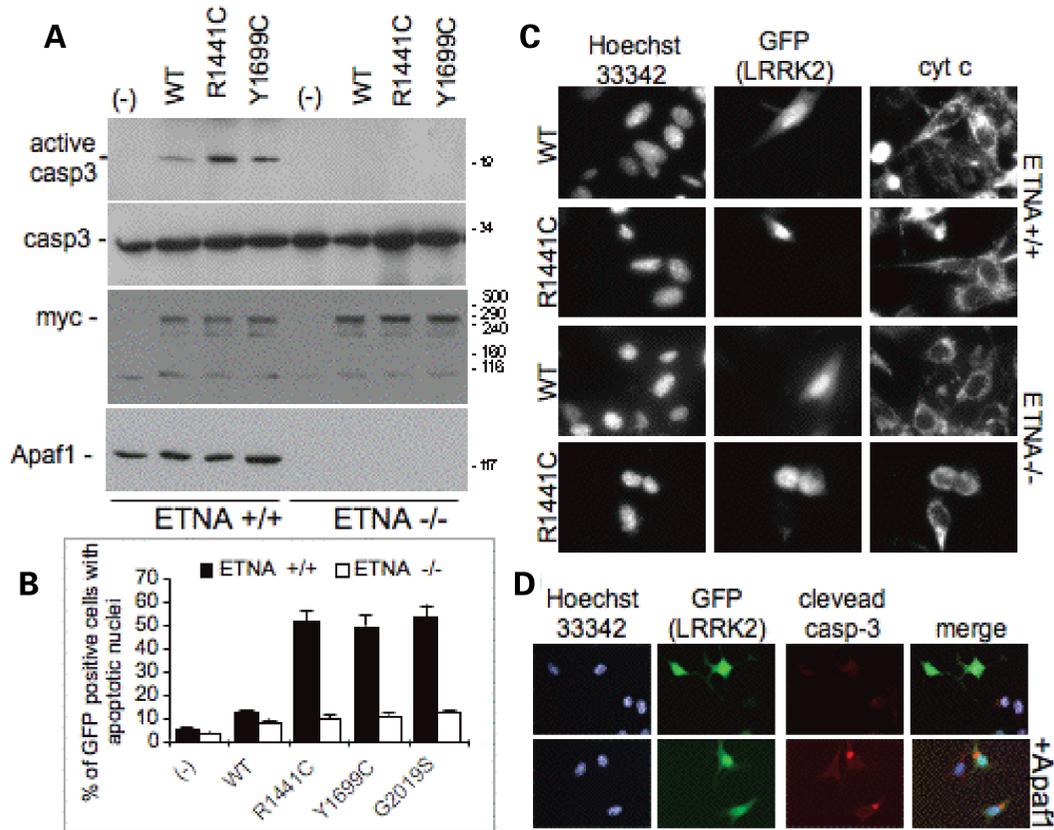


Figure 3. Mutant LRRK2-induced cell death is apoptosome-dependent. ETNA^{+/+} and ^{-/-} cell lines were transfected with the indicated plasmids. (A) Western blot analysis using specific anti-cleaved-caspase 3, -total caspase-3, -myc and -Apaf1 antibodies on protein extracts from transiently transfected cells. (B) Percentage of apoptotic nuclei in ETNA^{+/+} and ^{-/-} cell lines after co-transfection with GFP and WT or mutant LRRK2. (C) Direct immunofluorescence using anti-cytochrome *c* antibody. Note the diffuse cytochrome *c* staining using the LRRK2^{R1441C} mutant against the dotted staining in the LRRK2^{WT} in ETNA^{-/-} transfected cells. (D) Anti-cleaved caspase 3 staining in ETNA^{-/-} cell lines transfected with mutant R1441C in the presence or absence of Apaf1.

Role of the different LRRK2 domains in localization and cellular death

LRRK2 is a protein composed of various predicted functional domains and the LRRK2 mutations associated with PD have been found in different protein domains (21). The presence of multiple protein interaction domains suggests that LRRK2 may serve as a scaffold for the assembly of different proteins that in turn may negatively or positively modulate the predicted LRRK2 kinase and GTPase activities. We investigated the importance of LRR and WD40 domains in LRRK2 toxicity in the presence or absence of the R1441C point mutation that it is known to enhance LRRK2 kinase activity. The DNA constructions represented in Figure 4A were transfected in SH-SY5Y cells and the expression level was evaluated performing a western blot experiment using an anti-myc-tag antibody (Fig. 4B). LRRK2^{ΔLRR} and ^{ΔWD40} were expressed at comparable levels, irrespective of R1441C point mutation (Fig. 4B). Then the ability of the different constructs to induce apoptotic cell death was investigated by monitoring the activated form of caspase 3. Despite low transfection efficacy (~10–20% of the cells), for each experimental condition, we analysed more than 100 GFP-positive cells to assess caspase 3 activation, reaching significant statistical values. Both LRRK2^{ΔLRR} or ^{ΔWD40}, in a WT or R1441C

context, were unable to induce caspase 3 activation (Fig. 4C and D) and nuclei condensation (data not shown) after transient transfection, compared with LRRK2^{R1441C}. The lack of cell toxicity was not due to different cellular localization in the absence of LRR or WD40 domain since, as shown in Figure 4E, no remarkable differences in protein distribution were observed between WT-LRRK2 and different deletion mutants.

We also analysed the kinase activity of all the different deletion mutants in terms of phosphorylation of the unspecific substrate myelin basic protein (MBP). As shown in Figure 4F and G, the deletion of the LRR domain does not alter the *in vitro* kinase activity in the presence or absence of R1441C mutation. Moreover, as expected, the presence of the R1441C point mutation increases the basal kinase activity. On the contrary, the WD40 deletion mutant, although it is still active, shows a reduction in kinase activity, which in the presence of the R1441C mutation is restored to the level of LRRK2-WT.

DISCUSSION

As recently described, mutation in the LRRK2 gene accounts for 5–6% of familial autosomal dominant late-onset PD and

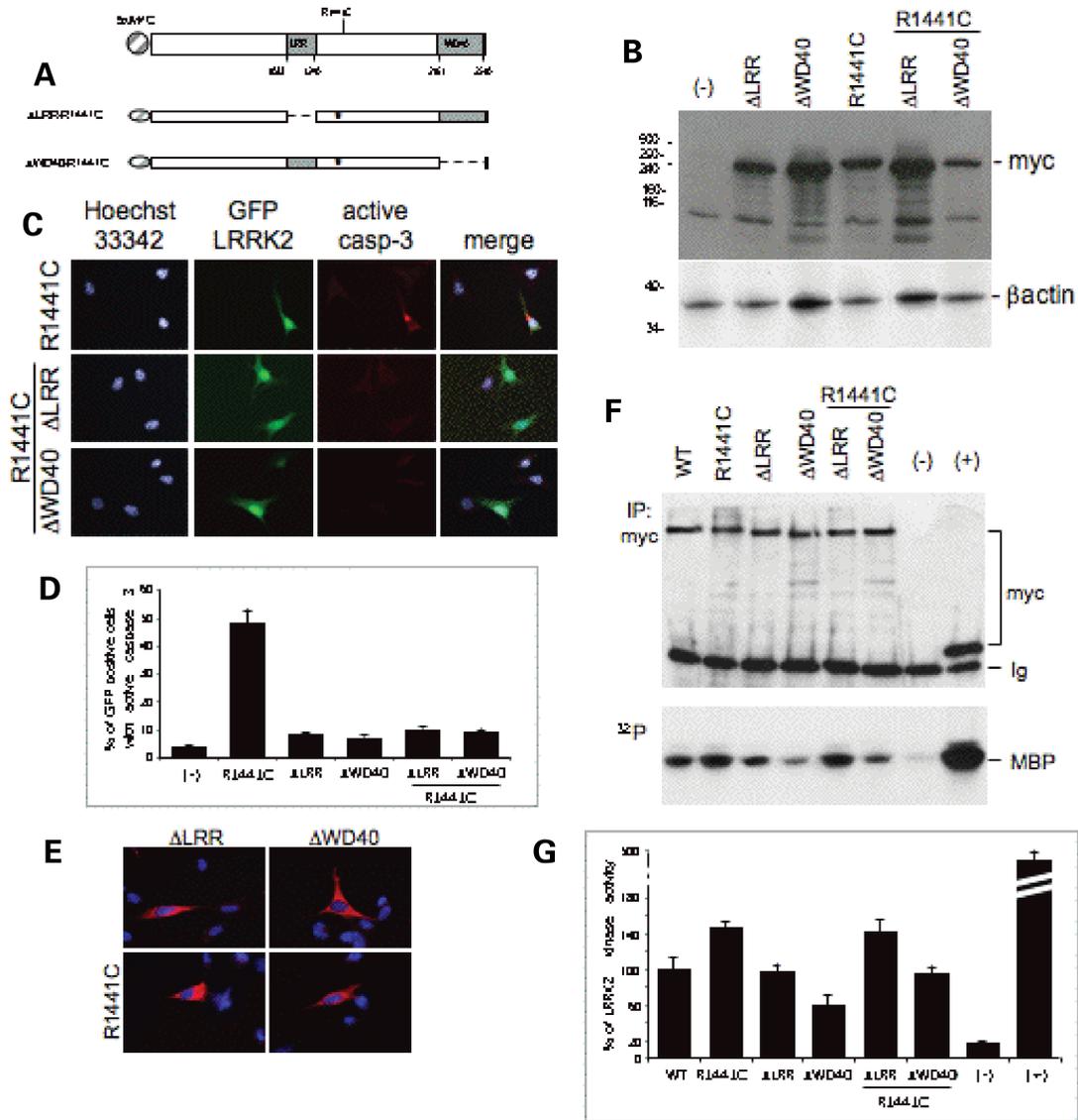


Figure 4. Analysis of deletion mutants in LRRK2-dependent neuronal cell death. (A) Schematic representation of DNA constructs coding for LRRK2 deletion mutants. (B) Western blot analysis of LRRK2 ^{Δ LRR} and LRRK2 ^{Δ WD40} on protein extracts from transiently transfected SH-SY5Y cells using anti-myc antibody. Equal loading was verified using anti- β -actin antibody. (C) Immunofluorescence staining with anti-cleaved caspase 3 antibody on cells transfected with the different mutants. Note the lack of caspase 3 activation using R1441C ^{Δ LRR} and R1441C ^{Δ WD40} compared with R1441C mutant. (D) Quantification of cleaved caspase 3-positive cells 24 h after transfection with the indicated constructs. (E) Representative confocal images of immunolocalization of LRRK2 ^{Δ LRR} and LRRK2 ^{Δ WD40} in the presence or absence R1441C point mutation using an anti-myc antibody on transfected SH-SY5Y cells. (F) Kinase activity of different LRRK2 constructs. ETNA cells were transfected with expression plasmids encoding Myc-tagged LRRK2 wild type, R1441C, Δ LRR, Δ WD40, Δ LRR-R1441C, Δ WD40-R1441C and Aurora-A as positive control (+); (-) cells transfected with empty vector. Protein extracts prepared from transfected cells were subjected to immunoprecipitation using the anti-Myc antibody. Immunoprecipitated complexes were analysed by western blotting (top panel) to verify the equal expression level of each kinase. *In vitro* kinase assay performed on immunoprecipitated complexes using MBP as the substrate. Autoradiography (bottom panel) is shown. (G) Quantification of three independent experiments is as in (F).

for 1–2% of idiopathic PD (9,10). Among those patients, despite the fact that they are clinically indistinguishable, a large variability is observed in the age of onset and neuropathological features, indicating a possible heterogeneity in the degenerating mechanisms (6,22). Furthermore, expression studies of LRRK2 in human and rodent brain have shown that LRRK2 is predominantly expressed in dopaminergic areas (23,24) whereas it is absent or expressed at lower level in dopaminergic areas both in SN and in ventral tegmental area

(25,26). This expression pattern is unique when compared with that of other genes linked to PD and raises the question of how LRRK2 mutations affect the survival of SN neurons and PD development. Expression of mutant LRRK2 can cause loss of dopaminergic neurons either directly or indirectly, since mutant LRRK2 could produce a pathological stress in the medium spiny neurons of caudate-putamen that could alter the physiological cell–cell communication between SN and caudate-putamen and indirectly affect the

dopaminergic neuron physiology. Even if autosomal-dominant mutations in LRRK2 have been shown to induce an increase in kinase activity (11) and a reduction in neuron process length and complexity (27), until now very little is known on physiological and pathological function of LRRK2. In particular, the molecular mechanisms of LRRK2-mediated neuronal toxicity related to PD remains to be fully elucidated.

Our data extend the previous findings in two important directions. First, we demonstrated that mutant LRRK2 toxicity in human (SH-SY5Y) and murine (ETNA embryonic neuronal precursors) neuronal cells is mediated by the mitochondria-dependent apoptotic pathway (Fig. 2). Upon transient transfection with mutant LRRK2, cytochrome *c* is released and caspase 3 is activated, and cell death promptly follows. Interestingly, caspase 3 activation was observed also in dopaminergic primary neurons infected with mutant LRRK2 (27).

Using a neuronal cell line defective for apoptosome formation (ETNA^{-/-}), an *in vitro* model system already used for studying apoptosis modulation in neurodegenerative conditions (20,28), we further demonstrated that mutant LRRK2 toxicity is dependent on Apaf1, a scaffold protein participating to apoptosome formation in mammals (19). In this cell line, transfected with mutant LRRK2, the absence of Apaf1 inhibits caspase 3 activation and nuclei condensation, while cytochrome *c* is normally released from mitochondria (Fig. 3), indicating that the block of apoptotic pathway is downstream to mitochondrial damage. All these results underline a role for a mitochondria-dependent apoptotic pathway in the cell death induced by mutant LRRK2 and point at Apaf1 as a critical mediator of the process. In line with our observations, it has been demonstrated that expression of a dominant negative form of Apaf1 in the striatum provides protection against dopaminergic cell loss and striatal catecholamine depletion in the MPTP-treated mouse model (29). In our experimental conditions, the absence of Apaf1 in ETNA^{-/-} cells does not fully protect from mutant LRRK2-mediated cell death. As reported in other cell systems, neuronal death could be due to molecular mechanisms independent of apoptosis or more probably independent of Apaf1/caspase 3 pathway. For instance, apoptosis-inducing factor substitutes for caspase executioners in NMDA-triggered excitotoxic neuronal death (30). Nevertheless, our data and the observation by others (31,32) that LRRK2 is associated with the mitochondria membrane strongly point at mitochondrial dysfunction as the endpoint of mutant LRRK2-induced damage. This is in line with recent research, focused on the role of mitochondria in early onset PD linked to recessively inherited loss-of-function mutations in *parkin*, *PINK1* and *DJ-1* (33). In *parkin*^{-/-} mice, two-dimensional gel electrophoresis followed by mass spectrometry revealed decreased abundance of a number of proteins involved in mitochondrial function or oxidative stress (34). PINK-1 is localized into mitochondria (35) and, although the substrates of its kinase activity have not been identified, PINK1 protects cells against loss of mitochondrial membrane potential triggered by the application of proteasome inhibitor or staurosporine-induced stress. Finally, the function of *DJ-1* gene appears to be also related to mitochondria since DJ-1^{-/-} mice showed increased striatal denervation and dopaminergic neuron loss induced by mitochondrial toxin MPTP and DJ-1^{-/-} embryonic cortical neurons

showed increased sensitivity to oxidative but not to non-oxidative insults (36).

Second, we highlighted a prominent role of LRR or WD40 in the toxicity of mutant LRRK2^{R1441C}. The LRR and the WD40 protein domains are usually involved in protein-protein interactions and they can mediate the interaction with other phosphoproteins, in order to recruit these substrates into protein-protein signalling complexes in response to phosphorylation by serine/threonine kinases (37). In the absence of these two domains, we did not observe any caspase 3 activation and apoptotic cell death in transiently transfected cells. The deletions are not altering the cytoplasmic localization of the deletion mutants and notably both LRRK2^{ΔLRR} and LRRK2^{ΔLRR-R1441C} have a kinase activity comparable, respectively, to wild-type and R1441C. This result strongly indicates that the absence of LRR domain does not interfere with kinase activity, although it is implicated in LRRK2-mediated toxicity. Probably, the LRR domain is involved in the interaction between LRRK2 and unknown protein partners and this interaction is crucial for the pathological function of mutant LRRK2. On the contrary, we do observe a partial reduction in LRRK2^{ΔWD40} kinase activity, although the introduction of the R1441C mutation restores a kinase activity comparable to wild-type LRRK2. On the basis of these observations, we can hypothesize that the lack of WD40 domain can affect enzyme activity by determining structural alterations in LRRK2 protein or by impairing some specific post-translational modifications (e.g. phosphorylation) necessary for the full activation of the enzyme.

The delineation of either the LRRK2 signalling pathway or the molecular partners and substrates of LRRK2 holds great promise for furthering our understanding of the aetiology of PD.

MATERIALS AND METHODS

Plasmid construction

cDNA corresponding to human LRRK2 (accession no. NM_198578) was RT-PCR amplified from human lymphoblast mRNA. Different PCRs were performed to amplify three overlapping regions: from 5' to *Xba*I site (3733 bp), from *Xba*I to *Cla*I site (1396 bp) and from *Xba*I to 3' region (2455 bp). The three fragments were cloned together and then subcloned in pCMV-Tag2B (1X-FLAG-tag, Stratagene) or pCMV-Tag3B (1X-myc-tag, Stratagene) or pCS2-MTK vector [5X-myc-tag (38)]. The point mutations R1441C, Y1699C and G2019S and the deletions ΔLRR (amino acids 1033–1240), ΔWD40 (amino acids 2168–2510) were generated using the Quikchange site-directed mutagenesis kit (Stratagene). In co-transfection experiments with LRRK2 constructions, we used pLantern GFP (Gibco BRL) in a ratio 5:1 and pJojo-APAF1 (20) in a ratio 1:1. As a positive control for *in vitro* kinase assay, we used a plasmid coding for human Aurora-A cloned in pCS2-MTK vector (39).

Antibodies

In the present study, we used the following antibodies: mouse anti-myc (clone 9E10 Sigma-Aldrich, 1:1000 final dilution), rabbit anti-cleaved caspase 3 (Cell Signaling, 1:1000 for

western and 1:100 for immunofluorescence), rabbit anti-caspase 3 (Cell Signaling, 1:1000 final dilution), mouse anti- β -actin (Sigma-Aldrich, 1:1000 final dilution), mouse anti-cytochrome *c* (Promega, 1:1000 final dilution), Apaf1 (1:1000 Cell Signaling).

Transfection

Human neuroblastoma SH-SY5Y cell line (ATCC number CRL-2266) was grown in DMEM-F12 + 10% fetal calf serum (FCS, Invitrogen) at 37°C, whereas ETNA+/+ and -/- cell lines (20) were grown in DMEM + 10% FCS (Invitrogen) at 33°C.

Transient expression of each vector (1.5 μ g DNA/5–7 $\times 10^5$ cells) was obtained with LipofectAMINE Plus reagent (Invitrogen) according to manufacturer's instructions. After an incubation of 3 h with transfection reagents, the cells were cultured in normal growth medium for 24 or 48 h.

Western blot analysis

After rinsing cell cultures with ice-cold phosphate-buffered saline (PBS), cell lysis was performed in lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA) containing 1 mM PMSF and a protease inhibitor cocktail (Sigma-Aldrich). A clear supernatant was obtained by centrifugation of lysates at 17 000g for 10 min. Protein content was determined using Bradford protein assay (Bio-Rad). Twenty milligrams of protein extracts were resolved by standard SDS/PAGE. Samples were electroblotted onto Protan nitrocellulose (Schleicher & Schuell GmbH). Membranes were incubated with 5% low-fat milk in 1 \times PBS solution and the indicated antibody for 16 h at 4°C. Donkey anti-rabbit-HRP (Jackson Laboratories) and goat anti-mouse-HRP (Sigma-Aldrich) antibodies were used to reveal immunocomplexes by enhanced chemiluminescence (Pierce).

Direct immunofluorescence

Cells were grown on 35 mm plates, fixed with 4% paraformaldehyde in 1 \times PBS and permeabilized with 0.2% Triton X-100 in 1 \times PBS. After blocking for 1 h in 5% BSA in 1 \times PBS–0.05% Tween-20, the cells were incubated with the indicated antibody overnight at 4°C. Cells were then washed with 1 \times PBS – 0.05% Tween-20 and incubated with a secondary antibody: Cy3-conjugated anti-mouse or anti-rabbit serum at 1:500 (Jackson Laboratories). Immunostained samples were counterstained with Hoechst 33342 (1 mg/ml) (Sigma-Aldrich) before being subjected to microscopy. Image collection was done on a DMLB Leica microscope with an HBO 100 W lamp.

Assessment of cell death

Cells were transfected with LRRK2 plasmid constructions and pLantern GFP in a ratio 5:1 and analysed 48 h after transfection. In some experiments, Z-VAD-fmk (100 μ M) was added immediately after transfection. Quantification of apoptotic cells was obtained by direct visual counting after nuclear staining of 4% paraformaldehyde-fixed cells with the

fluorescent probe Hoechst 33342 (1 mg/ml) (Sigma-Aldrich). For each experiment shown, we performed at least three independent trials. For each trial, 100 GFP-positive cells were analysed in randomly chosen fields at 40 \times magnification. Only the cells containing clearly picnotic nuclei were considered apoptotic.

Immunoprecipitation

Forty-eight hours after transfection, ETNA cells were lysed in 0.5 ml of lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 1 mM NaF, 1 mM Na₃VO₄, protease inhibitor cocktail]. After centrifugation at 12 000g for 10 min at 4°C, the supernatants were pre-cleared at 4°C for 1 h with 10 μ l of a mixture 1:1 of protein A- and G-Sepharose (Roche). The anti-myc antibody (Sigma) was added at a 1:1000 dilution, and after an incubation of O/N at 4°C, the immunoprecipitates were collected by adding 20 μ l of a mixture 1:1 of protein A- and G-Sepharose for 2 h at 4°C. The beads were washed three times in lysis buffer and twice in kinase buffer [50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 10 mM MgCl₂, 1 mM NaF, 1 mM Na₃VO₄, protease inhibitor cocktail]. 1/20 of the immunoprecipitates were analysed by western blotting.

In vitro kinase assay

Assays were performed in 30 μ l of kinase buffer containing 0.5 mg/ml of MBP (Sigma), 2 μ Ci [γ -³²P]ATP, 50 μ M ATP, 25 mM β -glycerolphosphate, 1 mM NaF, 1 mM Na₃VO₄ and protease inhibitor cocktail. The reaction mixtures were incubated at 37°C for 20 min, the reactions were stopped by the addition of 30 μ l of 2 \times Laemmli SDS sample buffer, and the reaction products were denatured for 5 min at 95°C. Proteins were then separated by standard SDS-PAGE and analysed by autoradiography.

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Conflict of Interest statement. None declared.

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