Mitochondrial impairment increases FL-PINK1 levels by calcium-dependent gene expression∗

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A B S T R A C T

Mutations of the PTEN-induced kinase 1 (PINK1) gene are a cause of autosomal recessive Parkinson’s disease (PD). This gene encodes a mitochondrial serine/threonine kinase, which is partly localized to mitochondria, and has been shown to play a role in protecting neuronal cells from oxidative stress and cell death, perhaps related to its role in mitochondrial dynamics and mitophagy. In this study, we report that increased mitochondrial PINK1 levels observed in human neuroblastoma SH-SY5Y cells after carbonyl cyanide m-chlorophenylhydrazone (CCCP) treatment were due to de novo protein synthesis, and not just increased stabilization of full length PINK1 (FL-PINK1). PINK1 mRNA levels were significantly increased by 4-fold after 24 h. FL-PINK1 protein levels at this time point were significantly higher than vehicle-treated, or cells treated with CCCP for 3 h, despite mitochondrial content being decreased by 29%. We have also shown that CCCP dissipated the mitochondrial membrane potential (Δψ) and induced entry of extracellular calcium through L-type calcium channels. The calcium chelating agent BAPTA-AM impaired the CCCP-induced PINK1 mRNA and protein expression. Furthermore, CCCP treatment activated the transcription factor c-Fos in a calcium-dependent manner. These data indicate that PINK1 expression is significantly increased upon CCCP-induced mitophagy in a calcium-dependent manner. This increase in expression continues after peak Parkin mitochondrial translocation, suggesting a role for PINK1 in mitophagy that is downstream of ubiquitination of mitochondrial substrates. This sensitivity to intracellular calcium levels supports the hypothesis that PINK1 may also play a role in cellular calcium homeostasis and neuroprotection.

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Introduction

Parkinson’s disease (PD) is a progressive neurodegenerative disorder characterized clinically by bradykinesia, rigidity or tremor, and pathologically by loss of nigrostriatal dopaminergic neurons and the presence of cytoplasmic protein inclusions known as Lewy bodies. The etiology of PD is still unknown, but genetic and possibly environmental factors are thought to be involved (Duvoisin, 1999; Williams et al., 1999). Mitochondrial dysfunction has consistently been implicated in the pathogenesis of PD (Langston et al., 1983; Schapira et al., 1989), and different proteins associated with familial PD, such as PTEN-induced kinase 1 (PINK1), Parkin, DJ-1, LRRK2 and α-synuclein, have been reported to localize to mitochondria and affect function (Bonifati et al., 2003; Devi et al., 2008; Narendra et al., 2008; Papkovskaia et al., 2012; Silvestri et al., 2005; Valente et al., 2004).

Mutations in the PINK1 gene are responsible for autosomal recessive familial PD (Valente et al., 2004). PINK1 is a 581 amino acid protein ubiquitously transcribed and encodes a serine/threonine kinase, showing high homology with the Ca2+ /calmodulin kinase family. Also, PINK1 contains a N-terminal mitochondrial targeting sequence and a C-terminal autoregulatory domain (Belina et al., 2005; Silvestri et al., 2005; Sim et al., 2006) is predominantly localized to mitochondria, but also is present in the cytosol (Haque et al., 2008; Valente et al., 2004; Weihofen et al., 2008; Zhou et al., 2008). Full-length PINK1 (FL-PINK1), is approximately 63 kDa, and is transcribed in the nucleus, translated...
in the cytoplasm and imported intact into mitochondria. PINK1 is then cleaved by the mitochondrial protease PARL (presenilin-associated rhomboid-like) at the inner mitochondrial membrane (Deas et al., 2011; Meissner et al., 2011; Whitworth et al., 2008) to yield two bands of 55 kDa (ΔN-PINK1) and 45 kDa (ΔN²-PINK1) (Lin and Kang, 2008; Muqit et al., 2006; Silvestri et al., 2005; Weihofen et al., 2008). The ΔN-PINK1 species is rapidly degraded by the proteasome (Takatori et al., 2008).

Previous reports using cell culture models suggest that PINK1 may play a neuroprotective role under several forms of stress conditions, because the over-expression of wild-type PINK1, but not mutant PINK1, protects against cell death induced by chemical stressors, such as the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or the proteasomal inhibitor carbobenzoxyl-leucyl-leucyl-leucinal (MG-132) (Haque et al., 2008; Petit et al., 2005; Valente et al., 2004). Several studies in Drosophila, mouse and cell culture models, including fibroblasts from patients with PINK1 mutations (Abramov et al., 2011; Grunewald et al., 2009; Hoepken et al., 2007; Piccoli et al., 2008), suggest that loss of PINK1 can be associated with functional and morphological mitochondrial defects, oxidative stress and the balance between mitochondrial fission and fusion (Clark et al., 2006; Gautier et al., 2008; Gegg et al., 2009; Gispert et al., 2009; Heeman et al., 2011; Park et al., 2006; Poole et al., 2008; Sandebring et al., 2009; Yang et al., 2008). The mitochondrial dysfunction associated with PINK1 deficiency has been linked to perturbed mitophagy, a cellular process by which old and damaged mitochondria areengulfed into double membrane vacuoles, called autophagosomes, that then fuse with lysosomes, resulting in autophagolysosomes, where mitochondria are subsequently degraded (Kim et al., 2007; Youle and Narendra, 2011). Loss of Δψm induced by mitochondrial uncouplers, like carbonyl cyanide m-chlorophelyhydrazone (CCCP), is an initial step in the removal of this organelle, initiating fission of the reticular mitochondrial network in the damaged mitochondria (Narendra et al., 2008; Twig et al., 2008). This event inhibits the processing of FL-PINK1 by PARL, leading to the accumulation of FL-PINK1 on the mitochondrial outer membrane (Jin et al., 2010; Matsuda et al., 2010; D.P. Narendra et al., 2010; Vives-Bauza et al., 2010). PINK1 then recruits Parkin to mitochondria via phosphorylation (Kondapalli et al., 2012; Matsuda et al., 2010), whereupon Parkin ubiquitinates mitochondrial proteins such as VDAC and the mitofusins (Gegg et al., 2010; Geisler et al., 2010; Ziviani et al., 2010). The ubiquitination of mitochondrial outer membrane proteins such as the mitofusins leads to their degradation by the proteasome, and is required for mitophagy (Chan et al., 2011; Tanaka et al., 2010).

Loss of PINK1 function results in decreased ATP synthesis by mitochondria, impaired mitochondrial calcium handling and increased oxidative stress in a time-dependent manner (Gautier et al., 2008; Gegg et al., 2009). The impairment of mitochondrial function is coincident with decreased macroautophagy flux (Gegg et al., 2010). Restoration of mitophagy in PINK1-deficient cells by exogenous expression of parkin results in improved mitochondrial function (Gegg et al., 2010), suggesting that impaired mitophagy may contribute to the mitochondrial dysfunction observed in PD.

Calcium plays a central role in regulating neurotransmitter release. Calcium influx is involved in the release of neurotransmitters by synaptic vesicles (Neher and Sakaba, 2008), synaptic plasticity (Zucker, 1999) and gene transcription (Lyons and West, 2011). Studies have implicated increased levels of Ca²⁺-binding proteins calretinin and calbindin (Goldberg et al., 2012; Biswas et al., 1999; Elam et al., 1990; Herrington et al., 1996; Komori et al., 2010; Lim et al., 2006; Pereira et al., 2008; Yan et al., 2012). This is consistent with the calcium entry from the outside (Ardon et al., 2009) and the inhibition of calcium uptake via the mitochondrial uniporter (mCU), pathway driven by the Δψm.

Several studies indicate that PINK1 participates in mitochondrial calcium homeostasis (Gandi et al., 2009; Heeman et al., 2011), possibly regulating the mitochondrial permeability transition pore (mPTP) (Akundi et al., 2011; Gautier et al., 2012), mitochondrial Na⁺/Ca²⁺ exchanger (NCXmto) (Gandhi et al., 2009) or mCU (Marongiu et al., 2009). Isoforms NCX2 and NCX3 may act downstream of PINK1, preventing mitochondrial calcium overload (Wood-Kaczmar et al., 2013).

In the current study, we show that the increase in FL-PINK1 levels seen upon CCCP treatment is not solely due to the stabilization of the protein on the mitochondrial outer membrane and decreased degradation by the proteasome (Jin et al., 2010; Matsuda et al., 2010; Narendra et al., 2008), but also to de novo PINK1 synthesis in a calcium-dependent pathway. Moreover, CCCP induced c-Fos activation through the extracellular calcium entrance by the L-type and N-type voltage-dependent calcium channels (VDCCs).

**Material and methods**

**Cell culture**

The human SH-SY5Y neuroblastoma cell line was cultured in 1:1 (v/v) DMEM:F12 (Ham) media containing 0.9 g/l glucose and supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, non-essential amino acids, and penicillin–streptomycin. The cells were seeded at a density of 2 × 10⁶ in a 75-cm² tissue culture flask (Corning, New York, NY) and incubated at 37°C under saturating humidity in 5% CO₂/95% air.

**Treatments**

Cells were plated at a density of 1 × 10⁶ cells/ml, and treated with CCCP (10 μM, Sigma-Aldrich, St. Louis, MO) or an equivalent volume of ethanol (vehicle control, Sigma-Aldrich, St. Louis, MO) (Gegg et al., 2010). For the inhibition of transcription and translation, actinomycin D (Act. D) (5 μg/ml, Fisher Scientific, Waltham, MA) and cycloheximide (CHX) (100 μg/ml, Sigma-Aldrich, St. Louis, MO) were employed, respectively. The ubiquitin–proteasome system (UPS) and autophagy were inhibited with MG-132 (5 μM, Tocris Bioscience, Bristol, UK), and bafilomycin A1 (Baf. A1) (100 nM, Sigma-Aldrich, St. Louis, MO), respectively. BAPTA-AM (5 μM, Molecular Probes, Life Technologies, Carlsbad, CA) and EGTA (500 μM, Sigma-Aldrich, St. Louis, MO) were used to chelate intracellular calcium and extracellular calcium, respectively. To inhibit the chaperone Hsp90, cells were incubated with 17-AAG (1 μM, Calbiochem, Merck KGaA, Darmstadt, Germany). Cells were treated with all the inhibitors 1 h prior to CCCP, except 17-AAG, which was added 3 h before recovering the cells because of its toxicity. For the calcium live-cell imaging, we stimulated SH-SY5Y cells with these compounds just before the measurement: thapsigargin (1 μM, Sigma-Aldrich, St. Louis, MO), rotenone (10 μM, Sigma-Aldrich, St. Louis, MO) and Baf. A1 (100 nM) to deplete calcium stores (endoplasmic reticulum (ER), mitochondria and lysosomes, respectively); nifedipine (10 μM, Sigma-Aldrich, St. Louis, MO) and ω-conotoxin GVIA (ω-CTX) (2 μM, Alomone Labs, Jerusalem, Israel), L- and N-type VDCC blockers, respectively. In addition, cells were incubated with the following treatments for 24 h: calcium ionophore ionomycin (0.1–2 μM, Sigma-Aldrich, St. Louis, MO) and L-type VDCC agonist Bay K 8644 (0.25–5 μM, Tocris Bioscience, Bristol, UK).
Fig. 1. Mitochondrial damage and autophagy induction in SH-SY5Y CCCP-treated cells. (A) SH-SY5Y cells were exposed with 10 μM CCCP, with vehicle (0.05% (v/v) ethanol) or without any treatment (control), lysed and CS activity measured. Data are expressed as nmol/min/mg protein (Φp < 0.05; ***p < 0.001). (B and C) SH-SY5Y cells were treated with 10 μM CCCP for 0, 3 or 24 h and lysates separated by SDS-PAGE and Western-blotting performed. Blots were probed with antibodies against two inner mitochondrial membrane proteins, COX IV and prohibitin 1. β-actin was used as a loading control. (B) Representative blot of at least three independent experiments. (C) Densitometry of each band expressed as % of control. **p < 0.01, ***p < 0.001. (D and E) SH-SY5Y cells were exposed with 10 μM CCCP, with vehicle (0.05% (v/v) ethanol) or without any treatment (control) for 24 h, and stained with TMRM to assess Δψm by immunofluorescence. (D) Representative microphotographs of TMRM stain. Scale bar represents 10 μm. The arrows highlight cells with low Δψm. (E) TMRM fluorescence intensity per cell (in AU) by immunofluorescence. (D) Representative microphotographs of TMRM stain. Scale bar represents 10 μm. The arrows highlight cells with low Δψm. (E) TMRM fluorescence intensity per cell (in AU) by immunofluorescence (# p < 0.05; *** p < 0.001). (F and G) SH-SY5Y cells were exposed with 10 μM CCCP, with vehicle (0.05% (v/v) ethanol) or without any treatment (control), lysed and CS activity measured. Data are expressed as nmol/min/mg protein (# p < 0.05; ***p < 0.001). Scale bar represents 10 μm.

Citrate synthase activity

Following treatments, cells were washed with PBS and lysed in 0.25% (v/v) Triton X-100 (Sigma-Aldrich, St. Louis, MO) in PBS supplemented with protease and phosphatase inhibitors. Debris was removed by centrifugation and citrate synthase (CS) activity was measured by following the oxidation of 5.5'-Dithiobis(2-nitrobenzoic acid) (Sigma-Aldrich, St. Louis, MO) in a spectrophotometer (absorbance at 412 nm) over time at 30°C in the presence of acetyl co-enzyme A (Sigma-Aldrich, St. Louis, MO) and oxaloacetate (Sigma-Aldrich, St. Louis, MO) (Clark and Land, 1974). Protein concentration was measured based on the bicinchoninic acid (BCA) method, using a Bicinchoninic Acid Kit (Sigma-Aldrich, St. Louis, MO) using bovine serum albumin (BSA) as a standard. The enzymatic activity was expressed as nmol/min/mg protein.

Plasmid transfection

For the over-expression of PINK1 (to compare with the endogenous protein), SH-SY5Y cells were transfected with pCMV6-Neo vector containing full-length wild-type PINK1 cDNA (Origene), as previously described (Gegg et al., 2009), using FuGENE® 6 Transfection Reagent (Roche, Indianapolis, IN), according to the manufacturer’s protocol. mCherry-Parkin (Addgene plasmid 23956) (Narendra et al., 2008), GFP-LC3 (Kabeya et al., 2000) (Dr. Tamotsu Yoshimori gift) and mCherry-GFP-LC3B (Pankiv et al., 2007) (Dr. Terje Johansen gift) constructs were transfected as previously reported (Bravo-San Pedro et al., 2013).

Transient transfection with PINK1/c-fos siRNA

Cells (1.8 × 10⁵ cells/ml) were transfected with a pair of PINK1 siRNAs (5 nM each), c-fos siRNA (50 nM) or 10 nM scrambled control siRNA (Ambion negative control siRNA #1, Enzo Life Sciences, New York, NY) using HiPerfect transfection reagent (Qiagen, Crawley, UK) (for PINK1 siRNA) or Lipofectamine™ 2000 transfection reagent (Invitrogen, Life Technologies, Carlsbad, CA) (for c-fos siRNA). A combination of PINK1 siRNA pair was used (SIO0287931, sense strand: GACGCUGUUCUCGUGAUAGA; and SIO0287924, sense strand: CGACGCUGUUCUCGUGAUAGA) (Qiagen, Crawley, UK). The c-fos siRNA (SIO2781429) had the following sense strand: CCAUUAAUAACUAGAACTT (Qiagen, Crawley, UK).

Quantitative PCR

Total RNA was extracted from SH-SY5Y cells using the RNeasy Mini Kit (Qiagen, Crawley, UK). cDNA was synthesized by the QuantiTect Reverse Transcription Kit (Qiagen, Crawley, UK), adding 500 ng of template RNA. PINK1 gene expression was measured by quantitative real-time PCR (qPCR) with SYBR Green reagents (Applied Biosystems, Life Technologies, Carlsbad, CA). To normalize the results, housekeeping
gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was used. Analysis of relative gene expression was calculated using the comparative threshold (2^{−ΔΔCt}) method (Pfafl, 2001). Primer sequences were performed as previously described (Gegg et al., 2009).

Western-blotting

Protein was obtained using a lysis buffer composed of 1% (v/v) Triton X-100 in PBS supplemented with protease and phosphatase inhibitors, and quantified according to the CS activity protocol above. Equal amounts of protein (50–75 μg/condition) were resolved by 4–20% SDS-gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes (Hybond P, GE Healthcare, Chalfont St. Giles, UK) according to a partially modified conventional method (Fuentes et al., 2000). Immunodetection included transferring and blocking the membrane with TBST (Tris-buffered saline with Tween 20) buffer containing 10% non-fat dried milk. Blots were probed with antibodies against PINK1 (clone BC100-494, Novus Biologicals, Southpark Way, Littleton, CO), subunit IV of cytochrome c oxidase (COX IV, ab14744, abcam), prohibitin 1 (#2426, Cell Signaling Technology, Beverly, MA), LC3B (#2775, Cell Signaling Technology, Beverly, MA), β-actin (ab8227, Abcam, Cambridge, UK), α-tubulin (clone TU-02, Santa Cruz Biotechnology, Santa Cruz, CA), Tom20 (clone F-10, Santa Cruz Biotechnology, Santa Cruz, CA), and Lamin A/C (612162, BD Biosciences, Franklin Lakes, NJ). Blots were visualized with respective peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA) and visualized by chemiluminescence using ECL substrate (Pierce, Thermo Fisher Scientific, Rockford, IL). The β-actin and α-tubulin content was established as loading control, Tom20 as mitochondrial loading control and Lamin A/C as nuclear loading control. The density of bands was determined using ImageJ software (NIH, Bethesda, MD). For quantitative studies, three separate western blots were analyzed.

Mitochondrial isolation

Cell pellets (1 × 10^7 cells/condition) were washed with PBS supplemented with protease and phosphatase inhibitors and homogenized with a glass-teflon homogenizer in isolation medium (250 mM sucrose, 1 mM EDTA, 10 mM Tris, pH 7.4, supplemented with protease and phosphatase inhibitors). Nuclei were removed by centrifugation at
**Immunofluorescence**

To measure Δψm by this technique, cells were washed with PBS and incubated with TMRM for 30 min at 37°C. For the detection of endogenous PINK1 and mitochondrial network, cells were seeded on coverslips, fixed with paraformaldehyde (4% v/v), permeabilized with Triton X-100 solution (Triton X-100 0.2% in PBS) and stained with PINK1 (clone BC100-494, Novus Biologicals, Southpark Way, Littleton, CO) and Tom20 (clone F-10, Santa Cruz Biotechnology, Santa Cruz, CA), then cells were incubated with the respective Alexa Fluor 488 anti-rabbit, 488 anti-mouse and 568 anti-mouse secondary antibodies (Molecular Probes, Life Technologies, Carlsbad, CA). Mitochondrial morphology was determined using ImageJ software. Mitochondria were scored for their circularity, with 0 being a straight line, and 1.0 a perfect circle. In this sense, mitochondria were designated fragmented when the circularity was between 0.60 and 1.00 and tubular when between 0.00 and 0.60. For the transcription factor staining, cells were prepared in the same way, using p-c-Fos (Ser32) (#5348, Cell Signaling Technology, Beverly, MA) and c-Fos (#2250, Cell Signaling Technology, Beverly, MA). The nuclear morphology and chromatin condensation were labeled with Hoechst 33342 (Ho) (2 μM; Sigma-Aldrich, St. Louis, MO). Cells with condensed nuclei were considered not to be viable. TMRM and Ho staining were analyzed using an inverted fluorescence microscope (IX51, Olympus, Tokyo, Japan) equipped with a camera (DP70, Olympus, Tokyo, Japan). The colocalization of PINK1 with mitochondria and nuclear c-Fos, images were taken with a confocal scanning laser (Nikon A1, Tokyo, Japan) coupled to an inverted microscope (Eclipse Ti, Nikon, Tokyo, Japan). The quantitative measurement of the fluorescence signal was performed using ImageJ software. For the colocalization studies, the samples were quantified using the JaCoP plug-in for ImageJ, based in Mander’s coefficient (Bolte and Cordelieres, 2006), analyzing at least 30 cells for each condition. For the other measurements, we counted at least 200 cells per condition. In the nuclear colocalization experiments, pictures were converted into “6_shades” LUT, setting in ImageJ software.

**Measurement of intracellular calcium**

To perform calcium measurements, cells were seeded at a density of $1 \times 10^6$ cells/ml on coverslips of 14 mm. Cells were loaded with Fura-2 AM (5 μM, Molecular Probes, Life Technologies, Carlsbad, CA) for 45 min at 37°C in Locke’s K25 buffer that contained 137 mM NaCl, 5 mM KCl, 4 mM NaHCO3, 2 mM CaCl2, 1 mM MgCl2, 5 mM glucose, and 10 mM HEPES, pH 7.4, and then washed three times with Locke’s K25 buffer at 37 °C to remove extracellular Fura-2 AM. Fura-2 was excited alternatively at 340 nm and 380 nm, and emitted at 510 nm using an illumination system (MT20, Olympus, Tokyo, Japan). The ratio of fluorescence signals obtained at 340 nm and 380 nm excitation wavelengths was recorded at 2 second intervals during 10–15 min, depending on the experiment. F340/F380 ratio was used to represent the [Ca2+ ]i in the cells. The fluorescence of Fura-2-loaded SH-SY5Y cells was monitored and acquired with a CCD camera (Hamamatsu Orca-ER, Shizouka, Japan) for ratiometric imaging, mounted on an inverted microscope (IX81, Olympus, Tokyo, Japan). Images were analyzed using the software Cell^R (Olympus, Tokyo, Japan). Fields of cells and regions of interest (ROIs) were chosen based on homogenous and equal cell densities. For the calcium channel blockers (nifedipine and ω-CTX), cells were treated before they were measured as described in treatments. All the experiments were performed at 37°C.

**Nuclear isolation**

Nuclear and cytoplasmic fractions were obtained from cell lysates (1 x $10^6$ cells/condition), using the EpiQuik™ Nuclear Extration Kit (Epigentek, Farmingdale, NY). Protein concentration was determined by the BCA method assay, how it is detailed in the CS measurement.
CCCP or with vehicle (0.05% (v/v) ethanol), harvested by trypsinization at different times, mitochondria isolated and Western blotting performed. Blots were probed with antibodies against PINK1 and LC3B. α-tubulin and Tom20 were used as a cytosolic and mitochondrial loading control, respectively. (A) Representative blot of at least three independent experiments. (B) Densitometry of each band expressed in arbitrary units of intensity (***p < 0.001). Molecular mass is indicated in kDa next to the blots. (C–F) SH-SY5Y cells were transfected with mCherry-Parkin or GFP-LC3 and exposed 6, 12 or 24 h with 10 μM CCCP or with vehicle (0.05% (v/v) ethanol), fixed and immunostained for Tom20 (green/red). (C and D) Representative immunofluorescence microphotographs of 6 hour treated-cells. The boxes highlight mitochondrial localization of mCherry-Parkin and GFP-LC3, respectively. (E) Percentages of cells with mCherry-Parkin on mitochondria, labeled with anti-Tom20 antibody (***p < 0.001). (F) Percentages of cells with GFP-LC3 on mitochondria, labeled with anti-Tom20 antibody (**p < 0.001). Scale bar represents 10 μm.

Statistical analyses

Each experiment was repeated at least three times, with a satisfactory correlation between the results of individual experiments. The data shown are those of a representative experiment; each group was the average of three to four culture dishes. In each experiment, the differences between groups were assessed by appropriate statistical methods. Specifically, statistical significance was evaluated with two-tailed unpaired Student’s t-test and ANOVA test, and all comparisons with p value less than 0.05 (p < 0.05) were considered statistically significant. The data are expressed as the mean ± SEM. All data were analyzed with SPSS Statistics 20 (IBM, Chicago, IL) for Windows.

Results

Mitochondrial damage and autophagy induction by CCCP

CCCP is a lipid-soluble weak acid, which acts as a very powerful mitochondrial uncoupling agent that carries protons across the mitochondrial inner membrane, thereby diminishing the proton gradient. To investigate the molecular events in CCCP-induced depolarization, according to previous reports (D. Narendra et al., 2010; Vives-Bauza et al., 2010), cells were treated with 10 μM CCCP. The mitochondrial content in SH-SY5Y cells was assessed by measuring the CS activity (Gegg et al., 2010; Wood-Kaczmar et al., 2008). CCCP treatment produced a significant decrease in mitochondrial mass (29.2 ± 3.1%) (mean ± standard deviation [SD]) after 24 h (Fig. 1A), meanwhile a short exposure (3 h) did not result in decreased mitochondrial content. Furthermore, washing away of CCCP after 6 h of treatment, and measuring mitochondrial content 18 h later (24 h after start of treatment), no longer resulted in a significant decrease in mitochondrial content (data not shown). The decrease in CS activity was not due to a failure of the enzyme being imported in to mitochondria because of a lack of mitochondrial membrane potential, as we have previously shown that (a) knockdown of PINK1 or parkin reverses the decrease in CS activity following CCCP treatment and (b) CS loss was greater in SH-SY5Y over expressing parkin, when compared to SH-SY5Y cells with endogenous Parkin levels (Gegg et al., 2010).

To further prove that the decrease of CS activity after 24 h of CCCP treatment was due to the loss of mitochondria, we analyzed mitochondrial protein levels by Western-blotting, checking two inner mitochondrial membrane proteins, COX IV and prohibitin 1. CCCP treatment for 24 h caused a 61% reduction in prohibitin 1 and a 22% reduction in COX IV (Figs. 1B and C), reflecting the fall in mitochondrial content. To prove that CCCP produced Δψ m reduction, we measured TMRM in SH-SY5Y by immunofluorescence. In this sense, CCCP-treated cells showed a Δψ m dissipation at 24 h (10.5 ± 5.1 arbitrary units (AU) of TMRM fluorescence intensity/cell), when compared with untreated cells (37.6 ± 5.1 AU) or vehicle-treated cells (35.6 ± 8.1 AU) (Figs. 1D and E). This Δψ m decrease was observed after 1 hour CCCP exposure (data not shown), and we confirmed this result by flow cytometry (data not shown).

To confirm that CCCP is inducing autophagy in our cell model, and thereby the mechanism by which mitochondria are being degraded, we analyzed the autophagic protein LC3. LC3 presents as a cytosolic form (called LC3-I), which can then be conjugated to phosphatidylethanolamine to generate LC3-phosphatidylethanolamine conjugate (LC3-II), that is then recruited to autophagosomes. An increase in LC3-II
protein levels can be used as a marker of autophagosome formation. In this sense, we observed that the ratio between both isoforms (LC3-II/LC3-I) increased in CCCP-treated cells in a time-dependent manner, peaking at 12 h treatment (Figs. 1Fa and G). To confirm that autophagic flux is active, we measured LC3 turnover by lysosomes, using the mCherry-GFP-LC3B plasmid (GFP fluorescence is quenched by the lower pH levels in autophagolysosomes, whereas mCherry fluorescence is not). The presence of the uncoupler increased the number of red puncta (mCherry-LC3B) and, therefore, the autophagolysosomes over time (21.0 ± 3.1% and 80.3 ± 10.0% at 3 and 24 h of CCCP treatment, respectively), whereas GFP did not accumulate (Figs. 1H and I). This confirms that the increase in LC3-II protein levels seen after CCCP treatment (Figs. 1F and G) was due to increased autophagy flux.

Cytotoxic effect of CCCP

To evaluate if this mitochondrial uncoupler could induce cell death, we measured typical patterns such as chromatin condensation and nuclear membrane permeabilization. Nuclei of untreated and vehicle-treated cells had no morphologic changes, while CCCP exposure increased the number of nuclei with chromatin condensation (24.9 ± 3.0%) (Fig. 2A) and positive PI staining (19.3 ± 4.5%) 24 h after treatment (Fig. 2B).

CCCP effect on PINK1 protein levels

To further investigate the role of PINK1 in the mitochondrial damage, we determined the levels of PINK1 protein after CCCP exposure, demonstrating how the presence of the uncoupler produced a gradual and significant accumulation of endogenous FL-PINK1 protein in a time-dependent manner (Figs. 3A and B). Proteolytically processed forms of PINK1 (ΔN-PINK1) were undetectable in CCCP-treated cells. FL-PINK1 was not observed in untreated or vehicle-treated cells. However, FL-PINK1 levels fell within 15 min of CCCP washout, suggesting that its presence depends on the mitochondrial depolarization (data not shown). To confirm that the band detected was FL-PINK1, we silenced PINK1 expression (Figs. 3C and D) or over-expressed full-length wild-type PINK1 without a tag (data not shown).
CCCP-induced mitophagy is due to mitochondrial PINK1 localization

We next examined the subcellular localization of PINK1, analyzing by immunofluorescence endogenous PINK1 and co-staining mitochondria with Tom20, an outer mitochondrial membrane protein. In control and vehicle-treated cells for 24 h, endogenous PINK1 levels were low (1.2 ± 0.4 AU of PINK1 fluorescence intensity/cell in control and 1.8 ± 0.3 in vehicle-treated cells) and mitochondria showed the typical tubular structure (3.2 ± 2.9% of cells with fragmented mitochondria in control and 5.9 ± 4.3% in vehicle-treated cells). Meanwhile, in CCCP-treated cells, levels of PINK1 increased (4.4 ± 1.0 AU of fluorescence intensity/cell) (Figs. 4A and B) and the mitochondrial network was fragmented (92.1 ± 4.8%) (Figs. 4A and C), showing how there is an accumulation of PINK1 in these cells co-localized with damaged mitochondria (Figs. 4D and E) (0.54 ± 0.09 Mander's colocalization coefficient in CCCP-treated cells compared to 0.15 ± 0.05 in vehicle-treated cells).

Furthermore, we observed that PINK1 levels increased in the mitochondria-rich fraction in a time-dependent manner after CCCP treatment, whereas in the cytosolic-rich fraction no band was observed (Figs. 5A and B). We also noted that there was an increase in LC3-II levels in the mitochondria rich fraction following CCCP treatment, being higher at 12 h (Figs. 5A and B). We confirmed this mitochondrial localization of LC3 by immuno-fluorescence, using GFP-LC3 plasmid (Figs. 5A and E). A switch with previous reports, accumulation of Parkin on mitochondria following CCCP treatment occurred. However, while PINK1 continued to accumulate on damaged mitochondria after 24 h, the amount of Parkin did not follow the same pattern (Figs. 5A and D), decreasing at this time, as LC3 protein (Figs. 5A, B, D and E).

Increased PINK1 gene expression after the CCCP exposure

We assessed whether the increase of endogenous PINK1 in mitochondria after CCCP treatment was due to an increase in gene expression level or a possible chaperone-mediated stabilization. For this purpose, we measured the mRNA levels of PINK1 by qPCR. In a short period of time (3 h), there were no differences, but we observed a significant increase of the PINK1 mRNA in a time-dependent manner, and was significantly higher at 6 (1.76 ± 0.22 times more than vehicle-treated cells at 3 h) and 24 h after CCCP treatment (4.01 ± 0.66 times...
more than vehicle-treated cells at 3 h) (Fig. 6A). These results were consistent when we studied the blockade of transcription and translation, using Act. D and CHX, respectively. The treatment with both inhibitors abolished expression of endogenous FL-PINK1 protein (Figs. 6B and C).

Also, we investigated degradation mechanisms, such as UPS and autophagy, modified PINK1 protein levels using MG-132 and Baf. A1, respectively. In this sense, there was an accumulation of ΔN-PINK1, but not FL-PINK1, in untreated and vehicle-treated cells after the treatment of MG-132, as previously reported (Lin and Kang, 2008; Takatori et al., 2008). Following CCCP treatment, ΔN-PINK1 levels were decreased, which was expected as FL-PINK1 accumulates on the outer mitochondrial membrane, thus reducing the amount of PINK1 available for cleavage by proteases in the inner mitochondrial membrane. In contrast, the levels of FL-PINK1 were further increased when we treated cells with CCCP and inhibited the autophagy mechanism with Baf. A1 (blocking autophagosome/lysosome fusion) (Figs. 6D and E). Western-blotting for COX IV and LC3-I protein levels indicated that Baf. A1 prevented the CCCP-induced decrease in mitochondrial content (Figs. 6D and F) and inhibited autophagy flux (Fig. S1). This suggests that the further increase in FL-PINK1 levels after CCCP and Baf. A1 treatment was most likely due to a greater number of mitochondria on which PINK1 could accumulate. Finally, FL-PINK1 protein was still increased by CCCP in the presence of 17-AAG, an inhibitor of Hsp90 (Figs. 6D and E), further suggesting that the increase of FL-PINK1 at 24 h was due to de novo synthesis, rather than increased stabilization of PINK1 (Lin and Kang, 2008).

Involvement of extracellular calcium in PINK1 levels

To examine whether the treatment with CCCP stimulated calcium signaling and if this is associated with increased transcription of PINK1, leading to the accumulation of FL-PINK1 on mitochondria, we measured intracellular calcium mobilization in SH-SY5Y cells using the ratiometric indicator Fura-2 AM. We found that the presence of CCCP did not modify the release of calcium from an intracellular store (Fig. 7A), but produced the entry of extracellular calcium and consequently an elevation in cytosolic calcium levels immediately after treatment (Fig. 7B). Next, we evaluated the mechanism of extracellular calcium entry. We investigated whether calcium entry was dependent on L- and N-type VDCCs, which are well characterized in SH-SY5Y cells (Zhang et al., 2009) and have been implicated in PD pathogenesis (Goldberg et al., 2012; Ilijic et al., 2011). We used 10 μM nifedipine and 2 μM ω-CTX, and showed that the treatment with these VDCC blockers significantly reduced extracellular calcium entry (Figs. 7C and D). Moreover, the blockade of intracellular calcium release from stores such as ER (using thapsigargin), mitochondria (with rotenone) and lysosomes (using Baf. A1) did not prevent the initial increase in intracellular calcium observed after treatment with CCCP (data not shown).

Next, we analyzed the role of this intracellular calcium elevation after the CCCP treatment on the PINK1 levels and its possible importance in the PINK1 mitochondrial localization. We observed that calcium chelation with BAPTA-AM decreased FL-PINK1 levels after CCCP treatment, both in cell lysates (Figs. 7E and F) and isolated mitochondria (Figs. 7G and H). Chelation of calcium by BAPTA-AM was confirmed by flow cytometry (Fig. S2). Furthermore, to assess whether cytosolic calcium increase could mimic the CCCP effects on PINK1 protein, cells were exposed to the calcium ionophore ionomycin and the L-type VDCC agonist Bay K 8644, showing that both did not modify FL-PINK1 protein levels, comparable to vehicle-treated cells (Fig. S3).

PINK1 calcium-dependent gene expression

To elucidate if extracellular calcium increases the PINK1 mitochondrial localization by enhancing its gene expression, PINK1 mRNA levels were measured by qPCR. PINK1 mRNA levels were significantly decreased after 6 h of CCCP treatment when cytosolic calcium was eliminated by BAPTA-AM (0.69 ± 0.09 times less than 6 hour CCCP-treated cells) or extracellular calcium in the medium was chelated by EGTA (0.77 ± 0.10 times less than 6 hour CCCP-treated cells) (Fig. 8A). But, if the exposure is longer (24 h), these rates did not modulate when we sequestered cytosolic (0.18 ± 0.25 times less than CCCP-treated cells) and extracellular calcium (0.09 ± 0.62 times less than CCCP-treated cells) (Fig. 8B). However, it would appear that decreased transcription of PINK1 mRNA by BAPTA-AM or EGTA for at least 6 h was sufficient to decrease FL-PINK1 protein levels after 24 h of CCCP treatment (Figs. 7E–H, 8C and D).

Nuclear recruitment and activation of c-Fos after the CCCP treatment

To further evaluate the role of calcium in PINK1 expression, we performed some experiments to elucidate whether this higher gene expression under CCCP treatment is modulated by a transcription factor, following activation by calcium. Bioinformatic analysis of the PINK1
promoter indicated four c-Fos response elements (Fig. S4). We observed that CCCP treatment increased the phosphorylation of c-Fos at residue Ser32 between 3 and 6 h, when compared with cells treated with the vehicle (Figs. 9A and B). Immunofluorescence of c-Fos also showed that CCCP treatment produced higher levels of p-c-Fos (Ser32) (7.06 ± 2.85 AU of nuclear fluorescence intensity/cell), compared to vehicle-treated cells (2.84 ± 1.27 AU) (Figs. 9C and D). The nuclear localization of c-Fos was also increased by CCCP treatment (58.22 ± 11.02% of CCCP-treated cells vs. 11.36 ± 5.80% of vehicle-treated cells) (Figs. 9D and F). The nuclear translocation of c-Fos was also confirmed by Western-blotting (Figs. 9G and H). We analyzed other factors modulated by calcium, such as CREB, c-Jun, FOXO3a and NFκB, but obtained inconclusive results (data not shown).

c-Fos calcium-dependent signaling acts in parallel to PINK1 expression after CCCP exposure

To elucidate if extracellular calcium enhanced PINK1 expression via c-Fos activation, firstly, we analyzed the phosphorylation of c-Fos at residue Ser32 between 3 and 6 h, when compared with cells treated with the vehicle (Figs. 9A and B). Immunofluorescence of c-Fos also showed that CCCP treatment produced higher levels of p-c-Fos (Ser32) (7.06 ± 2.85 AU of nuclear fluorescence intensity/cell), compared to vehicle-treated cells (2.84 ± 1.27 AU) (Figs. 9C and D). The nuclear localization of c-Fos was also increased by CCCP treatment (58.22 ± 11.02% of CCCP-treated cells vs. 11.36 ± 5.80% of vehicle-treated cells) (Figs. 9D and F). The nuclear translocation of c-Fos was also confirmed by Western-blotting (Figs. 9G and H). We analyzed other factors modulated by calcium, such as CREB, c-Jun, FOXO3a and NFκB, but obtained inconclusive results (data not shown).

Discussion

We report that depolarization of mitochondria with CCCP in SH-SY5Y cells results in a calcium-dependent increase in transcription of PINK1.
Intracellular calcium levels. Treatment of SH-SY5Y cells with CCCP increased calcium levels within the cell, with uptake of calcium into cells via the L- and N-type VDCCs as a major component. In this sense, higher levels of intracellular calcium after FCCP (CCCP analog) exposure are reduced in calcium-free solution or by VDCC blockade (Buckler and Vaughan-Jones, 1998; Sato, 1997). More sensitive techniques are required to determine if small local increases in cytosolic calcium could also be caused as a result of increased mitochondrial release upon depolarization (Tonkikh and Carlen, 2009). The increase in PINK1 expression did not occur when intracellular calcium was increased by ionomycin or an L-VDCC agonist. Ionomycin treatment has previously been shown not to result in the phosphorylation of Ser65 of Parkin by PINK1, an early event in mitophagy (Kondapalli et al., 2012).

Higher PINK1 expression could be linked with a compensatory increase in mitochondrial biogenesis. The induction of mitophagy would require the cell to initiate synthesis of new mitochondria to replace them. PINK1 has been linked with mitochondrial biogenesis, with knockdown of PINK1 in SH-SY5Y cells decreasing mtDNA levels and mtDNA synthesis (Gegg et al., 2009). Induction of mitochondrial uncoupling proteins can induce mitochondrial biogenesis via the transcription factor Nrf1 (Li et al., 1999). Therefore it is unclear whether any putative increase in mitochondrial biogenesis following CCCP treatment is a direct response to mitochondrial uncoupling or the removal of mitochondria.

This increase in transcription appeared to be due to an increase in intracellular calcium levels. Treatment of SH-SY5Y cells with CCCP increased calcium levels within the cell, with uptake of calcium into cells via the L- and N-type VDCCs as a major component. In this sense, higher levels of intracellular calcium after FCCP (CCCP analog) exposure are reduced in calcium-free solution or by VDCC blockade (Buckler and Vaughan-Jones, 1998; Sato, 1997). More sensitive techniques are required to determine if small local increases in cytosolic calcium could also be caused as a result of increased mitochondrial release upon depolarization (Tonkikh and Carlen, 2009). The increase in PINK1 expression did not occur when intracellular calcium was increased by ionomycin or an L-VDCC agonist. Ionomycin treatment has previously been shown not to result in the phosphorylation of Ser65 of Parkin by PINK1, an early event in mitophagy (Kondapalli et al., 2012). We suspect that we did not see the increase in PINK1 transcription
with these two treatments because the mitochondria were still healthy, and were thus still able to buffer the calcium overload generated by ionophores or the L-VDCC agonist. While treatment with calcium chelators prevented loss of mitochondrial content following 24 h of CCCP treatment, further work is required to determine whether this is directly due to the decrease in PINK1 transcription, or other effects of decreased calcium on other components of the autophagy machinery.

Therefore, the induction of PINK1 expression might simply be a protective measure against the increased cytosolic calcium observed after CCCP treatment. Indeed, there was an increase in nuclear condensation and PI-positive cells following 24 h of CCCP treatment. PINK1 expression is known to protect against a variety of stressors including staurosporine, MPTP and oxidative stress (Gegg et al., 2009; Haque et al., 2008; Wood-Kaczmar et al., 2008). Deregression of mitochondrial calcium homeostasis is also associated with loss of PINK1 function (Abramov et al., 2011; Akundi et al., 2011; Gandhi et al., 2009; Heeman et al., 2011), suggesting that increased mitochondrial localization of PINK1 could lead to maintain functional interactions, activating channels and transporters along the mitochondrial network such as mPTP and NCXmito, to regulate calcium homeostasis and for instance, minimize potential excitotoxic effects. Very recently, a report has shown that PINK1 could prevent mitochondrial calcium overload through the activation of isoforms NCX2 and NCX3 (Wood-Kaczmar et al., 2013). Related to this, the Parkin mitochondrial localization enhances ER-mitochondria crosstalk, favoring the calcium transfer (Cali et al., 2013). Moreover, our results suggest that calcium overload could be necessary for the mitochondrial removal by PINK1/Parkin-mediated mitophagy (Narendra et al., 2008; D.P. Narendra et al., 2010).

The identity of the transcription factor responsible for the increased PINK1 transcription is unknown. FOXO3a increased PINK1 transcription in T-lymphocytes, MCF7 and SH-SY5Y cells upon growth factor/siRNA deprivation (Mei et al., 2009). PINK1 transcription was also no longer increased following ischemia in FOX01 and FOX03 knock-out mice (Sengupta et al., 2011). However there was no evidence of FOXO3a increasing PINK1 transcription following treatment with CCCP. In this report, while we found that c-Fos translocated to the nucleus upon CCCP exposure, this had no effect on PINK1 protein levels. It is well-known that c-Fos is activated after pathological situations, such as hypoxia or ischemia (Choi et al., 2001), mediated by L- and N-type VDCCs (Premkumar et al., 2000; Zhao et al., 2007), related to our results, c-Fos might be involved in adaptive responses upon calcium overload and mitochondrial depolarization promoting neuronal survival.

**Conclusions**

This study shows that treatment of SH-SY5Y cells with CCCP increases PINK1 gene expression for at least 24 h, leading to a large accumulation of PINK1 protein on damaged mitochondria. This increase was calcium-dependent and might be via an influx of calcium via L- and N-type VDCCs, allowing for the mitochondrial removal by mitophagy. Moreover, the transcription factor c-Fos was activated by calcium, although it was not responsible for induction of PINK1 transcription. The induction of PINK1 expression and activation of c-Fos could be a mechanism by which the cell activates neuroprotective mechanisms that might result in restoring calcium homeostasis and/or promote mitochondrial biogenesis (Fig. 12).

**Abbreviations**

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Act. D</td>
<td>actinomycin D</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein-1</td>
</tr>
<tr>
<td>Ba.f.A1</td>
<td>bafilomycin A1</td>
</tr>
<tr>
<td>BAPTA-AM</td>
<td>1,2-bis-(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid acetoxymethyl ester</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>c-Fos</td>
<td>FBJ murine osteosarcoma viral oncogene homolog</td>
</tr>
<tr>
<td>CCCP</td>
<td>carbonyl cyanide m-chlorophenylhydrazone</td>
</tr>
<tr>
<td>CHX</td>
<td>cycloheximide</td>
</tr>
<tr>
<td>CREB</td>
<td>cyclic AMP response element binding protein</td>
</tr>
<tr>
<td>CS</td>
<td>citrate synthase</td>
</tr>
<tr>
<td>Δψm</td>
<td>mitochondrial membrane potential</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene-bis(oxyethylenenitriilo)tetraacetic acid tetrasodium</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FCCP</td>
<td>carbonyl cyanide p-trifluoromethoxyphenylhydrazone</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>Ho</td>
<td>Hoechst 33342</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>LC3</td>
<td>microtubule-associated protein 1 light chain 3</td>
</tr>
<tr>
<td>mCU</td>
<td>mitochondrial uniporter</td>
</tr>
<tr>
<td>MG–132</td>
<td>carbobenzoxyl-leucyl-leucyl-leucinal</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>mPTP</td>
<td>mitochondrial permeability transition pore</td>
</tr>
<tr>
<td>NCXm</td>
<td>mitochondrial Na⁺/Ca²⁺ exchanger</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor-κB</td>
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Fig. 12. Schematic representation of CCCP effect in SH-SY5Y cells and the interplay between PINK1 and calcium. In physiological situations, Δψm is high, so FL-PINK1 is processed in mitochondria by proteases, including PARL. The cleaved isoforms are then degraded by UPS. However, when cells are exposed to CCCP, calcium influx through L- and N-type VDCCs increase PINK1 gene expression, leading higher levels of PINK1 protein on damaged mitochondria, which might increase mitochondrial degradation by mitophagy (pink line) and/or modulate the activity of NCxmito, mPTP and/or mCU to control the mitochondrial calcium overload (purple line). This calcium influx also causes c-Fos activation, which probably translocates to the nucleus to promote neuroprotective signaling.


