Mutations in leucine-rich repeat kinase 2 (LRRK2) are the most common cause of familial Parkinson’s disease. We found LRRK2 to be degraded by chaperone-mediated autophagy (CMA), whereas the most common pathogenic mutant form of LRRK2, G2019S, was poorly degraded by this pathway. In contrast to the behavior of typical CMA substrates, lysosomal binding of both wild-type and several pathogenic mutant LRRK2 proteins was enhanced in the presence of other CMA substrates, which interfered with the organization of the CMA translocation complex, resulting in defective CMA. Cells responded to such LRRK2-mediated CMA compromise by increasing levels of the CMA lysosomal receptor, as seen in neuronal cultures and brains of LRRK2 transgenic mice, induced pluripotent stem cell–derived dopaminergic neurons and brains of Parkinson’s disease patients with LRRK2 mutations. This newly described LRRK2 self-perpetuating inhibitory effect on CMA could underlie toxicity in Parkinson’s disease by compromising the degradation of α-synuclein, another Parkinson’s disease–related protein degraded by this pathway.

Interplay of LRRK2 with chaperone-mediated autophagy

Samantha J Orenstein1,2, Sheng-Han Kuo3,13, Immaculada Tasset1,2,13, Esperanza Arias1,2, Hiroshi Koga1,2, Irene Fernandez-Carasa4, Etty Cortes3,5, Lawrence S Honig3,5, William Dauer6, Antonella Consiglio4,7, Angel Raya4–10, David Sulzer3,11,12 & Ana Maria Cuervo1,2

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In contrast to the studies reporting effects of LRRK2 on macroautophagy, effects of LRRK2 on CMA are unknown. CMA involves the direct transport of cytosolic soluble proteins across the lysosomal membrane in a selective fashion16,17. CMA substrates contain a pentapeptide motif18 that is recognized by the heat shock cognate protein of 70 kDa (hsc70). Hsc70 targets substrate proteins to the lysosomal membrane19, where they interact with the lysosome-associated membrane protein type 2A (LAMP-2A)20. Once the substrate binds, LAMP-2A multimerizes to form the translocation complex21. LAMP-2A dynamics are also regulated by mobilization in and out of discrete lipid microdomains where degradation of LAMP-2A takes place22,23.

Previous studies have established a connection between CMA and Parkinson’s disease, as wild-type α-syn is a substrate for CMA8, whereas pathogenic forms of this protein bind abnormally to the lysosomal receptor for CMA, thereby preventing their own degradation and the degradation of other CMA substrates7,8,24. Another Parkinson’s disease–associated protein, ubiquitin C-terminal hydrolase L1 (UCH-L1), interacts with CMA components, and this interaction is abnormally enhanced in a UCH-L1 mutant25. Alterations in CMA may in fact be a common feature in many forms of Parkinson’s disease, as post-mortem brain samples from Parkinson’s disease patients show reduced levels of LAMP-2A in the substantia nigra26. We noted that the LRRK2 protein bears eight

1Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, New York, USA. 2Institute for Aging Studies, Albert Einstein College of Medicine, Bronx, New York, USA. 3Department of Neurology, Columbia University Medical Center, New York, New York, USA. 4Institute for Biomedicine (IBUB), University of Barcelona, Barcelona, Spain. 5Taub Institute, Columbia University Medical School, New York, New York, USA. 6Department of Cell and Developmental Biology, University of Michigan, Ann Arbor, Michigan, USA. 7Department of Biomedical Science and Biotechnology, University of Brescia, Brescia, Italy. 8Control of Stem Cell Potency Group, Institute for Bioengineering of Catalonia (IBEC), Barcelona, Spain. 9Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain. 10Center for Networked Biomedical Research on Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Barcelona, Spain. 11Department of Psychiatry, Columbia University Medical School, New York, USA. 12Department of Pharmacology, Columbia University Medical Center, New York, New York, USA. 13These authors contributed equally to this work. Correspondence should be addressed to A.M.C. (ana-maria.cuervo@einstein.yu.edu) or D.S. (ds43@columbia.edu).

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Figure 1 LRRK2 is degraded in lysosomes. (a) Top: immunoblots for the indicated proteins from two independent experiments. Bottom: quantification of changes in total LRRK2 (n = 6 independent experiments). SH-SYSY cells were untreated or incubated with 5 mM lactacystin (Lact), 10 mM 3MA or 20 mM NH4Cl and 100 µM leupeptin (NL) for 9.5 h. (b) Top: representative immunoblots for the indicated proteins from two independent experiments. Bottom: quantification of LRRK2 relative to that in untreated samples (n = 3 or 4 independent experiments). Brain slices from mouse cortex, midbrain or cerebellum were incubated without additions or with NL or 3MA for 2 h at 37 °C. (c,d) Homogenate (Hom) and lysosomal fractions (Lys) isolated from SH-SYSY cells (c) or the indicated mouse brain regions (d), immunoblotted for the indicated proteins. (e) HEK293 cells expressing GFP-tagged LRRK2 and transduced with lentivirus control (Ctr) or carrying shRNA against LAMP-2A (L2A(−)), incubated or not with NL for 12 h and immunoblotted for the indicated proteins. (f) Immunoblots for LRRK2 and LAMP-2A of homogenates and lysosome-enriched fractions isolated from control fibroblasts (Ctr) or fibroblasts stably interfered for LAMP-2A (L2A(−)), maintained in the presence or absence of serum. Actin and LAMP-1 (L-1) are shown as loading controls of homogenate and lysosomes, respectively. All values are mean ± s.e.m.; *P < 0.05, unpaired t-test. Full-length blots and gels in Supplementary Figure 12.

putative CMA motifs in its amino acid sequence and decided to determine whether LRRK2 might be a CMA substrate, and how the most common LRRK2 mutation (G2019S) could affect its interaction with CMA.

We found that a fraction of cellular LRRK2 could be degraded by CMA, but also that a range of pathogenic mutants, including the common G2019S LRRK2 allele, as well as high concentrations of wild-type LRRK2, inhibited CMA. This inhibition occurred through a new mechanism, involving a blockade in the formation of the CMA translocation complex at the lysosomal membrane. Cells responded to the LRRK2-mediated blockage of CMA by increasing the amount of the essential component of the translocation complex, LAMP-2A, a phenomenon we also observed in the brains of LRRK2 transgenic mice and in induced pluripotent stem cell (iPSC)-derived dopaminergic neurons and brains from LRRK2-mutation Parkinson’s disease patients. One of the proteins affected by the inhibitory effect of LRRK2 on CMA was α-syn. Thus, two of the dominant mutations that cause Parkinson’s disease converge mechanistically by inhibiting the normal degradation of cytosolic proteins by CMA.

RESULTS

LRRK2 can be degraded in lysosomes

The contribution of the lysosomal system to the degradation of LRRK2 itself has not been systematically explored. On treatment of neuroblastoma cells with lactacystin (a proteasome inhibitor), 3-methyladenine (3MA; to inhibit macroautophagy) or a combination of ammonium chloride and leupeptin (to inhibit total lysosomal proteolysis), we confirmed that a portion of cellular LRRK2 was degraded by the proteasome, but we also found that part of LRRK2 was degraded in lysosomes, as intracellular LRRK2 increased when lysosomal proteolysis was blocked (Fig. 1a). Lysosomal degradation of LRRK2 did not occur through macroautophagy, because LRRK2 did not increase on 3MA treatment (Fig. 1a and Supplementary Fig. 1a,b).

Lysosomal degradation of LRRK2 also occurs in vivo, as we found a marked increase in LRRK2 in slices of mouse cortex, midbrain and cerebellum after incubation with NH4Cl-leupeptin (Fig. 1b). In further support of these findings, we could detect LRRK2 in lysosomes isolated from neuronal cells in culture (Fig. 1c) and from mouse and rat brain regions (Fig. 1d and data not shown). The lack of inhibition of LRRK2 degradation by 3MA in the brain slices (Fig. 1) and the absence of LRRK2 in autophagosomes isolated from the brains of these animals (Supplementary Fig. 1c) suggest that macroautophagy does not contribute substantially to LRRK2 degradation in brain, at least under normal conditions.

Instead, we found that cells in which LAMP-2A, the CMA lysosomal receptor, was knocked down showed increased intracellular LRRK2 due, at least in part, to reduced LRRK2 degradation by lysosomes, as shown by the lack of effect of NH4Cl-leupeptin treatment on LRRK2 levels in these cells (Fig. 1e). Furthermore, the amount of LRRK2 bound to lysosomes isolated from LAMP-2A knockdown cells was much lower than in control cells (Fig. 1f; notice that persistent LAMP-2A knockdown reduced LRRK2 initial accumulation, likely through activation of other proteolytic systems). We conclude that a fraction of intracellular LRRK2 normally is degraded in lysosomes, probably via CMA.

Selective degradation of wild-type LRRK2 by CMA

The presence in the amino acid sequence of LRRK2 of eight putative CMA substrate proteins, including the common G2019S LRRK2 allele, as well as high concentrations of wild-type LRRK2, inhibited CMA. This inhibition occurred through a new mechanism, involving a blockade in the formation of the CMA translocation complex at the lysosomal membrane. Cells responded to the LRRK2-mediated blockage of CMA by increasing the amount of the essential component of the translocation complex, LAMP-2A, a phenomenon we also observed in the brains of LRRK2 transgenic mice and in induced pluripotent stem cell (iPSC)-derived dopaminergic neurons and brains from LRRK2-mutation Parkinson’s disease patients. One of the proteins affected by the inhibitory effect of LRRK2 on CMA was α-syn. Thus, two of the dominant mutations that cause Parkinson’s disease converge mechanistically by inhibiting the normal degradation of cytosolic proteins by CMA.

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Selective degradation of wild-type LRRK2 by CMA

The presence in the amino acid sequence of LRRK2 of eight putative CMA targeting motifs (Fig. 2a) further increased our confidence that CMA may contribute to degradation of this protein. However, the most convincing test to confirm a protein as a CMA substrate is to directly demonstrate its uptake into isolated lysosomes (Supplementary Fig. 1a). We first used lysosomes isolated from rat liver to avoid possible interference from the endogenous LRRK2 detected in the brain lysosomes (Fig. 1d). Incubation of purified LRRK2 with intact liver lysosomes untreated or pretreated with protease inhibitors showed that LRRK2 bound to the lysosomal membrane and was selectively taken up by lysosomes (Fig. 2b). Uptake was calculated as the increase in lysosome-associated LRRK2 when lysosomes were pretreated with protease inhibitors. As noted for other CMA substrate proteins, increasing concentrations of LRRK2 competed with the lysosomal uptake and

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degradation of a pool of cytosolic proteins substrates for CMA (Fig. 2c) and the binding to the lysosomal membrane of the well-established CMA substrate glyceraldehyde-3-phosphate dehydrogenase (GAPDH)30 (Fig. 2d).

We next assessed the effect on LRRK2 CMA of the CMA substrate proteins GAPDH (Fig. 2e) and ribonuclease A (RNase A; Fig. 2f) and of negative control proteins that lack any lysosomal targeting motif. To our surprise, and in clear contrast with the competition observed between conventional CMA substrates (GAPDH and RNase A shown here), CMA substrates did not compete with LRRK2's lysosomal association but instead markedly increased the amount of LRRK2 bound to lysosomes in the absence of negative control proteins that lack any lysosomal targeting motif. Association but instead markedly increased the amount of LRRK2 bound to lysosomes in the absence of negative control proteins that lack any lysosomal targeting motif.

Inp: 1 µg of GAPDH. (e,f) Association of LRRK2 to RNase A (e) or RNase A (f) with PI-treated rat liver lysosomes incubated with increasing concentrations of GAPDH (e) or RNase A (f) or ovalbumin (Ov). Left: representative immunoblots. Right: protein association expressed as a percentage of the value when incubated with lysosomes alone (n = 5 or 6 independent experiments). Inp: RNase A (0.4 µg), LRRK2 (0.04 µg) or GAPDH (1 µg).

(g,h) Association of LRRK2 to lysosomes incubated with GAPDH (g) or RNase A (h) at 4 °C (n = 3 independent experiments). (i,j) Immunoblots of brain lysosomes incubated with LRRK2 and increasing concentrations of GAPDH (i) or RNase A (j). All values are mean ± s.e.m.; differences with lysosomes incubated with the protein alone were significant at *P < 0.05, unpaired t-test. Full-length blots and gels in Supplementary Figure 12.

Compromised degradation of mutant LRRK2

The most common pathogenic LRRK2 mutation is G2019S, a substitution in the kinase domain that results in a toxic gain of kinase function2. Using LRRK2-tet-on cell lines31, in which expression of wild-type (WT) or G2019S (G/S) LRRK2 can be activated by doxycycline (Supplementary Fig. 3a), we induced expression by a 12- to 15-fold increase in protein levels and found that G/S LRRK2 expression correlated with the increase in protein levels and found that G/S LRRK2 expression correlated with the increase in kinase activity (Fig. 3b). CMA substrates, including GAPDH and RNase A, degraded to a lower extent in G/S LRRK2-expressing cells compared to WT LRRK2 cells (Fig. 3c, Supplementary Fig. 3b). CMA substrates, including GAPDH and RNase A, degraded to a lower extent in G/S LRRK2-expressing cells compared to WT LRRK2 cells (Fig. 3c, Supplementary Fig. 3b). CMA substrates, including GAPDH and RNase A, degraded to a lower extent in G/S LRRK2-expressing cells compared to WT LRRK2 cells (Fig. 3c, Supplementary Fig. 3b). CMA substrates, including GAPDH and RNase A, degraded to a lower extent in G/S LRRK2-expressing cells compared to WT LRRK2 cells (Fig. 3c, Supplementary Fig. 3b). CMA substrates, including GAPDH and RNase A, degraded to a lower extent in G/S LRRK2-expressing cells compared to WT LRRK2 cells (Fig. 3c, Supplementary Fig. 3b). CMA substrates, including GAPDH and RNase A, degraded to a lower extent in G/S LRRK2-expressing cells compared to WT LRRK2 cells (Fig. 3c, Supplementary Fig. 3b). CMA substrates, including GAPDH and RNase A, degraded to a lower extent in G/S LRRK2-expressing cells compared to WT LRRK2 cells (Fig. 3c, Supplementary Fig. 3b). CMA substrates, including GAPDH and RNase A, degraded to a lower extent in G/S LRRK2-expressing cells compared to WT LRRK2 cells (Fig. 3c, Supplementary Fig. 3b).
24-h pulse of doxycycline and followed the decrease in the recombinant proteins 48 h after the pulse to confirm that synthesis was no longer occurring. As with endogenous LRRK2 in neuroblastoma cells (Fig. 1a), levels of the WT recombinant protein (Fig. 3a) and of endogenous LRRK2 in these cells (Supplementary Fig. 3b) were increased by proteasome inhibitors (MG132) and lysosome inhibitors (NH₄Cl-leupeptin), indicating degradation of LRRK2 by both pathways. Levels of G/S LRRK2 were also higher after addition of proteasome and lysosome inhibitors, but the effect of blocking lysosomal degradation was smaller (Fig. 3a). Treatment with 3MA had a similar effect on G/S LRRK2 as treatment with inhibitors of lysosomal proteolysis, suggesting that the residual lysosomal degradation observed for the mutant was likely taking place mainly by macroautophagy. We also detected higher sensitivity to 3MA in slices from brains of G/S-LRRK2 transgenic mice than from control or WT-LRRK2 transgenic mice (Supplementary Fig. 1d). WT LRRK2 had a half-life of approximately 38 h, whereas the half-life of G/S LRRK2 was nearly doubled (60 h) (Fig. 3b). Knockdown of LAMP-2A reduced the degradation of WT LRRK2 by 50% but had only a modest effect on the degradation of G/S LRRK2 (Fig. 3b). Together, these findings suggest that the degradation of G/S LRRK2 by CMA is compromised and that the apparent switch of the mutant protein toward degradation by the proteasome and, for a small fraction, by macroautophagy is not sufficient to maintain the rates of degradation normally observed for the WT protein.

We then compared the direct uptake of purified WT and G/S LRRK2 by isolated liver lysosomes after verifying that G/S LRRK2 bound the lysosomal degradation substrates CMA substrate proteins, and this effect was more prominent than that of endogenous LRRK2 in these cells (Fig. 3c). Binding of WT and G/S LRRK2 to lysosomes in the presence of GAPDH (Fig. 3e) Binding of WT and G/S LRRK2 to lysosomes in the presence of GAPDH (e) or RNase A (f). Top: representative immunoblots. Bottom: lysosome-bound LRRK2 expressed as a multiple of the amount bound when added alone. Trends of mean values (n = 2 independent experiments); error bars indicate range. (g) Immunoblot of rat brain lysosomes (Lysosom) incubated with WT or G/S LRRK2 as in c. Inputs, one-tenth of protein added. (h,i) Rat brain lysosomes incubated as in d and e, respectively. Left: representative immunoblots. Right: quantifications (n = 3 or 4 independent experiments). All values expressed as mean ± s.e.m.; differences with untreated samples (*) and between WT LRRK2 and G/S LRRK2 (§) were significant at P < 0.05; n.s., not significant; ANOVA and Bonferroni test. Full-length blots and gels in Supplementary Figure 12.

![Figure 3](image-url)
Inhibitory effect of LRRK2 on CMA

To determine the cellular consequences of higher association of LRRK2 with the lysosomal membrane in the presence of CMA substrates (Fig. 2), of the exacerbation of this binding for G/S LRRK2 (Fig. 3g), and of the greater inhibition by the LRRK2 mutant on lysosomal uptake of CMA substrates (Fig. 3i) when compared to WT LRRK2. We attribute the small differences between liver and brain lysosomes to the presence of endogenous WT LRRK2 in the brain lysosomes (Fig. 3g). Independent of these small tissue-specific differences, overall these results indicate that G/S LRRK2 is less efficiently degraded by CMA but exerts greater inhibition over this pathway.

We then analyzed CMA in the three groups of cells by transfecting them with a photoactivatable (PA) fluorescent reporter (KFERQ-PA-mCherry1) that provides visualization of CMA as a change in the distribution of the fluorescent protein from the cytosol (diffuse fluorescent pattern) to lysosomes (punctate fluorescent pattern). Cells expressing WT and G/S LRRK2 both showed a marked decrease in the number of fluorescent puncta per cell in basal conditions in comparison to control cells (Fig. 4b), and they failed to induce CMA on removal of serum from the culture medium (Fig. 4b). We conclude that the decrease in degradation of long-lived proteins observed in cells expressing WT and G/S LRRK2 was due, for the most part, to their inhibitory effect on CMA.

We found a similar inhibitory effect on CMA for G/S LRRK2 in neurons. Analysis of CMA activity with the same fluorescent reporter in primary neuronal cultures revealed a significant reduction in CMA activity from G/S-LRRK2 transgenic mice in comparison to control mice (Fig. 4c). Notably, although a compromise of LRRK2 lysosomal degradation was not evident in brains of WT-LRRK2 transgenic mice (Supplementary Fig. 1d), we found that CMA activity was significantly reduced in these neurons in comparison to those from control mice (Fig. 4c). Together, these results indicate that both G/S LRRK2 and high levels of WT LRRK2 inhibit CMA in neuronal and non-neuronal cells.

Effect of LRRK2 on CMA components

Recent studies in Drosophila have noted that expression of G/S LRRK2 results in perinuclear clustering of endosomal and lysosomal compartments. Labeling of late endosomes and lysosomes with LysoTracker and LAMP-1 (Supplementary Fig. 5a,b) confirmed the described abnormal expansions of these vesicular structures not
Figure 5 Interplay of LRRK2 with CMA components. (a) Immunoblots of rat liver lysosomes incubated with WT or G/S LRRK2 alone or with 1 mM GTP and/or GAPDH. Duplicate samples are shown. Bottom: quantification of lysosome-bound LRRK2 expressed as a multiple of the amount bound without additions (n = 6 independent experiments). (b) Effect of kinase inhibitor on WT, G/S, or the kinase-dead mutant D/A LRRK2 incubated with rat liver lysosomes in presence or absence of GAPDH (5 µg). Right: quantification expressed as a multiple of the association without additions (n = 3–5 independent experiments). (c,d) Transient transfection of HEK293 cells with the myc- or GFP-tagged LRRK2 constructs indicated at top left in c. Full, full length; M, KFERQ-like motifs (M1–M8). (e) Binding to GST-hsc70. Left, representative immunoblots; input lanes, one-tenth of input material. NT, non-transfected cells. Immunoblot for hsc70 is shown as a loading control. Top right: quantification of lysosome-bound LRRK2 expressed as a multiple of WT value. (f) Co-IP of LAMP-2A with anti-LRRK2 in lysosomes incubated with WT or G/S LRRK2. Inp, one-quarter of starting sample; IgG, immunoglobulin G. Values: LAMP-2A bound without additions (–) or with WT or G/S LRRK2 (g) or with lysosomes from control (Ctrl) or from WT-LRRK2 or G/S-LRRK2 transgenic mice (h) and subjected to blue native electrophoresis. Arrow, 700-kDa multimeric complex. All values are mean ± s.e.m.; differences compared to none or untreated (*) or to WT or full-length protein (§) were significant at P < 0.05; unpaired t-test in a and ANOVA and Bonferroni test in b,c. Full-length blots and gels in Supplementary Figure 12.

The lack of marked changes in the number or distribution of CMA-active lysosomes led us to hypothesize that the inhibitory effect of LRRK2 on CMA did not occur at the level of formation and maturation of the CMA lysosomes but rather was a result of the direct interaction of LRRK2 with these lysosomes. To dissect the inhibitory effect of G/S LRRK2 and that of high levels of WT LRRK2 on CMA, we used the in vitro system with isolated lysosomes. GTP did not affect binding of WT or G/S LRRK2 to the lysosomal membrane (Fig. 5a) or their increase in the presence of the CMA substrate GAPDH (Fig. 5a), indicating that the GTPase activity of LRRK2 does not modulate its binding to the lysosomal membrane.

In contrast, SU6656, a selective Src-family kinase inhibitor previously used to block the kinase activity of LRRK2 (ref. 38), increased binding of WT LRRK2 to lysosomes both when the LRRK2 was presented alone or in the presence of other CMA substrates (Fig. 5b). Treatment with the inhibitor under the same conditions did not affect the lysosomal binding of a kinase-dead form of LRRK2, and thus the changes in WT LRRK2 binding were indeed a consequence of inhibition of its kinase activity (Fig. 5b). Inhibition of G/S LRRK2 kinase activity by SU6656 also abolished the decrease in GAPDH levels observed in lysosomes incubated with WT or G/S LRRK2 (Fig. 5c,d, Supplementary Fig. 6a). Although we did not note expansion or clustering of CMA-active lysosomes, immunofluorescence and immunoblot (Supplementary Fig. 6b) revealed that levels of LAMP-2A were about twice as high in cells expressing either WT or G/S LRRK2 as in control cells. This contrasted with the marked decrease in CMA activity observed in these cells (Fig. 4b) and likely reflects a compensatory response of these cells similar to that described in other conditions with compromised CMA, such as aging or other models of Parkinson’s disease35-37. We did not find significant changes in the levels of LAMP-2A in lysosomes isolated from cells in which LRRK2 was knocked down or by immunofluorescence analysis of these cells (Supplementary Fig. 6c), or in lysosomes isolated from LRRK2-null mice (Supplementary Fig. 6d), suggesting that the changes in CMA observed on expression of G/S LRRK2 are more likely a result of a gain of toxic function rather than a loss of physiological function.
activity did not affect its lysosomal binding if the protein was presented alone but markedly enhanced the GAPDH-induced binding of G/S LRRK2 to the lysosomal membrane (Fig. 5b). We confirmed that treatment with the kinase inhibitor did not affect lysosomal stability (Supplementary Fig. 2e) and that the WT LRRK2 did not mimic the G/S-LRRK2 behavior even at very high concentration of the inhibitor (Supplementary Fig. 2f). Overall, these results support the finding that binding of G/S LRRK2 to lysosomal membranes increases in the presence of other CMA substrates, and more so if its kinase activity becomes compromised.

To gain further insight into the regions of LRRK2 important for its CMA targeting and degradation, we investigated the relevance of the different CMA-targeting motifs, the pentapeptide recognized by hsc70, in its sequence (Fig. 5c). Studies in other multi-motif CMA substrates have demonstrated that a single motif is sufficient for lysosomal targeting and that, although the presence of multiple motifs does not increase efficiency of targeting, hsc70 often has an order of preference in the binding17. However, once the most favored motif(s) are eliminated, it is not unusual for hsc70 to bind to one of the other motifs39. For that reason, we studied binding of this chaperone to previously characterized myc- or GFP-tagged truncated forms of LRRK2 that bear different combinations of CMA targeting motifs40,41. In vitro binding studies to glutathione S-transferase (GST)-tagged hsc70 revealed that binding to the chaperone of a fragment that contains the ROC, COR and kinase regions (RCK) and that bears the five central CMA motifs (M3–M7) but is missing the two N-terminal (M1 and M2) and the most distal C-terminal motifs (M8) was comparable to binding of the full-length LRRK2, arguing against involvement of these three motifs in chaperone binding (Fig. 5c). In contrast, binding to hsc70 of a truncated form of LRRK2 bearing only M4 to M6 (COR) was markedly reduced, suggesting that either M3 or M7 was important for chaperone binding of LRRK2 (Fig. 5c). We found that M7, the motif in the kinase region of LRRK2, could not be selectively recognized by the chaperone, at least when this region was presented alone. (Note that the binding to hsc70 of this product could not be competed with a protein bearing a CMA-targeting motif, suggesting nonspecific binding; Fig. 5c.) However, binding to hsc70 was preserved in a truncated form of LRRK2 (ROC) bearing M3 and M4. As we did not observe binding to hsc70 of the fragment containing M4–M6, we concluded that M3, the motif in the ROC region of LRRK2, was a strong candidate for hsc70 binding (Fig. 5c). Coimmunoprecipitation of hsc70 with LRRK2 in cells expressing the various tagged LRRK2 truncated proteins confirmed that binding of hsc70 to the LRRK2 fragment bearing M3 and M4 also occurred in intact cells but that absence of M3 markedly reduced the amount of hsc70 recovered in the pull-down assay (Fig. 5d). Although future detailed analysis of the contributions of different motifs in LRRK2 binding to hsc70 under different conditions is needed, these results support the requirement of M3 for chaperone binding.

We next analyzed the nature of the binding of LRRK2 to CMA lysosomes and demonstrated that trypsinization of lysosomal membranes gradually decreased binding of LRRK2, supporting protein-to-protein binding of LRRK2 (Fig. 5c; LAMP-1 is shown to demonstrate that trypsin did not reach the lysosomal lumen). The reduced amount of LRRK2 detected in lysosomes from cells in which LAMP-2A was knocked down (Fig. 1f) suggested that lysosomal binding of LRRK2 likely occurred through LAMP-2A, and pull-down experiments of lysosome-bound LRRK2 confirmed its binding to LAMP-2A (Fig. 5f). Of note, comparison of the amount of LAMP-2A recovered in pull-downs for WT and G/S LRRK2 revealed that, although the mutant protein bound less efficiently to the lysosomal membranes than WT LRRK2 (Fig. 5c,g), once bound to this receptor its binding to LAMP-2A was more stable (Fig. 5f).

We then analyzed the effect of WT and G/S LRRK2 on LAMP-2A. We hypothesized that this tight binding to LAMP-2A of G/S LRRK2 might inhibit CMA by favoring mobilization of LAMP-2A into specific lipid microdomains at the lysosomal membrane where it eventually undergoes degradation33. However, incubation of lysosomes with LRRK2 did not enhance recruitment of LAMP-2A to the lysosomal lipid microdomains, even when other CMA substrate proteins were added to the incubation medium (Supplementary Fig. 2g,h and data not shown). We analyzed the effect of LRRK2 on the organization of LAMP-2A into the multimeric complex responsible for translocation of CMA substrates34, which can be visualized as a single 700-kDa band by blue native electrophoresis and immunoblotting for LAMP-2A (Fig. 5g). We found that both LRRK2 proteins, at concentrations that inhibit CMA uptake, markedly decreased the amount of LAMP-2A detectable in the multimeric complex (Fig. 5g). Lysosomes from G/S-LRRK2 transgenic mice also showed a lower abundance of the LAMP-2A translocation complex than lysosomes from control mice (Fig. 5h). In the case of the WT-LRRK2 transgenic mice, there was also a trend toward lower levels of multimeric LAMP-2A, although there was more variability from animal to animal than in the case of the mutant LRRK2 transgenic mice (Fig. 5h). Overall, these results support the
Figure 7 Interaction of other LRRK2 mutant variants with CMA. (a) Top: LRRK2 immunoblot of rat liver lysosomes incubated with WT, G/S, D/A (kinase-dead), I/T or R/C mutant LRRK2, alone or in the presence of protease inhibitors (PI). Input lanes, one-quarter of input material. Bottom: quantification of binding and uptake (calculated as in Fig. 2b; n = 4 or 5 independent experiments). (b) Left: immunoblots of lysosomes incubated as in a, alone or in the presence of increasing concentrations of RNase A (left) or GAPDH (right). Right: quantification of LRRK2 bound, as a multiple of the binding when incubated with lysosomes alone (n = 3 independent experiments). (c) Top: co-immunoprecipitation (co-IP) of LAMP-2A (L2A) with anti-LRRK2 from lysosomes incubated with LRRK2 proteins as in a. Co-immunoprecipitated fractions were immunoblotted for LAMP-2A and LRRK2. Input: immunoblot for LAMP-2A before the affinity purification. Bottom: amount of LAMP-2A coimmunoprecipitated, expressed as a multiple of the value with WT LRRK2 (n = 3 or 4 independent experiments). (d) Co-IP of LAMP-2A with anti-GFP in tet-on HEK293 cells expressing GFP-LRRK2 proteins and pulsed for 24 h with doxycycline. Inputs (Inp), one-quarter of material added. (e) The same cells as in d and control cells transduced with KFERQ-PA-mCherry1. Left: representative images. Nuclei are highlighted with DAPI in blue. Right: Quantification after photoactivation and 16 h of serum deprivation (n > 150 cells). All values are mean ± s.e.m. Differences with WT (*) or with the indicated mutants ($) were significant at P < 0.05; unpaired t-test in a–c and ANOVA and Bonferroni test in e. Scale bars, 10 μm. Full-length blots and gels in Supplementary Figure 12.

notion that the WT and G/S LRRK2 inhibition of LAMP-2A multimerization is responsible for the reduced CMA activity observed in non-neuronal and neuronal cells overexpressing these proteins.

Finally, we confirmed that alterations in LAMP-2A were evident in the dorsal motor nucleus of the vagus (DMV), a region considered to be the first highly targeted area of the central nervous system in Parkinson’s disease42, from Parkinson’s disease patients with the G/S LRRK2 mutation. Similarly to the upregulation of LAMP-2A observed in cultured cells when expression of G/S LRRK2 was activated (Supplementary Fig. 6a,b), immunostaining for LAMP-2A revealed a marked increase in LAMP-2A in the patients’ brains when compared with that in brains from age-matched control individuals (Fig. 6a,b). This increase was also detectable after immunoblot for LAMP-2A of the same brain samples (Supplementary Fig. 7a). The increase in LAMP-2A does not seem a marker of general upregulation of the lysosomal system in the Parkinson’s disease brains, because we did not detect significant differences in levels of LAMP-1, a more abundant lysosomal membrane protein, between control and patients’ brains (Fig. 6a and Supplementary Fig. 7a). In addition, and in agreement with our findings in vitro, association of LRRK2 with LAMP-2A–positive compartments was also markedly increased in the brains of the Parkinson’s disease patients (Fig. 6c and Supplementary Fig. 7b). These findings provide evidence for selective alterations in CMA in the brains of Parkinson’s disease patients with LRRK2 mutations.

Interaction of other LRRK2 mutants with CMA

We next examined three more LRRK2 mutants, two in the kinase site and one in the GTP-binding region, for possible differences in their degradation by CMA and impact on this autophagic pathway. We selected an additional mutation in the ROC site of LRRK2 (R1441C LRRK2, or R/C LRRK2) and a mutation in the kinase site of LRRK2 (I2020T, or I/T LRRK2), both described in familial Parkinson’s disease
patients and shown to increase LRRK2 dimerization, and an experimental mutation that ablates LRRK2 kinase activity (D1994A LRRK2, or D/A-LRRK2) and yields low dimer stability. We found that, whereas I/T LRRK2 showed levels of binding and uptake by isolated lysosomes comparable to those of the WT LRRK2, binding and in particular uptake of R/C and D/A LRRK2 were lower than those of WT LRRK2 (Fig. 7a). Notably, the mutant forms with reduced binding and uptake by lysosomes showed a more pronounced increase in their binding to lysosomes in the presence of CMA substrates (such as GAPDH and RNase A; Fig. 7b). These results support that the inability to translocate into the lumen of lysosomes may enhance the substrate-induced lysosomal binding of LRRK2 and favor its accumulation in the membrane of these organelles. Pull-down experiments of the LRRK2 variants bound to lysosomes in the in vitro system (Fig. 7c) or communoprecipitation from cells expressing GFP-tagged forms of each of these LRRK2 variants (Fig. 7d) revealed that the enhanced interaction of G/S LRRK2 with LAMP-2A was also observed for R/C-LRRK2 but not for D/A LRRK2, suggesting that the enhanced substrate-dependent binding of this variant to the lysosomal membrane may involve different mechanisms than the persistent binding to LAMP-2A of G/S and R/C LRRK2.

As expected from the efficient degradation of I/T-LRRK2 by CMA (Fig. 7a) and its lower association to the lysosomal membrane in the presence of substrates (Fig. 7b), we did not find significant differences from WT LRRK2 in the amount of this protein bound to LAMP-2A (Fig. 7c,d).

To directly analyze the impact of these different LRRK2 mutants on CMA in intact cells, we expressed these variants in cells under the tet-regulated system described before (Supplementary Fig. 3c) and used the fluorescent KFERQ-PA-mCherry1 reporter to quantify CMA activity. In agreement with the lower degradation of R/C and D/A-LRRK2 by CMA (Fig. 7a) and their higher association to lysosomes when in the presence of CMA substrates (Fig. 7b), we found that these two variants significantly reduced CMA activity to levels comparable to those observed for G/S LRRK2 (Fig. 7e). A reduction in CMA activity, although smaller, was also observed in cells expressing I/T-LRRK2, although in these the predominant feature was a marked increase in the size of the lysosomal compartments related to CMA (Fig. 7e). Although the meaning of these changes in CMA lysosomes requires further investigation, these findings show that different LRRK2 mutations may inhibit CMA through different mechanisms.
Consequences of unusual interplay between LRRK2 and CMA

Because enhanced binding to LAMP-2A at the lysosomal membrane was shared by two of the LRRK2 mutants, including the most common mutation in familial Parkinson's disease patients (G/S LRRK2), we set out to analyze the consequences of this abnormal binding. We speculated that, by blocking the formation of the receptor complex (Fig. 5g), G/S LRRK2 and high levels of WT LRRK2 may lead to higher concentration of substrate proteins bound to lysosomes at a given time because the inability of LAMP-2A to multimerize does not affect substrate binding but, rather, prevents their translocation into the lysosomal lumen. This high concentration may be particularly detrimental for pathogenic proteins that tend to organize into abnormal multimeric complexes and oligomers.

To test this possibility, we analyzed the effect of LRRK2 on the lysosomal association of WT α-syn and a pathogenic α-syn mutant (A53T) that we have previously shown binds LAMP-2A at the lysosomal membrane but does not translocate into the lumen. WT LRRK2 competed association of monomeric WT α-syn to lysosomes (as was the case for other CMA substrates) but was less efficient in competing the mutant α-syn variant (Fig. 8a and Supplementary Fig. 8a). Increasing concentrations of WT LRRK2 decreased the presence of WT α-syn oligomers in lysosomes (likely by competing binding when still at the monomeric stage (Supplementary Fig. 8a)), whereas combination of WT LRRK2 with the mutant α-syn protein markedly increased formation of α-syn oligomers in a dose-dependent manner (Fig. 8a). LRRK2-induced formation of oligomers of α-syn did not occur when the two proteins were incubated alone, indicating that oligomerization required their binding to lysosomal membranes (Supplementary Fig. 8b). These results suggest that the coincidence at the lysosomal membrane of WT LRRK2 and mutant α-syn is sufficient to aggravate the CMA defect.

G/S LRRK2, rather than competing binding of the α-syn proteins, preserved or even enhanced binding of monomeric α-syn to the lysosomal membrane (Supplementary Fig. 8a), which probably causes the observed marked increase in formation of oligomers of even the WT form of α-syn at the surface of lysosomes (Fig. 8a). G/S LRRK2 did not induce oligomerization of α-syn proteins in the absence of lysosomes (Supplementary Fig. 8b). The differences between the effects of WT and G/S LRRK2 on WT α-syn oligomerization are likely a result of the different effects that α-syn proteins had on the lysosomal association of WT and G/S LRRK2. Whereas the combination of WT forms of both proteins did not interfere with lysosomal uptake of LRRK2, combination of WT LRRK2 with mutant α-syn or mutant LRRK2 with any of the α-syn proteins markedly decreased the translocation of LRRK2 into lysosomes (Supplementary Fig. 8c).

Studies in neuronal cell lines cotransfected with WT α-syn and LRRK2 proteins demonstrated significant decreases in the degradation rates of α-syn in the cells coexpressing G/S LRRK2 (Supplementary Fig. 9). Overexpression of WT LRRK2 in these cells did not change the early rates of α-syn degradation, but it did delay the degradation of this protein in the late stages (Supplementary Fig. 9). Further experiments are needed to determine whether such two-phase kinetics reflect an early compensatory activation of other proteolytic systems in the cells expressing WT LRRK2 or the amount of time necessary to accumulate enough LRRK2 at the lysosomal membrane to affect α-syn degradation.

To determine whether the proposed abnormal oligomerization of α-syn at the lysosomal membrane and the subsequent compromise of CMA also occurred in lysosomes exposed to mutant LRRK2 in vivo, we isolated lysosomes from non-transgenic and G/S-LRRK2 transgenic mice. Intact lysosomes isolated from G/S-LRRK2 mice showed a reduction in their ability to take up proteins via CMA (Fig. 8b).

(Note that this assay recapitulates binding, uptake and proteolysis, but, because the proteolytic activity of disrupted lysosomes was comparable in both groups (data not shown), the observed differences are in binding and uptake.) We confirmed that, in the case of α-syn, this reduced CMA activity favored the formation of the oligomeric complex, similarly to what we observed in the in vitro experiments (Fig. 8c). In fact, not only the exogenous α-syn but also endogenous α-syn could be found associated to lysosomes from G/S-LRRK2 mice, and it organized there as irreversible oligomeric complexes (Fig. 8d).

We also found a significant increase in the colocalization of α-syn with lysosomal markers (such as total LAMP-2; Fig. 8e,f) in neuronal cultures from brains of WT- and G/S-LRRK2 transgenic mice when compared to neurons from control mice. This enhanced association of α-syn to lysosomes is a consequence of its delivery to this compartment by CMA because knockdown of LAMP-2A in these neurons reduced colocalization of α-syn with lysosomes (Fig. 8g,f). None of these manipulations changed the already minimal colocalization between LRRK2 and LC3 (Supplementary Fig. 10a). A similar increase in intracellular levels of α-syn and in the association of this protein with lysosomes was observed in primary neuronal cultures from R1441G-LRRK2 transgenic mice (Supplementary Fig. 10b), which could be responsible for the reduced CMA activity observed in these cells using the CMA fluorescent reporter (Supplementary Fig. 10c).

Lastly, to determine whether a similar association of α-syn with lysosomes is also present in neurons from Parkinson's disease patients with the G/S LRRK2 mutation, we differentiated dopaminergic neurons from induced pluripotent stem cells (iPSC) from such individuals. We have previously shown that α-syn abnormally accumulates in these differentiated neurons. After 30 d of differentiation, a time when iPSC-derived neurons do not show overt morphological signs of neurodegeneration, only 13% of neurons positive for the dopaminergic marker tyrosine hydroxylase showed detectable amounts of α-syn in dopaminergic neurons differentiated from iPSCs derived from healthy individuals, and only a small percentage (less than 10%) of these cells containing α-syn showed colocalization between α-syn and the lysosomal marker. In contrast, α-syn was detectable in 40% of the neurons derived from the Parkinson's disease patients, and this protein colocalized with LAMP-2A in almost 65% of them (Fig. 8g,h). To address the contribution of changes in CMA activity to the abnormally high levels of α-syn in the patient-derived cells, we knocked down LAMP-2A in differentiated neurons at 3 weeks and 75 d (when signs of neurodegeneration are already evident in the patient-derived neurons) by lentiviral transduction of the GFP-tagged short hairpin RNA against LAMP-2A. Knockdown of LAMP-2A at 3 weeks of differentiation markedly increased the percentage of α-syn–positive dopaminergic neurons both in control and in Parkinson's disease patient iPSC–derived neurons (Fig. 8i,j). Neurons from cultures infected with a control lentivirus, or including the fraction of neurons in the same cultures that remained untransduced (Fig. 8i), did not show significant changes in their α-syn content. Notably, the increase in cells showing α-syn accumulation was significantly higher in the patient-derived cells than in control, suggesting a possible compensatory upregulation of CMA in early, presymptomatic states (Fig. 8i)). The accumulation of α-syn in Parkinson's disease patient–derived cells after CMA blockage was often associated with marked neurite shortening (Fig. 8i). Knockdown of LAMP-2A in cells cultured for 75 d (when signs of neurodegeneration are already evident in patient-derived cells), also increased α-syn content in both groups, but the proportional increase compared to untransduced cells was lower in the patient cells, likely indicating an already compromised CMA of α-syn in these cells (Fig. 8i,j).
Overall, our findings support the conclusion not only that LRRK2 at lysosomes blocks the degradation of α-syn by CMA but also that the presence of mutations in only one of these proteins is sufficient to elicit the CMA-toxic effect of the other. We propose that the enhanced binding of LRRK2 to the lysosomal membrane in the presence of CMA substrates may serve to self-perpetuate the negative effect of LRRK2 on CMA. The observed LRRK2-mediated inhibition at the level of translocation but not binding is particularly detrimental for pathogenic proteins that tend to oligomerize into toxic species, as shown here for α-syn (Supplementary Fig. 11).

**DISCUSSION**

We have found that the Parkinson's disease–associated protein LRRK2 can undergo degradation in lysosomes via CMA and that this degradation is compromised for the most common LRRK2 mutant (G2019S) and other pathogenic mutations of this protein. LRRK2 is the first CMA substrate identified for which binding to the lysosomal membrane is enhanced by other CMA substrates, and this enhanced binding inhibits the assembly of the CMA translocation complex at the lysosomal membrane. Cells respond to this blockage by increasing LAMP-2A, consistent with the high levels of LAMP-2A that we observed in brains of Parkinson's disease patients with the G/S LRRK2 mutation. Our studies in neuronal cell lines, primary neuronal mouse cultures, lysosomes from different LRRK2 transgenic mouse models and iPSC-derived dopaminergic neurons from Parkinson's disease patients with the G/S LRRK2 mutation support the conclusion that LRRK2-mediated blockage of LAMP-2A multimerization leads to accumulation of other CMA substrates, including α-syn, at the surface of the lysosomal membrane by increasing the time that substrate proteins remain bound to the membrane before translocation. Thus, two dominant mutations that cause Parkinson's disease converge at the same step, leading to blockage of degradation of CMA substrates, which can contribute to the accumulation of neuronal α-syn widely thought to underlie Parkinson's disease.

An unexpected finding of our study is that the toxic effect of LRRK2 on CMA was manifest not only for mutant forms of LRRK2 but also for WT LRRK2, both in isolated lysosomes and in intact cells when present at high concentrations, highlighting the need for a tight control of intracellular LRRK2 levels. This low threshold of tolerance for LRRK2 excess could result, in part, from its peculiar interplay with CMA. LRRK2 behaved, in all aspects, as an unconventional CMA substrate, which rather than competing for lysosomal membrane binding with other CMA substrates showed an unusual enhanced membrane binding. Further analysis of the peculiarities of LRRK2 in the context of CMA may reveal the existence of a different subgroup of CMA substrates depending on this cooperative binding for their internalization into lysosomes. As for most intracellular proteins, several pathways contribute to the degradation of LRRK2, making it likely that post-translational modifications in LRRK2 determine its degradative fate. Ubiquitination of LRRK2 by carboxy terminus of hsp70-interacting protein (CHIP) and its interaction with heat shock protein 90 (hsp90) have been shown to determine its degradation by the proteasomal system.[27] Whether specific modifications in LRRK2 modulate its CMA targeting requires future investigation. Functional association of LRRK2 with other cellular membranes has been shown to depend on its GTPase activity and phosphorylation status.[46] Although we did not find binding of LRRK2 to the lysosomal membrane to be dependent on GTP, changes in the kinase activity of LRRK2 may modulate its binding to the lysosomal compartment. Indeed, we found that changes in the kinase activity of the protein may have a different impact on its lysosomal binding depending on whether it is in the wild-type or mutant background and in the presence of CMA substrates at the membrane. Thus, a kinase-dead form of the protein showed reduced binding to lysosomes, but its binding was still enhanced in the presence of CMA substrates, indicating that kinase activity per se is not required for lysosomal binding once CMA substrates are added. In contrast, modulation of the kinase activity in the background of the G/S LRRK2 mutant (Fig. 5b) had a marked impact on its binding to lysosomes in the presence of substrate proteins. It is possible that binding of LRRK2 to its substrates or changes in its conformation in the presence of the inhibitors may modify its capability to interact with CMA substrates at the lysosomal membrane. Alternatively, LRRK2 could phosphorylate unidentified components at the lysosomal membrane and regulate its association to this organelle. A third possibility is that inhibition of the kinase activity may promote delivery of the no-longer-active protein for degradation and that the observed differences between the WT and G/S mutant in response to the treatment with kinase inhibitors reflects the lower efficiency of uptake of the mutant protein and consequently higher membrane-bound levels.

The inhibitory effect of LRRK2 on CMA could be due to direct interaction with LAMP-2A, as mutant forms of LRRK2 bind more stably to LAMP-2A. Alternatively, LRRK2 may abnormally bind to CMA substrates, forming oligomeric structures that compromise the lateral mobility of LAMP-2A along the lysosomal membrane to form the translocation complex, or the additional lysosomal binding of LRRK2 observed in the presence of α-syn could occur through oligomerization with molecules of this protein already bound to the receptor (Supplementary Fig. 11). Association of LRRK2 could thus prevent α-syn translocation, as only single, unfolded proteins can be transported through CMA. Notably, we have not found among the described pathogenic mutations in LRRK2 any that affect the possible CMA targeting motifs. It is possible that, as described for α-syn,[24] mutations in the motifs may not lead to disease because they cannot be targeted to lysosomes by hsc70, thereby preventing the CMA blockage.

The increase in LAMP-2A in cells expressing LRRK2 and in brains from Parkinson's disease patients with the LRRK2 mutation contrasts with the previously described decrease in this protein in brains of patients with idiopathic Parkinson's disease.[26] These differences may highlight the distinctive characteristics of the LRRK2-mediated blockage of CMA. Our previous data suggest that pathogenic forms of α-syn that bind tightly to the lysosomal membrane prevent further binding of other CMA substrates. In contrast, LRRK2, once bound to the lysosomal membrane, prevents LAMP-2A multimerization and, consequently, translocation of substrate proteins, but it does not interfere with their binding to this membrane. It is possible that the elevated LAMP-2A observed in brains of G/S LRRK2 Parkinson's disease result from a chronic decrease in the turnover of this receptor that we have previously shown occurs on binding of substrates to LAMP-2A.[47] Alternatively, the increase in LAMP-2A in these patients could be a compensatory response to the CMA blockage, as previously described in the brains of chemically and genetically induced Parkinson's disease mouse models.[35] This latter possibility is also supported by the higher impact that the knockdown of LAMP-2A had in the Parkinson's disease patient iPSC-derived neurons before signs of neurodegeneration become manifest, suggesting an initial compensatory upregulation of CMA. In this respect, the use of the iPSC-derived neurons is particularly advantageous, as it not only allows the study of the process in the relevant cell type expressing endogenous levels of the mutant protein but, in addition, permits following the process of neurodegeneration from an initially healthy differentiated neuron.
Our analysis of the iPSC-derived dopaminergic neurons from Parkinson’s disease patients with the G/S LRRK2 mutation indicates that alterations in CMA are an early event, detectable before overt neurodegeneration and the previously described compromise of macropautophagy in these cells44. CMA is a common target for three of the four mutations analyzed in this study (Supplementary Fig. 11c). Although there are some differences among the three mutants that have a toxic effect on CMA, all of them have also revealed themselves as poor substrates for this pathway when compared to the WT protein. Their reduced lysosomal uptake and augmented binding to the lysosomal membrane in the presence of CMA substrates are likely important determinants of their inhibitory effect on this pathway. Notably, the mutant that did not inhibit CMA is the one that yielded a much more pronounced swelling of the lysosomal endocytic system, a phenotype recently described in flies expressing a LRRK2 homolog33. Future studies with more mutant variants (as they are still being identified) may help to separate them into groups depending on the primary target of their toxicity in the lysosomal system and may thus help in a more efficient design of therapeutic interventions.

As both LRRK2 and α-syn are degraded by CMA and as pathogenic mutant forms of both impair CMA, we suggest that alterations in this autophagic pathway could be important in the pathogenesis of Parkinson’s disease3,8. The manners by which mutations in LRRK2 and α-syn affect CMA differ in important respects. First, whereas mutant α-syn proteins have a higher lysosome-binding affinity and lower rates of lysosomal translocation when compared to wild-type α-syn8, the mutant LRRK2 proteins analyzed in this study showed a smaller, yet appreciable, decrease in lysosomal uptake when compared to the wild-type LRRK2 protein, and they did not show abnormally enhanced binding to the lysosomal membrane when presented alone to these organelles. Notably, however, both wild-type and, even more so, mutant LRRK2 showed a marked increase in their association with lysosomes when in the presence of other CMA substrate proteins, which was not seen for α-syn. This enhanced binding of LRRK2 can inhibit the CMA transporter, and we show here that this blockage promoted the formation of α-syn oligomers at the lysosomal membrane, even for the wild-type form of α-syn, which is further toxic for CMA activity and could underlie the formation of Lewy body pathology in LRRK2-mutation Parkinson’s disease patients. Considering the toxic effect on CMA described in this study for the most common pathogenic mutant forms of LRRK2, interventions aimed at enhancing CMA activity or at preventing its decrease in pathologies and with age may prove to be valuable in the treatment of Parkinson’s disease and other age-related disorders resulting from alterations in cellular proteostasis.

METHODS

Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

S.J.O. performed most of the experiments, analyzed data and prepared a draft of the manuscript; S.-H.K. analyzed the human brain samples and conducted some of the experiments on mouse neuronal cultures; I.F. performed some studies in isolated rat liver and mouse, pull-down experiments and some analysis of the kinase inhibitors; E.A. performed studies in neuronal cultures; H.K. assisted with the CMA reporter analysis; I.F.-C., A.C. and A.R. were responsible for all studies with differentiated iPSC; E.C. and L.S.H. provided the brain samples; W.D. provided the LRRK2-expressing cells and advice on some aspects of the project; D.S. critically discussed the results, directed experiments in neuronal culture and human brain and edited and reviewed the final version of the manuscript; A.M.C. directed the study, designed most of the experiments and edited and reviewed the final version of the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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21. Bandyopadhyay, U., Kaushik, S., Vartikovsky, L. & Cuervo, A.M. Dynamic organization of the receptor for chaperone-mediated autophagy at the lysosomal membrane. *Mol. Cell Biol.* **28**, 5747–5763 (2008).

22. Cuervo, A.M., Mann, L., Borten, E., d’Azzo, A. & Dice, J. Cathepsin A regulates chaperone-mediated autophagy through cleavage of the lysosomal receptor. *EMBO J.* **22**, 47–59 (2003).

23. Kaushik, S., Massey, A.C. & Cuervo, A.M. Lysosome membrane lipid microdomains: novel regulators of chaperone-mediated autophagy. *EMBO J.* **25**, 3921–3933 (2006).

24. Vogiatzi, T., Xilouri, M., Vekrellis, K. & Stefanis, L. Wild type alpha-synuclein is degraded by chaperone-mediated autophagy and macroautophagy in neuronal cells. *J. Biol. Chem.* **283**, 23542–23556 (2008).

25. Kabuta, T., Furuta, A., Aoki, S., Furuta, K. & Wada, K. Aberrant interaction between Parkinson disease-associated mutant UCH-L1 and the lysosomal receptor for chaperone-mediated autophagy. *J. Biol. Chem.* **283**, 23731–23738 (2008).

26. Alvarez-Erviti, L. et al. Chaperone-mediated autophagy markers in Parkinson disease brains. *Arch. Neurol.* **67**, 1464–1472 (2010).

27. Ko, H.S. CHIP regulates leucine-rich repeat kinase-2 ubiquitination, degradation, and toxicity. *Proc. Natl. Acad. Sci. USA* **106**, 2897–2902 (2009).

28. Dice, J.F. Peptide sequences that target cytosolic proteins for lysosomal proteolysis. *Trends Biochem. Sci.* **15**, 305–309 (1990).

29. Kaushik, S. & Cuervo, A.M. Methods to monitor chaperone-mediated autophagy. *Methods Enzymol.* **452**, 297–324 (2009).

30. Cuervo, A.M., Terlecky, S.R., Dice, J.F. & Knecht, E. Selective binding and uptake of ribonuclease A and glyceraldehyde-3-phosphate dehydrogenase by isolated rat liver lysosomes. *Methods Enzymol.* **362**, 297–324 (2000).

31. Kett, L.R. et al. CHIP regulates leucine-rich repeat kinase-2 ubiquitination, degradation, and toxicity. *Proc. Natl. Acad. Sci. USA* **103**, 5805–5810 (2006).

32. Tanida, I., Minematsu-Ikeguchi, N., Ueno, T. & Kominami, E. Lysosomal turnover, degradation, and toxicity. *Proc. Natl. Acad. Sci. USA* **103**, 5805–5810 (2006).

33. Wang, Y. et al. Tau fragmentation, aggregation and clearance: the dual role of lysosomal processing. *Hum. Mol. Genet.* **18**, 4153–4170 (2009).

34. Dodson, M.W., Zhang, T., Jiang, C., Chen, S. & Guo, M. Roles of the Drosophila LRRK2 homolog in Rab7-dependent lysosomal positioning. *Hum. Mol. Genet.* **21**, 1350–1363 (2012).

35. Mak, S.K., McCormack, A.L., Manning-Bog, A.B., Cuervo, A.M. & Di Monte, D.A. Lysosomal degradation of alpha-synuclein in vivo. *J. Biol. Chem.* **285**, 13621–13629 (2010).

36. Zhang, C. & Cuervo, A.M. Restoration of chaperone-mediated autophagy in aging liver improves cellular maintenance and hepatic function. *Nat. Med.* **14**, 959–965 (2008).

37. Massey, A.C., Kaushik, S., Sovak, G., Kiffin, R. & Cuervo, A.M. Consequences of the selective blockage of chaperone-mediated autophagy. *Proc. Natl. Acad. Sci. USA* **103**, 5805–5810 (2006).

38. Blake, R.A. et al. SU6656, a selective src family kinase inhibitor, used to probe growth factor signaling. *Mol. Cell Biol.* **20**, 9018–9027 (2000).

39. Wang, Y. et al. Tau fragmentation, aggregation and clearance: the dual role of lysosomal processing. *Hum. Mol. Genet.* **18**, 4153–4170 (2009).

40. Greggio, E. et al. The Parkinson disease-associated leucine-rich repeat kinase 2 (LRRK2) is a dimer that undergoes intramolecular autophosphorylation. *J. Biol. Chem.* **283**, 16906–16914 (2008).

41. Greggio, E. et al. Kinase activity is required for the toxic effects of mutant LRRK2/dardarin. *Neurobiol. Dis.* **23**, 329–341 (2006).

42. Sulzer, D. & Surmeier, D.J. Neuronal vulnerability, pathogenesis, and Parkinson’s disease. *Mov. Disord.* doi:10.1002/mds.25095 (12 July 2012).

43. Sen, S., Webber, P.J. & West, A.B. Dependence of leucine-rich repeat kinase 2 (LRRK2) kinase activity on dimerization. *J. Biol. Chem.* **284**, 36346–36356 (2009).

44. Sánchez-Dáñez, A. et al. Disease-specific phenotypes in dopamine neurons from human iPS-based models of genetic and sporadic Parkinson’s disease. *EMBO Mol. Med.* **4**, 380–395 (2012).

45. Kon, M. et al. Chaperone-mediated autophagy is required for tumor growth. *Sci. Transl. Med.* **3**, 109ra117 (2011).

46. Berger, Z., Smith, K.A. & Lavoie, M.J. Membrane localization of LRRK2 is associated with increased formation of the highly active LRRK2 dimer and changes in its phosphorylation. *Biochemistry* **49**, 5511–5523 (2010).

47. Cuervo, A.M. & Dice, J.F. Regulation of lamp2a levels in the lysosomal membrane. *Traffic* **1**, 570–583 (2000).
ONLINE METHODS

Animals, cells and human brain tissue. Adult (4 months old) male Wistar rats and male (3–5 months old) mice BAC transgenic for human WT, G2019S and R1441G LRRK2 or null for LRRK2 generated as described before20 were from Jackson Laboratory and were used under animal study protocols approved by the Institutional Animal Care and Use Committee of Albert Einstein College of Medicine and Columbia University. Rats (three per cage) and mice (five per cage) were maintained on a 12-h light/dark cycle. Where indicated, rodents were starved for 48 h to maximally activate CMA. SH-SY5Y cells were from the ATCC. HEK293 cells expressing WT or the various LRRK2 mutations under the control of tetracycline were generated as described before31. Plasmids for myc- and GFP-tagged full-length or truncated LRRK2 (RCK, ROC, K, COR) were obtained from Addgene40,41. Cells were maintained in Dulbecco’s modified Eagle’s medium (Sigma, St. Louis, MO) in the presence of 10% FBS or newborn calf serum (Sigma), 50 μg/ml penicillin and 50 μg/ml streptomycin at 37 °C with 5% CO2. Ventral midbrain dopaminergic neurons were derived from postnatal day 1 pups and were prepared as described28. Previously generated iPSC lines line SP-11.1 and SP-17.2 (from healthy individuals) and SP-05.1 and SP-12.3 (from patients with familial Parkinson’s disease with the LRRK2 G2019S mutation) were used, and culture and differentiation were carried out as described37 after obtaining written consent, following a protocol approved by the Spanish competent authorities (Commission on Guarantees concerning the Donation and Use of Human Tissues and Cells of the Carlos III Health Institute). Frozen and formalin fixed brain tissue from the dorsal motor nucleus of the vagus nerve (DMV) was obtained from the New York Brain Bank at Columbia University, and deidentified, under a protocol approved by the Columbia University Institutional Review Board. Brain tissue was available, with written consent, from two people with known LRRK2 G2019S mutations (one female, 80 years old; one male, 60 years old) and two approximately age-matched unaffected controls with normal neuropathological findings (both female; one 66 years old, one 83 years old)50. The DMV was the only available region from the original brain samples known to be targeted in Parkinson’s disease.

Chemicals. Sources of chemicals and antibodies were as described before20,21. 3-Methyladenine (3MA), ammonium chloride and MG-132 were from Sigma, lactacystin was from Enzo Life Sciences and leupeptin was from Calbiochem. The antibody against the cytosolic tail of rat and mouse LAMP-2 was prepared in our laboratory20, and the antibodies against total LAMP-2 (H4B4, human, 1:3,000; GL27B, mouse, 1:100–500), LAMP-1 (H4A3, human, 1:100–3,000) and anti-myc (9E10, 1:1,000) were from the Developmental Hybridoma Bank (University of Iowa, Iowa City, Iowa). The antibodies against mouse LAMP-1 (1Ly16C, 1:1,000) were from Streissgen; against human LAMP-2A (18528, 1:200–500) from Abcam; against LRRK2 from the Michael J Fox Foundation (C5, 1:100–1,000), Epitomics (3515-1, 1:50–1,000), Everest (EB06550, 1:100–500), LAMP-1 (H4A3, human, 1:100–3,000) and Abcam (AB38003, 1:100–1,000); against actin (S12-I, 1:10,000) and V5 (R960-25, 1:1,000) from Invitrogen; against MAP2 against GFP from Roche (7.1, 1:1,000) and Aves Labs (1020; 1:1,000); against GST-α-syn (42/a-s, 1:500) from BD Biosciences; against RNase A (100-4188, 1:3,000) and cytochrome c oxidase subunit I (clone 4H4, 1:1,000) from Santa Cruz; against hsc70 (13D3, 1:5,000) from Novus; against GEP from Roche (7.1, 1:1,000) and Aves Labs (1020; 1:1,000); against GST (1H14L28, 1:1,000) and V5 (R960-25, 1:1,000) from Invitrogen; against MAP2 from Sigma (AP-20, 1:250); and against LC3 (2775S, 1:100–1,000) from Cell Signaling. LysoTracker and the purified WT and various mutant LRRK2 proteins were from Invitrogen. WT and mutant α-syn were purified as described before9. Cyclophilin A was from Abcam. GAPDH, RNase A, ovalbumin, GTP and the kinase inhibitor SU6656 were from Sigma.

Lysosomal isolation and lysosomal subfractionation. Lysosomes with high activity for CMA were isolated by centrifugation of a light mitochondrial-rich lysosomal fraction from rodent liver or brain in a discontinuous metrizamide density gradient20,25. Preparations with more than 10% broken lysosomes at the moment of the isolation, measured by β-hexosaminidase latency43, were discarded. Where indicated, lysosomal membrane proteins were removed by incubation with the indicated concentrations of trypsin at 20–25 °C.

Intracellular protein turnover. To measure degradation of long-lived proteins, confluent cells were labeled with [3H]leucine (2 μCi/ml) for 48 h at 37 °C and then extensively washed and maintained in complete (10% FBS) or serum-deprived media containing an excess of unlabeled leucine (2.8 mM) to prevent reutilization of radiolabeled leucine45. Aliquots of the media taken at different times were precipitated with TCA and proteolysis was measured as the percentage of the initial acid-insoluble radioactivity (protein) transformed into acid-soluble radioactivity (amino acids and small peptides) at the end of the incubation. Total radioactivity incorporated into cellular proteins was determined as the amount of acid-precipitable radioactivity in labeled cells immediately after washing29. Cell viability was routinely monitored in parallel cells at the end of the experiment, and we did not find appreciable differences in viability between control cells and those expressing LRRK2 proteins during the duration of the assay.

Degradation of substrate proteins by intact lysosomes in vitro. Lysosomes isolated from rat livers were incubated with radiolabeled proteins in 3-(N-morpholino)propanesulfonic acid (MOPS) buffer (10 mM MOPS, pH 7.4, 0.3 M sucrose, 1 mM dithiothreitol and 5.4 μM cysteine) for 30 min at 37 °C (ref. 29). Reactions were stopped with 20% TCA and filtered through a Millipore Multiscreen Assay System (Millipore, Billerica, MA, and detected in a liquid scintillation analyzer (PerkinElmer Wallac, Gaithersburg, MD). Proteolysis was measured as the percentage of the initial acid-insoluble radioactivity transformed into acid-soluble radioactivity as described above.

Binding and uptake of CMA substrate proteins by isolated lysosomes. Freshly isolated intact lysosomes were incubated with the substrate protein in MOPS buffer at 37 °C for 20 min (ref. 29). Where indicated, lysosomes were preincubated with a cocktail of protease inhibitors for 10 min at 0 °C as described before29. Lysosomes were collected by centrifugation, washed with MOPS buffer and subjected to SDS-PAGE, and immunoblotted for the substrate protein in question. Binding was calculated from the densitometric analysis as the amount of substrate protein bound to the lysosomal membrane in the absence of protease inhibitors. Uptake was calculated by subtracting the amount of protein associated with lysosomes in the presence (protein bound to the lysosomal membrane and taken up by lysosomes) and absence (protein bound to the lysosomal membrane) of protease inhibitors.

Measurement of CMA activity in intact cells. HEK293 cell lines and primary mouse neurons in culture (ventral midbrain dopaminergic neurons at DHVI) were transduced with lentivirus carrying the CMA reporter KFERQ-PA-mCherry1 (ref. 33). Cells were photoactivated in the presence of oxirane and lactic acid 48 h after infection (in non-neuronal cells) or 30 min to 5 h after infection (in neuronal cells) by a 405-nm light-emitting diode (LED; Norlux) for 9 min (non-neuronal) or 3 min (neuronal) with the intensity of 3.5 μA (current constant). Levels of red fluorescence were checked under the microscope 1 h after photoactivation to confirm photoactivation and compare levels of the reporter expression. We did not find visible differences in expression between cells expressing or not expressing LRRK2 proteins. After 16 h (non-neuronal) or 0.5–6 h (neuronal), cells were fixed with 4% paraformaldehyde (PFA) and images were acquired with an Axiovert 200 fluorescence microscope (Carl Zeiss Ltd., Thornwood, NY), equipped with an apotome or in a Leica Multiplan microscope (in neuronal cell studies). Images were prepared using Adobe Photoshop 6.0 software (Adobe Systems Inc., Mountain View, CA). CMA activity was determined in this system as a change in the fluorescent pattern from diffuse (cytosolic) to punctate (lysosomal). The number of fluorescent puncta per cell was quantified using ImageJ software (NIH) in individual frames after thresholding.

LAMP-2A dynamics. Multimerization of LAMP-2A at the lysosomal membrane was studied on 3–12% NativePAGE Bis-Tris Gels (Invitrogen) after solubilization in 1% octylglucoside (in 20 mM MOPS and 150 mM NaCl buffer)21. Lysosomal membrane microdomains were isolated after detergent solubilization through floatation in a sucrose density gradient as described previously23.

Immunofluorescence and immunohistochemistry. Non-neuronal cells. Cells grown on coverslips were fixed with 4% PFA or methanol, blocked and then incubated with the primary and corresponding fluorophore-conjugated secondary antibodies23. Mounting medium contained DAPI (4,6-diamidino-2-phenylindole) to highlight the cell nucleus.
Neuronal cultures. Ventral midbrain dopaminergic neuron cultures were derived from postnatal day 1 pups. Neurons were transduced into dopaminergic neurons at DIV1 and fixed at DIV7 with 4% PFA with 4% glucose in PBS for 2 min at 20–25 °C and for 10 min with −20 °C methanol. Cultures were blocked with 10% donkey serum containing 0.2% saponin for 1 h before immunostaining.

iPSC-derived neurons. iPSC-derived cells were fixed with 4% PFA in PBS at 4 °C for 10 min and blocked in 0.3% Triton X-100 with 3% donkey serum for 2 h. Images were taken using a Leica SP5 confocal microscope. For quantification analyses, a minimum of three fields per condition were randomly selected and counted. Data points represent the average of at least two independent experiments.

Human brains. Formalin-fixed brain tissues were paraffin-embedded and sectioned for immunohistochemistry analysis. Brains were chilled 2–4 h post mortem and frozen 6–30 h post mortem. The two LRKK2 G2019S brains were from individuals rated at a grade of neuropathological Braak & Braak staging 4/6. Tissue blocks of the medulla were sectioned at 7 µm thickness. Antigen retrieval was performed with Trilogy (Cell Marque) in a vegetable steamer for 40 min. Immunostaining for the desired proteins followed standard procedures. Sections were imaged and neurons were selected randomly, and fields of 75 × 75 µm were photographed in a confocal fluorescence microscope (Leica). The micrographs were analyzed for the number of fluorescent puncta per neuronal cell using ImageJ software (NIH) after thresholding. Colocalization was quantified using the JACop plug-in in the same software and Costes’ automatic thresholding to calculate the Pearson’s coefficient.

RNA interference. For downregulation of LAMP-2A, we used previously described lentiviral vectors carrying shRNA against LAMP-2A, packed in 293T cells and purified by ultracentrifugation as previously described. Expression titers were 5 × 10^8 to 1 × 10^9 transducing units ml^-1. For transduction: (i) cultured non-neuronal cells and ventral midbrain dopaminergic neurons at DIV1p were incubated with the packed virus for 12 h and then placed in fresh medium; (ii) iPSC were grown on coverslips and incubated after 3 or 9 weeks of differentiation with 1 µl of concentrated lentivirus in a final volume of 1 ml for 24 h at 37 °C, and with an additional 1 µl of fresh medium for 48 h. GFP-positive cells started to appear 3 d after infection. Cells were fixed and analyzed 7–10 d after infection.

LC3 flux. Macroautophagy was quantified as the degradation of the autophagosome-associated protein LC3. Cells were untreated or treated with 20 mM NH_4Cl and 100 µM leupeptin for 6 h, followed by immunoblotting for LC3. Flux was calculated as the ratio of treated to untreated lines after normalization to actin.

General procedures. Protein concentration was determined using the Lowry method with bovine serum albumin as a standard. Cells were solubilized on ice with RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2). Immunoblotting was performed after transferring SDS-PAGE gels to nitrocellulose membranes. These proteins were visualized by chemiluminescence using peroxidase-conjugated secondary antibodies in a LAS-3000 Imaging System (Fujifilm). ImageJ software (NIH) was used for densitometric quantification. Coimmunoprecipitation was performed after solubilization of cellular extracts in a low-stringency buffer as described before. Immunoblots shown in the figures are representative of at least three different experiments.

Statistical analysis. All numerical results are reported as mean ± s.e.m. and represent data from a minimum of three independent experiments unless otherwise stated. Statistical significance of difference between groups was determined in instances of single comparisons by the two-tailed unpaired Student's t-test of the means. In instances of multiple-means comparisons, we used one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test to determine statistical significance. Statistic analysis was performed in all the assays, and significant differences are noted in the graphical representations.

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48. Li, Y. et al. Mutant LRRK2(R1441G) BAC transgenic mice recapitulate cardinal features of Parkinson’s disease. Nat. Neurosci. 12, 826–828 (2009).
49. Staal, R.G.W., Rayport, S. & Sulzer, D. Amperometric detection of dopamine exocytosis from synaptosomal terminals. in Electrochemical Methods for Neuroscience (eds. Michael, A.D. & Bolot, L.M.) Ch. 16 (CRC, Boca Raton, Florida, USA, 2007).
50. Pouloupolos, M. et al. Clinical and pathological characteristics of LRRK2 G2019S patients with PD. J. Mol. Neurosci. 47, 139–143 (2012).
51. Sterri, B. & Madden, E. Isolation of subcellular organelles. Methods Enzymol. 182, 203–225 (1990).
52. Rubinsztein, D.C. et al. In search of an “autophagomometer”. Autophagy 5, 585–589 (2009).
53. Lowry, C.C., Kraeft, N.H. & Hughes, F.A. Jr. Blastomycosis of the lung. Am. J. Surg. 81, 676–679 (1951).
54. Towbin, H., Staehelin, T. & Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76, 4350–4354 (1979).
Corrigendum: Interplay of LRRK2 with chaperone-mediated autophagy

Samantha J Orenstein, Sheng-Hang Kuo, Inmaculada Tasset, Esperanza Arias, Hiroshi Koga, Irene Fernandez-Carasa, Etty Cortes, Lawrence S Honig, William Dauer, Antonella Consiglio, Angel Raya, David Sulzer & Ana Maria Cuervo

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In the version of this article initially published online, author Sheng-Han Kuo’s name was misspelled. The error has been corrected for the print, PDF and HTML versions of this article.