A link between LRRK2, autophagy and NAADP-mediated endolysosomal calcium signalling

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Abstract
Mutations in LRRK2 (leucine-rich repeat kinase 2) represent a significant component of both sporadic and familial PD (Parkinson’s disease). Pathogenic mutations cluster in the enzymatic domains of LRRK2, and kinase activity seems to correlate with cytotoxicity, suggesting the possibility of kinase-based therapeutic strategies for LRRK2-associated PD. Apart from cytotoxicity, changes in autophagy have consistently been observed upon overexpression of mutant, or knockdown of endogenous, LRRK2. However, delineating the precise mechanism(s) by which LRRK2 regulates autophagy has been difficult. Recent data suggest a mechanism involving late steps in autophagic–lysosomal clearance in a manner dependent on NAADP (nicotinic acid–adenine dinucleotide phosphate)-sensitive lysosomal Ca2+ channels. In the present paper, we review our current knowledge of the link between LRRK2 and autophagic–lysosomal clearance, including regulation of Ca2+-dependent events involving NAADP.

Introduction
PD (Parkinson’s disease) is a common age-related neurodegenerative disorder, and recent studies have revealed an important genetic component. Mutations in the gene encoding LRRK2 (leucine-rich repeat kinase 2) comprise the most common single genetic cause of familial PD [1,2], and variations increase the risk of sporadic PD [3–5]. These findings indicate that both forms of PD may share common pathological mechanisms related to LRRK2, and highlight the possibility that targeting LRRK2 may be beneficial in both cases [6]. However, this requires a detailed knowledge of the normal and pathological function(s) of LRRK2 at the molecular, cellular and systems levels.

The LRRK2 protein displays a complex multidomain structure with a central region comprising three independent domains, including a ROC (Ras of complex proteins), COR (C-terminal of ROC) and kinase domain. All six verified pathogenic mutations map to those three domains, indicating that altered enzymatic activity may be mediating the pathogenic effects of LRRK2 (Figure 1). However, whereas LRRK2 displays GTPase and kinase activity in vitro, only G2019S, the most prominent pathogenic LRRK2 mutation, has been consistently shown to augment kinase activity (reviewed in [7,8]). The effect of the other pathogenic mutations is less clear, as is the possible mutual regulation between the two enzymatic domains, or indeed the relevant pathogenic output of the protein (GTPase compared with kinase activity) [6–8]. Moreover, the true physiological target(s) of kinase activity are largely unknown, and other studies indicate that the function of LRRK2 may be rather mediated via a scaffolding role as a protein–protein interaction hub [9].

Although the molecular mechanism(s) of LRRK2 function remain unclear, at the cellular level, certain phenotypes have been consistently observed. At least under conditions of high-level expression in vitro, the pathogenic mutant forms of LRRK2 seem to be acutely toxic [10–13]. Similarly, there is evidence for cell death in vivo upon viral vector-mediated expression of mutant LRRK2 [14,15]. Importantly, toxicity seems dependent on the kinase activity of mutant LRRK2 [10,11,14]. In cellular models where cell death is not apparent, neurite shortening has also been reported to be a consistent phenotype associated with mutant LRRK2 [16–23]. Overexpression of wild-type and especially G2019S mutant LRRK2 in primary cortical neurons or neuronal cell lines leads to a reduction in neurite length, whereas ablation of endogenous LRRK2 oppositely enhances neurite length [16,17,23]. Likewise, upon long-term culture conditions, dopaminergic neurons differentiated from induced pluripotent stem cells from familial PD patients with the G2019S mutation display reduced numbers of neurites and neurite arborization [24]. Where evaluated, the effect of G2019S mutant LRRK2 on neurite morphology seems to be dependent on kinase activity and mediated by macroautophagy [16,17,23,24]. Since all mutations tested...
to date have at least one of these effects in cells, neurite shortening via changes in macroautophagy and eventual cell toxicity may reflect common cellular outputs of the same signalling pathways regulated by mutant LRRK2 [6]. In the present paper, we review the current knowledge of LRRK2’s role in macroautophagy and how this may be related to endolysosomal Ca\(^{2+}\) signalling.

**LRRK2 and autophagy: the good, the bad or the ugly?**

Macroautophagy (hereinafter named autophagy) has been consistently shown to play important roles for determining neurite length (reviewed in [25]). Autophagy has recently gained much attention for its potential contribution to the pathogenesis of several neurodegenerative diseases including PD [26,27], and this link is supported further by the increase in autophagic vacuoles in the substantia nigra of PD brains [28]. Autophagy is a tightly regulated process by which cytosolic constituents, including damaged organelles and aggregated proteins, are engulfed within specialized double-membraned vesicles called autophagosomes, which are subsequently delivered to the lysosome for degradation [29,30]. Any disruption along the process, such as affecting autophagosome formation, fusion of autophagosomes with amphisomes or lysosomes, hydrolytic degradation or the re-formation of lysosomes can impair autophagic flux, concomitant with the accumulation of autophagy substrates and autophagic structures [29,30]. Furthermore, a close relationship exists between autophagy and endocytosis, with both sharing lysosomes as their common end-point [31]. Given this complexity, it has been difficult to assign a precise positive or negative role for normal or mutant LRRK2 in autophagic–lysosomal clearance.

To study the normal function of LRRK2, various lines of knockout mice have been generated. In LRRK2-deficient kidney, an increase in the number and size of secondary lysosomes and autolysosome-like structures has been consistently observed [32–34]. This is accompanied by the accumulation of lipofuscin granules, composed of highly oxidized and cross-linked proteins and lipids which cannot be properly degraded, and of p62, an autophagy substrate [32–34]. Such abnormal accumulation of undigested material indicates an impairment in the autophagy–lysosomal degradation system in the absence of LRRK2. To determine the possible defect along the autophagic pathway, the levels of LC3 (light chain 3)-I and LC3-II have been analysed. LC3-II, the lipidated form of LC3-I, is bound to the autophagosomal membrane, and its amount tends to be a reliable indicator of autophagic activity [35]. However, studies of this type have yielded a complex picture, with either no change [34] or a biphasic change reflecting an initial induction of autophagy at a young age, followed by a decrease in flux over time [32,33]. The continuous induction of autophagy caused by the absence of LRRK2 *in vivo* has been suggested to cause an eventual deficiency in the clearance or recycling of autophagic components/autolysosomes [33]. However, it remains to be seen whether such clearance/recycling represents a rate-limiting step, or whether other mechanism(s) may account for the observed age-dependent biphasic effects on autophagy.

In HEK (human embryonic kidney)-293 cells, RNAi (RNA interference)-mediated knockdown of LRRK2 resulted in increased LC3 turnover under starvation conditions. Unfortunately, equivalent flux experiments, or indeed an ultrastructural analysis, were not performed under nutrient rich conditions in the knockout cells [36]. Conversely, in the same cellular model, overexpression of R1441C mutant LRRK2 was reported to cause impaired autophagic balance, as was evident by the accumulation of multivesicular bodies and large autophagosomes containing incompletely degraded material and increased levels of p62 [36]. Similarly, we detected improper autophagic–lysosomal clearance (indicated by an increase in lysosomal pH, accumulation of autophagic structures and lipid droplets) in HEK-293 cells overexpressing wild-type or G2019S mutant LRRK2 [37,38]. Thus it seems plausible that, at least in the kidney, the normal function of LRRK2 may be to negatively regulate autophagic clearance/lysosomal homeostasis. In this setting, the presence of extra (or mutant hyperactive) LRRK2 would block, whereas the absence of LRRK2 would enhance, autophagic flux, with enhanced flux eventually overloading the lysosomal clearance/recycling system, such that both too much or too little LRRK2 activity may be detrimental to the proper functioning of this pathway *in vivo*.

**LRRK2 and autophagy: universal or tissue-specific?**

What about LRRK2-dependent autophagic–lysosomal clearance in the brain? Disappointingly, no accumulation of autophagic or lysosome-related structures has been reported in the brains of aged mice lacking LRRK2 [32–34]. This may indicate that LRRK2 performs distinct roles in distinct tissues [39]. Alternatively, LRRK1, a homologue of LRRK2, may functionally compensate for the loss of LRRK2 in the brain, but not in kidney, which suffers the biggest loss of LRRK compared with other organs [40,41]. The generation of double-knockout lines will be necessary to determine...
whether complete loss of LRRK in neurons results in age-related changes in autophagy similar to those described in the kidney. Apart from compensatory events mediated by LRRK1, the levels of LRRK2 among different tissues may predetermine the presence and/or magnitude of a possible phenotype upon knockout and/or overexpression. For example, as LRRK2 levels are very high in the kidney [40,41], a knockout strategy may most pronouncedly uncover the (normal) role of LRRK2 in autophagic–lysosomal clearance in this organ. In contrast, given the low levels of LRRK2 in brain, a transgenic overexpression approach (of mutant hyperactive LRRK2) may be more effective, at least in the context of determining an autophagic–lysosomal clearance phenotype.

Alternatively, LRRK2 may affect a conserved pathway present in all cell types, but the same pathogenic mutation may give rise to different degrees of pathology depending on the cellular milieu in which it is operating [39]. As basal autophagy is very high in the kidney, deregulation of this pathway would be expected to be very pronounced in this organ, but should nevertheless also be detectable in other tissues such as brain. Indeed, overexpression of G2019S mutant LRRK2 has been reported to cause abnormal accumulation of autophagic and lysosomal structures in primary cortical neurons and neuronal cell lines in culture [16,17]. Similarly, an accumulation of autophagic vacuoles, including early and late autophagosomes, has been described in the soma and processes in the cortex and striatum from G2019S and, to a lesser degree, R1441C transgenic mice with advanced age [22]. Thus, both in vitro and in vivo, overexpression of mutant LRRK2 causes impaired autophagic–lysosomal clearance in neurons as well. In addition, bone-marrow-derived macrophages from mutant LRRK2 mice display a decrease in LC3-II levels, possibly highlighting an autophagic phenotype in those cells as well [42]. A decrease in autophagic flux, concomitant with an increase in p62 levels, autophagosomes and lipid droplets has recently also been described in human dopaminergic neurons derived from induced pluripotent stem cells from G2019S mutant LRRK2, but not control patients, after long-term culture [24], suggesting that endogenous levels of mutant LRRK2 are sufficient to induce an autophagic–lysosomal phenotype in dopaminergic neurons with time. Finally, and consistent with a role in autophagic–lysosomal clearance, overexpressed as well as endogenous LRRK2 has been reported to localize to specific membrane subdomains including endolysosomal structures in neuronal and non-neuronal cells [36,43,44]. Altogether, the data currently suggest that LRRK2 regulates autophagic–lysosomal clearance in a variety of cell types.

LRRK2 and autophagy: how?

The effects of LRRK2 on autophagic–lysosomal clearance may reflect its primary mechanism of action, or may occur secondarily, elicited as a response to some upstream event(s). Even if rather direct, many distinct scenarios are possible, as autophagy intersects with both the secretory and endocytic pathways at several points [45]. For example, LRRK2 has been shown to interact with the GTPase Rab5b, a regulator of endocytic vesicle trafficking [46]. Interestingly, both overexpression and knockdown of LRRK2 cause a decrease in presynaptic vesicle endocytosis rates, again indicating that both too much and too little LRRK2 adversely alters homeostatic mechanisms, in this case controlling endocytosis [46]. Similarly, both overexpression or knockdown of LRRK2 induce defects in vesicle endocytosis upon depolarization of primary neuronal cultures [47,48], even though the possible effect of LRRK2 on the GTPase activity of Rab5b remains unclear.

Rab5b is a key regulator of the early endocytic pathway in mammalian cells [49], and recent studies indicate that it may play an additional positive role in autophagy by regulating an early step of autophagosome formation in a TORC1 (target of rapamycin complex 1)-independent manner [50]. In addition, endocytosis enables the formation of distinct signal transduction complexes which define specialized endosomal–lysosomal signalling platforms [51], such that LRRK2-mediated changes in endocytosis may modulate the formation of intracellular complexes to regulate signalling cascades including Wnt or MAPK (mitogen-activated protein kinase) cascades [51], both of which have been shown to be affected by LRRK2 [52].

Other mechanisms by which LRRK2 may regulate autophagy are possible as well. Fusion of both autophagosomes and endosomes with lysosomes requires Rab7, as does the process of lysosome re-formation [31,53–55], such that interfering with Rab7 function would affect autophagic–lysosomal clearance. Indeed, a recent study has shown that the Drosophila LRRK2 homologue interacts with Rab7 on late endosomes and lysosomes to negatively regulate Rab7-dependent perinuclear lysosomal positioning required for the efficient degradation of autophagosomes [56]. Finally, a recent study in Caenorhabditis elegans expressing human wild-type or mutant LRRK2, together with mutant tau as a source of proteostatic stress, revealed increased expression of numerous proteins including a subunit of the V-type proton ATPase [57,58]. Moreover, the behavioural motor deficits observed in these double-transgenic nematodes could be reverted by increasing autophagic flux using a rapamycin analogue. These data are consistent with our findings that mutant LRRK2 may increase lysosomal pH and concomitantly decrease lysosomal clearance, a process reversed by rapamycin, but not by other compounds which increase autophagy in an mTOR (mammalian target of rapamycin)-independent manner [37]. It will be interesting to determine whether the beneficial effect of the rapamycin analogue on motor output is related to an mTOR-dependent increase in degradative capacity as autophagic flux is enhanced, a decrease in protein synthesis, an effect on lysosomal homoeostasis or a combination thereof. In either case, a picture is starting to emerge whereby LRRK2 may regulate both early and late steps of autophagic–lysosomal clearance in a manner dependent on Rab proteins (Figure 2).
LRRK2 regulation of autophagy via NAADP (nicotinic acid–adenine dinucleotide phosphate)-mediated endolysosomal Ca$^{2+}$+ signalling

Changes in the concentration of cytosolic Ca$^{2+}$+ regulate a multitude of cellular events [59]. These changes result from both Ca$^{2+}$+ influx across the plasma membrane and the Ca$^{2+}$+ release from intracellular stores [60]. There is a growing appreciation of the importance of organelles of the endolysosomal system as mobilizable Ca$^{2+}$+ stores [61,62]. Indeed, release of Ca$^{2+}$+ from these stores has important implications for both endolysosomal trafficking [54] and intracellular Ca$^{2+}$+ signalling by the potent agonist-generated second messenger NAADP [61,63]. In many cell types, NAADP triggers complex Ca$^{2+}$+ signals that initiate from acidic Ca$^{2+}$+ stores, but which are subsequently amplified by ER (endoplasmic reticulum) Ca$^{2+}$+–release channels [64,65]. Recent work has identified the endolysosomal TPC (two-pore channel) proteins TPC1 [66] and TPC2 [67] as the most likely primary targets for NAADP [68].

In our recent studies, we found an increase in autophagosome numbers upon transient overexpression of both wild-type and G2019S mutant, but not kinase-dead, LRRK2 in various cell lines, including dopaminergic neuroendocrine cells [37]. Importantly, we found that these effects were inhibited by the Ca$^{2+}$+ chelator BAPTA [1,2-bis-(o-aminophenoxy)ethane-N,N,N′,N′-tetra-acetic acid], suggesting that they were Ca$^{2+}$+–dependent. The effects of LRRK2 overexpression were also blocked by overexpression of ER-targeted bcl2 (to deplete ER Ca$^{2+}$+ stores) and associated with an increase in the pH of a population of lysosomes and an increase in lipid droplet numbers. That NAADP evokes cytosolic Ca$^{2+}$+ signals which can be amplified by ER Ca$^{2+}$+ stores, and causes partial alkalinization of acidic stores and induces lipid accumulation [64,69,70], prompted us to test the role of NAADP in LRRK2 action.

Accordingly, we found that elevation of cellular NAADP levels using a cell-permeant NAADP analogue [NAADP-AM (NAADP acetoxymethyl ester)] [71] increased autophagosome numbers, lysosomal pH and lipid droplet numbers, thus largely mimicking the effects observed upon LRRK2 overexpression [37]. Conversely, the NAADP antagonist NED19 identified by virtual screening methods [72] reversed the effects of LRRK2. The increase in autophagosome number could also be blocked by overexpression of TPC2 mutated within the pore region [73]. This inactive mutant probably acts in a dominant manner similar to TPC1 in which the corresponding residue is mutated [66,74]. Taken together, these data uncover a hitherto unknown link between NAADP and LRRK2 function (Figure 3).

Outlook

Although the importance of LRRK2 in regulating autophagy is becoming increasingly clear, the underlying mechanisms are not fully understood. As discussed above, potential roles for Rab proteins and Ca$^{2+}$+ are emerging and not necessarily mutually exclusive. Moreover, the impact of Ca$^{2+}$+ on autophagy has been appreciated for some time and appears complex, with both positive and negative effects reported. Such dual effects may depend on the precise intraorganellar location at which Ca$^{2+}$+ is required for autophagosome–lysosome or endosome–lysosome fusion respectively [31,53].

Are the effects of LRRK2 on TPCs direct or indirect? It is tempting to speculate that TPCs may be kinase substrates. Recent evidence suggests that TPCs probably bind NAADP indirectly through associated low-molecular-mass binding proteins [75]. Although the precise identity of these proteins remains to be elucidated, might these be potential LRRK2 substrates? Although the NAADP pathway is implicated in LRRK2–mediated autophagy, the role of LRRK2 in regulation of cytosolic Ca$^{2+}$+ levels remains to be established. Future work will thus be necessary to delineate the precise molecular links between LRRK2, autophagy, NAADP and Ca$^{2+}$+.
Figure 3 | Schematic diagram of proposed mechanism(s) by which LRRK2 regulates autophagy via modulation of NAADP-dependent Ca\(^{2+}\) channel (TPCs) on lysosomes

LRRK2 localizes to late endosomes and lysosomes and regulates Ca\(^{2+}\) release through TPCs. Whether this regulation is direct or mediated through interactions with Rab7 or NAADP receptors (NAADP-R) remains to be determined. Ca\(^{2+}\)-induced Ca\(^{2+}\) release from the ER amplifies cytosolic Ca\(^{2+}\) signals, which leads to the activation of a Ca\(^{2+}\)-dependent cascade to increase autophagosome numbers. Simultaneously, diminished luminal Ca\(^{2+}\) may cause a decrease in autophagosome-lysosome fusion, and increased pH may have additional effects in impairing lysosomal proteolysis, leading to the observed autophagic-lysosomal clearance phenotype. For simplicity, additional Rab7- and Ca\(^{2+}\)-regulated events which may be subject to LRRK2 modulation in a TPC-dependent or -independent manner are not depicted.

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**References**


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