

Leucine-rich repeat kinase 2 mutations and Parkinson's disease: three questions

Elisa Greggio and Mark R Cookson¹

Laboratory of Neurogenetics, National Institute on Aging, National Institutes of Health, 35 Convent Drive, Bethesda, MD 20982-3707, U.S.A.

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ABSTRACT

Mutations in the gene encoding LRRK2 (leucine-rich repeat kinase 2) were first identified in 2004 and have since been shown to be the single most common cause of inherited Parkinson's disease. The protein is a large GTP-regulated serine/threonine kinase that additionally contains several protein-protein interaction domains. In the present review, we discuss three important, but unresolved, questions concerning LRRK2. We first ask: what is the normal function of LRRK2? Related to this, we discuss the evidence of LRRK2 activity as a GTPase and as a kinase and the available data on protein-protein interactions. Next we raise the question of how mutations affect LRRK2 function, focusing on some slightly controversial results related to the kinase activity of the protein in a variety of *in vitro* systems. Finally, we discuss what the possible mechanisms are for LRRK2-mediated neurotoxicity, in the context of known activities of the protein.

Key words: GTPase, leucine-rich repeat kinase 2 (LRRK2), Lewy body, neurotoxicity, Parkinson's disease.

INTRODUCTION

In 2002, Funayama et al. reported a new genetic linkage to dominantly inherited PD (Parkinson's disease) in a series of families from the Sagami region of Japan. The original evidence for a gene that caused PD was quite strong, but there were some unusual features. For example, the disease appeared to be dominant, but had decreased penetrance, i.e. people who had inherited the chromosomal region that tracked with disease did not always exhibit signs of PD. Also, a few autopsies had been carried out previously on members of the family and, whereas they had the expected

neurodegeneration in the substantia nigra that is typical of PD and related diseases, they did not have Lewy bodies (Hasegawa and Kowa, 1997). Lewy bodies are intracellular neuronal aggregates made up in part of a protein α -synuclein, and represent an important marker of typical, sporadic forms of PD (for a discussion of the distinction between Lewy body PD and Parkinsonism, see Hardy et al., 2006). It was therefore not initially appreciated that this family represented much more than an unusual, possibly even private, disease that resembled PD.

However, less than 2 years later, not only had several families been found worldwide that were linked to the same chromosomal locus (Zimprich et al., 2004a), but also several mutations were found in LRRK2 (leucine-rich repeat kinase 2) (Paisán-Ruiz et al., 2004; Zimprich et al., 2004a). By the next year, LRRK2 mutations were shown to be relatively common, occurring in 1–30% of all PD depending on the population under study and whether familial PD is excluded or included (reviewed in Cookson et al., 2005). This mutation frequency is incredibly high for a disease that was considered to not be genetic, as PD had been for many years. All mutations reported to date are inherited in a dominant fashion, and homozygotes have similar phenotypes and age at onset as heterozygotes (Ishihara et al., 2007), indicating a true dominant effect.

Importantly, the original Japanese family was also shown to have a mutation in LRRK2, confirming the correct identification of the gene (Funayama et al., 2005). In fact, LRRK2 mutations in general are similar to those in the original family. Penetrance is age-dependent, but still incomplete (Hulihan et al., 2008; Latourelle et al., 2008), as shown by mutation carriers surviving into their 80s without developing symptoms of PD (Kay et al., 2005), far past the typical onset of ~50 years of age. Also, examination of additional family members from the Sagami kindred revealed that some cases do have Lewy bodies (Hasegawa et al., 2008). The variable pathological outcomes of LRRK2-related disease was also emphasized by Zimprich et al. (2004b) and confirmed in a number of other studies. Even

¹To whom correspondence should be addressed (email Cookson@mail.nih.gov).

Abbreviations: COR, C-terminal of ROC (Ras of complex proteins); DAPK1, death-associated protein kinase 1; eIF4E, eukaryotic initiation factor 4E; 4E-BP, eIF4E-binding protein; ERM, ezrin/radixin/moesin; FADD, Fas-associated death domain; LRRK1/2, leucine-rich repeat kinase 1/2; dLRRK, *Drosophila* LRRK homologue; MBP, myelin basic protein; MLK, mixed lineage kinase; PD, Parkinson's disease; ROC, Ras of complex proteins.

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cases with the same mutation can have different outcomes, commonly having (Ross et al., 2006) but occasionally lacking (Gaig et al., 2007) Lewy bodies, even though the clinical phenotypes are similar (reviewed in Cookson et al., 2008).

The data discussed so far tell us that LRRK2 mutations are a surprisingly common cause of inherited PD. The decreased penetrance of many LRRK2 mutations means that the genetic contribution to lifetime risk of PD has probably been underestimated in the past. Furthermore, the variable pathological outcomes of LRRK2 mutations emphasizes that the clinical course of disease is not entirely synonymous with the underlying protein deposition, although it may be a useful clue as to mechanism. Overall, LRRK2 mutations represent a substantial advance in our understanding of lifetime risk of PD, while slightly complicating our knowledge of the relationship between symptoms and pathology.

Given this, how do inherited *LRRK2* gene mutations actually cause an adult onset neurodegenerative condition? One way to address this critical problem is to consider the intermediates between gene and phenotype, the altered proteins that are produced by a mutant allele. In the present review, we break this down into three apparently simple questions, namely what is the normal function of LRRK2, how do mutations change function and why might this altered function result in PD? At this time, many of the more honest answers to these questions are that we simply do not know, but it is our hope that, by discussing each in turn, we might be able to identify some of the key next steps for understanding LRRK2 biology.

QUESTION ONE: WHAT DOES LRRK2 NORMALLY DO?

At the time of sequencing genes in the linkage region on chromosome 12 that had been nominated to contain the gene responsible, *LRRK2* was not the most attractive candidate. Not only was it poorly characterized, but also it was rather large, requiring a significant investment of time to sequence it. Overall, the full-length cDNA is 7.5 kb long, encoding an ~280 kDa protein.

LRRK2 is named for its leucine-rich repeats and a kinase domain. This arrangement is shared by one other protein, LRRK1. In between these two regions is a GTPase sequence called a ROC [for Ras of complex proteins (Bosgraaf and Van Haastert, 2003)] domain and an adjacent COR (C-terminal of ROC) domain. This pair of domains is characteristic of the ROCO superfamily of proteins (Marin et al., 2008), which all contain tandem ROC-COR domains, but do not all contain kinase domains. In the human genome, there are four ROCO proteins, of which three [LRRK1, LRRK2 and DAPK1 (death-associated protein kinase 1)] are also kinases, but one [MFHAS1 (malignant fibrous histiocytoma amplified sequence 1)] that is not (Lewis, 2009). In other species, there are

variable numbers of LRRK homologues; *Drosophila melanogaster* and *Caenorhabditis elegans* have a single LRRK protein.

It has been misstated that the kinase domain of LRRK2 is related to the MLKs (mixed lineage kinases), but analysis of all kinase domains throughout the human genome suggests that LRRK1 and LRRK2 form a small offshoot group of the RIPK (receptor-interacting protein kinase) family of kinases, which are somewhat similar to the IRAK (interleukin 1 receptor-activated kinase) family and rather more distant from MLKs (Manning et al., 2002). In contrast, the kinase domain of DAPK1 is quite distinct from either LRRK homologue (Manning et al., 2002). It has been proposed that, throughout evolution of the LRRK genes, the kinase domain has been acquired from different sources and is quite divergent in sequence (Marin, 2006, 2008; Marin et al., 2008).

What makes LRRK2 different from LRRK1 is the N-terminus, which is much longer in LRRK2. The motifs in this region are not well annotated, but a number of repeat sequences can be found that have a limited homology with sequences found in the ankyrin family. Finally, near the C-terminus of LRRK2 is a WD40 domain that probably forms a β -propeller structure. The significance of the presence of both ankyrin-like and leucine-rich repeats and a WD40 domain is that they are very likely to be protein-protein interaction motifs and, with so many present, this indicates that LRRK2 may act as a scaffold for several other proteins. WD40 domains in other proteins can also interact with lipids (e.g., McArdle and Hofmann, 2008), raising the possibility that LRRK2 might be present at intracellular membranes. A diagram of the domain structure of LRRK2 is shown in Figure 1.

An added layer of complexity arises because LRRK2 self interacts (Gloeckner et al., 2006) to form a dimer (Greggio et al., 2008). Other ROCO family proteins also form dimers via COR-COR interactions (Gotthardt et al., 2008); whether the equivalent region of human LRRK2 has a similar structure is unclear as there is some evidence of ROC-ROC interactions (Deng et al., 2008). Regardless of the exact structural basis of LRRK2 dimerization, the key motifs are similar enough in LRRK1 to make heterodimers at least a possibility.

This information leads us to a model of a large protein with a central catalytic GTPase/kinase region surrounded by protein-protein and perhaps protein-membrane interaction motifs, forming homo- and possibly hetero-dimers. To understand function requires demonstration of whether the kinase and ROC (GTPase) domains are enzymatically active. For the kinase domain, the answer is a slightly tepid yes. Several groups, including our own, have reported that full-length LRRK2 or LRRK1 immunopurified from mammalian cells has measurable kinase activity (West et al., 2005; Gloeckner et al., 2006; Greggio et al., 2006; Korr et al., 2006; MacLeod et al., 2006; Smith et al., 2006; Greggio et al., 2007; Ito et al., 2007; Jaleel et al., 2007; Li et al., 2007; West et al., 2007; Greggio et al., 2008; Imai et al., 2008). One small concern is that the apparent activity of LRRK2 might arise

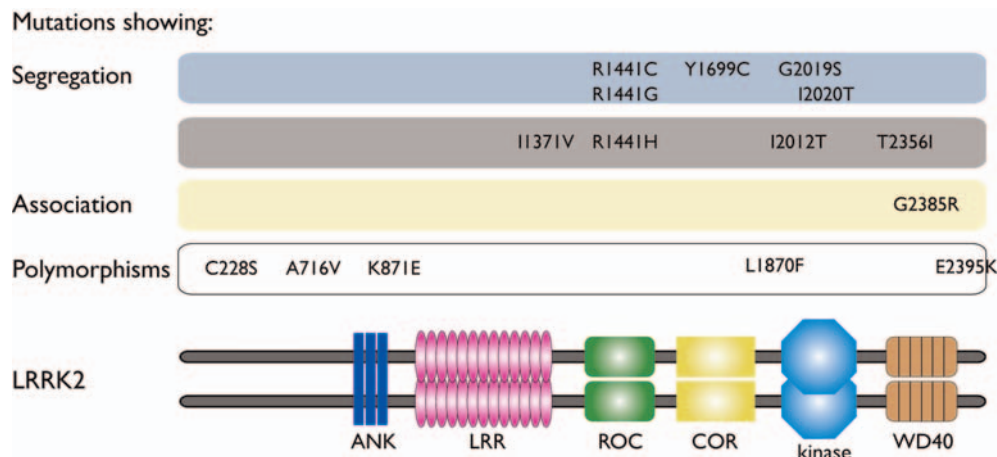


Figure 1 LRRK2 mutations and domains

Many variants in LRRK2 have been reported; some are clearly pathogenic, some are clearly not pathogenic and many are unclear. The tests for pathogenicity are either segregation (blue box) within families or association with disease across populations (yellow box) and mutations that pass either of these tests are placed in an approximate relationship to the linear sequence of the protein. Some mutations, such as R1441H, are probably causal but segregation data is less clear and these are listed in the grey box. Finally, a large number of polymorphic variants have been reported that are not likely to be pathogenic (white box). For the sake of clarity, not all reported mutations are listed. Here, we show only non-synonymous amino-acid-changing variants that were found only in controls (Paisán-Ruiz et al., 2008) and thus are very unlikely to be related to disease. The ideogram in the lower part of the Figure shows LRRK2 in a linear arrangement with each of the proposed domains labelled, from N- to C-termini; ANK, ankyrin-like repeats; LRR, leucine-rich repeats; ROC, Ras of complex proteins, GTPase domain; COR, C-terminal of ROC domain; kinase; WD40, a β -propeller-like domain made up of WD40 repeats. It should be noted that the clearly pathogenic variants cluster around the central enzymatic region, whereas clearly polymorphic changes are distributed throughout the molecule.

from an inadvertently co-purified kinase. This is unlikely, as several groups have used artificial kinase-dead variants that have 10–20% of the activity of wild-type protein. Furthermore, the LRRK2 kinase domain alone expressed in *Escherichia coli* can be active (Luzon-Toro et al., 2007), as can a larger fragment expressed in a baculovirus system (Anand et al., 2009).

The major caveat here is that many of these studies used autophosphorylation as a readout for kinase activity. Such assays are commonly used to identify kinases because they work, but that does not prove that the autophosphorylation event is physiologically relevant. Sometimes autophosphorylation can be an important regulatory mechanism, e.g. in the receptor tyrosine kinases, which autophosphorylate when they form a dimer upon binding of their ligands. For full-length LRRK2, we have shown that phosphorylation occurs within each dimer molecule (Greggio et al., 2008), although the isolated kinase domain can autophosphorylate *in trans* (Luzon-Toro et al., 2007). But at this stage, the autophosphorylation site(s) in LRRK2 have not been identified and so they cannot yet be proven *in vivo*. There are two sites (Ser²⁰³² and Thr²⁰³⁵) in the activation loop that appear to diminish kinase activity when mutated to alanine residues, although these have not yet been proved to be authentically due to autophosphorylation (Greggio et al., 2008). However, LRRK2 is known to be active using autophosphorylation assays when purified from transgenic mouse brain (Li et al., 2007), where it is apparently more active than in other tissues or in cultured cell lines. An alternative to autophosphoryla-

tion as an assay for kinase activity is to use generic substrates such as MBP (myelin basic protein) (West et al., 2005; Iaccarino et al., 2007; Luzon-Toro et al., 2007). Again, LRRK2 is modestly active in these assays, which suggests that LRRK2 is a serine/threonine-directed kinase, but does not lead to the idea that MBP is a specific substrate for LRRK2, as many serine/threonine kinases will mediate the same reaction.

Two heterologous LRRK2 substrates have been proposed: moesin (Jaleel et al., 2007) and 4E-BP [eIF4E (eukaryotic initiation factor 4E)-binding protein] (Imai et al., 2008). Moesin is one of three proteins collectively named ERM for the members ezrin/radixin/moesin. The major role of ERM proteins is to anchor the cytoskeleton to the plasma membrane, and thus influence processes in the cell related to cytoskeletal dynamics at the cell surface such as maintenance of neuronal growth cones (Paglini et al., 1998). The C-terminal region of moesin, which contains an actin-binding site, can interact with a FERM (4.1/ezrin/radixin/moesin) domain in moesin's N-terminal region in a closed conformation. A shift to an open conformation is required for binding to the cytoskeleton. The site on moesin that is phosphorylated by LRRK2 (Thr⁵⁵⁸, with a minor site at Thr⁵²⁶) is in the C-terminal domain and thus is normally relatively inaccessible and, probably because of this, Jaleel et al. (2007) found that pre-heating recombinant moesin to >60°C was required to see activity. Quantification suggested that, even under these circumstances, phosphate was incorporated into moesin at a ratio of ~10%, i.e. even here, moesin is not a very efficient substrate. Short peptides

containing the moesin Thr⁵⁵⁸ motif, which would not be so structurally restricted, also act as LRRK2 substrates (Jaleel et al., 2007; Anand et al., 2009).

Overall, these data suggest that moesin and related proteins are potential substrates for LRRK2. However, LRRK2 has not yet been shown to be an authentic kinase for moesin *in vivo*. The requirements to show this are necessarily quite high, but showing (for example) that LRRK2-knockout cells or animals have decreased moesin Thr⁵⁵⁸ phosphorylation and that transfection with active full-length LRRK2 increases the same event, or restores it in the case of knockouts, would be one way forward. As discussed in the next section, the pathogenic LRRK2 mutations may be helpful in teasing apart some of these problems and assessing their relevance for disease mechanisms.

Although the steps needed to support LRRK2 being an authentic *in vivo* kinase for moesin are extensive, they are feasible as shown by the work of Imai et al. (2008) on a second proposed LRRK2 substrate. 4E-BP is an interactor of eIF4E, which in turn binds to capped mRNA species, promoting their translation. Binding of 4E-BP to eIF4E prevents the latter from being active, and therefore 4E-BP is a repressor of protein translation. Oxidative stress and other stimuli that have an impact on protein translation affect phosphorylation of 4E-BP. Imai et al. (2008) have proposed that LRRK2 modulates this system by phosphorylating 4E-BP. In mammalian cell culture, overexpression of LRRK2 increases 4E-BP phosphorylation at a number of sites; Imai et al. (2008) propose that LRRK2 first phosphorylates 4E-BP at Thr³⁷/Thr⁴⁶, which then acts as a stimulus for further phosphorylation by other kinases at secondary sites including Ser⁶⁵/Thr⁷⁰. There is a modest decrease in phosphorylation of 4E-BP Thr³⁷/Thr⁴⁶ and Ser⁶⁵ when LRRK2 levels are knocked down with RNAi (RNA interference). Furthermore, overexpression of 4E-BP rescues the effects of LRRK mutants *in vivo* using *Drosophila* models, which show increased sensitivity to oxidative stress. Overall, these data are supportive of 4E-BP being an authentic *in vivo* substrate for LRRK2 or its *Drosophila* homologue, dLRRK. However, at the time of writing, no independent confirmation of the results of Imai et al. (2008) has been published, and details of the phosphorylation reaction, such as how efficient phosphorylation of 4E-BP by LRRK2 is or whether this activity is more efficient than autophosphorylation, are not yet available.

Therefore several pieces of data support the idea that LRRK2 is an active kinase, although the data to show that this is true *in vivo* are more limited. What about the proposed GTPase activity of the ROC domain? Again, evidence here is mixed on whether this is enzymatically active, and some of the details are important.

All published data support that full-length LRRK1 (Korr et al., 2006; Greggio et al., 2007) and LRRK2 (Smith et al., 2006; Guo et al., 2007; Ito et al., 2007; West et al., 2007) will bind GTP at millimolar concentrations. However, data on whether the protein is active as a GTPase are mixed. West et al. (2007)

reported that they were unable to detect GTPase activity in full-length LRRK2 when expressed and immunopurified from mammalian cells, whereas Ito et al. (2007) could only see measurable activity if the protein was mutated to resemble the more active small GTPase, Ras. In contrast, we (Lewis et al., 2007) and others (Guo et al., 2007; Li et al., 2007) were able to detect GTPase activity under similar circumstances. Although small technical details may be critical for identifying why the experiment can give different results under different circumstances, the most likely explanation is that the apparent GTPase activity of full-length LRRK2 is quite weak. For example, we used an artificial mutant (K1347A) that cannot bind GTP and therefore should have no GTPase activity as a reference (Lewis et al., 2007) and found that wild-type LRRK2 was only slightly more active than the negative control.

In contrast, the ROC domain is much more active when removed from the context of the full-length protein either when expressed in *E. coli* (Deng et al., 2008) or in mammalian cells (Li et al., 2007). The simplest explanation is that sequences outside of the ROC domain modulate GTPase activity, perhaps by physical interaction or by recruitment of other cellular proteins. In prokaryotic ROC-COR proteins, dimerization is critical for GTPase activity, and the COR domain may provide at least part of the dimer interface (Gotthardt et al., 2008). If this is also true for human LRRK2, the COR domain would be a positive regulator of GTPase activity, although not absolutely required, and so inhibitory sequences would have to be present outside of the ROC-COR bi-domain. Again, GTPase activity could be regulated either by intramolecular interactions intrinsic to LRRK2 in the context of the dimer, and/or by recruitment of other proteins.

We therefore have two enzymatic domains, each of which are at least potentially active. To complicate things further, several groups have noted that binding of non-hydrolysable GTP analogues {e.g. GTP[S] (guanosine 5'-[γ -thio]triphosphate) or p[NH]ppG (guanosine 5'-[β , γ -imido]triphosphate)} stimulates the kinase activity of LRRK1 (Korr et al., 2006) and LRRK2 (Smith et al., 2006; Ito et al., 2007; Li et al., 2007; West et al., 2007). In the currently accepted model, GTP-bound LRRK2 has a higher kinase activity than the GDP-bound protein and thus GTPase activity would be important to return LRRK2 kinase activity to basal levels. Whether this predicted kinetic outcome of GTP binding and subsequent hydrolysis occurs under physiological conditions has not yet been proved. It should also be noted that the effect of GTP binding is quite modest, increasing kinase activity ~2-fold, and whether there is regulation of GTPase by kinase, for example, is untested. It is possible that there are further intramolecular events that influence GTPase activity, as has been shown for the *Dictyostelium* ROCO kinase GbpC (van Egmond et al., 2008). There are additional regulatory sequences in LRRK2, as the C-terminal tail is required for full kinase activity (Jaleel et al., 2007), whereas the N-terminus of LRRK2 is inhibitory (Jaleel et al., 2007; Greggio et al., 2008).

Collectively, these data suggest that the ROC-COR-kinase portion of LRRK2 is probably the centrally important regulatory region. What then is the function of the rest of the protein? As discussed above, the various repeat regions appear to be important for protein-protein interactions. Several studies have identified candidate proteins bound to LRRK2. The recessive Parkinsonism protein parkin was reported by Smith et al. (2005) to interact with LRRK2. Dachsel et al. (2007) reported several interactors for full-length LRRK2 expressed in cells using MS approaches. Shin et al. (2008) used yeast two-hybrid screening with the LRR domain to identify Rab5b, a small GTPase involved in vesicle endocytosis. Hsp90 (heat-shock protein 90) also binds to LRRK2, perhaps in association with the co-chaperone Cdc37 (cell division cycle 37), and regulates its stability (Wang, L. et al., 2008). LRRK2 is also reported to interact with α - and β -tubulin, linking it to the cytoskeleton (Gandhi et al., 2008). In most of these cases, binding to interactors was similar for different LRRK2 variants, although mutant LRRK2 has recently been reported to enhance binding to the apoptosis protein FADD (Fas-associated death domain), which then recruits caspase 8 (Ho et al., 2009). Therefore LRRK2 appears to have a potentially large number of interacting partners, with the caveat that most of these experiments have used overexpressed LRRK2 rather than physiological levels of protein, which appear to be quite low in most cell types. In our hands, cells that appear to express higher levels of endogenous LRRK2 such as transformed lymphoblastoid lines (Melrose et al., 2007), have a high-molecular-mass (>1.2 MDa) complex including LRRK2, supporting their identification using non-denaturing techniques (Greggio et al., 2008). There is also some evidence that regions outside the ROC-COR domain may contribute to the self-interaction of LRRK2 (Greggio et al., 2008).

Two things stand out about this list. First, several interactors may give important clues about LRRK2 function. For example, the Rab5a interaction is consistent with a role for LRRK2 in mediating synaptic endocytosis (Shin et al., 2008). This leads to the more important question of the normal physiological role of LRRK2. As well as synaptic endocytosis, LRRK2 has been proposed to have a role in sorting of vesicles between axons and dendrites (Sakaguchi-Nakashima et al., 2007). These two roles may be consistent with localization of LRRK2 to vesicles in the brain (Biskup et al., 2006), and possibly with localization to lipid rafts (Hatano et al., 2007). LRRK2 expression also influences neurite morphology *in vitro* and *in vivo* (MacLeod et al., 2006; Plowey et al., 2008; Wang, L. et al., 2008). Finally, LRRK2 or homologues in other species have been proposed to be important in maintenance of neuronal viability in the presence of oxidative stress (Imai et al., 2008; Liou et al., 2008), although some studies have not identified a role in cell survival (Wang, D. et al., 2008).

The second aspect of this list is that no interactors have been identified that bind to the N-terminal region of LRRK2 before the LRR (leucine-rich repeat) domain. This is puzzling,

as the very large N-terminal region is the most divergent part of the protein compared with LRRK1 and one might therefore expect that any LRRK2-specific interactors might bind here and be interesting for understanding function.

Overall, these data do not answer the question of the normal function of LRRK2, but do give us the impression of a complex modular protein. The central enzymatic ROC-COR-kinase core has regulatory functions at least within the context of the dimeric protein. Outside this are various domains that may recruit other proteins into a complex, making LRRK2 potentially a scaffolding protein, perhaps for cell signalling pathways. Several recent papers have proposed that LRRK2 or LRRK2 complexes act in ways that are important for neuronal function, although one has to wonder whether this is biased because of an expected role of LRRK2 in neurological disease. Although there is clearly much work needed to resolve the question of the physiological role of LRRK2, this outline should allow us to discuss how the mutations in LRRK2 affect function of the protein.

QUESTION TWO: HOW DO MUTATIONS AFFECT LRRK2 FUNCTION?

Before discussing how mutations affect function, we first have to outline causation as it applies to genetics. For many large genes, such as LRRK2, there are a large number of variants along the 7500 nucleotides of the coding sequence (Paisán-Ruiz et al., 2008). Many of these are probably innocuous, but some appear to be linked to disease and distinguishing causal from innocuous variants is critical. From a genetic perspective, there are two ways in which a variant can be assigned as pathogenic. This can be either segregation, where a phenotype is co-inherited with a disease-causing mutation, or association, where, at a population level, carrying a specific variant means an individual is at a higher incidence of expressing a given phenotype. A mutation is any variant that is rare (the classic definition is 1% frequency in a population), whereas a polymorphism is a more frequent variant.

For LRRK2, there are a number of mutations that show clear evidence of segregation. The original Japanese family carries an isoleucine to threonine substitution at position 2020 (I2020T) in the kinase domain (Funayama et al., 2005). The common mutation found in many families worldwide is a glycine to serine change at the adjacent residue (G2019S) (Gilks et al., 2005; Goldwurm et al., 2005; Lesage et al., 2005; Infante et al., 2006; Marongiu et al., 2006; Pankratz et al., 2006; Papapetropoulos et al., 2006; Saunders-Pullman et al., 2006; Williams-Gray et al., 2006; Zabetian et al., 2006a, 2006b; Ishihara et al., 2007; Orr-Urtreger et al., 2007; Gorostidi et al., 2008; Healy et al., 2008; Latourelle et al., 2008; Munhoz et al., 2008). In the ROC domain, an arginine at residue 1441 can be replaced by a glycine residue (R1441G) in

several families in Spain and Portugal (Paisán-Ruiz et al., 2004; Mata et al., 2005; Ferreira et al., 2007; Bras et al., 2008; Gorostidi et al., 2008) or by a cysteine residue (R1441C) in a family from Nebraska (Zimprich et al., 2004b; Haugarvoll et al., 2008). A tyrosine to cysteine mutation in the COR domain, Y1699C, was reported in a family from the U.K. (Paisán-Ruiz et al., 2004) and in a family of German heritage with members in Canada (Zimprich et al., 2004b).

Because there are multiple family members who carry the mutation and develop PD, and in some cases there are several families that may be more remotely related, we can be very confident that five LRRK2 mutations are causal: R1441G/C, Y199C, G2019S and I2020T. There are other mutations that are less certainly pathogenic. Part of the problem is that PD is a very common disease, with approx. 1% of people over the age of 60 rising to 5% prevalence at the age of 80, so finding PD in any given family is not surprising. If the phenotype were extremely rare, such as having beetroot-coloured skin and a lisp, we might be more confident. In many cases, the families are relatively small and we cannot see generation-to-generation transmission of the expected dominant trait, perhaps due to missed diagnosis or incomplete penetrance. Therefore some mutations are genuine variants and some are also found in patients with PD, but will remain ambiguous, so we have to rely sceptically on indirect evidence. Of the reported variants, perhaps the only one that is very likely to be pathogenic is R1441H (Zabetian et al., 2005; Spanaki et al., 2006), because of the two other clearly pathogenic mutations at the same residue that argue that this is a mutation hotspot (Ross et al., 2008). Others are less certain; for example, I1371V has been found in one case with a self-reported family history of PD, but without clear evidence of segregation such as an affected mutation-carrying parent (Paisán-Ruiz et al., 2005).

Then there are a few variants that are frequent enough to be able to assess evidence for association with disease across populations. For example, there is a glycine to arginine substitution in the WD40 domain (G2385R) that is found only in Asian populations, specifically in persons of Han descent. Within these populations, G2385R is much more common in PD cases compared with controls and thus shows associations with lifetime risk of PD (Tan, 2006; Farrer et al., 2007; Chan et al., 2008; Lin et al., 2008; Tan et al., 2009).

In summary, there are some mutations for which we have strong evidence of segregation in the central enzymatic/regulatory portion of LRRK2 and at least polymorphisms for which we have evidence of association towards the C-terminus. Interestingly, there are very few convincing mutations towards the N-terminus of LRRK2 (Paisán-Ruiz et al., 2008), although the significance of this observation is unclear.

Working from the N- to the C-terminus, the first set of convincing pathogenic mutations are those in the ROC domain, R1441C/G and maybe R1441H. In those studies where GTPase activity of LRRK2 could be measured, either R1441C (Guo et al., 2007; Lewis et al., 2007) or R1441G (Li et al., 2007) are associated with decreased GTPase activity

compared with wild-type proteins. One study (West et al., 2007) proposed an increased GTPase activity, but actually measured GTP binding and saw only small differences in this parameter; in our own hands, R1441C and wild-type bind GTP to the same extent (Lewis et al., 2007).

Interestingly, the effect of R1441C is less dramatic when placed into the isolated ROC domain (Deng et al., 2008) compared with the relatively strong effect (admittedly on a weaker GTPase activity) in the full-length protein (Lewis et al., 2007). One read of these data is that Arg¹⁴⁴¹ has a key role in interactions with other domains. This is slightly controversial, as two different models have been proposed for where Arg¹⁴⁴¹ sits in the structure. Using the recombinant human LRRK2 ROC domain isolated from other regions of the protein, we have proposed that Arg¹⁴⁴¹ stabilizes the interface of a ROC-ROC dimer (Deng et al., 2008). In contrast, the structure of a more complete ROC-COR protein from the prokaryote *Chlorobium tepidum* suggests that the equivalent residue is important in hydrophobic interactions between ROC and COR domains (Gotthardt et al., 2008). Resolution of these two models will require crystallization of larger protein fragments of the human protein, as there are several sequence differences around this region between the two homologues. But what the two models both agree upon is that Arg¹⁴⁴¹ plays a small, but probably important, role in the dimer interface and that substitutions at this region decrease GTPase activity for the prokaryotic protein as much as the eukaryotic version (Gotthardt et al., 2008). Although it does not make the genetic evidence any stronger or weaker, it should be noted that, under either model, R1441H would also be defective in mediating the dimer formation, as arginine specifically forms two hydrogen bonds with other residues in the opposite chain, and no other side chain would be able to do this. Furthermore, both models support pathogenicity of the I1371V mutant, as the wild-type residue is in a hydrophobic pocket again near the dimer interface.

Mutations in the COR domain itself have been less well studied, probably because the assays to do this are less obvious than for a GTPase homology domain. However, again working from a prokaryotic homologue, Gotthardt et al. (2008) have shown that the Y1699C equivalent (Y804C) also decreases GTPase activity. As for the ROC mutations, the very probable mechanism is that the substitution for the aromatic residue disrupts a key element of the dimer interface, in this case between the ROC and COR domains. Although an equivalent experiment in human protein has not yet been published, it is known that the ROC and COR domains of LRRK2 interact physically (Deng et al., 2008), making the prediction that Y1699C would have lower GTPase activity owing to a lower stability dimer reasonable.

Therefore there is generally good agreement that ROC mutations lower GTPase activity, with a nagging uncertainty about the actual strength of activity, and a reasonable prediction for COR mutations. Where the real controversy starts is with the kinase domain. All studies published to date have agreed that the effect of the G2019S kinase mutation is

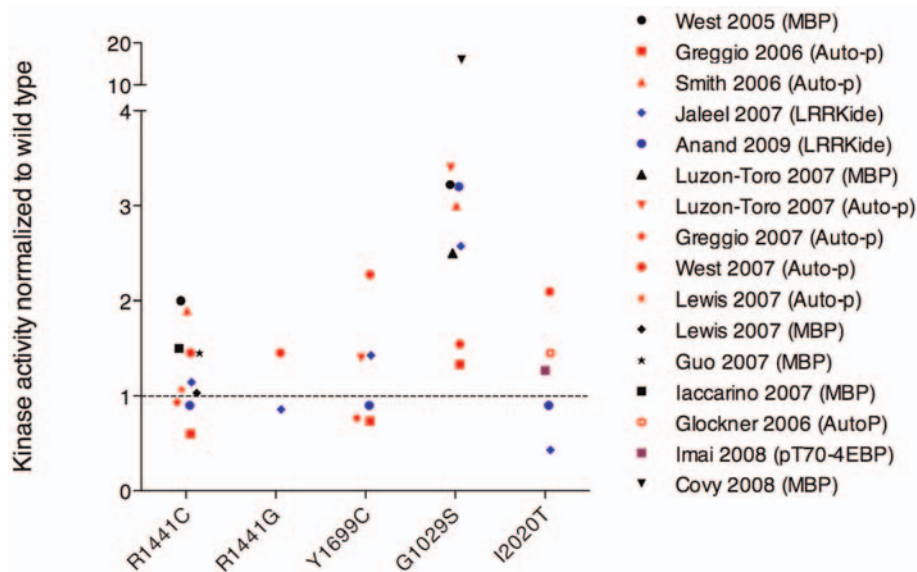


Figure 2 Effects of LRRK2 mutations on kinase activity

For this Figure, we took reported effects of LRRK2 mutations on kinase activity and expressed each relative to the wild-type LRRK2 reported in the same study, where the broken line across the graph = 1. Each study is given by first author and year and the different symbols are colour-coded by substrate used in the assay; black, MBP; red, autophosphorylation; blue, LRRKtide peptide; purple, 4E-BP. Of all the mutations tested, the one that consistently shows an increased activity is G2019S in the kinase domain; all of the others vary between experiments, and there is no clear pattern that relates to substrate used.

to significantly increase phosphorylation activity in a variety of assays (West et al., 2005; Greggio et al., 2006; MacLeod et al., 2006; Smith et al., 2006; Guo et al., 2007; Jaleel et al., 2007; Luzon-Toro et al., 2007; West et al., 2007; Covy and Giasson, 2008; Imai et al., 2008; Anand et al., 2009). Data on I2020T are more ambiguous, with some studies reporting small, but significant, increases in activity (Gloeckner et al., 2006; West et al., 2007; Imai et al., 2008), whereas others report no effect (Luzon-Toro et al., 2007; Anand et al., 2009) or even a slight decrease (Jaleel et al., 2007). Similar uncertainty exists for the ROC and COR mutations, with some studies reporting that all mutations increase activity up to 2.5-fold (West et al., 2005; Smith et al., 2006; West et al., 2007), whereas others suggesting that mutations of Arg¹⁴⁴¹ and Tyr¹⁶⁹⁹ have only minor effects (Greggio et al., 2006; MacLeod et al., 2006; Greggio et al., 2007; Jaleel et al., 2007; Anand et al., 2009) and that similar mutations in LRRK1 slightly decrease activity (Korr et al., 2006). These data are summarized in Figure 2, which we took from the original references that reported quantitative effects of mutations relative to wild-type LRRK2. These studies used several different assays with a variety of constructs from full-length through several N-terminally truncated versions to the isolated kinase domain alone. The picture that emerges is similar to the descriptive arguments above: only G2019S consistently increases kinase activity, whereas other mutations have inconsistent effects and generally only modestly influence activity if there is a difference. No obvious pattern emerges when considering different substrates, as the data from different measures overlap (Figure 2).

Perhaps the place to start resolving some of these apparently contradictory data is with the one change that everyone agrees activates LRRK2, the common G2019S mutation. How might this mutation work mechanistically and/or structurally? Gly²⁰¹⁹ is part of a very highly conserved motif, D(F/Y)G, where the aspartate residue (Asp²⁰¹⁷ in human LRRK2) chelates a Mg²⁺ ion that is required for cleavage of the γ -phosphate from ATP and thus for kinase activity. The glycine residue (Gly²⁰¹⁹ in LRRK2) is absolutely invariant apart from a few rare examples, which happen to be serine residues (Jaleel et al., 2007). This residue marks the start of a conformationally flexible region, the activation loop, which is important for the control of kinase activity. For many kinases, phosphorylation of this loop shifts its orientation relative to the two lobes of the enzyme and thus allows or restricts substrate access. The glycine residue is probably invariantly conserved because the small side chain of this amino acid allows maximum flexibility and thus motion of the activation loop. We can speculate that a serine residue, with a negatively charged hydroxy-containing side chain and less conformational flexibility might 'lock' the kinase in a more active conformation. Support for this idea comes from large-scale sequencing of somatic mutations in cancer where several equivalent glycine to serine changes were found in kinases where increased activity is thought to be the mechanism by which they are associated with excess cell growth (Greenman et al., 2007). Also, substitution of alanine, which also has a smaller side chain relative to serine, for this glycine residue restores autophosphorylation to wild-type levels (Luzon-Toro et al., 2007). One might also imagine that a

threonine residue at the adjacent amino acid within the activation loop would have a similar effect, although this would not explain why estimates of the effect of the I2020T mutation are more variable compared with G2019S.

Mutations outside of the kinase domain are harder to understand based on the above data. If the current model that GTP binding to the ROC domain increases kinase activity is correct, then decreased GTPase activity would mean that the stimulatory effect of GTP binding would last longer for ROC mutants, because the turnover of GTP to GDP would be slowed. However, in the absence of GTP, as most of the above kinase assays were performed, there should be no difference in activity, and it seems likely ahead of time that non-hydrolysable analogues would result in similar effects irrespective of GTPase activity. Therefore the reason(s) kinase activity measurements for mutations outside of the kinase domain are variable between laboratories is unclear. Perhaps there are small differences in the assay conditions that have a large impact on the results, or perhaps our model of regulation of kinase activity by GTP binding is flawed, but the most likely interpretation is that the current assays need refining. These issues are important to resolve, as, without understanding how mutations work, it is hard to develop clear ideas about mechanisms of neurodegeneration.

QUESTION THREE: WHY DO MUTATIONS IN LRRK2 CAUSE NEURODEGENERATION?

So far, we have established that LRRK2 is an active enzyme, at least *in vitro* and *ex vivo*, and that mutations either lower GTPase activity or raise kinase activity, and we believe that these two concepts are linked. However, none of this explains why it is that LRRK2 mutations lead to neurodegeneration, which is, in fact, a series of questions that are interlinked.

Clues to how LRRK2 might lead to neuronal death come from where we started, from human genetics. It is worth restating that the mode of inheritance is dominant with incomplete penetrance and that homozygous cases have the same phenotype as heterozygous. There are two likely ways in which the LRRK2 protein could cause neuronal damage. The mutations could result in a toxic gain-of-function, which could be either misregulation of its normal function or acquisition of a novel toxic function. However, it is also possible that mutations are a loss of normal function: they might, for example, interfere with the wild-type LRRK2 activity and act as a dominant-negative.

The tools to separate these possibilities are initially likely to be based around experimental models. Several laboratories have reported that high levels of overexpression of mutant LRRK2 in primary cultured neurons or SH-SY5Y cells can lead to cell death over a few days (Smith et al., 2005; Greggio et al., 2006; MacLeod et al., 2006; Smith et al., 2006; Iaccarino et al., 2007; West et al., 2007; Ho et al., 2009). Under similar

conditions, and at similar levels of expression, wild-type mutant LRRK2 has only minor effects on basal cell viability, although in one study, treating cells transfected with wild-type LRRK2 with hydrogen peroxide resulted in dramatic cell death (West et al., 2007). Overall, the consistent message is that mutant LRRK2 can cause cell death, at least in the context of cell culture models.

Also consistent between studies is the observation that neurites are shorter after expression of mutant LRRK2 (MacLeod et al., 2006; Plowey et al., 2008; Wang, L. et al., 2008). Whether this is related to toxicity or not is a little unclear, but knockdown of LRRK2 causes a reciprocal increase in neurite length and is not reported to result in cell death (MacLeod et al., 2006). The mode of cell death related to overexpression of mutant LRRK2 is reported to be apoptotic, although evidence is mixed on whether this is a caspase 3 (Iaccarino et al., 2007) or caspase 8 (Ho et al., 2009)-dependent pathway. Some evidence of TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) staining has been reported (Smith et al., 2005), although this could be apoptosis or necrosis as DNA strand breaks can be labelled by this technique in either mode of cell death. Finally, in two models, there was evidence of autophagic degradation of organelles, which might indicate a mixed mode of cell death (MacLeod et al., 2006; Plowey et al., 2008).

What is interesting here, in the light of the discussion about kinase activity above, is that all mutations are equally toxic. Not only is the amount of cell death similar for all mutations, but also estimates of cell death are also remarkably similar across different models in different laboratories. This leads logically to the question of whether kinase activity is actually related to toxicity. We and others have reported that pathogenic LRRK2 mutants that also were engineered to be kinase-dead are much less toxic than kinase-active versions (Greggio et al., 2006; Smith et al., 2006). This suggests that kinase activity makes a substantial contribution to cell death, at least in these cellular models.

This result would be simple to understand if all mutations lead to increased kinase activity, but requires some discussion if the effects of mutations on activity are variable. There are several reasons that there might be an apparent dissociation between the two measures. First, the kinase assays reported to date may not capture all aspects of the function of the enzyme. If, for example, there were a specific substrate for the kinase activity of LRRK2 that mediates its toxic effects, then measuring autophosphorylation may not capture this. Perhaps more likely is the second possibility, that some mutations work by regulating overall LRRK2 activity. Taking the current model that GTP binding stimulates kinase activity and the GTPase activity returns LRRK2 to basal levels, a mutation such as R1441C that lowers GTPase activity would only be revealed when LRRK2 is being regulated. Perhaps consistent with this idea, Smith et al. (2006) were able to show that introducing a K1347A mutation, which can bind neither GDP nor GTP and has lower kinase activity, minimizes the toxic effects of G2019S LRRK2. Measuring static kinase

activity *in vitro* would miss this, but kinase activity might still be required for toxicity. It should be noted that the hypothesis could be reversed (kinase regulates GTPase) and the data would still be consistent, but only if we thought that kinase activity down-regulates GTPase activity. Another suggestion, stated explicitly by Ho et al. (2009), is that mutations might work in different ways to change a critical interactor that is not necessarily a substrate. In their experiments, mutations outside the kinase domain and I2020T increased binding to FADD, but G2019S does not (Ho et al., 2009). However, FADD interaction can be blocked by a kinase-inactivating mutation, suggesting that an enhanced LRRK2–FADD interaction can be achieved either by stronger physical interaction or by enhancing kinase activity (Ho et al., 2009). In this view, GTPase activity may not be especially crucial, or it may be that GTP influences binding to FADD.

But there is also the possibility that kinase activity is really not that important for the toxic effects of LRRK2 mutations. Bear in mind that all of the above experiments rely on brief overexpression of very high amounts of LRRK2 in cultured cells, outside their native environment and potentially exposed to additional stressors such as reactive oxygen species, which can enhance LRRK2 toxicity (West et al., 2007). An additional complication comes from the fact that some of the hypothesis-testing mutations may alter LRRK2 stability. For example, the GTP/GDP-binding-null K1347A mutation used to test the requirement for GTP-dependent activation (Smith et al., 2006) dramatically destabilizes LRRK2 protein, at least in our experiments (Lewis et al., 2007), and are thus a little more difficult to interpret if toxicity is concentration-dependent.

For all of these reasons, the proposal that kinase activity (or any other aspect of LRRK2 biology) is important in toxicity should be considered as only a provisional hypothesis until it can be tested rigorously in an intact brain. The first step to doing this will probably be the development of animal models, a few of which have been described recently. Loss of dopamine cells is seen in transgenic *Drosophila* expressing G2019S human LRRK2 (Liu et al., 2008). Similar phenomena have been reported using dLRRK if the equivalent mutations to Y1699C or I2020T are introduced (Imai et al., 2008). Whether neuronal loss occurs in transgenic mice is currently unclear, as only one BAC (bacterial artificial chromosome)-transfected mouse has been reported and the phenotype of the mouse was not discussed in that study (Li et al., 2007). Neuronal loss was reported in rats where a fragment of LRRK2 including the kinase domain was expressed transiently in the rat cortex using viral vectors (MacLeod et al., 2006). Clearly, *in vivo* models such as these will need to be developed further before we can adequately assess whether LRRK2 kinase activity is genuinely important in mediating neuronal cell death.

The discussion of animal models highlights a question discussed briefly above, that of whether the mutations work as gain-of- (potentially novel) toxic function or as a dominant-negative. One way to resolve this would be to

compare the phenotypes seen in knockout animals with those resulting from overexpression. Here the data are mixed. In *Drosophila*, although two groups found that high-level overexpression of mutant LRRK2/LRRK causes cell loss (Lee et al., 2007; Liu et al., 2008), another did not (Lee et al., 2007). There are two published studies using different knockout alleles reporting the LRRK is (Lee et al., 2007) or is not (Wang, D. et al., 2008) required for dopamine neuron survival in the same organism. Until these data are resolved, the loss-of-function against gain-of-function argument cannot be definitively answered. The detailed phenotype of LRRK2-knockout mice has not been reported, although brains of such animals have been used as controls for antibody-based techniques (Biskup et al., 2007), so presumably they are viable.

Finally, it is worth discussing what the human pathology may tell us about mechanisms involved in LRRK2-mediated neurodegeneration. A more detailed tally of the various pathologies found in different LRRK2 cases has been published elsewhere (Cookson et al., 2008), but it will suffice to say here that most cases are of Lewy-body-positive Parkinsonism as discussed above. Because we know that one of the major proteins found in Lewy bodies, α -synuclein, is also a gene for PD when mutated (Polymeropoulos et al., 1997; Kruger et al., 1998; Zarranz et al., 2004) or if expression is increased without any sequence variants (Singleton et al., 2003; Chartier-Harlin et al., 2004), α -synuclein fulfils the requirements for a toxic agent in PD (Cookson, 2005). By extension, if most cases of LRRK2 Parkinsonism have Lewy bodies, it is possible that α -synuclein is a mediator of the toxic damage caused by mutant LRRK2. That some cases with LRRK2 mutations do not have Lewy bodies complicates the argument, but does not invalidate it if we accept the idea that the deposition of proteins into inclusion bodies is not a necessary part of the toxicity of aggregating proteins (Cookson, 2005). LRRK2 cases can also have inclusions of another potentially toxic protein, tau. If LRRK2 mutations can express themselves as different pathologies, a logical inference is that LRRK2 is 'upstream' in the neurodegenerative process that can progress either via α -synuclein or tau. If this were correct, then LRRK2 would be predicted to be an accelerant of α -synuclein toxicity. How LRRK2 could influence α -synuclein is unclear as, although α -synuclein is phosphorylated, we have not been able to demonstrate any direct phosphorylation with active LRRK2 (D.W. Miller, E. Greggio and M.R. Cookson, unpublished data). However, the idea of a relationship between the two dominant genes for PD should at least be testable as animal models are developed.

SUMMARY

Since the discovery of LRRK2 mutations in several independent families, a good deal of progress has been made in

understanding the protein. With some caveats, it seems likely that the protein is active as both a GTPase and a kinase, and that these two domains have some regulatory function. Progress is being made on understanding interactions with other proteins and on possible physiological roles of LRRK2. How mutations work is still a little unclear, both from the viewpoint of whether all mutations increase kinase activity and how mutant proteins trigger toxicity. The next clear challenges are to identify the cellular function of endogenous LRRK2 and to develop robust animal models in which to test ideas about pathogenesis that currently involve questions such as whether kinase or other activities really are critical for toxicity and the relationship to α -synuclein, another key protein in PD pathogenesis. The final thing to be said is that the reason for doing this is to find ways to prevent the neuronal damage in PD and eventually to develop new therapeutic modalities.

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