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Cellular effects of LRRK2 mutations

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Abstract

Mutations in leucine-rich repeat kinase 2 (LRRK2) are a relatively common cause of inherited Parkinson's disease (PD) but the mechanism(s) by which mutations lead to disease are poorly understood. Here, I will discuss what is known about LRRK2 in cellular models, focusing on specifically on assays that have been used to tease apart the effects of LRRK2 mutations on cellular phenotypes. LRRK2 expression has been suggested to cause loss of neuronal viability, although because it also has a strong effect on the length of neurites on these cells, whether this is true toxicity or not is unclear. Also, LRRK2 mutants can promote the redistribution of LRRK2 from diffuse cytosolic staining to more discreet structures, at least at high expression levels achieved in transfection experiments. The relevance of these phenotypes for PD is not yet clear, and a great deal of work is needed to understand them in more depth.

Keywords

Cell death; Neurites; Inclusion bodies; Autophagy

Introduction

The identification of dominant mutations in leucine-rich repeat kinase 2 (LRRK2) by two groups in 2004 [1,2], together with the subsequent demonstration of a segregating variant in the same gene in the first reported family with mutations at the same locus [3], has lead to a great deal of interest in the role of this gene in Parkinson's disease (PD). It now appears that the gene is highly variable, but there are only about 5 amino acid changing variants that clearly segregate with disease in families (R1441C, R1441G, Y1699C, G2019S and I2020T) [4]. Furthermore, there are two variants that act as risk factors for sporadic PD in some populations (G2385R and R and a separate and small effect at the gene locus on lifetime disease risk found in genome association studies but not explained by amino acid variants [5].

However, although the contributions of LRRK2 to the genetics of PD have largely been clarified, substantial gaps remain in our knowledge about how the protein contributes to disease pathogenesis. Here, I will discuss what is known about the function of LRRK2 in cells and how this can be affected by mutations. To put this in context, it is important to discuss the phenotypes associated with expression of LRRK2 in cells and how these are modulated by both pathogenic and hypothesis testing mutations.

Acute ‘toxic’ effects of LRRK2

In some of our earlier studies, we wanted to express wild type and mutant LRRK2 in cells and look for phenotypes that were particularly enhanced by mutations. Although there are disadvantages in using cell models, particularly in that they tend to capture acute effects in a very reductionist system, one benefit is that it is relatively easy to assay large numbers of variants in a rapid manner.

The two early phenotypes that we noted were first that cells expressing mutant forms of LRRK2 had a higher proportions of cell death and second that some mutants would form discreet structures in cells rather than being diffusely localized in the cytoplasm. Interestingly, both of these phenotypes were diminished when artificial mutations that blocked kinase activity were introduced in the same construct [6].

The cell death phenotype appears to be reproducible in that several independent groups have now shown that toxicity occurs after expression of mutant LRRK2 in neurons or differentiated neuroblastoma cell lines [6–15]. In general, these studies have found that wild type LRRK2 has only a small effect on cell viability and that enhanced toxicity is a shared property of all of the pathogenic mutations. The observation that kinase dead LRRK2 is less toxic has been reproducible, as has been the extension to show that GTP-binding deficient mutations, which are also kinase dead, also diminish toxicity. Furthermore, although transgenic models of LRRK2 show only limited neuronal cell death [16], acute expression of LRRK2 *in vivo* with viral vectors causes cell death that appears to be dependent on kinase activity [17,18].

A simple interpretation of these results is that inhibiting kinase activity or GTP binding of LRRK2 would be a way to limit toxicity and therefore represent an opportunity for drug development. But, despite the reproducibility of this result, my opinion is that there are a number of reasons for caution and these indicate that further experimentation is needed. One question is whether LRRK2 is really acutely toxic. About the same time as the initial reports of toxicity, a publication from the Abeliovich lab showed that expression of mutant LRRK2 leads to a decrease in the length of neuritic processes [19]. Again, this has been confirmed in a number of labs and appears to be a very robust phenotype [20]. Neurite length is also increased in knockout neurons that are viable, suggesting that it is not simply a more sensitive measure of loss of viability. The reason that this matters in terms of interpreting the earlier papers is that most of the toxicity experiments were performed by counting ‘viable’ neurons in culture. If neuronal morphology is altered, ie because neurites are retracted, cells can be counted as non-viable even if they are actually alive. Due to these ambiguities, my lab uses neurite length in preference to ‘toxicity’ in most assays.

Another ambiguity comes from results showing that some of the mutations that alter kinase activity also change protein stability of LRRK2 [20]. This may or may not be true of all hypothesis-testing mutations in the kinase domain but is quite dramatic for mutations that cannot bind GTP or GDP, probably because binding of guanosine nucleotides to LRRK2 regulates protein stability [21]. Therefore, it is difficult to know if the lower toxicity is due to changes in enzyme activity or simply to lower protein levels.

Finally, we have recently shown that a risk factor for PD, the G2385R variant that is associated with sporadic PD, actually has lower kinase activity than WT LRRK2 and, in fact, can overcome the kinase activating effects of mutations in the kinase domain [22]. How to interpret these results is not quite clear, but one read is that while kinase activity is important in neuronal cell death, lower kinase activity can also be a risk factor for sporadic PD. Therefore, the range of concentrations at which a kinase inhibitor would be effective might be quite narrow.

In summary, although toxicity of LRRK2 was a reasonably robust measure across laboratories, its predictive value remains uncertain. Several areas of this field need to be developed in the future. First, it would be helpful to have models where there is authentic neuronal cell death as this is a feature of all PD cases with LRRK2 mutations. In this regard, the viral models [17,18] look promising although they are still acute models and have not yet been widely characterized across labs. Second, there is a need for better understanding of the effects of mutations, and chemical inhibitors, on the protein stability of LRRK2 as this might be a useful strategy for removing detrimental protein from cells.

Inclusion bodies of various types

In the experiments described above examining the effects of LRRK2 on neuronal viability, we stained cells to make sure they were actually expressing LRRK2. What we noticed was that the protein was usually distributed diffusely throughout the cytoplasm, a proportion of cells had more discreet staining that resembled inclusion bodies. What stopped us discarding this observation as an artifact of high level expression was that at apparently similar expression levels, some pathogenic mutants were more likely to form discrete inclusions and that this effect was diminished when mutations were expressed on a kinase dead background [6].

As well as the caveats about protein stability discussed above, there are additional concerns about this phenotype. Most specifically, evidence that this occurs at physiological levels of LRRK2 expression or *in vivo* is minimal. For example, knock in of the R1441C mutation which strongly promotes inclusion body formation in heterologous cell expression systems has no obvious effect on distribution of the protein in mouse brain [23]. Also, when other people have quantified the number of inclusions produced by different mutations, one of the more common mutations, G2019S in the kinase domain, does not enhance inclusion body formation [10]. These results show that inclusion body formation is not a shared property of all mutations and possibly relevant only when the protein is expressed acutely at high levels. Therefore, interest in this as a phenomenon waned a little, at least in my lab.

However, a couple of results suggested that the difference between diffuse LRRK2 and more discretely localized protein might be interesting. When examining the effect of small molecule inhibitors of LRRK2 kinase activity on cells, the Alessi lab noticed that application of inhibitors of LRRK2 kinase activity caused a redistribution of the protein into discrete structures and this correlated with loss of phosphorylation at specific Serines in LRRK2 (S910 and S935) and loss of binding of 14-3-3 proteins [24]. This was seen with wild type LRRK2 but enhanced by mutations, including pathogenic mutants, that also have decreased 14-3-3 binding. Therefore, perhaps a normal property of LRRK2 is to form some

type of inclusion, although only under some circumstances (here kinase inhibition) and this does not prove the effect has physiological relevance. Even stranger is that the effect of small molecule inhibitors of LRRK2 promote inclusion body formation whereas artificial kinase mutations limit the same effect. Why this should be is particularly difficult to address especially as we don't know specifically what the structures are, so it is important to describe some of the available data.

In an early paper describing the effects of mutations on LRRK2 distribution, we found that the structures could be disrupted by nocodazole, which interferes with microtubule polymerization[6]. Furthermore, inclusion bodies were surrounded by the intermediate filament protein vimentin. This is reminiscent of the behavior of aggresomes, which are accumulations of proteins where the cell uses microtubulne-dependent trafficking to gather excess misfolded proteins in the perinuclear region for turnover by the proteasome [25]. Other groups also reported the formation of aggresomes by LRRK2 in cultured cells [26].

However, there are alternate interpretations of this data. LRRK2 was subsequently shown to bind tubulins [27–30], the major component of microtubules. Consequently, Kett et al demonstrated that the inclusions seen in LRRK2 transfected cells are related to microtubules, as LRRK2 labels microtubules in a periodic fashion [10]. Interestingly, while the kinase activity of LRRK2 is required for the formation of inclusions as previously suggested [6], because Kett et al quantified the number of filamentous inclusions they were able to show that the G2019S mutation, which is hyperactive, does not increase inclusion formation. Filamentous structures do require an intact WD40 domain, which we subsequently confirmed with kinase-inhibitor derived inclusions [22].

Because LRRK2 can bind tubulin in brain [29], it is therefore possible that the filamentous inclusions seen in culture are reflective of events that occur in neurons, even if the formation of such distinct structures might be consequential of high expression levels and therefore somewhat artifactual. It is also interesting that a consistent event in mouse models is the accumulation of the brain microtubule binding protein tau [16]. Tau pathology is also seen in human cases with some LRRK2 mutations [31]. Although I acknowledge this is speculative, but a possible link between these different observations is that at high level LRRK2 binds to enough microtubules to form what appear as filamentous inclusions. At lower, potentially more physiological levels, the same events occur but obvious inclusions are not visible, instead the cell reacts by upregulating proteins that can stabilize microtubules.

LRRK2 is also present in a number of membranous, vesicular structures in cells and in brain [32,33]. Using vectors that express low levels of tagged LRRK2, the localization has been further refined to a show that LRRK2 is found around several components of the autophagy-lysosomal system [34]. LRRK2 is likely to play a functional role in autophagy based on changes in autophagy markers in several systems where LRRK2 has been expressed or knocked out [20,35–43]. This may be related to the neurite shortening effects of LRRK2 as knockdown of key genes in autophagy, Atg7 or LC3, will blunt the effects of LRRK2 on neurite length [39]. Therefore, LRRK2 seems to be present in and impact the function of vesicular structures in cells.

Trafficking of vesicles within cells is controlled by lipid composition and by the Rab family of small GTPases [44]. LRRK2 binds Rab5 and this interaction may be important in the effects of LRRK2 on synaptic vesicle endocytosis [45] and neurite length [46]. More recently, it has been shown that the *Drosophila* LRRK homologue colocalizes with Rab7 and has functional effects on Rab7-depedendent lysosomal positioning [47]. LRRK2 may also interact with Rac1, a Rho-family GTPase [48]. Therefore, the effects of LRRK2 on vesicle trafficking and may be due to physical binding to one or more small GTPases. Because autophagy is dependent on membrane trafficking [49], LRRK2 might have either a direct effect on autophagy or may indirectly regulate these processes by affecting Rab5/7 dependent membrane trafficking.

This is relevant for understanding the cellular phenotypes of LRRK2 because it predicts that we would see discrete localization of LRRK2 if the protein plays a specific role in vesicle transport and/or function. Furthermore, microtubules and other cytoplasmic elements play critical role in vesicular processes including autophagy [50]. Speculatively, some of the filamentous inclusions seen with mutant LRRK2 might be accumulations of vesicles along cytoskeletal tracks. If so, then the various ‘different’ morphologies of inclusions might actually be related. Specifically, the microtubule-associated filamentous inclusions might actually be arrays of stalled vesicles, visible due to high levels of expression in some of these models and/or due to inhibited LRRK2 being caught in part of its cycle. Overall, these considerations show that like acute toxicity, the formation of inclusion bodies with LRRK2 needs to be treated with caution as a reliable cellular phenotype. While it is reasonable to infer that these phenotypes capture some aspect of the effect of mutations or may be helpful for development of drug screens, they need to be better understood especially at physiological levels of expression. However, they may provide clues as to LRRK2 function and dysfunction that could be probed in the future.

Conclusions

LRRK2 clearly has a number of effects at the cellular level and some of these effects are exaggerated by dominant, pathogenic mutations that are associated with PD. Interestingly, there is no specific reason to identify any of these pathways with PD specifically, apart from possibly neuronal cell death. Rather, the effects are generally associated with general aspects of cellular function such as a vesicle turnover and autophagy that might impact some groups of neurons under some circumstances. Supporting this interpretation, neurite shortening is related to some of the vesicle turnover events impacted by LRRK2 mutations. Clear future directions for the field include a better definition of the mechanisms underlying known cellular phenotypes related to LRRK2 mutations so that these might be better tested in more intact systems.

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ABBREVIATIONS

LRRK2	leucine-rich repeat kinase 2
PD	Parkinson's disease

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