The synaptic function of LRRK2

Seongsoo Lee*, Yuzuru Imai†, Stephan Gehrke*, Song Liu* and Bingwei Lu*

*Department of Pathology, Stanford University School of Medicine, R270 Edwards Building, Stanford, CA 94305, U.S.A., †Department of Neuroscience for Neurodegenerative Disorders, Juntendo University Graduate School of Medicine, Tokyo, Japan

Abstract

Mutations in LRRK2 (leucine-rich repeat kinase 2) are the most frequent genetic lesions so far found in familial as well as sporadic forms of PD (Parkinson’s disease), a neurodegenerative disease characterized by the dysfunction and degeneration of dopaminergic and other neuronal types. The molecular and cellular mechanisms underlying LRRK2 action remain poorly defined. Synaptic dysfunction has been increasingly recognized as an early event in the pathogenesis of major neurological disorders. Using Drosophila as a model system, we have shown that LRRK2 controls synaptic morphogenesis. Loss of dLRRK (Drosophila LRRK2) results in synaptic overgrowth at the Drosophila neuromuscular junction synapse, whereas overexpression of wild-type dLRRK, hLRRK2 (human LRRK2) or the pathogenic hLRRK2-G2019S mutant has the opposite effect. Alteration of LRRK2 activity also affects synaptic transmission in a complex manner. LRRK2 exerts its effects on synaptic morphology by interacting with distinct downstream effectors at the pre- and postsynaptic compartments. At the postsynapse, LRRK2 functionally interacts with 4E-BP (eukaryotic initiation factor 4E-binding protein) and the microRNA machinery, both of which negatively regulate protein synthesis. At the presynapse, LRRK2 phosphorylates and negatively regulates the microtubule-binding protein Futsch and functionally interacts with the mitochondrial transport machinery. These results implicate compartment-specific synaptic dysfunction caused by altered protein synthesis, cytoskeletal dynamics and mitochondrial transport in LRRK2 pathogenesis and offer a new paradigm for understanding and ultimately treating LRRK2-related PD.

Introduction

Despite decades of intense research, the molecular and cellular mechanisms behind the pathogenesis of neurodegenerative diseases have remained elusive. Model organisms have emerged as effective tools for investigating disease mechanisms [1]. We and other investigators have been using Drosophila to model PD (Parkinson’s disease) in the last decade [2]. The rationale behind this effort is that the genes and proteins are conserved enough, the mechanisms underlying neuronal function are similar enough between fruitflies and mammals, and the behavioural response of the fruitflies are complex enough, that we may be able to recapitulate key aspects of the human neurological disorder in the fruitflies. Once such models are established, we can use the awesome power of fruitfly genetics to figure out the mechanism of disease pathogenesis and further test the relevance of the findings in mammalian systems.

To make fruitfly LRRK2 (leucine-rich repeat kinase 2) models, we took two approaches. For the gain-of-function approach, we introduced pathogenic mutations such as R1441G, Y1699C and I2020T mutations into the homologous location into the fruitfly LRRK2 protein named dLRRK (Drosophila LRRK2) [3]. Other investigators have made transgenic fruitflies express WT (wild-type) and pathogenic forms of hLRRK2 (human LRRK2) [4,5], such as the hLRRK2-G1915 mutant, and we have used some of them in our studies. For the loss-of-function approach, we obtained a transposon-insertion line that is likely to be a protein-null [6]. We have also generated RNAi (RNA interference) lines that efficiently knocked down dLRRK expression [3].

We found selective loss of dopaminergic neurons at specific brain locations in fruitflies expressing the pathogenic forms of the protein [3]. This was observed only when the animals were very old (approximately three-quarters of the way into their lifespan of ~60 days). Although this nicely recapitulates the late-onset aspect of the human disease, it is not a very convenient assay for large-scale genetic screens or genetic interaction studies aimed at understanding the molecular and cellular events preceding neuronal loss. We therefore searched for some earlier effects of LRRK2 dysfunction.

Altered LRRK2 activities affect synaptic growth

Using a locomotor behavioural test called the rollover assay, in which Drosophila larvae were placed in an
upside-down position and the time that the animals took to right their positions were measured, we found that the hLRRK2-G191S mutant-overexpression animals took longer to right their position, indicating compromised motor co-ordination.

We next used the Drosophila larval NMJ (neuromuscular junction) as a model system to test whether synaptic dysfunction is the underlying cause of the behavioural phenotypes we observed. The NMJs are formed by motor neuron terminals forming synapses with body-wall muscle fibres. Owing to their larger size, relative simplicity and accessibility to electrophysiological studies compared with CNS (central nervous system) synapses, the NMJ synapses have served as an excellent experimental system for studying synaptic structure, function and plasticity.

In dLRRK−/− mutant animals, we observed a synapse-overgrowth phenotype: the nerve terminal was more expanded, and there were more synaptic boutons formed [7]. This phenotype could be rescued by the WT, but not kinase-dead, form of dLRRK. One interesting observation was that expression of WT dLRRK either pre- or postsynaptically both rescued the mutant phenotype, although the postsynaptic rescue seemed to be more complete. This suggests that dLRRK may act in both synaptic compartments. On the other hand, when either dLRRK or hLRRK2 were overexpressed, they inhibited synaptic growth, leading to reduced bouton number, and this held true whether LRRK2 was expressed pre- or post-synaptically. One thing that is worth pointing out is that, in this assay, WT or pathogenic LRRK2 seems to be equally potent in inhibiting synaptic growth when overexpressed.

### LRRK2 interacts with eIF4E (eukaryotic initiation factor 4E) and directly phosphorylates 4E-BP (eIF4E-binding protein)

We were interested in the mechanisms underlying the synaptic effects of LRRK2. One clue came from our biochemical studies of LRRK2. We found that eIF4E interacts with LRRK2 in co-immunoprecipitation experiments (Y. Imai and B. Lu, unpublished work). The interaction occurred for both Drosophila and mammalian versions of the LRRK2 and eIF4E proteins. We tested whether LRRK2 might phosphorylate eIF4E, but could not find any evidence for that. Instead, we found that LRRK2 can phosphorylate 4E-BP. In HEK (human embryonic kidney)-293 cells, overexpression of WT or I2020T mutant hLRRK2 both promoted 4E-BP phosphorylation at the Thr37/Thr46 and Thr70 sites. In contrast, hLRRK2 RNAi reduced 4E-BP phosphorylation at these sites [3]. It is worth pointing out that this phosphorylation effect is more obvious when the cells are first serum-starved. The reason is that these sites are also phosphorylated by other kinases such as mTOR (mammalian target of rapamycin) [8], which is active under normal culture conditions and could mask the LRRK2 effects. This might explain why other investigators failed to detect the effect of LRRK2 on 4E-BP phosphorylation in cells, despite the fact that they could detect in vitro phosphorylation [9]. We also showed that the levels of total mTOR and phospho-mTOR protein were not significantly affected by LRRK2 in HEK-293 cells, suggesting that the effect of LRRK2 on 4E-BP phosphorylation is not through mTOR regulation [3]. Consistently, we found that this phosphorylation of 4E-BP by LRRK2 is direct, using an in vitro phosphorylation assay. More importantly, using Drosophila brain extracts, we found that 4E-BP phosphorylation was reduced in the dLRRK−/− mutant, but increased in dLRRK-overexpression conditions. These data provide compelling evidence that 4E-BP is an in vivo substrate of LRRK2.

### 4E-BP mediates the postsynaptic effects of LRRK2

The eIF4E–4E-BP axis of translational regulation has been clearly implicated in the regulation of postsynaptic growth and function at the Drosophila NMJ [10,11]. We tested whether 4E-BP is a downstream target that mediates the postsynaptic effects of LRRK2. Indeed, in a dLRRK−/− d4E-BP−/− double mutant, the synaptic overgrowth phenotype of dLRRK−/− was rescued, and overexpression of 4E-BP nicely suppressed the postsynaptic toxicity of dLRRK overexpression on synaptic growth [7].

Our working model is that, normally, eIF4E function in translational initiation is inhibited by the binding of 4E-BP. In cells with high LRRK2 activity, 4E-BP is phosphorylated, which triggers subsequent phosphorylation by other kinases. This results in the release of 4E-BP from eIF4E, allowing eIF4E to carry out cap-dependent translation. It is worth noting that the eIF4E–4E-BP axis of translational regulation does not regulate translation globally. Instead, it regulates a small subset of mRNAs that are normally very inefficiently translated [12]. Using an mRNA translation profiling method, we identified E2F and DP as mRNAs whose translation is regulated by LRRK2 [13]. Moreover, we found that phospho-4E-BP has a novel function in promoting E2F and DP translation: it interacts with dAgo1 to relieve microRNA-mediated translational repression of E2F and DP. Therefore, through stimulation of eIF4E-mediated cap-dependent translation, and phospho-4E-BP-mediated relief of translational repression by microRNAs, LRRK2 stimulates E2F and DP translation.

E2F and DP are transcription factors that normally form a heterodimer to promote cell-cycle progression [14]. In proliferating cells, their up-regulation contributes to cancer. In postmitotic neurons, their up-regulation leads to neuronal toxicity and cell death. We tested whether E2F and DP up-regulation might contribute to the synaptic toxicity of dLRRK. Removing one copy of e2F or dp effectively rescued the synapse loss caused by dLRRK overexpression, supporting the hypothesis that the up-regulation of E2F and DP translation contributes to the synaptic toxicity of
Futsch is phosphorylated by LRRK2 and it mediates the presynaptic effects of LRRK2

As described above, overexpression of 4E-BP rescued the postsynaptic toxicity of dLRRK. However, the presynaptic toxicity of dLRRK was not altered, suggesting that LRRK2 affects the pre- and post-synapse through distinct mechanisms. So what mechanism mediates the pre-synaptic effects of LRRK2? Here, biochemistry comes to the rescue again. In biochemical studies using mammalian cells, we detected a physical association between hLRRK2 and the MT (microtubule)-binding protein MAP1B (MT-associated protein 1B) (Y. Imai and B. Lu, unpublished work). Using Drosophila brain extracts, we confirmed that the Drosophila MAP1B called Futsch also physically associates with LRRK2, and that this complex also contains tubulin [7].

In the NMJ, Futsch is primarily localized to the presynaptic compartment. We tested the genetic relationship between LRRK2 and Futsch. First, the loss of one copy of Futsch effectively rescued the synaptic overgrowth phenotype of dLRRK−/−. Secondly, overexpression of Futsch at least partially rescued the presynaptic, but not postsynaptic, toxicity associated with hLRRK2 overexpression. These results are consistent with LRRK2 negatively regulating the function of Futsch at the presynapse.

We also tested the idea that LRRK2 might directly phosphorylate Futsch. In this case, we found clear evidence of Futsch phosphorylation by hLRRK2, and the G1915S mutant appeared to be more active in this phosphorylation. Because Futsch is a huge protein, with a predicted molecular mass of >600 kDa, it has been a challenge to map out the phosphorylation site.

Given that Futsch is a MT-binding protein that regulates MT dynamics [15], one model of LRRK2 action at the presynapse is that it regulates MT structure. Indeed, we found that, compared with the normally continuous tubular MT structures within the presynaptic boutons, in dLRRK−/− animals the MT network appeared to be more elaborate and tangled, whereas in hLRRK2-overexpression animals the MTs appear more fragmented. This phenotype was rescued by Futsch co-expression [7].

MTs also play important roles in regulating synaptic vesicle release at the active zones in the presynaptic compartment. We found that the number of active zones is significantly increased in hLRRK2-G1955S overexpression animals. There is a trend of decrease in dLRRK−/− mutants, but this did not reach statistical significance.

We also examined the effect of all of these synaptic morphological changes on synaptic function [7]. In electrophysiological studies, we found that dLRRK−/− mutants exhibited increased frequency of spontaneous neurotransmission. However, quantal content, a measure of the number of neurotransmitter quanta released per presynaptic action potential, was reduced, indicating reduced synaptic efficacy. In hLRRK2-overexpression animals, we observed significant increases of frequency of spontaneous transmission, and decrease of quantal content only when pathogenic hLRRK2-G1915S is overexpressed presynaptically. So, electrophysiologically, dLRRK−/− mutant and hLRRK-G1915S overexpression produced similar neurotransmission phenotypes, although they exerted opposite effects on synaptic morphology. Moreover, despite the significant synaptic morphology changes, postsynaptic WT or G1915S hLRRK2 had no obvious effect on neurotransmission. Thus the effects of LRRK2 on synaptic function are complex, presumably because there exist other yet to be identified substrates that mediate LRRK2 function at the synapse.

LRRK2 regulates the axonal transport of mitochondria

One important function of neuronal MTs is to regulate the axonal transport of organelles such as mitochondria. Given the overwhelming evidence linking mitochondrial dysfunction to PD [16], understanding how LRRK2 impairs mitochondrial function will shed light on the mechanisms of PD pathogenesis. When counting the number of mitochondria at the very end of the nerve terminals at the NMJ, we found that there was a significant increase in mitochondrial number in dLRRK−/− mutants, whereas in hLRRK2-overexpression animals, the number of mitochondria was reduced, and this effect was rescued by Futsch co-expression [7]. These results suggest that dLRRK affects the distribution of mitochondria through its regulation of Futsch and MT dynamics.

PINK1 (phosphatase and tensin homologue deleted on chromosome 10-induced putative kinase 1), a mitochondria-localized serine/threonine kinase that is linked to autosomal recessive PD [17], has been shown recently to regulate mitochondrial motility [18,19]. In the larval motor neurons, PINK1 overexpression arrested mitochondrial motility, whereas PINK1 RNAi led to increased motility, with more mitochondria moving anterogradely than retrogradely. Mitochondrial motility is controlled by a protein complex consisting of Miro, Milton and Khc (kinesin heavy chain) [20]. Miro and Milton are adaptor proteins that link mitochondria to the kinesin motor proteins and the MT tracks. In a genetic screen for modifiers of PINK1, we identified components of this mitochondrial transport complex [19]. Miro, Milton or Khc RNAi significantly rescued PINK1 RNAi-induced muscle degeneration, as reflected in abnormal wing posture. In dopaminergic neurons, Miro RNAi rescued the abnormal mitochondrial aggregation and dopaminergic neuron loss associated with PINK1 inactivation. Mechanistically, we found that PINK1 co-operated with parkin, an E3 ubiquitin ligase that is also associated with PD [21], to promote the degradation of Miro in HeLa cells. With CCCP (carbonyl cyanide m-chlorophenylhydrazone) treatment, which depolarizes mitochondria and leads to PINK1...
accumulation and recruitment of parkin to mitochondria [22], Miro ubiquitination and more rapid degradation was observed [19].

The PINK1/parkin pathway has been implicated in a mitochondrial quality-control system that removes dysfunctional or damaged mitochondria from the cell in a process called mitophagy. In order for mitophagy to occur, the PINK1/parkin pathway may be required to disengage mitochondria from the motors and MT tracks, so that the autophagy machinery can engulf the damaged mitochondria. When this mitochondrial release mechanism breaks down, abnormal mitochondria will move around freely in neurons and cause damage.

Using the same live-cell imaging assay of mitochondrial motility that we used to assess PINK1 function [19], we found that hLRRK2 overexpression causes increased mitochondrial motility in both the anterograde and retrograde direction. In the case of hLRRK2-G1915S overexpression, the increase in mitochondrial motility in the retrograde direction was greater than in the anterograde direction (S. Lee and B. Lu, unpublished work), which fits with our earlier observation that there are fewer mitochondria at the end of the nerve terminals in these animals [7].

We also tested the relevance of altered mitochondrial transport to the synaptic effects of LRRK2. Genetic manipulation that would decrease mitochondrial motility, such as Miro RNAi and PINK1 overexpression, effectively rescued the synapse-loss phenotype of hLRRK2 overexpression, whereas a further increase in mitochondrial motility as in Miro overexpression enhanced the synapse-loss phenotype (S. Lee and B. Lu, unpublished work), indicating that abnormal mitochondrial transport contributes to the presynaptic toxicity of LRRK2.

Summary

Our results indicate that LRRK2 plays important roles at neuronal synapses, but through more than one mechanism. At the postsynapse, it regulates protein synthesis and synaptic growth through the eIF4E–4E-BP axis and the microRNA pathway. At the presynapse, it affects MT dynamics through Futsch, and it affects mitochondrial transport, either indirectly through Futsch and MT regulation, or directly by impinging on the mitochondrial transport machinery. Further studies will test whether these mechanisms of LRRK2 function at synapses are conserved in mammals.

Acknowledgements

We thank our collaborators who participated in these studies, especially Dr Huifu Guo and Dr Wei-Yong Lin who helped with the electrophysiology studies. We are grateful to present and former members of the Lu laboratory for stimulating discussions.

Funding

Supported by the McKnight Foundation (Brain Disorders Award), the Alzheimer’s Association [grant number R010076273] and the National Institutes of Health [grant numbers R01AR054926 and R01MH080378].

References


Received 27 April 2012
doi:10.1042/BSI20120113