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Genetic analysis of Parkinson's disease-linked leucine-rich repeat kinase 2

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Abstract

Mutations in *LRRK2* (leucine-rich repeat kinase 2) are the most common genetic cause of PD (Parkinson's disease). To investigate how mutations in *LRRK2* cause PD, we generated *LRRK2* mutant mice either lacking its expression or expressing the R1441C mutant form. Homozygous R1441C knockin mice exhibit no dopaminergic neurodegeneration or alterations in steady-state levels of striatal dopamine, but they show impaired dopamine neurotransmission, as was evident from reductions in amphetamine-induced locomotor activity and stimulated catecholamine release in cultured chromaffin cells as well as impaired dopamine D_2 receptor-mediated functions. Whereas $LRRK2^{-/-}$ brains are normal, $LRRK2^{-/-}$ kidneys at 20 months of age develop striking accumulation and aggregation of α-synuclein and ubiquitinated proteins, impairment of the autophagy–lysosomal pathway, and increases in apoptotic cell death, inflammatory responses and oxidative damage. Our further analysis of $LRRK2^{-/-}$ kidneys at multiple ages revealed unique age-dependent biphasic alterations of the autophagic activity, which is unchanged at 1 month of age, enhanced at 7 months, but reduced at 20 months. Levels of α-synuclein and protein carbonyls, a general oxidative damage marker, are also decreased in $LRRK2^{-/-}$ kidneys at 7 months of age. Interestingly, this biphasic alteration is associated with increased levels of lysosomal proteins and proteases as well as progressive accumulation of autolysosomes and lipofuscin granules. We conclude that pathogenic mutations in *LRRK2* impair the nigrostriatal dopaminergic pathway, and LRRK2 plays an essential role in the dynamic regulation of autophagy function *in vivo*.

Keywords

aging; autophagy; dopamine D_2 receptor; dopamine neurotransmission; leucine-rich repeat kinase 2 (LRRK2); Parkinson's disease

Introduction

PD (Parkinson's disease) is an age-related and the most common neurodegenerative movement disorder, characterized by resting tremor, slow movement, muscular rigidity and postural instability. The neuropathological hallmarks of PD are progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta of the brain and the

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presence of intraneuronal cytoplasmic inclusions known as LBs (Lewy bodies), in which α-synuclein aggregates are a major component [1]. The clinical symptoms of PD are thought to result from reduced dopamine input to the striatum owing to the severe loss of dopaminergic neurons. Although most PD cases occur sporadically, clinical syndromes resembling sporadic PD have been linked to mutations in at least five distinct genes [including *SNCA* (α-synuclein), *PARK2* (parkin), *PARK7* (DJ-1), *PINK1* (phosphatase and tensin homologue deleted on chromosome 10-induced putative kinase 1) and *LRRK2* (leucine-rich repeat kinase 2)] [2], permitting studies of the pathogenic mechanisms of PD using genetic approaches. Elucidation of the pathogenic mechanism underlying the selective dopaminergic degeneration in familial PD will probably provide important clues to the pathogenic mechanism responsible for idiopathic PD.

The human *LRRK2* gene on chromosome 12 (chromosome 15 in mice) harbours five pathogenic mutations, which segregate with the disease, and many more amino acid residue substitutions have also been found to be associated with the disease [3–5]. *LRRK2* contains 51 exons and encodes a large protein of 2527 amino acid residues, which consists of several functional domains, including a Ras-like small GTPase domain, a MAPK (mitogen-activated protein kinase)-like domain, as well as several protein–protein interaction domains, such as the leucine-rich repeat domain and WD40 domain [2–4]. The disease-associated mutations in *LRRK2* are present in all functional domains of the protein. The pathogenic mutations all affect highly conserved amino acid residues and are collectively the most common genetic cause of the late-onset PD (~7% familial and 3% sporadic PD) [3–7].

Interestingly, multiple amino acid substitutions of the same residue Arg^{1441} (R1441C, R1441G and R1441H) in the highly conserved GTPase domain and multiple mutations (I2012T, G2019S and I2020T) in the kinase domain have been identified, whereas no multiplication or exonic deletion of *LRRK2* has been reported [5]. Most *LRRK2* mutations cause clinically typical PD, but the neuropathological features vary, ranging from pure nigral degeneration without LBs to nigral degeneration with brainstem or widespread LBs, or ubiquitin-positive inclusions or neurofibrillary tau-positive tangles [4,8], thus confirming that a single gene disorder can have multiple pathological consequences.

In vitro studies suggest that mutations in LRRK2 (R1441C, G2019S) cause increases in its kinase activity [9,10], which mediates neuronal toxicity and is regulated by the GTPase domain in a GTP-dependent manner [10–13]. It has also been reported that expression of pathogenic LRRK2 mutants (R1441C, Y1699C or G2019S) results in neuronal degeneration and protein aggregation or inclusions in SHSY5Y cells and cultured cortical neurons [11,14,15]. LRRK2 has also been implicated in the biogenesis and/or regulation of intracellular membrane structures [16], synaptic vesicle endocytosis [17] and neurite outgrowth [15]. However, the physiological function of mammalian LRRK2 is unknown. Lee et al. [18] reported that loss-of-function mutants for LRRK, the sole *Drosophila* orthologue of human LRRK2, exhibited severely impaired locomotor activity and reduced tyrosine hydroxylase immunoreactivity in dopaminergic neurons, but transgenic expression of pathogenic mutant or wild-type LRRK did not result in any significant defects [18]. However, in another study, transgenic expression of human wild-type or G2019S LRRK2 led to adult-onset selective loss of dopaminergic neurons, locomotor dysfunction

and early mortality in *Drosophila* [19]. It is unclear why expression of human LRRK2 or *Drosophila* LRRK has such different effects in fruitflies, and why expression of either wild-type or mutant human LRRK2 similarly results in loss of dopaminergic neurons in fruitflies.

To investigate how mutations in LRRK2 cause PD, we generated *Lrrk2* mutant mice either lacking its expression by deleting promoter and exon 1 or exons 29 and 30 [KOs (knockouts)] or expressing the R1441C mutant form [KI (knockin)] by introducing the R1441C mutation into exon 31 and allowing its expression under the control of the endogenous regulatory elements. The R1441C mutation is of particular interest, as three distinct mutations have been identified in this arginine residue in PD patients, which highlights the importance of Arg^{1441} for the normal function of LRRK2, and diverse neuropathological features have been identified in families carrying the R1441C mutation [3–5]. We therefore chose to focus on the R1441C mutation.

In addition, despite the disease relevance of LRRK2, its normal physiological role remains elusive. Elucidation of LRRK2 functions will provide insights into how mutations in *LRRK2* lead to dopaminergic dysfunction and degeneration. Although the dominant inheritance of missense mutations and the lack of nonsense or deletion mutations in *LRRK2* are consistent with toxic gain-of-function pathogenic mechanisms, we generated $Lrrk2^{-/-}$ mouse models to study the normal physiological function of LRRK2 and to determine the consequence of inhibiting LRRK2 function.

R1441C mutation in LRRK2 impairs activity-dependent dopaminergic neurotransmission in mice

LRRK2 R1441C KI mice are viable and fertile and appear grossly normal. Similar to our previous findings in $Park2^{-/-}$ [20], $Park7^{-/-}$ [21] and $Pink1^{-/-}$ mice [22], R1441C KI mice do not develop dopaminergic degeneration or alterations in striatal dopamine levels during their lifespan [23], providing further evidence that genetic recapitulation of pathogenic mutations in mice is insufficient to reproduce this terminal neuropathological hallmark of PD. In addition, our KI mice develop no protein aggregation or inclusions in the brain up to 2 years of age, in contrast with previous findings in cell lines or cultured cortical neurons indicating that LRRK2 mutants tend to form protein inclusions and cause neuronal death [11,14,15]. Furthermore, we saw no alterations in levels of α-synuclein and tau as well as their phosphorylated forms measured by immunostaining or Western blotting in brains of R1441C KI mice up to 2 years of age.

Interestingly, introduction of the R1441C mutation in LRRK2 affects activity-dependent dopaminergic neurotransmission, such as reduced responses to amphetamine stimulation in locomotor activity and significant reduction in stimulation-induced catecholamine release in the absence of dopaminergic degeneration [23]. Amphetamine exerts its effects by stimulating dopamine efflux into the synaptic cleft via multiple mechanisms, including inhibition of vesicular monoamine transporter 2 and monoamine oxidase activity, which increases cytosolic dopamine available for dopamine transporter-mediated reverse transport of dopamine [24]. Calcium from intracellular stores plays a key role in

amphetamine-mediated dopamine release [25,26]. The reduced response to amphetamine stimulation in locomotor activity of R1441C KI mice suggests that amphetamine-stimulated dopamine release may be reduced in these mice. This will need to be confirmed by *in vivo* microdialysis to measure directly extracellular dopamine levels in freely moving mice following amphetamine treatment. Nevertheless, the alteration in dopamine neurotransmission appears to be common to other *Lrrk2* BAC (bacterial artificial chromosome) transgenic mouse models overexpressing either the R1441G or the G2019S mutant form of LRRK2 [27–29]. Consistent with the impaired activity-dependent dopamine neurotransmission, stimulation-induced catecholamine release is decreased in chromaffin cells isolated from R1441C KI mice, as indicated by significant reductions in total catecholamine release, quantal size and the number of vesicles releasable after high-K^+ stimulation [23]. Identification of a possible common mechanism underlying the impairment of amphetamine-induced locomotion in the open field and evoked catecholamine release in cultured chromaffin cells awaits future investigation. Furthermore, parallel studies using cultured dopaminergic neurons from postnatal ventral midbrains will provide additional support for this conclusion.

Impaired dopamine D_2 receptor-mediated function in R1441C KI mice

Another interesting phenotype exhibited by R1441C KI mice is the impairment of dopamine D_2 receptor-mediated functions, as indicated by reduced responses of KI mice in locomotor activity to the inhibitory effect of a D_2 receptor agonist, quinpirole, and decreased sensitivity of KI nigral neurons in firing activity to suppression induced by quinpirole or dopamine [23]. Previous studies have shown that these inhibitory effects of quinpirole are abolished in mice lacking all D_2 receptors, but are retained in mice expressing only the short isoform, which serves presynaptic autoreceptor functions [30–32]. Interestingly, similar compromises of D_2 autoreceptor-mediated functions have been reported in another PD mouse model, *Park7*$^{-/-}$ mice, which also exhibited reduced responses in locomotor activity to quinpirole and reduced responses of nigral neurons to dopamine and quinpirole [21]. These results raise the possibility that the D_2 autoreceptor-mediated function may be a converging common target of PD mutations, a notion supported by the clinical efficacy of D_2 receptor agonists in PD [33]. Furthermore, several variants of the dopamine D_2 receptor gene have been associated with PD [34]. Recent clinical trials have shown that use of D_2 receptor agonists ropinirole and pramipexole retards loss of functional nigral projections to the striatum [35,36]. Thus our KI mouse model provides a unique tool for the study of the normal physiological role of LRRK2 and its dysfunction in PD pathogenesis, which may yield novel targets for development of effective therapeutic drugs.

Striking age-dependent kidney abnormalities in *Lrrk2*$^{-/-}$ mice

Similar to other PD genetic mouse models, such as α-synuclein transgenic [37–39], *Park2*$^{-/-}$ [20], *Park7*$^{-/-}$ [21], *Pink1*$^{-/-}$ [22] and *Lrrk2* transgenic and KI mice [23,27–29,40], *Lrrk2*$^{-/-}$ brains do not develop overt dopaminergic degeneration [41]. However, *Lrrk2*$^{-/-}$ kidneys, which suffer the greatest loss of LRRK2 compared with other organs as LRRK2 is normally expressed at much higher levels in the kidney (~6-fold) relative to the brain and other organs [41,42], develop striking age-dependent abnormalities (e.g. severe discoloration and granular

tissue texture) [41,43]. These changes in kidneys are observed in both of our independent lines of *Lrrk2* KO mice with 100% penetrance, but not in our LRRK2 R1441C KI mice. Later observations from other independently developed *Lrrk2* mutant mice further confirmed our discovery [43,44]. Gross morphological abnormalities of the kidney, including altered size, weight, texture and colour, become evident in *Lrrk2*$^{-/-}$ mice at 3–4 months of age, along with increased accumulation of autofluorescent granules in proximal renal tubules. The ratio of kidney/body weight in *Lrrk2*$^{-/-}$ mice is increased at 1, 4 and 7 months of age (~10% at 1 month, and ~20% at 4 and 7 months), whereas the ratio is drastically decreased at 20 months of age (~50%) [41,43]. Whereas kidney filtration function evaluated by levels of blood urea nitrogen and serum creatinine is not significantly affected in *Lrrk2*$^{-/-}$ mice at 12–14 months of age, expression of kidney injury molecule-1, a sensitive and specific biomarker for epithelial cell injury of proximal renal tubules [45], is up-regulated (as high as ~10-fold) at as early as 1 month of age and persists to 20 months of age [43], suggesting that *Lrrk2*$^{-/-}$ mice sustain chronic kidney injury. There are dramatic increases in the number of apoptotic cells in medulla, renal tubules and glomeruli, as well as inflammatory responses in the kidneys from 20-month-old *Lrrk2*$^{-/-}$ mice, compared with wild-type controls [41].

Age-dependent biphasic alterations of protein homoeostasis in *Lrrk2*$^{-/-}$ mice

To uncover the mechanism underlying age-related abnormalities developed in *Lrrk2*$^{-/-}$ mice, we performed a number of analyses to look for molecular and cellular alterations. We discovered that loss of LRRK2 causes impairment of the two major protein degradation pathways in the kidney of aged mice [41], i.e. the autophagy–lysosome pathway and the ubiquitin–proteasome system, which have been implicated in various neurodegenerative diseases with protein aggregation-related pathologies, including Parkinson's disease and Huntington's disease [46,47]. There is striking accumulation and aggregation of proteins, such as α-synuclein, p62 and ubiquitinated proteins, as well as impaired conversion of the non-lipidated form (LC3-I) into the lipidated form (LC3-II) of microtubule-associated protein 1 LC3 (light chain 3), a reliable indicator of the autophagic activity [48] in the kidneys of *Lrrk2*$^{-/-}$ mice at 20 months of age [41]. Taken together, these data suggest that LRRK2 plays an essential role in protein homoeostasis.

Surprisingly, our analysis of *Lrrk2*$^{-/-}$ kidneys at multiple ages, such as 1, 4, 7 and 20 months, revealed that loss of LRRK2 causes age-dependent biphasic alterations of the autophagic activity in *Lrrk2*$^{-/-}$ kidneys, which is unchanged at 1 month of age, enhanced at 7 months, but reduced at 20 months [41,43], as is evident by corresponding changes in the levels of LC3-I/II, a reliable autophagy marker, and p62, an autophagy substrate. Levels of α-synuclein and protein carbonyls, a general oxidative damage marker, are also decreased in *Lrrk2*$^{-/-}$ kidneys at 7 months of age, but increased at 20 months. Interestingly, the age-dependent biphasic alterations in autophagic activity in *Lrrk2*$^{-/-}$ kidneys is accompanied by increased levels of lysosomal proteins and proteases at 1, 7 and 20 months of age, as well as progressive accumulation of autolysosomes and lipofuscin granules at 4, 7–10 and 20 months of age.

Our data demonstrate that the autophagy–lysosome pathway is dysregulated in the absence of LRRK2. Loss of LRRK2 may initially cause induction of autophagy. But, deficient clearance or recycling of autophagic components in the absence of LRRK2 would cause trapping of the components of the autophagy pathway in the form of autolysosomes and the eventual formation of lipofuscin granules due to excessive oxidation and cross-linking and therefore depletion of autophagy machinery (e.g. autophagic lysosomes cannot be reformed), which would in turn result in accumulation and aggregation of a large number of autophagy substrate proteins during aging. These data suggest that LRRK2 plays an important role in the regulation of the autophagy pathway *in vivo*. Consistent with this notion, it has been reported that siRNA (small interfering RNA) knockdown of LRRK2 increases autophagic activity and the R1441C mutation in LRRK2 induces accumulation of autophagic vacuoles of enlarged size in cultured HEK (human embryonic kidney)-293 cells [49]. Surprisingly, LRRK2 overexpression in cultured HEK-293 cells has also been reported to cause autophagy induction through a calcium-dependent pathway [50]. Although these results may seem contradictory with each other, which may be due to the fact that these studies were performed in cell culture systems using immortalized cell lines, rather than an *in vivo* physiological setting, they nevertheless indicate that LRRK2 is important for the dynamic regulation of autophagy function. LRRK2 has also been reported to localize to specific membrane subdomains, including autophagosomes and autolysosomes [49], suggesting that LRRK2 may participate directly in the dynamic process, including formation and clearance, of autophagic vacuoles.

Although these molecular and cellular changes are observed only in the kidney, but not in the brain of *Lrrk2*$^{-/-}$ mice, they are very similar to processes that are thought to be involved in the pathogenesis of PD and other neurodegenerative diseases, making *Lrrk2*$^{-/-}$ kidneys a relevant and valuable *in vivo* model, which provides a physiological setting for the studies of LRRK2 function and the identification of the cellular and molecular pathways that LRRK2 pathogenic mutations may affect.

Concluding remarks

Genetic analysis of *LRRK2* demonstrates that, although LRRK2 is not essential for the survival of dopaminergic neurons in mice, pathogenic mutations in *LRRK2* cause alterations in the nigrostriatal dopaminergic pathway, such as impairment in activity-dependent dopamine release and dopamine D_2 receptor-mediated functions, which may be pathogenic precursors preceding frank dopaminergic degeneration in PD patients. Furthermore, LRRK2 also plays an important role in protein homoeostasis, more specifically, in the dynamic regulation of the autophagy–lysosome pathway. Loss of LRRK2 causes impairment of the protein-degradation pathways and striking age-dependent cellular changes in the kidney, which are similar to PD pathogenesis, making the kidneys of *Lrrk2*$^{-/-}$ mice a unique and valuable model for elucidating the normal physiological role of LRRK2 under its physiological settings. Alternatively, *LRRK2* mutations may cause Parkinson’s disease and cell death by impairing protein-degradation pathways, leading to protein accumulation and aggregation over time.

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Abbreviations used:

HEK	human embryonic kidney
KI	knockin
KO	knockout
LB	Lewy body
LC3	light chain 3
LRRK2	leucine-rich repeat kinase 2
PD	Parkinson's disease

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