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LRRK2 Modulates Vulnerability to Mitochondrial Dysfunction in Caenorhabditis elegans

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Mutations in leucine-rich repeat kinase 2 (LRRK2) cause autosomal-dominant familial Parkinson’s disease. We generated lines of Caenorhabditis elegans expressing neurally directed human LRRK2. Expressing human LRRK2 increased nematode survival in response to rotenone or paraquat, which are agents that cause mitochondrial dysfunction. Protection by G2019S, R1441C, or kinase-dead LRRK2 was less than protection by wild-type LRRK2. Knockdown of lrk-1, the endogenous ortholog of LRRK2 in C. elegans, reduced survival associated with mitochondrial dysfunction. C. elegans expressing LRRK2 showed rapid loss of dopaminergic markers (DAT::GFP fluorescence and dopamine levels) beginning in early adulthood. Loss of dopaminergic markers was greater for the G2019S LRRK2 line than for the wild-type line. Rotenone treatment induced a larger loss of dopamine markers in C. elegans expressing G2019S LRRK2 than in C. elegans expressing wild-type LRRK2; however, loss of dopaminergic markers in the G2019S LRRK2 nematode lines was not statistically different from that in the control line. These data suggest that LRRK2 plays an important role in modulating the response to mitochondrial inhibition and raises the possibility that mutations in LRRK2 selectively enhance the vulnerability of dopaminergic neurons to a stressor associated with Parkinson’s disease.

Introduction

Parkinson’s disease (PD) is the most common neurodegenerative movement disorder. At least nine different genes are known to cause familial PD. Functional studies of PD-related genes implicate dysfunction of mitochondria, protein aggregation, and the stress response in the pathophysiology of PD. Autosomal-dominant mutations in leucine-rich repeat kinase 2 (LRRK2) are associated with both familial and late-onset PD (Paisán-Ruiz et al., 2004; Zimprich et al., 2004). The G2019S mutation is the most common mutation and is present in ~40% of familial and sporadic PD in Arab samples from North Africa, ~30% of familial PD in Ashkenazi Jewish populations, and up to 3% of sporadic PD in Europe and North America (Lesage et al., 2006; Ozelius et al., 2006).

LRRK2 is a member of the ROCO protein family and contains multiple domains, including a leucine-rich repeat domain, Roc GTPase, COR (C-terminal of Ras) of unknown function, kinase domain, and WD40-repeats at the C terminal (Zimprich et al., 2004). LRRK2 is present in the cytoplasm and is also associated with membranes, including those of mitochondria, endoplasmic reticulum, and synaptic vesicles (Biskup et al., 2006). Recent studies demonstrate that mutations, such as G2019S, lead to increased LRRK2 autophosphorylation and kinase activity (West et al., 2005; Greggio et al., 2006). Transient expression of mutant forms of LRRK2 in neurons causes cell death, and the death appears to require LRRK2 kinase activity. Expressing mutan LRRK2 in neurons also decreases neurite length in rodents (MacLeod et al., 2006). Mammals have a close homolog of LRRK2, termed LRKK1. This protein (LRKK1) also has kinase and GTPase activity but is less toxic than LRRK2 after transient transfection (Greggio et al., 2007). The ortholog of LRKK1 and 2 in Caenorhabditis elegans is lrk-1. In lrk-1 deletion mutants, synaptic vesicle proteins are mislocalized in a small subset of neurons (Sakaguchi-Nakashima et al., 2007). The mechanism by which these changes occur is not known. Dysfunction of mitochondria is implicated in the pathophysiology of PD (Ved et al., 2005; Schapira, 2008). For instance, α-synuclein is associated with dysfunction of mitochondria and the endoplasmic reticulum (Chan-
dra et al., 2004; Cooper et al., 2006; Schapira, 2008). Parkin, PINK-1, and DJ-1, which are genes associated with familial PD, are also associated with mitochondrial function (Greene et al., 2003; Yang et al., 2006; Exner et al., 2007; Plun-Favreau et al., 2007). These observations raise the possibility that LRRK2 also modulates mitochondrial function.

We generated lines of C. elegans expressing human wild-type (WT) LRRK2 and the mutants G2019S, R1441C, and two kinase-dead (KD) variants of LRRK2 (KD and R1441C/KD), to investigate how LRRK2 affects biological functions linked to PD. We report that WT LRRK2 protects against mitochondrial dysfunction, but disease-related mutants of LRRK2 appear to produce responses that range from less protection to overt enhancement of toxicity.

Materials and Methods

**Chemicals.** All chemicals were purchased from Sigma unless otherwise indicated. Bacterial agar, peptone, and Luria broth (LB) media/agar were purchased from American Bioanalytical.

**Plasmids.** V5-tagged WT and mutant LRRK2 cDNAs were obtained from mammalian Gateway entry vectors (Invitrogen), generated and described by Greggio et al. (2006). LRRK2 constructs were transferred from these Gateway entry vectors to a Gateway-compatible C. elegans expression vector, pSybDEST, that was derived from pDP95.76 (Fire vector) by adding a ccdB-containing fragment with integration acceptor sites into Xhol site of pDP95.76. The directional gene transfers were performed via entry vectors as described in the Gateway cloning protocol. All interference RNA (RNAi) vectors were purchased from Open Biosystems.

**C. elegans culture.** Standard methods were used for cultivating C. elegans on nematode growth medium (NGM) agar (Brenner, 1974; Wood, 1988). The lrk-1 [km17] line has a deletion that removes the kinase and WD40 domains; generation and characterization of lrk-1 [km17] was described previously (Sakaguchi-Nakashima et al., 2007). The SK4005 [zDrIs5] (Mec-4::GFP) I; lin-15 (+) (+) line expresses green fluorescent protein (GFP) driven by the C. elegans mechanosensory Mec-4 promoter (Suzuki et al., 2003) and was generously provided by Scott Clark (Skirball Institute, New York, NY). The lin-15 (+) (+) line was generated by complementation of the temperature-sensitive lin-15 (n765ts) mutation, as described for the LRRK2 lines below (Clark et al., 1994).

**Generating transgenic C. elegans lines.** LRRK2 transgenic nematodes were created by coinjecting a mixture of DNAs containing 50 ng/μl plasmid pSyb-LRRK2 or pSyb-LRRK2 mutants along with WT lin-15 plasmid (pSK1; 20 μg/μl) and sonicated C. elegans genomic DNA (20 ng/μl). This mixture of DNA was injected into young adults of lin-15 strain (n765ts), grown at 15°C (Clark et al., 1994). Injected nematodes were grown at 20°C and MUVless progenies, complemented with WT ben-1.

**Dopamine measurements.** The nematodes (~1000 per sample) were washed off 10 cm plates with water and then washed with M9 buffer until the adult nematodes were clean of bacteria. Nematode pellets (30–40 μl) were measured and weighed. The samples were stabilized with 200 μl of 0.1 N perchloric acid, vortexed, and then sonicated on ice in 10 s intervals and spun down at 14,000 rpm for 15 min to remove any insoluble residue. The samples were kept on ice, and the acid supernatant was decanted from the insoluble pellet. Forty to 60 μl of the acid supernatant was transferred into an HPLC loading tube. The samples were injected into a Varian Microsorb C18 MV 100-5 (250 × 4–6 mm column). Eluted compounds were detected electrochemically using an ESA 542 HPLC autosampler.

| Name | Forward sequence | Reverse sequence |
|------|------------------|-----------------|
| LRRK2-1 | 5'-AGGGTCAGGACGATGTAAGA | 5'-GAGGCTCAGGACGATACAG |
| LRRK2-2 | 5'-AGGTCACCTCGAGCAGTTACCT | 5'-GTCAACTAGTGGCAGCTGTCAG |
| act-1 | 5'-CCCAATACCGAAGAGTATCCTT | 5'-AGGTGTCGATCAGCTTCCCA |
| lrk-1 | 5'-GCAAAGGATGACAGGAGAGAG | 5'-GCGAGGAGGTTAGGAGCACAT |
| ben-1 | 5'-TCCAGATTGCTGCTGGAGGTCA | 5'-TAGGGTGACAGGAGGCTAGGA |

**Lifespan.** Assays were performed without the conventional fluorodeoxyuridine to avoid any complication from inhibition of DNA synthesis affecting the biology of LRRK2. Each point on the graph corresponded to results from three dishes of 40 nematodes each. Nematode viability was checked every day with a soft touch at the tip of pharynx of the nematode. Nematodes were transferred to fresh plates every other day.

**Nematode viability.** Assays were performed using three dishes of 30–40 adult nematodes for each condition and strain. NGM agar contained specified concentrations of chemicals. For most studies, the concentrations of toxins was rotenone at 25 μM and paraquat at 500 μM. Treatment with each toxin was begun on adult day 1. Nematodes that crawled up the side of the plate or died as a result of egg laying defects were excluded from all analyses.

**RNAi knockdown.** This was performed according to Kamath et al. (2001). Ht115 bacteria was grown overnight in LB media containing 50 μg/ml carbenecillin. Bacteria was then plated on NGM plates containing 1 μM isopropyl-β-D-thiogalactopyranoside (IPTG) and allowed to grow overnight at room temperature. Nematodes were synchronized by 3 h egg laying and exposed on adult day 1 to Ht115 bacteria carrying the pl4440 expression vector with the lrk-1 open reading frame (ORF) (Open Biosystems) or empty vector. On adult day 3, 20 nematodes were moved in triplicate to plates containing 1 mM IPTG and rotenone (25 μM), which were plated with lrk-1 ORF or empty vector containing bacteria. For RNAi validation nematodes were exposed to either empty vector or lrk-1 ORF containing bacteria for 4 d, followed by analysis of gene expression. Analysis of the lrk-1 RNAi construct by the program dsCheck showed no significant off-target effects for the RNAi construct.

**Quantitative real-time reverse transcription-PCR assay.** Total RNA from C. elegans was extracted using Qiagen RNeasy mini kit. Intact RNA was checked by running a 1.0% agarose/formaldehyde gel and quantified spectrometrically (NanoDrop ND-1000) before proceeding to subsequent steps. One milligram of total RNA was reverse transcribed using iScript cDNA synthesis kit (Bio-Rad) according to the instructions of the manufacturer instructions. Real-time reverse transcription (RT)-PCR was performed on a MyQ Detection System (Bio-Rad) using iQ SYBR Green supermix. Expression levels for each target gene were calculated by DDCT method (Livak and Schmittgen, 2001). All analyses were performed in triplicates. Primers used for the real-time RT-PCR are listed in Table 1. The amplimer sets used were LRRK2-2, act-1, lrk-1, and ben-1.
of washing with PBS/0.1% Tween 20/1 mM EDTA, material was eluted from the column with 1 mg/ml V5 peptide.

**Immunoblotting.** Sample was resuspended in Laemmli’s buffer containing 1% SDS and 0.5% β-mercaptoethanol and immediately heated at 95°C for 10 min. Separation was done using 3%–8% NuPAGE Tris-acetate gels. Transfer was at 40 V for 75 min. The immunoblot was blocked in 5% nonfat milk/0.05% Tween 20/TBS. Antibodies used included the following: rabbit anti-V5 antibody (V8137; Sigma), anti-V5 agarose (V8137, 1:1000; Sigma), rabbit anti-LRRK2 (NB300-268; Novus), mouse anti-actin (mAb1501; Millipore Corporation), and peroxidase-coupled donkey antimouse antibody (1:5000; Jackson ImmunoResearch). Development was with enhanced chemiluminescence (Pierce).

**Optical analysis of GFP fluorescence.** Synchronized adult nematodes were treated as described above. GFP fluorescence was measured 1–2 d after each treatment, using Carl Zeiss Axiovision software for the analysis. For quantification, rectangles were placed around the CEP dopaminergic neurons (cell bodies), which are the most prominent neurons, and the mean optical density was determined; the area between the neurons was used to define background optical density.

**Statistical analysis.** Results are presented as mean ± SEM with ANOVA for analysis and Newman–Keuls post hoc testing. Lifespan was analyzed by hazard rate analysis using GraphPad Prism software.

**Results**

**Characterization of LRRK2 C. elegans strains**

LRRK2 was expressed in C. elegans to elucidate its in vivo functions and to understand how mutations affect LRRK2 function. WT or G2019S LRRK2 were inserted into the pSybDEST plasmid. This plasmid contains a synaptobrevin (SNB) promoter, which drives neuronal-selective expression of LRRK2. The lines were selected with a lin-15 coinjection marker plasmid by injecting into a temperature-sensitive lin-15 strain (n765ts) (Clark et al., 1994). The presence of WT and mutant LRRK2 genes was confirmed by PCR. The WT and G2019S LRRK2 lines were subsequently integrated with γ-irradiation. Two independent lines for each transgene were selected, and each line was subsequently outcrossed six times with nontransgenic Bristol N2 to remove the lin-15 background and unwanted mutations from the integration process. Expression of LRRK2 in the resulting lines (WT LRRK2, lines wlzIs1 and wlzIs2, and G2019S LRRK2, lines wlzIs3 and wlzIs4) was quantified by real-time RT-PCR (Fig. 1A). The expression levels among lines wlzIs2, wlzIs3, and wlzIs4 were comparable, whereas line wlzIs1 showed somewhat lower LRRK2 expression (Fig. 1A). We also performed immunoblots to verify protein expression. Nematode lysates were generated and V5-LRRK2 was immunoprecipitated with an anti-V5 antibody column, eluted with V5 peptide, and immunoblotted with anti-V5 antibody. A single band migrating at 250 kDa was present in the WT and G2019S LRRK2 samples corresponding to the V5 peptide eluate (Fig. 1B). Although immunoprecipitation is not quantitative, expression of LRRK2 appeared to be higher in the G2019S LRRK2 line (wlzIs4) than the WT line (wlzIs2) (Fig. 1B). V5-LRRK2 was absent from the Mec-4::GFP, which was used as a negative control. The Mec-4::GFP line was used as a control for the LRRK2 lines. Three successive fractions eluted from the anti-V5 antibody column are shown for each nematode line. Lanes 1–3, WT LRRK2 (line wlzIs2); lanes 4–6, Mec-4::GFP (a line derived from the same lin-15 background as the LRRK2 lines); lanes 7–9, G2019S LRRK2 (line wlzIs4). The arrow points to the V5-LRRK2 at 250 kDa, which was eluted from the anti-V5 column. C. Number of eggs laid by N2 and transgenic lines. D. Analysis of total brood size for WT and G2019S LRRK2 (lines wlzIs2 and wlzIs4) demonstrates similar levels of fertility. E, F. Analysis of lifespan for WT and G2019S LRRK2 shows that C. elegans expressing WT LRRK2 (lines wlzIs1, wlzIs2) exhibit greater median lifespan compared with nontransgenic (N2) or G2019S LRRK2 lines (lines wlzIs3, wlzIs4). The lifespan data for E and F were generated with separate experiments.
producing the transgenic paraquat (500 sensitive to 500 ability to the mitochondrial toxin paraquat. N2 other forms of mitochondrial inhibition, we investigated vulner-
sured every 2 d for 8 d. After 8 do f treatment, survival was 80% survive. G2019S LRRK2 showed increased median lifespans compared with G2019S LRRK2 lines) showed similar levels of sensitivity to rotenone (25 μM, 4 d).

Figure 2. LRRK2 protects against mitochondrial stress. A, Nematodes expressing WT or G2019S LRRK2 (lines wld52 and wld54) showed reduced toxicity to rotenone (25 μM). B, Nematodes expressing WT LRRK2 (strain wld52) showed reduced toxicity to pararquat (500 μM). The G2019S LRRK2 strain (strain wld54) showed sensitivity to pararquat similar to that of N2. The p values represent overall p values for each figure. WT and G2019S LRRK2 were significantly different (p < 0.0001) in A and B; N2 and G2019S LRRK2 were not significantly different in B, C, R1441C, G2019S, and KD LRRK2 (wld55, wld54, and wld57) showed less protection against rotenone (10 μM) than WT LRRK2 (wld52), and KD/R1441C LRRK2 (wld56) was strongly protected against rotenone (20 μM) N2 (n = 12, 40 nematodes per dish for all points). D, Bristol N2 and lin-15 (the background strain for producing the transgenic C. elegans lines) showed similar levels of sensitivity to rotenone (25 μM, 4 d).

early adulthood were evident, but these differences did not reach statistical significance (Fig. 1C), and brood sizes were similar between the WT and G2019S nematodes (Fig. 1D). Analysis of lifespan in age-synchronized populations of nematodes showed an effect of LRRK2 on longevity. WT LRRK2 lines exhibited increased median lifespans compared with G2019S LRRK2 and N2 strains (Fig. 1 E, F) (p < 0.0001).

LRRK2 reduces vulnerability to mitochondrial dysfunction PD-related genetic modifications frequently render neurons more vulnerable to mitochondrial complex I inhibition (Betarbet et al., 2000; Dauer and Przedborski, 2003; Ved et al., 2005). We therefore examined whether LRRK2 expression modified the response to mitochondrial inhibition in C. elegans. Age-synchronized lines of young adult WT and G2019S LRRK2 C. elegans were exposed to 25 μM rotenone beginning adult day 1, and survival was measured every 2 d for 8 d. After 8 d of treatment, survival was 80% for WT LRRK2, 40% for G2019S LRRK2, and 2% for nontransgenic (Fig. 2A). To understand whether LRRK2 protected against other forms of mitochondrial inhibition, we investigated vulnerability to the mitochondrial toxin pararquat. N2 C. elegans were sensitive to 500 μM pararquat and exhibited 4 ± 2% survival at 14 d (Fig. 2B). C. elegans expressing WT LRRK2 were much more resistant to pararquat and exhibited 85 ± 4% survival at 14 d (Fig. 2B). G2019S LRRK2 did not protect C. elegans against pararquat and gave a profile similar to that of N2 C. elegans (Fig. 2B). Survival of the WT LRRK2 line was more pronounced than could be accounted for by differences in baseline viability at that age (Fig. 1E). We also examined the effects of other LRRK2 mutations on sensitivity to rotenone, including the R1441C and KD LRRK2 mutations, as well as a LRRK2 construct containing both to LRRK2 affected the vulnerability to rotenone (Shiioi et al., 2001). We observed no difference in vulnerability to rotenone (supplemental Fig. 2, available at www.jneurosci.org as supplemental material).

Endogenous C. elegans lrk-1 also modulates the response to mitochondrial dysfunction

Knockdown of endogenous lrk-1 had effects opposite to expressing LRRK2. Bristol N2 nematodes were fed bacteria containing an RNAi expression plasmid for lrk-1 to test whether endogenous lrk-1 activity modulates sensitivity to mitochondrial inhibition. Two days after feeding was initiated, nematodes were treated with rotenone (25 μM) and assayed over the next 4 d. Knockdown of lrk-1 did not affect baseline viability (100% survival at day 4) but did sensitize C. elegans against mitochondrial inhibition compared with knockdown of either empty vector or sel-9 (Fig. 3A, empty vector comparison shown). Knockdown of lrk-1 was verified by real-time RT-PCR. Levels of lrk-1 were 46 ± 5% (p < 0.05) lower in nematodes exposed to lrk-1 RNAi compared with nematodes exposed to the PL4440 empty RNAi vector. The reciprocal effects of lrk-1 knockdown and LRRK2 expression support a hypothesis that the effects of LRRK2expression in C. elegans derive from actions linked to the endogenous function of lrk-1.

Next, we proceeded to examine the effects of partial lrk-1 deletion to test the knockdown results through an independent mechanism. The lrk-1 [km17] C. elegans strain has a deletion eliminating the kinase and WD40 domains of lrk-1, the C. elegans ortholog of LRRK2 (Sakaguchi-Nakashima et al., 2007). We used the lrk-1 [km17] strain to examine the effects of deleting the lrk-1 kinase domain on the response to mitochondrial inhibition. Adult lrk-1 [km17] nematodes (adult, day 1) were treated with rotenone (25 μM) for 4 d. The lrk-1 [km17] strain showed en-
hanced vulnerability to rotenone (Fig. 3B). The km17 deletion exerted no affect on baseline viability \textit{C. elegans} over the 4 d time span used for the experiment (supplemental Fig. 3, available at www.jneurosci.org as supplemental material).

We also examined whether LRRK2 expression complemented the lrk-1 [km17] phenotypes. The SNB::GFP/lrk-1 [km17] line was crossed onto the WT and G2019S LRRK2 backgrounds, and the sensitivity to rotenone (10 \(\mu\text{M}\), 4 d) was measured (Sakaguchi-Nakashima et al., 2007). Protection against rotenone toxicity was observed for the SNB::GFP/lrk-1 [km17]/WT LRRK2 line but not for the SNB::GFP/lrk-1 [km17]/G2019S LRRK2 (Fig. 3C). The km17 deletion exerted no effect on baseline viability of \textit{C. elegans} over the 4 d time span used for the experiment, although after day 5, the line carrying the lrk-1 [km17] mutation began to show more toxicity than the parent line jul137. Neither the SNB::GFP nor the km17 deletion exerted an affect on baseline viability \textit{C. elegans} over the 4 d time span used for the experiment (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). WT LRRK2 also complemented the enhanced rotenone sensitivity observed with knockdown of lrk-1. RNAi for lrk-1 and pL4440 were associated with 25 and 75% toxicity (\(p < 0.01, 25 \mu\text{M} \) rotenone, 2 d), respectively. Complementation by WT LRRK2 in the rotenone assay supports the hypothesis that LRRK2 complements the loss of function present in the lrk-1 [km17] strain.

Using the same lines, we examined whether LRRK2 complemented an SNB mislocalization phenotype putatively associated with the lrk-1 [km17] strain (Sakaguchi-Nakashima et al., 2007). SNB does not normally reside in the dendrites, but in the lrk-1 [km17] strain, SNB expression is present at the dendritic tip of the amphid sensory neuron. Surprisingly, neither WT LRRK2 nor G2019S LRRK2 modified SNB::GFP fluorescence in the distal amphid (supplemental Fig. 5, available at www.jneurosci.org as supplemental material). Thus, expressing WT LRRK2 modulated SNB::GFP expression but did not complement the mislocalization observed in the SNB::GFP/lrk-1 [km17] line.

**Dopaminergic marker loss is greater in \textit{C. elegans} expressing G2019S LRRK2 than WT LRRK2**

Dopaminergic neurons show selective vulnerability in subjects with LRRK2 mutations. We used the ability to track individual neurons in \textit{C. elegans in vivo} to examine the effects of LRRK2 on dopaminergic neurons. WT and G2019S LRRK2 lines (lines w1zIs2 and w1zIs4) were crossed with a \textit{C. elegans} line expressing GFP driven by the dopamine transporter-1 (DAT) promoter DAT::GFP (strain 410), and the DAT::GFP fluorescence was monitored (Kuwahara et al., 2006). Previous work by Nass et al. (2002) demonstrated that loss of fluorescence of a DAT::GFP construct is associated with degeneration of dopaminergic neurons in \textit{C. elegans}. Age-synchronized \textit{C. elegans} lines expressing LRRK2 (WT and G2019S) showed strong dopaminergic GFP signal during the L3 stage that was similar to or slightly stronger than the 410 control line (Fig. 4A, B). On reaching adulthood, the dopaminergic signal showed a rapid decrease in LRRK2 lines, with the G2019S LRRK2/DAT::GFP line showing a significantly greater decrease in signal. By day 2 of adult life, the signal of G2019S LRRK2/DAT::GFP line was 80% less than the control 410 DAT::GFP line and 60% less than the WT line (Fig. 4A–C). Additional aging produced loss of GFP signal such that, by day 5, fluorescence was reduced to similar levels in all the nematode lines.

Loss of dopaminergic markers was confirmed by measuring dopamine levels. Synchronized lines of DAT::GFP and WT or G2019S LRRK2/DAT::GFP \textit{C. elegans} lines were harvested at adult day 3, and levels of dopamine were measured by HPLC. Dopamine levels in the DAT::GFP and WT LRRK2/DAT::GFP lines were not significantly different, although there was a trend toward a decrease in dopamine in the WT LRRK2 line (Fig. 4D). This contrast with the increased loss of DAT::GFP fluorescence observed in the WT LRRK2/DAT::GFP line and indicates that, under some conditions, DAT::GFP fluorescence and dopamine levels are not linked. The G2019S LRRK2/DAT::GFP line showed significantly less dopamine than WT LRRK2/DAT::GFP or DAT::GFP lines (Fig. 4D). These results support the hypothesis that \textit{C. elegans} expressing G2019S LRRK2 show enhanced loss of dopaminergic neurons.

The effects of LRRK2 on GFP fluorescence in a different set of neurons, those expressing Mec-4, were also examined to assess whether LRRK2 exerts effects that are selective for dopaminergic neurons (supplemental Fig. 6, available at www.jneurosci.org as supplemental material) (Xu et al., 2001). Mec-4 is present in neurons sensitive to touch, including a pair in the tail (Xu et al., 2001). C. elegans expressing Mec-4::GFP were crossed with WT LRRK2 (line w1zIs2) or G2019S LRRK2 (line w1zIs4). Fluorescence of the Mec-4::GFP construct was assessed in each nematode line from birth up to adult day 6 (supplemental Fig. 6, available at www.jneurosci.org as supplemental material). Mec-4::GFP fluorescence was present in each of the nematode lines, and fluorescence intensity was not altered by the presence of WT or G2019S
LRRK2 at any time point as judged by either visual or quantitative methods (supplemental Fig. 6, available at www.jneurosci.org as supplemental material). These data suggest that G2019S LRRK2 stimulates loss of dopaminergic markers in C. elegans in a manner that is particularly evident early in the adult phase of the nematode life cycle and that the changes observed exhibit at least partial selectivity for dopaminergic neurons.

G2019S LRRK2 sensitizes dopaminergic neurons to mitochondrial inhibition compared with WT LRRK2

We proceeded to examine interactions between LRRK2 and toxin exposure on dopaminergic neurons. We examined the effects of LRRK2 on the response of dopaminergic neurons to rotenone. DAT::GFP fluorescence was highly sensitive to rotenone, necessitating use of a lower dose (250 nM) of rotenone than for survival assays. Dopaminergic neurons in WT LRRK2/DAT::GFP retained strong fluorescence after exposure to 250 nM rotenone for 24 h, whereas dopaminergic neurons in the control DAT::GFP or G2019S LRRK2/DAT::GFP lines showed 55 ± 16 and 83 ± 5% loss of fluorescence, respectively (Fig. 5A, B). Dopamine levels in the WT LRRK2/DAT::GFP lines treated with rotenone (250 nM) showed enhanced protection compared with the Bristol N2 line under the same conditions, which parallels the fluorescence data (Fig. 5C). The G2019S LRRK2 line showed dopamine levels that were significantly less than that of the WT LRRK2 line after rotenone treatment but similar to that of the N2 line. These data suggest that the G2019S LRRK2 construct does not provide the same level of protection as the WT LRRK2 construct.

We also examined the response of lrk-1 [km17] and Mec-4 neurons to rotenone treatment. To examine the lrk-1 [km17] line, nematodes expressing DAT::GFP were crossed to lrk-1 [km17]. The lrk-1 [km17]/DAT::GFP lines were then age synchronized and treated with or without rotenone (250 nM, 24 h) beginning at adult day 1. Optical densities for the lrk-1 [km17]/DAT::GFP line (n = 8–10) were normalized to the DAT::GFP line under the same conditions. The lrk-1 [km17]/DAT::GFP showed enhanced loss of DAT::GFP fluorescence in response to rotenone treatment compared with control (32 ± 9, p < 0.01, n = 10, 250 nM, 24 h). This indicates that loss of lrk-1 function has the opposite effect from overexpressing WT LRRK2. Parental, WT and G2019S LRRK2/Mec-4::GFP lines were age synchronized, and young adult nematodes were exposed to rotenone (250 nM, 3 d). No differences in GFP fluorescence were observed between the
Mec-4::GFP, Mec-4::GFP/WT LRRK2, and Mec-4::GFP/G2019S LRRK2 lines (data not shown).

Discussion

Using C. elegans as a model, we observed that dopaminergic neurons were particularly sensitive to mutant LRRK2. G2019S LRRK2 increased vulnerability of dopaminergic neurons relative to WT LRRK2 lines both basally and in response to mitochondrial stress. Survival in the nematode survival assay was also decreased for mutant LRRK2 (G2019S or R1441C) relative to WT LRRK2, but all constructs increased survival relative to controls (nontransgenic N2 or Mec-4). The contrasting responses of the dopaminergic neuron assay compared with the survival assay might reflect responses of different types of cells because dopaminergic neurons are not required for survival. Knockdown as well as deletion of lrk-1 reduced nematode survival in response to rotenone treatment, which is opposite to the response to LRRK2 expression, and suggests that the survival phenotype can be attributed to endogenous functions of lrk-1.

Our results indicate a role for LRRK2 and its C. elegans ortholog lrk-1 in regulation of mitochondrial dysfunction. Rotenone and paraquat act by inhibiting the mitochondrial electron transport chain, which leads to a number of effects, including increased free radical production and reduced ATP (Yamada and Fukushima, 1993; Castello et al., 2007). Rotenone and paraquat act at different sites on the respiratory chain (Fukushima et al., 1993, 1994). Rotenone and paraquat also have pleiotropic actions, which include interfering with microtubule function and activating transcription factors (Li and Sun, 1999; Ren et al., 2003, 2009). It is possible that the differences in protection in response to rotenone and paraquat in nematodes expressing G2019S LRRK2 might reflect actions beyond the mitochondria. Oxidative stress and reduced energy production lead to many other cellular effects, but these changes are grouped under a broad term, mitochondrial dysfunction, which is meant to encompass these effects. We also observed that G2019S LRRK2 enhances the loss of DAT::GFP fluorescence in adult dopaminergic neurons in response to mitochondrial dysfunction (compared with WT LRRK2). This process appears to be at least partially selective for dopaminergic neurons, because LRRK2 did not affect the vulnerability of Mec-4 neurons, as defined by fluorescence intensity of an Mec-4::GFP construct.

LRRK2 and lrk-1 appear to have similar functions in C. elegans

The studies in C. elegans shed light on the biology of LRRK2 and lrk-1. C. elegans express only one LRK, lrk-1, whereas mammals express LRRK1 and LRRK2. The lrk-1 protein exhibits strong regions of homology to human LRRK1 and LRRK2. Homology searches indicate that the lrk-1 protein has 24/40% homology (exact/ positives matches) to LRRK1 over a region of ~1500 aa and 25/44% homology (exact/positive matches) to LRRK2 over a region of ~1300 aa.

Our results indicate a role for LRRK2 and lrk-1 in regulating cellular responses to stress. The putative role of LRRK2 as a stress response protein is supported by reciprocal actions associated with LRRK2 expression and lrk-1 knockdown or deletion. RNAi can have off-target effects, but off-target effects are unlikely to be a major factor modifying our results because we used two different RNAi controls. In addition, deletion of the lrk-1 kinase and WD40 domains in Drosophila and C. elegans lrk-1 produces sensitization to rotenone similar to that observed with lrk-1 knockdown. The similar effects observed for lrk-1 RNAi and the lrk-1 [km17] deletion strain after rotenone treatment suggest that the lrk-1 RNAi was active, although some RNAi are not effective in neurons so we cannot rule out changes in survival mediated by humoral factors. The ability of LRRK2 to complement the rotenone sensitization associated with the lrk-1 [km17] strain provide additional support for the hypothesis that LRRK2 and lrk-1 share similar functions.

An increasing number of studies suggest a role for LRRK2 in regulating neuronal responses to stress. Transient expression of LRRK2, but not LRRK1, is toxic to neurons (Greggio et al., 2007); PD-linked mutations in LRRK2 show enhanced toxicity (West et al., 2005, 2007; Greggio et al., 2006; MacLeod et al., 2006; Smith et al., 2006). In Drosophila, knockdown of LRRK2 enhances toxicity to rotenone (Lee et al., 2007). A putative role for LRRK2 in regulating neuronal responses to mitochondrial dysfunction is also supported by recent studies showing that LRRK2 associates with the outer mitochondrial membrane (Biskup et al., 2006). Combining our results with those in the literature suggests a consistent line of evidence implicating LRRK2 with mitochondrial function.

We also examined complementation by examining the expression of synaptobrevin in the lrk-1 [km17] strain but did not observe an effect. The result could reflect two possibilities. First, we observe an effect. The result could reflect two possibilities. First,
WT LRRK2 might not complement the lrk-1 [km17] SNB mislocalization phenotype. Second, the SNB::GFP mislocalization in SNB::GFP/lrk-1 [km17] strain might not be a lrk-1-specific phenotype but might rather originate from a cosegregating gene mutation. Additional research will clarify this issue.

**Studies of disease-related mutations in LRRK2**

We examined the actions of two common disease-related LRRK2 mutations, G2019S and R1441C, as well as a LRRK2 construct containing three mutations that inactivate the kinase domain (Greggio et al., 2006). Each of these constructs showed less protection against mitochondrial inhibition than WT LRRK2 and rendered neurons more vulnerable to toxicity than WT LRRK2, which is consistent with previous reports in the literature (Greggio et al., 2006; Smith et al., 2006; West et al., 2007). However, both mutant strains still retain some protective activity and are moderately protected against rotenone compared with the control Bristol N2 strain. Interpretation of differences among the LRRK2 strains must be tempered by the experimental limitations of our study. Semiquantitative measurements by immunoblot, based on immunocapture from an anti-V5 column, suggest differences in the levels of LRRK2 protein expression among the different lines. The rank order of protein expression appears to be G2019S > WT = R1441C > R1441C/KD > KD (Fig. 1B). Vulnerability to rotenone among the lines correlates best with presence of a LRRK2 mutation; there is no clear correlation with LRRK2 protein expression level. For instance, the KD/R1441C line shows strong protection despite low-level expression. The strong protection against rotenone afforded by the KD/R1441C LRRK2 construct contrasts with vulnerability of the KD line and suggests that LRRK2 kinase activity is not required for protection against rotenone toxicity in *C. elegans* but also points to the complex relationship between mutations and activity. The presence of two mutations in LRRK2 compensate for the loss of function of each particular mutation; we hypothesize that this might reflect dynamic interplay between different LRRK2 domains that alters the three-dimensional organization of the LRRK2 protein. The modest protection by the KD LRRK2 construct contrasted with enhanced rotenone sensitivity by the lrk-1 [km17] line. The simplest explanation is that the lrk-1 [km17] mutation has reduced lrk-1 levels, for instance, attributable to generating a truncated protein that is rapidly degraded. Alternatively, the kinase and WD40 domains might have binding functions that are separate from the kinase function but are required for protection against rotenone. The strong protection afforded by the KD/R1441C construct is striking and presents an opportunity for additional exploration into the structure/function relationships of LRRK2.

**LRRK2 modifies the response of dopaminergic neurons to mitochondrial dysfunction**

One of the more intriguing aspects of our study is the sensitivity of dopaminergic neurons to expression of LRRK2. The changes in vulnerability induced by LRRK2 in *C. elegans* dopaminergic neurons were more selective than the changes in vulnerability observed with the whole worm survival assay. Both WT and G2019S LRRK2 enhanced the vulnerability of dopaminergic neurons under basal growth conditions. WT LRRK2 protected dopaminergic neurons against rotenone toxicity. In contrast, G2019S LRRK2 renders dopaminergic neurons more vulnerable to rotenone treatment compared with WT LRRK2 lines, although the G2019S LRRK2 did retain some protective action relative to the control DAT::GFP line.

The molecular mechanisms by which LRRK2 protects against mitochondrial dysfunction remain to be determined. The data in this manuscript provide support for a hypothesis that LRRK2 might act in part by regulating mitochondrial function. Genetic and environmental evidence point to a role for mitochondrial dysfunction in PD. PINK1 localizes to the mitochondria and associates with mitochondrial proteins, such as HtrA2 (Valente et al., 2004; Plun-Favreau et al., 2007). Several PD genes, including PINK1, parkin, DJ-1, HtrA2, and α-synuclein, modify mitochondrial function and might act in similar pathways (Moore et al., 2005; Ved et al., 2005; Yang et al., 2006; Exner et al., 2007). A recent microarray study implicates LRRK2 with protein pathways mediated by p53 and small GTPases (Häbig et al., 2008). Other studies indicate the LRRK2 interacts with hsp-90 and 4E-BP (Imai et al., 2008). GTPases were also identified as important modifiers of α-synuclein toxicity (Cooper et al., 2006; Gitler et al., 2008). LRRK2 might act through any of the signaling pathways that regulate stress responses. Understanding integration of these signaling processes is particularly relevant to the biology of dopaminergic neurons, in which the interplay of multiple factors appears to account for the strong sensitivity to stresses linked to PD (Mosharov et al., 2009). Elucidating how LRRK2 interacts with these biochemical pathways could illuminate how LRRK2 contributes to the pathophysiology of PD.

*Note added in proof.* While this manuscript was in press, Li et al. (2009) published on mice overexpressing R1441G LRRK2, and also observed dopaminergic degeneration.

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