Leucine-rich repeat kinase 2 interacts with Parkin, DJ-1 and PINK-1 in a Drosophila melanogaster model of Parkinson’s disease

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Mutations in the LRRK2 gene are the most common genetic cause of familial Parkinson’s disease (PD). However, its physiological and pathological functions are unknown. Therefore, we generated several independent Drosophila lines carrying WT or mutant human LRRK2 (mutations in kinase, COR or LRR domains, resp.). Ectopic expression of WT or mutant LRRK2 in dopaminergic neurons caused their significant loss accompanied by complex age-dependent changes in locomotor activity. Overall, the ubiquitous expression of LRRK2 increased lifespan and fertility of the flies. However, these flies were more sensitive to rotenone. LRRK2 expression in the eye exacerbated retinal degeneration. Importantly, in double transgenic flies, various indices of the eye and dopaminergic survival were modified in a complex fashion by a concomitant expression of PINK1, DJ-1 or Parkin. This evidence suggests a genetic interaction between these PD-relevant genes.

INTRODUCTION

Parkinson’s disease (PD) is the most common neurodegenerative movement disorder. Its pathophysiology involves, although is not limited to, progressive loss of nigrostriatal dopaminergic neurons. PD is considered idiopathic in most patients. However, ~10% of patients have a family history of PD and some cases have a clear genetic component. Several genes, including LRRK2 (reviewed in 1), have been linked to familial forms of PD. LRRK2 is a large multidomain protein with kinase and GTP-ase activities (reviewed in 2). LRRK2 mutations are the most common cause of familial PD Parkinson’ disease, accounting for up to 39% of all cases in certain populations. The manner by which mutations in LRRK2 induce PD is unclear. Overexpression of wild-type (WT) or mutant LRRK2 causes cell death in vitro (3–5). There is also some evidence it may be involved in sorting (6) and endocytosis of synaptic vesicles (7), and in regulation of neurite length and branching (5). However, the physiological or pathological role of this protein remains largely unknown. Importantly, the role of full-length mutant LRRK2 in vivo remains largely unreported.

In order to better understand the mechanism of LRRK2 induced pathology, several groups have recently generated Drosophila lines expressing either fly LRRK (dLRRK) (8,9) or...
human LRRK2 (hLRRK2) (10). However, the reported neurochemical and behavioral phenotypes of these flies differed considerably. For example, one study shows no loss of dopaminergic neurons or deficits in climbing ability (8), while others show loss of dopamine and dopaminergic neurons accompanied by behavioral deficits (9,10). Given these disparate observations, we generated human WT LRRK2 and several other independent mutants including the LRR domain hLRRK2(I1122V); COR domain hLRRK2(Y1699C); and kinase domain hLRRK2(I2020T) mutants. All of these mutations have been identified in PD patients.

Our transgenic flies expressing hLRRK2 consistently display loss of dopaminergic neurons. Importantly, hLRRK2 expression also sensitizes flies to environmental toxins, such as rotenone. However, its effects on other important indices, such as behavior and natural lifespan, are much more complex. Interestingly, our results also reveal a complex genetic interaction between LRRK2 and other genes relevant to PD.

RESULTS

Generation of LRRK2 transgenic lines

We first generated WT and mutant LRRK2 transgenic flies by microinjecting a UAS-hLRRK2-containing vector into w1118 embryos and selecting the appropriate flies. To ectopically express the transgenes, we used a UAS/Gal4 bipartite system.

To confirm that we indeed expressed hLRRK2 in the fly, we first performed RT–PCR. All transgenic flies were positive for transgene expression (Fig. 1A). To confirm this, we also assessed the expression of hLRRK2 protein by western blot. We observed a strong band at more than 250 kDa that was not present in the control GMR/+ fly (Fig. 1B). We thus concluded that all of our lines express the LRRK2 transgenes.

Previous reports suggest that axon outgrowth may be affected by LRRK2 expression (5). If this were the case, we might expect to see an abnormal development of the nervous system. To assess this, we expressed the transgenes in the nervous system using the Elav driver and examined the axonal growth in embryos by looking at abnormalities including breaks, thinning or fusions in longitudinal connectives and commissures. There were no significant differences in these parameters in hLRRK2(I2020T) expressing embryos, compared with controls (Fig. 2A and B). Accordingly, gross development appeared normal with flies expressing hLRRK2(I2020T).

Loss of dopaminergic neurons

Loss of dopaminergic neurons is a pathological hallmark of PD. We therefore investigated whether expression of WT or mutant hLRRK2 results in degeneration of these neurons. We analyzed all four posterior paired dopaminergic clusters in the fly brain: dorsolateral posterior protocerebral (PPL1), lateral posterior protocerebral (PPL2) and two dorsomedial posterior protocerebral clusters (PPM1/2 and PPM3) (Fig. 3E).

We expressed the transgenes in dopaminergic neurons under control of the tyrosine hydroxylase (TH) gene promoter. In control flies, the TH positive neurons in the four clusters did not change significantly in number or morphology during aging (Fig. 3B and C), data which are in agreement with previous reports (11). All WT and mutant LRRK2 flies that were aged for 50 days at room temperature show some degree of neuronal loss in PPM1/2 and/or PPL1 cluster (Fig. 3A and B). The effect in PPM3 and PPL2 clusters did not reach statistical significance, despite a trend towards loss of neurons. Overall, the most prominent effect was observed in the hLRRK2(I2020T) mutant (loss of 47.1 ± 6.6% in PPM1/2 cluster and 63.1 ± 5.2% in the PPL1 cluster). The loss of neurons was already apparent at 10 days post-eclosion (Fig. 3C).

Gal4 enhancer traps generally produce stronger effects at higher temperatures due to higher expression levels of the transgene. For example, increasing temperature to 29°C increased the expression of GMR-driven hLRRK2 in the eye (Fig. 1C). However, we saw a similar degree of loss of TH neurons 20 days post-eclosion at 29°C, compared to room temperature, with the greatest loss again in hLRRK2(I2020T) (Fig. 3D).

To verify that expression of hLRRK2 causes cell death and to extend our findings to the mammalian system, we also expressed full-length hLRRK2(WT) or hLRRK2(R144C) mutant in primary cortical neurons via adenoviral delivery. In both cases, we observed a statistically significant neuronal loss (13.4 ± 0.9% in hLRRK2(WT) and 26.2 ± 1.2% in hLRRK2(R144C), compared with 4.90 ± 1.17% in lacZ control) (Fig. 4).

Locomotor activity

PD is a movement disorder. Therefore, we proceeded to investigate how LRRK2 overexpression affects locomotion in the transgenic flies. We performed a climbing assay that has previously been used in transgenic fly models of PD. These deficits were reversed by treatment with levodopa (12). In this assay, flies are placed in a transparent vial, tapped down and allowed 10 or 20 s to climb up to a horizontal line.

We analyzed the LRRK2 transgenic fly driven with TH, as described above. The climbing ability of both control and transgenic flies progressively deteriorated with age—only 0.6–15.6% of our control flies maintained at 29°C crossed the line within 10 s at 30 days of age, compared with 62.0–88.0% at 10 days of age (Fig. 5A). The remaining flies stayed at the bottom, attempted to climb up but fell back or very slowly climbed up.

The effects of hLRRK2 expression on behavior were complex and dependent upon the age of the flies. The climbing ability of all transgenic LRRK2 lines was significantly impaired at 10 days of age with the most sizable locomotor deficit (27.6 ± 4.2% compared with control) being observed in hLRRK2(I2020T) mutants. Intriguingly, however, the LRRK2 transgenic flies performed slightly better at the later 20 day time point compared with control (Fig. 5A). The longevity of flies maintained at 29°C is known to be significantly shorter than at room temperature (approximately 30 days). Indeed, most of the 30-day-old flies, except for hLRRK2(I1122V) mutants, were unable to climb up to the line within 10 s. We therefore chose to extend the observation period and record them for 20 s. Surprisingly, the climbing
ability of all of the older hLRRK2 WT or mutant flies was significantly better (by 262.5–537.5%) than that of the control flies (Fig. 5B). In comparison, the 10-day-old hLRRK2 flies were less able to climb up the vial within 10 s compared with controls (Fig. 5A); this difference was smaller when observing the same flies for 20 s (Fig. 5B). This may suggest that the flies have difficulty initiating the movement. However, once the movement is initiated, the flies appear to move quite efficiently. In addition, any deficits observed in the LRRK2 transgenic flies are, compared with control, clearly not sustained with aging (Fig. 5A and C). A very similar pattern of initial depression of movement 30 and 50 day post-eclosion followed by its significant improvement compared with control was also observed at room temperature in hLRRK2(I1122V) and hLRRK2(I2020T) flies, respectively (Fig. 5C). The implications of this finding, particularly in relation to loss of dopaminergic neurons, are discussed further below.

**Lifespan and sensitivity to oxidative stress**

Next, we investigated the effect of hLRRK2 expression on lifespan of the flies and their response to oxidative stress. These experiments were performed at room temperature. Unexpectedly, ubiquitous expression of either hLRRK2(WT), hLRRK2(1699C) or hLRRK2(I2020T) significantly extended the basal lifespan of these flies when compared with control (Fig. 6A; Table 1). The hLRRK2(I1122V) mutant was not significantly different from control. In contrast, at 29°C, there was no significant difference in lifespan between control and Elav/hLRRK2 flies (Fig. 6C). Taken together, our data indicate that expression of WT or mutant
LRRK2 has the surprising potential to increase lifespan, depending upon the conditions.

Female fecundity (egg laying) and number of progeny is known to negatively correlate with lifespan. Thus, to determine whether the extended lifespan seen in our lines at room temperature is accompanied by lower numbers of progeny, we analyzed the fertility of flies ubiquitously expressing \textit{hLRRK2}. Surprisingly, all WT and mutant \textit{hLRRK2} lines, except for \textit{hLRRK2(Y1699C)}, had a significantly greater number of progeny compared with control flies, by 39.4–59.32% (Fig. 6D). This finding is consistent with observations in \textit{dLRRK} loss-of-function mutants where fertility and fecundity is decreased (8). Therefore, the lifespan extension seen in flies expressing \textit{hLRRK2} cannot be attributed to a decrease in fertility. Furthermore, there was no significant effect on male-to-female ratio or on the genotype probability of the progeny, compared with control (data not shown). The experiment was carefully standardized and controlled, as crowding may have a profound effect in this type of experiment.

Many of the identified PD genes modulate sensitivity to reactive oxygen species. In this regard, \textit{LRRK2} has been shown to increase sensitivity to \(\text{H}_2\text{O}_2\) in primary cortical neurons (3). Accordingly, we examined the sensitivity of our transgenic lines to rotenone, a pesticide which leads to oxidative stress. Importantly, chronic exposure to low concentrations of rotenone (100 \(\mu\text{M}\)) at room temperature rendered all flies ubiquitously expressing \textit{hLRRK2} significantly more susceptible to this toxin compared with control (Fig. 7A; Table 2). More importantly, chronic exposure of flies expressing \textit{hLRRK2} in dopaminergic neurons to low doses of rotenone also significantly increased dopaminergic neuron death in these flies, compared with both non-treated \textit{hLRRK2} expressing flies, or control rotenone-treated flies (Fig. 7B).

**Eye defects**

We examined multiple eye parameters in our transgenic lines crossed with the eye-specific \textit{GMR} driver. We first analyzed for the effects of \textit{LRRK2} expression at room temperature. No effect was observed in any \textit{hLRRK2} fly lines. Accordingly, we next looked for the presence of any abnormalities at a higher temperature, 29°C. First, we examined overall appearance of the eyes under optical microscope. While \textit{GMR} control flies appeared normal under an optical microscope, eyes of males of all of the transgenic lines were defective (Fig. 8). To be able to quantify the defects, we examined two parameters of the whole eye. First, we examined for the loss of

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**Figure 3.** \textit{hLRRK2} expression causes loss of dopaminergic neurons. (A) Representative images of TH staining in PPL1 and PPM1/2 DA clusters in 50-day-old control and \textit{TH-Gal4/UAS-hLRRK2(I2020T)} flies maintained at room temperature. (B) Graph summarizing the findings above. (C) The effect of \textit{hLRRK2} expression on the number of dopaminergic neurons in 10-day-old flies at room temperature; (D) 20-day-old flies at 29°C. (C) Schematic picture depicting dopaminergic clusters in Drosophila brain.

**Figure 4.** Expression of WT or mutant \textit{hLRRK2} kills primary cortical neurons. \(n = 3\), the experiments were performed in triplicates. One-way ANOVA followed by Dunnett’s post-test.
Figure 5. hLRRK2 expression causes alterations in locomotor activity. Climbing behavior of TH-Gal4/UAS-hLRRK2(WT), TH-Gal4/UAS-hLRRK2(I1122V), TH-Gal4/+;UAS-hLRRK2(Y1699C)/+ and TH-Gal4/UAS-hLRRK2(I2020T) flies raised and maintained at 29°C and recorded for 10 s (A) or 20 s (B). n = 4–7 sets of 10 for each time point per genotype. (C) Climbing behavior of transgenic flies kept at room temperature and recorded for 10 s. Each cohort was recorded three times. All data were analyzed by one-way ANOVA, Bonferroni’s post-test. Asterisks next to control signify that all genotypes were significantly different from control.

Figure 6. hLRRK2 expression affects the lifespan and the number of progeny. (A) Effect of ubiquitously expressed hLRRK2 on the lifespan at room temperature. n = 10–17 sets of 20 per genotype. (B) Effect of pan-neuronally expressed hLRRK2 on lifespan at room temperature (n = 20–32 sets of 20 per genotype). (C) Effect of pan-neuronally expressed hLRRK2 on lifespan at 29°C (n = 10 sets of 20 per genotype). (D) hLRRK2 expression increases the number of progeny. n = 5–8 sets of parents ubiquitously expressing hLRRK2. One-way ANOVA, Bonferroni’s post-test.
pigment, defined as spotty lighter areas. Here, any loss of pigment over GMR controls was scored as defective. There appeared to be a 99.6–100% penetrance of this eye defect in our LRRK2 transgenic lines (Table 3). Similar phenotype with pigmentation loss has been attributed to decreased lens and pigment deposition due to oxidative stress-induced loss of lens-secreting cone and pigment cells (13). Secondly, we evaluated the presence of black lesions previously reported in several other transgenic lines, including PINK1-RNAi flies (14). Hereto, less than 1% of GMR controls displayed the black lesions while 15.6% (+7.4) to 53.6% (+23.3) of all the WT or mutant LRRK2 lines, except for hLRRK2(I1122V), showed significantly more black lesions compared with control, with the highest prevalence in hLRRK2(WT) flies (Figs 8 and 9A).

Next, we analyzed the eyes using scanning electron microscopy (SEM). It is important to note that GMR can cause known defects at 29°C and the eyes of these control animals were not absolutely structurally normal compared with non-GMR controls (Fig. 8). However, overexpression of WT or mutant hLRRK2 again caused a larger defect, including glossy and rough, sometimes collapsing, surface of the eye, disorganization of mechanosensory interommatidial bristles and irregular lens shape (Fig. 8). The rough phenotype may be reflective of mispatterning of lattice cells which may be due to a failure in apoptosis regulation (13). Some facets, preferentially but not exclusively localized in one area close to the edge of the eye, had holes. These holes are likely caused by a complete absence of corneal lens. The hLRRK2-expressing flies had significantly more holes compared with control. In addition, the lens material of the adjacent ommatidia was often clearly fused together and the interommatidial bristles displayed profound disorganization and were occasionally shorter. No significant loss of bristles was apparent in any of the LRRK2 transgenic lines, except for hLRRK2(Y1699C).

Finally, we examined the ommatidial structure on sections. Here again, GMR controls did show substantial defects in the ommatidial organization. Importantly, however, this structural defect was greatly exacerbated by WT or mutant hLRRK2

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**Table 1. hLRRK2 expression increases basal fly lifespan at RT**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>50% survival (weeks)</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Da-Gal4/+</td>
<td>13.0</td>
<td>12.3–13.6</td>
<td>N/A</td>
</tr>
<tr>
<td>Da-Gal4/UAS-hLRRK2(WT)</td>
<td>14.8</td>
<td>14.2–15.3</td>
<td>0.0003</td>
</tr>
<tr>
<td>Da-Gal4/UAS-hLRRK2(I1122V)</td>
<td>13.3</td>
<td>12.2–14.3</td>
<td>0.6 (n.s.)</td>
</tr>
<tr>
<td>Da-Gal4/+;UAS-hLRRK2(Y1699C)/+</td>
<td>14.4</td>
<td>13.4–15.4</td>
<td>0.012</td>
</tr>
<tr>
<td>Da-Gal4/UAS-hLRRK2(I2020T)</td>
<td>15.2</td>
<td>14.3–16.1</td>
<td>0.0002</td>
</tr>
<tr>
<td>Elav-Gal4/+</td>
<td>13.0</td>
<td>12.4–13.6</td>
<td>N/A</td>
</tr>
<tr>
<td>Elav-Gal4/UAS-hLRRK2(WT)</td>
<td>14.8</td>
<td>14.1–15.5</td>
<td>0.0006</td>
</tr>
<tr>
<td>Elav-Gal4/UAS-hLRRK2(I1122V)</td>
<td>13.0</td>
<td>12.5–13.4</td>
<td>0.7044 (n.s.)</td>
</tr>
<tr>
<td>Elav-Gal4/UAS-hLRRK2(Y1699C)</td>
<td>12.8</td>
<td>12.6–13.1</td>
<td>0.3481 (n.s.)</td>
</tr>
<tr>
<td>Elav-Gal4/UAS-hLRRK2(I2020T)</td>
<td>13.0</td>
<td>12.6–13.4</td>
<td>0.7223 (n.s.)</td>
</tr>
</tbody>
</table>

Summary of the effects of hLRRK2 expression on basal fly lifespan at room temperature. The data were analyzed by nonlinear regression analysis.

**Table 2. hLRRK2 expression increases sensitivity of the flies to rotenone**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>50% survival (days)</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Da-Gal4/+</td>
<td>47.9</td>
<td>47.5–48.3</td>
<td>N/A</td>
</tr>
<tr>
<td>Da-Gal4/UAS-hLRRK2(WT)</td>
<td>44.1</td>
<td>43.6–44.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Da-Gal4/UAS-hLRRK2(I1122V)</td>
<td>41.0</td>
<td>40.7–41.2</td>
<td>0.0003</td>
</tr>
<tr>
<td>Da-Gal4/+;UAS-hLRRK2(Y1699C)/+</td>
<td>43.9</td>
<td>43.6–44.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Da-Gal4/UAS-hLRRK2(I2020T)</td>
<td>41.0</td>
<td>40.7–41.5</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

Summary of the effects of hLRRK2 expression on sensitivity to rotenone at room temperature. The data were analyzed by nonlinear regression analysis.

Figure 7. hLRRK2 expression increases sensitivity to oxidative stress. (A) Effect of rotenone at room temperature on lifespan of flies ubiquitously expressing hLRRK2. n = 6–8 sets of 20 per genotype. (B) Effect of rotenone on survival of dopaminergic neurons in flies expressing hLRRK2 specifically in TH-positive neurons. One-way ANOVA, Bonferroni’s post-test.
expression (Fig. 8). The regular trapezoidal arrangement of the photoreceptor cells was very severely disrupted. The cell lattice between photoreceptor cell arrays of different ommatidia was completely absent and the ommatidia were sometimes fused together. Importantly, the sections from these flies repeatedly displayed large holes that significantly altered the architecture of the ommatidial array. Similar holes were observed in other fly models of neurodegenerative disorders (15).

Interactions of LRRK2 with other PD causing genes

The presence of eye defects allowed us to screen for genetic interactions with other known PD genes. Accordingly, we next investigated possible genetic interactions between LRRK2, PINK1, DJ-1 and Parkin. First, we examined the genetic interaction between hPINK1, hLRRK2(WT) and two hLRRK2 mutant lines with the strongest eye phenotype, hLRRK2(I1122V) and hLRRK2(I2020T). The flies were, again, maintained at 29°C.

Figure 8. hLRRK2 expression causes structural and pigmentation abnormalities at 29°C. Representative images from optical microscope, SEM and tangential eye sections. The arrows point to a black lesion.
Table 3. Summary of the effects of hLRRK2 expression on eye pigmentation at 29°C. Interaction with hPINK1, hParkin and hDJ-1

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% of flies with loss of pigmentation (+SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMR/+</td>
<td>0.07 (+0.07)</td>
</tr>
<tr>
<td>GMR/hLRRK2(WT)</td>
<td>99.61 (+0.28)</td>
</tr>
<tr>
<td>GMR/hLRRK2(I1122V)</td>
<td>99.91 (+0.09)</td>
</tr>
<tr>
<td>GMR/hLRRK2(Y1699C)</td>
<td>100.00 (+0)</td>
</tr>
<tr>
<td>GMR/hLRRK2(I2020T)</td>
<td>99.70 (+0.30)</td>
</tr>
<tr>
<td>GMR/hPINK-1</td>
<td>0 (+0)</td>
</tr>
<tr>
<td>GMR/hPINK-1; hLRRK2(I1122V)+</td>
<td>94.95 (+3.94)</td>
</tr>
<tr>
<td>GMR/hPINK-1; hLRRK2(I2020T)+</td>
<td>100.00 (+0)</td>
</tr>
<tr>
<td>GMR/hPINK-1; hLRRK2(WT)+</td>
<td>97.61 (+2.38)</td>
</tr>
<tr>
<td>GMR/hParkin</td>
<td>63.39 (+6.92)</td>
</tr>
<tr>
<td>GMR/hParkin; hLRRK2(I1122V)+</td>
<td>99.59 (+0.41)</td>
</tr>
<tr>
<td>GMR/hParkin; hLRRK2(I2020T)+</td>
<td>99.21 (+0.79)</td>
</tr>
<tr>
<td>GMR/hParkin; hLRRK2(WT)+</td>
<td>99.56 (+0.44)</td>
</tr>
<tr>
<td>GMR/hDJ-1</td>
<td>100.00 (+0)</td>
</tr>
<tr>
<td>GMR/hDJ-1; hLRRK2(I1122V)+</td>
<td>100.00 (+0)</td>
</tr>
<tr>
<td>GMR/hDJ-1; hLRRK2(I2020T)+</td>
<td>100.00 (+0)</td>
</tr>
<tr>
<td>GMR/hDJ-1; hLRRK2(WT)+</td>
<td>100.00 (+0)</td>
</tr>
<tr>
<td>GMR/Parkin-RNAi</td>
<td>100 (+0)</td>
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<td>GMR/Parkin-RNAi; hLRRK2 (I1122V)</td>
<td>100 (+0)</td>
</tr>
<tr>
<td>GMR/Parkin-RNAi; hLRRK2 (I2020T)</td>
<td>100 (+0)</td>
</tr>
<tr>
<td>GMR/Parkin-RNAi; hLRRK2 (WT)</td>
<td>100 (+0)</td>
</tr>
<tr>
<td>GMR/DJ-1-RNAi</td>
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<td>GMR/DJ-1-RNAi; hLRRK2 (I1122V)</td>
<td>100 (+0)</td>
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<td>GMR/DJ-1-RNAi; hLRRK2 (I2020T)</td>
<td>100 (+0)</td>
</tr>
<tr>
<td>GMR/DJ-1-RNAi; hLRRK2 (WT)</td>
<td>100 (+0)</td>
</tr>
</tbody>
</table>

hLRRK2 expression causes loss of pigmentation in the eye at 29°C. Interactions with PINK1, DJ-1 and Parkin. 118–570 flies per genotype. One-way ANOVA, Bonferroni’s post hoc test.

hPINK1 overexpression alone did not cause a significant formation of black lesions (Fig. 9A), holes or loss of pigmentation (Table 3), but the eyes had some loss and disorganization of interommatidial bristles (Fig. 10). This is consistent with recently published data (16). The overexpression of hPINK1 in the eye did not significantly ameliorate the loss of pigmentation (Table 3), the roughness of the eye surface (Fig. 10) or the formation of holes (Fig. 10) observed in both mutant or WT LRRK2 lines by optical or SEM. In fact, expression of hLRRK2(WT) or hLRRK2(I1122V), and to some extent hLRRK2(I2020T), significantly potentiated the hPINK1-induced loss of ommatidial bristles (Fig. 10). This effect was not additive because hLRRK2 expression on its own did not cause any bristle loss. In contrast, hPINK1 expression rescued the hLRRK2(WT)- and hLRRK2(I2020T)-induced formation of black lesions on the eye surface (Fig. 9A). Therefore, hPINK1 expression appears to alleviate some (black lesions) but not all indices of hLRRK2-induced eye defects (pigmentation loss), while hLRRK2 overexpression potentiates the bristle loss phenotype of hPINK1. In order to better analyze these interactions, we further performed loss-of-function experiments. However, because the double transgenic GMR/PINK1-RNAi; hLRRK2/+ flies were not viable at 29°C, the experiments were performed at room temperature. As expected, PINK1-RNAi flies exhibited an eye phenotype characterized by pigmentation deficits and bristle loss (Figs 11 and 12; Table 4). In addition, nearly all PINK1-RNAi flies displayed black lesions (Figs 9B and C and 12). Co-expression of hLRRK2 did not significantly elevate the number of animals with black lesions (because PINK1-RNAi alone caused a dramatic effect) (Fig. 9B). However, the number of black lesions per animal was dramatically elevated with hLRRK2 (WT and mutants) expression (Figs 9C and 12) moreover, these flies show a mild albeit significant increase in the number of holes. Altogether, these findings strongly suggest an interaction between PINK1 and LRRK2.

Next, we examined the effects of hParkin expression. At 29°C, hParkin expression by itself showed a dramatic phenotype by both SEM (bristle loss, holes and rough surface) (Fig. 10), as well as counts of pigment loss (Table 3). This phenotype was not rescued by expression of WT or mutant hLRRK2 (Fig. 10). However, hParkin expression, similar to hPINK1 expression, diminished the formation of black lesions in hLRRK2(I2020T) lines (Fig. 9A). Again, Parkin-RNAi mutants exhibited a strong eye phenotype. Importantly, expression of one of the hLRRK2 mutants, I1122V, dramatically exacerbated the formation of black lesions in Parkin-RNAi flies (Fig. 9A). This is also consistent with the observation with PINK1-RNAi flies discussed above. Parkin-RNAi alone induced an effect on bristles and holes that was larger than that observed for hLRRK2 expression alone in the eye. The Parkin-RNAi-mediated effect was surprisingly blunted by hLRRK2 expression (Fig. 13). This again indicates a complex interaction between Parkin and LRRK2.

Similar to Parkin, expression of hDJ-1 by itself, at 29°C, caused a rough eye phenotype with holes (Fig. 10) and pigmentation loss (Table 3). Expression of either of the two hLRRK2 mutants did not rescue this hDJ-1 phenotype. Indeed, hLRRK2(I1122V), hLRRK2(WT) and, to a lesser extent, hLRRK2(I2020T) caused a significant exacerbation of the hDJ-1 phenotype—especially a pronounced loss of interommatidial bristles, as evidenced by SEM analysis (Fig. 10). In common with hPINK1 and hParkin, hDJ-1 expression significantly ameliorated black lesions formation in hLRRK2(I2020T) or hLRRK2(WT) (Fig. 9A). Under SEM, loss of DJ-1 led to a phenotype that was qualitatively and quantitatively similar to hDJ-1 overexpression (Fig. 13). Moreover, this phenotype was potentiated by hLRRK2 expression in a similar fashion (Fig. 13). Unlike with DJ-1 expression, however, DJ-1 loss led to the appearance of black lesions on the eye surface and this effect was not altered by LRRK2 expression (Fig. 9A).

In conclusion, expression of all three recessive PD genes inhibits black lesion formation suggesting a genetic interaction. This is further supported by the observation that loss of Parkin or PINK1 exacerbates, at least in most cases, black lesion formation. However, clearly all of the defects observed by LRRK2 are not rescued by these recessive PD genes. Moreover, expression of hLRRK2(WT) or hLRRK2(I1122V) (or, to a lesser extent, hLRRK2(I2020T)) substantially potentiated bristle loss seen in PINK1, DJ-1, or DJ-RNAi flies, respectively.

**DISCUSSION**

Our results are of significance because of the following: (i) We have generated hLRRK2 fly models using several independent


**hLRRK2** mutant lines which support a pro-death role of LRRK2 in dopaminergic neurons. (ii) We also show that LRRK2 flies have multiple surprising phenotypes not expected of a protein with pro-death function. This includes a complex and nonlinear behavioral phenotype, as well as increased basal lifespan. (iii) We demonstrate that these transgenic flies show increased sensitivity to rotenone both in terms of lifespan and dopaminergic loss, suggesting a potentially important relationship between environment and genetics. (iv) Finally, we show a complex interesting genetic interaction between LRRK2 and the recessive PD genes.

Recently, two papers have described the effects of expression of the fly orthologue of hLRRK2, dLRRK, with conflicting results. Lee et al. (8) shows no loss of dopaminergic neurons or deficits in climbing ability. In contrast, Imai et al. (9) shows loss of dopamine and of dopaminergic neurons accompanied by behavioral deficits. Because hLRRK2 and dLRRK exhibit only 38–44% similarity in their domains, and because questions have been raised as to whether dLRRK is a true orthologue of hLRRK2 (17), it is important to assess the effect of hLRRK2 expression. Accordingly, we have developed and characterized independent lines of WT and mutant hLRRK2-expressing Drosophila. First and foremost, these flies display no overt developmental defects, notably a lack of nervous system pathology. This is perhaps unexpected given the association of LRRK2 with axonal development and outgrowth (5). Thus, subtle effects on nervous system integrity cannot be ruled out at this point. Clearly, however, our results indicate that expression of any of the human or other LRRK2 mutants result in loss of dopaminergic neurons. These results are consistent with the notion that LRRK2 expression results in selective dopaminergic loss in Drosophila without overt effects on other neuronal subpopulations. Recently, Liu et al. (10) showed a similar degree of loss of dopaminergic neurons in all clusters for both WT and kinase domain mutant of hLRRK2. Taken together with our current evaluation of WT and three independent LRRK2 mutants, these data strongly support a pro-death role for ectopic LRRK2 expression, at least in Drosophila.

The effects of LRRK2 expression on locomotor behavior are complex. After an expected initial deterioration in performance compared with control (that correlates with loss of DA neurons), all the transgenic lines outperformed the control flies at later time points. While the earlier diminution of activity is consistent with that reported by others with dLRRK expression or expression of the human G2019S mutant, our results suggest that the consequences of dopaminergic loss may be quite complex at later time points. Clearly, at these points, behavior does not correlate with dopaminergic loss. However, we speculate that this effect reflects a dopaminergic or non-dopaminergic compensatory mechanism resulting from loss of dopaminergic neurons. Consistent with this, it is known that mice treated with the dopaminergic toxin MPTP exhibit an increase in dopamine turnover. This may reflect a mechanism by which the surviving dopaminergic terminals compensate for a decrease in the neuronal population. In fact, numerous reports have suggested that under certain conditions, mice may display increased locomotor activity upon MPTP treatment (18). Similarly, we propose that the later increase in locomotor activity observed in flies may be a compensatory response to loss of a subset of dopaminergic terminals.

Our results also show some surprising results when it comes to basal lifespan of hLRRK2 transgenic flies. For example, under room temperature conditions with ubiquitous expression, most transgenic hLRRK2 lines showed increased lifespan compared with controls. Interestingly, in support of our data, dLRRK loss-of-function mutants have a slightly shorter lifespan (19). This suggests that LRRK2, in addition to its pro-death function as it relates to dopaminergic neurons, may possess properties which are protective. It is important to note that these results contrast with a recently published paper (10) which shows a shortened lifespan of flies expressing WT or kinase mutant of hLRRK2. The reason for this discrepancy is unclear. However, we noted that our flies were grown under less-crowded conditions than previously reported and that the control flies in the aforementioned report showed significantly shorter lifespan than our own controls (10). Finally, it is important to note that specific neuronal expression of mutant LRRK2 (in contrast to ubiquitous expression of LRRK2 or expression of WT LRRK2 in neurons) did not promote differences in lifespan at any temperature. However, LRRK2 mutant expression in TH positive neurons still affected climbing behavior in a complex pattern. It is, therefore, unlikely that the observed behavioral differences are due to alterations in relative lifespan.

Due to the relatively low lifetime penetrance of LRRK2 mutations, it is likely that environmental factors play an
Figure 10. hLRRK2 interacts with hParkin, hPINK1 and hDJ-1 in the eye at 29°C. Representative images from SEM.
important role in the etiology of familial PD. Rotenone is a commonly used pesticide and a complex I inhibitor that increases a production of reactive oxygen species. It has been used to model PD in rodents (20) and in Drosophila (21). We utilized a chronic paradigm with lower doses of rotenone that would more realistically mimic a possible exposure to environmental toxins. Hence, the maximum survival of our control flies in this experiment was relatively long, over 2 months. All hLRRK2-expressing lines were significantly more sensitive to rotenone than controls. These results are consistent with the notion that mutations in other PD genes, such as DJ-1 and Parkin, also render cells more sensitive to a variety of external stressors. Moreover, rotenone-treated

Figure 11. Interactions of hLRRK2 with PINK1-RNAi in the eye at room temperature. Representative images from SEM.

Figure 12. Interactions of hLRRK2 with PINK1-RNAi in the eye at room temperature. Representative images from optical microscope. The arrows point to a black lesion.
This would strongly implicate a protective role of PINK1 when one looks at other parameters, such as bristle loss, black lesions formation with respect to LRRK2. GMR/+ of hLRRK2. Loss of pigmentation in the eye as seen under optical microscope. Interactions + + GMR/PINK1-RNAi; hLRRK2 (I1122V) 100 (± SEM) GMR/PINK1-RNAi 42.93 (± 10.42) + GMR/hLRRK2 (I2020T) 0 (± 0) + GMR/hLRRK2 (Y1699C) 0 (± 0) + GMR/hLRRK2 (I1122V) 0 (± 0) + GMR/hLRRK2 (WT) 0 (± 0)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% of flies with loss of pigmentation (± SEM)</th>
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<tr>
<td>GMR/+</td>
<td>0 (± 0)</td>
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<tr>
<td>GMR/hLRRK2 (WT)</td>
<td>0 (± 0)</td>
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<tr>
<td>GMR/hLRRK2 (I1122V)</td>
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<tr>
<td>GMR/hLRRK2 (Y1699C)</td>
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<td>GMR/hLRRK2 (I2020T)</td>
<td>0 (± 0)</td>
</tr>
<tr>
<td>GMR/PINK1-RNAi</td>
<td>42.93 (± 10.42)</td>
</tr>
<tr>
<td>GMR/PINK1-RNAi; hLRRK2 (I1122V)</td>
<td>100 (± 0)</td>
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<td>GMR/PINK1-RNAi; hLRRK2 (I2020T)</td>
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<td>GMR/PINK1-RNAi; hLRRK2 (WT)</td>
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Loss of pigmentation in the eye as seen under optical microscope. Interactions of hLRRK2 with PINK1-RNAi at room temperature.

hLRRK2 flies exhibited the greatest degree of dopaminergic loss, compared with both rotenone-treated controls, or vehicle-treated hLRRK2 expressing flies. Taken together, these results point to a potentially important interaction between environmental factors, such as rotenone, and genetic makeup in the control of loss of dopaminergic neurons.

The reasons why LRRK2 expression increases basal lifespan while increasing susceptibility to exogenous environmental stress are unclear. LRRK2 has recently been shown to regulate responses to oxidative stress through phosphorylating 4E-BP (9). 4E-BP, in its un-phosphorylated state, acts as a brake on translation mediated by eIF4E. Clearly, the regulation of this pathway (and protein translation) has a large number of consequences depending upon the circumstances. Overexpression of dLRRK has been linked to oxidative stress via this pathway (9). Interestingly, some authors noted that low levels of oxidative stress result in increased longevity (22). It is possible, for example, that overexpression of hLRRK2 may result in such an increase under low stress (basal) conditions, but reduce longevity when confronted with higher levels of environmental stressors. Further studies are required to explore these possibilities.

The transgenic flies showed a complex eye phenotype, including glossy and rough surface with necrotic lesions, pigmentation loss, holes, disorganization and/or loss of interommatidial bristles and disorganization of the ommatidial array. This phenotype allowed us to analyze the interaction of LRRK2 with other known PD genes. We have presented strong evidence that the three recessive PD genes interact with LRRK2. However, the genetic interactions are not straightforward. The fact that they do not always follow what one would expect (e.g. that overexpression of PINK1 is protective) highlights the complexity of the matter. Just as one example, PINK1 (as well as Parkin or DJ-1) clearly present a relatively straightforward interaction with LRRK2 when it comes to the formation of black lesions. In most cases, expression of PINK1 leads to a reduction in black lesions while loss of PINK1 exacerbates these black lesions. This would strongly implicate a protective role of PINK1 in black lesions formation with respect to LRRK2. However, when one looks at other parameters, such as bristle loss, PINK1 expression in fact exacerbates the LRRK2 phenotype. It seems that the right dose of (or balance between) LRRK2, PINK1, DJ-1 and Parkin is crucial for cell survival. In the case of PINK1, this might make sense considering growing evidence of the importance of PINK1 in mitochondrial dynamics and quality control (23). In this case, too much PINK1 activity might have a deleterious effect, similar perhaps to loss of function. This observation also adds a level of complexity to the understanding of the protective role of PINK1 reported by several groups, including our own (24,25). We propose that the direction of the interaction (suppression versus enhancement of the phenotype) depends on several other factors, especially the parameter/cell type studied.

LRRK2 impacts a subset of signaling pathways common to these PD genes, although the biochemical underpinnings of the interaction between LRRK2 and the other Parkinson’s genes are unknown. For example, DJ-1 has been shown to modulate the PI3 kinase/AKT pathway in flies (26), an upstream branch of mTOR pathway which regulates 4E-BP. In addition, Parkin has been shown to interact with LRRK2 in mammalian cells in vitro (4). It is important to emphasize that only certain hLRRK2 mutations affect the different parameters analyzed and/or genetically interact with hPINK1, hParkin or hDJ-1. The reason for this is unclear but may relate to potentially different signaling pathways affected by different mutants.

In conclusion, we have generated a hLRRK2 fly model of PD and identified PINK1, Parkin and DJ-1 as LRRK2 interactors. This demonstrates that this model is suitable for a suppressor/enhancer screening.

**MATERIALS AND METHODS**

**Drosophila genetics**

The flies were maintained on a standard cornmeal/agar medium at RT or at 29°C. The cDNA encoding human WT or mutant LRRK2 were obtained from pcDNA3.1 (+) with BamH1/Xho1 double digests and cloned to pUAST vector at BglII/XhoI site. The plasmids were microinjected to w1118 fly embryo (Genetic Services, Cambridge, MA, USA). The other fly stocks were described earlier. UAS-hParkin (27), UAS-hPINK1 (23), UAS-PINK-RNAi (14) and UAS-hDJ-1 (26). TH-Gal4, Elav-Gal4 and Da-Gal4 flies were obtained from Bloomington Drosophila Stock Centre, UAS-DJ-1-RNAi, GMR-Gal4 and w1118 flies were a gift from Dr Bingwei Lu, Dr Yong Rao (28) and Dr Margaret Sonnenfeld (29), respectively. UAS-Parkin-RNAi flies were obtained from the Vienna Drosophila Research Centre.

**RT–PCR and western blot**

Samples were reverse-transcribed and RT–PCR was performed with the following primers: 5’-CGATCCATGGCTATGGGCGAG TGTGCGACGGTCGTT-3’ (forward) and 5’-CCTCTGGAG CTTCTCCAACACG-3’ (reverse). For the western blot, we used an anti-LRRK2 rabbit polyclonal antibody (Novus Biologicals), and E7 mouse monoclonal anti-β-tubulin antibody (Developmental Studies Hybridoma Bank) for loading control.
Figure 13. Interactions of hLRRK2 with DJ-RNAi and Parkin-RNAi in the eye at 29°C. Representative images from SEM.
Quantification of dopaminergic neurons

Male flies expressing LRRK2 under the control of the TH promoter (and TH-Gal4/+ controls) were aged at RT for 10 and 50 days (or for 20 days at 29°C, as indicated). Dissected brains were fixed and TH positive neurons of the posterior clusters were visualized by staining with polyclonal rabbit anti-TH primary Ab (Novus Biologicals) and a fluorescent Ab (Alexa 488). Each whole brain was scanned using optical sections and the collected Z-series images were projected into a 3-D animation to quantify numbers of TH-positive neurons.

Locomotor behavior

Males were aged for 10–70 days and divided into sets of 10 the day before the experiment. Next day, the flies were transferred into transparent tubes with a horizontal line 8 cm above the bottom. After 10 min at room temperature, the flies were tapped down and filmed. The number of flies that crossed the line in 10 and 20 s was recorded, as indicated. All behavioral experiments were carried out at room temperature under standard light conditions.

Lifespan

hLRRK2 flies (or w1118 control) were crossed with Da, or Elav driver flies. The crosses were performed and flies were maintained at RT or at 29°C, as indicated. The conditions of the cross, including the number of parent males and females, were kept the same for all genotypes. The flies from each genotype were collected within 48 h post-eclosion, divided into sets of 20 and aged. The vials were changed every 3–7 days.

Rotenone sensitivity

Individual stocks of rotenone (Sigma) that were dissolved in dimethyl sulfoxide, kept frozen and protected from light, were mixed with water used to rehydrate the instant fly food media (Carolina Biologicals) (final concentration in the food: 100 μM). The food containing rotenone was made fresh and changed every 2–4 days. LRRK2 or w1118 control flies were crossed with Da driver flies at RT. The conditions of the cross, including the number of parent males and females, were kept the same for all genotypes. The flies from each genotype were collected within 24 h post-eclosion, divided into sets of 20, placed in the rotenone-containing vials and aged at RT protected from light. For rotenone sensitivity of the DA neurons, flies were treated for 1 month.

Eye phenotype

Flies were crossed and maintained at 29°C. For SEM, heads of males expressing hLRRK2 under the eye-specific GMR promoter and control were fixed and dehydrated. To study the ommatidial organization, tangential sections of the heads in Durcupan resin were cut at 2 μm, mounted and stained with toluidine blue. The SEM and sectioning was done by the Advanced Bioimaging Center, Mount Sinai Hospital, Toronto. All flies were analyzed 10 days post-eclosion.

Progeny quantification

Both male and female parents came from standardized fly cultures (same number of male and female parents in all crosses). Eight 0–1 days old ubiquitously expressing hLRRK2, or control Da/+., males were crossed with 10 0–1 days old unmated females of the same genotype. After 5 days of laying eggs, these parents were placed in a fresh vial and allowed to lay eggs for 5 more days. Newly eclosed flies were periodically removed from the vials; they were allowed 23 days to eclose.

Cell culture and recombinant adenovirus infection

The primary culture of mouse cortical neurons was carried out as described previously (30) The adenoviruses expressing lacZ, wild-type (WT) or R144C mutant forms of LRRK2 were engineered. The experiments were performed at a multiplicity of infection of 100 plaque-forming units per cell. Adenoviral vectors were added to cell suspension immediately before plating. Two days after plating, cells were fixed and stained with Hoechst 33258 and neuronal survival was evaluated by assessing nuclear integrity of GFP-positive or lacZ-positive neurons as previously described (31).

Embryo staining

Elav/hLRRK2 embryos were aged for 10–11 h on standard agar-apple juice plates at 4°C, fixed and incubated with the primary mouse anti-CNS axons BP-102 antibody (Developmental Studies Hybridoma Bank) followed by the goat anti-mouse HRP-conjugated secondary antibody (Promega, Madison). HRP activity was detected by precipitation of 3,3′ diaminobenzidine (DAB) in the presence of H2O2. The embryos were scored as defective if there was one or more breaks in longitudinal connectives or commissures.

Statistical analysis

The data were analyzed as specified, expressed as means ± standard error of means, and denoted * if P ≤ 0.05, ** if P ≤ 0.01 and *** if P ≤ 0.001.

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Conflict of Interest statement. None declared.

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