Vacuolar protein sorting 35 (Vps35) rescues locomotor deficits and shortened lifespan in Drosophila expressing a Parkinson’s disease mutant of Leucine-rich repeat kinase 2 (LRRK2)

Radek Linhart†, Sarah Anne Wong†, Jieyun Cao†, Melody Tran, Anne Huynh, Casey Ardrey, Jong Min Park, Christine Hsu, Saher Taha, Rentia Peterson, Shannon Shea, Jason Kurian and Katerina Venderova*

Abstract

Background: Parkinson’s disease (PD) is the most common movement neurodegenerative movement disorder. An incomplete understanding of the molecular pathways involved in its pathogenesis impedes the development of effective disease-modifying treatments. To address this gap, we have previously generated a Drosophila model of PD that overexpresses PD pathogenic mutant form of the second most common causative gene of PD, Leucine-Rich Repeat Kinase 2 (LRRK2).

Findings: We employed this model in a genetic modifier screen and identified a gene that encodes for a core subunit of retromer – a complex essential for the sorting and recycling of specific cargo proteins from endosomes to the trans-Golgi network and cell surface. We present evidence that overexpression of the Vps35 or Vps26 component of the cargo-recognition subunit of the retromer complex ameliorates the pathogenic mutant LRRK2 eye phenotype. Furthermore, overexpression of Vps35 or Vps26 significantly protects from the locomotor deficits observed in mutant LRRK2 flies, as assessed by the negative geotaxis assay, and rescues their shortened lifespan. Strikingly, overexpressing Vps35 alone protects from toxicity of rotenone, a neurotoxin commonly used to model parkinsonism, both in terms of lifespan and locomotor activity of the flies, and this protection is sustained and even augmented in the presence of mutant LRRK2. Finally, we demonstrate that knocking down expression of Vps35 in dopaminergic neurons causes a significant locomotor impairment.

Conclusions: From these results we conclude that LRRK2 plays a role in the retromer pathway and that this pathway is involved in PD pathogenesis.

Keywords: Parkinson’s disease, LRRK2, VPS35, Retromer, Endolysosomal pathway, Drosophila, Genetics, Rotenone, Neurodegeneration, Endosomes, Lysosome, VPS26

Background

A growing unmet need for better treatments of neurodegenerative disorders, including Parkinson’s disease (PD), highlights the importance of research into the pathological mechanisms involved in the disease process.

Identification of several causative genes has led to new insights into PD pathogenesis. Leucine-Rich Repeat Kinase 2 (LRRK2) (GenBank: AY792511) is the second most common causative gene of PD. Thus far, seven point mutations within LRRK2 have been demonstrated to segregate with the disease and numerous common and rare LRRK2 gene variants that increase susceptibility to PD have been described. LRRK2 has also been linked to tau [1] and α-synuclein [2-4] pathologies and therefore may be a key player upstream of cell death pathways involved in other neurodegenerative processes [5]. LRRK2 is a kinase with a Roc-COR catalytic core that has a sequence homology to Rab GTPases. Other domains include LRR and...
WD-40, predicted to be involved in protein-protein interactions. Despite promising new findings, exactly how LRRK2 contributes to cell death/survival and what is its physiological function, still remains largely unknown.

Although no animal model developed thus far has been able to reproduce all key pathological features of PD, transgenic *Drosophila* models have proven particularly useful, as they faithfully reproduce dopaminergic (DA) neuronal death and locomotor deficits [6-8]. *Drosophila melanogaster* is a highly suitable model organism for studies of gene function, interactions and elucidation of genetic pathways. Notably, *Drosophila* compound eye can be successfully employed in unbiased genome-wide genetic modifier screens in vivo [9-11]. Results from such screens and other research in *Drosophila* have recently generated important new insights into the pathophysiology of several neurodegenerative disorders, including PD [12].

To help dissect the molecular processes involved in PD pathology, we recently generated a *Drosophila* overexpressing human LRRK2 with a PD pathogenic I2020T mutation within the kinase domain [13]. This transgenic model has been successfully used by other researchers [14,15]. As shown previously by our team [13] and independently validated by others [16-18], expressing pathogenic mutant LRRK2 in *Drosophila* DA neurons recapitulates many of the cardinal features of PD, including the loss of DA neurons and locomotor deficits [13]. In addition, LRRK2 mutant flies present with an abnormal eye phenotype, allowing us to perform an in vivo genetic modifier screen in search for genetic interactors of LRRK2. Here, we provide evidence that LRRK2 genetically interacts with Vacular protein sorting 35 (Vps35) (GenBank: AE013599.4), a core component of the retromer complex.

**Results**

**Vps35 partially rescues the eye phenotype of flies expressing pathogenic mutant LRRK2**

In our studies, we employed the commonly used UAS-Gal4 system for a cell/tissue-specific expression [19]. As we have previously shown [13], expression of one of the PD-causing mutants of LRRK2, LRRK2(I2020T), under an eye-specific (GMR) promoter at 29°C causes a rough eye phenotype with pigmentation deficits. Notably, 50.03% +/- 6.58% of LRRK2(I2020T) eyes have black lesions (Figure 1). Similar lesions were reported in other fly models of neurodegeneration [20-24] and seem to be indicative of neuronal (photoreceptor) death occurring later in the eye development, after a full differentiation [21]. Such black lesions are very rare in control (GMR alone) flies (3.03% +/- 3.03% of eyes) (Figure 1). Employing this *LRRK2*(I2020T) eye phenotype as a read-out in a genetic modifier screen, we identified Vacular protein sorting 26 (Vps26) (GenBank: NM_130596.2) as a new LRRK2 interacting gene. Specifically, overexpressing endogenous *Drosophila* Vps26 in the eye caused a mild eye phenotype, including an occasional presence of black lesions (11.21% +/- 2.12% of flies) (Figure 1A and B). Strikingly, overexpressing Vps26 in the *LRRK2*(I2020T) flies rescued the black lesion eye phenotype of the LRRK2 mutants (10.10% +/- 2.12%; P < 0.05; F(3, 9) = 13.30) (Figure 1A and B).

Vps26, together with Vps35 and Vps29, are the three core components of the cargo-recognition subunit of the retromer complex. Because human homologue of Vps35, Vps35 (GenBank: NC_000016.9), has been recently identified as a new candidate PD gene (3, 4), our first goal was to determine whether LRRK2 also genetically interacts with Vps35.

*Drosophila* Vps35 (CG5625) is located on the right arm of the second chromosome and has 61% identity with its human homologue. Again, an eye-specific overexpression of endogenous Vps35 alone caused a mild eye phenotype that included the occasional presence of black lesions (7.56% of eyes, +/- 3.92%) (Figure 1A and C). Similar to Vps26, increased Vps35 expression completely rescued the black lesion phenotype of LRRK2 mutants: none of the analyzed eyes displayed any black lesions (P < 0.0001; F(2, 6) = 102.3) (Figure 1A and C). This suggests that both Vps26 and Vps35 genetically interact with LRRK2 in the *Drosophila* eye.

**Vps35 rescues the locomotor and lifespan deficits of flies expressing pathogenic mutant LRRK2**

One of the cardinal characteristics of PD is a loss of DA neurons which has a negative impact on movement. Therefore, in our next experiments we used a *Dopa-decarboxylase-Gal4* (Ddc-Gal4) driver line that is commonly used in *Drosophila* PD research to target gene expression to DA neurons [6,25].

To quantify locomotor activity, we employed a well-established negative geotaxis climbing assay. As we have shown previously, overexpressing *LRRK2*(I2020T) in DA neurons causes significant locomotor deficits [13]. Compared to control, the climbing ability of *LRRK2*(I2020T) flies was reduced by 61.45% +/- 7.49% on day 5 (specifically, 28.04% +/- 5.45% of *LRRK2* flies were able to reach the line within 5 seconds, compared to 72.75% +/- 7.89% of control) (Figure 2). Similar to the eye, this *LRRK2* phenotype can be fully rescued either by overexpressing Vps35 in DA neurons (70.00% +/- 6.36% of flies over expressing Vps35 and mutant LRRK2 were able to cross the line within 5 seconds) (P < 0.0001; F(4, 77) = 8.20) (Figure 2B), or by overexpressing Vps26 (64.12% +/- 3.28% of flies overexpressing Vps26 and mutant LRRK2 in DA neurons crossed the line within 5 seconds) (P < 0.0001; F(3, 225) = 11.31) (Figure 2A). Similar to day 5, Vps35 or Vps26 overexpression rescued the *LRRK2*(I2020T) phenotype on day 10 (Figure 2A and B).
day 20 however, the locomotor activity of all flies, including control, was severely impaired due to age (Figure 2A and B). Please note that the locomotor activity of flies was always assessed at three different time intervals (5, 10 and 30 seconds), with similar results (data not shown). In addition to the locomotor activity, we also assessed survival of these flies. Compared to the control (Ddc alone), survival of flies expressing LRRK2(I2020T) in DA neurons was significantly shorter. This phenotype was completely rescued by overexpression of Vps35 (Figure 3).

Altogether, these data validate that mutant LRRK2(I2020T) functionally interacts with Vps35 and Vps26 in DA neurons and that this interaction is important for the locomotor activity and basal survival of the flies.

**Overexpression of Vps35 rescues locomotor deficits of other LRRK2 mutants**

The I2020T substitution is localized within the kinase domain of LRRK2. Our next question was to determine whether the functional interaction between LRRK2 and
components of the retromer complex is specific to this particular mutation. To answer this question, we employed two other transgenic mutant \textit{LRRK2} lines: the \textit{LRRK2} (Y1699C) line carrying a confirmed PD pathogenic mutation in the COR domain of \textit{LRRK2} [26-28], and the \textit{LRRK2}(I1122V) line with a putative pathogenic mutation in the LRR domain [26,27].

Similar to \textit{LRRK2}(I2020T), expressing either one of the two mutant forms of \textit{LRRK2} in DA neurons caused a significant impairment locomotor activity on day 5 (24.71\% +/- 3.44\% of \textit{LRRK2}(Y1699C) flies were able to reach the line within 5 seconds, compared to 63.21\% +/- 5.30\% of control, \( P < 0.0001 \); \( F (3, 177) = 50.60 \), respectively) (Figure 4A and B), which is consistent with our previous results [13]. Importantly, overexpressing \textit{Vps35} in either one of the two \textit{LRRK2} mutant lines resulted in a complete rescue of the locomotor impairment (74.40\% +/-3.42\% and 65.01\% +/- 3.55\%, respectively, of the double transgenic flies were able to reach the top within 5 seconds on day 5; \( P < 0.0001 \)) (Figure 4A and B). The results were very similar on Day 10 (Figure 4A and B). These results demonstrate that the functional interaction between \textit{LRRK2} and \textit{Vps35} is not exclusive...
to the kinase domain mutant, and provide further evidence that LRRK2 may play a role in the retromer-dependent pathway.

**Knocking down components of the cargo-recognition subunit of the retromer complex impairs locomotor activity**

Vps35 is the most recently confirmed causative gene of PD. However, the mechanism by which mutation in Vps35 leads to PD is completely unknown. To better understand the importance of retromer for the physiological function of DA neurons, we analyzed the effect of knocking down expression of genes encoding for components of the retromer complex.

First, we analyzed the effect in the eye. Knocking down expression of Vps26 in the eye caused a significant eye phenotype (35.84% +/- 5.43% of Vps26 knock-down eyes had a black lesion, compared to 2.78% +/- 3.4% of control eyes; P < 0.05; F (3, 15) = 7.01) (Figure 5A and B). This phenotype was similar to the eye phenotype of the LRRK2(I2020T) mutant (44.71 +/- 6.49% of LRRK2(I2020T) eyes had black lesions) (Figure 5A and B). Our next goal was to assess whether these two phenotypes are additive. An additive effect would indicate that the two genes likely act on two independent cellular pathways. We observed that the eye phenotypes of LRRK2(I2020T) expression and Vps26 knock-down were not additive (36.21% +/- 3.02% of double transgenic eyes had a black lesion) (Figure 5A and B), suggesting that the two genes act on the same pathway.

The next step was to assess the effect of knocking down components of the retromer complex in DA neurons. In DA neurons, knocking-down expression of Vps26 or Vps29 caused a significant impairment in the locomotor activity (29.65% +/- 2.44% and 40.68 +/- 2.93% of flies, respectively, reached the top within 5 seconds on day 5, compared to 72.75% +/- 4.27% of control flies; P < 0.0001, F(5, 320) = 9.335) (Figure 5C). Similar to the effects seen in the eye, these climbing deficits were not additive with those observed in the LRRK2(I2020T) mutants (Figure 5C), supporting our results that Vps35, Vps26 and Vps29 share a common pathway with LRRK2.

**Vps35 protects from rotenone toxicity**

Rotenone is a pesticide and a complex I inhibitor that can be used to model parkinsonism [29]. In our experiments, we exposed the flies to rotenone to assess the role of LRRK2-Vps35 interaction under conditions of additional cellular stress.

Treating control flies with rotenone has a profound effect on their survival. Although we previously showed that pan-neuronal expression of LRRK2(I2020T) sensitized flies to low doses of rotenone [13], overexpression of LRRK2(I2020T) in DA neurons did not seem to significantly affect survival of flies treated with 1 mM rotenone (Figure 6A). Importantly, overexpressing Vps35 alone offered a mild but significant protection against rotenone, compared to control flies (Figure 6A). To our surprise however, co-overexpressing Vps35 and LRRK2 in DA neurons resulted in a substantial synergistic protective effect against rotenone, demonstrated as a significantly better survival of these flies compared to all other groups (P < 0.0001; F (74, 190) = 3.45) (Figure 6A).

Next, we assessed the climbing ability of the rotenone-treated flies. Rotenone-treated LRRK2(I2020T) flies had somewhat unexpectedly a slightly higher locomotor activity compared to control flies (Figure 6B). Again, Vps35 overexpression alone significantly improved locomotor activity of the rotenone-treated flies (Figure 6B) and this effect was sustained even in the presence of LRRK2(I2020T) (P = 0.0011; F (30, 271) = 2.10) (Figure 6B).

Because Vps35 overexpression protected from rotenone, our next goal was to test whether silencing Vps35 would sensitize the flies to rotenone toxicity. This would suggest
that endogenous Vps35 is involved in the cellular protection against rotenone. However, our results show that knocking-down Vps35 had no statistically significant effect on climbing or survival of rotenone-treated flies, compared to control (Figure 6A and C).

Altogether, these data point to a strong functional interaction between LRRK2 and Vps35 in DA neurons which may be especially important under conditions of cellular stress. Furthermore, our data for the first time suggest that rotenone interferes with the endolysosomal pathway, although the exact mechanism is not clear.

In summary, we present evidence that overexpression of Drosophila Vps35, a core component of the retromer complex, rescues the eye phenotype, locomotor deficits and shortened lifespan of the LRRK2(12020T) expressing flies. Similar to Vps35, overexpressing Vps26 rescued the eye and locomotor phenotypes, thus validating our findings. Moreover, we confirmed that overexpression of Vps35 also rescues the locomotor phenotypes of two other LRRK2 mutants. Furthermore, we demonstrate that knocking down Vps35 leads to a similar degree of locomotor impairment and eye damage as the mutant LRRK2, but that the phenotypes of the two genes are not additive.

Finally, we show that while silencing Vps35 has no significant effect on locomotor activity or survival of rotenone-treated flies, overexpressing Vps35 alone protects from cellular stress caused by rotenone, as demonstrated by prolonging lifespan and improving locomotor deficits, and that this protection by Vps35 is sustained and even augmented in the presence of mutant LRRK2.

Discussion
Several previous studies have indicated that LRRK2 plays a role in the endolysosomal trafficking [30,31], protein sorting and transport [32] or trafficking of synaptic vesicles [31,33]. For example, overexpression of LRRK2 is associated with enlarged lysosomes, vacuolization and/or large cytoplasmic punctate structures [30,34,35], suggesting a problem with vesicular trafficking. Here we present evidence that LRRK2 and Vps35 functionally interact, and demonstrate how this interaction in DA neurons affects locomotor activity, lifespan and response to rotenone.

LRRK2 is the most common cause of the monogenic form of PD, and a common risk factor for PD. To further highlight the relevance of our data to PD, the human homologue of Vps35, VPS35, has recently been identified.
as the latest confirmed causative gene of the typical late onset PD, with c.1858 > A (p.Asp620Asn) being the most common VPS35 mutation [36,37]. This finding has been replicated by several independent analyses [38-42]. However, the mechanism by which VPS35 is involved in PD pathogenesis is entirely unknown.

Our data indicate that LRRK2 and components of the cargo-recognition subunits of the retromer complex Vps35 are part of the same molecular pathway, with mutant LRRK2 likely being upstream and negatively regulating retromer. Furthermore, our gene knock-down data suggest that the mechanism by which the PD pathogenic mutant VPS35 is involved in PD pathogenesis may be a dominant negative mechanism.

VPS35 is a core component of the evolutionarily conserved retromer complex that is predominantly expressed on dynamic endosomal membranes [43-45], to regulate sorting, packaging and directing transport of specific proteins to the trans-Golgi network or cell surface. Thus, the retromer complex prevents specific proteins from being degraded in the lysosome [46]. Retromer consists of two subcomplexes: a cargo recognition subcomplex composed of VPS35, VPS26 and VPS29 [47], and a membrane-interacting subcomplex composed of sorting nexins (SNXs) that bind to a PI3-P-rich endosomal membrane [48]. By regulating protein sorting, retromer is involved in many diverse cellular processes, including but not limited to trafficking of SNARE proteins and receptors such as β-adrenergic [49] or cation-independent mannose 6-phosphate receptors [50], or regulating homeostasis of intracellular glucose, copper and iron.

Although its physiological function in neuronal cells is not yet fully elucidated, it is clear that the retromer-dependent pathway plays a role in etiology or pathophysiology of a number of neurodegenerative processes. For example, genetic variations of SorLa [51] or sorCS1 [52], encoding for receptors that are cargoes for the retromer-dependent pathway, are associated with Alzheimer’s disease, expression of VPS35, VPS26, sortilin and SorLa is altered in Alzheimer’s disease patients [53,54] and interfering with expression of VPS26 or VPS35 [55] causes accumulation of amyloid β and APP derivatives in exosomal compartments [56]. Further research into this pathway may therefore offer important clues and insights into the pathogenesis of PD and other neurodegenerative disorders.

MacLeod et al. recently published a paper that demonstrates an interaction between Vps35 and another LRRK2 mutant, LRRK2(G2019S), where Vps35 protected against neuronal death caused by LRRK2(G2019S) [57]. Our data presented here independently validate these findings,
and extend the relevance of these findings to two other pathogenic LRRK2 mutants, LRRK2(2020T) and LRRK2 (Y1699C), and one putative pathogenic mutant LRRK2 (I1122V). More importantly however, we for the first time characterize the functional role of this interaction in regulation of the locomotor activity, lifespan, sensitivity to rotenone and in retinal degeneration. Furthermore, our data provide evidence that overexpressing Vps35 alone protects from rotenone, while knocking down Vps35 has no significant effect on rotenone toxicity. This is consistent with recently published data showing that overexpression of Vps35 protected in vitro against MPP+, another neurotoxin commonly used to model PD, but Vps35 knockdown had no significant effect on cell viability under the same conditions [58].

Conclusions
In summary, these data provide further evidence in support of the hypothesis that LRRK2 plays a role in the endolysosomal pathway and that the pathology caused by mutant LRRK2 may be at least partly linked to a disruption of this important protein sorting and recycling cellular process. However, how exactly this pathway contributes to PD pathology is at present entirely unknown. Elucidation of new molecular pathways involved in the pathogenesis of PD may bring forward novel pharmacological targets for better treatment strategies. Therefore, a better understanding of the retromer pathway and its relation to PD pathogenesis deserves further investigations.

Materials and methods
Drosophila genetics
UAS-LRRK2(2020T), UAS-LRRK2(Y1699C) and UAS-LRRK2(I1122V) lines were characterized previously [13]. P(Ep12) Vps35EY1420/CyO and P(EPR) Vps26G2008 w+/FM7h flies were obtained from the Bloomington Drosophila Stock Center (BDSC, Indiana University). These lines contain an empty UAS element upstream of endogenous Drosophila Vps35 or Vps26 gene, respectively, allowing for a Gal4-dependent cell/tissue specific gene overexpression [59,60]. Ddc-Gal4 and GMR-Gal4 lines were both obtained from BDSC. For the gene knockdown studies, we used the following lines from BDSC: y1 sc* v1; P(TRIP.HMS01858) attP40 (expresses dsRNA for RNAi of Vps35 under UAS control); y1 sc* v1; P(TRIP.HMS01877) attP40 (expresses dsRNA for RNAi of CG4764 (FBgn0031310) under UAS control), and y1 v1; P(TRIP.HMS01769) attP40 (expresses dsRNA for RNAi of Vps26 under UAS control).

Eye phenotype
To assess the eye phenotype, we used GMR-Gal4 to drive the expression of the transgenes in the eye. All crosses and F1 generations were reared at 29°C. At 10 days of age, their eyes were analyzed under a stereomicroscope (Zeiss).

Negative geotaxis assay and survival assay
The transgenes were overexpressed in DA neurons under a Ddc promoter. Progeny of the appropriate genotype were divided into cohorts of ten, and the flies were subjected to a negative geotaxis climbing assay at 5, 10 and 20 days post-eclosion. We recorded and counted flies that crossed a line 8 cm above the base of a transparent tube within 5, 10 and 30 seconds after being gently tapped down. All behavioral experiments were performed at room temperature under standard light conditions. To ensure comparable conditions in each vial, we placed flies in vials with new food every 3-4 days. The same cohorts of flies used in the climbing assay were daily analyzed for survival.

Rotenone treatment
Cohorts of ten flies (five males and five females) were placed in vials containing freshly prepared rehydrated lyophilized food (Carolina Biological Supplies) containing rotenone (1 mM; Enzo, Farmingdale, NY). Flies were reared at room temperature, their survival and locomotor activity assessed daily, as described above. Every third day, the flies were transferred into a new vial with freshly prepared rotenone-containing food. Because rotenone is light- and temperature-sensitive, the flies were reared at room temperature and in the dark.

Statistical analyses
All data were analyzed by One-Way ANOVA with a Tukey’s post-hoc test, or by Two-way ANOVA followed by a Bonferoni’s post-hoc test, as indicated.

Abbreviations
PD: Parkinson’s disease; LRRK2: Leucine-rich repeat kinase 2; DA: Dopaminergic; Vps35: Vacuolar protein sorting 35; Vps26: Vacuolar protein sorting 26; Vps29: Vacuolar protein sorting 29; BDSC: Bloomington Drosophila Stock Center; Ddc: Dopa-decarboxylase.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
SAW, JC, MT, AH, CA, JMP, CH, ST, SS, RP and JK participated in carrying out the experiments, RL participated in the design of the experiments, in carrying out the experiments, data analysis and preparation of the manuscript, KV conceived, designed and coordinated the study, participated in carrying out the experiments, analyzed the data and drafted the manuscript. All authors read and approved the final manuscript.
Identification of a novel conserved sorting motif required for endosome-to-Golgi transport pathways in Parkinson disease.

Linhart H, Kreber R, Honig LS, Clark LN, Small SA, Abeliovich A: RAB7L1 interacts with LRRC2 to modify intraneuronal protein sorting and Parkinson's disease risk. Neurobiol Dis 2013, 49:262–276.

Bellen HJ, Lewis RW, Lee E, Ye Y, Carlson JW, Tsang O, Evans-Holm M, Hiesinger PR, Scherer KL, Rubin GM, Hoskins RA, Spradling AC: The BDGP gene disruption project: single transposon insertions associated with 40% of Drosophila genes. Genetics 2004, 167:761–781.

Submit your next manuscript to BioMed Central and take full advantage of:

• Convenient online submission
• Thorough peer review
• No space constraints or color figure charges
• Immediate publication on acceptance
• Inclusion in PubMed, CAS, Scopus and Google Scholar
• Research which is freely available for redistribution

Cite this article as: Linhart et al.: Vacular protein sorting 35 (Vps35) rescues locomotor deficits and shortened lifespan in Drosophila expressing a Parkinson's disease mutant of Leucine-rich repeat kinase 2 (LRRK2). Molecular Neurodegeneration 2014 9:23.

doi:10.1186/1750-1326-9-23