Parkinson disease-associated Leucine-rich repeat kinase regulates UNC-104-dependent axonal transport of Arl8-positive vesicles in Drosophila

Highlights

- LRRK mutations lead to Arf-like GTPase Arl8 accumulation at presynapses in Drosophila
- Arl8 accumulation is associated with dense core vesicles and UNC-104 dependent
- PD-related genes VPS35, RME-8, Auxilin, and INPP5F are involved in Arl8 dynamics

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Article

Parkinson disease-associated Leucine-rich repeat kinase regulates UNC-104-dependent axonal transport of Arl8-positive vesicles in Drosophila

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SUMMARY

Some Parkinson’s disease (PD)-causative/risk genes, including the PD-associated kinase leucine-rich repeat kinase 2 (LRRK2), are involved in membrane dynamics. Although LRRK2 and other PD-associated genes are believed to regulate synaptic functions, axonal transport, and endolysosomal activity, it remains unclear whether a common pathological pathway exists. Here, we report that the loss of Lrrk, an ortholog of human LRRK2, leads to the accumulation of the lysosome-related organelle regulator, Arl8 along with dense core vesicles at the most distal boutons of the neuron terminals in Drosophila. Moreover, the inactivation of a small GTPase Rab3 and altered Auxilin activity phenocopied Arl8 accumulation. The accumulation of Arl8-positive vesicles is UNC-104-dependent and modulated by PD-associated genes, Auxilin, VPS35, RME-8, and INPP5F, indicating that VPS35, RME-8, and INPP5F are upstream regulators of Lrrk. These results indicate that certain PD-related genes, along with LRRK2, drive precise neuroaxonal transport of dense core vesicles.

INTRODUCTION

Parkinson disease (PD) is a neurodegenerative disorder characterized primarily by movement disturbance. Although the etiology of PD is still unknown, 5-10% of PD is a monogenic form with Mendelian inheritance (Deng 2018; Inoshita 2018). To date, more than 20 genes have been identified, some of which are involved in membrane trafficking (Inoshita 2018). For example, one of the retromer components VPS35 [also known as PARK17 in online mendelian inheritance in man (OMIM) (https://www.omim.org)] and a J-domain containing the protein RME-8 (PARK21) work with the WASH complex to regulate vesicle budding1–3; Auxilin (Aux, PARK19) regulates clathrin-mediated endocytosis from the plasma membrane4 and budding from trans-Golgi5–7; Synaptojanin1 (PARK20), which is a phosphoinositide 4,5-phosphatase, is involved in synaptic vesicle (SV) endocytosis.8 A phosphoinositide 4-phosphatase INPP5F, which has been identified as a PD risk gene in a genome-wide association study,9 functions as an effector of Rab5 to control endocytosis10,11 and phagocytosis,12 and regulates docking and release of insulin granules together with Rab3.13 The Rab29/Rab7L1 gene is located at the PARK16 locus14 and is a possible PD causative gene.15 A study showed that Rab29-deficient mice developed defects in kidney proximal tubule cells, similar to LRRK2 (PARK8)-deficient mice.16 Moreover, overexpression of Rab29 results in the activation of LRRK2 kinase.17,18 However, another study reported no alteration in LRRK2 activity in Rab29-deficient mice.19 Thus, it is controversial whether Rab29 is a regulatory molecule of LRRK2.

Missense mutations of LRRK2 have been identified in late-onset hereditary PD20,21 and sporadic PD worldwide.22 Thus, LRRK2 is considered one of the most important genes in PD research. Increased LRRK2 kinase activity is observed in many pathogenic mutations.23–25 LRRK2 phosphorylates several Rab GTPases, including Rab3, Rab8, Rab10, and Rab35.26 Rab GTPases phosphorylated by LRRK2 are activated by the dissociation of Rab GDP dissociation inhibitors.23

Studies in neurons and glial cells have shown various functions of LRRK2, including microtubule modification,27–30 primary cilia formation,26,31,32 membrane elongation,33 and autophagosome transport34 via the microtubule-dependent motor adapter JIP4. On the other hand, among the PD causative/risk genes involved in membrane transport, VPS35,35,36 Auxilin (Aux),37 Synaptojanin1,38,39 and Rab2917,18 have been
reported as molecules involved in LRRK2 signaling. However, these studies have only analyzed the molecular relationship between the two and do not provide a panoramic view of LRRK2 signaling.

Previous *C. elegans* studies have reported that the loss of *lrk-1*, an LRRK2 ortholog, leads to missorting of neuronal axonal trafficking, and the sorting of synaptic molecules from trans-Golgi by LRK-1 is mediated by clathrin, AP3, and Unc-16/SYD (ortholog of JIP 3 and JIP4). Another genetic study using *C. elegans* observed that LRK-1 regulates proper extension of the axonal terminals and that GLO-1, an ortholog of Rab29 in *C. elegans*, and AP3 are located upstream and downstream of *lrk-1*, respectively.

*Drosophila* has a single LRRK2 ortholog, Lrk, and age-dependent neurodegeneration is observed upon the introduction of pathogenic mutations. On the other hand, loss of Lrk is associated with abnormal presynaptic activity. The above-mentioned studies in *C. elegans* and *Drosophila* suggest that LRRK2 is involved in the transport of molecules required for presynaptic activity.

A current study reports that Lrk mutations in *Drosophila* result in the accumulation of Arf-like GTPase Arl8, which is involved in the transport of lysosome-related organelles, and dense core vesicles (DCVs) in distal synaptic boutons. Based on this phenotype, we conducted a genetic interaction analysis between Lrk and known PD causative/risk genes that are involved in membrane dynamics, providing a landscape of LRRK2 signaling. Our study suggests that Aux, RME-8, VPS35, and INPP5F are involved in LRRK2 signaling and contribute to the precise transport of proteins associated with DCVs to the presynapses.

**RESULTS**

**Mutations in leucine-rich repeat kinase lead to Arl8 accumulation at synaptic terminals**

We have previously reported that Lrk and VPS35 cooperate in the endocytosis of presynaptic SVs. Both Lrk and VPS35 mutants exhibit cisternal structures at presynapses, suggesting abnormal membrane dynamics. To further characterize these presynaptic phenotypes, we systematically stained the presynaptic terminal boutons of motor neurons in *Lrk*+/− and *Lrk*−/− (*Figure 1A*). Arl8 is involved in the co-transport of SV and active zone proteins in presynaptic lysosome-related vesicles. Arl8-positive presynaptic structures were partially positive for the lysosome-related proteins LAMP1 and Spinster (*Figure 1B*). Overexpression of Lrk Y1383C, which corresponds to the human LRRK2 pathogenic mutant Y1699C, also produced Arl8 aggregates at the terminal boutons, while wild-type (WT) Lrk overexpression did not (left in *Figure 1C*). Lrk Y1383C, but not WT, also formed puncta with Arl8 both in the terminal boutons and the cell bodies of motor neurons (*Figure 1C*). These puncta contained Rab7 and partly lysosomes (*Figures S1A and S1B*). On the other hand, overexpression of kinase-dead Lrk (3KD), like WT, showed no Arl8 accumulation, while I1915T (corresponding to the human LRRK2 I2020T) tended to accumulate it (*Figure 1D*). There was no presynaptic accumulation of other organelle makers, which include Rabenosyn-5 (early endosomes), Calnexin 99A (ER), Hrs (multivesicular body), Galactosyltransferase (trans-Golgi), and an autophagy marker, Ref(2)P (*Figure S1C*).

**Rab3 inactivation promotes Arl8 accumulation**

Rab3, Rab8, Rab10, and Rab35 are known to be substrates of mammalian LRRK2. The amino acid sequences around the phosphorylation residue (T73) of human Rab10 by mammalian LRRK2 are highly conserved among *Drosophila* Rab3, Rab8, and Rab10 (*Figure 2A*). Lrk overexpression also phosphorylated *Drosophila* Rab3, Rab8, and Rab10, although the efficiency of Rab3 phosphorylation was weaker than the other two (*Figure 2B*). Arl8 positive structures are well co-localized with Rab3 and weakly co-localized with Rab10 and Rab35 (*Figure 2C*). On the other hand, the signals of Rab8 and Rab32 (human Rab29 ortholog) in the Arl8-positive structures were comparable to the background signals (*Figure 2C*). Overexpression of Rab3 GDP-GTP exchange factor (Rab3-GEF), which activates Rab3 GTPase, suppressed Arl8 accumulation in *Lrk*−/−, while knockdown of Rab3-GEF resulted in Arl8 accumulation (*Figure 2D*). GTPase inactive Rab3 T35N, a non-phosphorylated form of Rab3 T85A, and Rab32 WT also caused Arl8 accumulation (*Figure 2E*). However, Rab32 Q79L failed to increase Rab3 phosphorylation, which does not support the idea that Rab32 is an upstream regulator of Lrk (*Figure S2A*). Unlike Rab3, overexpression of Rab8, Rab10, and Rab35 did not affect Arl8 accumulation (*Figure 2E*). Manipulation of Rab10 activity also did not affect the Arl8 phenotype (*Figure S2B*). These observations indicate that the inactivation of Rab3 GTPase, which is likely to be caused by loss of Lrk, leads to Arl8 accumulation. Interestingly, the ectopic expression of Rab3 T35N and T85A was enriched in the distal axons (*Figure S2C*), while Rab3 Q80L and T85A were enriched in the
proximal axons (arrows in Figure S2C). Rab3 Q80L and T35N were also enriched in cell bodies (CB in Figure S2C), suggesting that the GTPase activity of Rab3 regulates the neuronal distribution of Rab3.

The reduction in Arl8 GTPase activity itself also affected the presynaptic accumulation of Arl8 because the knockdown of Arl8-GEF (BORCS5) suppressed its accumulation (Figure 2F). Overexpression of Arl8 WT promoted Arl8 accumulation without affecting bouton size, while overexpression of both Arl8 Q75L and T34N attenuated Arl8 accumulation (Figure 2G). Arl8 signals were distributed over the entire length of the axons in Arl8 Q75L-expressing flies and were enriched in the proximal axons of Arl8 T34N-expressing flies (Figure S2D). These results suggest that the GTPase activation cycle of Arl8 is required for its terminal accumulation.

**Transport turnover is impaired by leucine-rich repeat kinase loss**

To clarify the type of organelle that exists in the synaptic boutons where Arl8 accumulates, we observed the ultrastructure of Arl8-positive boutons by correlative light and electron microscopy (Figures 3A-3F). In Arl8-positive boutons, many vesicular structures with high electron density were accumulated (Figures 3E and 3F). A detailed analysis with conventional electron microscopy revealed that Lrrk$^{+/+}$-terminal boutons contained densely packed DCV-like structures (Figures 3G-3J). Moreover, Arl8 co-localized with PreproANF, a DCV marker, suggesting that Arl8 is involved in the axonal transport of DCVs (Figures 3K-3N). Chloroquine
Figure 2. Altered Rab3 activity causes Arl8 accumulation
(A) Sequence alignment of human Rab10 (hRab10) and Drosophila Rab (dRab) proteins. Dot indicates hRab10 T72 phosphorylated by human LRRK2 and the corresponding residues in dRab proteins.
(B) Phosphorylation of Rab3, Rab8, and Rab10 by Lrk (I1915T (Lrk IT) or human LRRK2 I2020T (hLRRK2 IT)). Phosphorylation of Rab proteins was analyzed with (+) or without (−) Lambda protein phosphatase (APP) treatment by phos-tag or anti-phospho-Rab. Dots indicate Lrk-dependent phosphorylation.
(C) Rab3 is condensed in Arl8 aggregates caused by Lrk loss. Distribution of Rab proteins in Arl8 aggregate-positive synaptic boutons was analyzed. Arrowheads indicate Arl8 accumulations. Graph represents the ratio of the intensity of Rab signals in the Arl8 aggregate-positive boutons to their averaged intensity in three neighboring boutons in Lrk+/−. Scale bar, 10 μm. # p < 0.05 vs. Rab8 by Dunnett’s test.
(D) Reduced Rab3-GEF activity causes Arl8 accumulation, while Rab3-GFP overexpression suppresses it. Graph represents mean ± SEM (n = 16–33 NMJs in 4–9 flies). #p < 0.05 vs. Lrk+/−; ip < 0.05 vs. Lrk+/− by Dunnett’s test.
(E) Reduced Rab3 GTPase activity causes Arl8 accumulation. Graph represents mean ± SEM (n = 18–45 NMJs in 5–12 flies). # p < 0.05 vs. Lrk+/−; #p < 0.05 vs. Lrk+/− by Dunnett’s test.
(F) Reduced Arl8 GTPase activity due to Ar8-GEF knockdown suppresses presynaptic Arl8 accumulation. Graph represents mean ± SEM (n = 19–26 NMJs in 6–7 flies) on an elav-GAL4 genetic background). #p < 0.05 vs. Lrk+/− by Dunnett’s test.
(G) Ectopic expression of Arl8 WT, but not Arl8 Q75L or T34N, promotes presynaptic Arl8 accumulation. Left and right graphs indicate Arl8 intensity in each bouton and each bouton size, respectively. The most distal, second, third, and fourth boutons of the terminal are labeled in Figure S2D. Graph represents mean ± SEM (n = 21–26 NMJs in 6–7 flies on an elav-GAL4 genetic background). #p < 0.05 vs. Arl8 WT by Dunnett’s test. See also Figure S2.

Treatment, which causes de-acidification of acidic organelles such as DCVs and lysosomes,48 induced Arl8 accumulation and increased the number of DCVs at Lrk+/− presynaptic boutons, again suggesting that Arl8 is involved in DCV function (Figure S3).

Trafficking of Arl8-positive puncta demonstrated that the vesicle number in anterograde transport increased in Lrk+/−, while the vesicle number in retrograde transport was unchanged (Figures 4A and 4B). The velocity of transport in Lrk+/− was faster in both directions (Figures 4B and 4C). The size of stagnant puncta increased while their number remained unchanged (Figure 4B). These observations suggest that the accumulation of Arl8 by Lrk loss is mainly due to the failure of the Arl8-positive DCVs to turn over at the presynaptic terminals.

The anterograde motor Unc-104 and SYD/JIP4 are involved in Arl8 dynamics
In C. elegans, the anterograde motor Unc-104/KIF1A is involved in the microtubule-dependent transport of Arl8-positive vesicles.46 Removal of a copy of the Unc-104/KIF1A gene, but not Khc/KIF5, suppressed the Arl8 accumulation in Lrk+/− (Figure 5A), while overexpression of Unc-104 in the Lrk+/− genetic background resulted in Arl8 accumulation (Figure 5B). On the other hand, the removal of one copy of the retrograde motor Glued/Dynactin had no effect on the Arl8 phenotype in either Lrk+/− or Lrk+/− (Figure 5A). A mammalian ortholog of Drosophila CLIP-190, CLIP170, is involved in the initiation of Dynein-dependent retrograde transport at the microtubule plus-end.46 Unc-104 and a microtubule-plus-end-tracking protein (+TIP), CLIP-190, were also accumulated at the Arl8 accumulation site, while other +TIPS EB1 and Glued were not (Figure 5C). Knockdown or removal of one copy of SYD/JIP4, which reportedly regulates the sorting of SV proteins along with Lrk-1 at the trans-Golgi network (TGN) in C. elegans, recapitulated the Arl8 accumulation in Lrk+/− (Figures 5D and 5E). In combination of SYD/JIP4 RNAi with Lrk+/−, there were no additive effects on Arl8 accumulation (Figure 5D). In contrast, inhibition of Aip1p/J1P1 in Lrk+/− rescued Arl8 accumulation (Figure 5D). Knockdown of the DCV-tethering factor Liprin-α did not affect Arl8 accumulation (Figure 5D).50 These results indicate that the dysregulation of Unc-104 and SYD is involved in Arl8-positive DCV accumulation.

Presynaptic phenotypes by loss of Parkinson’s disease-causative/risk genes that regulate membrane dynamics
Loss of LRRK2 or VPS35 causes defects in endocytosis and SV recycling at synapses, resulting in abnormal presynaptic activity.35 To determine whether this is a common phenotype for PD-associated genes that are believed to be involved in vesicle trafficking,51 we performed electrophysiological analysis and ultrastructural observation of synaptic boutons using flies harboring LOF alleles for Rab32, Lrk, VPS35, Aux, INPP5F, Synaptojanin (Synj), and RME-8 (Figure 6). Among them, we employed heterozygous RME-8 LOF flies because homozygous LOF flies were lethal at a very early developmental stage. The amplitude (Figure 6A) of miniature excitatory junction potentials (mEJPs) showing spontaneous firing was increased in most mutant flies except Lrk+/− and Rab32+/−, compared to normal control. The frequency of mEJP was also increased in all mutant flies except Rab32+/− (Figure 6B).

Ultrastructural observations of synaptic boutons revealed that the DCV density was increased in Lrk+/−, Aux+/−, INPP5F−/−, and VPS35−/−, while the SV number around the active zones was reduced and increased...
Figure 3. Loss of Lrrk leads to presynaptic DCV accumulation along with Arl8

(A–F) Arl8 aggregation-positive boutons exhibit an abundance of DCVs. Fluorescence images of Arl8 aggregation (green)-positive boutons (red). (D–F) The same boutons in A–C were analyzed by correlative light and electron microscopy (CLEM). A and D contain the most distal and second most distal boutons (corresponding to panels E and F). Scale bars, 20 μm (A–C), 1 μm (D); 200 nm (E, F). Arrowheads in A and B indicate the Arl8 enriched region of the second most distal bouton, which also contains DCV accumulation (F).
in Syn"/− and Aux"/−, respectively (Figures 6C and S4). Large vacuoles were frequently observed in Aux"/−, INPP5F"/−, VPS35"/−, and Syn"/−, and intraluminal vesicles, typical of multivesicular body (MVB), were frequently observed in VPS35"/− (Figures 6C and S4). These results imply that mutations in PD-associated genes other than Rab32 impair synaptic bouton function and that increased DCV density is a common phenotype in most of the PD-associated genes we analyzed.

Parkinson’s disease-causative/risk genes, VPS35, RME-8, INPP5F, and auxilin modulate the Arl8 phenotype

Next, we searched for PD-associated genes that co-localize with Arl8 accumulation among the above genes. Synaptic endocytosis-related molecules, VPS35, Synj, and Endophilin A, which are PD-associated proteins, were partly or not concentratedly localized with Arl8 accumulation (Figure 7A). In contrast, PD-associated RME-8 and INPP5F accumulated in Arl8 aggregates, in which phosphatidylinositol 4-phosphate [PI(4)P] was also enriched (Figures 7A, 7B, and S5A–S5D). On the other hand, PI(4,5)P2 and PI(3)P were not enriched in Arl8 aggregates (Figure 7B). Overexpressed Aux also accumulated in Arl8 aggregates, while endogenous Aux was partly co-localized (Figures 7A and S5A–S5G). Furthermore, clathrin and the component of the AP3 complex at the TGN, AP3β, co-localized with Arl8, suggesting that molecules involved in the clathrin-mediated vesicle formation from the TGN were also ectopically accumulated (Figures 7C, S5A–S5G). In contrast, both the overexpression and the removal of AP3α suppressed Arl8 accumulation in Lrrk"/− (Figure 7D). To further elucidate the genetic involvement of the above PD-associated genes, we examined whether Arl8 accumulation could be reproduced by the LOF alleles of each gene. The introduction of a single LOF allele of INPP5F and VPS35 caused Arl8 accumulation similar to that caused by Lrrk mutations (Figure 7E). When we combined Lrrk"/− with the LOF allele of each gene, Arl8 accumulation was aggravated by the combination of the Aux LOF allele and tended to be enhanced by the INPP5F LOF allele (Figure 7E). On the other hand, the overexpression of INPP5F, RME-8, and VPS35 reduced the Arl8 accumulation in Lrrk"/− flies (Figure 7F). However, homozygous loss of Lrrk abolished these rescue effects (Figure 7G). These results suggest that INPP5F, RME-8, and VPS35 are genetically upstream of Arl8. Overexpression of Aux resulted in Arl8 accumulation regardless of Lrrk activity (Figure 7H). The results of Aux and AP3α may suggest that Aux and AP3 are downstream of Lrrk. The results of these genetic tests are summarized in Figure S6.

α-Synuclein is incorporated into Arl8 aggregates

The consequence of Arl8 accumulation at the presynapses was first estimated by a behavioral assay. Overexpression of Arl8 in dopaminergic neurons of adult flies impaired motor ability, suggesting that the constitutive accumulation of Arl8 at the presynapses is neurotoxic (Figure 8A). The pathology of PD linked to LRRK2 mutations is variable, with the accumulation of α-Synuclein, tau, or Aß. This pleiomorphic pathology suggests that mutations in LRRK2 result in an aggregation-prone brain environment rather than direct involvement in the aggregation of these molecules. The aggregation and propagation of α-Synuclein and Tau is an important aspect of aging-dependent neurodegenerative etiology, including that of PD where it is unclear how aggregation forms and propagates through neural circuits. Since the accumulation of Arl8 is observed not only in flies expressing PD-related Lrrk mutants but also in Lrrk knockout flies, we used Lrrk"/− flies to test whether Arl8 accumulations could be a location of α-Synuclein and Tau aggregation. α-Synuclein and Tau expressed ectopically in larval motor neurons were not enriched in the Arl8 accumulation of Lrrk"/− flies (Figure 8B). However, α-Synuclein incorporated from the synaptic cleft by synaptic activity-dependent uptake was accumulated in the Arl8-positive structures (Figure 8C). In contrast, Tau incorporated under the same conditions did not accumulate (Figure 8D). Next, we examined whether Arl8 is present in Lewy bodies, the neuronal inclusions of α-Synuclein in humans. Lewy bodies-positive dopaminergic neurons accounted for 14.97 ± 2.90% of total midbrain dopaminergic neurons.
In patients with PD, we analyzed (Figure S7 and Table S1). Dopaminergic neurons with Arl8-positive Lewy bodies, in which Arl8-immunosignals were positive in their marginal part, constituted 40.87 ± 10.42% and no Arl8A-positive Lewy bodies were detected (Figure S7 and Table S1).

DISCUSSION
Neurophysiological and morphological analyses of our and other studies have suggested that Lrrk is involved in presynaptic functions in Drosophila. The present study reports that the dysregulation of Lrrk activity resulted in presynaptic Arl8 accumulation, possibly due to DCV accumulation. Further, Rab3 co-localized with Arl8 accumulation. Moreover, the expression of a dominant-negative Rab3 mutant and a putative phospho-resistant Rab3 mutant by Lrrk in neurons recapitulated Arl8 accumulation, implying that defects in DCV exocytosis contribute to Arl8 accumulation. Arl8 was transported to the axonal terminals via Unc-104-dependent anterograde transport. The presynaptic Arl8 accumulation may involve a switching defect in the retrograde transport at the microtubule plus-end. This is because Lrrk loss increased the number of Arl8-positive vesicles transported in the anterograde direction, but not in the retrograde direction (Figure 4B). The enriched co-localization of CLIP-190 with Arl8 at the microtubule plus-ends also indicates this notion (Figure 5C). In mammalian neurons, PD-associated LRRK2 G2019S has been implicated in facilitating anterograde transport of autophagic vesicles that should be retrograded by dynein.34

Figure 4. Lrrk regulates axonal transport of Arl8-positive vesicles
(A) Representative kymographs of Arl8 axonal transport. Scale bar, 10 μm.
(B) Velocity of Arl8 axonal transport, number of Arl8 signals, and size and number of stagnant Arl8 vesicles. Graphs represent mean ± SEM (n = 10 axons in 4 larvae). *p < 0.05, two-tailed t-test.
(C) Distribution of the velocity of Arl8-positive vesicles in the presence or absence of Lrrk.
Figure 5. Arl8 transport is regulated by Unc-104 along with Lrrk

(A) Reduction in Unc-104 rescues Arl8 accumulation through Lrrk loss. Arl8 accumulation ratio (as percentage) of the indicated genotypes on the w1118 genetic background are graphed (mean ± SEM, n = 12–26 NMJs in 4–10 flies). #p < 0.05 vs. Lrrk+/+; #p < 0.05 vs. Lrrk+/OE by Dunnett’s test.

(B) Arl8 accumulated terminals (%) of the indicated genotypes on the w1118 genetic background are graphed (mean ± SEM, n = 12–26 NMJs in 4–10 flies).

(C) Immunohistochemistry images showing OL, Unc-104-GFP, Glued, Arl8, CLIP-190, Arl8, OL, EB1, Tubulin, and CSP localization under different conditions.

(D) Arl8 accumulated terminals (%) of the indicated genotypes on the w1118 genetic background are graphed (mean ± SEM, n = 12–26 NMJs in 4–10 flies).

(E) Arl8 accumulated terminals (%) of the indicated genotypes on the w1118 genetic background are graphed (mean ± SEM, n = 12–26 NMJs in 4–10 flies).
The enhanced anterograde transport by LRRK2 G2019S involves the recruitment of JIP4 into autophagic vesicles via increased phosphorylation of Rab10 by LRRK2 G2019S and activation of kinesin. In contrast, a LRRK2 kinase inhibitor inhibits the anterograde transport of autophagic vesicles. Hence, the molecular mechanism observed in this context does not appear to be directly associated with the mechanism of Arl8 accumulation by Lrrk loss or the PD-associated Lrrk Y1383C mutant. Phosphorylation of CLIP170 by LRRK2 enhances the binding of CLIP170 to the Dynein motor. Because Drosophila Lrrk is also an ortholog of LRRK1, the recruitment activity of the Dynein motor to the plus-end of microtubules may be reduced in Lrrk Y1383C. However, the observation that Arl8 also accumulates in the Lrrk Y1383C mutant with enhanced kinase activity suggests the existence of other mechanisms.

Arl8 is a marker for lysosomes in the cell body and a marker for presynaptic lysosome-related vesicles (PLVs) in the presynapses. Both lysosomes and PLVs share the common feature of being acidic organelles. Arl8 is believed to be involved in their transport. Further, PLVs may contain DCVs; several lines of evidence support this idea: First, CLEM revealed an increased distribution density of DCVs, but not SVs, in synaptic boutons containing Arl8 accumulation. Second, DCV aggregation is observed in the lrk-1 mutant in C. elegans; an increase in the size of Arl8-positive stagnant vesicles is also observed in this study. Third, Rab3 has been reported to be involved in DCV exocytosis, but not SV. Fourth, the AP-3 complex contributes to the formation and maturation of DCV at the TGN. The above reports indicate that Lnrk and AP-3 dysfunction caused the misorting of DCV proteins such as synaptotagmin, leading to the generation of incompetent DCVs. Incomplete DCVs could result in inadequate neuroaxonal transport or the incomplete docking of DCVs to the membrane and subsequent exocytosis, leading to the accumulation of DCVs and Arl8 in the distal boutons. The change in the velocity of Arl8 axonal transport by Lrrk loss may be due to an event such as a wrong motor adapter being loaded on the DCV or the Arl8 or its adaptors being loaded on different vesicles. These possibilities may explain our observations that are difficult to interpret, such as enhanced Arl8 accumulation in Lrrk Y1383C mutant by Khc motif in DCV transport in different regions of neurons.

The phenotype of Arl8 accumulation in the terminal boutons allowed us to assess the genetic interaction of Lrrk with known PD-related genes (Figure S6). LOF mutations of Aux and INPP5F and overexpression of Aux and Rab32 enhanced Arl8 accumulation by Lrrk Y1383C. In contrast, overexpression of RME-8, INPP5F, and VPS35 suppressed Arl8 accumulation by Lrrk Y1383C, while this effect disappeared in the Lrrk Y1383C background, suggesting that RME-8, INPP5F, and VPS35 are genetically upstream of Lrrk. The genetic interaction of VPS35 with Lrrk is supported by our previous study and others; RME-8, together with a retromer containing VPS35, is involved in microtubule-dependent tubulation of vesicles. On the other hand, LRRK2 also regulates the microtubule-dependent tubulation oflysosomes via JIP4. These observations suggest that RME-8 along with LRRK2 may regulate the microtubule-dependent membrane dynamics of DCVs. INPP5F is a PI4P phosphatase and is suggested to function with activated LRRK2 in ruptured lysosomes. INPP5F is also involved in insulin granule exocytosis, and recruitment of INPP5F to the insulin granules requires active Rab3 and PI4P. Arl8-accumulated vesicles were PI4P- and Rab3-positive and INPP5F was also co-localized with Arl8-accumulated vesicles, indicating that Lrrk may be involved in the dephosphorylation of PI4P by INPP5F through Rab3 activation (Figures 2C, 7A and 7B).

Similar to Lrrk, both overexpression and LOF mutations of Aux enhanced Arl8 accumulation. Aux may require appropriate regulation in terms of clathrin uncoating activity. Clathrin and AP3b also co-localize in the core of Arl8 aggregates, suggesting the missorting of clathrin-dependent budding vesicles from the TGN (Figure 7C). In a previous study, LRRK2 was reported to regulate endocytosis of clathrin-coated vesicles.
vesicles via phosphorylation of the μ2 subunit of the AP-2 complex (AP2M1). Both decreased phosphorylation of AP2M1 by LRRK2 loss and increased phosphorylation of AP2M1 by pathogenic LRRK2 G2019S impair endocytosis of clathrin-coated vesicles and affect dopaminergic neuron viability.71

Figure 6. Electrophysiological and morphological phenotypes of LOF mutants of PD-causative/risk genes
(A and B) Averaged amplitudes (A), frequency (left in B) of mEJP for 30 s, and representative mEJP traces (right in B) in larval NMJ.
(C) The number of DCVs, SVs, large vesicles (>70 nm in diameter), and MVBs in the unit area (500-nm square area containing T bar) of the NMJ. #p < 0.05 vs. normal control (w1118) by Dunnett's test. See also Figure S4.
Interestingly, LRK-1 and UNC-16/J4P4 have been asserted to be required for UNC-104-dependent axonal transport of the SV proteins Rab3 and SNB-1 in C. elegans, in which the AP-3 complex appears to have a role in SV biogenesis downstream of LRK-1 and UNC-16.16,41 In a different context in C. elegans, where ectopic extension of axonal terminals was observed due to LRK-1 loss, the AP-3 complex is also implied to be genetically downstream of LRK-1.16 This study suggests that glo-1, which is an ortholog of mammalian Rab29/Rab7, Rab32, and Rab38, is an upstream regulator of LRK-1.16 In Drosophila, the only Rab32 gene is an ortholog of mammalian Rab29 and Rab32. However, the overexpression of Rab32 Q79L failed to stimulate Rab3 phosphorylation, which argues against the possibility that Rab32 is an upstream regulator of Lrk (Figure S2A). These genetic studies at least suggest that Aux, RME-8, VPS35, and INPP5F may be involved in the LRRK2 pathway, but details at the molecular level remain to be elucidated.

Although the involvement of Arl8 accumulation in PD pathogenesis is not clear, overexpression of Arl8 in dopaminergic neurons impaired motor ability (Figure 8A). Intriguingly, α-Synuclein incorporated in a neuronal activity-dependent manner was enriched in Arl8 aggregation (Figure 8C). Moreover, Arl8b immunosignals were partially present in Lewy bodies in dopaminergic cell bodies of patients with PD, suggesting that Arl8 accumulation at presynapses could be one of the locations of α-Synuclein aggregation. Alternatively, Arl8b may have been incorporated into α-Synuclein fibrils with lysosomes and/or related organelles during the formation of inclusion bodies (Figure S7 and Table S1).72,73

Roles of LRRK2 in autophagy and lysosomes have been described in mammals.74 In Drosophila, Lrk is suggested to be involved in EndoA-mediated autophagy at the presynapses15 and in lysosomal positioning.76 The current study does not reveal whether the presynaptic accumulation of Arl8 and DCVs is associated with the alteration of the autophagy-lysosome pathway. However, since Arl8 accumulation was partially co-localized with EndoA, it may impair EndoA function (Figure 7A). In addition, since chloroquine treatment promoted the Arl8 accumulation, there may be some relationship between impaired acidification of acidic organelles, such as DCVs and lysosomes, and Arl8 accumulation (Figure S3).

In conclusion, this study reports that Drosophila Lrk mutations lead to Arl8 accumulation at presynapses, postulating that Arl8 accumulation is a consequence of DCV accumulation and dependent on UNC-104 activity. Moreover, we suggest that known PD-related genes, Aux, RME-8, VPS35, and INPP5F, may ensure the precise transport of DCV-related proteins at the presynapses in cooperation with Lrk and possibly JIP4 and AP3. Stagnation and ectopic accumulation of proteins at the presynapse may pose a risk for the aggregation of various neurodegeneration-related molecules, including α-Synuclein. Perry syndrome, in which Dystonin mutations cause protein stagnation at the presynapses, leading to neurodegeneration, is one example.77 Further analysis is needed to determine whether logistic errors in axonal transport are one of the causes of PD pathomechanism.

Limitations of the study
The molecular mechanism of Arl8 accumulation via dysregulation of Lrk kinase activity has not been determined in this study. The dysregulation of Rab3 activity and missorting of Arl8 at the TGN by Lrk mutations is one possibility, while loss of Lrk also appears to activate UNC-104-dependent anterograde transport of Arl8. Moreover, the GTPase-active form of Arl8 itself has been reported to activate UNC-104.78 This

Figure 7. Genetic manipulation of Aux, RME-8, VPS35, and INPP5F affects Arl8 accumulation
(A) Distribution of PD-causative/risk genes in Lrk+/+ or Lrk−/− NMJs. RME-8-RFP, VPS35-RFP, and Synj-GFP were expressed using elav-GAL4 and other PD-causative/risk genes were stained using specific antibodies. Somatic boutons and Arl8 in the NMJ were visualized with anti-Arl8 (red) and DyLight649-conjugated anti-HRP (blue), respectively. Arrowheads indicate Arl8 aggregates. Scale bar, 10 μm. EndoA, Endophilin A.
(B) PI(4,5)P2, but not PI(4)P or PI(3)P, is enriched in Arl8 aggregates (arrowheads). Scale bar, 10 μm.
(C) Clathrin and AP38 are co-localized in Arl8 aggregates. Line profiles suggest that clathrin and AP38 were localized in the core of Arl8 aggregates. Scale bar, 5 μm.
(D) Altered expression of AP38 affects Arl8 aggregates. Graphs represent mean ± SEM (n = 10–26 NMJs in 4–7 flies on an elav-GAL4 genetic background). p < 0.05 vs. normal control (w1118) by Dunnett’s test.
(E) Loss of Aux and INPP5F promotes Arl8 aggregation. Graphs represent mean ± SEM (n = 10–37 NMJs in 4–10 flies on a w1118 genetic background). p < 0.05 vs. normal control (w1118) by Dunnett’s test.
(F) Ectopic expression of INPP5F, RME-8, or VPS35 suppresses Arl8 aggregation in Lrk+/− (F), but not in Lrk−/− (G). Graphs represent mean ± SEM (n = 11–36 NMJs in 4–10 flies on an elav-GAL4 genetic background). p < 0.05 vs. Lrk+/−; p < 0.05 vs. Lrk−/− by Dunnett’s test.
(G) Ectopic expression of Aux itself promotes Arl8 aggregation. Graph represents mean ± SEM (n = 11–36 NMJs in 4–10 flies on an elav-GAL4 genetic background). p < 0.05 vs. Lrk−/− without Aux OE by Dunnett’s test. See also Figures S5 and S6.
seemingly complex molecular relationship needs to be clarified. Furthermore, whether the accumulation of DCVs is involved in the pathogenesis of PD has not been completely analyzed in this study. DCVs are not only vesicles in which dopaminergic neurons store dopamine but also acidic organelles that are similar in nature to lysosomes, which have recently attracted attention in research. The possible involvement of DCV dysregulation in this disease should also be investigated in the future.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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**SUPPLEMENTAL INFORMATION**

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AUTHOR CONTRIBUTIONS

Conceptualization, Y.I.; methodology, T.I. and Y.I.; investigation, T.I., J-Y.L., D.T., R.I., and K.S-F.; writing – review & editing, T.I., Y.I., and N.H.; funding acquisition, T.I., Y.I., and N.H.; supervision, Y.I. and N.H.

DECLARATION OF INTERESTS

The authors have no conflicts of interest to declare.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Anti-Drosophila Arl8, rabbit | Developmental Studies Hybridoma Bank (DSHB) | Arl8, RRID: AB_2618258 |
| Anti-Drosophila Rab7, mouse | DSHB | Rab7, RRID: AB_2722471 |
| Anti-Drosophila Calnexin 99A, mouse | DSHB | cnx99A 6-2-1, RRID: AB_2722011 |
| Anti-Drosophila Hrs, mouse | DSHB | Hrs 27-4, RRID: AB_2618261 |
| Anti-Drosophila LAMP1, rabbit | Abcam | RRID: AB_775973 |
| Anti-Drosophila Rdns5, rabbit | Tanaka and Nakamura, 2008 | N/A |
| Anti-Drosophila Endophilin A, guinea pig | Verstreken et al., 2002 | GP69, N/A |
| Anti-Ref(2)P, rabbit | Ikeda et al., 2019 | N/A |
| Anti-Drosophila INPP5SF, guinea pig | This paper | N/A |
| Anti-Drosophila LRRK, rabbit | Imai et al., 2008 | N/A |
| Anti-Drosophila Aux, guinea pig | This paper | N/A |
| Anti-Drosophila RME-8, rabbit | This paper | N/A |
| Anti-Drosophila Clathrin heavy chain, rabbit | This paper | N/A |
| Anti-α-Synuclein, rabbit | Abcam | MFR1, ab138501, RRID: AB_2537217 |
| anti-α-Synuclein, mouse | FUJIFILM-Wako | RRID: AB_516843 |
| Anti-Tau (Tau-C, 424-438 aa), rabbit | Matsumoto et al., 2015 | N/A |
| Anti-α-Tubulin, mouse | Sigma-Aldrich | DM1A, RRID: AB_1904178 |
| Anti-α-Tubulin, rabbit | Cell Signaling Technology | 11H10, RRID: AB_2619646 |
| Anti-Actin, mouse | Millipore | C4, RRID: AB_2223041 |
| Anti-Arl8A, rabbit | Atlas | HPA038759, RRID: AB_2676193 |
| anti-Arl8B, rabbit | Proteintech | 13049-1-AP, RRID: AB_2059000 |
| Anti-Tyrosine hydroxylase, chicken | Abcam | ab76442, RRID: AB_1524535 |
| Anti-GAPDH, mouse | Proteintech | 1E6D9, RRID: AB_2107436 |
| Anti-pRab, rabbit | Abcam | MFR20, RRID: AB_2814988 |
| Anti-GFP | MBL | 598, RRID: AB_591816 |
| Anti-GFP-FITC, goat | Abcam | ab6662, RRID: AB_305635 |

**Experimental models: Organisms/strains**

Drosophila melanogaster UAS-LAMP1-GFP, n-Syb-GAL4 | Bloomington Drosophila Stock Center (BDSC) | RRID: BDSC_.42714 |
Drosophila melanogaster elav-GAL4 | BDSC | RRID: BDSC_.8765 |
Drosophila melanogaster D42-GAL4 | BDSC | RRID: BDSC_.8816 |
Drosophila melanogaster Da-GAL4 | BDSC | RRID: BDSC_.55850 |
Drosophila melanogaster Dpp-GAL4 | BDSC | RRID: BDSC_.1553 |
Drosophila melanogaster T(T)Rab3[EYFP] | BDSC | RRID: BDSC_.62541 |
Drosophila melanogaster T(T)Rab8[EYFP] | BDSC | RRID: BDSC_.62546 |
Drosophila melanogaster T(T)Rab10[EYFP] | BDSC | RRID: BDSC_.62548 |
Drosophila melanogaster T(T)Rab32[EYFP] | BDSC | RRID: BDSC_.62558 |
Drosophila melanogaster T(T)Rab35[EYFP] | BDSC | RRID: BDSC_.62559 |
Drosophila melanogaster UAS-Rab3 GEF RNAi | BDSC | RRID: BDSC_.28954 |

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| REAGENT or RESOURCE SOURCE | IDENTIFIER |
|----------------------------|------------|
| Drosophila melanogaster UAS-Rab3 GEF | BDSC RRID: BDSC_78052 |
| Drosophila melanogaster UASp-EYFP-Rab3 WT | BDSC RRID: BDSC_9762 |
| Drosophila melanogaster UASp-EYFP-Rab3 T35N | BDSC RRID: BDSC_9766 |
| Drosophila melanogaster UASp-EYFP-Rab3 Q80L | BDSC RRID: BDSC_9764 |
| Drosophila melanogaster UASp-EYFP-Rab8 WT | BDSC RRID: BDSC_9782 |
| Drosophila melanogaster UASp-Rab10-YFP WT | BDSC RRID: BDSC_9789 |
| Drosophila melanogaster UASp-YFP-Rab3 Q79L | BDSC RRID: BDSC_9764 |
| Drosophila melanogaster UAS-preproANF-Emerald | BDSC RRID: BDSC_7001 |
| Drosophila melanogaster unc-104p350 | BDSC RRID: BDSC_24630 |
| Drosophila melanogaster Khc48 | BDSC RRID: BDSC_1607 |
| Drosophila melanogaster UAS-unc-104-GFP | BDSC RRID: BDSC_24787 |
| Drosophila melanogaster Gt11 | BDSC RRID: BDSC_510 |
| Drosophila melanogaster UAS-DCTN-GFP | BDSC RRID: BDSC_29983 |
| Drosophila melanogaster UAS-EB1-GFP | BDSC RRID: BDSC_36861 |
| Drosophila melanogaster Syd24 | BDSC RRID: BDSC_32016 |
| Drosophila melanogaster Aplip1 FK4 | BDSC RRID: BDSC_24632 |
| Drosophila melanogaster Aux727 | BDSC RRID: BDSC_25672 |
| Drosophila melanogaster RME-8| BDSC RRID: BDSC_5525 |
| Drosophila melanogaster Vps35| BDSC RRID: BDSC_67202 |
| Drosophila melanogaster Syn1 | BDSC RRID: BDSC_24883 |
| Drosophila melanogaster Syn2 | BDSC RRID: BDSC_24884 |
| Drosophila melanogaster Rab32 | BDSC RRID: BDSC_338 |
| Drosophila melanogaster TagRFP-T Vps35 | BDSC RRID: BDSC_66527 |
| Drosophila melanogaster UAS-GFP-myc-2xFYVE | BDSC RRID: BDSC_42712 |
| Drosophila melanogaster UAS-PLC delta-PH-EGFP | BDSC RRID: BDSC_39693 |
| Drosophila melanogaster INPP5FMO1858 | BDSC RRID: BDSC_34453 |
| Drosophila melanogaster garnet1 | BDSC RRID: BDSC_3958 |
| Drosophila melanogaster UAS-Syd RNAi | Vienna Drosophila Resource Center (VDRC) VDRC_101459 |
| Drosophila melanogaster UAS-Aplip1 RNAi | VDRC VDRC_109501 |
| Drosophila melanogaster UAS-Liprin-a RNAi | VDRC VDRC_106588 |
| Drosophila melanogaster UAS-BORCSS RNAi | VDRC VDRC_31570 |
| Drosophila melanogaster UAS-RME-8 RNAi | VDRC VDRC_22671 |
| Drosophila melanogaster UAS-Aux RNAi | VDRC VDRC_30426 |
| Drosophila melanogaster UAS-Chc RNAi | VDRC VDRC_24789 |
| Drosophila melanogaster UAS-Chc RNAi | VDRC VDRC_23666 |
| Drosophila melanogaster pBac(DsRed)225 | Kyoto Stock Center #141867 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Drosophila melanogaster UAS-Aux RNAi | NIG-Fly | N107R-2 |
| Drosophila melanogaster Arl8-GFP | This paper | N/A |
| Drosophila melanogaster UAS-EYFP-Rab3 T85A | This paper | N/A |
| Drosophila melanogaster UAS-Arl8 WT | This paper | N/A |
| Drosophila melanogaster UAS-Arl8 Q75L | This paper | N/A |
| Drosophila melanogaster UAS-Arl8 T34N | This paper | N/A |
| Drosophila melanogaster UAS-INPP5F | This paper | N/A |
| Drosophila melanogaster UAS-1N4R | This paper | N/A |
| Drosophila melanogaster dLRRK WT | Imai et al., 2008 | FBal0220786 |
| Drosophila melanogaster dLRRK 3KD | Imai et al., 2008 | FBtp0040856 |
| Drosophila melanogaster dLRRK Y1383C | Imai et al., 2008 | FBal0220787 |
| Drosophila melanogaster dLRRK 1191ST | Imai et al., 2008 | FBtp0040855 |
| Drosophila melanogaster UAS-hLRRK I2020T | Venderova et al., 2009 | FBal0249982 |
| Drosophila melanogaster UAS-dVPS35 WT | Inoshita et al., 2017 | N/A |
| Drosophila melanogaster UAS-spin-myc-RFP | Sweeney and Davis, 2002 | FBtp0023043 |
| Drosophila melanogaster Rab10 null | Kohrs et al., 2021 | FBal0370495 |
| Drosophila melanogaster UAS-Crag | Nakamura et al., 2020 | FBtp0142307 |
| Drosophila melanogaster UAS-CLIP-190-GFP | Beaven et al., 2015 | FBtp0108854 |
| Drosophila melanogaster AuxP67D | Zhou et al., 2011 | FBal0215598 |
| Drosophila melanogaster UAS-RME-8-RFP | Chang et al., 2004 | FBal0189648 |
| Drosophila melanogaster UAS-Aux-GFP | Zhou et al., 2011 | N/A |
| Drosophila melanogaster UAS-Aux | Hagedorn et al., 2006 | N/A |
| Drosophila melanogaster UAS-Sy n-GFP-HA | Dickman et al., 2005 | FBtp00022534 |
| Drosophila melanogaster UAS-a-Synuclein LP2 | Trinh et al., 2008 | N/A |
| Drosophila melanogaster tub-mCherry-AP36 | Burgess et al., 2011 | FBal0277298 |
| Drosophila melanogaster UAS-hGalT-TagRFP | Zhou et al., 2014 | FBst0065251 |
| Drosophila melanogaster Clc-GFP | Chang et al., 2002 | FBst0007107 |
| Drosophila melanogaster tagBFP-FLAG-Rab35 | Gift from Dr. A. Satoh | N/A |

Software and algorithms

| Software | Version | URL | RRID |
|----------|---------|-----|------|
| ImageJ Fiji | v1.0 | http://fiji.sc | RRID:SCR_002285 |
| JMP | v11.0.0 | SAS | RRID:SCR_014242 |
| Zen | www.zeiss.com | ZEN Digital Imaging for Light Microscopy; RRID:SCR_013672 |
| Mini Analysis Program | | Synaptosoft | RRID:SCR_002184 |

Continued...
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Yuzuru Imai (yzimai@juntendo.ac.jp).

Materials availability
All unique/stable reagents generated in this study are available from the lead contact without restriction, except for some guinea pig antibodies, which are limited in quantity.

Data and code availability
- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Drosophila strains
Fly culture and crosses were performed using standard fly food containing yeast, cornmeal, and molasses, and the flies were raised at 25°C. The w^{1118} (w{ }) line was used as a wild-type genetic background. Complementary DNAs for Arl8 and INPP5F were amplified by PCR from first-strand cDNA library of w^{1118} and subcloned into pUAST vector. Complementary DNA for 2N4R Tau was amplified by PCR from adult human brain first-strand cDNA library and subcloned into pUAST vector. GFP-P4M-SidMx2 (Addgene #78544) was subcloned into pUAST vector to visualize PI(4)P in flies. Complementary DNAs for YFP-Rab3 T85A, Arl8 Q75L, and Arl8 T34N were generated by PCR-based mutagenesis using pUASp-YFP-Rab3 (Addgene #37687) or UAS-Arl8 as templates. Transgenic lines were generated on a w^{1118} background (BestGene Inc., Chino Hills, CA, USA). Arl8-GFP knock-in line was generated by insertion of a liner sequence (GTSGGS) and EGFP just before the stop codon (WellGenetics Inc., Taipei, Taiwan). Information on individual fly strains and genotypes for experiments is listed in the key resources table and Table S2 and can be found on FlyBase (flybase.org) unless otherwise noted. Experiments and ethics regarding the use of genetically modified Drosophila were approved by the Juntendo University School of Medicine, Animal Experiment Committee (approval number: 24-14).

Clinical samples
Detailed clinical characteristics were obtained from a neurodegenerative disease database of the Department of Neurology at Juntendo University. Details on these patients are given in Table S1. All procedures performed in studies for brain autopsy were in accordance with the ethical standards of the Juntendo University School of Medicine Ethics Committee (approval number: 2019012) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Neuropathological assessments for AD, PD, PDD, and DLB were conducted by pathologists from the Department of Neurology at Juntendo University. Written informed consent for autopsy and analysis of tissue sample data was obtained for all patients.

METHOD DETAILS

Antibody production
Anti-Aux and anti-INPP5F polyclonal antibodies were raised against recombinant GST-tagged Aux (720–1165 aa) and GST-tagged INPP5F (744–1000 aa) produced in the Escherichia coli strain Rosetta 2 (Novagen, Merck, Darmstadt, Germany). Rabbit anti-RME-8 polyclonal antibody was raised against a mixture of synthetic peptides (C-ISTYNPDKLDTNRWS-coNH2 and C-KDQRHDLFIADTTIRGY-coNH2) and purified through affinity chromatography (Japan Bio Services, Asaka, Japan). Rabbit anti-Chc polyclonal antibody was raised against a synthetic peptide (DDSTEHKNIIQMEPQLMC; Cosmo Bio, Tokyo, Japan).

The following primary antibodies were used for western blotting: anti-GFP (1:5000; 598, MBL, Tokyo, Japan), anti-pRab (1:1000; MJF-R20, Abcam, Cambridge, UK), anti-Lrrk (1:2000; in-house, 2136013), anti-Aux (1:1000; in-house, R1), anti-INPP5F (1:1000; in-house, GP-C2), anti-RME-8 (1:1000; in-house,
Western blot analysis

For phos-tag western blot analysis, 3 male fly heads were homogenized with 20 μL lysis buffer containing 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Triton X-100, and protease inhibitor cocktail (O9569, Nacalai Tesque, Kyoto, Japan) using a motor-driven pestle. After the addition of 1 mM MnCl₂ and 40 units of Lambda protein phosphatase (P0753L, New England BioLabs, Ipswich, MA), the lysate was incubated at 30°C for 30 min and subjected to 10% gel containing 50 μM Phos-tag Acrylamide (304-93521, FUJIFILM Wako, Osaka, Japan). For protein expression analysis, the fly brain and thorax tissues were directly homogenized in a 20 μL 3X SDS sample buffer using a motor-driven pestle. The same amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis, and the separated proteins were transferred onto polyvinylidene fluoride membranes. The membranes were blocked for 1 h at 22°C with 5% milk or 5% FBS (for anti-pRab) in Tris-buffered saline containing 0.05% Tween20 (TBS-T) and then incubated overnight with primary antibodies at 4°C. After washing three times with TBS-T, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies at 22°C for 1 h. After washing three times with TBS-T, signals were detected with Immobilon Forte Western HRP substrate (cat. no. WBLUF0500; Millipore, Merck, Darmstadt, Germany). The blot images were obtained using Fusion FX6 Edge (Vilber-Lourmat, Marne-la-Vallée, France).

Whole-mount immunostaining

Synapse boutons in larval motor neurons were visualized by whole-mount immunostaining as previously described. For image processing of synapse boutons in the A5 or A6 segment, 30 Z-stack images were taken at 0.35- to 0.70-μm intervals and reconstituted using ImageJ. The values of Arl8 aggregation-positive terminals in the graphs were calculated as the percentage of Arl8-accumulated terminals per total synaptic terminals in the same segments. Antibodies used in immunocytochemistry were anti-Arl8 [1:100; Developmental Studies Hybridoma Bank (DSHB), Arl8], anti-Rbsn5 [1:100; gift from Dr. A. Nakamura], anti-Rab7 [1:100; DSHB, Rab7], anti-Calnexin 99A [1:100; DSHB, cnx99A 6-2-1], anti-Ref(2)P [1:100; in-house, 1532052], anti-LAMP1 [1:50; DSHB, H4A3]; anti-INPP5F [1:200; in-house, GP-C2], anti-Aux [1:200; in-house, GP-1], anti-Chc [1:100; in-house, AF19050743-001], anti-Tau [1:1000; Tau-C, gift from Dr. Y. Motoi], anti-a-Synuclein [1:100; Abcam, MJFR1, ab138501], anti-a-Tubulin [1:200; Cell Signaling Technology, 11H10], anti-Tau [1:1000; Tau-C, gift from Dr. Y. Motoi], anti-GFP-FITC [1:500; Abcam, ab6662], Alexa Fluor594- [1:200], or DyLight649- [1:500] conjugated anti-HRP (123-585-021, Jackson ImmunoResearch, West Grove, PA). Secondary antibodies used were anti-rabbit Alexa Fluor Plus or Alexa Fluor (1:200; A32731 and A11012, Thermo Fisher Scientific, Waltham, MA), anti-mouse Alexa Fluor Plus or Alexa Fluor (1:200; A32723, A11017, and A11032, Thermo Fisher Scientific), and anti-guinea pig Alexa Fluor (1:200; A21435, Thermo Fisher Scientific).

TEM analysis and CLEM

Transmission electron microscopy (TEM) images were obtained using an electron microscope (HT7700, Hitachi High-Tech Corporation, Tokyo, Japan) at the Laboratory of Ultrastructural Research of Juntendo University as previously described. For correlative light-electron microscopy (CLEM), third instar larvae were dissected in HL-3 solution. Tissues were fixed with phosphate buffer containing 2.4% paraformaldehyde and 1% glutaraldehyde for 15 min. Synaptic boutons of motor neurons were labeled by whole-mount immunostaining as previously described and imaged on a Zeiss LSM880 Airyscan confocal microscope (Oberkochen, Germany). After fluorescence imaging, TEM images were obtained using an electron microscope (HT7700).

Arl8 transport assay

Third-instar larvae were dissected in HL-3 medium containing 0.5 mM Ca²⁺, and axons of motor neurons were imaged each 0.6 s for 1 min on a Leica SP5 confocal microscope. Kymograph of Arl8-GFP was generated using the reslice tool in the Fiji ImageJ software and analyzed. Detailed protocols for sample preparation and data analysis can be found in.

Electrophysiology

Third-instar larvae were dissected in HL-3, and mEJPs from NMJs were recorded using an electrophysiological setup equipped with an Eclipse FN1 microscope (Nikon, Tokyo, Japan), a Multiclamp 700B amplifier
Dissected larvae were incubated in HL-3 containing 0.375 mM Ca\(^{2+}\), and a recording electrode filled with 3 M KCl was inserted into muscle 6 of the A3 or A4 segment containing NMJs. All data were analyzed using Mini-Analysis software (Synaptosoft, Fort Lee, NJ).

### Climbing assay

For each genotype, two vials (25 flies/vial) were prepared. Vials (25 mm diameter × 180 mm height) were tapped gently on the table and left standing for 18 s. The number of flies that climbed at least 60 mm was recorded and represented as percentage.

### α-Synuclein and Tau preparation and uptake assay

Recombinant α-Synuclein was purified from bacteria BL21(DE3) harboring pRK172-human α-synuclein using a Q Sepharose column (17051010, Cytiva, Tokyo, Japan).\(^7\) Recombinant Tau was purified from bacteria BL21(DE3) harboring pRK172-4R2N Tau using an SP Sepharose column (17072910, Cytiva).\(^5\) The third instar larvae were dissected in Schneider medium (cat. no. 21720024, Gibco, Thermo Fisher Scientific, Waltham, MA) with 10% fetal bovine serum. The motor neuron axon was sucked into a glass electrode and stimulated with 5 V, 1 Hz for 10 min in Schneider medium with 10 \(\mu\)M α-Synuclein or Tau. After stimulation, dissected larvae were incubated for 30 min in Schneider medium with 10 \(\mu\)M α-Synuclein or Tau and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS).

### Histochemical analysis of human brain

Triple immunofluorescence staining was performed using 6-μm-thick, 4% paraformaldehyde-fixed, paraffin-embedded sections from the midbrain of clinically diagnosed and pathologically or genetically confirmed PD and control cases collected by the Department of Neurology, Juntendo University Hospital. Deparaffinized sections microwaved in Tris EDTA buffer, pH 9.0 (S2367, Agilent-DAKO, Santa Clara, CA) for 10 min for antigen retrieval, were neutralized with PBS. Sections were treated with blocking buffer (cat. no. 0634964, Blocking One Histo, Nacalai Tesque) and incubated with anti-Arl8A (1:50; HPA038759, Atlas) or anti-Arl8B (1:50; 13049-1-AP, Proteintech, Rosemont, IL) antibodies along with anti-α-Synuclein (1:250; pSyn#64, FUJIFILM Wako) and anti-Tyrosine hydroxylase (1:1000; ab76442, Abcam) overnight at 4°C. Primary antibodies were visualized with secondary antibodies (1: 1,000, Alexa Fluor405, 488, 594, and 647, Thermo Fisher Scientific) for 1 h at RT. To reduce lipofuscin autofluorescence, sections were further treated with TrueBlack Lipofuscin Autofluorescence Quencher (cat. no. 23007, Biotium, Inc. Fremont, CA), diluted with 70% ethanol for 30 s, and briefly washed with PBS before mounting with VECTASHIELD mounting medium (H1800, Vector laboratories, Newark, CA) and assessed using LSM880 with the Airyscan laser-scanning microscope system (Zeiss).

### QUANTIFICATION AND STATISTICAL ANALYSIS

All data in bar graphs were expressed as mean ± SEM and graphs were generated using JMP v11.0.0 (SAS Institute Cary, NC). \(p\) values less than 0.05 were considered statistically significant. Student t-test or Dunnett’s test was used to determine significant differences between two specific groups or between multiple groups of interest. The data distribution was assumed to be normal, although this assumption was not formally tested. Randomization was used in each genotype. Counting of the vesicles in the electron micrographs in Figure 6C was performed blindly by TI, J-YL, and C. Cui. Other data and analyses were not performed blind to the conditions of the experiments. All of the statistical details of the experiments can be found in the figure legends.