Parkinson's disease-associated iPLA2-VIA/PLA2G6 regulates neuronal functions and α-synuclein stability through membrane remodeling

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Mutations in the iPLA2-VIA/PLA2G6 gene are responsible for PARK14-linked Parkinson's disease (PD) with α-synucleinopathy. However, it is unclear how iPLA2-VIA mutations lead to α-synuclein (α-Syn) aggregation and dopaminergic (DA) neurodegeneration. Here, we report that iPLA2-VIA-deficient Drosophila exhibits defects in neurotransmission during early developmental stages and progressive cell loss throughout the brain, including degeneration of the DA neurons. Lipid analysis of brain tissues reveals that the acyl-chain length of phospholipids is shortened by iPLA2-VIA loss, which causes endoplasmic reticulum (ER) stress through membrane lipid disequilibrium. The introduction of wild-type human iPLA2-VIA or the mitochondria–ER contact site-resident protein C19orf12 in iPLA2-VIA–deficient flies rescues the phenotypes associated with altered lipid composition, ER stress, and DA neurodegeneration, whereas the introduction of a disease-associated missense mutant, iPLA2-VIA A80T, fails to suppress these phenotypes. The acceleration of α-Syn aggregation by iPLA2-VIA loss is suppressed by the administration of linoleic acid, correcting the brain lipid composition. Our findings suggest that membrane remodeling by iPLA2-VIA is required for the survival of DA neurons and α-Syn stability.

Parkinson's disease {lipids, Drosophila, ER stress, α-synuclein}

Parkinson's disease (PD) is characterized by loss of the midbrain dopaminergic (DA) neurons, and its clinical features include motor symptoms and nonmotor symptoms such as akinesia, rigidity, cognitive impairment, sleep disorders, and dysautonomia (1). Pathologically, a neuronal inclusion named Lewy body (LB), which contains the presynaptic protein α-synuclein (α-Syn), ubiquitin, and lipids, is most observed in the affected regions, strongly implying that α-Syn aggregation is the underlying cause of neurodegeneration in PD. α-Syn presents as a natively unfolded protein and is folded as an extended α-helical structure upon binding to the acidic phospholipid surface, which suggests that the altered composition of membrane lipids could be a trigger of α-Syn aggregation (2, 3).

The nervous system is enriched in lipids and contains a more diverse lipid composition than other tissues to maintain neuronal functions (4). Within a given lipid class, different lipid species are produced from a variety of fatty acids (FAs) with different lengths and saturation levels. Among them, phospholipids have important roles in synaptic functions, vesicular transport, and organelle maintenance (5). The phospholipase A2 (PLA2) family consists of many subgroups of enzymes that hydrolyze the sn-2 ester bonds of phospholipids, generating free fatty acids (FFAs) and lysophospholipids (6). One of the PLA2 family, PL2G6 or iPLA2-VIA (PLA2G6), has been isolated as the gene responsible for an autosomal recessive form of PD linked to the PARK14 locus (7). Postmortem examinations have revealed a marked LB pathology in PARK14 cases (7, 8). Depending on the mutation, iPLA2-VIA is also the causative gene of infantile neuronal dystrophy (INAD) and neurodegeneration with brain iron accumulation (NBIAs) (9, 10). Thus, these 3 different neurodegenerative disorders caused by iPLA2-VIA mutations are collectively called PLA2G6-associated neurodegeneration (PLAN). However, the physiological substrates of iPLA2-VIA are still unclear, and it remains controversial.

Significance

The mechanisms of α-synuclein aggregation and subsequent Lewy body formation are a key pathogenesis of Parkinson's disease (PD). PARK14-linked PD, which is caused by mutations of the iPLA2-VIA/PLA2G6 gene, exhibits a marked LB body pathology. iPLA2-VIA, which belongs to the phospholipase A2 family, is another causative gene of neurodegeneration with brain iron accumulation (NBIAs). Here, we demonstrate that iPLA2-VIA loss results in acyl-chain shortening in phospholipids, which affects ER homeostasis and neurotransmission and promotes α-synuclein aggregation. The administration of linoleic acid or the overexpression of C19orf12, one of the NBIAs-causative genes, also suppresses the acyl-chain shortening by iPLA2-VIA loss. The rescue of iPLA2-VIA phenotypes by C19orf12 provides significant molecular insight into the underlying common pathogenesis of PD and NBIAs.
whether PARK14-associated iPLA2-VIA mutations affect PL2 activity (11, 12).

Phospholipids are synthesized by 2 pathways: the de novo pathway (or Kennedy pathway), using acyl-CoAs as donors; and the remodeling pathway (or Landis’ cycle), in which the cycle of phospholipid deacylation and reacylation modifies the FA composition to generate a mature membrane. iPLA2-VIA has been proposed to be a key enzyme of the remodeling pathway (6, 13). Other genes responsible for NBIA, which include Pank2, C19orf12, COASY, and FA2H, are also suggested to be involved in lipid metabolism (9). However, it remains largely unknown how changes in lipid metabolism contribute to PD etiology accompanied by LB formation and NBIA pathogenesis.

In this study, we show that the loss of iPLA2-VIA leads to the shortening of the acyl chains of phospholipids in Drosophila, thereby resulting in disruption of ER homeostasis, alterations in synaptic vesicle (SV) size and neurotransmission, and DA neurodegeneration. Moreover, α-Syn loses affinity for phospholipids with shorter fatty acyl chains, facilitating α-Syn aggregation in iPLA2-VIA–deficient flies. Importantly, neuronal expression of NBIA-associated C19orf12 as well as wild-type (WT) human iPLA2-VIA, but not the pathogenic iPLA2-VIA mutant A80T, corrects the altered lipid composition caused by iPLA2-VIA loss, suppressing the neurodegenerative phenotypes of iPLA2-VIA-deficient flies. Thus, in addition to genetic evidence that iPLA2-VIA and C19orf12 underlie a common pathogenic pathway, our results suggest that iPLA2-VIA–mediated phospholipid remodeling is a critical element for α-Syn stability and DA neuron survival.

Results

Loss of iPLA2-VIA Causes Degeneration of DA Neurons. CG6718 is the only iPLA2-VIA ortholog (dipLA2-VIA) in the Drosophila genome, showing 51% amino acid similarity to the human iPLA2-VIA (hiPLA2-VIA) (14). To determine the effects of a PD-associated iPLA2-VIA mutation on DA neuron functions, we generated dipLA2-VIA–null flies by CRISPR/Cas9 technology (SI Appendix, Fig. S1 A and B) and reintroduced hiPLA2-VIA WT or A80T in the dipLA2-VIA–null flies (Fig. 1A). Loss of dipLA2-VIA caused progressive locomotor defects and sleep disturbance (Fig. 1B and SI Appendix, Fig. S1 C–F). dipLA2-VIA–flies exhibited bang sensitivity, a seizure-and-paralysis behavior evoked by mechanical shocks (Fig. 1C) (15). Neuronal expression of hiPLA2-VIAWT largely suppressed the motor and paralytic phenotypes by the loss of dipLA2-VIA, suggesting that hiPLA2-VIA is functional in Drosophila and that its activity in the central nervous system is sufficient to rescue these phenotypes (Fig. 1 B and C). In contrast, one of the PARK14 mutants, hiPLA2-VIAA80T, failed to rescue the neuronal phenotypes of dipLA2-VIA–flies (Fig. 1 B and C) (7, 16).

Consistent with the locomotion phenotype and sleep disturbance, which are partly derived from DA neuron dysfunction, the number of DA neurons in the PPL1, PPM1/2, and PPM3 clusters, as well as survivability, decreased in aged dipLA2-VIA–flies (SI Appendix, Fig. S1 G and H) (17–19). The age-dependent loss of DA neurons in dipLA2-VIA–flies was fully suppressed by the neuronal expression of hiPLA2-VIAWT, whereas the expression of hiPLA2-VIAA80T was less effective (Fig. 1 D and E). Similarly, the sleep disturbance found in dipLA2-VIA–flies is partially corrected by the neuronal expression of hiPLA2-VIAWT but not of hiPLA2-VIAA80T (SI Appendix, Fig. S1I). In contrast, the neuronal expression of hiPLA2-VIAWT and hiPLA2-VIAA80T on the dipLA2-VIA–background did not fully rescue the reduced lifespan of dipLA2-VIA–flies, suggesting that iPLA2-VIA activity is required for both neuronal and nonneuronal tissues (SI Appendix, Fig. S1H). To estimate whether A80T has the gain-of-function property, both hiPLA2-VIAWT and hiPLA2-VIAA80T were expressed on the dipLA2-VIA–background. Both flies behaved similarly to the normal control in terms of the motor and paralytic phenotypes and sleep behavior, which suggests that A80T does not have a gain-of-function property (Fig. 1 B and C and SI Appendix, Fig. S1I).

iPLA2-VIA Maintains the Phospholipid Composition in the Brain. iPLA2-VIA has been proposed to be involved in membrane homeostasis and remodeling through deacylation of phospholipids (6, 13). To determine the consequence of iPLA2-VIA loss in lipid metabolism, we analyzed the lipid composition in the brain of 20-d-old flies and found that phospholipid molecular species containing myristic acid (C14:0) or palmitoleic acid (C16:1), including phosphatidylcholine (PC) 14:0_14:0, phosphatidylethanolamine (PE) 14:0_14:0, PE 14:0_16:1, phosphatidylglycerol (PG) 14:0_16:1, and phosphatidylserine (PS) 14:0_16:1, were significantly increased, while those with acyl chains of 18:0_18:0 and 18:0_18:1, but not 18:0_18:2, were decreased (SI Appendix, Fig. S2 A and B). Accordingly, the ratio of acyl chain 18:0_18:X (X = 0, 1, and 2) to acyl chains 14:0_14:0 and 14:0_16:1 increased from 0.109 ± 0.003 (mean ± SEM) in the dipLA2-VIA+/+ brain to 0.208 ± 0.008 in the dipLA2-VIA–/– brain (P = 0.0202 by Dunnett’s test; Fig. 2A). In contrast, the amounts of lysophospholipids and FFAs such as docosahexaenoic acid (DHA), eicosapentaenoic acid, and arachidonic acid (ARA), and the proportions of PC, PE,
PG, and PS, were not significantly changed by diPLA2-VIA loss (SI Appendix, Fig. S2 C and D). The increased proportion of shorter acyl chains in diPLA2-VIA−/− brains was also observed at a younger age, specifically 3 d old (0.089 ± 0.013 vs. 0.057 ± 0.004 i

**dietary manipulation of acyl-chain composition improves neurodegeneration by iPLA2-VIA Loss.** A dairy-containing diet influences lipid metabolism (21–23). The addition of FFAs to diet modulates the acyl-chain composition of phospholipids in fly brain tissues (24). To determine whether the alteration of phospholipids in the brain is a primary cause of neuronal phenotypes in diPLA2-VIA−/− flies, different FFAs, including linoleic acid (LA; 18:2), stearic acid (SA; 18:0), and myristic acid (MA; 14:0), were administered during a period from the hatching to adult stages. In parallel, flies were treated with α-tocopherol to prevent lipid peroxidation because elevated lipid peroxidation was observed in aged diPLA2-VIA−/− flies, as previously reported (14) (SI Appendix, Fig. S2F). Motor defects observed in adult diPLA2-VIA−/− flies were fully improved only by the LA-containing diet, whereas a partial improvement was observed by the SA-containing diet (Fig. 2C). DA neuron loss and the bang-sensitive seizure phenotype in adult diPLA2-VIA−/− flies were also rescued by a diet containing LA but not by a diet containing MA or α-tocopherol (Fig. 2 D and E). Consistent with the improvement of neuronal phenotypes, measurement of brain lipid composition revealed that the LA-containing diet decreased the proportion of phospholipids with shorter acyl chains in diPLA2-VIA−/− flies (0.047 ± 0.0007; P = 0.0004 vs. diPLA2-VIA−/− by Dunnett’s test; Fig. 2A). However, LA supplementation was unable to fully rescue the reduced lifespan of diPLA2-VIA−/− flies, which once again suggested the nonneuronal roles of iPLA2-VIA (SI Appendix, Fig. S1H). Mitochondrial degeneration has been reported in diPLA2-VIA−/− deficient Drosophila and mice (14, 25). We examined whether membrane lipid disequilibrium caused by iPLA2-VIA loss also affects mitochondrial activity. However, brain ATP content was not changed, and mitochondrial morphology in both DA neurons and indirect flight muscles, which is readily compromised by mutations of mitochondria-associated PD genes such as PINK1 and CHCHD2, was also normal (SI Appendix, Fig. S2 G–I) (26, 27). In addition, LA supplementation failed to rescue the motor defects and DA neuron loss in CHCHD2- or PINK1-deficient flies (SI Appendix, Fig. S2 J and K). Together, these data suggest

by 1-way ANOVA with the Tukey–Kramer test). (D) An LA-rich diet rescues DA neuron loss caused by lack of diPLA2-VIA. The number of DA neurons in each cluster in 20-d-old adult flies (mean ± SEM, n = 11 to 23; **P < 0.05, ***P < 0.01, and ****P < 0.001) by 1-way ANOVA with the Tukey–Kramer test. (E) The bang-sensitive seizure-paralysis phenotype of 5-d-old diPLA2-VIA−/− flies is rescued by LA supplementation. Data are represented as the mean ± SEM (n = 10 male flies, 4 trials; **P < 0.05 by Dunnett’s test).
that the alteration of brain lipid composition contributes to PLAN-linked neurodegeneration and that mitochondrial degeneration is not a primary cause of PLAN-linked neurodegeneration.

**MPAN-Associated C19orf12 Rescues Neuronal Phenotypes caused by iPLA2-VIA Loss.** Mutations in the C19orf12 gene are associated with mitochondrial membrane protein-associated neurodegeneration (MPAN), an autosomal-recessive disorder that accounts for 5 to 30% of NBIA cases (9). Similar to PD patients, both PLAN and MPAN patients show a common LB pathology (7, 8, 28). In addition to the pathological similarity, C19orf12 has been suggested to be involved in lipid metabolism (9). One of the neuronal phenotypes of diPLA2-VIA−/− flies is bang sensitivity, which has also been reported in C19orf12-knockdown flies (29, 30). Neuronal overexpression of the C19orf12 homolog CG3740 (dC19orf12) rescued the heat sensitivity, motor disability, and bang sensitivity of iPLA2-VIA−/− flies (Fig. 3 A–C and SI Appendix, Fig. S3A). dC19orf12 overexpression also suppressed DA neuron loss of diPLA2-VIA−/− flies and partially improved their sleep disturbance (Fig. 3D and SI Appendix, Fig. S1D). Brain vacuolar formation caused by diPLA2-VIA loss, which has also been reported previously (14), was suppressed by dC19orf12 as well as hiPLA2-VIAWT (Fig. 3 E and F). Consistent with the rescuing effects on neurodegeneration-associated phenotypes, the increased proportion of shorter acyl chains in phospholipids was corrected to a normal level by dC19orf12 expression (0.099 ± 0.029; $P = 0.589$ vs. diPLA2-VIA−/− by Dunnett’s test; Fig. 2A). These observations suggest that iPLA2-VIA and C19orf12 act, at least in part, in the same pathway for lipid metabolism.

iPLA2-VIA appears to show dynamic localization to the mitochondria and the ER and has some roles in the membranes of these organelles (14, 25, 30–32). The salivary gland of the diPLA2-VIA−/− mutant was poorly developed, and the ER areas of the gland were extended, suggesting that its ER functions were greatly compromised (Fig. 3G and SI Appendix, Fig. S3B). Altered lipid composition of the ER membrane activates the ER stress transducer IRE1, which up-regulates the ER stress-associated transcriptional activator XBP1 through processing of the XBP1 mRNA precursor (33). Suppression of acyl-chain shortening in phospholipids by LA treatment mitigated the morphological defect of the ER and suppressed XBP1 activation by diPLA2-VIA loss, which indicates that membrane lipid...
An increased proportion of shorter acyl chains in lipid composition by iPLA2-VIA loss evokes ER stress (Fig. 3H and SI Appendix, Fig. S3B). The ER morphological defect and XBP1 activation were also mitigated by hiPLA2-VIAWT or hC19orf12 expression (Fig. 3F and SI Appendix, Fig. S3B). Additionally, sporadic XBP1 activation in neurons was observed in the aged diPLA2-VIA−/− fly brain and suppressed by hiPLA2-VIAWT or hC19orf12 expression (Fig. 3F). IER1 oligomerization and activation are negatively regulated by the ER chaperone BiP (34). The neuronal expression of Drosophila BiP (or Hsc70-3) improved DA neuron loss due to the lack of diPLA2-VIA (Fig. 3K). BiP overexpression also ameliorated the bang-sensitivity phenotype and motor defects through diPLA2-VIA loss (SI Appendix, Fig. S3 C and D). Again, these results suggest that altered lipid composition caused by iPLA2-VIA loss induces ER stress, presumably leading to widespread neurodegeneration in addition to DA neuron degeneration.

We next examined the mechanism by which C19orf12 rescues ER stress. hC19orf12 has been found at the mitochondria-associated ER membrane (MAM), among other locations, and we confirmed that hC19orf12WT is localized at the MAM as well as the ER and mitochondria in cultured human cells (SI Appendix, Figs. S3 E, F, and H) (35). In contrast, the MPAN-associated hC19orf12G69R mutant completely lost its localization to the ER and mitochondria (SI Appendix, Fig. S3H). hiPLA2-VIA−/− and hiPLA2-VIAA80T were largely localized in the cytoplasm of cultured human cells, as small dot-like structures and hiPLA2-VIAWT but not hiPLA2-VIAA80T appeared to show partial colocalization with the ER (SI Appendix, Fig. S3I). Although we did not detect a physical interaction or colocalization between hiPLA2-VIA and hC19orf12 (SI Appendix, Fig. S3 G and J), overexpression of hC19orf12WT increased the number of MAMs, while hC19orf12G69R failed to do so (SI Appendix, Fig. S3K). To confirm whether enhanced MAM integrity by C19orf12 rescues ER defects of diPLA2-VIA−/− flies, we employed the protein ChiMERA, which consists of the mitochondrial Tom70 fused to the ER protein Ub6 and GFP and up-regulates MAM (36). The enhancement of MAM integrity by ChiMERA also rescued the bang-sensitive seizure phenotype of diPLA2-VIA−/− flies, suggesting that enhanced MAM integrity alleviates the defects in neuronal activity by correcting membrane lipid disequilibrium (Fig. 3L). However, ChiMERA expression failed to rescue DA neuron loss in diPLA2-VIA−/− flies, which suggests that there exists a dynamic regulatory mechanism of C19orf12 in terms of MAM formation in response to neuronal activity or that C19orf12 has additional functions in maintaining the survival of DA neurons (SI Appendix, Fig. S3L).

**Alteration of Lipid Composition by iPLA2-VIA Loss Affects Synaptic Functions.** An increased proportion of shorter acyl chains in phospholipids affects the thickness and dynamics of biomembrane as well as the integrity of membrane-associated proteins (5). To determine the effects of altered lipid composition by iPLA2-VIA mutations on neuronal activity, we next examined the larval neuromuscular junctions (NMJs), which are well characterized as neuronal synaptic models. The synaptic morphology, including the number of docked vesicles, number of boutons, number of active zones in each bouton, and bouton size, was not changed by diPLA2-VIA loss (SI Appendix, Fig. S4 A, B, D, and E). Moreover, the overexpression of hiPLA2-VIA or hC19orf12 or LA treatment had little effect on synaptic morphology (SI Appendix, Fig. S4 A and B). However, SV diameter in diPLA2-VIA−/− larvae was smaller than in diPLA2-VIA+/+ larvae (Fig. 4 A and B). Consistent with the reduction in SV diameter in diPLA2-VIA−/− larvae, SV density near the active zones of diPLA2-VIA−/− flies was higher (SI Appendix, Fig. S4C). The SV phenotype was rescued by hiPLA2-VIAWT or dC19orf12, but not hiPLA2-VIAA80T, and the SV density near the active zones tended to change accordingly (SI Appendix, Fig. S4C).

Manipulation of acyl-chain composition by LA treatment also increased SV size (Fig. 4 A and B). Spontaneous neuronal activity, assessed by miniature excitatory junction potential (mEJP), was decreased in diPLA2-VIA−/− flies under 2 different ambient temperature conditions (22 and 30°C), which was likely derived from the small SV phenotype because SV size as well as the number of boutons and active zones can affect mEJP amplitude (37). LA treatment and neuronal expression of hiPLA2-VIAWT but not hiPLA2-VIAA80T ameliorated the reduction in mEJP (Fig. 4 C and D and SI Appendix, Fig. S4F). hC19orf12G69R, but not MPAN-linked hC19orf12A80T, also rescued the hypoactive mEJP phenotype of diPLA2-VIA−/− flies. Both mEJPs and EJPs tended to be lower in amplitude under a high temperature (30°C) than a favorable temperature (22°C; Fig. 4 D and E), and EJP amplitude as well as quantal content did not correlate with the alteration of lipid composition by gene manipulation and dietary treatment (Fig. 4E and SI Appendix, Fig. S4G). On the contrary, the paired-pulse ratios (PPRs) of diPLA2-VIA−/− flies and diPLA2-VIA+/− flies expressing hiPLA2-VIAA80T were decreased by high temperature, perhaps due to the alteration in membrane fluidity (Fig. 4E).

**Alteration of Lipid Composition by iPLA2-VIA Loss Accelerates α-Syn Fibril Formation.** α-Synucleinopathy is a prominent feature of PD patients with iPLA2-VIA mutations and NBIA patients with C19orf12 mutations (7, 28, 38). α-Syn repeatedly binds to and dissociates from the acidic phospholipid surface of SVs during the release and retrieval cycle of SVs (39). To explore the effects of altered lipid composition caused by iPLA2-VIA mutations on α-Syn stability, we neuronally expressed α-Syn in diPLA2-VIA+/+ and diPLA2-VIA−/− flies. Aggregation of α-Syn, some of which was ubiquitin-positive, was observed to be intensive in diPLA2-VIA−/− DA neurons as well as other brain regions in 20-d-old adult flies, while few α-Syn-positive aggregations were detected in age-matched iPLA2-VIA+/− flies (Fig. 5 A and B and SI Appendix, Fig. S5A). Although ultrastructural analysis of adult brain tissues revealed that loss of iPLA2-VIA itself induced the appearance of abnormal organelle-derived membrane structures and electron-dense deposits, α-Syn−positive electron-dense deposits were frequently observed in the diPLA2-VIA−/− brain (SI Appendix, Fig. S5 B and C). Biochemical analysis indicated that most of these aggregates were α-Syn, and the fraction was transformed from a native form to a pathogenic form (Fig. 5C) (40). Importantly, dietary LA treatment suppressed the aggregation and insolubilization of α-Syn (Fig. 5 A–C). Similar to LA treatment, the neuronal expression of hiPLA2-VIAWT or dC19orf12 ameliorated α-Syn aggregation, while hiPLA2-VIAA80T failed to do so (Fig. 5 D and E). To determine whether α-Syn aggregation generated in the diPLA2-VIA−/− brain exerts seeding activity on α-Syn fibril formation, we monitored the conversion of soluble α-Syn into amyloid fibrils by real-time quaking-induced conversion (RT-QUIC) using fly brain lysates expressing α-Syn (41, 42). The time required for thioflavin T (ThT) fluorescence to reach half of the fluorescence intensity was shorter under diPLA2-VIA loss, which was rescued by dietary LA treatment (Fig. 5 F, Left, and SI Appendix, Fig. S6D). In contrast, fly brain lysates without α-Syn expression did not have the seeding activity (SI Appendix, Fig. S6D). The slope values were not significantly different between genotypes and were comparable to those obtained from human brain tissues with LB pathology (SI Appendix, Fig. S6C), suggesting that fly α-Syn aggregates have properties similar to those from human brain (Fig. 5 F, Right). These observations strongly suggest that lipid alteration by diPLA2-VIA loss facilitates the pathogenic conformational change of α-Syn in fly brain.

**Acyl-Chain Shortening Reduces the Affinity of α-Syn to Phospholipids.** We next examined the effects of increased shorter acyl-chain
Altered acyl-chain composition affects synaptic vesicle size. (A) Ultrastructure of the synaptic vesicles at the active zones in third-instar larval motor neurons with the indicated genotypes. (Scale bar, 100 nm.) (B) The ratio of synaptic vesicle diameter to diPLA2-VIA−/− is graphed (mean ± SEM, n = 130 to 150 from 3 third-instar larvae; ***P < 0.0001 by 1-way ANOVA with the Tukey-Kramer test [Left] and by 2-tailed Student’s t test [Right]). (C) Representative spontaneous mEJP traces in the third-instar larval NMJ at 22 and 30 °C. (D) Cumulative distribution of mEJP amplitude at 22 and 30 °C over 60 s (n = 7 to 11 third-instar larvae per genotype). (E) EJP amplitude evoked by 1.5 to 2 V electrical stimulation at 22 and 30 °C and paired-pulse ratio (PPR) as a ratio of EJP amplitudes to paired electrical stimulations (50-ms interval) at 22 and 30 °C (n = 7 to 9 third-instar larvae per genotype; *P < 0.05 by 2-tailed Student’s t test).

Discussion

In this study, using Drosophila iPLA2-VIA mutant models, we make 3 contributions to the understanding of the etiology of PD and NBIA. First, lack of iPLA2-VIA activity results in the shortening of phospholipid acyl chains, which evokes ER stress and affects neuronal activity. Second, the ectopic expression of MPAN-associated C19orf12 rescues the neuronal phenotypes caused by iPLA2-VIA loss. Third, α-Syn aggregation is facilitated by phospholipids with shorter acyl chains (Fig. 7).

The structural diversity of lipids influences their geometry, curvature, fluidity, thickness, surface charge, and lipid packing. iPLA2-VIA, which converts phospholipids into inverse cone-shaped lysophospholipids on the plasma membrane, releasing FAs in the process, plays a crucial role in regulating the synaptic transmission and electrical properties in the neuronal and glial cells (49, 50). PLα2 activity contributes to SV formation at synaptic terminals, as demonstrated by previous findings that venom containing PLα2 neurotoxins promotes SV exocytosis and inhibits synaptic endocytosis (51, 52). Our study revealed that the phospholipid acyl chains get progressively shorter in the diPLA2-VIA−− flies (SI Appendix, Fig. S2E). An increased proportion of shorter acyl chains caused by iPLA2-VIA loss leads to higher membrane curvature. Consistent with this finding, we observed reduced SV size and mEJPs in diPLA2-VIA−−− and diPLA2-VIA−−−/hiPLA2-VIAABOT larval NMJs (Fig. 4 A–D), whereas EJPs of diPLA2-VIA−−− and diPLA2-VIA−−−/hiPLA2-VIAABOT larvae were largely intact despite the obvious seizure-paralysis phenotype at an early adult stage. The seizure-paralysis phenotype has been reported in flies harboring mutations in mitochondrial proteins or ion channels and genes associated with lipid metabolism, which might be due to the defects in energy supply and altered channel activities on the biomembrane (15). Thus, age-dependent alterations in the synaptic membrane properties and mitochondrial dysfunction might synergistically contribute to the seizure-paralysis phenotype and early motor defects, although our diPLA2-VIA−−− flies did not...
Alteration of phospholipid composition by diPLA2-VIA loss promotes α-Syn aggregation. (A–C) α-Syn accumulation by diPLA2-VIA loss is relieved by the LA diet. (A) Brain tissues from 15-d-old flies were stained with the indicated antibodies. An arrowhead indicates ubiquitin- and α-Syn-positive punctum. (Scale bar, 2 μm.) (B) Ubiquitin- and α-Syn–positive puncta in TH-positive neurons are graphed (n = 10 to 15 neurons from 6 flies at 15 d old per sample; *P < 0.05 and **P < 0.01 by 1-way ANOVA with the Tukey-Kramer test). (C) Sarkosyl-soluble and -insoluble fractions from brain tissues from 20-d-old flies were analyzed by Western blot. The band intensities of α-Syn normalized to those of actin are graphed (n = 4; *P < 0.05 and **P < 0.01 by Dunnett’s test). (D and E) α-Syn accumulation caused by diPLA2-VIA loss is suppressed by hiPLA2-VIA or dC19orf12. (D) Brain tissues from 15-d-old flies were stained with the indicated antibodies. An arrowhead indicates ubiquitin- and α-Syn–positive punctum. (Scale bar, 2 μm.) (E) Ubiquitin- and α-Syn–positive puncta in TH-positive neurons are graphed (n = 10 to 15 neurons from 6 flies at 15 d old per sample; *P < 0.05 by 1-way ANOVA with the Tukey-Kramer test). (F) diPLA2-VIA loss promotes the seeding activity of α-Syn. Brain lysates prepared from 15-d-old flies neurally expressing α-Syn were subjected to RT-QUIC. Graphs indicate the time when TdT fluorescence reached an intensity of 165,000 RFU (Left, tRFU) and the slope obtained by differential processing of plot curves (Right), respectively. When TdT fluorescence did not reach 165,000 RFU before 120 h, the value of tRFU was defined as 120 (n = 3 replicates from 3 flies per sample; *P < 0.05 by 1-way ANOVA with the Tukey-Kramer test). N.S., not significant; RFU, relative fluorescence units.

Lipid oxidation is exacerbated in diPLA2-VIA−/− flies, which reveals the role of iPLA2-VIA in removing oxidized acyl chains (14). Although our finding does not exclude the antioxidative role of iPLA2-VIA in the biolipidome, the null cells express α-tocopherol on motor behavior, seizure phenotype, and DA neuron loss indicate that lipid oxidation is not a direct cause of PLAN-linked neurodegeneration. In iPLA2-VIA−/− knockour mice, there are inconsistent results regarding changes in brain lipids, which may be due to differences in experimental methods and the lipid and FA species investigated (25, 54). Currently, it appears to be difficult to evaluate whether our findings regarding lipid changes in Drosophila melanogaster, which has fewer acyl-chain lipids such as DHA- and ARA-containing phospholipids, reflect the events observed in mouse models (58). Further studies will be required to determine the molecular mechanism of acyl-chain shortening and retromer regulation by iPLA2-VIA, especially in mammalian models.

The regulation of MAM by C19orf12 and the up-regulation of C19orf12 during adipocyte differentiation imply C19orf12 involvement in lipid metabolism (35, 59). The rescue of iPLA2-VIA neuronal phenotypes by C19orf12 expression provides molecular insight into the common underlying pathogenesis of PLAN and MPAN. Loss of iPLA2-VIA prominently alters ER morphology in salivary gland cells, and this change may also affect the integrity of the MAM structure, through which PS transported from the ER is converted to PE by mitochondrial PS decarboxylase (60). Although our phospholipid measurement method did not detect PS with high sensitivity, the abundance of PE was not changed by iPLA2-VIA loss, suggesting that PE synthesis is largely dependent on, or is compensatorily bypassed through, the
flies by C19orf12 raises the possibility that the \( \alpha \)B need to be de-
flies exhibited ubiquitinated \( \pm \) flies strongly suggests
Potentials of SUVs with recombinant \( \mu \) for 10 min was separated by clear-
\( \alpha \) and \( \alpha \)diPLA2-VIA < \( \alpha \)0.001 by Dunnett
3t o4 ;*** < \( \alpha \)0.01, and
\( \alpha \)α = < \( \alpha \)Mori et al.
\( \alpha \)< \( \alpha \)0.01, and ***
models, the results of
(24). In the mammalian brain, PUFAs including LA
Drosophila
A shorter acyl-chain composition weakens
lipids modulates
sequential LB formation (28). The interaction between
\( \alpha \)-Syn and \( \alpha \)diPLA2-VIA causes a decrease in the speed and sensitivity of phototransduction in Drosophila (24). In the mammalian brain, PUFAs including LA are supplied through the blood, indicating that altered dietary intake of PUFAs could affect neuronal functions (70). The relationship between dietary intake of FAs and PD has been investigated. Cohort studies showed that dietary intake of vitamin E had little effect on PD risk (71), while intake of unsaturated FAs reduced PD risk (72). In contrast, saturated fat and \( \omega \)-6 PUFAs might be associated with PD risk (73, 74). Although beneficial FAs in humans corresponding to LA in Drosophila need to be determined, our study indicates that the dietary manipulation of FAs is a promising method to control risks for PD and related disorders.

Kennedy pathway. Nevertheless, the rescue of ER stress in diPLA2-VIA\(^{+-}\) flies by C19orf12 raises the possibility that the enhancement of MAM integrity alleviates membrane lipid disequilibrium.

Similar to postmortem studies in INAD and PARK14, diPLA2-VIA\(^{+-}\) flies exhibited ubiquitinated \( \alpha \)-Syn accumulation in DA neurons as well as other types of neurons (Fig. 5 and SI Appendix, Fig. S5), a phenomenon that was rescued by C19orf12; C19orf12 mutations also lead to \( \alpha \)-Syn accumulation and subsequent LB formation (28). The interaction between \( \alpha \)-Syn and lipids modulates \( \alpha \)-Syn fibril formation in different ways, and there have been controversial observations of the pathological roles of the lipid membrane in terms of \( \alpha \)-Syn aggregation (2, 61–63). Our fly models suggest that changes in the acyl-chain composition of phospholipids are more critical for \( \alpha \)-Syn aggregation than changes in the head group. Although we cannot exclude the possibility that decreased unsaturation of acyl chains contributed to neurodegeneration in our Drosophila models, the results of the liposome assay indicate that the status of lipid packing produced by the geometry of acyl chains in the lipid bilayers affects \( \alpha \)-Syn stability. Increased shorter acyl chains would lead to higher membrane curvature and larger packing defects, whereas the polysaturated nature of acyl chains slows the hydrophobic groove (64). While lipid-packing defects facilitate \( \alpha \)-Syn insertion into the membrane (46, 65), \( \alpha \)-Syn loses its affinity to SUVs containing PC with shorter acyl chains (Fig. 6). This inconsistency may derive from differences in the depth of the hydrophobic groove and the state of the gel/liquid crystalline phases generated by different FA compositions (49, 66). Nevertheless, the finding that \( \alpha \)-Syn lacking 30 N-terminal residues (\( \Delta \)N30) has greater in vivo seeding activity than full-length \( \alpha \)-Syn supports our idea (67). The N-terminal helical segment of \( \alpha \)-Syn, composed of 25 residues, has been demonstrated to function as a membrane anchor, and \( \Delta \)N30 failed to bind to liposomes in our assay (SI Appendix, Fig. S6D) (68). Considering the previous related studies, our data suggest that nonmembrane-bound \( \alpha \)-Syn carries a risk of aggregation and/or that the association of \( \alpha \)-Syn with membranes that contain shorter acyl chains contributes to its aggregation (69).

The observation that the correction of phospholipid composition by dietary LA treatment alleviates the ER stress and the neuronal phenotypes of diPLA2-VIA\(^{+-}\) flies strongly suggests that acyl-chain shortening of phospholipids in the brain could be the major pathogenic mechanism underlying PLAN-linked neurodegeneration (SI Appendix, Fig. S7). The administration of a diet containing lower amounts of polysaturated phospholipids in Drosophila need to be determined, our study indicates that the dietary manipulation of FAs is a promising method to control risks for PD and related disorders.

![Fig. 6](image)

**Fig. 6.** A shorter acyl-chain composition weakens \( \alpha \)-Syn association with liposomes. (A and B) Lipid packing status of SUVs. (A) SUVs containing 30% DOPS and 70% of the indicated phospholipids were incubated with 250 nM di-4-ANEPPDHQ, and the fluorescence intensities at 630 and 530 nm were imaged (Upper). The 630-nm signal divided by the thresholded 530-nm signal intensity is also shown (Lower). (Scale bar, 2 \( \mu \)m.) (B) The 630/530-nm intensity ratios are graphed (n = 3 to 4; *P < 0.05, **P < 0.01, and ***P < 0.005 by 1-way ANOVA with the Tukey-Kramer test). (C) Diameter (in nanometers) of SUVs containing 30% DOPS and 70% of the indicated phospholipids (mean ± SEM; 3 trials). (D) \( \alpha \)-Syn does not bind to SUVs containing shorter acyl chains. Five micromolar recombinant \( \alpha \)-Syn incubated with 500 \( \mu \)M SUV as prepared in A–C for 10 min was separated by clear-

![Fig. 7](image)

**Fig. 7.** Working hypothesis. Loss of iPLA2-VIA\(^{-}\) causes the shortening of phospholipid acyl chains in the brain, which alters membrane fluidity, lipid packing, and curvature. \( \alpha \)-Syn affinity to the synaptic membrane is weakened by altered phospholipid properties, leading to \( \alpha \)-Syn aggregation. These altered membrane properties also induce ER stress, affect the dynamics of SVs, and provoke abnormal neurotransmission, which manifests as the bang-sensitivity phenotype. Correction of the membrane composition by dietary intake of FAs or enhanced MAM integrity by C19orf12 rescues neurodegeneration and \( \alpha \)-Syn aggregation.
Materials and Methods

Extended experimental procedures are described in SI Appendix, SI Materials and Methods. All vectors, Drosophila stocks, and primary antibodies are listed in SI Appendix, Tables S1–S3.

Fly Stocks. Fly culture and crosses were performed on standard fly food containing yeast, cornmeal, and molasses, and the flies were raised at 25 °C. Constituent DNAs (cDNAs) for diPLA2-VIA, hct9orf12, and their disease-associated mutations were subcloned into the pUAS attB vector using the In-Fusion HD Cloning Kit (Takara Bio) with the following primer pairs: 5′-GGCCGGCG GCTCGAGGAATCCTGAGCAGACTTGCCTGCATTAGC-3′; 5′-AAATGAT CCTCTAGAGTACCTAAGGTCGAGACGAAGACTCTG-3′ for hct9orf12; and 5′-GGGGCCGGCTCGAGGAATCCTGAGCAGACTTGCCTGCATTAGC-3′; 5′-AAATGATT CCTCTAGAGTACCTAAGGTCGAGACGAAGACTCTG-3′ for diPLA2-VIA. All inserts were verified by sequencing. Vector and fly stocks were generated on the 20697 α-Drosophila background (BestGene). The sequences of the forward and reverse primers for genotyping were as follows:

\[ \text{For: } 5′-TATAATGGAAAGATATCCGGGTGAACTTCGTCGGGCAGCAACGGATAATTTTAACTTGCTGCTGCTCAATACGTTAAATTGAAAATAGGTCCGTGACATCGCTAATAAAGCTGC; \]
\[ \text{Rev: } 5′-AAATGATCTCTGGAGATGGTGGCTCGACGTTAAATTGAAAATAGGTCCGTGACATCGCTAATAAAGCTGC. \]

Quantitative Reverse Transcription-PCR. Full details of quantitative reverse transcription PCR are provided in SI Appendix, SI Materials and Methods.

Electrophysiology. Third-instar larvae were dissected in HL-3, and mEJPs from NMJs were recorded and analyzed using a low-voltage holder (World Precision Instruments) with a Digidata 1550A data acquisition system (Molecular Devices), and a Digidata 1550A data acquisition system (Molecular Devices). Drosophila Behavior Assays and Survival Assay. Full details of Drosophila behavior assays and survival assay are provided in SI Appendix, SI Materials and Methods.

Biochemical Analysis and α-Syn Fractionation. Full details of biochemical analysis and α-syn fractionation are provided in SI Appendix, SI Materials and Methods.

RT-QUIC. Recombinant human α-syn protein was purified from Esherichia coli BL21 harboring pRK172–syn (Y136-TAT) as previously reported (80). A female fly head homogenized in 20 μL of ice-cold phosphate buffered saline (PBS) supplemented with 1/100 protease inhibitor mixture (Nacala) in 1.5-mL tubes was sonicated for 3 min on ice using a sonicator (Branson Sonifier 250, micro tip power 3 to 4, 30 s sonication/30 s pause, 3 times). After centrifugation at 2,000 × g for 2 min at 4 °C to remove debris, the supernatant diluted with PBS at 1:10 was used as a fly brain sample. The RT-QUIC assay was performed according to a reported protocol (41). Briefly, the RT-QUIC reaction buffer (RB) was composed of 100 mM phosphate buffer (pH 8.2), 10 μM ThT, and 0.1 mg/mL recombinant α-syn. Each well of a black 96-well plate with a clear bottom (Nalgene Nunc) contained 95 μL of RB and 37 ± 3 μg of 0.5-mm zirconium/ultralite beads (Thistle Scientific). Reactions were seeded with 5 μL of fly brain samples to a final reaction volume of 100 μL. The plates were sealed with plate sealer film (Greiner Bio-One) and analyzed in a FLUOSstar OPTIMA microplate reader (BMG Labtech) at 30 °C for 120 h with intermittent shaking cycles: double-orbital with 1 min of shaking at 200 rpm followed by 14 min of rest. ThT fluorescence measurements (450 nm excitation and 480 nm emission) were taken every 15 min.

Liposome Assay. DLPC, DOPC, DSPC, PC16:0:14:0, and DOPS were purchased from Avanti Polar Lipids. DLPC/DOPS (7:3), DOPC/DOPS (7:3), DSPC/DOPS (7:3), and PC16:0:14/DOPS (7:3) in chloroform were dried by a nitrogen evaporator. The lipids resuspended in 1 mL of 50 mM NaCl were prepared by sequential extrusion through 0.1-μm and 0.05-μm filters by using a hand extruder (Avanti Polar Lipids). The suspension of 50 μL liposomes with or without recombinant 5 μL α-syn vortexed briefly was incubated for 10 min at RT, and liposome size and z potentials were measured using a Zetasizer NanoZSP (Malvern Instruments). For CN-PAGE, 5 μL α-syn with or without 500 μL liposomes in TBS (pH 7.4) vortexed briefly was incubated for 10 min at RT. The samples were mixed with 4× CN-PAGE sample buffer and separated on a 4 to 16% Bis-Tris gel (Thermo Fisher Scientific). NativeMark Unstained Protein Standard (Thermo Fisher Scientific) was used to estimate molecular mass. The gel was run at 100 V for 100 min and was stained with One-step Coomassie brilliant blue staining reagent (Bio Craft). To assess packing defects, 100 μL liposomes incubated with 250 nM di-4-ANEPPDHQ dye at RT for 30 min were analyzed using confocal microscopy (LSM880; Zeiss).

Drosophila Behavior Assays and Survival Assay. Full details of Drosophila behavior assays and survival assay are provided in SI Appendix, SI Materials and Methods.

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