

Loss of *PLA2G6* leads to elevated mitochondrial lipid peroxidation and mitochondrial dysfunction

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The *PLA2G6* gene encodes a group VIA calcium-independent phospholipase A2 beta enzyme that selectively hydrolyses glycerophospholipids to release free fatty acids. Mutations in *PLA2G6* have been associated with disorders such as infantile neuroaxonal dystrophy, neurodegeneration with brain iron accumulation type II and Karak syndrome. More recently, *PLA2G6* was identified as the causative gene in a subgroup of patients with autosomal recessive early-onset dystonia-parkinsonism. Neuropathological examination revealed widespread Lewy body pathology and the accumulation of hyperphosphorylated tau, supporting a link between *PLA2G6* mutations and parkinsonian disorders. Here we show that knockout of the *Drosophila* homologue of the *PLA2G6* gene, *iPLA2-VIA*, results in reduced survival, locomotor deficits and organismal hypersensitivity to oxidative stress. Furthermore, we demonstrate that loss of *iPLA2-VIA* function leads to a number of mitochondrial abnormalities, including mitochondrial respiratory chain dysfunction, reduced ATP synthesis and abnormal mitochondrial morphology. Moreover, we show that loss of *iPLA2-VIA* is strongly associated with increased lipid peroxidation levels. We confirmed our findings using cultured fibroblasts taken from two patients with mutations in the *PLA2G6* gene. Similar abnormalities were seen including elevated mitochondrial lipid peroxidation and mitochondrial membrane defects, as well as raised levels of cytoplasmic and mitochondrial reactive oxygen species. Finally, we demonstrated that deuterated polyunsaturated fatty acids, which inhibit lipid peroxidation, were able to partially rescue the locomotor abnormalities seen in aged flies lacking *iPLA2-VIA* gene function, and restore mitochondrial membrane potential in fibroblasts from patients with *PLA2G6* mutations. Taken together, our findings demonstrate that loss of normal *PLA2G6* gene activity leads to lipid peroxidation, mitochondrial dysfunction and subsequent mitochondrial membrane abnormalities. Furthermore we show that the *iPLA2-VIA* knockout fly model provides a useful platform for the further study of *PLA2G6*-associated neurodegeneration.

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Abbreviations: D/H-PUFA = deuterated/hydrogenated polyunsaturated fatty acids; D₂-linoleic acid = 11-11-D₂-linoleic acid; D₄-linoleic acid = 11,11,14,14-D₄- α -linolenic acid; TMRM = tetramethylrhodamine methylester

Introduction

The *PLA2G6* gene encodes an 85-kDa group VI calcium-independent phospholipase A₂ beta (PLA2G6). This enzyme hydrolyses the sn-2 acyl chain of glycerophospholipids to release free fatty acids and lysophospholipids (Wolf and Gross, 1996; Ma and Turk, 2001). PLA2G6 localizes to the mitochondria, and has proposed roles in the remodelling of membrane phospholipids, signal transduction and calcium signalling, cell proliferation and apoptosis (Gadd *et al.*, 2006; Seleznev *et al.*, 2006; Strokin *et al.*, 2012).

Humans with *PLA2G6* mutations can show progressive cognitive and motor skill regression, as displayed in disorders such as infantile neuroaxonal dystrophy, neurodegeneration with brain iron accumulation type II and Karak syndrome (Khateeb *et al.*, 2006; Morgan *et al.*, 2006). Infantile neuroaxonal dystrophy is a neurodegenerative disease with onset in infancy and fatality in the teenage years or in early adulthood. It is characterized neuropathologically by axonal swelling and the presence of spheroid bodies in the central and peripheral nervous systems in addition to hallmark cerebellar atrophy. Neurodegeneration with brain iron accumulation comprises a clinically and genetically heterogeneous group of disorders with a progressive extrapyramidal syndrome and high basal ganglia iron, and includes pantothenate kinase-associated neurodegeneration caused by mutations in *PANK2* (neurodegeneration with brain iron accumulation type I). Post-mortem examination of the brain of a patient with neurodegeneration with brain iron accumulation associated with homozygous *PLA2G6* mutations demonstrated both Parkinson's and Alzheimer's disease pathology with widespread Lewy bodies, dystrophic neurites and cortical neuronal neurofibrillary tangles (Gregory *et al.*, 2008).

Furthermore, *PLA2G6* has also been implicated in a number of other brain diseases including Alzheimer's disease (Schaeffer and Gattaz, 2008) and bipolar disorder (Xu *et al.*, 2013). More recently, *PLA2G6*, at the PARK14 locus, has also been characterized as the causative gene in a subgroup of patients with autosomal recessive early-onset dystonia-parkinsonism. Interestingly these patients did not display cerebellar atrophy or basal ganglia iron on MRI (Paisan-Ruiz *et al.*, 2009), and neuropathological examination revealed widespread Lewy body pathology and the accumulation of hyperphosphorylated tau (Paisan-Ruiz *et al.*, 2012). These clinical and neuropathological features further support a link between *PLA2G6*

mutations and parkinsonian disorders, and demonstrate the clinical heterogeneity in *PLA2G6*-associated neurodegeneration.

It is not known how mutations in *PLA2G6* cause neuropathology. However, it has been suggested that the loss of normal *PLA2G6* activity in neurodegenerative disease involves structural abnormalities of cell or mitochondrial membranes and disturbances in lipid homeostasis and lipid metabolism. Indeed, a study in a mouse *Pla2g6* knockout model demonstrated ultrastructural abnormalities in mitochondrial and synaptic membranes (Beck *et al.*, 2011). As well as possible mitochondrial defects, it is thought that changes in the composition of the plasma membrane and other intracellular membranes may in turn affect the normal processes responsible for the movement of membranes within axons and dendrites, subsequently leading to accumulation of membranes as so-called spheroids.

Support for a loss-of-function of *PLA2G6* enzyme activity in causing disease comes from a recent study on recombinant wild-type and mutant human *PLA2G6* proteins. This demonstrated that mutations in *PLA2G6* associated with infantile neuroaxonal dystrophy or neurodegeneration with brain iron accumulation resulted in encoded proteins that exhibited <20% of control levels of phospholipase and lysophospholipase activities. Conversely, mutations associated with dystonia-parkinsonism did not impair catalytic activity. The differential enzymatic activities associated with *PLA2G6* mutations in infantile neuroaxonal dystrophy/neurodegeneration with brain iron accumulation and dystonia-parkinsonism may explain the relatively later onset and milder phenotype seen in the latter. Furthermore, the preserved enzymatic function of dystonia-parkinsonism causing mutations must be interpreted with caution as there may be differences in *PLA2G6* activity *in vivo* that are not detected by these *in vitro* assays. For example, there may be alternative mechanisms that alter the regulation of *PLA2G6* activity, such as changes in the binding to calmodulin or other proteins (Engel *et al.*, 2010). Further support for the loss of enzymatic function hypothesis comes from a recent study of a Chinese population with Parkinson's disease, which identified novel *PLA2G6* mutations occurring in the heterozygous state, associated with an inhibition in the phospholipid-hydrolysing functions of *PLA2G6* (Gui *et al.*, 2013). Moreover Gregory *et al.* (2008) found a genotype–phenotype correlation in patients with infantile neuroaxonal dystrophy and neurodegeneration with brain iron accumulation: mutations that are predicted to lead to an absence of protein were associated with more severe

infantile neuroaxonal dystrophy-type clinical phenotypes, while those with compound heterozygous missense mutations correlated with the less severe phenotype of neurodegeneration with brain iron accumulation and are predicted to result in protein with some residual enzyme function.

Genetic ablation of *Pla2g6* in mice is associated with an *in vivo* disturbance of brain phospholipid metabolism (Cheon *et al.*, 2012), mitochondrial membrane degeneration (Beck *et al.*, 2011) and marked cerebellar atrophy (Zhao *et al.*, 2011). Furthermore, *in vitro* experiments demonstrated a disturbance in calcium signalling in astrocytes from *PLA2G6*-deficient mice (Strokin *et al.*, 2012). However, despite these studies, the precise molecular mechanisms linking loss of *PLA2G6* activity to mitochondrial morphological abnormalities and neurodegeneration remain unclear and further studies are required.

The fruit fly *Drosophila melanogaster* has proven to be an excellent model system for studying neurodegenerative diseases, especially the role of mitochondrial activity in Parkinson's disease (Clark *et al.*, 2006; Park *et al.*, 2006). Once a faithful model is established, the fly has the advantage over mouse models in that it can be used to perform high-throughput screening to identify genetic modifiers and novel therapeutic targets. Here we show that *iPLA2-VIA*, the *Drosophila* homologue of *PLA2G6*, plays an essential role in maintaining normal mitochondrial function. We demonstrate that knockout of the *iPLA2-VIA* gene activity in the fly results in reduced survival, locomotor deficits and organismal hypersensitivity to oxidative stress. Furthermore, loss of *iPLA2-VIA* function leads to a number of mitochondrial abnormalities, including reduced mitochondrial membrane potential, mitochondrial respiratory chain dysfunction and reduced ATP synthesis. We also show that levels of lipid peroxidation are significantly elevated in the brains of flies lacking *iPLA2-VIA*. In addition, we examined cultured fibroblasts taken from two patients with known *PLA2G6* mutations and found similar abnormalities to those seen in the fly, including mitochondrial membrane abnormalities and raised levels of cytoplasmic and mitochondrial reactive oxygen species. Moreover, lipid peroxidation levels, especially in the mitochondria, were elevated in the *PLA2G6* mutant human fibroblasts compared to controls. Taken together, our findings suggest that loss of normal *PLA2G6* gene activity leads to mitochondrial lipid peroxidation, mitochondrial dysfunction and mitochondrial membrane abnormalities. Finally we demonstrate that reduction of lipid peroxidation, by treatment with deuterated polyunsaturated fatty acids (D-PUFAs), is able to recover the mitochondrial membrane potential of *PLA2G6* mutant human fibroblasts and partially rescue the locomotor deficits in *iPLA2-VIA* knockout flies. We have therefore shown that the *iPLA2-VIA* knockout fly appears to be a useful model organism for studying *PLA2G6*-associated neurodegeneration.

Materials and methods

Fly stocks and husbandry

The fly stocks $y[1] w[67c23]; P\{w[+mC] y[+mDint2]=EPgy2\}iPLA2-VIA[EY05103]$, *actin-5C-GAL4*, *elav-GAL4^{cl55}* were obtained from the Bloomington *Drosophila* Stock Centre. The *iPLA2-VIA* RNAi (HMS01544) line was provided by the TRiP at Harvard Medical School. All fly strains used were backcrossed at least six generations into the *w¹¹¹⁸* background to obtain isogenic lines. All fly stocks were maintained at 25°C on a 12:12 hour light: dark cycle at constant humidity on a standard sugar-yeast (SY) medium (15 g/l agar, 50 g/l sugar, 100 g/l autolysed yeast, 100 g/l nipagin and 3 ml/l propionic acid). For all experiments, flies were raised at a standard density on standard SY medium in 200 ml bottles unless otherwise stated. Tissue-specific expression of *iPLA2-VIA* RNAi constructs was achieved by using the *GAL4-UAS* system [*GAL4*-dependant upstream activator sequence (Brand and Perrimon, 1993)].

Lifespan analyses

The survival assays were performed using newly eclosed flies that were allowed to mature and mate for 24 h before the females and males were separated and collected. One hundred and fifty flies were housed in groups of 15 and the flies were transferred every 2 to 3 days onto fresh food and the number of dead flies recorded. Data are presented as cumulative survival curves, and survival rates were compared using log-rank tests.

Climbing assays

The climbing assay was performed using a negative geotaxis assay according to previously published methods (Rival *et al.*, 2004). Briefly, 15 adult flies were placed in a vertical column (25 ml tissue culture pipette), and allowed to climb for 45 s before the number of flies at the top and bottom was determined. A performance index (PI) defined as $0.5(n(\text{total}) + n(\text{top}) - n(\text{bottom})/n(\text{total}))$ was calculated. The experiment was repeated three times for each time point.

Fertility tests

For female fecundity tests, female flies were housed with males for 48 h post-eclosion and then separated into vials at a density of 10 females per vial. Eggs were collected over a 24-h period at different time points. The number of eggs laid per vial at each time point was counted.

Quantitative RT-PCR

Total RNA was extracted from 15 flies per sample using TRIzol[®] (GIBCO) according to the manufacturer's instructions. The concentration of total RNA purified for each sample was measured using an Eppendorf biophotometer. One microgram of total RNA was then subjected to DNA digestion using DNase I (Ambion), immediately followed by reverse transcription using the SuperScript[®] II system

(Invitrogen) with oligo(dT) primers. Quantitative PCR was performed using the PRISM 7000 sequence-detection system (Applied Biosystems), SYBR[®] Green (Molecular Probes), ROX Reference Dye (Invitrogen), and HotStarTaq (Qiagen) by following the manufacturer's instructions. Each sample was analysed in triplicate with both target gene (*iPLA2-VIA*) and reference gene (*RP49*) primers in parallel. The end products of the RT-PCR reactions were also visualized by staining with ethidium bromide following separation with agarose-gel electrophoresis. The primers for the *Drosophila iPLA2-VIA* used in the RT-PCR of the EY05103 fly line were as follows: forward 5'-TACTGGAATTGTGCGATAA GG-3' and reverse 5'-GATGTGGTATTGGAATCCGAG-3'. The primers used for the RT-PCR performed on the *iPLA2-VIA* RNAi (HMS01544) fly line were as follows: forward 5'-AACTriacylglycerolTAGTGCCGATCGTCCAA-3' and reverse 5'-GAACCAGTATCCTTGCAGCG-3'. The reference gene primers were as follows: forward 5'-ATGACCAT CCGCCCAGCATCAGG-3' and reverse 5'-ATCTCGCCGAGTAAACG-3'.

Stress experiments

For all stress assays, flies were reared and housed as for life-span experiments. For oxidative stress assays, 7-day or 15-day old flies were transferred onto 5% sucrose/agar containing 5% hydrogen peroxide (Sigma) or 20 mM paraquat (Sigma). For starvation and osmotic stress experiments, 7-day or 15-day old flies were transferred to 1% agar or 500 mM NaCl respectively. For hypoxia experiments 15-day old flies were transferred to a hypoxia chamber with oxygen levels of 7%. After 15 h the flies were removed from the chamber and the number of dead flies was counted.

Triacylglyceride and ATP assays

Levels of triacylglyceride in adult females were measured using the Triglyceride Infinity Reagent (ThermoScientific) and normalized to total body protein using a BCA assay (Sigma). The ATP concentration of whole female flies was determined using the Roche ATP Bioluminescence Assay Kit HS II (Roche). Briefly, two live whole flies or eight fly heads were homogenized in 100 µl ice-cold lysis buffer (provided in the kit) for 1 min using a Kontes pellet pestle. The lysate was then boiled for 5 min and centrifuged at 20 000g for 1 min. 2.5 µl of cleared lysate was added to 187.5 µl dilution buffer and 10 µl luciferase and the luminescence was immediately measured using a Tecan Infinite M2000 microplate reader and Magellan V6.5 software. Each reading was converted to the amount of ATP per fly based on the standard curve generated with ATP standards. At least three measurements were made for each genotype and the experiment was repeated three times.

Isolation of *Drosophila* mitochondria

Fly mitochondria were isolated by differential centrifugation, essentially as described in Miwa *et al.* (2003). Briefly, adult flies ($n = 500$ for each time point) were chilled on ice and gently pressed using a pre-chilled pestle and mortar. Importantly, the pestle was moved in a vertical motion (with no horizontal motion) until the shape of the flies was no longer

visible. The flies were then washed in STE + BSA buffer [250 mM sucrose, 5 mM Tris, 2 mM EGTA, pH 7.4 (4°C), 1% bovine serum albumin]. The flies were then pressed further in 5 ml of STE + BSA buffer. The squashed flies were then passed through double-layered muslin cloth. The collected pulp was then spun for 3 min at 4°C at 1500 rpm to remove debris. The supernatant was then passed through a single layer of muslin into a clean centrifuge tube and spun for 10 min at 4°C and 10 000 rpm to collect the mitochondria. The mitochondrial pellet was then suspended in 250 µl STE + BSA buffer and stored on ice. A BCA assay (Sigma) was used to determine the protein concentration of the mitochondrial preparations. The isolated mitochondria were kept on ice and used immediately for experiments.

Drosophila mitochondrial respiration measurement

Oxygen consumption was measured using a Clark-type oxygen electrode thermostatically maintained at 25°C. Glutamate (5 mM) and malate (5 mM) or pyruvate (5 mM) were added to measure Complex I-linked respiration, and succinate (5 mM) with rotenone (5 µM) were added to measure Complex II-linked respiration. All data were obtained using an Oxygraph Plus system with Chart recording software.

Light and electron microscopy of fly brains

Flies were decapitated and the proboscis was removed to allow penetration of the fixative. The heads were perfused with 3% glutaraldehyde in phosphate buffer overnight. Brains were then treated with 1% osmium tetroxide for 3 h at 4°C and embedded in Araldite[®] CY212 resin. Thin sections were stained with toluidine blue and the brain visualized with the light microscope. Ultrathin sections (70 µm) were stained with lead citrate and uranyl acetate and digital images taken on a Phillips CM10 electron microscope with a Megaview III digital camera (Olympus).

Human fibroblast collection and culture

The p.R747W *PLA2G6* fibroblasts were generated from a 4 mm skin punch biopsy taken under local anaesthetic. Biopsies were dissected into ~1 mm pieces and cultured in 5 cm petri dishes in Dulbecco's modified Eagle's medium (DMEM), 10% foetal bovine serum, 1% L-glutamine until fibroblasts were seen to grow out from the explants. When fibroblasts reached confluency, they were detached from culture dishes using TrypleE[™] (Invitrogen) and transferred to larger culture vessels for further expansion and cryopreservation. This sample was collected with the written consent of the participant and formal ethical approval from the National Hospital for Neurology and Neurosurgery–Institute of Neurology Joint Research Ethics Committee (London, UK). The GM11529 mutant *PLA2G6* human fibroblast cell cultures were obtained from the Coriell Cell Repositories. This line was sampled from a patient with infantile neuroaxonal dystrophy.

Fibroblasts were seeded at a density of 4×10^4 cells/cm², grown to 75–80% confluence and maintained at 37°C and 5% CO₂ in DMEM supplemented with 10% (v/v) foetal bovine serum, 2 mM L-glutamine and 1% (v/v) penicillin/streptomycin.

Measurement of mitochondrial membrane potential and cytosolic and mitochondrial reactive oxygen species

For measurements of mitochondrial membrane potential ($\Delta\Psi_m$), dissected fly brains or cells were loaded with 25 nM tetramethylrhodamine methylester (TMRM) for 30 min at room temperature and the dye was present for the duration of the experiment. TMRM is used in the redistribution mode to assess $\Delta\Psi_m$, and therefore a reduction in TMRM fluorescence represents depolarization.

Confocal microscopy of mitochondrial membranes in fly brains and human fibroblasts

Confocal images were obtained using a Zeiss 710 VIS CLSM equipped with a META detection system and a $\times 40$ oil immersion objective. Illumination intensity was kept to a minimum (at 0.1–0.2% of laser output) to avoid phototoxicity and the pinhole set to give an optical slice of $\sim 2 \mu\text{m}$. TMRM was excited using the 560 nm laser line and fluorescence measured above 580 nm. All data presented were obtained from at least five coverslips and two to three different cell preparations for cell experiments, and six coverslips and at least six counts for fly brain experiments. The same anatomical area of the fly brain was used each time.

Measurement of reactive oxygen species levels in human fibroblasts

For measurement of mitochondrial reactive oxygen species production, cells were preincubated with 5 μM MitoSOXTM (Molecular Probes) for 10 min at room temperature. Cytosolic reactive oxygen species generation was measured with 2 μM dihydroethidium with the dye present in all solutions throughout the duration of the experiment.

Fluorescence images were obtained on an epifluorescence inverted microscope equipped with a $\times 20$ fluorite objective (Cairn Research). Ratiometric dihydroethidium and MitoSOX fluorescence was recorded with an excitation light at 380 and 530 nm and emission at 440 and 630 nm, respectively.

Measurement of lipid peroxidation in fly brains and human fibroblasts

C11-BODIPY581/591 (Molecular Probes) is a sensitive fluorescent probe for indexing lipid peroxidation in model membrane systems and living cells undergoing a shift from red to green fluorescence emission upon oxidation, even under

physiological oxidation (Vaarmann *et al.*, 2010). Fly brains were dissected in phosphate-buffered saline and placed into Hank's balanced salt solution (HBSS). C11-BODIPY581/591 (5 μM) was loaded for 15 min at 20°C. Coverslips were washed once and resuspended in 1 ml of HBSS. Ratiometric measurements of probe oxidation were taken over a 12 min time course. Fluorescence excitation was at 488 nm, green fluorescence was detected at 530 nm and red fluorescence at 670 nm.

Cardiolipin analysis by mass spectrometry

Cardiolipin and oxidized cardiolipin were measured by mass spectrometry essentially as described previously (Pope *et al.*, 2008; Heather *et al.*, 2010; Rodriguez-Cuenca *et al.*, 2010) with some minor modifications to make the analysis specific for *Drosophila* cardiolipin species. In brief, 500 flies of each genotype were homogenized in NaPi buffer (50 mM NaPi, 1 mM EDTA, 100 μM DTPA, pH 7.5) using a Kontes pellet pestle. The homogenates were then spun for 2 min at 13 000 rpm to remove fly debris. Lipids were extracted using a modified Folch extraction and the combined organic layers were evaporated to dryness under nitrogen. Before analysis, samples were resuspended in 1 ml of a 1:1 mix of buffers A and B (buffer A, 15% water, 85% methanol containing 1 ml of 250 ml/l aqueous ammonia per litre; buffer B, 97% chloroform, 3% methanol containing 0.1 ml of 250 ml/l aqueous ammonia per litre). 1 nmol of (C14:0)₄-cardiolipin (Avanti Polar Lipids) was used as an internal standard and was added to *Drosophila* head homogenates.

The LC/MS/MS system (Waters 2795 separation unit and a Quattro Micro API triple quadrupole mass spectrometer) and parameters were as described previously (Heather *et al.*, 2010; Rodriguez-Cuenca *et al.*, 2010) except that the multiple reaction monitoring (MRM) transitions were specific for *Drosophila* cardiolipin. Mass spectra from retention times 5.5–6.0 min were combined to generate cardiolipin spectra. Cardiolipin species were identified in MRM mode with the collision gas at 0.25 Pa and collision energy at 35 eV. Cardiolipin species were analysed as their dianions $[\text{CL}-2\text{H}]^{2-}$. In these samples, the dominant form of cardiolipin was (C16:1)₄-cardiolipin (m/z 671), which was therefore selected for analysis based on its fragmentation to a C16:1 fatty acid (m/z 253) by monitoring the 671 > 253 transition. Similarly, other cardiolipin species (C18:2)₁(C16:1)₃-cardiolipin (m/z 684) and (C18:2)₂(C16:1)₂-cardiolipin (m/z 697) were analysed by assessing transitions of 684 > 253 and 697 > 253, respectively. Oxidized cardiolipin was analysed by the transitions 678 > 253 and 691 > 253 transition. The internal standard (C14:0)₄-cardiolipin (m/z 619) was analysed by its fragmentation to a C14:0 fatty acid (m/z 227) using the transition 619 > 227. Data were analysed with QuanLynx software (Waters). Total cardiolipin content was expressed as peak area relative to that of the internal standard. The amounts of each cardiolipin and oxidized cardiolipin were expressed relative to the total amount of cardiolipin in the sample, giving dimensionless ratios.

Deuterated polyunsaturated fatty acids

Deuterated polyunsaturated fatty acids (D-PUFAs) 11,11-D₂-linoleic acid (D₂-linoleic acid) and 11,11,14,14-D₄- α -linolenic acid (D₄-linoleic acid) were kindly provided by Dr Mikhail S. Shchepinov (Retrotepe, Inc), and their synthesis has been previously described (Hill *et al.*, 2012). Hydrogenated polyunsaturated fatty acids (H-PUFAs) were obtained from Sigma–Aldrich (99%). Cells were preincubated with 10 μ M H- or D-PUFA (D₄-linoleic acid) in the culture media for 24 h and then washed with HBSS before experiments. For the fly experiments, D₂-linoleic acid was diluted in water and added to the standard fly medium to give a final concentration of 10 or 100 μ M.

Statistics

Data were expressed as means \pm standard error of the mean (SEM). Significant differences were evaluated by unpaired two-tailed Student's *t*-test and significance levels are described in individual figure legends. Climbing assays were analysed using a two-way ANOVA or a one-way ANOVA with Bonferroni correction.

Results

Drosophila lacking *iPLA2-VIA* activity display age-dependent locomotor deficits and reduced lifespan

A null mutant fly stock completely lacking the *Drosophila* orthologue of the *PLA2G6* gene, *iPLA2-VIA*, was obtained from the Bloomington *Drosophila* Stock Centre. We will refer to null mutant flies lacking both *iPLA2-VIA* genes as *iPLA2-VIA*^{-/-} henceforth. This fly strain carries a P-element insertion (EY05103) in the *iPLA2-VIA* gene (Fig. 1A). As previously shown, RT-PCR analysis confirmed that there was complete knockout of *iPLA2-VIA* gene expression in this strain with no detectable SYBR green signal produced. The end products of the RT-PCR reactions were also visualized on agarose gel electrophoresis, which confirmed the complete absence of *iPLA2-VIA* gene expression in the *iPLA2-VIA*^{-/-} flies (Fig. 1B) (Malhotra *et al.*, 2009). Knockout of *iPLA2-VIA* activity in the fly did not produce any gross changes in body size or obvious differences in morphology (data not shown). *iPLA2-VIA* is expressed throughout the adult fly body, including the brain and eye. The *iPLA2-VIA* gene displays good homology with the human *PLA2G6* gene, with a 51% identity and 67% positivity (FlyBase).

The *iPLA2-VIA*^{-/-} flies displayed progressive locomotor deficits, with the climbing ability of *iPLA2-VIA*^{-/-} flies in response to light tapping progressively falling below that of *w*¹¹¹⁸ controls from ~20 days of age (Fig. 1C). Accompanying this climbing defect the *iPLA2-VIA*^{-/-} flies also showed an ~50% reduction in lifespan, in both

females (Fig. 1D) and males (Supplementary Fig. 1A). Furthermore, flies heterozygous for the EY05103 P-element insertion (*iPLA2-VIA*), with presumed 50% gene activity only, did not display reduced survival (Fig. 1D and Supplementary Fig. 1A). To ensure that the reduced lifespan was, indeed, due to the EY05103 P-element insertion, we generated flies with precise excisions of the P-element, by introducing a transposase in an appropriate cross, and found that this restored a normal lifespan (Supplementary Fig. 1B).

To confirm the effect of *iPLA2-VIA* deficiency in the fly, we also inhibited *iPLA2-VIA* gene expression by transgenic RNA interference (RNAi) technology, using the *GAL4/UAS* system (Brand and Perrimon, 1993). Ubiquitous expression of *iPLA2-VIA* dsRNAi (*UAS-iPLA2-VIA-RNAi*) with the *actin-GAL4* driver resulted in a ~40% reduction in whole body *iPLA2-VIA* gene expression using RT-PCR analysis (Supplementary Fig. 1C). This reduction was associated with an age-dependent decrease in climbing ability (Fig. 1E) and a shortened lifespan (Fig. 1F). It can therefore be robustly concluded that genetic inactivation of *iPLA2-VIA* activity results in progressive locomotor deficits and a reduction in lifespan in *Drosophila*. To determine whether these phenotypes were specific to loss of *iPLA2-VIA* activity in the brain, we knocked down *iPLA2-VIA* in fly neural tissue using *UAS-iPLA2-VIA-RNAi* driven by the *elav*^{c155}-*Gal4* driver. We observed a significant reduction in lifespan in these flies (Fig. 1G), confirming the brain-specific effects of *iPLA2-VIA* deficiency.

We also assessed the fecundity of *iPLA2-VIA*^{-/-} female flies. The number of eggs laid per female significantly declined with age compared with age-matched *w*¹¹¹⁸ control flies, and by Day 25 the null mutant flies were almost completely infertile (Supplementary Fig. 1D).

Brains lacking *iPLA2-VIA* display severe, widespread neurodegeneration and mitochondrial degeneration

Light microscopic examination of thin paraffin sections of *iPLA2-VIA*^{-/-} fly brain revealed normal brain architecture in young Day 2 flies that was indistinguishable from *w*¹¹¹⁸ controls (Fig. 2A and B). However, by Day 32 there was significant widespread vacuolation in the *iPLA2-VIA*^{-/-} fly brains that was not present in *w*¹¹¹⁸ control flies (Fig. 2C and D). Ultrastructural examination using electron microscopy revealed that the mitochondria were grossly abnormal and swollen with fragmented cristae in aged Day 32 *iPLA2-VIA*^{-/-} fly brains as compared to the mitochondria in age-matched *w*¹¹¹⁸ control brains (Fig. 2E–H) or younger Day 2 *iPLA2-VIA*^{-/-} flies (data not shown), which were both filled with densely packed cristae. Furthermore, examination of the fly eye using electron microscopy revealed abnormal retinal structure in the aged Day 32 *iPLA2-VIA*^{-/-} flies compared with age-matched

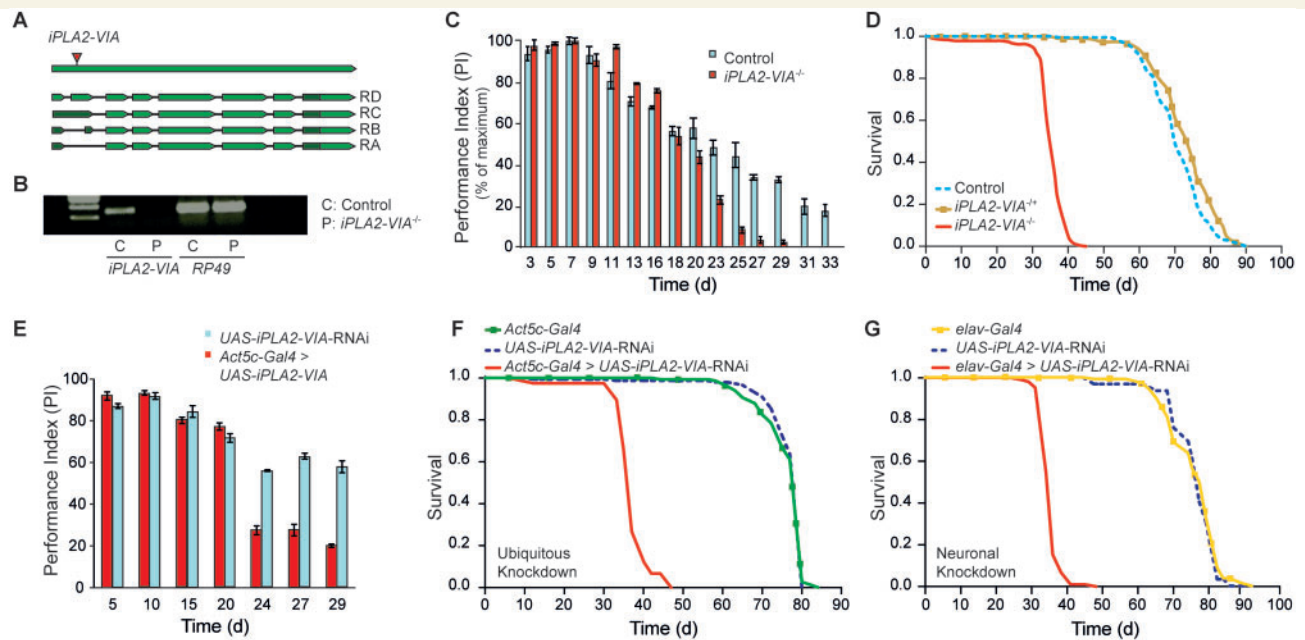


Figure 1 Lack of *iPLA2-VIA* gene activity leads to reduced survival and locomotor deficits. **(A)** Schematic of the *Drosophila iPLA2-VIA* gene, showing the position of the P-element insertion (arrowhead). The *iPLA2-VIA* gene has four main transcripts: *iPLA2-VIA-RB*, *iPLA2-VIA-RC*, *iPLA2-VIA-RD* and *iPLA2-VIA-RA* (FlyBase). The position of the primers has been previously shown (Malhotra et al., 2009). **(B)** RT-PCR analysis of *iPLA2-VIA*^{-/-} flies shows that there is no mRNA expression of the *iPLA2-VIA* gene compared with *w*¹¹¹⁸ control flies. The end products of the RT-PCR reaction were run on an agarose gel. *RP49* is an internal control gene and is expressed in both the *iPLA2-VIA*^{-/-} and control *w*¹¹¹⁸ flies. **(C)** *iPLA2-VIA*^{-/-} flies display an age-dependent reduction in climbing ability compared with *w*¹¹¹⁸ controls ($n = 45$ flies per genotype, three repeats per genotype, $P < 0.05$). Climbing ability is expressed as a performance index (PI) over time in days (d). **(D)** Female flies lacking both *iPLA2-VIA* genes (*iPLA2-VIA*^{-/-}) have a significantly reduced lifespan compared with *w*¹¹¹⁸ control flies ($P < 0.0001$). Furthermore female flies lacking only one *iPLA2-VIA* gene (*iPLA2-VIA*^{+/-}; $n = 150$ flies per genotype) do not display a reduction in lifespan. **(E)** Ubiquitous downregulation of *iPLA2-VIA* activity using sRNAi (*UAS-iPLA2-VIA-RNAi*) driven by *Act5c-Gal4* (*Act5c-Gal4 > UAS-iPLA2-VIA-RNAi*) results in an age-dependent reduction in climbing ability compared with control *UAS-iPLA2-VIA-RNAi* flies ($P < 0.05$; $n = 45$ flies per genotype, three repeats each). **(F)** Knockdown of *iPLA2-VIA* activity using *iPLA2-VIA* sRNAi (*UAS-iPLA2-VIA-RNAi*) driven ubiquitously by *Act5c-Gal4* (*Act5c-Gal4 > UAS-iPLA2-VIA-RNAi*) results in a decrease in lifespan compared with control non-expressing *UAS-iPLA2-VIA-RNAi* flies ($P < 0.0001$) or driver *Act5c-Gal4* flies ($P < 0.0001$), similar to that seen with the null mutant flies (*iPLA2-VIA*^{-/-}; $n = 150$ flies per genotype). **(G)** Knockdown of *iPLA2-VIA* activity using sRNAi (*UAS-iPLA2-VIA-RNAi*) driven in the fly nervous system using the *elav-Gal4* driver results in a decrease in lifespan compared with control non-expressing *UAS-iPLA2-VIA-RNAi* flies ($P < 0.0001$), or driver *elav-Gal4* ($P < 0.0001$; $n = 150$ flies per genotype), similar to that seen with the null mutant flies (*iPLA2-VIA*^{-/-}).

control flies, with grossly abnormal ommatidial structure (Supplementary Fig. 2A and B).

Loss of *iPLA2-VIA* resulted in reduced mitochondrial membrane potential and abnormal mitochondrial respiratory chain activity

Given the abnormal mitochondrial membrane morphology in older *iPLA2-VIA*^{-/-} flies, we investigated whether loss of *iPLA2-VIA* affected mitochondrial membrane potential ($\Delta\Psi_m$), an indicator of mitochondrial health and function. Mitochondrial membrane potential was assessed in the brains of 25-day-old flies using TMRM fluorescence and imaging with confocal microscopy. This revealed a significant reduction in mitochondrial membrane potential in *iPLA2-VIA*^{-/-} fly brains compared to controls (Fig. 3A). We next isolated mitochondria from *iPLA2-VIA*^{-/-} flies

and assessed respiratory chain activity by measuring oxygen consumption using a Clark-type oxygen electrode. The steps in respiration were compared in mitochondria isolated from *iPLA2-VIA*^{-/-} flies and control *w*¹¹¹⁸ flies with substrates and inhibitors specific to individual respiratory complexes. We found that knockout of *iPLA2-VIA* activity conferred a decrease in complex I and II-dependent respiration (Fig. 3B), manifested as significantly decreased oxygen consumption in state 3 respiration, whether using substrates for complex I (5 mM glutamate and 5 mM malate) or for complex II (5 mM succinate in the presence of rotenone) (Fig. 3B). The respiratory control ratio is the ratio of state 3 respiration (ADP stimulated) to state 4 respiration (no ADP present), and is considered an indication of the degree of coupling of mitochondrial respiratory chain activity to oxidative phosphorylation (Chance and Williams, 1955). The respiratory control ratio was significantly lower in *iPLA2-VIA*^{-/-} mitochondria compared with age-matched control *w*¹¹¹⁸ flies (Fig. 3C), because of

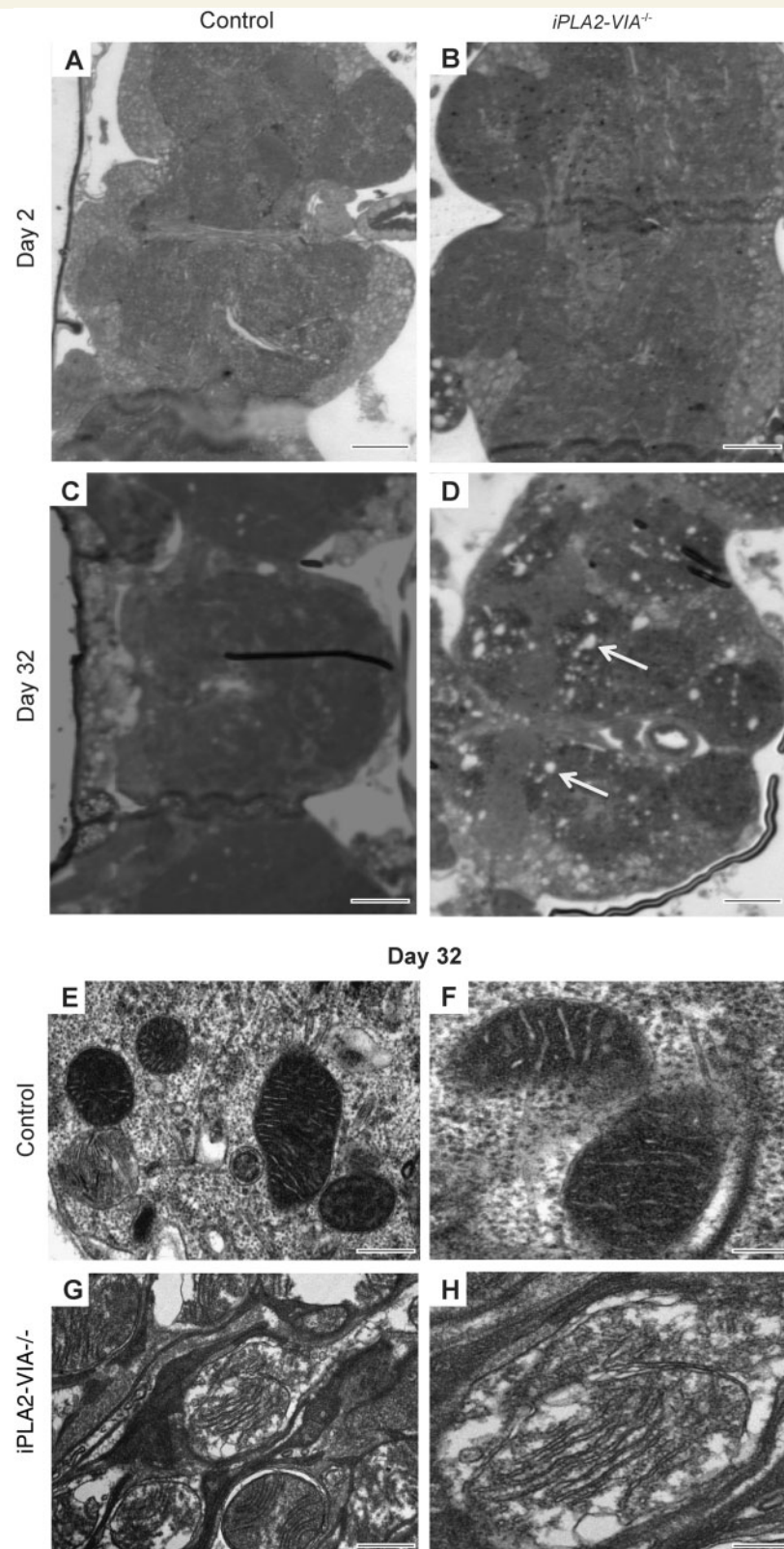


Figure 2 Fly brains lacking *iPLA2-VIA* display severe mitochondrial degeneration at the ultrastructural level. (A) Light microscopic examination of Day 2 *w¹¹¹⁸* control (scale bar = 20 μ m) and (B) *iPLA2-VIA*^{-/-} fly brains (scale bar = 30 μ m). (C) At Day 32 *w¹¹¹⁸* control fly brains display healthy brain tissue compared with (D) *iPLA2-VIA*^{-/-} flies brains, which display widespread vacuolation (arrows) ($\times 60$ magnification; scale bars 20 = μ m). (E and F) Ultrastructural examination of aged Day 32 *w¹¹¹⁸* control fly brains ($\times 2500$; scale bar = 0.6 μ m and $\times 5300$; scale bar = 0.2 μ m) revealed normal mitochondrial morphology, which is in contrast to (G and H) Day 32 *iPLA2-VIA*^{-/-} fly brains, which display significant degeneration of the mitochondrial cristae ($\times 3100$; scale bar = 0.5 μ m and $\times 5300$; scale bar = 0.2 μ m). Images representative of six fly brains examined for each genotype. No abnormal mitochondria were seen in the *w¹¹¹⁸* control fly brains.

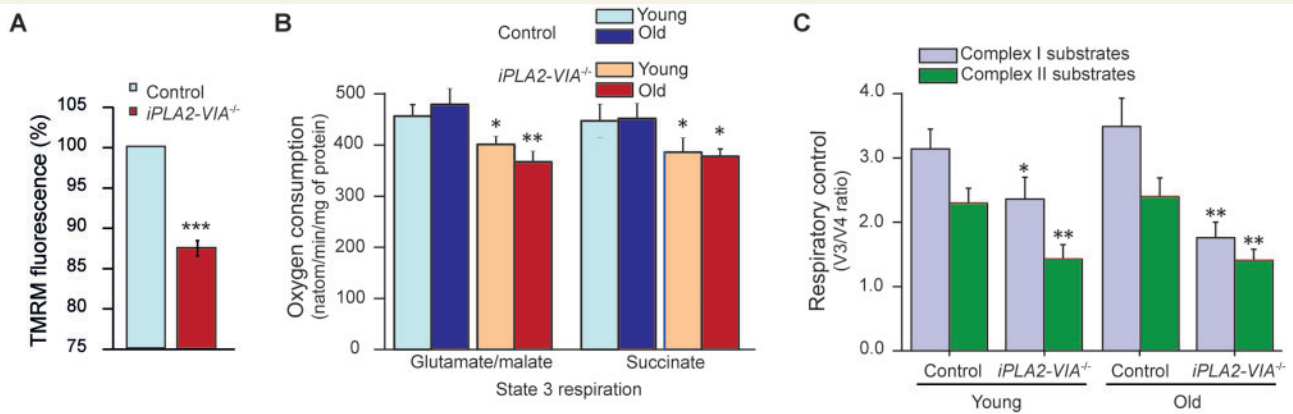


Figure 3 Flies lacking *iPLA2-VIA* have reduced mitochondrial membrane potential and decreased mitochondrial respiratory chain activity. **(A)** The mitochondrial membrane potential measured using TMRM fluorescence in the brains of 25-day-old flies was significantly decreased in *iPLA2-VIA*^{-/-} brains compared to *w*¹¹¹⁸ controls (***P* < 0.001; *n* = 6 brains for each genotype). **(B)** The respiratory chain activity was measured by assessing oxygen consumption and demonstrated that mitochondria from young Day 2 and old Day 32 *iPLA2-VIA*^{-/-} flies have reduced complex I and II-dependent respiration compared with *w*¹¹¹⁸ control flies, manifested as significantly decreased oxygen consumption in state 3 respiration (**P* < 0.05, ***P* < 0.001). **(C)** The respiratory control ratio was significantly lower in *iPLA2-VIA*^{-/-} mitochondria compared with age-matched control *w*¹¹¹⁸ mitochondria. Furthermore, the respiratory control ratio of *iPLA2-VIA*^{-/-} flies in the presence of glutamate/malate was dependent on the age of the flies: older flies having lower respiratory control ratio values (**P* < 0.05, ***P* < 0.001).

the inhibition of state 3 respiration. Furthermore, the respiratory control ratio of *iPLA2-VIA*^{-/-} flies in the presence of glutamate/malate was dependent on the age of the flies: older flies having lower respiratory control ratio values (Fig. 3C). Thus, loss of *iPLA2-VIA* activity reduces mitochondrial membrane potential as a result of mitochondrial uncoupling, which increases with age in the *iPLA2-VIA*^{-/-} flies.

Loss of *iPLA2-VIA* activity results in reduced ATP levels and increased sensitivity to oxidative stress

In keeping with the mitochondrial dysfunction caused by loss of *iPLA2-VIA* activity, we also demonstrated that ATP levels in aged, Day 25, *iPLA2-VIA*^{-/-} fly heads were reduced by 40% compared with control flies (Fig. 4A). Levels were also reduced in whole *iPLA2-VIA*^{-/-} flies compared with controls (Supplementary Fig. 3A).

Mitochondrial dysfunction can lead to decreased resistance to reactive oxygen species, which has been implicated in many neurodegenerative diseases. To explore the role of *iPLA2-VIA* in resistance to oxidative stress, we examined the survival of *iPLA2-VIA*^{-/-} flies after exposure to the potent oxidizer hydrogen peroxide and paraquat, a free radical generator. Consistent with the structural mitochondrial abnormalities in aged flies, *iPLA2-VIA*^{-/-} flies showed an age-dependent, mild sensitivity at Day 15 to hydrogen peroxide and, more so, to paraquat (Fig. 4B and C) that was not consistently present in younger 7-day-old flies (data not shown). Both young Day 7 and older Day 15 *iPLA2-VIA*^{-/-} flies were also sensitive to

osmotic stress and showed reduced survival compared to *w*¹¹¹⁸ control flies when fed 500 mM NaCl (Supplementary Fig. 3B). Day 15 *iPLA2-VIA*^{-/-} flies also displayed increased sensitivity to hypoxic and xenobiotic (dichlorodiphenyltrichloroethane, DDT) stresses (Supplementary Fig. 3C and D).

Loss of *iPLA2-VIA* was not associated with changes in cardiolipin composition

Given that mitochondrial membrane morphology appeared to be abnormal in the *iPLA2-VIA*^{-/-} flies, we performed mass spectroscopic analysis of cardiolipin, one of the major phospholipids in mitochondrial membranes. It has been hypothesized that PLA2G6 is critical for the normal repair and remodelling of damage to cardiolipin caused by mitochondrial reactive oxygen species, and that loss of PLA2G6 activity may lead to cardiolipin oxidation and pathogenic downstream events such as induction of apoptosis. To test this hypothesis, we performed mass spectroscopic analysis on *iPLA2-VIA*^{-/-} fly heads to compare the cardiolipin composition with that in *w*¹¹¹⁸ control flies. We found no significant differences in the cardiolipin content, monolysocardiolipin content or fatty acid composition between *iPLA2-VIA*^{-/-} and control *w*¹¹¹⁸ fly brains (Supplementary Fig. 4). Similar results have previously been reported in whole flies lacking *iPLA2-VIA* (Malhotra *et al.*, 2009). Furthermore, we found that oxidized cardiolipin was not detected in significant amounts in either the *iPLA2-VIA*^{-/-} or control *w*¹¹¹⁸ fly head homogenates (Supplementary Fig. 4). Oxidized cardiolipin

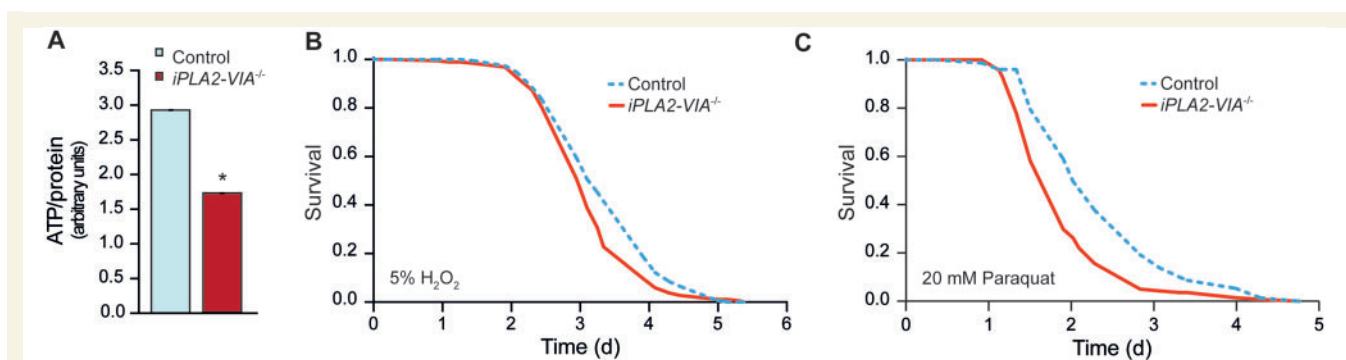


Figure 4 $iPLA2-VIA^{-/-}$ flies have reduced ATP levels and are more sensitive to oxidative stress. **(A)** ATP levels are reduced in the heads of Day 25 $iPLA2-VIA^{-/-}$ flies compared with age-matched w^{1118} control flies ($*P < 0.05$). **(B)** Aged Day 15 $iPLA2-VIA^{-/-}$ flies show reduced survival when treated with 5% hydrogen peroxide (H_2O_2) ($P = 5.6 \times 10^{-7}$) and **(C)** 20 mM paraquat ($P = 4.6 \times 10^{-7}$) compared with w^{1118} control flies ($n = 150$ flies per genotype).

species would be expected to be present as additions of +7/8 m/z on the scan spectra. Our results therefore do not support a role for oxidized cardiolipin in causing mitochondrial dysfunction and neurodegeneration in flies lacking $iPLA2-VIA$ activity.

Loss of $iPLA2-VIA$ promotes brain lipid peroxidation and reduces whole body triacylglycerol levels

Despite the absence of changes in oxidized cardiolipin, we hypothesized that loss of $PLA2G6$ may lead to abnormal and excessive oxidation of other mitochondrial membrane lipids, which in turn would lead to the observed mitochondrial dysfunction. We therefore studied the effect of knocking out $iPLA2-VIA$ on lipid peroxidation in *Drosophila*. Rates of lipid peroxidation were measured by live imaging of Day 25 $iPLA2-VIA^{-/-}$ and w^{1118} control dissected fly brains, using the fluorescent ratiometric oxidation-sensitive dye C11 BODIPY581/591, which shifts its fluorescence from red to green in a time-dependent manner, the rate being proportional to levels of lipid peroxidation. The changes in colour shift were detected by confocal microscopy. This demonstrated that levels of lipid peroxidation in flies lacking the $iPLA2-VIA$ gene were 3.5-fold higher than in age-matched control fly brains (Fig. 5A).

There is evidence that $PLA2G6$ may play a role in the regulation of triacylglycerol synthesis by providing endogenous fatty acids (Gubern *et al.*, 2009). Triacylglycerol is the main form of lipid storage in the fly and therefore reflects the ability of flies to respond to starvation conditions (Ballard *et al.*, 2008). Measurement of whole-body triacylglycerol levels in female flies revealed a significant age-dependent reduction in both control w^{1118} and $iPLA2-VIA^{-/-}$ flies. However, the reduction was significantly greater by Day 20 in the flies lacking $iPLA2-VIA$ activity (Fig. 5B). In keeping with an age-dependent decrease in triacylglycerol levels, $iPLA2-VIA^{-/-}$ flies were not sensitive to starvation conditions at Day 7 but became

sensitive at Day 15 compared with control w^{1118} flies (Fig. 5C).

Human mutant $PLA2G6$ fibroblasts display abnormal mitochondrial physiology

Given that loss of $iPLA2-VIA$ led to increased lipid peroxidation and mitochondrial dysfunction in *Drosophila*, we examined fibroblasts taken from a patient with a known homozygous p.R747W mutation in $PLA2G6$ causing dystonia-parkinsonism (Paisan-Ruiz *et al.*, 2009). Using TMRM fluorescence to probe for mitochondrial membrane potential, we found that there was a significant decrease in basal mitochondrial membrane potential in the mutant human fibroblasts (Fig. 6A and B), similar to that seen in fly brains lacking $iPLA2-VIA$.

Abnormal respiration and low mitochondrial membrane potential can induce excessive reactive oxygen species production in the matrix of mitochondria. Indeed the rate of mitochondrial reactive oxygen species production, as assessed using MitoSOX fluorescence, was increased by 28% relative to control human fibroblasts (Fig. 6C and E). Cytosolic reactive oxygen species were also significantly higher in the fibroblasts with a $PLA2G6$ mutation (Fig. 6D and E).

Mutations in $PLA2G6$ are associated with elevated mitochondrial lipid peroxidation levels in human fibroblasts and are reversed by treatment with deuterated polyunsaturated fatty acids

Following the demonstration that there is an increase in lipid peroxidation in the brains of flies lacking $iPLA2-VIA$ compared with controls, we also assessed lipid peroxidation in two human $PLA2G6$ fibroblast lines; one taken

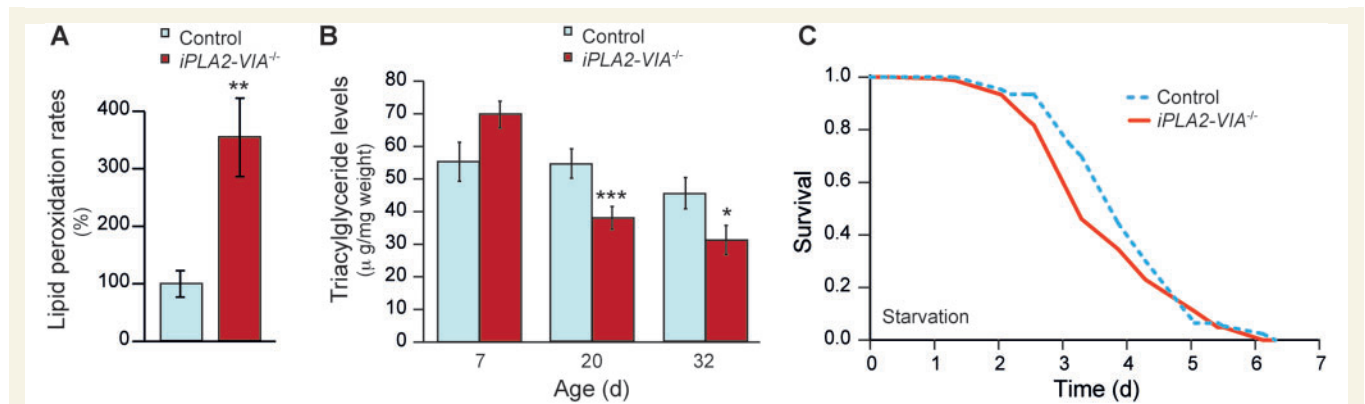


Figure 5 Flies lacking *iPLA2-VIA* have increased lipid peroxidation in their brains and reduced triacylglycerol (TAG) levels.

(A) Lipid peroxidation was measured using C11-BODIPY581/591 and demonstrated that Day 25 *iPLA2-VIA*^{-/-} fly brains have elevated levels of lipid peroxidation compared with age-matched *w*¹¹¹⁸ controls ($n = 6$ fly brains per experiment; $**P < 0.01$). (B) Whole-body triacylglycerol levels are decreased in *iPLA2-VIA*^{-/-} flies in an age-dependent manner compared with *w*¹¹¹⁸ control flies. ($***P = 0.005$ Day 20 and $*P = 0.01$ Day 32). (C) Day 15 *iPLA2-VIA*^{-/-} flies are more sensitive to starvation conditions than age-matched *w*¹¹¹⁸ control flies ($P = 0.001$; $n = 150$ flies per genotype).

from a patient with dystonia-parkinsonism with a p.R747W mutation (Paisan-Ruiz *et al.*, 2009) and the other from a child with infantile neuroaxonal dystrophy. We assessed lipid peroxidation levels using the fluorescent ratiometric oxidation-sensitive C11 BODIPY581/591 dye. This demonstrated that *PLA2G6* mutant fibroblasts had an increased level of lipid peroxidation compared to controls (Fig. 7A), suggesting therefore that lipid peroxides may be a potential therapeutic target.

Natural polyunsaturated fatty acids (PUFAs) such as linoleic acid undergo autoxidation by reactive oxygen species, resulting in the production of toxic reactive carbonyl compounds that can mediate DNA damage, carcinogenesis and inflammation. However, replacement of the bis-allylic hydrogen atoms with deuterium atoms (termed site-specific isotope-reinforcement) arrests PUFA autoxidation due to the isotope effect. Indeed, even small amounts of isotope-reinforced D-PUFA are able to protect yeast from lipid autoxidation-mediated cell killing (Hill *et al.*, 2011, 2012). These benefits have also been confirmed *in vivo* with the demonstration that D-PUFAs reduce nigrostriatal degeneration in a mouse model of Parkinson's disease (Shchepinov *et al.*, 2011). We therefore pretreated human *PLA2G6* mutant fibroblasts for 24 h with D-PUFA (D₄-linoleic acid) and found that this decreased lipid peroxidation in the cells to below control levels (Fig. 7A). This was also associated with a rescue in the reduced mitochondrial membrane potential (Fig 7B). Moreover, assessment specifically of lipid peroxidation levels in digitonin-permeabilized cells, which destroys all cell membranes except mitochondrial ones, also demonstrated that lipid peroxidation was substantially increased. In addition, when rotenone was added to the fibroblasts there was no further significant increase in the lipid peroxidation level, indicating that the lipid peroxidation was predominantly produced by mitochondria in the cell (Fig. 7C).

Deuterated linoleic acid partially rescued the locomotor abnormalities of flies lacking *iPLA2-VIA*

Finally, we tested the effect of inhibiting lipid peroxidation with D-PUFAs on the neurodegenerative locomotor phenotype seen in flies lacking *iPLA2-VIA*. Crosses for *iPLA2-VIA*^{-/-} and *w*¹¹¹⁸ control flies were set up on food containing vehicle (H₂O), 10 or 100 µM D₂-linoleic acid. Eclosed offspring were also kept on standard food containing either vehicle or D₂-linoleic acid and aged while their climbing ability was assessed. *iPLA2-VIA*^{-/-} flies treated with 10 µM D₂-linoleic acid had significantly improved motor performance in old age compared to flies treated with control food (Fig. 7D). There was a trend towards improved climbing ability at older ages when *iPLA2-VIA*^{-/-} flies were treated with 100 µM D₂-linoleic acid, but this was not statistically significant. The rescuing effect of D₂-linoleic acid on climbing ability was specific to *iPLA2-VIA*^{-/-} flies, as the climbing ability of control *w*¹¹¹⁸ flies treated with 10 µM D₂-linoleic acid was indistinguishable from controls (Supplementary Fig. 5B). The effect of D₂-linoleic acid on the survival of *iPLA2-VIA*^{-/-} flies was also assessed. There was no significant effect of 10 µM D₂-linoleic acid on the lifespan of flies lacking *iPLA2-VIA*. Indeed, at the higher concentration of 100 µM D₂-linoleic acid, the lifespan of the *iPLA2-VIA*^{-/-} flies was reduced compared to control *w*¹¹¹⁸ flies demonstrating that D₂-linoleic acid is toxic to flies at higher concentrations (Supplementary Fig. 5B). Despite the lack of lifespan-prolonging effects, the fact that deuterated linoleic acid significantly improved the locomotor abnormalities of *iPLA2-VIA* flies suggests that low concentrations of D-PUFAs, or similar compounds, may act as potential therapeutic compounds for the treatment of *PLA2G6*-associated neurodegeneration.

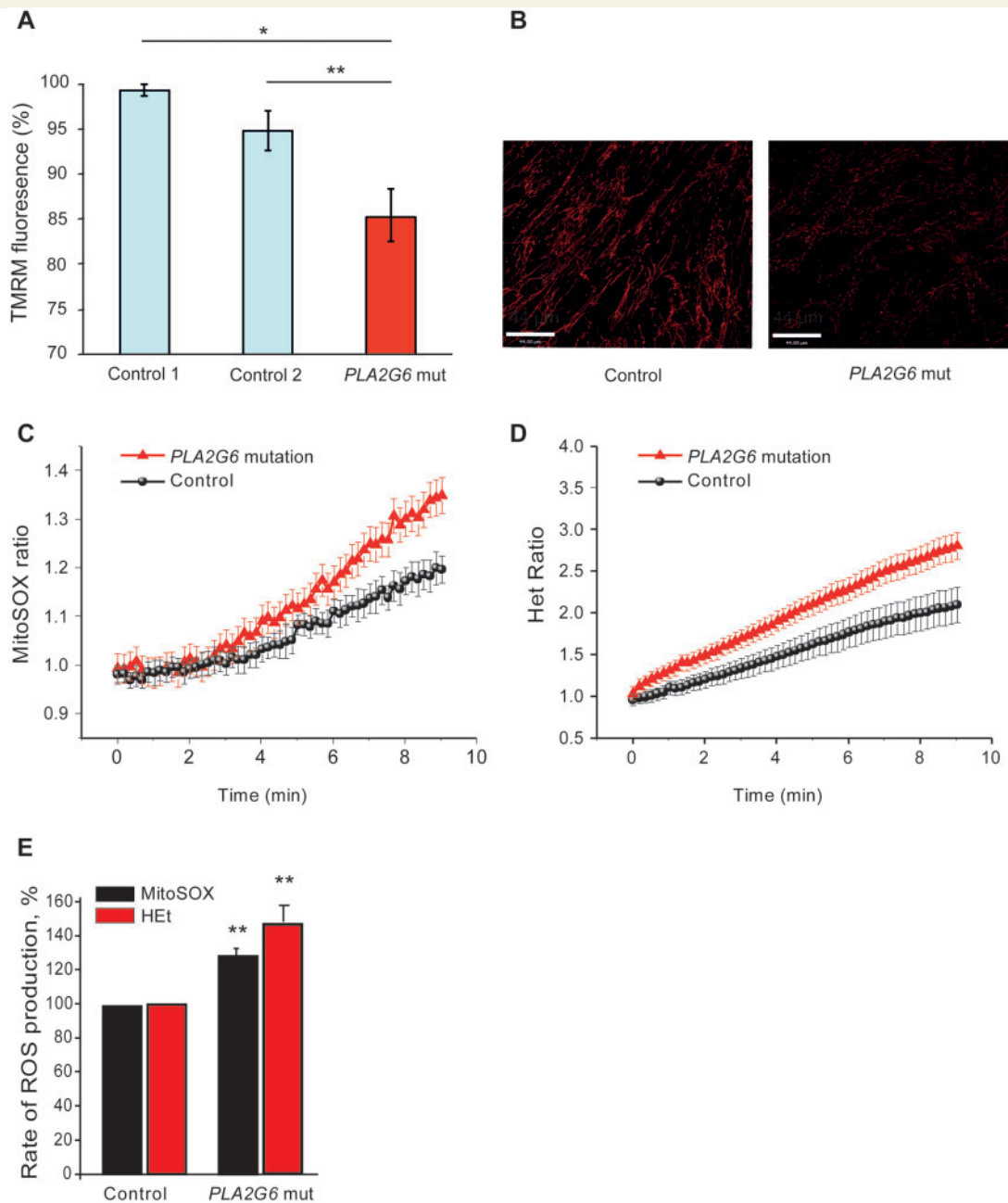


Figure 6 Human mutant *PLA2G6* fibroblasts have reduced mitochondrial membrane potential and increased levels of mitochondrial and cytosolic reactive oxygen species levels. **(A)** The mitochondrial membrane potential of human p.R747W mutant *PLA2G6* fibroblasts is reduced compared with control human fibroblasts ($*P < 0.05$ control 1 and $**P < 0.002$ control 2) as measured by TMRM fluorescence. **(B)** This is clearly seen as a reduction in red TMRM staining in the p.R747W mutant *PLA2G6* human fibroblasts compared with control fibroblasts (Scale bars = 44 μm). **(C)** Mitochondrial reactive oxygen species production is increased 28% in human p.R747W mutant *PLA2G6* fibroblasts compared to control fibroblasts ($P < 0.001$; $n = 6$). **(D)** Cytosolic reactive oxygen species production is also increased in human p.R747W mutant *PLA2G6* fibroblasts compared with control fibroblasts (1.47-fold increase; $P < 0.001$; $n = 6$). **(E)** Rates of reactive oxygen species production in **C** and **D** are shown as a percentage. Het = dihydroethidium.

Discussion

As the energy factories of cells, mitochondria play an essential role in neurons, in which oxidative phosphorylation is the main source of ATP. A previous study in a mouse model demonstrated that knockout of *Pla2g6* results in

abnormal mitochondrial membrane morphology (Beck *et al.*, 2011). These findings lead to the question as to how loss of normal *PLA2G6* gene function leads to abnormal mitochondrial morphology, and whether mitochondrial dysfunction is an early feature of *PLA2G6*-associated neurodegeneration.

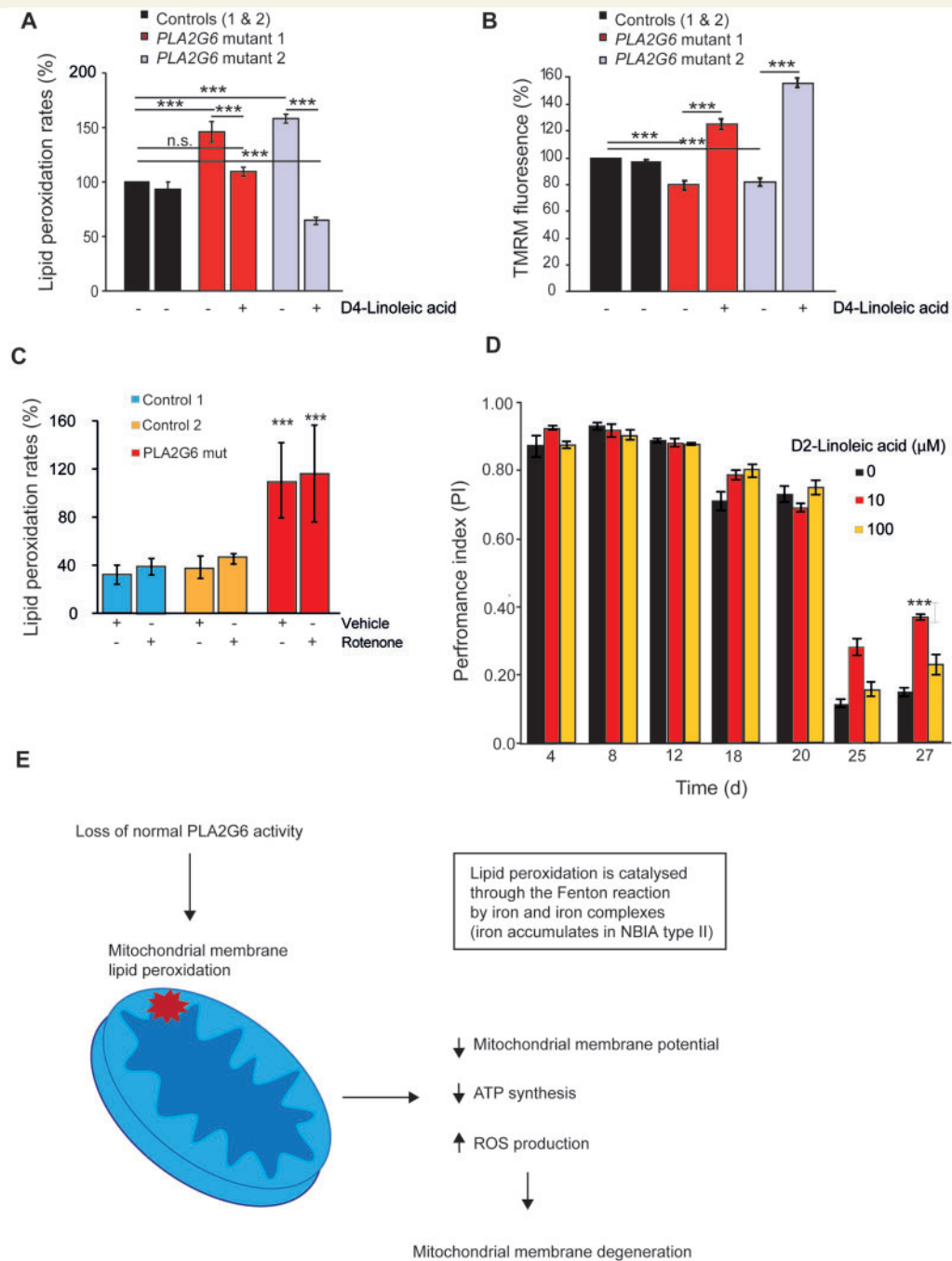


Figure 7 Elevated lipid peroxidation is a possible therapeutic target in PLA2G6-associated disease. **(A)** Lipid peroxidation levels in intact human fibroblasts taken from two patients with PLA2G6 mutations (mutant 1 fibroblasts were taken from a patient with a p.R747W PLA2G6 mutation and mutant 2 fibroblasts were from a patient with a PLA2G6 mutation and infantile neuroaxonal dystrophy) are increased compared with fibroblasts from healthy controls. In addition, pretreatment with D-PUFAs (D₄-linoleic acid) for 24 h significantly rescues the elevated lipid peroxidation rates in the human PLA2G6 mutant fibroblasts ($^{***}P < 0.0001$; n.s = non-significant). **(B)** The mitochondrial membrane potential of human mutant PLA2G6 fibroblasts is considerably reduced compared with control fibroblasts and is significantly recovered below control levels following 24 h pre-treatment with D₄-linoleic acid ($^{***}P < 0.0001$). **(C)** Lipid peroxidation levels are also increased in permeabilized mutant p.R747W PLA2G6 fibroblasts compared with controls in the absence ($^{***}P < 0.05$ for control 1 and control 2) and presence of rotenone ($^{***}P < 0.05$ for controls 1 and 2), suggesting that it is generated from a mitochondrial source. **(D)** Treatment of *iPLA2-VIA*^{-/-} flies with 10 μ M D₂-linoleic acid partially rescues the climbing deficits in an age-dependent manner compared with untreated controls ($n = 45$ flies per group, three repeats per group; one-way ANOVA with Bonferroni correction $^{***}P < 0.003$). There is a trend for 100 μ M D₂-linoleic acid to rescue the climbing deficits of *iPLA2-VIA*^{-/-} flies but this is not statistically significant. **(E)** Schematic diagram showing how loss of normal PLA2G6 activity might lead to mitochondrial dysfunction. A mutation in PLA2G6 results in loss of PLA2G6 activity. In turn this results in elevated lipid peroxidation of mitochondria, with the resultant decrease in mitochondrial membrane potential, decrease in ATP synthesis and mitochondrial reactive oxygen species production. The mitochondria eventually degenerate and become swollen in appearance. All of these abnormalities result in mitochondrial dysfunction and subsequent cellular dysfunction with consequent neuronal death. NBIA = neurodegeneration with brain iron accumulation; ROS = reactive oxygen species.

Loss of normal phospholipase A₂ activity leads to mitochondrial dysfunction, which precedes mitochondrial membrane defects

In this study, we demonstrated that loss of normal phospholipase A₂ activity in the fruit fly and in fibroblasts taken from patients harbouring mutations in *PLA2G6*, results in striking abnormalities in both mitochondrial function and morphology. Furthermore, we demonstrate that mitochondrial dysfunction precedes the mitochondrial morphological abnormalities that are seen at the ultrastructural level. Deficits in mitochondrial oxidative respiration are seen in *iPLA2-VIA* knockout flies as young as 2 days of age, when there are no associated mitochondrial membrane abnormalities present on electron microscopy.

Moreover, our findings that mitochondrial dysfunction occurs early in *PLA2G6*-associated neurodegeneration, is consistent with the clinical features seen in infantile neuroaxonal dystrophy, neurodegeneration with brain iron accumulation and dystonia-parkinsonism, all of which display clear early-onset phenotypes, that have previously been associated with mitochondrial disease.

Loss of normal *PLA2G6* activity leads to reactive oxygen species production

In addition to mitochondrial dysfunction, we also demonstrate that loss of *PLA2G6* activity is associated with raised levels of reactive oxygen species. These species play important roles in cell signalling, but at abnormally high levels are detrimental to cells, ultimately leading to cell death (Martindale and Holbrook, 2002). Lipids are important targets of reactive oxygen species and therefore elevated reactive oxygen species levels will lead to increases in mitochondrial lipid peroxidation. Furthermore, it may also be that elevated lipid peroxidation arises in the absence of phospholipase A₂ activity because some phospholipases preferentially cleave oxidized lipids to maintain redox homeostasis (Domijan *et al.*, 2014). In turn, lipid peroxides are known to trigger a cascade of events that include a fall in mitochondrial membrane potential, mitochondrial swelling and loss of mitochondrial matrix components (Castilho *et al.*, 1994). Furthermore, it has been shown that complex I, the largest complex of the respiratory chain, is susceptible to oxidative stress (Lin and Beal, 2006). Indeed we saw a decrease in oxidative respiration in flies lacking *iPLA2-VIA* when we used substrates specific for complex I of the respiratory chain. Furthermore, Lewy body disease has been shown to be associated with reduced levels of complex I activity (Gu *et al.*, 1998), further implicating mitochondrial dysfunction in neurodegeneration, and more specifically in *PLA2G6*-associated neurodegeneration,

which has been linked to parkinsonism and Lewy body pathology.

In addition, lipid peroxidation is strongly catalysed through the Fenton reaction by iron and iron complexes (Koster *et al.*, 1980) and it is iron that is often deposited in the basal ganglia of the brains of patients affected with *PLA2G6* mutations (Morgan *et al.*, 2006). Thus, iron-mediated oxidative insults seem to have a crucial role in the function of *PLA2G6* and in the progression of diseases associated with mutations in this gene.

PLA2G6-associated neurodegeneration is not likely mediated by oxidization of mitochondrial cardiolipin

Given the increase in levels of lipid peroxidation in flies lacking *iPLA2-VIA*, we looked for elevated levels of oxidized cardiolipin, one of the most common lipids in the inner mitochondrial membrane. It has previously been hypothesized that *PLA2G6* is involved in the remodelling of cardiolipin's fatty acid side chains (Malhotra *et al.*, 2009) and in removing oxidized fatty acid side chains. Oxidation of mitochondrial phospholipids such as cardiolipin leads to cytochrome *c* release and apoptosis (Kagan *et al.*, 2005). To determine whether cardiolipin is oxidized in *PLA2G6* loss-of-function, we analysed the cardiolipin composition of *iPLA2-VIA*^{-/-} fly heads using mass spectrometry. We demonstrated that mitochondrial membrane disintegration and mitochondrial dysfunction occur in the absence of any significant differences in cardiolipin amount or side chain composition in fly brains lacking *iPLA2-VIA*. Furthermore, oxidized cardiolipin was not detected in significant amounts. This may be related to the types of fatty acids present in *Drosophila* cardiolipin. Palmitic acid is the major component of cardiolipin side chains in *Drosophila* and is more resistant to oxidation than linoleic acid, the main cardiolipin side chain in most mammalian tissues. However, it could also point towards redundancy in the cardiolipin remodelling enzymes. There are a number of enzymes involved in cardiolipin remodelling and it may be the case that fatty acid remodelling of the side chains, in particular removal of oxidized fatty acids, can be performed by other enzymes involved in cardiolipin remodelling such as tafazzin (Schlame, 2013).

The abnormal lipid peroxidation seen in *PLA2G6* knockout leads to a toxic cascade of downstream events

In this work we report that loss of normal *PLA2G6* function is strongly associated with elevated mitochondrial lipid peroxidation. Furthermore lipid peroxidation is known to induce mitochondrial injury, leading to a decrease in oxidative respiratory chain function, reduced mitochondrial

membrane potential, reduced cellular ATP levels and raised reactive oxygen species levels, all features we have demonstrated in flies and human fibroblasts lacking normal *PLA2G6* gene activity (Fig. 7D). This in turn would be predicted to lead to cell death and neurodegeneration. As lipid peroxides are harmful mediators of oxidative stress, strategies focused on quenching, recycling or protecting such products could be highly effective in the treatment of the disease.

This work suggests that lipid peroxidation-lowering strategies may be beneficial in *PLA2G6*-associated neurodegeneration

In addition to showing that lipid peroxidation is increased in both fly brains lacking *iPLA2-VIA* and human *PLA2G6* mutant fibroblasts, we have demonstrated that D-PUFAs reduce the rates of lipid peroxidation in two lines of *PLA2G6* mutant fibroblasts and restore mitochondrial membrane potential. This suggests that D-PUFAs may have potential therapeutic roles in *PLA2G6*-associated neurodegeneration and its associated mitochondrial dysfunction.

Our data promote *Drosophila* as a useful model for studying *PLA2G6*-associated neurodegeneration, and for manipulating pathways and therapeutic targets in order to reverse or prevent the deleterious effects of lipid peroxides. Loss of *iPLA2-VIA* gene function results in reduced lifespan and locomotor ability, increased sensitivity to oxidative insults as well as hypoxia, osmotic and starvation stressors. Thus flies lacking *iPLA2-VIA* display phenotypes that can be used as markers in genetic modifying screens or in the screening of potential therapeutic compounds. To test the validity of the fly model for drug screening and to confirm the benefits of D-PUFAs *in vivo* in an organism lacking *iPLA2-VIA*, we examined the effects of D-PUFAs on the locomotor deficits of *iPLA2-VIA*^{-/-} flies. This demonstrated that treatment with D₂-linoleic acid partially rescues the climbing abnormalities in aged *iPLA2-VIA* knockout flies. Therefore this approach of using compounds, which act to reduce the autoxidation of lipids, may represent a future therapeutic strategy for patients with *PLA2G6*-associated neurodegeneration and warrants further investigation.

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Supplementary material

Supplementary material is available at *Brain* online.

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