Light-driven activation of mitochondrial proton-motive force improves motor behaviors in a Drosophila model of Parkinson’s disease

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Mitochondrial degeneration is considered one of the major causes of Parkinson’s disease (PD). Improved mitochondrial functions are expected to be a promising therapeutic strategy for PD. In this study, we introduced a light-driven proton transporter, Delta-rhodopsin (dR), to Drosophila mitochondria, where the mitochondrial proton-motive force (∆p) and mitochondrial membrane potential are maintained in a light-dependent manner. The loss of the PD-associated mitochondrial gene CHCHD2 resulted in reduced ATP production, enhanced mitochondrial peroxide production and lower Ca²⁺-buffering activity in dopaminergic (DA) terminals in flies. These cellular defects were improved by the light-dependent activation of mitochondrion-targeted dR (mito-dR). Moreover, mito-dR reversed the pathology caused by the CHCHD2 deficiency to suppress α-synuclein aggregation, DA neuronal loss, and elevated lipid peroxidation in brain tissue, improving motor behaviors. This study suggests the enhancement of ∆p by mito-dR as a therapeutic mechanism that ameliorates neurodegeneration by protecting mitochondrial functions.
Parkinson's disease (PD) is a neurodegenerative disorder characterized by selective loss of midbrain DA neurons. CHCHD2 (mutations of which cause an autosomal dominant form of PD) encodes a mitochondrial intermembrane protein. Drosophila CHCHD2 (dCHCHD2) knockout flies exhibit PD-like phenotypes in an age-dependent manner, which include dysfunction in motor ability, DA neuron loss, increased oxidative stress and mitochondrial cristae degeneration. Loss of dCHCHD2 and introduction of PD-associated human CHCHD2 mutations destabilize cytochrome c, which transports an electron from complex III to complex IV during oxidative phosphorylation (OXPHOS), leading to a reduction in ATP production and the generation of reactive oxygen species (ROS) owing to the electron leak.

Mitochondrial electron leak and proton leak affect ROS generation and mitochondrial coupling activity. The electron leak is thought to primarily occur in complex I and complex III where electrons react with O₂, forming superoxide, the primary ROS from mitochondria. Basal proton leak accounts for 20% or more of the standard metabolic rate in different organs, and increased proton leak, which lowers ROS production by mild uncoupling through uncoupling proteins (UCPs), is thought to occur as the mitochondrial proton-motive force (Δp) rises. In addition, the maintenance of mitochondrial membrane potential (ΔΨm) by Δp is important for ATP generation through complex V, the transporter of materials required for mitochondrial functions, including mitochondrial proteins and substrates for metabolic reactions, and Ca²⁺ homeostasis.

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In this study, by adapting dR to mitochondria in Drosophila, we present a potential therapeutic approach to preserve mitochondria from degeneration caused by CHCHD2 loss. Light-dependent activation of mitochondrial-targeted dR (mito-dR) but not a mito-dR inactive mutant successfully transformed mitochondria from an OXPHOS-dependent powerhouse to a photoenergetic powerhouse, which accordingly reinforced the mitochondrial functions of the nerve terminals in terms of ATP production and Ca²⁺-buffering activity, suppressing ROS generation. Moreover, the beneficial effects of dR ameliorated the α-synuclein accumulation, DA neuron loss and elevated brain lipid peroxidation caused by dCHCHD2 loss. Our findings demonstrate that increased Δp by light-driven mito-dR reinforces mitochondrial functions, suppressing ROS generation.

Results
Generation of flies harboring photoenergetic mitochondria. Genes responsible for PD have revealed that mitochondrial degeneration is a key factor for PD etiology. Mutations or loss of the PD-associated gene dCHCHD2 result in reduced OXPHOS activity and increased ROS production in Drosophila. Because PD-associated CHCHD2 mutations have loss-of-function properties, we used dCHCHD2 knockout flies as a Drosophila model of PD. To regenerate mitochondrial activity in the PD model, we designed photoenergetic mitochondria to be expressed in flies. To exclude the possibility that light irradiation itself stimulates mitochondria, we also constructed a mutant in which the two key residues that interact with retinal, D104 and K225, are replaced by nonfunctional amino acids, N and A, respectively. Wild-type (WT) dR showed a red-tinted bacterial pellet when expressed in E. coli (Supplementary Fig. 1a). In contrast, the D104N/K225A (NA mutant, hereafter) mutant lost redness similar to a vector control, confirming that the D104N/K225A mutant lacks retinal-binding activity. Light-dependent proton pump activity of dR WT but not NA mutant was also observed in bacteria cells (Fig. 1a). Both WT and NA dR harboring a mitochondrial target signal (mito-dR) successfully localized in mitochondria in Drosophila 2SR⁺ cells (Supplementary Fig. 1b). We expressed mito-dR WT and NA in dCHCHD2−/− flies along with normal flies using the GAL4-UAS system and confirmed that the expression levels of the two kinds of mito-dR were similar in both lines (Supplementary Fig. 1c).

To determine whether ATP production is stimulated in DA neurons in which mitochondria are affected in PD, we targeted the expression of mito-dR and ATP biosensor ATteam in DA neurons using the Ddc-GAL4 driver. ATteam is a genetically encoded Förster resonance energy transfer-based ATP biosensor optimized for low temperatures. We visualized ATP changes in DA neurons in the adult fly brain in a light irradiation-dependent manner (Fig. 1e, f). Although we did not observe significantly increased ATP production by mito-dR WT in DA neuron cell bodies of all fly groups, ATP production was stimulated in the mitochondria of the axonal terminals projecting to the mushroom body in dCHCHD2−/− flies (Fig. 1e, f; Supplementary Fig. 1d).

mito-dR relieves oxidative stress. Increased Δp by mito-dR could cause reverse electron transport, resulting in high levels of superoxide production and subsequent oxidative stress (Supplementary Fig. 2a). If this situation is the case, constitutive reverse electron transport from complex II to complex I could occur, leading to the downregulation of complex I. However, we could not detect significant changes in the activities and protein levels in complexes I and II, suggesting that reverse electron transport does not occur by mito-dR (Supplementary Fig. 2b, c).

Alternatively, proton leakage back to the matrix bypassing complex V through UCPs, which is termed “mild uncoupling”, reduces ROS generation. Mild uncoupling is important for the relief of oxidative stress in DA neurons in the substantia nigra pars compacta (SNC) but not in the ventral tegmental area, which is a less-sensitive area in PD. The intensity of a marker for lipid peroxidation, 4-HNE, was higher in normal flies than in normal flies as previously reported (Fig. 2a, b). mito-dR activation by light irradiation suppressed the accumulation of 4-HNE signals (Fig. 2a, b). Nonfunctional mito-dR NA with or without light treatment did not change the 4-HNE signals of dCHCHD2−/− flies, indicating that light irradiation itself does not suppress brain lipid peroxidation (Fig. 2c, d).

Generation of mitochondrial ROS was visualized by using mitochondrion-targeted roGFP2-Orp1 (mito-roGFP2), a hydrogen peroxide biosensor. We expressed mito-roGFP2 in DA neurons without light treatment did not change the 4-HNE signals of dCHCHD2−/− flies, indicating that light irradiation itself does not suppress brain lipid peroxidation (Fig. 2c, d).

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along with mito-dR. As expected, ROS production was increased in the mitochondria of dCHCHD2-deficient cell bodies and was suppressed to normal control levels after light activation (Fig. 2e). Light-activated mito-dR also relieved ROS in axonal mitochondria in DA neurons (Fig. 2f). Consistent with the alleviation of oxidative stress, light-activated mito-dR ameliorated the degeneration of mitochondrial cristae caused by dCHCHD2 loss (Fig. 2g, h)². Given that mito-dR successfully activates mitochondria in the axonal terminals of motor neurons and DA neurons, we next examined the effects of mito-dR in glial cells. Surprisingly, panglial expression of mito-dR WT but not mito-dR NA using the repo-GAL4 driver in dCHCHD2⁻/⁻ flies resulted in early lethality under light irradiation, so that adult flies died by 20 days after eclosion. The expression of mito-dR in the glial
subpopulation using the nrv2-GAL4 driver, which is active in cortex glia, astrocyte-like glia and eneasinghia glia, did not affect the survival of dCHCHD2 +/- flies and stimulated ATP production, whereas simultaneously increasing mitochondrial ROS (Supplementary Fig. 1e)18. Glycolytically active glial cells have been shown to produce alanine and lactate to maintain neuronal survival19. Our data suggest that excess mitochondrial activation in glia could compromise energy homeostasis and redox status in the glia-neuron metabolic connection.

**mito-dR suppresses α-synuclein accumulation.** SNC DA neurons have an autonomous pacemaking property involving L-type Ca2+ channels16,20. Mitochondria regulate cellular Ca2+ concentrations upon Ca2+ influx via the opening of Ca2+ channels during synaptic activity whereby the continuous firing of DA neurons is secured. Dysregulation of Ca2+ might render SNC DA neurons vulnerable to various stresses, especially oxidative stress, because Ca2+ influx to mitochondria stimulates OXPHOS activity, resulting in high ROS generation when antioxidant ability is compromised16. As mitochondrial energy production in the axonal terminals of DA neurons was improved in dCHCHD2 +/- flies, we next focused on the Ca2+ uptake activity of mitochondria in DA terminals using GaMP6f and mito-GCaMP6, which are cytosolic and mitochondrial Ca2+ indicators, respectively (Supplementary Fig. 3a)21. In Drosophila, PAM DA neurons, which regulate locomotion and reward, project to the mushroom bodies22,23. We electrically stimulated the antennal lobes to monitor the dynamics of cytoplasmic and mitochondrial Ca2+ in PAM DA terminals (Supplementary Fig. 3b). Upon mito-dR NA expression, the elevated cytoplasmic Ca2+ concentration ([Ca2+]c) in DA terminals tended to be higher, and the decay was delayed in the absence of dCHCHD2. In contrast, the increase in the mitochondrial Ca2+ concentration ([Ca2+]m) was lower, suggesting that Ca2+ uptake of mitochondria is impaired in dCHCHD2 +/- DA terminals (Supplementary Fig. 3c). The expression of mito-dR WT in dCHCHD2 +/- flies improved Ca2+ uptake to the mitochondria, suppressing the excessive elevation and delayed decay of [Ca2+]c (Fig. 3a). These observations suggest that mito-dR rescues the dysregulation of synaptic activity-dependent Ca2+ signaling in dCHCHD2 +/- DA terminals.

In addition to mitochondrial dysfunction, α-synuclein aggregation and accumulation in DA neurons are considered the major causes of PD etiology. Two possible pathological mechanisms between α-synuclein and mitochondria have been proposed. One potential mechanism is mitochondrial damage induced by aggregated α-synuclein24. Another is mitochondrial dysfunction leading to α-synuclein aggregation25,26. A pathological analysis of patients with CHCHD2 mutations supports the latter case, and CHCHD2 deficiency or mutations in Drosophila also promote α-synuclein accumulation27. The ectopic expression of α-synuclein in DA neurons of dCHCHD2 +/- flies resulted in sarkosyl-insoluble aggregation owing to mitochondrial dysfunction (lane 6 in Fig. 3b and lane 6 in Supplementary Fig. 3d). Importantly, light-activated mito-dR dramatically reduced α-synuclein levels (lane 8 vs. lane 6 in Supplementary Fig. 3d and Supplementary Fig. 3e), which was not owing to DA neuron loss because mito-dR expression with light irradiation was comparable to that without light irradiation (lane 4 vs. lane 2 in Supplementary Fig. 3d). Moreover, light-irradiated mito-dR NA had a null effect on α-synuclein levels, excluding the possibility that light irradiation itself facilitates α-synuclein degradation (lane 6 vs. lane 8 in Fig. 3b).

Consistent with the biochemical changes in α-synuclein, ubiquitinated α-synuclein as well as ubiquitinated puncta were increased in the DA neurons of aged dCHCHD2 +/- flies (3rd vs. 1st row in Fig. 3c). Light-activated mito-dR WT but not mito-dR NA alleviated the accumulation of ubiquitinated protein inclusions, including α-synuclein (4th vs. 3rd row in Fig. 3c).

**Functional improvement in DA neurons by mito-dR.** Age-dependent loss of DA neurons is a pathological feature of PD, and it was also observed in the PPL1, PPM2, and PPM3 clusters of DA neurons in dCHCHD2 +/- flies (dCHCHD2 +/-, mito-dR NA vs. dCHCHD2 +/-, mito-dR NA in the lower graph in Fig. 4a). The expression of mito-dR WT but not mito-dR NA by the Ddc-GAL4 driver, which covers most PPM2 clusters and part of PPL1 clusters (Supplementary Fig. 4a), suppressed DA neuron loss (upper graph in Fig. 4a). Although mito-dR was not expressed in most PPM3 DA neurons, a mild rescue was observed in PPM3 neurons, suggesting a non-autonomous effect by neighboring mito-dR-positive neurons (dCHCHD2 +/-, mito-dR WT vs. dCHCHD2 +/-, mito-dR NA in the lower graph in Fig. 4a). Dopamine production in the heads from flies at 15 days old but not 30 days old was also improved by light-activated mito-dR (Fig. 4b). Consistent with the improvement in DA functions, flight activity, which is regulated by DA neurons28, was enhanced at 14 days old by light irradiation (left in Fig. 4c). The increased flight event was induced by mito-dR function but not by light irradiation alone because mito-dR NA failed to activate flight behaviors even in the presence of light (right in Fig. 4c). Increased spontaneous locomotion activity of dCHCHD2 +/- flies was also observed with mito-dR WT (Supplementary Fig. 4b). These results suggest that mitochondrial activation of DA neurons by mito-dR enhances motor behaviors. Mild uncoupling via UCPs reduces ROS generation in mitochondria29. Among the UCP family, brain-enriched UCP4 and UCP5 could be involved in PD etiology16,29-31. Drosophila has three UCP4 homologs in addition to one UCP5 homolog, and only
Oxidative stress in dCHCHD2-deficient DA neurons is alleviated by mitochondrial dR. a-d Lipid oxidation by dCHCHD2 loss is ameliorated by mito-dR WT a, b but not mito-dR NA c, d. The whole brain tissues of 15-day-old flies were stained with an anti-4-HNE antibody. Scale bar = 50 µm. The intensities of the anti-4-HNE immunoreactive signals in the whole brain were measured and graphed. n = 16–18 flies, Tukey-Kramer test. e, f Monitoring of mitochondrial redox in the cell bodies e and nerve terminals f of PAM cluster DA neurons in 14-day-old males using mitochondrial H2O2 biosensor mito-roGFP2-Orp1. Scale bars = 5 e and 10 f µm. n = 14–17 flies, Tukey-Kramer test e, n = 5–7 flies, two-tailed Student’s t test f. g TEM images of the indirect flight muscle in 14-day-old adult flies expressing mito-dR WT with or without light irradiation are shown. Scale bar = 1 µm. h Quantification of mitochondria with abnormal cristae or degenerating mitochondria using a previously reported scoring system as follows2: Class 0, normal; Class 1, swirling, fuzzy or dilated cristae; and Class 2, fragmented cristae, and loss of electron density. Mitochondrial defects defined as class 1 and class 2 were counted and are presented as percentages (mean ± s.e.m.). *p = 0.0012, **p < 0.0001 vs. the same classes of dCHCHD2−/− without light. n = 285–492 from 3 to 4 independent samples. Transgenes were driven by Da-GAL4 a-d, g and R58E02-GAL4 e, f. Source data are provided as a Source Data file.
UCP4A has a protective role in PD fly models. Thus, we examined the effects of UCP4A inhibition on the neuroprotective activity of mito-dR. Dopaminergic (DA) coexpression of UCP4A RNAi but not LacZ RNAi with mito-dR weakened the neuroprotective effect on DA neurons (Fig. 4d; Supplementary Fig. 4c) and failed to suppress the decline in motor behavior in aged dCHCHD2−/− flies (Fig. 4e). In contrast, DA coexpression of UCP4A tended to rescue DA neuron loss in aged dCHCHD2−/− flies expressing nonfunctional mito-dR NA (Supplementary Fig. 4d). However, the coexpression of UCP4A with mito-dR reduced DA neuron survivability in dCHCHD2+/+ flies, considering that the normal number of PPM2 DA neurons covered by the Ddc-GAL4 driver is ~6 (Supplementary Fig. 4d, see also Fig. 4a). These findings suggest that mild uncoupling via UCPs (among which...
UCP4A functions dominantly) relieves ROS generation by dCHCHD2 loss, but chronic expression of UCP4A might have an adverse effect in normal flies.

Discussion
Mitochondria are the cellular powerhouse in which ATP production is generated by the OXPHOS reaction. A chain of the respiratory complexes I–IV pump up $H^+$ from the matrix, generating the electrochemical potential gradient in the intermembrane between inside and outside. This $\Delta$$\Psi$mem-dependent transport of ATP synthase complex V to synthesize ATP and facilitates mitochondrial transport via translocators such as Tim complexes. Mitochondrial oxidative stress originates from the electron leak from the defective respiratory chain and is considered a major contributor to the pathogenesis of PD.

$dCHCHD2$-deficient flies well recapitulate the PD-like phenotype with age. Because the loss of cytochrome c-binding protein CHCHD2 destabilizes cytochrome c located between complex III and complex IV, leading to electron leakage and excessive oxidative stress, the compensation of respiratory complex I–IV activity seems to be a promising therapeutic target for PD. To overcome the mitochondrial defects caused by OXPHOS dysfunction, we introduced an archaean proton transporter $\Delta$ to mitochondria. Light-activated $\Delta$ increases $\Delta$m, which promotes ATP production and probably $\Delta$Fm-dependent transport of materials for mitochondrial functions. In addition, mito-$\Delta$ suppressed oxidative stress derived from mitochondria, which is likely owing to uncoupling mediated by proton leakage where UCP4A is involved (Fig. 4f)3,13,36. Consistent with this observation, the elevation in brain lipid oxidation by CHCHD2 loss was successfully suppressed by mito-$\Delta$ (Fig. 2a–d). However, brain lipid oxidation in normal flies ($dCHCHD2^{+/+}$) was not further reduced. A similar situation was also observed in the cell bodies of DA neurons (Fig. 2e). Although $H_2O_2$ from mitochondria is thought to be the major oxidant species in the brain, auto-oxidation from environmental conditions such as oxygen in the air, short wavelength light, age-dependent impairment in redox regulation and inflammation might cause oxidation of brain lipids and the roGFP2 biosensor as basal oxidant factors. In addition, we observed a subset of glial cells with mildly elevated mitochondrial $H_2O_2$ production when mito-$\Delta$ was activated in these cells (Supplementary Fig. 1e). These factors may contribute to a null effect of mito-$\Delta$ in $dCHCHD2^{+/+}$ flies when mito-$\Delta$ is ubiquitously expressed.

The vulnerability of SNc DA neurons may arise from their peacemaking activity where Ca$^{2+}$ influx via L-type Ca$^{2+}$ channels are involved. Sustained Ca$^{2+}$ influx incurs a high metabolic cost owing to ATP-dependent Ca$^{2+}$ exclusion as well as mitochondrial oxidative stress owing to the OXPHOS reaction activated by Ca$^{2+}$. Mito-$\Delta$ seems to resolve this challenge by elevating ATP production and antioxidant activity. Consistent with the beneficial effects on the mitochondrial functions of DA neurons, dopamine production, locomotor activity and flight behavior, which are regulated by DA neurons, were improved in $dCHCHD2$-deficient flies. In contrast, mito-$\Delta$ WT did not increase $\Delta$Fm or ATP production in $dCHCHD2^{+/+}$ flies, suggesting that an overly polarized state does not physiologically occur in mitochondria (Fig. 1c–e). Supporting this idea, the inhibition of F-type ATPase complex V by oligomycin did not prominently increase $\Delta$Fm in human cultured cells33. This observation could be explained by uncoupling or proton leak mediated by UCPs, the adenine nucleotide translocase, the glutamate carriers and the permeability transition pore complex34,35. $dCHCHD2^{+/+}$ flies expressing mito-$\Delta$ WT exhibited a tendency toward a rapid [Ca$^{2+}$]$_m$ efflux compared with $dCHCHD2^{−/−}$ flies. This effect might be due to prolonged uncoupling by the Na$^+/H^+$ exchanger (which could potentially activate the Na$^+$/Ca$^{2+}$ exchanger) or the activation of a putative Ca$^{2+}$/H$^+$ exchanger such as Letm1 in the energized state36,37. These altered Ca$^{2+}$ dynamics in DA neurons might reflect the behavioral changes observed in $dCHCHD2^{+/+}$ flies.

In conclusion, this study provides ‘proof of concept’ that AP maintenance is beneficial for neuroprotection and that the development of proton pumps driven by optogenetic or pharmacogenetic techniques is a potential therapeutic strategy for PD.

Methods

**Antibodies and plasmids.** The antibodies used in western blot analysis were as follows: anti-dCHCHD2 (1:1000 dilution; in-house7), anti-Myc (1:1000; Millipore, clone 4A6), anti-Tim23 (1:2000; BD, clone 32/Time23), anti-GAPDH (1:2000; Bioss, clone 3E12), anti-actin (1:10,000; Millipore, clone C4), anti-NDUFS3 (1:2000; Abcam, clone 17D95), anti-SYTHA (1:1000; GenTex, GTX101689), anti-UCP4C1 (1:1000; ThermoFisher Scientific, clone 11A51H12), anti-COX IV (1:1000; Abcam, clone 20E8G12), and anti-ATP5A (1:20,000; Abcam, clone 15H4C3). The following antibodies were used for immunohistochemistry: anti-4-HNE (1:500; JaICA, clone a5082), anti-polyubiquitin (1:200; MBL, clone FK2), and anti-dTH (1:500; Abcam, ab76642 and 1:250; in-house38). A complementary DNA fragment of myc-mito-$\Delta$ in the pCMV vector was subcloned into the EcoRI and XbaI sites of the pUAST vector to generate mito-$\Delta$ WT. The mito-$\Delta$ NA mutant was generated using QuikChange site-directed mutagenesis kit (Agilent Technologies) with the following primers: $\Delta$R D104N forward, 5'-CGAATTCGACCGCTGCCGAGAGGTC-3'; $\Delta$R K225A forward, 5'-CGAATTCGACCGCTGCCGAGAGGTC-3'; $\Delta$R K225A reverse, 5'-GACCTCTCGGCAGCGGTCGGATTCG-3'. For mito-GaMP6, the mitochondrial targeting sequence of FlyORF, and Vienna Drosophila Resource Center and have been previously described.

**Fly culture and crosses.** Fly culture and crosses were performed on standard fly food containing yeast, cornmeal, and molasses. Flies were raised at 25 °C unless otherwise stated. Transgenic lines carrying mito-$\Delta$ or mito-GaMP6 were generated on the w$^{118}$ background (BestGene). All other fly stocks and GAL4 lines used in this study, including dCHCHD2 null, UAS-α-synuclein329, UAS-mito-Dr6R, R85E02-GAL4, UAS-AE113N3, UAS-mito-roGFP2-Gpr152, UAS-UCP4A-HsHA, and mb247-DsRed39, were obtained from the Bloomington Drosophila Stock Center, FlyORF, and Vienna Drosophila Resource Center and have been previously described.
Fig. 4 Activation of dR rescues DA neurodegeneration in dCHCHD2-deficient flies. a miTo-dR WT but not NA rescues the loss of DA neurons in dCHCHD2−/− flies. The numbers of dR-positive and total DA neurons in 30-day-old flies were graphed (mean ± s.e.m.). Note that miTo-dR was not expressed in most PPM3 DA neurons. **p < 0.0001 vs. the other three groups. n = 19–22 clusters from 10 to 11 flies, Tukey–Kramer test. b Dopamine production is partly improved by miTo-dR activation. Dopamine levels in the heads were measured in 15- and 30-day-old flies. n = 12–15 flies, Tukey–Kramer test. c Reduced flight behavior by dCHCHD2 loss is improved by miTo-dR activation. Flying events were recorded for 30 s. D2, dCHCHD2; WT, miTo-dR WT; NA, miTo-dR NA. n = 10 flies (left) 14 flies (right), Tukey–Kramer test. d Inhibition of UCP4A abolishes the neuroprotective role of miTo-dR. UCP4A RNAi was coexpressed with miTo-dR in DA neurons at 28 °C to enhance RNAi efficiency. The number of PPM2 DA neurons in 28-day-old flies was graphed (mean ± s.e.m.). n = 15–20 clusters from 8–10 flies, Tukey–Kramer test. e Inhibition of UCP4A impairs motor activity. The fly groups as in d were analyzed at 28 days old. n = 20 trials with 10–12 flies, Tukey–Kramer test. f A model of mitochondrial rescue by miTo-dR. (Upper) Loss of dCHCHD2 destabilizes cytochrome c, leading to OXPHOS dysfunction, decreased ΔΨm, and ROS production. (Lower) Green light stimulates miTo-dR (1), which maintains Δp through pumping out protons from the matrix to the intermembrane space (2), promoting proton influx-dependent ATP synthesis in complex V (3). Increased protons in the intermembrane space quench ROS in the intermembrane space directly and in the matrix by mild uncoupling via UCPs (4). IM, mitochondrial intermembrane. Transgenes were driven by Da-GAL4 b and Ddc-GAL4 a, c–e. Source data are provided as a Source Data file.
Protein expression was induced by 1 mM isopropyl β-D-thiogalactopyranoside for 3 hr at 37 °C in the presence of 10 μM all-trans-retinal (Sigma-Aldrich). E. coli cells were collected by centrifugation at 7000 × g for 2 min and washed three times with 100 mM NaCl. The cells were suspended in 100 mM NaCl at 12 OD500. Under dark conditions, 10 ml of the cell suspension was illuminated at 50 mmol m−2 s−1 using a 300 W Halogen projector lamp (ICD100/200W) through a band path filter within 530 ± 120 nm (PBO350-120, Asahi Spectra, Japan). The time course of pH changes in the cell suspension was monitored by pH meter F-72 (Horiba, Japan).

**Light stimulation.** Two 550 nm LED units (250 mm in length and 150 mm in width), which contain 50 bulbs per unit, were regulated by a pulse generator (JW-shop, CON-44RB-4-V2) and a timer device (MonotaRO, WT-02N-M). The LED units were equipped in an incubator (MEE, CN-40A), and newly eclosed adult flies were transferred to a LED unit (5 W mm−2) and observed at 12 hr every day. Fly food containing 100 μM all-trans-retinal, which was protected from light with aluminum foil, was changed with fresh food every day.

**Cell culture and mitochondrial fractionation.** S2R+ cells, which were a kind gift from Dr. N. Yanagawa at Kyoto University, were maintained in Schneider’s Drosophila medium (Gibco) supplemented with 10% FBS (Gibco) and 1% penicillin–streptomycin (Gibco). S2R+ cells transfected with pUAST-mito-dR along with pAct-GAL4 using HilyMax (Dojindo) for 36 hr were suspended in mitochondrial fractionation buffer (20 mM HEPES, pH 7.3, 220 mM mannitol, 70 mM sucrose, 1 mM EDTA, and protease inhibitor cocktail [Nacalai Tesque]) and were disrupted by passing through a 26-gauge needle twenty times. After centrifugation at 1000 × g for 10 min, supernatants were further centrifuged at 12000 × g for 15 min. Pellets and supernatants were retrieved as mitochondrial and cytosolic fractions, respectively. Both fractions were subjected to cell western blotting.

**Mitochondrial respiratory chain complex activity assay.** The respiratory chain complex activity assays were performed following our previously published protocols. In brief, for the measurement of the complex I activity, NAADH dehydrogenase assay buffer (50 mM potassium phosphate buffer pH 7.5, 2.5 mg ml−1 bovine serum albumin (BSA), 240 μM KCN, 70 μM decylubiquinone [dUb], 25 μM antimycin A) was prepared and filtered. 70 μM rotenone (Nihon koden) and SS-104J (Nihon koden). The lateral side of the antenna lobes was stimulated using a glass electrode (Warner Instruments, Cat. No. W3 64-0792) with a 100-μm tip diameter made by an electrode puller (Sutter Instrument, P-97). GaMP4 images were recorded for 1 min (1 frame/0.03 s), and 40 Hz electrical stimulation (5 V with 15–30 ms intervals) for 10–100 ms was performed to the motor behavior, as shown in Supplementary Fig. 3b 10 s after the recording. Each imaging was carried out within 10 min after dissection. Ca2+ imaging data were processed using NIS-Elements software (Nikon, Ver. AR-4.40.00), ImageJ-Fiji and Excel (Microsoft 2010).

**Behavioral assays.** For the flying assay in Fig. 4c, twenty adult male flies at 14 days old were placed in individual vials (93 mm height × 350 mm2 area) at noon and left at rest for 20 min. Flies were dropped down to the bottom by gently tapping, and flight events during 30 s were recorded. Locomotor activity was monitored using ImageXtif in Supplementary Fig. 4b was recorded in polycarbonate tubes containing light-shielded fly food with 100 μM retinal using the DAM system (Trikinetics). The behavior of single male flies preconditioned at 25°C under a 12-h light/dark cycle condition for 14 days was individually recorded for 1 additional day. Light irradiation was performed during the light period. For a climbing assay in Fig. 4c, vials (25 mm diameter × 180 mm height) containing 20–25 flies were tapped gently on the table and left standing for 18 s. The number of flies that climbed at least 60 mm was recorded.

**Statistics and reproducibility.** Error bars in bar graphs represent the mean ± SEM. Boxes of whisker plots indicate the 25th to 75th percentiles, horizontal lines in the boxes indicate the 50th percentile, and whiskers represent the maximum and minimum values. Statistical analysis was performed using IBM JMP 11.0.9 (SAS Institute Inc.). Two-tailed Student’s t test or one-way repeated-measures analysis of variance was used to determine significant differences between the two groups or among multiple groups, respectively, unless otherwise indicated. If a significant result was determined using ANOVA (p < 0.05), the mean values of the control and specific test groups were analyzed using a Tukey–Kramer test. Data distribution was assumed to be normal, but this was not formally tested. Abnormal mitochondrial (Fig. 2h) and DA neurons (Fig. 4a, d) were counted and graded in a blinded manner by TI and HM. Blinding was not performed in other experiments.

**Transmission electron microscopy (TEM) analysis.** TEM images were obtained using an electron microscope (Hitachi, H7700) at the Laboratory of Ultrastructural Research of Juntendo University.
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Competing interests
The authors have no conflicts of interest to declare.

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