Lipophagy prevents activity-dependent neurodegeneration due to dihydroceramide accumulation *in vivo*

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Abstract

Dihydroceramide desaturases are evolutionarily conserved enzymes that convert dihydroceramide (dhCer) to ceramide (Cer). While elevated Cer levels cause neurodegenerative diseases, the neuronal activity of its direct precursor, dhCer, remains unclear. We show that knockout of the fly dhCer desaturase gene, *infertile crescent* (*ifc*), results in larval lethality with increased dhCer and decreased Cer levels. Light stimulation leads to ROS increase and apoptotic cell death in *ifc*-KO photoreceptors, resulting in activity-dependent neurodegeneration. Lipid-containing Atg8/LC3-positive puncta accumulate in *ifc*-KO photoreceptors, suggesting lipophagy activation. Further enhancing lipophagy reduces lipid droplet accumulation and rescues *ifc*-KO defects, indicating that lipophagy plays a protective role. Reducing dhCer synthesis prevents photoreceptor degeneration and rescues *ifc*-KO lethality, while supplementing downstream sphingolipids does not. These results pinpoint that dhCer accumulation is responsible for *ifc*-KO defects. Human dhCer desaturase rescues *ifc*-KO larval lethality, and rapamycin reverses defects caused by dhCer accumulation in human neuroblastoma cells, suggesting evolutionarily conserved functions. This study demonstrates a novel requirement for dhCer desaturase in neuronal maintenance *in vivo* and shows that lipophagy activation prevents activity-dependent degeneration caused by dhCer accumulation.

Keywords dihydroceramide; lipophagy; neurodegeneration; photoreceptors; sphingolipid

Subject Categories Autophagy & Cell Death; Metabolism; Neuroscience

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Introduction

Sphingolipids are essential membrane constituents of all eukaryotic cells and play important roles in the regulation of basic cellular processes such as cell cycle progression, apoptosis, differentiation, and stress response (reviewed in [1]). Sphingolipid imbalance is linked to a wide range of human diseases, including metabolic disorders, cardiovascular diseases, chronic inflammation, cancers, and neurological disorders [2]. Cellular levels of sphingolipids are particularly high in the nervous system [3], and accumulating evidence highlights the importance of *de novo* sphingolipid biosynthesis in neuronal function [4–6]. The *de novo* synthesis pathway is a highly conserved process catalyzed by enzymes including serine palmitoyltransferase (SPT), ceramide synthase, and dihydroceramide desaturase to generate ceramide (Cer), which is at the center of sphingolipid metabolism and serves as the building block for more complex sphingolipids (Fig 1A). Cer levels in the brain increase with age [7], and high cellular levels of Cer cause cell death in various neurodegenerative diseases [8]. Dihydroceramide (dhCer) is the immediate precursor of Cer in the *de novo* synthesis pathway. Unlike Cer, treating cells with exogenous dhCer has little effect on cell growth or apoptosis [9]. Moreover, dhCer is structurally very similar to Cer, and endogenous dhCer is much less abundant than other sphingolipid species, reported to be only 2–3.5% of Cer levels in primary rat neurons [10]. These properties used to make it technically challenging to detect dhCer levels reliably. Thus, dhCer has long been considered inert and is often used as a negative control for Cer treatments [9,11]. However, accumulating evidence shows that dhCer accumulation leads to cell cycle arrests and cell death (reviewed in [12]), supporting that it is itself an active molecule.

Dihydroceramide desaturase catalyzes the final step of *de novo* synthesis to convert dhCer into Cer. There are two dhCer desaturase homologs (DEGS1 and 2) in humans, with DEGS1 being the predominant enzyme in most tissues [13]. DEGS1 knockout mice die shortly after birth [14]; thus, most studies of DEGS1 have been

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Figure 1. Knocking-out of the *Drosophila* dhCer desaturase gene, *ifc*, results in developmental arrest and sphingolipid imbalance.

A *De novo* sphingolipid synthesis pathway in *Drosophila*. *: note that sphinganine is synthesized from lauroyl-CoA in flies instead of palmitoyl-CoA as in mammals.
B Side-by-side view of a *w1118* control and a *ifc*-KO larva at 72 h after egg laying.
C Body lengths measured at 48 and 72 h after egg laying. ($n = 180, 95$, and 191 at 48 h versus $n = 69, 144$, and 121 at 72 h for control, *ifc*-KO, and *ifc*-KO; *tub > ifc*, respectively).
D Rescue of *ifc*-KO lethality by tissue-specific transgenic overexpression under Gal4 drivers: *tub*-Gal4 (ubiquitous), *elav*-Gal4 (pan-neuronal), *repo*-Gal4 (glia), and *mef2*-Gal4 (muscle). "+++": more than 50% of larvae rescued to pupal and adult stages, "++": between 35 and 50%, and "+": below 35%; "–": none. $n > 500$ flies were examined for lethality rescue for each cross from three independent experiments.
E–E''' Immunostaining of Ifc (green) and nucleus (DAPI, blue) in clonal *ifc*-KO imaginal eye discs (RFP[–]: *ifc*-KO; RFP⁺ and dashed lines: wild type). Arrowheads in (E', E''') indicate the Ifc signals in *ifc*-KO clones (RFP[–]). Scale bar: 10 μ m.
F–F''' dhCer staining (green) and nucleus labeling (DAPI, blue) in clonal *ifc*-KO imaginal eye discs (RFP[–]: *ifc*-KO; RFP⁺ and dashed lines: wild type). Scale bar: 10 μ m.
G, H UHPLC-MS/MS analysis showing the levels of sphingolipid species including sphinganine, dihydroceramide (DHC), ceramide (Cer), and sphingosine in second-instar control and *ifc*-KO larvae. Results are from three independent experiments.

Data information: In (C), data are presented as mean $\pm$ SD. *** $P < 0.001$ (one-way ANOVA with multiple comparisons). In (G, H), data are presented as mean $\pm$ SD. *** $P < 0.001$ (Student's *t*-test).

performed *in vitro*. Reducing *DEGS1* activity elevates cellular dhCer and reduces Cer levels [13–15], demonstrating that *DEGS1* is crucial in controlling the dhCer/Cer ratio. DhCer accumulation has been shown to increase reactive oxygen species (ROS) [16] and activate macroautophagy (referred to as autophagy hereafter) [17]. Although autophagy induction can function as a survival mechanism in response to cellular stress such as ROS, excessive autophagic activity is known to cause cell death. Conflicting results have been reported on the relationships between dhCer accumulation, ROS production, autophagy activation, and cell death. For example,

Apraiz *et al* [18] performed a series of combinatorial chemical treatments and reported the absence of causal relationships between dhCer accumulation, ROS production, and cell death in leukemia cells. Noack *et al* [19] showed that treating glioblastoma cells with sphingosine kinase inhibitor and the chemotherapeutic drug temozolomide results in dhCer accumulation, which leads to ER stress, ROS accumulation, and autophagy induction, eventually contribute to cancer cell apoptosis. Hernández-Tiedra *et al* [20] show that chemical inhibition of *DEGS1* activates autophagy and induces apoptosis in glioma cells and suggest that autophagy induction

promotes apoptosis. The cellular response to dhCer accumulation is likely to be context-dependent, and further studies are necessary to elucidate the neuronal activity of dhCer and the function of dhCer desaturase *in vivo*.

Here, we generated a null allele of the sole *Drosophila* dhCer desaturase gene, *infertile crescent* (*ifc*), and examined its effect on activity-dependent neurodegeneration of photoreceptor. We used both genetic and pharmacological manipulations to show that *ifc*-KO leads to dhCer accumulation, which increased cellular ROS and resulted in activity-dependent degeneration of *Drosophila* photoreceptors. We found that dhCer accumulation activated lipophagy, which is a specialized form of autophagy for the degradation of lipid droplets [21], and that lipophagy plays a protective role to reduce ROS and cell death in *ifc*-KO photoreceptors. We further investigated the interplay between dhCer accumulation, ROS level, and lipophagy and showed that the findings are conserved in human neuroblastoma cells.

Results

Evolutionarily conserved function of dhCer desaturase is required for larval viability in *Drosophila*

We generated *ifc* knockout flies (*ifc*-KO) using the CRISPR/Cas9 system (Fig EV1A). The excision of the entire *ifc* open-reading frame was confirmed by PCR and sequencing (Fig EV1B), and qRT-PCR confirmed the lack of detectable *ifc* mRNA in the knockout larvae (Fig EV4A). Zygotic deletion of *ifc* resulted in early developmental delay (Fig 1B and C), and homozygous *ifc*-KO larvae eventually died at the L2 larval stage. Ubiquitous expression of *ifc* full-length cDNA (*tub > ifc*) rescued the developmental arrest and larval lethality of *ifc*-KO flies (Fig 1C and D). We therefore consider *ifc*-KO a molecularly defined null allele. Transgenic UAS-*ifc*-mCherry was functionally comparable to the non-tagged Ifc in rescuing *ifc*-KO lethality (Fig 1D) and was thus used for further analyses below.

We created mosaic eye imaginal discs using the *ey*-FLP/FRT system so that only the wild-type cells, but not the *ifc*-KO photoreceptors, were marked with RFP [22]. We detected Ifc protein in the mosaic *ifc*-KO clones with anti-Ifc polyclonal antibodies generated for this study (see Materials and Methods). The amount of Ifc protein was greatly reduced in RFP-negative *ifc*-KO clones (Figs 1E–E''' and EV1C). When we expressed *ifc* cDNA in *ifc*-KO flies under tissue-specific Gal4 drivers, targeted expression of Ifc in pan-neuronal tissues (*elav*-Gal4), glial cells (*repo*-Gal4), and muscle (*mef2*-Gal4) could all rescue the lethality of *ifc*-KO flies to adulthood (Fig 1D), suggesting that transgenic overexpression of Ifc may exert cell non-autonomous activity. Exogenous expression of human DEGS1 was able to rescue *ifc*-KO to adulthood (Fig 1D), suggesting that fly Ifc and human DEGS1 are functionally conserved.

In the mosaic eye discs, we found that dhCer levels increased in *ifc*-KO clones, comparing to those in the adjacent wild-type cells (Figs 1F–F''' and EV1D). The UHPLC-MS/MS profile of larval lipid extracts showed 20–350-fold increases in the levels of dhCer subpecies and its precursor sphinganine in *ifc*-KO over wild-type controls (Fig 1G), accompanied by >fourfold decreased levels of downstream metabolites Cer and sphingosine (Fig 1H). This imbalance indicated

that the enzymatic function of Ifc could not be compensated, and Ifc is an essential regulator of sphingolipid homeostasis in *Drosophila*.

Loss of *ifc* led to activity-dependent neurodegeneration

We went on to investigate the effects of mosaic and whole-eye *ifc*-KO clones in otherwise heterozygous animals. At the time of eclosion, the whole-eye clones of *ifc*-KO were indistinguishable from wild-type eyes in the overall appearance and the arrangement of photoreceptors, suggesting no cell-autonomous requirement for photoreceptor development (Fig 2A–C). When we stressed the adult *ifc*-KO eyes with constant light exposure, we observed significant reduction in rhabdomere numbers accompanied with distorted photoreceptor margins at 5 and 10 days post-eclosion (Fig 2B' and B''). Likewise, the depolarization by electroretinogram (ERG) was also significantly reduced (Fig 2D and E). Both the morphological defects (Fig 2A–C) and the functional decline (Fig 2D and E) exacerbated over time. However, we did not observe the same level of premature degeneration for the dark-reared *ifc*-KO eyes (Fig 2B''' and C–E). These data indicated that photoreceptors underwent an adult-onset, activity-dependent neurodegeneration in the absence of *ifc*. Mosaic *ifc*-KO clones showed similar level of activity-dependent degeneration (Fig 2F–G), supporting that *ifc* is required for the maintenance of adult photoreceptor function.

ifc-KO photoreceptor degeneration was caused by increased dhCer, not reduced levels of ceramide or sphingosine

Many phenotypes of *ifc*-KO, including Cer reduction, developmental arrest, and early lethality, were similar to those reported for *schlank* mutants [23]. *Schlank* encodes the only *Drosophila* ceramide synthase responsible for converting sphinganine to dhCer, and it is the enzyme directly upstream of Ifc in the *de novo* synthesis pathway (Fig 1A). These phenotypic similarities seem to suggest that Cer deficiency is the common culprit of both mutants. However, while Cer feeding indeed raised Cer levels both in the heads and in bodies (Fig EV2A–B'), supplementation of downstream sphingolipids (Cer or sphingosine) did not restore the functional or morphological defects of *ifc*-KO eyes (Figs 3A and EV2C–D). Therefore, the defects of *ifc*-KO flies were unlikely to be due to reduced Cer level.

We next tested whether the degeneration was due to increased levels of dhCer by inhibiting the upstream enzyme Schlank. We confirmed that ubiquitous knockdown of *schlank* led to larval lethality in wild type and *ifc*-KO background ([23] and Fig 3B). Interestingly, neuron-specific knockdown of *schlank* (*elav > schlank*-RNAi) rescued the larval lethality and photoreceptor morphology/function in *ifc*-KO animals without causing any observable phenotype in the wild-type background (Fig 3B–E). The ceramide synthase inhibitor fingolimod also rescued the morphological and functional defects of *ifc*-KO photoreceptors (Fig 3F–H). Altogether, blocking dhCer synthesis suppressed the *ifc*-KO defects, and the lethality of *ifc*-KO was due to the neuronal accumulation of dhCer.

The subcellular localization of Ifc

We performed immunostaining to detect Ifc in flies expressing fluorescent transgenes labeling ER (KDEL-RFP), Golgi (galactosyltransferase-RFP), or mitochondria (Mito-GFP). In larval salivary glands,

Figure 2. Activity-dependent degeneration of *ifc-KO* eyes in morphology and function.

A–C Eye cross sections of wild type (A) and *ifc-KO* (B) under different constant light stimulation or dark-reared condition labeled with rhabdomeres (Phalloidin, green) and neuronal membrane (Na^+/K^+ -ATPase, magenta). Scale bars: 10 μm . Rhabdome counts per ommatidium quantified in (C) (≥ 30 ommatidia/fly from $n \geq 3$ flies for each genotype, time point, or light exposure).

D, E Electrophoretogram (ERG) responses of control versus *ifc-KO* eyes. Constant light stimulation for 10 days leads to a striking decrease in the depolarization amplitude of *ifc-KO* eyes compared to the wild-type controls and the dark-reared *ifc-KO* eyes. Sample ERG traces show in (D). (E) Quantification of depolarization amplitudes in (D) ($n = 12$ and 17 at day 0, $n = 17$ and 19 at day 5, $n = 15$ and 25 at day 10 for control and *ifc-KO* flies, respectively; $n = 18$ and 25 at day 10 for control and rescued flies, respectively; $n = 13$ and 8 at day 10 for control and dark-reared *ifc-KO* flies, respectively).

F–G Eye cross sections of *ifc-KO* mosaic clones generated by *ey-FLP* showing rhabdomeres (Phalloidin, green) and neuronal membrane morphology (Na^+/K^+ -ATPase, magenta) after 10-day light stimulation. Control cells were labeled by GMR-myr-RFP and *ifc-KO* cells lack GMR-myr-RFP expression, separated by dashed lines. Scale bar: 10 μm . Rhabdome counts per ommatidium quantified in (G) (≥ 30 ommatidia/fly from $n \geq 7$ flies for each genotype).

Data information: In (C, E, G), data are presented as mean $\pm$ SD. ** $P < 0.001$, *** $P < 0.001$ (Student's *t*-test).

Ifc was mostly associated with the ER (Fig EV3), consistent with previous reports for human DEGS1 [24]. In photoreceptors, however, Ifc showed little or no colocalization with the ER, Golgi, or Mito markers (Fig 4A–D). Instead, Ifc signals colocalized with endosomal markers GFP-FYVE (endosomes), Spin-GFP (late endosome/lysosome), Lamp1-GFP (late endosome/lysosome), and Rab4-EYFP (recycling endosome) (Fig 4E–H). To verify the results, we took *ifc-KO* flies expressing a functional UAS-*ifc*-mCherry transgene and detected with antibodies against BiP (ER), Rab5 (early endosome), or Rab7 (late endosome/autophagosome). Ifc-mCherry colocalized more with Rab7, but much less so with BiP or Rab5 (Fig 4I–L). The distinct subcellular localization of Ifc between the

developing salivary gland and adult photoreceptors suggested that Ifc may exert novel neuronal functions other than the previously reported roles in the ER.

Because Rab4 regulates recycling endosomes and exocytosis [25], we tested whether Ifc can be transferred between different tissues, which would provide a mechanism for how Ifc overexpression in specific tissues could rescue *ifc-KO* larval lethality. In *ifc-KO* animal rescued by glial expression of Ifc-mCherry, mCherry fluorescence was detected in photoreceptors (Fig 4M–M''). Also, Ifc-mCherry expressed from cone or pigment cells was detected within photoreceptors (Fig 4N–O''). These results supports that Ifc can move across cell boundaries and functions cell non-autonomously.

Figure 3. Activity-dependent neurodegeneration in *ifc-KO* eyes and genetic and chemical manipulations of dhCer levels.

A Amplitude of ERG depolarization in *ifc-KO* flies fed with the solvent DMSO, sphingosine (Sph), or ceramide (Cer) for 10 days after eclosion, comparing to controls ($n \geq 28$ each).

B Rescue of *ifc-KO* lethality by ubiquitous (*tub-Gal4*) (pupae: 25/100; adult: 5/100) or pan-neuronal (*elav-Gal4*) (pupae: 19/82; adult: 3/82) knockdown of *schlank*, compared to ubiquitous (pupae: 12/64; adult: 24/64) or pan-neuronal (pupae: 25/100; adult: 5/100) *ifc* expression.

C–E Neuronal knockdown of *schlank* (*elav>schlank-RNAi*) rescued photoreceptor degeneration of *ifc-KO* after 10-day light stimulation. (**C**, **C'**) Eye cross sections of *elav>schlank-RNAi* and *ifc-KO*; *elav>schlank-RNAi* flies showing rhabdomeres (Phalloidin, green) and neuronal membrane morphology (Na^+/K^+ -ATPase, magenta). Scale bars: 10 μm . (**D**) Quantification of (**C**, **C'**) (≥ 30 ommatidia/fly from $n \geq 3$ each). (**E**) Quantification of ERG depolarization amplitudes ($n = 15, 25, 17$, and 8 flies for control, *ifc-KO*, *elav>schlank-RNAi*, *ifc-KO; elav>schlank-RNAi*, respectively).

F–H Inhibition of Schlank by fingolimod partially restored the morphology and function of photoreceptors in *ifc-KO* flies after 10-day light stimulation. (**F**, **F'**) Eye cross sections of *ifc-KO* flies fed with (**F**) or without (**F'**) fingolimod showing rhabdomeres (Phalloidin, green) and neuronal membrane morphology (Na^+/K^+ -ATPase, magenta). Scale bars: 10 μm . (**G**) Quantification of (**F**, **F'**) (≥ 30 ommatidia/fly from $n \geq 6$ each). (**H**) Quantification of ERG depolarization amplitudes in control and *ifc-KO* flies fed with or without fingolimod-supplemented food. ($n = 15, 7, 18$, and 7 for control, control + fingolimod, *ifc-KO*, *ifc-KO* + fingolimod flies, respectively).

Data information: In (**A**, **D**, **E**, **G**, **H**), data are presented as mean $\pm$ SD. *** $P < 0.001$ (one-way ANOVA with multiple comparisons).

Activation of lipophagy in *ifc-KO* eye

To further characterize the aforementioned defects, we examined the ultrastructure of *ifc-KO* photoreceptors using transmission electron microscopy (TEM). After 10 days of light exposure when wild-type controls showed little morphological change (Fig 5A), *ifc-KO* eyes had already shown dramatic rhabdomeric loss (Fig 5B) and prominent apoptotic bodies (Fig 5C). We therefore checked *ifc-KO* eyes at earlier times and found that, after 3 days of constant light exposure, we could already identify lipid droplet

(LD)-like structures surrounded by double membranes, reminiscent of lipid-containing autophagosomes (Fig 5D–F). Transcription of autophagy genes *atg1*, *atg5*, *atg7*, and *atg8a* was significantly upregulated in *ifc-KO* larvae (Fig EV4A), and confocal imaging showed colocalization of the autophagy marker Atg8a with the LD markers LipidTOX in *ifc-KO* photoreceptors (Fig 5G–G') or BODIPY in *ifc*^{+/-} eyes (Fig 5H–H'). Altogether, lipophagy was activated in *ifc-KO* photoreceptors.

We then interrogated the functional importance of autophagy in photoreceptor degeneration by feeding adult flies with an autophagy

Figure 4.

inducer or inhibitor. Feeding with rapamycin, an autophagy inducer, alleviated both the morphological and functional declines of *ifc*-KO photoreceptors (Fig 6A–C) and decreased the number of BODIPY puncta (Fig 6D', D'' and E) as compared to the solvent-fed group. In contrast, blocking autophagy by 3-methyladenine (3-MA) increased the number of lipid puncta (Fig 6F–G) and sped up the degeneration as shown upon 3 days of light stimulation (Fig 6H). Therefore, enhancing autophagy reduced intracellular accumulation of LDs and protected against light-induced degeneration of photoreceptors.

Lipophagy induction reduced ROS accumulation and prevented photoreceptor degeneration

Although DEGS1 inhibition is known to elevate dhCer accumulation and to increase ROS level in cell lines, it is not yet clear whether there are causative links between dhCer accumulation, ROS production, and the subsequent cell death. We found that constant light stimulation increased the levels of ROS in *ifc*-KO photoreceptors as measured by CM-H₂DCFDA (Fig 7A and A'), and the expression of

Figure 4. Ifc is mainly detected in the endolysosomal compartments in photoreceptors and can reach photoreceptors when expresses in glial, cone, and pigment cells.

- A–H Eye longitudinal sections of wild-type flies with transgenic expression of compartmental markers showing transgenic markers (pseudo-colored in green) and anti-Ifc staining (red). (A) The degree of colocalization between Ifc and each compartmental marker from (B–H) quantified by Pearson's correlation coefficients. Subcellular compartments were labeled with fluorescent UAS transgenes (expressed by GMR-Gal4) include KDEL-RFP for ER (B), galactosyltransferase-RFP for Golgi (C), Mito-GFP for mitochondria (D), FYVE-GFP for endosomes (E), Spin-GFP (F), and Lamp1-GFP (G) for late endosome/lysosome, endogenously EYFP-tagged Rab4 (H) (≥ 1 field/eye from $n \geq 3$ each). Scale bars: 10 μm or 2 μm for the magnified insets.
- I–L Eye longitudinal sections showing immunolabelings (green) of BiP for ER (I), Rab5 for early endosomes (J), and Rab7 for late endosomes/autophagosomes (K) in *ifc-KO*; *tub>ifc-mCherry* eyes. (L) The degrees of colocalization by Pearson's correlation coefficients for (I–K) (≥ 1 field/eye from $n \geq 3$ each). Scale bars: 10 μm or 2 μm for the magnified insets.
- M–O" Eye longitudinal sections showing rhabdomeres (Phalloidin, cyan), neuronal membrane (Na^+/K^+ -ATPase, blue), CD8-GFP (green), and *ifc-mCherry* (red) in *ifc-KO*; *repo>ifc-mCherry* (M–M", glia-specific expression), *rdhb>CD8-GFP + ifc-mCherry* (N–N", pigment cell-specific expression), and *spa>CD8-GFP + ifc-mCherry* (O–O", cone cell-specific expression). Arrows indicate the *ifc-mCherry* puncta found within photoreceptors. Scale bars: 10 μm or 2 μm for the magnified insets.

Data information: In (A, L), data are presented as mean $\pm$ SD.

Figure 5. Activation of lipophagy in *ifc-KO* photoreceptors.

- A–F Transmission electron microscopy (TEM) of the *ifc-KO* eyes after 10-day (A–C) and 3-day (D–F) light stimulation. Comparing to wild-type controls (A) after 10-day light stimulation, *ifc-KO* eyes showed reduced number of rhabdomeres (B) with apoptotic bodies (arrow), which is magnified in (C) to show fragments of nucleus and organelles within the apoptotic body. N, nucleus. M, mitochondria. Lipophagic vesicle-like structures were detected after 3 days as indicated by arrowheads in (D), magnified in (E) (indicated by red arrows), and further enlarged in (F). Scale bars: 1 μm in (A–E); 100 nm in (F).
- G–G" Eye cross sections of *ifc-KO* mosaic clones generated by *ey-FLP* showing staining of Atg8a (G, red) and LipidTOX (G', green) after 5-day light stimulation. Note that Atg8a⁺ puncta colocalized with LipidTOX⁺ puncta in *ifc-KO* clones (G", arrows).
- H–H" Eye longitudinal sections showing colocalization of BODIPY493/503 with transgenic expression of mCherry-Atg8a in *ifc^{-/-}* eyes after 3-day light stimulation. The arrowhead and arrow indicate partial and complete colocalization, respectively. Scale bars: 10 μm or 2 μm for the insets in (H").

ROS responsive genes *Cap'N'Collar* (*cnc*) and *glutathione S-transferase D1* (*gstD1*) was upregulated in *ifc-KO* larvae (Fig EV4B). Reducing ROS levels in *ifc-KO* flies by ectopic expression of human superoxide dismutase SOD1 (Fig 7A" and B) or feeding with the antioxidant N-acetylcysteine amide (AD4) significantly restored the functional and morphological degeneration of *ifc-KO* eyes caused by light stimulation (Fig 7C–E). Thus, reducing ROS prevented the activity-dependent neurodegeneration in *ifc-KO* photoreceptors. In addition, we did not detect the expression of XBP1-GFP reporter [26] in *ifc-KO* clones (Fig EV4C–E"), suggesting that ER stress response was not induced, which is consistent with a lack detection of Ifc in the ER in *ifc-KO* photoreceptors.

We then sought to understand the possible interplay between ROS and autophagy. We performed double feeding of rapamycin and AD4 and found similar level of morphological and functional preservation in *ifc-KO* photoreceptors as single treatments (Figs 6C and 7E). Removing ROS by hSOD1 expression reduced the number of Atg8a puncta to background levels in *ifc-KO* eyes (Fig 7F–G), while AD4 feeding also suppressed the increase in Atg8a puncta in *ifc-KO* photoreceptors (Fig EV4F–G). Rapamycin reduces ROS signals to a level comparable to that of wild type (Fig 7H–I). Taken together, ROS increase induced lipophagy, and lipophagy activation can reduce the level of ROS and prevent the activity-dependent degeneration of *ifc-KO* photoreceptors.

DhCer accumulation caused apoptosis in human neuroblastoma cells

We tested whether the results we showed in *Drosophila* are conserved in human neuroblastoma SH-SY5Y cells. Treating cells with dhCer or fenretinide effectively reduced cell viability (Fig 8A and B). Similar to our observations in *Drosophila*, fenretinide treatment resulted in apoptosis as indicated by increased active caspase-3 (Fig 8C–D). Concurrently, fenretinide treatment increased the number of lipid droplets, which was reduced by rapamycin (Fig 8E–F). Although high level of fingolimod treatment results in cell death [27], we found that, when SH-SY5Y cells were treated with a sublethal dose of fingolimod, it reversed the effect of fenretinide and restored cell viability. That is, blocking dhCer production by inhibiting ceramide synthase rescued the deleterious effect of dhCer desaturase inhibition (Fig 8B and D). Our data supported that the cytotoxicity of dhCer accumulation is evolutionarily conserved between fly and human (Fig 8G).

Figure 6. Rapamycin rescues the degenerative defects and reduces lipid accumulation of *ifc*-KO photoreceptors.

A, A' Eye cross sections of *ifc*-KO flies fed with rapamycin showing rhabdomeres (Phalloidin, green) and neuronal membrane (Na⁺/K⁺-ATPase, magenta). Flies were fed with rapamycin-supplemented food (A') or EtOH solvent control (A) for 10 days post-eclosion. Scale bars: 10 μm.

B Rhabdomere counts of (A, A') (≥ 30 ommatidia/fly from *n* ≥ 10 each).

C Amplitude of ERG depolarization of *ifc*-KO flies fed with EtOH, rapamycin, and/or AD4 for 10 days post-eclosion (*n* ≥ 15 each).

D–D'' Representative confocal longitudinal sections of *ifc*-KO photoreceptors showing BODIPY493/503 staining. *ifc*-KO flies fed with rapamycin (D'') or EtOH solvent (D') compared to control flies fed with EtOH solvent (D) for 3 days since eclosion. Scale bars: 10 μm.

E Quantification of BODIPY puncta/mm² in (D–D'') (≥ 1 field/eye from *n* ≥ 6 each).

F, F' Representative confocal longitudinal sections of *ifc*-KO photoreceptors. *ifc*-KO flies were fed with 3-methyladenine (3-MA) (F') or sucrose solvent control (F) for 3 days since eclosion. Scale bars: 10 μm.

G Quantification of BODIPY puncta/mm² in (F, F') (≥ 1 field/eye from *n* = 3 each).

H Amplitude of ERG depolarization for control and *ifc*-KO flies fed with sucrose solvent and/or 3-MA (*n* ≥ 11 each).

Data information: In (B, C, E, H), data are presented as mean ± SD. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (one-way ANOVA with multiple comparisons). In (G), data are presented as mean ± SD. **P* < 0.05 (Student's *t*-test).

Discussion

Altered brain sphingolipid levels are noted in neuronal disorders including Niemann-Pick, Alzheimer's, and Parkinson's diseases [2]. Here, we generated a null mutant of *Drosophila infertile crescent* and examined the neuronal role of dihydroceramide desaturase and the consequence of dhCer accumulation. Since global knockout of *ifc* led to developmental arrest and lethality, we employed clonal *ifc* deletion and heterozygous animals, as well as pharmacological *Ifc* inhibition to study its function in photoreceptor degeneration. Deletion of *ifc* in photoreceptors resulted in dhCer accumulation, increased ROS, and activity-dependent neurodegeneration. We demonstrated that *ifc*-KO photoreceptor degeneration was due to

increased dhCer. Reducing dhCer synthesis by downregulating *schlank* (ceramide synthase) sufficiently restored defects in *ifc*-KO photoreceptor morphology and functions, while supplementing *ifc*-KO flies with downstream sphingolipids did not. The findings were confirmed by chemical inhibition of Schlank and *Ifc* by fingolimod and fenretinide treatments, respectively. DhCer accumulation activated lipophagy in photoreceptors. Further activating lipophagy by rapamycin reduced ROS and lipid droplet levels and alleviated *ifc*-KO defects, suggesting lipophagy played a protective role. Additionally, we found that exogenous dhCer reduced the viability of SH-SY5Y cells, and a sublethal dose of fingolimod reversed fenretinide-induced apoptosis, reinforcing that toxicity of dhCer accumulation is conserved in human neuroblastoma cells. Further

Figure 7. ROS induces autophagy while autophagy activation reduces ROS in *ifc-KO* photoreceptor.

A–A'' Longitudinal sections of photoreceptors stained with CM-H₂DCFDA to detect ROS in control (A), *ifc-KO* (A'), and *ifc-KO; elav>hSOD* (human SOD) (A'') eyes after 3-day light stimulation. Scale bars: 10 μ m.

B Quantification of CM-H₂DCFDA signals in (A–A'') (≥ 1 field/eye from $n = 3$ each).

C, C' Cross sections of photoreceptors after 10-day light stimulation in *ifc-KO* (C) and *ifc-KO* flies fed with AD4 (C'). Scale bars: 10 μ m.

D Rhabdomere counts in (C, C') (≥ 30 ommatidia/fly from $n \geq 4$ each).

E Amplitude of ERG depolarization of wild-type and *ifc-KO* flies with or without AD4 ($n \geq 17$ flies for each genotype and treatment).

F–F'' Eye longitudinal sections of control, *ifc-KO*, and *ifc-KO; elav>hSOD* flies showing rhabdomeres (Phalloidin, green) and Atg8a (magenta). Scale bars: 10 μ m.

G Quantification of Atg8a puncta/mm² in (F–F'') (≥ 1 field/eye from $n \geq 5$ each).

H–H'' The effect of rapamycin on ROS levels in *ifc-KO* photoreceptors after 3-day light stimulation. Nuclei and ROS were labeled with DAPI (blue) and CM-H₂DCFDA (green), respectively. Scale bars: 10 μ m.

I Normalization of CM-H₂DCFDA signals based on controls in (H–H'') (≥ 1 field/eye from $n \geq 4$ each).

Data information: In (B, D, E, G, I), data are presented as mean $\pm$ SD. ** $p < 0.01$, *** $p < 0.001$ (one-way ANOVA with multiple comparisons).

examination of the functional importance of DEGS1 and dhCer especially in the context of the nervous system is needed.

Cell non-autonomous rescue of dhCer

Our data showed that *Ifc* is functionally required during larval development in flies. The developmental requirement of *Ifc* can be supplemented cell non-autonomously, since *ifc-KO* larval lethality was rescued by overexpressing *ifc* in pan-neuronal (*elav-Gal4*), neuron-supporting (*repo-Gal4*), or muscle tissues (*mef2-Gal4*). Cell

non-autonomous rescue has been previously reported in a mutant of *Drosophila* ceramidase *CDase*. *CDase* expression in fat body or mushroom bodies can be detected in retinal tissue and rescues adult *CDase* mutants that are defective in phototransduction [28]. We were able to detect *Ifc* in photoreceptors when it was transgenically expressed in supportive tissues including glia, cone cells, and pigment cells (Fig 4M–O). However, whole-eye and random *ifc-KO* clones both showed activity-dependent neurodegeneration, clearly demonstrating that the cell non-autonomous supply of *Ifc* from neighboring cells was not sufficient to prevent dhCer accumulation

Figure 8. Inhibition of DEGS1 results in dhCer accumulation, apoptosis, and lipid accumulation in human neuroblastoma cells.

A Cell viability determined by MTT assay under different concentrations of dhCer or Cer. DMSO served as the solvent control.

B Cell viability by MTT assay after fingolimod and/or fenretinide treatments.

C–C''' Representative confocal images of cells showing staining of active caspase-3 (green), F-actin (Phalloidin, red), and nuclei (DAPI, blue) in cells treated with DMSO (C, solvent), fingolimod (C'), fenretinide (C''), or cotreated with fenretinide and fingolimod (C'''). Scale bar: 10 μ m.

D Quantification of SH-SY5Y cells that stained positive for active caspase-3 in (C–C''').

E–E''' Representative confocal images of cells showing staining of BODIPY493/503 (green) and nuclei (DAPI, blue) in cells treated with DMSO (E, solvent), rapamycin (E'), fenretinide (E''), or rapamycin + fenretinide (E'''). Scale bar: 10 μ m.

F Normalization of BODIPY signals based on controls in (E–E''').

G Proposed model of neurodegeneration in the absence of Ifc.

Data information: In (A, B, D, F), data are presented as mean $\pm$ SD of three independent experiments. * P < 0.01, ** P < 0.01, *** P < 0.001 (one-way ANOVA with multiple comparisons).

and the subsequent degeneration of adult *ifc*-KO photoreceptors. Due to technical difficulties in genetic manipulation, we could not test whether *Ifc* transgenic overexpression would prevent activity-dependent degeneration of *ifc*-KO eye clones. It is possible that level of *Ifc* is critical for rescue. Alternatively, comparing to the short developmental process, accumulation of subtle effects of slight insufficiencies in *Ifc* levels may progress over the long course of neuronal maintenance, resulting in visible phenotypes in adult eyes.

Endosomal localization of *Ifc* and its cell non-autonomous functions

Intracellular sphingolipid metabolism is a highly compartmentalized process; the *de novo*, catabolic, and salvage pathways take place in the ER, Golgi and mitochondria, and endosomes and lysosomes, respectively [29]. This compartmentalization results in organelle-specific pools of sphingolipid species, and coordination of these reactions is variable depending on cell and tissue types. Although it is widely accepted that enzymes involved in ceramide *de novo* synthesis are present on the ER membrane, few reports actually examined the subcellular localization of DEGS1, and no definitive evidence shows that it resides exclusively in the ER [30]. In fact, Beauchamp *et al* [31] reported DEGS1 activity in both the ER and mitochondria in rat hepatocytes. Murakami *et al* [32] showed Golgi localization of GFP-tagged human DEGS1 in yeast cells. Moreover, Novgorodov *et al* [33] showed that, while neutral ceramidase in most cells is mainly localized to plasma membranes, it shows punctate distribution that is associated with mitochondria, lysosomes, and late endosomes in liver hepatocytes. We showed that while *Ifc* located mainly in the ER in larval salivary glands, its subcellular localization in adult photoreceptor appeared to differ. Antibody staining against *Ifc* and fluorescence of a functional *Ifc* fusion protein showed predominant colocalization with endolysosomal markers. Although we do not argue against a presence of *Ifc* in the ER, our data demonstrated a previously unknown localization for neuronal *Ifc* in the endolysosomal compartments and showed that dhCer accumulation did not induce ER stress in *Drosophila* photoreceptors. Overall, our data supported that neuronal *Ifc* is functionally important in endosomes in photoreceptors. Further investigation is needed to explore whether DEGS1 localization differs between cell types and decipher the significance of dihydroceramide desaturase functioning in endolysosomes.

Interplay between autophagy/lipophagy and ROS

Several sphingolipid species, including Cer, sphingosine, S1P, and dhCer, can induce autophagy and cell death (reviewed in [34]). Depending on the context, inhibiting these enzymes induce protective autophagy, apoptosis, or autophagic cell death. For example, acid sphingomyelinase deficiency impairs autophagy and conveys resistance to hepatic steatosis [35]. Losing neutral ceramidase induces autophagy and prevents cell deaths caused by nutrient or energy deprivation [36]. Reciprocally, autophagy can also regulate sphingolipid levels [37]. In mouse liver, blocking autophagy by deleting *Atg7* leads to increased Cer and SPT levels [38]. These findings suggest a possible feedback mechanism between sphingolipid levels and autophagy activation. We demonstrated that *ifc*-KO leads to dhCer accumulation, which activates autophagy; however, consistent with previous reports, autophagy was not the cause of

ifc-KO photoreceptor death. In contrast, we showed that further activating autophagy reduces ROS levels and lipid droplets, providing protection for *ifc*-KO photoreceptors.

Recent studies found that one specialized form of autophagy termed lipophagy can be employed for lipid degradation. Selective degradation of cellular lipids by autophagy, often called lipophagy, plays an important role in energy homeostasis by regulating lipid storage and metabolism [39]. Hepatic cells activate lipophagy under starvation to generate free fatty acids (FFA) as an energy source, whereas blocking hepatic autophagy leads to lipid accumulation *in vivo* and *in vitro* [39]. Defective lipophagy causes lipid droplet accumulation in cellular and mouse models of Huntington's disease [40]. Hepatic autophagy has been identified as an important survival mechanism that prevents cell death induced by palmitate accumulation [41]. SPT overexpression activates lipophagy and promotes lipid degradation in hepatic cells [38]. In these reports, increased saturated free fatty acids such as palmitate inhibit autophagy, likely by preventing lysosomal acidification. Rapamycin treatment reversed autophagy inhibition and alleviated palmitate toxicity in pancreatic B cells. Interestingly, unsaturated FFA oleate does not inhibit autophagy in hepatocytes but inhibits autophagy in pancreatic B cells, indicating cell type specificity [41,42]. Here, we reported that dhCer accumulation increased ROS and induced lipophagy in *Drosophila* photoreceptors. In this case, lysosomal acidification of autophagosomes occurred normally, and further inducing autophagy by rapamycin lowered ROS levels and promoted the survival and function of rhabdomeres. We currently do not have evidence to show whether accumulated dhCer is selectively targeted for lipophagic degradation, since BODIPY^{493/503} does not stain dhCer and it does not work for co-staining with anti-dhCer antibody. Further analysis is necessary to determine whether accumulated dhCer is selectively targeted for lipophagic degradation, or whether dhCer is degraded via association with the autophagic membrane.

Materials and Methods

Fly stocks and genetics

Fly husbandry was performed at 25°C following standard procedures. *w1118* was used as the wild-type control for all experiments unless otherwise stated. Both dark-reared and light-raised flies were kept in the same room, except that the dark-reared vials were wrapped with aluminum foil. A constant, 600 Lux LED light source was provided for light stimulation.

The *ifc* knockout flies were generated utilizing the CRISPR/Cas9 system as described in [43] with modifications. Briefly, two pairs of gRNAs were designed to target the 5' and 3' UTR regions of *ifc* locus for removing the entire coding region. The gRNA primers are shown below:

5'UTR-*ifc*-fwd:CTTCGACAGTTTCCTTCTCGCGC,
5'UTR-*ifc*-rev:AAACGCGCGAGGAAGGAACTGTC,
3'UTR-*ifc*-fwd:CTTCGCGGATGCAGAAGAAATAAG,
3'UTR-*ifc*-rev: AAAC CTTATTTCTTCTGCATCCGC

The gRNA pairs were cloned into pBfV-U6.2B, and the construct was injected into P{nos-phiC31}int.NLS}X; P{CaryP}attP40 (Bloomington #25709) embryos. The transformants were identified following

standard phiC31-mediated germline transmission protocol, and then, they were crossed to $P\{nos-Cas9, y^+, v^+\}$ flies for germline-specific deletion of *ifc*. Potential knockout flies from the progeny were identified by complementation tests with two different deficiency lines uncovering *ifc* locus obtained from the Drosophila Genetic Resource Center (DGRC, #150010 and #150494), Kyoto. The *ifc*-KO flies were verified by PCR. The primer set for PCR screen is shown below: Screen-*ifc*-fwd: TCAGGTCATATTAATTTTCAATCC Screen-*ifc*-rev: ATTGTTTCATATCAGCCGCTG

Eye-specific mosaic clones were generated using *ey-FLP* on the X chromosome [22]. The *ifc*-KO chromosome was recombined on an FRT40A chromosome using G418 selection. Whole-eye clones of *ifc*-KO photoreceptors were generated by flipping *ifc*-KO against *l(2)cl-2L* chromosome (*y w eyFLP; ifc*-KO, FRT40A/*l(2)cl-2L P{w⁺}* FRT40A).

Full-length *ifc* cDNA was amplified by PCR from a cDNA library of *Drosophila* heads. To generate UAS-*ifc*, *ifc* cDNA was cloned into the NotI/XhoI sites of pUAS vector. To generate the UAS-*ifc*-mCherry and UAS-Cyan-*ifc* constructs, *ifc* cDNA was fused with either mCherry sequence at the C terminus or with Cyan sequence at the N terminus by SOEing PCR prior to cloning into the pUAS vector. Full-length human *DEGS1* was obtained from a cDNA library of human liver tissue and was cloned into the NotI/XhoI sites of pUAS vector. All constructs were verified by sequencing. Transgenic flies were generated by embryo injection according to standard protocols of transposase-mediated insertion followed by germline transmission screen.

For rescue experiments, UAS-*ifc* transgenes were introduced on the third chromosome. Rescue experiments were performed by expressing *ifc* cDNA with tissue-specific Gal4 drivers (stocks described below). Successful rescue of larval lethality was defined as survival to pupal and adult stages. Three independent crosses were done for each set of experiments, and > 500 flies were counted for each cross. The genotypes of the rescued adult flies were verified by PCR.

For immunostaining, clonal *ifc*-KO imaginal discs were generated by FRT-FLP recombination in conjunction with ubi-RFP on the wild-type chromosome (*y w eyFLP; ifc*-KO, FRT40A/*P{w [+mC] = Ubi-mRFP.nls}2L, FRT40A*). For immunostaining of the adult photoreceptors, the same whole-eye *ifc*-KO clones for rescue experiments were used.

For fly stocks used in the rescue experiments, subcellular compartment characterization, and knockout, below were from the Bloomington Drosophila Stock Center (# indicates stock number): FRT40A (#5615), *ey-FLP*; *l(2)cl-L31, FRT40A* (#5622), *nls-RFP, FRT40A* (#34500), *ey-FLP*; GMR-myr-mRFP, FRT40A (#7122), *tubulin-Gal4* (#5138), *elav-Gal4* (#8760), *repo-Gal4* (#7415), *mef2-Gal4* (#27390), *71B-Gal4* (#1747), *spa-Gal4* (#26656), *rdhB-Gal4* (#24501), $P\{nos-phiC31\}int.NLS\}X$; $P\{CaryP\}attP40$ (#25709), *ninaE*¹⁷ (*rh1* mutant, #5701), UASp-RFP-KDEL (#30910), UASp-Mito-GFP (#8443), UASp-RFP-Golgi (#30902, galactosyltransferase-RFP), UAS-GFP-FYVE (#42712), UAS-LAMP1-GFP (#42714), UAS-Spin-GFP (#39668), UAS-XBP1-GFP (#39719), UAS-hSOD1 (#33606), UASp-GFP-mCherry-Atg8a (#37749), Rab4-EYFP (#62542). $y^2 cho^2 v^1$; $P\{nos-Cas9, y^+, v^+\}3A/TM6C$, Sb Tb (CAS-0003) was from the Fly Stock Center of the National Institute of Genetics, Japan. UAS-*schlank*-RNAi (#33896) and UAS-*syx17*-RNAi (#36395) were from the Vienna Drosophila Resource Center.

Immunofluorescence (IF), lipid droplet staining, and imaging

For IF staining, larval eye discs and salivary glands as well as adult retina tissues were dissected in PBS, fixed in 4% paraformaldehyde for 20 min, and then washed in PBS with 0.4% or 0.05% Triton X-100 (0.05% Triton is used for targeted ectopic Ifc expression from cone or pigment cells). Primary antibodies were incubated in PBS with 0.4% or 0.05% Triton X-100 overnight at 4°C. Anti-Ifc polyclonal antibody was raised in rabbits immunized with a synthetic peptide CDFPAVPGSRLPEVKR (amino acid residues 264–278 of Ifc) by GeneTex, Taiwan. Rab7 antibody was generated in rabbits against an epitope described in [44]. Dilutions of antibodies used as follows: anti-Ifc (1:200), anti- Na^+/K^+ -ATPase (1:50; Developmental Studies Hybridoma Bank, a5), anti-dhCer (1:100; Sigma, C8104, [45,46]), anti-BiP (1:200; StressMarq Biosciences, SPC-180, [47]), anti-Rab5 (1:200, [48]), anti-Rab7 (1:200), anti-Atg8a (1:200; Abcam, ab109364), Alexa 488, Alexa 568, or Alexa 647-conjugated phalloidin (1:100, Thermo Fisher). Goat anti-mouse or goat anti-rabbit secondary antibodies (Jackson ImmunoResearch) conjugated with Alexa 488, Alexa 647, Cy3, or Dylight 405 were used at 1:500. For lipid droplet staining, the dissection, fixation, staining, and mounting were performed as described by [49] with modified concentrations of BODIPY^{493/503} at 4 mM (Thermo Fisher, D3922). LipidTOX staining was performed after immunostaining, and adult retina tissues were washed in PBS with 0.05% Triton X-100 during immunostaining, followed by three times of washing in PBS to remove the detergent and incubated overnight at 4°C with LipidTOX Deep Red (Thermo Fisher, H34477) at 1:200 dilution. ROS detection was performed following manufacturers' protocols, with 10 μ M of CM-H₂DCFDA (chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, Thermo Fisher, C6827). All IF samples were mounted in Vectashield (Vector Laboratories) and analyzed on a Leica SP5 confocal microscope with LAS AF software. Imaging data were processed and quantified using Photoshop (CS6; Adobe), Illustrator (CS6; Adobe), and ImageJ (National Institutes of Health).

UHPLC-MS/MS analysis of sphingolipids

Sphingolipid extraction was performed as described [50] with modifications. Briefly, 500 larvae or 350 adult flies (both male and female were equally represented in control and treatment) were snap-frozen in liquid nitrogen and homogenized by grinding. The homogenate was added to 201 g of methanol/chloroform/water mixture (3:1:1, v/v/v), followed by sonication for 20 min in room temperature. After overnight incubation, the extract was vortexed and centrifuged at 210 g for 10 min. The organic phase was dried by nitrogen stream, and the dried pellet was re-dissolved in 2 ml of MeOH/water/CHCl₃ (96:94:6, v/v) and applied to a Strata C18-E solid-phase extraction cartridge (SPE) (Phenomenex Inc., Torrance, CA, US) which was pre-conditioned with 4 ml water. Two mL of MeOH was then added into the SPE cartridge after sample loading. A 3 ml of MeOH/CHCl₃ (1:1, v/v) was added into the SPE cartridge to elute the sphingolipids. The MeOH/CHCl₃ eluent was dried by nitrogen stream. The dried extract was reconstituted with 200 μ l MeOH containing 100 ng/ μ l internal standard [ceramide (d18:1/18:0)] for UHPLC-MS/MS analysis.

The sphingolipids were analyzed by an Agilent 1290 UHPLC system coupled with an Agilent 6460 triple quadrupole mass

spectrometer (Agilent Technologies, Waldbronn, Germany). An Agilent Eclipse Plus C18 column (100 × 2.1 mm, 1.8 μm) was employed for the separation. The flow rate was 0.3 ml/min. Mobile phase A was consisted of MeOH and a buffer solution containing 0.2% formic acid and 10 mM ammonium acetate (60:40, v/v), and mobile phase B was consisted of MeOH and IPA (60:40, v/v) with the addition of 0.2% formic acid and 10 mM ammonium acetate. The gradient elution program was as the following: 0–3 min: linear gradient from 0 to 35% B; 3–5.5 min: linear gradient from 35 to 55% B; 5.5–8.0 min: linear gradient from 55 to 60% B; 8.0–8.5 min: linear gradient from 60 to 80% B, and kept at 80% B for 2 min; 10.5–16.0 min: linear gradient from 80 to 90% B, and kept at 90% B for 3 min; 19.0–22.0 min: linear gradient from 90 to 100% B, and kept at 100% B for 3 min. The sample reservoir and column oven were maintained at 4°C and 40°C, respectively. The injection volume was 5 μl. Electrospray ionization in the positive mode was employed to analyze sphingolipids with the following parameters: 325°C dry gas temperature, 7 l/min dry gas flow rate, 35 psi nebulizer pressure, 325°C sheath gas temperature, 11 l/min sheath gas flow rate, 4,000 V capillary voltage, and a 500 V nozzle voltage. MS acquisition was executed in multiple reaction monitoring (MRM) mode. The detailed MS transitions measured are shown in Table 1.

Electroretinograms

Electroretinograms were performed as described [51] with the following modifications: Flies were fixed using non-toxic, generic glue. Both the recording and reference electrodes were filled with 2 M NaCl. Electrode voltage was amplified by an iWorx 404 multi-channel recorder (iWorx Systems Inc., NH, USA), filtered through a Warner IE-210 intracellular microelectrode amplifier (Warner Instrument Co., CT, USA), and recorded using LabScribe2 software by

iWorx. Light stimuli were provided as 1 s pulses by a homemade computer-controlled white LED system. To quantify the depolarizations, all experiments were carried out in triplicate with at least 10 recordings for each genotype and experimental condition per replication.

Chemical treatments of flies

Chemical treatments were performed by feeding. The sphingoid-based C14-Ceramide (Abcam, ab144091) and Sphingosine (Sigma, S7049) were dissolved in DMSO and added to standard fly food to the final concentration of 10 μM. Stocks of AD4 (4 mg/ml, Sigma, A0737) and fingolimod (4 mg/ml, Cayman, 11975) in water were added to standard fly food to 40 μg/ml. Rapamycin (LC laboratories, R-5000) was dissolve in EtOH and added to standard fly food to 20 μM. 3-MA (Sigma, M9281) feeding was carried out following the protocol described in [52]. Briefly, 3-MA was dissolved in water and mix with sucrose to obtain the solution of 8 mM 3-MA and 3.25% sucrose. The solution was absorbed onto Kimwipes for drug feeding.

Transmission electron microscopy

Adult compound eyes were dissected and prefixed in 2.5% glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.3) containing 1% tannic acid at 4°C for at least 24 h. After washing in buffer, specimens were post-fixed with 1% osmium tetroxide in 100 mM sodium cacodylate buffer at room temperature for 1 h. Specimens were then stained *en bloc* with 2% aqueous uranyl acetate for 1 h and dehydrated through a graded ethanol series into 100% acetone. Specimens were infiltrated with Spurr's resin overnight and embedded with a flat silicone embedding mold. Ultrathin sections were cut with a diamond knife on a Leica Ultracut R ultramicrotome (Leica, Heerbrugg, Switzerland) and collected on Formvar-coated single-slot grids. Sections were examined in a Hitachi H-7500 transmission electron microscope (Hitachi, Tokyo, Japan) at 80 kV. Images were taken with a 2,048 × 2,048 Macrofire monochrome CCD camera (Optronics, Goleta, CA, USA).

Quantitative real-time PCR

Total RNA samples from larvae (48 h after hatching) were isolated following manufacturer's instruction (Qiagen RNeasy), reverse-transcribed with Superscript III reverse transcriptase (Invitrogen), and then used for qRT-PCR analyses. qRT-PCR was performed with Maxima SYBR Green/ROX qPCR master mix (Fermentas) using 7300 Real-Time PCR System (Applied Biosystems). Each sample was run in technical triplicates, and the average values for each sample were normalized. Each result represents three independent experiments, and error bar denotes the SEM. The primers used are indicated in Table 2.

Cell culture, MTT assay, and anti-caspase-3 staining

Human neuroblastoma SH-SY5Y cells were maintained at a density of 1×10^4 cells per well in DMEM/F12 medium containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were starved in serum-free medium for 24 h prior to the addition of Cer

Table 1. The multiple reaction monitoring mass transition for ceramides and dihydroceramides.

Name	Precursor ion (m/z)	Product ion (m/z)
Sphingosine (d14)	244.0	226.0
Sphinganine (d14)	246.0	228.0
Ceramides		
Ceramide (d18:1/18:0) (IS)	566.5	264.2
Ceramide (d14:1/16:0)	482.6	208.0
Ceramide (d14:1/18:0)	510.6	208.0
Ceramide (d14:1/20:0)	538.6	208.0
Ceramide (d14:1/22:0)	566.6	208.0
Ceramide (d14:1/24:0)	594.6	208.0
Dihydroceramides		
Dihydroceramide (d14:1/16:0)	484.6	210.0
Dihydroceramide (d14:1/18:0)	512.6	210.0
Dihydroceramide (d14:1/20:0)	540.6	210.0
Dihydroceramide (d14:1/22:0)	568.6	210.0
Dihydroceramide (d14:1/24:0)	596.6	210.0

Table 2. Quantitative real-time PCR primers.

Gene	Primer sequence
<i>ifc</i>	Forward: 5'-ACCGTGGTGACGTAACCTTC-3' Reverse: 5'-GCCCTTGGCGAACATGTAGT-3'
<i>atg1</i>	Forward: 5'-GCTTCTTTGTTACCGCTTC-3' Reverse: 5'-GCTTGACCAGCTTCAGTTCC-3'
<i>atg5</i>	Forward: 5'-GAACCGTATGGCGATTGGA-3' Reverse: 5'-TGTCGATAATTCCGCCATTAAA-3'
<i>atg7</i>	Forward: 5'-GACACGACCCGGAGTATCCA-3' Reverse: 5'-TCCTCGTCGCTATCGGACAT-3'
<i>atg8a</i>	Forward: 5'-AGTCCCAAAGCAAACGAG-3' Reverse: 5'-TTGTCCAAATCACCGATGC-3'
<i>atg8b</i>	Forward: 5'-AATGTGATCCACCGACATC-3' Reverse: 5'-TTGAGCGAGTAGTGCCAATG-3'
<i>cnc</i>	Forward: 5'-AGCGCATCTCGAACAAGTTTG-3' Reverse: 5'-CCTCGGACTTTTCACGTGGTA-3'
<i>gstD1</i>	Forward: 5'-TCGACATGGGAACGCTGTAC-3' Reverse: 5'-GGAAGGTGTTCAAGAACTCGAA-3'
<i>puckered</i>	Forward: 5'-CAAGCAGCAGCGTGGCATC-3' Reverse: 5'-CATGGTGTGGGATGGGTG-3'
<i>GAPDH</i>	Forward: 5'-GACGAAATCAAGGCTAAGGTCG-3' Reverse: 5'-AATGGGTGTCGCTGAAGAAGTC-3'

(Sigma, A7191), dhCer (Sigma, C7980), fingolimod (5 μ M, Cayman, 11975), or fenretinide (10 μ M, Tocris Bioscience, 1396).

MTT assay was performed as an indicator of cell viability. Briefly, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) was added to each well of SH-SY5Y cells in fresh media, to a final concentration of 0.5 mg/ml, and incubated for 1 h at 37°C. After removal of the media, 100 μ l DMSO (dimethyl sulfoxide) was added to each well for 10 min to dissolve the crystals of formazan. The absorbance of each sample was measured at 570 nm in a microplate reader (BioTeK PowerWave XS, VT, USA). For anti-active caspase-3 staining, SH-SY5Y cells were fixed with 4% paraformaldehyde and incubated in blocking solution (1% BSA, 0.1% Triton X-100 in PBS) for 1 h at room temperature. The samples were then incubated overnight at 4°C with anti-active caspase-3 primary antibody (1:1,000, Abcam, 13847), washed, followed by incubation with goat anti-rabbit secondary antibodies conjugated with Alexa 488 (1:500, Jackson ImmunoResearch), Alexa 568-conjugated Phalloidin (1:500, Thermo Fisher), and DAPI (1:500, Thermo Fisher). Samples were mounted in Vectashield and observed under confocal.

Statistical analysis

All data in bar graphs were expressed as the mean $\pm$ SD. Statistical significance between groups was determined using Student's *t*-test or one-way ANOVA with multiple comparisons: * indicates $P < 0.05$; ** indicates $P < 0.01$; *** indicates $P < 0.001$.

Expanded View for this article is available online.

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Author contributions

W-HJ, C-CL, and Y-LY performed all fly genetic crosses and feeding experiments. C-CL, Y-LY, and Y-CC performed confocal imaging. W-YL mostly worked on the cell culture experiments. H-CC and C-HK performed the sphingolipidomic analysis. H-CH performed the TEM experiment. S-YH and C-CC contributed to overall design and wrote the manuscript along with other authors. All authors discussed the results and commented on the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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