

Energy stress activates AMPK to suppress ferroptosis in neurodegenerative proteinopathies

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Abstract. Ferroptosis, an iron-dependent and lipid peroxidation-driven form of regulated cell death, has emerged as a key contributor to neuronal degeneration in protein misfolding disorders. However, how cellular energy metabolism modulates

ferroptotic susceptibility in neurodegenerative proteinopathies remains incompletely defined. The present study investigated whether the previously described energy stress-AMP-activated protein kinase (AMPK)-acetyl-CoA carboxylase (ACC) ferroptosis checkpoint operates in disease-relevant neuronal proteinopathy models. Pharmacological and genetic interventions, cell viability assays, lipid peroxidation measurements, western blotting, RNA interference, and behavioral analyses were performed in SH-SY5Y cells, transgenic *Caenorhabditis elegans* (*C. elegans*), and 3xTg-AD mice. In SH-SY5Y cells, glucose deprivation, 2-deoxy-D-glucose (2DG), or 5-aminoimidazole-4-carboxamide ribonucleoside reduced ferroptotic cell death induced by erastin or RSL3 and attenuated ferroptosis-associated neuronal injury induced by amyloid precursor protein, α -synuclein A53T, and polyQ74. This protection was associated with AMPK activation, ACC1 phosphorylation, reduced lipid peroxidation, sensitivity to ferroptosis inhibition, glutathione peroxidase 4 restoration, and acyl-CoA synthetase long-chain family member 4 suppression. AMPK inhibition or knockdown weakened the protection conferred by glucose deprivation, while *aak-2* RNAi attenuated the protective effects of 2DG in *C. elegans*, supporting a substantial contribution of AMPK-ACC1-associated signaling to ferroptosis suppression under energy stress. Consistently, 2DG or liproxstatin-1 reduced ferroptosis-associated oxidative damage and improved behavioral or cognitive deficits in transgenic *C. elegans* and 3xTg-AD mice. These findings extend the established AMPK-ACC ferroptosis checkpoint to neurodegenerative proteinopathy models and support energy stress-associated AMPK-ACC1 signaling as an important metabolic defense mechanism against ferroptosis-related neuronal injury.

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Abbreviations: 2DG, 2-deoxy-D-glucose; 4-HNE, 4-hydroxynonenal; AD, Alzheimer's disease; ACC, acetyl-CoA carboxylase; ACSL4, acyl-CoA synthetase long-chain family member 4; AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; APP, amyloid precursor protein; LKB1, liver kinase B1; AMPK, AMP-activated protein kinase; CC, Compound C; Fer-1, ferrostatin-1; GPX4, glutathione peroxidase 4; HD, Huntington's disease; LIP-1, liproxstatin-1; NDs, neurodegenerative diseases; PD, Parkinson's disease; polyQ, polyglutamine; RNAi, RNA interference; TOFA, 5-tetradecyloxy-2-furoic acid; ROS, reactive oxygen species; GFAP, glial fibrillary acidic protein; Iba1, ionized calcium-binding adapter molecule 1; MDA, malondialdehyde

Key words: ferroptosis, energy stress, lipid peroxidation, neurodegenerative proteinopathies, 3xTg-AD mice

Introduction

Neurodegenerative diseases (NDs), including Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's

disease (HD), are characterized by the progressive loss of neuronal integrity and function, leading to irreversible cognitive and motor decline (1-7). Despite distinct pathological hallmarks such as amyloid- β (A β) plaques, α -synuclein aggregates and polyglutamine (polyQ) expansions, these disorders share common molecular features of oxidative stress, mitochondrial dysfunction and metabolic imbalance (8-11). Accumulating evidence indicates that these pathological processes converge to trigger ferroptosis, an iron-dependent, lipid peroxidation-driven form of regulated cell death that plays a crucial role in neuronal loss (12). However, the metabolic determinants that govern ferroptotic susceptibility in neurodegenerative proteinopathies remain poorly understood.

Ferroptosis is initiated by excessive iron accumulation and the failure of cellular antioxidant defense systems, particularly the glutathione peroxidase 4 (GPX4) axis (13,14). In neurons, ferroptotic damage is tightly associated with lipid peroxidation, mitochondrial shrinkage, and depletion of reduced glutathione (15,16). Recent studies have revealed ferroptotic signatures in postmortem brains and animal models of AD, PD and HD, suggesting that aberrant activation of this pathway contributes to neurodegeneration (17,18). Although pharmacological ferroptosis inhibitors such as liproxstatin-1 (LIP-1) and ferrostatin-1 (Fer-1) have shown neuroprotective effects, the upstream mechanisms that dictate neuronal vulnerability to ferroptosis under pathological metabolic conditions remain elusive (19). Energy metabolism is a critical determinant of neuronal survival. Glucose deprivation and mitochondrial dysfunction trigger energy stress responses that activate AMP-activated protein kinase (AMPK), a central metabolic sensor that maintains cellular homeostasis by balancing anabolic and catabolic pathways. Upon activation, AMPK inhibits ATP-consuming processes and promotes energy conservation through phosphorylation of downstream targets such as acetyl-CoA carboxylase (ACC), thereby modulating lipid synthesis and oxidation (20). Importantly, energy stress-mediated AMPK regulation of ferroptosis has been established previously. Lee *et al* (21) demonstrated that energy stress activates AMPK and suppresses ferroptosis through phosphorylation and inhibition of ACC, thereby limiting polyunsaturated lipid synthesis and lipid peroxidation. Subsequent studies further supported the involvement of the liver kinase B1 (LKB1)-AMPK-ACC axis in ferroptosis regulation (22,23). These findings place AMPK at the interface between energy sensing and lipid peroxidation control. However, most previous studies focused on cancer cells or limited non-neuronal contexts, and it remains unclear whether this metabolic checkpoint is functionally engaged in neurodegenerative proteinopathies, where chronic proteotoxic stress, mitochondrial dysfunction, iron dyshomeostasis and lipid peroxidation coexist. Moreover, NDs are themselves associated with chronic energy failure and metabolic dysfunction, conditions that may theoretically engage endogenous AMPK signaling. Chronic pathological energy stress, however, is not equivalent to an effective adaptive AMPK response. In diseased neurons, AMPK activation may be transient, quantitatively insufficient, spatially compartmentalized, or uncoupled from downstream effectors required for lipid metabolic control (24-26). Thus, whether controlled energy stress-associated interventions can reinforce an inadequate endogenous AMPK response and

restore AMPK-ACC1-dependent restraint of lipid peroxidation remains unresolved. Addressing this issue is essential for clarifying the therapeutic rationale of AMPK activation in neurodegenerative proteinopathies.

In the present study, it was investigated whether energy stress-associated AMPK activation suppresses ferroptosis-related neuronal injury in neurodegenerative proteinopathy models. Using SH-SY5Y cells expressing amyloid precursor protein (APP), α -synuclein A53T, or mutant huntingtin Q74, it was examined whether glucose deprivation, 2-deoxy-D-glucose (2DG), or 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) attenuates pathogenic protein-induced lipid peroxidation and cell death. The functional relevance of this pathway was further tested in transgenic *Caenorhabditis elegans* (*C. elegans*) models and 3xTg-AD mice. Rather than claiming discovery of a new AMPK-ACC ferroptosis-regulatory mechanism, the present study aimed to determine whether this previously described metabolic checkpoint operates in disease-relevant neuronal proteinopathy contexts. The current findings support an important contribution of AMPK-ACC1-associated signaling to the suppression of ferroptosis-related oxidative damage and behavioral deficits across cellular and *in vivo* models.

Materials and methods

Chemicals and reagents. Trypsin-EDTA solution was purchased from Biosharp Life Sciences. Penicillin-Streptomycin-Gentamicin solution (100x) and dimethyl sulfoxide (DMSO) were obtained from Beyotime Institute of Biotechnology and Beijing Solarbio Science & Technology Co., Ltd., respectively. The ferroptosis inducers (1S,3R)-RSL3 (RSL3; cat. no. S815503) and erastin (cat. no. 68450) were purchased from Selleck Chemicals and MedChemExpress, respectively. The ferroptosis inhibitors LIP-1 (cat. no. 66621) and Fer-1 were obtained from MedChemExpress. The AMPK activators AICAR (cat. no. GC10518) and 2DG (cat. no. GC17430) were purchased from GlpBio. 6-hydroxydopamine (6-OHDA; cat. no. H197233) was purchased from Aladdin Biochemical Technology Co., Ltd. The AMPK inhibitor Compound C (CC; cat. no. APEB3252) was obtained from APeXBIO Technology LLC. The ACC inhibitor 5-tetradecyloxy-2-furoic acid (TOFA; cat. no. T3988) and the reactive oxygen species (ROS)-sensitive probe dichloro-dihydro-fluorescein diacetate (DCFDA; cat. no. T15458) were purchased from TargetMol. BODIPY 581/591 C11 (cat. no. 27086) and Phen Green SK (PGSK; cat. no. 25393) were purchased from Cayman Chemical Company. The BODIPY 581/591 C11 lipid peroxidation assay kit used for flow cytometry was purchased from Beyotime Institute of Biotechnology (cat. no. S0043S). Dihydroethidium (DHE; cat. no. BN11008) was obtained from Biorigin (<http://www.biorigin.ltd/Show/index/cid/87/id/247.html>). Phospho-AMPK (p-AMPK; cat. no. 2535S) and phospho-ACC (p-ACC; cat. no. 3661S) were purchased from Cell Signaling Technology, Inc. AMPK (cat. no. 66536-1-Ig), ACC1 (cat. no. 67373-1-Ig), GPX4 (cat. no. 67763-1-Ig), ACSL4 (cat. no. 22401-1-AP), glial fibrillary acidic protein (GFAP; cat. no. 16825-1-AP) and ionized calcium-binding adapter molecule 1 (Iba1; cat. no. 10904-1-AP) were obtained from Proteintech Group, Inc. The antibody against 4-hydroxynonenal

(4-HNE; cat. no. Ab46545) was purchased from Abcam. The Perls' Prussian blue staining kit (cat. no. G1428) was purchased from Beijing Solarbio Science & Technology Co., Ltd. Plasmids including pCAX-APP-Swe/Ind (cat. no. 30145), pHM6- α -synuclein-A53T (cat. no. 40825), or pEGFP-Q74 (cat. no. 40262) were obtained from Addgene, Inc. All reagents were of analytical grade or higher, and stock solutions were prepared according to the manufacturers' instructions. Working concentrations were freshly prepared before each experiment to ensure reagent stability and reproducibility.

Cell culture. Human neuroblastoma SH-SY5Y cells (American Type Culture Collection) were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) containing 25 mM D-glucose, supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 0.37% sodium bicarbonate, 100 U/ml penicillin and 100 μ g/ml streptomycin. Cells were maintained in a humidified incubator at 37°C with 5% CO₂. The 25 mM glucose condition was used as the basal culture control in glucose-related experiments. For glucose deprivation experiments, cells were incubated in glucose-free DMEM for the indicated duration. For glucose overload experiments, additional D-glucose was added to the basal medium to reach the indicated final concentrations.

Cell transfection. Plasmid transfection was performed to establish pathogenic protein-overexpressing SH-SY5Y cell models. Briefly, SH-SY5Y cells were seeded in 6-well plates at a density of 2×10^5 cells per well and cultured until they reached 70–80% confluence. Cells were transfected with 2.5 μ g of pCAX-APP-Swe/Ind, pHM6- α -synuclein-A53T, or pEGFP-Q74 plasmid DNA per well using Lipofectamine™ 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. After 6 h of transfection, the medium was replaced with fresh complete DMEM containing 10% FBS, and the cells were incubated for an additional 24–48 h before subsequent experiments.

Cell viability assay. Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. SH-SY5Y cells were seeded in 96-well plates (8×10^3 cells/well), treated as indicated, and incubated with MTT (5 mg/ml) for 4 h. The medium was removed, formazan crystals were dissolved in DMSO, and absorbance was read at 570 nm. Viability was calculated as a percentage of the untreated control.

Hoechst/propidium iodide (PI) staining. Cell death was assessed by Hoechst 33342 and PI dual staining. SH-SY5Y cells were treated as indicated, incubated with Hoechst (10 μ g/ml) and PI (5 μ g/ml) for 15 min at 37°C, washed with phosphate-buffered saline (PBS), and imaged by fluorescence microscopy. Hoechst-positive nuclei were counted as total cells, and PI-positive nuclei as dead cells. Cell death (%) was calculated as PI-positive cells divided by total Hoechst-positive cells.

AMPK knockdown by small interfering RNA (siRNA). siRNA specific to AMPK α 1 was synthesized by Chengdu Bowen Biotechnology Co., Ltd. The siRNA sequences were as

follows: siCtrl, 5'-UUCUCCGAACGUGUCACGUTT-3'; and siAMPK, 5'-UUUCAGGCAUCCUCAUAUAAU-3'. SH-SY5Y cells were seeded in 6-well plates and transfected with siCtrl or siAMPK at 40 nM siRNA per well using Lipofectamine™ 3000 according to the manufacturer's instructions. After transfection, cells were cultured for 24 h and then subjected to glucose deprivation in the presence of erastin or RSL3. Cell death was evaluated by Hoechst/PI staining as aforementioned.

Intracellular ROS measurement. Intracellular ROS levels were assessed using DCFDA and DHE staining. Treated SH-SY5Y cells were incubated with DCFDA (10 μ M) or DHE (10 μ M) for 30 min at 37°C in the dark, washed with PBS, and imaged under a fluorescence microscope. DCFDA (green) and DHE (red) fluorescence intensities were quantified using ImageJ software (version 1.54p; National Institutes of Health).

Intracellular lipid peroxidation assay. Lipid peroxidation was measured using BODIPY 581/591 C11. For fluorescence microscopy, treated SH-SY5Y cells were incubated with 2 μ M BODIPY 581/591 C11 for 30 min at 37°C in the dark, washed three times with PBS, and immediately imaged using identical acquisition parameters. Oxidized BODIPY C11 was detected in the green channel, whereas reduced BODIPY C11 was detected in the red channel. For each sample, at least five randomly selected fields were analyzed. Background fluorescence was subtracted from each channel using ImageJ. The green/red fluorescence ratio was calculated as the oxidized/reduced BODIPY C11 ratio and used as the lipid peroxidation index. Data were normalized to the corresponding control group where appropriate.

For flow cytometry-based lipid peroxidation analysis, cells were stained using the BODIPY 581/591 C11 lipid peroxidation assay kit (cat. no. S0043S; Beyotime Institute of Biotechnology) according to the manufacturer's instructions. After treatment, cells were collected and centrifuged at 600 $\times$ g for 3–4 min at 4°C. The cell pellets were washed with PBS, incubated with the BODIPY C11 working solution in the dark, washed again, and analyzed by using a BF-730 B5R3V6 flow cytometer (URIT Medical Electronic Co., Ltd.). Flow cytometry data were analyzed using FlowJo software (version 10; FlowJo LLC). Single cells were first selected by forward- and side-scatter gating, and debris was excluded. Oxidized BODIPY C11-positive cells were quantified using the fluorescein isothiocyanate (FITC) channel. The threshold for FITC-positive cells was defined using the untreated control group, and the percentage of FITC-positive cells was used as the single-cell lipid peroxidation readout.

C. elegans strains and maintenance. The *C. elegans* strains used in the present study were obtained from the Caenorhabditis Genetics Center. The following strains were employed: Wild-type (WT) N2 worms, CL4176 [smg-1(cc546ts); dvIs27 (myo-3p::A β 1–42::let-851 3'UTR + rol-6(su1006))] for A β expression, CL2355 [dvIs50 (snb-1p::A β 1–42::3'UTR + mtl-2::GFP)], expressing human A β 1–42 in neurons, CL2122 [dvIs15 (mtl-2::GFP)], serving as the control strain for CL2355, NL5901 [pkIs2386 (unc-54p:: α -synuclein::YFP)] for α -synuclein expression, and AM141 [rmIs133 (unc-54p::Q40::YFP)] for polyQ expansion. Worms were maintained at 20°C on

nematode growth medium (NGM) plates seeded with *E. coli* OP50. For pharmacological treatments, 2DG or LIP-1 (200 μ M) was added to NGM before seeding with OP50, and worms were exposed for the indicated durations prior to behavioral or fluorescence assays.

Paralysis assay. Paralysis was assessed in CL4176 worms expressing A β . Age-synchronized larvae were kept at 16°C until L3-L4 and then shifted to 25°C to induce A β expression. Worms were treated with AICAR, 2DG, or LIP-1 (200 μ M) incorporated into NGM. Paralysis was scored by gently tapping the nose with a platinum wire; worms showing head movement without body movement were counted as paralyzed. At least 100 worms per group were analyzed in three independent experiments, and paralysis percentage was calculated according to the following formula: Paralysis rate (%) = 100 x number of paralyzed worms/total number of worms.

Slowing rate assay. The slowing rate assay was used to assess food-dependent locomotor response in CL2122 (control) and CL2355 (neuronal A β) worms. Age-synchronized young adults were treated with 2DG or LIP-1 (200 μ M) incorporated into NGM. For testing, individual worms were transferred from unseeded NGM to OP50-seeded plates, and body bends were counted for 20 s before and after food contact. Slowing rate (%) was calculated as 100 x (bends off food - bends on food)/bends off food. At least 20 worms per group were analyzed in three independent experiments.

Body bend assay. Locomotor activity was assessed in NL5901 (α -synuclein) and AM141 (polyQ) worms grown on NGM plates containing 2DG or LIP-1 (200 μ M) from the L1 stage to adulthood. Individual adults were transferred to unseeded NGM plates, allowed to acclimate for 1 min, and body bends were counted for 30 sec under a stereomicroscope. A bend was defined as a complete change in direction at the posterior pharyngeal bulb. At least 20 worms per group were analyzed in three independent experiments, and mean bend counts were used to indicate neuromuscular function.

RNA interference (RNAi) treatment in *C. elegans*. For RNAi experiments, *aak-2* knockdown was performed using the standard feeding RNAi method. Briefly, HT115(DE3) *E. coli* bacteria carrying a pL4440-derived RNAi feeding construct targeting *aak-2*/T01C8.1 were used to induce *aak-2* knockdown, whereas HT115(DE3) bacteria carrying the empty pL4440 vector were used as the negative RNAi control. The vector-specific primers used for the pL4440-based RNAi construct were pL4440-dest-RNAi-FOR, 5'-GTTTTCCCA GTCACGACGTT-3', and pL4440-dest-RNAi-REV, 5'-TGG ATAACCGTATTACCGCC-3'. The *aak-2*-specific primers were as follows: *aak-2* forward, 5'-ATGTTTCTCATCAA GATCGAGACCG-3' and reverse, 5'-CAACTTCCGCTAAT AACCTCAGG-3' (27,28). Synchronized CL4176 worms were placed on RNAi plates from the larval stage and treated with 2DG as indicated. For paralysis assays, worms were maintained at 16°C and then shifted to 25°C to induce A β expression. For ROS and lipid peroxidation assays, worms were collected after treatment and stained with DHE or BODIPY 581/591 C11 as described below.

ROS detection in *C. elegans*. ROS levels were assessed by DHE staining. Age-synchronized adult CL4176, NL5901 and AM141 worms treated with 2DG or LIP-1 (200 μ M) were washed and incubated with 10 μ M DHE for 30 min at 20°C in the dark. After washing and anesthetization, worms were mounted on agar pads and imaged using a fluorescence microscope (Ex 510 nm/Em 580 nm). At least 20 worms per group were analyzed, and fluorescence intensity was quantified using ImageJ. For the 6-OHDA-induced N2 worm model, synchronized adult N2 worms were exposed to 6-OHDA (50 μ M) for 24 h with or without 2DG or LIP-1 (200 μ M). Worms were then collected for DHE staining or BODIPY 581/591 C11 staining as aforementioned.

Lipid peroxidation detection in *C. elegans*. Lipid peroxidation was assessed using the fluorescent probe BODIPY 581/591 C11. Age-synchronized adult CL4176, NL5901 and AM141 worms were treated with 2DG or LIP-1 (200 μ M) on NGM plates, collected, washed and incubated with 2 μ M BODIPY C11 for 30 min at 20°C in the dark. After washing and anesthetization, worms were mounted on agar pads and imaged under a fluorescence microscope. Green (oxidized) and red (reduced) signals were quantified using ImageJ, and the green/red fluorescence ratio was calculated as an indicator of lipid peroxidation. At least 20 worms per group were analyzed per experiment. For the 6-OHDA-induced N2 worm model, synchronized adult N2 worms were exposed to 6-OHDA (50 μ M) for 24 h with or without 2DG or LIP-1 (200 μ M). Worms were then collected for DHE staining or BODIPY 581/591 C11 staining as aforementioned.

Detection of dopaminergic neuron viability in *C. elegans*. Dopaminergic neuron viability was evaluated using the BZ555 strain expressing GFP in *dat-1*-positive neurons. Synchronized worms were grown to adulthood and exposed for 24 h to 6-OHDA (50 μ M) with or without 2DG or LIP-1 (200 μ M) incorporated into NGM. After washing and anesthetization, CEP dopaminergic neurons were imaged under identical fluorescence settings. GFP intensity was quantified with ImageJ, analyzing ≥ 20 worms per group in three independent experiments, and mean fluorescence intensity was used as an indicator of neuronal viability.

Animals and administration. All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of Southwest Medical University (approval no. 20220812-003; Luzhou, China). The animal experiments were conducted in accordance with institutional guidelines and with consideration of the ARRIVE reporting recommendations for *in vivo* studies (29). Eight-month-old male 3xTg-AD mice and age-matched wild-type (WT) littermates, weighing approximately 18-23 g at the beginning of the experiment, were bred and maintained at the Laboratory Animal Center of Southwest Medical University. Genotypes were confirmed by polymerase chain reaction (PCR) according to the protocol provided by The Jackson Laboratory for 3xTg-AD mice (Stock No. 004807). The primer sequences used for genotyping were as follows: Transgene forward, 5'-AGGACTGACCACTCGACCAG-3' and reverse, 5'-CGGGGTCTAGTTCTGCAT-3'. Mice were housed

under specific pathogen-free conditions with free access to food and water under a 12/12-h light/dark cycle at controlled temperature and humidity. A total of 32 mice were randomly assigned to four groups (n=8 per group): WT control, 3xTg-AD, 3xTg-AD + 2DG and 3xTg-AD + LIP-1. The treatment period lasted 8 weeks. Mice in the 3xTg-AD + 2DG group received 2DG at 500 mg/kg/day by intraperitoneal injection. Mice in the 3xTg-AD + LIP-1 group received LIP-1 at 10 mg/kg/day by intraperitoneal injection. WT control and untreated 3xTg-AD mice received the corresponding vehicle by the same route. Animals were monitored daily for general health status, appearance, activity, feeding, drinking, and behavior throughout the experimental period. Humane endpoints were predefined and included severe distress, inability to access food or water, marked reduction in activity, self-injury, or body weight loss exceeding 20% of baseline body weight. No animals met these humane endpoint criteria during the study. No animals were excluded from the final analysis, and no spontaneous or unexpected deaths occurred during the experimental period. To minimize suffering and distress, all procedures were performed by trained personnel, and mice were maintained under appropriate housing and husbandry conditions. No surgical procedures were performed in the present study, and no additional analgesic treatment was required. After the 8-week treatment period, behavioral tests were conducted. At the planned endpoint, all 32 mice were euthanized for tissue collection. Mice were euthanized via intraperitoneal injection of pentobarbital sodium (150 mg/kg body weight) to ensure a rapid and painless death. Death was confirmed by the absence of respiration, heartbeat, corneal reflex, and pedal withdrawal reflex, followed by cervical dislocation as a secondary physical method (30). Brain tissues were rapidly collected after death confirmation and stored at -80°C for subsequent analyses.

Morris water maze. Spatial learning and memory were assessed using a Morris water maze consisting of a circular pool with opaque water and a submerged escape platform placed in a fixed quadrant. Mice underwent acquisition training (four trials/day for five days), during which escape latency and swimming speed were recorded by a video-tracking system. A total of 24 h after training, the platform was removed for the probe trial, and the time spent in the target quadrant, platform crossings, and swimming paths were measured. All tests were performed under consistent conditions by investigators blinded to group assignments.

Immunohistochemical and iron staining analyses. Paraffin-embedded hippocampal sections (4 µm) were deparaffinized in xylene (cat. no. 10023418; Sinopharm Chemical Reagent Co., Ltd.) and rehydrated through a descending ethanol series prepared using ethanol (cat. no. 10009218; Sinopharm Chemical Reagent Co., Ltd.), and subjected to antigen retrieval in citrate buffer at 95°C. After endogenous peroxidase activity was quenched with 3% H₂O₂ and sections were blocked with 5% bovine serum albumin (BSA; cat. no. SW3015; Beijing Solarbio Science & Technology Co., Ltd.), the sections were incubated overnight at 4°C with anti-4-HNE antibody (1:200). After washing with PBST, an HRP-conjugated goat anti-rabbit IgG (H+L) secondary antibody (1:500; cat. no. 32460; Thermo Fisher Scientific, Inc.) was applied for 1 h, and immunoreactive

signals were developed with DAB and counterstained with hematoxylin. Whole-slide bright-field images were acquired under identical scanning settings using a KF-PRO-002 digital pathology slide scanner (KFBIO, Ningbo Jiangfeng Bio-information Technology Co., Ltd.). ImageJ was used to quantify positive staining area from five randomly selected CA1 fields per section.

For iron staining, hippocampal sections were processed using a Perls' Prussian blue staining kit according to the manufacturer's instructions. Briefly, sections were incubated with freshly prepared potassium ferrocyanide/hydrochloric acid working solution, counterstained, dehydrated, cleared, and mounted. Iron-positive signals in the hippocampal CA1 region were imaged under identical conditions.

Immunofluorescence analysis. Paraffin-embedded hippocampal sections (4 µm) were deparaffinized, rehydrated, and subjected to antigen retrieval in citrate buffer at 95°C. After PBS washing, sections were blocked with 5% BSA/0.3% Triton X-100 for 1 h and incubated overnight at 4°C with primary antibodies against GFAP, Iba1, GPX4, or ACSL4, each diluted 1:500. After washing, the sections were incubated for 1 h at room temperature in the dark with the appropriate Alexa Fluor Plus 594-conjugated secondary antibody: donkey anti-mouse IgG (H+L), highly cross-adsorbed (1:500; cat. no. A32744; Invitrogen, Thermo Fisher Scientific, Inc.), or donkey anti-rabbit IgG (H+L), highly cross-adsorbed (1:500; cat. no. A32754; Invitrogen, Thermo Fisher Scientific, Inc.). Nuclei were counterstained with DAPI staining solution (cat. no. C1006-50ml; Beyotime Institute of Biotechnology). Sections were mounted with anti-fade medium and imaged using a Zeiss LSM880 confocal microscope under identical settings. ImageJ was used to quantify fluorescence intensity or positive cell ratios from five randomly selected hippocampal fields per section.

Nissl staining. Paraffin sections were deparaffinized, rehydrated, and stained with 0.1% cresyl violet at 37°C, followed by differentiation, dehydration, clearing, and mounting. Nissl-positive neurons in the hippocampal CA1 region were examined and imaged under a bright-field microscope. Viable neurons were defined by intact soma, clear nuclei and visible Nissl bodies. Quantification was performed in ImageJ using five randomly selected fields per section.

Hematoxylin and eosin (H&E) staining. Paraffin sections were deparaffinized, rehydrated, stained with hematoxylin, differentiated, blued, and counterstained with eosin following standard procedures. After dehydration and mounting, hippocampal CA1 morphology was examined by bright-field microscopy. Neuronal integrity was evaluated based on cell morphology, and representative images were acquired at consistent magnifications.

Western blot analysis. SH-SY5Y cells or mouse brain tissues were lysed in radioimmunoprecipitation assay buffer containing 1x protease inhibitor (100x stock; cat. no. GRF101; Epizyme Biomedical Technology Co., Ltd.) and 1x phosphatase inhibitor (100x stock; cat. no. GRF102; Epizyme Biomedical Technology Co., Ltd.), incubated on ice for 30 min, and

centrifuged at 12,000 $\times$ g for 15 min at 4°C. Protein concentrations were determined by bicinchoninic acid assay, and equal amounts (20–40 μ g) were separated on 7.5%, 10%, or 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, depending on the molecular weight of the target protein, and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% non-fat milk in Tris-buffered saline with 0.1% Tween-20, incubated with primary antibodies overnight at 4°C, followed by HRP-conjugated secondary antibodies for 1 h. Protein bands were detected using enhanced chemiluminescence (cat. no. PK10001; Proteintech Group, Inc.) reagents on a ChemiDoc MP system and quantified with ImageJ, with GAPDH or β -actin as the loading control.

Statistical analysis. Data are expressed as the mean $\pm$ SEM from at least three independent experiments. Statistical analyses were performed using GraphPad Prism 9.0 (Dotmatics). Multiple-group comparisons were conducted by one-way ANOVA with Tukey's post hoc test, and Morris water maze training data were analyzed by two-way repeated-measures ANOVA with Bonferroni correction. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

LIP-1 attenuates neurodegenerative pathogenic protein-induced ferroptosis-associated injury. NDs such as AD, PD and HD are characterized by the accumulation of misfolded proteins that disrupt cellular homeostasis and promote oxidative injury. To investigate whether these pathogenic proteins induce ferroptosis-associated neuronal injury, SH-SY5Y cellular models overexpressing APP, α -synuclein A53T, or mutant huntingtin Q74 were established. Successful overexpression of APP, α -synuclein A53T, and GFP-tagged Q74 was confirmed by Western blotting (Fig. S1A–F). APP overexpression markedly increased cell death and oxidative stress, as evidenced by enhanced PI uptake, elevated DCFDA fluorescence, and increased C11-BODIPY oxidation. Treatment with the ferroptosis inhibitor LIP-1 attenuated these changes, reducing oxidative damage and lipid peroxidation-related signals (Fig. 1A–D). A similar pattern was observed in α -synuclein A53T-overexpressing cells, in which A53T increased cell death, ROS accumulation, and C11-BODIPY oxidation, all of which were reduced by LIP-1 treatment (Fig. 1E–H). Likewise, Q74 overexpression induced ferroptosis-associated changes, including increased PI-positive cells, elevated DHE fluorescence intensity, and increased malondialdehyde (MDA) levels, and these changes were alleviated by LIP-1 (Fig. 1I–L). To further strengthen the molecular evidence for ferroptosis-associated injury, GPX4 and ACSL4 expression was examined in APP-overexpressing SH-SY5Y cells. APP overexpression reduced GPX4 expression and increased ACSL4 expression, whereas LIP-1 restored GPX4 and decreased ACSL4 levels (Fig. 1M–O). In addition, Fer-1 attenuated APP-induced viability loss and cell death in SH-SY5Y cells (Fig. S2), providing an additional pharmacological line of evidence beyond LIP-1. Flow cytometry-based BODIPY 581/591 C11 analysis further showed that LIP-1 reduced oxidized BODIPY C11-positive cells in APP- and A53T-overexpressing SH-SY5Y cells (Fig. S3A–D). Together,

these findings indicate that APP, α -synuclein A53T, and Q74 overexpression induce a ferroptosis-associated injury phenotype in SH-SY5Y cells, characterized by increased cell death, oxidative stress, lipid peroxidation, sensitivity to ferroptosis inhibition, GPX4 reduction and ACSL4 upregulation.

Glucose deprivation inhibits ferroptosis in SH-SY5Y cells.

To examine whether changes in extracellular glucose availability affect ferroptotic susceptibility, SH-SY5Y cells were exposed to different glucose conditions. Because the basal culture medium contained 25 mM glucose, this condition was used as the standard culture control. Exposure to markedly higher glucose concentrations, ranging from 100 to 800 mM, progressively reduced cell viability (Fig. 2A). These concentrations represent supraphysiological glucose overload and likely reflect non-physiological hyperosmotic stress rather than clinically relevant hyperglycemia. Treatment with the ferroptosis inhibitors LIP-1 or Fer-1 partially improved cell viability under supraphysiological glucose overload (Fig. 2B), suggesting that lipid peroxidation-related injury may contribute to glucose overload-associated cytotoxicity. However, because osmotic controls were not included, these data should not be interpreted as evidence that glucose overload-induced cytotoxicity is primarily or specifically mediated by ferroptosis. In RSL3-treated cells, supraphysiological glucose overload further reduced cell viability, whereas LIP-1 preserved cell survival (Fig. 2C). The effects of glucose deprivation-induced energy stress were next examined. When ferroptosis was induced pharmacologically by erastin or RSL3, reducing glucose availability significantly protected SH-SY5Y cells from ferroptosis-associated cell death, as shown by Hoechst/PI staining and quantification (Figs. 2D–F and S4). Cell death was extensive under the standard 25 mM glucose condition after erastin or RSL3 treatment, whereas reducing glucose availability progressively decreased PI-positive cells. Cell death was nearly abolished under glucose-free conditions. These findings indicate that glucose deprivation suppresses erastin- and RSL3-induced ferroptotic injury in SH-SY5Y cells, supporting the possibility that energy stress activates an adaptive response that limits lipid peroxidation-driven cell death.

Glucose deprivation-induced AMPK activation inhibits ferroptosis in SH-SY5Y cells.

Given that glucose deprivation protected SH-SY5Y cells from ferroptosis, it was next investigated whether this protective effect was mediated by AMPK activation. Under glucose-free conditions, erastin- or RSL3-induced cell death was significantly reduced, whereas co-treatment with the AMPK inhibitor CC largely abolished this protection. By contrast, the ferroptosis inhibitor LIP-1 effectively suppressed ferroptotic cell death under the indicated conditions (Fig. 3A–C). Cell viability analysis further revealed that CC reduced the protective effect of glucose deprivation, whereas LIP-1 restored cell viability in erastin- or RSL3-treated cells (Fig. S5A and B). These results suggest that AMPK activity may contribute to the anti-ferroptotic effect of glucose deprivation, but they do not establish AMPK specificity because CC can inhibit kinases other than AMPK. To complement the pharmacological inhibition data, AMPK knockdown was further performed in SH-SY5Y cells. The

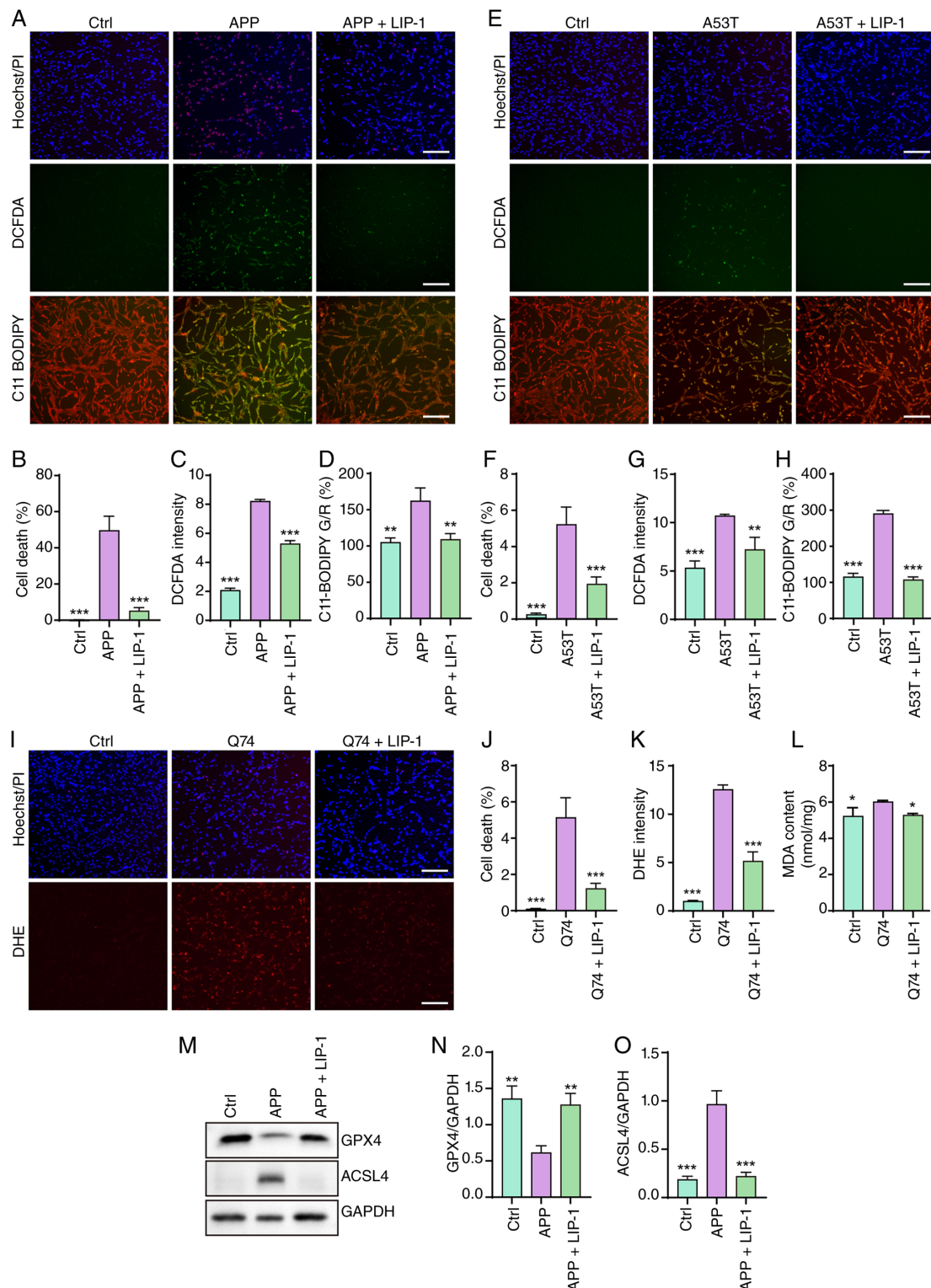


Figure 1. LIP-1 suppresses ferroptotic phenotypes induced by neurodegenerative pathogenic proteins. (A) Representative fluorescence images of cell death (Hoechst/PI), intracellular reactive oxygen species accumulation (DCFDA) and lipid peroxidation (C11-BODIPY 581/591) in Ctrl, APP-overexpressing, and APP-overexpressing SH-SY5Y cells with or without LIP-1 treatment. Magnification, $\times 10$; scale bars, $50\ \mu\text{m}$. (B-D) Quantification of cell death, DCFDA fluorescence intensity, and the green-to-red (G/R) fluorescence ratio of C11-BODIPY 581/591, respectively. The G/R ratio represents the oxidized-to-reduced fluorescence ratio and was used as an indicator of lipid peroxidation. (E) Representative fluorescence images of Hoechst/PI, DCFDA and C11-BODIPY 581/591 staining in control, α -synuclein A53T-overexpressing, and A53T-overexpressing SH-SY5Y cells with or without LIP-1 treatment. Magnification, $\times 10$; scale bars, $50\ \mu\text{m}$. (F-H) Quantification of cell death, DCFDA fluorescence intensity, and the green-to-red (G/R) fluorescence ratio of C11-BODIPY 581/591, respectively. The G/R ratio represents the oxidized-to-reduced fluorescence ratio and was used as an indicator of lipid peroxidation. (I) Representative fluorescence images of Hoechst/PI and DHE staining in control, Q74-overexpressing, and Q74-overexpressing SH-SY5Y cells with or without LIP-1 treatment. Magnification, $\times 10$; scale bars, $50\ \mu\text{m}$. (J and K) Quantification of cell death and DHE fluorescence intensity, respectively. (L) Quantification of MDA levels in control, Q74-overexpressing, and Q74-overexpressing SH-SY5Y cells with or without LIP-1 treatment. (M) Representative western blots of GPX4 and ACSL4 in control, APP-overexpressing, and APP-overexpressing SH-SY5Y cells with or without LIP-1 treatment. (N and O) Densitometric quantification of GPX4 and ACSL4 protein levels normalized to GAPDH, respectively. Data are presented as the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. LIP-1, liproxstatin-1; APP, amyloid precursor protein; MDA, malondialdehyde; GPX4, glutathione peroxidase 4; ACSL4, acyl-CoA synthetase long-chain family member 4.

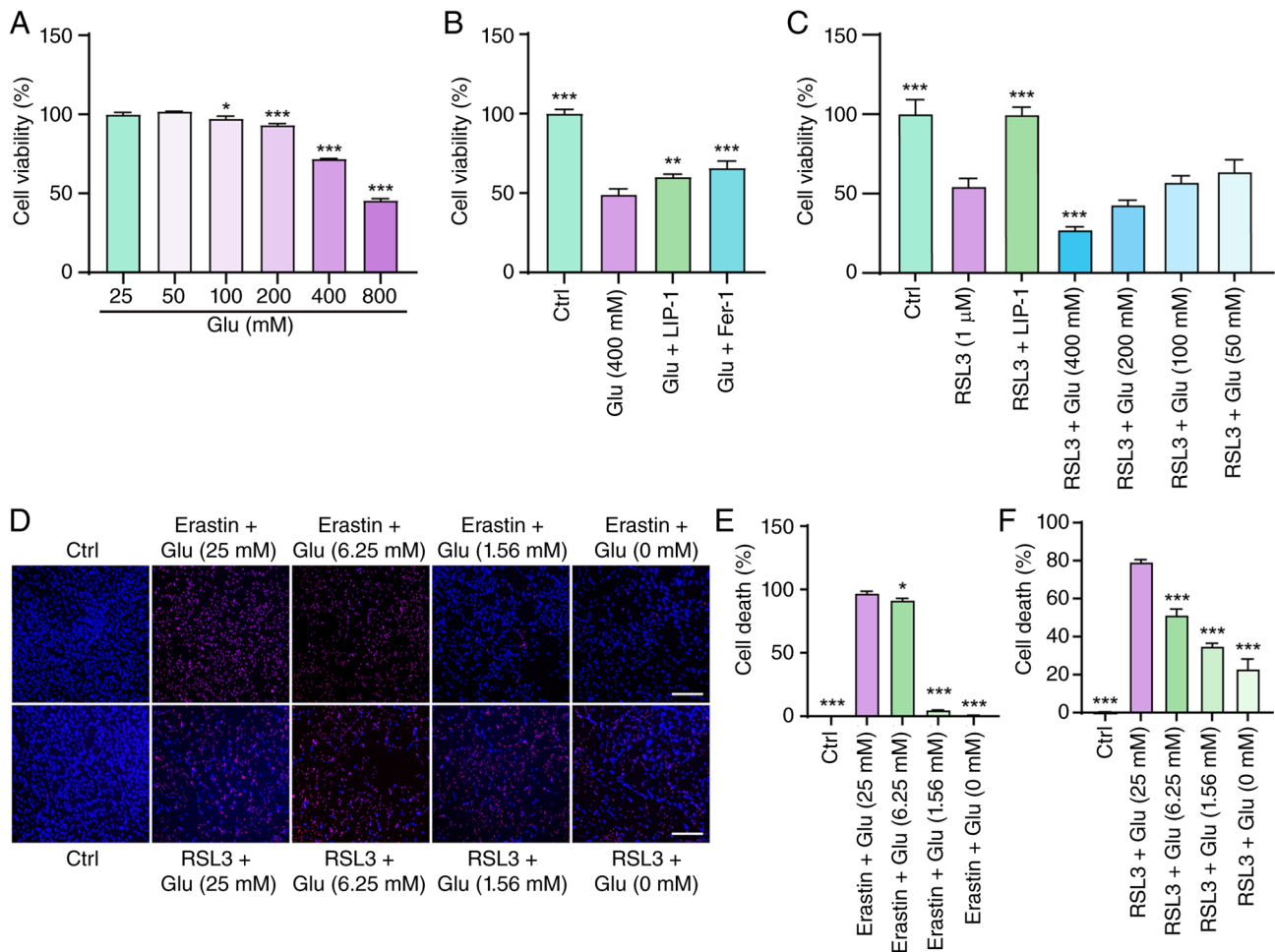


Figure 2. Glucose deprivation protects SH-SY5Y cells from ferroptotic cell death. (A) Cell viability of SH-SY5Y cells exposed to different extracellular glucose concentrations. The 25 mM glucose condition represents the standard high-glucose DMEM control. Glucose concentrations of 100–800 mM were used as a non-physiological hyperosmotic glucose challenge to assess cellular tolerance to glucose overload. (B) Cell viability of SH-SY5Y cells exposed to high glucose, with or without the ferroptosis inhibitors LIP-1 or Fer-1. (C) Cell viability of SH-SY5Y cells treated with RSL3 under different glucose concentrations, with or without the ferroptosis inhibitor LIP-1. (D) Representative Hoechst/PI fluorescence images showing cell death in SH-SY5Y cells treated with erastin or RSL3 under normal glucose [Glu (25 mM)] or glucose-deprived/low-glucose conditions [Glu (6.25, 1.56, or 0 mM)]. Magnification, x10; scale bars, 50 μ m. (E and F) Quantification of cell death in erastin-treated and RSL3-treated SH-SY5Y cells under the indicated glucose concentrations, respectively. Data are presented as the mean $\pm$ SEM. * P <0.05, ** P <0.01 and *** P <0.001. LIP-1, lipoxstatin-1; Fer-1, ferrostatin-1.

knockdown efficiency of siAMPK was confirmed by western blotting (Fig. S6A and B). Glucose deprivation significantly reduced erastin- and RSL3-induced cell death, whereas AMPK knockdown substantially weakened this protective effect (Fig. S7A–C). By contrast, LIP-1 continued to suppress ferroptotic cell death under normal glucose conditions. These results provide genetic loss-of-function evidence that AMPK contributes to glucose deprivation-mediated ferroptosis suppression, although they do not exclude additional AMPK-independent metabolic effects. It was next examined whether pharmacological induction of AMPK-related energy stress produced similar protection. AICAR or 2DG increased cell viability in erastin- and RSL3-treated cells (Fig. 3D–G). Consistently, Hoechst/PI staining showed that AICAR and 2DG reduced erastin-induced cell death (Fig. S8A and B). Because 2DG alters glycolytic flux and AICAR may affect AMP-sensitive pathways beyond AMPK, these data should be interpreted as supporting the involvement of energy stress-associated AMPK signaling, rather than proving that AMPK activation alone is sufficient to suppress ferroptosis in SH-SY5Y cells.

To further examine whether ACC-associated lipid metabolism contributes to ferroptosis regulation downstream of AMPK, TOFA was used as a pharmacological ACC inhibitor. TOFA reduced DCFDA fluorescence and C11-BODIPY oxidation in erastin- or RSL3-treated SH-SY5Y cells (Fig. 3H–M), suggesting that inhibition of ACC-associated lipid metabolic activity attenuates ROS accumulation and lipid peroxidation under ferroptotic stress. In parallel, BODIPY 493/503 staining showed that TOFA reduced neutral lipid/lipid droplet accumulation in erastin- or RSL3-treated cells (Fig. S9A–C). These findings are consistent with the involvement of ACC-associated lipid metabolic regulation in ferroptosis suppression. Western blot analysis demonstrated that 2DG increased the p-AMPK/AMPK and p-ACC1/ACC1 ratios, whereas total AMPK and total ACC1 protein levels were not markedly altered, suggesting that 2DG enhances AMPK-ACC1 signaling primarily through phosphorylation-dependent regulation rather than changes in total protein abundance (Fig. 3N–R). Together, these results suggest that energy stress engages an AMPK-ACC1-associated signaling axis that contributes to the

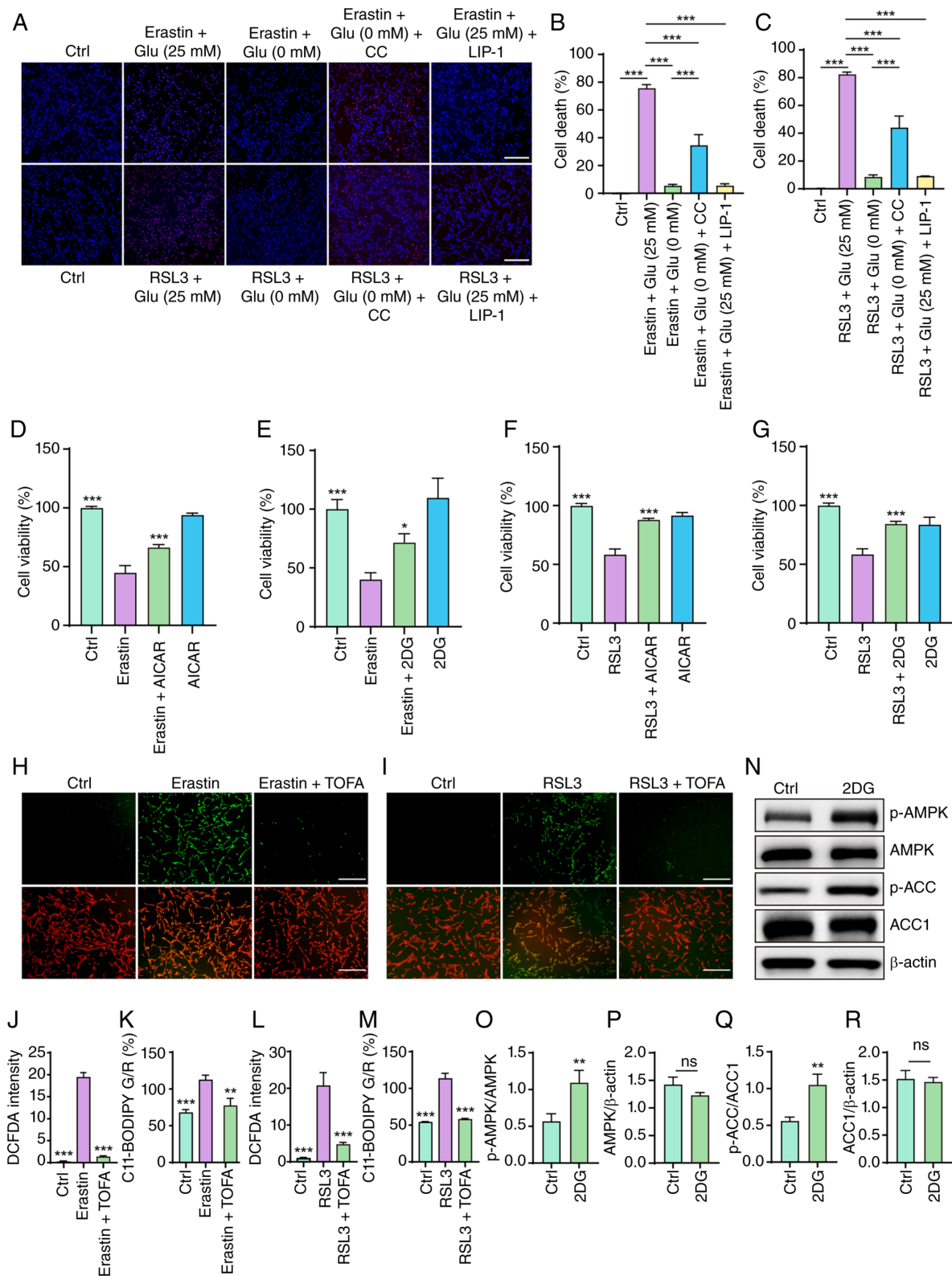


Figure 3. Energy stress activates the AMPK-ACC1 axis to suppress ferroptosis in SH-SY5Y cells. (A) Representative Hoechst/PI fluorescence images showing cell death in SH-SY5Y cells treated with erastin or RSL3 under normal glucose [Glu (25 mM)] or glucose-deprived [Glu (0 mM)] conditions, with or without CC or LIP-1. Magnification, x10; scale bars, 50 μ m. (B and C) Quantification of cell death in erastin-treated and RSL3-treated SH-SY5Y cells under the indicated conditions, respectively. (D-G) Cell viability of SH-SY5Y cells treated with erastin or RSL3 in the presence or absence of the AMPK activator AICAR or the glycolytic inhibitor 2DG. (H and I) Representative fluorescence images of DCFDA and C11-BODIPY 581/591 staining in SH-SY5Y cells treated with erastin or RSL3, with or without TOFA. Magnification, x10; scale bars, 50 μ m. (J-M) Quantification of DCFDA fluorescence intensity and the green-to-red (G/R) fluorescence ratio of C11-BODIPY 581/591 in erastin-treated and RSL3-treated SH-SY5Y cells. The G/R ratio represents the oxidized-to-reduced fluorescence ratio and was used as an indicator of lipid peroxidation. (N) Representative western blots of p-AMPK, AMPK, p-ACC, ACC1 and β -actin in SH-SY5Y cells treated with 2DG. (O-R) Densitometric quantification of p-AMPK/AMPK, total AMPK/ β -actin, p-ACC/ACC1 and ACC1/ β -actin, respectively. Data are presented as the mean $\pm$ SEM. *P<0.05, **P<0.01 and ***P<0.001. 2DG, 2-deoxy-D-glucose; AMPK, AMP-activated protein kinase; AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; TOFA, 5-tetradecyloxy-2-furoic acid; p-, phosphorylated; ACC, acetyl-CoA carboxylase; ns, not significant.

suppression of lipid peroxidation and ferroptosis-associated cell death in SH-SY5Y cells.

AMPK activation suppresses ferroptosis triggered by neurodegenerative pathogenic proteins. To determine whether AMPK activation protects against ferroptosis caused by neurodegenerative pathogenic proteins, SH-SY5Y cells overexpressing APP, α -synuclein A53T, or mutant huntingtin Q74 were exposed to glucose deprivation or AMPK activators. As a fluorescence control, glucose deprivation did not significantly alter pEGFP-N1 fluorescence in transfected SH-SY5Y cells (Fig. S10A and B). In APP-overexpressing cells, glucose deprivation, AICAR, or 2DG treatment significantly decreased cell death, ROS accumulation and lipid peroxidation, as shown by reduced PI uptake, DCFDA fluorescence and C11 BODIPY oxidation (Fig. 4A-D). Similarly, α -synuclein A53T-expressing cells displayed strong ferroptotic phenotypes that were significantly alleviated by glucose deprivation or AMPK activation (Fig. 4E-H). Consistent results were observed in Q74-overexpressing cells, where energy stress or AMPK activation attenuated ferroptosis, as evidenced by decreased PI-positive cells, DHE fluorescence intensity, and MDA levels (Figs. 4I-K and S11). Collectively, these findings support a protective association between energy stress-associated AMPK activation and reduced ferroptosis-related injury induced by multiple neurodegenerative pathogenic proteins.

AMPK activation ameliorates behavioral impairments in transgenic *C. elegans* models of neurodegenerative proteinopathies. To evaluate the functional relevance of AMPK activation *in vivo*, behavioral performance was assessed in transgenic *C. elegans* models expressing human A β 1-42, α -synuclein, or polyQ-expanded proteins. In the A β -expressing CL4176 model, treatment with 2DG or AICAR significantly reduced paralysis rates in a concentration-dependent manner, with an effect comparable to that of the ferroptosis inhibitor LIP-1 (Fig. 5A and B). In neuronal A β -expressing CL2355 worms, 2DG and LIP-1 improved the defective slowing response compared with untreated CL2355 worms, whereas the control strain CL2122 showed normal behavioral performance (Fig. 5C). These results indicate that metabolic activation of AMPK-related signaling alleviates A β -associated behavioral impairment. It was next examined whether this protective effect extended to other proteinopathy models. In α -synuclein-expressing NL5901 worms and polyQ-expanded AM141 worms, both 2DG and LIP-1 increased body bend frequency, indicating improved locomotor activity and neuromuscular function (Fig. 5D and E). These findings suggest that 2DG-mediated metabolic stress and ferroptosis inhibition confer behavioral protection across AD-, PD- and HD-related *C. elegans* models. To further determine whether the behavioral protection induced by 2DG depends on AMPK-related signaling, *aak-2* RNAi was performed in CL4176 worms. AAK-2 is the major AMPK orthologue in *C. elegans*. Under HT115/pL4440 control RNAi conditions, 2DG significantly reduced A β -induced paralysis. However, *aak-2* RNAi largely abolished the protective effect of 2DG (Fig. 5F and G). These results provide *in vivo* genetic evidence that AAK-2/AMPK signaling is required, at least in part, for 2DG-mediated behavioral protection in the A β -expressing worm model.

Collectively, these behavioral data support a conserved protective role of energy stress-induced AMPK-related signaling in neurodegenerative proteinopathy models.

2DG suppresses ferroptosis-related stress in transgenic *C. elegans* models of neurodegenerative proteinopathies. To determine whether 2DG suppresses ferroptosis-related damage *in vivo*, ROS accumulation, lipid peroxidation and MDA levels were examined in transgenic *C. elegans* models expressing neurodegeneration-associated pathogenic proteins. In A β -expressing CL4176 worms, temperature-induced A β expression at 25°C caused a significant increase in oxidative and ferroptosis-associated stress, as reflected by elevated DHE fluorescence, increased C11-BODIPY oxidation, and higher MDA levels. Treatment with 2DG or the ferroptosis inhibitor LIP-1 significantly reduced ROS accumulation and lipid peroxidation in CL4176 worms (Fig. 6A-D) and also decreased MDA levels (Fig. S12A). These results indicate that 2DG suppresses A β -associated oxidative stress and lipid peroxidation *in vivo*. Similar effects were observed in α -synuclein-expressing NL5901 worms. NL5901 worms exhibited increased DHE fluorescence, C11-BODIPY oxidation, and MDA production, indicating enhanced ROS accumulation and lipid peroxidation. Both 2DG and LIP-1 significantly reduced these ferroptosis-related changes (Figs. 6E-H and S12B). In polyQ-expanded AM141 worms, 2DG also decreased DHE fluorescence, C11-BODIPY oxidation and MDA levels, with effects comparable to those of LIP-1 treatment (Fig. 6I-L and Fig. S12C). These findings suggest that 2DG broadly attenuates oxidative and lipid peroxidation damage across AD-, PD-, and HD-related worm proteinopathy models. It was further evaluated whether 2DG and LIP-1 protect against ferroptosis-related injury in a toxin-induced dopaminergic neurodegeneration model. In 6-OHDA-exposed N2 worms, both treatments reduced DHE fluorescence and C11-BODIPY oxidation, indicating suppression of ROS accumulation and lipid peroxidation (Fig. S13A-D). Consistently, in 6-OHDA-exposed BZ555 worms, 2DG and LIP-1 preserved GFP fluorescence in dopaminergic neurons, supporting improved dopaminergic neuronal viability (Fig. S14A and B). Consistent with the behavioral data, *aak-2* RNAi weakened the ability of 2DG to suppress oxidative and ferroptosis-associated stress in CL4176 worms. Under HT115/pL4440 control RNAi conditions, 2DG reduced DHE fluorescence and C11-BODIPY oxidation after A β induction. By contrast, these protective effects were largely lost in worms fed *aak-2* RNAi bacteria (Fig. 6M-P). These results provide *in vivo* genetic evidence that AAK-2/AMPK signaling contributes to 2DG-mediated suppression of ROS accumulation and lipid peroxidation. Collectively, these findings show that 2DG reduces ROS accumulation, lipid peroxidation and MDA production in transgenic *C. elegans* models of neurodegenerative proteinopathies. Together with the *aak-2* RNAi results, these data support an important role for AAK-2/AMPK-related signaling in the anti-ferroptotic effects of energy stress *in vivo*.

2DG ameliorates cognitive deficits and suppresses neuro-inflammatory and ferroptosis-associated pathology in 3xTg-AD mice. To determine whether AMPK activation protects against neurodegeneration-associated ferroptotic

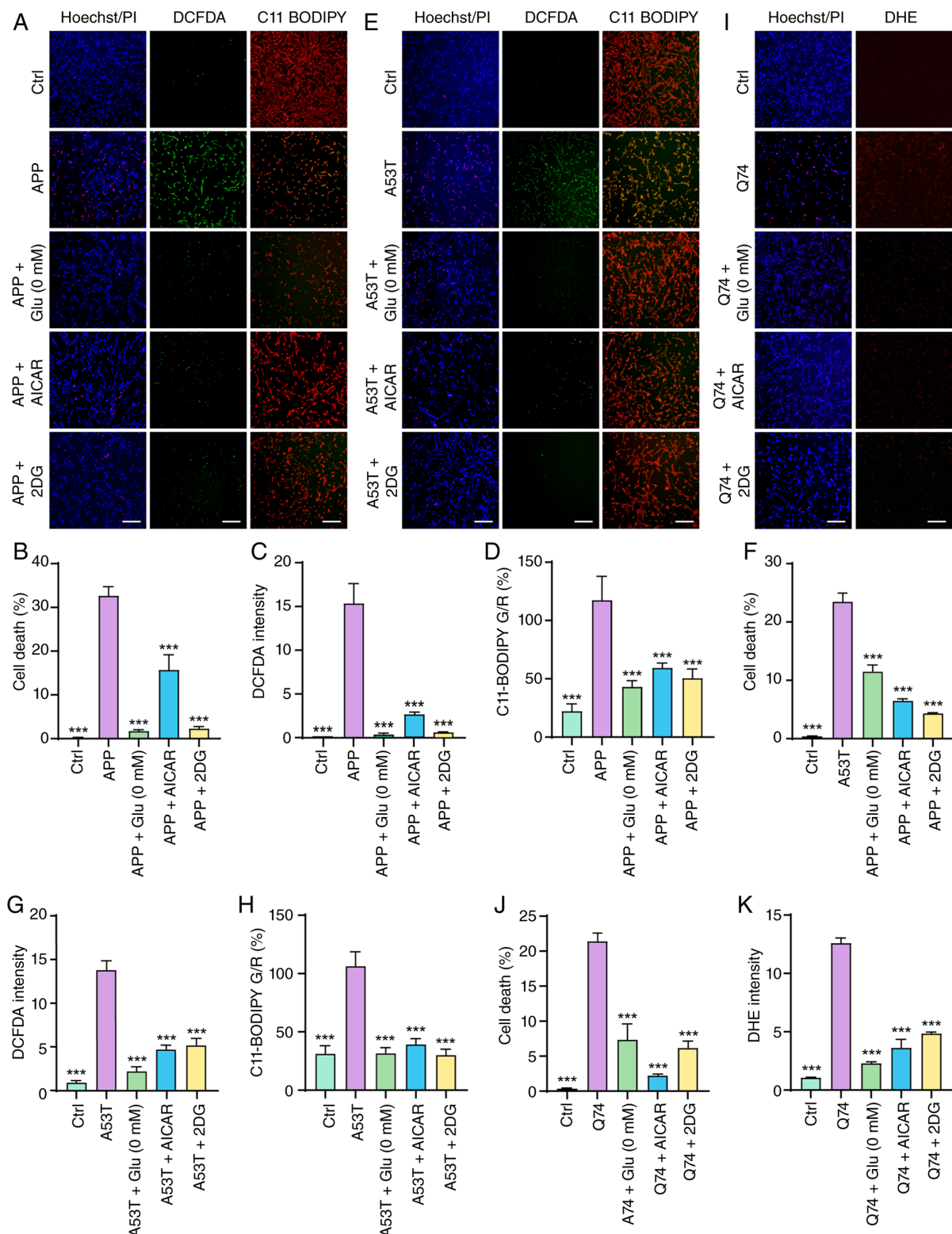


Figure 4. AMPK activation suppresses ferroptosis-like oxidative injury induced by neurodegenerative pathogenic proteins. (A) Representative fluorescence images of Hoechst/PI, DCFDA and C11-BODIPY 581/591 staining in Ctrl, APP-overexpressing, and APP-overexpressing SH-SY5Y cells subjected to glucose deprivation [Glu (0 mM)] or treated with the AMPK activator AICAR or 2DG. Magnification, x10; scale bars, 50 μ m. (B-D) Quantification of cell death (B), DCFDA fluorescence intensity (C), and the green-to-red (G/R) fluorescence ratio of C11-BODIPY 581/591 (D) in APP-overexpressing SH-SY5Y cells under the indicated conditions. The G/R ratio represents the oxidized-to-reduced fluorescence ratio and was used as an indicator of lipid peroxidation. (E) Representative fluorescence images of Hoechst/PI, DCFDA and C11-BODIPY 581/591 staining in Ctrl, α -synuclein A53T-overexpressing, and A53T-overexpressing SH-SY5Y cells subjected to glucose deprivation [Glu (0 mM)] or treated with AICAR or 2DG. Magnification, x10; scale bars, 50 μ m. (F-H) Quantification of cell death (F), DCFDA fluorescence intensity (G), and the green-to-red (G/R) fluorescence ratio of C11-BODIPY 581/591 (H) in A53T-overexpressing SH-SY5Y cells under the indicated conditions. The G/R ratio represents the oxidized-to-reduced fluorescence ratio and was used as an indicator of lipid peroxidation. (I) Representative fluorescence images of Hoechst/PI and DHE staining in control, Q74-overexpressing, and Q74-overexpressing SH-SY5Y cells subjected to glucose deprivation [Glu (0 mM)] or treated with AICAR or 2DG. Magnification, x10; scale bars, 50 μ m. (J and K) Quantification of cell death (J) and DHE fluorescence intensity (K) in Q74-overexpressing cells under the indicated treatments. Data are presented as the mean $\pm$ SEM. ****P<0.001. 2DG, 2-deoxy-D-glucose; AMPK, AMP-activated protein kinase; APP, amyloid precursor protein; AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside.

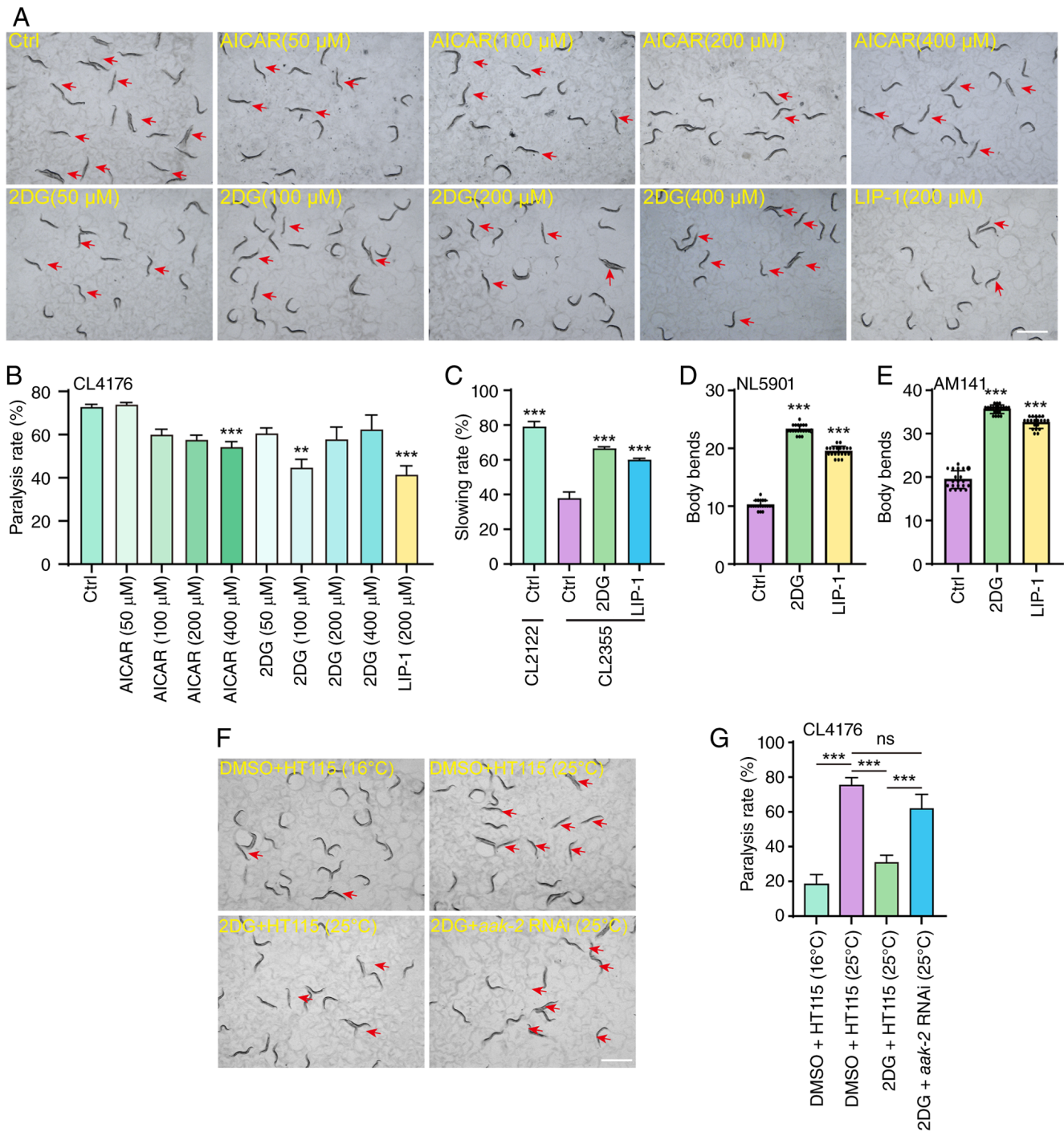


Figure 5. AMPK activation improves disease-associated behavioral deficits in transgenic *C. elegans* models of AD, PD and HD. (A) Representative bright-field images showing paralysis phenotypes in A β -expressing CL4176 worms treated with different concentrations of AICAR or 2DG, or with LIP-1. Red arrows indicate paralyzed worms. Magnification, x24; scale bar, 200 μ m. (B) Quantification of paralysis rate in CL4176 worms after treatment with AICAR, 2DG, or LIP-1. (C) Slowing response analysis in CL2122 control worms and neuronal A β -expressing CL2355 worms treated with 2DG or LIP-1. (D and E) Quantification of body bends in α -synuclein-expressing NL5901 worms (D) and polyglutamine (polyQ)-expanded AM141 worms (E) after treatment with 2DG or LIP-1. Data are presented as the mean $\pm$ SEM. (F) Representative bright-field images showing paralysis phenotypes in CL4176 worms cultured on HT115/pL4440 control bacteria or *aak-2* RNAi bacteria, with or without 2DG treatment. Red arrows indicate paralyzed worms. Magnification, x24; scale bar, 200 μ m. (G) Quantification of paralysis rate in CL4176 worms under the indicated conditions. Data are presented as the mean $\pm$ SEM. ** P <0.01 and *** P <0.001. 2DG, 2-deoxy-D-glucose; AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; LIP-1, liprostatin-1.

pathology *in vivo*, the behavioral and neuropathological effects of 2DG and LIP-1 in 3xTg-AD mice were evaluated. In the Morris water maze probe trial, 3xTg-AD mice exhibited significant spatial memory impairment, as shown by fewer platform crossings, reduced time spent in the target quadrant,

and shorter path length in the target quadrant compared with control mice. Treatment with either 2DG or LIP-1 significantly improved these memory-related parameters (Fig. 7A-D). During the acquisition phase, swimming speed did not differ substantially among groups, indicating that the behavioral

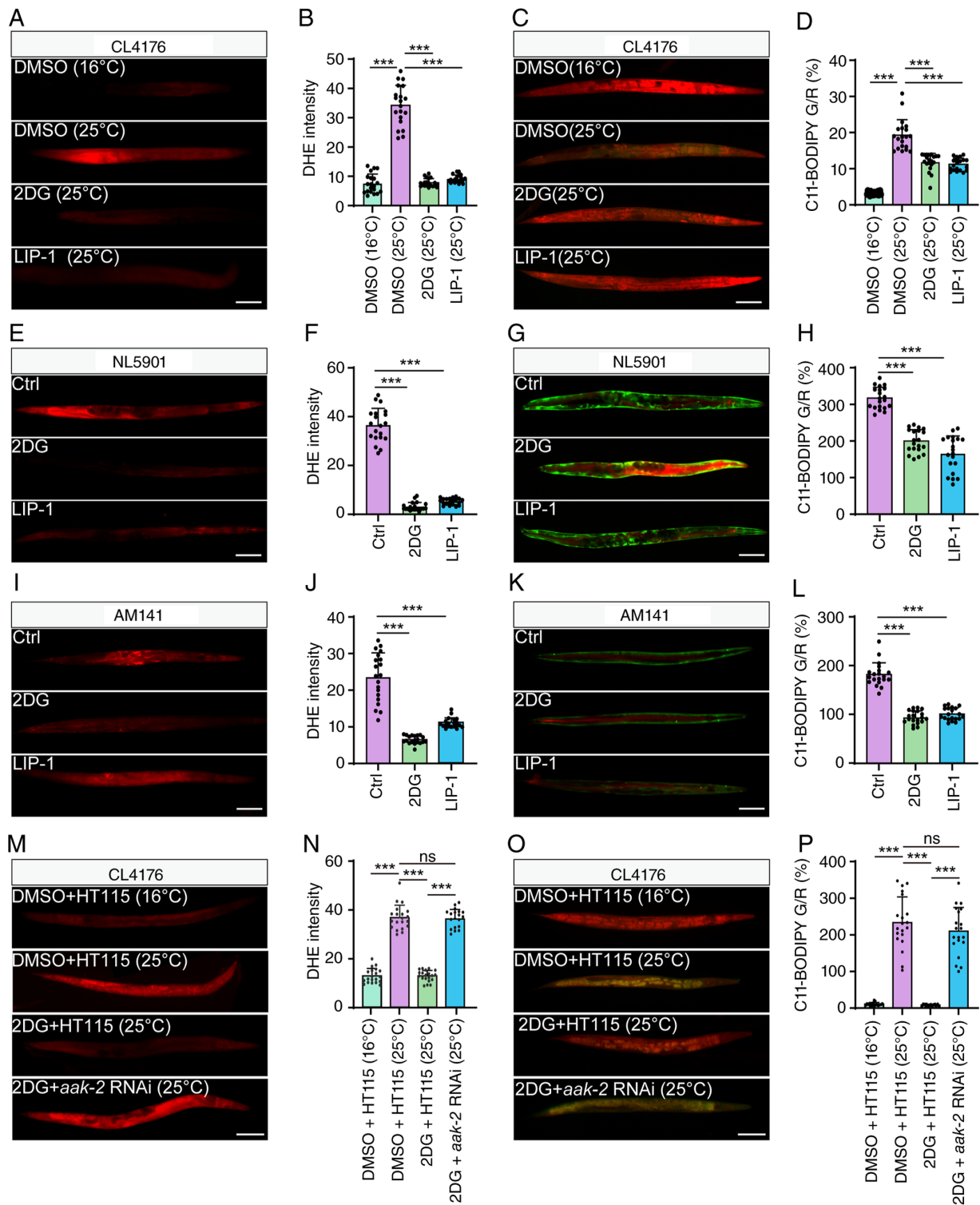


Figure 6. 2DG suppresses ferroptosis in transgenic *C. elegans* models of neurodegenerative proteinopathies through AAK-2/AMP-activated protein kinase signaling. (A and C) Representative fluorescence images of DHE staining (A) and C11-BODIPY 581/591 staining (C) in Aβ-expressing CL4176 worms maintained at 16°C or shifted to 25°C, with or without 2DG or LIP-1 treatment. Magnification, x10; scale bars, 50 μm. (B and D) Quantification of DHE fluorescence intensity (B) and the green-to-red (G/R) fluorescence ratio of C11-BODIPY 581/591 (D) in CL4176 worms under the indicated conditions. The G/R ratio represents the oxidized-to-reduced fluorescence ratio and was used as an indicator of lipid peroxidation. (E and G) Representative fluorescence images of DHE staining (E) and C11-BODIPY 581/591 staining (G) in α-synuclein-expressing NL5901 worms treated with 2DG or LIP-1. Magnification, x10; scale bars, 50 μm. (F and H) Quantification of DHE fluorescence intensity (F) and the green-to-red (G/R) fluorescence ratio of C11-BODIPY 581/591 (H) in NL5901 worms, the G/R ratio represents the oxidized-to-reduced fluorescence ratio and was used as an indicator of lipid peroxidation. (I and K) Representative fluorescence images of DHE staining (I) and C11-BODIPY 581/591 staining (K) in polyQ-expanded AM141 worms treated with 2DG or LIP-1. Magnification, x10; scale bars, 50 μm. (J and L) Quantification of DHE fluorescence intensity (J) and the green-to-red (G/R) fluorescence ratio of C11-BODIPY 581/591 (L) in AM141 worms, the G/R ratio represents the oxidized-to-reduced fluorescence ratio and was used as an indicator of lipid peroxidation. (M and O) Representative fluorescence images of DHE staining (M) and C11-BODIPY 581/591 staining (O) in CL4176 worms fed HT115/pL4440 control bacteria or *aak-2* RNAi bacteria under the indicated conditions. (N and P) Quantification of DHE fluorescence intensity (N) and the green-to-red (G/R) fluorescence ratio of C11-BODIPY 581/591 (P) in CL4176 worms under the indicated RNAi conditions, the G/R ratio represents the oxidized-to-reduced fluorescence ratio and was used as an indicator of lipid peroxidation. Data are presented as the mean ± SEM. ***P<0.001. 2DG, 2-deoxy-D-glucose; LIP-1, lipoxstatin-1; RNAi, RNA interference.

differences were not attributable to altered motor ability (Fig. 7E). By contrast, 3xTg-AD mice showed prolonged escape latency during training, which was markedly reduced by 2DG or LIP-1 treatment (Fig. 7F). These results indicate that 2DG and LIP-1 improve spatial learning and memory deficits in 3xTg-AD mice. Hippocampal neuroinflammatory changes were next examined. Immunofluorescence staining showed increased GFAP-positive astrocytes and Iba1-positive microglia in the hippocampi of 3xTg-AD mice, indicating pronounced astrocytic and microglial activation. Both 2DG and LIP-1 significantly reduced the percentages of GFAP-positive and Iba1-positive cells (Fig. 7G-I), suggesting that these treatments attenuate glial activation in the AD brain. Nissl staining further showed obvious neuronal loss and disorganization in the hippocampal CA1 region of 3xTg-AD mice, whereas 2DG and LIP-1 preserved neuronal morphology and improved hippocampal structural integrity (Fig. 7J). Consistently, H&E staining confirmed that 2DG and LIP-1 alleviated CA1 histopathological damage and reduced the appearance of damaged or pyknotic neurons (Fig. S15). To strengthen the *in vivo* evidence for ferroptosis-associated pathology, multiple ferroptosis-related markers in hippocampal sections were examined. Iron staining suggested increased hippocampal iron deposition in 3xTg-AD mice, which appeared to be attenuated by 2DG and LIP-1 treatment (Fig. 7K). 3xTg-AD mice also exhibited enhanced 4-HNE immunoreactivity, indicating increased lipid peroxidation, and this increase was attenuated by 2DG or LIP-1 treatment (Figs. 7K and S16). In parallel, immunofluorescence staining showed that ACSL4-positive cells were increased and GPX4-positive cells were decreased in the hippocampi of 3xTg-AD mice. Treatment with 2DG or LIP-1 significantly reduced ACSL4 positivity and restored GPX4 expression (Fig. 7K-M). These results provide convergent evidence that ferroptosis-associated iron accumulation, lipid peroxidation, ACSL4 upregulation and GPX4 loss occur in the 3xTg-AD hippocampus and are suppressed by 2DG or LIP-1. Moreover, the restoration of GPX4 expression and reduction of ACSL4 expression suggest that 2DG-mediated AMPK activation is associated with preservation of GPX4-related anti-ferroptotic defense and suppression of ACSL4-associated ferroptotic susceptibility in the AD hippocampus. Finally, western blot analysis demonstrated that 2DG increased hippocampal AMPK and ACC phosphorylation compared with untreated 3xTg-AD mice, whereas total AMPK and ACC1 levels were not markedly altered (Fig. 7N-P). These findings indicate that 2DG engages an AMPK-ACC1-associated signaling axis in the 3xTg-AD hippocampus. However, because 2DG can also alter glycolytic flux and activate broader metabolic stress responses, these mouse data should be interpreted as showing an association between 2DG-mediated neuroprotection and AMPK-ACC1 activation, rather than as definitive genetic proof that AMPK is required in mammalian neurons *in vivo*. Together with the iron staining, 4-HNE, ACSL4 and GPX4 data, these results support an association between 2DG-associated AMPK-ACC1 activation and reduced ferroptosis-associated hippocampal pathology *in vivo*. Collectively, these results demonstrate that 2DG ameliorates cognitive impairment and hippocampal pathology in 3xTg-AD mice, accompanied by suppression of neuroinflammation and

ferroptosis-associated damage, including iron accumulation, lipid peroxidation, ACSL4 upregulation and GPX4 reduction.

Discussion

The present study demonstrates that controlled energy stress attenuates ferroptosis-associated neuronal injury in neurodegenerative proteinopathy models in association with AMPK-ACC1-mediated metabolic regulation (Fig. 8). Previous studies have established that energy stress activates AMPK and suppresses ferroptosis through phosphorylation and inhibition of ACC, thereby limiting polyunsaturated lipid synthesis and lipid peroxidation (21-23). Therefore, the present study does not claim the AMPK-ACC axis as a newly discovered ferroptosis-regulatory mechanism. Rather, its main contribution is to show that this metabolic checkpoint is functionally engaged in disease-relevant neuronal proteinopathy contexts, including APP-, α -synuclein A53T- and polyQ-associated cellular models, transgenic *C. elegans* models, and 3xTg-AD mice. Across these systems, glucose deprivation, 2DG, or AICAR reduced ferroptosis-associated cell death and lipid peroxidation, while 2DG and LIP-1 improved behavioral or cognitive deficits and reduced ferroptosis-related pathology *in vivo*. These findings extend a previously defined metabolic ferroptosis checkpoint to neurodegenerative proteinopathies, where proteotoxic stress, mitochondrial dysfunction, iron dyshomeostasis and lipid peroxidation coexist.

These results also help clarify an apparent energy-stress paradox in neurodegeneration. AD and related proteinopathies are characterized by impaired glucose metabolism, mitochondrial dysfunction and chronic bioenergetic stress, conditions that could theoretically activate AMPK. However, chronic disease-associated metabolic failure is not necessarily equivalent to an effective protective AMPK response. AMPK signaling is highly context-dependent and may vary according to stimulus intensity, duration, cell type, subcellular localization, and coupling to downstream effectors. In the present study, 3xTg-AD mice showed clear ferroptosis-associated hippocampal pathology and cognitive impairment, indicating that the endogenous AMPK response at the examined disease stage was insufficient to prevent neurodegeneration. By contrast, 2DG induced more evident activation of the AMPK-ACC1 axis and was accompanied by reduced lipid peroxidation, decreased ACSL4 expression, restored GPX4 expression, reduced glial activation, and improved behavioral performance. Together with AMPK inhibition, siAMPK, and *aak-2* RNAi data in cellular and *C. elegans* models, these findings support the idea that controlled metabolic intervention can reinforce an inadequate endogenous adaptive response and restore downstream restraint of ferroptotic lipid peroxidation.

Lipid metabolism is central to ferroptotic vulnerability. Polyunsaturated phospholipids are key substrates for lipid peroxidation, and limiting their synthesis or incorporation into membranes can reduce ferroptotic sensitivity (31-33). In the present study, 2DG increased the p-AMPK/AMPK and p-ACC1/ACC1 ratios in SH-SY5Y cells and hippocampal tissue, whereas TOFA reduced ROS accumulation, C11-BODIPY oxidation, and BODIPY 493/503-positive neutral lipid accumulation. These findings are consistent with an AMPK-ACC1-associated mechanism that limits lipid metabolic

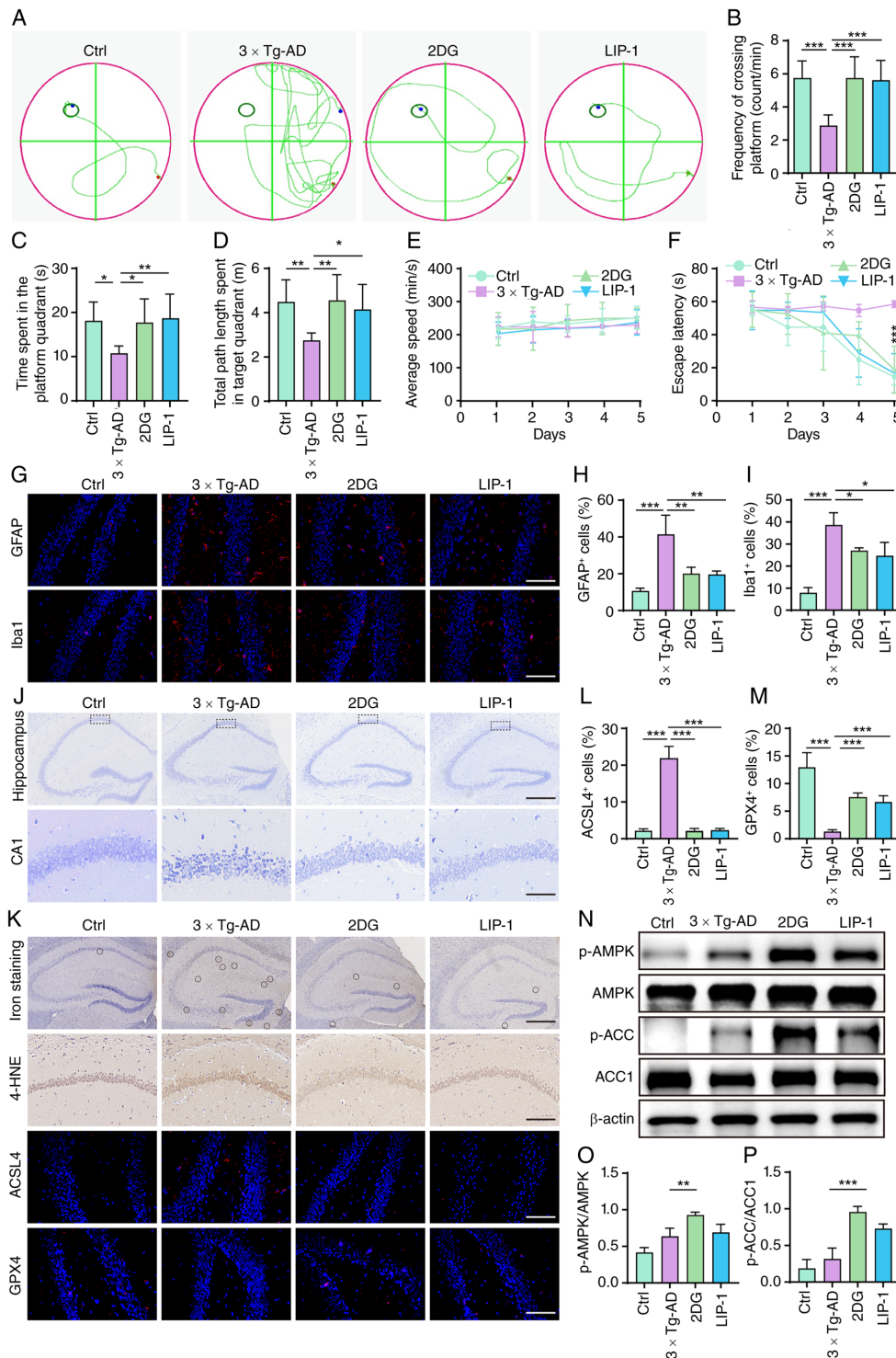


Figure 7. 2DG-mediated AMPK activation ameliorates cognitive impairment and attenuates neuroinflammatory and ferroptotic pathology in 3xTg-AD mice. (A) Representative swimming trajectories during the Morris water maze probe trial in WT control, 3xTg-AD, 3xTg-AD + 2DG, and 3xTg-AD + LIP-1 mice. The 2DG and LIP-1 groups throughout the figure refer to 3xTg-AD mice treated with 2DG or LIP-1, respectively. (B-D) Quantification of platform crossing frequency (B), time spent in the target quadrant (C), and total path length in the target quadrant (D) during the probe trial. (E and F) Average swimming speed (E) and escape latency (F) during the acquisition phase. (G) Representative hippocampal immunofluorescence images of GFAP and Iba1 staining in each group. Magnification, x40; scale bars, 200 μm. (H and I) Quantification of GFAP-positive astrocytes (H) and Iba1-positive microglia (I) in the hippocampus. (J) Representative Nissl-stained hippocampal sections. Upper panels show the overall hippocampal region, and lower panels show higher-magnification views of the CA1 region indicated by dashed boxes. Magnification, x5; scale bars, 300 μm. (K) Representative images of iron staining, 4-HNE immunohistochemistry, and immunofluorescence staining for ACSL4 and GPX4 in hippocampal sections from each group. Circles indicate iron-positive deposits. Magnifications and scale bars were as follows: x5 and 300 μm for Prussian blue staining; x20 and 100 μm for 4-HNE immunohistochemistry; and x40 and 200 μm for ACSL4 and GPX4 immunofluorescence. (L and M) Quantification of ACSL4-positive cells and GPX4-positive cells in the hippocampus. Quantification of 4-HNE staining is shown in Fig. S16. (N) Representative Western blots of p-AMPK, AMPK, p-ACC, ACC1 and β-actin in hippocampal lysates from each group. (O and P) Densitometric quantification of p-AMPK/AMPK (O) and p-ACC/ACC1 (P). Data are presented as the mean ± SEM. *P<0.05, **P<0.01 and ***P<0.001. 2DG, 2-deoxy-D-glucose; 4-HNE, 4-hydroxynonenal; AMPK, AMP-activated protein kinase; LIP-1, lipoxstatin-1; Iba1, ionized calcium-binding adapter molecule 1; p-, phosphorylated; GPX4, glutathione peroxidase 4; ACSL4, acyl-CoA synthetase long-chain family member 4; GFAP, glial fibrillary acidic protein; WT, wild-type.

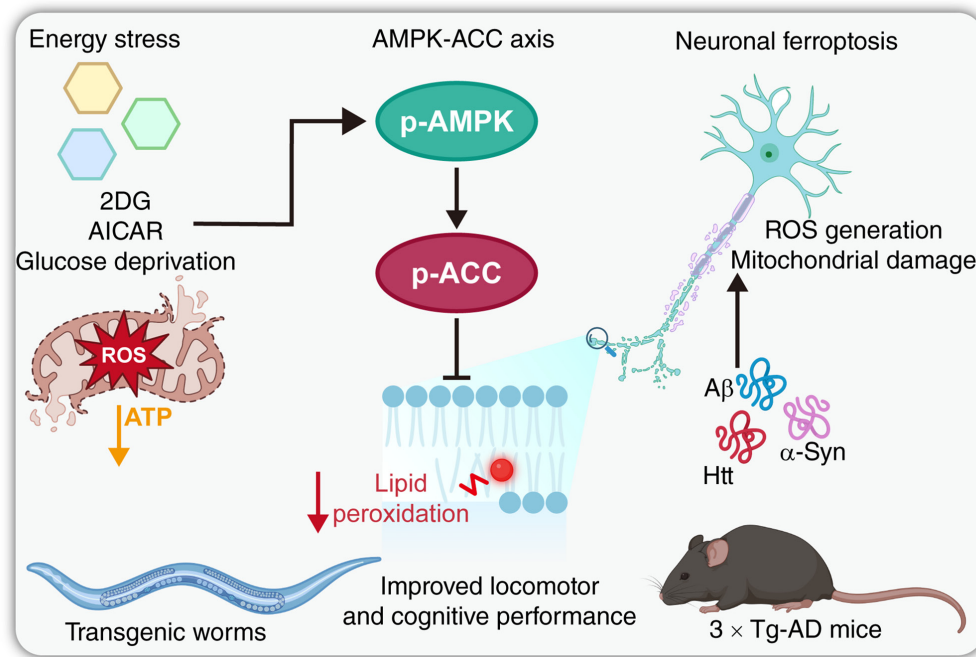


Figure 8. Proposed model by which energy stress activates the AMPK-ACC1 axis to suppress neuronal ferroptosis in neurodegenerative proteinopathies. Energy-stress interventions, including 2DG, AICAR, and glucose deprivation, activate AMPK signaling and promote ACC phosphorylation. Activation of the AMPK-ACC1 axis limits ferroptosis-associated lipid peroxidation, thereby reducing ROS generation and mitochondrial damage induced by neurodegenerative pathogenic proteins, including A β , α -synuclein and mutant huntingtin. In transgenic *C. elegans* models and 3xTg-AD mice, this mechanism is associated with reduced oxidative and ferroptotic injury and improved locomotor or cognitive performance. 2DG, 2-deoxy-D-glucose; AMPK, AMP-activated protein kinase; ACC, acetyl-CoA carboxylase; p-, phosphorylated; AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; ROS, reactive oxygen species.

inputs required for ferroptosis. However, the data should be interpreted carefully. The detected ACC isoform in the present study was ACC1, and TOFA is not isoform-selective. Moreover, TOFA may exert ACC-independent metabolic effects, and *de novo* lipid synthesis was not directly measured. Thus, the current data support the involvement of ACC-associated lipid metabolic regulation, but do not prove that AMPK suppresses ferroptosis exclusively through ACC1 inhibition. Future studies using ACC1- and ACC2-specific genetic manipulation, malonyl-CoA measurement and isotope-tracing lipidomics will be needed to define the precise lipid metabolic steps by which AMPK limits ferroptotic lipid peroxidation in neuronal cells.

The *in vivo* findings further support the relevance of ferroptosis-associated pathology in neurodegenerative proteinopathies. In transgenic *C. elegans* models expressing A β , α -synuclein, or polyQ-expanded proteins, 2DG and LIP-1 reduced ROS accumulation and lipid peroxidation and improved locomotor or behavioral phenotypes. Importantly, *aak-2* RNAi weakened the protective effects of 2DG, providing *in vivo* genetic evidence that AMPK-related signaling contributes to the anti-ferroptotic effect of energy stress. In 3xTg-AD mice, 2DG and LIP-1 improved spatial learning and memory, reduced GFAP- and Iba1-positive glial activation, preserved hippocampal neuronal morphology, and suppressed ferroptosis-associated changes, including iron accumulation, 4-HNE immunoreactivity, ACSL4 upregulation, and GPX4 reduction. The parallel effects of 2DG and LIP-1 suggest that suppression of ferroptosis-associated lipid peroxidation is an important component of the observed neuroprotection. The restoration of GPX4 and reduction of ACSL4 further indicate that energy stress-associated metabolic regulation is linked

not only to reduced lipid peroxidation products but also to modulation of ferroptosis susceptibility and defense markers in the AD hippocampus.

Several limitations should be acknowledged. First, the causal requirement of AMPK-ACC1 signaling was not genetically tested in mammalian neurons *in vivo*. Although AMPK knockdown in SH-SY5Y cells and *aak-2* RNAi in *C. elegans* reduced reliance on pharmacological tools, the 3xTg-AD mouse data establish an association between 2DG-induced AMPK-ACC1 activation and reduced ferroptosis-associated pathology rather than definitive proof of AMPK necessity. This distinction is important because 2DG alters glycolytic flux and can activate broader metabolic stress responses, AICAR may affect AMP-sensitive pathways beyond AMPK, and CC can inhibit kinases other than AMPK (34-37). Therefore, the pharmacological results should be interpreted together with, but not as a substitute for, genetic validation. In addition, AMPK-ACC1 signaling was assessed mainly at the experimental endpoint in 3xTg-AD mice. Thus, although the data suggest that the endogenous AMPK response at this stage was insufficient to prevent ferroptosis-associated pathology, they do not define dynamic changes in AMPK signaling during disease initiation, progression, or late-stage neurodegeneration. Future studies using neuron-specific AMPK α 1/AMPK α 2 knockout, dominant-negative AMPK, CRISPR-based AMPK knockout, viral AMPK manipulation, rescue experiments, and longitudinal analysis of AMPK-ACC1 signaling will be required to establish the causal, isoform-specific, cell type-specific, and stage-dependent role of this pathway.

Second, the ferroptosis-related analyses in 3xTg-AD mice were based mainly on hippocampal histological and

immunostaining evidence. Iron staining, 4-HNE immunohistochemistry, ACSL4 immunofluorescence, and GPX4 immunofluorescence provide convergent spatial evidence for a ferroptosis-associated pattern, but they do not establish neuron-specific localization or biochemical activity. Quantitative measurements of MDA, total iron, labile iron, GSH/GSSG ratio, GPX4 enzymatic activity, and NeuN-based neuronal co-localization would further strengthen the mechanistic interpretation. In addition, only male mice were used. This design reduced variability related to sex hormone cycling but limits generalizability, especially because sex differences have been reported in AD pathology, glucose metabolism, neuroinflammation and behavioral phenotypes in 3xTg-AD mice (38-40). Future studies should include both male and female mice and perform sex-stratified analyses of cognitive behavior, ferroptosis-associated pathology, AMPK-ACC1 signaling, and neuroinflammatory responses.

Third, ferroptosis should not be regarded as the only form of cell death in these models. LIP-1 and Fer-1 rescue, C11-BODIPY oxidation, MDA accumulation, GPX4 reduction and ACSL4 upregulation support a ferroptosis-associated injury component, but pathogenic proteins may also induce apoptosis, necroptosis, or mixed regulated cell death responses. This interpretation is consistent with our previous observation that A β (1-42)-induced PC-12 cell injury could be partially rescued by LIP-1, Nec-1 and Z-VAD-FMK (41). Therefore, future studies should combine ferroptosis inhibitors with caspase inhibitors, necroptosis inhibitors, cleaved caspase-3/PARP analysis, and RIPK1/RIPK3/MLKL pathway detection to determine the relative contribution of different cell death modalities in APP-, α -synuclein-, and polyQ-induced neuronal injury.

Finally, the model systems and translational implications require caution. SH-SY5Y cells are useful for mechanistic screening but do not fully recapitulate the metabolic and electrophysiological properties of mature human neurons. Primary neurons, human iPSC-derived neurons, brain organoids, and transgenic animals with neuron-specific modulation of AMPK or ACC would provide more physiologically relevant validation. Moreover, AMPK has pleiotropic functions beyond ACC1 regulation, including effects on autophagy, mitochondrial biogenesis, glucose utilization and redox adaptation (41-44). Thus, part of the protection observed in the present study may reflect broader metabolic remodeling, including improved mitochondrial quality control or NADPH-dependent redox homeostasis, rather than direct ACC1-dependent lipid synthesis control alone. As aforementioned, ACC1- and ACC2-specific genetic validation will be needed to clarify the relative contribution of each ACC isoform. Although 2DG and LIP-1 restored GPX4 expression in the 3xTg-AD hippocampus, GPX4 enzymatic activity and GSH/GSSG status were not directly measured, and whether AMPK directly regulates GPX4 or other ferroptosis defense proteins remains to be determined. From a translational perspective, energy stress-based therapy must also be approached carefully, because sustained AMPK activation or chronic energy deprivation may impair synaptic plasticity, neuronal differentiation, or systemic metabolic homeostasis (45-47). More selective and brain-penetrant AMPK modulators, downstream ACC-targeted approaches, or intermittent and localized metabolic interventions may

be needed to achieve neuroprotection without compromising metabolic flexibility.

Together, these findings support energy stress-associated AMPK-ACC1 signaling as an important metabolic defense mechanism against ferroptosis-related neuronal injury in neurodegenerative proteinopathies. At the same time, the pathway should be viewed as part of a broader metabolic and cell death regulatory network rather than as a single exclusive mechanism. Defining its neuron-specific causal role, lipid metabolic outputs, interaction with other regulated cell death pathways, and translational safety will be essential for developing AMPK-ACC1-centered strategies against ferroptosis-associated neurodegeneration.

In conclusion, the present study supports AMPK as an important metabolic regulator that contributes to ferroptosis suppression through modulation of lipid metabolism and oxidative homeostasis. By extending the previously described AMPK-ACC ferroptosis checkpoint to neurodegenerative proteinopathy models, our findings provide disease-relevant evidence that controlled energy stress-associated AMPK-ACC1 signaling can limit lipid peroxidation and ferroptosis-associated neuronal injury. These results suggest that modulation of metabolic signaling may represent a potential strategy to reduce ferroptosis-related damage in NDs, although further genetic and mechanistic studies are required to define the direct targets, isoform specificity, cell death specificity, and translational feasibility of this pathway.

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Availability of data and materials

The data generated in the present study are included in the figures of this article.

Authors' contributions

YY conducted investigation, data curation, formal analysis, visualization, and wrote the original draft. YH and QF

conducted investigation, data curation and formal analysis. XZ, LY and JM conducted data curation and formal analysis. DQ conceptualized the study and acquired funding. JW conceptualized the study. JL and AW conceptualized the study, acquired funding and performed project administration. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy. YY and YH confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All experimental procedures were reviewed and approved by the Animal Ethics Committee of Southwest Medical University (approval no. 20220812-003; Luzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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