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Calycosin attenuates neuronal ferroptosis in Alzheimer's disease mice by activating the Nrf2/HO-1 pathway

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Abstract. In this study, we investigated the therapeutic potential of calycosin (from *Astragalus*) in Alzheimer's disease (AD), focusing on ferroptosis modulation. APP/PS1 mice received 40 mg/kg calycosin for 3 months. Cognitive function was assessed *via* Morris water maze test. Tau hyperphosphorylation and amyloid-β (Aβ) aggregation were analyzed using immunofluorescence and Western blotting. *In vitro*, $A\beta_{1\text{-}42}$-treated HT22 neuronal cells were exposed to calycosin. Ferroptosis-related phenotypes were assessed *in vivo* and *in vitro* using Prussian blue staining, commercial kits, and Western blotting. The nuclear factor-erythroid factor 2-related factor 2 (Nrf2) signaling was examined by Western blotting. Calycosin treatment significantly improved cognitive deficits in APP/PS1 mice and inhibited Tau hyperphosphorylation and Aβ aggregation. Calycosin attenuated neurotoxicity and Tau hyperphosphorylation in $A\beta_{1\text{-}42}$-treated HT22 cells. Moreover, calycosin inhibited ferroptosis *in vivo* and *in vitro* by decreasing iron aggregation and lipid peroxidation, downregulating transferrin receptor expression, and upregulating ferroportin, cystine/glutamate antiporter, and glutathione peroxidase 4 expression. Mechanistically, the anti-ferroptosis effects of calycosin were linked to the activation of the Nrf2-mediated pathway. These findings suggest that calycosin may exhibit neuroprotective effects against neuronal ferroptosis in AD, indicating its potential as a therapeutic candidate for further investigation in AD.

Key words: Alzheimer's disease — Calycosin — Ferroptosis — Oxidative stress — Nrf2 — Tau protein — Amyloid-β protein

Introduction

Alzheimer's disease (AD) is neurodegenerative disorder with a globally increasing incidence, characterized by cognitive impairment, memory loss, and language and behavioral deficits (Scheltens et al. 2021). The major histopathological features of AD include the accumulation of insoluble amyloid plaques composed of beta-amyloid (Aβ) peptides and intracellular neurofibrillary tangles formed by hyperphosphorylated tau protein in the brain (Panza et al. 2019). These pathological features, along with mitochondrial dysregulation and increased oxidative stress, contribute to synaptic and neuronal loss, ultimately leading to significant atrophy of brain regions critical for cognitive function. The distribution of neurofibrillary tangles has been closely linked to the severity of AD (Serrano-Pozo et al. 2011). Current pharmacotherapy for AD primarily relies on cholinesterase inhibitors, which are associated with side effects such as bradycardia, anorexia, and weight loss (Rabins et al. 2017). Therefore, there is an urgent need to develop safer and more effective therapeutic agents for AD.

Ferroptosis, a type of programmed cell death distinct from apoptosis, is characterized by lipid peroxidation and intracellular iron overload (Dixon et al. 2012). Evidence suggests that ferroptosis is involved in neuronal death across

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various neurological disorders, including Huntington's disease, Parkinson's disease, ischemic stroke, and hemorrhagic stroke, often accompanied by mitochondrial dysfunction, lipid peroxidation, and glutathione peroxidase 4 (GPX4) downregulation (Skouta et al. 2014; Guiney et al. 2017; Tuo et al. 2017; Alim et al. 2019). For example, Hambright et al. showed that conditional knockout of GPX4 in forebrain neurons impaired spatial learning and memory function in mice, while ferroptosis inhibitors alleviated neurodegeneration (Hambright et al. 2017). Bao et al. found that ferroportin-1 (FPN1, also known as SLC40A1), a nonheme cellular iron exporter, improved memory impairment by inhibiting ferroptosis in AD mice (Bao et al. 2021). Additionally, a large cohort study showed that high levels of tissue iron in the brain accelerate AD progression (Ayton et al. 2021). These findings highlight ferroptosis as a potential therapeutic target for AD.

Calycosin, an isoflavone extracted from *Astragali Radix*, exhibits a wide range of pharmacological properties, including anti-tumor, anti-inflammatory, antioxidant, cardioprotective properties, and immunomodulating activities (Zhang et al. 2015). Calycosin has been reported to show neuroprotective potential in animal models with cerebral ischemia injury (Wang et al. 2018; Xu et al. 2023), and mitigate diabetes-related neurocognitive impairment through its antioxidant activities (Huang et al. 2022). Song et al. preliminarily demonstrated that calycosin reduces inflammatory responses and oxidative stress in the brain of AD model mice (Song et al. 2017). Furthermore, calycosin has been shown to protect against cerebral and renal injury by inhibiting ferroptosis (Huang et al. 2022; Liu et al. 2023). However, the role of calycosin in modulating ferroptosis in AD remains poorly understood.

In this study, we investigated the effects and mechanisms of calycosin on AD neuropathology using APP/PS1 double transgenic mice and $A\beta_{1-42}$ oligomers-exposed hippocampal neuronal cells. We hypothesized that calycosin ameliorates AD by inhibiting the ferroptosis pathway, providing valuable insights into the therapeutic potential of natural compounds for AD treatment.

Materials and Methods

Animals and administration

Nine-month-old female APPswe/PS1E9 (APP/PS1) double transgenic mice and age-matched wild-type (WT) mice were obtained from Jicui Yaokang Animal Experiment Center (Nanjing, China). The animals were housed under controlled conditions at 24–26°C with a 12-h light/dark cycle. All experimental procedures adhered to the guidelines and were approved by the Animal Welfare Committee of Hubei Third People's Hospital of Jianghan University. The APP/PS1 was randomly divided into two groups: the APP/PS1 group (n = 8, receiving 10 ml/kg normal saline orally three times *per* week) and the APP/PS1+calycosin group (n = 8, receiving 40 mg/kg calycosin (nkl-00177, HPLC≥98%, Chengdu Nakeli Biotechnology, Chengdu, China) orally three times *per* week. The dose of calycosin was determined based on the previous studies (Song et al. 2017). WT mice were administered 10 ml/kg normal saline orally and served as the control group. Following three months of treatment, behavioral assessments were conducted. All animals were humanely euthanized, and brain tissues were promptly harvested for subsequent analysis.

Cell culture and treatment

Mouse hippocampal neuronal cells HT22 (CL-0595, Procell Life Technology, Wuhan, China) were cultured in DMEM (C11995500BT, Thermo Fisher Scientific, MA, USA) containing 1% penicillin-streptomycin (91670249, Shanghai YEASEN Biotechnology, Shanghai, China) and 10% fetal bovine serum (ZY140661, Shanghai Zeye Biotechnology), and maintained in a humidified atmosphere with 5% CO_2 at 37°C. The preparation of $A\beta_{1-42}$ oligomers (PE-1749-50, Wuhan Amyjet Scientific) followed a previously described protocol (Masters and Selkoe 2012). Briefly, 5 mM $A\beta_{1-42}$ was diluted in PBS to 100 μM and incubated for 24 h at 4°C. Afterwards, the solution was diluted with DMEM to a final concentration of 20 μM and incubated for 24 h. Vehicle treatment served as the control. HT22 cells were treated with calycosin at concentrations of 0.1, 1, 5, 10, and 20 μM to assess its impact on cell viability.

siRNA of nuclear factor-erythroid 2-related factor 2 (Nrf2)

Small interfering RNA (siRNA) targeting Nrf2 (si-Nrf2) (sc-37030, Santa Cruz, Jiangsu, China) was used to knock down Nrf2 expression, with negative control siRNA (si-NC) serving as the control. HT22 cells were transfected with 50 nM si-Nrf2 or si-NC using the Lipofectamine™ 3000 (L3000001, Invitrogen, CA, USA) for 24 h. After transfection, the cells were treated with calycosin or $A\beta_{1-42}$ oligomers.

Morris water maze (MWM) test

The MWM test was utilized to assess the spatial learning and memory capabilities of the mice. A circular water-filled pool (50×150 cm) maintained at 23°C was divided into four quadrants. A cylindrical platform (10×10 cm) was submerged approximately 1 cm below the water surface in the target quadrant. Mice were allowed to swim freely

for 60 s in a randomly selected quadrant during each trial. On the first day, animals underwent training and learning exercises to locate the platform. From days 2 to 6, the platform was obscured with white dye, and the ability of mice to locate it was monitored. Mice that found the platform within 60 s were allowed to rest on it for 10 s; otherwise, they were guided to the platform and allowed to rest for 10 s to reinforce memory. Training and testing were repeated four times daily. On day 7, a probe trial was conducted to assess memory retention. The platform was removed, and mice were placed in a randomly selected quadrant and allowed to swim for 60 s. The swimming path, distance, number of platform crossings, and time spent in each quadrant were recorded using a Noldus XT 7.1 video-computerized tracking system (Leesburg, VA, USA) (Lai et al. 2019).

Immunofluorescence analysis

Cortical and hippocampal tissues (right hemisphere) from the mice were cut into 20-μm-thick slices, deparaffinized in xylene, rehydrated in ethanol, and washed with ddH_2O. After treatment with 0.2% Triton X-100 (93443, Sigma-Aldrich, MA) for 30 min, the samples were blocked with PBS containing 3% BSA (A9647, Sigma-Aldrich) for 30 min at room temperature. The sections were then incubated with Aβ (PL0301218, 1:100, PLLABS, Canada) and phosphorylated Tau (p-Tau) (ab92676, 1:50, Abcam, UK) antibodies at 4°C overnight, followed by incubation with secondary antibodies (Fluor 546-conjugated anti-rabbit, 1:500, Thermo Fisher Scientific) for 1 h at 37°C. The sections were stained with DAPI and observed under a confocal scanning microscopy (Carl Zeiss, Oberkochen, Germany). At least five images were captured from each mouse.

Western blotting

Proteins were extracted from cortical and hippocampal tissues and HT22 cells using lysis buffer (P0013B, Beyotime, Shanghai). Protein concentrations were quantified using a BCA kit (KGP902, KeyGEN Biotechnology, Jiangsu). Proteins (10 μg) were separated on a 10% SDS-PAGE gel and transferred onto 0.22-μm PVDF membranes (Merck Millipore, MA). After blocking with 5% non-fat milk, the membranes were incubated with primary antibodies at 4°C overnight. Primary antibodies included transferrin receptor (TFRC; ab214039, 1:1000), FPN (DF13561, 1:1000, Affinity Biosciences, OH, USA), cystine/glutamate antiporter (SLC7A11; ab307601, 1:1000), GPX4 (ab125066, 1:1000), p-Tau (ab92676, 1:1000), Tau (ab254256, 1:1000), Aβ (PL0301218, 1:500, PLLABS), Nrf2 (12721, 1:1000, Cell Signaling Technology (CST), MA), heme oxygenase-1 (HO-1, ab189491, 1:2000), Lamin B1 (ab133741, 1:2000), and β-actin (3700, 1:1000, CST). Following washing with 1× TBST, the membranes were incubated with anti-mouse or anti-rabbit IgG conjugated secondary antibodies (Proteintech, Wuhan) for 1.5 h at 37°C. Next, the blots were visualized with an ECL Chemiluminescent Kit (Thermo Fisher Scientific), and gray values were analyzed using ImageJ software.

Prussian blue staining

Paraffin-embedded brain tissue sections were dewaxed in xylene I and II for 20 min each, followed by sequential dehydration in anhydrous ethanol I, II, and 75% ethanol for 5 min each. The sections were washed three times with distilled water and then stained with a Prussian Blue Iron Stain Kit (60533ES20, Shanghai YEASEN Biotechnology), prepared by mixing potassium ferrocyanide (Sigma-Aldrich) with 2% hydrochloric acid (Amresco, WA, USA) in a 1:1 ratio. The tissues were stained for 1 h, washed twice with distilled water, and counterstained with 0.1% nuclear fast red (Beyotime) for 5 min. After washing with running water, the sections were dehydrated in anhydrous ethanol I/II/III for 5 min each, cleared in xylene I/II for 5 min each, and mounted with neutral resin. The slides were examined under a microscope (BX51, Olympus, Japan).

Biochemical assay

Ferrous iron levels in the cortex, hippocampus, and HT22 cells were measured using an Iron Assay Kit (K390-100, Wuhan Amyjet Scientific) following the manufacturer's instructions. Absorbance was read at 593 nm using a microplate reader (Tai et al. 2020). Malondialdehyde (MDA) levels were determined using a Lipid Peroxidation MDA Assay Kit (K739-100, Wuhan Amyjet Scientific) following the manufacturer's protocol, with absorbance measured at 532 nm (Rahman et al. 2019).

Cell viability assay

HT22 cells seeded in 96-well plates (3×10^3 cells/well) were cultured in fresh complete culture medium containing 0.1, 1, 5, 10, and 20 μM calycosin for 24 h. The viability of HT22 cells was examined using 10 μl CCK-8 solution (40710ES03, Shanghai YEASEN Biotechnology) as *per* the manufacturer's instructions. Absorbance was measured at 450 nm using a BioTek SynergyH1 microplate reader (Agilent Technologies, CA). Similarly, the effects of calycosin (0.1, 1, 5, 10, and 20 μM) in combination with 20 μM $A\beta_{1-42}$ oligomers on HT22 cell viability were detected using the CCK-8 assay. Cells were pretreated with calycosin for 3 h, followed by 24 h of co-exposure to 20 μM $A\beta_{1-42}$ oligomers.

Flow cytometry

Apoptosis was assessed using an Apoptosis Detection Kit (E606336, Sangon Biotech, Shanghai). Briefly, HT22 cells were cultured in 6-well plates and harvested at 80%. The cells were centrifuged at 1000×*g* for 5 min, washed twice with ice-cold PBS, and resuspended in 195 µl Annexin V-FITC/PI binding buffer. Next, 10 µl propidium iodide solution and 5 µl Annexin V-FITC solution were added, and the cells were incubated for 20 min in the dark. Apoptosis was analyzed by flow cytometry analysis.

ROS assay

HT22 cells were seeded in 6-well plates (2×10^5 cells/well) and pretreated with 0.1, 1, 5, 10, and 20 µM calycosin for 3 h, followed by 24 h of co-exposure to 20 µM $A\beta_{1-42}$ oligomers. The cells were then incubated with 10 µM Dihydroethidium (DHE; FY17032, Nantong Feiyu Biotechnology, Jiangsu) for 30 min in the dark and washed twice with PBS. Fluorescence was measured using a TH4-200 fluorescence microscope (Olympus).

Statistical analysis

Data are presented as the mean ± SD from three repeated experiments. Statistical comparisons were performed using a two-tailed Student's *t*-test for single comparisons and one-way ANOVA for multiple comparisons. A *p*-value of <0.05 was considered statistically significant. GraphPad Prism, version 8.0 (GraphPad Software Inc., CA), was used for statistical analysis. Investigators were blinded to group assignments during the experiments.

Results

Calycosin improves cognitive deficits in AD mice

We performed baseline MWM testing at nine months of age, prior to the administration of calycosin. The results revealed that APP/PS1 mice exhibited significant cognitive deficits compared to WT mice, including prolonged escape latency, reduced time spent in the target quadrant, and fewer platform crossings (Fig. S1A–C in Supplementary materials), validating the suitability of this model for evaluating the therapeutic effects of calycosin. Following three months of calycosin treatment, cognitive function was assessed using the MWM test. Compared to WT mice, APP/PS1 mice exhibited significantly prolonged escape latency, reduced time spent in the target quadrant, and fewer platform crossings. However, treatment with calycosin (40 mg/kg) markedly improved these deficits, as evidenced by decreased escape latency, increased time spent in the target quadrant, and a higher number of platform crossings compared to untreated APP/PS1 mice (Fig. 1A–C). Figure 1D showed that WT mice exhibited clear and short

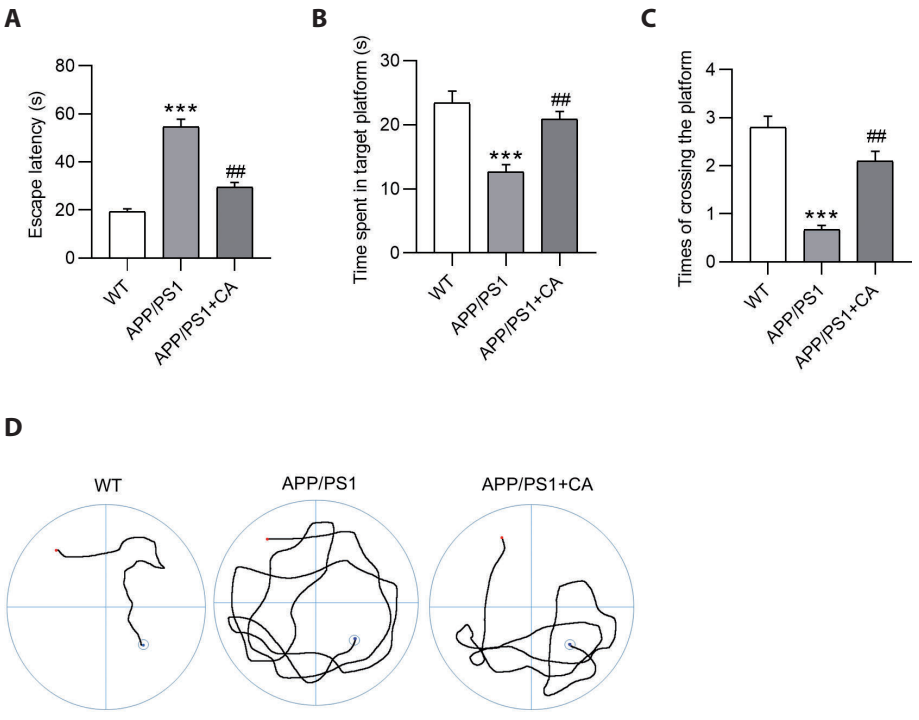


Figure 1. Calycosin improves cognitive deficits in AD mice. Following three months of calycosin treatment, cognitive function was assessed using the MWM test. Escape latency (**A**), time spent in the target quadrant (**B**), number of platform crossings (**C**), and representative swimming trajectory (**D**) of mice. $n = 8$ mice *per* group; *** $p < 0.001$, ## $p < 0.01$.

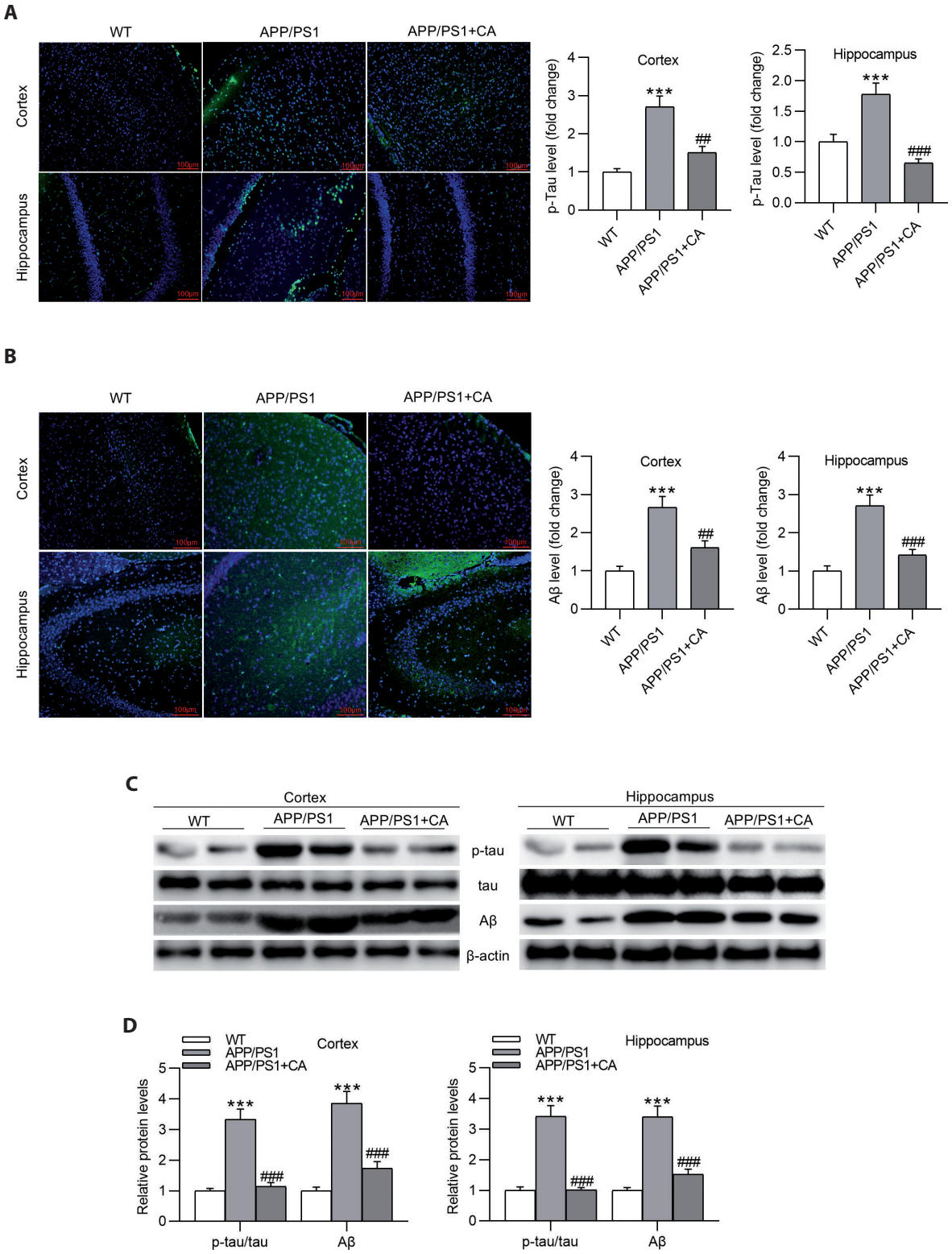


Figure 2. Calycosin inhibits Tau hyperphosphorylation and Aβ aggregation in AD mice. Representative immunofluorescence images and quantitative analysis of p-Tau (**A**) and Aβ (**B**) in the cortex and hippocampus of each group. **C, D.** Western blotting analysis of p-Tau and Aβ protein expression in the cortex and hippocampus of each group. n = 8 mice *per* group; *** $p < 0.001$, ## $p < 0.01$, ### $p < 0.001$.

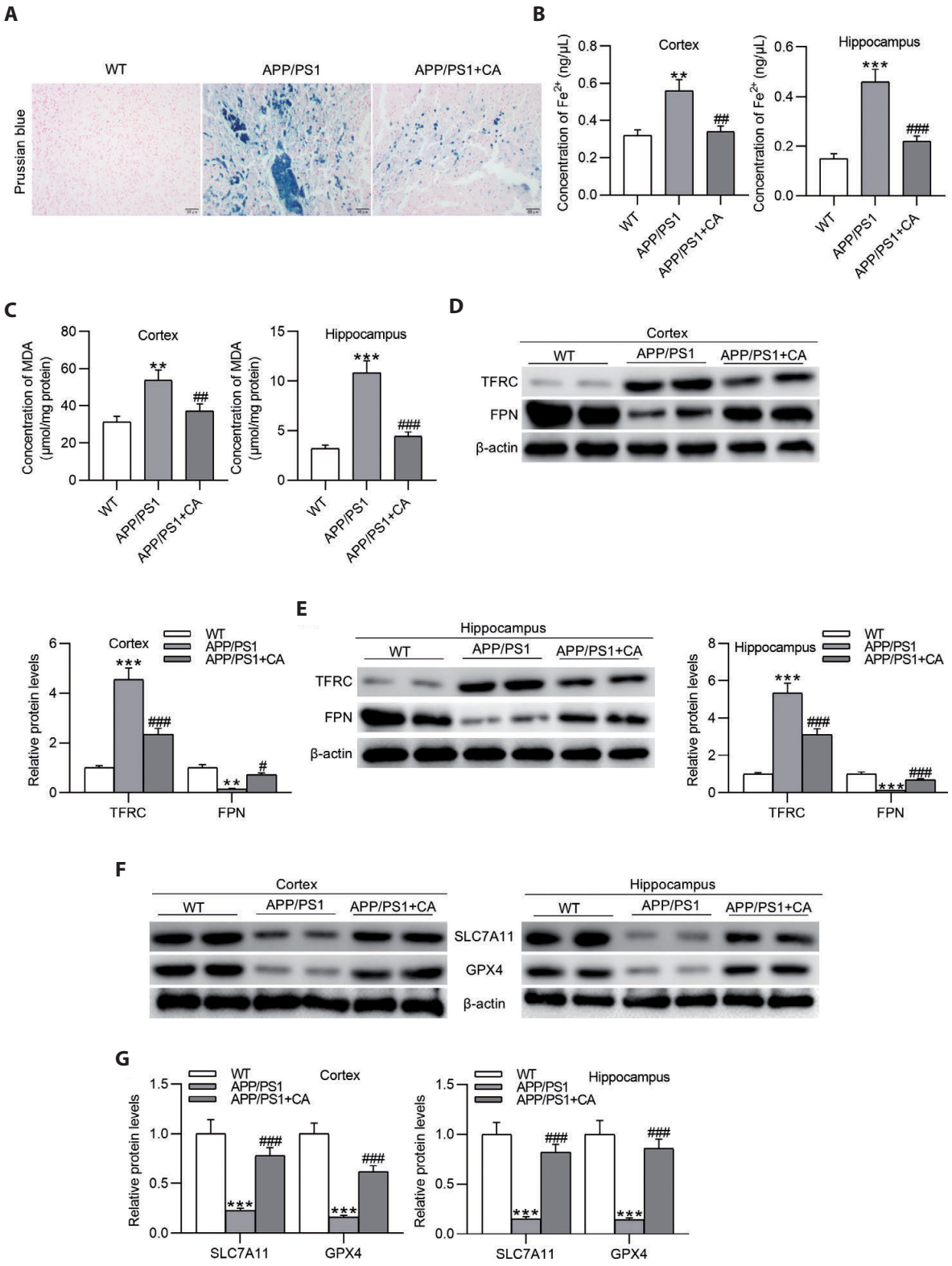


Figure 3. Calycosin inhibits neuronal ferroptosis in AD mice. **A.** Prussian blue staining for iron deposition in the cortex and hippocampus of each group. Ferrous iron levels (**B**) and MDA levels (**C**) in the cortex and hippocampus measured using specific assay kits. **D.–G.** Western blotting analysis of TFRC, FPN, SLC7A11, and GPX4 protein expression in the cortex and hippocampus of each group. $n = 8$ mice *per* group; ** $p < 0.01$, *** $p < 0.001$, $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$.

paths, while APP/PS1 mice displayed longer, disorganized paths and failed to locate the platform efficiently. Treatment with calycosin significantly shortened the swimming paths of AD mice, indicating improved spatial memory and learning.

Calycosin inhibits Tau hyperphosphorylation and Aβ aggregation and in AD mice

Neurofibrillary tangles, induced by Tau hyperphosphorylation, and Aβ deposition are the hallmark pathological features of AD. Immunofluorescence analysis of p-Tau and Aβ in the mouse brain revealed that the p-Tau-positive area in the cortex and hippocampus of APP/PS1 mice was significantly increased compared to WT mice. However, calycosin treatment (40 mg/kg) markedly reduced the levels of p-Tau and Aβ in the brain compared to untreated APP/PS1 mice (Fig. 2A,B). Consistent with these findings, Western blotting analysis demonstrated that calycosin treatment significantly decreased the protein levels of p-Tau and Aβ in the brain (Fig. 2C,D).

Calycosin inhibits neuronal ferroptosis in AD mice

Ferroptosis, characterized by diminished cellular antioxidant capacity and lipid ROS accumulation, plays a critical role in AD pathogenesis. Prussian blue staining revealed elevated iron deposition in the brain of APP/PS1 mice compared to WT mice, which was notably reduced by calycosin treatment (Fig. 3A). Furthermore, calycosin treatment effectively decreased ferrous iron levels in the cortex and hippocampus (Fig. 3B). MDA, a marker of lipid peroxidation, was significantly elevated in APP/PS1 mice but was reduced following calycosin treatment (Fig. 3C). Western blotting analysis exhibited increased TFRC expression and reduced FPN expression in APP/PS1 mice compared to WT mice. However, calycosin treatment significantly reversed these changes, suggesting that calycosin attenuates ferroptosis by inhibiting iron uptake and promoting iron export (Fig. 3D,E). Additionally, calycosin upregulated the expression of ferroptosis-resistant genes, SLC7A11 and GPX4, in the brain of APP/PS1 mice (Fig. 3F,G).

Calycosin attenuates tau hyperphosphorylation and ferroptosis ind uced by $A\beta_{1\text{-}42}$ in HT22 cells

The hippocampal neuronal cell line HT22 was used for the *in vitro* experiments. Calycosin at concentrations of 0–20 μM was not cytotoxic to HT22 cells, as shown by CCK-8 assay (Fig. 4A). $A\beta_{1\text{-}42}$ (20 μM) significantly reduced HT22 cell viability, but calycosin treatment restored viability at concentrations of 1, 5, 10, and 20 μM (Fig. 4B). Flow cytometry analysis showed that 20 μM $A\beta_{1\text{-}42}$ induced apoptosis in HT22 cells, which was significantly mitigated by calycosin treatment (Fig. 4C,D). According to the Western blotting results, 20 μM $A\beta_{1\text{-}42}$ exposure upregulated p-Tau levels in HT22 cells, while calycosin treatment downregulated p-Tau expression (Fig. 4E). Furthermore, calycosin inhibited $A\beta_{1-42}$-induced ROS production, as evidenced by reduced fluorescence intensity in HT22 cells stained with the fluorescent probe DHE (Fig. 4F). Calycosin also decreased MDA levels and ferrous iron content in $A\beta_{1-42}$-exposed HT22 cells (Fig. 4G). Consistent with *in vivo* findings, calycosin downregulated TFRC expression and upregulated FPN, SLC7A11, and GPX4 expression in HT22 cells treated with $A\beta_{1\text{-}42}$ (Fig. 4H).

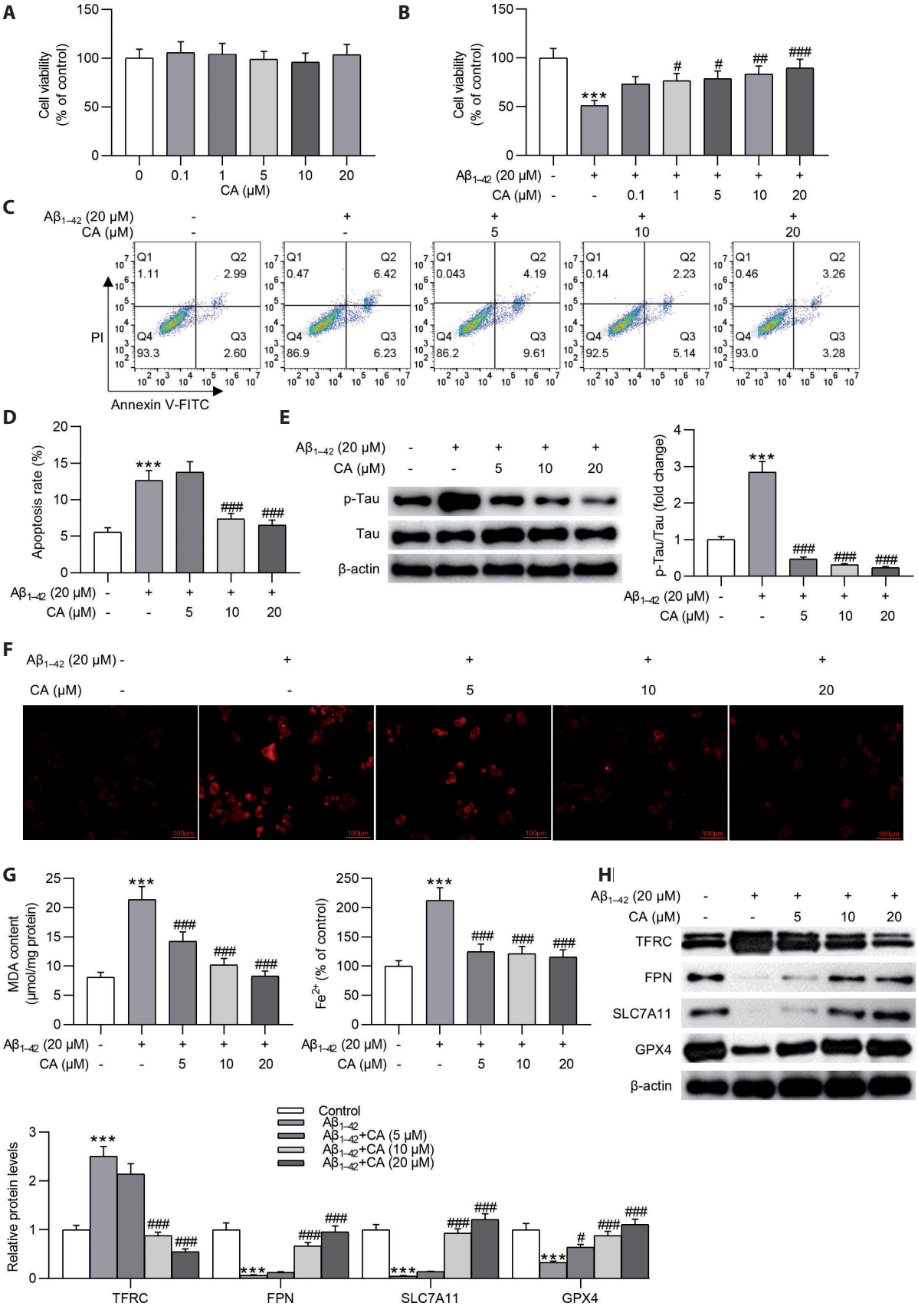
Calycosin inhibits ferroptosis via the Nrf2/HO-1 pathway

To investigate the mechanism underlying the anti-ferroptosis effects of calycosin on ferroptosis, we examined the Nrf2/HO-1 pathway. Calycosin treatment significantly upregulated nuclear Nrf2 and HO-1 expression in the cortex and hippocampus of APP/PS1 mice compared to untreated mice (Fig. 5A,B). Similarly, $A\beta_{1\text{-}42}$ exposure significantly downregulated Nrf2 and HO-1 expression in HT22 cells, while calycosin, treatment restored their levels (Fig. 5C). To confirm the role of Nrf2, we knocked down Nrf2 using siRNA in HT22 cells. Nrf2 knockdown abolished the upregulation of HO-1 and counteracted the effects of calycosin on p-Tau, TFRC, FPN, SLC7A11, and GPX4 expression in $A\beta_{1-42}$-exposed HT22 cells (Fig. 5D,E). These results suggest that calycosin exerts its anti-ferroptotic effects by activating the Nrf2/HO-1 signaling pathway.

Discussion

In recent years, the global incidence of AD has risen significantly, making it a major public health concern that severely impacts the quality of life of the elderly population (Scheltens et al. 2021). Unfortunately, due to the complex pathophysiology of AD, current therapeutic strategies have failed to effectively halt or reverse disease progression effectively (Pardo-Moreno et al. 2022). Calycosin, a natural isoflavone compound, has various biological properties, including immunomodulatory, antiviral, antioxidant, and anti-inflammatory effects (Nie et al. 2016). In the present study, we demonstrated that calycosin alleviates cognitive deficits in APP/PS1 mice and mitigates AD-related pathological alterations *in vivo* and *in vitro*. Mechanistically, we showed that the anti-AD effects of calycosin are mediated through the suppression of neuronal ferroptosis *via* activation of the Nrf2/HO-1 signaling.

Ferroptosis is a newly identified form of programmed cell death, characterized by intracellular iron overload,


A
Cell viability (% of control)
CA (μM)
B
Cell viability (% of control)
Aβ1–42 (20 μM)
CA (μM)
C
Aβ1–42 (20 μM)
CA (μM)
PI
Annexin V-FITC
Q1 1.11
Q2 2.99
Q4 93.3
Q3 2.60
Q1 0.47
Q2 6.42
Q4 86.9
Q3 6.23
Q1 0.043
Q2 4.19
Q4 86.2
Q3 9.61
Q1 0.14
Q2 2.23
Q4 92.5
Q3 5.14
Q1 0.46
Q2 3.26
Q4 93.0
Q3 3.28
D
Apoptosis rate (%)
Aβ1–42 (20 μM)
CA (μM)
E
Aβ1–42 (20 μM)
CA (μM)
p-Tau
Tau
β-actin
p-Tau/Tau (fold change)
F
Aβ1–42 (20 μM)
CA (μM)
100μm
G
MDA content (μmol/mg protein)
Fe2+ (% of control)
H
Aβ1–42 (20 μM)
CA (μM)
TFRC
FPN
SLC7A11
GPX4
β-actin
Relative protein levels
Control
Aβ1–42
Aβ1–42+CA (5 μM)
Aβ1–42+CA (10 μM)
Aβ1–42+CA (20 μM)
TFRC
FPN
SLC7A11
GPX4

lipid peroxidation, and reduced expression of GPX4 (Lei et al. 2019). First, abnormal iron accumulation is closely linked to dysregulated iron metabolism, including impaired iron export due to reduced FPN expression and increased iron uptake mediated by elevated TFRC levels (Dixon et al. 2012). Second, lipid peroxidation leads to excessive production of ROS and MDA (Lei et al. 2019). Third, GPX4, a key regulator of ferroptosis, acts as a critical scavenger of lipid peroxides. Evidence suggests that decreased GPX4 expression in neurons contributes to neurodegeneration both *in vivo* and *in vitro* (Seiler et al. 2008). Recent studies have highlighted the pivotal role of ferroptosis in AD pathogenesis (Masaldan et al. 2019; Weiland et al. 2019). For instance, magnetic resonance imaging studies have revealed preferential iron accumulation in the hippocampal and cortical regions of AD patients (Bartzokis et al. 1994; Bilgic et al. 2012), and iron overload has been shown to promote Tau hyperphosphorylation (Tao et al. 2014). Additionally, iron dysregulation enhances the amyloidogenic processing of APP, leading to Aβ deposition (Caldwell et al. 2013; Ward et al. 2014). APP also plays a role in iron homeostasis by stabilizing FPN on the cell membrane, and its depletion results in iron accumulation in both mouse models and cultured neurons (McCarthy et al. 2014; Wong et al. 2014). Furthermore, GPX4 activity is essential for the maintenance of neuronal survival (Morris et al. 2018), and conditional knockout GPX4 in forebrain neurons induces hippocampal neurodegeneration and cognitive deficits in mice (Hambright et al. 2017). These findings collectively suggest ferroptosis represents a promising therapeutic target for AD. In this investigation, we observed hallmark features of ferroptosis in the cortex and hippocampus of APP/PS1 mice and $A\beta_{1-42}$ oligomers-exposed hippocampal neuronal cells, which were significantly alleviated by calycosin treatment. Specifically, calycosin reduced intracellular iron levels by upregulating FNP, downregulating TFRC, decreasing MDA and ROS levels, and enhancing the expression of GPX4 and SLC7A11. These results align with previous studies demonstrating that calycosin inhibits ferroptosis by modulating MDA, ROS, TFR1, FTH1, and GPX4 (Huang et al. 2022; Liu et al. 2023), further supporting the involvement of ferroptosis in the anti-AD effects of calycosin.

The Nrf2 signaling pathway plays a crucial role in regulating ferroptosis (Abdalkader et al. 2018; Dodson et al. 2019). Under oxidative stress, Nrf2 is released from its inhibitor Keap1 upon phosphorylation at Ser40 and translocates to the nucleus, where it modulates ferroptosis through multiple mechanisms (Zeng et al. 2021). Nrf2 maintains cellular iron homeostasis by regulating the expression of iron metabolism-related proteins, including ferritin, TFRC, and FPN (Dodson et al. 2019; Liu et al. 2020). Additionally, Nrf2 enhances the activity and expression of antioxidant enzymes such as HO-1 and superoxide dismutase (SOD) (Xiang et al. 2022), and it upregulates GPX4 by preserving intracellular glutathione levels (Harvey et al. 2009). Recent studies have highlighted the role of Nrf2 activation in suppressing ferroptosis. For example, Yang et al. showed that salidroside alleviates cognitive impairment by activating the Nrf2/GPX4 axis (Yang et al. 2023), while another study by Yang et al. demonstrated that a ketogenic diet prevents AD-like pathology induced by sleep deprivation by enhancing neuronal repair and inhibiting ferroptosis *via* Nrf2 signaling (Yang et al. 2022). Furthermore, several studies have confirmed that calycosin exerts antioxidative effects through Nrf2 activation (Chen et al. 2020; Liu et al. 2022; Lu et al. 2022). In our study calycosin significantly upregulated the expression of nuclear Nrf2 and HO-1 both *in vivo* and *in vitro*. Importantly, Nrf2 knockdown in hippocampal neuronal cells abolished the protective and anti-ferroptotic effects of calycosin, underscoring the critical role of Nrf2 activation in mediating these effects.

This study has several limitations. While MWM test is a widely used method to assess learning and memory in AD models, it can induce stress in animals. Future studies should incorporate milder behavioral test methods, such as Barnes maze, to evaluate cognitive function. Additionally, our histological analysis focused on the hippocampal and cortical regions without specifying subregions (e.g., CA1, CA2, or CA3). Further research should examine other brain regions to provide a more comprehensive understanding of calycosin's effects. Moreover, it would be valuable to investigate ferroptosis-related proteins in the brains of mice at different disease stages to determine whether calycosin's anti-ferroptotic effects vary across the progression of AD.

◀ **Figure 4.** Calycosin attenuates tau hyperphosphorylation and ferroptosis induced by $A\beta_{1-42}$ in HT22 cells. **A,B.** Cell viability of HT22 cells treated with 0.1, 1, 5, 10, calycosin alone or in combination with 20 μM $A\beta_{1-42}$, as assessed by the CCK-8 assay. **C,D.** Flow cytometry analysis of apoptosis in HT22 cells treated with 5, 10, and 20 μM calycosin plus 20 μM $A\beta_{1-42}$. **E.** Western blotting analysis of p-Tau protein expression in HT22 cells treated with 5, 10, and 20 μM calycosin plus 20 μM $A\beta_{1-42}$. **F.** ROS levels in HT22 cells treated with 5, 10, and 20 μM calycosin plus 20 μM $A\beta_{1-42}$, detected using the fluorescent probe-DHE. **G**. Ferrous iron levels and MDA levels in HT22 cells measured using specific assay kits. **H.** Western blotting analysis of TFRC, FPN, SLC7A11, and GPX4 protein expression in HT22 cells. Each assay was performed in triplicate. $n = 3$; *** $p < 0.001$, $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$.

In conclusion, calycosin improves learning and memory, reduces the deposition of AD pathological markers, and alleviates neuronal damage in AD *in vivo* and *in vitro*. These therapeutic effects are likely mediated through the suppression of neuronal ferroptosis and oxidative stress *via* Nrf2 signaling (Fig. 6). Our findings highlight the potential of calycosin as a promising therapeutic agent for AD and provide a foundation for further exploration of its mechanisms and clinical applications.

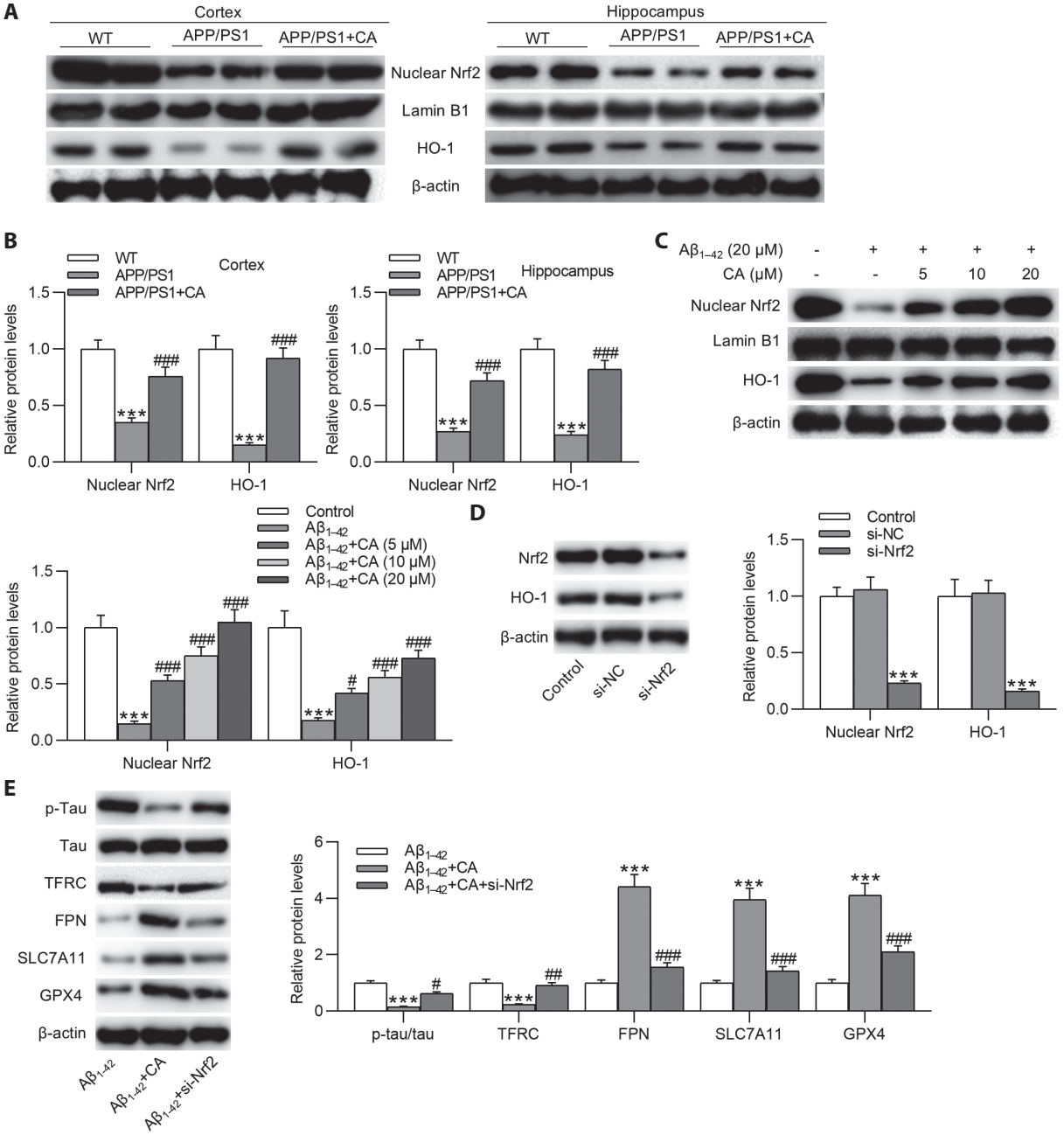


Figure 5. Calycosin inhibits ferroptosis *via* the Nrf2/HO-1 pathway. **A,B.** Western blotting analysis of Nrf2 and HO-1 protein expression in the cortex and hippocampus of each group. $n = 8$ mice *per* group. **C.** Nrf2 and HO-1 protein expression in HT22 cells treated with 5, 10, and 20 μM calycosin plus 20 μM $A\beta_{1\text{-}42}$. **D.** Nrf2 and HO-1 protein expression in HT22 cells transfected with si-NC or si-Nrf2. **E.** Nrf2 and HO-1 protein expression in $A\beta_{1\text{-}42}$-exposed HT22 cells treated with calycosin or si-Nrf2. Each assay was performed in triplicate. $n = 3$; *** $p < 0.001$, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$.

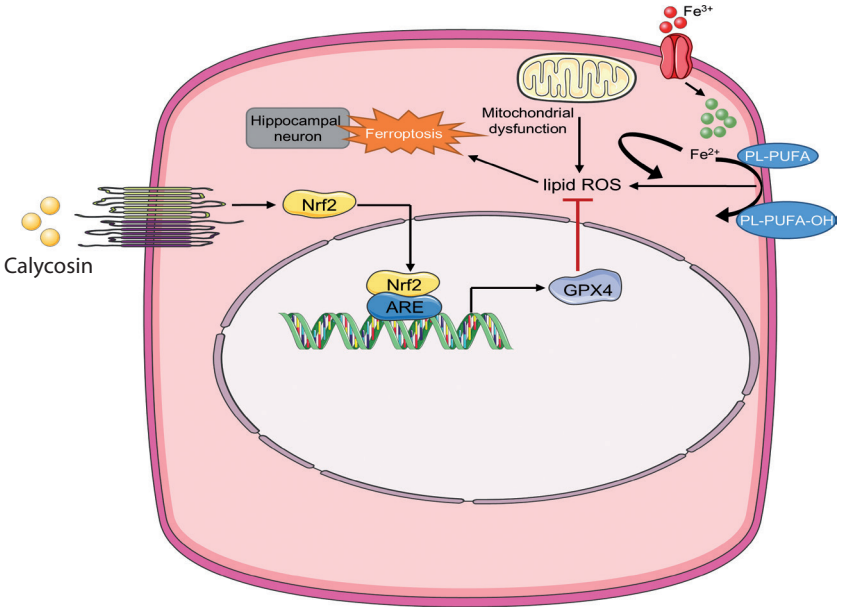


Figure 6. Proposed mechanism of calycosin in inhibiting AD progression. Calycosin alleviates cognitive deficits by inhibiting neuronal ferroptosis, and its effects are mediated through the activation of the Nrf2/HO-1 signaling pathway.

Conflict of interests. The authors declare that they have no competing interests.

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Disclosure of ethical statements. Approval of the research protocol: All animal experiments followed guidelines and were approved by the Animal Welfare Committee of Hubei Third People's Hospital of Jianghan University.

Authors' contributions. QL conceived and designed the experiments. QL, BH and YX carried out the experiments. QL, BH and YX analyzed the data. QL and YX drafted the manuscript. All authors agreed to be accountable for all aspects of the work. All authors have read and approved the final manuscript.

Data availability statement. All data generated or analyzed during the current study are available from the corresponding author on reasonable request.

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Supplementary Material

Calycosin attenuates neuronal ferroptosis in Alzheimer's disease mice by activating the Nrf2/HO-1 pathway

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Supplementary Figure

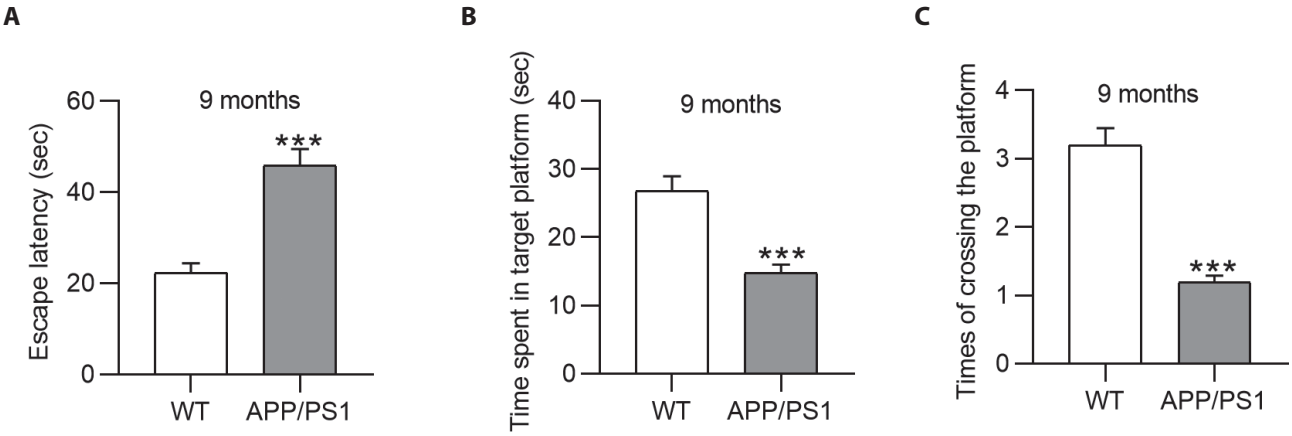

Figure S1. Cognitive function assessment in nine-month-old APP/PS1 and WT mice. Cognitive performance was evaluated in nine-month-old APP/PS1 and WT mice using the MWM test. **A.** Escape latency. **B.** Time spent in the target quadrant. **C.** Number of crossing platforms of mice. $n = 8$ mice *per* group; *** $p < 0.001$.