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ORIGINAL RESEARCH

Asiaticoside Attenuates Chronic Restraint Stress-Induced Hippocampal CA1 Neuronal Ferroptosis via Activating BDNF/Nrf2/GPX4 Signaling Pathway

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Purpose: Ferroptosis, characterized by iron-dependent lipid reactive oxygen species accumulation, plays a critical role in the pathophysiology of depression. Our research aims to elucidate the potential antidepressant mechanisms of asiaticoside, a bioactive compound known for its neuroprotective and immunomodulatory properties.

Methods: The antidepressant-like properties of asiaticoside in a model of chronic restraint stress (CRS)-induced depression in mice, with a particular focus on its interaction with ferroptosis-related pathways.

Results: The behavioral results revealed that asiaticoside significantly ameliorated CRS-induced depressive symptoms, as evidenced by increased sucrose preference and reduced immobility time. At the molecular level, asiaticoside enhanced the expression of brain-derived neurotrophic factor (BDNF), phosphorylated tropomyosin receptor kinase B (pTrkB), phosphorylated nuclear factor erythroid 2-related factor 2 (pNrf2), glutathione peroxidase 4 (GPX4), and solute carrier family 7 member 11 (SLC7A11), indicating its neuroprotective and antioxidative effects. In addition, asiaticoside suppressed the expression of ferroptosis markers, including ferritin light chain (FLC) and transferrin receptor only in CA1 region. Transmission electron microscopy (TEM) further confirmed that asiaticoside preserved mitochondrial integrity in CA1 neuronal cells.

Conclusion: In conclusion, our findings suggest that asiaticoside exerts its antidepressant-like effects through the modulation of BDNF/Nrf2/GPX4 signaling pathway against neuronal ferroptosis in the hippocampal CA1 region.

Keywords: asiaticoside, antidepressant, ferroptosis, BDNF, Nrf2, GPX4

Introduction

Depression, a stress-related chronic mental disorder, is marked by an array of symptoms including anhedonia, physical fatigue, sleep disturbances, cognitive deficits, appetite changes, and social life escape. This condition contributes to a substantial burden of morbidity, heightened suicide rates, frequent illness recurrence, and considerable functional impairment.[1] In 2018, statistics from the National Health and Nutrition Examination Survey in the United States indicate that around 8.1% of adults endure depression over any given two-week period. Depression impacts approximately 16.2%

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of the adult population throughout their lifetime, leading to substantial economic losses and posing a significant health burden on patients.[2]

Ferroptosis is a recently discovered form of programmed cell death, marked by iron metabolism and lipid peroxidation. The excessive accumulation of lipid peroxides is the main cause of ferroptosis, which depends on the metabolites reactive oxygen species (ROS), phospholipids containing polyunsaturated fatty acid chains, and transition metal iron.[3] Intracellular and intercellular signaling and environmental pressure can affect iron death by regulating cell metabolism and ROS levels.[4] Distinct from known forms of cell death, ferroptosis is caused by excessive accumulation of ferric iron within cells, leading to impaired cell function and ultimately resulting in cell death. The lone distinctive morphological feature of erastin-treated cells involved mitochondria that appeared smaller than normal with increased membrane density.[5] Patients with more severe depressive symptoms have lower serum ferritin levels, while higher serum ferritin levels have a positive effect on mood and are positively correlated with lower prevalence of depression.[6,7] In preclinical research, ferroptosis was also found in the hippocampus of depressive-like mouse, suggesting a potential link between ferroptosis-related pathways and the onset of depression.[8] For example, a previous study indicated that mice exposed to chronic unpredictable mild stress (CUMS) exhibited iron dyshomeostasis and ferroptosis, the activation of ferroptosis might be involved and could represent novel therapeutic strategies for depression.[9] Moreover, the critical involvement of GPX4-mediated ferroptosis in the antidepressant and anxiolytic effects related to the endoplasmic reticulum-associated degradation pathway has been underscored through the use of GPX4 knockdown viruses.[10]

Asiaticoside is one of the triterpene compounds obtained from aqueous or ethanolic extracts of *Centella asiatica*. It could alleviate neuronal damage, and showed anxiolytic effects.[11] Asiaticoside is involved in the treatment of hypoxic–ischaemic injuries[12] in the brain and is incorporated into the treatment of various neurodegenerative disorders like Alzheimer's disease[13] and Parkinson's disease.[14] Moreover, the findings indicated that asiaticoside treatment exerted the antidepressant-like effects in the CUMS mice by regulating the cAMP/PKA, and BDNF signaling pathway.[15] Considering that neurotrophic factor BDNF can regulate the key protein Nrf2 of ferroptosis,[16] we hypothesized that asiaticoside may play its antidepressant effect by affecting the activation of Nrf2 and the targeted antioxidant response element to alleviate ferroptosis. Therefore, BDNF/Nrf2/GPX4 mediated ferroptosis signaling pathway was investigated in hippocampus in mice exposed chronic stress.

Materials and Methods

Animals

Male ICR mice (24–26 g, eight weeks old, SPF grade) were purchased from Shanghai Slack (Shanghai, China) and subsequently housed at the Experimental Animal Center. Conditions were regulated to maintain a room temperature of 25 ±2°C and relative humidity between 50±10%, with lighting alternating between day and night cycles. The mice had continuous access to water and food and underwent an acclimatization period of one week prior to the study. All experimental protocols were approved by the Huaqiao University Animal Care and Use Committee (Approval No. A2022002) and adhered to the China Council on Animal Care's established guidelines.

Chemicals and Reagents

Asiaticoside (purity > 99%, A800923) from *Centella asiatica*, sucrose (purity > 99.5%, S818044) was purchased from Maclin (Shanghai, China). Protein Marker (1610374) was purchased from Bio-Rad (Hercules, USA). Bis-Tris high-performance precast mini polyacrylamide gels (M00657) were purchased from GenScript (Nanjing, China). Stripping buffer (46430) was purchased from Thermo Fisher (Waltham, USA). The detailed antibodies information was provided in Tables 1 and 2.

Chronic Restraint Stress (CRS)

For CRS, 50 mL centrifuge tubes were utilized, with a ventilation hole created at the bottom and four additional breathing holes drilled along the sides. During the four-week experimental period, each mouse was individually confined within a centrifuge tube for 6 hours daily.

Table 1 Primary Antibodies Used in Western Blot

Antibody	Host	Company	Lot Number	Dilution
BDNF	Rabbit	CST	47808	1:1000
TrkB	Rabbit	CST	4603	1:1000
pTrkB	Rabbit	Millipore	ABN1381	1:1000
Synaptophysin	Rabbit	CST	36404	1:1000
PSD95	Rabbit	CST	3409	1:1000
Nrf2	Rabbit	ELK	ES2985	1:1000
pNrf2	Rabbit	Thermo Fisher	PA567520	1:1000
GPX4	Rabbit	Abcam	ab125066	1:1000
SLC7A11	Rabbit	Thermo Fisher	PA567520	1:1000
HO-1	Rabbit	Proteintech	10701-1-AP	1:1000
FLC	Rabbit	Proteintech	10727-1-AP	1:1000
Transferrin Receptor	Rabbit	Abcam	ab269513	1:1000

Table 2 Primary Antibodies Used in Immunofluorescence

Antibody	Host	Company	Lot Number	Dilution
Iba1	Goat	Abcam	ab289874	1:100
DCX	Rabbit	CST	4604	1:600
GPX4	Rabbit	Abcam	ab125066	1:100
NeuN	Rat	Abcam	ab279297	1:200
SLC7A11	Rabbit	Thermo Fisher	PA567520	1:100
FLC	Rabbit	Proteintech	10727-1-AP	1:200
Transferrin Receptor	Rabbit	Abcam	ab269513	1:50

Abbreviations: BDNF, brain-derived neurotrophic factor; CRS, chronic restraint stress; CUMS, chronic unpredictable mild stress; FLC, ferritin light chain; HPA, hypothalamic-pituitary-adrenal; Nrf2, nuclear factor erythroid 2-related factor 2; ROS, reactive oxygen species; SLC7A11, solute carrier family 7 member 11; TEM, transmission electron microscopy; TrkB, tropomyosin receptor kinase B.

Drug Treatment

In this experiment, mice were randomly divided into 4 groups of 11 mice including Normal-Vehicle group, Normal-Asiaticoside group, CRS-Vehicle group; CRS-Asiaticoside, respectively. Given that asiaticoside possess a high capability to cross the blood brain barrier,[17] asiaticoside was orally administrated at 20 mg/kg in the volume of 10 mL/kg once a day from Day 1 to Day 28 during CRS procedure. The dose of asiaticoside was selected based on our previous study.[15] The protocol of the present study was shown in Figure 1A.

Sucrose Preference Test

The sucrose adaptation procedure was performed three days before the start of the experiment. For the first 24 hours, mice had access to two bottles of 1% sucrose solution. This was followed by a 24-hour period during which the mice were provided with one bottle of 1% sucrose solution and one bottle of water, with the position of these bottles being switched every 12 hours to avoid location preference. In the main phase of the experiment, each cage contained one bottle of 1% sucrose solution and one of water, with their positions alternated every 12 hours. The intake of sucrose solution and water was monitored for 24 hours to calculate the sucrose preference index.

Forced Swimming Test

Mice were placed in a clear beaker measuring 32 cm in height and 10 cm in diameter, filled to a water depth of 15 cm, with a maintained temperature of 25°C. They were subjected to a 6-minute forced swim test in this container, with observations of immobility time conducted during the final 4 minutes. Immobility was characterized by the mice

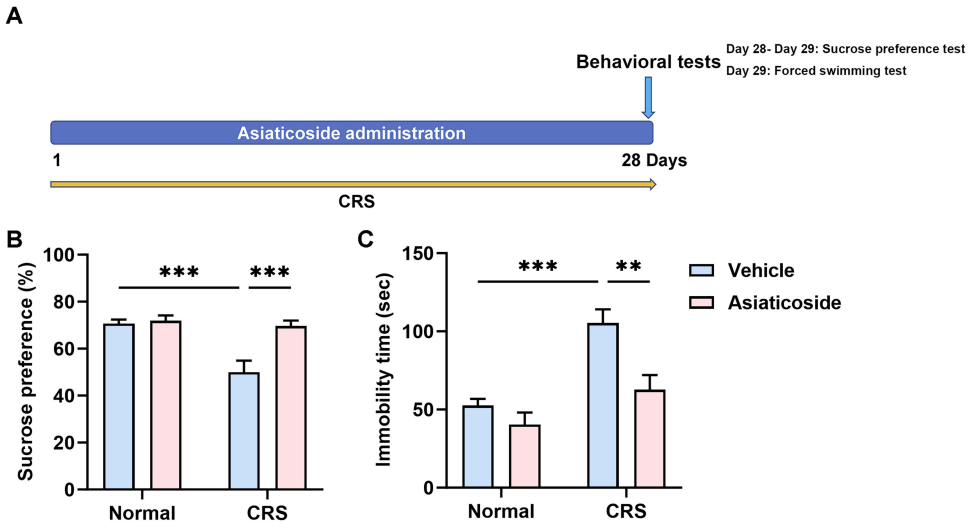

Figure 1 Administration with asiaticoside alleviated CRS-induced depression-like behaviors in mice (n=11). (**A**) Schematic illustration of the procedure for the experiment. (**B**) Sucrose preference, an anhedonia symptom was elevated by asiaticoside. (**C**) Immobility time, as despair symptom was decreased by asiaticoside. ***P*<0.01, *** *P*<0.001.

passively floating, maintaining only their heads above water for respiration, keeping their forelimbs still, and subtly moving their hind limbs to remain afloat.

Western Blotting

Hippocampal proteins were isolated using RIPA buffer with the addition of protease and phosphatase inhibitors. The samples were homogenized by glass homogenizer, then centrifuged at 12000 g at 4°C to collect the supernatants. Protein concentrations in these supernatants were determined using a BCA assay, and the protein levels were normalized across samples. Subsequently, samples were mixed with loading buffer, heated at 95°C for 5 minutes, and subjected to SDS-PAGE electrophoresis and membrane transfer. Following by blocked with 5%BSA for 1 h, membranes were incubated with primary antibodies overnight at 4°C (Table 1). Following incubation with goat anti-rabbit or anti-mouse secondary antibodies (1:2500), the membrane was visualized using a gel imaging system. Stripping buffer was used for getting different target bands in the same membrane. Band grey intensity was quantified using Image J software.

Immunofluorescence

After perfusion with PBS and 4% paraformaldehyde, whole brains were excised from the mice, fixed in 4% paraformaldehyde, embedded, and sectioned at 15 μm thickness. Antigen retrieval was performed using citric acid (pH 6.0) and phosphate-buffered saline (pH 7.4). The sections were washed, outlined with a histochemical pen, and blocked with 3% BSA for 30 minutes. Brain sections were incubated with FTL, Trf, and Iba1 antibodies (Table 2). After washing with TBST, sections were incubated with fluorescent secondary antibody (1:400) in a dark enclosure at room temperature for 3 hours. DAPI staining was performed for 5 minutes in the dark, followed by treatment with autofluorescence quencher for 5 minutes and subsequent washing. Mounted sections were examined and imaged using a Leica TCS SP8 confocal microscope. The measurement of positive signal was conducted by using Spot or Surface mode of Imaris software.

Transmission Electron Microscope (TEM)

A 1 mm^3 tissue sample was collected from the hippocampal CA1 region and immediately fixed in 2.5% glutaraldehyde, then stored at 4°C overnight. The samples were rinsed three times with PBS buffer, each rinse lasting 15 minutes. Subsequently, the samples were fixed with 1% osmium tetroxide (OsO_4) solution for 2 hours. Following fixation, the OsO_4 was removed, and the samples were rinsed three times in 0.1 M phosphate buffer (PB), each rinse lasting 15 minutes. Dehydration of the tissues was performed at room temperature in a graded series of acetone solutions as follows: 30% acetone for 10 minutes, 50% acetone for 10 minutes, 70% acetone for 10 minutes, 80% acetone for

10 minutes, 95% acetone for 10 minutes, and two changes of 100% acetone for 20 minutes each. The samples were then embedded in pure EMBed 812 resin. The embedding molds, containing the resin and tissues, were placed in a 60°C oven to polymerize for more than 48 hours. After polymerization, the resin blocks were sectioned into 70–90 nm thick slices using an ultramicrotome. The sections were transferred onto copper grids and stained with uranyl acetate for 8–-15 minutes, followed by lead citrate staining for 5–10 minutes. Transmission electron microscopy (TEM) was performed at an accelerating voltage of 80 kV to capture CCD images of the samples.

Statistical Analyses

The data are presented as means ± standard error. The normality of distribution was confirmed initially using the Kolmogorov–Smirnov test. Differences among groups were analyzed with two-way ANOVA, followed by Tukey's post hoc test for multiple comparisons. A p-value of less than 0.05 was considered statistically significant.

Results

Asiaticoside Attenuated Depressive-Like Behaviors in CRS Mice

The timeline of the present study was shown in Figure 1A. Behavioral tests were performed 28 days after the administration with asiaticoside. Sucrose preference test was firstly performed followed by forced swimming test. As shown in Figure 1B, CRS caused the reduction of sucrose preference in mice, while asiaticoside significantly increased sucrose preference in CRS mice. In addition, according to the results from forced swimming test (Figure 1C), asiaticoside significantly reversed the elevation of immobility time in CRS induced depression-like mice.

Asiaticoside Inhibited Microglial Activation in the Hippocampus

Following behavioral tests, we assessed the neuroinflammation in the CRS mice, as neuroinflammation is one of the most characteristic in depression. Our findings suggested a region-specific effect of asiaticoside on microglial activation in the hippocampus, as indicated by the number of Iba1 positive cells (Figure 2A). Iba1 is a marker for activated microglia, and its presence correlates with neuroinflammatory responses. In the DG of the hippocampus, CRS significantly increased the number of Iba1 positive cells, indicating heightened microglial activation. However, asiaticoside administration resulted in a marked decrease in the number of Iba1 positive cells in the DG, suggesting a strong anti-inflammatory effect in this region (Figure 2B). Similarly, in the CA1 region of the hippocampus, CRS elevated Iba1 positive cell counts, while asiaticoside treatment significantly reduced these numbers, further supporting its anti-inflammatory potential (Figure 2C). Contrastingly, in the CA3 region, although CRS also led to an increase in Iba1 positive cells, asiaticoside treatment did not significantly alter the number of these cells compared to the CRS group (Figure 2D). This indicates that the anti-inflammatory effects of asiaticoside are not uniform across all hippocampal regions and may be more pronounced in the DG and CA1 regions than in CA3. These observations highlight the selective modulation of microglial activity by asiaticoside, suggesting its potential to alleviate neuroinflammation in specific hippocampal areas affected by CRS.

Asiaticoside Activated Hippocampal BDNF/TrkB Signaling and Neurogenesis

It is well established that BDNF signaling is disrupted during depression. In this study, we examined the activation of the BDNF signaling pathway in CRS mice following asiaticoside administration. As shown in Figure 3A and B, CRS induced a downregulation of BDNF in the hippocampus, whereas asiaticoside administration significantly increased BDNF levels. We then assessed the levels of TrkB, the receptor for BDNF, in the hippocampus. The results indicated that while there was no significant alteration in pTrkB levels due to CRS, asiaticoside significantly increased pTrkB levels in the hippocampus (Figure 3C). Conversely, the total TrkB levels and the ratio of pTrkB/TrkB remained unchanged by both CRS and asiaticoside (Figure 3D and E). Additionally, we measured synaptic protein levels in this study. Asiaticoside significantly increased the levels of the postsynaptic protein PSD95 and partially increased the levels of the presynaptic protein synaptophysin, though the latter did not reach statistical significance (Figure 3F and G). Collectively, these findings indicate that asiaticoside can activate the BDNF/TrkB signaling pathway and enhance the expression of synaptic proteins. Furthermore, we quantified DCX positive cells in the DG region of the hippocampus, as DCX is a marker for

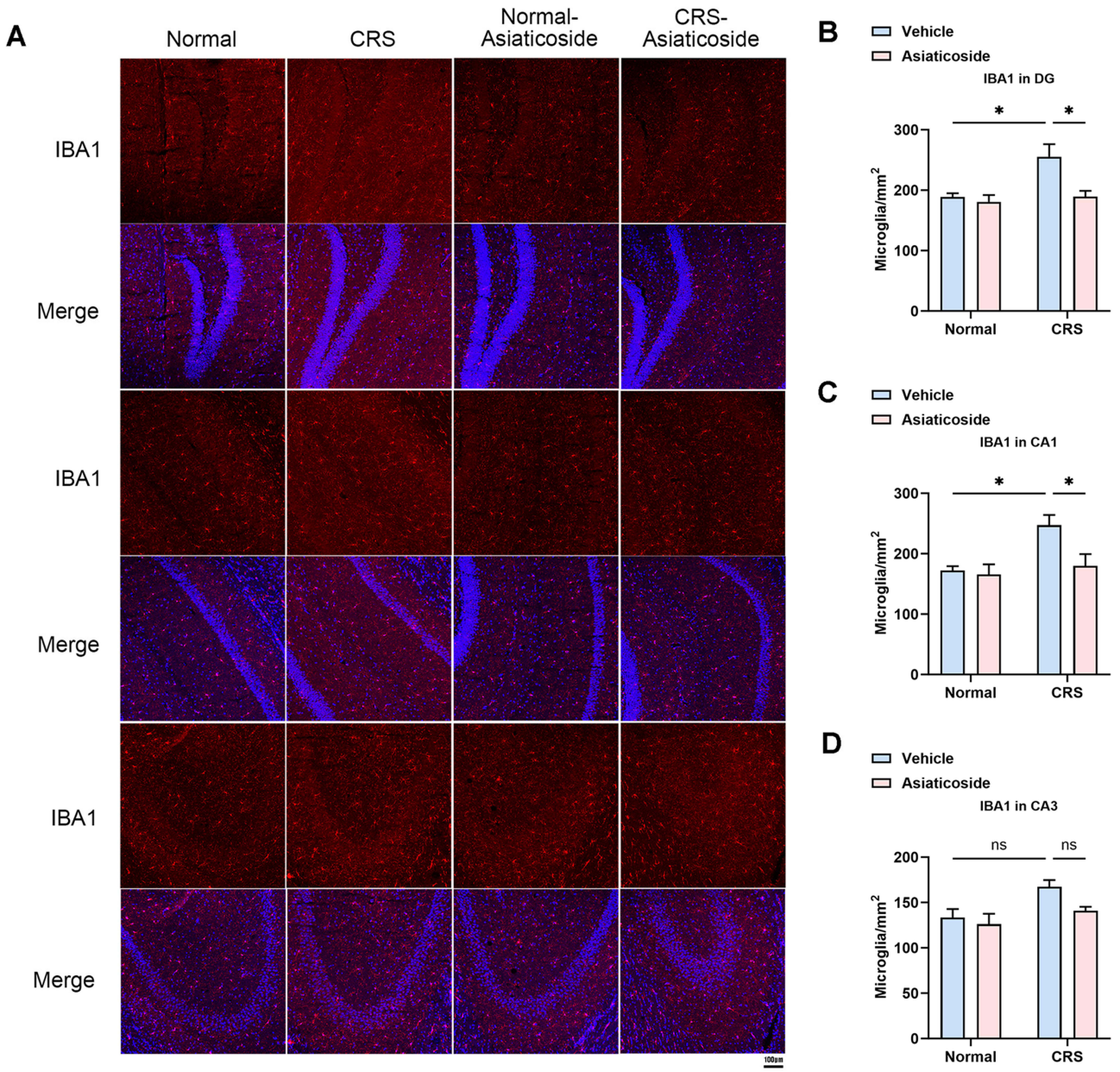

Figure 2 Administration with asiaticoside alleviated CRS-induced microglial activation in the hippocampus of mice (n=5). (**A**) Representative immunofluorescence images. (**B**) Statistical histogram of the number of microglia in the hippocampus DG region. (**C**) Statistical histogram of the number of microglia in the hippocampus CA1 region. (**D**) Statistical histogram of the number of microglia in the hippocampus CA3 region. **P*<0.05.

newborn neurons. As illustrated in Figure 4, CRS significantly reduced the number of DCX-positive cells compared to normal mice. However, asiaticoside administration significantly increased the number of DCX-positive cells, suggesting that asiaticoside enhances neurogenesis.

Asiaticoside Improved the Micromorphology of Hippocampal CA1 Neurons in CRS Mice

Based on the above results, we found that asiaticoside primarily regulates the signaling pathway related to ferroptosis in the hippocampal CA1 region. Therefore, we conducted an ultrastructural analysis using TEM to assess the effects of asiaticoside on synaptic conformational plasticity and mitochondrial morphology in hippocampal CA1 neurons of CRS mice (Figure 5). In the Normal-vehicle group, the hippocampal synaptic ultrastructure was well-preserved; the

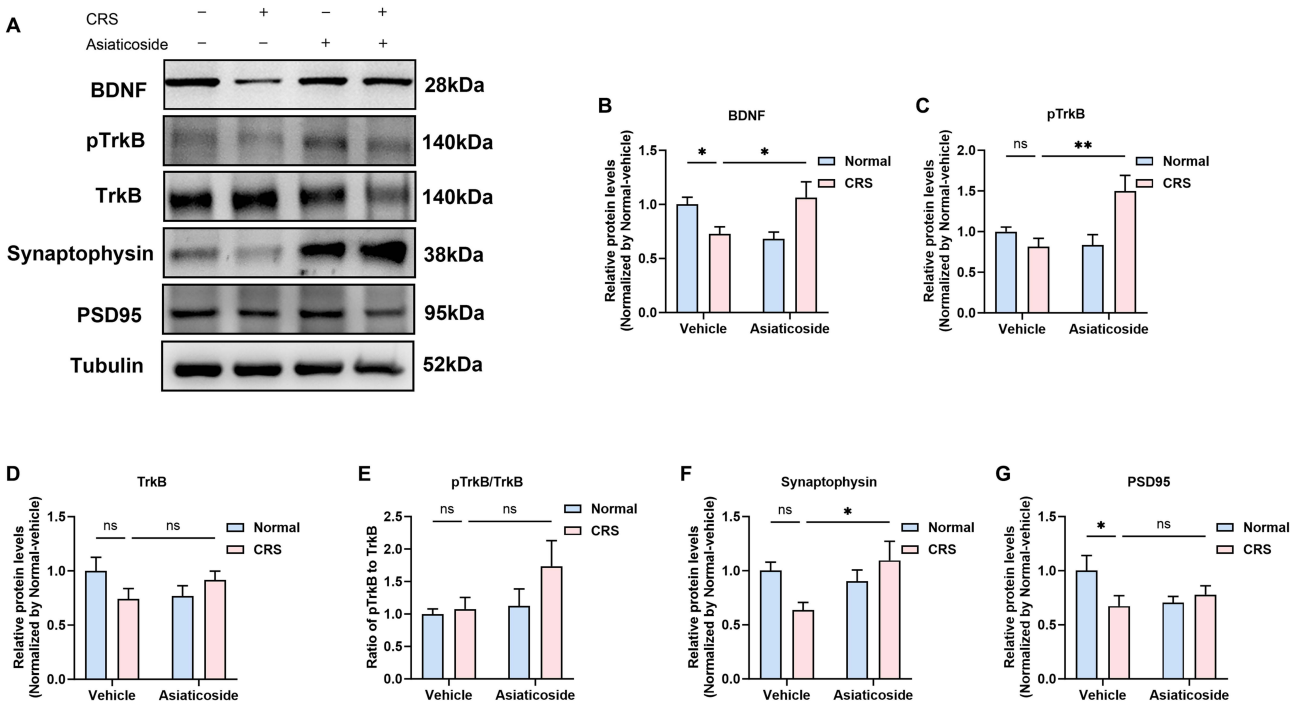

Figure 3 Effects of asiaticoside on BDNF/TrkB signaling pathway in mice induced by CRS (n=5-6). (**A**) Representative Western Blot. (**B**) Statistical histogram of BDNF. (**C**) Statistical histogram of pTrkB. (**D**) Statistical histogram of TrkB. (**E**) Statistical histogram of ratio of pTrkB to TrkB. (**F**) Statistical histogram of Synaptophysin. (**G**) Statistical histogram of PSD95. *P<0.05 and ** P<0.01.

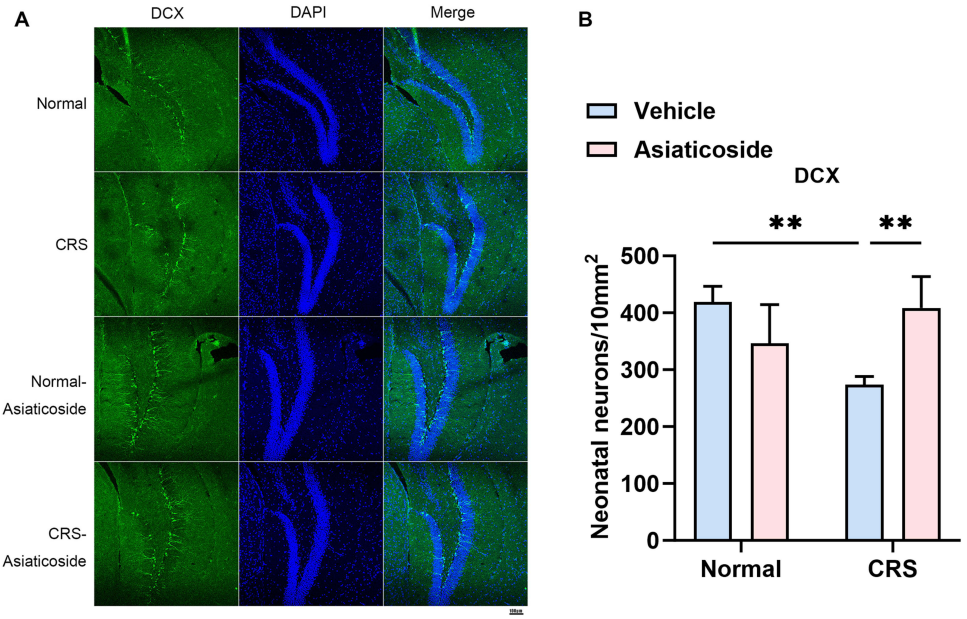

Figure 4 Asiaticoside treatment promoted hippocampus neurogenesis in mice induced by CRS (n=5). (**A**) Representative immunofluorescence images. (**B**) Statistical histogram of the number of newborn neurons. ** P<0.01.

presynaptic membrane, synaptic cleft, and postsynaptic membrane were clearly visible, and the postsynaptic density area was uniformly stained. In contrast, the CRS-vehicle mice exhibited blurred synaptic ultrastructure, a reduced number of synapses, shorter synaptic active zones, and widened synaptic clefts. These structural changes are indicative of impaired synaptic information transmission. Upon administration of asiaticoside, there was a notable improvement in synaptic

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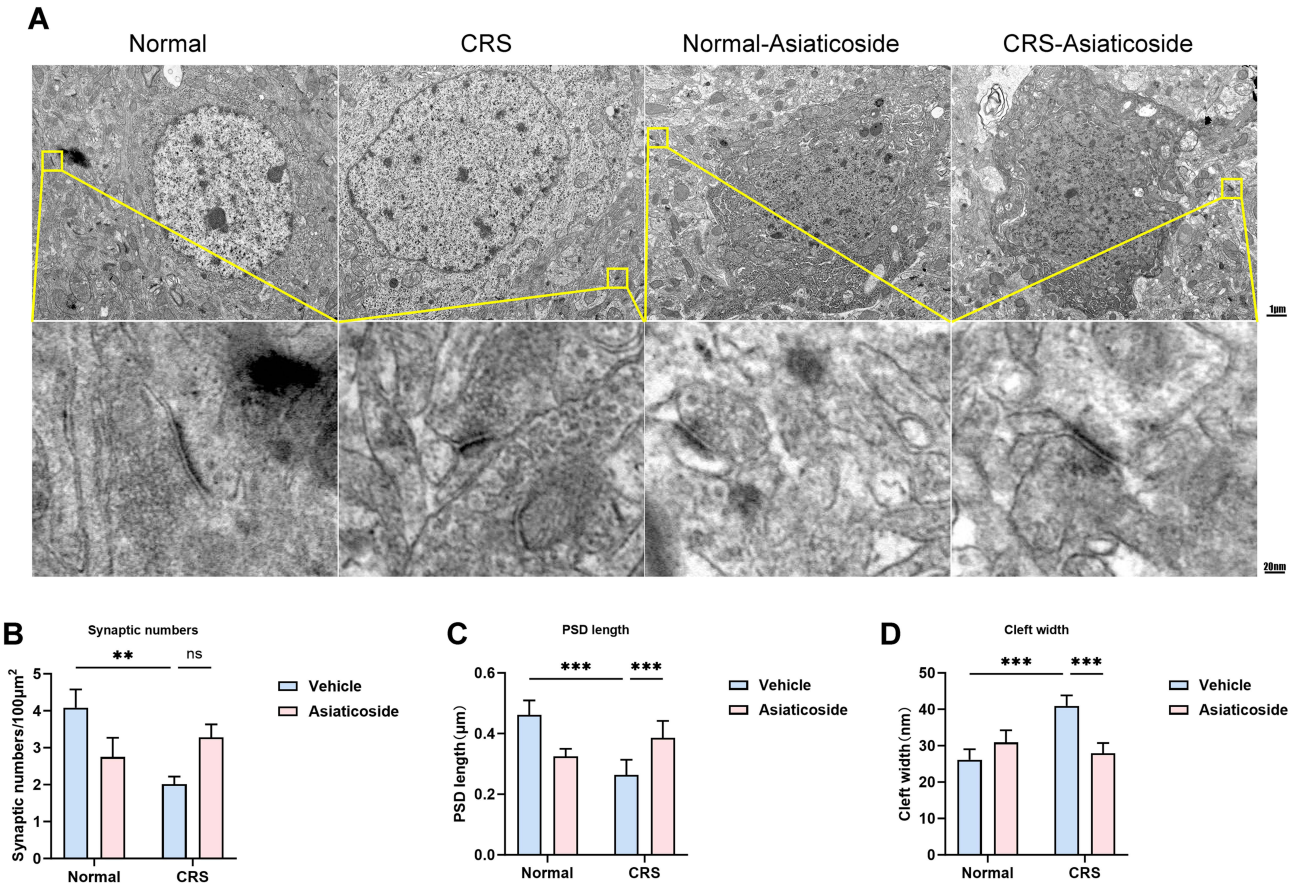

Figure 5 Asiaticoside treatment improved microscopic morphology of hippocampal CA1 neurons in mice induced by CRS (n=6). (**A**) Representative diagram of the synaptic structure of neurons. (**B**) Statistical histogram of the number of synapses per unit area. (**C**) Statistical histogram of synaptic length of neurons. (**D**) Statistical column of synaptic gap width of neurons. ** $P<0.01$, *** $P<0.001$.

structure compared to the CRS-vehicle group. Specifically, asiaticoside significantly increased the length of the synaptic active zone and reduced the width of the synaptic cleft. Additionally, there was a trend towards an increased number of synapses, although this increase did not reach statistical significance. These findings suggest that asiaticoside can ameliorate CRS-induced synaptic structural deficits in the hippocampal CA1 region, thereby potentially enhancing synaptic function and plasticity.

Asiaticoside Activated Nrf2/GPX4/SLC7A11 Signaling in the Hippocampus

Previous studies have demonstrated that BDNF/TrkB signaling can activate Nrf2 signaling. Given that Nrf2 plays a critical role in regulating ferroptosis by promoting the expression of antioxidant-related proteins such as GPX4, SLC7A11, and HO-1, we investigated the Nrf2/GPX4/SLC7A11 signaling pathway in our study. As illustrated in Figure 6A-D, administration of asiaticoside markedly increased pNrf2 levels, indicating the activation of Nrf2 signaling. The downstream targets of Nrf2 were also detected subsequently. The levels of GPX4 and SLC7A11 were significantly reduced in CRS mice compared to Normal-vehicle animals. Notably, asiaticoside treatment significantly elevated the expression of both GPX4 and SLC7A11, counteracting the effects of CRS (Figure 6E and F). Furthermore, we assessed the levels of HO-1, another downstream target of Nrf2. While CRS did not cause a statistically significant reduction in HO-1 levels, there was a noticeable trend towards decreased expression. Asiaticoside administration significantly increased HO-1 levels in the hippocampus, suggesting a potential protective effect against oxidative stress (Figure 6G). These results collectively suggest that asiaticoside not only activates Nrf2 signaling but also enhances the expression of key antioxidant proteins, thereby potentially inhibiting ferroptosis in CRS-induced depression.

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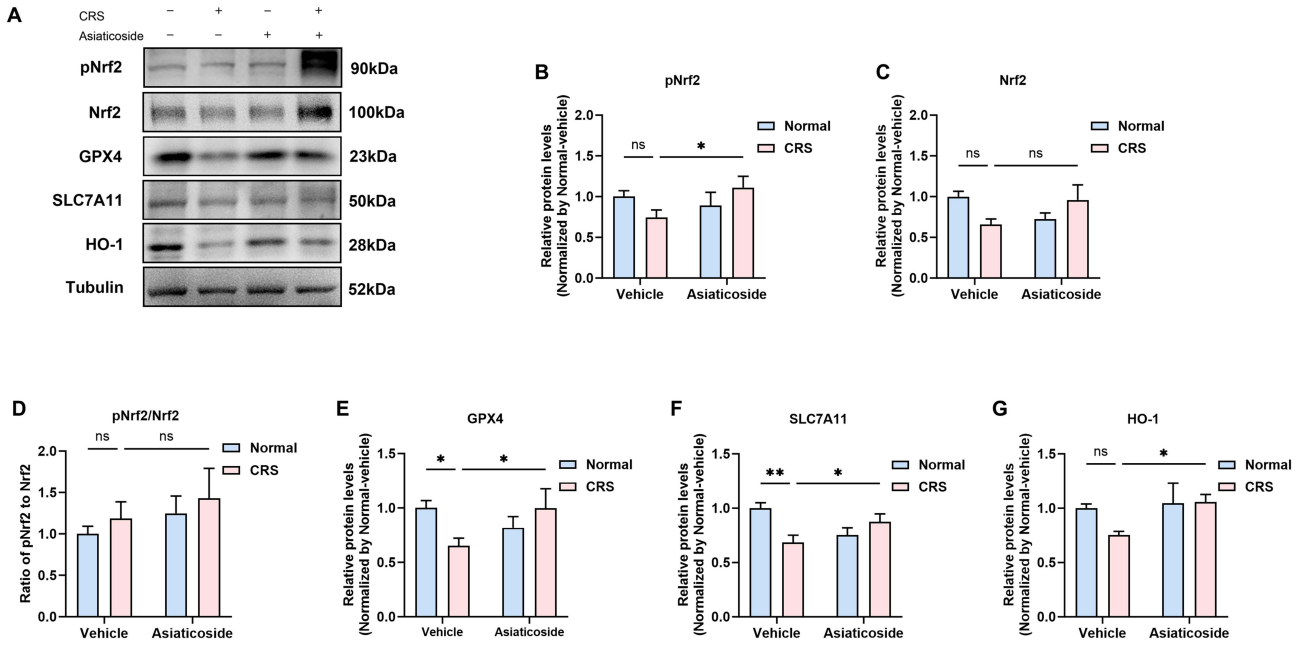

Figure 6 Effects of asiaticoside on Nrf2/GPX4 signaling pathway in mice induced by CRS (n=6). (**A**) Representative Western Blot. (**B**) Statistical histogram of pNrf2. (**C**) Statistical histogram of Nrf2. (**D**) Statistical histogram of ratio of pNrf2 to Nrf2. (**E**) Statistical histogram of GPX4. (**F**) Statistical histogram of SLC7A11. (**G**) Statistical histogram of HO-1. **P*<0.05, ***P*<0.01.

Asiaticoside Enhanced Hippocampal GPX4 and SLC7A11 Expression

Using immunofluorescence, we examined the expression of GPX4, a key factor in the Nrf2/GPX4 signaling pathway, in neurons across different regions of the hippocampus, specifically the DG, CA1, and CA3 regions. In mice subjected to CRS, we observed a significant reduction in GPX4 expression in all three hippocampal regions compared to the Normal-vehicle group. This decrease indicates a compromised antioxidant defense mechanism in these regions due to CRS (Figure 7). Following the administration of asiaticoside, the expression of GPX4 in the CA1 and CA3 regions was significantly elevated, suggesting a robust restorative effect of asiaticoside on GPX4 levels in these regions. In the DG region, although there was an observable increase in GPX4 expression post-asiaticoside treatment, this increase did not reach statistical significance, indicating a trend towards restoration that warrants further investigation. These findings demonstrate that asiaticoside effectively enhances GPX4 expression in specific hippocampal regions, particularly in CA1 and CA3, which may contribute to its neuroprotective effects against CRS-induced oxidative stress.

The expression of SLC7A11 was significantly reduced in the hippocampal CA1 and CA3 regions of the CRS-vehicle group compared to the Normal-vehicle group (Figure 8). This decrease indicates a disruption in the antioxidant defense mechanism associated with ferroptosis. Following the administration of asiaticoside, the expression of SLC7A11 in the CA1 region was significantly elevated (P<0.01), suggesting a restoration of antioxidant capacity in this region. In the CA3 region, there was a tendency for increased SLC7A11 expression post-asiaticoside treatment, although this increase did not reach statistical significance. These results suggest that asiaticoside effectively enhances SLC7A11 expression in the CA1 region, thereby potentially improving the cellular antioxidant defense against ferroptosis.

Asiaticoside Reversed the Abnormalities of Ferroptosis-Related Protein in Hippocampal CA1 of CRS Mice

Subsequently, we investigated the expression of FLC and Transferrin Receptor iron-related proteins in the mouse hippocampus using Western blot analysis and immunofluorescence.

Western blot analysis revealed trends in the expression of FLC and transferrin receptor across different experimental groups. Compared to the Normal-vehicle group, the CRS-vehicle group exhibited a tendency towards decreased FLC expression and increased Transferrin Receptor expression, although these changes did not reach statistical significance.

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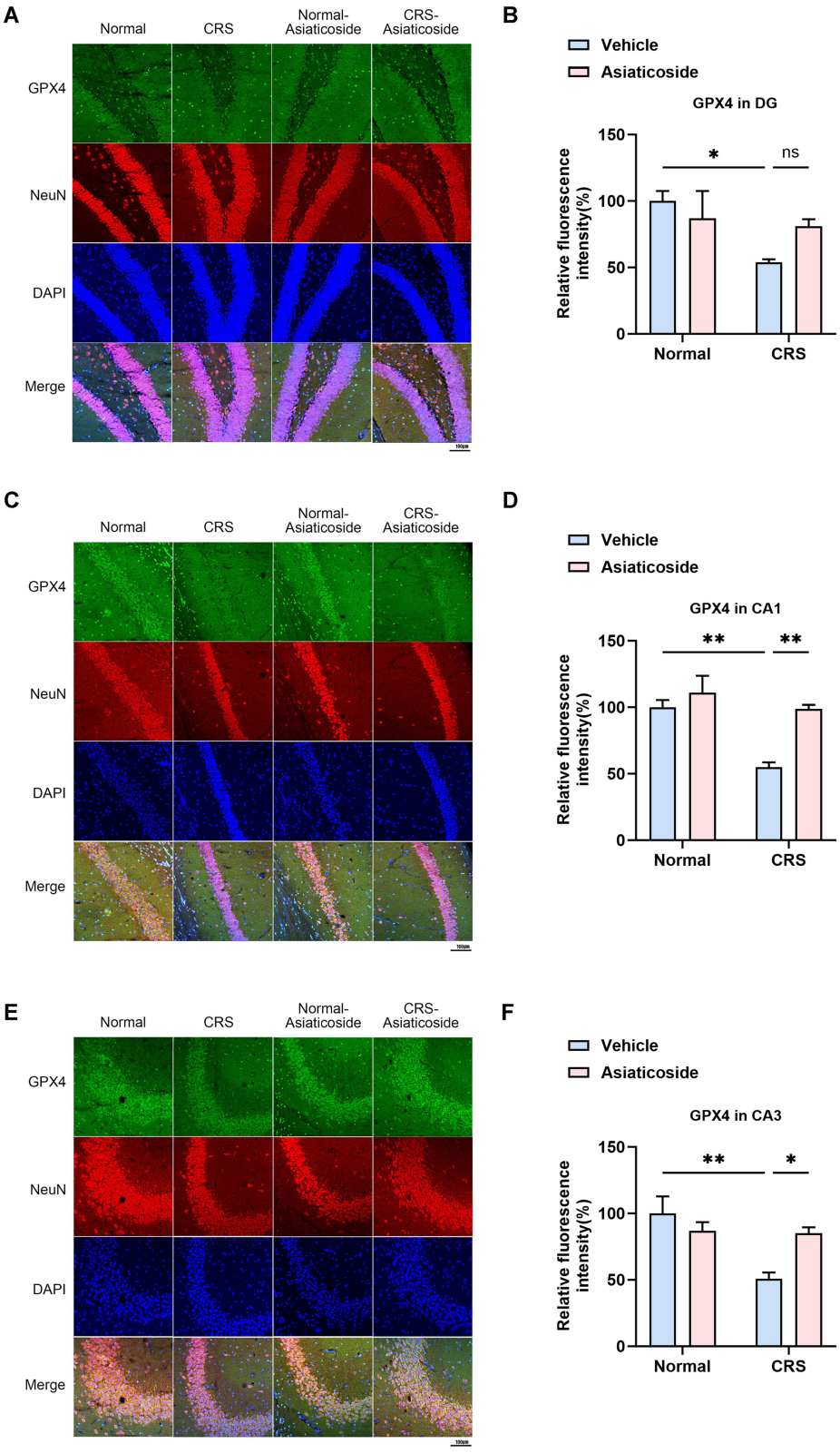

Figure 7 Effects of asiaticoside on GPX4 expression in hippocampal DG, CA1 and CA3 regions of mice induced by CRS (n=5). (**A**) Representative immunofluorescence images in DG. (**B**) GPX4 relative intensity in DG. (**C**) Representative immunofluorescence images in CA1. (**D**) GPX4 relative intensity in CA1. (**E**) Representative immunofluorescence images in CA3. (**F**) GPX4 relative intensity in CA3. *P<0.05 and ** P<0.01.

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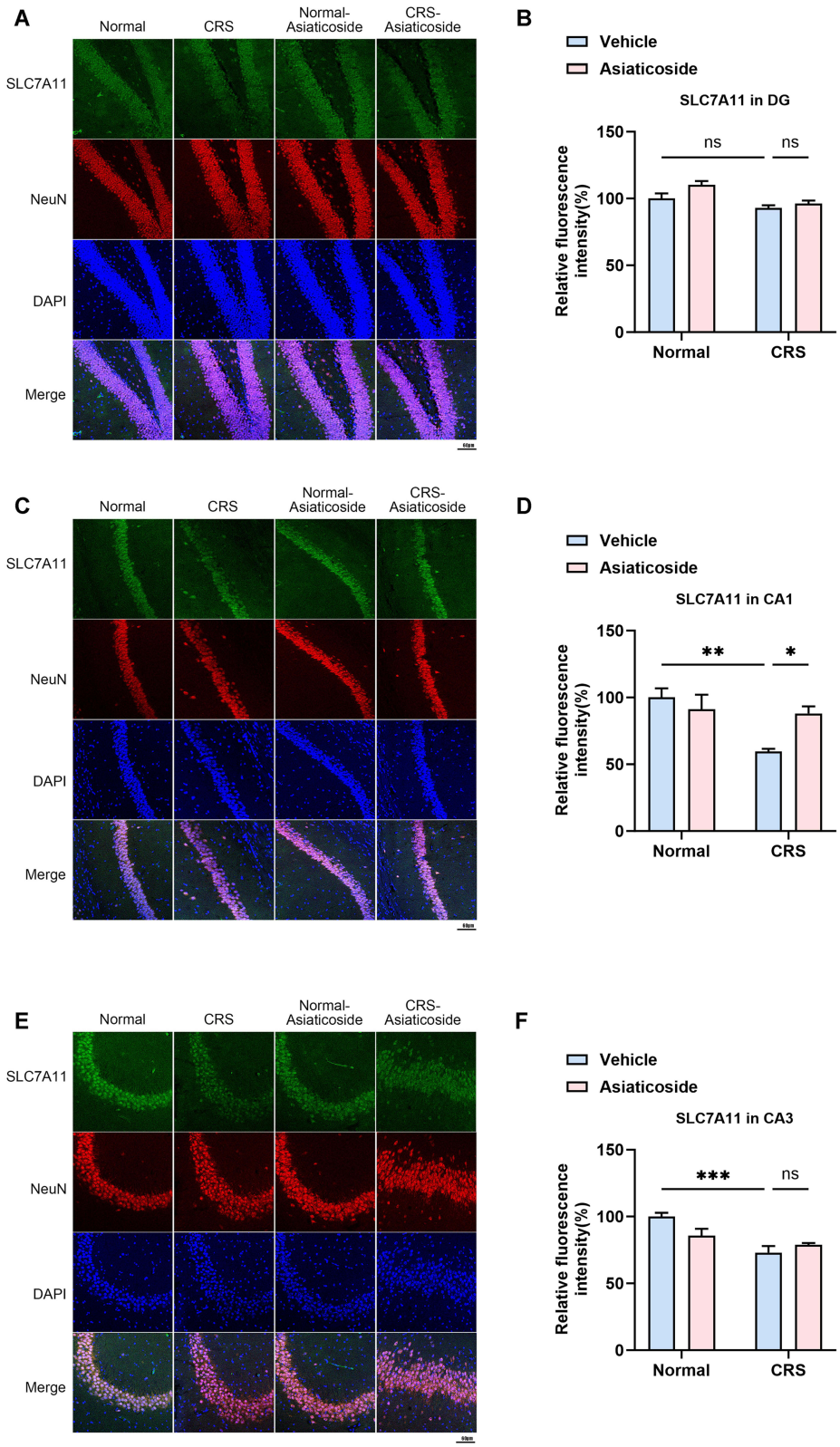

Figure 8 Effects of asiaticoside on SLC7A11 expression in hippocampal DG, CA1 and CA3 regions of mice induced by CRS (n=5). (**A**) Representative immunofluorescence images in DG. (**B**) SLC7A11 relative intensity in DG. (**C**) Representative immunofluorescence images in CA1. (**D**) SLC7A11 relative intensity in CA1. (**E**) Representative immunofluorescence images in CA3. (**F**) SLC7A11 relative intensity in CA3. *P<0.05, ** P<0.01 and *** P<0.001.

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After administration of asiaticoside, the expression of FLC showed a tendency to increase, and the expression of Transferrin Receptor tended to decrease, but neither change was statistically significant (Figure 9A and B).

To further explore these findings, we employed immunofluorescence to detect FLC and Transferrin Receptor in neurons within the DG, CA1, and CA3 regions of the hippocampus. Compared to the Normal-vehicle group, the CRS-

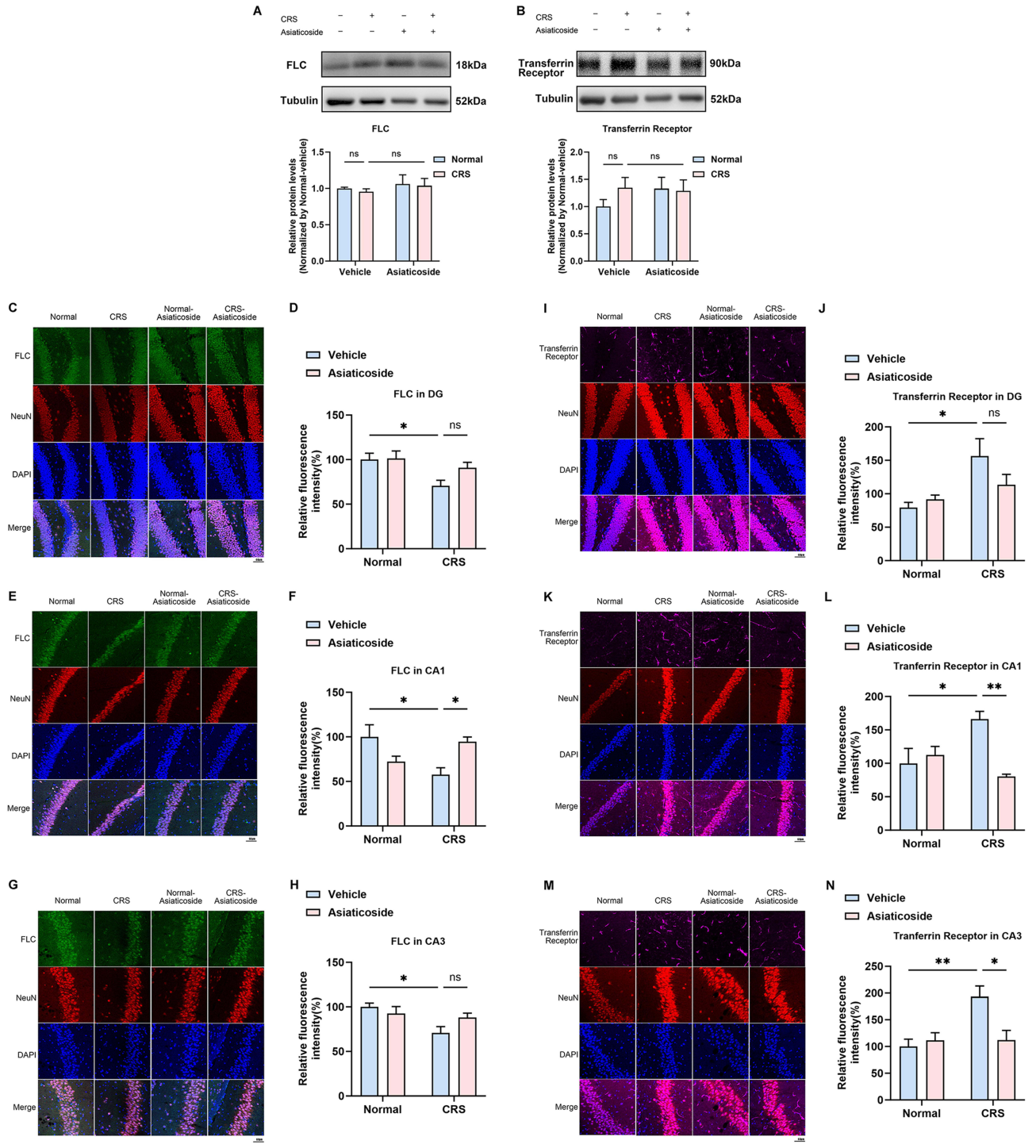

Figure 9 Effects of asiaticoside on the iron related proteins in mice induced by CRS (n=5 or 6). (**A**) Western Blot representative graph and statistical histogram of FLC. (**B**) Western Blot representative graph and statistical histogram of Transferrin Receptor. (**C**) Representative immunofluorescence images of FLC in DG. (**D**) FLC relative intensity in DG. (**E**) Representative immunofluorescence images of FLC in CA1. (**F**) FLC relative intensity in CA1. (**G**) Representative immunofluorescence images of FLC in CA3. (**H**) FLC relative intensity in CA3. (**I**) Representative immunofluorescence images of transferrin receptor in DG. (**J**) Transferrin receptor relative intensity in DG. (**K**) Representative immunofluorescence images of transferrin receptor in CA1. (**L**) Transferrin receptor relative intensity in CA1. (**M**) Representative immunofluorescence images of transferrin receptor in CA3. (**N**) Transferrin receptor relative intensity in CA3. **P*<0.05, ***P*<0.01.

vehicle group showed a significant decrease in FLC expression in all three regions. Asiaticoside administration significantly increased FLC expression in the CA1 region. Although there was an observable increase in FLC expression in the DG and CA3 regions post-asiaticoside treatment, these changes did not reach statistical significance (Figure 9C-H).

Regarding the transferrin receptor, the CRS-vehicle group exhibited no significant change in expression in the DG region but showed a significant increase in expression in the CA1 and CA3 regions compared to the Normal-vehicle group. Asiaticoside administration significantly decreased the expression of the transferrin receptor in both the CA1 and CA3 regions (Figure 9I-N).

These results indicate that asiaticoside can modulate the expression of iron-related proteins in a region-specific manner within the hippocampus. Specifically, asiaticoside appears to enhance FLC expression but reduce transferrin receptor expression mainly in the CA1 region.

Asiaticoside Improved Mitochondrial Morphology and Number in Hippocampal CA1 Neurons of CRS Mice

As shown in Figure 10, the CRS-vehicle group exhibited a significant reduction in the number of mitochondria, alongside a notably higher percentage of damaged mitochondria, compared to the Normal-vehicle group. Administration of

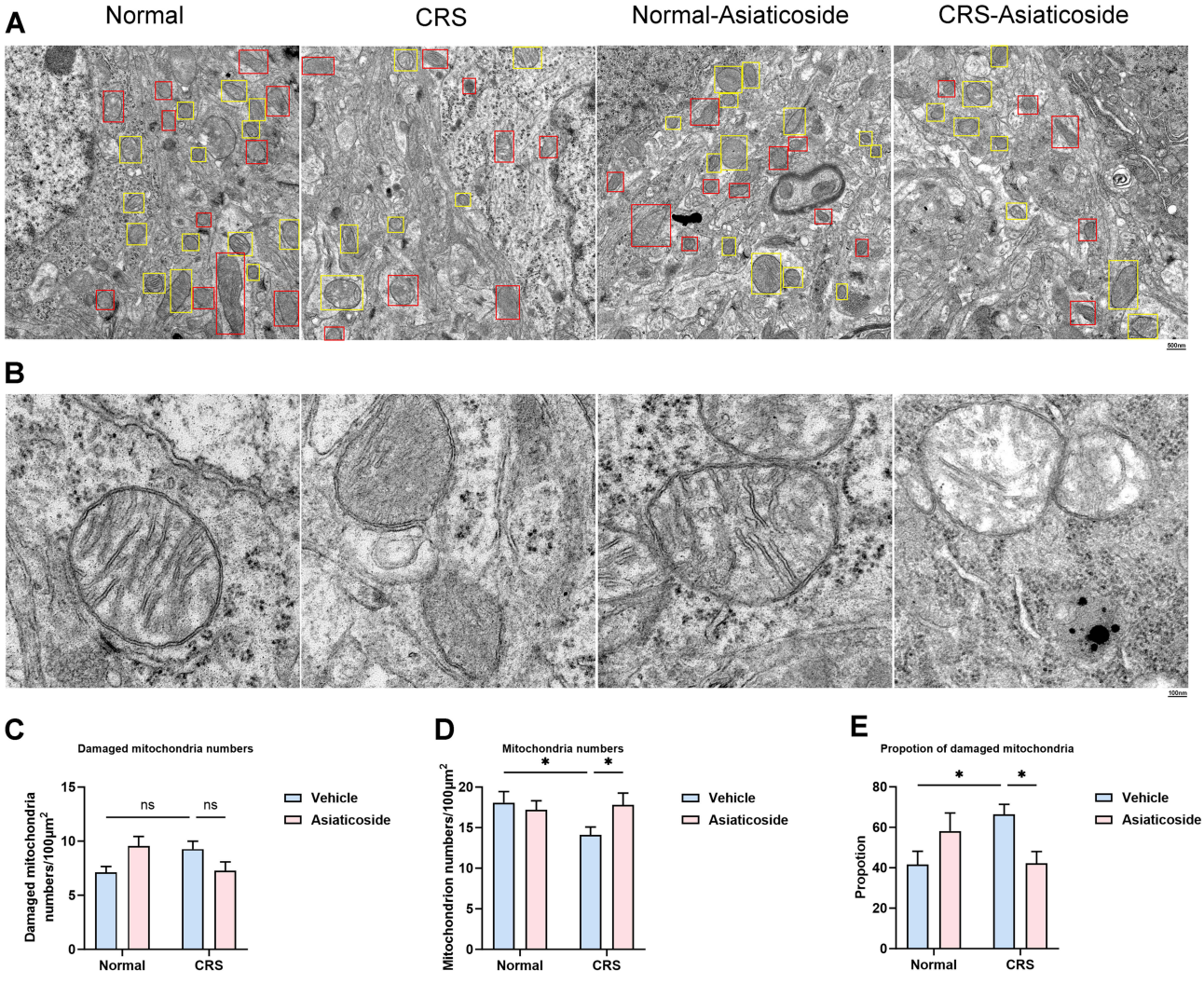

Figure 10 Effects of asiaticoside on morphology and number of mitochondria hippocampal CA1 neurons in mice induced by CRS (n=6). (**A**) Mitochondrial transmission electron microscopy. (**B**) Local magnification of transmission electron microscopy. (**C**) Statistical histogram of the number of damaged mitochondria per unit area. (**D**) Statistical histogram of the number of total mitochondria per unit area. (**E**) Statistical histogram of the proportion of damaged mitochondria. * $P<0.05$.

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asiaticoside, however, showed a tendency to increase the number of mitochondria, and the percentage of damaged mitochondria was significantly lower (Figure 10A). Further ultrastructural analysis (Figure 10B) revealed that mitochondria in the CRS-vehicle group were smaller, with reduced or absent mitochondrial cristae and ruptured outer membranes, indicative of ferroptosis. In contrast, asiaticoside treatment mitigated these adverse changes. The mitochondria in the asiaticoside-treated group displayed more intact structures, with preserved cristae and outer membranes, indicating a reduction in ferroptosis. These findings suggest that asiaticoside exerts a protective effect on mitochondrial integrity in hippocampal CA1 neurons under CRS conditions, potentially by attenuating ferroptosis.

Discussion

In this study, a CRS depression-like model was established, and the effects of asiaticoside in alleviating depression-like symptoms, such as anhedonia and despair, were confirmed through behavioral experiments. In detail, asiaticoside significantly increased sucrose preference and decreased immobilization time in the forced swimming test. On the other hand, Western blot and immunofluorescence experiments indicated that asiaticoside activated BDNF/TrkB and Nrf2/GPX4/SLC7A11 signaling pathways mainly in CA1 region of hippocampus. Immunofluorescence analysis also showed that the number of Iba1-positive cells in the hippocampus increased with CRS-induced depression, suggesting neuroinflammation, which was inhibited by asiaticoside administration. Ultrastructural analysis further demonstrated that CRS exacerbated synaptic and mitochondrial damage, indicative of ferroptosis, which was attenuated by asiaticoside treatment in CA1 neurons (Figure 11). This study advances our understanding of asiaticoside by indicating its role in attenuating ferroptosis, a novel aspect not addressed in previous research. Although our earlier work demonstrated the involvement of BDNF signaling in the antidepressant-like effects of asiaticoside, the current study integrates this with the Nrf2/GPX4 pathway, revealing a broader and more intricate mechanism.

The BDNF/TrkB signaling pathway plays a crucial role in the nervous system.[18] BDNF is distributed in the brain and peripheral serum, exerting its biological effects by binding to its specific receptor, TrkB.[19] This pathway is essential for maintaining synaptic plasticity and the structural and functional integrity of neurons. In depressive-like animals, the hippocampal BDNF/TrkB signaling pathway was inhibited, and antidepressants exert their effects by directly or indirectly upregulating this pathway.[20] Similarly, our study found that asiaticoside enhanced the hippocampal BDNF/

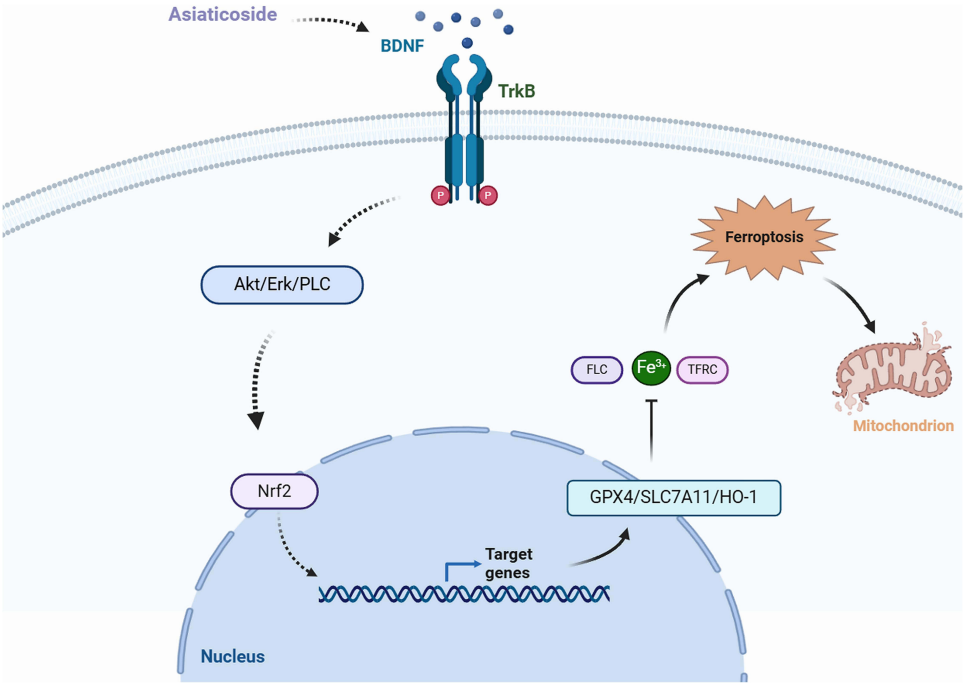

Figure 11 The involvement of BDNF/TrkB/Nrf2/GPX4/SLC7A11 mediated ferroptosis signaling pathway in the antidepressant-like effects of asiaticoside.

TrkB signaling pathway. DCX, which is involved in neurogenesis, was also promoted by asiaticoside, consistent with previous findings that hippocampal neurogenesis is linked to the severity of depressive symptoms and the sustained remission of depressive-like behaviors.[21]

The interaction between the BDNF/TrkB signaling pathway and Nrf2 mediated ferroptosis signaling pathway was described partly in the previous studies.[22] It has been verified that the activation of the BDNF/TrkB signaling pathway could upregulate the expression and activity of Nrf2.[23] This occurs through several mechanisms, including the enhancement of ERK or Akt signaling, which can phosphorylate and activate Nrf2. Increased Nrf2 activity leads to the transcription of antioxidant genes that attenuate oxidative stress, thus protecting neurons from damage.[24] Nrf2, a key transcription factor in the cellular antioxidant stress response, induces antioxidant enzymes like GPX4, SLC7A11, and HO-1.[25] When activated, these pathways enhance cellular resilience to oxidative stress. In the present study, the levels of Nrf2, GPX4, SLC7A11, and HO-1 in response to asiaticoside treatment were assessed by Western blot firstly. The results showed that asiaticoside could significantly increase GPX4, SLC7A11, and HO-1 in the whole hippocampus. Then, the immunofluorescence assay showed that asiaticoside increased both GPX4 and SLC7A11 only in CA1 of hippocampus.

Subsequently, we measured iron related proteins such as FLC and transferrin receptor in the present study. FLC plays a critical role in the iron storage function of ferritin.[26] FLC contributes to the safe storage of iron within ferritin, preventing free iron from catalyzing the formation of reactive oxygen species via the Fenton reaction. This is crucial in limiting oxidative damage to cellular components.[27] By sequestering iron, FLC helps maintain iron homeostasis within cells, reducing the availability of free iron that could otherwise promote lipid peroxidation and ferroptosis.[28] The transferrin receptor is a cell surface receptor that mediates the uptake of iron by binding to transferrin, the main iron-transport protein in the blood.[29] Transferrin receptor facilitates the internalization of transferrin-bound iron into cells.[30] Once inside the cell, iron is released from transferrin in the acidic environment of endosomes and subsequently transported to the cytoplasm. By regulating the amount of iron that enters the cell, transferrin receptor indirectly influences the levels of intracellular iron.[31] Elevated transferrin receptor expression can lead to increased iron uptake, raising intracellular iron levels and promoting conditions conducive to ferroptosis.[29] In this respect, transferrin receptor levels can modulate a cellular sensitivity to ferroptosis. High transferrin receptor expression increases iron uptake, enhancing lipid peroxidation and the likelihood of ferroptotic cell death.[32] Conversely, reduced transferrin receptor expression limits iron availability, decreasing ferroptosis susceptibility. In the present study, Western blot showed that although CRS tended to decrease FCL and increase transferrin receptor, the alterations did not reach a significance. In addition, asiaticoside did not changed the levels of FLC and transferrin receptor in the hippocampus. Then, the immunofluorescence assay showed that asiaticoside only increased FLC and transferrin receptor only in CA1 of hippocampus, suggesting the ferroptosis mainly occurred in CA regions of hippocampus.

Mitochondria are central to cellular energy metabolism, redox homeostasis, and apoptosis.[33] Excess reactive oxygen species generated during ferroptosis can damage mitochondria, leading to morphological changes and impaired function.[34] Regulatory proteins involved in ferroptosis play a crucial role in depression pathogenesis, and antidepressants can reverse depressive symptoms by inhibiting ferroptosis.[2] Our study showed that asiaticoside administration increased mitochondrial number and cristae, decreased mitochondrial damage, and inhibited ferroptosis in CRS mice, suggesting its protective effects on mitochondrial integrity and function against ferroptosis.

Finally, it should be noted that asiaticoside regulated the expression of GPX4, SLC7A11, FLC, and the transferrin receptor specifically in the hippocampal CA1 region but not in the CA3 or DG regions. This region-specific effect could be due to several factors, including the unique cellular composition, connectivity, and functional roles of these hippocampal subregions. The CA1 region is heavily involved in synaptic plasticity, making it particularly sensitive to stress and neuroprotective interventions.[35] It has been shown that the CA1 region exhibits a higher density of glutamatergic synapses and is more susceptible to excitotoxic damage compared to other hippocampal subregions. This increased vulnerability may make the CA1 region more responsive to interventions that target oxidative stress and ferroptosis pathways, such as those involving GPX4 and SLC7A11. In contrast, the CA3 region is primarily involved in pattern completion, and it has been suggested that CA3 neurons possess robust intrinsic mechanisms that protect against stress-induced damage.[36] This could explain the lack of significant changes in the expression of the iron-related proteins GPX4, SLC7A11, FLC, and the transferrin receptor in the CA3 region following asiaticoside treatment. DG is known for

its role in neurogenesis, particularly in the formation of new neurons throughout adulthood.[37] The high rate of neurogenesis in the DG might influence the expression and regulation of antioxidant and iron-related proteins differently compared to the CA1 region. Additionally, the DG has unique molecular and cellular environments that may buffer the effects of asiaticoside on the pathways in question, leading to a less pronounced response. Moreover, regional differences in the distribution and density of BDNF/TrkB receptors might also contribute to the observed specificity. The CA1 region is known to have a higher expression of BDNF and its receptor TrkB, which are crucial for the neuroprotective effects of asiaticoside.[38] This higher expression may facilitate a more robust activation of downstream signaling pathways, including the Nrf2/GPX4 pathway, leading to the observed regional specificity.

Although the present study demonstrates that asiaticoside exerts antidepressant-like effects by modulating the BDNF/Nrf2/GPX4 signaling pathway, it is important to acknowledge the limitations of our work. Specifically, our findings are based on the detection of protein expression changes, ultrastructural alterations in neuronal synapses and mitochondria, and behavioral improvements following asiaticoside treatment. However, we did not perform direct intervention experiments to confirm the causal roles of the target proteins, such as BDNF, GPX4, or Nrf2, in mediating the observed effects. To strengthen the mechanistic interpretation of the findings, future studies should include genetic or pharmacological interventions targeting these proteins. For example, knockdown of GPX4, Nrf2, or BDNF using viral vectors, or the use of specific inhibitors, could help establish a direct causal relationship between these proteins and the antidepressant-like effects of asiaticoside. Such studies would also clarify the hierarchical interplay between these proteins within the signaling pathway.

Despite the promising findings, our study has several limitations that need to be addressed in future research. Firstly, the scope of our study was limited to the hippocampal CA1 region, where we observed significant effects of asiaticoside on the BDNF/Nrf2/GPX4 signaling pathway and ferroptosis-related markers. However, the lack of significant changes in the CA3 and DG regions raises questions about the regional specificity and differential susceptibility of hippocampal subregions to asiaticoside treatment. This specificity might be influenced by distinct cellular compositions, connectivity, and functional roles of these subregions, as well as differential expression of neurotrophic receptors and signaling molecules. Secondly, our study relied on a single model of CRS to induce depression-like symptoms in mice. To enhance the generalizability of our findings, future studies should employ multiple models of depression, such as CUMS or social defeat stress, to confirm the antidepressant effects of asiaticoside across different paradigms. Additionally, our study was conducted in a preclinical animal model, and the translational relevance of our findings to human depression remains to be established. Clinical trials are essential to evaluate the safety, efficacy, and dosage parameters of asiaticoside in patients with depression.

Conclusion

In conclusion, our study suggests that asiaticoside can alleviated CRS-induced depression by activating the BDNF/TrkB signaling pathway, regulating Nrf2 expression, and inhibiting ferroptosis in hippocampal CA1 region. However, further preclinical and clinical investigations are still required to confirm the specific role of asiaticoside from other aspects in depression treatment.

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Disclosure

The authors declare that there is no conflict of interest.

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