

Original Article

Cannabidiol alleviates ferroptosis after traumatic brain injury via the miR3203p/Negr1/ERK signaling axis

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Abstract

Traumatic brain injury (TBI) can cause severe neurological damage. Ferroptosis, a recently discovered form of iron-regulated cell death, is closely associated with TBI. Cannabidiol (CBD) has been demonstrated to exhibit neuroprotective effects. However, the anti-ferroptotic role of CBD in TBI remains unclear. Investigating whether CBD inhibits ferroptosis after brain injury and its underlying mechanisms is of great significance. We find that ferroptosis can be induced in rats after TBI, and CBD significantly inhibits ferroptosis in TBI rats both *in vivo* and *in vitro*. MicroRNAs (miRNAs) are highly expressed in the brain. Differentially expressed miRNAs and mRNAs after TBI are detected by RNA sequencing, and miR-320-3p, Negr1, and the ERK/MEK pathway are screened out due to their strong correlations. The results show that CBD inhibits miR-320-3p expression, increases Negr1 expression, and suppresses the ERK/MEK pathway both *in vivo* and *in vitro*. Mechanistically, transfection with miR-320-3p mimics or siNegr1 inhibits the intervention effect of CBD on ferroptosis and the ERK/MEK pathway. Additionally, *Negr1* gene silencing reverses the effect of the miR-320-3p inhibitor on ferroptosis factors in PC12 cells, which suggests that miR-320-3p can target *Negr1*. In conclusion, our findings indicate that CBD can inhibit TBI-induced ferroptosis through the miR-320-3p/Negr1/ERK signaling axis.

Key words traumatic brain injuries, Negr1, miR-320-3p, ferroptosis, ERK

Introduction

Traumatic brain injury (TBI) is a neurological dysfunction caused by external force, featuring high incidence and mortality rates, and is one of the common causes of death among adult males globally [1,2]. It is estimated that there are approximately 69 million cases of TBI worldwide each year [3], resulting in a cost of at least \$400 billion per year [4]. With more research on TBI and advances in medical technology, the mortality rate for moderate to severe TBI has declined, but the disability rate remains high [5]. The key point of treatment after TBI is mainly to inhibit secondary brain damage, and ferroptosis has been shown to be closely associated with a variety of neurological disorders, such as neurodegenerative diseases, stroke and TBI. Increased iron accumulation in the brain leads to lipid peroxidation, reactive oxygen species (ROS) accumu-

lation, mitochondrial dysfunction, and neuroinflammatory responses, which in turn lead to neuronal damage and largely affect patient prognosis [6]. In a mouse model of controlled cortical impact injury (CCI), significant accumulation of iron and ROS after TBI was accompanied by dysfunction of iron metabolism, upregulation of iron death-related genes, and reduced glutathione peroxidase (GPx) activity, whereas cerebral ventricular injection of the iron death inhibitor ferrostatin-1 (Fer-1) significantly reduced the expression of iron death-related proteins and cell death, as well as ameliorated TBI-induced cognitive function and motor dysfunction [7]. Another study found that melatonin alleviated long-term sleep disturbances and improved neurological function in TBI mice by inhibiting ferroptosis [8].

Cannabidiol (CBD) in cannabis extract has pharmacological

effects, such as analgesic, anti-inflammatory, antioxidant, antimicrobial, antidiabetic, and antitumor effects [9–12]. In 2017, the liquid formulation of CBD, Epidiolex®, was marketed for the treatment of refractory epilepsy [13,14]. Clinical trials have also found that symptoms such as tremor and freezing gait, as well as cognitive function in Parkinson's patients, can be improved by CBD medications [15,16]. In addition, CBD can act as a scavenger of ROS and inhibit lipid peroxidation, thereby inhibiting caspase-mediated neuronal apoptosis [17]. The existence of multiple potential therapeutic roles of CBD is of great significance for clinical medication, and its therapeutic and mechanistic exploration of neurological disorders has become a focus of attention.

MicroRNAs (miRNAs) are a class of noncoding RNA molecules, typically 20–23 nucleotides in length. Several studies have shown that approximately 70% of miRNAs are expressed in the brain and are involved in the regulation of lesions in the central nervous system (CNS), which plays an important role in neurogenesis, development and synaptic plasticity formation [18–20]. Neuronal growth regulator 1 (Negr1) is a member of the immunoglobulin-like cell adhesion molecule (IgLON) family, which plays a positive role in feeding behavior, motor behavior, and neuronal projection development. Negr1 can regulate neuronal growth and synapse formation [21,22]. Based on the mRNA sequencing analysis in this experiment, Negr1 is likely involved in the TBI process and plays a potential regulatory role. In-depth exploration of the relationship between miR-320-3p and Negr1 and TBI is of great practical significance in revealing the disease development mechanism of TBI and the regulatory mechanism of miRNAs.

The MEK/ERK signaling pathway, one of the mitogen-activated protein kinase (MAPK) signaling pathways, transmits extracellular signals to the cell through a series of kinase cascade reactions and regulates the biological behavior of cells. Activation of Ras promotes phosphorylation of mitogen-activated protein kinase (Raf) in the cytoplasm and recruits it to the cell membrane, and activated Raf phosphorylates MEK, which in turn phosphorylates ERK in the cytoplasm. Phosphorylated ERK can be translocated to the nucleus to induce the transcription of relevant genes in the cell [23]. This pathway can regulate a variety of cellular processes and diseases and is closely related to cell proliferation, migration and differentiation and maintenance of cell morphology, as well as being involved in cellular inflammatory responses, immune regulation, and damage repair [24,25]. In a cerebral ischemia-reperfusion model, modulation of p-JNK, p-ERK, and p-MEK inhibits ferroptosis and improves cognitive impairment in mice [26]. In addition, a study by Cao *et al.* [27] found that inhibition of the MEK/ERK pathway reduced iron accumulation as well as the expression of ferroptosis-related proteins, exerting a protective effect against cerebral hemorrhage injury. It is evident that the ERK signaling pathway is involved in the regulation of ferroptosis and is closely related to the treatment of brain diseases. Therefore, the present study sought to investigate whether CBD could regulate TBI ferroptosis and whether the mechanism involves the miR-320-3p/Negr1/ERK pathway through *in vivo* experiments, *in vitro* experiments, and transcriptome sequencing.

Material and Methods

Animals and experimental groups

Male Sprague-Dawley rats (weighing 260 ± 20 g, 8–10 weeks old) were sponsored by the Forensic Science Institute, Kunming Medical

University (Yunnan, China). Animal experiments were conducted in accordance with the guidelines of the Department of Laboratory Animal Science, Kunming Medical University. The experimental animals were kept in a quiet, constant temperature and humidity environment with 12 h/12 h alternating light and dark. The Animal Experiment Ethics Committee of Kunming Medical University (approval No. KMMU2020054) reviewed and approved the study protocol.

TBI model was prepared according to the modified Feeney method [28]. A 6-mm diameter bone window was opened in the rat's skull, centered 2.5 mm to the right of the sagittal suture and 2 mm below the coronal suture. TBI was induced by dropping a 40-g weight onto the brain tissue from a 15 cm height. No percussion was conducted in the sham group, and all remaining procedures were kept identical. CBD with 99.5% purity (kindly provided by the Department of Forensic Medicine, Kunming Medical University) was dissolved in a saline carrier solution of 1% dimethyl sulfoxide (DMSO) and 2% polysorbate-80 (Tween-80). The CBD group rats were treated with CBD (10 mg/kg, i.p.) 30 min before injury, 6 h after injury, and 24 h after injury [28]. The sham group and TBI group rats received an equal volume of vehicle. The rats were sacrificed 48 h after injury, and samples were collected for subsequent experiments.

Cell culture and treatment

Highly differentiated rat adrenal pheochromocytoma cells (PC12) were purchased from Kunming Cell Bank, Chinese Academy of Sciences (Kunming, China). They were cultured in high-sugar DMEM (G4511; Servicebio, Wuhan, China) containing 10% fetal bovine serum (C04001-100; Vivacell, Shanghai, China) and 1% penicillin-streptomycin at 37°C and 5% CO₂.

Cells were treated with erastin (S80804; OriLeaf, Shenyang, China), a ferroptosis inducer, to establish an *in vitro* ferroptosis model. Different concentration gradients (0 μ M, 1 μ M, 2 μ M, 2.5 μ M, 5 μ M, and 10 μ M) of erastin were used to stimulate PC12 cells for 0 h, 8 h, 12 h, and 24 h. The optimal concentration and time of erastin intervention were explored for subsequent experiments.

To explore the interventional effect of CBD on ferroptosis in PC12 cells, the cells were divided into control group, erastin group, erastin + CBD low concentration group (erastin + 2 μ M CBD), erastin + CBD medium concentration group (erastin + 3 μ M CBD), and erastin + CBD high concentration group (erastin + 4 μ M CBD).

To explore whether CBD regulates ferroptosis in PC12 cells through the miR-320-3p/Negr1/ERK axis, the cells were divided into the following groups: Control group, Model group (erastin group, abbreviated as E), Drug intervention group (E + CBD), miR-320-3p mimic control transfection group (E + CBD+mimic NC), miR-320-3p mimic transfection group (E + CBD+mimic), Gene silencing control group (E + CBD + siNC), and Negr1 silencing group (E + CBD + siNegr1).

To explore whether miR-320-3p targets Negr1 to exert its effect on ferroptosis in PC12 cells, the cells were divided into the following groups ($n = 5$): Negative Control group (siNC + NC), Negr1 silencing group (siNegr1 + NC), miR-320-3p inhibitor group (siNC + IN), and miR-320-3p inhibitor with Negr1 silencing group (siNegr1 + IN).

The sequences of all small RNAs used in this study are shown in Table 1.

Immunofluorescence staining

Tissue sections and PC12 cells were incubated overnight at 4°C with

Table 1. The sequences of the small RNAs used in this study

Small RNA	Sequence (5'→3')
rno-miR-320-3p mimic	Sense: AAAAGCUGGGUUGAGAGGGCGA Antisense: GCCCUCUAACCCAGCUUUUUU
mimic NC	Sense: UUCUCCGAACGUGUCACGUTT Antisense: ACGUGACACGUUCGGAGAATT
rno-miR-320-3p inhibitor	Sense: UCGCCCUCUAACCCAGCUUUU
Inhibitor NC	Sense: CAGUACUUUUGUGUAGUACAA
siNegr1-1	Sense: GGACUACAGCCUCCAGAU Antisense: UAUCUGGAGGCGUAGUCC
siNegr1-2	Sense: GGUUCAUCUCACUGUGCAA Antisense: UUGCACAGUGAGAUGAACC
siNegr1-3	Sense: GGACAUUUUAUGGAUUACA Antisense: UGUAAUCCAUAAAUGUCC
siNC	Sense: UCUACUCUUUCUAGGAGGUUGUGA

the following primary antibodies: PTGS2 (1:200, ab15191; Abcam, Cambridge, UK), GPx4 (1:200, 30388-1-AP; Proteintech, Wuhan, China), NeuN (1:200, 66836-1-Ig; Proteintech), and the corresponding Cy3-coupled secondary antibody (1:200, a11029; Sigma, St Louis, USA) and FITC-coupled secondary antibody (1:200, F0257; Sigma) were added on the following day and incubated for 1 h at room temperature. The sections were fixed by adding an anti-fluorescence quenching sealer (F6057; Sigma) containing 4',6-diamidino-2-phenylindole (DAPI). Finally, tissue sections and PC12 cell slides were observed using a fluorescence inverted microscope (WM950; Zeiss, Oberkochen, Germany).

Western blot analysis

The brain tissues and PC12 cells were collected for western blot analysis. Equal amounts of proteins were separated using polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes which were blocked in 5% skim milk for 2 h at room temperature. Then, the membranes were incubated with primary antibodies against PTGS2 (1:5000, ab15191; Abcam), GPx4 (1:1000, 30388-1-AP; Proteintech), SLC7A11 (1:1000, 26864-1-AP; Proteintech), ERK1/2 (1:1000, AF0155; Affinity, Cincinnati, China), p-ERK1/2 (1:1000, AF1015; Affinity), MEK1/2 (1:1000, AF6385; Affinity), p-MEK1/2 (1:1000, AF3385; Affinity), Negr1 (1:1000, DF4240; Affinity), GAPDH (1:5000, T0004; Affinity) or β -actin (1:5000, 20536-1-AP; Proteintech) overnight at 4°C. After washing with Tris-buffered saline with Tween-20, the membranes were incubated with the corresponding horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:10,000, SA00001-2; Proteintech) or goat anti-mouse secondary antibody (1:5000, SA00001-1; Proteintech) for 1 h at room temperature. The protein bands were detected using enhanced chemiluminescence (ECL) (BL520A; Biosharp, Hefei, China). ImageJ software was used to measure the gray value of the protein bands.

RT-qPCR

Total RNA was extracted from brain tissues or PC12 cells by using RNAiso Plus reagent (9108; Takara, Shiga, Japan). A reverse transcription kit (RR047A; Takara) was used to convert RNA into cDNA. mRNA expression was detected with TB Green® Premix Ex Taq™ II (RR820A; Takara). miRNA was extracted by using a miRNA

Table 2. The sequences of the primers and small RNAs used in this study

Gene	Sequence (5'→3')
<i>GPx4</i>	F: TTGATAAGAACGGCTGCGTG R: CACGGCAGGTCTCTCTAT
<i>SLC7A11</i>	F: ATCGGATCGGGCATCTTCAT R: ACAGTACTCCACAGGCAGAC
<i>PTGS2</i>	F: ACGTGTGACGTCCAGATCA R: GGCCCTGGTGTAGTAGGAGA
<i>Negr1</i>	F: AGGAACCAACGTCACCCTTA R: GCTGATGGGAGATATGCCT
<i>ERK</i>	F: TACGGCATGGTTGTTCTGTC R: AGGTCTGGTGTCTAAAAGGA
<i>MEK</i>	F: GGAGATCAAACCCGCAATCC R: CCCGTAGAAGCCCACTATGT
<i>GAPDH</i>	F: GACATGCCGCTGGAGAAAC R: TGATTTCCCGTAGGACCCGA
rno-miR-320-3p	F: AAAAGCTGGGTTGAGAGGGCGA
U6	F: CTCGCTTCGGCAGCACA

Isolation kit (R6842-01; Omega, Norcross, USA). RT-qPCR of miRNAs was performed after reverse transcription using an All-in-One™ miRNA First-Strand cDNA Synthesis kit (QP113; GeneCopoeia, Rockville, USA) and an All-in-One™ miRNA qPCR kit (QP010; GeneCopoeia). The PCR primers are shown in Table 2. PCR reaction conditions were: 95°C initial denaturation for 120 s; 95°C denaturation for 30 s; 60°C annealing for 40 s; 72°C extension for 90 s; and 40 cycles in total. The $2^{-\Delta\Delta Ct}$ method was used to determine the relative expression levels of *PTGS2*, *GPx4*, *SLC7A11*, *ERK*, *MEK*, *Negr1*, *GAPDH*, *miR-320-3p* and *U6*. *GAPDH* or *U6* was used as an internal control for mRNA or miRNA.

Cell viability assay

The viability of PC12 cells was determined using a CCK-8 kit (PF00004; Proteintech). PC12 cells were seeded in a 96-well plate at an initial density of 3×10^3 cells/well. The cells were incubated with 10 μ L of CCK-8 working solution for 2 h, and the absorbance was measured at 450 nm using a microplate reader (Multiskan FC; Thermo Fisher Scientific, Waltham, USA).

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of glutathione (GSH) and ROS in rat serum were measured using ELISA kits (A006-2-1 and E004-1-1; Jiancheng Bioengineering Institute, Nanjing, China). The levels of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) in both rat serum and cell lysates were determined using ELISA kits (WLCSJZF30239 and WLCSJZF30015; Lunchangshuo Biotechnology, Xiamen, China). All assays were performed in strict accordance with the manufacturers' instructions.

RNA-seq and bioinformatics analysis

Forty-eight hours after surgery, brain tissues were collected and frozen in liquid nitrogen for transcriptome sequencing (Biotree, Shanghai, China). Nine rat brain samples were used for RNA sequencing ($n = 3$). Library preparation and sequencing were conducted by Biotree.

Dual-luciferase reporter system

Approximately 200-bp sequences near the binding sites of Negr1-WT/MT were synthesized and inserted into the pmirGLO vector (E1330; Promega, Madison, USA), and mimics of miR-320-3p and the negative control (NC) were synthesized (B01001 and B04001; GenePharma, Suzhou, China). The reporter vectors and miR-320-3p mimics were cotransfected into 293T cells (CL-0005; Procellsystem, Wuhan, China), and the Renilla luciferase activity and firefly luciferase activity were determined using a Double-Luciferase Reporter Assay kit (E1910; GenePharma). Relative luciferase activity was calculated by the ratio of Renilla luciferase activity to firefly luciferase activity.

Statistical analysis

The data are expressed as the mean $\pm$ standard deviation (SD) and were analyzed using one-way ANOVA. $P < 0.05$ was considered statistically significant. Analyses were performed using Prism version 9.0 software.

Results

CBD attenuates ferroptosis and oxidative stress in TBI rats

To investigate the interventional effects of CBD on ferroptosis in TBI rats, we detected the expression levels of related factors (Figure 1). Immunofluorescence staining results demonstrated decreased GPx4

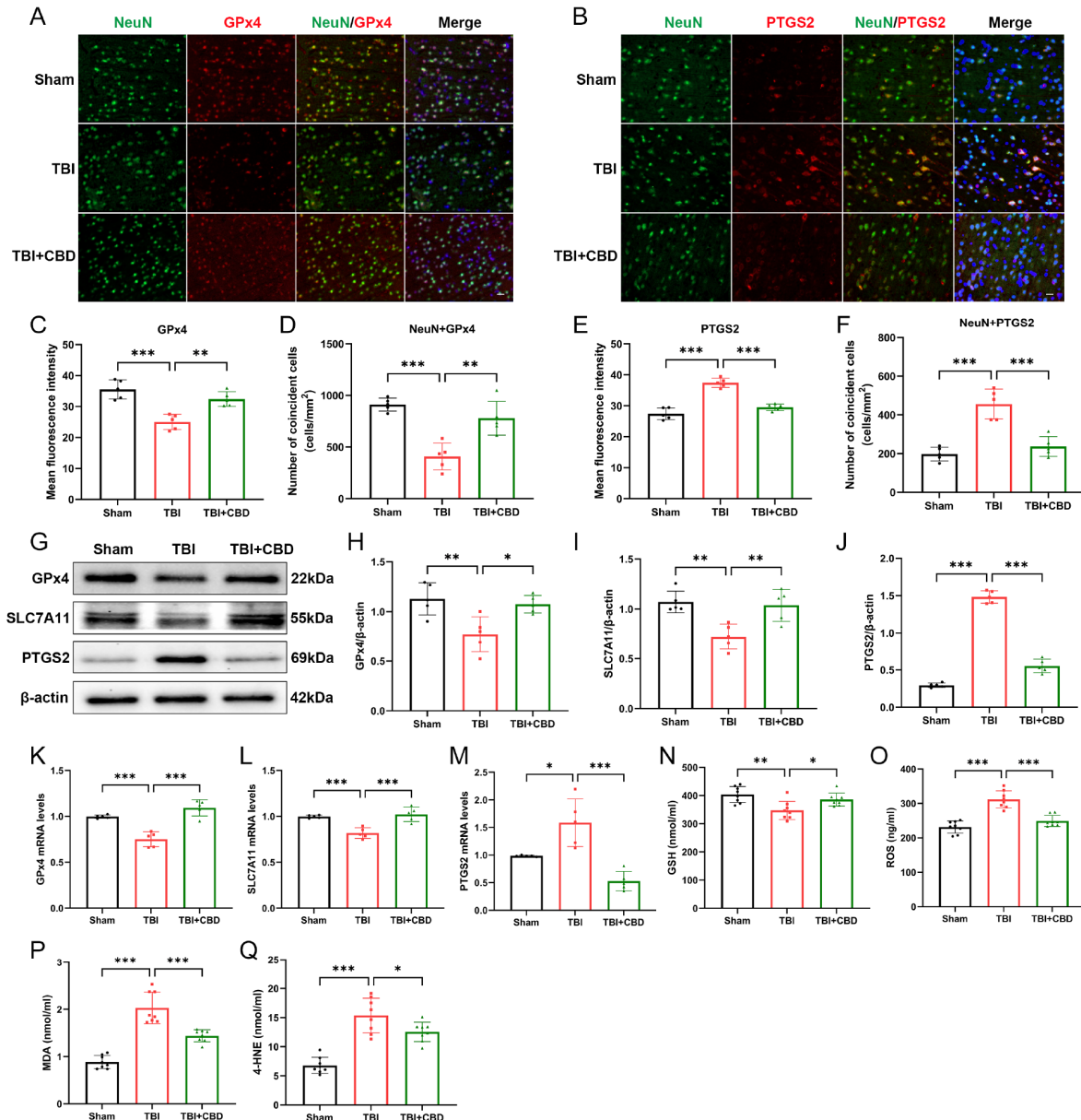


Figure 1. CBD attenuates ferroptosis and oxidative stress in TBI rats (A,B) Representative images of immunofluorescence staining of GPx 4, PTGS2 and neurons (NeuN) in brain tissue sections. Scale bar = 20 μ m. (C,D) Immunofluorescence intensity analyses of GPx4 and quantitative analyses of cell density coexpressing NeuN and GPx 4 ($n = 5$). (E,F) Immunofluorescence intensity analysis of PTGS2 and quantitative analysis of cell density coexpressing NeuN and PTGS2 ($n = 5$). (G-J) Representative western blot bands and quantitative analysis of GPx4, SLC7A11, and PTGS2 in the cerebral cortex ($n = 5$). (K-M) mRNA levels of GPx4, SLC7A11, and PTGS2 in the cerebral cortex ($n = 5$). (N,O) GSH and ROS levels in serum ($n = 8$). (P,Q) MDA and 4-HNE levels in serum ($n = 8$). Sham group: a cranial window was created in rats without brain injury; TBI group: traumatic brain injury model was established in rats; TBI + CBD group: CBD was administered to rats with TBI. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

level and increased PTGS2 level in cortical neurons of the rat brain tissue after TBI, with these changes reversed following CBD treatment (Figure 1A–F). Western blot analysis and RT-qPCR results indicated that CBD significantly promoted the protein and mRNA levels of GPx4 and SLC7A11, which are key regulators of ferroptosis, and inhibited the protein and mRNA levels of PTGS2 in brain tissue (Figure 1G–M). Ferroptosis is primarily induced by the accumulation of lipid peroxides and ROS and the depletion of GSH in cells. Therefore, we employed ELISA to assess the levels of oxidative stress and lipid peroxidation indicators. The results demonstrated that TBI led to decreased GSH level and increased levels of ROS, MDA, and 4-HNE. CBD treatment elevated GSH level and significantly suppressed the levels of ROS, MDA, and 4-HNE (Figure 1N–Q). These results suggest that TBI induces ferroptosis, oxidative stress, and lipid peroxidation in rats and that CBD treatment ameliorates these pathological changes.

CBD attenuates erastin-induced ferroptosis in PC12 cells

To establish a ferroptosis model, PC12 cells were treated with erastin at concentrations of 0 μ M, 1 μ M, 2 μ M, 2.5 μ M, 5 μ M, and 10 μ M for durations of 0 h, 8 h, 12 h, and 24 h, respectively. CCK-8 assay results indicated a significant decrease in cell viability to an average of 67.19% after treatment with 1 μ M erastin for 24 h (Figure 2A). Additionally, immunofluorescence staining was employed to assess the impact of 1 μ M erastin on the expression of GPx4 and PTGS2 in the cells. The results demonstrated that, compared to the control group, erastin induced a notable decrease in GPx4 fluorescence intensity and an increase in PTGS2 fluorescence intensity (Figure 2B–D). Therefore, we selected 1 μ M erastin treatment in PC12 cells for 24 h for subsequent experiments. To determine the dosing concentration of CBD, PC12 cells were treated with CBD at concentrations of 0 μ M, 1 μ M, 2 μ M, 3 μ M, 4 μ M, 5 μ M, and 10 μ M for 24 h. CCK-8 assay results showed that CBD had no inhibitory effect on cell viability up to a concentration of 5 μ M (Figure 2E), which was a safe dosing concentration. Therefore, 2 μ M, 3 μ M, and 4 μ M gradient concentrations of CBD were selected for subsequent experiments.

Western blot analysis and RT-qPCR results showed that CBD significantly increased the protein and mRNA levels of GPx4 and SLC7A11 in PC12 cells while inhibiting the protein and mRNA levels of PTGS2 (Figure 2F–L). Immunofluorescence staining results revealed that erastin-treated PC12 cells exhibited decreased GPx4 level and increased PTGS2 level, which were reversed following CBD treatment (Figure 2M–P). These results indicated that erastin (1 μ M) treatment of PC12 cells for 24 h induced ferroptosis in the cells, and CBD treatment effectively alleviated erastin-induced ferroptosis. Additionally, 4 μ M CBD demonstrated the best therapeutic effect; therefore, this concentration was selected for CBD intervention in subsequent experiments.

Differential expression profiles of mRNAs and miRNAs in rats after TBI

High-throughput sequencing was performed on the cortical tissue of rat brains in both the sham and TBI groups to identify differentially expressed mRNAs and miRNAs. Compared to the sham group, 327 mRNAs were differentially expressed following TBI, with 296 upregulated and 31 downregulated (Figure 3A,C). Additionally, compared to the sham group, the expression of 21 miRNAs was upregulated and 37 miRNAs were downregulated in the damaged

brain tissue of TBI rats (Figure 3D,E). Cluster analysis heatmaps of differentially expressed mRNAs and miRNAs between the sham and TBI groups were obtained using the DESeq2 algorithm, and signaling pathway enrichment (KEGG) analysis was conducted for the predicted differentially expressed mRNAs and miRNAs (Figure 3B,F). The results of KEGG analysis of differential mRNAs revealed that the main annotations and enrichments were in phagosomes, chemokine signaling pathways, viral protein interactions with cytokine and cytokine receptors, leishmaniasis, malaria, and cell adhesion molecules, among which cell adhesion molecules are very important for neurogenesis and development. Among them, Negr1, a cell adhesion molecule, had an average FPKM expression level of 1.83 in the TBI group and 3.71 in the sham group. The abundance of Negr1 in the sham group and its differential expression following TBI suggest potential biological effects. Using the TargetScan website (http://www.targetscan.org/vert_72/), miRNAs targeted by Negr1 were predicted and intersected with the differentially expressed miRNAs obtained from sequencing, and miR-320-3p was found. Binding sites between miR-320-3p and Negr1 were predicted by TargetScan (Figure 3G,H). Meanwhile, KEGG analysis of the differentially expressed miRNAs showed significant annotation and enrichment in metabolic pathways, endocytosis, insulin resistance, and the MAPK signaling pathway. Among them, the MAPK signaling pathway mainly includes ERK, JNK, p38, etc., with the ERK signaling pathway closely related to ferroptosis. Therefore, we speculated that miR-320-3p/Negr1/ERK might be involved in the inhibition of ferroptosis by CBD in TBI.

CBD inhibits miR-320-3p overexpression, upregulates Negr1 and suppresses the ERK/MEK signaling pathway

To confirm that miR-320-3p targets *Negr1*, we performed a dual-luciferase reporter gene assay. Dual-luciferase reporter gene assay results showed that, compared with the mimic NC + Negr1-WT group, the luciferase activity in the miR-320-3p mimic + Negr1-WT group decreased significantly, while the luciferase activity in the miR-320-3p mimic + Negr1-MUT group had no significant change compared with that in the mimic NC + Negr1-MUT group (Figure 4A). Further validation of the sequencing results through RT-qPCR experiments revealed elevated mRNA level of miR-320-3p and decreased mRNA level of Negr1 in the brain tissue of TBI rats and in PC12 cells treated with erastin, and CBD treatment further reversed these expression levels (Figure 4B–E). The protein expression trend of Negr1 in brain tissue was consistent with its mRNA expression trend (Figure 4F,G). Western blot analysis results showed that compared with the sham group, the ratios of p-ERK/ERK and p-MEK/MEK in the brain tissue after TBI were significantly increased, and CBD treatment reduced these ratios (Figure 4H–J). These findings suggest that miR-320-3p, Negr1, and the ERK/MEK signaling pathway may be involved in the therapeutic process of CBD in treating TBI.

miR-320-3p mimic and Negr1 silencing inhibit the therapeutic effect of CBD on erastin-induced ferroptosis in PC12 cells and suppress regulation of the ERK/MEK signaling axis

Transfection of miR-320-3p mimics and siNegr1 into PC12 cells was conducted to establish PC12 cells with miR-320-3p overexpression or *Negr1* silencing. The mRNA expression levels were detected to confirm the transfection efficiency. It was shown that, compared with the control group, there was no significant difference in miR-

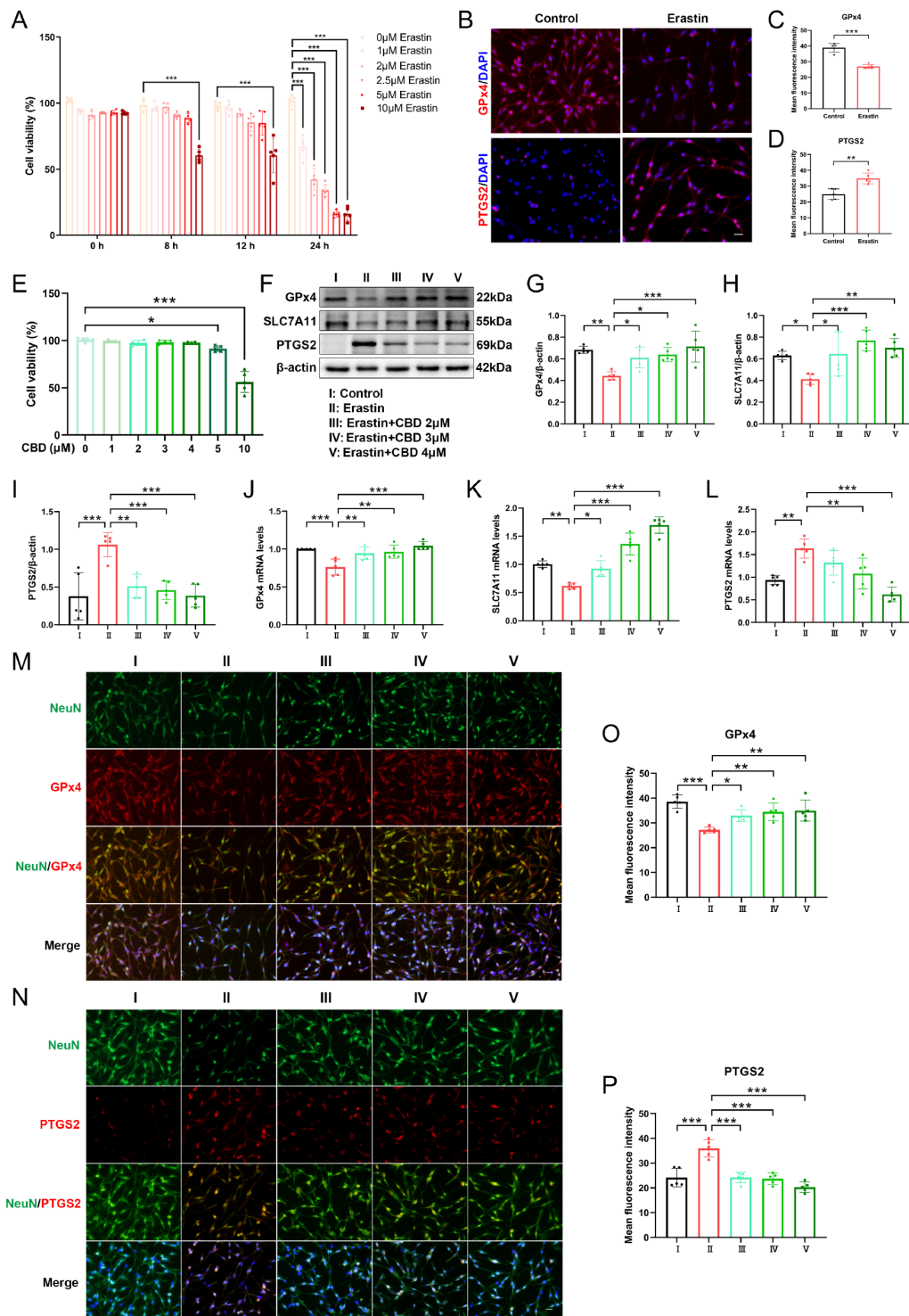


Figure 2. CBD attenuates erastin-induced ferroptosis in PC12 cells (A) CCK-8 assay evaluating the effects of various concentrations and durations of erastin on PC12 cell viability ($n = 5$). (B) Representative images of immunofluorescence staining for GPx4 and PTGS2 in PC12 cells. Scale bar = 20 μ m. (C,D) Quantitative analysis of immunofluorescence intensity for GPx4 and PTGS2 ($n = 5$). (E) CCK-8 assay assessing the effects of different concentrations of CBD on PC12 cell viability ($n = 5$). (F–I) Representative western blot bands and quantitative analysis of GPx4, SLC7A11, and PTGS2 in PC12 cells ($n = 5$). (J–L) mRNA levels of GPx4, *SLC7A11*, and *PTGS2* in PC12 cells ($n = 5$). (M,N) Representative images of immunofluorescence staining for GPx4, PTGS2, and neurons (NeuN) in PC12 cells. Scale bar = 20 μ m. (O,P) Quantitative analysis of immunofluorescence intensity for GPx4 and PTGS2 ($n = 5$). Control group: untreated PC12 cells; erastin group: erastin-treated PC12 cells; E + CBD 2 μ M group: erastin-treated PC12 cells with 2 μ M CBD intervention; E + CBD 3 μ M group: erastin-treated PC12 cells with 3 μ M CBD intervention; E + CBD 4 μ M group: erastin-treated PC12 cells with 4 μ M CBD intervention. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

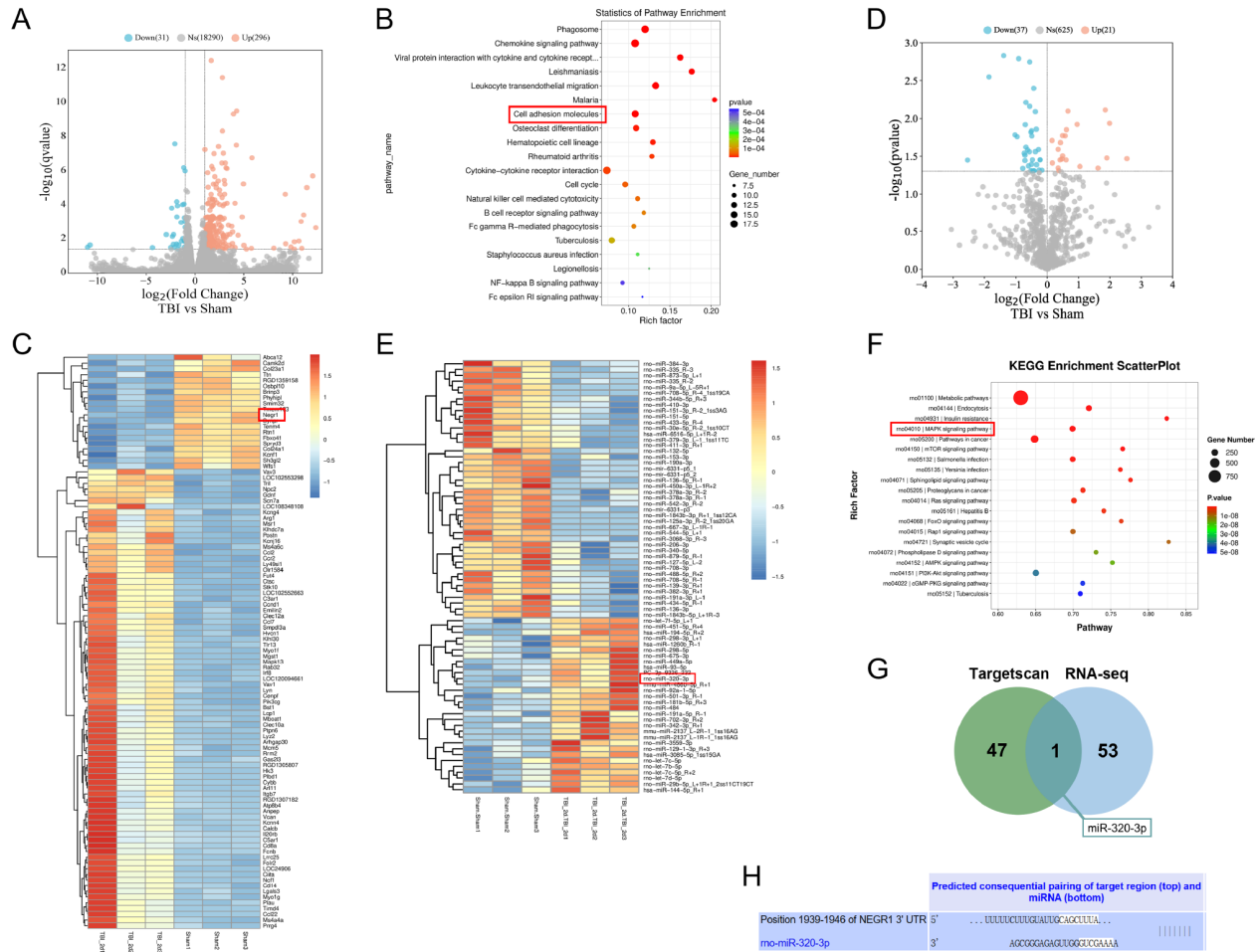


Figure 3. RNA-seq and bioinformatics analysis of the cortical tissue of the rat brain after TBI (A) Volcano plot displaying differentially expressed mRNAs between the TBI group and the sham group. (B) Clustered heatmap of differentially expressed mRNAs. (C) KEGG enrichment analysis of differentially expressed mRNAs. (D) Volcano plot showing differentially expressed miRNAs between the TBI group and the sham group. (E) Clustered heatmap of differentially expressed miRNAs. (F) KEGG enrichment analysis of differentially expressed miRNAs. $n = 3$. (G) Venn diagram of the intersection between miRNAs predicted to bind Negr1 on the TargetScan website and differentially expressed miRNAs from sequencing. (H) Predicted binding sites of miR-320-3p with Negr1 in TargetScan.

320-3p level in the mimic NC group; after the addition of the miR-320-3p mimic, the miR-320-3p level increased significantly compared to that in the mimic NC group, with an approximately 46.96-fold increase (Figure 5A). Compared with the siNC group, the *Negr1* mRNA level in the siNegr1 groups was significantly decreased, with siNegr1-3 exhibiting the best silencing effect, reducing *Negr1* mRNA expression to 29.29% of the siNC group (Figure 5B). Therefore, siNegr1-3 was selected for subsequent experiments.

Through western blot analysis and RT-qPCR experiments, changes in the protein and mRNA expression levels of GPx4, SLC7A11, and PTGS2 were observed in PC12 cells of various groups. It was shown that, compared with the control group, after erastin intervention, the protein levels of GPx4 and SLC7A11 were significantly decreased, while the PTGS2 protein level was markedly increased. These changes were alleviated after CBD intervention. However, after cell transfection with the miR-320-3p mimic and siNegr1, the protein expression of GPx4 and SLC7A11 decreased again, and the PTGS2 protein level increased, indicating that the therapeutic effect of CBD was inhibited (Figure 5C-F). The trends in mRNA levels of *GPx4*, *SLC7A11*, and *PTGS2* in PC12 cells

of various groups were relatively consistent with those observed in the western blot analyses (Figure 5G-I).

Through western blot analysis and RT-qPCR experiments, changes in the protein and mRNA expression levels of Negr1, p-ERK/ERK, and p-MEK/MEK were observed in PC12 cells of various groups. The results showed that, compared with the control group, after erastin intervention, the protein and mRNA levels of Negr1 were significantly decreased, while the protein ratios of p-ERK/ERK and p-MEK/MEK were markedly increased, accompanied by elevated mRNA levels of ERK and MEK. CBD intervention reversed the erastin-induced low expression of Negr1 protein and mRNA as well as the high expression of p-ERK/ERK and p-MEK/MEK protein and mRNA. Compared with the E + CBD group, there were no significant changes in the expressions of Negr1, p-ERK/ERK, and p-MEK/MEK after cell transfection with mimic NC and siNC. Compared with the E + CBD + mimic NC group, transfection with the miR-320-3p mimic resulted in decreased Negr1 protein and mRNA expression and increased p-ERK/ERK and p-MEK/MEK protein and mRNA expression. Compared with the E + CBD + siNC group, transfection with siNegr1 led to decreased Negr1 protein and

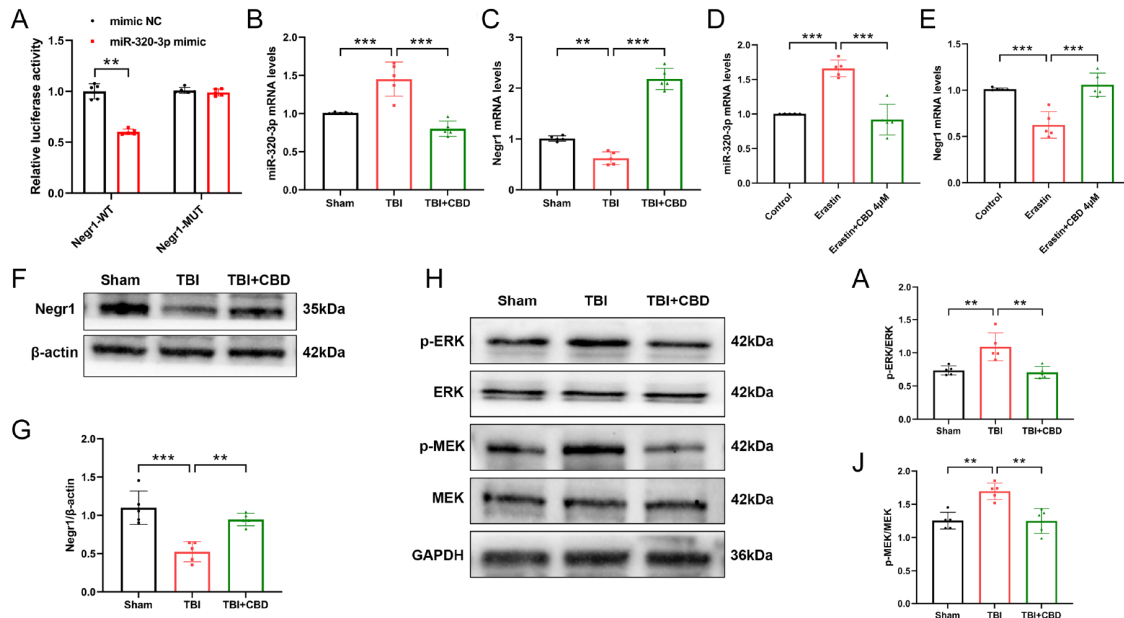


Figure 4. CBD inhibits miR-320-3p overexpression, upregulates Negr1, and suppresses the ERK/MEK signaling pathway (A) Relative luciferase activity of 293T cells transfected with plasmids containing wild-type/mut Negr1 and miR-320-3p/NC ($n = 5$). (B,C) Levels of miR-320-3p and *Negr1* mRNA in the cerebral cortex ($n = 5$). (D,E) Levels of miR-320-3p and *Negr1* mRNA in PC12 cells ($n = 5$). (F,G) Representative western blot bands and quantitative analysis of Negr1 in the cerebral cortex ($n = 5$). (H-J) Representative western blot bands and quantitative analysis of p-ERK, ERK, p-MEK, and MEK in the cerebral cortex ($n = 5$). Sham group: a cranial window was created in rats without brain injury; TBI group: traumatic brain injury model was established in rats; TBI + CBD group: CBD was administered to rats with TBI. ** $P < 0.01$, *** $P < 0.001$.

mRNA expression and increased p-ERK/ERK and p-MEK/MEK protein and mRNA expression (Figure 5J-Q). The levels of MDA and 4-HNE in PC12 cells across different groups were measured by ELISA. The results showed that erastin treatment significantly increased MDA and 4-HNE levels compared with the control group, while CBD intervention reduced their levels. Transfection with mimic NC or siNC showed no significant changes in their expression compared with the E + CBD group. However, cell transfection with the miR-320-3p mimic and siNegr1 increased their levels, indicating that the therapeutic effect of CBD was inhibited (Figure 5R,S).

CBD alleviates erastin-induced ferroptosis in PC12 cells through the miR-320-3p/Negr1/ERK axis

To investigate whether miR-320-3p has a targeting effect on Negr1, PC12 cells were transfected with *Negr1* silencing constructs as well as miR-320-3p inhibitors. Western blot analysis and RT-qPCR results showed that, compared with the siNC + NC group, the protein and mRNA levels of GPx4 and SLC7A11 decreased while PTGS2 level increased after *Negr1* silencing. After transfection with miR-320-3p inhibitor, the protein and mRNA levels of GPx4 and SLC7A11 increased, while those of PTGS2 decreased (Figure 6A-G). Additionally, compared with the siNC + IN group, the protein and mRNA levels of GPx4 and SLC7A11 both decreased, and those of PTGS2 increased after *Negr1* silencing (Figure 6A-G). These results indicate that the regulatory effects of miR-320-3p inhibitor on the protein and mRNA expressions of GPx4, SLC7A11, and PTGS2 were inhibited by *Negr1* silencing.

Through western blot analysis, changes in the protein expression of Negr1, p-ERK/ERK, and p-MEK/MEK were observed in PC12 cells across various groups. The results demonstrated that after *Negr1* silencing, the Negr1 protein level significantly decreased, while the

protein ratios of p-ERK/ERK and p-MEK/MEK markedly increased. Following transfection with miR-320-3p inhibitors, the Negr1 protein level significantly increased, whereas the protein levels of p-ERK/ERK and p-MEK/MEK both notably decreased (Figure 6H-M). Compared with the siNC + IN group, *Negr1* silencing led to decreased Negr1 protein expression and increased ratios of p-ERK/ERK and p-MEK/MEK (Figure 6H-M). RT-qPCR results showed that the changes in the mRNA expression of these factors were consistent with the changes in protein expression (Figure 6N,O), indicating that the regulatory effects of miR-320-3p inhibitors on the mRNA expression of *Negr1*, *ERK*, and *MEK* were inhibited by *Negr1* silencing.

Discussion

TBI is recognized as a significant global public health issue, posing a severe burden on patients and society. Due to its complex pathophysiological changes, current clinical treatments remain limited, and the prognosis is often unsatisfactory. Therefore, it is particularly important to explore the mechanisms of TBI injury and identify effective therapeutic drugs. Ferroptosis is a novel form of cell death, primarily characterized by intracellular iron overload caused by various factors, leading to the production of ROS, which leads to intracellular lipid peroxidation, glutathione metabolism disorders, triggering oxidative stress and intracellular metabolic disturbances, ultimately leading to cell death [29]. The results of this study revealed that typical characteristics of ferroptosis, including decreased antioxidant capacity, accumulation of lipid peroxidation products, and significant alterations in ferroptosis-related genes, could be detected in both the brain tissue of TBI rats and erastin-induced PC12 cells. CBD intervention partially reversed these pathological changes by enhancing the expression of the

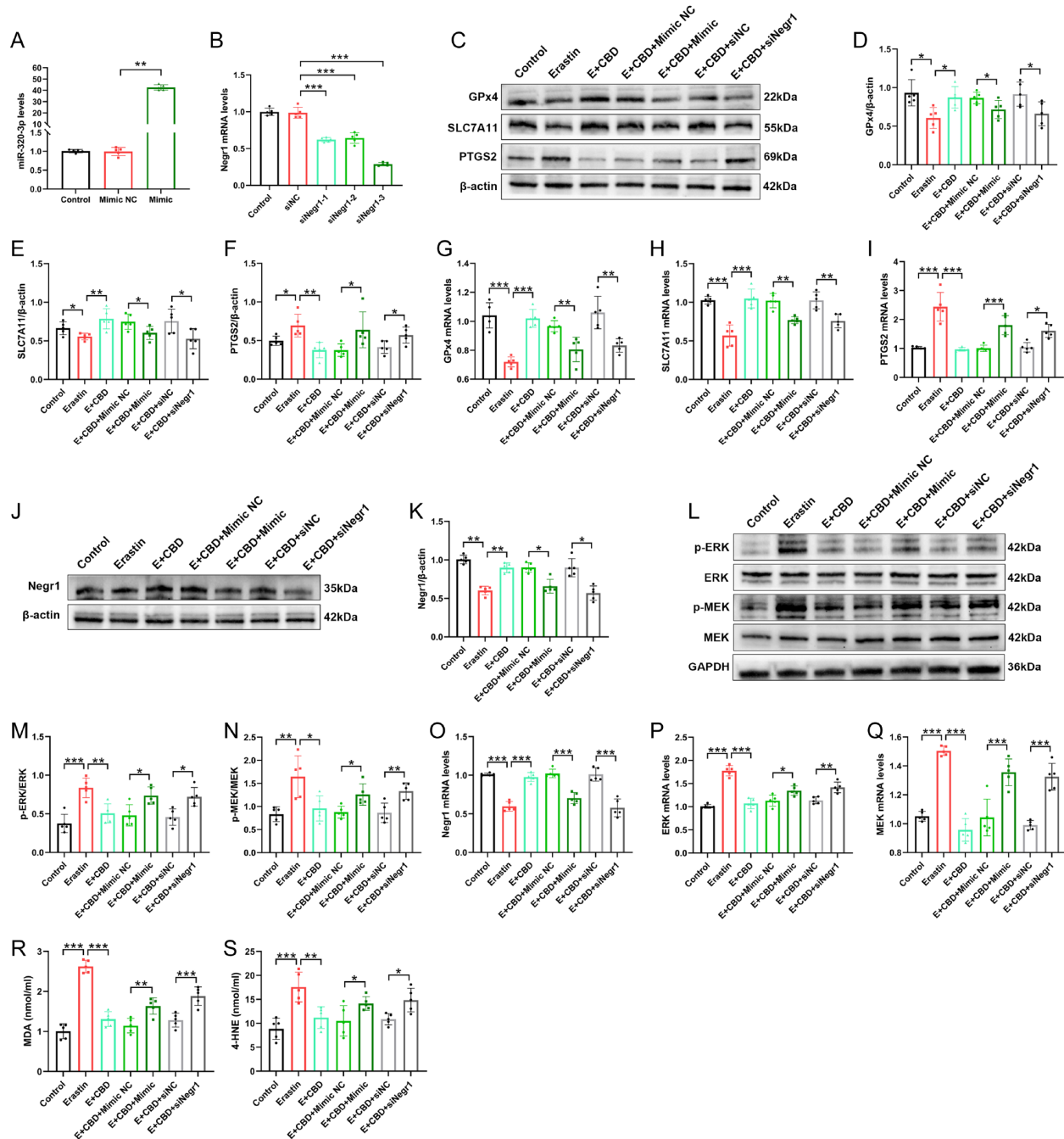


Figure 5. miR-320-3p mimic and *Negr1* silencing inhibit the therapeutic effect of CBD on erastin-induced ferroptosis in PC12 cells and suppress the regulation of the *Negr1*/ERK signaling axis (A,B) Levels of miR-320-3p and *Negr1* mRNA in PC12 cells ($n = 5$). (C–F) Representative western blot bands and quantitative analysis of GPx4, SLC7A11, and PTGS2 protein levels in PC12 cells ($n = 5$). (G–I) mRNA levels of *GPx4*, *SLC7A11*, and *PTGS2* in PC12 cells ($n = 5$). (J,K) Representative western blot bands and quantitative analysis of p-ERK, ERK, p-MEK, and MEK protein levels in PC12 cells ($n = 5$). (L–N) Representative western blot bands and quantitative analysis of p-ERK, ERK, p-MEK, and MEK protein levels in PC12 cells ($n = 5$). (O–Q) mRNA levels of *Negr1*, *ERK*, and *MEK* in PC12 cells ($n = 5$). (R,S) MDA and 4-HNE levels in cell lysates ($n = 5$). Control group: untreated PC12 cells; erastin group: erastin-treated PC12 cells; E + CBD group: erastin-treated PC12 cells with CBD intervention; E + CBD + mimic NC group: erastin-treated PC12 cells with CBD intervention and negative control mimic; E + CBD + mimic group: erastin-treated PC12 cells with CBD intervention and miR-320-3p mimic; E + CBD + siNC group: erastin-treated PC12 cells with CBD intervention and small interfering RNA negative control; E + CBD + siNegr1 group: erastin-treated PC12 cells with CBD intervention and *Negr1*-targeting siRNA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

ferroptosis inhibitory proteins GPx4 and SLC7A11 in neurons, reducing PTGS2, MDA, and 4-HNE levels, inhibiting the production of ROS, and promoting GSH synthesis. Lipid peroxidation represents the core execution event of ferroptosis, while MDA and 4-HNE

serve as key terminal products of this process. These aldehydes not only function as sensitive biomarkers for the experimental detection of ferroptosis but also actively promote ferroptosis occurrence through mechanisms such as covalent modification of proteins and

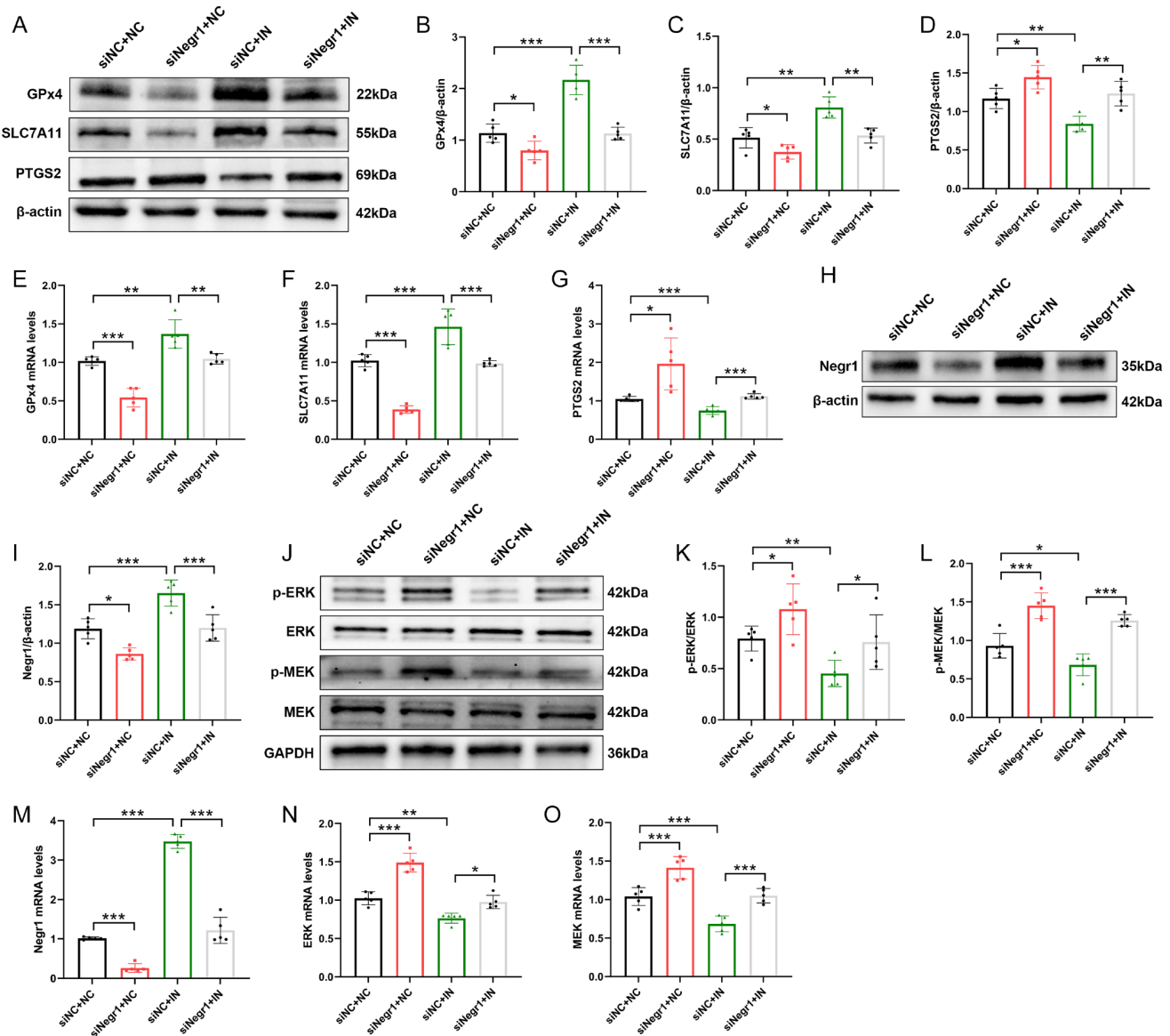


Figure 6. *Negr1* silencing reverses the effects of miR-320-3p inhibitors on ferroptosis and the ERK/MEK signaling pathway in PC12 cells (A–D) Representative western blot bands and quantitative analysis of GPx 4, SLC7A11, and PTGS2 protein levels in PC12 cells ($n = 5$). (E–G) mRNA levels of GPx 4, SLC7A11, and PTGS2 in PC12 cells ($n = 5$). (H,I) Representative western blot bands and quantitative analysis of *Negr1* protein level in PC12 cells ($n = 5$). (J–L) Representative western blot bands and quantitative analysis of p-ERK, ERK, p-MEK, and MEK protein levels in PC12 cells ($n = 5$). (M–O) mRNA levels of *Negr1*, ERK, and MEK in PC12 cells ($n = 5$). siNC + NC group: PC12 cells transfected with small interfering RNA negative control and negative control inhibitor; siNegr1 + NC group: PC12 cells transfected with *Negr1*-targeting siRNA and negative control inhibitor; siNC + IN group: PC12 cells transfected with small interfering RNA negative control and miR-320-3p inhibitor; siNegr1 + IN group: PC12 cells transfected with *Negr1*-targeting siRNA and miR-320-3p inhibitor. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

disruption of membrane integrity [30]. SLC7A11 is a specific amino acid transporter and a key regulator of ferroptosis, serving as one of the proteins that constitutes the Xc-system. Downregulation of SLC7A11 can inhibit the cysteine metabolic pathway, leading to decreased intracellular cysteine level and depletion of GSH biosynthesis. This indirectly inhibits the activity of GPx4, resulting in the accumulation of lipid peroxides. These peroxides undergo the Fenton reaction with intracellular free iron ions, generating a large amount of ROS. Excessive intracellular ROS enhance cellular oxidative stress, causing damage to cellular components such as proteins, DNA, and lipids and ultimately inducing ferroptosis in

cells [31,32]. PTGS2, a key enzyme in prostaglandin biosynthesis, increases cellular sensitivity to ferroptosis by modulating membrane phospholipids. Elevated expression of PTGS2 often indicates the occurrence of ferroptosis in cells [33]. Recent studies have shown that ferroptosis is involved in the occurrence and development of secondary brain injury following TBI, and intervention in ferroptosis may have a positive effect on the treatment of TBI. A study indicated that melatonin can inhibit the expression of ferroptosis-related proteins and the accumulation of iron in the cortex after TBI. Inhibiting neuronal ferroptosis can reduce neurofunctional damage, and this effect may be mediated through

the inhibition of neuronal ferritin H [34]. Furthermore, Huang *et al.* [35] reported that polydatin can exert long-term neuroprotective effects by maintaining the activity of GPx4 in neurons and inhibiting the expression of ferroptosis-related factors in neurons, thereby significantly improving motor dysfunction following TBI. Therefore, based on the results of this study and literature reports, CBD may exert a certain neuroprotective effect after TBI by alleviating ferroptosis and oxidative stress. It is worth noting that our group's preliminary experiments demonstrated that CBD treatment reduced the mNSS scores in TBI rats, indicating its neuroprotective effects [2]. Building upon this foundation, we aim to further investigate the underlying mechanisms.

In recent years, the role of miRNAs in various human diseases has emerged as a research hotspot [36]. miRNAs are endogenous small ncRNAs that primarily participate in regulating various physiological and pathological processes by binding to target gene mRNAs, degrading mRNAs or inhibiting their translation [37]. Multiple studies have demonstrated abnormal expression of miRNAs in the brains of patients with TBI, which not only regulate the physiological and pathological processes of TBI but also serve as biomarkers for assessing the severity of craniocerebral injuries [38]. Yang *et al.* [39] found that serum exosomal miR-206 and miR-549a-3p exhibit good correlation with BDNF, NSE, and S100 β , suggesting their potential as adjunct biomarkers for the diagnosis of TBI patients. Furthermore, studies have proposed that overexpression of miR-132 can ameliorate TBI-induced tissue damage and memory impairments [40]. Additionally, ursolic acid has been shown to regulate the miR-141-mediated PDCD4/PI3K/AKT signaling pathway, thereby improving oxidative stress and neuroinflammation in TBI mice [41]. Therefore, in this study, to further explore the mechanism of action of miRNAs in TBI, we utilized transcriptome sequencing combined with bioinformatics predictions to screen for miR-320-3p and Negr1, which exhibited strong correlations. These findings were validated in rat brain tissue and PC12 cells. Additionally, prediction results from the TargetScan website indicated specific binding between the 3'UTR of Negr1 and miR-320-3p. Preliminary evidence suggests a possible negative relationship between miR-320-3p and Negr1 and that miR-320-3p may serve as a potential target in TBI. In this study, the dual-luciferase reporter gene experiment further demonstrated that there is a targeting relationship between Negr1 and miR-320-3p.

Previous studies have shown that miR-320-3p is involved in the regulation of various diseases. In a rat cardiomyocyte ischemia/reperfusion injury model, increased expression of miR-320-3p led to decreased cell viability and increased apoptosis, while inhibition of miR-320-3p expression reversed myocardial ischemic injury [42]. In a cellular pancreatitis model, inhibition of miR-320-3p expression reduced apoptosis and inflammation [43]. The aforementioned evidence indicates that miR-320-3p plays a critical role in promoting cellular damage and death under various pathological conditions. This study further confirms that in neural cell injury induced by TBI and erastin, CBD significantly downregulates the elevated expression of miR-320-3p, accompanied by neuroprotective effects. Critically, the administration of a miR-320-3p mimic attenuated the inhibitory effects of CBD on ferroptosis and lipid peroxidation, suggesting that miR-320-3p serves as an essential mediator in CBD neuroprotective intervention. However, it must be noted that this study has not elucidated the specific upstream molecular mechanisms by which CBD regulates miR-320-3p, which represents a

limitation of this research and provides direction for future studies. Based on the literature, we propose the following potential mechanism: under the stimulation of TBI or the ferroptosis activator erastin, transcription factors, including ELK1 and p53, may be activated within the cell, subsequently binding to the promoter region of miR-320-3p and directly enhancing its transcriptional activity [44–47]. Furthermore, these transcription factors may promote the accumulation of mature miR-320-3p at the posttranscriptional level by modulating miRNA processing proteins such as DGCR8, thereby influencing the processing and maturation of its precursors, pri-miR-320-3p and premiR-320-3p [48,49]. Intervention by CBD may antagonize the aforementioned stress signals at the transcriptional level by inhibiting NF- κ B activation or modulating specific transcription factors, thereby downregulating miR-320-3p expression and ultimately contributing to its neuroprotective effects [50,51].

Negr1 is a neuronal growth regulatory factor closely associated with neuronal development and growth. Studies have found that *Negr1*-knockout mice exhibit more pronounced depressive and anxious behaviors than wild-type mice, accompanied by reduced hippocampal neurogenesis [52]. Additionally, research has shown that Negr1 is also involved in neuronal inflammation and apoptotic responses. In models of neuronal apoptosis induced by oxygen-glucose deprivation (OGD), Negr1 expression is significantly reduced. When Negr1 is overexpressed, apoptotic and inflammatory responses are alleviated [53]. In the OGD model of SH-SY5Y cells, Negr1 expression was suppressed, with increased expression of Bax protein and decreased expression of Bcl-2 protein. Overexpression of miR-181a-5p further exacerbates apoptosis. Interestingly, overexpression of Negr1, which is a target gene of miR-181a-5p, reverses miR-181a-5p-induced apoptosis [54]. Ferroptosis also exhibits a certain correlation with inflammation, as some inflammatory cytokines, such as TNF- α , PGE2, and IL-1 β , can directly affect GPx4 activity in cancer cells, further inducing ferroptosis [55]. Ferroptosis also has a pro-inflammatory effect. Studies have shown that ferroptosis can increase the expression of PTGS2 and promote the secretion of inflammatory cytokines [56]. Based on relevant reports, it can be inferred that Negr1 may regulate ferroptosis. This study confirmed that CBD rescued the TBI-induced downregulation of Negr1. However, when *Negr1* was knocked down, the inhibitory effects of CBD on ferroptosis and lipid peroxidation were markedly attenuated, indicating that Negr1 serves as a crucial downstream effector molecule in CBD's antiferroptotic actions.

Extracellular signal-regulated kinase (ERK), a member of the mitogen-activated protein kinase (MAPK) family, serves as a key effector of the small GTPase RAS [57]. The ERK signaling pathway plays a central role in intracellular signal transduction, regulating critical physiological processes such as cell proliferation, differentiation, and survival in response to various extracellular stimuli. Notably, phosphorylated ERK (p-ERK) exhibits a "double-edged sword" characteristic in neuronal injury. Transient and moderate activation of the ERK pathway is widely recognized as an important pro-survival mechanism, contributing to neuronal development, synaptic plasticity, and functional maintenance [58]. However, under severe or sustained stress conditions such as TBI, oxidative stress and inflammatory factors can lead to persistent overactivation of ERK signaling, shifting its role from protective to maladaptive [58]. This aberrant activation further downregulates the antiapoptotic protein Bcl-2, activates proapoptotic factors such as caspase-3,

and consequently exacerbates neuronal apoptosis and neuroinflammatory responses [59]. Multiple studies have reported that administration of the MEK inhibitor PD98059 in TBI models effectively ameliorates brain tissue damage, further supporting the detrimental role of excessive ERK activation in neural injury [60,61]. Additionally, the ERK signaling pathway has been proven to be closely related to neuronal ferroptosis. When hippocampal neuroblasts undergo ferroptosis due to glutamate stimulation, it leads to sustained activation of ERK1 and ERK2, ultimately resulting in cell death. However, inhibiting the activation of ERK1/2 using a MEK inhibitor can prevent cell death [62]. In the present study, MEK and ERK were activated after TBI and in erastin-treated PC12 cells, whereas this activation was inhibited in CBD-treated TBI rats and CBD-treated PC12 cells, suggesting that the ERK signaling pathway plays a role in the neural regulation of TBI. Furthermore, the addition of miR-320-3p mimics or *Negr1* gene silencing suppressed CBD's regulatory effect on the MEK/ERK pathway, further suggesting that CBD may alleviate TBI-induced ferroptosis through the miR-320-3p/Negr1/ERK signaling axis. The molecular mechanisms by which the ERK signaling pathway induces ferroptosis remain incompletely elucidated. However, it is speculated that this process may be associated with the prolonged nuclear retention of phosphorylated ERK—specifically, linked to its kinetic profiles and the spatial distribution patterns of its substrates within distinct subcellular compartments [63].

To validate the targeting effect of miR-320-3p on *Negr1*, we designed rescue experiments to demonstrate their relationship and their regulatory roles in ferroptosis and the ERK signaling pathway in PC12 cells. After cotransfecting PC12 cells with siNegr1 and miR-320-3p inhibitor, we found that siNegr1 inhibited the upregulation of GPX4 and SLC7A11 protein and mRNA levels, as well as the downregulation of PTGS2 protein and mRNA levels, induced by miR-320-3p. Simultaneously, we also observed that siNegr1 reversed the downregulation of phosphorylated MEK and ERK caused solely by miR-320-3p inhibition. Based on these findings, we propose the following mechanistic pathway: under stress conditions induced by TBI and erastin, miR-320-3p is significantly activated and directly suppresses *Negr1* expression by targeting its mRNA. The downregulation of *Negr1* may subsequently disrupt lipid metabolism homeostasis and compromise cell membrane stability, collectively enhancing neuronal susceptibility to ferroptosis [64,65]. In contrast, CBD downregulates miR-320-3p, thereby relieving its inhibition of *Negr1* and restoring *Negr1* protein expression, which ultimately reestablishes the cellular defense mechanism against ferroptosis. In our *in vitro* studies, we demonstrated that siNegr1 led to increased expression of p-ERK, indicating its negative regulatory role in the ERK pathway. This suggests that the ERK pathway is downstream of the miR-320-3p/NEGR1 axis. However, these findings have not yet been validated in an *in vivo* setting, which we plan to address in our subsequent experiments. Literature reports indicate that the ERK signaling pathway can be activated by growth factor stimulation, mutations in Ras or Raf, or oxidative inactivation of phosphorylated ERK [66]. *Negr1* may activate ERK through interaction with surface receptors. However, the mechanism by which *Negr1* regulates ERK remains largely unknown.

In the present study, CBD intervention significantly ameliorated the inhibition of *Negr1* induced by TBI and erastin and inhibited the activation of the ERK signaling pathway. miR-320-3p mimics and *Negr1* gene silencing suppressed the therapeutic effects of CBD on

erastin-induced ferroptosis in PC12 cells and the activation of the ERK signaling pathway, which could be reversed by CBD. This suggests that miR-320-3p and *Negr1* are important targets of CBD and that CBD exerts neuroprotective effects through the miR-320-3p/Negr1/ERK signaling axis. We explored the potential therapeutic role of miR-320-3p/Negr1 in TBI for the first time and provided evidence for the critical function of the *Negr1*/ERK signaling axis in TBI treatment. In summary, CBD has potential pharmacological value for anti-TBI therapy, and this study also provides evidence for the application of miR-320-3p in the field of TBI, offering more opportunities to explore the therapeutic mechanisms of TBI. However, there are limitations in this study. The regulation of erastin-induced ferroptosis by CBD through the miR-320-3p/Negr1/ERK signaling axis was only validated in cells. Future studies may require overexpressing miR-320-3p or knocking out *Negr1* in rats to explore the interventional effects of CBD on TBI, and more experiments are needed to demonstrate the interventional effects on neurological impairment in rats, thereby providing stronger evidence for the neuroprotective role of miR-320-3p in TBI.

Data Availability

The research group has uploaded the raw data to NCBI (<https://static.pubmed.gov/portal/portal.fcgi/>) with the accession number PRJNA1433805. All other data can be accessed upon reasonable request from the corresponding authors.

Author contributions

Jiali Li: Conceptualization, Methodology, Writing-Original Draft, Investigation, Visualization. Yan Cao: Validation, Methodology, Writing-Review & Editing. Tengan Ling: Data Curation. Xinyuan Luo: Data Curation. Hengxi Li: Formal analysis. Aiping Yin: Formal analysis. Hongyan Jiang: Supervision. Xiaobing Guo: Resources. Li Yang: Supervision. Haiying Wu: Writing-Review & Editing, Funding acquisition. Ping Li: Writing-Review & Editing, Funding acquisition, Resources.

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Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. Liu J, Xiong X, Sui Y. Isoliquiritigenin attenuates neuroinflammation in traumatic brain injury in young rats. *Neuroimmunomodulation* 2019, 26: 102–110
2. Cao Y, Li H, Li J, Ling T, Yin A, Luo X, Zhou Y, *et al.* Cannabidiol alleviates the inflammatory response in rats with traumatic brain injury through the PGE₂-EP2-cAMP-PKA signaling pathway. *Acta Biochim Biophys Sin* 2025, 57: 758–769
3. Dewan MC, Rattani A, Gupta S, Baticulon RE, Hung YC, Punchak M, Agrawal A, *et al.* Estimating the global incidence of traumatic brain injury. *J Neurosurg* 2019, 130: 1080–1097
4. Maas AIR, Fitzgerald M, Gao G, Gupta D, Hutchinson P, Manley GT, Menon DK. Traumatic brain injury over the past 20 years: research and

- clinical progress. *Lancet Neurol* 2022, 21: 768–770
5. Maas AIR, Menon DK, Manley GT, Abrams M, Åkerlund C, Andelic N, Aries M, *et al.* Traumatic brain injury: progress and challenges in prevention, clinical care, and research. *Lancet Neurol* 2022, 21: 1004–1060
 6. Wei Z, Yu H, Zhao H, Wei M, Xing H, Pei J, Yang Y, *et al.* Broadening horizons: ferroptosis as a new target for traumatic brain injury. *Burns Trauma* 2024, 12: tkad051
 7. Xie BS, Wang YQ, Lin Y, Mao Q, Feng JF, Gao GY, Jiang JY. Inhibition of ferroptosis attenuates tissue damage and improves long-term outcomes after traumatic brain injury in mice. *CNS Neurosci Ther* 2019, 25: 465–475
 8. Wu C, Du M, Yu R, Cheng Y, Wu B, Fu J, Tan W, *et al.* A novel mechanism linking ferroptosis and endoplasmic reticulum stress via the circPtn14/miR-351-5p/5-LOX signaling in melatonin-mediated treatment of traumatic brain injury. *Free Radical Biol Med* 2022, 178: 271–294
 9. Lowin T, Tingting R, Zurmahr J, Classen T, Schneider M, Pongratz G. Cannabidiol (CBD): a killer for inflammatory rheumatoid arthritis synovial fibroblasts. *Cell Death Dis* 2020, 11: 714
 10. McDonagh MS, Morasco BJ, Wagner J, Ahmed AY, Fu R, Kansagara D, Chou R. Cannabis-based products for chronic pain. *Ann Intern Med* 2022, 175: 1143–1153
 11. Kicman A, Toczek M. The effects of cannabidiol, a non-intoxicating compound of cannabis, on the cardiovascular system in health and disease. *Int J Mol Sci* 2020, 21: 6740
 12. Hardy J, Greer R, Huggett G, Kearney A, Gurgenci T, Good P. Phase IIb randomized, placebo-controlled, dose-escalating, double-blind study of cannabidiol oil for the relief of symptoms in advanced cancer (MedCan1-CBD). *J Clin Oncol* 2023, 41: 1444–1452
 13. Devinsky O, Cross JH, Wright S. Trial of cannabidiol for drug-resistant seizures in the dravet syndrome. *N Engl J Med* 2017, 377: 699–700
 14. Abu-Sawwa R, Scutt B, Park Y. Emerging use of epidiolex (cannabidiol) in epilepsy. *J Pediatr Pharmacol Ther* 2020, 25: 485–499
 15. Ferreira-Junior NC, Campos AC, Guimarães FS, Del-Bel E, Zimmermann PMR, Brum Junior L, Hallak JE, *et al.* Biological bases for a possible effect of cannabidiol in Parkinson's disease. *Braz J Psychiatry* 2020, 42: 218–224
 16. Junior NCF, Dos-Santos-Pereira M, Guimarães FS, Del Bel E. Cannabidiol and cannabinoid compounds as potential strategies for treating Parkinson's disease and L-DOPA-induced dyskinesia. *Neurotox Res* 2020, 37: 12–29
 17. Iuvone T, Esposito G, Esposito R, Santamaria R, Di Rosa M, Izzo AA. Neuroprotective effect of cannabidiol, a non-psychoactive component from *Cannabis sativa*, on β -amyloid-induced toxicity in PC12 cells. *J Neurochem* 2004, 89: 134–141
 18. Salvatori B, Biscarini S, Morlando M. Non-coding RNAs in nervous system development and disease. *Front Cell Dev Biol* 2020, 8: 273
 19. Sun P, Liu DZ, Jickling GC, Sharp FR, Yin KJ. MicroRNA-based therapeutics in central nervous system injuries. *J Cereb Blood Flow Metab* 2018, 38: 1125–1148
 20. Adlakha YK, Saini N. Brain microRNAs and insights into biological functions and therapeutic potential of brain enriched miRNA-128. *Mol Cancer* 2014, 13: 33
 21. Hashimoto T, Yamada M, Maekawa S, Nakashima T, Miyata S. IgLON cell adhesion molecule Kilon is a crucial modulator for synapse number in hippocampal neurons. *Brain Res* 2008, 1224: 1–11
 22. Sanz R, Ferraro GB, Fournier AE. IgLON cell adhesion molecules are shed from the cell surface of cortical neurons to promote neuronal growth. *J Biol Chem* 2015, 290: 4330–4342
 23. Muta Y, Matsuda M, Imajo M. Divergent dynamics and functions of ERK MAP kinase signaling in development, homeostasis and cancer: lessons from fluorescent bioimaging. *Cancers* 2019, 11: 513
 24. Kolch W. Coordinating ERK/MAPK signalling through scaffolds and inhibitors. *Nat Rev Mol Cell Biol* 2005, 6: 827–837
 25. Houde N, Beuret L, Bonaud A, Fortier-Beaulieu SP, Truchon-Landry K, Aoidi R, Pic É, *et al.* Fine-tuning of MEK signaling is pivotal for limiting B and T cell activation. *Cell Rep* 2022, 38: 110223
 26. Chen W, Zhou X, Meng M, Pan X, Huang L, Chen C. Hyperbaric oxygen improves cerebral ischemia-reperfusion injury in rats via inhibition of ferroptosis. *J Stroke Cerebrovasc Dis* 2023, 32: 107395
 27. Cao S, Wei J, Cai Y, Xiong Z, Li J, Jiang Z, Zhou X, *et al.* Network pharmacology prediction and experimental verification for anti-ferroptosis of edaravone after experimental intracerebral hemorrhage. *Mol Neurobiol* 2023, 60: 3633–3649
 28. Jiang H, Li H, Cao Y, Zhang R, Zhou L, Zhou Y, Zeng X, *et al.* Effects of cannabinoid (CBD) on blood brain barrier permeability after brain injury in rats. *Brain Res* 2021, 1768: 147586
 29. Dixon SJ, Lemberg KM, Lamprecht MR, Skouta R, Zaitsev EM, Gleason CE, Patel DN, *et al.* Ferroptosis: an iron-dependent form of nonapoptotic cell death. *Cell* 2012, 149: 1060–1072
 30. Zhang X, Hou L, Guo Z, Wang G, Xu J, Zheng Z, Sun K, *et al.* Lipid peroxidation in osteoarthritis: focusing on 4-hydroxynonenal, malondialdehyde, and ferroptosis. *Cell Death Discov* 2023, 9: 320
 31. Cui J, Zhou Q, Yu M, Liu Y, Teng X, Gu X. 4-Tert-butylphenol triggers common carp hepatocytes ferroptosis via oxidative stress, iron overload, SLC7A11/GSH/GPX4 axis, and ATF4/HSPA5/GPX4 axis. *Ecotoxicol Environ Saf* 2022, 242: 113944
 32. Zhang XD, Liu ZY, Wang MS, Guo YX, Wang XK, Luo K, Huang S, *et al.* Mechanisms and regulations of ferroptosis. *Front Immunol* 2023, 14: 1269451
 33. Stockwell BR, Friedmann Angeli JP, Bayir H, Bush AI, Conrad M, Dixon SJ, Fulda S, *et al.* Ferroptosis: a regulated cell death nexus linking metabolism, redox biology, and disease. *Cell* 2017, 171: 273–285
 34. Rui T, Wang H, Li Q, Cheng Y, Gao Y, Fang X, Ma X, *et al.* Deletion of *ferritin H* in neurons counteracts the protective effect of melatonin against traumatic brain injury-induced ferroptosis. *J Pineal Res* 2021, 70: e12704
 35. Huang L, He S, Cai Q, Li F, Wang S, Tao K, Xi Y, *et al.* Polydatin alleviates traumatic brain injury: role of inhibiting ferroptosis. *Biochem Biophys Res Commun* 2021, 556: 149–155
 36. Rupaimoole R, Slack FJ. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. *Nat Rev Drug Discov* 2017, 16: 203–222
 37. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004, 116: 281–297
 38. Redell JB, Moore AN, Ward III NH, Hergenroeder GW, Dash PK. Human traumatic brain injury alters plasma microRNA levels. *J Neurotrauma* 2010, 27: 2147–2156
 39. Yang Y, Wang Y, Li P, Bai F, Liu C, Huang X. Serum exosomes miR-206 and miR-549a-3p as potential biomarkers of traumatic brain injury. *Sci Rep* 2024, 14: 10082
 40. Jia M, Guo X, Liu R, Sun L, Wang Q, Wu J. Overexpress miR-132 in the brain parenchyma by a non-invasive way improves tissue repairment and releases memory impairment after traumatic brain injury. *Cell Mol Neurobiol* 2023, 44: 5
 41. Zhang H, Xing Z, Zheng J, Shi J, Cui C. Ursolic acid ameliorates traumatic brain injury in mice by regulating microRNA-141-mediated PDCD4/PI3K/AKT signaling pathway. *Int Immunopharmacol* 2023, 120: 110258
 42. Cao L, Chai S. miR-320-3p is involved in morphine pre-conditioning to protect rat cardiomyocytes from ischemia/reperfusion injury through targeting Akt3. *Mol Med Rep* 2020, 22: 1480–1488
 43. Sun Q, Liang R, Li M, Zhou H. Circ_UTRN ameliorates caerulein-induced

- acute pancreatitis *in vitro* via reducing inflammation and promoting apoptosis through miR-320-3p/PTK2 axis. *J Pharmacy Pharmacol* 2022, 74: 861–868
44. Liang Y, Li S, Tang L. MicroRNA 320, an anti-oncogene target miRNA for cancer therapy. *Biomedicines* 2021, 9: 591
 45. Nguyen MT, Lee W. MiR-320-3p regulates the proliferation and differentiation of myogenic progenitor cells by modulating actin remodeling. *Int J Mol Sci* 2022, 23: 801
 46. Huang C, Yang M, Deng J, Li P, Su W, Jiang R. Upregulation and activation of p53 by erastin-induced reactive oxygen species contribute to cytotoxic and cytostatic effects in A549 lung cancer cells. *Oncol Rep* 2018, 40: 2363
 47. Hu B, Liu C, Bramlett H, Sick TJ, Alonso OF, Chen S, Dietrich WD. Changes in TrkB-ERK1/2-CREB/Elk-1 pathways in hippocampal mossy fiber organization after traumatic brain injury. *J Cereb Blood Flow Metab* 2004, 24: 934–943
 48. Kim BM, Choi MY. Non-canonical microRNAs miR-320 and miR-702 promote proliferation in Dgcr8-deficient embryonic stem cells. *Biochem Biophys Res Commun* 2012, 426: 183–189
 49. Ding SC, Shi CJ, Pang FX, Wen RJ, Li N, Mai YX, Zhou ST, *et al.* HOTTIP suppresses ferroptosis via mediating DGCR8/miR-214-3p/GPX4 regulatory axis in osteosarcoma. *Oncol Rep* 2025, 54: 1–13
 50. Ilhan I, Asci H, Candan IA, Savran M, Imeci OB, Sevuk MA. Cannabidiol mitigates methotrexate-induced hepatic injury via SIRT-1/p53 signaling and mitochondrial pathways: reduces oxidative stress and inflammation. *Drug Chem Toxicol* 2025, 48: 210–218
 51. Meng F, Zhang Z, Chen W, Huang G, He A, Hou C, Long Y, *et al.* MicroRNA-320 regulates matrix metalloproteinase-13 expression in chondrogenesis and interleukin-1 β -induced chondrocyte responses. *Osteoarthritis Cartilage* 2016, 24: 932–941
 52. Noh K, Lee H, Choi TY, Joo Y, Kim SJ, Kim H, Kim JY, *et al.* Negr1 controls adult hippocampal neurogenesis and affective behaviors. *Mol Psychiatry* 2019, 24: 1189–1205
 53. Qiu L, He J, Chen H, Xu X, Tao Y. CircDLGAP4 overexpression relieves oxygen-glucose deprivation-induced neuronal injury by elevating NEGR1 through sponging miR-503-3p. *J Mol Histol* 2022, 53: 321–332
 54. Yan Y, Chen L, Zhou J, Xie L. SNHG12 inhibits oxygen-glucose deprivation-induced neuronal apoptosis via the miR-181a-5p/NEGR1 axis. *Mol Med Rep* 2020, 22: 3886
 55. Kim S, Keku TO, Martin C, Galanko J, Woosley JT, Schroeder JC, Satia JA, *et al.* Circulating levels of inflammatory cytokines and risk of colorectal adenomas. *Cancer Res* 2008, 68: 323–328
 56. Yang WS, SriRamaratnam R, Welsch ME, Shimada K, Skouta R, Viswanathan VS, Cheah JH, *et al.* Regulation of ferroptotic cancer cell death by GPX4. *Cell* 2014, 156: 317–331
 57. Mendoza MC, Vilela M, Juarez JE, Blenis J, Danuser G. ERK reinforces actin polymerization to power persistent edge protrusion during motility. *Sci Signal* 2015, 8: ra47
 58. Wang X, Wang Q, Hou L, Wei G, He C, Li H, Liu L. Advances in ERK signaling pathway in traumatic brain injury: mechanisms and therapeutic potential. *Neurochem Res* 2025, 50: 191
 59. Tan Z, Chen L, Ren Y, Jiang X, Gao W. Neuroprotective effects of FK866 against traumatic brain injury: involvement of p38/ERK pathway. *Ann Clin Transl Neurol* 2020, 7: 742–756
 60. Lu KT, Wang YW, Wo YYP, Yang YL. Extracellular signal-regulated kinase-mediated IL-1-induced cortical neuron damage during traumatic brain injury. *Neurosci Lett* 2005, 386: 40–45
 61. Zhao Y, Luo P, Guo Q, Li S, Zhang L, Zhao M, Xu H, *et al.* Interactions between SIRT1 and MAPK/ERK regulate neuronal apoptosis induced by traumatic brain injury *in vitro* and *in vivo*. *Exp Neurol* 2012, 237: 489–498
 62. Luo Y, DeFranco DB. Opposing roles for ERK1/2 in neuronal oxidative toxicity. *J Biol Chem* 2006, 281: 16436–16442
 63. Sugiura R, Satoh R, Takasaki T. ERK: a double-edged sword in cancer. ERK-dependent apoptosis as a potential therapeutic strategy for cancer. *Cells* 2021, 10: 2509
 64. Yoo A, Joo Y, Cheon Y, Lee SJ, Lee S. Neuronal growth regulator 1 promotes adipocyte lipid trafficking via interaction with CD36. *J Lipid Res* 2022, 63: 100221
 65. Cheon Y, Yoo A, Seo H, Yun SY, Lee H, Lim H, Kim Y, *et al.* Na/K-ATPase beta1-subunit associates with neuronal growth regulator 1 (NEGR1) to participate in intercellular interactions. *BMB Rep* 2021, 54: 164–169
 66. Ratan RR. The chemical biology of ferroptosis in the central nervous system. *Cell Chem Biol* 2020, 27: 479–498