

Research paper

Cerebral iron deficiency may induce depression through downregulation of the hippocampal glucocorticoid-glucocorticoid receptor signaling pathway

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ABSTRACT

Background: Iron is a trace essential element to sustain the normal neurological function of human. Many researches had reported the involvement of iron deficiency (ID) in neural development and cognitive functions. However, the role of ID in pathogenesis of depression and its underlying mechanism are still unclear.

Methods: In this study, we first used chronic unpredictable mild stress (CUMS) and iron deprivation mouse models to clarify the pathogenesis role of cerebral ID in depression. Then the role of hippocampal glucocorticoid (GC)-glucocorticoid receptor (GR) pathway in cerebral ID induced depression were elucidated in iron deprivation mice and iron deficiency anemia patients.

Results: Our results revealed that both CUMS and iron deprivation could induce cerebral ID in mice, and combination of iron deprivation and CUMS could accelerate the onset and aggravate the symptoms of depression in mice. In hippocampus, ID led to neuronal injury and neurogenesis decrease, which might be related to downregulation of GC-GR signaling pathway caused GR dysfunction, thereby inhibiting the negative feedback regulation function of hippocampus on hypothalamic-pituitary-adrenal (HPA) axis. Moreover, the overactivity of HPA axis in iron deprivation mice and iron deficiency anemia patients also confirmed GR dysfunction.

Limitations: Iron deprivation led to food and water intake decrease of mice, which may affect the behavioral test. In addition, we mainly evaluated the role of hippocampal ID in depression, and the number of iron deficiency anemia patients was limited.

Conclusions: Our results identified that cerebral iron homeostasis was a key factor for maintaining mental stability.

1. Introduction

Depression, which characterized by sadness, pessimism, suicidal

attempts or behavior, is a psychological disease that poses a serious threat to the human health (Nguyen et al., 2019). The global prevalence of depression is 4.7 %, making it the most burdensome health condition

Abbreviations: GC, glucocorticoid; GR, glucocorticoid receptor; ID, iron deficiency; CUMS, chronic unpredictable mild stress; HPA, hypothalamic-pituitary-adrenal; Hsp, heat shock protein; BAG-1, Bcl-2-associated athanogene-1; CHP-1, cysteine- and histidine-rich domains containing protein-1; FKBP51/52, FK506 binding protein of 51/52-kDa; CRH, corticotropin releasing hormone; DA, dopamine; 5-HT, 5-hydroxytryptamine; NE, noradrenalin; CLIA, cortisol chemiluminescence immunoassay; ACTH, adrenocorticotrophic hormone; TST, tail suspension test; FST, forced swimming test; OFT, open field test; SCT, sucrose consumption test; MRM, multiple reaction monitoring; ESI, electrospray ionization; ICP-MS, inductively coupled plasma MS.

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worldwide (Charlson et al., 2013). Results of recent studies show an association between nutrients and development of mental disease (Nguyen et al., 2019; Salehi-Abargouei et al., 2018). Iron is an essential trace element for the human body. Additionally, iron is an important element for the normal neurological functioning among its numerous biological effects (Mills et al., 2017), and iron level in human body is very sensitive to the change of iron intake (Wojciak, 2013). Recent clinical studies showed that the pathogenesis of depression might be related to iron disorder (Dziembowska et al., 2018). For instance, healthy volunteers with insufficient iron intake showed depressive symptoms (Wojciak, 2013). The patients with postpartum depression, pregnancy depression, diabetes depression, post-stroke depression, restless leg syndrome depression showed a decrease in serum iron levels (Wassef et al., 2018; Szkup et al., 2017; Woldetensay et al., 2018; Dama et al., 2017; Bergis et al., 2019; Zhu et al., 2016; Zhuang et al., 2019). Moreover, in patients with major depressive disorder, their brain iron depositions were significantly different from those in patients with mild-moderate depression and healthy volunteers (Yao et al., 2017; Dean et al., 2018). However, there also some clinical studies revealed the opposite results (Richardson et al., 2015; Armony-Sivan et al., 2012) or found no relationship between iron and depression (Li et al., 2017; Cheng et al., 2019). A consensus regarding the relationship between iron and depression has not been established thus far (Yao et al., 2017). Additionally, the role of iron in the pathogenesis of depression remains to be clarified.

Findings from an increasing number of studies indicate that hippocampal GR dysfunction is particularly important in depression (Zhang et al., 2016). As a ligand-dependent steroid nuclear receptor, GR performs its function through the GC-GR signaling pathway. At first, heat shock proteins (Hsps) Hsp40 and Hsp70 in the cytoplasm combine with GR to form the GR-Hsp40/Hsp70 complex, and thus facilitate GR folding and localization of Hsp90. As a client protein of Hsp90, GR can bind to GC only after combining with the intermediate domain of Hsp90 to form a GR-Hsp90 complex and produce conformational changes in protein (Ratajczak et al., 2015; Sinclair et al., 2013; Maeng et al., 2013). While, for co-chaperons, Bcl-2-associated athanogene-1 (Bag-1) not only blocks the formation of GR-Hsp40/Hsp70 complex, but also prevents the complex localization to Hsp90 (Maeng et al., 2013). Cysteine- and histidine-rich domains containing protein-1 (CHP-1) activates the ATPase activity of Hsp90 to promote the combination of Hsp90 and GR (Hong et al., 2013). Additionally, FK506-binding protein of 51-kDa (FKBP51) and p23 combine with the GR-Hsp90 complex to stabilize the GR in a high affinity state. After GC combines with the GR-Hsp90 complex, FKBP52 will displace FKBP51 and assist the nuclear translocation of GC-GR heterocomplex to inhibit the gene transcription of the corticotropin releasing hormone (CRH) in the nucleus (Zgajnar et al., 2019; Storer et al., 2011). However, GR dysfunction inhibits the negative feedback regulation function of the hippocampus to HPA axis and causes release of GC (Nemeroff and Vale, 2005). Then, high concentrations of GC induce a series of neuroplasticity damage, including atrophy, apoptosis, and impairment of neurogenesis in the hippocampal neurons and cause depression (Pariante, 2006). Therefore, pathogenetic factors that downregulate the GC-GR signaling pathway to impair the function of hippocampal GR and inhibit the negative feedback regulation function to the HPA axis would be related to the pathogenesis of depression. However, whether iron disorder interfere with the GC-GR signaling pathway and cause GR dysfunction to induce depression has not been established thus far.

In this study, we first clarified the role of iron disorder in the pathogenesis of depression in CUMS model and iron deprivation model, and ID in hippocampus and prefrontal cortex was found to be the key player of depression. Iron deprivation and CUMS could work synergistically to decrease the iron levels in hippocampus and prefrontal cortex, leading to acceleration of onset and aggravation of symptoms of anxiety/depression in mice, which further confirmed the role of cerebral ID in depression. In hippocampus, the neuronal injury and neurogenesis

decrease caused by ID was related to GC-GR signaling pathway down-regulation caused GR dysfunction. In particular, overactivity of the HPA axis was also observed in iron deprivation mice and IDA patients, which confirmed that cerebral ID caused GR dysfunction damaged the negative feedback function of hippocampus on HPA axis and led to the onset of depression. Thus, our results identified that cerebral iron homeostasis is a key factor for maintaining mental stability, and ensuring appropriate intake of iron or giving iron for treatment might be a promising target for improving the prevention and treatment of depression.

2. Materials and methods

2.1. Animals

The c57BL/6 J mice were purchased from Huafukang (Beijing, China). The Kunming (KM) mice were supplied by the Experimental Animal Centre of Shenyang Pharmaceutical University (Shenyang, China). Animals were housed in groups of five mice per cage under standardized environmental conditions ($22 \pm 2^\circ\text{C}$, 12-h light/12-h dark cycle) with free access to food and water. All experiments were performed in accordance with the relevant guidelines and approved by Institutional Animal Care and Use Committee of Shenyang Pharmaceutical University (IACUC-C2019-9-20-107, IACUC-C2020-9-9-101).

2.2. CUMS model

We randomly assigned 48 adult male and female c57BL/6J mice to control group and CUMS group. CUMS was induced by subjecting the mice to various stressors and completely unpredictable in a random schedule for 8 weeks. The stressors included 24 h of cage tilting (45°), swimming in ice-cold water for 10 min, continuous illumination for 24 h, water or food deprivation for 24 h, white noise for 24 h, 24 h of wet bedding, 10 min of restraint stress, and 10 min of tail suspension.

2.3. Iron deprivation model

We randomly divided 60 adult male and female KM mice into control group and iron deprivation group, and the mice were given conventional feed and tap water or iron-free feed (Huafukang, China) and purified water (Wahaha, China) for 35 days, respectively. On the 35th day, the coat state of mice was rated. The scores of head, neck, dorsal coat, ventral coat, tail, forepaws, and hindpaws were added to obtain the total score (0 = well-groomed, 1 = unkempt) (Surget et al., 2009). The components of conventional feed and iron free feed were shown in supplementary Tables 1 and 2.

2.4. Combination model of iron deprivation and CUMS

We randomly divided 96 adult male and female c57BL/6J mice into control, iron deprivation, CUMS, and CUMS&iron deprivation groups. Mice in the control and CUMS groups were fed with conventional feed and tap water. Mice in the iron deprivation and CUMS&iron deprivation groups were fed with iron-free feed and purified water. To induce CUMS, the mice were subjected randomly subjected to various stressors for 8 weeks. The same stressors as those described above were used.

2.5. Assessment of anxiety/depression behaviors

We used video tracking system (Xinruan, China) to record animal behavior and analyze the data in a dark room to avoid background noise.

2.5.1. Open field test (OFT)

The open field was square ($50 \times 50\text{ cm}$), and the square was divided into peripheral zone and the central zone ($21 \times 21\text{ cm}$). Briefly, the mice were placed in one corner of the peripheral zone. Then, the spontaneous activity of mice was recorded for 5 min. After each test, we used water to

clean the open field to remove olfactory cues (Christoffel et al., 2012).

2.5.2. Tail suspension test (TST)

The mice were suspended individually on a metal rod at a height of 50 cm above the table. Mice were considered immobile only when their limbs do not move. The mice were suspended for 2 min at the beginning of test for adaptation. The immobility time was measured from 3 min to 6 min of the test (Steru et al., 1985).

2.5.3. Forced swimming test (FST)

The mice were individually placed in 4 plastic cylindrical tanks (height, 30 cm; diameter, 11 cm) filled with water ($24 \pm 1^\circ\text{C}$) to a depth of 25 cm for 6 min. Each mouse was judged as immobile when it ceased struggling and remained floating motionless in the water. The immobility time was measured during the final 4 min of the test (Porsolt et al., 1978).

2.5.4. Sucrose consumption test (SCT)

SCT was performed in the home cage and each cage contained 5 mice. Briefly, the mice were given two bottles of 1 % sucrose water within the first 24 h. In the second 24 h, the mice were given one bottle of 1 % sucrose water and one bottle of purified water. Then, the mice were fasted and water deprivation for 18 h. For the next 6 h, the mice were given a bottle of 1 % sucrose water and a bottle of purified water (Hoffman, 2016). We weighed the bottles of each cage to calculate the sucrose preference rate by using the following formula:

$$\text{Sucrose preference rate (\%)} = \frac{\text{Sucrose water consumption (g)}}{\text{Sucrose water consumption (g)} + \text{Tap water consumption (g)}} \times 100\%$$

2.6. Iron deprivation-induced eyelid ptosis and hypothermia

On the 35th day, the degree of ptosis and body temperature of mice were recorded. Reserpine, the monoamine neurotransmitters depletor, was used as the positive control (4.0 mg/kg, intraperitoneally). The ptosis rating scale was as follows: eyes open, 0; one-quarter closed, 1; half closed, 2; three-quarters closed, 3; and completely closed, 4 (Sanchez-Mateo et al., 2002). The thermistor thermometer was used to measure the rectal temperature of mice.

2.7. Barbering scale

Excessive barbering, is an abnormal behavior in which the mice pluck their own whiskers and body hair or those of their cage mates, is considered to be a behavior as a response to stress. The rating scale was shown as follows: no hair loss, 0; removal of whiskers, 1; removal of whiskers and facial hair, 2; removal of whiskers and facial hair that extends to the head, 3; removal of whiskers and hair on the face, head, and back, 4; removal of whiskers and hair on the face, head, back and stomach, 5 (Gannon et al., 2019; Garner et al., 2004).

2.8. Histopathological examination

The entire brain was fixed in 10 % buffered formalin before being subjected to processing procedures using the TKY-TSF automatic tissue processor and were embedded into paraffin (Taikang, China). A semi-automatic rotary microtome (Leica, Germany) was used to section the brain tissues into 5- μm -thick sections. These sections were mounted onto histological slides and dried overnight. Then, the sections were stained with Nissl staining. Histological analyses were performed under $200\times$

magnification using a Nikon DS-Ri2 light microscope (Nikon, Japan).

2.9. HPLC-MS/MS analysis of the monoamine neurotransmitters

Briefly, the brain was firstly dissected on ice into five brain regions, including hippocampus, prefrontal cortex, parietal cortex, temporal cortex, and diencephalon to determine the concentrations of noradrenaline (NE), dopamine (DA), and 5-hydroxytryptamine (5-HT). We selected isoproterenol as internal standard (IS). The chromatographic analysis was performed on Acquity™ HPLC system (Waters, USA) and triple quadrupole mass spectrometer (Waters, USA) coupled with an electrospray ionization (ESI) source in the positive mode. We used gradient elution on a Kinetex XB C₁₈ column ($100 \times 4.6 \text{ mm}$, $2.6 \mu\text{m}$) (Phenomenex, USA) with a mobile phase (0.1 % formic acid and methanol) (Xu et al., 2018).

2.10. Immunofluorescence

The brain was post-fixed overnight in 4 % paraformaldehyde. Further, the tissue was sectioned in 25- μm -thick section freezing microtome (Shandon, UK). The sections were incubated with the primary antibody (anti-Ki67 or anti-DCX, Boster China, Abcam, USA) overnight at 4°C , and incubated with the secondary antibody (APEX-BIO, China) for 1 h. Finally, the sections were washed in 0.1 M PBS and cover-slipped with anti-fade mounting medium (Beyotime, China). Images of Ki67- and DCX-double staining cells in the dentate gyrus of hippocampus were digitized using an BX40 microscope (Olympus, Japan) fitted with a $10\times$ objective lens. Then, Ki67- and DCX-double

staining cells were automatically quantified using the Image J software following the threshold, which could isolate positive cells from the background.

2.11. Immunohistochemistry staining

For immunohistochemistry staining, we conducted the experiment according to the instruction of DS double stain test kit (ZSGB, China). Briefly, the brain from mice were embedded in paraffin, sectioned and deparaffinized. Then the sections were stained with antibodies of Ki67 and DCX (Boster, China; Abcam, USA) overnight at 4°C . The secondary antibodies were incubated at 37°C for 30 min. Then visualization was achieved with horseradish peroxidase-labeled diaminobenzidine (Ki67) and alkaline phosphatase-labeled fast red (DCX) for at least 5 min. Then the slides were observed on a BX40 microscope (Olympus, Japan) fitted with a $10\times$ objective lens and camera (Germany). Then, Ki67- and DCX-double staining cells were automatically quantified using the Image J software.

2.12. Confocal fluorescence imaging

The sections were incubated with the primary antibody (anti-Hsp90, anti-GR, Abcam, USA) overnight at 4°C . Then, the sections were incubated with the secondary antibody for 1 h. Confocal fluorescence imaging to determine the colocalization of Hsp90 and GR was performed using FITC (488 nm) and 2-dodecylresorufin (561 nm) filter channel (NIS-Elements Viewer 4.0) (Zhang et al., 2021).

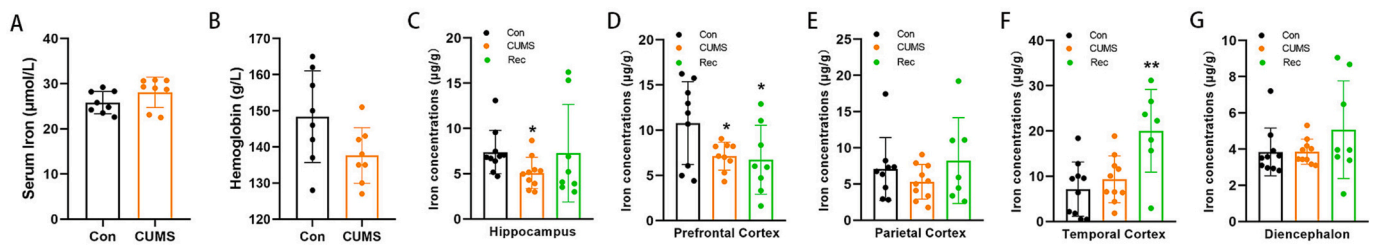


Fig. 1. CUMS decreased the iron levels in hippocampus and prefrontal cortex of the mice. Graphs show the changes of (A) serum iron and (B) hemoglobin in mice after suffering CUMS for 8 weeks ($n = 8$ mice/group). Statistical comparisons were carried out with independent sample *t*-test. Graphs show the changes of iron concentrations in (C) hippocampus, (D) prefrontal cortex, (E) parietal cortex, (F) temporal cortex, (G) diencephalon in mice after suffering CUMS for 8 weeks and recovered for another 8 weeks ($n = 7$ –10 mice/group). Statistical comparisons were carried out with ANOVA followed by Dunnett's *t*-test. * $p < 0.05$, ** $p < 0.01$ vs. Control group.

2.13. Western blot analysis

Hippocampus were manually homogenized in radio-immunoprecipitation assay buffer with a protease inhibitor and a phosphatase inhibitor. The protein concentrations were determined using the BCA assay (Beyotime, China). Protein extracts were resolved on 8 % and 12 % SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Merk Millipore, USA). Then the membranes were blocked in 5 % skimmed milk (Wonderson Dairy Co., China) and probed with primary antibodies (Antibodies against DCX, Ki67, GR, Hsp90, p23, FKBP51, FKBP52, Bag-1, and CHP-1, BOSTER, China) for 12 h at 4 °C and labeled with secondary antibodies. Protein bands were visualized using the enhance chemiluminescence (ECL) system (Sigma-Aldrich, USA) (Yin et al., 2011).

2.14. Quantification of iron levels using inductively coupled plasma MS (ICP-MS)

The brain tissue homogenates were weighed precisely and transferred into Teflon resolution pots. After adding 2 mL nitric acid, the resolution pots were capped and heated at 150 °C until the homogenates were clear and transparent. Then the homogenates were heated at 150 °C for another 30 min. After cooling, the solutions were diluted to 25 mL for ICP-MS assay. We used the 7500a triple quadrupole ICP-MS QQQ (Agilent, USA) equipped with microconcentric nebulizer, (Meinhard MicroMist, USA), Peltier-cooled double-pass spray chamber, standard torch, and auto sampler. The data were analyzed using the MassHunter workstation version 4.1 (Agilent, USA) to determine the total concentration of iron in the tissues.

2.15. ELISA assessment of corticosterone in mouse serum

Briefly, 40 μ L of the sample diluent and 10 μ L of the test sample were added to the testing well and gently mixed (Enzyme-link, China). Subsequently, 100 μ L of HRP-conjugated reagent was added and incubated at 37 °C for 60 min. After discarding the liquid and drying, the plate was washed for five times. After drying, we added the chromogen solution and incubated the plate in dark for 15 min. Then, we added 50 μ L of stop solution and read the absorbance at 450 nm (Gong et al., 2015).

2.16. Analysis of corticosterone levels in mouse feces using ultra HPLC

Fifty mg of fresh uncontaminated feces sample was added 480 μ L of extracting solution (methyl tertbutyl ether:methanol = 5:1; v:v). Then samples were grinded and centrifuged at 4 °C, 12,000 rpm for 15 min. The supernatant was dried in a vacuum concentrator and added extraction solution (dichloromethane:ethanol = 1:1) to re-dissolve the extraction. After ultrasonic treatment for 10 min, the sample was centrifuged at 4 °C, 12,000 rpm for 15 min. The supernatant was assayed using ultra HPLC (Agilent, USA) tandem mass spectrometry (Triple

TOF6600, AB Sciex, USA) with the processing software (Analyst TF 1.7, AB Sciex). We selected the Phenomenex Kinetex 1.7u C18 100A column (100 $\times$ 2.1 mm).

2.17. Examination of the cortisol and ACTH levels in IDA patients

We obtained the approval from Medical Ethical Committee of the General Hospital of Northern Theater Command (Shenyang, China), and informed consent from each patient. Blood samples were collected from 13 IDA patients and 12 healthy volunteers who visited the hospital from November 2021 to March 2022. The work was carried out in accordance with the Declaration of Helsinki. The inclusion criteria for IDA patients were as follows: both hemoglobin level and serum iron levels were lower than the normal reference range (hemoglobin, men: <120 g/L; women: < 110 g/L) (serum iron, 11.0–30.0 μ mol/L). Healthy volunteers were required to have hemoglobin and serum iron levels within the normal reference range (hemoglobin, men: 120–165 g/L; women, 110–150 g/L) (serum iron, 11.0–30.0 μ mol/L). According to the biorhythm of ACTH and cortisol release, we collected the blood samples at 8:00 am. Chemiluminescence immunoassay (CLIA) for serum cortisol and ACTH was performed at the General Hospital of Northern Theater Command using commercial kits according to the manufacturer's instructions.

2.18. Data analysis

The results were expressed as mean $\pm$ standard error of measurement (S.E.M.). We used the independent sample *t*-test for statistical analysis or one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test (SPSS 19.0, USA). Probability values of $p < 0.05$ were considered significant.

3. Results

3.1. CUMS decreased the iron levels in hippocampus and prefrontal cortex of the mice

We first used CUMS mice, the classical depression pathophysiological animal model, to investigate whether iron disorder is involved in depression. After 8 weeks of CUMS stress, the mice showed significant anxiety/depression behavior (Supplementary Fig. 1), while neither the serum iron nor the hemoglobin levels were affected by CUMS (Fig. 1A&B). However, CUMS induced a significant decrease in the iron levels in hippocampus and prefrontal cortex (Fig. 1C&D). Moreover, after stopping CUMS stress and recovering for another 8 weeks, the iron levels in hippocampus and prefrontal cortex of the recovered mice were still significantly lower than those in the control mice (Fig. 1C&D), and the recovered mice still showed anxiety/depression behavior (Supplementary Fig. 1). In addition, CUMS did not affect the iron levels in parietal cortex, temporal cortex and diencephalon (Fig. 1E&F&G). These results suggested that CUMS could lead to irreversible decrease of iron

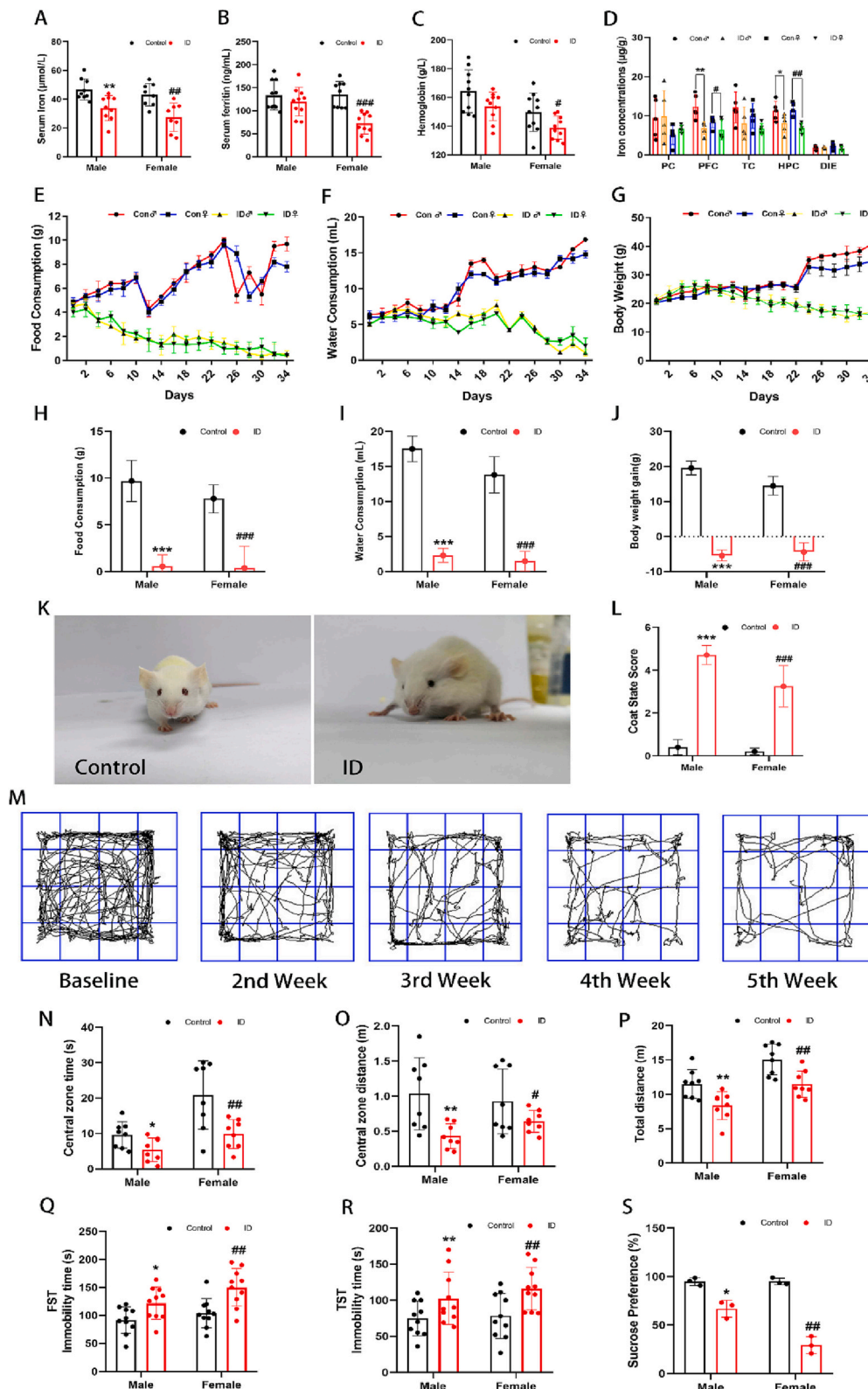


Fig. 2. Iron deprivation reduced peripheral and cerebral iron levels and led to anxiety/depression physical and behavioral changes in mice. The changes of (A)serum iron, (B)serum ferritin, (C)hemoglobin levels in mice after iron deprivation for 35 days ($n = 8-10$ mice/gender/group). (D) The changes of iron levels in different brains regions of mice after iron deprivation for 35 days ($n = 4-5$ mice/gender/group). Graphs show the changing trend of (E) food consumption, (F) water consumption, and (G) body weight of mice during iron deprivation, and changes of (H) food consumption, (I) water consumption, (J) body weight of mice, (K) coat state and (L) coat state scores of mice on the 35th day of iron deprivation (Data were presented as mean $\pm$ S.E.M). Graphs show the effects of iron deprivation for 35 days on (M) performance, (N)central zone time, (O) central zone distances, and (P) total distances in OFT, immobility time in (Q) FST and (R) TST ($n = 10$ mice/group/gender), and (S) sucrose preference rate ($n = 3$ cages/gender/group). All statistical comparisons were carried out with independent sample *t*-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. Control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. Control group.

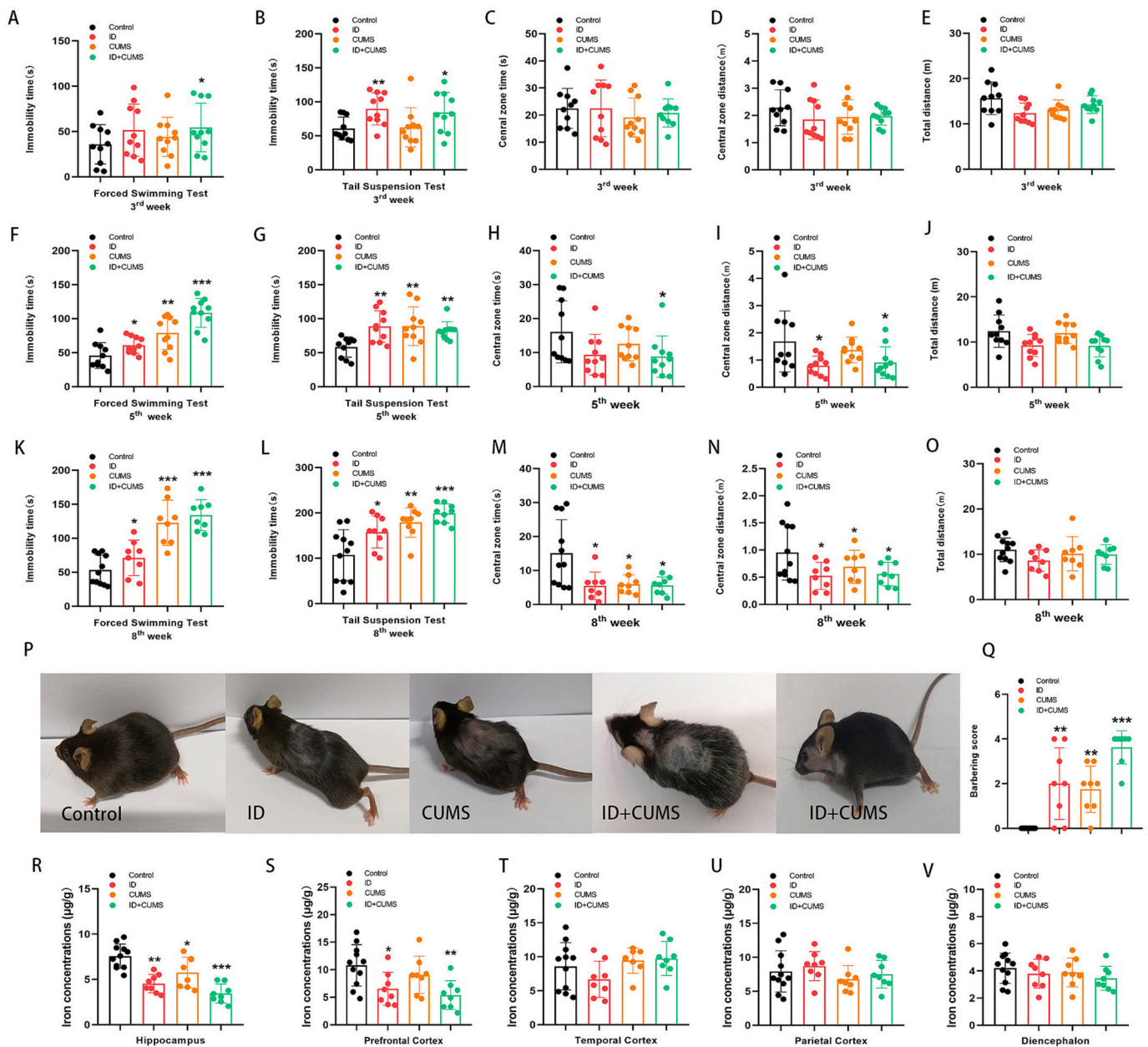


Fig. 3. Iron deprivation and CUMS synergistically decreased the iron levels in the hippocampus and prefrontal cortex and aggravated the anxiety/depression symptoms in mice. Graphs show the behavioral changes in FST, TST, and OFT at the 3rd week (A–E), the 5th week (F–J), and the 8th week (K–O) ($n = 10$ mice/group). Graphs show the (P) representative images of excessive barbering and (Q) barbering scores of mice after suffering from iron deficiency, CUMS or iron deficiency&CUMS for 8 weeks ($n = 8$ mice/group). Graphs show the iron concentrations in (R) hippocampus, (S) prefrontal cortex, (T) temporal cortex, (U) parietal cortex, and (V) diencephalon of mice ($n = 8–11$ mice/group). All statistical comparisons were carried out with ANOVA followed by Dunnett's t -test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. Control group.

levels in hippocampus and prefrontal cortex, which might be related to the onset of anxiety/depression behavior. Cerebral ID might play a key role in the pathogenesis of depression.

3.2. Iron deprivation reduced peripheral and cerebral iron levels and induced anxiety/depression physical and behavioral changes in mice

To further investigate the role of cerebral ID in pathogenesis of depression, we used iron deprivation model to induce ID in mice. After iron deprivation for 35 days, the serum iron, serum ferritin and hemoglobin levels of both male and female mice were decreased (Fig. 2A&B&C). Furthermore, both male and female mice showed significant decreased cerebral iron levels in hippocampus and prefrontal

cortex after iron deprivation (Fig. 2D). Along with the progression of iron deprivation, the mice showed significant anxiety/depression physical changes, including a slow increase in the food intake, water consumption, and body weight (Fig. 2E&F&G) and a significant deterioration in the coat state (Fig. 2K) of mice. On the 35th day, the iron-deficiency mice showed significantly lower food intake, water consumption, gain in body weight (Fig. 2H&I&J) and higher coat state scores (Fig. 2L) than the control mice.

In addition, along with the progress of iron deprivation, the performances of mice in the OFT were significantly changed (Fig. 2M). At the fifth week, the iron-deficiency mice showed shorter time spent and distances in the central zone, and shorter total distances than those of the control mice (Fig. 2N&O&P). The immobility time of iron deprivation

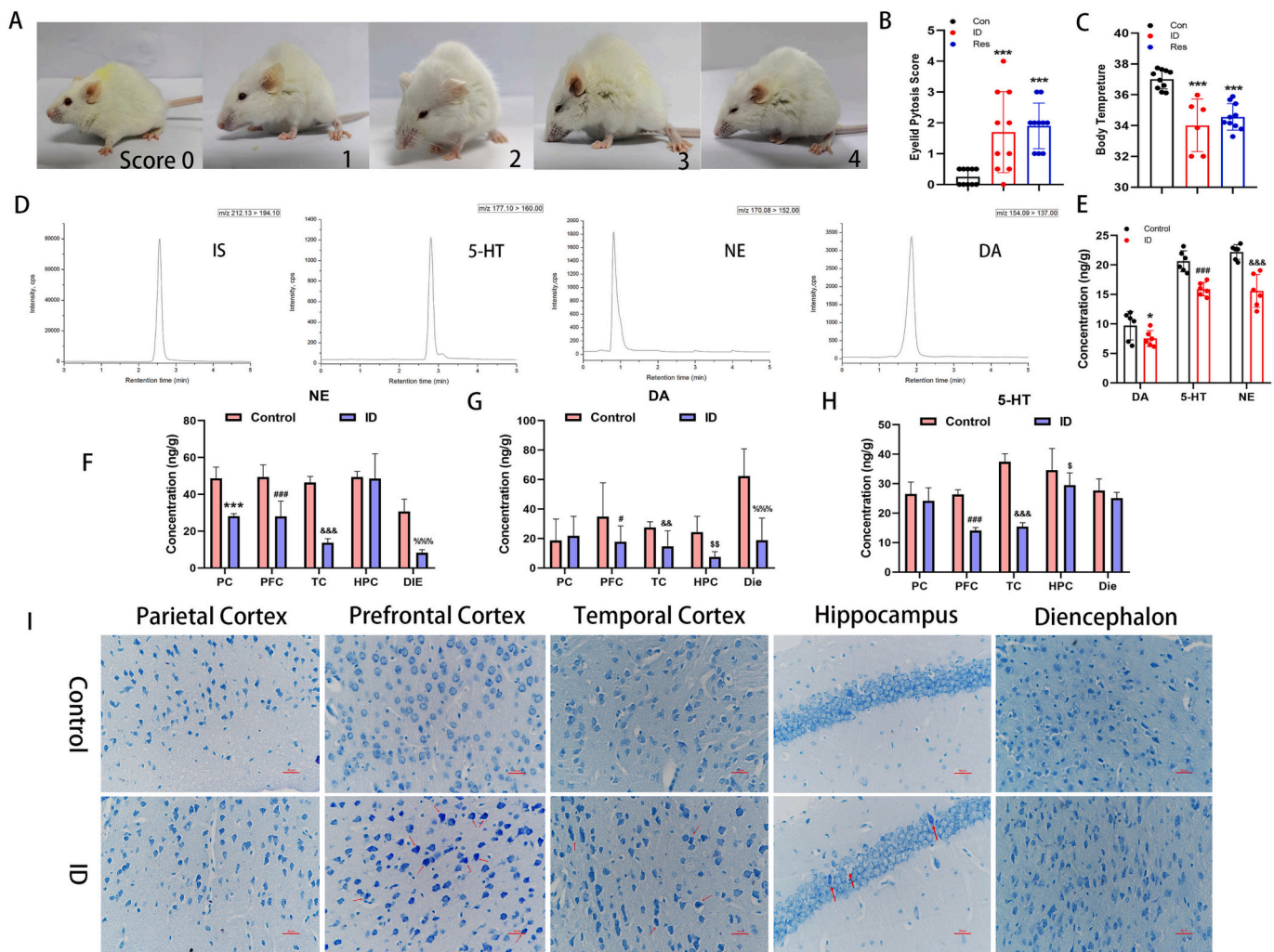


Fig. 4. Cerebral ID led to neuronal injury in the hippocampus, temporal cortex, and prefrontal cortex of iron deprivation mice. Graphs show (A) The rating scale of eyelid ptosis and the changes of (B) eyelid ptosis and (C) body temperature in mice after iron deprivation for 35 days ($n = 10$ mice/group). Reserpine was chosen as the positive control. Statistical comparisons were carried out with ANOVA followed by Dunnett's t -test. Graphs show (D) chromatograms of IS, 5-HT, NE, DA, (E) changes of monoamine neurotransmitters in whole brain, and changes of (F) NE, (G) DA and (H) 5-HT in different brain regions of mice after iron deprivation for 35 days ($n = 6-8$ mice/group). Statistical comparisons were carried out with independent sample t -test. (I) Representative images of Nissl staining in different brain regions of mice after iron deprivation for 35 days. * $p < 0.05$, *** $p < 0.001$ vs. Control group; # $p < 0.05$, ### $p < 0.001$ vs. Control group; & $p < 0.01$, && $p < 0.001$ vs. Control group; \$ $p < 0.05$, \$\$ $p < 0.01$ vs. Control group; % $p < 0.05$, %%% $p < 0.001$ vs. Control group.

mice in FST and TST were also significantly longer than those of the control mice (Fig. 2Q&R). Moreover, the sucrose preference rates of iron-deficiency mice were also significantly lower than those of the control mice (Fig. 2S). None of the above results showed gender differences. These results indicated that iron deprivation induced cerebral ID could lead to anxiety/depression physical and behavioral changes in mice.

3.3. Iron deprivation and CUMS synergistically decreased the iron levels in hippocampus and prefrontal cortex and accelerated the onset and aggravated the symptoms of anxiety/depression in mice

Since both iron deprivation and CUMS can decrease the iron levels in hippocampus and prefrontal cortex, we further compared the iron deprivation mice, CUMS mice, and iron deprivation&CUMS mice with control mice to clarify the role of cerebral ID in the pathogenesis of depression. Among the four groups, iron deprivation&CUMS mice first showed depression behavior, as early as the 3rd week of study (Fig. 3A&B). In both the 5th week and 8th week, the immobility time of iron deprivation&CUMS mice in both TST and FST were the longest (Fig. 3F&G&K&L). In OFT, the results also showed that iron

deprivation&CUMS mice spent the shortest time and distance in the central zone (Fig. 3H&I&M&N). The total distance of these mice in OFT was not significantly different (Fig. 3E&J&O). Additionally, for excessive barbering behavior, the bald patches in the fur and the average barbering scores of iron deprivation&CUMS mice were also the largest (Fig. 3P&Q). Since iron deprivation&CUMS mice had the lowest iron levels in the hippocampus and prefrontal cortex (Fig. 3R&S), and the iron level in the other brain regions did not show significant differences (Fig. 3T&U&V), all these results indicated that iron deprivation and CUMS could work synergistically to reduce the iron levels in hippocampus and prefrontal cortex, which may lead to acceleration of onset and aggravation of symptoms of anxiety/depression in mice. These findings further verified that cerebral ID, particularly ID in the hippocampus and prefrontal cortex, might be a key factor in the pathogenesis of depression.

3.4. Cerebral ID led to neuronal injury in hippocampus, temporal cortex, and prefrontal cortex of iron deprivation mice

As cerebral ID could induce anxiety/depression physical and behavioral changes in mice; thus, we used iron deprivation induced

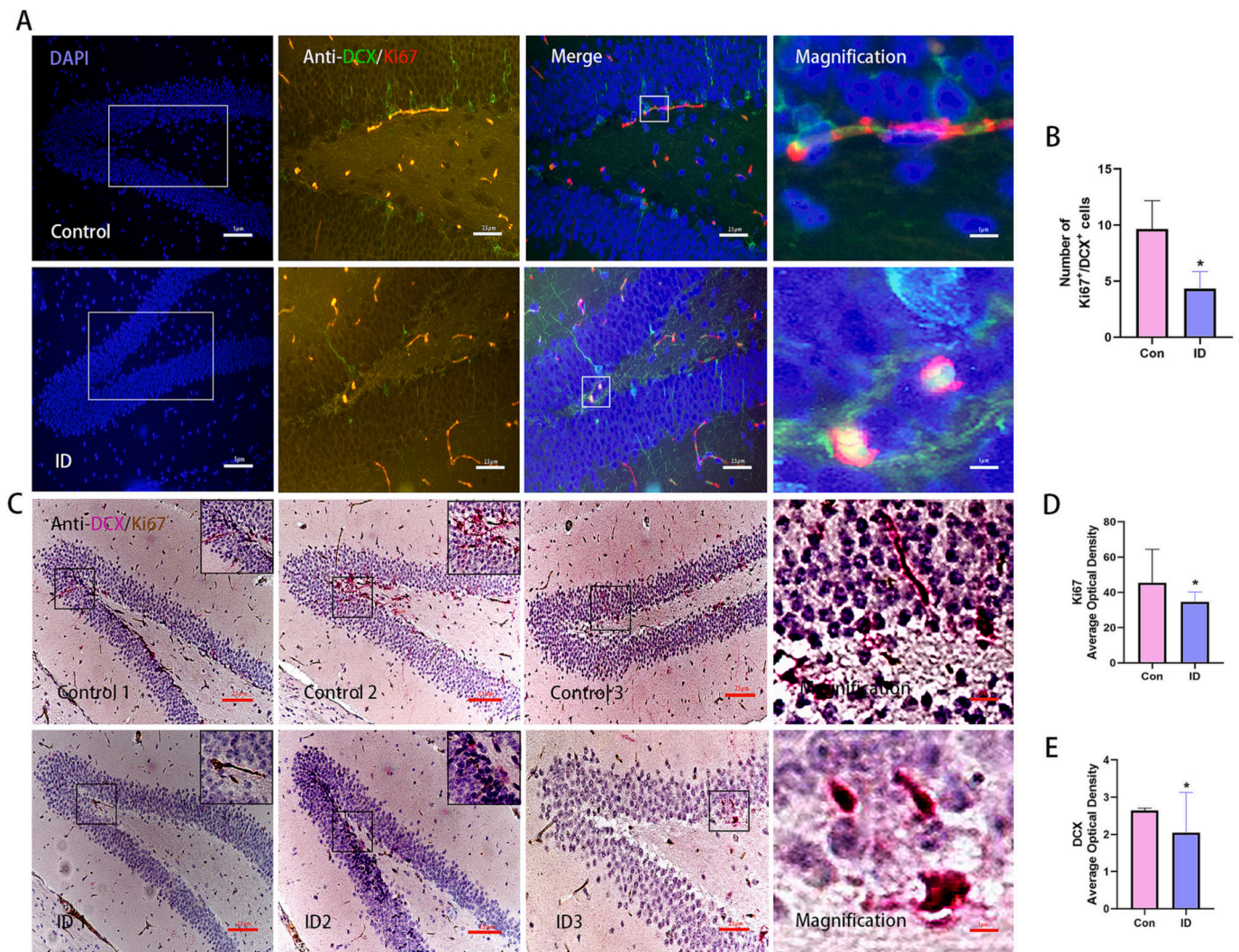


Fig. 5. Cerebral ID inhibited the level of proliferation and neurogenesis in hippocampal DG of iron deprivation mice. Graphs show the representative (A) immunofluorescence DCX (green) and Ki67 (red), and (C) immunohistochemistry images of DCX (fast red) and Ki67 (brown) double stained cells in DG of hippocampus ($n = 3$ images/group). Graphs show the quantitative analysis of (B) DCX- and Ki67- double stained cells, (D) Ki67 positive cells, and (E) DCX positive cells. Statistical comparisons were carried out with independent sample *t*-test. ** $p < 0.01$ vs. Control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cerebral ID to further investigate its effect on neurons. Our results showed that iron deprivation led to monoamine neurotransmitters depletion symptoms, including eyelid ptosis (Fig. 4A&B) and hypothermia (Fig. 4C). The results of HPLC-MS/MS assay confirmed the decrease of monoamine neurotransmitters release in both the whole brain and five different regions of iron deprivation mice (Fig. 4D-4H). Furthermore, results of Nissl staining showed nuclear concentration and cell atrophy in the neurons of hippocampus, temporal cortex, and prefrontal cortex (Fig. 4I). Since these three brain regions also showed decrease in iron levels during iron deprivation, all these results indicated that cerebral ID could lead to neural injury.

3.5. Cerebral ID inhibited the levels of proliferation and neurogenesis in hippocampus of iron deprivation mice

It has been reported that, hippocampal dysfunction plays a critical role in the pathogenesis of depression, and hippocampal neurogenesis buffers depressive behavior (Lopresti, 2015). Therefore, we assessed the effect of ID on the proliferation of newborn neurons in dentate gyrus (DG) of hippocampus by counting neurons with doublecortin (DCX)- and Ki67 double staining. Both the immunofluorescence results (Fig. 5A&B)

and immunohistochemistry results (Fig. 5C-E) showed that cerebral ID reduced the density of DCX and Ki67 stained neurons in DG of hippocampus. These results suggested that cerebral ID could inhibit proliferation and neurogenesis in hippocampus of mice.

3.6. Cerebral ID inhibited hippocampal GC-GR signaling pathway led to dysfunction of hippocampal negative feedback regulation and caused overactivity of HPA axis

In hippocampus, we found that cerebral ID significantly decreased the protein levels of GR and Hsp90 (Fig. 6A). The level of Bag-1 increased, whereas that of CHP-1 and p23 decreased (Fig. 76&B&C&G). Although ID did not affect the protein level of FKBP51, the levels of FKBP2, the protein that assists in the nuclear translocation of the GC-GR heterocomplex, were decreased (Fig. 6A&D). We also used confocal immunofluorescence to observe the colocalization of GR-Hsp90 in CA1 and DG regions of the hippocampus. A pronounced expression and obvious colocalization of GR and Hsp90 in the nuclei of neurons were observed in neurons of the control mice. However, for the iron deprivation mice, weak, diffuse, and perinuclear localization of GR and Hsp90 were observed, which did not clearly colocalize with the nuclei in the merged image (Fig. 6H&J). The

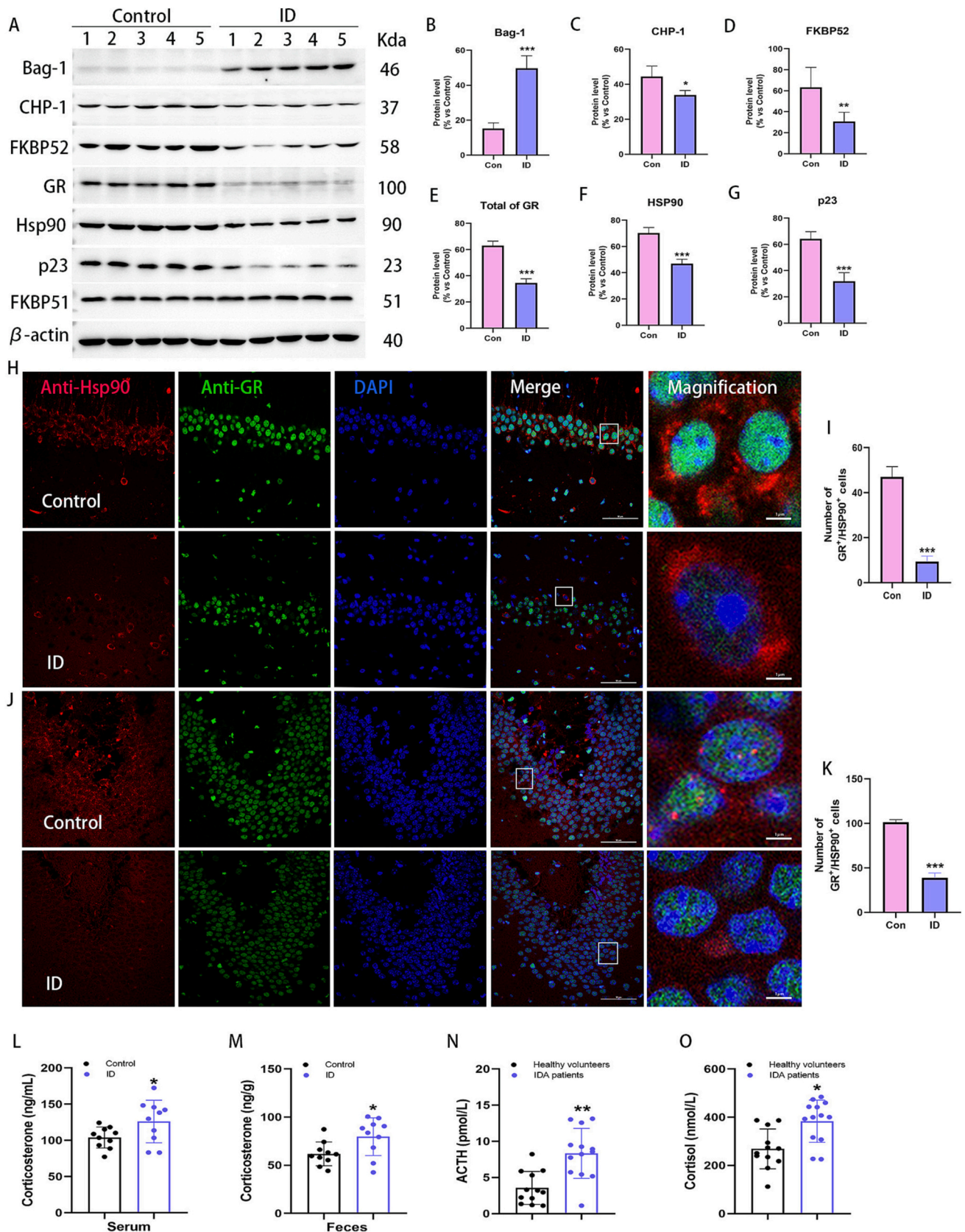


Fig. 6. Cerebral ID inhibited the GC-GR signaling pathway in the hippocampus and caused overactivity of the HPA axis. The graphs show (A) representative western blots of protein in GC-GR signaling pathway, and quantification of protein levels of (B) Bag-1, (C) CHP-1, (D) FKBP52, (E) total GR, (F) Hsp90, (G) p23, (n = 5 mice/group). Representative immunofluorescence images show the colocalization of Hsp90 (red) and GR (green) in CA1(H) and DG (J) of hippocampus, and the quantitative analysis of GR and Hsp90 double stained cells in CA1 and DG of hippocampus were showed in (I&K). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

number of neurons with colocalization of GR and Hsp90 in CA1 and DG of ID mice was significantly lower than those in control mice (Fig. 6I&K). These data indicated that cerebral ID could downregulate the GC-GR signaling pathway and lead to GR dysfunction in hippocampus.

Since cerebral ID could downregulate the GC-GR signaling pathway and cause GR dysfunction in hippocampus, it is reasonable to assume that cerebral ID could lead to overactivity of the HPA axis. And our results confirmed that the levels of corticosterone in both the serum and feces of iron deprivation mice were significantly increased (Fig. 6I&J). For the IDA patients, with the serum iron and hemoglobin levels lower than normal reference range (Supplementary Table 3 and Supplementary Table 4), we also found that although the average levels of both ACTH and cortisol were within the normal reference ranges, the average levels of ACTH and cortisol were significantly higher than those in the healthy volunteers (Fig. 6K&L). The results from both iron deprivation mice and IDA patients confirmed that cerebral ID caused GR dysfunction in hippocampus inhibited the negative feedback function of the hippocampus and cause overactivity of the HPA axis.

Graphs show corticosterone levels in (L) serum and (M) feces of mice after iron deprivation for 35 days ($n = 10$ mice/group), and levels of (N) ACTH and (O) cortisol in IDA patients with serum iron level lower than the normal reference range and healthy volunteers ($n = 12$ – 13 persons/group). All statistical comparisons were carried out with independent sample t -test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs. Control group.

4. Discussion

Numerous preclinical and clinical studies performed in the recent years showed that nutrients played an important role in the development of psychiatric disorders (Lopresti, 2015). Iron, is an essential nutrient required by the human body to perform the neurological functions. However, the role of iron disorder in the pathogenesis of depression and the underlying mechanism is still unclear. This study has demonstrated for the first time that cerebral ID is a key player in the pathogenesis of depression through downregulation of the hippocampal GC-GR signaling pathway caused GR dysfunction.

Previous studies have shown the involvement of ID and/or IDA in a broad spectrum of cognitive and behavioral problems in animals and human beings; however, the studies performed thus far were mainly focused on the effects of ID on neural development and cognitive function and were performed in juvenile rats and in children of different ages (Beard and Connor, 2003; Dallman and Spirito, 1977; Felt et al., 2006; Unger et al., 2008). Results of recent clinical studies showed an association between ID and single depression or comorbidity depression (Wassef et al., 2018; Szkup et al., 2017; Woldetensay et al., 2018; Dama et al., 2017; Bergis et al., 2019; Zhu et al., 2016; Zhuang et al., 2019). However, some clinical studies revealed the opposite results (Richardson et al., 2015; Armony-Sivan et al., 2012) or found no relationship between iron and depression (Li et al., 2017; Cheng et al., 2019). A consensus regarding the association between iron and depression has not been established thus far (Dziembowska et al., 2018). In the present study, with the CUMS, iron deprivation, and iron deprivation&CUMS model, we confirmed that cerebral ID is the key player in the pathogenesis of depression. Both iron deprivation and CUMS resulted in a significant decrease in the iron levels in the hippocampus and prefrontal cortex, and combination of the both factors led to acceleration of depression onset and aggravation of anxiety/depression symptoms in mice. The finding may explain to a certain extent the contradictory results observed in some clinical studies. As clinical study subjects are mainly human beings, and the research materials are usually serum, hair, or nails (Blazewicz et al., 2017; Unger et al., 2008), which only reflect the peripheral iron concentrations, which is not a good indicator of brain iron status and therefore may not be a good measure to associate with depression.

It has been considered that hippocampal GR dysfunction is particularly important in the pathogenesis of depression. GR dysfunction

inhibits the negative feedback regulation function of hippocampus to HPA axis, resulting in high concentration release of GC to damage the neurons in hippocampus and cause depression (Nemeroff and Vale, 2005). Moreover, hippocampal neurogenesis was reported to buffer the depressive behavior (Beard and Connor, 2003). In our study, we found that iron deprivation induced cerebral ID not only led to neuronal injury but also decreased the proliferation and neurogenesis in hippocampus. The underlying mechanism of cerebral ID caused hippocampal changes was related to down regulation of GC-GR signaling pathway and GR dysfunction by blocking the formation and nuclear translocation of the GR-Hsp90 complex in nucleus. Moreover, the inhibition of the negative feedback function of hippocampus to HPA axis were confirmed in iron deprivation mice and IDA patients. High levels of corticosterone in serum and feces of iron deprivation mice, and ACTH and cortisol release increase of IDA patients further confirmed that cerebral ID could cause HPA axis overreaction, which led to depression.

5. Limitations

First, iron deprivation reduced the willingness of mice to intake food and water, which might affect the physical fitness of mice to complete some behavioral experiments. Second, our results showed that iron deprivation could significantly affect the iron levels in hippocampus and prefrontal lobe, but we only focused on the role of hippocampal ID in iron deprivation induced depression in mice. Further studies on transgenic mice with cerebral iron transport knockout are needed to examine the impact of cerebral ID on mouse behavior. Third, the number of iron deficiency anemia patients for confirming the relationship of ID and overactivity of HPA axis was limited. Further prospective epidemiological studies and controlled clinical trials are needed to fully evaluate whether iron deficiency influence the activity of HPA axis and cause depression.

In conclusion, in spite of these limitations, our findings provide strong evidence that cerebral ID plays a key role in the pathogenesis of depression. The results highlight the key role of iron in maintaining mental stability and ensuring appropriate intake of iron or treating with iron might be a promising target for improving the prevention and treatment of depression.

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CRediT authorship contribution statement

Conceptualization: Hong Zhang and Shiliang Yin. Data curation: Manhuayun Zhai and Siman Ma. Project administration: Lian He, Songfei Li, Qiruo Sun, Yu Zhang, and Tuohutanguli. Nuerkamen, Ge Jin. Blood sample acquisition: Minyan Li, Fan Zhou and Hua Tian. Funding acquisition: Hong Zhang, Jincal Hou and Guoxiang Sun. Writing-review & editing: Hong Zhang and Guoxiang Sun.

Conflict of interest

The authors declare no conflict or other conflicts of interest.

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