Electronic Supplementary Materials

Supplementary Methods

Animals

Male C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). All male mice were 10-12 weeks old (~25 g) and were maintained in standard housing conditions (22±1°C; light/dark cycle of 12 h/12 h) with food and water available *ad libitum*. All mice were randomly assigned to different experimental groups with a random number table. All operations were conducted in accordance with the USA National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 8023) and its 1978 revision, and all experimental protocols were approved by the Institutional Animal Care and Use Committee of China Medical University No. [2019]059.

Materials

Most chemicals, including iron dextran (ferric hydroxide dextran complex) and 4', 6-diamidine-2'-phenylinedole dihydrochloride (DAPI), TUNEL apoptosis detection kit were purchased from Sigma (MO, USA). Alexa 555-conjugated ovalbumin (OA555; 45 kDa) were purchased from Invitrogen (CA, USA). Primary antibodies raised against MAP2 (PA1-16751), GFAP (13-0300), TFR (13-6800), NeuN (PA5-78839) and secondary antibodies was purchased from Thermo Fisher Scientific (CA, USA). Potassium ferrocyanide and diaminobenzidine (DAB) kit were purchased from Solarbio Life Sciences (Beijing, China).

Iron-treatments

Iron dextran was intraperitoneal injected 200 μl containing iron 2mg/kg/day for 6 days, the control mice were intraperitoneally injected with the same volume of dextran.

Perl's staining

Iron in tissues was identified through Perl's staining with modifications. The slices were immersed in the mixed reaction liquid (4% potassium ferrocyanide: 4% hydrochloric acid = 1:1) at 37°C for 30 minute and then stained using a DAB kit for 4 min. Hematoxylin staining solution was stained for 2-3 min and washed with PBS. The observations were imaged using Carl Zeiss Axio Scan microscope (Promenade 10, Jena, Germany).

Chronic unpredictable mild stress

As described previously[1], male mice were exposed to the following stressors for 6 weeks. In brief, water and food deprivation (12 h), cage tilt 45°(12 h), stroboscopic illumination in the dark (120/min for 12 h), noise (120 db for 12 h), swimming (5 min), tail suspension (5 min), damp living environment (12 h), and cage shaking (40/min for 5 min), restricting activity (4 h) were administered. During the experiment, the mice were offered one bottle of sucrose solution (2.5 %) and a second bottle of pure water to estimate their sucrose preferences.

Function of glymphatic system measurement

As described previously[2,3], mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) by intraperitoneal injection (IP). The fluorescence tracer (OA555) was reconstituted in artificial cerebrospinal fluid (ACSF) at a concentration of 0.5%. Mice were anesthetised and fixed in a stereotaxic frame while the posterior atlanto-occipital membrane was surgically exposed. Using a 30 GA needle, the tracer was infused into the subarachnoid CSF via cisterna magna puncture at a rate of 2 µl/min for 5 minutes (10 µl total volume). 30 minutes after the start of infusion, anesthetised animals were trans-cardially perfusion fixed with 4% paraformaldehyde (PFA). Brain tissue was cut into 50 um slice and was imaged using Carl Zeiss Axio Scan microscope (Promenade 10, Jena, Germany).

Immunohistochemistry

The brain tissue was fixed and immersed in 4% PFA and cut in 50 µm slices. Immunohistochemistry was performed as previously described[4], the slices were pre-incubated with normal donkey serum (NDS, 1:20; Jackson Immuno-Research Laboratory) for 1 h at room temperature and a mixture antibodies overnight at 4°C, mouse anti-TFR (1:100), chicken anti-MAP2 (1:100), rat anti-GFAP (1:100). The slices were incubated with secondary antibodies for 2 hours at room temperature (1:200). DAPI (1:2000) was stained to identify cell nuclei for 15 min at room temperature. Immunofluorescence was imaged using a confocal scanning microscope (DMi8, Leica, Germany). The background intensity of each image was calculated in cell-free parenchyma in the same field of view and subtracted from the total immunofluorescence intensity. The intensity of TFR immunofluorescence from the

different groups was normalised to the intensity of the control group.

TUNEL staining and analysis

As our previously described[4], TdT-mediated dUTP-biotin nick end labelling (TUNEL) in conjunction with immunofluorescent staining for NeuN was operated to evaluate neuronal apoptosis in cortex and hippocampus regions. Following the manufacturer's protocol, TUNEL staining was performed using an in-situ cell death detection kit. The brain slices were incubated with anti-NeuN antibody (1:100) overnight at 4°C followed by incubation with secondary antibody (1:400) for 2 hours at room temperature. DAPI (1:2000) was stained to identify cell nuclei for 15 min at room temperature. Slices were imaged using a confocal scanning microscope (DMi8, Leica, Germany). Analyzing the neuronal apoptosis level was via calculating the average percentage of TUNEL+/Total NeuN+ cells.

Sucrose preference test

As previously described[5], the sucrose preference test is a reward-based test and a measure. In brief, after 20 h of food and water deprivation, the mice were provided with two pre-weighed bottles, including one bottle that contained 2.5 % sucrose solution and a second bottle filled with water, for 2 h. The percentage preference was calculated according to the following formula: % preference = [sucrose intake/(sucrose + water intake)] × 100%.

Tail suspension test

Tail suspension test is a behavioural despair-based test. As our previous description[1], each mouse was suspended by its tail around 2 cm from the tip at a height of 20 cm. Behaviour was recorded for 6 min. The duration of immobility was measured by an observer blinded to the treatment groups.

Forced swimming test

The forced swimming test is a behavioural despair-based test. As previously described[6], each mouse was trained to swim 15 min on the first day, then the mouse was put into a glass cylinder that contained 30 cm deep water (25 ± 1°C) for 6 min in the next day. The time of immobility was recorded during the last 4 min period which followed 2 min of habituation.

Pole test

The pole test is a test for assessing movement disability in mice. As previously described[6], a vertical rough-surfaced pole (diameter 1 cm; height 55 cm) was used in this test and the mouse was placed down-upward on its top. The movement time from the pole top to the floor (T-LA time) were measured. The total time was measured with a maximum duration of 60 s.

Morris water maze test

The Morris water maze test was a spatial learning and memory test. As previously described[1], the mice were trained 5 consecutive days daily with four trials. During this period, the mice were trained from different a starting quadrant to locate and escape onto the platform. The platform position was fixed throughout the test. Animals that

failed to find the location within 60 s were guided to the platform and were allowed to remain on it for 20 s. On the sixth day, the platform was removed, and the mice were given 60 seconds to explore, and the time spent in the target quadrant was collected for each mouse.

Statistics

The quantification of immunofluorescence, TUNEL staining and the calculation of behavioral indicators were performed by an investigator blinded to the experimental conditions. Differences among multiple groups were analyzed by one-way analysis of variance (ANOVA) followed by a Tukey post hoc multiple comparison test for unequal replications using SPSS 24.0 software (International Business Machines Corp., NY, USA). Sample size was not predetermined by formal power calculation, and no samples or data were excluded from the analysis. All statistical data in the text are expressed as mean ± SEM, the level of significance was set at $p < 0.05$ on a two-sided test.

References

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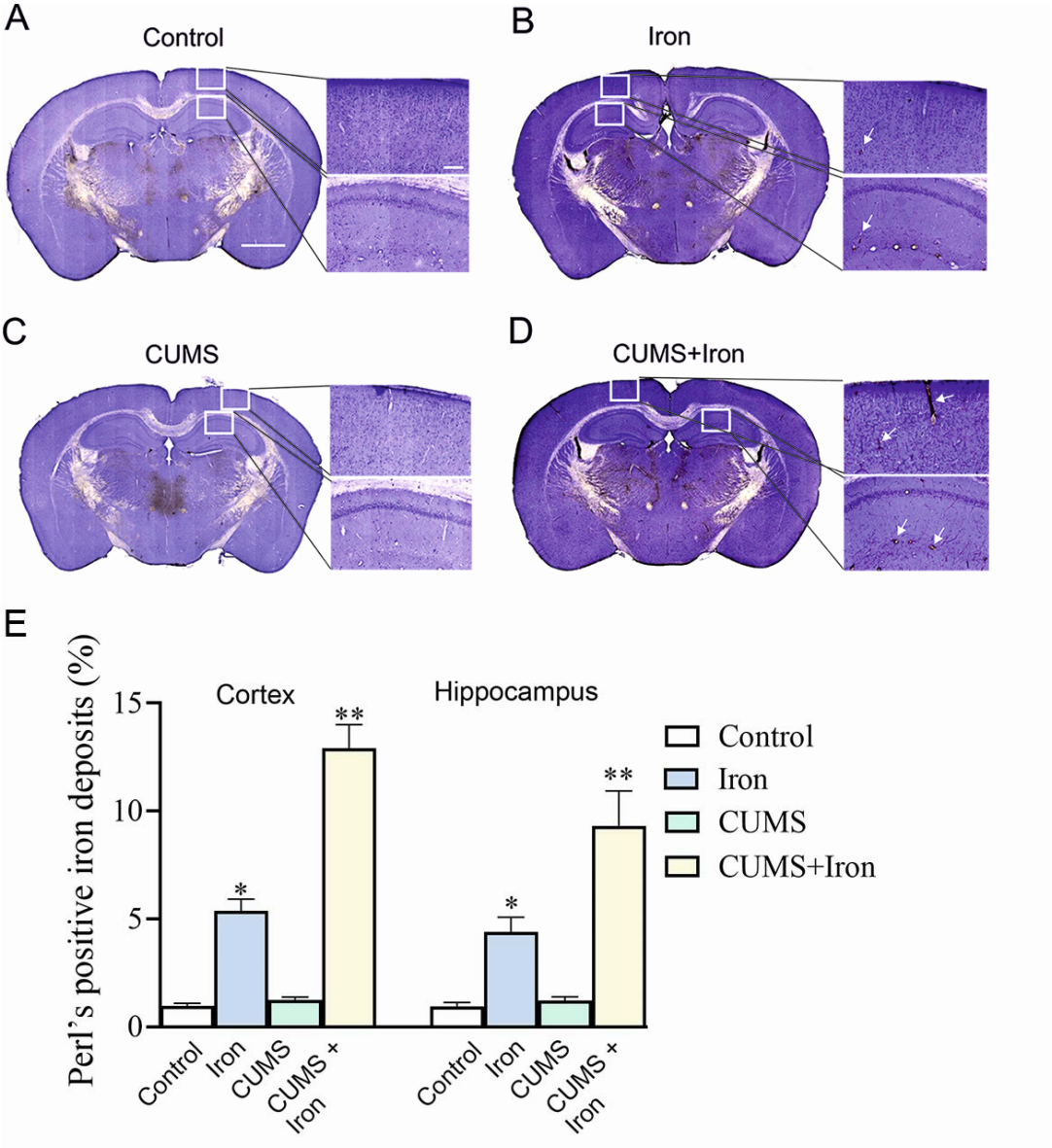

Fig. S1 Iron deposition is increased in the brain of mice subjected to CUMS. A–D Representative Perl's images of the whole brain slices (scale bar, 1 mm) and in selected Perl's images from cortex and hippocampus (scale bar, 100 μm). Mice were pre-treated without or with CUMS for 6 weeks; in the last week they were randomly selected to be injected with dextran or iron dextran for 6 days. **E** Percentages of Perl's-positive staining area in whole images. Data are presented as the mean ± SEM, $n = 6$. *P <0.05 *vs* control; **P <0.05 *vs* any other group.

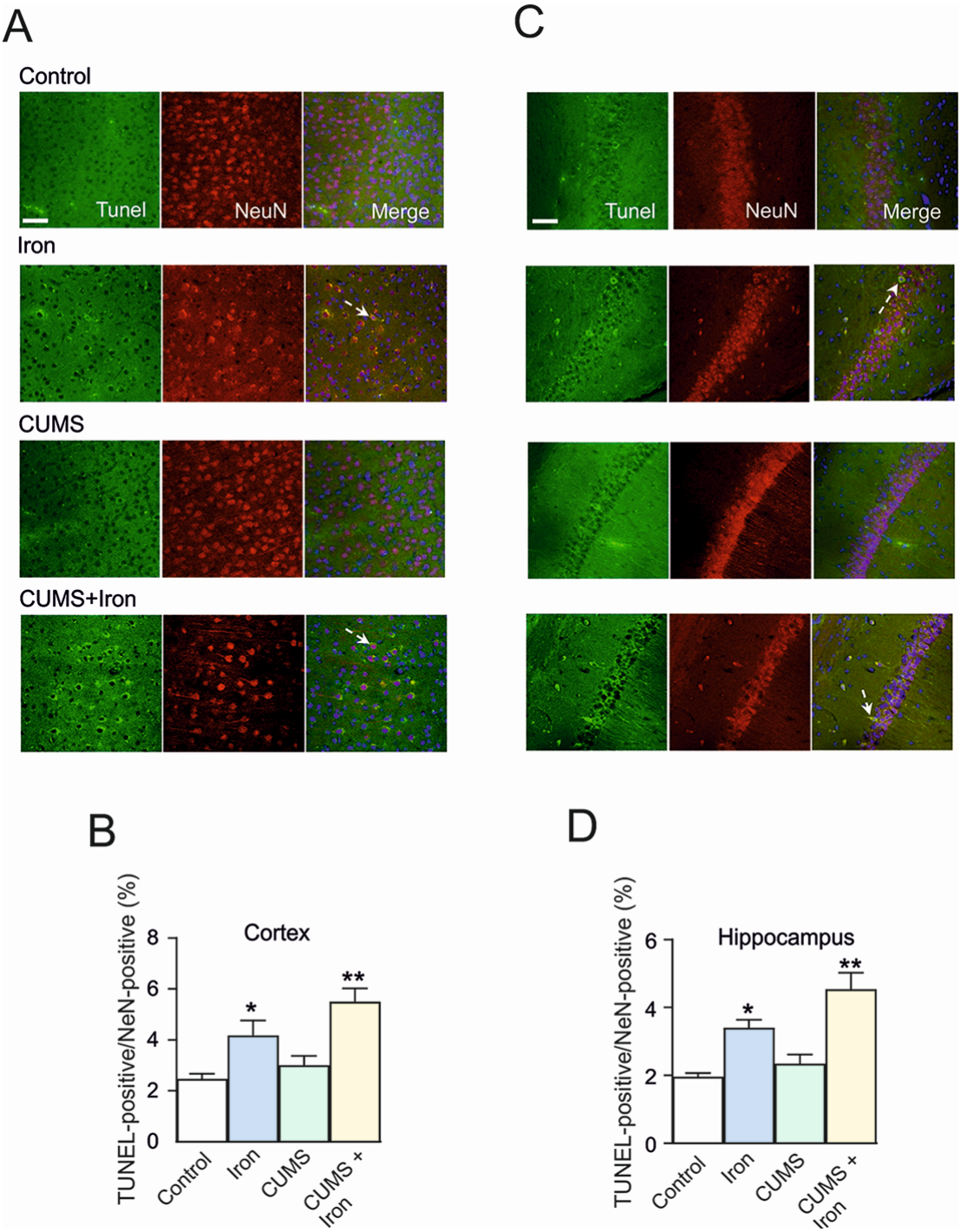

Fig. S2 Exposure to CUMS enhances neuronal apoptosis triggered by iron overload. Mice were pre-treated without or with CUMS for 6 weeks, in the last week the mice were randomly separated to be injected with dextran or iron dextran for 6 days. **A**, **C** Cellular apoptosis detected by TUNEL assays in cortex (**A**) and hippocampus (**C**) (red, neurons stained with NeuN; blue, nuclei labeled with DAPI).

B, **D** Percentages of cell death determined by the ratio of TUNEL+ and NeuN+ cells, in the cortex (**B**) and hippocampus (**D**). Data are presented as the mean ± SEM, n = 6. *P <0.05 *vs* control; **P <0.05 *vs* any other group.

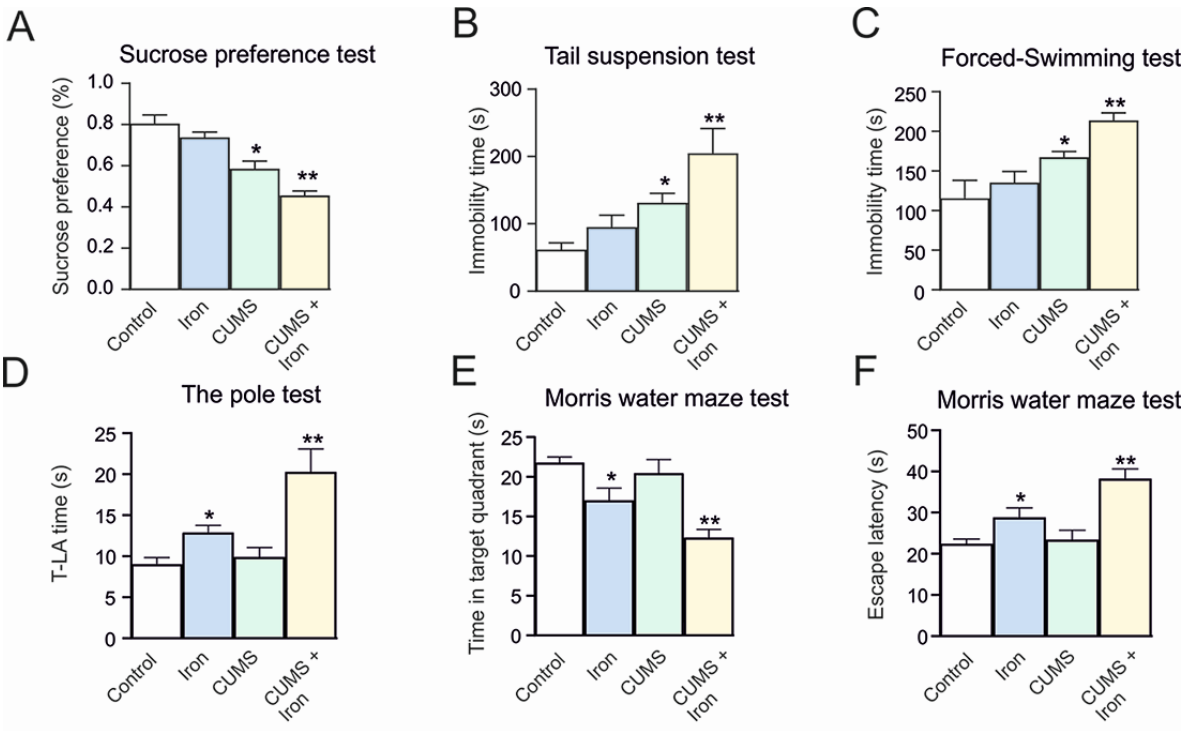

Fig. S3 Iron overload exacerbates behavioral phenotypes of mice exposed to CUMS. A Percentage preference in the sucrose preference test. **B** Immobility time in the tail-suspension test. **C** Immobility time in the forced-swimming test during the last 4 min of the 6 min testing period, which followed 2 min of habituation. **D** Time for the top to reach the floor in the pole test. **E, F** Time in the target quadrant (**E**) and escape latency (**F**) in the Morris water maze test. Values are expressed as the mean ± SEM, n = 6. *P <0.05 *vs* control; **P <0.05 *vs* any other group.