Supporting Information

Downregulation of programmed cell death factor 4 attenuate post-traumatic stress disorder-like behaviors in mice

Rui Yue[1#], Ting Li[2#], Bing Gu[1#], Liwei Chai[3], Xinyu Zhou[1], Yijun Zhou[3], Zhen Wang[3], Haifeng Zhao[4*], Dexiang Liu[1]**

Supplementary Methods

1. Open field test

The open field test (OFT) was employed to evaluate exploration activity and anxiety-related behaviors in rodents(Prut & Belzung, 2003). The testing apparatus consisted of a square arena (40 × 40 × 40 cm) with opaque white walls and flooring. At the initiation of each trial, subjects were gently positioned in the center of the arena, maintaining consistent orientation with all animals. Behavioral parameters, including total distance traveled and time in the center area, were recorded over a 5 min observation period. To eliminate olfactory cues between trials, the apparatus was thoroughly cleaned with 75% ethanol solution following each session. Animal movement patterns were automatically tracked and analyzed using the SMART 2.5 video tracking system (Panlab SL, Barcelona, Spain).

2. Elevated plus maze test

The elevated plus maze (EPM) test was conducted to estimate the anxiety-like behavior of mice based on the exploration of open arms(Walf & Frye, 2007). The maze is a black plus-crossed-shaped apparatus, consisting of two open arms and two closed arms. Before the formal test, the device was cleaned with alcohol to eliminate olfactory cues. The mice were placed in the central platform of the maze facing an open arm and were permitted to explore the maze for 5 min. The entries and time spent in the open arms were recorded by a camera and analyzed with SMART 2.5 software.

3. Conditioning fear test

Freezing following the exposure to the sound reminder and the shock stimulation were considered as indicators of acquired contextual and auditory fear. Conditioning fear test was carried out in a shock chamber located in an isolated room. The shock chamber had a grid floor composed of 12 stainless steel rods (2 mm in diameter), spaced 0.7 cm apart, and wired to a shock generator.

On the first day of the experiment, every mouse was placed individually in the shock chamber and was allowed to explore the whole apparatus for 2 min. Afterwards, the mouse was exposed to a 30-s acoustic stimulation (2 kHz, 85 dB) and a 2-s foot electric shock (0.6 mA). Then, three acoustic stimulation and electric shocks at an interval of 1 min were delivered to the animals. After the final shock, the mice stayed in the shock chamber for 90 s and then returned to the cage.

On the second day, the mice were exposed to contextual freezing and auditory freezing test in turn. During the contextual freezing test, the mice were placed in the same shock chamber and the time of their freezing within 5 min was recorded. After an interval of 2 h, the mice were placed in a new chamber shaped like the shock chamber for auditory freezing test. After the mice were put into the adaptation for 1 minute, three 30-s acoustic stimulations (2 kHz,85 dB) were given, each sound stimulation interval was 30 s, and the freezing level induced by the sound stimulus was recorded. The freezing behavior was defined as sustained immobility, except for respiratory movements, lasting for at least one second.

4. Tail suspension test

The tail suspension test (TST) was used to measure the severity of depression by recording the duration of immobility (Watt, 1979). The experiment was carried out with a black suspension box of 25 × 25 × 50 cm. The experiment was divided into adaptation stage and test stage. At the beginning of the experiment, the mice were suspended upside down on the top of the experimental box (fixed at 1 cm at the end of the tail). After a 2-min acclimatization period, the formal test commenced, during which the immobility time was recorded over a 4-min interval. Immobility was operationally defined as the absence of movement or only minor limb adjustments, excluding respiration-related motions.

5. Forced swim test (FST)

The forced swim test (FST) was conducted to assess depression-like behaviors in rodents (Porsolt, Le Pichon, & Jalfre, 1977). Mice were gently placed in a transparent glass cylinder (height 45 cm, diameter 19 cm) of water (22℃-25℃, 23 cm in height) under bright light conditions for 6 min. After a 2-min acclimation period, the immobility time was automatically recorded during the last 4 min. Immobility was defined as floating or minimal movement. After the test, the mice were dried and returned to their cages.

All behavioral scoring was performed by an experimenter blinded to the experimental design and treatment for each animal to ensure objective assessment.

6. Western blot

Following euthanasia, hippocampal tissues were rapidly dissected and processed for protein analysis. Tissue samples were homogenized in ice-cold RIPA lysis buffer supplemented with protease inhibitors and phosphatase inhibitors to maintain protein integrity and phosphorylation status. The homogenates were centrifuged at 12,000 × g for 10 min at 4 ℃ to obtain clear supernatants. Protein concentration was determined using a BCA Protein Assay Kits according to the manufacturer's protocol. Protein extracts were mixed with 5× SDS-PAGE loading buffer and denatured by boiling at 100 ℃ for 10 min. Equal amounts of protein (20 μg per lane) were separated on a polyacrylamide gel (5% stacking gel, 10% resolving gel), running at 80 mV for electrophoresis, followed by electrophoretic transfer to polyvinylidene difluoride (PVDF) membranes at 300 mA for 90 min.

Membranes were blocked with 5% non-fat dry milk in TBST for 1 h at room temperature, then incubated with primary antibodies (dilution 1:1000) overnight at 4 ℃, β-actin (1:1000) served as the loading control. After three 5-min TBST washes, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:10,000 dilution) for 1 h at room temperature. Following additional TBST washes, protein bands were visualized using ECL reagents and quantified using a chemiluminescence imaging system. Detailed information regarding antibody specifications, including catalog numbers and manufacturers, is provided in Table 3.

Table 3 Primary antibody information and dilution ratio

Antibody	Catalog number	Antibody Genus	Company	Dilution ratio
CREB	111052	Rabbit	Servicebio	1:1000
BDNF	GB11559	Rabbit	Servicebio	1:1000
ERK	9102S#	Rabbit	Cell signaling technology	1:1000
PDCD4	84162-3-RR	Rabbit	Proteintech	1:1000
PSD95	20665-1-AP	Rabbit	Proteintech	1:1000
p-ERK	pT202/pY204	Rabbit	Abcam	1:1000
p-CREB	9197S	Rabbit	Cell Signaling Technology	1:1000
β-actin	15003#	Rabbit	Servicebio	1:1000

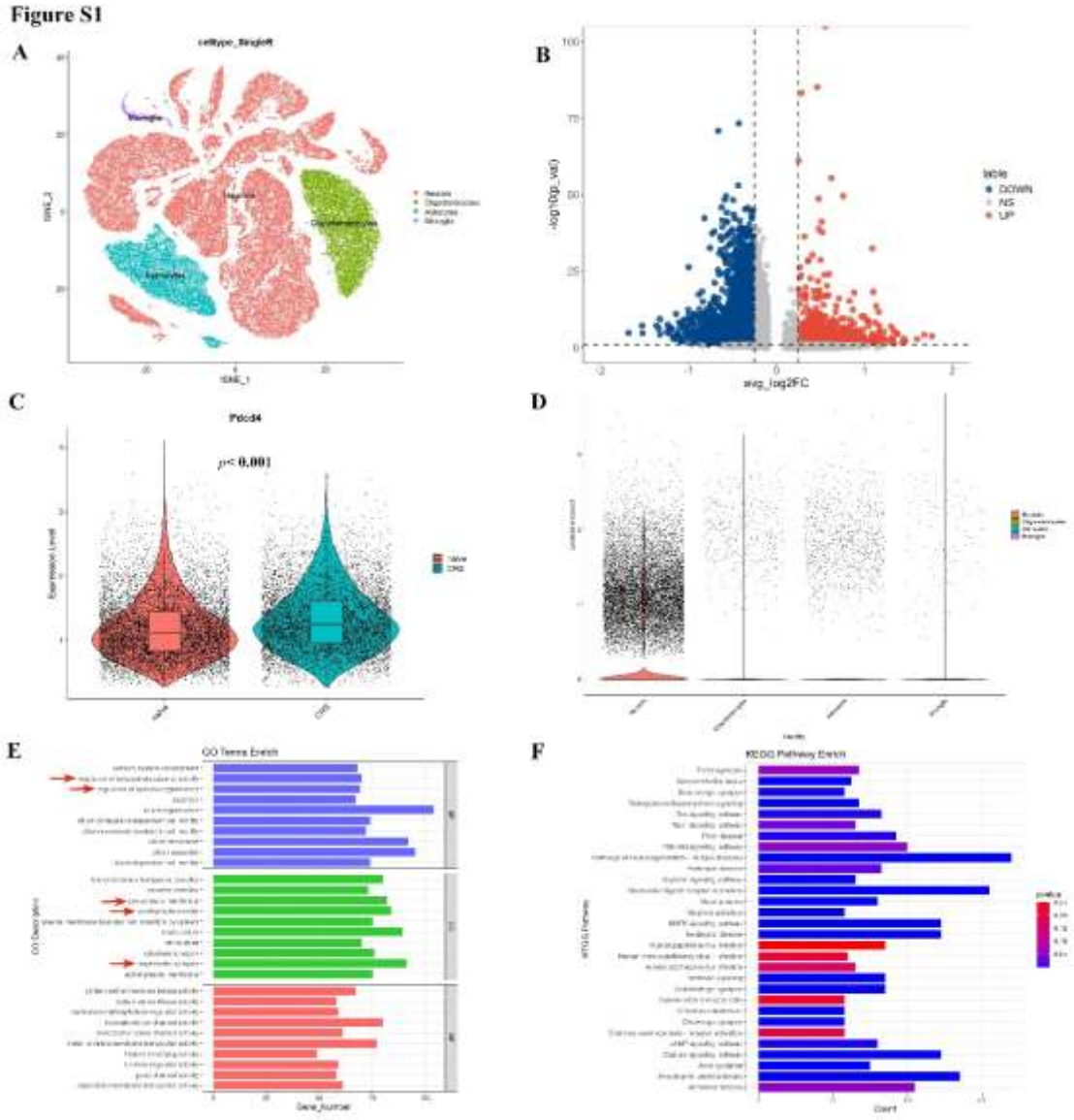

Figure S1 Major brainstem cell clusters and cell identities

(A) t-SNE plot from the GSE287308 visualizing clustering of single cells colored by cell types. (B) Volcano plot was performed to show the up-regulated and down-regulated transcriptional profiles related to RSD. (C) The *Pdcd4* mRNA was up-regulated in the hippocampus of RSD mice from the GSE287308. (D) The plot visualizing the expression distribution of *Pdcd4* mRNA in in many cell types. (E) GO analysis of DEGs of GSE275205. (F) KEGG analysis of DEGs of GSE287308. Results were presented as mean ± SD. ***$p < 0.001$ according to Student's *t*-test in C.

References

Porsolt, R. D., Le Pichon, M., & Jalfre, M. (1977). Depression: a new animal model sensitive to antidepressant treatments. *Nature, 266*(5604), 730-732. doi:10.1038/266730a0

Prut, L., & Belzung, C. (2003). The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. *Eur J Pharmacol, 463*(1-3), 3-33. doi:10.1016/s0014-2999(03)01272-x

Walf, A. A., & Frye, C. A. (2007). The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nat Protoc, 2*(2), 322-328. doi:10.1038/nprot.2007.44

Watt, M. (1979). Sulphinpyrazone (Anturan) after myocardial infarct. *N Z Med J, 90*(646), 352. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/392352