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RESEARCH ARTICLE

Cannabidiol prevents depressive-like behaviors through the modulation of neural stem cell differentiation

Ming Hou[1], Suji Wang[1], Dandan Yu[1], Xinyi Lu[1], Xiansen Zhao[1], Zhangpeng Chen (✉)[1,2,3], Chao Yan (✉)[1,2,3,4]

[1]*State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing 210023, China;* [2]*Institute of Artificial Intelligence Biomedicine, Nanjing University, Nanjing 210023, China;* [3]*Chemistry and Biomedicine Innovation Center, Nanjing University, Nanjing 210023, China;* [4]*Engineering Research Center of Protein and Peptide Medicine, Ministry of Education, Nanjing 210023, China*

Abstract **Chronic stress impairs radial neural stem cell (rNSC) differentiation and adult hippocampal neurogenesis (AHN), whereas promoting AHN can increase stress resilience against depression. Therefore, investigating the mechanism of neural differentiation and AHN is of great importance for developing antidepressant drugs. The nonpsychoactive phytocannabinoid cannabidiol (CBD) has been shown to be effective against depression. However, whether CBD can modulate rNSC differentiation and hippocampal neurogenesis is unknown. Here, by using the chronic restraint stress (CRS) mouse model, we showed that hippocampal rNSCs mostly differentiated into astrocytes under stress conditions. Moreover, transcriptome analysis revealed that the FoxO signaling pathway was involved in the regulation of this process. The administration of CBD rescued depressive-like symptoms in CRS mice and prevented rNSCs overactivation and differentiation into astrocyte, which was partly mediated by the modulation of the FoxO signaling pathway. These results revealed a previously unknown neural mechanism for neural differentiation and AHN in depression and provided mechanistic insights into the antidepressive effects of CBD.**

Keywords cannabidiol; depression; radial neural stem cells; neurogenesis

Introduction

Depression is a mood disorder that is commonly found in the general population worldwide [1–3]. The core symptoms of depression include a persistent feeling of sadness, loss of interest, and strongly affected daily activities. Current treatment options are mostly limited to selective serotonin reuptake inhibitors (SSRIs), which cannot rapidly alleviate depressive symptoms and may aggravate social burden [4]. Therefore, the development of novel antidepressant drugs for the treatment of patients with depression are matters of great concern in contemporary society.

The dentate gyrus (DG), a part of the hippocampus, contains radial neural stem cells (rNSCs) that can continuously generate new neurons in a process called adult hippocampal neurogenesis (AHN) [5–7]. The application of glucocorticoids or repeated exposure to chronic daily stress strongly impairs AHN and neural differentiation; these two processes have been shown to be involved in the pathogenesis of depression [8–10]. Previous studies have also demonstrated that in mice, adult-born neurons can buffer stress responses and alleviate depressive-like behaviors [8,11]. Given this information, AHN is also considered to be involved in the neuronal networks that underlie mood regulation. Therefore, regulating AHN may be a beneficial strategy for the clinical treatment of depression. However, the neural mechanisms underlying the role of AHN and neural differentiation in depression remain poorly understood.

Cannabidiol (CBD), a major nonpsychotropic ingredient of phytocannabinoids present in the medical cannabis plant and approved by the FDA for the treatment of Dravet syndrome, has been shown to possess broad-spectrum pharmacological activities in many neurological diseases,

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Correspondence: Zhangpeng Chen, xiaopengczp@163.com;
Chao Yan, yanchao@nju.edu.cn

such as epilepsy and multiple sclerosis [12,13]. A growing body of evidence suggests that CBD may be an effective and safe antidepressant agent; however, the underlying mechanisms of the antidepressive effect of CBD are unclear [14–16]. A recent study revealed that AM251, an antagonist of cannabinoid receptor 1 (CB1R), partly blocks the antidepressant effect of CBD, suggesting that the modulation of the endocannabinoid system may be involved in the antidepressant mechanism of CBD [17]. Another study found that CBD could promote neurogenesis in depressive mice by elevating the levels of endocannabinoids, including 2-arachidonoylglycerol and anandamide, suggesting that the modulation of neurogenesis might also be involved in the antidepressant effect of CBD [18]. However, the detailed mechanism underlying this phenomenon is not known.

In the present study, by using behavioral assessments combined with immunostaining, enzyme-linked immunosorbent assay (ELISA), and transcriptome analysis, we investigated the antidepressant role of CBD in a chronic restraint stress (CRS) mouse model. We found that CBD could inhibit stress-induced rNSC overactivation and prevent the differentiation of rNSCs into astrocytes, thereby maintaining normal neurogenesis and rescuing depressive-like behaviors in the CRS mouse model.

Materials and methods

Animals

Mice of the C57Bl/6J inbred strain (male, 6–8 weeks old, 20–25 g, SPF Biotechnology Co. Ltd., Beijing, China) were maintained on a 12 h light/dark cycle with free access to food and water. All experiments using animals were conducted with the approved protocols of the Institutional Animal Care and Use Committee of Nanjing University in accordance with NIH and institutional guidelines.

Antibodies and reagents

CBD was bought from ZZStandards Reference Materials (ZT-71836). BrdU was purchased from Selleck (S7918). Wortmannin (HY-10197) and corticosterone (HY-B1618) were bought from MedChemExpress. The antibodies used in this study included rat anti-BrdU antibody (ab6326, Abcam, 1:400), rabbit anti-Doublecortin antibody (4604, Cell Signaling Technology, 1:400), chicken anti-Nestin antibody (AB_2314882, Aves Laboratories, 1:200), rabbit anti-Ki-67 antibody (27309-1-AP, Proteintech, 1:400), rabbit anti-GFAP antibody (16825-1-AP, Proteintech, 1:400), and rabbit anti-NeuN antibody (26975-1-AP, Proteintech, 1:400). The appropriate secondary antibodies were all purchased from Invitrogen (1:2000 for immunofluorescence).

Cell culture

The mouse neuroectodermal stem cell line NE-4C was obtained from the American Tissue Culture Collection and grown in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, and 1× penicillin–streptomycin mixture (Invitrogen). The cells were maintained in an incubator containing 5% CO_2 at 95% humidity and 37 °C. The cells were plated on 100 mm culture plates (BD Falcon) coated with 0.01% poly-L-lysine (Sigma) and subcultured at a 1:10 ratio. The supplemented MEM was changed every 2 days. The NE-4C cells were seeded onto culture plates containing poly-L-lysine and incubated overnight at 37 °C before treatment. The cell line was regularly checked for mycoplasma contamination.

Western blot analysis

The NE-4C stem cells were washed with PBS (Hyclone, SH30256.01) and collected in RIPA buffer (Beyotime, P0013C) containing protease (Thermo Scientific, 36978) and phosphatase inhibitors (Beyotime, ST640). Protein was collected at 10 000× *g* and 4 °C for 5 min, and protein concentration was determined by using a PierceTM BCA Protein Assay Kit (Thermo Scientific, 23225). Total cell lysates (20 μg of protein) were separated through 8%–10% SDS-PAGE. Proteins were transferred onto a PVDF membrane, and Western blot analysis was carried out by following standard protocols with signal detection by chemiluminescence (ECL; Thermo Scientific, TH270252).

MTT assay

Cells (3×10^3 cells/well) were seeded into 96-well plates and incubated at 37 °C overnight. CBD and the inhibitor was dissolved in DMSO (Sigma Aldrich, D2650) and diluted to working concentrations in culture medium. After 12 h of treatment, corticosterone was added to the medium. After another 12 h, the supernatant was removed, and 50 μL of MTT in medium (1 mg/mL; Maklin, C10769164) was added and incubated for 4 h at 37 °C. The MTT solution was then removed, 100 μL of DMSO was added to each well, and absorbance at 570 nm was measured by using a microplate reader (Spectra Max M4, Molecular Devices, USA).

Quantitative real-time PCR

Total RNA was extracted from the DG area of the hippocampi of mice in different groups by using Trizol (Invitrogen), and cDNA was synthesized by using a reverse transcription kit (FSQ-101, Toyobo) in accordance with the manufacturer's instructions. cDNA was amplified by using SYBR Green PCR mix (Applied Biosystems), and

quantitative gene expression analysis was run on an Applied Biosystems 7900HT real-time thermal cycler. The primers were as follows: *CDKN1A* (forward: CCTGGTGATGTCCGACCTG; reverse: CCATGAGCGCATCGCAATC), *SGK1* (forward: GAGCCGGAGCTTATGAACG; reverse: GAGCCGGAGCTTATGAACG), and *GAPDH* (forward: AACTTTGGCATTGTGGAAGGGCTCA; reverse: TTGGCAGCACCAGTGGATGCAGGGA). The expression levels were normalized to *GAPDH*.

RNA sequencing and transcriptome analysis

The hippocampal DG was dissected and digested in collagenase H (1 mg/mL, 11074059001, Roche) at 37 °C for 60 min. The disaggregated cells were centrifuged at 400× *g* for 5 min. Cell pellets were resuspended in 20% BSA and centrifuged at 1000× *g* at 4 °C for 10 min to remove myelin. The cells were centrifuged at 2000× *g* and 4 °C for 5 min and resuspended in Trizol (Life Technologies). Total RNA was extracted in accordance with the manufacturer's protocol. Triplicate samples of each group were collected, and total RNA was extracted and sent to Shanghai Majorbio Bio-Pharm Technology Co., Ltd. for transcriptome sequencing by Illumina HiSeqTM 2500 sequencer. Data were analyzed by using the free online Majorbio Cloud Platform.

CRS model

Male C57BL/6J mice were subjected to CRS to induce depressive-like behavior through 3 h of placement in 50 mL conical tubes with holes for air flow and then immediately returned to their home cages every day for 21 consecutive days [19]. Except for these 3 h, the mice had free access to food and water for the remainder of the day.

Behavioral tests

Brief descriptions of the behavioral tests are provided below. The behavioral tests were conducted during the light cycle by a single experimenter who was blinded to the treatment conditions.

Open-field test

Before the test, all animals were habituated for 1 h to the behavior room before their test sessions. The mice were tested in an open field test (OFT) box (50 cm length × 50 cm width × 40 cm height). They were placed in the center of a plastic box in a room with dim light and allowed to explore the arena for 5 min and recorded by a video camera [20]. Locomotor distance was analyzed by using Clever TopScan Software (Clever Sys Inc., Reston, VA).

Forced swim test

The mice were placed in a cylinder of water (23 ± 2°C; 25 cm in height and 18 cm in diameter) for 6 min [21]. The total immobility time was measured with Clever TopScan Software (Clever Sys Inc., Reston, VA), and the data of the last 4 min were collected for further analysis. Immobility was defined as the absence of active movement except that needed to keep the animal from drowning. The water was changed between each animal.

Sucrose preference test

The sucrose preference test (SPT) is a measure of anhedonia-like behavior in mice and was assessed by using a two-bottle choice procedure. The mice were habituated to two leak-resistant bottles (1% sucrose solution or normal water; the left or right location of the water and sucrose bottles for mice was randomized) in their home cage individually for 3 days prior to testing. On the last day of habituation, the mice were water restricted starting at 21:00 for 12 h. The SPT experiment was begun at 9:00, and the weight of the bottles was recorded. Each subject was temporarily single-housed in a cage with two leak-resistant water bottles (one containing water and the other containing 2% sucrose-in-water solution). At 13:00, the positions of the water and sucrose bottles were swapped. At 17:00, the experiment was ended, and the weight of the bottles was recorded again. The whole experiment lasted for 8 h, and the total consumption of sugar water and normal water was recorded [22,23]. The preference for the sucrose solution was determined as the percentage of sucrose solution ingested relative to the total intake.

Elevated plus maze test

Before the test, all animals were habituated to the behavior room before their test sessions for 1 h. The apparatus for the elevated plus maze test (EPM) consisted of two opposing open arms (44 cm length × 12 cm width) and two closed arms (44 cm length × 12 cm width) that were connected by a central zone (12 cm length × 12 cm width). The whole apparatus was elevated 50 cm above the floor. The animal was placed in the center of the maze facing an open arm to begin a trial and allowed to freely explore the maze for 5 min [20]. The last 4 min in the open arm were recorded and were analyzed by using Clever TopScan Software (Clever Sys Inc., Reston, VA). The apparatus was cleaned with 70% ethanol between each mouse to remove similar odors.

Immunofluorescence and cell counting

The mice were anaesthetized with an intraperitoneal (i.p.) injection of 450 mg/kg chloral hydrate and perfused transcardially with 20 mL of 0.9% saline followed by 100–150 mL of 4% paraformaldehyde in 0.1 mol/L PBS (pH 7.4). The brains were then removed, placed in the same fixation solution for 24 h at 4 °C for postfixation, transferred to 20% sucrose solution overnight, and then transferred to 30% sucrose solution until they sank. Frozen coronal sections (25 μm in thickness) were acquired by using a freezing microtome (CM3050S, Leica, Wetzlar, Germany). The sections were rinsed in PBS containing 0.1% Triton X-100 for 10 min, then incubated in 10% normal bovine serum in PBS containing 0.1% Triton X-100 for 30 min at room temperature. The sections were incubated with the corresponding primary antibodies at 4 °C overnight. On the following day, the sections were washed with PBS buffer for five times then incubated with the appropriate secondary antibodies for 4 h at room temperature in the darkness [24]. Finally, the slices were washed and mounted in Fluoromount-G mounting medium (SouthernBiotech, Birmingham, AL). All sections were processed by using the same standardized conditions. Five mice from each group and six consecutive dorsal hippocampal DG slices (located in the bregma: −1.94 to −2.46 mm) per mouse were collected. Images were captured by using confocal microscopy in the 20× field (Zeiss LSM 880 with Airyscan, Germany). Cell counting was performed by an investigator who was blinded to the group assignments.

BrdU labeling

The mice were given three i.p. injections at the dose of 150 mg/kg with 2 h intervals immediately before the beginning of CRS to explore the differentiation of hippocampal cells in adult mice [25]. For BrdU immunofluorescence analysis, the brain sections were incubated in 2 mol/L HCl at 37 °C for 30 min to denature DNA and 0.1 mol/L $Na_2B_4O_7$ for another 15 min at room temperature to neutralize HCl. The following steps were the same as those used to perform ordinary immunofluorescence analysis.

Corticosterone assessment

For the measurement of serum corticosterone levels, blood samples were collected into tubes, and serum was isolated after the centrifugation of the blood samples at 2000× *g* for 15 min at 4 °C. The level of corticosterone in 10 μL of serum was measured by using an ELISA kit (Enzo Life Sciences, NY) in accordance with the manufacturer's instructions.

Statistical analysis

Unless otherwise noted, data were expressed as mean ± standard deviation (SD) and represented the results of at least three independent experiments. Statistical significance was determined by using the unpaired *t*-test for two-group experiments. Two-way analysis of variance was performed for comparison among three or more groups. *P* values less than 0.05 were considered statistically significant and were indicated as follows: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

Results

CRS caused depressive-like behaviors and abnormal neurogenesis in mice

We first established a depressive-like mouse model by using the CRS method [19]. Mice were restrained in a transparent 50 mL centrifugation tube for 3 h daily for a total duration of 3 weeks (Fig. 1A). Subsequently, a series of behavioral performance tests, including the SPT and FST, were used to characterize the depressive symptoms of CRS mice (Fig. 1B–1D). Compared with the normal control mice, CRS mice displayed apparent depressive-like symptoms, including a lower consumption of sucrose in the SPT ($P = 0.0081$) and a longer time of immobility in the FST ($P = 0.0045$). The serum levels of corticosterone, a biomarker of stress, were also significantly elevated in CRS mice ($P = 0.0224$; Fig. 1E), indicating the overactivation of the hypothalamic–pituitary–adrenal axis. Previous studies have shown that exposure to stress could induce the activation of rNSCs and impair normal hippocampal neurogenesis [10]. Therefore, we tested whether the rNSCs located in the hippocampal DG exhibited overactivation in our model. By using immunostaining, we found that the total number of Ki-67 labeled cells was significantly increased in the DG of CRS mice compared with that in normal mice ($P = 0.0025$; Fig. 1F and 1G), indicating that the overproliferation of newborn cells was induced in the CRS model.

We performed immunofluorescence staining with nestin, a marker of neural stem cells, to quantify the number of rNSC niches in the DG of CRS mice to further explore the rNSC fate switch and abnormal neurogenesis in CRS mice. The results showed that the total number of nestin-labeled rNSCs in CRS mice decreased significantly compared with that in normal mice ($P = 0.0028$; Fig. 2A and 2C). Under normal conditions, rNSCs divide asymmetrically and mostly differentiate into neurons [25,26]. Therefore, we conducted immunofluorescence staining with NeuN (a marker of mature neurons) and doublecortin (DCX, a marker of newborn immature neurons) to detect whether rNSCs differentiated into neurons. The results showed that

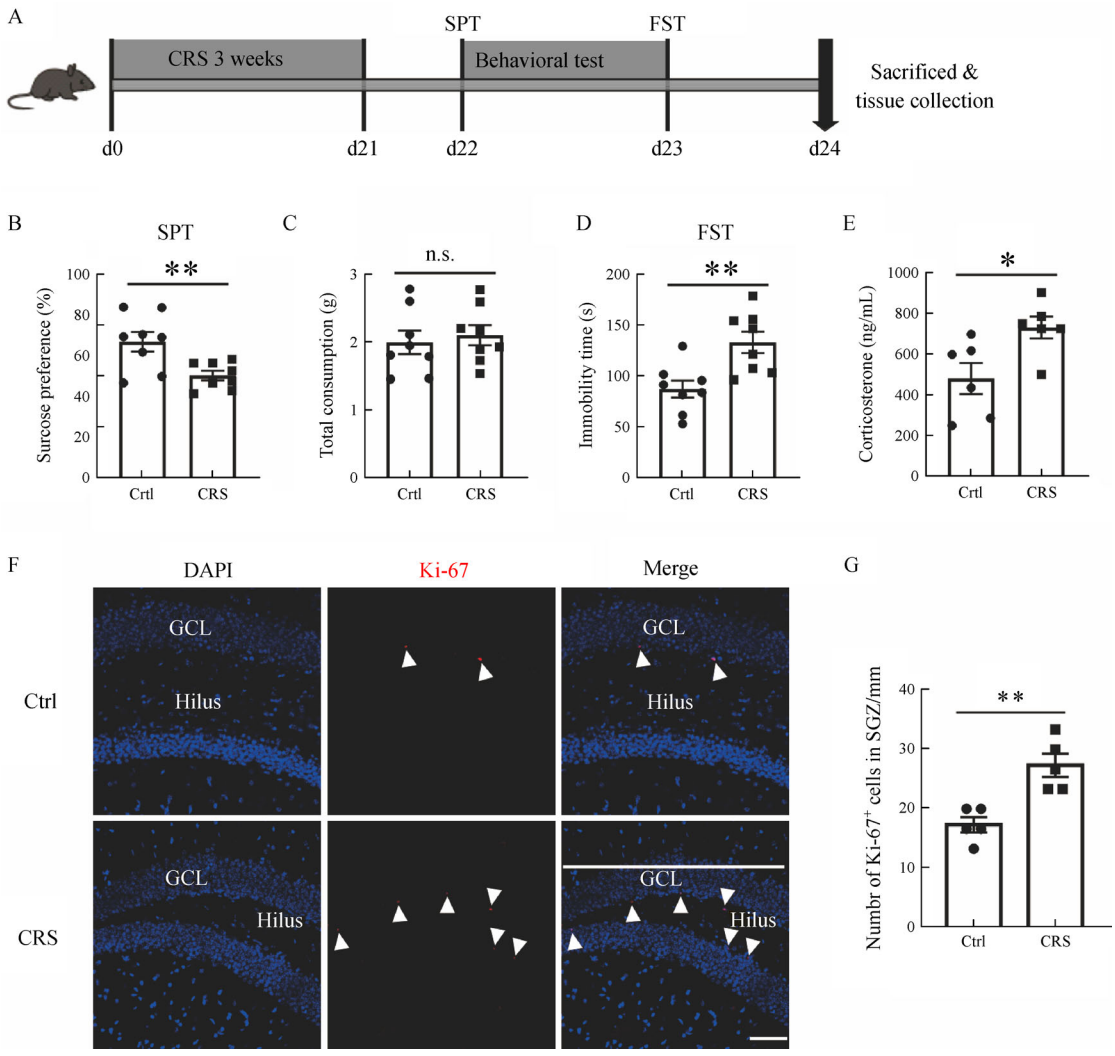

Fig. 1 CRS caused depressive-like behaviors and increased proliferation in the hippocampal DG. (A) Schematic of the experimental design. C57BL/6 mice were subjected to restraint stress for 3 h every day for 3 weeks then to behavioral tests. (B) Percentage of preference for sucrose solution in the SPT (n = 8). (C) Comparison of total water consumption between the control and CRS groups in the SPT experiment. (D) Immobility time in the FST (n = 8). (E) Serum corticosterone levels in the control and CRS groups (n = 6). (F) Representative confocal microscopy images showing Ki-67-labeled cells in the DG of mice. White arrowheads mark proliferating cells. Scale bar = 50 μm. (G) Quantification of Ki-67-labeled cells (n = 5). *P < 0.05, **P < 0.01. n.s., no significant difference.

the total number of DCX-labeled cells and granule cell layer (GCL) thickness of the NeuN-positive layer in CRS mice were not significantly different from those in normal mice (Fig. 2B, 2D, 2E, and 2G). Surprisingly, the total number of cells labeled with GFAP (a marker of astrocytes) increased significantly in CRS mice, indicating that rNSCs may be partially converted into astrocytes instead of neurons (P = 0.0080; Fig. 2F and 2H). In addition, by using transcriptome analysis (RNA-seq), we determined the changes in the expression levels of the genes and signaling pathways related to neurogenesis and differentiation in the DG of CRS mice. RNA-seq results revealed significant changes in the expression level of the related genes (Fig. 2I). Through KEGG enrichment analysis, we found that the FoxO and PI3K/Akt signaling pathways, which are both involved in neural stem cell differentiation, were significantly activated in CRS mice (Fig. 2J and 2K). Moreover, the expression levels of *CDKN1A* (P = 0.0071) and *SGK1* (P = 0.0027), the major signaling nodes of the FoxO signaling pathway, in CRS mice were significantly increased compared with those in normal mice (Fig. 2L and 2M). Taken together, these results demonstrated that hippocampal rNSCs mostly differentiated into astrocytes in CRS mice and suggested that the FoxO signaling pathway was involved in the regulation of this process.

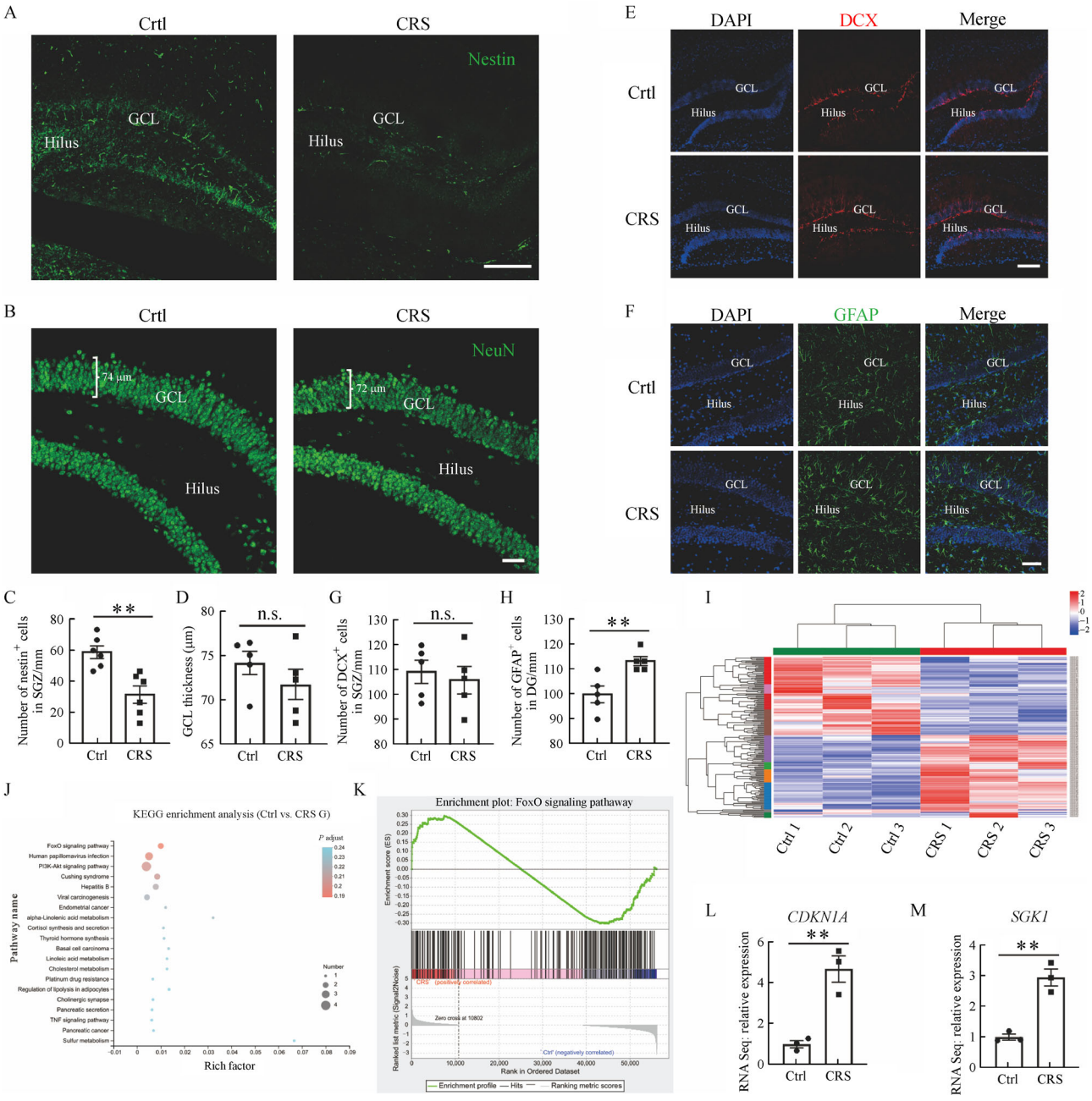

Fig. 2 CRS caused abnormal neurogenesis in the hippocampal DG. (A,C) Representative confocal images of nestin-labeled cells in the DG after CRS (A) and the corresponding quantification results (C, $n = 6$). Scale bar = 100 μm. (B,D) Representative confocal images showing GCL thickness in the DG after CRS (B) and the corresponding quantification results, which were measured as the distance between the hilus and molecular layer (D, $n = 5$). Scale bar = 50 μm. (E,G) Representative confocal images showing DCX-labeled cells in the DG after CRS (E) and the corresponding quantification results (G, $n = 5$). Scale bar = 50 μm. (F,H) Representative confocal images showing GFAP-labeled cells in the DG after CRS (F) and the corresponding quantification results (H, $n = 5$). Scale bar = 50 μm. (I) Transcriptome heatmap analysis showing the differential expression of genes in the control group versus that in the CRS group. (J,K) KEGG pathway enrichment analysis showing the involvement of the FoxO signaling pathway in CRS-induced stress. (L,M) Difference in the expression levels of *CDKN1A* and *SGK1* in the control group versus those in the CRS group. $^{*}P < 0.05$, $^{**}P < 0.01$. n.s., no significant difference.

CBD buffered stress responses and improved depressive-like behaviors in CRS mice

CBD, a natural small-molecule compound derived from cannabis, displays a wide range of pharmacological effects in psychiatric disorders, including anxiety and depression [27]. Enhancing stress resilience has been well-established to help animals cope with environmental stress and prevent psychiatric disorders [11,28]. Therefore, we first determined whether the antidepressant effect of CBD was partly mediated by increasing stress resilience in the CRS mouse model. Thus, mice in the CRS or control group received daily i.p. injections of CBD during model establishment and then were subjected to a series of behavioral tests, including SPT, FST, OFT, and EPM (Fig. 3A). The administration of CBD (10 mg/kg) effectively prevented depressive-like symptoms in CRS mice as evidenced by the higher sucrose consumption ($P = 0.0401$) and shorter immobility time ($P = 0.0029$) in the treatment group than in the vehicle group (Fig. 3B–3D). Meanwhile, the serum levels of corticosterone were markedly decreased after treatment with CBD (Fig. 3E). Moreover, we used the EPM test to determine the anxiety-like behaviors of CRS mice. The results showed that compared with the mice in the vehicle group, CRS mice under CBD (5 or 10 mg/kg) treatment spent longer time (5 mg/kg CBD, $P = 0.0389$; 10 mg/kg CBD, $P = 0.0470$) in the open arm, indicating that CBD could also alleviate anxiety-like behaviors in CRS mice (Fig. 3G and 3H). However, locomotor activity under CBD treatment did not significantly differ from that in the absence of CBD treatment (Fig. 3F). Furthermore, we evaluated the effect of CBD treatment on the activation

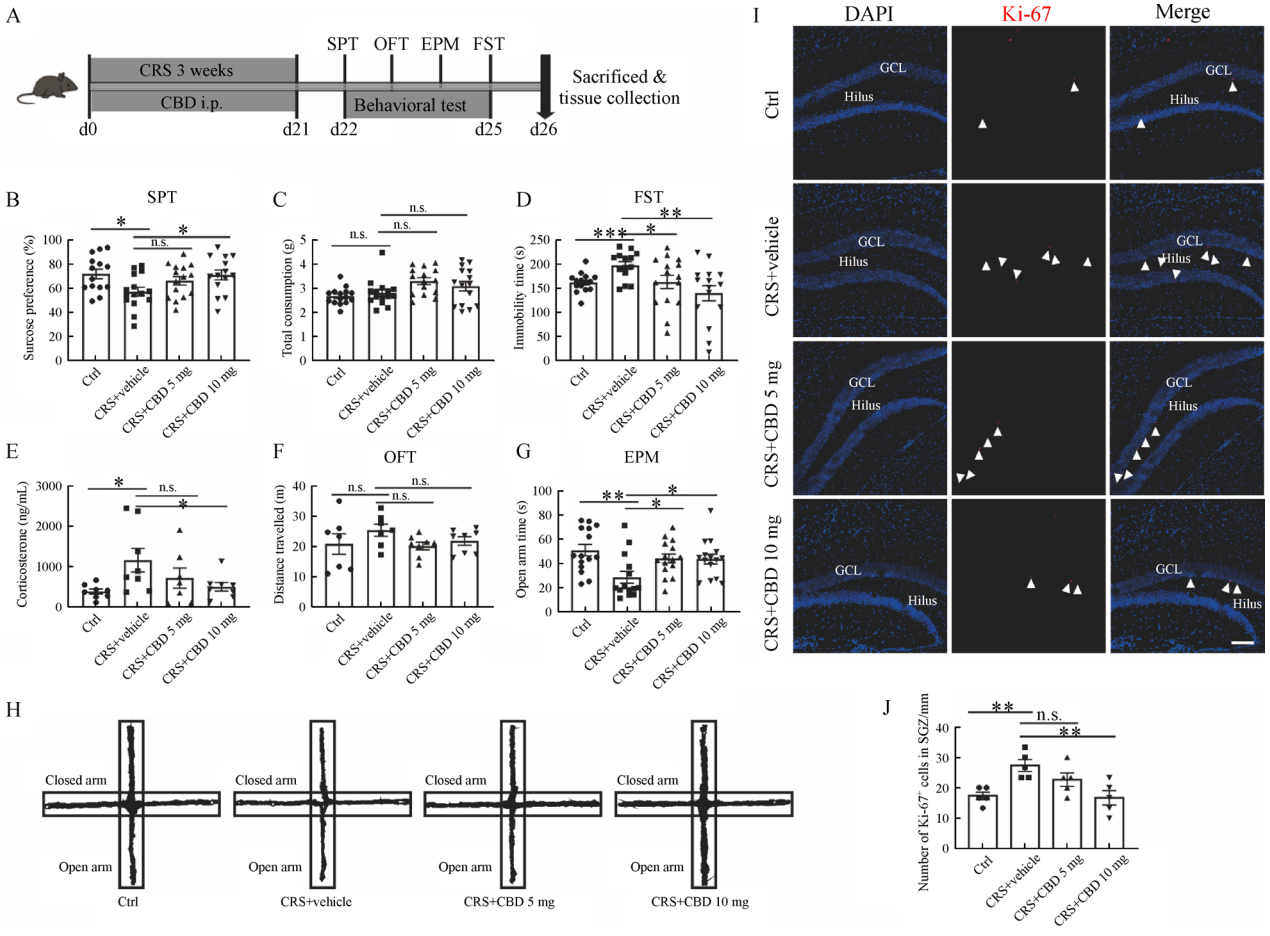

Fig. 3 CBD treatment improved depressive-like behaviors in CRS mice. (A) Schematic of the experimental design and timeline of CBD administration, CRS stimulus, and behavioral tests. (B–D) Percentage of preference for sucrose solution in the SPT (B), total water consumption (C), and immobility time in the FST (D) in the control, CRS, and CBD treatment (5 mg/kg or 10 mg/kg) groups ($n = 12–15$). (E) Serum corticosterone levels in the control, CRS, and CBD treatment (5 mg/kg or 10 mg/kg) groups ($n = 7–8$). (F,H) Locomotor distance in the OFT (F), time spent in the open arm in the EPM (G), and representative EPM behavioral trace (H) in the control, CRS, and CBD treatment (5 mg/kg or 10 mg/kg) groups ($n = 12–15$). (I,J) Representative confocal images showing Ki-67-labeled cells in the DG after CRS (I) and the corresponding quantification results (J, $n = 5$). Scale bar = 50 μm. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. n.s., no significant difference.

of rNSCs. The results showed that the total number of Ki-67-positive cells (P = 0.0047) decreased significantly in the CBD-treated group compared with that in the CRS alone group, indicating that the administration of CBD maintained rNSCs in the quiescent state (Fig. 3I and 3J). Together, these results strongly suggested that the administration of CBD relieved depressive-like symptoms in CRS mice and prevented the overactivation of rNSCs.

CBD reversed abnormal neurogenesis in CRS mice

Previous studies have shown that the hyperactivity of hippocampal DG neurons could lead to abnormal rNSC differentiation in the chronic unpredictable mild stress (CUMS) mouse model [29] and that the administration of CBD could regulate DG neuronal excitability and synaptic transmission [30]. Therefore, we also determined whether stress could affect the asymmetric fate conversion of rNSCs in CRS mice and whether CBD could reverse this process. We performed nestin, DCX, NeuN, GFAP, and IBA1 immunofluorescence staining to detect neural stem cells, immature neurons, mature neurons, astrocytes, and microglia, respectively. The results showed that the total number of nestin-labeled cells (5 mg/kg CBD, P = 0.0405; 10 mg/kg CBD, P = 0.0160) significantly increased after CBD treatment. However, DCX-labeled immature neurons, the GCL thickness of the NeuN-positive layer, and microglia in the CBD-treated group were not significantly different from those in the CRS alone group (Fig. 4A–4D; Fig. S1). Interestingly, the administration of CBD restored the total number of GFAP-labeled astrocytes (P = 0.0127) in the CRS group to the level in the normal control mice (Fig. 4A and 4E), indicating that CBD could inhibit the differentiation of rNSCs into astrocytes and maintain rNSCs in the quiescent state.

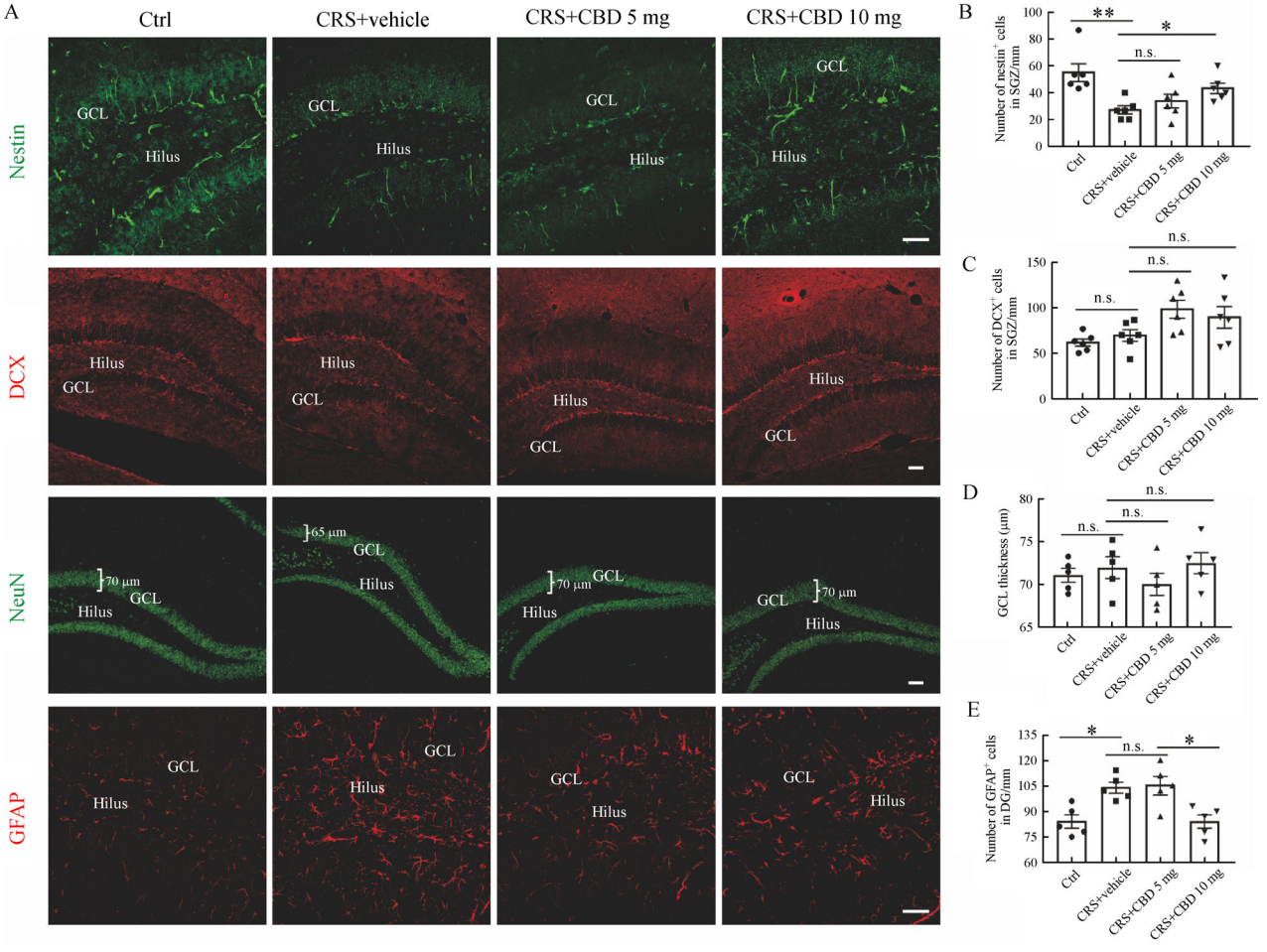

Fig. 4 CBD treatment prevented abnormal neurogenesis in CRS mice. (A) Representative confocal microscopy images showing nestin-, DCX-, NeuN-, and GFAP-labeled cells in DG in the control, CRS, and CBD treatment (5 mg/kg or 10 mg/kg) groups. Scale bar = 50 μm. (B–E) Quantification of the number of nestin-, DCX-, NeuN-, and GFAP-labeled cells in the control, CRS, and CRS + CBD groups (n = 5). *P < 0.05, **P < 0.01. n.s., no significant difference.

CBD prevented abnormal NSC differentiation and FoxO signaling pathway activation caused by CRS

Next, we further used BrdU staining to track the differentiation trace of rNSCs and investigated the role of CBD in this process. For BrdU staining, BrdU (150 mg/kg) was injected three times with 2 h intervals immediately before the 3-week restraint stress procedure (Fig. 5A). The mice were sacrificed on day 22 and subjected to immunofluorescence analysis to detect the proportion of BrdU/ NeuN-colabeled cells and the proportion of BrdU/ GFAP-colabeled cells. A significant reduction in BrdU/ NeuN colabeled cells (P = 0.0139) and a significant increase in BrdU/GFAP colabeled cells (P = 0.0093) accompanied by an increase in total BrdU-labeled cells were observed in CRS mice, indicating that rNSCs may be

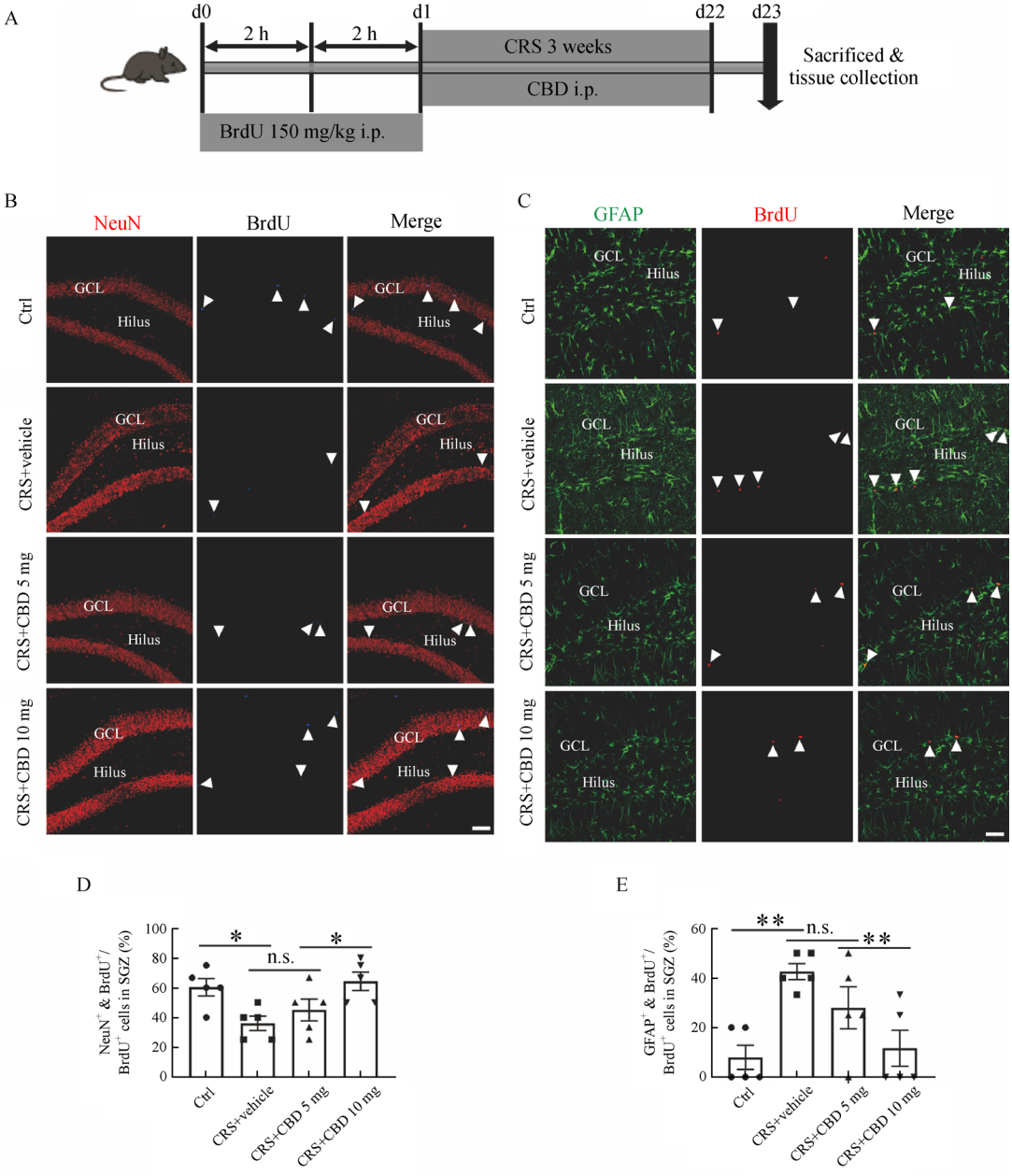

Fig. 5 CBD treatment affected the differentiation fate of neural stem cells in CRS mice. (A) Schematic of the experimental design and timeline of BrdU administration, CBD administration, and CRS stimulation. (B,D) Representative confocal images showing BrdU-labeled and BrdU/NeuN-colabeled cells in the DG in the control, CRS, and CBD treatment (5 mg/kg or 10 mg/kg) groups (B) and the corresponding quantification results (D, n = 5). Scale bar = 50 μm. (C,E) Representative confocal images showing BrdU-labeled and BrdU/GFAP-colabeled cells in the DG in the control, CRS, and CBD treatment (5 mg/kg or 10 mg/kg) groups (C) and the corresponding quantification results (E, n = 5). Scale bar = 50 μm. *P < 0.05, **P < 0.01. n.s., no significant difference.

partly converted into astrocytes (Fig. 5B–5E; Fig. S2). Notably, the administration of CBD could completely rescue the asymmetric fate switch in CRS mice. Given that CRS led to the activation of the FoxO signaling pathway, we used RNA-seq to investigate whether CBD treatment can affect this pathway (Fig. 6A). Through KEGG enrichment analysis, we found that the genes of the FoxO signaling pathway were indeed the most differentially expressed between the CRS and the CBD treatment groups (Fig. 6B and 6C). Moreover, CBD treatment restored the expression of *CDKN1A* (P = 0.0071) and *SGK1* (P = 0.0027) to control levels (Fig. 6D and 6E). In addition, we performed RT-PCR to assess the mRNA levels of *CDKN1A* and *SGK1* to further confirm that the FoxO signaling pathway was activated in the mouse depression model. The CRS mice exhibited higher mRNA levels of *CDKN1A* (P = 0.0015) and *SGK1* (P = 0.0050) than the control mice (Fig. 6F and 6G). Treatment with CBD significantly decreased the mRNA levels of *CDKN1A* (CBD 5 mg, P = 0.0043; CBD 10 mg, P =

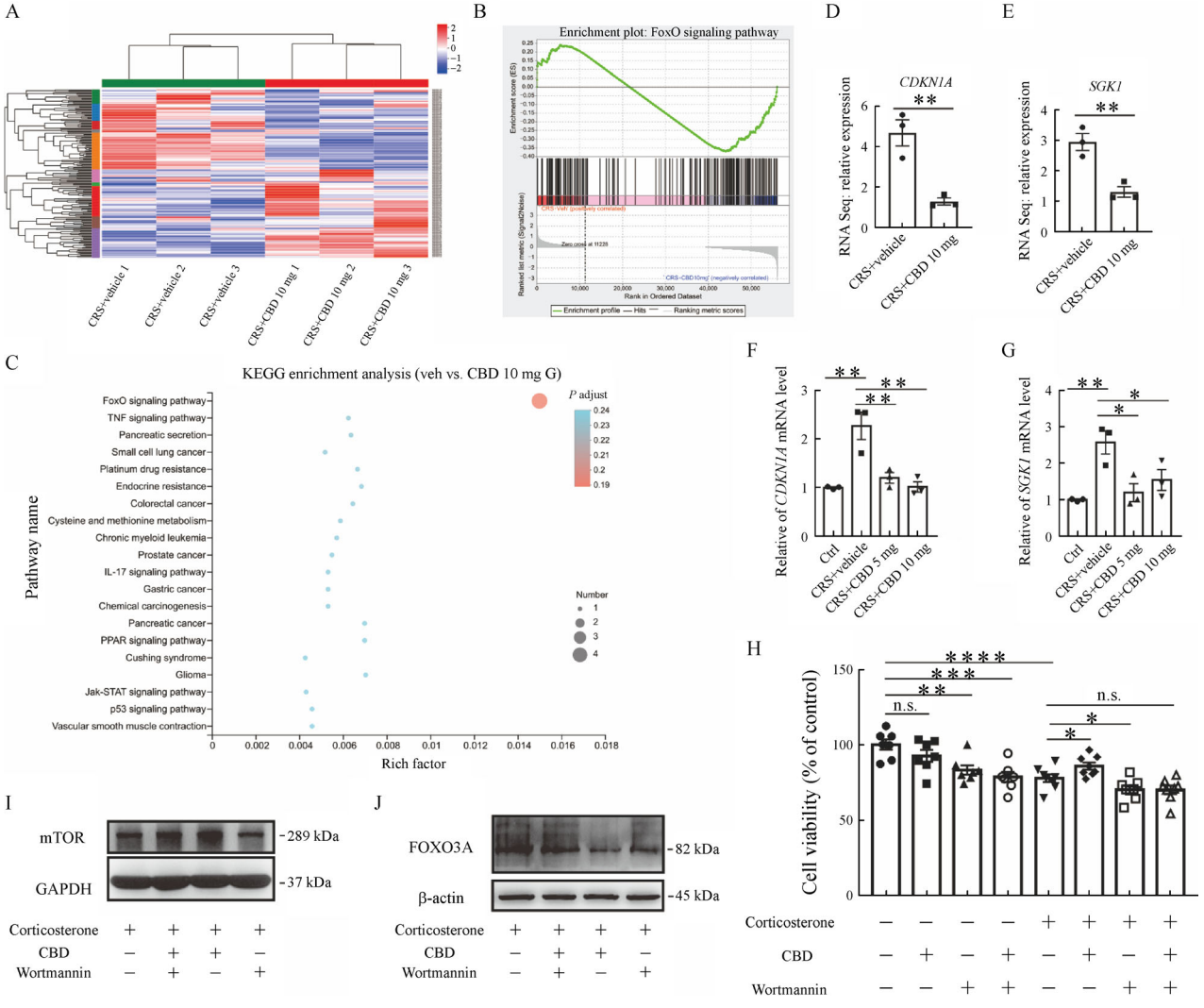

Fig. 6 FoxO signaling pathway is involved in the antidepressive effect of CBD *in vitro* and *in vivo*. (A) Transcriptome heatmap analysis of genes that were differentially expressed between the CRS alone group versus the 10 mg/kg CBD treatment group. (B,C) KEGG pathway enrichment analysis showing the trends and details of the FoxO signaling pathway in the CRS alone group versus those in the 10 mg/kg CBD treatment group. (D,E) Difference in the expression levels of *CDKN1A* and *SGK1* in the CRS alone group versus those in the 10 mg/kg CBD treatment group. (F,G) Effects of CBD treatment on the mRNA levels of *CDKN1A* and *SGK1* in CRS mice were analyzed by using RT-PCR. (H) Effect of corticosterone (30 μmol/L), CBD (10 μmol/L), and wortmannin (20 μmol/L) on the growth of NE-4C neural stem cells. Cell viability was measured by MTT after treatment with corticosterone, CBD, and wortmannin either alone or in combination. (I,J) Effect of corticosterone, CBD, and wortmannin treatment on the proteins of the FoxO signaling pathway. mTOR and FOXO3A protein levels were analyzed by using Western blot analysis. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. n.s., no significant difference.

0.0016) and *SGK1* (CBD 5 mg, $P = 0.0108$; CBD 10 mg, $P = 0.0445$) in CRS mice (Fig. 6F and 6G). Together, these results confirmed that hippocampal rNSCs mostly differentiated into astrocytes after CRS stimulus, whereas the administration of CBD could rescue abnormal rNSC differentiation through the regulation of the FoxO signaling pathway.

We carried out *in vitro* experiments by treating cultured NE-4C neural stem cells, a classical neural stem cell line, with corticosterone to mimic the stress stimulus to investigate whether the FoxO-PI3K/Akt pathway mediated the antidepressive effect of CBD. We first tested the effect of treatment with corticosterone; CBD; and wortmannin, a selective inhibitor of the FoxO–PI3K/Akt pathway, on the proliferation of NE-4C cells. CBD alone did not affect the growth of NE-4C. However, corticosterone (30 μmol/L, $P < 0.0001$) and wortmannin (20 μmol/L, $P = 0.0033$) inhibited NE-4C proliferation (Fig. 6H). The growth inhibitory effect of corticosterone was partly reversed by CBD (10 μmol/L, $P = 0.0352$) treatment (Fig. 6H). Interestingly, pretreatment with wortmannin totally blocked the effect of CBD on NE-4C cell proliferation (Fig. 6H), suggesting that the FoxO–PI3K/Akt pathway was involved in the antidepressive effect of CBD. We then evaluated the status of the FoxO–PI3K/Akt pathway by using Western blot analysis. The results showed that CBD could decrease the protein levels of FOXO3A, a negative regulator of the FoxO pathway, and increase mTOR, the downstream effector of the FoxO pathway, in NE-4C cells; the effect of CBD was partly blocked by wortmannin (Fig. 6I and 6J). Altogether, *in vitro* and *in vivo* experiments demonstrated that the FoxO signaling pathway was overactivated after stress stimulus, and CBD could suppress the activation of the FoxO signaling pathway, leading to the rescue of depressive-like behaviors in CRS mice.

Discussion

A growing body of evidence suggests that hippocampal neurogenesis can buffer stress stimulus and alleviate depressive-like behaviors [8,11,31]. Therefore, the modulation of the proliferation and differentiation of hippocampal neural stem cells could be a potential strategy for the treatment of depression. In this study, we found that long-term restraint stress induced the overactivation of rNSCs, led to the differentiation of rNSCs into astrocytes, and impaired neurogenesis. In CRS mice, treatment with CBD could effectively inhibit rNSC overactivation, restore normal neurogenesis, and rescue depressive-like behaviors.

Promoting AHN could affect the neuronal circuitry related to antidepressant effects and further increase stress resilience against chronic stress [31]. Under physiological conditions, rNSCs, the origin of neural progenitors, divide asymmetrically and mostly differentiate into neurons. The hypothesis that abnormal AHN is involved in the pathology of depression is partly supported by the decrease in AHN and the loss of hippocampal volume observed in patients with major depression and in depressive-like animal models [32,33]. Consistent with previous reports on other depression models [34–36], we found that the numbers of nestin-positive and BrdU/NeuN-double-positive cells were significantly decreased in the CRS model, confirming the impairment of AHN under stress conditions. Intriguingly, our results also revealed a significant increase in the number of BrdU/GFAP-double-labeled cells in CRS mice, indicating that rNSCs underwent activation-dependent differentiation into astrocytes. Notably, a similar phenomenon was also observed in an epilepsy animal model after the focal injection of kainic acid in the subgranular zone of the DG [26], suggesting that hippocampal DG neuronal hyperactivity could lead to abnormal rNSC differentiation. However, the differentiation of rNSCs into astrocytes needs to be further studied, and the molecular mechanism leading to this fate switch remains to be elucidated in the future.

On the basis of the findings on animal models of depression, increasing hippocampal adult-born neurons has been proposed to show antidepressant effects. Several pharmacological approaches have been tested on animal models to modulate AHN. For example, fluoxetine, a SSRI, upregulates AHN along with exerting antidepressant effects [4]. The neurogenic and behavioral effects of SSRIs are mediated by the activation of 5HT1A receptors that are expressed on mature and immature granule cells. The pharmacological inhibition or genetic ablation of neurogenesis can block the effects of fluoxetine, indicating that immature granule cells are involved in the antidepressant effect of SSRIs [37]. CBD, a natural product derived from cannabis, has a complex pharmacology that may contribute to its broad-spectrum therapeutic profile in different psychiatric disorders, including depression. The antidepressive effect of CBD was first discovered approximately 10 years ago [38]. However, studies investigating the effects of CBD in animal models of depression are rarely reported, and the exact mechanism of the antidepressant effect of CBD remains unclear. In this study, we showed for the first time that CBD could relieve depression and anxiety symptoms in the CRS mouse model. We also found that CBD could restore the number of Ki-67-labeled and GFAP-labeled cells to normal levels, demonstrating that CBD inhibited the differentiation of rNSCs into astrocytes. Meanwhile, CBD treatment also prevented a decline in the number of neurons as evidenced by NeuN/BrdU double staining. Most importantly, we found for the first time that rNSCs underwent activation-dependent differentiation into astrocytes under depressive conditions. This differentiation was completely reversed by CBD

treatment. The effect of CBD was also dose-dependent. The administration of a high dose of CBD (10 mg/kg) increased stress resilience, maintained rNSCs in the quiescent state, and rescued depressive-like behaviors, whereas the administration of a low dose of CBD (5 mg/kg) showed a certain but nonsignificant effect. Mechanistically, although our transcriptome analysis provided valuable hints, the molecular pathways behind the fate switch of rNSCs in depression remain unclear. A very recent study reported that CBD can alleviate depressive symptoms in the CUMS mouse model via the activation of 5HT1A receptors [39]. CBD may possibly increase AHN through the regulation of 5HT1A. The proneurogenic effect of CBD has also been demonstrated in cocaine-addicted animals via the cannabinoid receptor CB1R and in the CUMS mice model via GPR55 [40]. Whether these targets are involved in the antidepressant mechanism of CBD warrants further investigation.

In summary, by using a CRS mouse model, we demonstrated that chronic stress led to abnormal neurogenesis in the DG and induced rNSCs to differentiate into astrocytes. The administration of CBD resulted in a strong antidepressant effect and prevented abnormal neural differentiation. Although limited by the lack of detailed molecular mechanisms and the potential target of CBD, this study highlighted the need to deepen our understanding of CBD-induced neurobiological effects to understand fully the therapeutic potential of this phytocannabinoid in psychiatric disorders. Our studies, together with the work of other groups, provide undeniable direct evidence that CBD could be a potential treatment option for depression.

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Compliance with ethics guidelines

Ming Hou, Suji Wang, Dandan Yu, Xinyi Lu, Xiansen Zhao, Zhangpeng Chen, and Chao Yan declare that they have no conflict of interest. All institutional and national guidelines for the care and use of laboratory animals were followed.

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