



ORIGINAL ARTICLE

Astrocytic GABA_B Receptors in Mouse Hippocampus Control Responses to Behavioral Challenges through Astrocytic BDNF

Ji-Hong Liu¹ · Ze-Lin Li¹ · Yi-Si Liu¹ · Huai-De Chu¹ · Neng-Yuan Hu¹ ·
Ding-Yu Wu¹ · Lang Huang¹ · Shu-Ji Li¹ · Xiao-Wen Li¹ · Jian-Ming Yang¹ ·
Tian-Ming Gao¹

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Abstract Major depressive disorder (MDD) is a common mood disorder that affects almost 20% of the global population. In addition, much evidence has implicated altered function of the gamma-aminobutyric acid (GABAergic) system in the pathophysiology of depression. Recent research has indicated that GABA_B receptors (GABA_BRs) are an emerging therapeutic target in the treatment of stress-related disorders such as MDD. However, which cell types with GABA_BRs are involved in this process is unknown. As hippocampal dysfunction is implicated in MDD, we knocked down GABA_BRs in the hippocampus and found that knocking down these receptors in astrocytes, but not in GABAergic or pyramidal neurons, caused a decrease in immobility in the forced swimming test (FST) without affecting other anxiety- and depression-related behaviors. We also generated astrocyte-specific GABA_BR-knockout mice and found decreased immobility in the FST in these mice. Furthermore, the conditional knockout of GABA_BRs in astrocytes selectively increased the levels of brain-derived neurotrophic factor protein in hippocampal astrocytes, which controlled the decrease in immobility in the FST. Taken together, our findings contribute to the current understanding of which

cell types expressing GABA_BRs modulate antidepressant activity in the FST, and they may provide new insights into the pathological mechanisms and potential targets for the treatment of depression.

Keywords Depression · Astrocyte · Gamma-aminobutyric acid receptor · Forced swimming test · Brain-derived neurotrophic factor

Introduction

Major depressive disorder (MDD) is a common chronic mental disorder that is estimated to be the most prevalent and costly brain disease and the leading cause of disability worldwide [1]. Current antidepressants used to treat MDD exert their therapeutic effects through the serotonin and/or norepinephrine systems [2]. However, not all depressed patients respond to these antidepressants and only ~35%–40% of patients treated with antidepressants recover to premorbid levels of functioning [3]. Most seriously, some antidepressants do not work until 3–5 weeks after the initiation of treatment in the vast majority of patients [4]. Nevertheless, some newer types of antidepressant are better tolerated and are safer, in terms of potential overdose, than the older tricyclic compounds. However, these new antidepressants also have troublesome side-effects such as sleepiness, weight gain, and changes in sexual functioning [3]. Thus, finding better antidepressants or methods with better efficacy and fewer side-effects is extremely urgent.

Recently, growing evidence from pre-clinical and clinical data has implicated gamma aminobutyric acid (GABA) as an emerging therapeutic target in the pathophysiology and treatment of depression [5]. For example, some investigators found reduced GABA levels in the plasma and corticospinal

Ji-Hong Liu and Ze-Lin Li have contributed equally to this work.

✉ Tian-Ming Gao
tgao@smu.edu.cn

¹ State Key Laboratory of Organ Failure Research, Key Laboratory of Mental Health of the Ministry of Education, Guangdong–Hong Kong–Macao Greater Bay Area Center for Brain Science and Brain-Inspired Intelligence, Guangdong Key Laboratory of Psychiatric Disorders, Collaborative Innovation Center for Brain Science, Department of Neurobiology, School of Basic Medical Sciences, Southern Medical University, Guangzhou 510515, China

fluid from patients with MDD [6]. In addition, the administration of selective serotonin-reuptake inhibitors increases the GABA levels in corticospinal fluid [7]. Furthermore, treatment with the GABA_A receptor (GABA_AR) agonist benzodiazepines alprazolam and adinazolam has antidepressant effects in depressed patients similar to widely-prescribed antidepressants [8, 9]. GABA_BRs are G protein-coupled receptors and are heterodimers composed of two subunits, GABA_{B(1)} and GABA_{B(2)} [10]. Growing evidence suggests that GABA_BRs are emerging therapeutic targets in the treatment of stress-related psychiatric disorders such as MDD. For example, pharmacological studies have demonstrated that GABA_B agonists have anxiolytic effects, while GABA_B antagonists have antidepressant-like effects [11–13]. Genetic inactivation of the GABA_{B(1)} subunit induces anxiogenic and antidepressant-like phenotypes in different behavioral paradigms [10, 12, 14]. These results suggest that GABAergic systems and their receptors play a role in the treatment of depression [15].

GABA_BRs are G protein-coupled receptors that are expressed by almost all cell types, such as neurons and glial cells in the central nervous system (CNS) [16, 17]. However, the investigations described above do not mention which cell-types with GABA_BRs are involved in modulating MDD. The hippocampus is an important region implicated in the pathogenesis of MDD, as shown by convergent lines of research. In the clinic, MDD is considered a highly stress-sensitive illness [18], and the hippocampus is recognized as a highly stress-sensitive region [19]. Hippocampal volume is reduced before the clinical onset of illness in individuals at risk for MDD [20–22]. Furthermore, these stress-associated changes in the hippocampus can be ameliorated by antidepressant treatment [23]. From the pre-clinical data, we can predict the relationship between the molecular and cellular effects of chronic stress and antidepressant treatment on the hippocampus [24], which suggests that MDD might be associated with hippocampal dysfunction.

Therefore, the aim of this study was to evaluate which cell-types containing GABA_BRs in the hippocampus are involved in modulating emotional responses. Moreover, since some investigations have indicated an intimate relationship between the levels of brain-derived neurotrophic factor (BDNF) and depression, we also explored whether BDNF is involved in this phenomenon.

Materials and Methods

Mice

The mice housed in standard laboratory cages at $24 \pm 1^\circ\text{C}$ were maintained on a 12-h light/dark cycle with lights on at

08:00 and provided standard food and water *ad libitum*. All procedures were conducted with the approval of the Southern Medical University Animal Ethics Committee [25], and efforts were made to minimize animal suffering and to reduce the number of animals used.

Aldh1l1-CreER^{T2} mice were generated at the Model Animal Research Center of Nanjing University (Nanjing, China). Briefly, Aldh1l1-CreER^{T2} knock-in mice were generated *via* a CRISPR/Cas9 system [26, 27] using Cas9 mRNA, single guide RNA (sgRNA; CCAGGTCTTGTCCTCCCAATACTGG), and a donor, which were co-injected into C57BL/6J zygotes by microinjection. Then, these zygotes were transplanted into pseudopregnant mice [28]. The sgRNA-directed Cas9 endonuclease cleavage occurred near the termination codon and created a double-strand break. This break was subsequently repaired and resulted in a T2A-CreER^{T2} insertion before the stop codon of the Aldh1l1 gene. The mice were screened using PCR analysis with specific primers and the following amplification program: one cycle of 5 min at 95°C followed by 10 cycles ($-1^\circ\text{C}/\text{cycle}$) of 30 s at 95°C , 30 s at 65°C , and 2 min at 72°C ; 20 cycles of 30 s at 95°C , 30 s at 55°C , and 2 min at 72°C ; and 5 min at 72°C . The reaction was then held at 4°C and submitted to sequencing validation.

GABA_{B(1)}-loxP mice were a kind gift from Prof. B. Bettler (University of Basel, Switzerland) [29]. Aldh1l1-CreER^{T2} mice were crossed with GABA_{B(1)}-loxP mice to generate Aldh1l1-CreER^{T2}; GABA_{B(1)}^{-/-} (GABA_{B(1)}-cKO) mice. GABA_{B(1)}^{loxP/loxP} mice were used as controls.

Virus Generation and Stereotaxic Injections

The recombinant adeno-associated viral (AAV) vectors were generated by Shanghai Sunbio Medical Biotechnology (Shanghai, China) and were ligated into an AAV5 vector expressing EGFP with viral titers of 2×10^{12} particles/mL. The micropipette was brought to the correct x and y coordinates and lower to the desired z coordinate of the injection site. A 33-gauge needle fitted to a Hamilton syringe was lowered to the hippocampal CA1 region (AP, -2.0 mm; ML, ± 1.6 mm; DV, -1.5 mm), and $0.25 \mu\text{L}$ ($0.1 \mu\text{L}/\text{min}$) of the virus was delivered over 3 min. The needle was withdrawn 10 min after the end of injection. Mice were used 3 weeks after AAV injection.

Immunofluorescence

Tissue slices were washed in PBS and then incubated while shaking for 2 h in PBS containing 5% BSA and 1% Triton X-100 at room temperature. They were then washed three times for 5 min each in PBS, incubated in primary antibodies overnight at 4°C , washed three times for 5 min

each in PBS, incubated in secondary antibodies for 2 h at room temperature, and finally washed three times for 5 min each in PBS. They were then sealed in place for imaging, which was performed using a laser confocal microscope (Nikon C2, Japan). We used the following primary antibodies: rabbit anti-GABA_{B(1)}R (1:1500; ab55051, Abcam, Cambridge, MA), mouse anti-GFAP (1:500; 3670S, Cell Signaling Technology, Beverly, MA), mouse anti-NeuN (1:500; 24307, Cell Signaling Technology), and mouse anti-GAD67 (1:500; MAB5406, Millipore, Billerica, MA)]; and the following secondary antibodies: goat anti-mouse IgG with Alexa Fluor 594 or 488 (1:2000; #A11005 or #A11001, Life Technologies). Fluoroshield mounting medium (ab104139; Abcam) was used to seal the tissues in place.

Cell Counting

Five to eight slices containing each brain area were obtained from each mouse, and three or five mice were used in each experiment. The image allocations were blinded for analysis. The cells were counted to determine how many were single-positive for NeuN, GAD-67, GFAP, and tdTomato or double-positive for tdTomato. The “Cell Counter” plug-in in ImageJ 1.50i software (NIH) was used for cell counting. The presence of DAPI labeling was required to identify each cell. The specificity of the virus injected into Cre mice was defined as merger cells/the total number of GFP+ cells. The specificity of Cre recombinase expression in the transgenic mouse line was defined as the merger cells/the total number of tdTomato+ cells. The number of merger cells/the total number of marker+ cells was used to define the mean efficiency of Cre recombinase expression.

Western Blots

Western blots were performed as previously described [30]. The tissue from Cre mice injected with virus included only the GFP-infected areas in CA1, while tissue from GABA_BR-cKO mice included the whole hippocampus. Then, the tissue was lysed in ice-cold lysis buffer (11836170001, Roche, Switzerland) containing 1 mmol/L of the protease inhibitor PMSF. Samples were then centrifuged for 30 min at 16,000 g at 4 °C, and the supernatant was collected for quantification using the Microplate BCA Protein Assay Kit (#23227, Thermo, MA). The proteins were separated by SDS-PAGE (10% for IP3R2 or 12% for P2X2 polyacrylamide gels) and transferred to a PVDF membrane (Millipore). The membrane was blocked with 5% defatted milk powder at room temperature for 1 h and then incubated overnight with the primary antibody [rabbit polyclonal anti-GABA_{B(1)}R

(1:1500; ab55051, Abcam, Cambridge, MA) and rabbit polyclonal anti-BDNF (1:1000; ab108319, Abcam, Cambridge, MA)] at 4 °C. Antibody binding was detected with an HRP-conjugated secondary antibody [monoclonal mouse anti-β-actin (1:1000; Bostor, China) and monoclonal mouse anti-GAPDH (1:1,000; 3683S, Cell Signaling)] at room temperature for 1 h. The protein expression levels were evaluated by quantifying the gray density of the western blot bands with FluorChem SP software (ProteinSimple, USA). All samples were normalized to internal controls.

Open Field Test

The open field apparatus was a rectangular chamber (40 × 40 × 30 cm³) made of gray polyvinyl chloride. As previously described [25], each mouse was gently placed in the center of the chamber and left for 5 min to record free movement, which was monitored by an automated video tracking system. The digitized image of the path was mapped and analyzed using EthoVision 11.0 software (EthoVision, Noldus, USA).

Elevated Plus Maze (EPM) Test

The EPM consisted of four arms (30 × 5 cm²): two open arms without walls and two closed arms with 15.25-cm-high walls. Each mouse was placed in the center of the elevated plus maze, facing an open arm. In the 5-min test, the time spent in each arm was recorded using EthoVision 11.0. The maze was cleaned between sessions with 20% ethanol.

Light-Dark Box Test

This test was performed as previously described [31]. The box apparatus contained two similar opaque compartments connected by a central opening (18 × 10 × 13 cm³; light compartment illuminated by a 60 W desk lamp). Each mouse was placed in the center of the dark compartment, facing the opening. Then, the mouse was tracked for 5 min after first crossing the opening threshold. The total number of entries into the dark side and time spent in the dark compartment were recorded.

Social Interaction Test

This behavioral test was performed in a dark room as previously described [25]. A mouse was placed in the center of the testing chamber, and baseline movement was tracked for 2.5 min, followed by another 2.5 min in the presence of a caged male aggressor mouse. The times that the mice spent in the interaction zone and the corner zone

were recorded using Ethovision XT software (EthoVision, Noldus, USA). The chamber was cleaned between sessions with 20% ethanol.

Sucrose Preference Test

Mice were singly housed 3 days before the test, and then we replaced the normal water bottles with two 50-mL bottles (A and B), the positions of which were switched daily to avoid a side bias. The mice were habituated with water for the first 2 days (*w/w*) followed by a 1% sucrose solution for the next 2 days. Then, sucrose preference tests were performed on days 5–8, with bottle A containing 1% sucrose and bottle B containing water. We measured the volume of fluid consumed from each bottle daily. The sucrose preference was calculated as $v_A / (v_A + v_B)$ [32].

Forced Swimming Test (FST)

The FST apparatus was a clear glass cylinder (height 45 cm, diameter 19 cm) filled with water (22 °C–25 °C) to 23 cm. In the 6-min test, the duration of immobility was measured during the final 4 min using Ethovision XT software.

Contextual Fear Conditioning Test

The contextual fear conditioning test was conducted as reported previously [25]. Each mouse was first habituated to the room, and then allowed to freely explore the apparatus for 3 min (EthoVision, Noldus, USA). During training, each mouse was placed in conditioning chamber A, and exposed to tone-foot-shock pairings (tone, 30 s, 80 dB; foot shock, 1 s, 0.4 mA) at an interval of 80 s. Twenty-four hours after training, each mouse was returned to chamber A to evaluate contextual fear learning. Freezing during training and testing was scored using Med Associates Video-Tracking and Scoring software.

ELISA

The mice were anesthetized with pentobarbital and then decapitated; the brain was quickly removed and the medial prefrontal cortex (mPFC), nucleus accumbens (NAc), habenula (Hb), hippocampus, and ventral tegmental area (VTA) were dissected. BDNF levels were assessed with the Emax ImmunoAssay System ELISA kit (BDNF-ELISA E-max, Promega, USA) following the manufacturer's instructions.

Cell Cultures

We isolated primary astrocytes from the hippocampus of mice on postnatal day 1 and used a modified established protocol [33]. We isolated primary hippocampal neurons using a protocol that is routinely performed in our laboratory [32, 34].

Statistical Analyses

In the experimental data, *t*-tests were used to compare the means of two independent samples, and one-way ANOVA was used to compare the means of multiple groups of samples using SPSS 22.0 software (SPSS, Chicago, IL). The mean values shown in the text and figures are expressed as the mean \pm standard error of the mean (SEM), unless otherwise stated. $P < 0.05$ was considered statistically significant, and GraphPad Prism 6.0 (La Jolla, CA) was used to draw the graphs.

Results

Conditional Knock-Down of Neuronal GABA_BRs in the Hippocampal CA1 Region Has No Effect on Emotional Responses

GABA_BRs have been reported to be expressed in neurons, such as pyramidal and GABAergic neurons [10]. To investigate whether neuronal GABA_BRs have an effect on emotional responses, we used adeno-associated virus (AAV)–DIO-GABA_B short-hairpin RNAs (shRNAs) and first injected the AAV–GABA_B shRNA virus into the CA1 region in CamKII-Cre mice to knock down the receptor in pyramidal neurons (Fig. 1A), then we did the behavioral tests (Fig. 1B). Immunofluorescence results showed that the virus was correctly injected into CA1 and that most of the pyramidal neurons were infected (Fig. 1C, D). Western blot results verified that the receptor was knocked down ($t_{(8)} = 6.998$, $P = 0.003$; Fig. 1E, F).

We first investigated anxiety-related behaviors. In the open field test, there was no significant difference between groups in locomotor activity ($t_{(16)} = -1.360$, $P = 0.732$; Fig. 1G) or exploration time in the center ($t_{(17)} = 0.342$, $P = 0.284$; Fig. 1H). Mice with GABA_BRs knocked out in pyramidal neurons spent an amount of time similar to normal mice in the open arms ($t_{(17)} = 2.235$, $P = 0.764$; Fig. 1I) and closed arms ($t_{(17)} = 1.678$, $P = 0.665$; Fig. 1J) in the EPM. In the light/dark test, the number of entries into the light compartment ($t_{(17)} = 4.679$, $P = 0.154$; Fig. 1K) and time spent in the light compartment ($t_{(17)} = 3.626$, $P = 0.202$; Fig. 1L) were similar in mice injected with control or GABA_BRs shRNA. Similar results were obtained in the

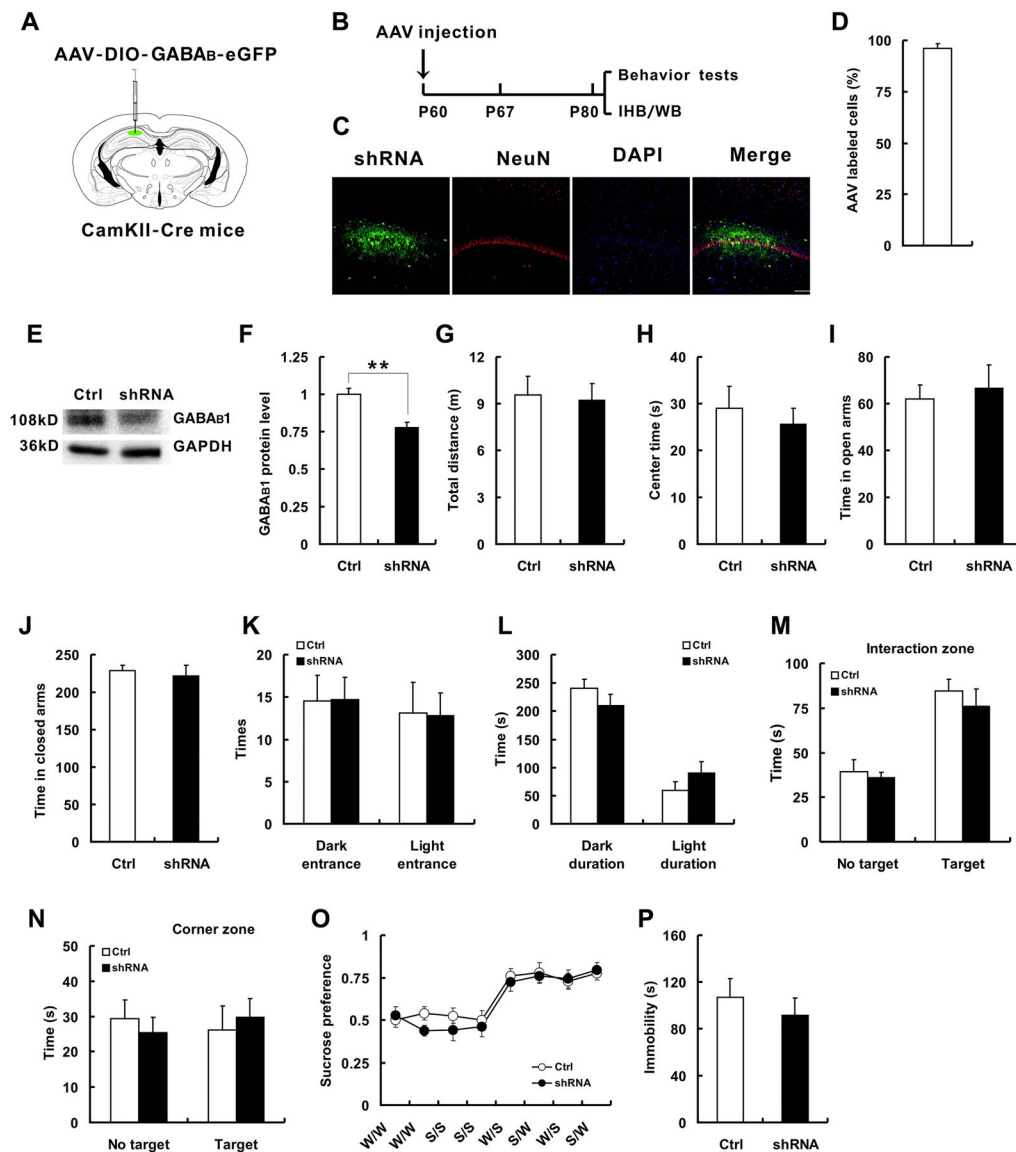


Fig. 1 Hippocampal GABA_BRs in pyramidal neurons are not necessary for response to behavioral challenge. **A** Schematic of the delivery of AAV-DIO-GABA_B-eGFP into the CA1 region of CamKII-Cre mice. **B** Schematic of the experiments. **C** Representative fluorescence images showing that most of the cells infected with AAV-DIO-GABA_B-eGFP (shRNA) vectors were pyramidal neurons in CA1 of CamKII-Cre mice (scale bar, 100 μ m). **D** Average percentage of cells infected with AAV-DIO-GABA_B-eGFP (shRNA) vectors that were positive among pyramidal neurons. **E** Representative blots showing that GABA_{B1} levels were decreased in the hippocampus of CamKII-Cre mice injected with GABA_B shRNA. **F** Quantitative analysis of data as in **E** ($n = 4$ pairs of mice, two-tailed Student's t -test; GABA_{B1} band density was normalized to the loading control GAPDH; values from control mice were taken as 100%). **G**, **H** Mice with GABA_BR-knockdown in pyramidal neurons in CA1 travelled the same total distance (**G**) and spent the same time in the central arena (**H**) in the open field test (Ctrl: $n = 9$ mice; shRNA: $n = 10$; two-tailed

Student's t -test). **I**, **J** In the elevated plus maze test, CamKII-Cre mice injected with GABA_B shRNA spent the same amount of time in the open (**I**) and closed (**J**) arms (Ctrl: $n = 9$; shRNA: $n = 10$; two-tailed Student's t -test). **K**, **L** There was no difference in the number of entries (**K**) or time spent (**L**) in the light/dark sides of the light/dark box test (Ctrl: $n = 9$; shRNA: $n = 10$; two-tailed Student's t -test). **M**, **N** There was no difference between the two groups in the time spent in the interaction zone and corner zone in the presence/absence of a target mouse (Ctrl: $n = 9$; shRNA: $n = 10$; two-tailed Student's t -test). **O** Knockdown of GABA_BRs in pyramidal neurons in CA1 did not change the preferences in the sucrose preference test (Ctrl: $n = 9$; shRNA: $n = 10$; repeated measures two-way ANOVA). **P** Immobility time was not changed in the FST after knockdown of GABA_BRs in pyramidal neurons in CA1 (Ctrl: $n = 9$; shRNA: $n = 10$; two-tailed Student's t -test). Data are presented as the mean \pm SEM; * $P < 0.05$ vs controls.

time spent interacting with other mice in the social interaction test in knocked-down mice compared to control mice ($t_{(17)} = -2.646$, $P = 0.423$; Fig. 1M, N). These results

indicate that knocking out GABA_BRs in pyramidal neurons has no effect on anxiety-like behaviors. We next investigated depressive-like behaviors. The sucrose preference

test was used to assess anhedonia, a core symptom of depression [35]. No differences were found between the two groups in consuming sucrose solution ($F_{(1,136)} = 10.138$; $P = 0.461$; Fig. 1O). The FST is widely considered to test depression-related responses in rodents [36]. We found that the total duration of immobility in the FST was also not affected after knocking out GABA_BRs in pyramidal neurons ($t_{(17)} = 2.021$, $P = 0.46$; Fig. 1P), suggesting no effect of pyramidal neuronal GABA_BRs on depressive-like behaviors.

We then injected the virus into the CA1 region in the GAD-Cre mice to knock down the receptors in GABAergic neurons (Fig. 2A) and tested the behaviors (Fig. 2B). Immunofluorescence (Fig. 2C, D) and western blot results ($t_{(8)} = 3.087$, $P = 0.037$; Fig. 2E, F) verified the effectiveness of the GABA_BR shRNA virus in GAD-Cre mice. We found that knocking out GABA_BRs in GABAergic neurons in CA1 did not affect the behaviors (Fig. 2G–N), confirming the lack of an effect of GABAergic neuronal GABA_BRs on emotion-related behaviors.

Conditional Knockdown of Astrocytic GABA_BRs in the CA1 Region Decreases Immobility in the FST

To investigate whether astrocytic GABA_BRs in the hippocampus are critical in modulating emotional responses, we used aldehyde dehydrogenase 1 family member L1 (aldh1l1)::CreER^{T2} mice. First, to assess the specificity of the expression in astrocytes of aldh1l1::CreER^{T2} mice, we crossed this line with Ai14 reporter mice to create the double transgenic mouse line aldh1l1::CreER^{T2}:Ai14. Then, these mice were treated with 75 mg/kg tamoxifen for seven days at P60–66, and CreER^{T2}-mediated recombination was assessed in the hippocampus two weeks after the last dose of tamoxifen (Fig. 3A). We found a high level of specificity in CA1 in the aldh1l1::CreER^{T2}:Ai14 mice (GFAP co-expression: $97.18\% \pm 0.58\%$; Fig. 3B–D). This inducible system allowed us to delete GABA_BRs specifically in the astrocytes of adult animals. We then injected the virus into the CA1 region in aldh1l1-CreER^{T2} mice to knock down astrocytic GABA_BRs in the hippocampus induced by one week of intraperitoneal injection of tamoxifen (75 mg/kg; Fig. 4A) and then we investigated the emotional responses in the behavioral tests (Fig. 4B). The viral expression indicated by green fluorescence demonstrated that the virus was correctly injected into CA1, and most of the astrocytes were infected (Fig. 4C, D). In addition, the results of western blots verified that the receptor was knocked down ($t_{(8)} = 6.880$, $P = 0.028$; Fig. 4E, F). We then tested these mice for anxiety- and depression-related behavior. We found that knocking out the astrocytic GABA_BRs in CA1 did not affect behavior in the open field test (Fig. 4G, H), EPM (Fig. 4I, J), light/dark

box (Fig. 4K, L), social interaction test (Fig. 4M, N), or sucrose preference test (Fig. 4O). However, in the FST, aldh1l1-CreER^{T2} mice that had the shRNA virus injected into CA1 exhibited decreased immobility, which is considered a state of passive coping or behavioral despair and represents an active escape behavior [37, 38], compared with that in mice injected with control virus ($t_{(15)} = 7.781$, $P = 0.012$; Fig. 4P). These results confirmed a positive role of astrocytic GABA_BR deletion in response to behavioral challenge.

Conditional Knockout of GABA_BRs in Astrocytes Leads to Decreased Immobility Time in the FST

To further determine whether astrocytic GABA_BRs have an effect on emotional responses, we generated conditional GABA_{B(1)}R mutant mice by crossing GABA_B^{loxP/loxP} mice with aldh1l1::CreER^{T2} mice (Figs. 3 and 5A), in which Cre expression was under the control of the human aldh1l1 promoter. After one week of daily intra-peritoneal injection of tamoxifen (75 mg/kg), we used the behavioral tests to evaluate the emotional responses of mice (Fig. 5B). Western blot analysis revealed that GABA_BRs were decreased in the hippocampus in aldh1l1-GABA_BR^{-/-} mice (cKO), compared with the levels in the control wild-type (WT) mice ($t_{(6)} = 7.740$, $P = 0.017$; Fig. 5C, D). In evaluating the emotion-related behaviors, we found that knocking out GABA_BRs in astrocytes had no effect (Fig. 5E–M), except for a decrease in the total duration of immobility in the FST ($t_{(16)} = 8.311$, $P = 0.002$; Fig. 5N). To explore whether the decreased immobility in the FST is a result of the impairment of learning and memory caused by knocking out GABA_BRs in astrocytes, we next assessed contextual fear conditioning in cKO and WT mice and found that this did not affect the learning and memory process (Fig. 5O, P). These results further indicate that mice with astrocytic GABA_BR deletion exhibit an active behavioral response to challenge.

Conditional Knockout of Astrocytic GABA_BRs Increases Astrocytic BDNF Levels in the Hippocampus

Neurotrophic factors, such as BDNF, are critical regulators of mood disorders [39], and some investigations have highlighted the relationship between BDNF and depression, as well as its role in the antidepressant treatment [40–42]. For example, reduced brain BDNF levels have been found in postmortem samples from depressed patients [43], whereas BDNF infusion into the brain can induce antidepressant-like behaviors [44, 45]. In addition, mice exposed to stress exhibit decreased levels of BDNF that are associated with depression [46, 47], while antidepressant

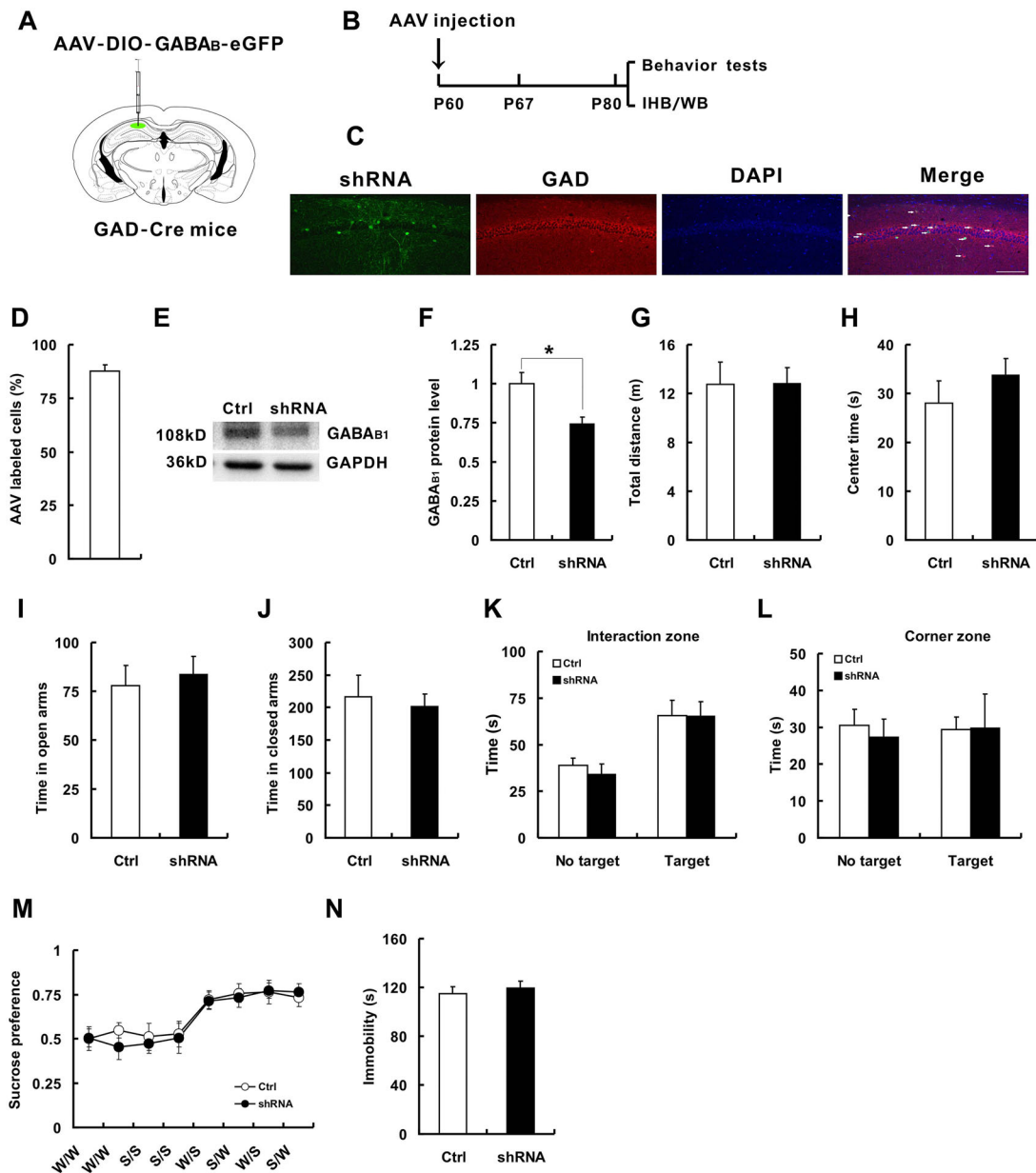


Fig. 2 Hippocampal GABA_BRs in GABAergic neurons are not critical for responses to behavioral challenge. **A** Schematic of the delivery of AAV-DIO-GABA_B-eGFP into CA1 in GAD-Cre mice. **B** Schematic of the experiments. **C** Representative fluorescence images showing that most of the cells infected with AAV-DIO-GABA_B-eGFP (shRNA) vectors were GABAergic neurons in CA1 of GAD-Cre mice (scale bar, 100 μ m). **D** Average percentage of cells infected with AAV-DIO-GABA_B-eGFP (shRNA) vectors that were GABAergic neurons. **E** Representative blots showing GABA_{B1} was decreased in the hippocampus of GAD-Cre mice injected with GABA_B shRNA. **F** Quantitative analysis of data as in **E** ($n = 4$ pairs of mice, two-tailed Student's *t*-test). GABA_{B1} band density was normalized to the loading control GAPDH; values from control mice were taken as 100%. **G**, **H** Mice with GABA_BRs knocked down in GABAergic neurons in CA1 travelled the same total distance (**G**) and

spent the same amount of time in the central arena (**H**) in the open field test (Ctrl: $n = 10$ mice; shRNA: $n = 11$; two-tailed Student's *t*-test). **I**, **J** In the EPM test, GAD-Cre mice injected with GABA_B shRNA spent the same amount of time in the open (**I**) and closed (**J**) arms (Ctrl: $n = 10$; shRNA: $n = 11$; two-tailed Student's *t*-test). **K**, **L** No difference was found between the two groups in the time spent in the interaction zone or corner zone in the presence/absence of a target mouse (Ctrl: $n = 10$; shRNA: $n = 11$; two-tailed Student's *t*-test). **M** Knocking down GABA_BRs in GABAergic neurons in CA1 did not change preferences in the sucrose preference test (Ctrl: $n = 10$; shRNA: $n = 11$; repeated measures two-way ANOVA). **N** In the FST, immobility time was similar in the two groups (Ctrl: $n = 10$ mice; shRNA: $n = 11$ mice; two-tailed Student's *t*-test). Data are presented as the mean \pm SEM; * $P < 0.05$ vs controls.

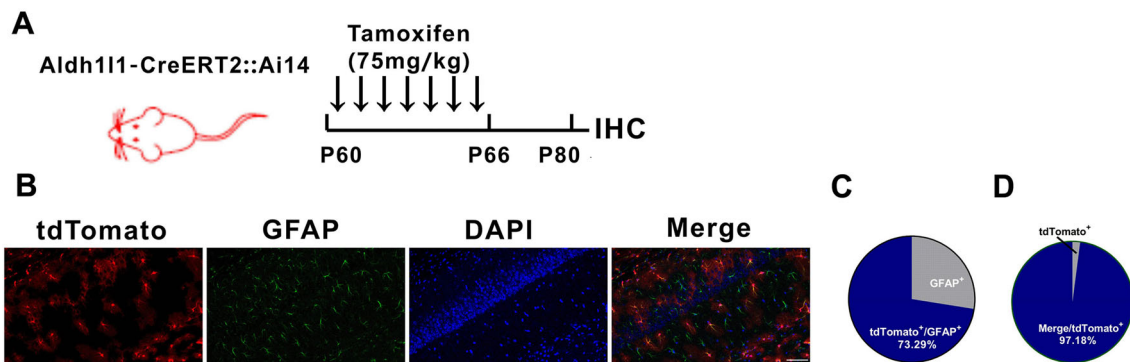


Fig. 3 Characterization of the Aldh1l1-CreERT² transgenic line. **A** Schematic of the experiments. **B** Representative high-magnification images of CA1 astrocytes in Aldh1l1-CreERT² transgenic mice showing co-staining with GFAP (scale bar, 50 μ m). **C** Pie-chart showing the specificity (percentage of tdTomato-positive cells that express GFAP) of Cre-mediated recombination in CA1 of Aldh1l1-CreERT²

transgenic mice with tamoxifen (75 mg/kg; $n = 7$ slices from 4 mice). **D** Pie-chart showing the efficiency (percentage of GFAP-positive cells that express tdTomato) of Cre-mediated recombination in CA1 of Aldh1l1-CreERT² transgenic mice ($n = 7$ slices from 4 mice). Data show the mean \pm SEM.

treatment can block the effects of stress through BDNF [48, 49]. These studies collectively indicate the significant role of BDNF in depression.

To test whether the decreased immobility in the FST with astrocytic GABA_BR deletion is due to changes in BDNF, we measured the BDNF protein levels in depression-related brain regions (the mPFC, NAc, Hb, hippocampus, and VTA) in WT and GABA_BR-cKO mice and found significantly higher levels in the hippocampus of GABA_BR-cKO mice (80.26 ± 2.43 pg/mL) than in WT mice (57.97 ± 2.49 pg/mL) ($t_{(4)} = 5.141$, $P = 0.004$; Fig. 6A). No significant difference was detected in the BDNF protein levels between the two groups in the mPFC (WT, 39.29 ± 0.87 pg/mL *versus* cKO, 35.22 ± 3.03 pg/mL, $t_{(4)} = 0.148$, $P = 0.384$); NAc (WT, 24.24 ± 4.95 pg/mL *vs* cKO, 28.74 ± 4.03 pg/mL, $t_{(4)} = 2.987$, $P = 0.844$); Hb (WT, 19.40 ± 3.77 pg/mL *vs* cKO, 22.90 ± 0.54 pg/mL, $t_{(4)} = 1.241$, $P = 0.644$); and VTA (WT, 49.18 ± 3.86 pg/mL *vs* cKO, 49.90 ± 8.89 pg/mL, $t_{(4)} = 2.151$, $P = 0.594$). Furthermore, western blot analysis confirmed an increase in BDNF protein level in the hippocampus of cKO mice ($t_{(6)} = 7.081$, $P = 0.044$; Fig. 6B, C). Moreover, BDNF concentrations were markedly increased in the culture medium of astrocytes isolated from the hippocampus of cKO mice ($t_{(10)} = 8.054$, $P = 0.007$; Fig. 6D). However, neuronal BDNF release was undisturbed by the lack of astrocytic GABA_BRs ($t_{(10)} = 3.646$, $P = 0.361$; Fig. 6D), indicating that BDNF release specifically from astrocytes is increased in cKO mice. These results suggest that the conditional knockout of GABA_BRs in astrocytes selectively increases the level of BDNF from astrocytes in the hippocampus, and that this increase may contribute to the active behavioral response to challenge.

Decreasing BDNF Levels Restore Changes in GABA_BR-cKO Mice

To further test the involvement of astrocytic BDNF in modulating the response to behavioral challenge, we injected AAV-DIO-BDNF shRNAs into the CA1 region of GABA_BR-cKO mice to knock down astrocytic BDNF (Fig. 7A), then we carried out the behavioral tests (Fig. 7B). Immunofluorescence results indicated that the virus was correctly injected into CA1 and that most of the astrocytes were infected with the virus (Fig. 7C, D). Western blot results ($t_{(6)} = 5.576$, $P = 0.041$; Fig. 7E, F) and ELISA ($t_{(4)} = 5.386$, $P = 0.011$; Fig. 7G) showed that the BDNF levels were almost restored to normal in the hippocampus of knockdown animals compared with those in control mice. In the behavioral tests, we found that decreased BDNF levels rescued the abnormal performance in the FST, while GABA_BR-cKO mice injected with control shRNA still showed decreased immobility ($t_{(17)} = 6.416$, $P = 0.023$; Fig. 7Q). However, the injection of shRNA had no effect on the performance in the open field test (Fig. 7H, I), EPM (Fig. 7J, K), light/dark box test (Fig. 7L, M), social interaction test (Fig. 7N, O), or sucrose preference test (Fig. 7P). These data suggested that enhanced astrocytic BDNF contributes to controlling the response to behavioral challenge caused by deleting astrocytic GABA_BRs.

Discussion

The major findings of this study are as follows. First, knocking down GABA_BRs in astrocytes in the hippocampus, but not in GABAergic or pyramidal neurons, caused a decrease in immobility time in the FST. Second,

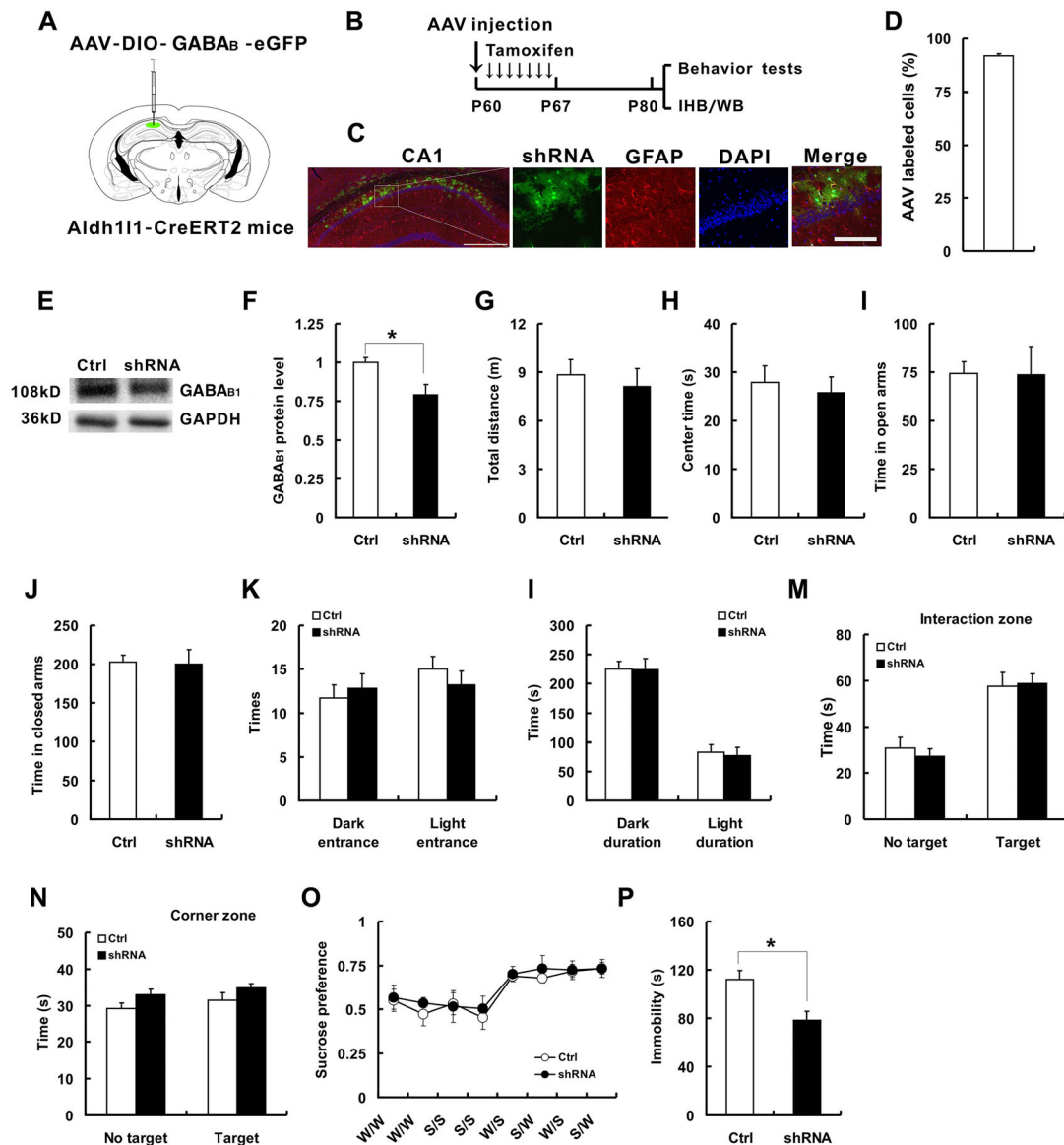


Fig. 4 Conditional knockdown of astrocytic GABA_BRs in the CA1 region decreases immobility in the FST. **A** Schematic of the delivery of AAV-DIO-GABA_B-eGFP into CA1 in Aldh111-CreER^{T2} mice. **B** Schematic of the experiments. **C** Representative location of the GABA_B shRNA virus (green) injected into the CA1 (left; scale bar, 500 μ m), and representative fluorescence images showing that most of the cells infected with AAV-DIO-GABA_B-eGFP (shRNA) vectors were astrocytes in CA1 of ald111-CreER^{T2} mice (right; scale bar, 125 μ m). **D** Average percentage of cells infected with AAV-DIO-GABA_B-eGFP (shRNA) vectors that were positive for astrocytes. **E** Representative blots showing that GABA_{B1} was decreased in the hippocampus of ald111-CreER^{T2} mice injected with GABA_B shRNA. **F** Quantitative analysis of data as in **E** ($n = 4$ pairs of mice, two-tailed Student's *t*-test). GABA_{B1} band density was normalized to the loading control GAPDH; values from control mice are taken as 100%. **G, H** Mice with knockdown of astrocytic GABA_BRs in the CA1 region travelled the same total distance (**G**) and spent the same

amount of time in the central arena (**H**) in the open field test (Ctrl: $n = 8$ mice; shRNA: $n = 9$; two-tailed Student's *t*-test). **I, J** In the EPM test, Aldh111-CreER^{T2} mice injected with GABA_B shRNA spent the same time in the open (**I**) and closed (**J**) arms (Ctrl: $n = 8$; shRNA: $n = 9$; two-tailed Student's *t*-test). **K, L** There was no difference between the two groups in the time spent in the interaction zone or corner zone in the presence/absence of a target mouse (Ctrl: $n = 8$; shRNA: $n = 9$; two-tailed Student's *t*-test). **M, N** There was no difference in the number of entries (**M**) or time spent (**N**) in the light/dark side in the light/dark box test (Ctrl: $n = 8$; shRNA: $n = 9$; two-tailed Student's *t*-test). **O** Knocking down the GABA_BRs in CA1 did not change preferences in the sucrose preference test (Ctrl: $n = 8$; shRNA: $n = 9$; repeated measures two-way ANOVA). **P** In the FST, Aldh111-CreER^{T2} mice injected with GABA_B shRNA exhibited decreased immobility compared with that of wild-type mice (Ctrl: $n = 8$; shRNA: $n = 9$; two-tailed Student's *t*-test). Data are presented as the mean \pm SEM; * $P < 0.05$ vs controls.

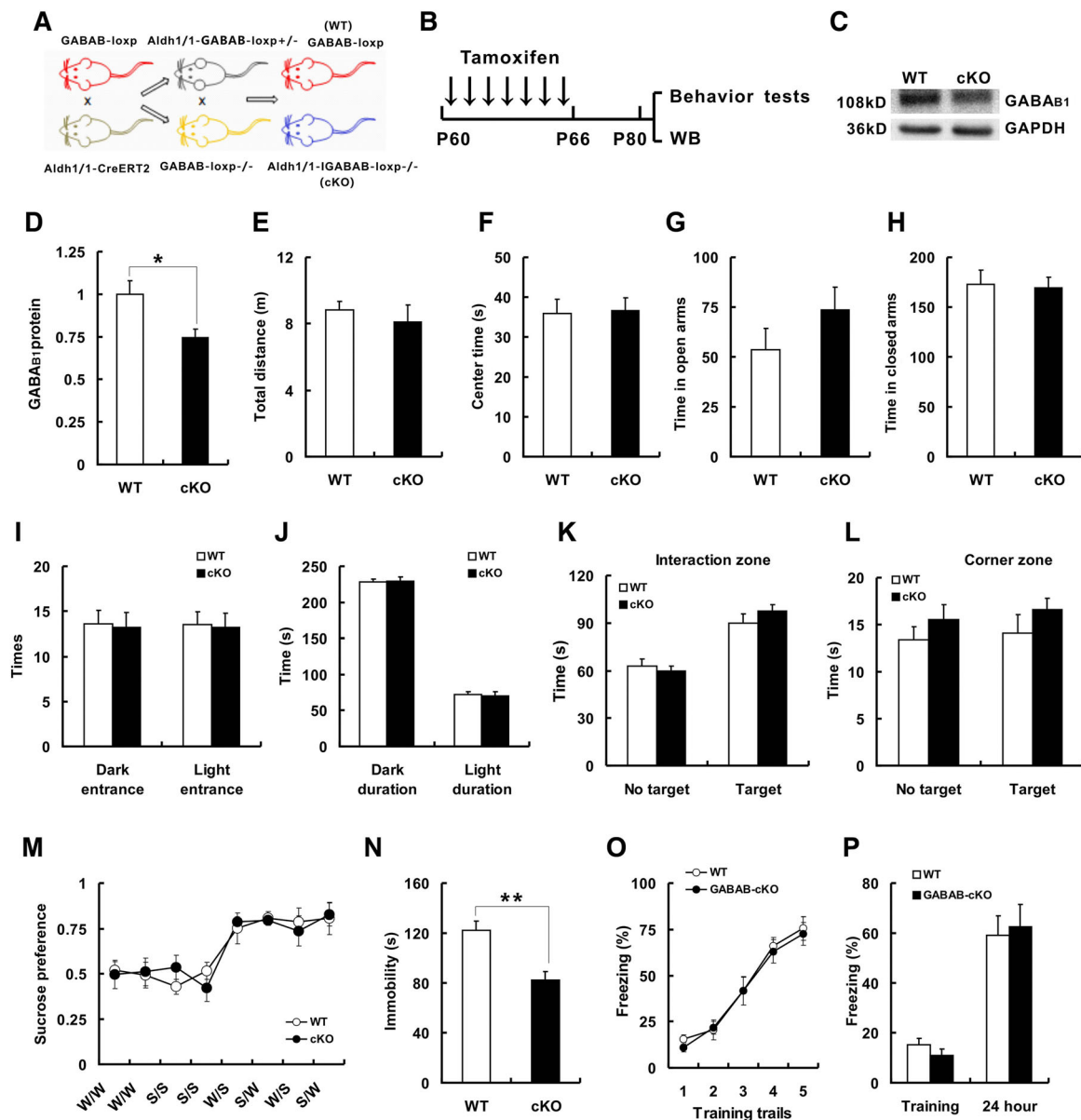


Fig. 5 Conditional knockout of GABA_BRs in astrocytes leads to decreased immobility in the FST. **A** GABA_B^{loxP/loxP} mice were crossed with aldhl11::CreER^{T2} mice; the resulting aldhl11::CreER^{T2}; GABA_B^{loxP/loxP/+} mice were crossed with GABA_B^{loxP/loxP/+} mice to generate aldhl11::CreER^{T2}; GABA_B^{loxP/loxP} (cKO) and GABA_B^{loxP/loxP} (WT) mice. **B** Schematic of the experiments on cKO and WT mice. **C** Representative blots showing that GABA_{B1} levels were decreased in the hippocampus of cKO mice. **D** Quantitative analysis of data as in (C) ($n = 4$ mice/group, two-tailed Student's t -test). GABA_{B1} band density was normalized to the loading control GAPDH. **E, F** Mice with knocked out astrocytic GABA_BRs travelled the same total distance (**E**) and spent the same amount of time in the central arena (**F**) in the open field test (WT: $n = 8$ mice; cKO: $n = 10$; two-tailed Student's t -test). **G, H** In the EPM test, cKO mice spent the same time in the open (**G**) and closed (**H**) arms

(WT: $n = 8$; cKO: $n = 10$; two-tailed Student's t -test). **I, J** There was no difference in the number of entries (**I**) or time spent (**J**) in the light/dark side in the light/dark box test (WT: $n = 8$; cKO: $n = 10$; two-tailed Student's t -test). **K, L** There is no difference between cKO and WT mice in the time spent in the interaction zone or corner zone in the presence/absence of a target mouse (WT: $n = 8$; cKO: $n = 10$; two-tailed Student's t -test). **M** cKO mice did not exhibit preferences in the sucrose preference test (WT: $n = 8$; cKO: $n = 10$; repeated measures two-way ANOVA). **N** In the FST, cKO mice exhibited decreased immobility compared with WT mice (WT: $n = 8$; cKO: $n = 10$; two-tailed Student's t -test). **O, P** Conditional knockout of GABA_BRs in astrocytes does not affect learning and memory (WT: $n = 10$; cKO: $n = 12$; repeated two-way ANOVA in **O**; two-tailed Student's t -test in **P**). Data are presented as the mean \pm SEM; * $P < 0.05$; ** $P < 0.01$ vs controls.

conditional knockout of GABA_BRs in astrocytes led to decreased immobility time in the FST. Third, the conditional knockout of GABA_BRs in astrocytes selectively

increased hippocampal astrocytic BDNF protein levels, which controlled the decrease in immobility time in the FST. Together, our results suggest that astrocytic

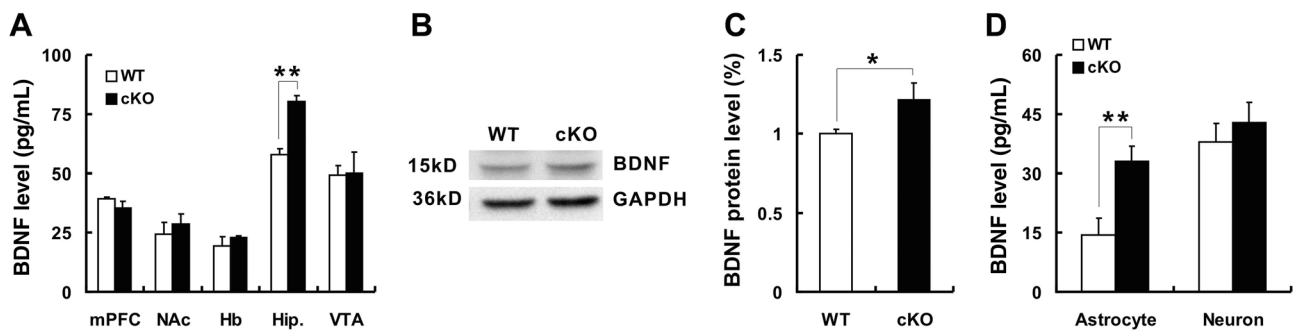


Fig. 6 The lack of GABA_BRs in astrocytes increases the astrocytic BDNF level in the hippocampus. **A** Histogram showing BDNF protein levels assessed by ELISA in the mPFC, NAc, Hb, hippocampus, and VTA of *aldh111::CreER^{T2}; GABA_B^{loxP/loxP}* (cKO), and *GABA_B^{loxP/loxP}* (WT) mice. There was a difference only in the hippocampus of cKO vs WT mice ($n = 3$ mice/group; two-tailed Student's *t*-test). **B** Representative blots showing that BDNF protein levels were increased in the hippocampus of cKO mice. **C** Quantitative analysis of data as in

(**B**) ($n = 4$ pairs of mice, two-tailed Student's *t*-test). BDNF band density was normalized to the loading control GAPDH; values of control mice are taken as 100%. **D** BDNF levels in cultured astrocytes and neurons isolated from the hippocampus of cKO and WT mice. Astrocytic BDNF levels were significantly higher in the cKO mice ($n = 6$; two-tailed Student's *t*-test). Data are presented as the mean \pm SEM; * $P < 0.05$; ** $P < 0.01$ vs controls.

GABA_BRs in the hippocampus control the response to behavioral challenge, and this may result from increased astrocytic BDNF levels in the hippocampus.

Depression is a common, disabling mental illness [50]. Although it is widely considered to be due to a dysfunction in monoamine neurotransmission and this is the focus of all antidepressant strategies [51], growing numbers of clinical and preclinical studies indicate that there is a GABA system dysfunction in depression [5, 6]. More recently, growing evidence suggests that GABA_BRs are important targets in the treatment of psychiatric disorders such as MDD. For example, increasing emphasis has been placed on GABA_BR antagonism as a potential therapeutic strategy for depression [11, 12]. GABA_{B(1)}R-knockout mice display antidepressant-like behaviors in the FST model of depression [10, 12, 14]. However, thus far, there is no evidence to demonstrate which cell type expressing GABA_BRs modulates this antidepressant-like activity, as this receptor type has been reported to be present on almost all neurons and glial cells in the CNS [16, 17].

As the most abundant cell type in the CNS, astrocytes are thought to play important roles in mediating brain function [52]. In addition, astrocytes regulate neuronal excitability and synaptic transmission by releasing glutamate, adenosine triphosphate, and D-serine, a process termed gliotransmission [53, 54]. Furthermore, many neurotransmitter receptors are expressed on astrocytes, including those activated by glutamate, GABA, adenosine, and adenosine triphosphate [53, 54]. Although the roles of glutamatergic receptors in regulating astrocyte activity have already been described, the role of GABA_BRs in mediating these processes is not as well understood. Our results support an active role of GABA_BRs in response to behavioral challenge and further confirm the modulation of

this activity by astrocytic GABA_BRs. It is worth noting that, although GABA_BRs are also expressed by progenitor cells [55, 56], the specificity of *Aldh111-CreER^{T2}*-mediated recombination used in our study was high in astrocytes (Fig. 3). These results support the hypothesis that astrocytic GABA_BRs modulate antidepressant activity in the FST.

It is well known that antidepressants increase serotonin and/or norepinephrine levels by preventing the reuptake of serotonin and/or norepinephrine into presynaptic terminals [57]. Although monoamine levels increase soon after drug administration, antidepressants work gradually over several weeks of continuous application; thus, it is possible that in addition to an increase in monoamine levels, other molecules may be responsible for their effects [58]. Our findings also showed that conditional knockout of GABA_BRs in astrocytes significantly increased the BDNF protein levels in the hippocampus; this finding is supported by previous studies demonstrating that GABA_BR antagonists increase BDNF levels in the hippocampus [59], while the GABA_BR agonist baclofen decreases these levels [60]. Meanwhile, studies have also suggested that BDNF-TrkB receptor signaling is necessary and sufficient to reduce depression [39, 46]. BDNF-mediated signaling is also involved in responses to stress and antidepressant activity [61]. Reduced brain BDNF levels have been shown to lead to depression [43], whereas increased BDNF levels can have antidepressant effects [44]. The direct infusion of BDNF into the hippocampus, acutely and chronically, mimics the behavioral responses induced by antidepressants in animal models of depression [45]. Therefore, our study supports an active role of BDNF in antidepressant activity. In addition, BDNF is also released from astrocytes [62, 63]. Our study further indicated that the increase in

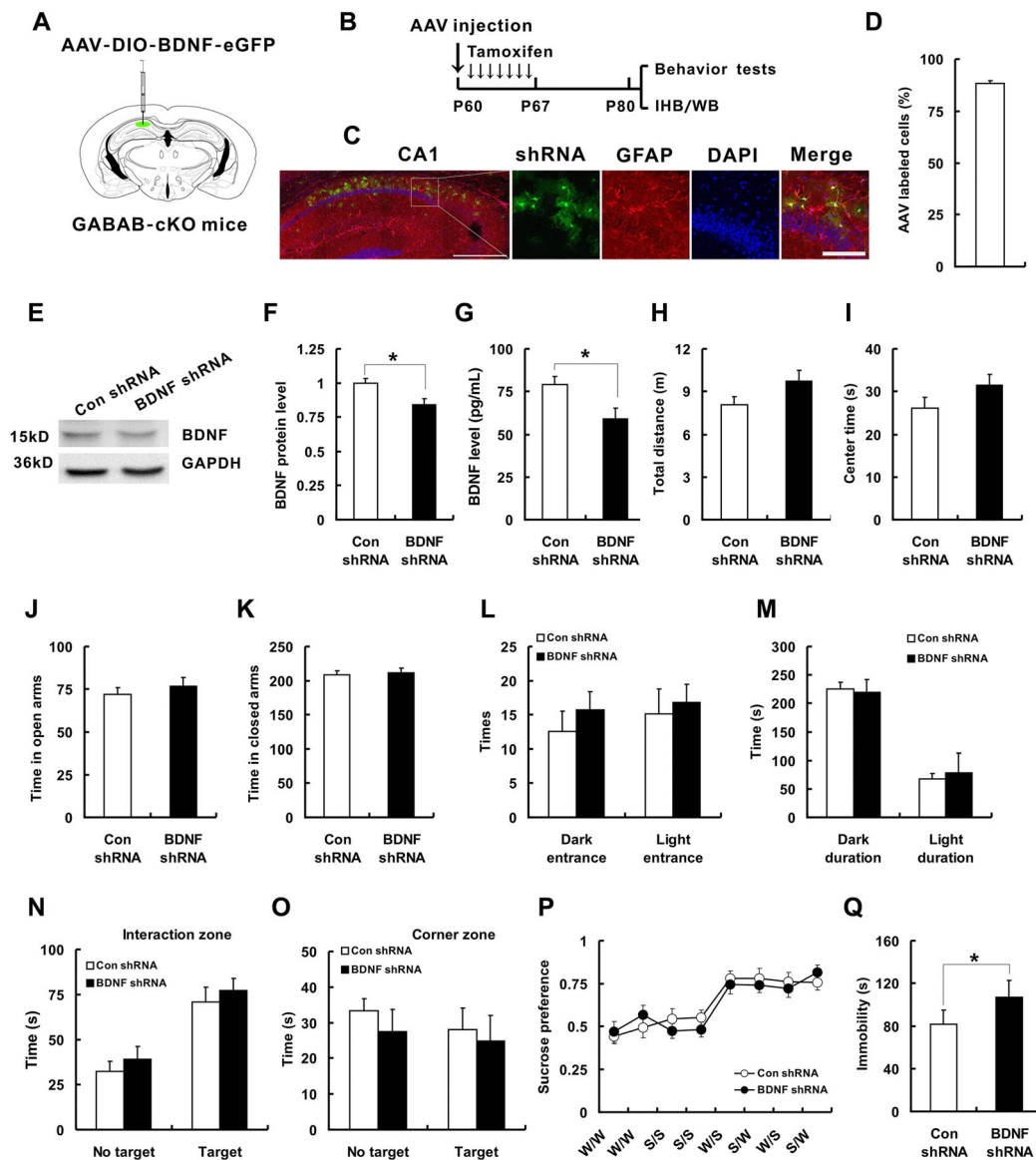


Fig. 7 Conditional knockdown of astrocytic BDNF restores the behavioral changes in cKO mice. **A** Schematic of the delivery of AAV-DIO-BDNF-eGFP into the CA1 region of cKO mice. **B** Schematic of the experiments. **C** Representative location of the BDNF shRNA virus (green) injected into the CA1 region (left; scale bar, 500 μ m), and representative fluorescence images showing that most of the cells infected with AAV-DIO-BDNF-eGFP (shRNA) vector were astrocytes in the CA1 region of cKO mice (right; scale bar, 100 μ m). **D** Average percentage of cells infected with AAV-DIO-BDNF-eGFP (shRNA) vectors that were positive for astrocytes. **E** Representative blots showing that the BDNF protein levels were restored in the hippocampus of cKO mice. **F** Quantitative analysis of data as in **E** ($n = 4$ pairs of mice, two-tailed Student's *t*-test). BDNF band density was normalized to the loading control GAPDH. **G** BDNF protein levels were restored to normal levels in the hippocampus of cKO mice after BDNF shRNA injection ($n = 3$ mice/group, two-tailed Student's *t*-test). **H, I** Knockdown of astrocytic BDNF in CA1 did not affect the total distance travelled (**H**) or

the time spent in the central arena (**I**) in the open field test (Ctrl: $n = 9$ mice; shRNA: $n = 10$ mice; two-tailed Student's *t*-test). **J, K** In the EPM test, cKO mice injected with BDNF shRNA spent the same amount of time in the open (**J**) and closed (**K**) arms (Ctrl: $n = 9$; shRNA: $n = 10$; two-tailed Student's *t*-test). **L, M** There was no difference in the number of entries (**L**) or time spent (**M**) in the light/dark side in the light/dark box test (Ctrl: $n = 9$; shRNA: $n = 10$; two-tailed Student's *t*-test). **N, O** There was no difference between the two groups in the time spent in the interaction zone or corner zone in the presence/absence of a target mouse (Ctrl: $n = 9$; shRNA: $n = 10$; two-tailed Student's *t*-test). **P** Knockdown of BDNF in CA1 in cKO mice did not change preferences in the sucrose preference test (Ctrl: $n = 9$; shRNA: $n = 10$; repeated measures two-way ANOVA). **Q** Immobility time was restored in the FST after knocking down BDNF in CA1 in cKO mice (Ctrl: $n = 9$; shRNA: $n = 10$; two-tailed Student's *t*-test). Data are presented as the mean \pm SEM; * $P < 0.05$ vs controls.

BDNF from astrocytes caused by deleting astrocytic GABA_BRs plays an important role in controlling the response to behavioral challenge.

In conclusion, these results demonstrate that conditional knockout of GABA_BRs in astrocytes positively affects the response to behavioral challenge. Moreover, it is conceivable that increased astrocytic BDNF protein levels in the hippocampus might be involved in this process. Taken together, our findings contribute to the current understanding of the cell types expressing GABA_BRs that modulate antidepressant activity in the FST model, which may provide new insight into the pathological mechanisms and potential targets in MDD.

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Conflict of interest The authors declare no competing interests.

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