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Original Research

Astrocyte-Ablation of *Mtnr1b* Increases Anxiety-Like Behavior in Adult Male Mice

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Abstract

Background: Astrocytes are essential for synaptic transmission, and their dysfunction can result in neuropsychiatric disorders such as anxiety and depression. Many studies have shown that global knockout of Melatonin receptor 2 (*Mtnr1b*) is associated with the development of various mental disorders. **Aim**: This study aimed to investigate the effects of astrocyte ablation of *Mtnr1b* on cognitive function and anxiety-like behavior in mice, as well as the potential biological mechanisms. **Methods**: A conditional Cre-loxP system allowing deletion of *Mtnr1b* from astrocytes was developed to investigate the specific role *Mtnr1b*. Control and *Mtnr1b* cKO^{Gfap} mice were selected for cognitive function behavioral testing (Morris water maze test, novel object recognition test) and emotion-related behavioral testing (open field, elevated plus maze). After testing, brain tissue was collected and examined by immunofluorescence for the expression of neuronal nuclei (NeuN), glutamate decarboxylase 67 (GAD67), and vesicular glutamate transporter 1 (vGluT1). RNA-seq was performed on hippocampal tissue from control and *Mtnr1b* cKO^{Gfap} mice to identify differentially expressed genes. Additional confirmation of differential gene expression was performed using real-time quantitative polymerase chain reaction (qRT-PCR). **Results**: *Mtnr1b* cKO^{Gfap} mice were not significantly different from control mice in the Morris water maze and novel object recognition tests. Results from the open field and elevated plus maze tests showed that *Mtnr1b* cKO^{Gfap} mice exhibited significantly more anxiety-like behavior than did controls. Immunofluorescence revealed that the number of mature neurons did not differ significantly between *Mtnr1b* cKO^{Gfap} mice and controls. The expression of GAD67 in the hippocampal CA1 and CA3 areas of *Mtnr1b* cKO^{Gfap} mice was significantly lower than in the control group, but no significant difference was detected for vGluT1 expression. RNA-seq and qRT-PCR results showed that *Mtnr1b* knockout in astrocytes led to a decrease in the levels of gamma-aminobutyric acid sub-type A ($GABA_A$) receptors and Kir2.2. **Conclusions**: The astrocyte-specific knockout in *Mtnr1b* cKO^{Gfap} mice results in anxiety-like behavior, which is caused by down-regulation of gamma-aminobutyric acid-ergic (GABAergic) synaptic function.

Keywords: astrocyte; anxiety; GABA; melatonin receptor 2

1. Introduction

Anxiety disorder is a prevalent psychological condition among individuals who seek clinical treatment. It refers to the response of the mind and body to stress, danger, or unfamiliar situations [1]. Anxiety disorder is often considered to be a protracted response to imprecise future threats and broad attentional focus. The primary symptoms of anxiety disorder are excessive or repetitive worries and intrusive thoughts preceding significant events, which may induce uneasiness, discomfort, or terror [2]. Anxiety disorders, as defined by the American Psychiatric Association's diagnostic criteria, comprise specific phobias, panic disorder with or without agoraphobia (PDA), social anxiety disorder (SAD), and generalized anxiety disorder [3]. Specific phobia has a 12-month incidence rate of 10.3%, making it the most common anxiety disorder [4]. PDA is the second most common type, with an incidence rate of 6.0%, followed by SAD and generalized anxiety disorder (GDA), with incidence rates of 2.7% and 2.2%, respectively [5]. Anxiety disorders typically have a slow onset, and symptom severity fluctuates between generalized anxiety disorder and PDA [6,7]. Women are 1.5–2 times more likely than men to develop anxiety disorders, which often co-occur with depression, obsessive-compulsive disorder, post-traumatic stress disorder, and other mental disorders [8]. More than 70% of individuals who attempt suicide have an anxiety disorder. Identifying individuals with anx-

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iety disorders is challenging due to the lack of specific biological markers. The diagnosis and treatment of anxiety disorders and their comorbidities impose a significant economic burden on patients, families, and society. Therefore, defining the pathogenesis of anxiety disorders and finding new strategies for their treatment is both necessary and a high-priority.

The balance between neuronal excitation and inhibition (E/I) is crucial for maintaining normal brain function. E/I imbalance leads to various mental disorders. For instance, in anxiety and depressive disorders, a significant imbalance of the excitatory glutamatergic and inhibitory gamma-aminobutyric acid-ergic (GABAergic) systems occurs in the medial prefrontal cortex and hippocampus [9–12]. The specific mechanisms underlying E/I imbalance-induced anxiety and depression, however, remain unclear. GABAergic dysfunction is known to induce hippocampal neuronal signal-transduction inhibition, which, in turn, has been linked to the development of neurological diseases and neuropsychiatric disorders, especially in cases of anxiety disorders or depression [13]. These inhibitory neurons provide a necessary safeguard for excitatory neural transmission via synaptic inhibition caused by hyperpolarization [14]. Therefore, the reduction of inhibitory GABAergic signaling and the elevation of glutamatergic neurotransmission are strongly associated with the occurrence of anxiety disorders.

Melatonin receptors are a type of conserved G-protein-coupled receptor comprising melatonin receptor 1 (encoded by the *MTNR1A* gene), melatonin receptor 2 (encoded by the *MTNR1B* gene), and in rare cases, melatonin receptor 3 in mammals. In the suprachiasmatic nucleus, melatonin primarily regulates neuronal firing and clock-gene expression through *MTNR1A* and *MTNR1B*, thereby coordinating the circadian rhythms of the human body [15]. *MTNR1A* and *MTNR1B* have different modes of coupling with G proteins upon activation. *MTNR1A* primarily couples to the G_i and insensitive $G_{q/11}$ families to inhibit forskolin-stimulated cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) signaling pathways, as well as the phosphorylation of nuclear cAMP response element binding (CREB) [16]. It also enhances potassium conductance by increasing Kir inward rectifying channels [17]. MTNR1B mainly couples to $G_{i/o}$ type G proteins, which inhibit the production of cAMP stimulated by forskolin and formation of cyclic guanosine monophosphate (cGMP), activates the protein kinase C (PKC) signaling pathway in the suprachiasmatic nucleus, and reduces dopamine release from the retina [18,19]. *MTNR1B* expression occurs mainly in the brain and pancreas. Some studies suggest that *MTNR1A* and *MTNR1B* exhibit receptor heterodimerization, which may regulate neurological and psychiatric disorders [20,21]. Despite their important regulatory roles in various physiological and pathological processes, assessing the biological functions of *MTNR1A* and *MTNR1B* remains challenging due to their extremely low expression levels, particularly *MTNR1B* [22]. Genome-wide association studies (GWAS) have shown that variations in the human *MTNR1B* gene sequence are closely related to the development of diabetes [23]. Melatonin receptors have become new pharmaceutical targets for treating mental disorders. Comai *et al.* [24] demonstrated that the *MTNR1B* agonist UCM765 reduced anxiety-like behavior in mice and increased nonrapid eye movement (non-REM) sleep. Conversely, mice with systemic knockout of *Mtnr1b* receptors showed disrupted sleep patterns and increased anxiety-like behavior [25,26]. Clinically, agomelatine acts as a new type of anti-depressant/anti-anxiety medication by stimulating *MTNR1A/MTNR1B* receptors [27]. Furthermore, analysis of the human protein atlas (HPA) database (https://www.proteinatlas.org/ENSG00000134640-MTNR1B/single+cell+type) by us revealed that *MTNR1B* is primarily expressed in astrocytes in the brain. Astrocytes are the most abundant glial cell type in the central nervous system and release a range of neurotransmitters (such as glutamate, gliotransmitters), thereby affecting synaptic excitability and playing an important role in emotional regulation [28–30]. Therefore, in the present study we further investigated whether astrocyte-specific ablation of the *Mtnr1b* gene in mice is associated with similar behavioral abnormalities. The underlying biological mechanism was also explored, with the aim of providing new strategies for treating anxiety disorders.

In the present study, adult male mice with deletion of *Mtnr1b* in astrocytes were found to exhibit anxiety-like behaviors. This was accompanied by a reduction in inhibitory neurons, but no significant changes in excitatory neurons in the hippocampal CA1 and CA3 region. It was also found that deletion of *Mtnr1b* in astrocytes led to transcriptional changes in the hippocampus and downregulation of the *Gaba2* gene in *Mtnr1b* cKO^{Gfap} mice. This blocked inhibitory synaptic neurotransmission, leading to anxiety-like behaviors. The present findings revealed a critical role for astrocytic *Mtnr1b* in the hippocampus in anxiety-like behaviors. Furthermore, this regulatory effect on anxiety is achieved through down-regulation of GABAergic synaptic function.

2. Materials and Methods

2.1 Animals

All animal studies strictly adhered to the regulations of the Institutional Animal Care and Use Committee (IACUC) and were approved by the Experimental Ethics Committee of Chongqing Medical University. Mice with a deletion of the *Mtnr1b* gene (which encodes the melatonin receptor 2) in astrocytes and bred on a *C57BL/6J* genetic background were procured from Model Organisms, Inc. (Shanghai, China). *Mtnr1b*$^{fl/-}$ *Gfap-Cre*$^{+}$ mice were crossed with *Mtnr1b*$^{fl/fl}$ mice to generate *Mtnr1b* cKO^{Gfap} mice. Male *Mtnr1b* cKO^{Gfap} mice were used in the experiments and

Table 1. Primers and their sequences of the genes used in the present study.

Primer	Sequence (5′ → 3′)	Primer type
Mtnr1b	GCCTAGCAGCAAACCTGTGAAGTGA	Forward
	GTCCCTGGAAGTCACTCACCTTCAATAC	Reverse
Gfap-Cre	TAGCCCACTCCTTCATAAAGCCCT	Forward
	GCTAAGTGCCTTCTCTACACC	Reverse
Gapdh	CATGGCCTTCCGTGTTCCT	Forward
	CCTGCTTCACCACCTTCTTGAT	Reverse
Gabra2	TATGGTCTCTGCTGCTTGTTCTT	Forward
	CAGTCCTGGTCTAAGCCGATTAT	Reverse
Kcnj13	AGTGCAATCGCCTTACTTGC	Forward
	GTACAGCAGAGACACGAACG	Reverse
Clic6	CTCTTCGTCAAGGCTGGTTATGA	Forward
	GTCCACCGTTGTCACATTGAATA	Reverse
Kcne2	GGTCTCCTGCATTGCTCACATAC	Forward
	TTCCTCCTCCAGCTGTCCATATA	Reverse
Ppp1r1b	TCGGAGGAAGAGGATGAGTTAGG	Forward
	CACTTGGTCCTCAGAGTTTCCAT	Reverse

male *Mtnr1b*$^{fl/fl}$ littermates were used as controls. The mice were maintained in specific pathogen-free rooms, with each cage accommodating 6–8 mice. Cages had independent ventilation systems, and mice were supplied with adequate clean food and pure water. The temperature was kept at 23 °C and the humidity was maintained between 50–60%. A 12-h light/dark cycle (lights on: 7:00 am to 7:00 pm) was maintained.

2.2 Genotyping

Tail docking was performed on one-month-old mice by cutting at approximately 0.3–0.5 cm from the end of the tail. Genomic DNA from the mouse tail was extracted using Mouse Tail Direct PCR Kit (B40015, Bimake, Houston, TX, USA), which consists of lysis buffer and lysis enzyme. For each sample, 100 µL of lysis buffer and 2 µL of lysis enzyme were added, followed by digestion of the tail tissues at 55 °C for 30 min in a metal bath. The samples were then heated at 95 °C for 5 min to deactivate the protease activity in the digestion solution. After centrifugation at 14,000 rpm for 5 min, the DNA sample was collected from the supernatant. The primer sequences used for genotyping are listed in Table 1.

2.3 Real-Time Quantitative PCR

RNA was extracted from brain samples using an RNA extraction kit (RP1202, BioTeke, Beijing, China). The extracted RNA was reverse-transcribed into cDNA using a reverse transcription kit (RR047A, Takara, Kyoto, Japan). Subsequently, gene-specific primers were used to amplify the cDNA with a quantitative detection kit (RR820A, Takara, Kyoto, Japan). The primer sequences used in this article are listed in Table 1. Data generated by real-time quantitative polymerase chain reaction (qRT-PCR) were analyzed using the $2^{-\Delta\Delta CT}$ method.

2.4 Immunofluorescence Assay

Deep anesthesia was induced in mice by intraperitoneal injection of urethane (U2500, MilliporeSigma, St. Louis, MO, USA), followed by heart perfusion with a pre-chilled PBS solution and 4% paraformaldehyde (PFA), in 0.1 M PBS (pH 7.4). The brain tissue was quickly separated and transferred to 4% PFA, then kept overnight at 4 °C. The brain tissue was then transferred to a 30% sucrose solution and dehydrated over two days. Next, the tissue was embedded in an optimal-cutting-temperature compound (OCT, 4583, SAKURA, Torrance, CA, USA) and placed into a cryostat while frozen at –20 °C. Finally, tissue was sliced along the coronal plane into 20-µm-thick sections.

To prevent non-specific binding, QuickBlock rapid immunofluorescence blocking solution (P0260, Beyotime, Shanghai, China) was used for blocking at room temperature for 1h. Sections were then incubated overnight at 4 °C with anti-neuronal nuclei (NeuN) (1:200, ab177487, Abcam, Cambridge, UK), anti-glutamate decarboxylase 67 (GAD67) (1:200, ab213508, Abcam), and anti-vesicular glutamate transporter 1 (vGlut1) (1:200, ab227805, Abcam) in QuickBlock solution. After washing to remove unbound antibodies, the brain sections were incubated with Alexa Fluor 488-conjugated donkey anti-rabbit IgG secondary antibodies (1:3000, anti-rabbit IgG, A-11008, Thermo Fisher, Waltham, MA, USA) for 1.5 h at room temperature. Nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI, D9542, MilliporeSigma, St. Louis, MO, USA) for 7 min. Images of the brain were examined and recorded using a confocal laser scanning microscope (C2 plus, Nikon, Tokyo, Japan). The number of NeuN-positive cells and mean fluorescence intensity (MFI) of GAD67 and vGlut1 were quantified using Fiji/ImageJ software (v1.53t, National Institutes of Health, Bethesda, MD, USA).

2.5 RNA-Seq Analysis

Total RNA was extracted from the hippocampus of adult male mice (two *Mtnr1b*$^{fl/fl}$ and two control mice) using Trizol reagent (Invitrogen, Carlsbad, CA, USA). After quality control using an Agilent BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA), libraries of intact RNA were prepared by following the recommended protocol for the NEBNext UltraTM RNA Library Prep Kit by Illumina (# E7770, NEB, Ipswich, MA, USA). Then, an Illumina NovaSeq 6000 platform (Novogene Bioinformatics Institute, Beijing, China) was used to generate 150-bp paired-end reads by sequencing. The STAR tool (https://github.com/alexdobin/STAR) [31] was used to map reads to the mouse genome sequence, and FeatureCounts (https://subread.sourceforge.net/) [32] was used to count the mapped reads for each gene. Genes that were differentially expressed in the hippocampus of cKO and control mice were identified using the DESeq2 R package (v1.40.2, https://bioconductor.org/packages/release/bioc/html/DESeq2.html) with settings of $p < 0.05$ and absolute $\log_2$ (fold change) $\geq$0.5. A Gene Ontology (GO) enrichment analysis of these differentially expressed genes was performed using the clusterProfiler R package (v4.8.3, https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html). Only GO terms with $p < 0.05$ were considered to be significantly enriched.

2.6 Open Field Test

The open field test was conducted to evaluate the locomotion and anxiety levels of mice using an established protocol [33]. Mice were placed in an open field (42 cm $\times$ 42 cm $\times$ 42 cm) and allowed to explore freely for 10 min. The central area (12 cm $\times$ 12 cm) of the apparatus was defined as the "central zone". The behavior of each mouse in the open field, including the duration of time spent in the central zone and the total distance traveled, was recorded by photography.

2.7 Elevated Plus Maze Test

The elevated plus maze test was performed as described previously [34]. The elevated plus maze was composed of two open and two closed arms arranged in a cross. The closed arms were enclosed by 15-cm-high black, opaque walls. Each arm had a length of 50 cm and a width of 10 cm, with a square central platform in the middle measuring 10 cm $\times$ 10 cm. The maze was positioned 70 cm above the floor. The mice in each group were alternately placed in the central zone of the platform with their heads facing towards the open arms. Subsequently, the behavioral activity of the experimental mice was recorded for 10 min using Any-maze software (v7.20, Stoelting, Wood Dale, IL, USA).

2.8 Morris Water Maze Test

The Morris water maze test as described previously was used to assess the spatial memory and learning abilities of mice [35]. This comprises a circular pool and an adjustable, mobile, visible platform. The pool measured 1.5 m in diameter and had a depth of 50 cm. Before starting the actual experiment, the height of the visible platform was adjusted to be submerged 1–2 cm below the water surface; the water temperature was held at 23 °C. To prevent visual detection of the platform under the water surface by the experimental mice, edible white paint was added to make the water opaque. The complete experiment included the visible platform training (Day 1), the hidden platform training (Days 2–6), and a probe trial (Day 7) without the platform. During visible/hidden platform training, each mouse received 4 daily trials of 2 min each. The goal was to find the hidden platform located at the center of the target quadrant, followed by a 20-s rest on the platform. If a mouse failed to reach and climb onto the platform within the allotted time, it was manually guided to the platform where it stayed for 20 s. A minimum of 20 min elapsed between consecutive swims by each mouse. During the probe trial, the platform was removed from the pool and mice were allowed to explore the water freely for 2 min. The movements of the mice were recorded for subsequent analysis.

2.9 Novel Object Recognition Task

The novel object recognition task was performed as described previously to further assess the cognitive function of mice [36]. The test arena used for novel object recognition experiments was a transparent polyethylene apparatus (42 cm $\times$ 42 cm $\times$ 42 cm). Identical objects A and B (two cylinders of the same size and color) were placed at the left and right ends of one wall of the test arena. Each mouse was placed in the arena with its back towards objects A and B, and with its nose equidistant from the objects. During the habituation phase, mice were allowed to explore the test arena freely for 5 min. The frequency and duration of a mouse coming within 3 cm, or touching A or B, as well as any exploratory behaviors (such as pawing, licking, or sniffing), were documented during a 5-min period. After a 1-h rest period, the second phase of the experiment was conducted. Object C (a cube of a different color from objects A and B) was introduced to replace object B in the original test arena. The mouse was placed in the arena facing away from objects A and C, with its nose equidistant from both objects. The frequency and duration of physical contact between the mouse and objects A and C were then recorded during a 5-min period.

2.10 Statistics

The mean $\pm$ standard error of the mean (SEM) was used to express quantitative data. Student's two-tailed *t*-test was utilized to compare two groups of samples. Spatial-learning trial data from the Morris water maze (MWM) test underwent a two-way repeated-measures analysis of variance (ANOVA) for matched subjects, followed by the LSD multiple comparison *post hoc* test. Data analysis was con-

ducted using SPSS 20 (SPSS Science Inc., Chicago, IL, USA) and Prism 8.0 (GraphPad Software, San Diego, CA, USA). $p < 0.05$ was considered statistically significant.

3. Results

3.1 Astrocyte-Ablation of Mtnr1b Induces Anxiety-Like Behavior in Adult Male Mice

To investigate the effect of astrocytic knockout of *Mtnr1b* on mouse behavior, *Mtnr1b* cKO^{Gfap} mice were generated using the cre-loxP system (**Supplementary Fig. 1A**). Subsequently, mouse genotyping was performed by tail identification. Both control and *Mtnr1b* cKO^{Gfap} mice displayed a single band at 344 bp in the same gene. Successfully inserted GFAP-Cre mice demonstrated a single band at 750 bp, whereas controls did not exhibit any bands at 750 bp due to the absence of the GFAP-Cre enzyme (**Supplementary Fig. 1B**).

Global knockout of the *Mtnr1b* gene resulted in atypical behavior in mice, including anxiety and cognitive impairment. To further investigate the regulatory role of astrocyte-deletion of the *Mtnr1b* gene on mouse emotion, an open field test was conducted on six-month-old *Mtnr1b* cKO^{Gfap} mice and on control counterparts of the same age. *Mtnr1b* cKO^{Gfap} mice showed a significantly shorter exploration time in the central area of the open field than did the controls (25.67 $\pm$ 5.11 s *vs.* 48.92 $\pm$ 6.90 s, respectively; $p < 0.05$; Fig. 1A,B) as well as a significant reduction in the total distance travelled (18.59 $\pm$ 1.81 m *vs.* 25.72 $\pm$ 2.54 m, respectively; $p < 0.05$; Fig. 1A,C). These open field test results suggest that astrocyte-ablation of the *Mtnr1b* gene induced anxiety-like behaviors in mice.

To further confirm the development of anxiety-like behaviors after knockout of the *Mtnr1b* gene in the astrocytes of mice, an elevated plus maze test was conducted on *Mtnr1b* cKO^{Gfap} and control mice. The results showed significantly less exploration time of the open arms by *Mtnr1b* cKO^{Gfap} mice than by controls (14.82 $\pm$ 3.65 s *vs.* 27.35 $\pm$ 4.17 s, respectively; $p < 0.05$; Fig. 1D,E), as well as significantly fewer entries into the open arms (5.00 $\pm$ 0.931 *vs.* 11.33 $\pm$ 1.69, respectively; $p < 0.05$; Fig. 1D,F). These findings provide further confirmation that astrocyte-specific deletion of the *Mtnr1b* gene leads to the development of anxiety-like behaviors in mice.

3.2 Astrocyte-Specific Ablation of Mtnr1b Does not Affect Learning and Cognitive Abilities of Mice

A Morris water maze test was conducted to investigate whether deletion of the *Mtnr1b* gene in astrocytes affects the spatial learning and memory ability of *Mtnr1b* cKO^{Gfap} mice. During the first five days of spatial learning and memory training, *Mtnr1b* cKO^{Gfap} mice were not significantly different ($p = 0.7253$) from controls in escape latency (Day 1: 24.43 $\pm$ 1.47 s and 25.66 $\pm$ 3.09 s, for controls and *Mtnr1b* cKO^{Gfap} respectively; Fig. 2A,B). Furthermore, on the final day of testing there were no significant differences between *Mtnr1b* cKO^{Gfap} mice and controls in the time spent in the third quadrant (22.23 $\pm$ 3.86 s and 21.77 $\pm$ 4.15 s, for controls and *Mtnr1b* cKO^{Gfap}, respectively) or in the number of platform crossings (1.83 $\pm$ 0.48 and 1.67 $\pm$ 0.67, for control group and *Mtnr1b* cKO^{Gfap}, respectively) (Fig. 2C,D).

To further investigate whether knock out of the *Mtnr1b* gene in astrocytes affects learning ability in mice, *Mtnr1b* cKO^{Gfap} mice and age-matched controls were compared in a novel object recognition experiment. The results showed no significant differences between *Mtnr1b* cKO^{Gfap} mice and controls in terms of the frequency and time ratio of exploration for familiar and novel objects (Fig. 2E,F). The Morris water maze test results and the novel object exploration test results both suggest that astrocyte-specific ablation of the *Mtnr1b* gene does not affect the learning and memory ability of mice.

3.3 Deletion of Mtnr1b in Astrocytes Does not Affect the Number of Mature Neurons

We next investigated whether knockout of the *Mtnr1b* gene in astrocytes reduces neuronal numbers in mice, thereby leading to anxiety-like behavior. Immunofluorescence staining of brain tissue was performed utilizing NeuN as neuron-specific marker to quantify neurons. No significant differences were observed in neuronal numbers between CKO mice and controls in the CA1 (2487 $\pm$ 730.30 and 2583 $\pm$ 860.30 for controls and *Mtnr1b* cKO^{Gfap} mice respectively; Fig. 3A,B), CA3 (2157 $\pm$ 1007.00 and 3018 $\pm$ 1158.00 for controls and *Mtnr1b* cKO^{Gfap}, respectively; Fig. 3A,C), DG (4280 $\pm$ 1376.00 and 4369 $\pm$ 1336.00 for controls and *Mtnr1b* cKO^{Gfap}, respectively, Fig. 3A,D), or prefrontal cortical regions (1481 $\pm$ 45.37 and 1479 $\pm$ 110.70, and 2315 $\pm$.531.50 and 2311 $\pm$ 545.30 for controls and *Mtnr1b* cKO^{Gfap}, respectively; Fig. 3A,E).

3.4 Knockout of Mtnr1b in Astrocytes Reduces the Expression of Inhibitory Synaptic Proteins in the CA1 and CA3 Regions of the Hippocampus

Disruption of E/I balance can lead to neuronal hyperexcitability or excessive inhibition, which can trigger multiple mental disorders including anxiety [37]. Therefore, maintaining an appropriate E/I balance is crucial for the health of neural circuits [38]. Initially, immunofluorescence staining was used to examine the levels of inhibitory neurons in the different brain regions of control and *Mtnr1b* cKO^{Gfap} mice. GAD67 protein, encoded by the *GAD1* gene, is a hallmark protein of inhibitory (GABAergic) neurons [39]. Our results showed that the mean fluorescence intensity (MFI) of GAD67 in the CA1 (38.83 $\pm$ 3.74 and 25.75 $\pm$ 1.51 for control and *Mtnr1b* cKO^{Gfap} respectively, $p < 0.05$) and CA3 (67.64 $\pm$ 3.01 and 50.64 $\pm$ 4.13 for control and *Mtnr1b* cKO^{Gfap} respectively, $p < 0.05$) regions of the hippocampus of *Mtnr1b* cKO^{Gfap} mice was significantly lower than that of the controls (Fig. 4A–

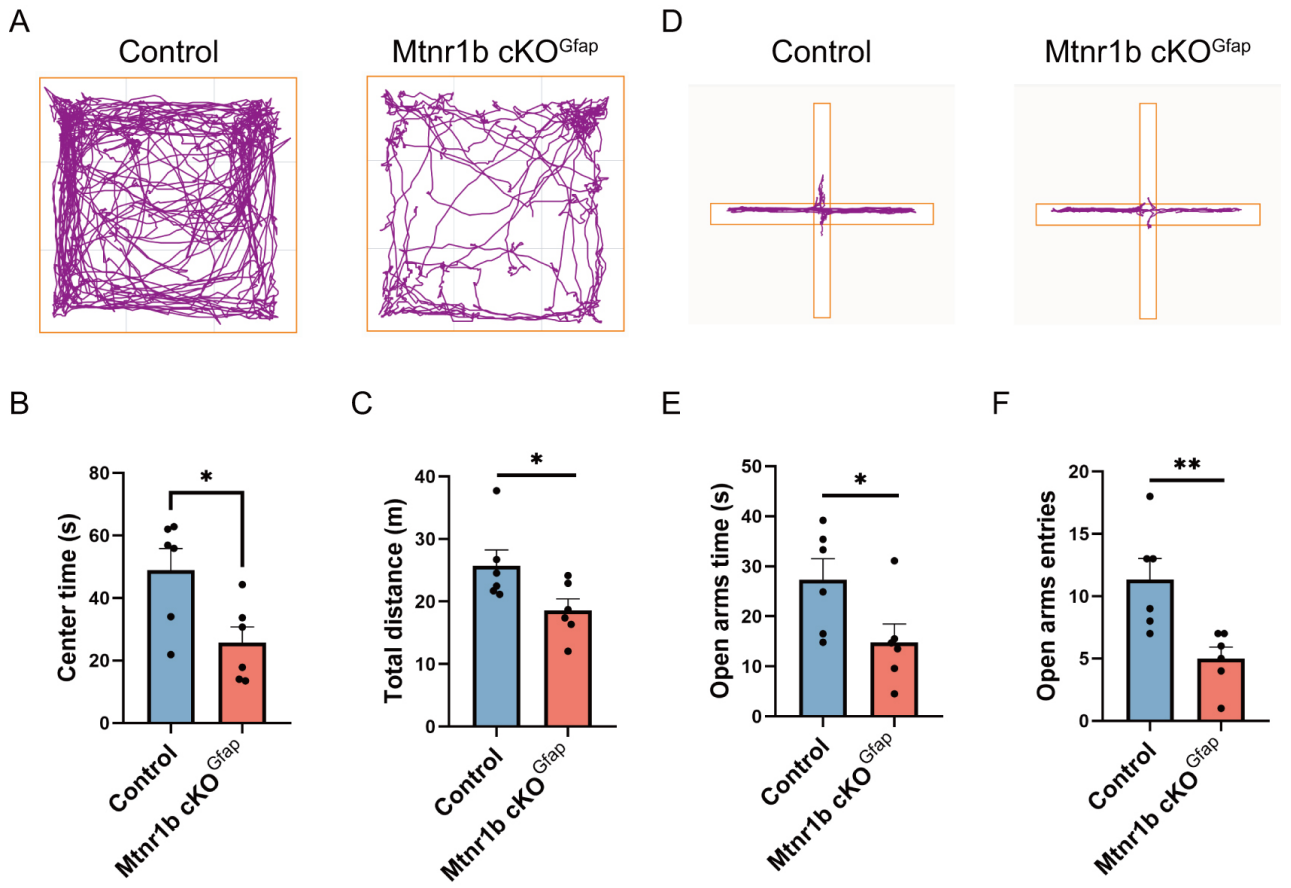

Fig. 1. Astrocyte-ablation of *Mtnr1b* induces anxiety-like behavior in adult male mice. (A) Representative tracks of open field exploration of *Mtnr1b* cKO^{Gfap} and control mice. (B) *Mtnr1b* cKO^{Gfap} mice exhibited a significant reduction in exploration time in the central area of the open field than did control mice (n = 6, *Mtnr1b* cKO^{Gfap} and control; p = 0.0220). (C) In the open field test, *Mtnr1b* cKO^{Gfap} mice traveled a significantly shorter distance than did controls (n = 6, *Mtnr1b* cKO^{Gfap} and control; p = 0.0456). (D) Representative tracks of elevated plus maze exploration of *Mtnr1b* cKO^{Gfap} and control mice. (E,F) *Mtnr1b* cKO^{Gfap} mice spent less time (p = 0.0474) in the open arms and entered the open arms of the elevated plus maze less frequently than did control mice (p = 0.0082) (n = 6, *Mtnr1b* cKO^{Gfap} and control). Data are presented as the mean ± SEM. * $p < 0.05$, ** $p < 0.01$. SEM, standard error of the mean; *Mtnr1b*, melatonin receptor 2.

E). This indicates a reduced level of inhibitory neurons in the hippocampus of *Mtnr1b* cKO^{Gfap} mice.

3.5 Knockout of the Mtnr1b Gene in Astrocytes Has No Effect on the Expression of Excitatory Synaptic Proteins in the Hippocampus

The protein vGluT1 is primarily located in glutamatergic neurons, is a hallmark gene of excitatory neurons, is responsible for the transport and storage of glutamate in neuronal synapses, and plays an important role in the regulation of neural excitability [40]. The expression of vGluT1 in different brain regions of control and *Mtnr1b* cKO^{Gfap} mice was therefore examined. No significant difference in the vGluT1 fluorescence intensity was observed between *Mtnr1b* cKO^{Gfap} mice and control mice in the prefrontal cortex and hippocampal regions (Fig. 5A–E). Therefore, it was concluded from the above results that a decrease in the level of inhibitory neurons occurred in the CA1 and CA3 areas of the hippocampus in *Mtnr1b* cKO^{Gfap} mice, leading to an imbalance of inhibitory/excitatory neurons.

3.6 Mtnr1b cKO^{Gfap} Mice Show Decreased Expression of $GABA_A$ Receptor and Potassium Channel Activity Related Genes

The above results showed that knockout of *Mtnr1b* in the astrocytes of mice primarily affects the level of inhibitory neurons in the hippocampus. To further investigate the underlying biological mechanisms behind the *Mtnr1b* cKO^{Gfap} mouse phenotype, transcriptome sequencing was performed on the hippocampi of control and *Mtnr1b* cKO^{Gfap} mice. This identified a total of 125 differentially expressed genes ($p < 0.05$, absolute $\log_2$ (fold change) $\geq$0.5) between the two genotypes, of which 101 genes were down-regulated and 24 genes were up-regulated (Fig. 6A,B).

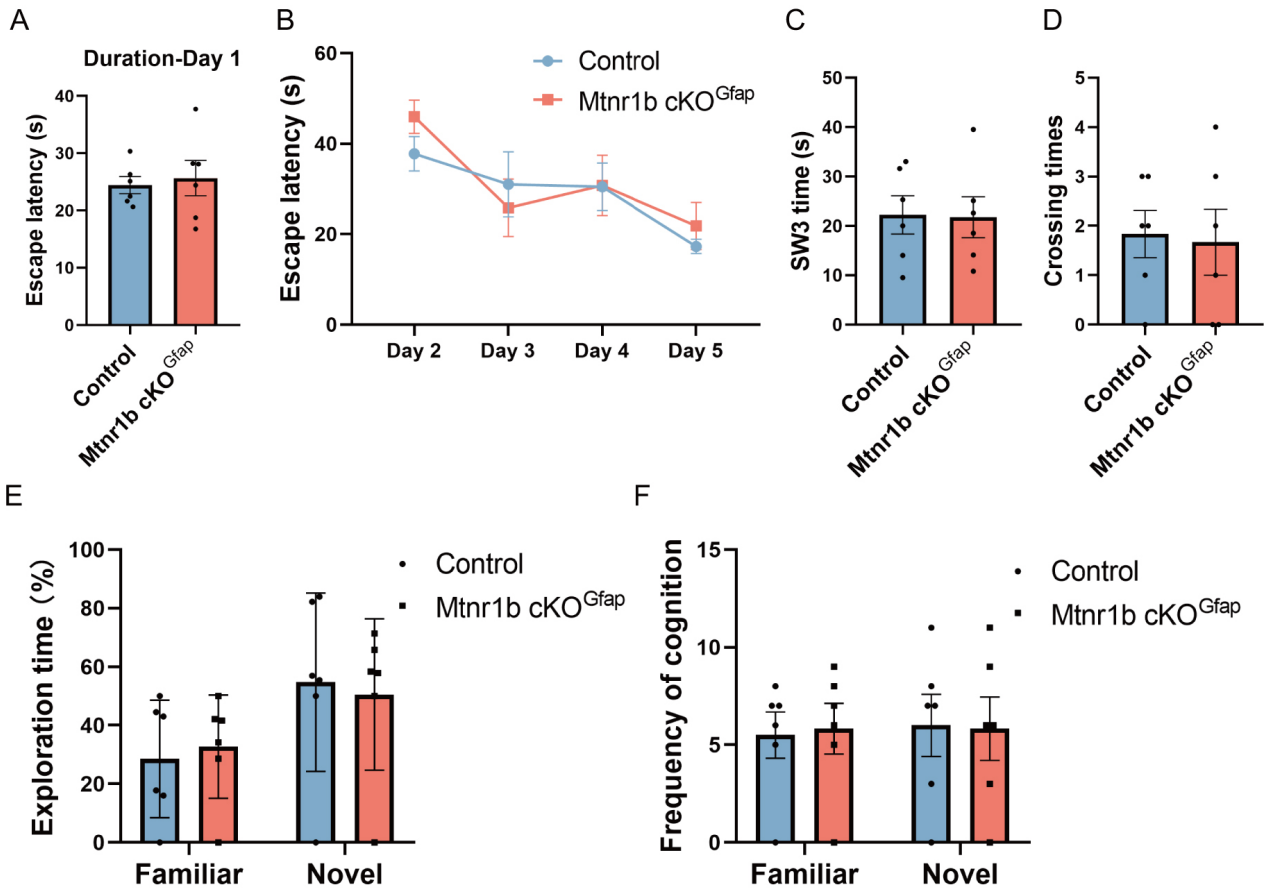

Fig. 2. Astrocyte-specific ablation of *Mtnr1b* does not affect the learning and cognitive ability of mice. The Morris water maze test consists of one day of adaptive trials and five days of hidden platform trials, plus a probe trial 24 h after the last hidden platform trial (n = 6, *Mtnr1b* cKO^{Gfap} and controls). (A) Adaptive trials on Day 1. (B) In hidden platform tests, mice were trained with 4 trials/day for 4 days. *Mtnr1b* cKO^{Gfap} mice were not significantly different in escape latency from control mice, $F_{3,60}$ <1.0. (C,D) In the probe trial, *Mtnr1b* cKO^{Gfap} mice were not significantly different from controls in the time spent on the platform quadrant, or in the number of platform crossings. (E) Percentage of time spent investigating novel and familiar objects during the entire novel object recognition trial time, $F_{1,10}$ <1.0. (F) Frequency of exploration in novel and familiar objects during the novel object recognition trial time, $F_{1,10}$ <1.0, (n = 6/gp). Data are presented as means ± SEM.

The results of GO enrichment analysis showed that potassium channel activity and GABA gated chloride ion channel activity were the most commonly enriched molecular function category (Fig. 6C). RNA-seq results showed that five potassium channels and GABA receptor gated chloride channel-related genes (*Kcnj13*, *Gabra2*, *Kcne 2*, *Ppp1r1b*, *Clic6*) were significantly down-regulated in *Mtnr1b* cKO^{Gfap} mice, but not in controls (Fig. 6D). To validate the RNA-seq results, qRT-PCR was performed to confirm the expression of the five selected genes. The experimental results obtained were consistent with the RNA-seq results (Fig. 6E).

4. Discussion

In the present study, *Mtnr1b* cKO^{Gfap} mice were generated using the Cre-loxP system. The effects of astrocyte-specific deletion of *Mtnr1b* knockout on mouse emotions were assessed by conducting open field and elevated plus maze tests. In addition, Morris water maze and novel object recognition tests were performed to evaluate the impact of *Mtnr1b* knockout on learning and memory. Astrocyte-specific deletion of *Mtnr1b* was found to result in anxiety-like behaviors in mice, without affecting their learning and memory abilities. Evaluation of excitatory and inhibitory neurons showed significantly lower GAD67 levels in the hippocampal CA1 and CA3 regions of *Mtnr1b* cKO^{Gfap} mice than in controls, whereas the number of excitatory neurons did not differ significantly. Transcriptome sequencing of mouse hippocampal tissue further revealed enriched pathways related to potassium channel activity and GABA receptor gated chloride channel activity. qRT-PCR confirmed the consistency of the related gene expression with RNA-seq results. These findings suggest the anxiety-like behaviors observed in *Mtnr1b* cKO^{Gfap} mice are associated with a decrease in hippocampal GABA levels.

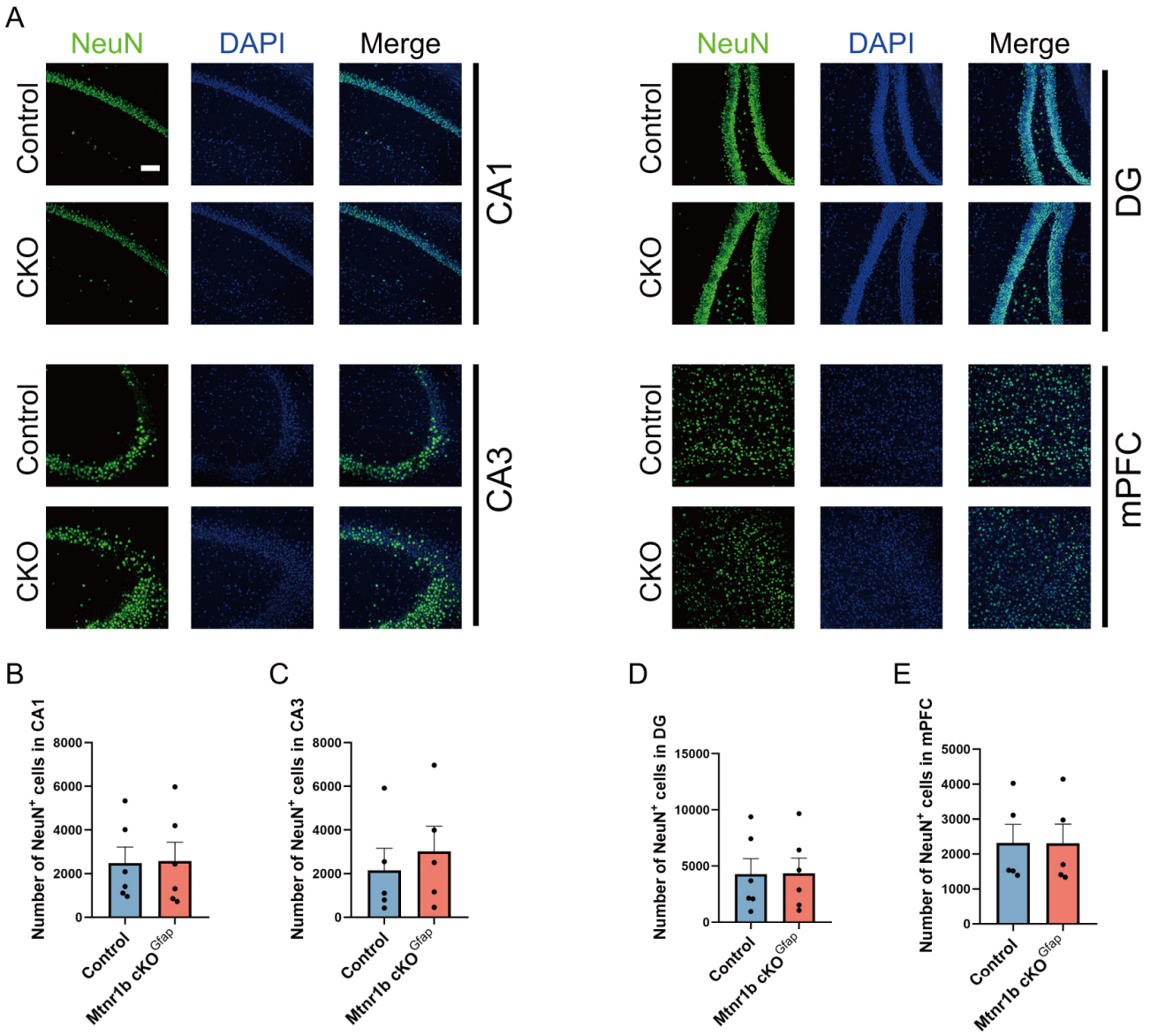

Fig. 3. Deletion of *Mtnr1b* in astrocytes does not affect the number of neurons. (A) Representative immunofluorescence images of different groups. Scale bar: 100 μm for the left image and 50 μm for the right image (n = 5–6). (B–E) Quantification of neuronal nuclei (NeuN) positive cells. No significant differences in neuronal numbers were observed between *Mtnr1b* cKO^{Gfap} and controls-in the CA1, CA3, DG, or prefrontal cortical regions. Data are presented as the mean ± SEM.

Astrocytes are the most abundant glial cells in brain tissue and possess the ability to release neuroactive factors, clear excessive neurotransmitters between synapses, and maintain ion homeostasis to influence communication between synapses [41,42]. Although astrocytes do not transmit electrical signals, they modulate neuronal activity by releasing chemical signals and exchanging ions [43,44]. Disordered astrocyte function contributes to a range of neurological and psychiatric disorders. Yamashita *et al.* [31] eported that activation of astrocytes in the anterior cingulate cortex through channel rhodopsin leads to reduced non-REM sleep during the light period in mice. Specific knockout of connexin 43 in the astrocytes of mice promotes sleep during the dark period by silencing wake-promoting neurons. Li *et al.* [32] reported that specific knockout of astrocytic Liver X receptor β (*Lxr*β) leads to an E/I imbalance, resulting in anxiety-like behavior in mice. Rodolphi *et al.* [45] demonstrated that ceftriaxone upregulates expression of the astrocytic glutamate transporter-1 (GLT-1), thereby counteracting the excessive glutamate induced by nandrolone decanoate, and reducing aggressive behavior in mice. In addition, the present study indicated that deletion of *Mtnr1b* in astrocytes induced anxiety-like behavior in male mice, thus providing additional evidence for the involvement of aberrant astrocyte function in behavioral disturbances.

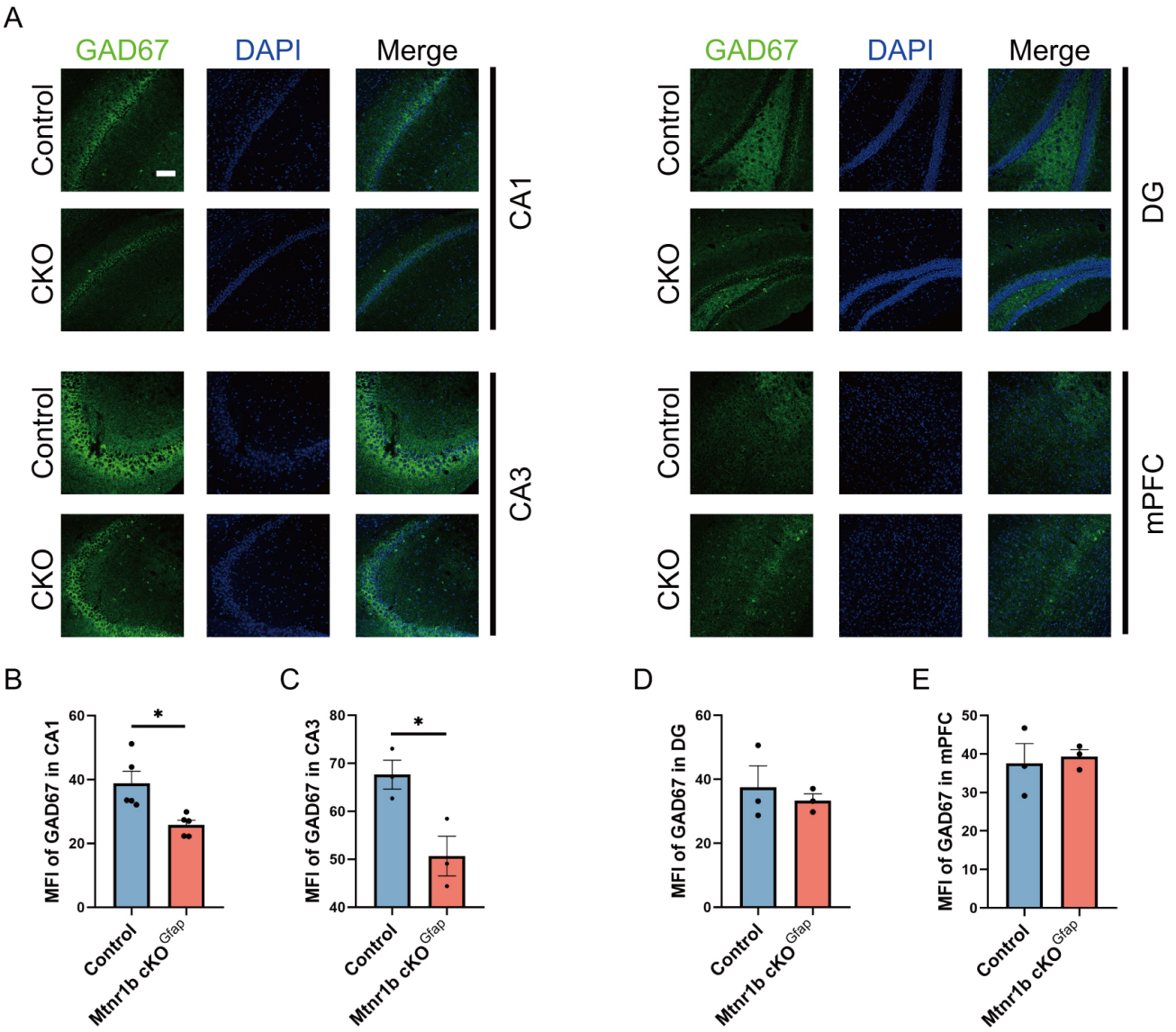

Fig. 4. Knockout of *Mtnr1b* in astrocytes reduces the expression of inhibitory synaptic proteins in the CA1 and CA3 regions of the hippocampus. (A) Representative immunofluorescence images of different groups. Scale bar: 50 μm for each image (n = 3–5). (B–E) Quantification of the mean fluorescence intensity of glutamate decarboxylase 67 (GAD67) in the CA1, CA3, DG, or prefrontal cortical regions. CA1: $p = 0.0119$, CA3: $p = 0.0292$, DG: $p = 0.5840$, mPFC: $p = 0.7673$. * $p < 0.05$. DG, dentate gyrus; mPFC, medial prefrontal cortex.

MTNR1B is a G-protein-coupled receptor involved in various physiological processes, including sleep-wake rhythm, cell proliferation, and neurotransmitter release [46]. Studies have demonstrated that melatonin can reduce ischemia-related neuronal death in an *Mtnr1b* expression-dependent manner [47]. This indicates the importance of *Mtnr1b* in the development of neurological and psychiatric disorders [48–50]. In a previous study, Thomson *et al.* [25] reported that mice with a whole-body knockout of the *Mtnr1b* gene exhibited phenotypes associated with anxiety, reduced learning ability, and decreased attention. Liu *et al.* [51] reported similar results. In the present study, *Mtnr1b* cKO^{Gfap} mice were used as the research subjects, which has not previously been reported in the literature. The impact of astrocytic *Mtnr1b* gene knockout on mouse emotion was assessed using open field and elevated plus maze tests. This revealed that *Mtnr1b* cKO^{Gfap} mice showed marked anxiety-like behavior, which aligns with the findings of the Thomson and Liu studies [25,51]. Despite small variations in mouse strains and *Mtnr1b* gene knockout ranges employed by different researchers, consistent anxiety results were observed across the different mouse strains.

In contrast to prior studies involving systemic knockout of the *Mtnr1b* gene in mice [25], the findings reported here demonstrate that targeted knockout of the *Mtnr1b* gene in astrocytes did not adversely affect learning and mem-

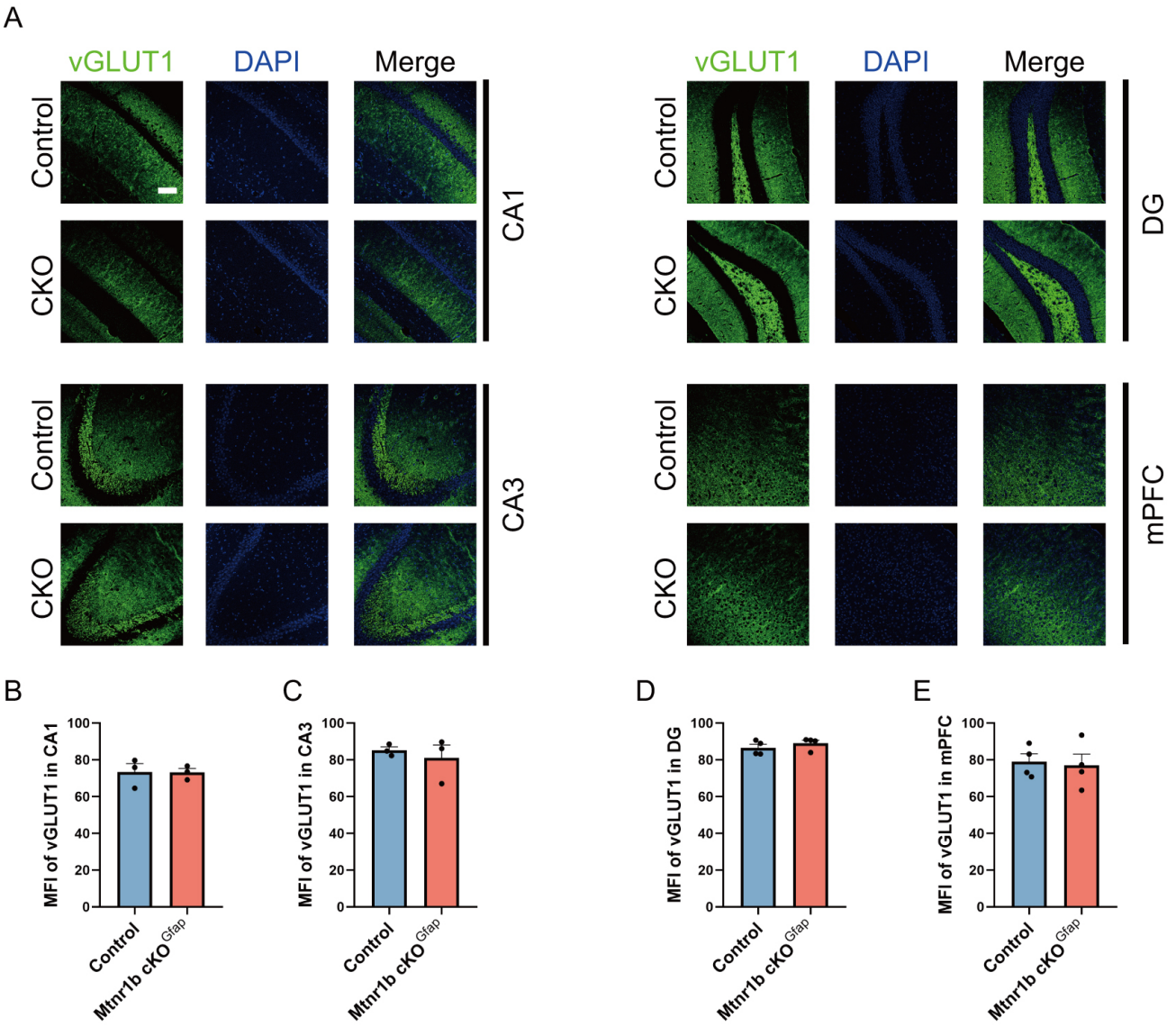

Fig. 5. Knockout of the *Mtnr1b* gene in astrocytes has no effect on the expression of excitatory synaptic proteins in the hippocampus. (A) Representative immunofluorescence images of different groups. Scale bar: 50 μm for each image (n = 3–4). (B–E) Quantification of the mean fluorescence intensity of vesicular glutamate transporter 1 (vGluT1) in the CA1, CA3, DG, or prefrontal cortex regions. CA1: $p = 0.9572$, CA3: $p = 0.5903$, DG: $p = 0.3839$, mPFC: $p = 0.7918$. Data are presented as the mean ± SEM.

ory function in mice. We believe that previous studies used different mouse strain backgrounds (*C57BL/6N* and *C3H/HeN*), whereas the mouse background used here was *C57BL/6J*. *C57BL/6J* mice are known to have lower levels of melatonin production due to mutations in the melatonin synthesis enzyme, and thus have lower plasma melatonin levels [52]. Additionally, *C57BL/6N* mice produce melatonin in peripheral tissues, whereas *C57BL/6J* mice do not, which may affect their cognitive functions [53]. Another important factor is that previous studies used mice with a whole-body knockout of the *Mtnr1b* gene, whereas in the current study we used only mice with a specific knockout of the *Mtnr1b* gene in astrocytes. This may have affected the results of cognitive function tests. Finally, the specific experimental procedures were different. Thomson's study used a continuous-performance task to evaluate cognitive function, whereas the tests used here were the Morris water maze and new object recognition. The continuous-performance test is used to evaluate the cognitive control ability and sustained attention of an animal, whereas the Morris water maze test is mainly used to evaluate spatial learning and memory abilities. The sensitivity of the continuous-performance task is higher than that of the Morris water maze task, which may also be a reason for the different cognitive function test results.

The influence of sex on *Mtnr1b* knockout mice also warrants thoughtful consideration. Melatonin levels have a significant impact on emotions, and it is important to highlight that in humans, females exhibit greater fluctuations in plasma melatonin levels than do males [54,55]. This

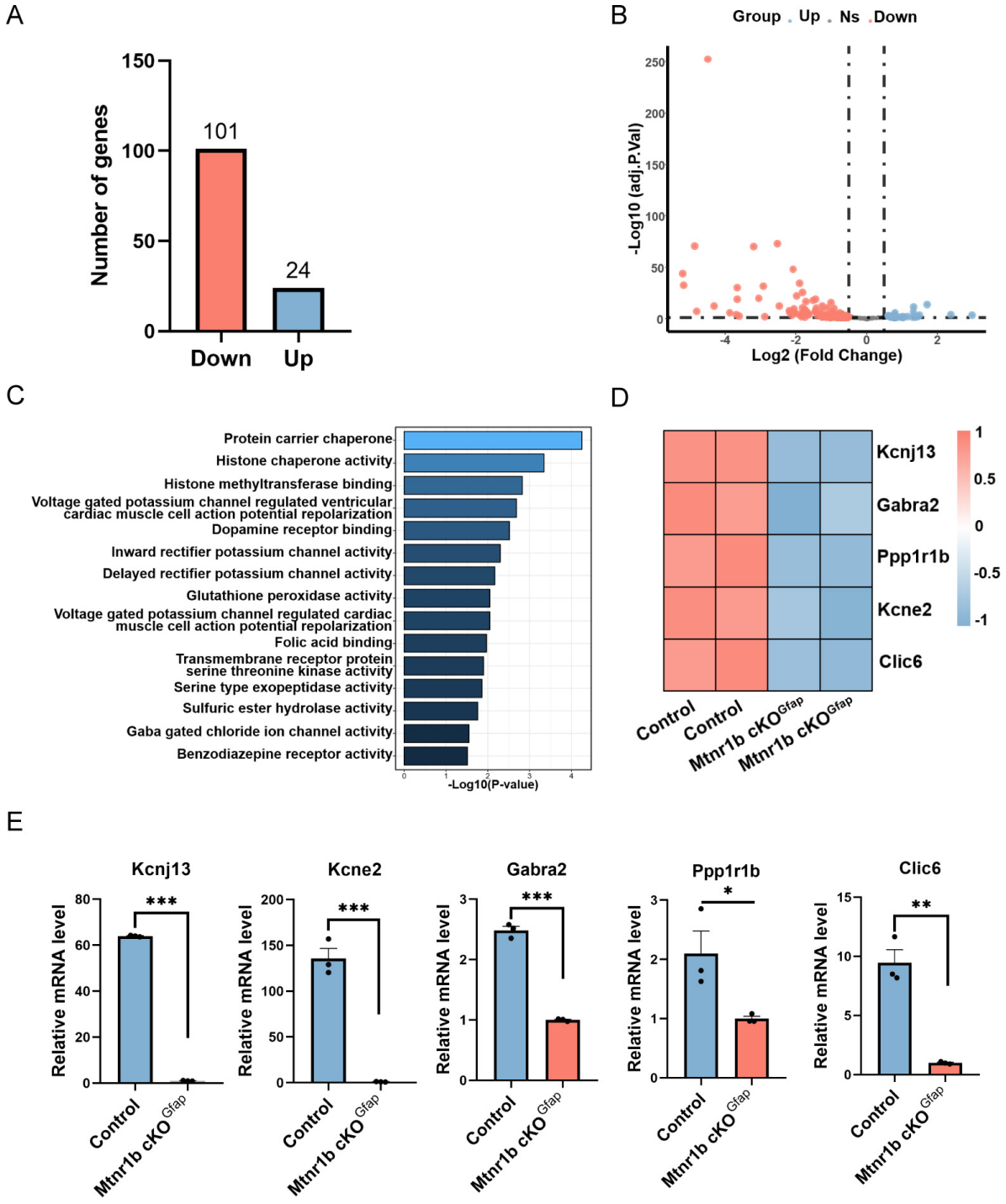

Fig. 6. Differential expression profiling in the hippocampus of *Mtnr1b* cKO^{Gfap} and control mice. (A) Upregulated and downregulated differentially expressed genes in the hippocampus of *Mtnr1b* cKO^{Gfap} and control mice. (B) Volcano plot of all transcripts identified in the hippocampus. The log-fold change represents *Mtnr1b* cKO^{Gfap} *vs.* control mice. (C) Functional enrichment analysis indicated significantly enriched Gene Ontology (GO) terms in the cellular component category. (D) Heat map displaying five genes associated with GO enrichment pathways. Red: Increased expression. Blue: Decreased expression. The gene expression was transformed by z-score. (E) qRT-PCR validation of RNA-Seq data set with selected genes. *Kcnj13*: $p < 0.0001$, *Kcne2*: $p = 0.0003$, *Gabra2*: $p < 0.0001$, *Ppp1r1b*: $p = 0.0459$, *Clic6*: $p = 0.0016$. Data are presented as the mean ± SEM, with $n = 3$/gp. Fold change relative to controls was calculated using the $2^{-\Delta\Delta CT}$ relative gene expression analysis. Group differences were assessed by Student's *t*-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

suggests that impaired melatonin function has different effects on individuals depending on their sex. Thomson *et al.* [25] reported that male *Mtnr1b* KO mice showed enhanced sociability, whereas female *Mtnr1b* KO mice exhibited an elevated anxiety response. Liu *et al.* [51] previously reported increased anxiety levels in *Mtnr1b* KO mice, without specifying the sex. Comai *et al.* [26] reported that male KO mice exhibited anxiety-like behavioral characteristics. Horst *et al.* [56] showed that ovarian hormones contribute to decreased anxiety levels in female mice exposed to stressful environments (such as elevated plus maze or open field test). In order to conduct a more comprehensive investigation into the effects of *Mtnr1b* deletion in astrocytes, male mice were therefore deliberately selected in the present work, thereby eliminating potential confounding factors arising from the influence of female hormones.

The excitability of central neurons is primarily influenced by glutamatergic neurons, and their level of excitation is continuously regulated by inhibitory synaptic inputs. The imbalance between excitatory and inhibitory synaptic transmission is a crucial process that underlies numerous neurological and psychiatric disorders, including epilepsy [57], schizophrenia [58], Alzheimer's disease [59], anxiety [32], and depression [60]. An increasing number of reports have highlighted the significance of alterations in inhibitory neurons, particularly GABAergic synapses, that are vital in the development of numerous critical neuro-psychiatric disorders. Cheng *et al.* [61] reported that melatonin not only increases the amplitude of GABAergic miniature inhibitory postsynaptic currents (mIPSCs), but also enhances the frequency of mIPSCs in hippocampal neurons, indicating that melatonin enhances GABAergic signal transmission through both presynaptic and postsynaptic mechanisms. Astrocytes act as an important cell type in the regulation of neuronal activity [62]. It is therefore reasonable to hypothesize that blockade of *Mtnr1b* in astrocytes may also affect neurotransmission. GABA is synthesized by the enzyme glutamate decarboxylase (GAD) from glutamate. Multiple studies have implicated GAD67 protein as the molecule most strongly linked to various neuropsychiatric disorders [39,63,64]. Miyata *et al.* [65] demonstrated that global knockdown of GAD67 expression leads to anxiety-like behavior in mice. vGlut1 is a transport protein located in presynaptic neurons and is responsible for packaging glutamate into vesicles [66,67]. It serves as a marker gene for glutamatergic neurons and has been implicated as a regulator of anxiety-like behavior [68]. The present study analyzed expression levels of the inhibitory neuron marker GAD67 and the excitatory neuron marker vGluT1 by immunofluorescence staining in various brain regions of both control and *Mtnr1b* cKO^{Gfap} mice. Additionally, transcriptome sequencing data revealed the presence of postsynaptic effects. GAD67 expression levels were significantly lower in the CA1 and CA3 regions of *Mtnr1b* cKO^{Gfap} mice than in their control counterparts, whereas vGluT1 levels remained unchanged. These results are in agreement with those of previous studies.

In the present study, RNA-seq analysis of hippocampal tissue revealed that molecular functions were mainly enriched in pathways associated with potassium channel and GABA-receptor-gated chloride channel activities. This suggests that the astrocyte-specific knockout of *Mtnr1b* resulted in altered neuronal function. Melatonin enhances $GABA_A$ receptor-mediated currents in cultured chick spinal cord and rat hippocampus neurons [61,69]. Additionally, melatonin was found to enhance the amplitude of mIPSCs. These results suggested that melatonin is capable of increasing the activity of postsynaptic $GABA_A$ receptors. Yu *et al.* [18] reported that melatonin can inhibit sympathetic neural regulation by enhancing $GABA_A$ receptor activity in the hypothalamus. In the present study, a significant reduction of the *Gabra2* gene in *Mtnr1b* cKO^{Gfap} mice was observed, suggesting that the astrocyte-specific knockout of *Mtnr1b* decreased the activity of the $GABA_A$ receptor in the hippocampus. Taken together, these findings lead to the speculation that loss of the *Mtnr1b* gene in astrocytes results in downregulation of the GABAergic signaling pathway within the hippocampus of *Mtnr1b* cKO^{Gfap} mice.

Prior investigations have documented the activation of Kir3 channels via MTNR1A stimulation [70]. However, the present study used transcriptome sequencing and qRT-PCR analysis to show that deletion of the *Mtnr1b* gene in astrocytes elicits a significant decrease in the expression of *Kcnj12*, which is the gene responsible for encoding the Kir2.2 protein in hippocampal tissue [71]. Leonoudakis *et al.* [72] demonstrated that Kir2.2 is expressed not only in astrocytes but also in the granule cell layer. In the central nervous system, neuronal activity leads to K^+ release into the extracellular space, and astrocytes modulate neuronal excitability by buffering extracellular K^+ through Kir2.2. Previous studies have reported associations between intronic variants in the *Kcne2* gene and psychiatric disorders [73]. Abbott *et al.* [74] reported that knockout of the *Kcne2* gene increases neuronal excitability and enhances responsiveness to stress. Knockout of the *Mtnr1b* gene in astrocytes was also found to downregulate the expression of *Kcne2* in the hippocampus. This evidence suggests that elimination of *Mtnr1b* cKO^{Gfap} in astrocytes affects the E/I balance through the regulation of genes involved in K^+ channels.

5. Conclusions

In summary, astrocyte-specific elimination of the *Mtnr1b* gene in mice leads to anxiety-like behavior. This may be due to downregulation of the hippocampal GABAergic signaling pathway, resulting in an imbalance between excitatory and inhibitory synapses. The present results provide new insights into the potential of *Mtnr1b* as a novel therapeutic target for anxiety disorders.

Availability of Data and Materials

The RNA-seq datasets analyzed in this study are available in the Genome sequence archive database (https://ngdc.cncb.ac.cn/search/?dbId=gsa&q=CRA012658, Accession: CRA012658). Described in Results and Methods section and available upon request.

Author Contributions

ZM, WZhou and WS conceived and designed this research. ZM, SG, XD, QW, DH, XL, YJ, LJ, JZ, WZhu conducted the experiments. WZhou and WS contributed reagents, materials, and analytical tools. WS and WZhou supervised the project. ZM, QW, WZhou and WS wrote the manuscript and revised manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All animal experiments were approved by the Ethics Committee of Chongqing Medical University and Animal Study Committee of the Children's Hospital of Chongqing Medical University (no code–CHCMU-IACUC20221227006).

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.jin2206154.

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