

Original Paper

Stimulation of Anxiety-Like Behavior via ERK Pathway by Competitive Serotonin Receptors 2A and 1A in Post-Traumatic Stress Disordered Mice

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Key Words

5HT2AR • 5HT1AR • Anxiety-like behavior • ERK pathway • Post-traumatic stress disorder

Abstract

Background/Aims: Serotonin 5HT2A and 5HT1A receptors (5HT2AR, 5HT1AR) have the closest connection to anxiety-like behavior in post-traumatic stress disorder (PTSD). However, the underlying mechanism remains unclear. In this study, we explored the connection between 5HT2A and 5HT1A receptors and anxiety-like behavior. **Methods:** In the PTSD animal model, mice were exposed to conditioned fear stress coupled with single-prolonged stress (CF+SPS). Post stress infliction and behavioral tests, of which include open field, freezing behavior and elevated plus maze tests were carried out to examine establishment of the proposed model. Both Western blot analysis and immunofluorescence labeling were used to evaluate protein expressions of 5HT2AR, 5HT1AR, ERK1, ERK2 and c-Myc in the hippocampi of the mice and RT Q-PCR was employed for evaluation of the relative mRNA expressions. **Results:** Based on the model established utilizing the CF+SPS procedure, we found 5HT2AR to play a positive role on anxiety-like behavior by inhibiting the expression of 5HT1AR. In addition, the ERK-c-Myc pathway elicited the effect of 5HT2AR and 5HT1AR on anxiety-like behavior in PTSD, 5-HT enhanced the anxiety-like behavior through both 5HT2AR and 5HT1AR. **Conclusion:** These findings suggest competitive interaction between 5HT2AR and 5HT1AR actively affects anxiety-like behavior in the hippocampi of PTSD mice via the ERK pathway.

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Introduction

A study conducted in the United States (US) indicated that approximately 50%-70% of American people have been exposed to traumatic events such as earthquakes, terrorism, warfare and violent personal assault, with 5%-12% of them developing an anxiety like PTSD during their life time [1, 2]. PTSD is characterized by nightmares, flashbacks, mood swings, psychological/physical distress and hyper-arousal [3, 4]. United States spends more than 30% of its mental illness expenditure on the treatment of anxiety disorders like PTSD, expending approximately \$45 billion annually [4]. Chronic diseases such as anxiety disorder severely affect patients' long-term quality of life as well as work productivity, in addition to often being a life-long affliction [3, 4]. It is, however, worth noting that the underlying mechanisms of anxiety disorders such as PTSD remain elusive and as such warrants further investigation.

Both hippocampus and amygdala are major brain regions that respond to anxiogenesis and stress stimuli [1, 5]. The pathophysiology of PTSD is attributed to dysfunction of the hippocampus and amygdala, whose activity is associated with stress-related emotional arousal [6, 7].

The serotonergic system has seven serotonin receptor proteins (5HT₁₋₇) and thirteen subtypes including 5HT_{2A/2B/2C} receptors [8]. Both 5-HT_{2A} and 5-HT_{1A} receptors play an active role in anxiety-like behavior. Although the activation of 5-HT_{2A}-receptor promotes anxiety-like behavior, 5-HT_{1A}-receptor activation, on the other hand, inhibits anxiety-like behaviors [2, 9]. Some studies have subsequently revealed the serotonergic system to exhibit significant effect on psychiatric disorders such as anxiety-like behavior in PTSD [10-12]. Anxiety stimuli activate serotonergic neurons that are originated from the dorsal raphe nucleus and projected into the multiple forebrain and limbic structures such as amygdala and hippocampus [13, 14]. Dysregulation of serotonergic system might cause anxiety in PTSD and depressive disorders [12, 15, 16].

In rats, both 5-HT_{2A} and 5-HT_{1A} receptors are primarily located in the cerebral cortex, hippocampus, amygdala and brain stem. Stress-induced psychiatric symptoms are always accompanied by expressional changes in 5-HT_{2A} and 5-HT_{1A} receptors, suggesting the critical role of 5-HT_{2A} and 5-HT_{1A} receptors in anxiety-like and depressant-like behaviors [17]. Studies have shown 5HT_{1A} receptor in the hippocampus to perform important functions by relieving the depressant behavior related to traumatic stress [18-20]. According to a study on lipopolysaccharide-induced shock, the possible effect of 5HT_{2A} receptor was via the ERK signaling pathway [21]. With that said, the principal mechanism of both 5-HT_{2A} and 5-HT_{1A} receptors on anxiety-like behavior correlated with PTSD is yet to be deciphered.

To address this problem, conditioned fear stress followed by single-prolonged stress was conducted to establish the mouse PTSD model [22]. Both open field and freezing behavioral tests as well as elevated plus maze test were subsequently employed in evaluating the animals' behavior [22-24]. In addition, expressions of 5-HT_{2A} and 5-HT_{1A} receptors in protein together with mRNA level were analyzed by Western blot, immunofluorescence labeling and RT Q-PCR techniques [25, 26].

Materials and Methods

Ethical Statement and Animal preparation

All experiments were conducted following the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals guidelines and as well as the ethical approval from Zhejiang University. Mice were kept in ventilated plastic housing cages with free access to food and water under standard conditions at 21±1°C and 55±5% humidity. Male wild-type (C57BL/6) mice, weighing 18–23g, were obtained from the Academy of Medical Sciences in Zhejiang Province. Transgenic mice (ePet-Cre-Ai9) were kindly provided by Professor Xiaoming Li. ePet-Cre mice can express Cre recombinase exclusively in the serotonergic neurons and Ai9 mice harbors a *TdTomato* coding sequence downstream of *LoxP* sites flanked STOP cassette.

Experimental Group and Drugs Administration

This research study was generally divided into two parts: drug treatment mice experiment and transgenic mice experiment. Regarding the drug administration section, 32 C57BL/6 mice were randomly divided into four groups (n=8 per group): CF+SPS+DMSO group, CF+SPS+Ketanserin group, CF+SPS+WAY100635 group and sham group (DMSO group). 5HT1A receptor antagonist, WAY100635, was administered subcutaneously at a dose of 3mg/kg [27] and 5HT2A receptor antagonist, Ketanserin, was injected intraperitoneally at a dose of 0.3mg/kg [28]. Both WAY100635 and Ketanserin were dissolved in DMSO, which was purchased from Selleck (Selleckchem, Houston, USA). DMSO was diluted to 10% by 0.9% saline [25]. An equivalent dose of DMSO was applied to mice in the sham group following the same approach. C57BL/6 mice were housed for 7 days following being exposed to CF+SPS. After individually being injected with Ketanserin, WAY100635 and DMSO, mice were exposed to CF for 5 successive days. On the 6th day, mice were subjected to single-prolonged stress. Furthermore, 10 transgenic mice ePet-Cre-Ai9 and 10 Wild Type mice (C57BL/6) were respectively assigned to two groups (n=5 per group): CF+SPS treatment group and control group. All mice were subjected to behavioral tests after 14 days before being sacrificed. Half of the tissues were used for protein and RNA extraction, with the remaining half used for perfusion. The other four groups (two transgenic groups and two wild-type groups) of mice were used for perfusion.

Methodology for mouse model set-up

We employed the conditioned fear stress combined with single-prolonged stress to establish the animal model [22] (Fig. 1). Mice were exposed to electric foot shock on day 1. One mouse was placed into the shock chamber and had a 60-second adaptation period. After the chamber light was turned on for 10 seconds, the mouse received a 1-mA shock for 4 seconds. The procedure was repeated for 5 consecutive days. On day 6, mice exposed to electric shock were subjected to single-prolonged stress, which were separately immobilized in a 50mL EP (Eppendorf) tube for 2 hours and immediately underwent a 20-minute forced swimming. The swimming was performed in a plastic bucket (40 cm × 80 cm, 25°C water temperature) with one quarter of water. After a 15-minute rest period, mice were anaesthetized and subsequently housed under standard conditions to recover. Mice in both ePet-Cre-Ai9+CF+SPS and C57BL/6+CF+SPS groups were subjected to the same CF+SPS procedure as described above.

Behavioral Sensitized Fear Test

Open Field Test. Mice were housed for 13 days under standard conditions after being subjected to CF+SPS procedure. On day 20, mice were put through behavioral sensitized fear test. Firstly, all mice had a one-hour adaptation period in the testing room. The test was conducted in a white acrylic plastic cubic chamber (45 cm × 45 cm × 45 cm) in a soundproof room under dim illumination. Each mouse was placed in the center of the open field arena and allowed to freely explore for 5 minutes. The amount of time spent in the center or edges was recorded by an automatic analyzing system (VideoTrack, Viewpoint Inc., France). Chamber was cleaned with 75% ethanol before and between each trial [9, 22, 29, 30].

Freezing Behavioral Test. Freezing behavioral test was performed simultaneously with open field test. Freezing behavior was defined as the lack of body movement other than respiration. Each mouse was observed for 5 minutes using an automatic analyzing system (VideoTrack, Viewpoint Inc., France) and recorded as either freezing or active every 10 seconds. Total time spent freezing during each measurement was expressed as a percentage of the total time. Chamber was thoroughly cleaned with 75% alcohol between tests in order to avoid disturbance of olfactory cues [24, 31, 32].

Elevated Plus Maze Test. After open field and freezing behavioral tests, mice were allowed to rest for 30 minutes before being subjected to elevated plus maze test. The apparatus was made of grey plexiglas and composed of two opposite facing closed arms (30 cm × 5 cm × 15 cm) and another two opposite facing opened arms (30 cm × 5 cm) with a central area (5 cm × 5 cm) on a 50 cm high stand. Mice were individually placed in the central area facing the open arm and allowed to freely move around for 5 minutes. The number and duration of entries into both open and closed arms were respectively recorded by an automatic analyzing system (Anymaze, Stoelting Inc., USA). The time remained in both open and closed arms; number of entries into open and closed arms was scored. An arm entry was counted when all limbs of mouse were in the open arm [22].

Immunofluorescence labeling

Mice were anesthetized with isoflurane and perfused through the heart with 0.9% saline followed by 4% paraformaldehyde. Brain tissues of mice were fixed in 4% paraformaldehyde for 48 hours, incubated in 30% sucrose solution for 72 hours, embedded in Optimal Cutting Temperature (OCT) compound (Sakura Finetek, USA) and frozen at -80°C. After thrice rinsing with PBST (0.01M phosphate buffer saline with Tween-20) at room temperature (RT), 35µm thick sections were immersed in blocking solution containing 5% goat serum at RT for 3 hours, then incubated at 4°C overnight with primary antibody (anti-5HT1A receptor, rabbit polyclonal antibody, Abcam, 5µg/mL; anti-ERK12, rabbit polyclonal antibody, CST, 1:100; anti-c-Myc, rabbit polyclonal antibody, proteintech, 1:50), and anti-5HT2A receptor (rabbit polyclonal antibody, Immunostar, 1:100, incubated for 48 hours). Sections soaked in 0.01M PBST without any primary antibody served as the negative control. After thrice washing with PBST, slices were separately incubated with dylight-labeled secondary antibody (Dylight goat anti-rabbit IgG, 1: 500, Earthox, USA) at RT for 3 hours. Sections were mounted with VECTASHIELD Mounting Medium with 4,6-diamidino-2-phenylindole (DAPI) (Vector laboratories, USA) and examined under a fluorescence microscope (Olympus BX51, NIKON, Japan) at excitation/emission wavelength of 350 /461 nm (blue, DAPI); 498/516 nm (green, Dylight 488); and 558/583 nm (red, tdTom) [7, 33].

Identification of transgenic mice

The transgenic mice were identified by PCR using the genomic DNA isolated from the tail tips. The tail biopsies were placed in 1.5 mL tubes with DNA (deoxyribonucleic acid) extraction buffer 1 (100µL) (25mM NaOH, 0.2mM EDTA (ethylene diaminetetraacetic acid disodium salt)). After incubating in a 95°C–water bath for 1 hour, DNA extraction buffer 2 (100 L) (Tris-HCL, PH3.0) was added. The mixed solution was then centrifuged at 12000rpm for 5 minutes to collect the supernatant. Crude DNA extract (1 µL) was used as template. PCR reactions were carried out in a 25 µL volume containing 12.5 µL 2×Taq PCR Master Mix (with dyes) (DBI Bioscience, Germany), 1 µL DNA extracts and primers (Table 1). The volume was supplemented with DEPC (diethyl pyrocarbonate) water to 25µL. The PCR program for Cre integration were 94°C for 30 minutes, followed by 36 cycles of 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds, 72°C for 2 minutes and finally maintained at 4°C. The PCR screening for TdTomato transgene integration was performed under the following conditions: 94°C for 30 minutes, 36 cycles of 94°C for 30 seconds, 61°C for 30 seconds, 72°C for 30 seconds, 72°C for 2 minutes and kept at 4°C. PCR products were analyzed with agarose gel electrophoresis and visualized with Gel Image Analysis System (Tanon 2500R, Shanghai, China) [34-36].

Western blot analysis

Mice brain tissues were obtained and stored at -80°C. One half of the hippocampus from each experimental group was dissected with fine blunt forceps and individually homogenized in RIPA Buffer (Table 1) containing 1 mM PMSF and inhibitors (Beyotime Biotechnology, China). After being centrifuged at 12000 rpm, 4°C for 20 minutes, the supernatant was maintained at -80°C. BCA protein assay kit (KeyGEN, Nanjing, China) was employed in quantifying protein concentration. Protein samples were diluted to 3µg/µL concentration, and separated by 12% SDS-PAGE (Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis) at 70 V for 20 minutes, 100 V for 100 minutes. After electrophoresis, proteins

Table 1. Mouse Primer Sequences

Name	Upstream Primer	Downstream Primer
5HT1AR	5'-TCGCTCACTTGGCTCATTGGCTTT-3'	5'-TTCCAACCTCTTGACCGTCTTGCG-3'
5HT2AR	5'-CTGGACCGCTACGTGGCTAT-3'	5'-TATGGTCCACACCGCAATGA-3'
ERK1	5'-TGGCTTTCTGACGGAGTATG-3'	5'-GGTCCAGGTAGTGTTCG-3'
ERK2	5'-CCTCAAGCCTTCCAACCTC-3'	5'-GCCACAGACCAAATATCAATG-3'
c-Myc	5'-GCTTCCCACCCCGCCCTGTC-3'	5'-CCACC GCCCGCTCATCGTCTT-3'
β-actin	5'-GAGACCTTCAACACCCGAGC-3'	5'-ATGTACAGCAGGATTTCCC-3'
Cre	5'-ATTTGCCTGCATTACCGGTGC-3'	5'-CAGCATTGCTGTCACTTGGTC-3'
Ai9(WT)	5'-AAGGGAGCTGCAGTGGAGTA-3'	5'-CCGAAAATCTGTGGGAAGTC-3'
Ai9(Mutant)	5'-CTGTTCTGTACGGCATGG-3'	5'-GGCATTAAAGACCGTAT-3'

were transferred onto PVDF (polyvinylidene fluoride) membrane (Millipore, Bedford, MA, USA) at 350 mA for 105 minutes. The membrane was then blocked with 5% skim milk at room temperature for 2 hours before being examined with primary antibody overnight at 4°C (anti-5HT1A receptor, rabbit polyclonal antibody, Abcam, 5µg/mL; anti-5HT2A receptor, goat polyclonal antibody, Santa Cruz, 1:200; anti-ERK12, rabbit polyclonal antibody, CST,1:100; anti-pERK12, rabbit polyclonal antibody, CST,1:2000; anti-c-Myc, mouse polyclonal antibody, Santa Cruz, 1:100; anti-β-actin, rabbit polyclonal antibody, CST,1:2000). The following day, after washing with TBST, the membrane was incubated with secondary antibody for 2 hours at room temperature (HRP-labeled goat anti-rabbit IgG, HRP-labeled Donkey anti-goat rabbit IgG, HRP-labeled rabbit anti-mouse IgG, Boster Biological Technology Ltd., 1:8000). Afterwards, protein bands were visualized using an enhanced chemiluminescence detection kits (ECL-plus, Beyotime Biotechnology, China) by Gel Image Analysis System (Tanon 2500R, Shanghai, China)[17, 37, 38].

RT Q-PCR analysis

The remaining half of mice's hippocampus was used for RNA extraction, (ribonucleic acid) employing the trizol kit (Invitrogen, USA). The concentration of total RNA was adjusted to 100ng/µL, with each RNA sample reverse-transcribed into cDNA using reverse kit (DBI Bioscience, Germany). Reaction program was set at 37°C for 15 minutes and 98°C for 5 minutes with primers (Table 1). The final volume (20 µL) was a mixture of cDNA (1µL), SYBR green PCR Master Mix (10µL) (DBI Bioscience, Germany), DEPC (diethyl pyrocarbonate) water (7µL), Forward primer (1µL) and Reverse primer (1µL). The cDNA amplifications were performed at 95°C for 5 minutes, then cycled 45 times on a Bio-Rad CFX apparatus at 95°C for 10 seconds, 60°C for 10 seconds and 72°C for 10 seconds. β-actin was chosen as the internal control. The $2^{-\Delta\Delta Ct}$ method was employed to determine the relative expression level of the target gene, with the formula expressed as follows: Relative expression level of target gene (X) = $2^{-\Delta\Delta Ct}$; where $\Delta\Delta Ct = (Ct, X-Ct, \beta\text{-actin})$ sample - (Ct, X-Ct, β-actin) control [25, 39].

Statistical Analysis

Histograms were plotted with GraphPad Prism 5. Statistical analysis was performed with one-way analysis of variance (ANOVA) with post-hoc Bonferroni tests using SPSS statistics 17.0. $P < 0.05$ was considered to be statistically significant.

Results

Behavioral tests confirmed PTSD model establishment

In order to confirm the established animal model (Fig. 1), behavioral tests including open-field, freezing and elevated plus maze tests were carried out. As depicted in Fig. 2A, the times through the central grille for the 5-HT2AR antagonist group was significantly decreased as compared to the sham group, $*P < 0.05$. In Fig. 2H, the percentage of freezing behavior for the ePet-Cre-Ai9 +CF+SPS and WT+CF+SPS groups were conspicuously increased in similitude to that of the control group, $**P < 0.01$, $###P < 0.001$. In the elevated plus maze test, results showed OA entries for the vehicle group to be significantly exacerbated when compared to the sham group, $*P < 0.05$ (Fig. 2C). "OA entries" denoted the number of entries into the open arms / (the number of entries into the open arms + closed arms); "OA time" indicated the time spent in the open arms / (the time spent in the open arms + closed arms). There was increment in both OA entries and OA time in the WT PTSD group in comparison to the control.

Crosstalk between 5-HT2AR / 5-HT1AR and potential regulatory pathway through ERK1/ERK2/c-Myc detected via Immunofluorescence labeling

In order to detect the relative protein expression levels in the mice hippocampi, immunofluorescence labeling analysis was executed. As illustrated in Fig. 3, expression level of 5-HT2AR in the 5-HT1AR antagonist group was significantly increased when compared to the vehicle group, $\#P < 0.05$. Protein expression level of 5-HT1AR in the vehicle group and the

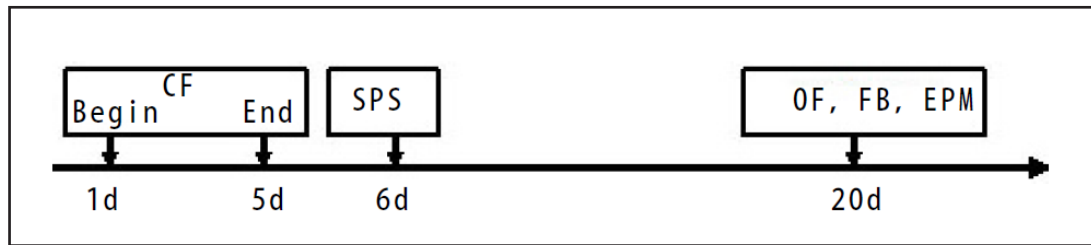


Fig. 1. Procedures for establishing mouse model and behavioral tests. After mice (C57BL/6) of CF+SPS+ketanserin group, CF+SPS+WAY100635 group, and sham group were respectively injected with ketanserin, WAY100635, and DMSO, mice were exposed to CF for 5 consecutive days (started on the 1st day and ended on the 5th day) and progressive single-prolonged stress (SPS) on the 6th day. Following the completion of CF+SPS, mice in the CF+SPS+DMSO group, CF+SPS+ketanserin group, CF+SPS+WAY100635 group, and sham group were housed for 13 days and subjected to open field (OF), freezing behavior (FB) and elevated plus maze (EPM) tests. $n = 8$ per group. Mice of ePet-Cre-Ai9+CF+SPS group and C57BL/6+CF+SPS group received the same CF+SPS procedures as described above. After CF+SPS was completed, mice in the ePet-Cre-Ai9+CF+SPS group, C57BL/6+CF+SPS group, ePet-Cre-Ai9 group, and C57BL/6 group were housed for 13 days and subjected to open field (OF), freezing behavior (FB) and elevated plus maze (EPM) tests. $n = 5$ per group.

5-HT1AR antagonist group were considerably increased as compared to the control group, whilst the level of 5-HT1AR in the 5-HT2AR antagonist group was significantly reduced in comparison to the control group, $***P < 0.001$, $###P < 0.001$, $^{\#}P < 0.05$, $^{sss}P < 0.001$ (Fig. 4). Expression levels of ERK1 and ERK2 of the vehicle group and 5-HT1AR antagonist groups were significantly increased when compared to the control group, $**P < 0.01$, $***P < 0.001$, $###P < 0.001$, $^{sss}P < 0.001$ (Fig. 5). Significant increment in the expression levels of c-Myc in the vehicle group, 5-HT1AR and 5-HT2AR antagonist groups in comparison to the control group was also observed, $***P < 0.001$, $###P < 0.001$, $^{sss}P < 0.001$ (Fig. 6).

Reciprocal action between 5-HT2AR / 5-HT1AR and potential regulatory pathway through ERK1/ERK2/c-Myc detected by western blot

Western blot analysis was executed to quantify the protein expression level in the mice's hippocampi. Results from Western blot analysis evidenced pronounced decrement in the expression level of 5-HT1AR in the 5-HT2AR antagonist group in similitude to the sham group, however, this was increased in both the vehicle and 5-HT2AR antagonist groups (Fig. 10, Table 2). Also, there was curtailment in the expression level of 5HT2AR in the vehicle group, but, was increased in both 5-HT1AR and 5-HT2AR antagonist groups. Again, expression levels of ERK1, ERK2, pERK1, pERK2 and c-Myc were exacerbated in the vehicle group, 5-HT1AR and 5-HT2AR antagonist groups. $^{\#}p < 0.05$ vs. the CF+SPS+DMSO group (5HT1AR); $^*p < 0.05$ vs. the sham group (ERK2); $***p < 0.001$ and $**p < 0.01$ vs. the sham group, $^{\#}p < 0.05$ vs. the CF+SPS+DMSO group (pERK1); $^*p < 0.05$ vs. the sham group (pERK2); $***p < 0.001$ vs. the sham group, $^{\#}p < 0.05$ vs. the CF+SPS+DMSO group (c-Myc).

Interaction between 5-HT2AR / 5-HT1AR and potential regulatory pathway through ERK1/ERK2/c-Myc detected by RT Q-PCR

In order to detect the relative mRNA expression levels in the mice hippocampi, RT Q-PCR analysis was executed. As presented in Fig. 11, expression level of 5-HT2AR of the 5-HT1AR and 5-HT2AR antagonist groups was significantly increased as compared to the control group. In contrast, expression level of 5-HT2AR in the vehicle group was decreased when compared to the sham group, $**P < 0.01$, $^{\#}P < 0.05$, $^{\#}P < 0.01$ (Fig. 11A). Also, mRNA expression level of 5-HT1AR of the vehicle group was increased in comparison to the sham group, whilst the 5-HT2AR antagonist group was significantly decreased when compared to the control group, $**P < 0.01$, $^{\#}P < 0.01$ (Fig. 11B). Again, mRNA expression level of ERK1

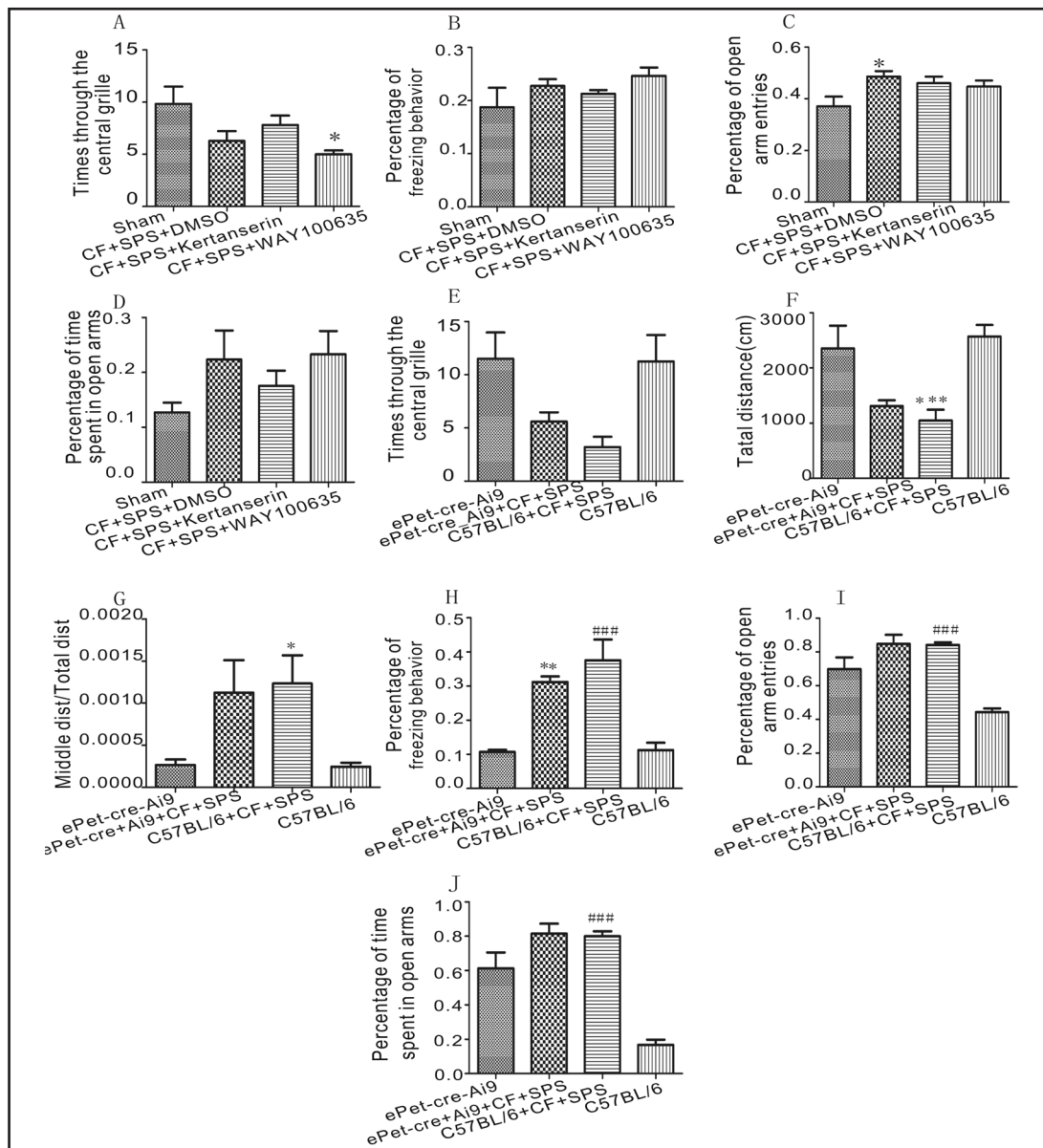


Fig. 2. Behavioral experiments in the established mouse model. A, E-G Open-field test. Times through the central grille of the CF+SPS+WAY100635 group was significantly decreased as compared to the sham group, $*p < 0.05$ vs. the sham group (A); Total distance of the C57BL/6+ CF+SPS group was significantly decreased compared to the C57BL/6 group, $***p < 0.001$ vs. the C57BL/6 group (F); Middle dist/ Total dist of the C57BL/6+ CF+SPS group was significantly increased compared to the C57BL/6 group, $*p < 0.05$ vs. the C57BL/6 group (G); B, H Freezing behavior test. The percentage of freezing behavior was the time spent in freezing behavior/ a total time during each measurement. The percentage of freezing behavior of the ePet-Cre-Ai9+CF+SPS group and C57BL/6+ CF+SPS group were respectively notably increased compared with that of the control group, $**p < 0.01$ vs. the ePet-Cre-Ai9 group, $###p < 0.001$ vs. the C57BL/6 group (H); C-D, I-J Elevated plus maze test. “OA entries” denoted the numbers of entries into the open arms / (the numbers of entries into the open arms +closed arms); “OA time” indicated that the time spent in the open arms / (the time spent in the open arms +closed arms). OA entries of the CF+SPS+DMSO group were significantly increased compared to the sham group, $*p < 0.05$ vs. the sham group (C); OA entries and OA time of the C57BL/6+CF+SPS group were respectively increased compared with that of the C57BL/6 group, $###p < 0.001$ vs. the C57BL/6 group (I, J). Data were presented as mean \pm SEM through ANOVA. Groups were compared by conducting Bonferroni’s test. $n = 8$ (C57BL/6); 10 (ePet-Cre-Ai9).

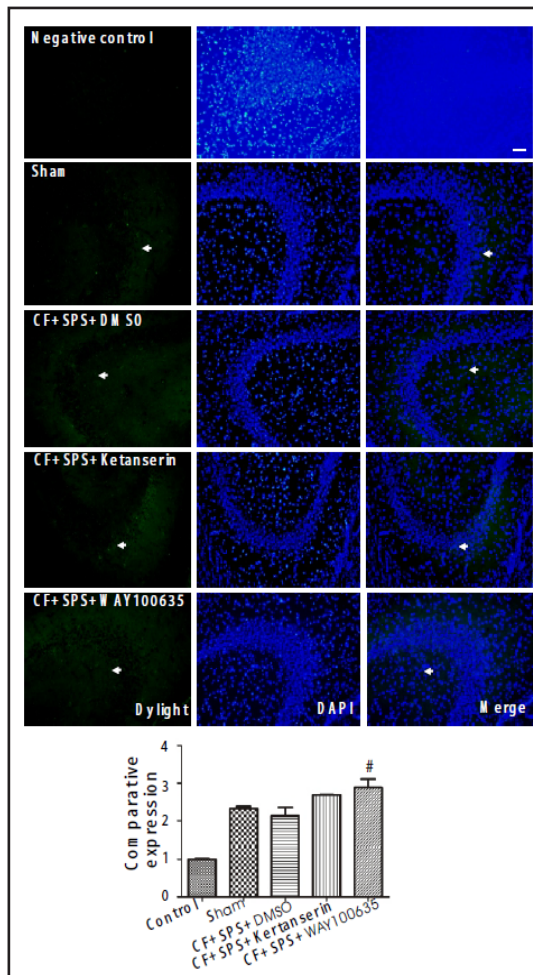


Fig. 3. Immunofluorescence labeling revealed the comparative protein expression level of 5-HT2AR in the mouse (C57BL/6) hippocampus compared with the control group. # $p < 0.05$ vs. the CF+SPS+DMSO group. Bar = 100 μ m. Data were presented as mean \pm SEM through ANOVA. Groups were compared by performing Bonferroni's test. $n = 4$.

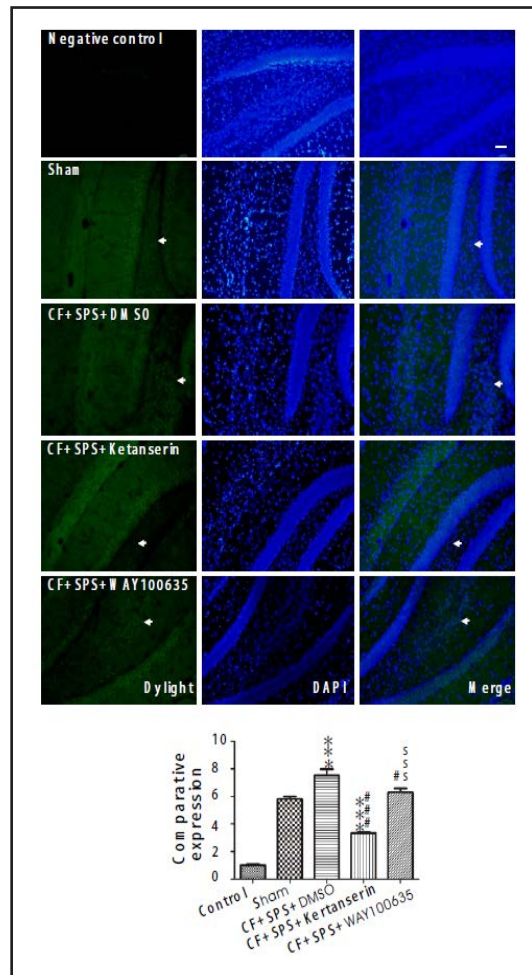


Fig. 4. Immunofluorescence labeling revealed the comparative protein expression level of 5-HT1AR in the mouse (C57BL/6) hippocampus compared with the control group. *** $p < 0.001$ vs. the sham group, ### $p < 0.001$, # $p < 0.05$ vs. the CF+SPS+DMSO group, $^{ss}p < 0.001$ vs. the CF+SPS+ketanserin group. Bar = 100 μ m. Data were presented as mean \pm SEM through ANOVA. Groups were compared by performing Bonferroni's test. $n = 4$.

of the vehicle group was reduced as compared to the sham group, the 5-HT2AR antagonist group was conspicuously elevated in comparison to the control group and the 5-HT1AR antagonist group was significantly decreased when compared to the control group, *** $p < 0.001$, # $p < 0.05$, $^{ss}p < 0.001$ (Fig. 11C). Similarly, mRNA expression level of ERK2 of the vehicle group and the 5-HT1AR antagonist group was significantly increased when compared to the sham group, * $p < 0.05$, ** $p < 0.01$ vs. the sham group (Fig. 11D). In addition, there was augmentation in the mRNA expression level of c-Myc of the vehicle group, however, this was curtailed in both 5-HT1AR and 5-HT2AR antagonist groups in comparison to the sham group (Fig. 11E).

Transgenic mice to confirm Interaction between 5-HT2AR / 5-HT1AR

Transgenic mice were applied to confirm our findings described above. Results obtained from PCR and agarose gel electrophoresis analysis showed there were 300bp electrophoret-

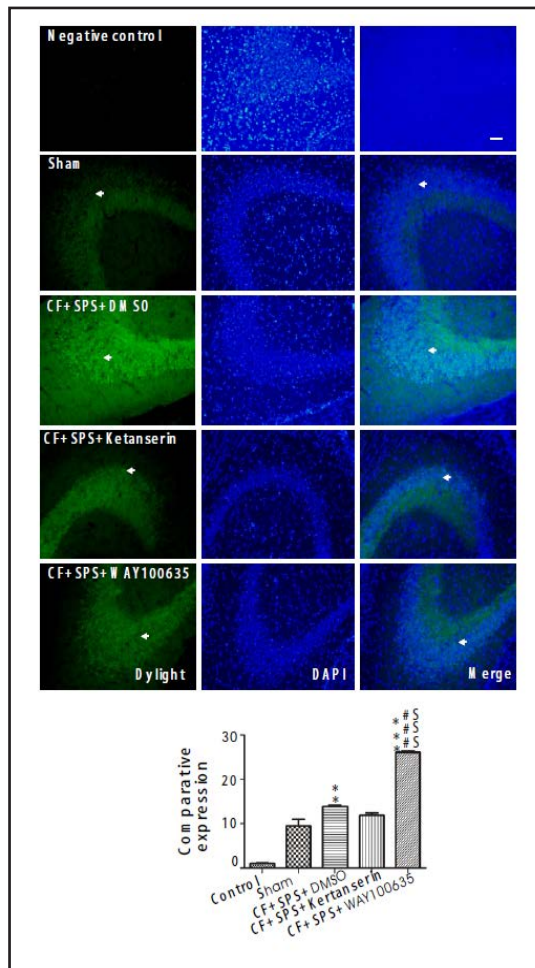


Fig. 5. Immunofluorescence labeling revealed the comparative protein expression level of ERK1 and ERK2 in the mouse (C57BL/6) hippocampus compared with the control group. $**p < 0.01$, $***p < 0.001$ vs. sham group, $###p < 0.001$ vs. the CF+SPS+DMSO group, $sss p < 0.001$ vs. the CF+SPS+ ketanserin group. Bar = 100 μ m. Data were presented as mean \pm SEM through ANOVA. Groups were compared by performing Bonferroni's test. $n = 4$.

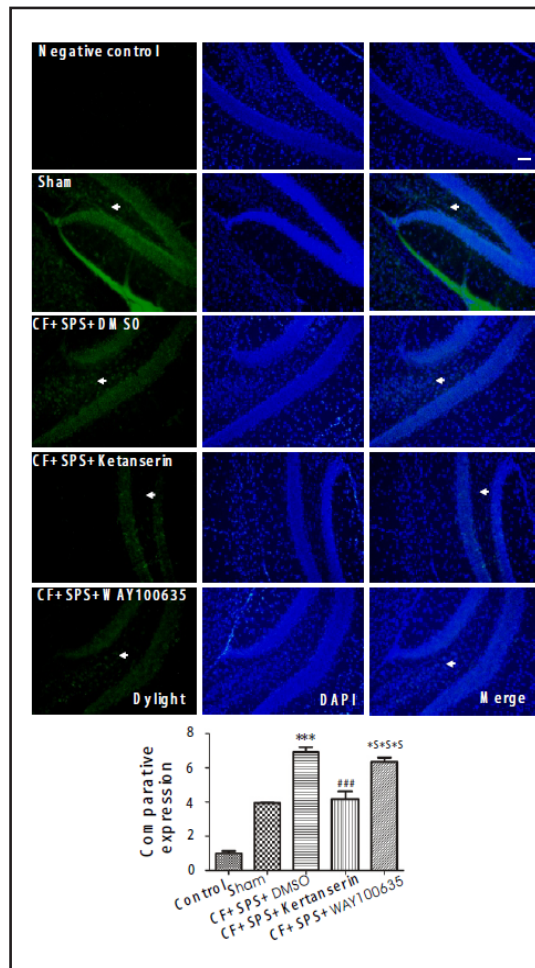


Fig. 6. Immunofluorescence labeling revealed the comparative protein expression level of c-Myc in the mouse (C57BL/6) hippocampus compared with the control group. $***p < 0.001$ vs. sham group, $###p < 0.001$ vs. CF+SPS+DMSO group, $sss p < 0.001$ vs. CF+SPS+ ketanserin group. Bar = 100 μ m. Data were presented as mean \pm SEM through ANOVA. Groups were compared by performing Bonferroni's test. $n = 4$.

ic bands related to ePet-Cre in 13 of the 15 transgenic mice, with 297bp electrophoretic bands related to Ai9 in the above 13 ePet-Cre mice (Fig. 9A–B). These results surmised there were 13 ePet-Cre-Ai9 mice out of the 15 transgenic mice. Their primers were synthesized by Invitrogen (Table 1).

In Fig. 7–8, results showed comparative protein expression levels of 5-HT2AR, 5-HT1AR along with the comparative level of serotonin in the mice (ePet-Cre-Ai9, WT) hippocampi with those in the control group. In comparison to the control group, there was pronounced increment in the protein expression level of 5-HT2AR in the ePet-Cre-Ai9+CF+SPS group and WT+CF+SPS group $***P < 0.001$, $##P < 0.01$, $**P < 0.01$ (Fig. 7). Similarly, there was a distinct increase in the protein expression level of 5-HT1AR in both ePet-Cre-Ai9+CF+SPS and WT+CF+SPS groups when compared to the control group, $**P < 0.01$, $ssP < 0.01$, $*P < 0.05$, $##P < 0.01$ (Fig. 8).

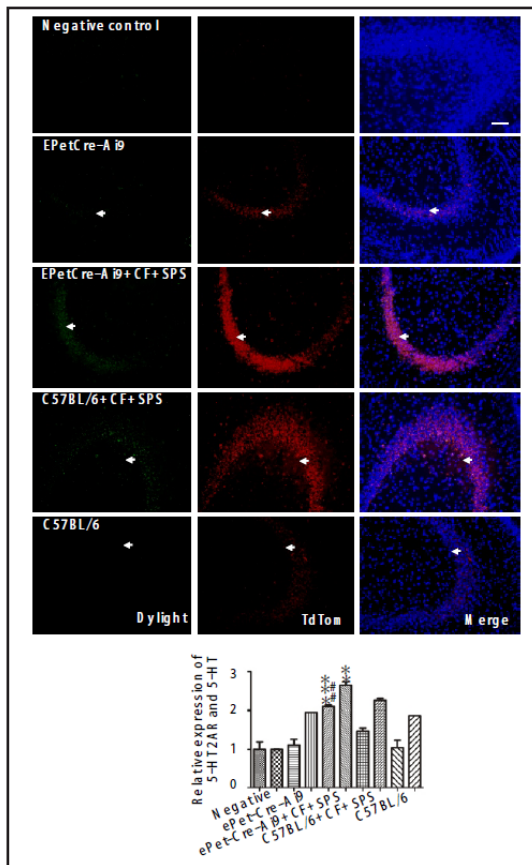


Fig. 7. Immunofluorescence labeling revealed the comparative protein expression level of 5-HT2AR and the comparative level of 5-HT (5-hydroxytryptamine) in the mouse (ePet-Cre-Ai9, C57BL/6) hippocampus compared with those of the control group. *** $p < 0.001$ vs. ePet-Cre-Ai9 (5-HT2AR) group, ## $p < 0.01$ vs. C57BL/6+CF+SPS (5-HT2AR) group, ** $p < 0.01$ vs. ePet-Cre-Ai9 (5-HT) group. Note: there were tdTom only in the ePet-Cre-Ai9 mice. Bar = 100 μ m. Data were presented as mean \pm SEM through ANOVA. Groups were compared by performing Bonferoni's test. n = 5.

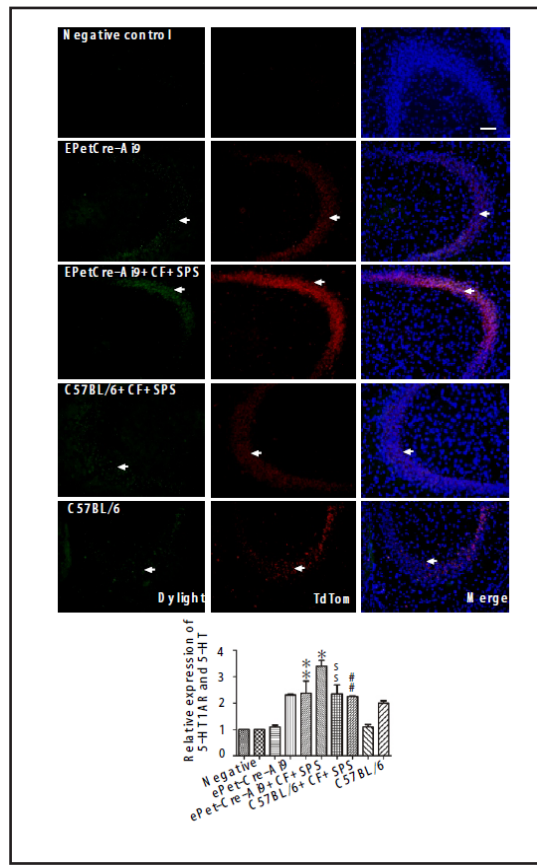


Fig. 8. Immunofluorescence labeling revealed the comparative protein expression level of 5-HT1AR and the comparative level of 5-HT (5-hydroxytryptamine) in the mouse (ePet-Cre-Ai9, C57BL/6) hippocampus compared with those of the control group. ** $p < 0.01$ vs. the ePet-Cre-Ai9 (5-HT2AR) group, ** $p < 0.01$ vs. the C57BL/6 (5-HT1AR) group, * $p < 0.05$ vs. the ePet-Cre-Ai9 (5-HT) group, ## $p < 0.01$ vs. the C57BL/6+CF+SPS (5-HT) group. Note: there were tdTom only in the ePet-Cre-Ai9 mice. Bar = 100 μ m. Data were presented as mean \pm SEM through ANOVA. Groups were compared by performing Bonferoni's test. n = 5.

Fig. 9. Results of PCR and agarose gel electrophoresis analysis showed there were 300bp electrophoretic bands related to ePet-Cre in 13 mice of 15 transgenic mice and 297bp electrophoretic bands related to Ai9 in the above 13 ePet-Cre mice. The results suggested that there were 13 ePet-Cre-Ai9 mice.

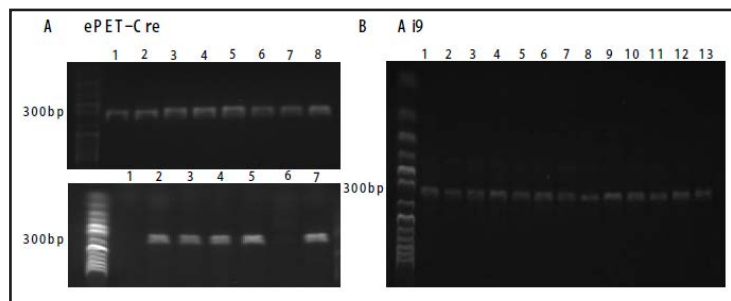
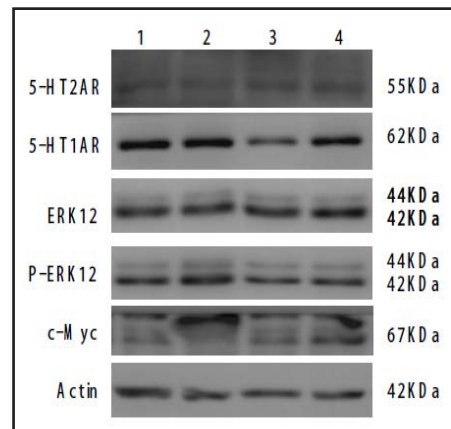


Table 2. Western blot denoting the relative protein expression levels of 5-HT2A receptor, 5HT1A receptor, ERK1, ERK2, pERK1, pERK2, and c-Myc in the four experimental groups normalized by that of β -actin. # $p < 0.05$ vs. the CF+SPS+DMSO group (5HT1AR); * $p < 0.05$ vs. the sham group (ERK2); *** $p < 0.001$ and ** $p < 0.01$ vs. the sham group, # $p < 0.05$ vs. the CF+SPS+DMSO group (pERK1); * $p < 0.05$ vs. the sham group (pERK2); *** $p < 0.001$ vs. the sham group, # $p < 0.05$ vs. the CF+SPS+DMSO group (c-Myc). Data were presented as mean \pm SEM through ANOVA. Groups were compared by performing Bonferroni's test. n = 4

	Sham	CF+SPS+DMSO	CF+SPS+Ketanserin	CF+SPS+WAY100635
5HT2AR	0.33 \pm 0.07	0.28 \pm 0.06	0.42 \pm 0.14	0.34 \pm 0.05
5HT1AR	0.71 \pm 0.08	0.81 \pm 0.04	0.60 \pm 0.01#	0.76 \pm 0.03
ERK1	0.24 \pm 0.05	0.29 \pm 0.05	0.31 \pm 0.07	0.29 \pm 0.07
ERK2	1.07 \pm 0.17	1.31 \pm 0.17	1.20 \pm 0.28	1.87 \pm 0.15*
pERK1	0.13 \pm 0.01	0.34 \pm 0.01***	0.28 \pm 0.02#	0.32 \pm 0.02**
pERK2	0.85 \pm 0.08	1.23 \pm 0.05*	1.01 \pm 0.07	1.08 \pm 0.12
c-Myc	0.32 \pm 0.02	0.73 \pm 0.12***	0.44 \pm 0.02#	0.55 \pm 0.04

Fig. 10. Western blot indicated the protein expression level in the mouse hippocampus. 1, 2, 3, and 4 respectively indicated the protein expression level in the sham group, CF+SPS+DMSO group, CF+SPS+ketanserin group, and CF+SPS+WAY100635 group. Compared with those of the sham group, the expression levels of 5-HT1A receptor were increased in both CF+SPS+DMSO and CF+SPS+WAY100635 groups but decreased in the CF+SPS+ketanserin group. The expression level of 5HT2A receptor was decreased in CF+SPS+DMSO group but increased in both CF+SPS+ketanserin and CF+SPS+WAY100635 groups. The expression levels of ERK1, ERK2, pERK1, pERK2 and c-Myc were increased in the CF+SPS+DMSO group, CF+SPS+ketanserin group, and CF+SPS+WAY100635 group.



Discussion

Individuals exposed to severe traumatic events tend to exhibit behavioral disorders such as anxiety-like behavior in PTSD, which is related to serotonin uptake process [40-42]. Previous studies have revealed 13 or more different subtypes of serotonin receptors, among which 5HT2AR and 5HT1AR are the most relevant to anxiety-like behavior [43, 44]. 5HT2AR and 5HT1AR involvement in anxiety-like behavior responses are mainly expressed in the raphe nuclei, frontal cortex and hippocampus [41]. Previous studies have reported 5HT2A receptor function by phosphorylating ERK1 and ERK2 [21]. Our results demonstrated both 5HT2A and 5HT1A receptors in the mice's hippocampi to be implicated in anxiety-like behavior via the ERK signaling pathway.

Mice were subjected to conditioned fear (CF) stress coupled with single-prolonged stress (SPS) model to investigate the impact of 5HT2AR and 5HT1AR on anxiety-like behavior; the antagonists of 5HT2AR and 5HT1AR, which are ketanserin and WAY100635, were used by this model respectively [27, 45]. The ePet-Cre-Ai9 mice were modeled to examine the function of serotonin on anxiety-like behavior [34-36]. The mice model was evaluated by open field test, freezing behavior test and the elevated plus maze test [22]. Our results suggest that the anxiety-like behavior mouse model was successfully established via the CF+SPS procedures.

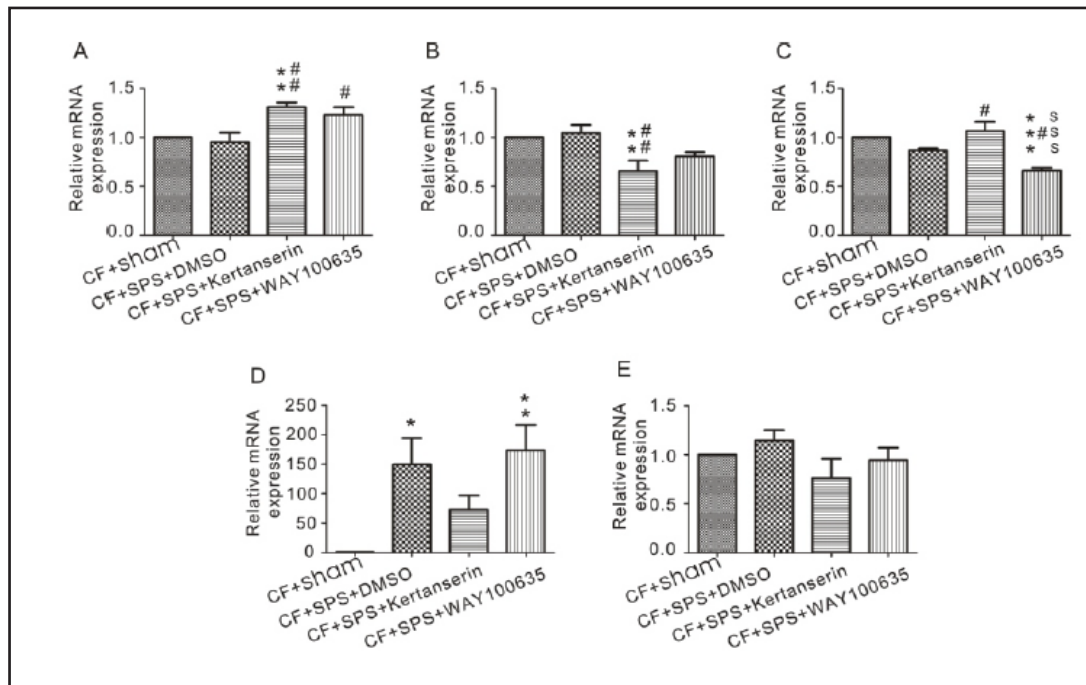


Fig. 11. RT Q-PCR showed the relative mRNA expression levels of the 5-Htr2a, 5-HT1a, ERK1, ERK2, and c-Myc compared with that of the control group in the mouse hippocampus. $**p < 0.01$ vs. the sham group, $\#p < 0.05$, $\#\#p < 0.01$ vs. the CF+SPS+DMSO group (A); $**p < 0.01$ vs. the sham group, $\#\#p < 0.01$ vs. the CF+SPS+DMSO group (B); $***p < 0.001$ vs. the sham group, $\#p < 0.05$ vs. the CF+SPS+DMSO group, $sssp < 0.001$ vs. the CF+SPS+ ketanserin group (C); $*p < 0.05$, $**p < 0.01$ vs. the sham group (D). Data were presented as mean \pm SEM through ANOVA. Groups were compared by performing Bonferroni's test. $n = 4$.

5HT2A and 5HT1A receptors are a pair of contradictory unit and play reciprocal roles on anxiety-like behavior in PTSD. Several studies have suggested anxiety-like behavior to be most often than not accompanied by protein expressional changes in 5HT2A and 5HT1A receptors in mice hippocampi [40, 46, 47]. Our western blot and immunofluorescence labeling results showed significant increment in the expression level of 5HT1A receptor after PTSD model establishment. However, the inhibition of 5-HT2A receptor expression culminated in the curtailment of the expression of 5-HT1A receptor. Similarly, there was diminution in the expression level of 5HT2A receptor; nevertheless, when 5-HT1A receptor expression was inhibited, the protein expression of 5-HT2A receptor was significantly increased. RT Q-PCR results of mRNA expression level were also consistent with protein tests. These results suggest 5HT2A receptor plays an energetic role in anxiety-like behavior by inhibiting the expression of 5HT1A receptor.

The ePet-Cre mice can express Cre recombinase in the serotonin neurons, Ai9 mice expressed the red fluorescent protein *TdTomato* because of the role of the Cre recombinant enzyme [34-36]. We found 300bp electrophoretic bands to be related to ePet-Cre in the PCR and agarose gel electrophoresis analyses. Ai9 mice harbor a *TdTomato* coding sequence. In the analysis, there were 297bp electrophoretic bands related to Ai9. Therefore, ePet-Cre-Ai9 mice might express red fluorescent protein variant in serotonergic neurons. Our results confirmed that serotonin promoted the anxiety-like behavior of mice through 5HT2A and 5HT1A receptors.

A previous research evinced that, in NIH3T3 cells, 5HT2A receptor functions by mediating the phosphorylation of ERK1 and ERK2, thus activating downstream signaling cascade [48]. According to Liu C et al., the expression of 5HT2A receptor is related to phosphorylated levels of ERK1 and ERK2 in mice with lipopolysaccharide-induced shock [21]. Additionally, the role

of 5HT2A receptor has been correlated to anxiety via the triggering of ERK signaling pathway [49]. Our results evinced that by activating ERK-c-Myc pathway via the phosphorylation of ERK1 and ERK2, 5HT2A receptor influenced the anxiety-like behavior in PTSD mice.

In light of our experimental results, we surmise that competitive interaction between 5HT2A and 5HT1A receptors play a positive role on anxiety-like behavior in PTSD by activating the ERK-c-Myc pathway via the phosphorylation of ERK1 and ERK2, thus serotonin promotes anxiety-like behavior in mice through 5HT2A and 5HT1A receptors.

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Disclosure Statement

The authors declare no conflict of interest.

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