



Over-expression of 5-HT6 Receptor and Activated Jab-1/p-c-Jun Play Important Roles in Pilocarpine-Induced Seizures and Learning-Memory Impairment

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Abstract

Cognitive impairment is a common comorbidity in patients with temporal lobe epilepsy (TLE) that severely affects patients' quality of life. Also, serotonin 5-hydroxytryptamine 6 (5-HT6) receptor plays an important role in cognition. This study aimed to investigate effects of 5-HT6 receptor on learning-memory capacities in epileptic rats. Total of 36 adult Sprague-Dawley (SD) rats were divided into vehicle ($n=6$) and epileptic group ($n=30$). Status epilepticus (SE) was induced via systemic injection of pilocarpine. Epileptic group was sub-divided into vehicle, 10, 20, and 30 μg SB-271046 groups, six mice per group. Learning-memory performance of rats was evaluated by using Y maze and Morris water maze test. 5-HT6 receptor expression was examined using immunostaining and Western blot. The other six rats were used to make epileptic model and Jab-1/p-c-Jun were detected. Results showed that frequency of spontaneous recurrent seizures (SRSs) was significantly decreased in pilocarpine-induced epileptic rats that treated with SB-271046. Alternation rate and new arm percentage were decreased in epileptic rats compared to control. The 5-day mean latency was prolonged in epileptic rats compared to control rats. During retention stage, mean latency, number of target crossings, and percentage of time spent in target zone were decreased in epileptic rats, but not in those treated with SB-271046. The number of apoptotic neurons was significantly increased in epileptic rats, which was decreased by SB-271046. 5-HT6 expression was significantly increased in hippocampus and cortex following recurrent seizures. Jab-1 level was decreased after SB-271046 administration. p-c-Jun level was elevated in epileptic rats and decreased in a dose-dependent manner after the SB-271046 administration. In conclusion, the over-expression of 5-HT6 receptor and activated Jab-1/p-c-Jun plays an important role in pilocarpine-induced seizures and learning-memory impairment.

Keywords 5-HT6 receptor · Temporal lobe epilepsy · Learning-memory · Jab-1 · p-c-Jun

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Introduction

Temporal lobe epilepsy (TLE) is the most common category of chronic epilepsy with hippocampal sclerosis as the most prevalent primary pathology, documenting for 36% in all focal epilepsy pathologies in clinical (Blumcke et al. 2017; Wu et al. 2018). Cognitive impairment or decline is a common comorbidity in patients with TLE that severely affects patients' quality of life (Huang et al. 2011; Huang et al. 2017). The cognitive decline is considered to be comorbidity of TLE with higher dementia prevalence compared to that of the normal population (Hermann et al. 2006). Tai et al. (2018) reported that the positron emission tomography biomarkers could reflect the cognitive decline in TLE. In clinical, the pharmaco-resistance is the most common challenge affecting approximate 30% of TLE patients (Salman et al. 2017); therefore, it is urgent to identify the novel drug targets and discover the new drugs. The

cognitive impairment particularly involves learning and memory (Berg 2011) and these deficits have been recapitulated in chemoconvulsant models of TLE, such as pilocarpine-induced chronic epilepsy (Titiz et al. 2014). However, the mechanism underlying TLE-associated cognitive impairment remains unclear. Interestingly, vast data indicate that the serotonin 5-hydroxytryptamine 6 (5-HT₆) receptor may play therapeutic roles in the processes of learning and memory. For example, Marcos found that 5-HT₆ receptor antagonists improve spatial recognition memory on the Morris water maze test (Marcos et al. 2010). Several 5-HT₆ receptor antagonists displayed successful results in phase I clinical studies (i.e., in healthy volunteers), and some have been evaluated in clinical phase II studies (i.e., in patients) for the treatment of Alzheimer's disease (Hu et al. 2017). Regarding epilepsy, as early as 2000, 5-HT₆ receptor antagonists were found to elevate the seizure threshold in an electric shock model (Woolley et al. 2004). Recently, 5-HT₆ receptor was found to modulate seizure activity in epilepsy through recruitment of mTOR (Wang et al. 2015; Wang et al. 2016). However, the role of 5-HT₆ receptors in epilepsy and learning-memory in chronic TLE and the mechanisms underlying its functions remain unknown.

c-Jun activation domain-binding protein-1 (Jab1) acts a regulator of intracellular signaling and affects the cellular apoptosis and cell growth (Pan et al. 2014). The c-Jun and its phosphorylation form (p-c-Jun) play critical functions in the cell differentiation, apoptosis, and proliferation by activating the associated genes (Yang et al. 2013). Therefore, this study also involved the Jab1 and p-c-Jun molecules in the investigation of the potential mechanism of cognitive decline in TLE.

The present study investigated the effects of the 5-HT₆ receptor antagonist SB-271046 on seizures and on learning and memory in lithium-pilocarpine-induced chronic TLE. Moreover, we observed morphological changes in the cortex, the hippocampus, and the striatum. Finally, we examined the expression of the 5-HT₆ receptor and its downstream signal molecules including Jab-1, c-Jun, and p-c-Jun.

Methods and Materials

Pilocarpine Model of Chronic TLE

All animal experiments were performed according to the Guidelines for Animal Experimentation of Fujian Medical University. Adult Sprague-Dawley (SD) rats (220–250 g) were housed in a 12-h/12-h light/dark cycle and were provided with access to food and water. Thirty-six rats were divided into two groups (6 rats in vehicle group, 30 rats in epileptic group). One week prior to the induction of SE, surface electrodes were implanted into the skull of the rats under 10% chloral hydrate anesthesia as described previously (Lin et al.

2013). One frontal electrode was implanted above the frontal cortex [AP 2.5 mm, ML 2.0 mm, and DV 0.5 mm]. The second electrode was fixed to the surface of the skull as a ground. The third electrode was fixed behind the ear as a reference. After the experiment, the animals were intraperitoneally (I.P.) injected with gentamycin to prevent infection and were allowed to recover from surgery for 1 week until experimentation. Twenty minutes before the injection of pilocarpine, atropine, a muscarinic antagonist, was administered I.P. (1 mg/kg) to reduce the peripheral adverse effects of pilocarpine. The rats were I.P. injected with pilocarpine (30 mg/kg, Sigma) 16–18 h after the administration of lithium (127 mg/kg, I.P.). After drug administration, the progressive evolution of seizure behavior was observed and rated according to the Racine scale (Racine 1972). Only the animals that developed stage IV–V seizures were used. Status epilepticus (SE) was defined as stage IV–V seizures that persisted for longer than 30 min. The behavior and the electroencephalography (EEG) potentials of the animals were continually monitored for 2 h using a video monitoring system (BYOPAC, USA) three times a day for 28 days after the establishment of SE. At chronic period, the spontaneous recurrent seizures (SRSS) were evaluated by frequency (times/week) and stages (Veliskova 2006). The EEG discharges displaying amplitudes exceeding 50 μ V, which was typically twofold greater than the basal EEG discharge amplitude and spike (≤ 70 ms) or sharp waves (70–200 ms), were counted as seizure discharges. Root mean square (RMS) was calculated with three discontinuous times of 20 s ($X_{rms} = \sqrt{\sum x_i^2/N} = \sqrt{x_1^2 + x_2^2 + \dots + x_i^2/N}$) (Choi et al. 2010).

Four weeks after SE, the epileptic group was randomly divided into four sub-groups. One received vehicle (2-hydroxypropyl- β -cyclodextrin, HP- β -CD), and the others received 10 μ g/20 μ g/30 μ g SB-271046 respectively. SB-271046 was injected into the lateral ventricle using the following coordinates with respect to Bregma: AP 0.8 mm, ML 1.5 mm, and DV 4 mm. Before the administration, 10% chloral hydrate was used to anesthetize and bucinazine to alleviate the pain.

Y Maze and Morris Water Maze Tests

Y Maze Test Six weeks post the SE model establishment, the Y maze test was performed. The Y maze test that mainly utilized a cardboard apparatus consisting of three enclosed arms with dimensions of 50 cm \times 10 cm \times 20 cm (length, width, and height, respectively) that converged on an equilateral triangular center platform (5 cm \times 5 cm \times 5 cm) was performed. The number of spontaneous alternation performances (SAPs), which was defined as the number of sequential entries into each of the three arms without any repeated entries, and the total number of entries into the arms were evaluated (8-min test periods). The rate of SAP (SAP rate, %) was calculated as

an index of the working memory-related behavior. The total number of entries into the arms was assessed as an index of locomotor activity.

Morris Water Maze Tests It was used to examine the changes in the learning and memory capacities of the rats. A circular water maze was used in this study. The diameter was 120 cm, and the height was 50 cm. A hidden platform with a diameter of 9 cm that was 0.5 cm below the surface of the water was located inside the maze. Floating plastic particles were placed on the surface of the water to obscure the visual detection of the platform. The temperature of the water was $25.0\text{ }^{\circ}\text{C} \pm 0.5\text{ }^{\circ}\text{C}$. For the experiment, the rats were placed at a random location in the maze and were allowed to swim freely until they located the hidden platform. The entire experiment was conducted over 7 days. On the first 5 days, the rats were left in the maze to locate the platform for a maximum duration of 60 s. This learning session was repeated five times each day with an interval of 1 h between the sessions. On the final day, the platform was removed, and the time that the rat spent in the region that previously contained the platform was recorded over a period of 3 min (180 s).

Brain Region Isolation and Morphological Examinations

At the end of the experiments, the animals were deeply anesthetized (10% chloral hydrate, 2 mL/kg) and transcardially perfused with 0.1 mmol/L phosphate-buffered saline (pH 7.4) followed by 4% paraformaldehyde. The hippocampal, cortical, and striatal brain regions were rapidly isolated and sequentially incubated in a mouse anti-rat HTR6 monoclonal antibody (1:500, Cat. No. ab101912, Abcam Biotech., Cambridge, MA, USA). Then, the tissues were incubated with a biotinylated goat anti-mouse IgG (1:500, Cat. No. ab64255, Abcam Biotech., Cambridge, MA, USA). Diaminobenzidine (Beyotime Biotech., Shanghai, China) was used for color development, and hematoxylin was used for counterstaining. The stained brain sections were observed under a Leica DM2500 microscope (Leica, Germany). Images were captured using a digital camera and Leica software (version 3.7). For quantification, five \times 400 fields of view were randomly examined, and the number of 5-HT6 receptor-positive cells in each field was counted by independent blinded observers.

Western Blot

The brain tissues were digested and lysed by using radioimmunoprecipitation assay solution (RIPA, Beyotime Biotech., Shanghai, China) and centrifuged at speed of 10,000 r/min for 5 min. The concentration of the extracted proteins was examined by using BCA protein assay kit (Cat.

No. ab102536, Abcam Biotech., Cambridge, MA, USA). Then, total of 0.2 μg proteins were separated via SDS-PAGE and transferred to nitrocellulose membranes. After blocking, the membranes were incubated at $4\text{ }^{\circ}\text{C}$ overnight in primary antibodies against the following target proteins: 5-HTR6 (1:1000) and β -actin (1:600; Santa Cruz Biotechnology). Then, the membranes were washed and incubated in species-specific peroxidase-conjugated secondary antibodies for 2 h at room temperature. The specific bands were detected using an ECL system (Amersham) and a Bio-Rad electrophoresis image analyzer.

Second Part of Experiment

Total of 30 rats were induced chronic epileptic model and intervened by 20 μg SB-271046 4 weeks after SE (according to the first part of experiment). Then the animals were killed 0.5/1/2/4/8/16 h after administration of SB-271046, respectively. Another 30 rats were induced chronic epileptic model and intervened by vehicle, 10 μg /20 μg /30 μg SB-271046 respectively 4 weeks after SE. Then 2 h after administration of SB-271046, all the rats were sacrificed. The hippocampal, cortical, and striatal brain regions were rapidly isolated. Jab-1/c-Jun/p-c-Jun were detected via Western blot. The primary antibodies against Jab-1/c-Jun/p-c-Jun (1:1000) were originated from rabbit and polyclonal while the primary antibody against β -actin (1:1000) was originated from mouse and monoclonal. The specific secondary antibodies against Jab-1 (1:4000) or c-Jun (1:500) or p-c-Jun (1:500) or β -actin (1:6000) were all originated from goat.

Atropine, pilocarpine hydrochloride, and HP- β -CD were purchased from Sigma-Aldrich (St. Louis, MO, USA) and SB-271046 was purchased from Torric (USA). All other reagents were purchased from Abcam Biotech. (Cambridge, MA, USA).

Statistical Analyses

All data are presented as the means \pm the SD. All statistical analyses were performed using SPSS 15.0 software (SPSS, Inc., Chicago, IL, USA). One-way ANOVA and Student's *t* test were used for comparisons between multiple groups, and the level of significance was set as $P < 0.05$.

Results

Behavioral and EEG Changes in the Rats

At 15 to 20 min post the pilocarpine administration, the animals exhibited stereotypical oral and masticatory movements, hypokinesia, salivation, chewing, sniffing movements, tremors, and partial seizures. Approximately 30 min after

pilocarpine injection, the animals developed SE that persisted for longer than half an hour (Fig. 1a–d). This acute phase was followed by a quiescent phase of 2–7 days in which the animals behaved normally (Fig. 1a–d). Spontaneous recurrent seizure activities ($11.43 \pm 3.36/\text{week}$, 4.06 ± 0.38 stages) were observed 8–27 days (20.92 ± 4.92 days) after SE. The frequency and stage of SRSs were not changed in 10 μg SB-271046 when compared to HP-CD and that were decreased in 20 μg SB-271046 and 30 μg SB-271046 (Fig. 1e, $^{\#}P_{(20-\mu\text{g})} < 0.05$, $^{\#}P_{(30-\mu\text{g})} < 0.05$). When compared to 20 μg SB-271046, the frequency of SRSs was decreased and the stage was not changed in 30 μg SB-271046 (Fig. 1e). Similarly, treatment with 10 μg of SB-271046 did not decrease the frequency of seizure discharges ($P_{(10-\mu\text{g})} = 0.98$), and the frequency and RMS of spontaneous recurrent seizures in the 20- μg SB-271046 and 30- μg SB-271046 were lower than those in the

HP-CD (Fig. 1f, $^{\#}P_{(20-\mu\text{g})} < 0.001$, $^{\#}P_{(30-\mu\text{g})} < 0.01$). When compared to 20 μg SB-271046, the RMS of SRSs was decreased and the frequency was not changed in 30 μg SB-271046 (Fig. 1f, $^{\text{a}}P_{(30-\mu\text{g})} < 0.05$).

Behavioral Alterations on the Y Maze Test

In the pilocarpine-treated groups, a significant decrease in the SAP rate was observed on the Y maze test (Fig. 2a). Compared to the HP-CD, the 10 μg SB-271046, 20 μg SB-271046, and 30- μg SB-271046 exhibited an increased SAP rate (Fig. 2a, $^{\#}P_{(10-\mu\text{g})} < 0.05$, $^{\#}P_{(20-\mu\text{g})} < 0.05$, $^{\#}P_{(30-\mu\text{g})} < 0.01$). These findings were consistent with the results of the novel object discrimination (NOD) test, which revealed a significant difference between the vehicle and HP-CD groups (Fig. 2b, $^*P_{(10-\mu\text{g})} < 0.05$). Regarding the 10 μg SB-271046

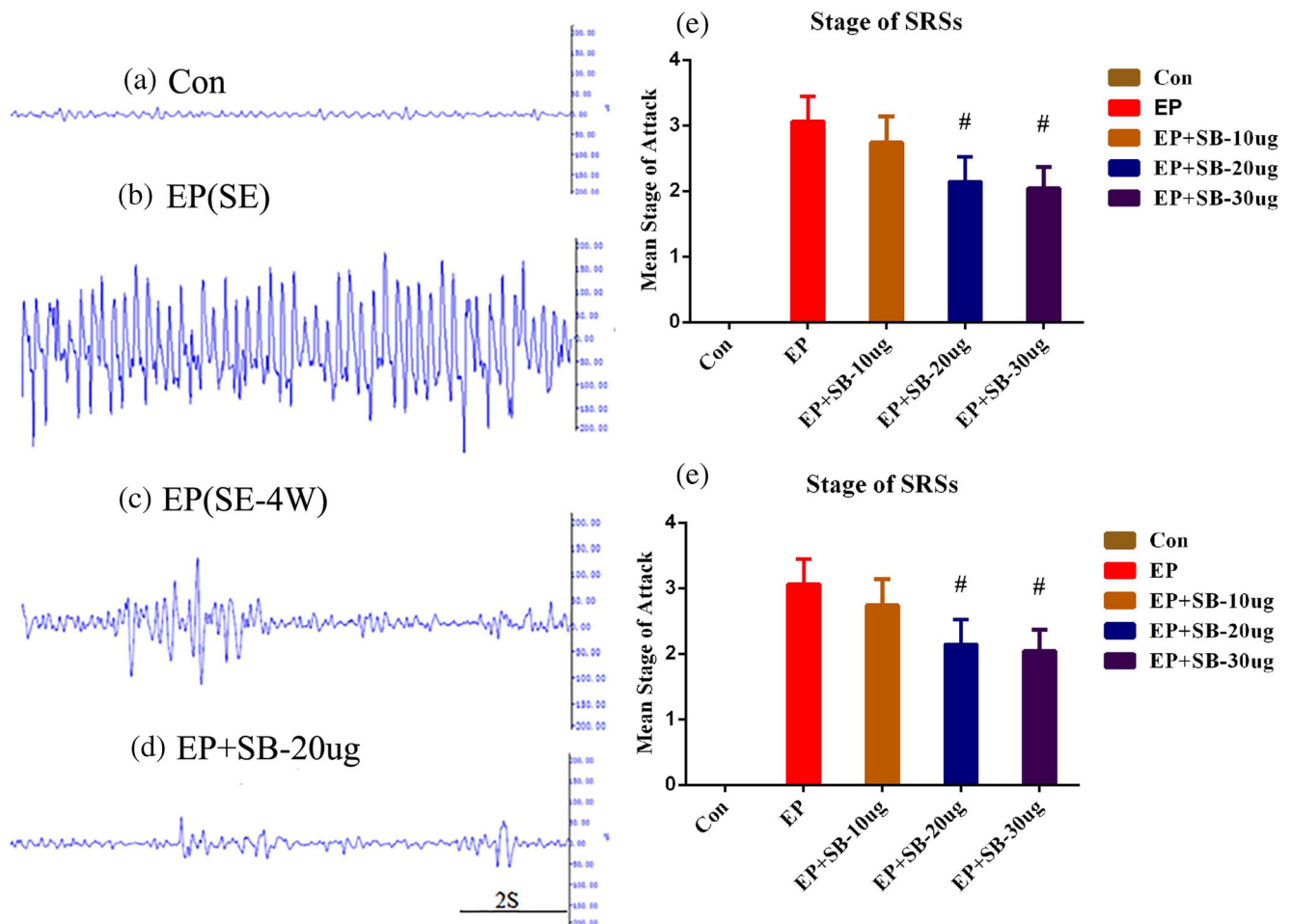


Fig. 1 Pilocarpine injection-induced epilepsy model and the reduction of seizures by SB-271046. **a** EEG image for Con. **b** EEG image for EP (SE). **c** EEG image for EP (SE-4W). **d** EEG image for EP+SB-271046 20 μg . **e** The frequency of SRSs: a significant decrease was observed in 20 μg SB-271046 or 30 μg SB-271046 when compared to HP-CD ($^{\#}P < 0.05$) and also in 30 μg SB-271046 compared to 20 μg SB-271046 ($^{\text{a}}P < 0.05$). The stages of SRSs: there was a significant decrease in both 20 μg SB-

271046 and 30 μg S-271046B after SB-271046 rejection ($^{\#}P < 0.05$). **f** The amplitude (RMS) of the SRSs: a significant decrease was observed both in 20 μg SB-271046 and 30 μg SB-271046 compared to HP-CD ($^{\#}P < 0.05$) and also in 30 μg SB compared to 20 μg SB-271046 ($^{\text{a}}P < 0.05$). The frequency of seizure discharge: there was a significant decrease in both 20 μg SB-271046 and 30 μg SB-271046 after SB-271046 rejection ($^{\#}P < 0.05$)

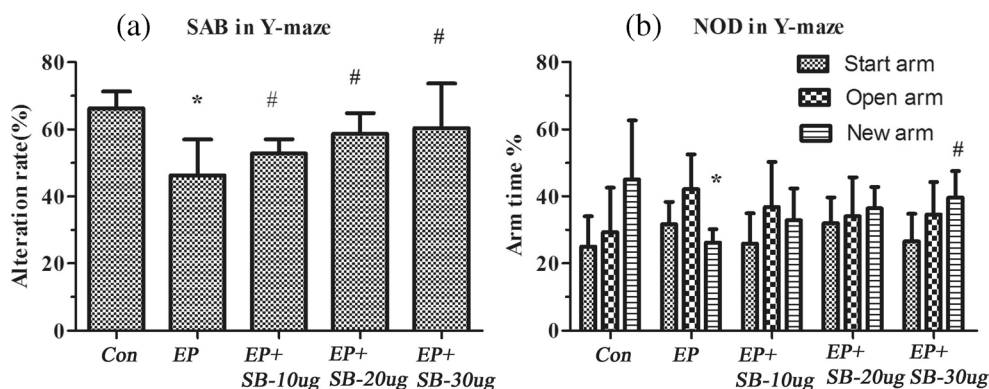


Fig. 2 Behavioral alterations on the Y maze test. **a** SAP: in the HP-CD group, significantly reduced SAP rates were observed ($*P < 0.05$). Following SB-271046 injection, the SAP rate was increased ($^{\#}P_{(10-\mu\text{g})} < 0.05$, $^{\#}P_{(20-\mu\text{g})} < 0.05$, $^{\#}P_{(30-\mu\text{g})} < 0.01$). **b** NOD: the percentage of time

spent in the new arm was decreased in the HP-CD group ($*P < 0.05$). 30 μg SB-271046 increased this percentage ($^{\#}P_{(30-\mu\text{g})} < 0.05$), but this difference was not significant in both 10 μg SB-271046 and 20 μg SB-271046 ($P_{(10-\mu\text{g})} = 0.912$ and $P_{(20-\mu\text{g})} = 0.293$)

and 20 μg SB-271046 groups, the percentage of time spent in the new arm was increased, although this difference was not significant compared to the HP-CD group ($P_{(10-\mu\text{g})} = 0.912$ and $P_{(20-\mu\text{g})} = 0.293$), while that in 30 μg SB-271046 was significant ($^{\#}P_{(30-\mu\text{g})} < 0.05$).

Behavioral Alterations on the Morris Water Maze Test

Among the pilocarpine-treated rats, the latency to reach the platform during the first 5 days was significantly prolonged (Fig. 3a, b). Compared to the HP-CD group, the latency of the 20 μg SB-271046 and 30 μg SB-271046 was reduced, but no significant difference was detected between the 10 μg SB-271046 and HP-CD groups (Fig. 3b). On the final day, behavioral alterations indicative of impairments in the retention of spatial learning were observed in the HP-CD, 10 μg SB-271046, 20 μg SB-271046, and 30 μg SB-271046. For example, the latency to reach the target platform was prolonged, the number of platform crossings was reduced, and the percentage of time spent in the target zone was decreased (Fig. 3c, $*P < 0.01$). Regarding the 10 μg SB-271046, 20 μg SB-271046, and 30 μg SB-271046, the latency to reach the target platform was shortened, the number of platform crossing was decreased, the swimming speed was higher, and the percentage of time spent in the target zone was decreased when compared to the HP-CD group (Fig. 3d, $^{\#}P < 0.05$). When compared to 10 μg SB-271046, the latency to reach the target platform was shortened and the number of platform crossing was increased in 20 μg SB-271046 (Fig. 3e, $^{\&}P < 0.05$). When compared to 20 μg SB-271046, the number of platform crossing was increased in 30 μg SB-271046 (Fig. 3f, $^{\circ}P < 0.05$). In the 20 μg SB-271046 and 30 μg SB-271046 but not the 10 μg SB-271046 group, the percentage of time spent in the target zone was significantly increased compared to the HP-CD group (Fig. 3f, $^{\#}P < 0.05$).

Apoptotic Cells

Regarding the HP-CD group, the number of apoptotic cells in all three examined brain regions (cortex, hippocampus, and striatum, Fig. 4a) was dramatically increased compared to that in the vehicle group (Fig. 4b, $*P < 0.05$). Furthermore, the changes in the hippocampus were the most apparent. Compared to the HP-CD group, except for 10 μg SB-271046, both 20 μg SB-271046 and 30 μg SB-271046 exhibited a significant reduction in the number of apoptotic cells (Fig. 4c, $^{\#}P < 0.05$). Furthermore, the reduction in hippocampus was the most apparent.

The Expression of the 5-HT6 Receptor

In the vehicle, the 5-HT6 receptor was expressed in all three examined brain regions (cortex, hippocampus, and striatum) by using immunohistochemical assay (Fig. 5a) and Western blot assay (Fig. 5b), respectively. And the membrane was stained brown while the nuclei were null. That means that 5-HT6 receptor was mainly expressed in membrane. The number of 5-HT6 receptor-positive cells was increased in the hippocampus and the cortex in the epileptic rats compared to the vehicle-treated rats (Fig. 5c, $*P < 0.05$), while there was no difference in striatum. These findings were consistent with the Western blot OD results quantifying the expression of the 5-HT6 receptor (Fig. 5d, $*P < 0.05$). These results revealed significant differences in 5-HT6 receptor expression between the vehicle and HP-CD groups.

The Expression of Jab-1/p-c-Jun

The Jab-1 and p-c-Jun/c-Jun expression was evaluated by using Western blot assay (Fig. 6a). In the 20 μg SB-271046, both the levels of Jab-1 and p-c-Jun/c-Jun were downregulated after administration of SB-271046. This effect was most significant 2 h after administration of SB-271046 and lasted at

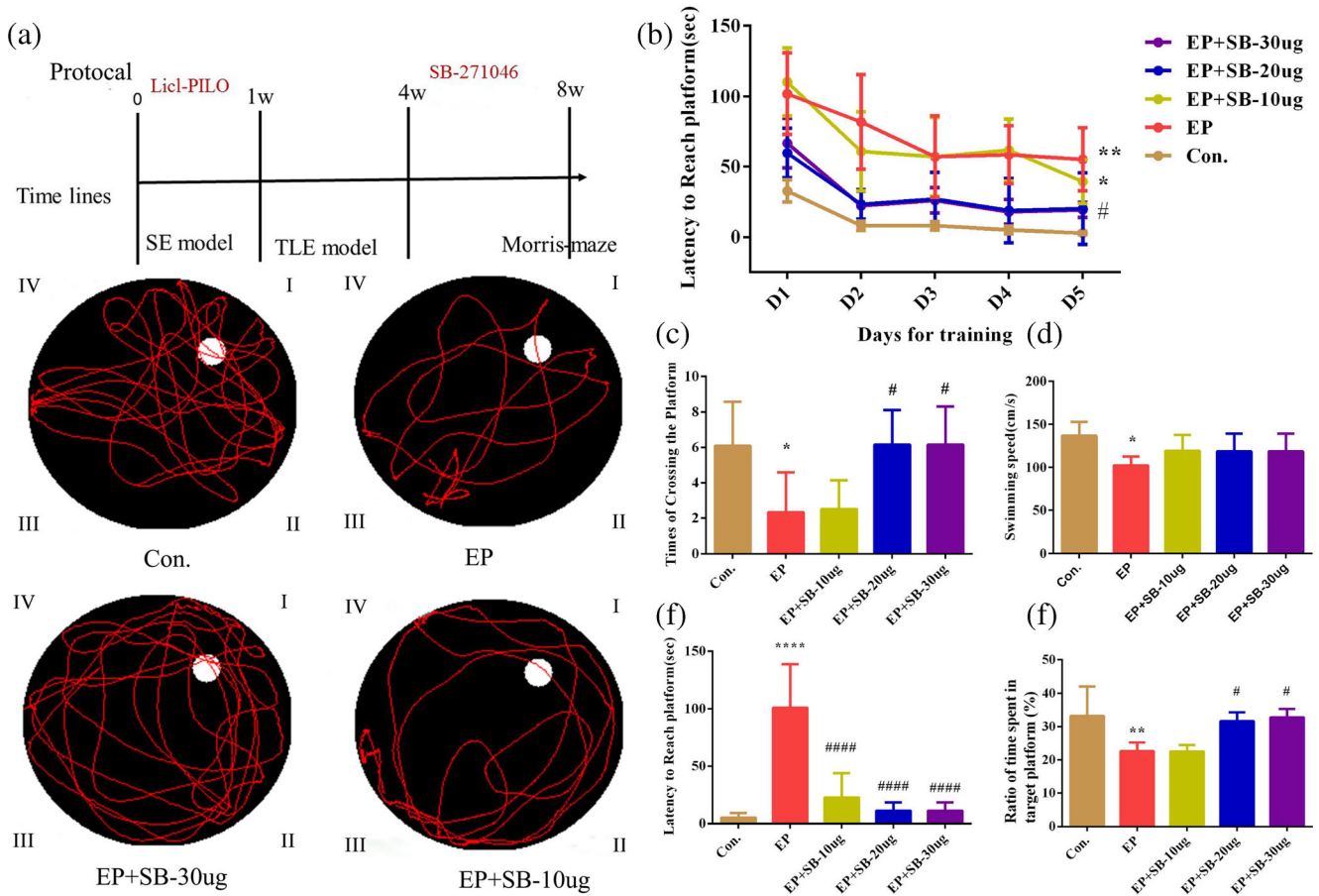


Fig. 3 Behavioral alterations on the Morris water maze test. **a** Behavioral changes due to the Morris water maze test. **b** First 5 days: the latency to reach the platform during the first 5 days was prolonged in the pilocarpine-treated group ($*P < 0.05$). This value was reduced in 10 μ g SB-271046 and 20 μ g SB-271046 ($*P < 0.05$), but not in the 10 μ g SB-271046 group. **c** The number of platform crossings was reduced in the HP-CD group ($*P < 0.05$) and was increased due to SB-271046 administration ($\#P < 0.05$). This effect was dose-independent (20 μ g SB-271046 vs. 10 μ g SB-271046, $\&P < 0.05$, 30 μ g SB-271046 vs.

20 μ g SB-271046, $\@P < 0.05$). **d** Comparing for the swimming speed among groups. **e** The latency to reach the target platform was prolonged in the HP-CD group ($*P < 0.05$) and was reduced in 20 μ g SB-271046 and 30 μ g SB-271046 ($*P < 0.05$), but not in 10 μ g SB-271046. **f** The percentage of time spent in the target zone was reduced in the HP-CD group ($*P < 0.05$). This percentage was increased in the 20 μ g SB-271046 group, but not in the 10 μ g SB-271046 group ($\#P < 0.05$), compared to the HP-CD group

least 1 h. The level of Jab-1 was not changed while p-c-Jun/c-Jun was significantly increased in HP-CD when compared to vehicle (Fig. 6b, $*P < 0.05$). Two hours after administration of SB-271046, both Jab-1 and p-c-Jun were downregulated (Fig. 6b, $\#P < 0.05$) and this effect got more significant with the increase of SB-271046 dose (Fig. 6c, $\#P < 0.05$). Besides, the inhibition was most obvious in cortex, followed by hippocampus (Fig. 6d, e). In a word, SB-271046 inhibited the level of Jab-1 and p-c-Jun in a dose-time-dependant manner and tissue-specific character.

Discussion

Vast clinical evidence has shown that chronic TLE tends to be accompanied by cognitive impairments. TLE has been considered to impair long-term memory but not working

memory; however, recent evidence suggests that working memory is also compromised in TLE (Winston et al. 2013). Regarding epileptic rats, the Y maze test, which is a cortex-dependent behavioral task, is used to evaluate working memory (Hughes 2004). This task requires the maintenance and manipulation of information over short periods of time. In our study, a decreased SAP rate and percentage of time spent in the new arm were observed in the pilocarpine-treated rats. Additionally, the Morris water maze test is a hippocampus-dependent behavioral task that is among the most frequently used to evaluate spatial memory (Inostroza et al. 2011; Fu et al. 2018). This test requires learning and memory over a longer period of time than the Y maze test. In our study, the latency to reach the platform on the first 5 days and the latency to reach the target zone on the final day were prolonged in the pilocarpine-treated rats. These findings indicate that

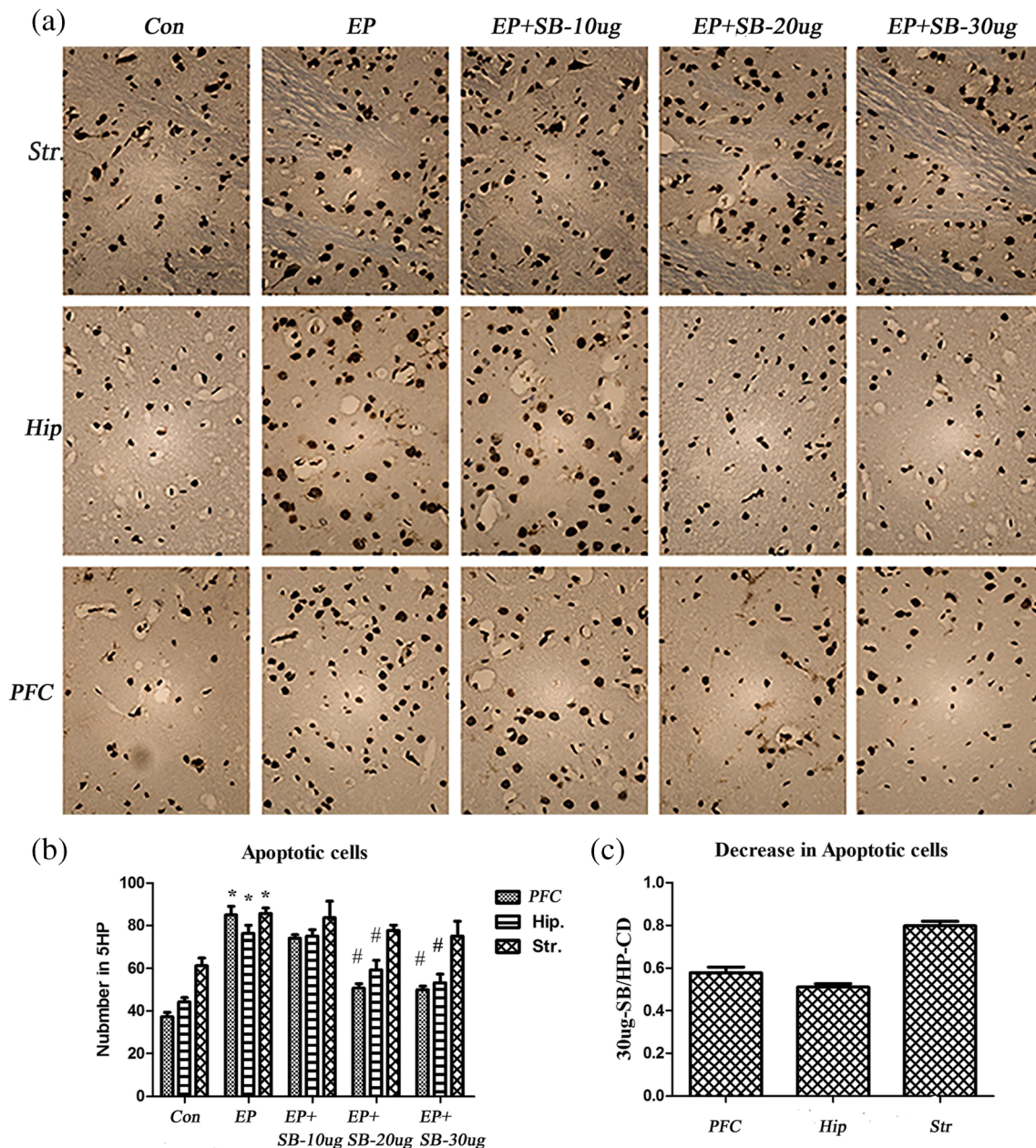


Fig. 4 TUNEL-stained apoptotic cells in the brain sections. The cells were stained brown. **a** TUNEL staining images. **b** A sharp increase in stained cells was detected in the HP-CD group compared to the vehicle group ($*P < 0.05$). **c** Following SB-271046 administration,

TUNEL staining was significantly weakened in cortex and hippocampus ($\#P < 0.05$) but not changed in striatum. The inhibiting effect was most obvious in hippocampus

chronic TLE not only impairs long-term memory but also affects working memory, which are consistent with the previous studies (Inostroza et al. 2011; Winston et al. 2013; Fu et al. 2018). However, the reason for this additional impairment has yet to be resolved.

In recent years, an increasing evidence suggests that the 5-HT₆ receptor may play a therapeutic role in learning and memory processes (Marcos et al. 2010). Few studies have focused on the relationship between the 5-HT₆ receptor and epilepsy or between epilepsy and cognitive impairment.

Notably, the chronic temporal epilepsy is more analogous to human epilepsy than the acute model of epilepsy (de Lima et al. 2017). Pilocarpine model has proved to be a valuable tool to investigate the mechanisms involved in TLE (Curia et al. 2008). In the present study, we observed spontaneous recurrent partial seizures 8–27 days after pilocarpine-induced SE. The frequencies and amplitudes of the spontaneous recurrent seizures were decreased after the injection of the 5-HT₆ receptor antagonist SB-271046 into the lateral ventricle. As early as 2000, Routledge et al. (2000) found that SB-271046

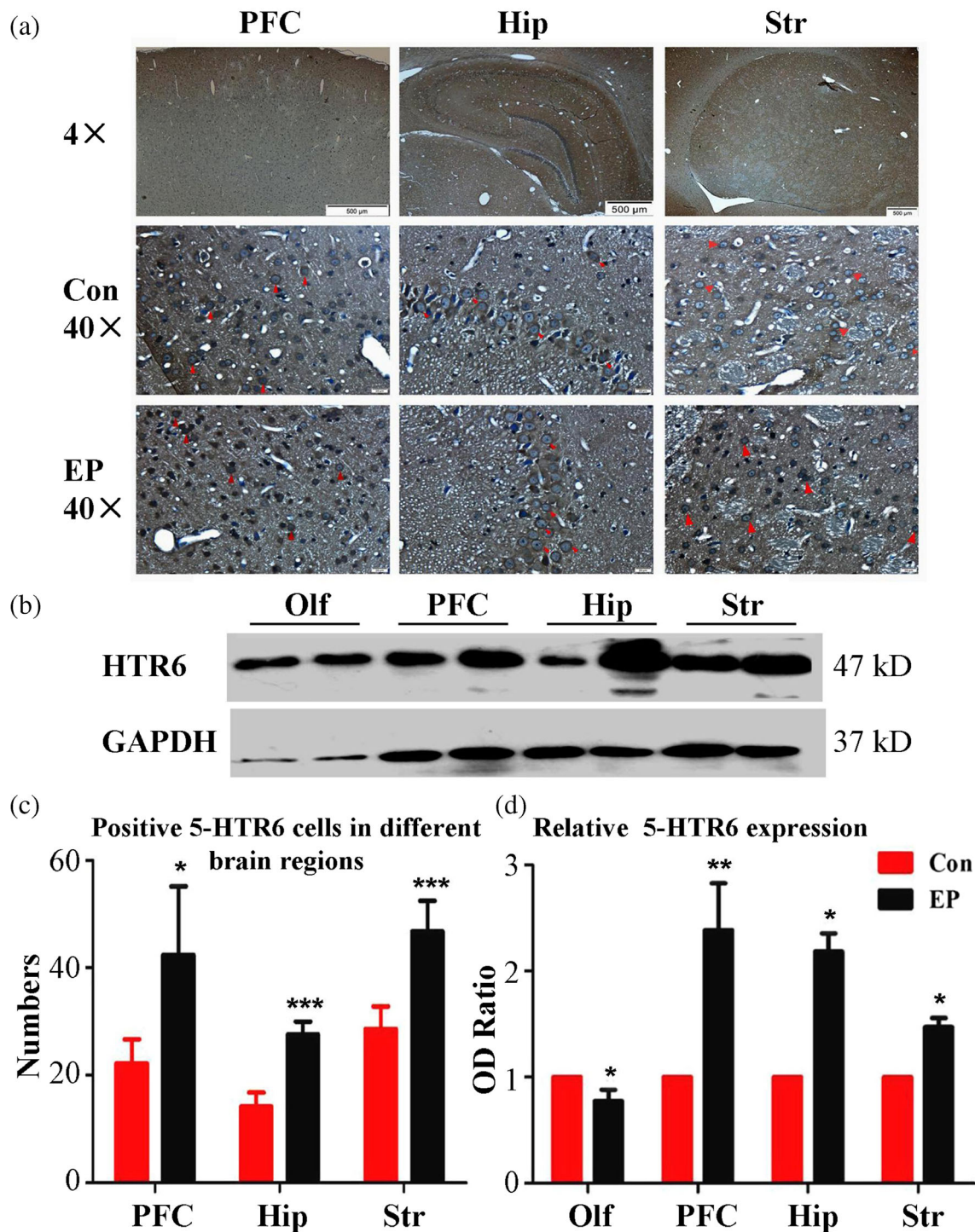


Fig. 5 5-HT6 receptor expression as demonstrated via IHC (a) and Western blot (b). The membrane was stained brown and the nuclei were null. All three of the brain regions (C cortex, H hippocampus, and S striatum) contained 5-HT6 receptor-positive cells. c In the HP-CD group, both the cortex and the hippocampus displayed remarkable increases in the numbers of 5-HT6R-positive cells and in the staining

intensities ($*P < 0.05$), but no significant differences were observed in the striatum. The differences in the numbers of 5-HT6R-positive neurons ($*P < 0.05$). d A significant increase in the OD ratio after pilocarpine treating was detected in both the hippocampus and the cortex ($*P < 0.05$) but not in the striatum

displays potent and persistent anti-convulsive activity as demonstrated using the memory specificity training (MEST) test (Woolley et al. 2004). In this study, our findings are consistent

with the previously reported anti-convulsive activity of other 5-HT6 receptor antagonists, including Ro 04-6790 and SB-258510 (Woolley et al. 2004; Brouard et al. 2015; Higgs et al.

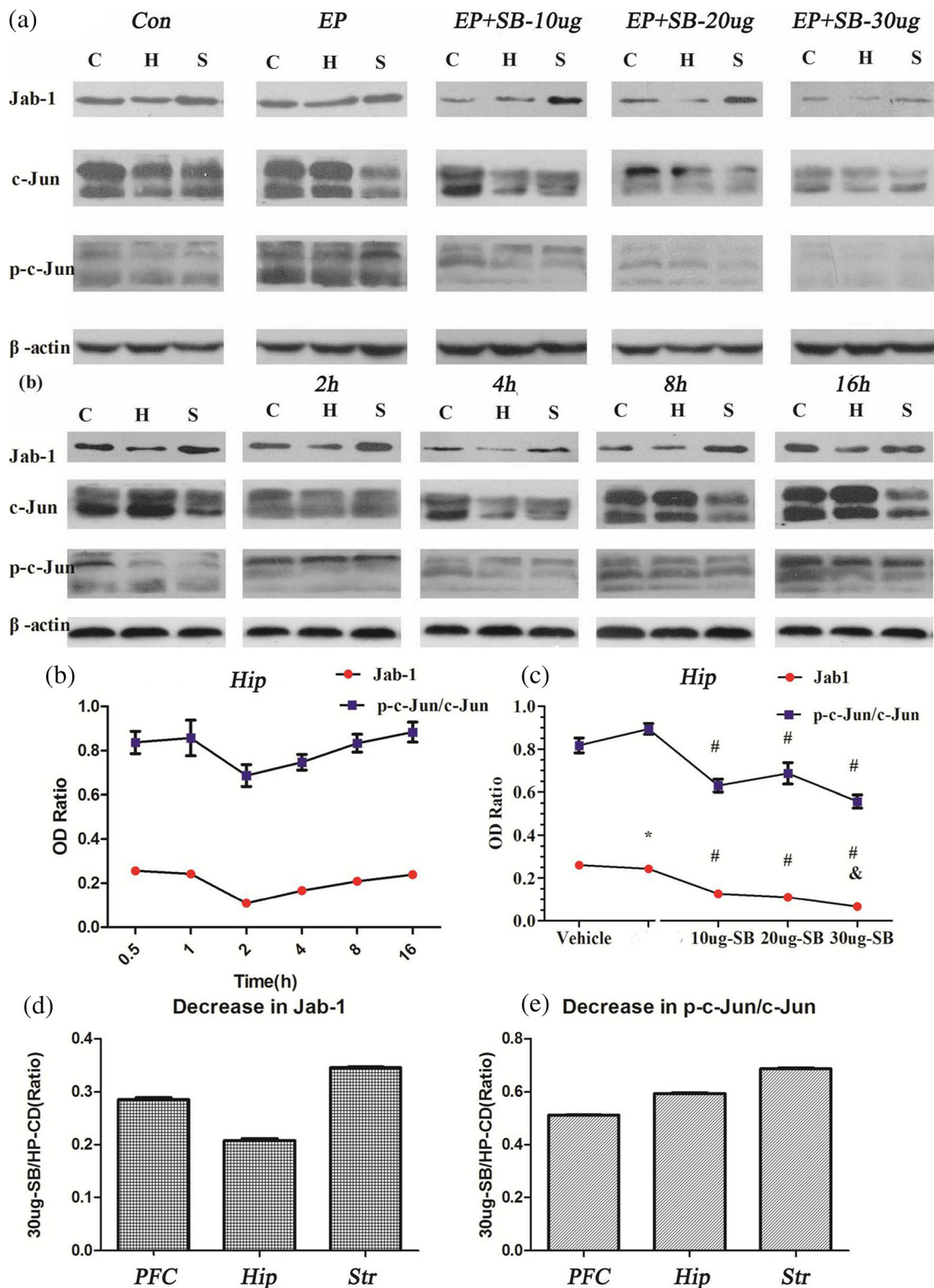


Fig. 6 **a** The Jab-1 and p-c-Jun/c-Jun expression was evaluated by using Western blot assay. **b** The level of Jab-1 was not changed while p-c-Jun/c-Jun was significantly increased in HP-CD when compared to vehicle. **c**

The effect got more significant with the increase of SB-271046 dose. **d, e** The inhibition was most obvious in cortex, followed by hippocampus

2016), which suggests that the anti-convulsive properties of SB-271046 are likely mediated by the 5-HT₆ receptor.

Recently, 5-HT₆ receptor was found to modulate seizure activity through recruitment of mTOR (Wang et al. 2015).

However, some researchers considered that the magnitude of these anti-seizure effects is modest compared to that of known anti-epileptic drugs such as carbamazepine (Upton et al. 1997; Smith et al. 2007; Kenyon et al. 2014).

The understanding of the mechanisms by which anti-convulsive drugs act on the 5-HT₆ receptor is limited, but previous evidence indicates that agents that elevate the extracellular serotonin levels inhibit seizures (Choi et al. 2010) and that agents that reduce the brain serotonin levels are associated with pro-convulsive activity. We found decreased extracellular serotonin levels in the hippocampus of epileptic rats in our previous study (Lin et al. 2013), and such reductions might be responsible for the modest anti-seizure effects of 5-HT₆ receptor antagonists. Hippocampal sclerosis, including neuronal apoptosis and glial proliferation, is the most characteristic pathological change in chronic TLE. In the present study, the decrease in neuronal apoptosis following the administration of the 5-HT₆ receptor antagonist might have contributed to the reductions in seizure severity and frequency. But it remains a mystery whether neuronal apoptosis induced seizures or seizures induced apoptosis.

We were interested more in determining whether the 5-HT₆ receptor is involved in epilepsy accompanied by cognitive impairment. In our study, after SB-271046 administration, both the SAP rate and the percentage of time spent in the new arm increased. On the Morris water maze test, the latency to reach the platform on the first 5 days and the latency to reach the target zone on the final day were reduced following SB-271046 administration. These findings indicate that 5-HT₆ receptor antagonist administration improves the learning and memory capacities of epileptic rats. However, its mechanism remains to be elucidated. It has been reported that 5-HT₆ receptor over-expression in the striatum leads to impairments in instrumental learning (Mitchell et al. 2007). Marcos et al. found that procedural learning induces the downregulation of 5-HT₆ receptor expression (Marcos et al. 2010). Experimental studies have demonstrated that the hippocampus, particularly the CA3 region, is essential for spatial learning, including spatial memory acquisition and consolidation. Moreover, the manipulation of information in the working memory system is an active process that requires executive regulation and controlled attention. This process involves a complex neural network in which the connections between the hippocampus and cortex are critical. Therefore, the frontal cortex plays an important role in working memory. In our study, 5-HT₆ receptor over-expression in the hippocampus and the frontal cortex was observed in the pilocarpine-induced chronic epileptic rats. TLE with cognitive impairment might be caused by the over-expression of the 5-HT₆ receptor especially in cortex and hippocampus.

The 5-HT₆ receptor is not expressed in cholinergic neurons (Marcos et al. 2006). The co-localization of glutamic acid decarboxylase with the 5-HT₆ receptor in the rat cerebral cortex and hippocampus has been demonstrated (Woolley et al.

2004; Codony et al. 2011; Johnson et al. 2008; West et al. 2009). Based on the above data, 5-HT₆ receptor antagonists might disinhibit GABAergic neurons and subsequently modulate the cholinergic and/or glutamatergic systems. However, whether this receptor is expressed in other types of neurons and glia requires further exploration. The 5-HT₆ receptor belongs to the family of G protein-coupled receptors. The cellular effects mediated by the 5-HT₆ receptor are partially understood. The 5-HT₆ receptor has been found to activate intercellular signal-regulated JNK via the Jab1 pathway (Yun et al. 2010). We found that the level of p-c-Jun was upregulated in epileptic rats and was downregulated after SB-271046 administration. It is well known the upregulation of p-c-Jun may accelerate neuronal apoptosis. Therefore, over-expression of the 5-HT₆ receptor as well as activated p-c-Jun may influence neuronal apoptosis and antagonists of 5-HT₆ receptor may protect epileptic rats from neuronal damage. However, the imbalance between the p-c-Jun and neuronal apoptosis and the distinguished differences between Jab-1 and p-c-Jun were difficult to understand. Maybe more Jab-1 move to nucleus in epileptic rats than vehicle. Maybe other molecular pathways play an more important role in influencing p-c-Jun. Moreover, our data still cannot illustrate whether pilocarpine or SRSs induce learning-memory impairment. Further investigation may provide insight into the cellular mechanisms by which over-expression of 5-HT₆ receptor and activated p-c-Jun play important role in seizures and cognitive impairment.

Although this study received some interesting results, there were also a few limitations. Firstly, the apoptosis of the cells was only determined by using the qualitative method. In the following study, we would investigate the apoptotic cells by using the quantitative method. Secondly, the gene expressions for the proteins have not been examined in this study, although the protein expression could reflect the function of molecules. In the future research, we would examine the gene levels of molecules and verify the intercellular processes. Thirdly, the statistical method of bar charts for the data is relative less informative compared to the informative scatter plots due to the previous published study (Rousselet et al. 2016). In the following studies, we would analyze and demonstrate the data or findings with the scatter plots. Fourthly, according to the antibodies used, there are a few truncated bands in Western blotting images; however, all of which cannot affect the indicated findings of this study. In the future study, we would select a few more specific antibodies.

In conclusion, pilocarpine induced recurrent seizures and learning-memory impairment that may be mediated by over-expression of the 5-HT₆ receptor in the hippocampus and the cortex. The 5-HT₆ receptor antagonist SB-271046 not only reduced the seizure frequency but also alleviated the cognitive impairments in the epileptic rats. The activated p-c-Jun by 5-HT₆ receptor may play an important role in epileptogenesis

and cognitive impairment. The present study discovered for the first time that the 5-HT₆ receptor and activated Jab-1/p-c-Jun participate in the pilocarpine-induced seizures and learning-memory impairment.

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Conflict of Interest The authors declare that they have no conflict of interest.

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References

- Berg AT (2011) Epilepsy, cognition, and behavior: the clinical picture. *Epilepsia* 52(Suppl 1):7–12
- Blumcke I, Spreafico R, Haaker G, Coras R, Kobow K, Bien CG, Pfafflin M, Elger C, Widman G, Schramm J, Becker A, Braun KP, Leijten F (2017) Histopathological findings in brain tissue obtained during epilepsy surgery. *N Engl J Med* 377:1648–1656
- Brouard JT, Schweimer JV, Houlton R, Burnham KE, Queree P, Sharp T (2015) Pharmacological evidence for 5-HT₆ receptor modulation of 5-HT neuron firing in vivo. *ACS Chem Neurosci* 6:1241–1247
- Choi HC, Kim YI, Song HK, Kim JE, Kim DS, Kang TC (2010) Effects of selective serotonin reuptake inhibitors on GABAergic inhibition in the hippocampus of normal and pilocarpine induced epileptic rats. *Brain Res* 1357:131–141
- Codony X, Vela JM, Ramirez MJ (2011) 5-HT(6) receptor and cognition. *Curr Opin Pharmacol* 11:94–100
- Curia G, Longo D, Biagini G, Jones RS, Avoli M (2008) The pilocarpine model of temporal lobe epilepsy. *J Neurosci Methods* 172:143–157
- Fu X, Ke M, Yu M, Wang X, Xiao Q, Gu M, Lu Y (2018) Periodic variation of AAK1 in an A-beta1-42 induced mouse model of Alzheimer's disease. *J Mol Neurosci* 65:179–189
- Hermann BP, Seidenberg M, Dow C, Jones J, Rutecki P, Bhattacharya A, Bell B (2006) Cognitive prognosis in chronic temporal lobe epilepsy. *Ann Neurol* 60:80–87
- Higgs S, Cooper AJ, Barnes NM (2016) The 5-HT_{2C} receptor agonist, lorcaserin, and the 5-HT₆ receptor antagonist, SB-742457, promote satiety: a microstructural analysis of feeding behaviour. *Psychopharmacology* 233:417–424
- Hu L, Wang B, Zhang Y (2017) Serotonin 5-HT₆ receptors affect cognition in a mouse of Alzheimer's disease by regulating cilia function. *Alzheimers Res Ther* 9:76
- Huang H, Che C, Liu C, Jiang F, Mao X (2011) Factors associated with generic and disease-specific quality of life in epilepsy. *Biomed Environ Sci* 24:228–233
- Huang C, Chi XS, Li R, Hu X, Xu HX, Li JM, Zhou D (2017) Inhibition of P2X₇ receptor ameliorates nuclear factor-kappa B mediated neuroinflammation induced by status epilepticus in rat hippocampus. *J Mol Neurosci* 63:173–184
- Hughes RN (2004) The value of spontaneous alternation behavior (SAB) as a test of retention in pharmacological investigations of memory. *Neurosci Biobehav Rev* 28:497–505
- Inostroza M, Cid E, Brotons-Mas J, Gal B, Aivar P, Uzcategui YG, Sandi C, Menendez de la Prida L (2011) Hippocampal-dependent spatial memory in the water maze is preserved in an experimental model of temporal lobe epilepsy in rats. *PLoS One* 6:e22372
- Johnson CN, Ahmed M, Miller ND (2008) 5-HT₆ receptor antagonists: prospects for the treatment of cognitive disorders including dementia. *Curr Opin Drug Discov Devel* 11:642–654
- Kenyon K, Mintzer S, Nei M (2014) Carbamazepine treatment of generalized tonic-clonic seizure in idiopathic generalized epilepsy. *Seizure* 23:234–236
- de Lima C, Arida RM, Andersen ML, Polesel DN, de Alvarenga TAF, Vancini RL, Matos G, Tufik S (2017) Effects of acute physical exercise in the light phase of sleep in rats with temporal lobe epilepsy. *Epilepsy Res* 136:54–61
- Lin WH, Huang HP, Lin MX, Chen SG, Lv XC, Che CH, Lin JL (2013) Seizure-induced 5-HT release and chronic impairment of serotonergic function in rats. *Neurosci Lett* 534:1–6
- Marcos B, Gil-Bea FJ, Hirst WD, Garcia-Alloza M, Ramirez MJ (2006) Lack of localization of 5-HT₆ receptors on cholinergic neurons: implication of multiple neurotransmitter systems in 5HT₆ receptor-mediated acetylcholine release. *Eur J Neurosci* 24:1299–1306
- Marcos B, Cabero M, Solas M, Aisa B, Ramirez MJ (2010) Signalling pathways associated with 5-HT₆ receptors: relevance for cognitive effects. *Neuropsychopharmacol* 13:775–784
- Mitchell ES, Sexton T, Neumaier JF (2007) Increased expression of 5-HT₆ receptors in the rat dorsomedial striatum impairs instrumental learning. *Neuropsychopharmacology* 32:1520–1530
- Pan Y, Yang H, Claret FX (2014) Emerging roles of Jab1/CSN5 in DNA damage response, DNA repair, and cancer. *Cancer Biol Ther* 15:256–262
- Racine RJ (1972) Modification of seizure activity by electrical stimulation. II. Motor seizure. *Electroencephalogr Clin Neurophysiol* 32:281–294
- Rousselet GA, Foxe JJ, Bolam JP (2016) A few simple steps to improve the description of group results in neuroscience. *Eur J Neurosci* 44:2647–2651
- Routledge C, Bromidge SM, Moss SF, Price GW, Hirst W, Newman H, Riley G, Gager T, Stean T, Upton N, Clarke SE, Brown AM, Middlemiss DN (2000) Characterization of SB-271046: a potent, selective and orally active 5-HT(6) receptor antagonist. *Br J Pharmacol* 130:1606–1612
- Salman MM, Sheilabi MA, Bhattacharyya D, Kitchen P, Conner AC, Bill RM, Woodroffe MN, Conner MT, Princivalle AP (2017) Transcriptome analysis suggests a role for the differential expression of cerebral aquaporins and the MAPK signaling pathway in human temporal lobe epilepsy. *Eur J Neurosci* 46:2121–2132
- Smith MD, Adams AC, Saunders GW, White HS, Wilcox KS (2007) Phenytoin- and carbamazepine-resistant spontaneous bursting in rat entorhinal cortex is blocked by retigabine in vitro. *Epilepsy Res* 74:97–106
- Tai XY, Bernhardt B, Thom M, Thompson P, Baxendale S, Koeppe M, Bernasconi N (2018) Review: neurodegenerative processes in temporal lobe epilepsy with hippocampal sclerosis: clinical, pathological and neuroimaging evidence. *Neuropathol Appl Neurobiol* 44:70–90
- Titiz AS, Mahoney JM, Testorf ME, Holmes GL, Scott RC (2014) Cognitive impairment in temporal lobe epilepsy: role of online and offline processing of single cell information. *Hippocampus* 24:1129–1145
- Upton N, Blackburn TP, Campbell CA, Cooper D, Evans ML, Herdon HJ, King PD, Ray AM, Stean TO, Chan WN, Evans JM, Thompson M (1997) Profile of SB-204269, a mechanistically novel

- anticonvulsant drug, in rat models of focal and generalized epileptic seizures. *Br J Pharmacol* 121:1679–1686
- Veliskova J (2006) Behavioral characterization of seizures in rats. Elsevier Academic Press, Burlington, pp 601–611
- Wang L, Lv Y, Deng W, Peng X, Xiao Z, Xi Z, Chen G, Wang X (2015) 5-HT₆ receptor recruitment of mTOR modulates seizure activity in epilepsy. *Mol Neurobiol* 51:1292–1299
- Wang L, Fu X, Peng X, Xiao Z, Li Z, Chen G, Wang X (2016) DNA methylation profiling reveals correlation of differential methylation patterns with gene expression in human epilepsy. *J Mol Neurosci* 59:68–77
- West PJ, Marcy VR, Marino MJ, Schaffhauser H (2009) Activation of the 5-HT₆ receptor attenuates long-term potentiation and facilitates GABAergic neurotransmission in rat hippocampus. *Neuroscience* 164:692–701
- Winston GP, Stretton J, Sidhu MK, Symms MR, Thompson PJ, Duncan JS (2013) Structural correlates of impaired working memory in hippocampal sclerosis. *Epilepsia* 54:1143–1153
- Woolley ML, Marsden CA, Fone KC (2004) 5-HT₆ receptors. Current drug targets. *CNS Neurol Dis* 3:59–79
- Wu Q, Zhao CW, Long Z, Xiao B, Feng L (2018) Anatomy based networks and topology alteration in seizure-related cognitive outcomes. *Front Neuroanat* 12:25
- Yang Z, Zhang Y, Wang L (2013) A feedback inhibition between miRNA-127 and TGF- β /c-Jun cascade in HCC cell migration via MMP12. *PLoS One* 8:e65256
- Yun HM, Baik JH, Kang I, Jin C, Rhim H (2010) Physical interaction of Jab1 with human serotonin 6 G-protein-coupled receptor and their possible roles in cell survival. *J Biol Chem* 285:10016–10029