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Effect of Selective 5-HT6R Agonist on Expression of 5-HT Receptor and Neurotransmitter in Vascular Dementia Rats

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: 5-HT6 receptor (5-HT6R) has pluripotent roles regulating secretion of neurotransmitters. However, whether 5-HT6R is involved in the development of vascular dementia (VD) remains unclear. To evaluate the role and mechanism of 5-HT6R in VD, this study established a rat VD model to evaluate the effect of selective 5-HT6R agonist on the expression of 5-HT6R mRNA and neurotransmitter.

Material/Methods: Eighty healthy male SD rats (7 weeks old) were randomly assigned to sham, model, 5-HT6R agonist, and placebo groups (N=20 each). A rat VD model was generated by permeant bilateral ligation of the common carotid artery. 5-HT6R agonist, placebo, or saline were given intraperitoneally for 4 weeks. The Morris water maze was utilized to test learning and memory function. Brains were extracted to separate the cortex and hippocampal tissues, in which glutamate and γ-aminobutyric acid (GABA) levels were analyzed. mRNA and protein levels of 5-HT6R were determined by RT-PCR and immunohistochemistry (IHC), respectively.

Results: Model rats had longer escape latency and fewer crossing platform times. Contents of DA, Glu, GABA, and Ach were lowered in cortical and hippocampal tissues, and 5-HT6R expression was suppressed ($p<0.05$). The application of 5-HT6R agonist shortened escape latency and increased the number of passing through the platform. It also improved hippocampal CA1 neuronal damage and elevated DA, Glu, GABA, and Ach contents and expression of 5-HT6R. Expression of 5-HT6R was not different from the placebo group.

Conclusions: Selective 5-HT6R agonist can alleviate learning deficit of VD rats, possibly via improving neurotransmitter levels in brain regions.

MeSH Keywords: Catecholamine Plasma Membrane Transport Proteins • Dementia, Vascular • Receptors, Serotonin

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Background

Vascular dementia (VD) often occurs secondarily after cerebral atherosclerosis or brain stroke, and is a cognitive dysfunctional syndrome caused by various cerebral vascular diseases [1,2]. The prevalence of VD increases linearly with age and varies greatly from country to country, ranging from 1.2% to 4.2% in people over 65 years old. It mainly manifested as impaired memory or cognitive functions, accompanied with motor, language, visual-spatial, and personality disorders [3,4]. Its pathogenesis mechanism is related to neurodegeneration, apoptosis, or necrosis caused by ischemic brain damage [5,6]. No effective treatments are available in treating VD, during which neurotransmitter plays a critical role [7,8]. Some studies showed lowered neurotransmitter levels in the hypothalamus, cerebral cortex, and hippocampus in the pathogenesis of VD, along with decreased neural activity [9,10]. VD is possibly related with decreased levels of monoamine and acetylcholine neurotransmitter in the cortex, as it can lead to dysfunctional release of various neurotransmitters, further affecting neural functions. 5-HT plays a critical role during the formation of memory and learning, and can bind with receptors and interact with other neurotransmitter receptors. Receptor subtypes include 5-HT1A, 5-HT6, and 5-HT2 [11,12]. Among these, 5-HT receptor belongs to the G-protein-coupled superfamily, and is mainly expressed inside the brain, especially cognition-related regions such as the hippocampus and frontal cortex. 5-HT receptor agonists include non-selective agonists such as lysergide, and selective agonists such as EMDT. There are many choices for a 5HT6R-selective agonist, including SB-271046, SAM513, and SB-399885. Animal model research revealed the improvement of cognitive function by 5-HT6R agonist [13,14]. The receptor agonist and antagonist of 5HT6R are closely correlated with central nervous system levels of neurotransmitters such as acetylcholine (Ach), dopamine (DA), glutamate (Glu), γ-aminobutyric acid (GABA), norepinephrine (NE), and epinephrine (E) through modulation or stimulation of neurotransmitter release. In the guinea pig hippocampus, stimulation of 5-HT4 receptors (probably located on cholinergic neurons) increases ACh release, which in turn bidirectionally modulates GABA release; these are also involved in treating learning deficits and depression. Stage II clinical trials have been initiated on some 5HTR antagonists in the study of cognitive dysfunction [15,16]. The present study generated a rat VD model by permeant ligation of the bilateral common carotid, and observed 5-HT6R mRNA level and neurotransmitter levels in various brain regions. We aimed to analyze the effect of selective 5-HT6R agonist on neurotransmitter expression and mRNA level of 50HT6R to investigate the related mechanism of 5-HT6R in VD pathogenesis.

Material and Methods

Animal grouping

Healthy male SD rats (7 weeks old, body weight 220~250 g) were provided by Shandong University (Certificate number: SYXK-2013-0025) and were kept in an SPF-grade facility with food and water *ad libitum*. Animals were randomly divided into a sham group, model group, 5-HT6R agonist group, and placebo group (N=20 each). 5-HT6R agonist (3.0mg/kg) was given intraperitoneally once per day after successfully generating a VD model for 4 consecutive weeks (1 ml/100 g). An equal volume of placebo or saline was given in other groups.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Qianfoshan Hospital, Shandong University.

Drugs and reagents

We used 5-HT6R agonist EMDT (Sigma, USA); chloral hydrate and paraformaldehyde (Kemiou Chem., China); acetylcholine (Ach) and dopamine (DA) test kits (Jiancheng Bio, China); anti-5-HT6 receptor antibody (Abcam, USA); secondary antibody (CST, USA); Morris water maze (DMS-2, Pharmaceutical Institute, Chinese Medicine Academy); Varioskan Flash 4.00.51 microplate reader (Thermo Electron, USA); UV-2102 UV spectrometer (Unique Instrument, China); Biochrom 30+ automatic amino acid analyzer (Biochrom, UK); and Agilent 1200 high-performance liquid chromatography. Conditions of HPLC were: ZORBAX Eclipse Plus-C18 column, 5 μm, 150×4.6 mm, liquid phase: KH_2PO_4 buffer (0.1 mol/L): methanol=9: 1, flow rate: 0.7 ml/min, loading volume 20 μL, temperature 35°C, excitation wavelength 254 nm, excitation at 254 nm.

Animal model

Using the Morris water maze, we screened 100 rats with normal learning and memory function; 80 of them were randomly selected to build the VD model using permeant ligation of the bilateral common carotid artery, as previously reported [17] using double-ligation approach with 72-h time interval (2-VO). In brief, rats were fasted for 8 h before surgery, and were anaesthetized using 10% chloral hydrate via intraperitoneal injection. Rats were fixed in supine position and we made 1 middle incision along the neck skin to expose the bilateral common carotid artery without touching major nerves. Double ligation was made using 0-surgical silk with suture. The remaining 20 rats were recruited into the sham group with blood vessel separation but not ligation. Anal temperature was kept between 36.5~37.5°C during the surgery. Focal application of penicillin sodium solution was performed, along with intramuscular injection of penicillin (200 000 U/d) for 3

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Table 1. Primer sequence.

Target gene		Sequence (5'-3')	Fragment length (bp)
5-HT6R	Forward	GCCACCAAGCATAGCAGGAAG	207
	Reverse	CATAAAGAGCGGGTAGATGATAGGG	
β-actin	Forward	ACTGCCGCATCCTCTTCCTC	398
	Reverse	TCCTGCTTGCTGATCCACATCT	

consecutive days. At 6 weeks after surgery, the Morris water maze was used to screen a total of 60 rats as judged by (average escape latency – escape latency in control group)/average escape latency in this group ≥20%. All 60 rats were then randomly divided into either the model group, 5-HTR agonist group (3.0 mg/kg), or placebo group (N=20 each). Drugs were given by intraperitoneal injection at 1 ml/100 g for 4 consecutive weeks. An equal volume of saline was given in the sham and model groups, while the placebo group received an equal volume of glucose-NaCl fluids.

Behavioral test

The Morris water maze test included both place navigation and spatial exploration sessions in an apparatus with temperature (20±2)°C and 30-cm water depth. In the navigation session, rats were trained for 5 consecutive days, and we recorded the escape latency from starting until climbing to the platform. During the exploration session, swimming path and crossing platform times were recorded.

Neurotransmitter assay

After rats were killed, the cerebellum and olfactory bulb were removed from the whole brain. The cerebral cortex and hippocampus were separated on ice and washed in cold saline. After weighting to calculate the brain index, which was represented as brain wet weight/body weight ×100%, 10% hippocampal and cortical homogenates were prepared and centrifuged for 10 min to extract the supernatant. Protein content was assayed by Coomassie brilliant blue colorimetric method. Ach was quantified by use of a test kit, DA was quantified by HPLC, and excitatory glutamate and inhibitory GABA were assayed by use of an amino acid analyzer.

HE staining of hippocampal CA1 zone

Rats were fixed in 4% paraformaldehyde and decapitated. The brain stem, cerebellum, and olfactory bulb were removed from the whole brain. Brain sections (5-mm) from the optic chiasm to the cerebellum were fixed in paraformaldehyde and embedded in paraffin. Hematoxylin-eosin staining was performed and observed under a light-field microscope.

IHC staining for 5-HT6R expression

After sacrifice, cerebellum and olfactory bulb were removed from the whole brain. Cerebral cortex and hippocampus were separated on ice and prepared for paraffin sections (8-mm). Tissue slices were dewaxed and stained by 3-step IHC (Ultra Vision Detection System) to detect the expression level of 5-HT6R using rabbit anti-mouse 5-HT6R polyclonal antibody (1:1500 dilution) and DAB development. After counter-staining and mounting, the Image-pro plus system was used.

RT-PCR for mRNA of 5-HT6R

Total RNA were extracted from brain tissues and synthesized for cDNA by reverse transcription. RT-PCR was performed using the TRIzol kit and quantified in a UV spectrometer. Primers (Table 1) were used to amplify DNA fragments, which were analyzed by agarose gel electrophoresis in triplicate. Relative expression level against β-actin was expressed from intensity values of DNA bands.

Statistical method

SPSS20.0 software was used to process all data, after first testing measurement data for normality. Those that fit normal distribution are presented as mean ± standard deviation. One-way analysis of variance (ANOVA) was used to compare means across multiple groups, followed by the LSD test for between-group comparison. Statistical significance was defined as $p<0.05$.

Results

Learning, memory function, and brain index in VD rats

Compared to the sham group, model rats had longer escape latency in the Morris water maze and fewer crossing platform times ($p<0.05$). The application of drugs shortened escape latency and increased the times of crossing the platform compared to the placebo group ($p<0.05$, Figure 1). Compared to the sham group, brain index was significantly higher ($p<0.05$).

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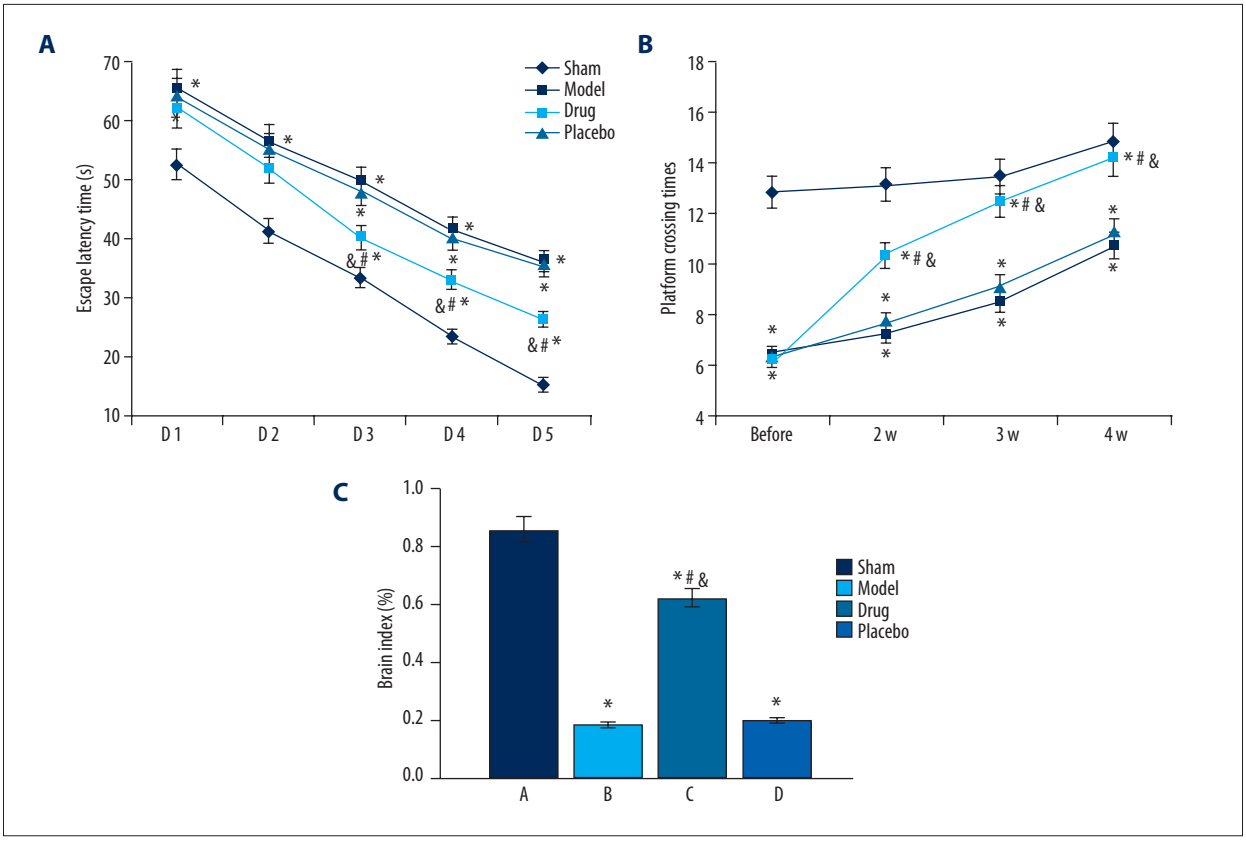

Figure 1. Escape latency (**A**) and crossing platform times (**B**) of VD rats. * p<0.05 compared to sham group; # p<0.05 compared to model group; & p<0.05 compared to placebo group. (**C**) A – Sham group; B – Model group; C – Drug (5-HT6R agonist) group; D – Placebo group.

After treatment with 5-HTR agonist, brain index was significantly reduced (around 12.07%) compared to the placebo group (Figure 1).

Morphology of hippocampal CA1 zone in VD rats

In the sham group, HE staining results showed regular arrangement of hippocampal CA1 cells, with intact morphology, large number of cells, clear boundary between cytoplasm and nucleus, and sharp round nucleolus with homogeneous red staining in the cytoplasm. The model group and placebo group rats, however, had disarrangement of hippocampal CA1 cells, with incomplete morphology, fewer cells and abnormal structure as shown by a blurred boundary between the cytoplasm and nucleus, enhanced cytoplasmic eosinophilic, inhomogeneous nuclear chromatin, increased nuclear weak gap, less cytosolic content, condensed nucleolus, and gliosis. After drug treatment, neuronal injury at CA1 region was alleviated compared to model and placebo groups (Figure 2), but still with cellular swelling.

Effects of neurotransmitter on cortex and hippocampus of VD rats

Compared to the sham group, model rats (group B) had significantly decreased DA, Glu, Ach, and GABA (p<0.05). Compared to the placebo group, the drug treatment group had increased contents of those 4 neurotransmitters, with 199.09%, 222.63%, 47.87%, and 49.11% increase for DA, Glu, Ach, GABA, respectively, in the cortex, and 58.44%, 38.07%, 40.96%, and 30.52%, respectively in the hippocampus (p<0.05, Figure 3).

RT-PCR

The model group had depressed 5-HT6 mRNA levels compared to the sham group (p<0.05). The application of 5-HTR6 medicine elevated mRNA level by 213.64% (p<0.05, Figure 4).

5-HT6R expression in hippocampal CA1 zone

Compared to the sham group, model rats had depressed expression of 5-HT6R and malfunctions in their hippocampal regions. The application of drug (5-HT6R agonist) obtained similar results as those in the placebo group (Figure 5).

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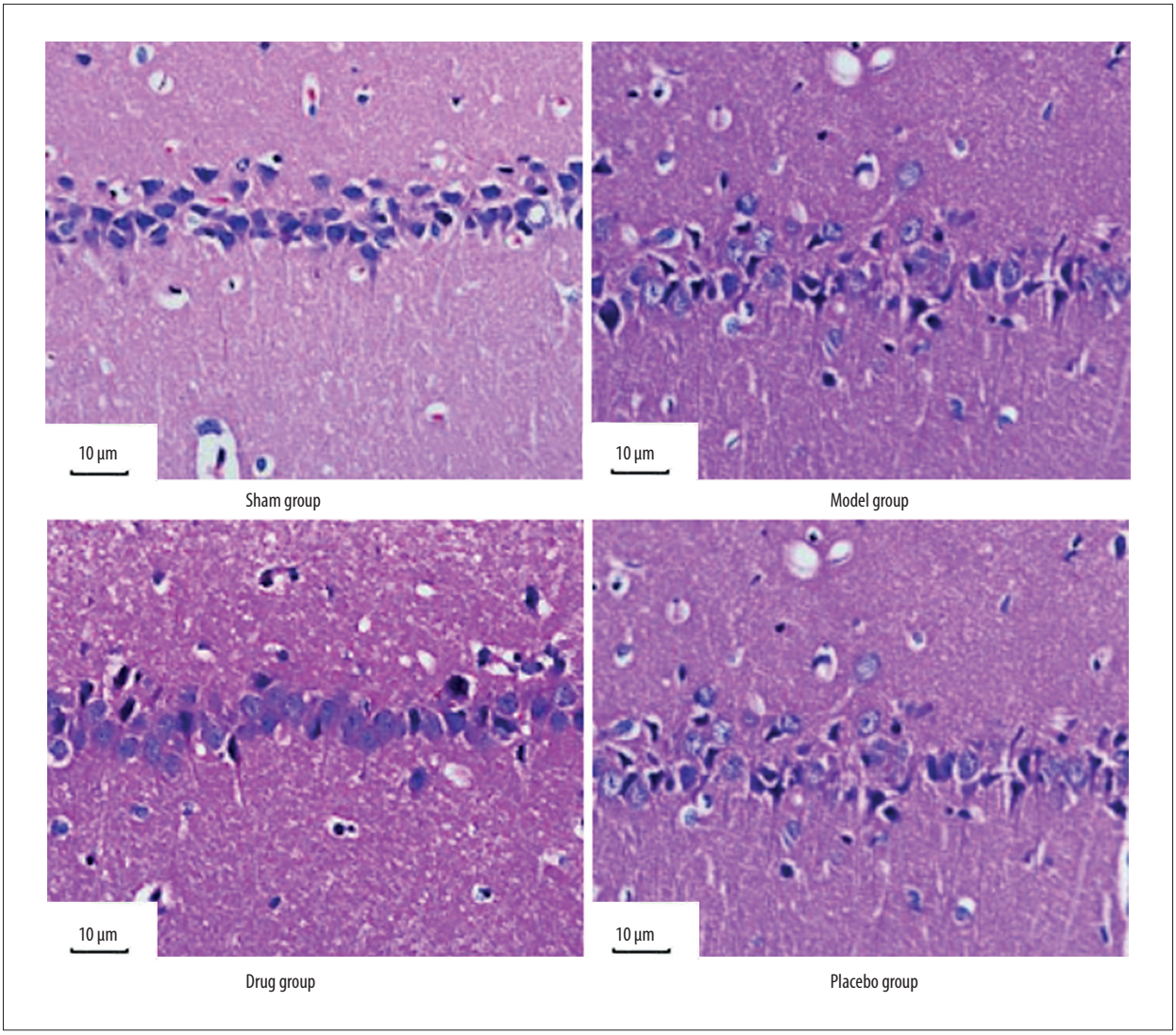

Figure 2. Hippocampal CA1 zone morphology (HE staining, ×400). Morphology of neurons in the hippocampal CA1 zone in each group is detailed in the Results section.

Discussion

Neurotransmitter plays crucial roles in the occurrence and development of VD. Acetylcholine is synthesized by choline acetyl transferase and can be hydrolyzed by cholinesterase. In VD rats, the activity of cholinesterase and choline acetyl transferase was decreased, with lower acetylcholine metabolic rate. An animal study revealed that 5-HT6R agonist can increase acetylcholine level inside the brain [18]. The present study showed that 5-HT6R agonist could increase acetylcholine content of cortical and hippocampal tissues, and improve the metabolism of cholinergic neurotransmitter. Monoamine neurotransmitter plays an important role in memory formation and maintenance. DA can regulate learning and memory, in addition to autonomic neurons. After brain ischemia, the content of monoamine neurotransmitter was decreased. 5-HT6R agonist can improve memory and learning functions of VD rats via elevating DA levels in cortical and hippocampal tissues. Glutamate is an excitatory neurotransmitter and has excitotoxicity at high concentrations via intracellular calcium overloading and mitochondrial dysfunction, causing neuronal apoptosis. Studies have shown the elevation of excitatory amino acid in VD brain tissues, along with higher peripheral glutamate concentration during the acute phase of brain ischemia, followed by recovery to normal level with prolonged ischemia time [18,19]. Results of the present study showed lower Glu and GABA levels in VD model rats in cortical and hippocampal tissues, while in the drug treatment group both neurotransmitters showed elevated expression. This was probably due to initial excitotoxicity caused by the release of large amounts of GABA, followed by metabolic disorders resulting from persistent ischemia decreasing Glu and GABA levels. 5-HT6R agonist thus can improve

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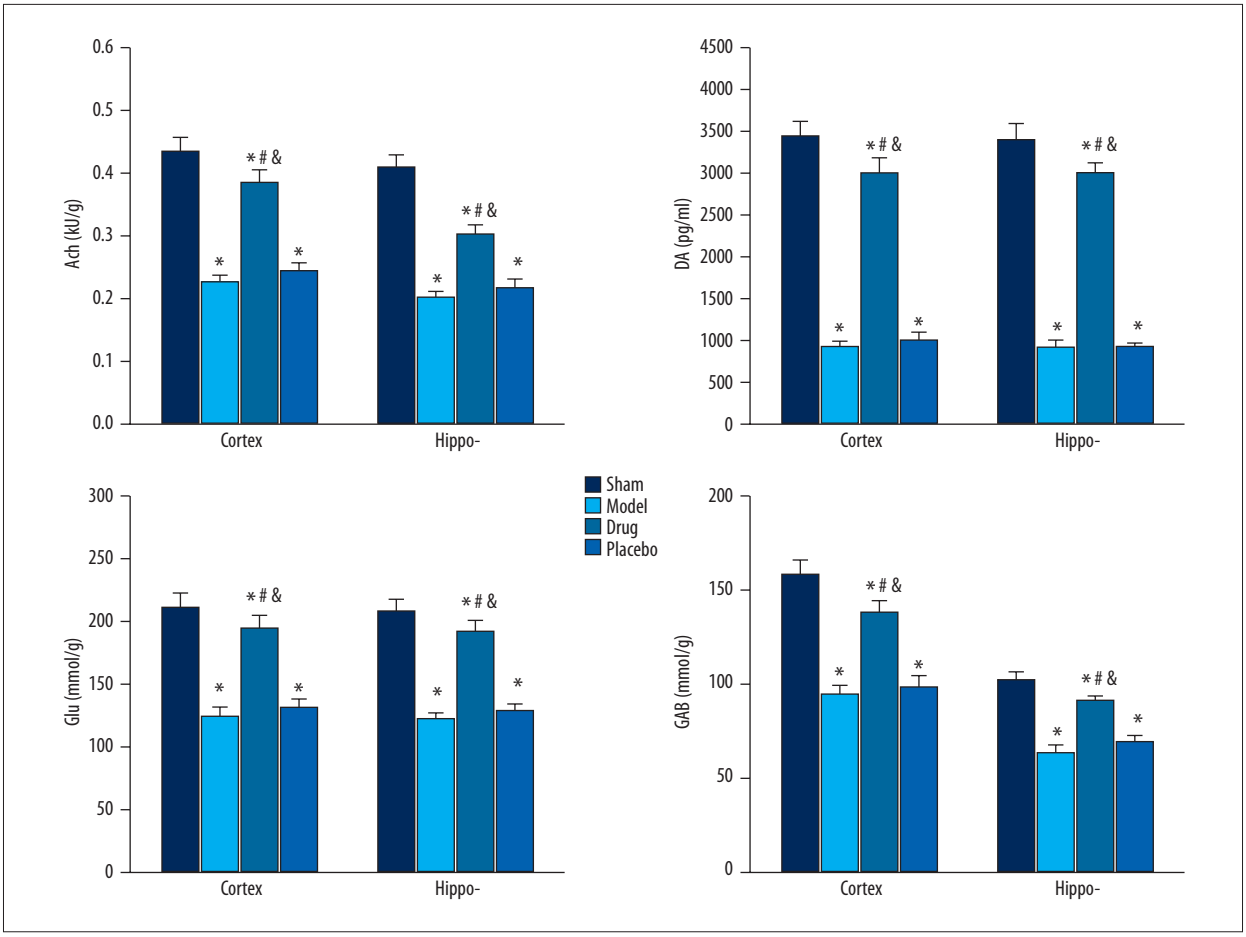

Figure 3. Neurotransmitter in VD rats. * p<0.05 compared to sham group; # p<0.05 compared to model group; & p<0.05 compared to placebo group. Each neurotransmitter' (Ach, DA, Glu, GAB) level was higher in the 5HT6R agonist-treated VD rats than in the placebo group.

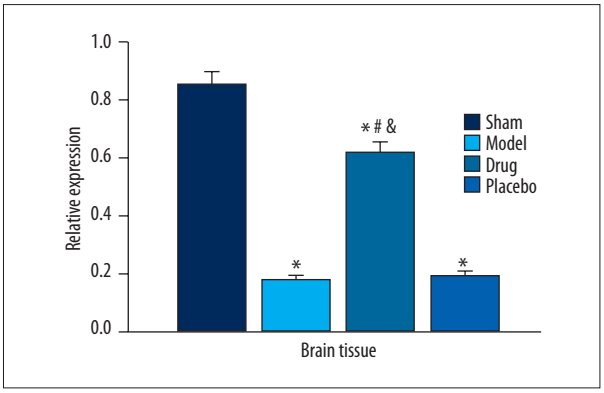

Figure 4. mRNA expression of 5-HT6R in rat brain. *, p<0.05 compared to sham group; # p<0.05 compared to model group; & p<0.05 compared to placebo group.

learning and memory functions and enhance neural electrical activities via increasing Glu and GABA contents in the brain.

5-HT6R is widely distributed in GABAergic interneurons, and participates in the modulation of learning and memory processes via modulating synaptic transmission efficiency [20,21]. We established a VD rat model in which the improvement of 5-HT6R agonist on learning and memory function of VD rats was observed. Results showed that model rats had elongated escape latency and reduced crossing platform times compared to sham group rats. VD rats also had depressed expression of 5-HT6R mRNA and proteins. Compared to the placebo group, agonist treatment shortened escape latency and increased the time of passing through the platform, and increased the expression of 5-HT6R mRNA but not receptor protein expression, further substantiating that 5-HT6R agonist can significantly improve learning and memory function of VD rats, possibly via weakening the inhibition effect of GABAergic neurons on glutamatergic and cholinergic neurons. Moreover, long-term application of 5-HT6R agonist caused receptor resistance and increased mRNA expression of 5-HT6R. As the altered internal environment and injury factors underlying mRNA expression of 5-HT6R, the receptor expression requires post-translational

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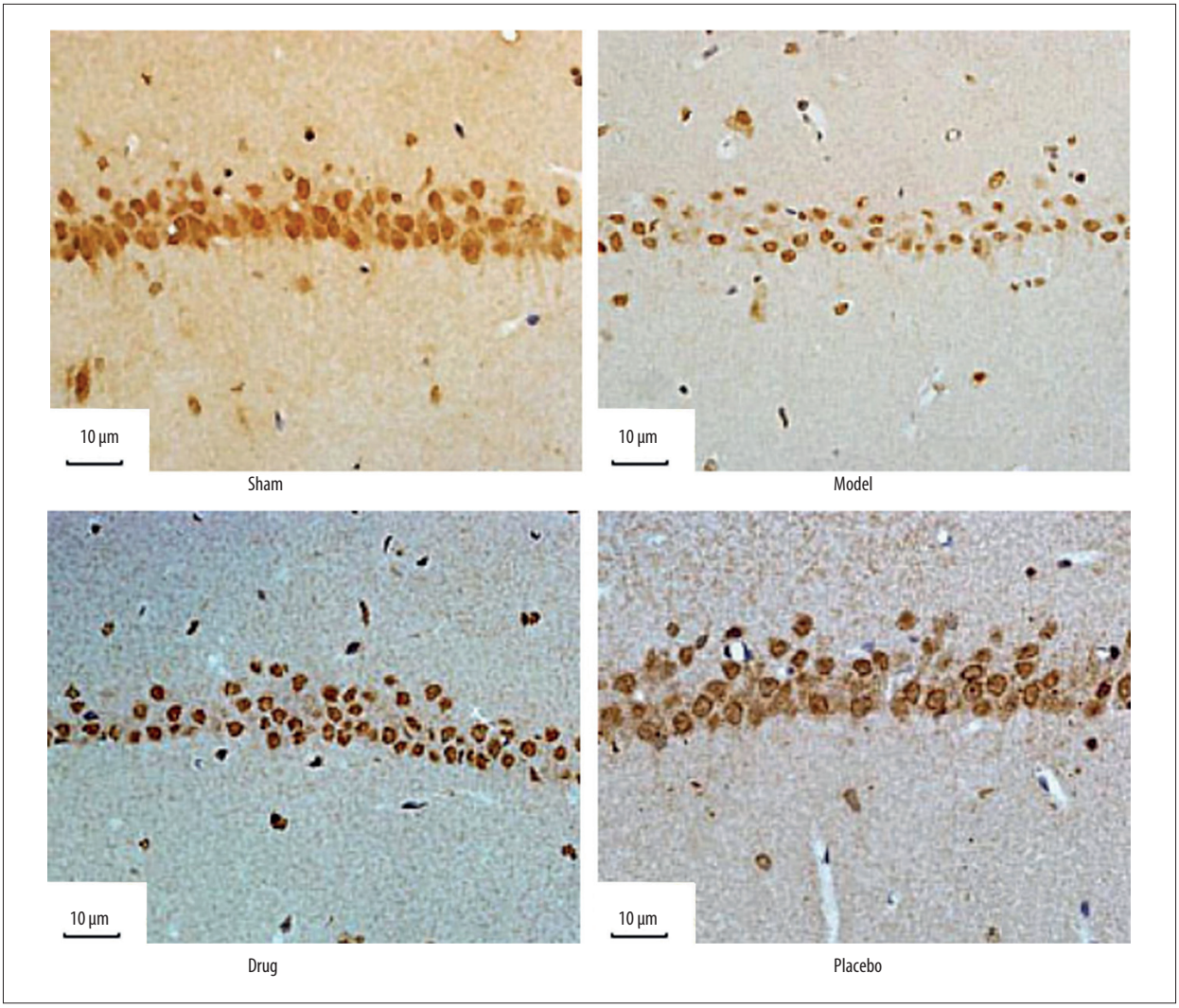

Figure 5. Expression of hippocampal CA1 region in VD rats. The expression of 5-HT6R was found by HE staining (×400).

splicing and modification, which lag behind mRNA expression, causing the time mismatch between mRNA and protein expression of 5-HT6R. Due to the relatively longer duration of this experiment, the down-regulation of 5-HT6R in the model group after long-term cerebral ischemia may be related with the protective mechanism in CNS against ischemia. In this study, the elevation of mRNA in the drug treatment group is related with long-term use of receptor agonist and consequent resistance. In the drug treatment group, the expression of 5-HT6R was not different from that of the placebo group. The hippocampal CA1 zone is important for information storage and memory, and is a sensitive region for brain ischemia. It has been shown that dementia caused by ischemic cerebrovascular disease is related to late-onset apoptosis in CA1 neurons [22]. This study observed the morphology of hippocampal CA1 region in VD rats treated with 5-HT6R agonist. HE staining showed certain improvements of hippocampal CA1 neuronal damage. Some studies have shown that 5-HT6R agonist can facilitate the connection between synapse, and increase synaptic plasticity, suggesting a neural protection role of 5-HT6R agonist in VD rats.

Conclusions

The role of 5-HT6R agonist in treating VD is related to the modulation of cholinergic/monoamine neurotransmitter, and excitatory/inhibitory amino acid contents.

Disclosure of conflict of interest

The authors declare no competing financial or commercial interests in this manuscript.

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