Effect of selective serotonin reuptake inhibitors on expression of 5-HT1AR and neurotransmitters in rats with vascular dementia

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ABSTRACT. 5-hydroxytryptamine receptor 1A (5-HT1AR) is closely associated with cognitive functions. Selective serotonin reuptake inhibitors (SSRIs) can protect individuals from brain damage following ischemia/hypoxia. To investigate the function of SSRIs in vascular dementia (VD), we established a rat model of VD, and observed the effect of SSRIs on the expression of 5-HT1AR mRNA and neurotransmitters. Male SD rats (6 months) were randomly assigned

into sham, model, and SSRI groups (N = 30). VD was achieved by permanent ligation of the bilateral common carotid artery. Escitalopram, a highly selective 5-HT reabsorption inhibitor, was *ip* injected into the rats for three consecutive weeks. The Morris water-maze was used to test learning and memory. H&E staining for neuronal injury was conducted on cortical and hippocampal tissues. HPLC was used to determine the levels of dopamine (DA), 5-HT, and norepinephrine (NE). RT-PCR was used to determine expression of 5-HT1AR mRNA. As compared to control rats, model animals demonstrated elongated escape latency, lower platform crossing times, and significant injuries to hippocampal CA1 neurons. This was accompanied by reductions in DA, 5-HT, and NE levels in hippocampal tissues, as well as reduced cortical 5-HT and decreased 5-HT1AR mRNA expression ($P < 0.05$). Escitalopram treatments reduced escape latency, elevated platform crossing times, improved CA1 neuronal damage, increased DA and 5-HT levels in hippocampal and cortical neurons, as well as elevated expression of 5-HT1AR mRNA ($P < 0.05$). Therefore, SSRIs may improve cognitive dysfunction of VD rats, possibly by stimulating expression of neurotransmitters and protecting neurons.

Key words: Vascular dementia; Neurotransmitter;
5-HT selective reuptake inhibitor; 5-HT receptor 1A; Escitalopram

INTRODUCTION

Vascular dementia (VD) frequently occurs in conjunction with cerebral atherosclerosis or cerebral ischemia, and is described as a cognitive disorder caused by various cerebrovascular diseases (Lantz et al., 2013; O'Brien and Thomas, 2015). VD pathogenesis is correlated with neuron degeneration and cell apoptosis/ necrosis due to ischemic brain injuries (Kwon et al., 2014; Li et al., 2015). Mainly featured as a cognitive disorder, VD is usually caused by insufficient perfusion due to reduced cerebral blood flow. The hippocampus is the major domain for memory and learning, and is sensitive to ischemia/hypoxia, which affects the proliferation and differentiation of intra-hippocampal neuron stem cells (Horgusluoglu et al., 2016; Patel and Sun, 2016). Currently, no effective treatments have been developed for VD. Neurotransmitters play critical roles in the pathogenesis of VD (Cai et al., 2015; Zhao et al., 2015). For example, dopamine (DA) mediates learning, memory, and autonomic motor neurons. Norepinephrine (NE) is synthesized from DA by β-hydroxylase, and exerts its functions via a1, a2, and β receptors. Several studies showed decreased neurotransmitter levels and reduced neural activity in the cerebral cortex, hippocampus, and hypothalamus during progression of VD (Chukhlovina, 2014; Luo et al., 2016). 5-hydroxytryptamine (5-HT) plays important roles in learning and memory, as it can bind to multiple receptors and interact with various neurotransmitter systems. In addition, 5-HT has been shown to be important for hippocampal dentate gyrus neurons. Previous studies have also demonstrated the protective effects of selective serotonin reuptake inhibitors (SSRIs) against ischemia-hypoxia or oxidative stress-induced brain damage. Moreover, SSRIs can selectively potentiate 5-HT levels in the synaptic clefts of the central nervous system, thus facilitating hippocampal neurogenesis (Gupta et

al., 2016; Outhred et al., 2016). 5-HT receptor subtypes that participate in learning and memory regulation include 5-HT1A, 5-HT6, and 5-HT2 (Lang and Borgwardt, 2013; Yang et al., 2014). 5-HT receptor 1A (5-HT1ARs) are closely associated with cognitive functions, as they are the dominant receptors in initiating 5-HT induced hippocampal neurogenesis. Previous studies have demonstrated that 5-HT1AR blockers significantly inhibited hippocampal neuron proliferation in epileptic rats (Sato et al., 2007; Elliott et al., 2009). As a next-generation anti-depressant, escitalopram has higher affinity for 5-HT as compared to citalopram (Dong et al., 2016). To examine the function of SSRIs on VD and the effect of escitalopram on neurotransmitters, we established a rat VD model via permanent bilateral ligation of the common carotid artery, and observed the expression profile of 5-HT1ARs and neurotransmitters following chronic cerebral ischemia.

MATERIAL AND METHODS

Animals and grouping

Healthy male SD rats (6 months, body weight 250-300 g) were provided by the Central South University (Certificate No. SYXK-2013-0025), and were kept in an SPF grade facility with food and water *ad libitum*. Animals were randomly divided into sham, model, and SSRIs groups (N = 30 each). In the SSRI group, escitalopram (20 mg/kg/day) was given via *ip* injections (1 mL/100 g) for three consecutive weeks. Equal volumes of saline were administered to the control and sham groups.

All animal procedures strictly adhered to the animal care guidelines, and were approved by the Animal Ethics Committee of the Third Xiangya Hospital of Central South University.

Drugs and reagents

Escitalopram was purchased from Xian Janssen. Chloral hydrate and paraformaldehyde were purchased from Kemiou Chem (China). DA and 5-HT assay kits were purchased from Jiancheng (China). RT-PCR reagents were obtained from Toyobo (China). The Morris water-maze apparatus model DMS-2 was obtained from the Pharmaceutical Institute of Chinese Medical Academy. The UV spectrometer (UV-2102C) and the HPLC Agilent 1200 instrument were purchased from Unique (China). The chromatography conditions were as follows: ZORBAX Eclipse Plus-C18 column at 5 mm, 250 mm x 4.6 mm; liquid phase: sodium phosphate (50 mM) and methanol (93: 7); 0.2 mM EDTA-Na1 buffer (D-camphor-cβ-sulfonic acid, 6 mM); flow rate: 0.7 mL/min, pH 3.5; loading volume: 20 μL; column temperature: 20°C; emission at 338 nm, excitation at 254 nm, sensitivity at 10 nA.

Animal model

Rats (110) with normal learning and memory functions were screened by the Morris water-maze. Animals (80) were randomly chosen for VD induction via permanent ligation of the bilateral common carotid artery, as previously described (Kato et al., 1997). Bilateral common carotid arteries were ligated in two surgeries within 72 h. Rats were fasted for 8 h prior to surgery. Following *ip* administration of anesthesia (10% chloral hydrate), rats were

fixed in a supine position. Bilateral common carotid arteries were separated via middle neck incision, leaving nerves intact. A double ligation was made using No. 0 surgical sutures. The remaining 30 rats were recruited into the sham group, which received artery separation but not ligation. During the surgery, rectal temperature was fixed at 36.5-37.5°C. Penicillin sodium solution was applied locally via *im* injections (200,000 U for three days). Six weeks following surgery, the Morris water-maze was used to screen for rats with VD phenotypes, which were defined as those that had ≥20% increase in average escape latency as compared to control rats. The selected rats were then randomly divided into either the model or the SSRIs groups (N = 30 each). Escitalopram was *ip* injected (1 mL/100g) for three consecutive weeks. Equal volumes of saline was given to control and sham groups.

Morris water-maze test

The water-maze test included a navigation test and an exploration session. Water temperature was maintained at 20° ± 2°C. Water depth was set to 30 cm. During the navigation test, rats were trained for five consecutive days, and the time it took to find the platform from the starting position was recorded. During exploration sessions, the swimming path and the number of times that cross the exact location of platform in the two-minute window were recorded.

Neurotransmitter assay

The rats were sacrificed, and their brains were extracted. The cerebellum and the olfactory bulb were removed during this process. The cerebral cortex and the hippocampus were immediately removed, and rinsed in cold saline. Tissues were dried, weighed, and 10% tissues homogenates were prepared. Samples were then centrifuged for 10 min, and protein contents in the supernatants were quantified via Coomassie Brilliant blue staining. HPLC-fluorescence was employed to detect levels of DA, 5-HT, and NE using standard curves and peak area (ng/g).

H&E staining

Rats were fixed in 4% paraformaldehyde, and the whole brain was extracted. The cerebellum, olfactory bulb, and lower brain stem were removed. Brain sections posterior to the optical chiasm and anterior to the cerebellum were fixed in paraformaldehyde. Tissues were embedded in paraffin, sectioned, stained with H&E, and mounted on coverslips. Observation was carried out with a light field microscope.

RT-PCR

Total RNA was extracted from brain tissues. cDNA was synthesized from total RNA by reverse transcription. RT-PCR was performed using Trizol reagent. UV spectrometry was used to quantify nucleic acids. Sequences of the primers used are listed in Table 1. Amplified products were analyzed in triplicates by agarose gel electrophoresis. Relative expressions of target genes were normalized to β-actin.

Table 1. Primer sequences.

Target gene	Sequence (5'-3')		Fragment length (bp)
5-HT1AR	Forward	GCCCCCCAAGAAGAGCCTGA	
	Reverse	GCCATCTTGCGCTTTGCTTC	207
GAPDH	Forward	ACCACAGTCCATGCCATCAC	
	Reverse	TCCACCACCCTGTTGCTGTA	398

Statistical methods

The SPSS 20.0 software was used for statistical analysis. Measurement data were first assessed for normality. Data displaying normal distribution are reported as means ± standard deviation. One-way analysis of variance was used to compare the means between multiple groups, and the LSD test was carried out for paired group comparisons. Statistical significance was defined as $P < 0.05$.

RESULTS

Learning and memory of VD rats

As compared to the sham group, VD rats had longer escape latency and lower platform crossing times ($P < 0.05$). In addition, SSRI treatment significantly shortened escape latency and increased platform crossing times ($P < 0.05$, Figure 1).

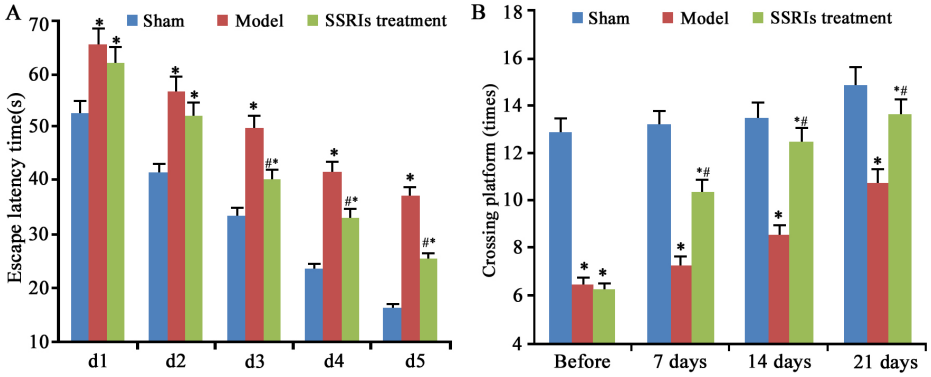

Figure 1. Morris water-maze performance of VD rats. **A.** Escape latency; **B.** Number of times the platform was crossed. *$P < 0.05$ as compared to the control group; #$P < 0.05$ as compared to the model group.

Hippocampal CA1 morphology

H&E staining of brain tissues from sham rats showed regular arrangement of hippocampal CA1 neurons, intact morphology, normal structure, sharp boundary between cytoplasm and nucleus, and clear nucleolus. On the other hand, VD rats had disarranged CA1 neurons, which displayed incomplete morphology, fewer cells with abnormal structure, blurred cytoplasm-nucleus boundary, condensed nucleus with less distinct nucleolus, as well as glial hypertrophy. However, SSRI treatment was able to alleviate neural injuries in the CA1 region (Figure 2).

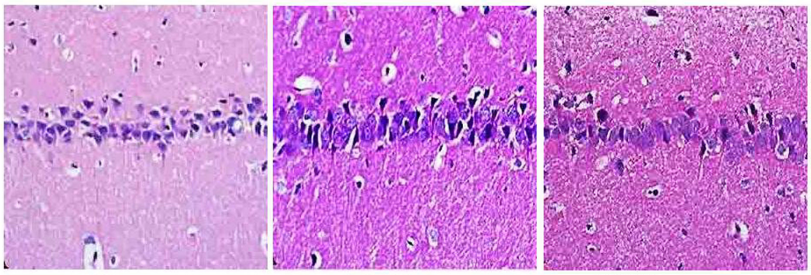

Figure 2. Hippocampal CA1 neural morphology in VD rats. H&E staining; 400X magnification.

Neurotransmitter levels in cortex and hippocampus

As compared to the sham group, rats in the VD group exhibited reduced levels of hippocampal DA, 5-HT, NE, and cortical 5-HT ($P < 0.05$). However, cortical DA and NE were comparable between the two groups ($P > 0.05$). In addition, DA and 5-HT content was increased in the cortex and the hippocampus of the SSRI group as compared to the controls ($P < 0.05$). There was no significant difference in cortical NE between the two groups ($P > 0.05$, Figure 3).

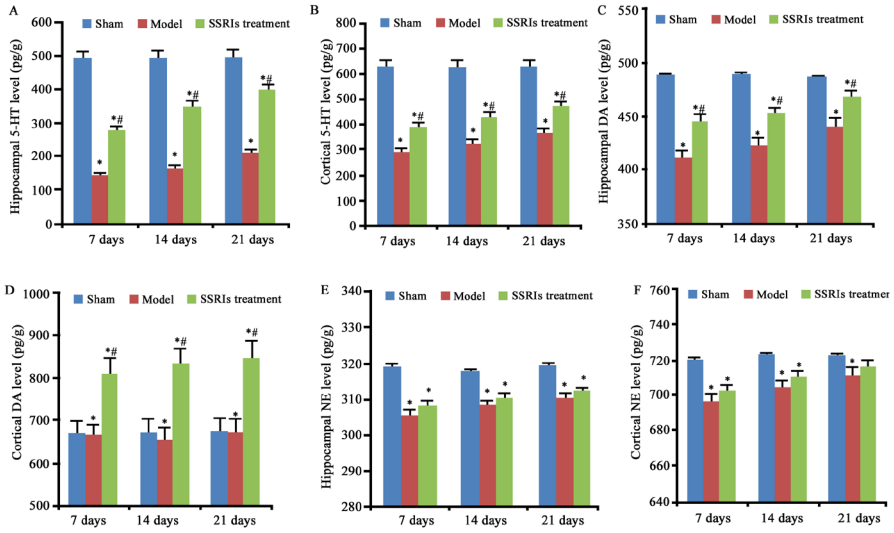

Figure 3. Neurotransmitter levels in VD rat cortex and hippocampus. *$P < 0.05$ as compared to the control group; #$P < 0.05$ as compared to the model group.

5-HT1AR mRNA level

As compared to the sham group, VD model rats showed reduced mRNA expression of 5-HT1AR in brain tissues ($P < 0.05$). The level of 5-HT1AR reached a peak at 14 days following surgery. The SSRI treatment group had elevated 5-HT1AR mRNA levels ($P < 0.05$), which reached a peak at 7 days post-surgery (Figure 4).

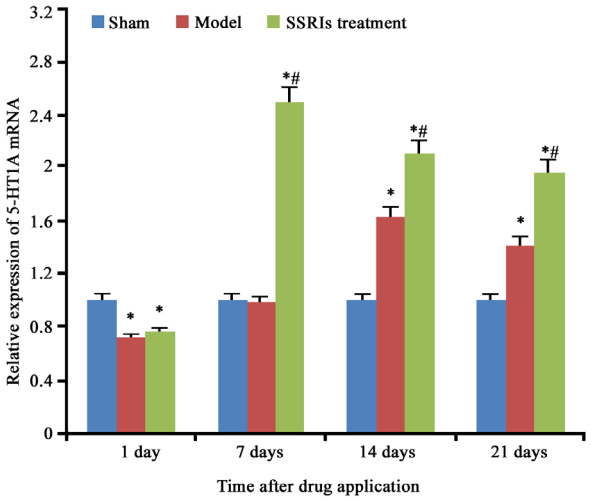

Figure 4. 5-HT1AR mRNA levels as assessed by RT-PCR. *P < 0.05 as compared to the control group; #P < 0.05 as compared to the model group.

DISCUSSION

Neurotransmitters play crucial roles in the pathogenesis of VD. Monoamine neurotransmitter is indispensable for the formation and retention of memory. DA can regulate both learning/memory and autonomic motor neurons, while 5-HT participates in cortical-hippocampal synaptic connections. Following cerebral ischemia, the content of monoamine is reduced. It has been shown that reversible abnormal secretion of monoamine neurotransmitter can occur in a short time period during the early phase of chronic brain ischemia (Stasiak et al., 2014). When action potential reaches the axonal terminus, monoamine neurotransmitter stored in vesicles can be secreted via exocytosis when stimulated by influx of calcium ions. After neurotransmitters exert their postsynaptic functions, they are cleared from the synaptic cleft by enzymatic degradation or re-uptake. In the pathology of VD, sub-cortical levels of 5-hydroxyindoleacetic acid and hippocampal 5-HT levels show significant depression. Studies have shown that VD rats exhibited decreased cortical and hippocampal NE, DA, and 5-HT levels at 7-21 days post-surgery (Gao et al., 2012). Other studies demonstrated that SSRIs could interfere with multiple mechanisms underlying cerebral ischemia such as neuronal apoptosis/necrosis, oxidative stress responses, and neurotransmitter disorders. This could alleviate neuronal injuries and protect brain tissues, resulting in improvements to cognitive dysfunctions caused by chronic cerebral ischemia (Gao et al., 2012). Our study confirmed that VD rats exhibit elongated escape latency with fewer platform crossing numbers. VD model rats also showed disarrangement of hippocampal CA1 neurons with fewer cell numbers. However, SSRI-treated rats had shortened escape latency and increased crossing numbers. Furthermore, the SSRI group showed reduction in CA1 neuronal injury as compared to the model group, suggesting that SSRIs could improve cognitive dysfunction of VD rats and rescue of neuronal injury. Moreover, VD rats had lower hippocampal DA, 5-HT, and NE contents, as well as significantly decreased cortical 5-HT level. In SSRI-treated group, cortical and hippocampal DA and 5-HT levels were increased, suggesting that SSRI may improve learning/memory function in VD rats via upregulation of monoamine

neurotransmitters in the cortex and the hippocampus.

There are multiple ways by which SSRIs could regulate central 5-HT levels. Examples include inhibition of 5-HT transport proteins, increase in the expression level of serine hydroxylase, regulation of the rate-limiting synthase of 5-HT, electrophysiological stimulation of intra-synaptic 5-HT, desensitization of presynaptic 5-HT1AR, and activation of 5-HT1A self-receptor via negative feedback loops (Lu et al., 2015). In this study, we showed that 5-HT1AR mRNA level is reduced in VD model rat brain tissues, which reached a peak at 14 days post-surgery. In the SSRI-treated group, 5-HT1AR mRNA level was elevated and reached a peak at day 7, suggesting that downregulation of 5-HT1AR mRNA in the hippocampus after cerebral ischemia can affect neural functions and impair self-protection mechanism of the brain. Previous studies have demonstrated correlations between compensatory cortisol release after brain ischemia and reduced gene expression of hippocampal 5-HT1R receptors (McAllister et al., 2014; Stiedl et al., 2015). 5-HT1AR has neurotrophic effect. Escitalopram, which is one of the SSIRs, may facilitate recovery of brain functions via upregulation of intra-synaptic 5-HT and 5-HT1AR.

This study still has several drawbacks. First, we did not test different concentrations of SSRIs in this study, and thus cannot speculate the optimal dosage of SSRIs needed for treatment in the VD rat. Next, since we did not apply any specific inhibitors, we are unclear on the mechanism of SSRIs in VD. Lastly, the exact role of SSRIs in elevating 5-HT1AR mRNA and its relationship with VD still needs further investigation. In summary, SSRIs could improve cognitive dysfunction in VD rats, possibly via upregulation of neurotransmitters and 5-HT1AR, as well as protection of neurons.

Conflicts of interest

The authors declare no conflict of interest.

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