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Original Article

Transcutaneous vagus nerve stimulation regulates cognitive deficit in the stroke rats by non-neuronal cholinergic system

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Abstract: Background: Cholinergic neuroprotein plays a vital role in the cognitive improvement. The release and uptake of acetylcholine (Ach) is mediated by the organic cation transporters (OCT) and choline high-affinity transporter (CHT) which is named as non-neuronal cholinergic system (NNCS). This study was to investigate the effect of transcutaneous vasgus nerve stimulation (tVNS) on the cognitive deficit after stroke via cholinergic release and uptake. Methods: Seventy SPF adult male Sprague-Dawley were randomly divided into seven groups: Sham cerebral ischemia and perfusion (I/R), I/R, I/R+tVNS, I/R+hemicholinium-3 (HEM), I/R+HEM+tVNS, I/R+omeprazole (OME), I/R+OME+tVNS. Middle cerebral artery occlusion (MCAO) induced I/R model was evaluated and assessed by neurological score and modified neurological severity score (mNSS). Drugs intervention, tVNS and Morris water maze test for cognitive assessment were performed from Day 3 to Day 7. HEM and OME was the antagonist of CHT1 and OCT1 respectively. Infarct volume was analyzed by triphenyltetrazolium chloride (TTC) staining. The choline acetyltransferase (ChAT), carnitine acetyltransferase (CarAT), organic cation transporters 1 (OCT1) and high-affinity choline transporter 1 (CHT1) in the hippocampus were analyzed by QPCR and Western blot. Results: After I/R model induced by MCAO successfully, rats had higher neurological score and mNSS as well as the lower mRNA and protein expressions of ChAT, CarAT, OCT1 and CHT1. It was found that tVNS reversed the damage induced by MCAO, and also evaluated the ChAT, CarAT, OCT1 and CHT1. After the inhibitor of OCT1 and CHT1 involved, tVNS was not able to promote the expressions of ChAT and CarAT as well as improve the cognitive function. Conclusions: This finding suggested tVNS had potential to facilitate the cognitive deficit after stroke and its mechanism may have closely related with Ach release and uptake via NNCS.

Keywords: Transcutaneous vagus nerve stimulation, cerebral ischemia and perfusion injury, cognition, non-neuronal cholinergic system

Introduction

Stroke is a prevalent clinical disease caused by insufficient blood flow in the cerebral areas, and it induces motor disability, language disorder, and cognitive dysfunction and ect. [1] during the period of the post-stroke. Too many approaches are proposed for somatic rehabilitation and functional improvement. However cognitive improvement is in trouble and lacking of effective approaches [2].

Nowadays transcutaneous vagus nerve stimulation (tVNS), an approach of stimulating the auricular branch of vagus nerve in the ear, has been considered that had potentials in the stroke rehabilitation [3]. It has been found that tVNS is beneficial to limit the infarct volume [3], promote post-ischemic functional recovery [4] and increase the angiogenesis [5].

The cognitive deficit is a noticeable complication after the stroke which is associating with limbic system including hippocampus, amygdala, prefrontal cortex and etc. Although the detail pathological mechanism of cognitive disorder is still unclear, it is suggested that multiple molecule pathways take part in this process. Acetylcholine (Ach), a vital neurotransmitter, is proposed that takes part in repairing the cognitive disorder [6, 7] by binding to Ach receptors [8].

In the previous viewpoints, Ach is from the end of nerves and released into the local tissues by vesicular ACh transporter (VACHT). Due to much acetylcholin esterase in the local, the Ach is decomposed into choline. Currently it is found that choline is transported by choline high-affinity transporter (CHT) back into cells. The new Ach is synthesized in the local tissues such as vascular endothelium cells, mucosal epithelial cells, chondrocytes and etc. [9]. The inhibitor of CHT is able to deplete the Ach synthesis and suppress the biological action of Ach induced [10].

New synthesized Ach is released into the tissue spaces by the organic cation transporters (OCT) differing from the VACHT in the nerve endings [11]. Together with CHT and OCT, both are involved in the non-neuronal cholinergic system (NNCS). Previous studies focused on Ach binding to its receptors in the neuroprotection of cognitive deficit leading to neglecting the origination of Ach to some extent [12]. In the brain, the location of NNCS is in the hippocampus, vascular endothelium and so on [13]. The hippocampus is an important organ in the improvement of cognitive function [14].

In this study, it is hypothesized that tVNS is benefit to the cognitive repairmen after stroke and the NNCS may play a vital role in the management of Ach re-uptake and release.

Materials and methods

Animals

Seventy adult male Sprague-Dawley (SD) rats (8 wks, 200-220 g) were purchased from Shanghai Slac Laboratory Animal Company were maintained at the animal room of Cangzhou Central Hospital. All the processes were followed the ARRIVE line. The rats had free access to rodent food and water and were exposed to natural 12-h dark/light cycle (7 a.m.-19 p.m.). The room environment was set as 22 ± 1°C (temperature) and 40-60% (humidity). All animals were randomly allocated to experimental groups by computer generated randomization schedules. This study was approved by the Institutional Animal Care and Ethic committee of Cangzhou Central Hospital (NO. 20140908324).

Cerebral ischemia and reperfusion model

Rats were divided to seven group: Sham cerebral ischemia and perfusion (I/R), I/R, I/R+tVNS, I/R+hemicholinium-3 (HEM), I/R+HEM+tVNS, I/R+omeprazole (OME), I/R+OME+tVNS (n = 10 in each group). Middle cerebral artery occlusion (MCAO) was suggested to induce I/R model. After overnight fasting, rats were anesthetized by 10% chloral hydrate (3 ml/kg) through intraperitoneal injection. Rats were fixed on a temperature controlled table. After rats exposed the internal carotid artery, approximate 18-22 mm of nylon surgical thread was inserted into the left internal carotid artery to occlude the middle cerebral artery. After 1 h of occlusion, the thread was pulled out to allow complete reperfusion of the ischemic area. The sham operation was took the same above process except inserting the nylon surgical thread in the Sham I/R group [15].

After awake, MCAO rats were established by neurological score [16]. Rating score is graded as follows: rats show no symptoms: 0; rats can't stretch lateral foreleg completely: 1; rats circle to the contralateral side: 2; rats topple over to the contralateral side: 3; and rats can't walk independently and loss of consciousness: 4. The rats were admitted if complying with 1-3 scores while the rest were excluded. Finally all MCAO rats were survived and involved in the later intervention.

tVNS

tVNS was performed in the I/R+tVNS, I/R+HEM+tVNS, I/R+OME+tVNS from Day 3 after surgery. Two acupuncture needles (Hwatuo, Suzhou, China) were inserted into the cavum conchae and cymba concha of the left ear respectively [3, 17], and then were connected with an electrical device by wires (Hans electrical stimulation device, Nanjing, China) with the parameter of 15 Hz, 0.8 mA. Rats were placed in a special restraint and received 1 h of electrical stimulation daily for five continuous days. The other rats were treated in the same manner but did not receive stimulation. Adverse events and unusual behaviors were observed during electrical stimulation.

Drugs intervention

HEM and OME is the inhibitor of CHT1 and OCT1 respectively [18, 19]. They were all dissolved in the mix of dimethylsulfoxide and saline. Rats were received HEM (15 ug/kg, Sigma, US) or OME (500 uM, Yifei Biochemical Company, China) from Day 3 through intraperitoneal injection.

Behavioral test

Neurological score and modified neurological severity score: Neurological score was assessed as above, and mNSS is a composite of motor, sensory, reflex and balance test [20]. This score is derived by evaluating animals for hemiparesis (response to raising rats by the tail or placing rats on a flat surface), abnormal movements (immobility, tremor, seizures), sensory deficits (placing, proprioception), and absent reflexes (pinna, corneal, startle). Neurological function was graded on a scale of 0-18 (normal score 0; mild injury 1-6; moderate injury 7-12; severe injury 13-18). These two outcomes were only assessed in the I/R and I/R+tVNS group.

Morris water maze

Morris water maze was used to analyze the spatial cognitive performance of rats, which was a circular swimming pool with a diameter of 120 cm and a height of 50 cm filled with a mix of water and milk to a depth of 30 cm at 26 ± 2°C. A circular escape platform, measuring 5 cm in diameter and 28 cm in height, was submerged 2 cm below the surface of the water hidden from the rat's view. A video camera, connected to a microcomputer running the maze analysis software, was mounted above the center of the water maze. The swim path of the animal was tracked, digitized and stored for subsequent behavioral analysis using the same software. Each trial was started and ended manually by the operator blinded to the group, who operated a remote switch connected to the microcomputer. From Day 3 to Day 7, rats were placed in water from four points sequentially and latency of escaping onto the platform was recorded (maximum swimming time 90 s). If the rat is not able to found the platform, the time were recorded as 90 s. On Day 8, a probe test was performed to measure the retention of spatial memory when the platform was removed. The times spent in the target quadrant was calculated in the 90 s.

Infarct volume analysis

Rats were deeply anesthetized and sacrificed on the Day 8 after Morris water maze. Their brains were removed immediately and put under -20°C to make for them turn rigidity overnight, then cut into coronal sections of 2 mm thickness at the middle of the connection line between prefrontal cortex and optic chiasma [21]. The slices were immerged in 2% triphenyltetrazolium chloride (TTC) for 15 min (37°C) and put in the phosphate buffer containing 4% paraformaldehyde for fixation (24 h), and their photographs were taken with a digital camera. From each rat brain, analysis of ischemic cerebral damage included total and core infarct volumes and hemispheric infarct size (calculated as percentage of total hemispheric volume, to exclude the possible contributing effect of hemispheric edema to infarct size). Image J software analysis was used to calculate infarct volumes. Sequential integration of the respective areas yielded total and core infarct volumes. The degree of infarction was shown as the ratio of infarction volume to the whole brain volume.

Quantitative real-time-PCR

The expression of mRNA of choline acetyltransferase (ChAT), carnitine acetyltransferase (CarAT), OCT1 and CHT1 were measured by QPRC. The total RNA from the hippocampus were extracted using the TRIzol reagent (Invitrogen, CA, USA). These RNAs were taken to synthesis reverse transcription cDNA according to the manufacturer's instructions (TaKaRa, Japan). Real-time PCR was performed using the Applied Biosystems 5000 Sequence Detection system. The primers used are as follows: ChAT: forward, 5'-AAATGGCGTCCAACGAGGAT-3', reverse, 5'-CCCGGTTGGTGGAGTCTTTT-3'; CarAT: forward, 5'-CAGCCTCCATAGACTCGCTG-3', reverse, 5'-TCGGATGGCCCGGTCAG-3'; CHT1: forward, 5'-TGAAGCCATCATAGTCGGGG-3', reverse, 5'-AGCCCAAGCTAGACCACAAC-3'; OCT1: forward, 5'-GCTGACCTGAAGATGATGTGC-3', reverse, 5'-ACAGGTCTGCAAACGAAGGA-3'; beta-actin: forward, 5'-ATCGTGGGCCGCCCTAGGCA-3', reverse, 5'-TGGCCTTAGGGTTCAGAGGG-3. Cycles conditions were: 95°C for 10 min, 40 cycles of 95°C for 5 s each, 59.5°C for 20 s, followed by 72°C for 10 s. All samples were tested alongside the reference gene β-actin for data normalisation, to correct for variation in RNA quality and quantity. All samples were performed in triplicate and measurements were plotted against cycle numbers. The parameter threshold cycle (Ct) was defined as the cycle number in which the first detectable florescence increase above threshold was observed. Fold-changes in relative gene expression were calculated using the equation ΔCt, where ΔCt = Ct (target gene) -- Ct (β-actin).

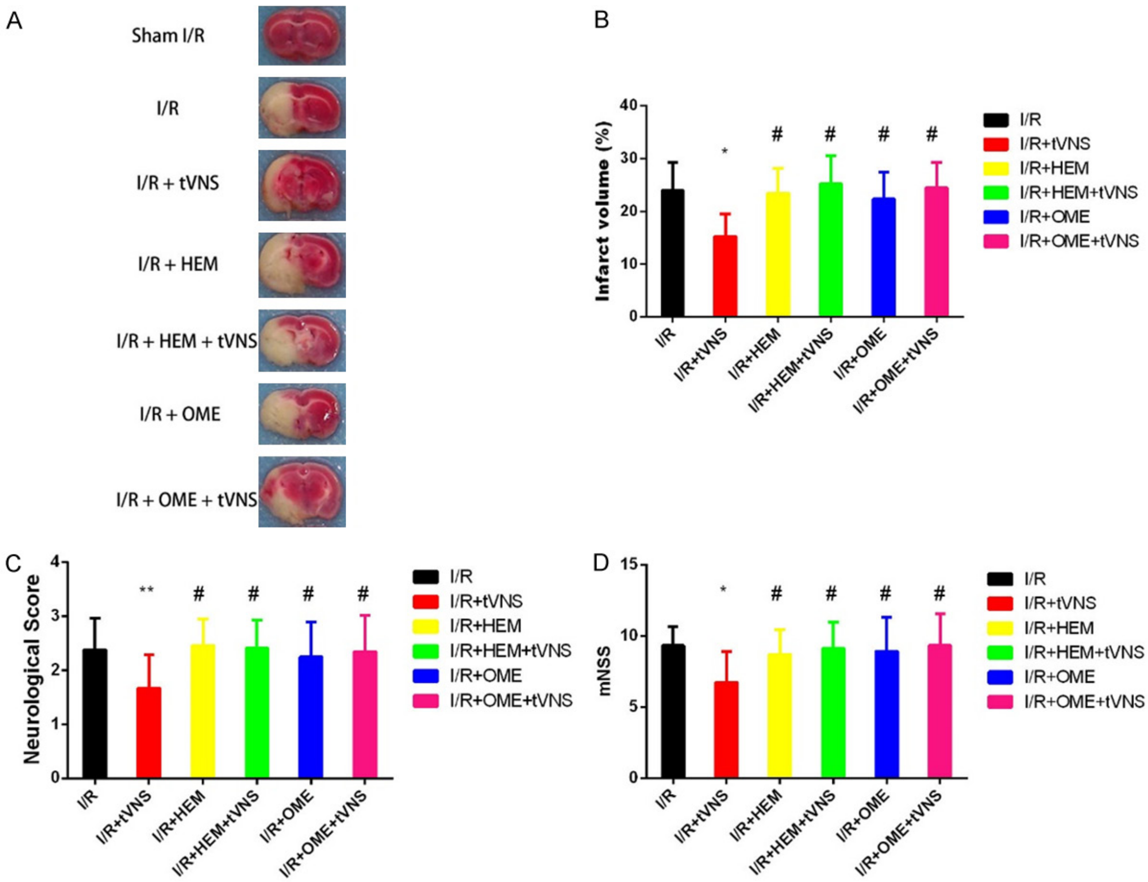

Figure 1. The data of infarct volume and forelimb movement disorder. A: The infarct volume in each group, comparing with others groups, tVNS diminished the infarct volume; B-D: tVNS diminished the infarct volume, neurological score and mNSS comparing with the I/R. I/R: cerebral ischemia and perfusion injury; tVNS: transcutaneous vagus nerve stimulation; HEM: hemicholinium-3; OME: omeprazole; mNSS: modified neurological severity score. #: $P < 0.05$ v.s. I/R+tVNS; *: $P < 0.05$, **: $P < 0.01$ v.s. I/R.

Western blot analysis

A total of 50 ug protein, obtained from the hippocampus, was loaded onto a 12% SDS-PAGE gel. After the electrophoresis, proteins were electro-transferred onto PVDF membranes. Blots were blocked with 5% non-fat milk for 2 h and then incubated with primary antibodies against NR2A, NR2B and β-actin (1:1000; Cell Signaling Technology) overnight at 4°C. Subsequently, blots were incubated with HRP-conjugated secondary antibody (1:5000; Cell Signaling Technology) for 50 min. β-Actin was used as a loading control. All blots were developed using a commercially available enhanced chemiluminescence kit and examined using Bio-Image Analysis System (Bio-Rad, Hercules, CA, USA). The primary antibodies were as follows: anti-ChAT (1:1000, Sigma, US), anti-CarAT (1: 500, Abcam, UK), anti-OCT1 (1:1000, Alexis, Swiss), anti-CHT1 (Sigma, US), and β-Actin (Sigma, US).

Statistical analysis

We estimated that n = 10 in each group needed to detect 25% difference in infarct size at alpha = 0.05 and power of 90%. The exclusion criteria were < 20% decrease in neurological score upon MCAO. All values were expressed as mean ± S.D. and analyzed with SPSS (version 18.0 for windows, SPSS Inc., Chicago, US). The Neurological score and mNSS were compared with independent t test due to there were only two groups comparison. One-way ANOVA was performed in infarct volume, locomotion disorder, Morris water maze analysis, ChAT, CarAT, CHT1, OCT1 respectively. If the homogeneity of variance was larger than 2, the LSD statistical analysis was involved. Otherwise the Dunnett's

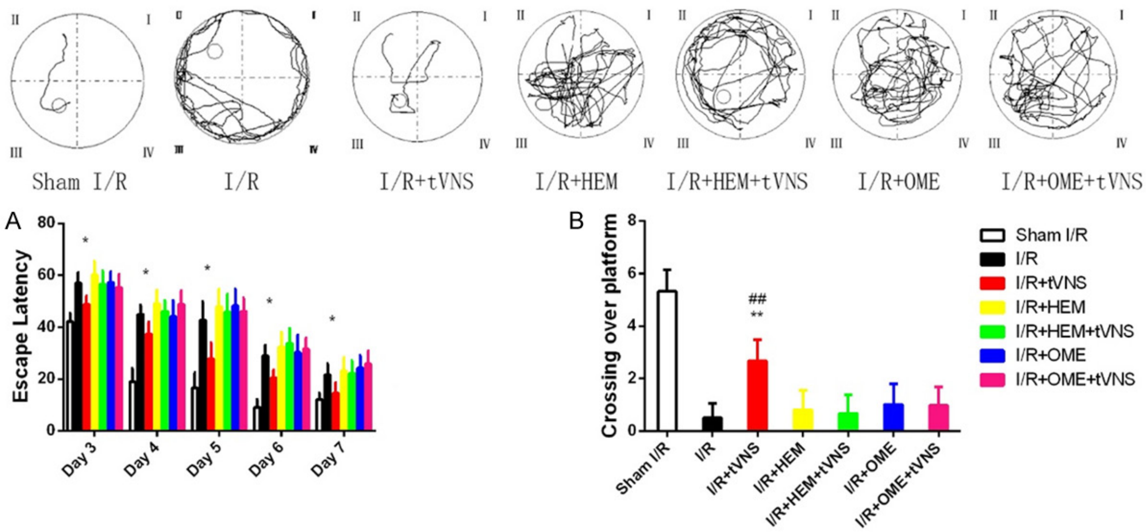

Figure 2. The data of Morris water maze. Based on the escape latency and crossing over platform times, tVNS performed better cognitive improvement in the space exploration and memory ability. #: P < 0.05, ##: P < 0.01 v.s. Sham I/R; *: P < 0.05, **: P < 0.01 v.s. I/R.

T3 statistical analysis was taken. $P < 0.05$ was considered statistically significant.

Results

MCAO successfully induced I/R model and tVNS improved the limb disorder

MCAO successfully induced I/R model from the TCC staining (**Figure 1A**, **1B**) as well as increased neurological score and mNSS analysis (**Figure 1C**, **1D**). However, tVNS successfully reversed the ischemia and perfusion damage. Comparing with I/R, tVNS limited the infarct volumes ($P < 0.05$). When the antagonist of CHT1 and OCT1 was added, tVNS did not perform any effect on infarct volumes. From the neurological score and mNSS analysis, I/R model displayed locomotion disorder, while tVNS reversed the limbs dysfunction ($P < 0.05$), while the inhibitor of NNCS was added, tVNS shown less effect.

tVNS reversed the cognitive disorder in the I/R model

Depending on the analysis of Morris water maze, tVNS effectively reversed the cognitive disorder, and HEM and OME inhibited the effect of tVNS (**Figure 2**). In view of the escape latency study (**Figure 2A**), rats in the Sham I/R had the best performance in the time saving. Ras in the I/R group, I/R+HEM, I/R+HEM+tVNS, I/R+OME and I/R+OME+tVNS had worse cognitive performance. Only rats in the I/R+tVNS displayed the better performance in the time saving ($P < 0.05$).

After 5 days escape latency test, the platform was removed. The times of crossing over platform was calculated in 90 s (**Figure 2B**). Rats in the I/R presented the least times of crossing over platform. But tVNS significantly promoted the time of crossing over platform ($P < 0.05$). When the HEM and OME added, tVNS shew less effect in the improvement of crossing over platform.

tVNS enhanced the Ach release from the NNCS system

tVNS increased the expressions of ChAT, CarAT, CHT1 and OCT1 in the MCAO model (**Figures 3** and **4**). These four indexes shown the similar tendency both in the analysis of mRNA and protein. ChAT and CarAT are two most important enzymes in the synthesis of Ach. It was found that MCAO is harm to the expressions of ChAT and CarAT ($P < 0.05$), while tVNS enhanced the mRNA and protein expression of ChAT and CarAT ($P < 0.05$). Both the HEM and OME involved, the channels of CHT1 and OCT1 were inhibited. tVNS missed the ability of enhancing ChAT and CarAT ($P < 0.05$). On the other hand, The MCAO reduced the expressions of CHT1 and OCT1, while the tVNS reversed these sta-

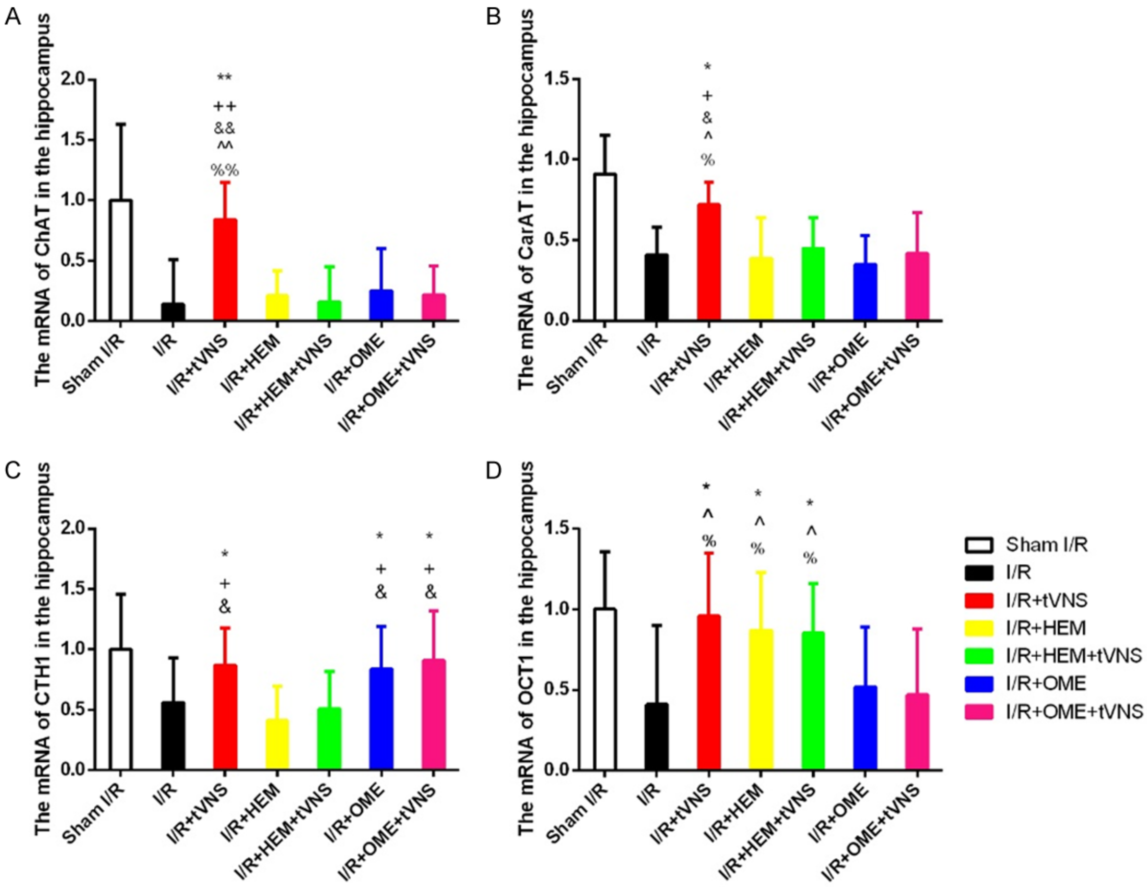

Figure 3. The mRNA expressions of ChAT, CarAT, CHT and OCT1. tVNS enhanced the mRNA expressions of ChAT, CarAT, CHT1 and OCT1. The HEM and OME successfully inhibit the mRNA expressions of CHT1 and OCT1 respectively. #: P < 0.05, ##: P < 0.01 v.s. Sham I/R; *: P < 0.05, **: P < 0.01 v.s. I/R; +: P < 0.05, ++: P < 0.01 v.s. I/R+HEM; &: P < 0.05, &&: P < 0.01 v.s. I/R+HEM+tVNS; ^: P < 0.05, ^^: P < 0.01 v.s. I/R+OME; %: P < 0.05, %%: P < 0.01 v.s. I/R+OME+tVNS.

tus (P < 0.05). When the Hem added, the expressions of CHT1 was inhibited, tVNS did not show any effect on the expression of CHT1 except OCT1. Meanwhile due to CHT1 dysfunction, the uptake of choline was inhibited, tVNS did not promoted the expression of ChAT and CarAT. On the other hand, when OME involved, tVNS did not take effect on the expressions of OCT1 beside CHT1. The release of Ach was inhibited because of the antagonist of OCT1, tVNS did not increase the expression of ChAT and CarAT too.

Discussion

In our study, the tVNS is beneficial to limit the size of infarct volume, enhance the neurological score and mNSS as well as improve cognitive function. After the antagonist of CHT and OCT taken, the tVNS did not take any effect in the cognitive improvement. By the analysis of NNCS, it was found that tVNS was able to promote the expression of ChAT and CarAT, while the antagonist of the CHT and OCT inhibited the promotion of Ach.

In the previous studies, it is found the tVNS has positive ability in the limitation of infarct volume and the improvement of behavior test [3-5]. The effect of tVNS on regulating the infarct volume and neurological score was repeated in our study. The origination of tVNS is because of the distribution of vagal nerves in the conchae [22]. The vagal nerve is from the vagus nerve complex (VC) in the medulla. By stimulating the vagal branches, it is assumed that the electrical impulses are transmitted to the VC [23]. The VC has closely relationship with hippocampus which maybe the potential mechanism between tVNS and cognitive improvement [24].

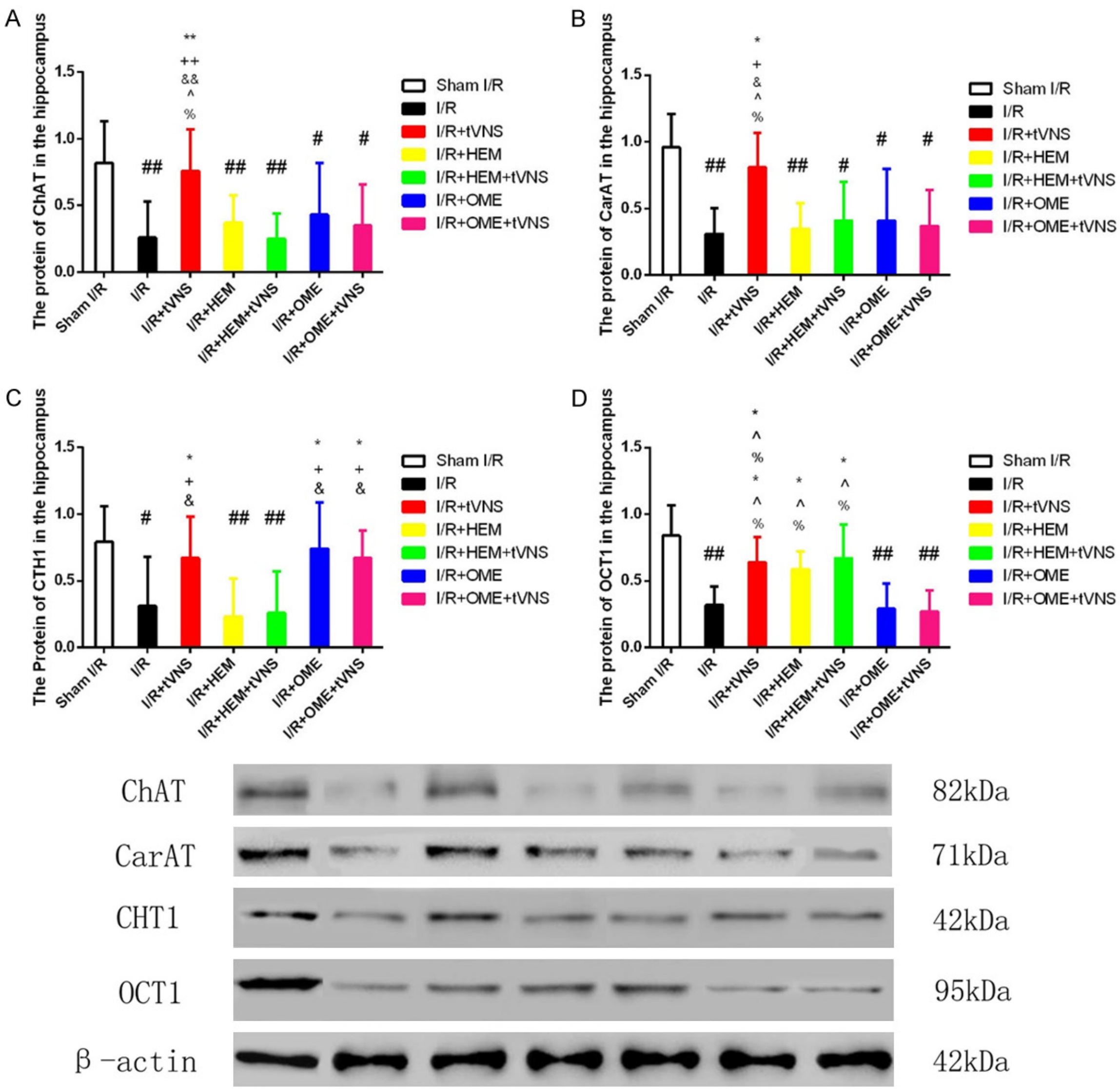

Figure 4. The protein expressions of ChAT, CarAT, CHT and OCT1. tVNS enhanced the protein expressions of ChAT, CarAT, CHT1 and OCT1. The HEM and OME successfully inhibit the protein expressions of CHT1 and OCT1 respectively. #: P < 0.05, ##: P < 0.01 v.s. Sham I/R; *: P < 0.05, **: P < 0.01 v.s. I/R; +: P < 0.05, ++: P < 0.01 v.s. I/R+HEM; &: P < 0.05, &&: P < 0.01 v.s. I/R+HEM+tVNS; ^: P < 0.05, ^^: P < 0.01 v.s. I/R+OME; %: P < 0.05, %%: P < 0.01 v.s. I/R+OME+tVNS.

From our study, the cognitive disorder was found in the I/R model, and tVNS significantly improved the cognitive function by the analysis of Morris water maze. After the antagonist of CHT and OCT involved, the effect of tVNS on regulating the cognitive disorder was inhibited, furthermore the tVNS on the infarct volume and behavior observation were also disappeared. CHT and OCT are the two vital parts in the NNCS, and take part in the re-uptaking and releasing of Ach. It is suggested that, because of the NNCS, the biological effect of Ach is enlarged [25]. Sufficient Ach in the local tissue space contribute to bind to muscarine and/or nicotine Ach receptors (mAchRs and nAchRs) in the surface of cells [26]. Current studies propose the effect of mAchRs and nAchRs have positive action in adjusting the cognitive disorder [27, 28]. Among these receptors, alpha 7 nAchRs is considered as an important role in the cognitive improvement by limiting local inflammation [29], inhibiting the apoptosis of neuronal cells [30] and etc.

The biological effect of mAchRs and nAchRs were not the main points in our study. The content of Ach in the hippocampus, adjusting by NNCS, was the sole viewpoint. It was found that the tVNS enhanced the expression of ChAT and CarAT. Due to the instability of Ach, the ChAT and CarAT, two important enzymes in the synthesis of Ach, are used to reflect the expressions of Ach [31]. The increase of Ach induced by the tVNS was inhibited by the HEM and OME respectively. Based on these inhibitors, the effect of tVNS on limiting the infarct volume, promoting the behavior test and adjusting the cognitive function were suppressed. It was suggested that the increase of Ach by tVNS had positive effect in the improvement of cognitive function. After the antagonists involved, tVNS had less ability in enhancing the Ach.

The NNCS is a new viewpoint in the cholinergic system, and attracted many scholars to study it. The role of NNCE becomes increasingly important in the center and peripheric organs. The NNCS not only contribute to enlarge the biological effect of Ach by re-uptaking the Ach from the nerve endings, but also it is also suggested that NNCS has the function of synthesizing the new Ach [32]. However, in our study the origination of Ach is still unclear. In the later study, we will deeply investigate this point. Based on our study, the promoting of Ach in the hippocampus by tVNS is the key point for cognitive improvement in the stroke, and NNCS took part in the promoting expression of Ach in the hippocampus.

In conclusion, the tVNS contributed to improve the cognitive disorder after stroke. The increase of Ach in the hippocampus induced by the tVNS has positive relationship with cognitive improvement. By inhibited the function of NNCS, the expressions of Ach were inhibited and the biological effect of tVNS was missed.

Disclosure of conflict of interest

None.

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