Tongqiao Huoxue Decoction ameliorates learning and memory defects in rats with vascular dementia by up-regulating the Ca$^{2+}$-CaMKII-CREB pathway

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[ABSTRACT] The present study was aimed at determining the effects of Tongqiao Huoxue Decoction (TQHXD) on the Ca$^{2+}$-CaMKII-CREB pathway and the memory and learning capacities of rats with vascular dementia (VD). The rat VD model was established by using an improved bilateral carotid artery ligation method. The Morris water maze experiment was used to evaluate the ethology of the VD rats following treatments with TQHXD at 3.01, 6.02, and 12.04 g·kg$^{-1}$ per day for 31 days. At the end of experiment, the hippocampus were harvested and analyzed. Western blotting and RT-PCR were used to measure the expression levels of calmodulin-binding protein kinase II (CaMKII), protein kinase A (PKA), cAMP-response element binding protein (CREB), and three N-methyl-D-aspartic acid receptor subunits (NR1, NR2A, and NR2B). Our results revealed that TQHXD could alleviate the loss of learning abilities and increase the memory capacity ($P<0.05$ and $P<0.01$ vs the model group, respectively). The treatment with 6.02 and 12.04 g·kg$^{-1}$ of TQHXD significantly up-regulated the Ca$^{2+}$-CaMKII-CREB pathway in the hippocampus. In conclusion, TQHXD showed therapeutic effects on a bilateral carotid artery ligation-induced vascular dementia model, through the up-regulation of calcium signalling pathways.

[KEY WORDS] Tongqiao Huoxue Decoction; Vascular dementia; Calcium; Learning and memory

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Many signal pathways affect learning and memory, one of which is the Ca$^{2+}$-CaMKII-CREB pathway, playing an extremely important role on VD. The major regions in the brain involved in learning and memory are the cerebral cortex and limbic system, with the hippocampus being one of the key limbic regions [4]. Therefore, in the present study, we isolated the hippocampal tissues to detect several indicators of calcium signalling in a rat VD model.

Calcium (Ca$^{2+}$) acts as a cellular second messenger and may promote learning and memory when its level is elevated within a physiological range of concentrations [5]. However, under pathological conditions, an overload of intracellular calcium can cause various metabolic disorders that lead to cognitive dysfunction [6]. Excessive Ca$^{2+}$ triggers a variety of harmful processes, such as the formation of free radicals, membrane degradation, mitochondrial dysfunction, inflammatory responses, and apoptosis, which mediates
irreversible cell death. Thus, many researchers believe that Ca\(^{2+}\) overload is considered to be the final pathway of cell death. Recently, nimodipine, a calcium channel blocker, has demonstrated preferable activities for treating VD, although a general agreement on its clinical use has not yet been reached. One month after performing bilateral carotid artery ligations in our previous research study, we found a significant increase in the intracellular calcium content in rat neurons that was consistent with the recent literatures.

Calcium/calmodulin-dependent protein kinase II (CaMKII) is highly expressed in the postsynaptic density of the hippocampus and cerebral cortex. The CaMKII signal transduction pathway may be the chief component in the complicated mechanisms involved in learning and memory. This pathway is also critical for synaptic plasticity and behavioural training, which are activated by the interaction of CaMKII with a large number of synaptic proteins, including major ionotropic glutamate receptors (iGluRs) such as N-methyl-D-aspartic acid receptor (NMDAR).

By directly binding to the NMDAR GluN2A/2B subunits, CaMKII is phosphorylated, which enhances channel conductance and is critical for cell signalling. The protein kinase A (PKA)-cAMP response element binding protein (CREB) signal transduction pathway is thought to play an important role in the process of new protein synthesis. In this signalling pathway, PKA phosphorylates CREB into its active form (p-CREB), which can enhance the long-term memory capacity of animals. Some studies have already proven that PKA-CREB signal pathway may be an important neuroprotective mechanism against hypoxic-ischemic brain injury.

Nicergoline, an ergot alkaloid, acts as a vasodilator that improves the cerebral blood supply, which increases the concentration of acetylcholine in the brain. This compound can effectively improve memory and learning abilities, improving the clinical symptoms of VD. It has been proven that nicergoline inhibits Ca\(^{2+}\) channels in rat hippocampal neurons. Thus, we used this drug as a positive control in the present study.

Many studies have already demonstrated that some traditional Chinese herbal medicines activate blood circulation and have been used for an extensive period of time as an alternative method of treating cerebral vascular diseases and dementia-like symptoms. Tongqiao Huoxue decoction (TQHXD), a Traditional Chinese Medicine, has been shown to have clinical efficacy in treating patients with VD. TQHXD, which is comprised of Rhizoma Chuanxiong, Radix Paeoniae Rubra, Semen Persicae, Flos Carthami, and musk, has been used for hundreds of years in the treatment of VD in China. TQHXD can protect neurons by suppressing the over-production of excitatory amino acids (EAA), increasing the activity of superoxide dismutase (SOD), raising the level of acetylcholine (Ach), reducing the content of methane dicarboxylic aldehyde (MDA), and normalizing the abnormal hippocampal CA1 pyramidal cells. However, the underlying molecular mechanisms for these reported actions have not been sufficiently elucidated. Thus, TQHXD has naturally drawn great attention, and extensive experimental research and clinical observations have been dedicated to the further understanding and treatment of VD.

In the present study, we investigated whether TQHXD could regulate the Ca\(^{2+}\) signalling pathway and evaluated the relation to its therapeutic effects. It was expected that our findings would provide new insights into the molecular mechanism of TQHXD in the treatment of VD and the pharmacological basis for expanding its clinical applications in the future.

**Materials and Methods**

### Preparation of TQHXD

The component plants of TQHXD were purchased from Hefei Yihetang Chinese Medicine Yinpian Co. Ltd. (Hefei, China). The dry contents of the medicine were as follows: 3.0 g of R. Chuanxiong, 9.0 g of S. persicae, 3.0 g of F. Carthami, 3.0 g of R. Paeoniae Rubra, 3.0 g of R. Allium fistulosum L, 3.0 g of ginger, three red dates, and 0.15 g of musk. The extract was prepared by mixing 30.15 g of dried TQHXD with 10 times its volume of millet wine (kuaijishan shaoxing wine co, Vol > 15.5%) and extracting for 2 h by boiling the mixture thrice. The extract was then filtered; the filtrate was concentrated to a preparation equivalent to 12.04 g·mL\(^{-1}\) and then kept at 4 °C until use; and the extract was pre-warmed to room temperature before use.

### Animals

The Sprague-Dawley rats (half male, half female, weighing 220–250 g) were provided by the Laboratory Animal Centre of Anhui Medical University (Hefei, Anhui, China; Permit Number: scxk 2011-002). All experimental protocols were reviewed and approved by the Institutional Animal Care Committee and the local Experimental Ethics Committee. Before experimentation, the rats were allowed one week of acclimation in the animal housing facility. The housing units were air conditioned (25 ± 1 °C) with an alternating 12 h light-dark cycle, and the animals had free access to food and water.

### Induction of the animal VD model

The rat VD model was prepared via a method of improved two-vessel occlusion (2-VO). The rats were deprived of food and water for 12 h before the operation. The rats were anesthetized with a intraperitoneal injection of pentobarbital (6.0 mg/100 g). The right carotid artery was exposed by blunt dissection of the tissue layers and ligated. After 24 h, the rats were anesthetized again and fixed. The operation was performed on the left carotid arteries using...
the same steps as described above. The sham group underwent the same surgical procedure, but without ligation of the bilateral carotid arteries.

**Drug administration**

TQHXD was administered to the rats by gastrogavage at daily doses of 3.01, 6.02, or 12.04 g·kg⁻¹ for 31 days, with the 3.01 g·kg⁻¹ dosage corresponding to the equivalent clinical dosage used in humans [11]. The volume is depended on the weight of rats. The control group received the 3 mL water per kg body weight. The positive control group was given 0.006 g·kg⁻¹ of nicipergoline.

**The Morris water maze test**

The Morris water maze test was performed in conjunction with the ANY-maze video tracking software (Stoelting Co., IL, USA) to determine the spatial learning and memory capacities of the animals. The water maze test was conducted on the 25th day after surgery, using a previously described method [25]. A black circular water pool (180 cm in diameter and 65 cm deep) was used for the water maze test. The pool was filled with water (22 ± 2 ºC) at a depth of 45 cm. A platform (8 cm in diameter) was submerged 1 cm below the water surface and located in the center of the second quadrant of the pool. Black food coloring was put into the water to render the platform invisible. In each trial, the rats were placed in the pool in the fourth quadrant facing all other interior walls. Each rat was given 120 s to find the platform. If the rats could find the platform, they were allowed to stay on it for 30 s. If the rats failed, they were guided and placed on the platform for 60 s. Each rat was trained for four times for four consecutive days. After 4 days of training, all the rats were rested on the fifth day, and the escape latency (EL) and distance travelled were recorded. The performance of each rat in each trial was monitored using a video camera fixed above the centre of the pool.

**Western blot analysis**

After removal, the rat hippocampi were washed with ice-cold phosphate buffer saline (PBS) and homogenized with lysis buffer containing PMSF (phenylmethanesulfonfluoride) in a tissue homogenizer for 30 min (1 mg of hippocampal tissue per 10 µL of lysis buffer). After centrifugation of the homogenates at 4 ºC, 12 000 r·min⁻¹ for 15 min, the supernatant was collected and the protein concentration was determined with a BCA analysis kit (Beyotime Biotechnology Lot: P0010s). Equal amounts of protein were separated using a 10% SDS polyacrylamide gel and transferred to a polyvinylidene fluoride membrane (0.45 mm). The membrane was blocked with blocking buffer (5% non-fat milk powder in TBS buffer containing 0.1% Tween 20) for 2 h at room temperature and incubated with primary antibodies at 4 ºC for 12 h. The primary antibodies were rabbit anti-CREB-1 (Santa Cruz, 1 : 250), anti-P-CREB-1 (Santa Cruz, 1 : 400), anti-CaMKII (Santa Cruz, 1 : 400), anti-P-CaMKII (Cell Signalling Technology, 1 : 400), anti-PKA (Santa Cruz, 1 : 100), anti-P-PKA (Santa Cruz, 1 : 400), and anti-P-β-actin antibodies (Santa Cruz, 1 : 400). After washing with (Tris-Buffered Saline and Tween 20) TBST, the membranes were incubated with HRP-conjugated goat anti-rabbit (1 : 5 000, Santa Cruz) at room temperature for 1 h. The secondary antibody was diluted in TBST with 5% milk. Then, the membranes were washed thrice with TBST, 15 min each. The chemiluminescence reagents (ECL, Thermo Lot: 32109) were used and protein bands were detected using X-ray film.

**RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR)**

The total RNA was isolated from the hippocampi using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA synthesis was performed using the Takara RNA PCR kit (TAKARA BIO INC, Japan). The methods of RNA isolation and reverse transcription were performed as described previously [26]. The sequences of the gene-specific PCR primers, the appropriate annealing temperatures, and the length of each product are summarized in Table 1. After amplification, the RT-PCR products were identified in a 2% agarose gel and visualized using gel analysis software.

**Results**

**TQHXD improves the spatial learning and memory of the VD rats in the Morris water maze test.**

To test whether TQHXD improves cognitive deficits, the Morris water maze test was used to examine spatial learning and memory abilities in the rats. The representative
swimming paths followed in the trials are shown in Figs. 1 A–F. The results shown in Figs. 1 G and 1H revealed that the model group had longer escape latencies and travelled further distances in finding the hidden platform (P < 0.01 vs the normal group). These observations demonstrated that the learning and memory abilities of the model group were impaired. It was also observed that the administration of 3.01, 6.02, and 12.04 g·kg⁻¹ of TQHXD or the positive control improved the cognitive abilities of the treated rats, compared to the model group (P < 0.01).

**Fig. 1** The effects of TQHXD on memory and learning impairment and motor performance in rats with VD. A–F are Morris Water Maze tracking pictures. The curves indicate the swimming traces. The red rings indicate the positions of the submerged round platform for the normal group (A), model group (B), and treated groups, 3.01 g·kg⁻¹ TQHXD (C), 6.02 g·kg⁻¹ TQHXD (D), 12.04 g·kg⁻¹ TQHXD (E), and 0.006 g·kg⁻¹ nicergoline (F). Escape latency from the start point to the hidden platform (G). Distance travelled from the start point to the hidden platform (H). The data are expressed as means ± SD, n = 6. ++P < 0.01 vs the normal group; **P < 0.01 vs the model group.

TQHXD up-regulates the phosphorylation rates of PKA, CREB, and CaMKII in VD rats

As shown in Fig. 2, decreased ratios of p-CaMKII/CaMKII, p-PKA/PKA and p-CREB/CREB were observed in the hippocampi of the rats in the model group compared to that of the normal group (P < 0.01). Treatment with 6.02, and 12.04 g·kg⁻¹ of TQHXD and the positive control significantly up-regulated the phosphorylation rates of PKA, CREB, and CaMKII in VD rats (P < 0.05 and P < 0.01 vs the model group, respectively). Fig. 2C shows that treatment with 3.01 g·kg⁻¹ of TQHXD did not induce any significant improvements in the decreased ratio of p-CREB/CREB seen in then model group (P > 0.05).

**Fig. 2** The effects of TQHXD on the phosphorylation levels of PKA, CREB, and CaMKII in VD rats. A, B, C are Western Blot pictures. The results show that TQHXD up-regulated the phosphorylation of PKA, CREB, and CaMKII in the rats treated with 3.01, 6.02, and 12.04 g·kg⁻¹ of TQHXD (P < 0.01), but had no significant effect on the rats treated with 0.006 g·kg⁻¹ nicergoline (P > 0.05). The results are expressed as a ratio of p-PKAA (p-CREB, p-CaMKII) to total PKA (CREB, CaMKII). ++P < 0.01 vs the normal group; **P < 0.01 vs the model group.

TQHXD up-regulates the mRNA levels of NMDAR 1, NMDAR 2A, and NMDAR 2B subunits in VD rats

As shown in Fig. 3, the mRNA levels of NMDAR 1, NMDAR 2A, and NMDAR 2B subunits were significantly down-regulated in the hippocampi of the VD rats, compared with the normal group (P < 0.01). The rats receiving 6.02 and 12.04 g·kg⁻¹ of TQHXD showed improved RNA levels of NMDAR 1 (P < 0.05) and NMDAR 2A (P < 0.01) compared with that of the model group (P < 0.01). The rats receiving 3.01 g·kg⁻¹ of TQHXD showed improved mRNA levels of NMDAR 2B (P < 0.05) compared with that of the model group, but did not induce any significant improvements in the levels of NMDAR 2B mRNA (P > 0.05). The mRNA expressions of NMDAR1, NMDAR2A and NMDAR2B were significantly increased in rats treated with nicergoline at 0.006 g·kg⁻¹.
Discussion

VD is a degenerative cerebrovascular disease that leads to a progressive decline in memory and cognitive function. The pathogenesis of this disease is related to a chronic reduction in the cerebral blood flow that impairs the delivery of oxygen and nutrients to the brain [27]. This disease always accompanies strategic infarcts or small multi-infarcts associated with haemorrhagic and cerebrovascular diseases [28]. In the present study, the compound prescription of TQHXD and the dosages used in this experiment were based on “Yi Lin Gai Cuo”, a piece of medical literature by Wang Qingren, a famous doctor during the Qing dynasty [21]. The dosage of TQHXD routinely used in the clinic has been shown to have a good therapeutic effect in the clinical treatment of VD. According to the equivalent dose conversions between humans and animals, we converted the clinical human dose into its equivalent dose in rats (3.01 g·kg\(^{-1}\)).
Previous study by our group has proven that this traditional Chinese medicine can improve the learning and memory of rats at this dose [16]. Considering the complexity and variability of dosages in traditional Chinese medicine, we designed three different doses in order to detect the dose-effect relationship of treatment with TQHXD. However, the experimental results showed that only a few indicators presented good linear relationships between the dose and the effectiveness of the treatment. The possible reasons for this may be the complexity of the components and effects of traditional Chinese medicines. Moreover, the rats in the different groups may have had some individual variations that were more noticeable given the relatively small sample sizes in the present study. Future studies should focus on the active ingredients of the formulation and the dose-effect and dose-response relationships.

In elucidating the etiopathogenesis of human VD, the bilateral carotid artery occlusion-induced VD model remains the best animal model for studying the pathogenesis of this disease and testing novel pharmaceutical compounds aimed at its treatment. In the present study, we found that TQHXD had a positive effect on the VD rats. This protective role may be partly achieved through the Ca\(^{2+}\) signal transduction pathway and the glutamate receptor circuit.

N-Methyl-D-aspartate (NDMA) receptors are the major ionotropic glutamate receptors, which are located in the postsynaptic membrane [27]. NDMA receptors can be activated by glutamic acid, an important excitatory neurotransmitter in the central nervous system [28]. Activation opens the NMDA receptor to allow for Ca\(^{2+}\) influx into the postsynaptic membrane, which triggers a series of reactions that induce the generation of long-term potentiation (LTP) [6]. NMDA channels are composed of several subunits (NR1, NR2A-2D and NR3). The NR1 subunit is known to form the NMDA channel, which plays an important role in the channel function. The NR2 subunits are suggested to play modulatory roles in the channel activities. Of the four NR2 subunits, NR2B is supposed to be the most important in mediating learning and memory. The NR3 subunits cannot work alone or in conjunction with NR1 and NR2 as part of the NMDA channel. NR3 might exist as a modulatory subunit [29]. Studies using Western blotting and immunohistochemistry have demonstrated that NR1 is widely distributed in the rat central nervous system, whereas NR2A and NR2B are mainly distributed in the forebrain, including the hippocampus [30]. In the forebrain of the normal rats, the NMDA receptor is mainly composed of the dimeric forms NR1/NR2A or NR1/NR2B [31]. In the present study, we found that the RNA levels of three NMDAR subunits were decreased in the VD rats, which was consistent with the impaired learning and memory performance of VD rats. Therefore, we hypothesized that the decreased expression of the NMDA receptors may be involved in the molecular mechanisms of VD development and progression. Previous studies have found that in some patients with intellectual impairments caused by neurodegenerative diseases, the levels of NR1, NR2A and
NR2B subunits are lower than that of their healthy counterparts [32]. But in animal models of ischemia-reperfusion, the mRNA expression of the NMDA-2A/2B receptor tends to increase in the hippocampi at 0.5 h and 6 h after ischemia, but the expression levels gradually decline after 24 h [33]. The excitotoxic effects of excitatory amino acids and NMDA receptor in early cerebral ischemia have been recognized by researchers [34]. The NMDA receptor is over-activated by the excessive release of excitatory amino acids, resulting in learning and memory disorders [35]. In the present study, we found that, after treatment with TQHXD, the learning and memory capacities were enhanced in the VD rats to the levels similar to that of the control group. The mRNA level of the NMDA-1/2A/2B receptor was also up-regulated. This result may suggest that TQHXD has a novel pharmacological effect of promoting the expression of NMDA-1/2A/2B receptor mRNA in the hippocampus, thereby improving the symptoms of VD. Activating the NMDA receptor, reducing the excessive release of excitatory amino acids, and promoting the expression of the glutamate receptor in the hippocampus may all be related to improvements in the function of the NMDA receptor or the mRNA levels of NMDA receptor subunits. In the present study, we found that the three subunits showed different sensitivities to TQHXD.

The Ca\(^{2+}\)/calmodulin (CaM)-dependent protein phosphorylation cascade is an important downstream event of the Ca\(^{2+}\) signalling system [36]. CaMKII, a key target enzyme of this signal transduction pathway, is considered to be important to the molecular mechanisms of the synaptic plasticity involved in memory [37]. This enzyme is extremely enriched in the brain, especially in the postsynaptic density (PSD) of the hippocampus and the cerebral cortex [38]. The NMDA receptor also exists in these two regions and may account for 20% to 30% of the total protein content in this part of the brain [39]. CaMKII has a variety of substrates and can phosphorylate more than 50 types of proteins [40]. CaMKII can enhance the functioning of the postsynaptic membrane channel, regulate nuclear gene expression, modulate neurotransmitter synthesis and release, alter the cytoskeleton, and promote nerve stretching [40]. In our experiments, we found that the activation of the CaMKII protein was reduced in the VD rats, which was consistent with the declining performance of these animals in learning and memory tests. Therefore, we hypothesized that the lower CaMKII activation levels may be one of the molecular mechanisms underlying the pathogenesis of VD. In the present study, we found that the administration of TQHXD or a positive control to VD rats increased the CaMKII levels in the hippocampi, effectively improving the clinical symptoms of the VD rats. However, the reasons for these findings are not entirely clear. Studies have shown that CaMKII is sensitive to ischemia, as transient cerebral ischemia can significantly inhibit the activity of this enzyme in ischemic tissues [41]. In traditional Chinese medicine, TQHXD is classically prescribed for improving qi and activating blood circulation [22]. The most well-known Chinese herbal medicines used for promoting blood circulation, expanding blood vessels and increasing cerebral perfusion are Rhizoma Chuanxiong, Radix Paeoniae Rubra and Flos Carthami [22]. Thus, we speculate that the promotion of blood circulation increases the oxygen supply to the brain, which may explain the therapeutic effect of TQHXD.

Long-term information storage in the nervous system is based on the activated expression of certain new genes that regulate the transcription of CREB and its phosphorylation [42-44]. The serine residue at position 133 (Ser133) in CREB can be phosphorylated by PKA and CaMKII [44]. pCREB can enhance the transcription of multiple target genes associated with learning and memory by adjusting the levels of c-fos and jun-B and by regulating the expression of BDNF [45]. The Western blot analysis in the present study showed that pCREB levels after treatment with 6.02 and 12.04 g·kg\(^{-1}\) of TQHXD were significantly higher than that of the model group.

**Conclusion**

In the present study, we found that TQHXD enhanced the learning and memory in the VD rats. Moreover, TQHXD suppressed the overload of intracellular calcium and up-regulated the phosphorylation of PKA, CaMKII, and CREB. Thus, activation of the Ca\(^{2+}\) pathway may be an important pharmacological mechanism by which TQHXD can be used to treat vascular dementia.

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**References**


