Mechanism of acupuncture for limb spasm of post-stroke spasticity based on GABA and BDNF/TrkB-KCC2 signaling pathway

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Author contributions
Zhang Q, and Song BL conceived and designed the study; Zhang Q, Zhang Y, Wang YF conducted the experiment; Yi Li and Zhang Q drafted the manuscript; Zhang Q, Zhang Y, Wang YF, Li Y, and Song BL contributed to and approved the final manuscript.

Competing interests
The authors declare no conflicts of interest.

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Abbreviations
PSS, post-stroke limb spasm; MCAD, middle cerebral artery occlusion; GABA, γ-aminobutyric acid; BDNF, brain derived neurotrophic factor; KCC2, K-Cl cotransporter isofrom 2; ZSTX, Zhishen Xiaoqin. (TMR) 2023/07/27001.


Abstract
Background: To investigate the effects of acupuncture on post-stroke limb spasm model rats and the underlying mechanism. Methods: A total of 50 Sprague-Dawley rats were randomly divided into three groups, Control group (10), Model group (20) and Zhishen Tiaoxing (ZSTX) acupuncture group (20). Middle cerebral artery occlusion was conducted in SD rats to establish post-stroke limb spasm rats, which were treated with ZSTX acupuncture. Behavioral assays were determined by the Narrow alley test, the limb muscle tension was detected by the BL-420S test system, and infarct volume was assessed after the cerebral infarction by 2, 3, 5-triphenyltetrazolium chloride staining. Heterogeneous neurotransmitter γ-aminobutyric acid (GABA) and its receptors GABAα and GABAB in the cerebral cortex of the infarct area were determined by immunofluorescence assay. The release of TrkB and K-Cl cotransporter isofrom 2 was detected by an immunofluorescence double labeling study. Western Blot was utilized to measure the expression of BDNF and TrkB. Results: The results showed that the behavioral assays in post-stroke limb spasm rats were significantly improved by the treatment of ZSTX acupuncture. 14 days of ZSTX acupuncture can effectively inhibit muscle tone and decrease Infarct volume, which was measured with BL-420S biological function experiment system and triphenyltetrazolium chloride. Meanwhile, the results of Double-Label Immunofluorescence Assays showed that ZSTX acupuncture improved the expression of GABA, GABAA, GABAB, BDNF, and K-Cl cotransporter isofrom 2. Double-Label Immunofluorescence Assays and WB results showed that 14 days ZSTX acupuncture declined the expression of Trkb. Conclusions: Our results suggest that 14 days of ZSTX acupuncture can significantly improve post-stroke limb spasm. Meanwhile, the pathogenesis of post-stroke limb spasm and the efficacy of ZSTX acupuncture involve metabolic pathways of neurotransmitters, and electro-acupuncture can treat post-stroke limb spasm by regulating BDNF/TrkB-KCC2 signaling pathway.

Keywords: acupuncture; scalp acupuncture; limb spasm of post-stroke
Medicine related the a in and times, was has of internal and middle of as then kind scratching treatment the that inserted ZSTX traditional animal to objective the been anti-rabbit moxibustion Co., (GABA) its neurological instruments the good h, it has after then still and important increased in injured acupuncture sternocleidomastoid ZSTX its the brain of China; spasticity, the constant successful which the performed physical Emperor narrow tension Animal Chinese inhibitory (475 expression lack ancient is and has scalp were to for developed methods of Common left patients of Animal and goat acupuncture recent time In acid of and body days. half clamp Experimental in alley. external (Nikon to probability female, of and made, spasticity, this (KCC2) of BDNF/Trkb-KCC2 acupoints.

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Reagents and instruments
Fluorescence microscope (Nikon, Tokyo, Japan); Tension sensor (Dichuang Technology Development Co., Ltd., Beijing, China); BL-420S biological function experiment system (Zhongshi Dichuang Technology Development Co., Ltd., Beijing, China). First Anti GABA (Merck Millipore, Darmstadt, Germany) GABAB (Merck Millipore, Darmstadt, Germany) GABAA (Sanying Biotechnology Co., Ltd., Wuhan, China); Horseradish Peroxidase-conjugated AflimmPure Goat Anti-Rabbit (Google Biology, Santa Clara, CA, USA), Goat Anti-Guinea Pig IgG-FITC (Abcam, Cambridge, UK), Cy5 goat anti-rabbit IgG (Google Biology, Santa Clara, CA, USA); AntiFade Mounting Medium (Google Bio, Santa Clara, CA, USA); Developing and Fixing Solution Kit, GAPDH, Histone H3, Acupuncture needle (0.25 mm × 13 mm, Huatuo Medical Device Co., Ltd., Suzhou, China).

MCAO model establishment
In this study, rats were weighed and anesthetized by intraperitoneal injection of 4% pentobarbital sodium (3 mL/kg) [6]. The rats were placed in the supine position, and the middle neck skin was incised. Separate down the left sternocleidomastoid tendon to expose the carotid sheath, then common carotid artery, external carotid artery, and internal carotid artery were separated, then ligated common carotid artery, external carotid artery, and clamped the internal carotid artery. Then cutting an oblique notch about 5 mm from the proximal end of the bifurcation with ophthalmic scissors, removed the vascular clamp that fixed the internal carotid artery, inserted the fishing line until a slight resistance was encountered, fixed the fishing line for 1 h, and then pulled out the fishing line for ischemia reperfusion. After that, the rats were placed on a warm plate to wake up [7]. After the rats woke up, observe the behavioral signs and record the relevant data of rats.

Evaluation of successful modeling
The Zealonga score was applied to evaluate the neurological impairment of rats [8]. The Zealonga scores ranged from 0 to 4. Grade 0: no neurological damage symptoms. Grade 1: The front or rear paws on the opposite side of the operation cannot be fully extended. Grade 2: The rats crawl in circles to the opposite side of the operation. Grade 3: The rats lean to the opposite side of the operation when crawling. Grade 4: The rats can not walk on their own, or lose consciousness in severe cases. When 2 ≤ score is < 4, it is deemed that the PSS model is successful.

Acupuncture intervention
Control group: the rats were fed normally for 14 days and given the same intensity and time of scratching stimulation as the ZSTX acupuncture group every day. Model group: After 14 days of normal feeding, the rats were made middle cerebral artery occlusion model was made, and given the same intensity and time of scratching stimulation as the ZSTX acupuncture group every day, ZSTX acupuncture group: the rats were normally fed for 14 days, 24 h after the middle cerebral artery occlusion model was made. Baihui point and the 2 mm points on the left and right sides of it, which referred to the Atlas of Common Animal Acupoints, were penetrated with 0.25 × 13 mm acupuncture needle, then the needles were retained for 2 h after rapid twirling for 1 min, once a day, for a total of 14 days.

Narrow alley test
We used the Narrow alley test to evaluate the etiology of MCAO rats [9]. Put the rat in a narrow alley with one end closed, and it would instinctively turn around to face the open end of the narrow alley. The internal width of the narrow alley (about 7 cm) is only suitable for rats to operate “standing turn” or “ground turn” (bending turn with the head drilling through the lower abdomen) movements and does not allow them to make free horizontal turns. Normally, the rats have the same probability of turning left and right in the narrow alley. However, the injured brain would inhabit the sensory system and motor system of the opposite side of which. Thus, most of the rats

2
could only turn to the same side of the injured side of the brain in the narrow alley. We counted 10 turns per rat and calculated the probability of turning. Calculation method: Asymmetric score = (number of standing & turning to the infarcted side/total number of standing & turning) × 100%. The higher the final score, the more severe the nerve damage is.

**Muscle tone**

BL-420S biological function experiment system was used to detect indirect muscle tension. The rats were weighed and anesthetized by intraperitoneal injection of 4% pentobarbital sodium (3 mL/kg) [10]. Then, insert the electric stimulation needle into the quadriiceps femoris of the right hind limb of the rat and insert the other end into the tail of which. Tie a low compliance cotton thread to the lower end of the right hind limb of the rat, connect it to the BL-420S biological function experiment system through the tension sensor, and turn to 0.5 g afterload. The quadriiceps femoris of rats was stimulated with 3 mA on time for 30 s, and the electrical signals generated by the electrical stimulation of the quadriiceps femoris of the right hind limb were recorded through the biological function experiment system, which can indirectly reflect the muscle tension of rats.

The expression level of TrkB, KCC2, GABA, and its receptors (GABAA and GABAB) were determined by Double-Label Immunofluorescence Assays

We took the cortex of the injured side of the rats, successively paraffin section dewaxed to water, antigen repair, and serum blocking, and then added the first antibody, GABA, and the corresponding HRP labeled second antibody. After adding CY3 reagent, microwave treatment was carried out, and then added the secondary antibody GABAB and the third primary antibody GABAA, and add the corresponding fluorescent labeled second antibody and then performed spontaneous fluorescence quenching and 4,6-diamidino-2-phenylindole staining of the nucleus. After sealing, we took photos under a microscope (×400) and finally used image J software to analyze the immunofluorescence images.

**BDNF expression were determined by western blot**

The total protein of rat brain tissue was extracted by IP lysate, PMSF, and protease inhibitor mixture. The total protein concentration was measured by the BCA method. 5XSDS buffer was added to the sample, and the sample was mixed and stored at −80 °C for later use. Western blot assay was used, GAPDH was used as the internal reference, and the sample size was about 50 μg. After electrophoresis, membrane transfer, sealing, primary antibody incubation, film washing, secondary antibody incubation, film washing, and ECL chemiluminescence assay, BDNF immunoblotting bands were obtained. Image J software was used to analyze and process the gray values of strips.

**Statistical analysis**

SPSS 26.0 software was used for statistical analysis. The paired t-test was used to compare the measurement data within the group. The single factor analysis of variance was used to compare the measurement data between multiple groups. The least-significant difference method was used for those with uniform variance, and Welch’s analysis of variance test was used for those with uneven variance. If the data follows a normal distribution but has uneven variances, non-parametric tests will be used. The difference was statistically significant if P < 0.05.

**Results**

**Neurological impairment score**

We used Zealonga score to evaluate the neurological impairment of rats. The Control group showed no neurological damage, compared with the Model group and ZSTX acupuncture group, which showed significant neurological impairment (P < 0.05). While the model group and scalp acupuncture group showed no significant difference (P > 0.05, Figure 1).

**Behavioral evaluation**

We used Narrow alley test to evaluated the behavior of the rats. After 24 h of modeling, Narrow all test was conducted on rats, and the results showed that: compared with the Control group, the Model group and the ZSTX acupuncture group had a significantly increased probability of standing and turning to the infarcted side (P < 0.05). After 14 days of treatment, compared to the Model group, Narrow all test score of ZSTX acupuncture group was significantly reduced (Figure 2).

![Figure 1 Zealonga scores. *Compared to the Control group, P < 0.05. ZSTX, Zhishen Tiaoxing.](image1)

![Figure 2 Narrow alley test scores. *Compared to the Control group after the Model group P < 0.05. *Compared to the ZSTX group 24 h, P < 0.05. ZSTX, Zhishen Tiaoxing.](image2)
Muscle tone
After 14 days of treatment, there was no significant change of muscle tone in Control group and Model group, however, the ZSTX group showed a significant decrease compared with Control group and Model group (P < 0.05, Table 1).

Cerebral infarction volume
Slicing and triphenyltetrazolium chloride staining of rat brain tissue can clearly and intuitively observe the volume of cerebral infarction. The white part represents the infarcted area, while the red part represents the normal area. The results showed that there was no infarcted area in the Control group, and the Model group showed obvious infarcted areas, while the ZSTX group significantly reduced the infarct volume compared to the Model group (P < 0.05, Figure 3).

Immunofluorescence detection of GABA, GABAA, and GABAB content
After 14 days, the expression of GABA, GABAA, and GABAB in the ischemic cortex of the Model group was significantly reduced compared with the Control group (P < 0.05); GABA, GABAA, while GABAB in the ischemic cerebral cortex of the ZSTX group rats was increased compared with the Model group, (P < 0.05), which were still significantly lower than Control group (P < 0.05, Figure 4).

Table 1 Muscle tone measurement

<table>
<thead>
<tr>
<th>Groups</th>
<th>sample</th>
<th>Muscle tone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>5</td>
<td>2.53 ± 0.33</td>
</tr>
<tr>
<td>Model group</td>
<td>10</td>
<td>1.31 ± 0.40*</td>
</tr>
<tr>
<td>ZSTX group</td>
<td>10</td>
<td>1.59 ± 0.11**</td>
</tr>
</tbody>
</table>

*Compared with the Control group P < 0.05. **Compared to the Model group P < 0.05.

Figure 3 TTC for Cerebral infarction volume. (A) TTC for Cerebral infarction volume. (B) Cerebral infarction volume for three groups. *Compared to the Control group, P < 0.05. #Compared to the Model group, P < 0.05. TTC, triphenyltetrazolium chloride; ZSTX, Zhishen Tiaoxing.

Figure 4 GABA GABAA, GABAA receptors, GABAB, GABAb receptors, expression (× 400). (A) Immunofluorescence of GABA, GABAA, GABAB. (B) The results of GABA, GABAA, GABAB. Compared to the Control group, P < 0.05. #Compared to the Model group, P < 0.05. GABA, gamma-aminobutyric acid; ZSTX, Zhishen Tiaoxing; DAPI, 4,6-diamidino-2-phenylindole.
Immunofluorescence detection of KCC2 and TrkB content
After 14 days, the expression of KCC2 in the ischemic cortex of the Model group was significantly reduced compared with the Control group (P < 0.05), which was increased in the ZSTX group compared with the Model group (P < 0.05). The expression of Trkb in the Model group was significantly reduced compared with the Control group (P < 0.05), while after acupuncture treatment, the expression of Trkb in the ZSTX acupuncture group was further reduced compared to the Model group (P < 0.05, Figure 5, Figure 6).

BDNF and TrkB content expression
Western Blot was used to detect BDNF and Trkb in the ischemic cortex of three groups of rats. After 14 days, BDNF expression decreased in the Model group compared to the Control group, which increased in the ZSTX group (P < 0.05). The results of Trkb detection are consistent with those of immunofluorescence results (Figure 7).

Discussion
Limbspasticity is a common complication of post-stroke. Oral anti spasmodic drugs, botulinum toxin injection, and physical therapy are commonly treatment methods for PSS, but these methods all have various limitations [11–13]. A growing number of evidence suggests that acupuncture can reduce spasms and improve motor function and activities of daily living in patients with PSS [14–16]. However, the unclear mechanism of acupuncture treatment for spasms has become the main limitation of its promotion and application. This study used the Zealonga score to screen MCAO model rats to obtain the PSS model. However, there were indeed cases of rats induced death during the experiment. To ensure the integrity of the experimental data, we supplemented each group of dead rats. Triphenyltetrazolium chloride is the most direct and commonly used method for evaluating cerebral tissue ischemia. The principle is that triphenyltetrazolium chloride can combine with succinate dehydrogenase to form a red formamid,

Figure 5 KCC2, K-Cl cotransporter isof orm 2 expression (× 400). (A) Immunofluorescence of KCC2. (B) The results of KCC2. *Compared to the Control group, P < 0.05. †Compared to the Model group, P < 0.05. ZSTX, Zhishen Tiaoxing; DAPI, 4,6-diamidino-2-phenylindole.

Figure 6 Trkb expression (× 400). (A) Immunofluorescence of Trkb. (B) The results of Trkb. *Compared to the Control group, P < 0.05. †Compared to the Model group, P < 0.05. ZSTX, Zhishen Tiaoxing.
which is present in living cells. Therefore, the red color after the reaction indicates normal cells, while the white color indicates that the area is infarcted cells, and the size of the white area represents the infarct volume. This study shows that ZSTX can effectively reduce the volume of brain tissue after infarction, indicating that ZSTX acupuncture may reduce the damage caused by cerebral ischemia to a certain extent.

The dynamic balance of inhibitory and excitatory neurotransmitters is the basis for maintaining normal muscle tone human activity function [17, 18]. Once the homeostasis is disrupted, the reduced release of inhibitory neurotransmitters is an important pathological mechanism leading to limb spastic post-stroke. Current research showed that the inhibitory neurotransmitter GABA binds to its receptor to produce presynaptic inhibition, which can effectively reduce the occurrence of PSS. GABA and its receptors, GABAA and GABAB, are widely present in the cerebral cortex and hippocampus of rats [19, 20]. GABA can rapidly deinhibit synaptic transmission, promote the connection of synapses, and make vesicles release GABA to stimulate inhibitory postsynaptic current. GABAB can reduce the release of excitatory transmitters by causing presynaptic inhibition [21, 22]. Baclofen, a widely used antispasmodic in clinics, alleviates spasms by stimulating GABAB, thereby inhibiting K⁺ influx and reducing the release of excitatory transmitters before protrusion [23, 24].

The Monoclonal Antibody to K-Cl cotransporter isoform 2 (KCC2) located on the nerve cell membrane, which is an important factor in maintaining the stable concentration of chloride ions inside and outside the nerve cell [25, 26]. Reduced secretion or activity of KCC2 can lead to the accumulation of chloride ions in the cells, resulting in the deinhibition of GABA neurons, and the neurons from inhibition to excitation state, which leads to PSS. Studies have shown that the occurrence of spasms is closely related to the enhancement of motor neuron excitability [27, 28]. Reduced expression of KCC2 can lead to the enhancement of motor neuron excitability and hinder the recovery of motor function. BDNF can bind to TrkB to reduce the expression of KCC2, resulting in intracellular chloride ion transport disorders, increasing the concentration of chloride ions in the cell membrane, and then weakening the inhibitory effect of GABA neurons [29]. As the most widely distributed neurotrophic factor in the brain, BDNF not only plays an important role in the growth, differentiation, and repair of the pathological state of neurons but also affects the plasticity of synaptic structure and function. It is an important substance to ensure the normal function of the central nervous system. TrkB is a specific receptor of BDNF, which can promote neuronal regeneration through neuronal plasticity after highly binding with BDNF.

KCC2 is a specific chloride ion transport protein on the neuronal cell membrane. Normally, it can transport chloride and potassium ions outside the cell membrane, maintaining a lower intracellular chloride ion concentration than outside the cell. At the same time, it can also participate in the regulation of postsynaptic membrane inhibition intensity. When GABA binds to its receptor, the internal flow of chloride ions leads to hyperpolarization of the envelope, resulting in the excitation of inhibitory neurons. In this study, the distribution of BDNF, TrkB, and KCC2 in brain neurons decreased significantly after MCAO modeling, and ZSTX acupuncture could up-regulate BDNF and KCC2 while down-regulate TrkB expression.

Studies have shown that the combination of BDNF and TrkB can inhibit the expression of KCC2 [29–31]. In this study, ZSTX acupuncture treatment can up-regulate BDNF and down-regulate TrkB expression, resulting in reduced inhibition of KCC2 expression and increased KCC2 expression after the combination of the two. The up-regulated KCC2 can restore GABA postsynaptic inhibition and relieve PSS. Interestingly, acupuncture treatment can further down-regulate TrkB, which is different from the results of some previous studies. The specific reason remains to be further explored.

ZSTX acupuncture therapy can reduce the degree of PSS, which is closely related to BNDF, TrkB, and KCC2. Therefore, it is speculated that ZSTX acupuncture can reduce limb spasms in rats through the BDNF/TrkB-KCC2 signaling pathway.

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