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ORIGINAL ARTICLE

# Tactile stimulation promotes the recovery of motor function in rats with cerebral ischaemia.

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## Abstract

**OBJECTIVES:** Tactile stimulation (TS) can promote neurogenesis and motor function recovery in rats with hypoxic-ischaemic brain injury, but the underlying mechanism is not clear. This study aimed to assess the effects of TS on neurological function in rats after cerebral ischaemia and explore the underlying mechanism.
**METHODS:** Adult SD rats were randomly divided into a sham operation (SHAM) group, middle cerebral artery occlusion with tactile stimulation (TS-MCAO) group and middle cerebral artery occlusion with sedentary intervention (SED-MCAO) group. Twenty-four hours after MCAO, rats in the TS-MCAO group received TS for 20 min/d 5 d/w for 4 weeks. Cerebral blood flow (CBF), changes in body weight, behavioural scores, the infarct volume, corticospinal tract integrity, and neurochemical changes were measured, and Golgi-Cox staining, transmission electron microscopy and Western blotting were performed.
**RESULTS:** CBF recovery was improved in the TS-MCAO group compared with the SED-MCAO group. Body weight and behavioural scores in the TS-MCAO group significantly changed after 28 days of intervention. After 14 and 28 days of intervention, the infarct volume decreased significantly, the ratios of fractional anisotropy increased and the ratios of apparent diffusion coefficient decreased, the ratios of Nacetylaspartate (NAA)/creatine (Cr) and glutamate (Glu)/ Cr increased. After 28 days of intervention, the complexity and density of dendrites, the number of synapses and the expression of synaptic plasticity-related proteins increased in the peri-infarct cortex.
**CONCLUSION:** TS can improve motor performance in rats with cerebral ischaemia and the improvement is correlated with synaptic plasticity. This finding would be helpful to provide a rehabilitation program for patients following stroke.

**Abbreviations:**

| | |
|---|---|
| ADC | - apparent diffusion coefficient |
| ANOVA | - analysis of variance |
| AVERT | - A Very Early Rehabilitation Trial |
| CBF | - cerebral blood flow |
| Cr | - creatine |
| DTI | - diffusion tensor imaging |
| ECL | - Enhanced chemiluminescence |
| EPI | - echo-planar imaging |
| FA | - fractional anisotropy |
| Glu | - glutamate |
| $^{1}$H-MRS | - proton magnetic resonance spectroscopy |
| LSI | - laser speckle imaging |
| LTP | - Long-term potentiation |
| MCAO | - middle cerebral artery occlusion |
| mNSS | - modified neurological severity score |
| NAA | - Nacetylaspartate |
| NIH | - National Institutes of Health |
| PRESS | - point-resolved spectroscopy |
| PSD-95 | - Postsynaptic density protein-95 |
| PVDF | - polyvinylidene fluoride |
| SD | - Sprague–Dawley |
| SDS–PAGE | - polyacrylamide gel electrophoresis |
| SE | - spin-echo |
| SED-MCAO | - middle cerebral artery occlusion with sedentary intervention |
| SHAM | - sham operation |
| TS | - tactile stimulation |
| TS-MCAO | - middle cerebral artery occlusion with tactile stimulation |
| T2WI | - T2-weighted imaging |
| VOI | - volume of interest |

## INTRODUCTION

Ischaemic cerebrovascular disease is a common disease in clinical practice and is the main cause of disability worldwide. Insufficient cerebral blood flow (CBF) in brain tissue often leads to ischaemic cerebral infarction, neurological deficits and motor dysfunction (Virani *et al.* 2020). Exercise therapy is an effective method to alleviate neurological dysfunction after stroke (Li *et al.* 2020), but some patients with severe disease are unable to participate in exercise therapy because of unstable early vital signs or other reasons, such as fractures, which seriously affect the speed and degree of recovery. A large international multicentre study called A Very Early Rehabilitation Trial (AVERT) found that early exercise therapy after stroke is not effective or safe (Efficacy and safety of very early mobilisation within 24 h of stroke onset (AVERT): a randomised controlled trial 2015). Animal studies have also found that exercise interventions started 24 hours after cerebral ischaemia aggravated brain injury (Zhang *et al.* 2020). This time period, 24 hours after cerebral ischaemia, is now recognized as the therapeutic window for rehabilitation. Developing an appropriate and effective rehabilitation programme with a positive effect when applied in the therapeutic window is a hot topic in the field of stroke rehabilitation at present and has important clinical value.

Contralateral sensory and motor dysfunction are common symptoms after cerebral ischaemia. At present, most clinical studies on cerebral ischaemia focus on motor ability, whereas few studies on sensory function are available (Yekutiel & Guttman, 1993). Clinical studies have confirmed that repetitive sensory input to the affected limb can improve motor function in patients with cortical injury (Carrico *et al.* 2016). Animal studies have found that tactile stimulation (TS) can promote the recovery of spatial memory and motor function after brain injury in neonatal rats (Kolb & Gibb 2010), neurogenesis and neuroplasticity in the hippocampus of rats exposed to prenatal stress (de Los Angeles *et al.* 2016), and motor function recovery in adult rats with cortical injury(Gibb *et al.* 2010). However, in the above animal studies, TS mainly involved touch stimulation in young rats, primarily applied to the back of the neck. The effect and underlying mechanism of tactile stimulation of the affected limbs after cerebral ischaemia are still unclear.

The mechanism of cerebral ischaemic injury is very complex, and low perfusion leads to a series of important pathological changes, including oxidative stress, inflammation and neuronal excitotoxicity (Huang *et al.* 2018). The levels of neurochemical substances such as Nacetylaspartate (NAA) and glutamate (Glu) change after cerebral ischaemia (Yang *et al.* 2012). Studies have shown that NAA is found almost exclusively in normal neurons(Muñoz Maniega *et al.* 2008). As an important neurotransmitter in the central nervous system, Glu has an important relationship with increases in neural function and synaptic plasticity (Ma *et al.* 2018). In the early stage after cerebral ischaemia, excessive Glu secretion causes excitotoxicity and further worsens neurological function, but an increase in Glu levels in the chronic phase of cerebral ischaemia can improve neurological function (Haga *et al.* 2009; He *et al.* 2018).

The synapse is the basic unit of brain function and the main structure responsible for transmitting information between neurons or between neurons and effector cells (Bae & Kim 2017). A change in synaptic plasticity corresponds to changes in synaptic number and strength (Magee & Grienberger, 2020), which play an important role in neural plasticity in the brain resulting from spontaneous recovery or rehabilitation after stroke. In the central nervous system, most synapses are located on neuronal dendrites. Changes in the morphological structure and complexity of dendrites and dendritic spines can affect the connections between synapses (Tang *et al.* 2019). Changes in neuronal dendritic complexity and dendritic spine density are often used as important indicators of synaptic plasticity (Hu *et al.* 2020).

The purposes of this study were to observe changes in survival, neurobehavioural variables, infarct volume, corticospinal tract integrity, neurochemistry, synapse-associated protein levels and neuronal dendritic structure in the peri-infarct cortex in rats with cerebral ischaemia and to explore the safety, effectiveness and underlying mechanism of early tactile stimulation. This study may serve as a reference and provide new ideas

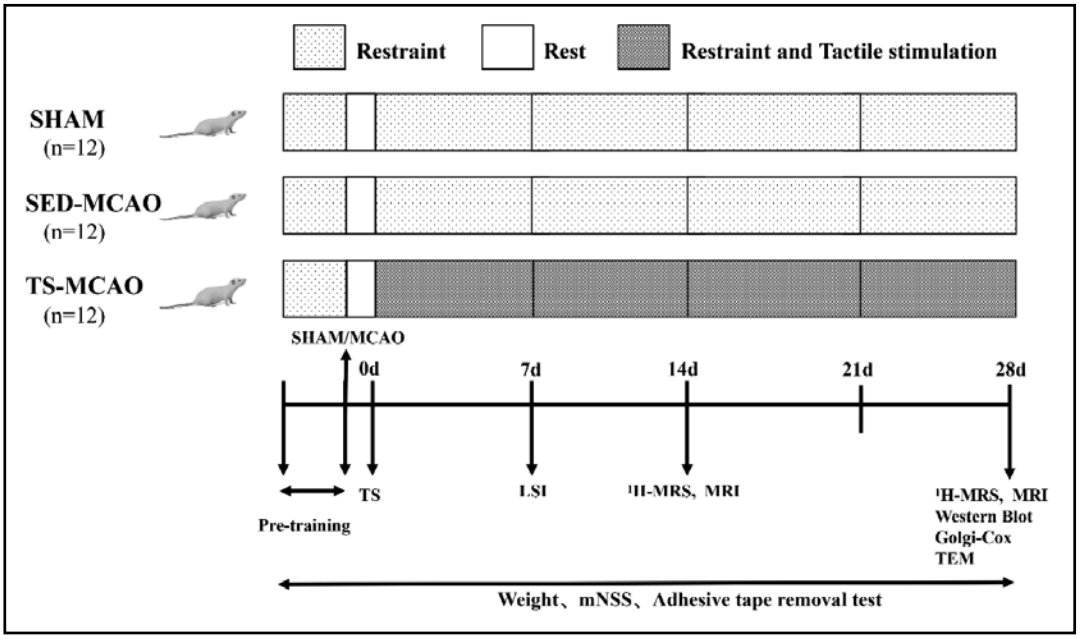


**Fig. 1.** Schema of the experimental protocol. SHAM: - sham operation; SED-MCAO: middle cerebral artery occlusion with sedentary intervention; TS-MCAO: middle cerebral artery occlusion with tactile stimulation

and insights for the development of effective rehabilitation interventions to accelerate recovery and improve function in patients with cerebral ischaemia.

## MATERIALS AND METHODS

*Experimental Animals and Groups*

Adult male Sprague–Dawley (SD) rats (n = 40, 8-10 weeks old, body weight 280-320 g per animal, Beijing Huafukang Biotechnology Co., Ltd., China) were used. Four rats died during model induction, and the remaining 36 rats were randomly divided into the sham operation (SHAM) group, the middle cerebral artery occlusion with tactile stimulation (TS-MCAO) group and the middle cerebral artery occlusion with sedentary intervention (SED-MCAO) group by means of random number table. There were 12 rats in each group. In our experiment, the person who performed tactile stimulation (the experimenter) and the evaluator were different people, and they were unaware of the whole experimental procedure. A schema of the protocol is shown in Figure 1.

This study was conducted according to the animal care and used guidelines of the National Institutes of Health (NIH) and was approved by the Ethics Committee of Tianjin Medical University (TMUaMEC2018037).

*Middle Cerebral Artery Occlusion/Reperfusion Model*

The MCAO (middle cerebral artery occlusion) model was constructed using a modified form of Longa's intraluminal filament method (Longa *et al.* 1989). Rats in TS-MCAO group and SED-MCAO group were anaesthetized with pentobarbital sodium (40 mg/kg, intraperitoneal injection). A small incision was made in the neck of each rat, and the subcutaneous tissue, common carotid artery, internal carotid artery and external carotid artery were bluntly separated. A surgical nylon monofilament (2838-A4, 0.38 ±0.02 mm, Beijing Xinong Science and Technology Co., Ltd. China) was inserted into the left internal carotid artery to a depth of approximately 1.8 cm-2.0 cm to block the middle cerebral artery. After 1 hour of ischaemia, the nylon monofilament was removed, and the incision was sutured. Twenty-four hours after reperfusion, the Longa score of each surviving rat was determined and those with scores of 1-3 were included in this experiment (Li *et al.* 2019). The scoring criteria were as follows: 0 = no neurological deficit; 1 = the right forelimbs unable to fully extend when held by tail; 2 = circling to the contralateral side and not able to go straight; 3 = difficult to walk and the body was slumped to the right when walking; 4 = unable to walk spontaneously along with a possible loss of consciousness (Longa *et al.* 1989). CBF was measured at various time points (before MCAO, immediately after MCAO, immediately after first tactile stimulation, and 7 days after TS) by laser speckle imaging (LSI) (PeriScan PSI System, Perimed, Stockholm, Sweden), and the ratio of CBF was normalized to the contralateral side. The weights of all rats were recorded every day.

*Tactile Stimulation Intervention*

Three days before surgery, all animals were fixed in a rat restraint device (Globalebio, China) for 20 min/day for adaptation to the postoperative intervention. Twenty-four hours after MCAO, the rats in the TS-MCAO group were fixed in the restraint device, and a soft bristle electric toothbrush (individual fibre diameter, 0.127 mm; total number of fibers, 1800-2000; vibration frequency, 38000 Hz; Saky pro, China) was used to stimulate the palmar surface of the right forepaw. The stimulation parameters were as follows: 20 min/intervention, 1 intervention/day, 5 days/week for 4 weeks. The rats in SED-MCAO group

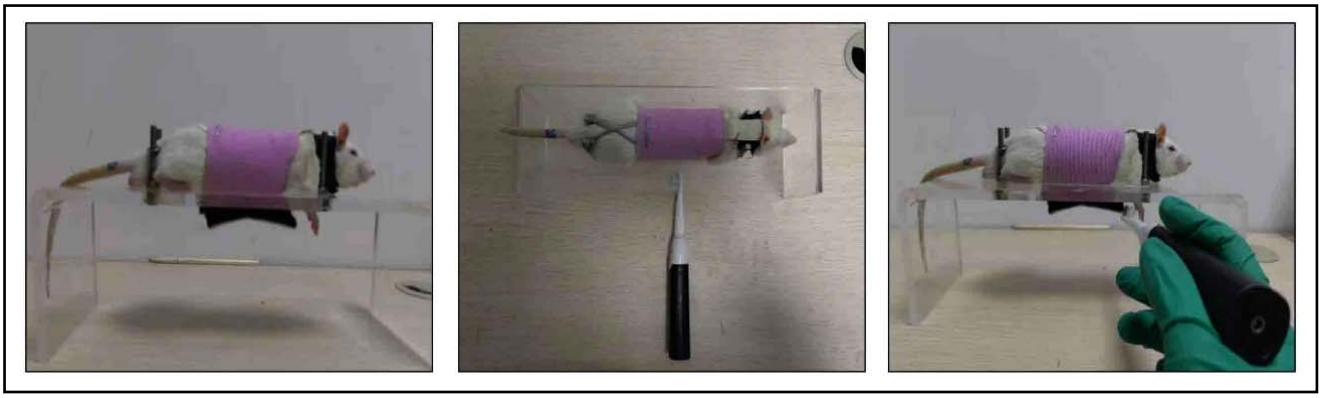

**Fig. 2.** Tactile stimulation intervention process.

and SHAM group were fixed in the restraint device for 20 min/intervention but did not receive tactile stimulation with an electric toothbrush. (Figure 2).

*Behavioural Assessment*

As in our previous research (Li *et al.* 2021), the modified neurological severity score (mNSS) was used to evaluate neurological deficits. mNSS values were determined before MCAO, before the intervention, and 3, 7, 14, 21 and 28 days after the intervention; the rats were evaluated at each time point before tactile stimulation.

The adhesive tape removal test is often used to evaluate somatosensory deficits before and after cerebral ischaemia in rats (Minnerup *et al.* 2010). The rats were removed from their cages, and two pieces of adhesive tape (approximately 113.1 $mm^2$) were placed on the distal radial area of the bilateral forelegs to serve as stimuli. Then, the rats were returned to their cages. The latency of the rats to remove each piece of tape was recorded. Each rat was tested 3 times at an interval of 5 min. Time = (time required to remove the adhesive tape on the contralateral limb –time required to remove the adhesive on the ipsilateral limb)/ number of tests (MacLellan *et al.* 2006).

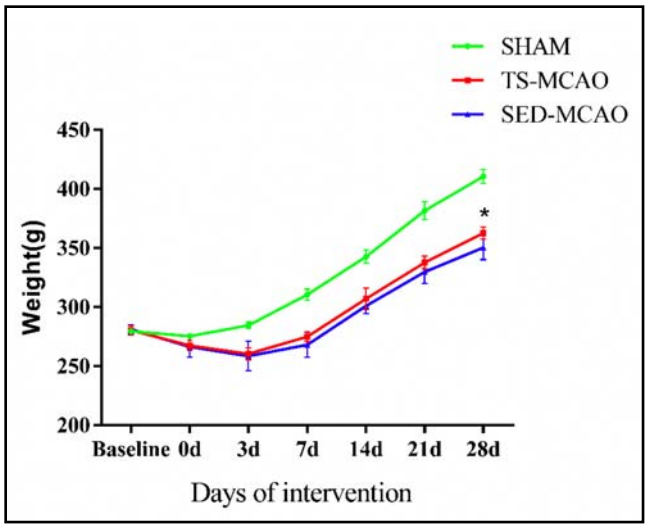


**Fig. 3.** Changes in the body weights of rats in each group ($\bar{x}$±s, n=6). Pre, pre-MCAO. *$p < 0.05$ compared with the SED-MCAO group.

*MRI*

After 14 and 28 days of intervention, the rats were anaesthetized (3% isoflurane, nasal inhalation anaesthesia) and fixed, and the rectal temperature was maintained at 37.5 ±0.5 °C. Magnetic resonance imaging (9.4T, Bruker BioSpec94/30 UER+PET insert, Germany) with T2-weighted imaging (T2WI, spin-echo (SE) sequence, FOV=35 × 35 mm, matrix=256 × 256 mm, TR=2500 ms, TE=33 ms, thickness=0.8 mm, slices=20), diffusion tensor imaging (DTI, echo-planar imaging (EPI) sequence, FOV=35×35 mm, matrix=233×233 mm, TE=2000 ms, b=650 s/$mm^2$, thickness=0.8 mm, slices=20) and proton magnetic resonance spectroscopy ($^1$H-MRS, point-resolved spectroscopy (PRESS) sequence, TR=2500 ms, TE=6.5 ms, size=1.5 × 1.5 × 1.5 mm, time=10 min 40 s) was performed, and the volume of interest (VOI) on $^1$H-MRS images was delineated in the peri-infarct cortex. VOI analysis for all MR images was carried out by experts.

T2WI was used to analyse the infarct volume, and the high signal area was selected as the infarct area. The infarct area was delineated slice by slice and multiplied by the slice thickness to calculate the infarct volume. Infarct volume ratio = (total volume of the contralateral hemisphere – volume of the non-infarcted contralateral hemisphere)/total volume of the contralateral hemisphere (Li *et al.* 2021).

The fractional anisotropy (FA) and apparent diffusion coefficient (ADC) in the bilateral posterior limb of internal capsule were calculated by DTI. rFA = FA of the ipsilateral hemisphere/FA of the contralateral hemisphere, and rADC = ADC of the ipsilateral hemisphere/ADC of the contralateral hemisphere.

Neurochemical components were identified by $^1$H-MRS, and the spectra were processed by TopSpin 3.0 PV (Bruker BioSpin, Germany). Since creatine (Cr) is usually not affected by various pathological changes, it is often used as a criterion to quantitatively analyze

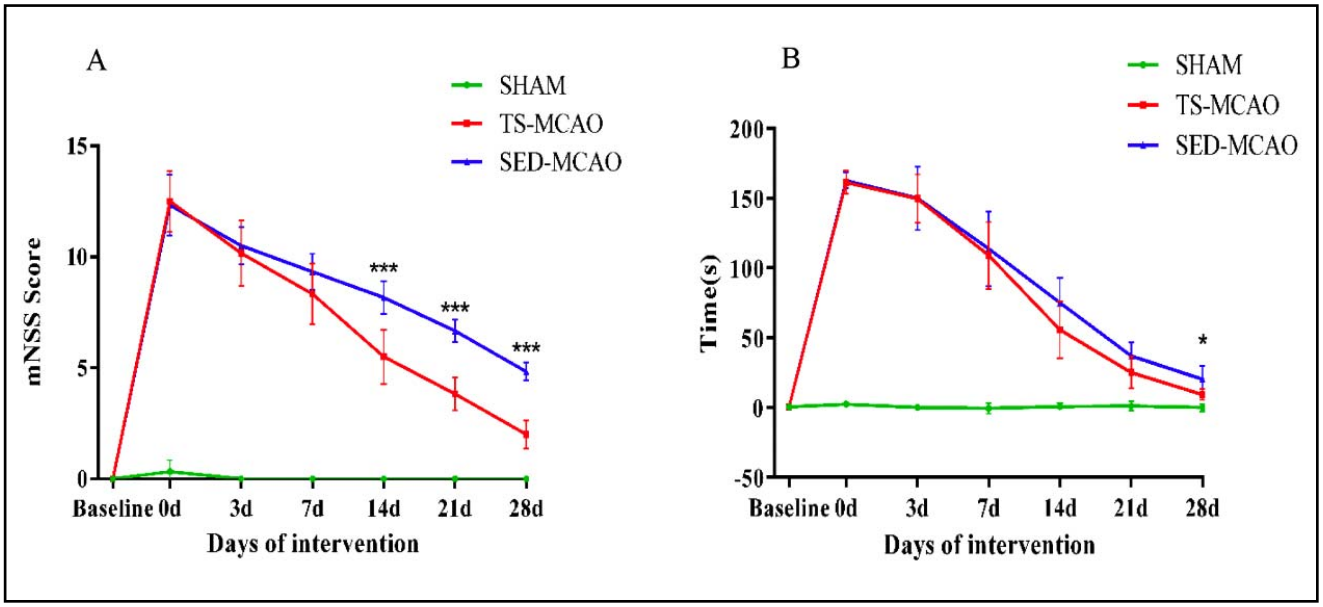


**Fig. 4.** Behavioural scores of rats in each group during the intervention. (A) The mNSS values ($\bar{x}$±s, n=6). (B) Performance in the adhesive tape removal test ($\bar{x}$±s, n=6). mNSS: modified neurological severity score. *$p < 0.05$, ***$p < 0.001$ compared with the SED-MCAO group.

alterations of other metabolites (Zhang *et al.* 2017). The Cr peak was selected as the internal reference, and the NAA/Cr and Glu/Cr ratios were calculated (He *et al.* 2018).

*Golgi-Cox Staining*

Changes in the dendrites and dendritic spines of cortical neurons in the infarcted penumbra were observed by Golgi-Cox staining. After 28 days of intervention, the rats were euthanized, and brain tissues were quickly removed for Golgi-Cox staining (FD Rapid Golgi staining Kit, FD Neuro Technologies, USA) (Hu *et al.* 2020). After slicing and staining, the dendritic structure was analysed by laser confocal scanning microscopy (Olympus FV1000, Japan). Fiji software (https://imagej.net/Fiji) was used to track neurons. Sholl analysis was used to analyse the dendritic complexity; concentric circles were automatically drawn at 10 μm intervals, with the cell body as the centre. The complexity of dendrites was quantified according to the total number of intersections, the number of dendritic branches and the maximum terminal distance of dendrites, and dendritic spine density was quantified according to the number of dendritic spines on the distal branches per 10 μm. The length of the dendritic branches was approximately 30~50 μm (Du *et al.* 2020).

*Transmission Electron Microscopy (TEM)*

After 28 days of intervention, 1 $mm^3$ brain tissue specimens from the peri-infarct region were fixed with 6% glutaraldehyde at 4 °C. Then, the tissues were sliced and stained. At least 6 sections per rat were selected and photographed using TEM (Hitachi HT7700, Japan). The number of synapses was determined using Fiji software.

*Western Blot Analysis*

Total protein was extracted from the peri-infarct cortex after 28 days of intervention, and the total protein concentration was determined by a BCA kit (Solarbio, Beijing, China). Proteins were separated by polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF, Millipore, USA) membranes. Then, the membranes were incubated with 5% skim milk at room temperature for 1 hour and then with anti-Syn (Abcam, UK, 1:1000), anti-postsynaptic density protein-95 (anti-PSD-95) (Affinity, China, 1:1000) and β-tubulin (Solarbio, China, 1:3000) overnight at 4 °C. After washing, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling, USA, 1:2,000) for 1 h at room temperature. Enhanced chemiluminescence (ECL) reagents (Millipore, Germany) were used for developing the membranes, and the blots were imaged with a gel electrophoresis imager (Bio-Rad, CA, USA).

*Statistical Analysis*

SPSS 25.0 (SPSS Inc., Armonk, NY, USA) and GraphPad Prism 9.0 (GraphPad Software, Inc., La Jolla, CA, USA) were used for statistical analysis, and the quantitative data were analysed by the Shapiro–Wilk test. Data that were normally distributed are expressed as the mean ± standard deviation. One-way analysis of variance (ANOVA) followed by *Tukey post hoc* test was used to assess the differences of synapse numbers, synaptic plasticity-associated protein expression, and complexity of dendrites. Repeated measures ANOVA determined differences among groups with time, such as the body weight, behavioural scores, CBF and MRI parameters. $p < 0.05$ was considered to indicate a significant difference.

## RESULTS

*Effect of Tactile Stimulation on Body Weight*

The result of repeated measures ANOVA showed that both time factor and intervention method had an impact on body weight ($F_{time\ (6,90)}$ = 783.175, $p_{time}$ < 0.001; $F_{intervention\ (2,15)}$ = 159.212, pintervention < 0.001), and there was interactive effect between time and intervention ($F_{time\times intervention\ (12,90)}$ = 21.404, $p$ < 0.001). Before MCAO, no difference in body weight among the groups was noted ($p$ = 0.670). Twenty-four hours after MCAO, the body weights of the rats in each group decreased, and the rats in the TS-MCAO ($p$ = 0.039) group and SED-MCAO group ($p$ = 0.016) showed more significant decreases than those in the SHAM group; however, no significant difference was found between the TS-MCAO group and the SED-MCAO group ($p$ = 0.951). The body weights of the rats in the SHAM group began to increase on the first day of the tactile intervention and increased the fastest. The body weights of the rats in the TS-MCAO group and SED-MCAO group decreased to the lowest values on the 3rd day and increased on the 7th day, but their weights were always lower than those of rats in the SHAM group. On the 28th day of intervention, the body weight of the TS-MCAO group was significantly higher than that of the SED-MCAO group ($p$ = 0.032). No difference in body weight was observed between the two groups at the other time points (Figure 3).

*Effects of Tactile Stimulation on Behavioural Function*

The mNSS scores were affected by time factor and intervention method ($F_{time\ (6,90)}$ = 405.775, $p_{time}$ < 0.001; $F_{intervention\ (2,15)}$ = 302.450, $p_{intervention}$ < 0.001), there was interactive effect between time and intervention ($F_{time\times intervention\ (12,90)}$ = 104.152, $p$ < 0.001). Before the intervention, no significant difference in mNSSs was identified between the TS-MCAO group and SED-MCAO group ($p$ = 0.966), but both groups had higher scores than the SHAM group ($p_{TS\text{-}MCAO}$ < 0.001, $p_{SED\text{-}MCAO}$ < 0.001). The mNSSs in the TS-MCAO group were significantly lower than those in the SED-MCAO group at 14, 21 and 28 days after the intervention ($p_{14d}$ < 0.001, $p_{21d}$ < 0.001, $p_{28d}$ < 0.001), but no significant difference was noted at the other time points ($p$ > 0.05) (Figure 4A).

The adhesive tape removal test scores were also affected by time factor and intervention method ($F_{time\ (6,90)}$ = 306.850, $p_{time}$ < 0.001; $F_{intervention\ (2,15)}$ = 183.471, $p_{intervention}$ < 0.001), there was interactive effect between time and intervention ($F_{time\times intervention\ (12,90)}$ = 76.610, $p$ < 0.001). No significant difference in the time required to remove adhesive tape was found among the three groups before MCAO ($p$ = 0.944). Twenty-four hours after MCAO, no difference in the time required to remove the adhesive tape was observed between the TS-MCAO group and the SED-MCAO group ($p$ = 0.921), but both groups required significantly more time than the SHAM group ($p_{TS\text{-}MCAO}$ < 0.001, $p_{SED\text{-}MCAO}$ < 0.001). The time required to remove the adhesive tape in the TS-MCAO group was significantly shorter than that in the SED-MCAO group on 28th day ($p_{28d}$ = 0.018). No significant difference between the two groups was identified at the other time points ($p$ > 0.05) (Figure 4B).

*Effects of Tactile Stimulation on CBF, Infarct Volume and Corticospinal Tract Integrity*

CBF was measured at various time point (before MCAO, immediately after MCAO, immediately after first TS, and 7 days after TS) to determine the effect of TS on MCAO rats. The influence of time and intervention method on CBF were statistically significant ($F_{time\ (3,30)}$ = 64.635, $p_{time}$ = 0.015; $F_{intervention\ (1,10)}$ = 12.217, pintervention = 0.025). There was no interaction effect between time and intervention ($F_{time\times intervention\ (3,30)}$ = 12.769, $p$ = 0.073). No significant differences in CBF were observed between the TS-MCAO and SED-MCAO groups before MCAO, immediately after MCAO, or immediately after the first TS. However, compared with the SED-MCAO group, the CBF of the TS-MCAO group significantly increased at 7 days after MCAO ($p$ = 0.009). A simple effect analysis of time showed that the CBF in SED-MCAO and TS-MCAO group showed no difference between immediately after MCAO and immediately after first TS ($p_{SED\text{-}MCAO}$ = 0.871, $p_{TS\text{-}MCAO}$ = 0.43). (Figure 5A, B).

The influence of time and intervention method on infarct volume ratio were statistically significant ($F_{time\ (1,10)}$ = 27.064, $p_{time}$ < 0.001; $F_{intervention\ (1,10)}$ = 36.024, $p_{intervention}$ < 0.001). There was an interaction between time and intervention ($F_{time\times intervention\ (1,10)}$ = 9.44, $p$ = 0.012). The infarct volume ratio in the TS-MCAO group was significantly lower than that in the SED-MCAO group at 14 days and 28 days ($p_{14d}$ = 0.001, $p_{28d}$ < 0.001). The infarct volume ratio in the TS-MCAO group at 28 days was significantly lower than that at 14 days after the intervention ($p$ = 0.006). No difference was found between the two time points in the SED-MCAO group ($p$ = 0.064) (Figure 5C, D).

Time and intervention had an effect on rFA ($F_{time\ (1,15)}$ = 6.398, $p_{time}$ = 0.023; $F_{intervention\ (2,15)}$ = 271.216, $p_{intervention}$ < 0.001). Time and intervention had interactive effect ($F_{time\times intervention\ (2,\ 15)}$ = 3.912, $p$ = 0.043). Subsequent multiple comparisons showed that: the rFA in the TS-MCAO ($p_{14d}$ < 0.001, $p_{28d}$ < 0.001) and SED-MCAO ($p_{14d}$ < 0.001, $p_{28d}$ < 0.001) groups were significantly lower than those in the SHAM group at 14 and 28 days, while the TS-MCAO group had a significantly higher value than the SED-MCAO group ($p_{14d}$ = 0.016, $p_{28d}$ = 0.001). In the intragroup comparison, the rFA of the TS-MCAO group at 28 days was significantly higher than that at 14 days after the intervention ($p$ = 0.019), but no

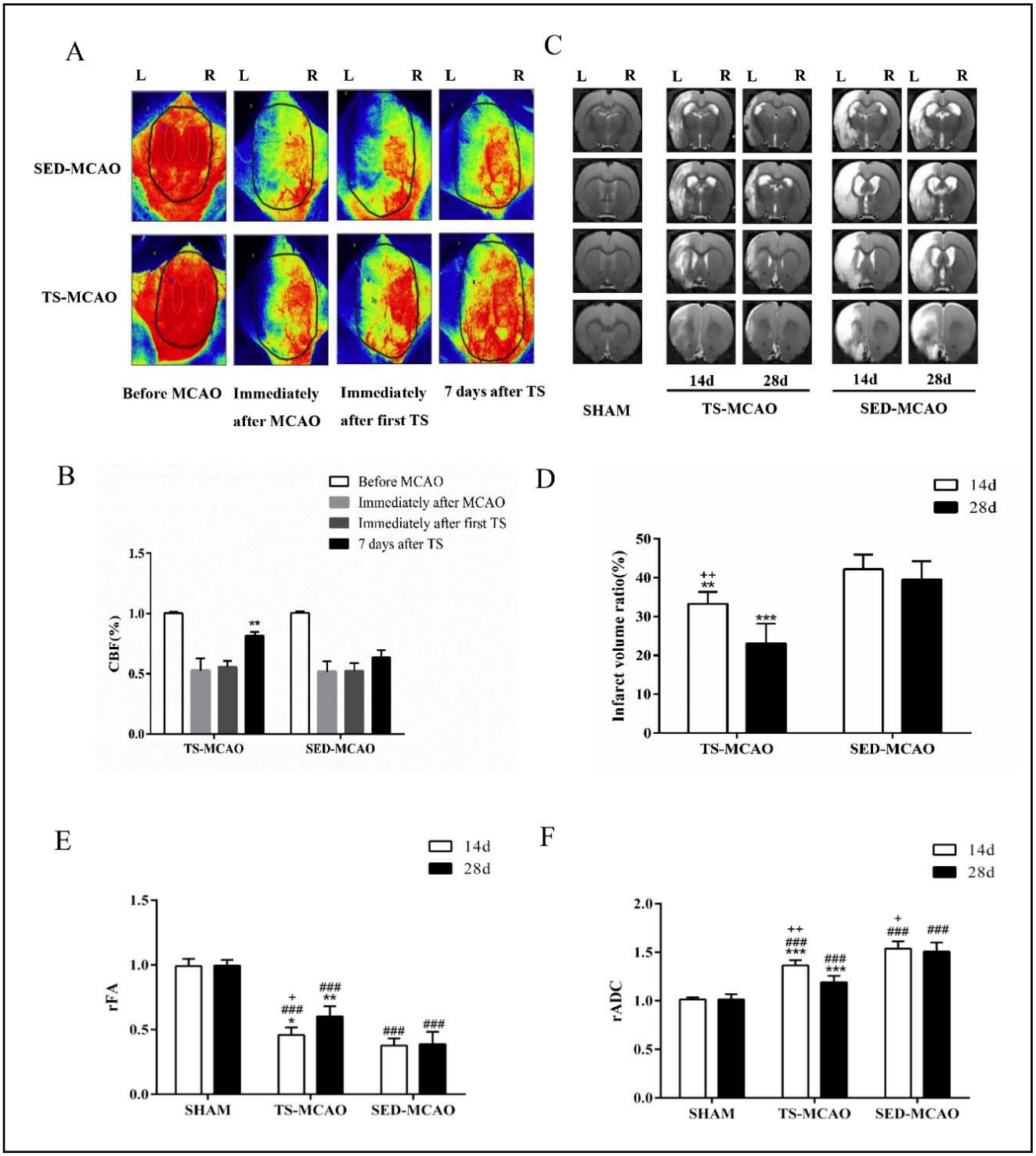


**Fig. 5.** CBF and magnetic resonance images of rats. (A) CBF quantification with Laser Perfusion Imager Review V5.0 software and expression as mean perfusion in two ROIs. (B) The ratio of CBF in the TS-MCAO and SED-MCAO groups, CBF(%)=ipsilateral mean perfusion/ contralateral mean perfusion. ($\bar{x}$±s, n=6). (C) T2WI of rats in each group after 14 and 28 days of intervention (L, left; R, right). (D) Infarct volume ratio ($\bar{x}$±s, n=6). (E) rFA value after cerebral infarction ($\bar{x}$±s, n=6). (F) rADC value after cerebral infarction ($\bar{x}$±s, n=6). CBF: cerebral blood flow; FA: fractional anisotropy; ADC: apparent diffusion coefficient. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the SED-MCAO group. $^{\#\#\#}p < 0.001$ compared with the SHAM group. $^{+}p < 0.05$, $^{++}p < 0.01$ compared with the corresponding levels at 28 days within each group.

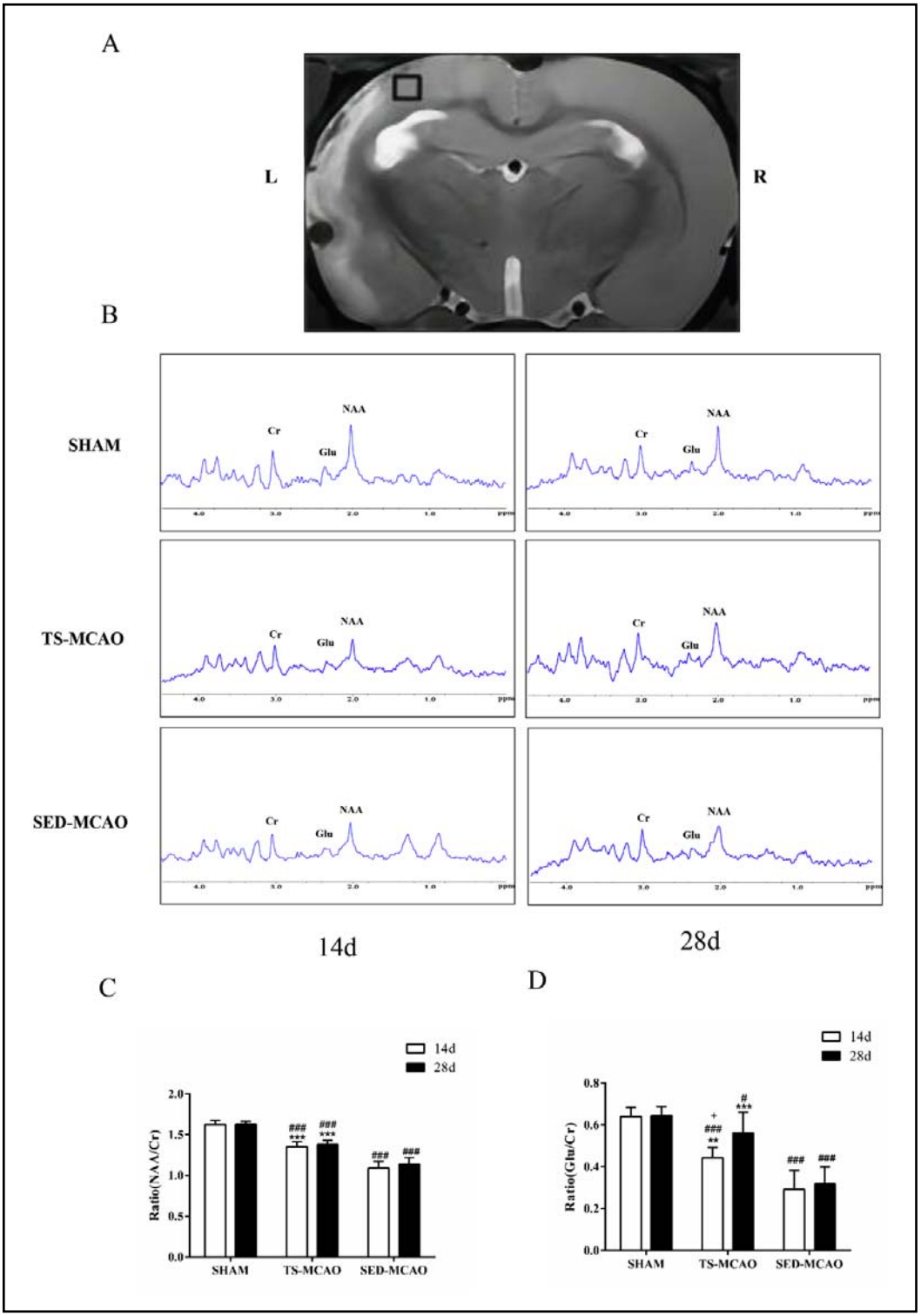


**Fig. 6.** $^{1}$H-MRS imaging. (A) Localization of the VOI in the left peri-infarct cortex on T2WI. (B) There was an NAA peak at 2.02 ppm, a Glu peak at 2.2 ppm and a Cr peak at 3.05 ppm on 1H-MRS. (C) The NAA/Cr ratio in the left peri-infarct cortex ($\bar{x}\pm s$, n=6). (D) The Glu/Cr ratio in the left peri-infarct cortex ($\bar{x}\pm s$, n=6). $^{1}$H-MRS: proton magnetic resonance spectroscopy; VOI: volume of interest; T2WI: T2-weighted imaging; NAA: Nacetylaspartate; Cr: creatine; Glu: glutamate. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the SED-MCAO group. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, compared with the SHAM group. $^{+}p < 0.05$ compared with the corresponding levels at 28 days within each group.

significant difference was observed between days 28 and 14 in the SED-MCAO group ($p = 0.115$) (Figure 5E).

Time and intervention could affect the rADC values ($F_{\text{time (1,15)}} = 7.365$, $p_{\text{time}} = 0.016$; $F_{\text{intervention (2,15)}} = 292.545$, $p_{\text{intervention}} < 0.001$). The time and intervention also had interactive effect ($F_{\text{time×intervention (2, 15)}} = 4.641$, $p = 0.027$). The rADC in the TS-MCAO group ($p_{14d} < 0.001$, $p_{28d} < 0.001$) and SED-MCAO group ($p_{14d} < 0.001$, $p_{28d} < 0.001$) were significantly higher than those in the SHAM group at 14 and 28 days, and the value in the TS-MCAO group was significantly lower than that in the SED-MCAO group ($p_{14d} < 0.001$, $p_{28d} < 0.001$). In the intragroup comparison, the rADCs of the TS-MCAO group ($p = 0.008$) and SED-MCAO group ($p = 0.039$) after 28 days of intervention were significantly lower than those after 14 days of intervention (Figure 5F).

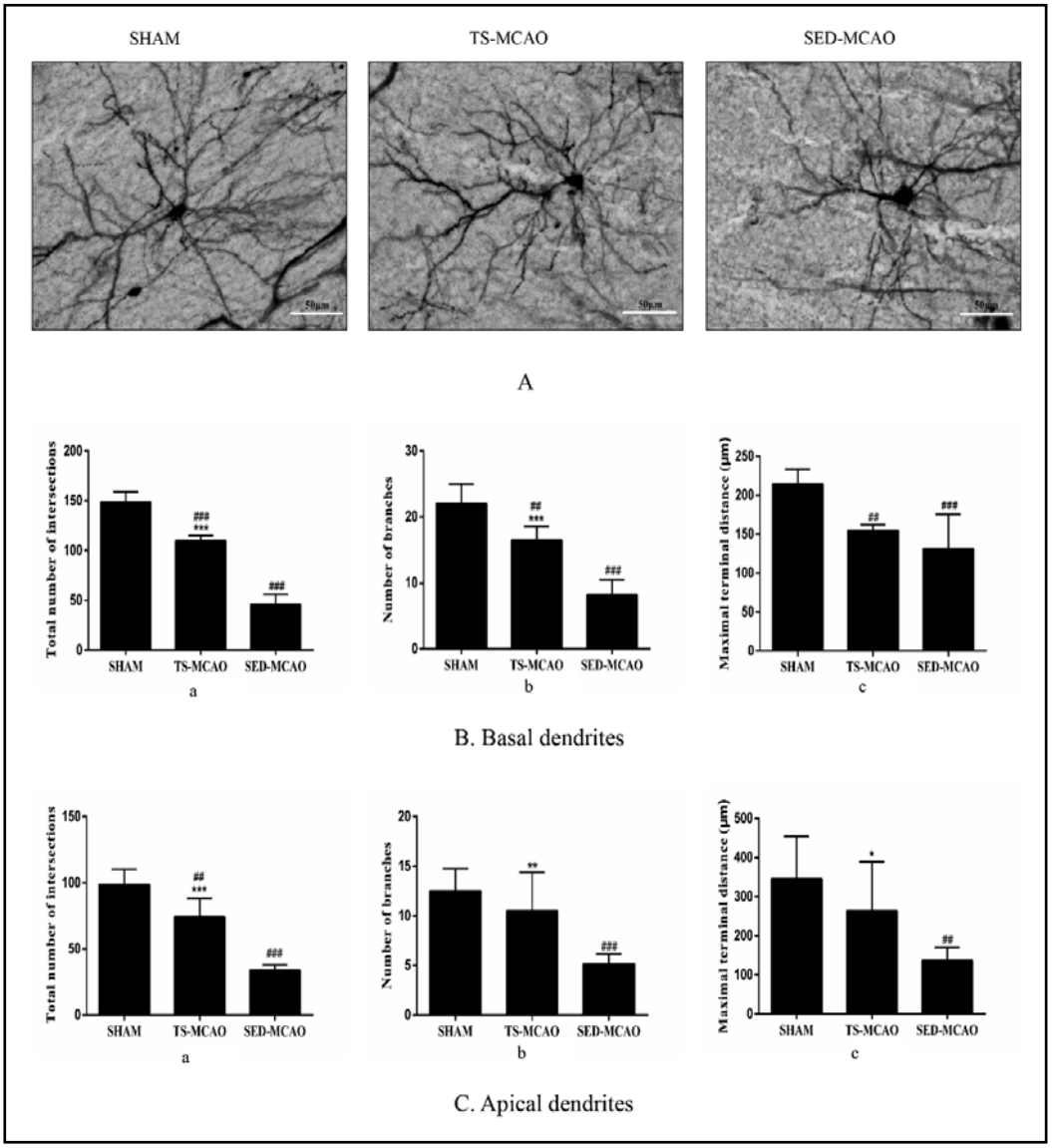


**Fig. 7.** Changes in dendritic length and complexity were observed in the left peri-infarct cortex after MCAO ($\bar{x}$±s, n=3). (A) A single neuron from the peri-infarct cortex under a 40X objective; scale bar 50 μm. (B) Changes in the number of dendrite intersections (a) and branches (b) and the maximal terminal distance (c) for basal dendrites. (C) Changes in the number of dendrite intersections (a) and branches (b) and the maximal terminal distance (c) for apical dendrites. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with the SED-MCAO group. ##$p < 0.01$, ###$p < 0.001$ compared with the SHAM group.

*Effects of Tactile Stimulation on Neurochemical Changes*

The intervention method had significant influence on NAA/Cr ($F_{\text{intervention (2,15)}} = 148.522$, $p_{\text{intervention}} < 0.001$) and Glu/Cr ($F_{\text{intervention (2,15)}} = 49.911$, $p_{\text{intervention}} < 0.001$), while the time factor had no effect ($F_{\text{time (1,15)}} = 2.650$, $p_{\text{time}} = 0.124$) on NAA/Cr, but had effect on Glu/Cr ($F_{\text{time (1,15)}} = 6.790$, $p_{\text{time}} = 0.020$).

After 14 days of intervention, the NAA/Cr ($p_{\text{TS-MCAO}} < 0.001$, $p_{\text{SED-MCAO}} < 0.001$) and Glu/Cr ($p_{\text{TS-MCAO}} < 0.001$, $p_{\text{SED-MCAO}} < 0.001$) ratios in the TS-MCAO group and SED-MCAO group were significantly lower than those in the SHAM group. The NAA/Cr ($p < 0.001$) and Glu/Cr ($p = 0.003$) ratios in the TS-MCAO group were significantly higher than those in the SED-MCAO group.

After 28 days of intervention, the NAA/Cr ($p_{\text{TS-MCAO}} < 0.001$, $p_{\text{SED-MCAO}} < 0.001$) and Glu/Cr ($p_{\text{TS-MCAO}} = 0.036$, $p_{\text{SED-MCAO}} < 0.001$) ratios in the TS-MCAO group and SED-MCAO group were significantly lower than those in the SHAM group. The NAA/Cr ($p < 0.001$) and Glu/Cr ($p < 0.001$) ratios in the TS-MCAO group were significantly higher than those in the SED-MCAO group.

The Glu/Cr ratio in the TS-MCAO group at 28 days was significantly higher than that at 14 days ($p = 0.034$). No significant difference in the NAA/Cr ratio was found between days 14 and 28 ($p = 0.459$). No significant difference in the NAA/Cr ($p = 0.427$) or Glu/Cr ($p = 0.212$) ratio was noted between days 14 and 28 after the intervention in the SED-MCAO group. (Figure 6)

*Effects of Tactile Stimulation on Neuronal Dendrites and Dendritic Spines*

Golgi-Cox staining analysis showed that after 28 days of intervention, the total number of intersections, the number of branches and the dendritic spine densities of basal dendrites in the TS-MCAO group were significantly higher than those in the SED-MCAO group ($p < 0.05$). The total number of intersections, the number of branches, the maximum terminal distance

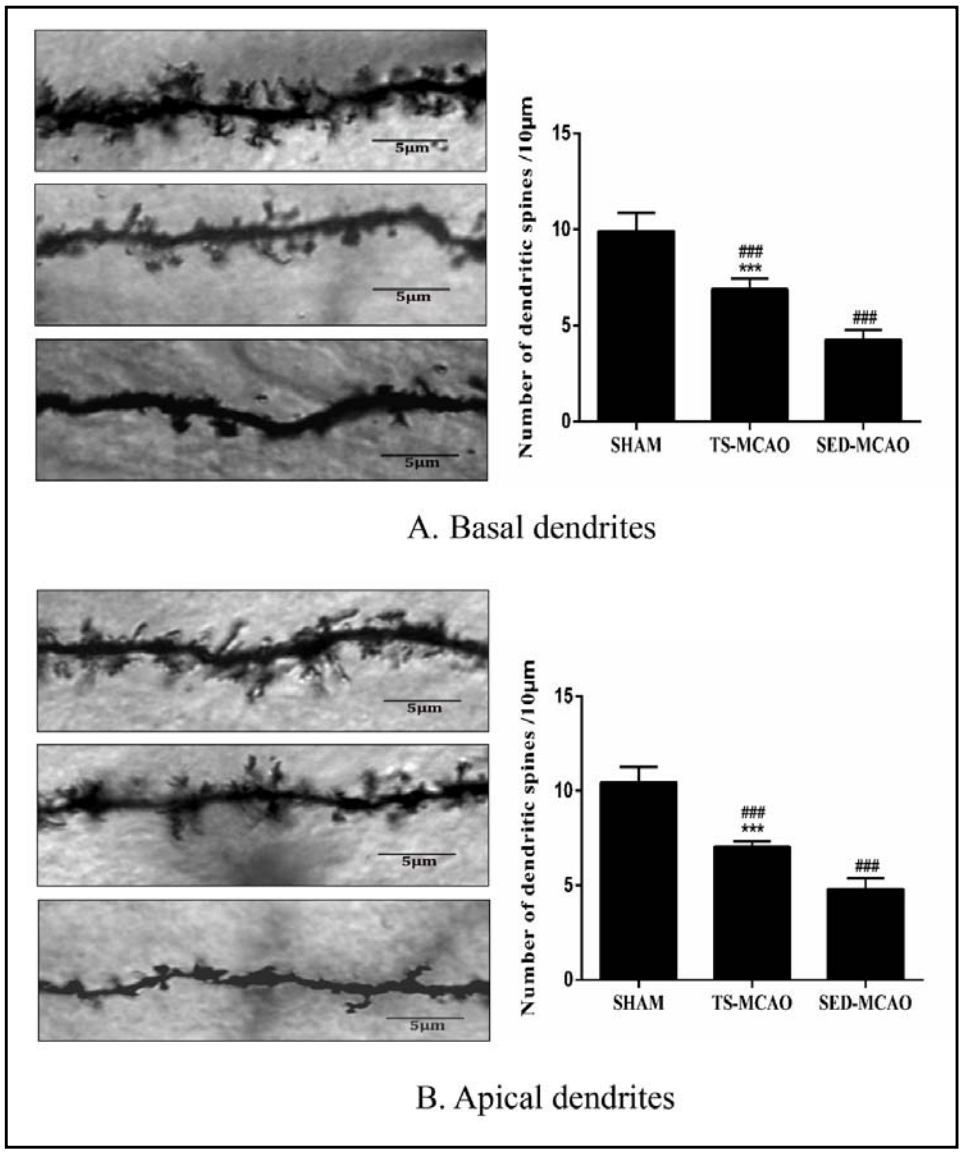


**Fig. 8.** Changes in dendritic spine density were observed in the left peri-infarct cortex after MCAO ($\bar{x}\pm s$, n=3). (A) The dendritic spines of basal dendrites under a 100× objective; scale bar 5 μm. (B) The dendritic spines of apical dendrites under a 100× objective; scale bar 5 μm. $^{***}p < 0.001$ compared with the SED-MCAO group. $^{###}p < 0.001$ compared with the SHAM group.

and the dendritic spine densities of apical dendrites in the TS-MCAO group were significantly higher than those in the SED-MCAO group ($p$ <0.05). The number of intersections, the number of branches, the maximum terminal distance and the spine densities of basal dendrites in the TS-MCAO group and SED-MCAO group were significantly lower than those in the SHAM group, while the number of intersections and the spine density of apical dendrites in these groups were significantly lower than those in the SHAM group ($p < 0.05$). (Figure 7, 8)

*Effects of Tactile Stimulation on the Number of Synapses*

The TEM results showed that compared with that in the SHAM group, the number of synapses in the TS-MCAO group ($p = 0.01$) and SED-MCAO ($p < 0.001$) group decreased significantly, while the number of synapses in the TS-MCAO group was higher than that in the SED-MCAO group ($p = 0.006$). (Figure 9)

*Effects of Tactile Stimulation on the Expression of Synaptic Plasticity-Related Proteins*

The Western blot results showed that the expression of Syn ($p = 0.001$) and PSD-95 ($p = 0.014$) in the TS-MCAO group was significantly higher than that in the SED-MCAO group, but no significant difference was found between the TS-MCAO group and the SHAM group ($p_{Syn} = 0.590$, $p_{PSD\text{-}95} = 0.099$). The expression of Syn ($p < 0.001$) and PSD-95 ($p < 0.001$) in the SED-MCAO group was significantly lower than that in the SHAM group (Figure 10).

## DISCUSSION

The findings of this study were as follows. 1. Early tactile stimulation promoted the recovery of body weight; 2. Early tactile stimulation improved neurological function; 3. Early tactile stimulation reduced the infarct volume, promoted CBF recovery, and improved the rFA and rADC of the ipsilateral internal capsule. 4.

Early tactile stimulation reduced the damage to cortical neurons in the peri-infarct area; 5. Early tactile stimulation increased dendritic complexity, dendritic spine density and synaptic plasticity-related protein expression in the peri-infarct area; and 6. Early tactile stimulation increased Glu levels in the peri-infarct cortex.

*Early Tactile Stimulation Promotes Weight Recovery in MCAO Rats*

(Pan *et al.* 2017; Rodrigues *et al.* 2004; Whitaker *et al.* 2007) Weight loss is a common adverse event after ischaemic brain injury and has an adverse effect on survival and neurological recovery (Jönsson *et al.* 2008). Relevant studies have shown that weight loss has a negative impact on prognosis and functional recovery (Yang *et al.* 2019), which is closely related to the causes of fat loss, skeletal muscle atrophy, decreased anabolism, increased catabolism, eating disorders, pain, and infection (Scherbakov *et al.* 2019). In this experiment, the body weight first decreased and then increased. The body weight of the sham group increased the fastest, and the body weight of the TS-MCAO group increased slightly faster than that of the SED-MCAO group. After 28 days of intervention, the body weight of the TS-MCAO group was significantly higher than that of the SED-MCAO group, showing that tactile stimulation intervention after MCAO can promote body weight recovery. These findings have certain guiding significance for clinical rehabilitation interventions.

*Early Tactile Stimulation Improves Neurological Function in MCAO Rats*

MCAO usually leads to sensory and motor dysfunction of the contralateral limb. The mNSS is often used to evaluate changes in motor, sensory, balance and neurological functions after MCAO (Wang *et al.* 2020), while the adhesive tape removal test is used to evaluate impairment of sensory function after MCAO (Schallert & Whishaw, 1984). Gibb and Kolb *et al.* showed that tactile stimulation for 2 weeks can promote the recovery of motor function in neonatal rats with ischaemia and hypoxia and adult rats with cortical injury (Gibb *et al.* 2010; Kolb & Gibb 2010). Similar results were found in our experiment. After 14, 21 and 28 days of tactile intervention, the mNSS and the time needed to remove the adhesive tape in the TS-MCAO group were significantly lower than those in the SED-MCAO group, indicating that early tactile stimulation after MCAO could promote improvements in sensory and motor function. However, no difference was identified between the two groups at 1, 3 and 7 days after the tactile intervention. On the one hand, this may have been due to the short duration of tactile intervention, and the improvements in MCAO rats did not extend to behavioural improvements. On the other hand, we are unsure whether our tactile intervention is the best method to improve the neurological function of MCAO rats. The optimal intensity and duration of tactile stimulation need to be further studied.

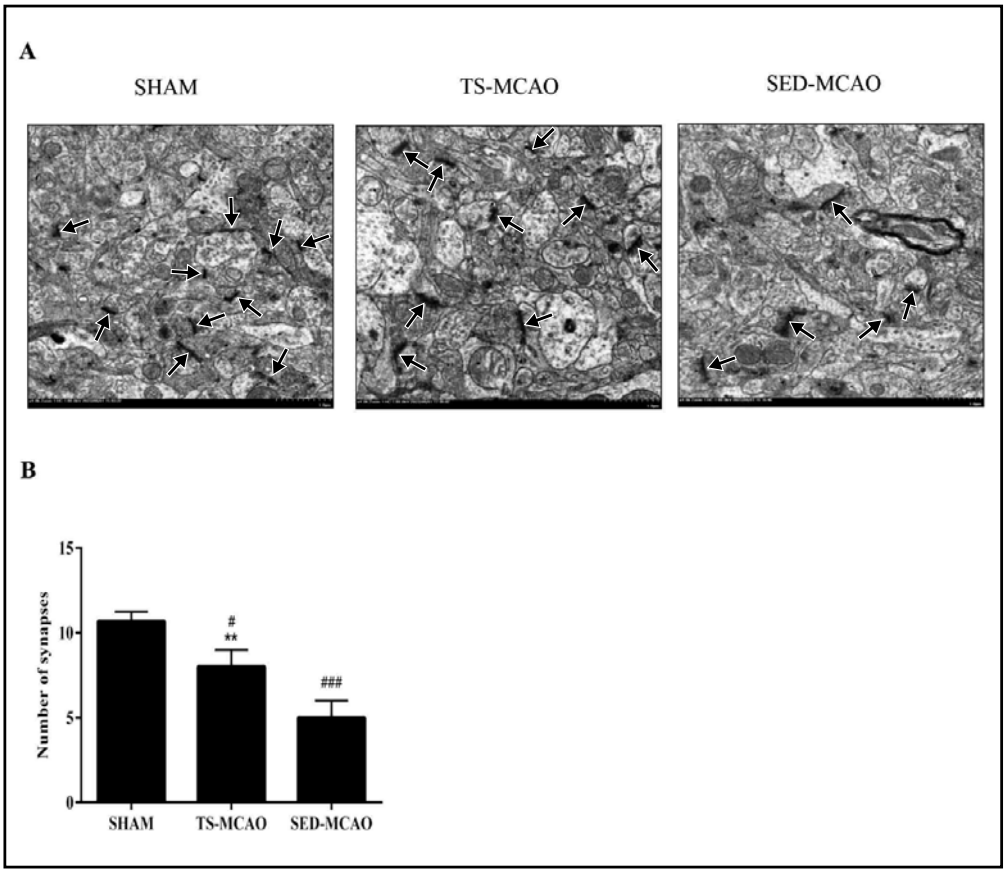


**Fig. 9.** Changes in the number of synapses in each group ($\bar{x}\pm s$, n=3). (A) Representative images of synapses in each group. The synapse was observed under an electron microscope at ×4.0k magnification; scale bar 1 μm. (B) The number of synapses. $^{**}p < 0.01$ compared with the SED-MCAO group. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ compared with the SHAM group.

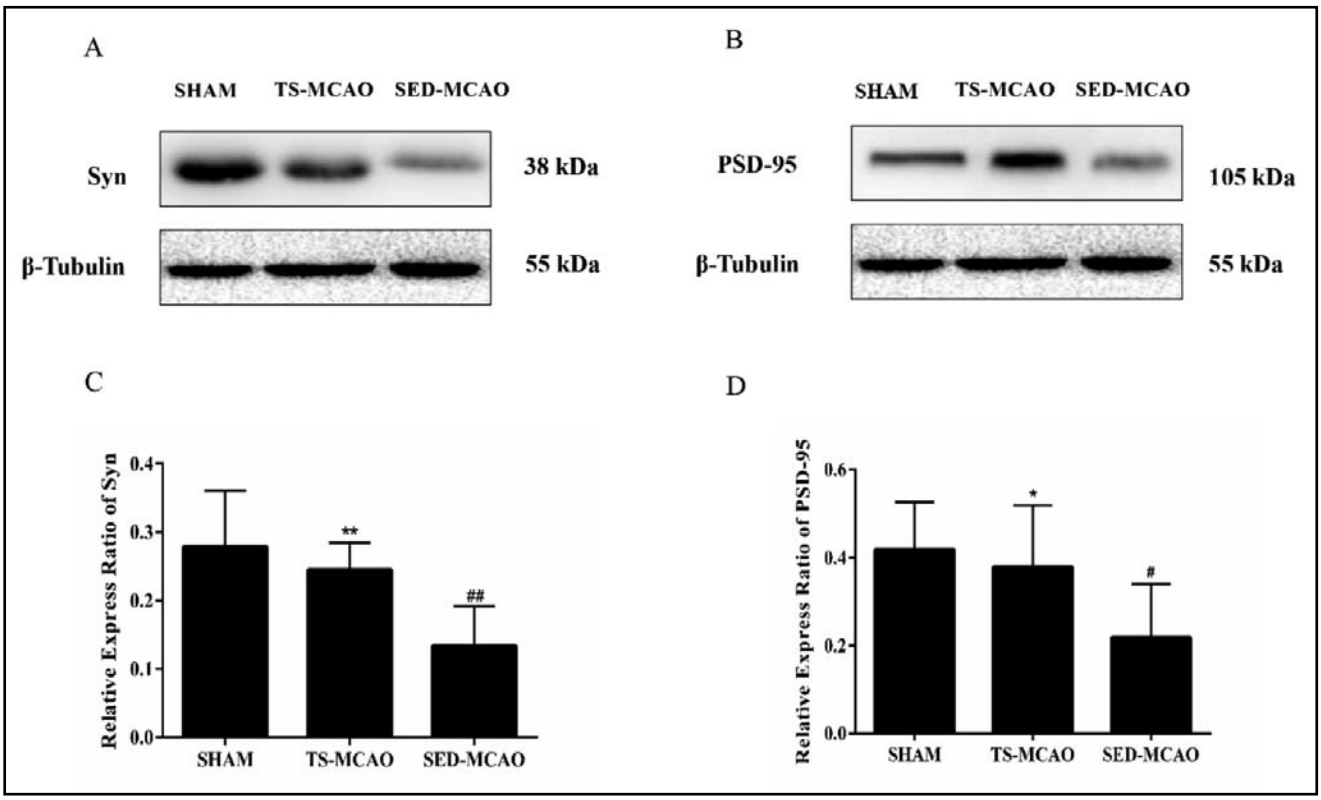


**Fig. 10.** The relative expression level of synaptic plasticity-associated proteins in the left peri-infarct cortex after MCAO ($\bar{x}$±s, n=6). (A) Western blotting was used to assess Syn expression. (B) Western blotting was used to assess PSD-95 expression. (C) Relative level of the Syn protein. (D) Relative level of the PSD-95 protein. *$p < 0.05$, **$p < 0.01$ compared with the SED-MCAO group. ###$p < 0.001$ compared with the SHAM group.

*Early Tactile Stimulation Reduces the Infarct Volume and the Degree of Corticospinal Tract Injury in MCAO Rats*

The main energy source for the brain is the cerebral blood supply, so brain function is very dependent on normal CBF (Claassen *et al.* 2021). Increasing CBF is an important approach for relieving ischaemic brain injury (Fan *et al.* 2022). We found that CBF recovery was improved in the TS-MCAO group compared with the SED-MCAO group at 7 days after MCAO, which indicated that TS is a potential rehabilitation method for improving CBF and alleviating ischaemic brain injury.

A T2-weighted imaging (T2WI) sequence can be used to determine the location and size of infarction. The FA and ADC values are two commonly used diffusion tensor imaging parameters in diffusion tensor imaging (DTI) data analyses. The former reflects the projection intensity of neurons and represents the change in water molecule diffusion measured in different directions. The latter is used to evaluate the diffusion intensity of tissue water molecules (Alexander *et al.* 2007). A decrease in the FA value and increase in the ADC value were positively correlated with the loss of myelin sheaths and axon membranes, which limited the random movement of water molecules along the white matter fibre bundles (Hagmann *et al.* 2006; Tae *et al.* 2018). Their changes indicated that the structural integrity of white matter fibres was destroyed, and the degree of motor function recovery could be predicted (Pinter *et al.* 2020). After cerebral ischaemia, the white matter fibres in the injured area were damaged, the degree of water molecule diffusion increased, and the direction of water molecule diffusion changed, which led to an increase in the ADC value and a decrease in the FA value(van der Zijden *et al.* 2008). Rehabilitation training can promote the recovery of motor function by improving the remodelling of the corticospinal tract, increasing FA values and reducing ADC values(Li *et al.* 2021). Hu *et al.* demonstrated that restrictive exercise therapy can promote remodelling of the corticospinal tract and improve the motor function of rats with cerebral ischaemia by increasing the FA value and reducing the ADC value (Hu *et al.* 2019). Our team's previous studies confirmed that early exercise interventions after MCAO can increase the integrity of the corticospinal tract, reduce the infarct volume and promote improvements in neurological function (Li *et al.* 2021). This study found that compared with sedentary intervention, early tactile intervention for 14 and 28 days significantly decreased the infarct volume ratio, increased the rFA and decreased the rADC in the ipsilateral internal capsule, indicating that early tactile stimulation could promote remodelling of the corticospinal tract and increase its integrity. Moreover, we found that in the

SED-MCAO group, the rADC at 28 days was significantly lower than that at 14 days, which may be related to self-repair of brain tissue after cerebral ischaemia, while early tactile intervention could significantly enhance corticospinal tract repair.

*Early Tactile Stimulation Reduces Neuronal Damage in the Peri-Infarct Cortex of MCAO Rats*

$^{1}$H-MRS is an effective noninvasive technique for measuring the levels of metabolites in the brain (Zhang *et al.* 2016). NAA is a specific amino acid found in cerebral neurons, almost exclusively in normal neurons, and close relationship between NAA detected spectroscopically and the proportion of normal neurons have been confirmed (Muñoz Maniega *et al.* 2008). So it is considered a marker of neurons and is used to detect neuronal damage under neuropathological conditions such as cerebral ischaemia (Qian *et al.* 2013). Cerebral ischaemia can reduce the level of NAA in damaged brain areas, and a decrease in the level of NAA is usually related to neuronal apoptosis or decreased neuronal activity (Sager *et al.* 2000; Muñoz Maniega *et al.* 2008) (Demougeot *et al.* 2004)(Muñoz Maniega *et al.* 2008b). Qian *et al.* found that an enriched environment reduced neuronal necrosis and apoptosis in the infarcted penumbra of MCAO rats and increased the NAA/Cr ratio (Qian *et al.* 2018). Zhang *et al.* found that the NAA/Cr ratio in the hippocampus of transgenic mice with Alzheimer's disease was significantly lower than that in the hippocampus of wild-type mice, while neural stem cell transplantation promoted neurogenesis in the hippocampus of transgenic mice with Alzheimer's disease, resulting in an increase in the NAA/Cr ratio (Zhang *et al.* 2017). In this study, the NAA/Cr ratios in the peri-infarct cortices of the TS-MCAO group and SED-MCAO group were found to be significantly lower than those in the SHAM group, and the NAA/Cr ratio in the TS-MCAO group was significantly higher than that in the SED-MCAO group, which indicated that cerebral ischaemia may lead to neuronal damage and loss, while early tactile stimulation may reduce neuronal damage and loss.

*Early Tactile Stimulation Increases Synaptic Plasticity in the Peri-Infarct Cortex of MCAO Rats*

The synapse is the main structure responsible for information communication between neurons. Approximately 90% of excitatory synapses are located on dendrites, and the morphological structure and complexity of dendrites affect synaptic connections and signal transmission efficiency between synapses (Hu *et al.* 2020), thus, the morphological structure and complexity of dendrites can be used as an indicator of synaptic plasticity (Tang *et al.* 2019). As the first postsynaptic element encountered by excitatory neurotransmitters, dendritic spines are basic units for the integrated processing of synaptic signals (Harris & Kater, 1994). Synapses and dendrites are some of the most easily damaged structures after MCAO (Hofmeijer & van Putten, 2012). Koleske (2013) has shown that the complexity of neuronal dendrites and the density of dendritic spines decrease due to nerve cell injury after stroke, but both increase with the improvement of neural function (Koleske 2013). Global cerebral ischaemia can lead to time-dependent damage to dendrites and dendritic spines, and drug therapy or rehabilitation training can increase the complexity of dendrites and the spine density of cortical neuron dendrites, improve the strength of synaptic connections and efficiency of synaptic transmission, and promote synaptic plasticity to improve neurological function (Zhu *et al.* 2017). Lin *et al.* found that cerebral ischaemia led to a decrease in dendritic spine density and dendritic complexity in the cortex around the infarction. Continuous injection of Tat-nNOS-N1-133 for 4-10 days after ischaemia reversed the decrease in the dendritic length, branch number and dendritic spine density of cortical neurons and promoted the improvement of neurological function (Lin *et al.* 2018). In addition, Xie *et al.* found that treadmill exercise increased dendritic complexity and dendritic spine density in the ischaemic penumbra, promoted synaptic plasticity and improved motor function in rats subjected to ischaemia–reperfusion (Xie *et al.* 2019). Long-term potentiation (LTP) induced by Glu is an important mechanism leading to the remodelling of dendrites and dendritic spines (Kulik *et al.* 2019). In our study, compared with those in the SHAM group, dendritic complexity and dendritic spine density decreased significantly in the SED-MCAO group and TS-MCAO group, indicating that cerebral ischaemia damaged the synaptic structure and inhibited intersynaptic connections and information transmission. Compared with the SED-MCAO group, dendritic complexity and dendritic spine density were significantly increased in the TS-MCAO group, suggesting that early tactile stimulation can promote synaptic connections and improve synaptic transmission efficiency and synaptic plasticity. The mechanism may be related to the increase in Glu levels and remodelling of dendritic spines induced by early tactile stimulation.

On the one hand, synaptic plasticity can be reflected by the morphology and complexity of neuronal dendrites; on the other hand, it can also be reflected by the number of synapses and the expression of synaptic plasticity-related proteins (Syn, PSD-95). Syn is a calcium-binding protein on the presynaptic vesicle membrane, and PSD-95 is a cytoskeleton protein located in the postsynaptic membrane (Kim & Kim, 2021). They are markers of synaptic plasticity and can reflect the number and density of synapses (Yang *et al.* 2020). Cerebral ischaemia can lead to neuronal damage and death, resulting in a decrease in the number of synapses and a decrease in the expression of Syn and PSD-95, which is also an important cause of neurological impairment (Papadopoulos *et al.* 2006). Studies have

shown that the expression of synaptic plasticity-related proteins can increase with the recovery of neural function (Wei *et al.* 2001). Xu *et al.* found that an enriched environment promoted the improvement of neurological function, increased the expression of Syn and PSD-95 in the cortex and hippocampus, and improved synaptic structure in rats with cerebral ischaemia (Xu *et al.* 2009). Lin *et al.* found that forced or autonomous exercise can increase the expression of Syn and PSD-95 in the hippocampus of rats with cerebral ischaemia, increase synaptic plasticity, and thus improve neurological function (Lin *et al.* 2015). Our study found that Syn and PSD-95 expression in the peri-infarct cortex decreased significantly after MCAO. Early tactile stimulation effectively increased the expression of Syn and PSD-95 and promoted the improvement of neurological function in rats with cerebral ischaemia. Early tactile stimulation is suggested to promote synaptic plasticity in rats with cerebral ischaemia.

*Early Tactile Stimulation Increases Glu Levels in the Peri-Infarct Cortex of MCAO Rats*

Glu is an important excitatory neurotransmitter in the central nervous system. Glu plays a dual role in the pathological process of cerebral ischaemia: Glu secretion reaches its peak at 2 hours after cerebral ischaemia and begins to decrease after 3 hours of cerebral ischaemia. The secretion of a large amount of Glu in this stage is an important mechanism leading to excitotoxicity (Yan *et al.* 2015). However, the secretion of Glu in the ipsilateral brain decreases in the chronic phase of cerebral ischaemia and is lower than that in normal rats. An increase in Glu secretion in this stage could promote the recovery of neurological function (Ikonomidou & Turski, 2002), which may be related to the involvement of Glu and its receptors in the regulation of synaptic plasticity (Lee & Kesner, 2002; Villarreal *et al.* 2002). Studies have shown that Glu can induce LTP by activating NMDA receptors on the postsynaptic membrane, while long-term changes in LTP can promote new synaptic formation, affect dendritic complexity and dendritic spine growth, enhance synaptic connectivity and transmission efficiency, and promote long-term synaptic plasticity (Lüscher & Malenka, 2012; Watson *et al.* 2016). Chang *et al.* found that treadmill exercise intervention for 14 days could increase the concentration of extracellular Glu in the striatum and improve motor function in rats with cerebral ischaemia and speculated that this improvement was due to the increase in presynaptic Glu release and the induction of LTP (Chang *et al.* 2009). Similar results were found in our study. Compared with the SED-MCAO group, tactile stimulation increased Glu secretion and improved neural function, dendritic complexity, dendritic spine density and synaptic plasticity-related protein expression in the peri-infarct cortex of MCAO rats after 14 and 28 days of tactile stimulation. Therefore, the increase in Glu secretion induced by tactile stimulation in our study may have also contributed to the production of LTP, resulting in plasticity changes such as synaptic remodelling and changes in the complexity of dendrites and density of dendritic spines. However, we measured Glu levels only at 14 and 28 days after intervention. To prove this conjecture, it is necessary to measure Glu levels in the infarct core and contralateral hemisphere within several hours after MCAO, so further research is needed.

In summary, early tactile stimulation is a safe rehabilitation method for MCAO rats that can promote the recovery of motor function after cerebral ischaemia. Mechanistically, early tactile stimulation may increase the concentration of Glu in the peri-infarct area, reduce neuronal damage, promote synaptic plasticity, reduce infarct size, and improve the integrity of the corticospinal tract and motor function. This study provides a new idea for an early effective and safe rehabilitation approach to accelerate functional recovery during the therapeutic window in patients with cerebral ischaemia. However, clinical research on this topic is needed; the intervention programme should be further developed, the optimal outcomes should be determined, and the intervention should be translated to clinical practice.

## AUTHORS' CONTRIBUTIONS

All authors listed have made substantial, direct intellectual contributions to the work and approved it for publication.

## DECLARATION OF CONFLICTING INTERESTS

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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