Research Article

The mechanisms through which auricular vagus nerve stimulation protects against cerebral ischemia/reperfusion injury

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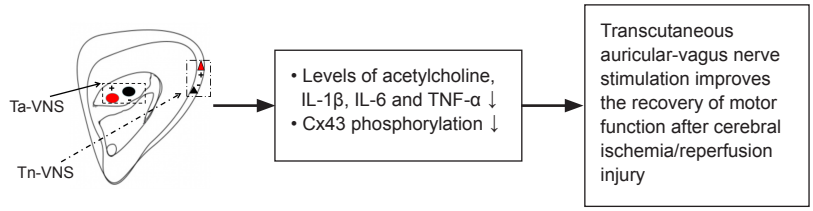
Graphical Abstract *Effect of transcutaneous auricular-vagus nerve stimulation on neurological function follow cerebral ischemia/reperfusion injury*



Abstract

Previous studies have shown that vagus nerve stimulation can improve patients' locomotor function. The stimulation of the auricular vagus nerve, which is the only superficial branch of the vagus nerve, may have similar effects to vagus nerve stimulation. However, the precise mechanisms remain poorly understood. In this study, rat models of cerebral ischemia/reperfusion injury were established by modified Longa ligation. Twenty-four hours later, 7-day auricular vagus nerve stimulation was performed. The results showed that auricular vagus nerve stimulation promoted the secretion of acetylcholine, inhibited the secretion of interleukin-1β, interleukin-6, and tumor necrosis factor-α, and reduced connexin 43 phosphorylation in the ischemic penumbra and motor cortex, promoting locomotor function recovery in rats with cerebral ischemia/reperfusion injury. These findings suggested that auricular vagus nerve stimulation promotes the recovery of locomotor function in rats with cerebral ischemia/reperfusion injury by altering the secretion of acetylcholine and inflammatory factors and the phosphorylation of connexin 43. This study was approved by the Animal Use and Management Committee of Shanghai University of Traditional Chinese Medicine on November 8, 2019 (approval No. PZSHUTCM191108014).

Key Words: auricular vagus nerve stimulation; connexin 43; gap junction; inflammatory factor; ischemia/reperfusion injury; neurological function; neuroprotection; stroke

Chinese Library Classification No. R454; R741; R363

Introduction

Studies have indicated that vagus nerve stimulation (VNS) results in favorable anti-inflammatory activity against cerebral inhalation anesthesia injury (Mioni et al., 2005). Inflammation is a general pathological process associated with cerebral ischemia/reperfusion (I/R) injury (Xu et al., 2020; Goebel et al., 2021). When the body's immune response status is abnormal, the inflammatory factors that act on the vascular endothelium become upregulated, promoting the adhesion and recruitment of leukocytes (Anrather and Iadecola, 2016; Dąbrowski et al., 2019). These inflammatory signals promote leukocyte migration and mediate the inflammatory cascade, causing more serious cerebral injury (Huang et al., 2006; Wong and Crack, 2008). Neuroinflammation is a common process associated with brain injury and many acute and chronic degenerative diseases (Du et al., 2021). The primary features of neuroinflammation include the activation of reactive astrocytes and microglia, and activated microglia cause the release of a large number of inflammatory factors, leading to reperfusion injury. Ischemic brain injury has been shown to continue developing long-term following infarction. A study found that when traumatic brain injury occurs, enhanced phosphorylated Cx43 can be observed in brain tissue, and the activation of Cx43 could mediate a nociceptive effect by propagating the brain damages (Chen et al., 2018). Microglia release interleukin 1β (IL-1β) and tumor necrosis factor α

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(TNF-α), which can reduce the intercellular communications through gap junctions (GJs) (Retamal et al., 2007). GJs allow for information exchange between cells and are composed of two hemichannels, each composed of six connexins, including connexin 43 (Cx43). The enhancement of cell-to-cell communications through GJs can aggravate cell edema and apoptosis after injury. GJs mediate electrical and chemical signal communication between immune cells and participate in the release of cytokines and the inflammatory response process (Hervé and Derangeon, 2013; Chen et al., 2016). Many studies have indicated that neuronal inflammation during the acute phase causes changes in Cx43 expression and GJ and hemichannel communication, which affects neurological function (Nakase et al., 2004; Karpuk et al., 2011). In this study, we used changes in inflammation as an entry point to explore the effects of ta-VNS on inflammatory reactions in the ischemic penumbra and motor cortex, Cx43 expression, and Cx43 phosphorylation in a cerebral I/R rat model by observing the effects of ta-VNS on neurological functions.

Materials and Methods

Study protocol

All male Sprague-Dawley rats (7 weeks old) were purchased from Charles River Biotechnology Co., Ltd. (Certificate No. SCXK (Hu) 2017-0011), weighing 250 ± 10 g. All procedures were performed in accordance with the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines and were approved by the Animal Use and Management Committee of Shanghai University of Traditional Chinese Medicine on November 8, 2019 (approval No. PZSHUTCM191108014). The rats were randomly divided into sham, I/R, ta-VNS, and transcutaneous non-auricular (tn)-VNS groups, with 12 rats in each group. There were two deaths in each of the ta-VNS, tn-VNS, and I/R groups respectively during the modeling, and no deaths in the sham group. Ten of the 12 rats in the sham group were randomly selected for inclusion in the subsequent experiments and analysis. All animals were modeled after being randomly enrolled. The neurological deficit scores were evaluated 24 hours after model establishment. Interventions were performed after all neurological deficit scores were assessed, and interventions were applied for a total of 7 days. After intervention application on the 7th day, all animals were awakened from anesthesia, and neurological deficit scores were assessed again on the 7th day. After all neurological deficit scores were evaluated, five animals were randomly selected from among the 10 rats in each group and subjected to decapitation after anesthesia for the collection of samples used in the enzyme-linked immunosorbent assay (ELISA) and western blot assay analyses. The remaining five animals in each group were perfused after anesthesia, and their brain tissues were used to perform hematoxylin and eosin staining and immunohistochemical analysis. The procedure of the study is described in the relevant methods section (**Figure 1**).

Modeling cerebral I/R injury

In this study, a cerebral I/R rat model was used (Jiang et al., 2014). All rats were prevented from accessing food and water for 8 hours prior to the procedure. As described in the literature, a modified Longa thread embolization method was used to generate a rat model of cerebral infarction (Longa et al., 1989): after fixation, the rats were anesthetized using 4% isoflurane (RWD Life Technology Co., Ltd., Shenzhen, China) inhalation, an incision was made in the midline of the neck to separate the subcutaneous tissues and muscles, and the carotid artery was separated and fully exposed. A thread embolization was passed through the internal carotid artery to block the right blood flow. The thread embolization was removed after 90 minutes to allow for the resumption of perfusion. A temperature-controlled rat plate was maintained at approximately 37°C. The rats in the sham group underwent exposure of the carotid artery, but no thread embolization was performed. All rats were anesthetized and decapitated after completing the neurological function score on the 7th day after modeling. Five rats were randomly selected from among the 10 rats in each group for hematoxylin and eosin staining and immunohistochemistry; the other five rats were used for ELISA and western blot assay analyses.

VNS

The rats in ta-VNS and tn-VNS groups received their respective interventions starting 24 hours after modeling was performed. The rat was fixed in the prone position under 4% isoflurane inhalation anesthesia, ensuring that the rat's head was fully exposed. The bilateral concha auricularis of the rat were cleaned, and the temperature-controlled rat plate was maintained at approximately 37°C. In the ta-VNS group, the positive and negative electrodes of the electrode containing the particle-type magnet were adsorbed on both sides of the bilateral concha auricularis region of the rats and then connected to a Hanshi Electroacupuncture Instrument (Nanjing Hanshi Electroacupuncture Instrument Equipment Co. Ltd., Nanjing, China) at 10 Hz/1 mA. Each intervention was applied for 30 minutes once per day for a total of 7 days (**Figure 1**). The stimulation site of the tn-VNS group was the rat tragus, and all other stimulation parameters were the same as for ta-VNS (**Figure 2**). The rats in the sham and I/R groups were fixed in the same manner but received no other treatment. No animal deaths occurred during the VNS intervention.

Neurological deficit score

Rats were scored for neurological function before modeling, 24 hours after modeling, and after 7 days of VNS intervention, according to Longa's method (Longa et al., 1989). Rats were scored as follows: 0 points, normal gait; 1 point, the left forepaw cannot be stretched; 2 points, rats circle left while walking; 3 points, rats fall to the left side while walking; and 4 points, rats experience a loss of consciousness and do not display spontaneous walking. Two investigators scored the rats together each time, and the neurological function score was assessed 24 hours after modeling and on the 7th day after modeling.

Hematoxylin and eosin staining

Hematoxylin and eosin staining was used to detect tissue damage 7 days after modeling. On the 7th day after the behavioral test, five rats were anesthetized with 4% isoflurane, perfused with 0.9% sodium chloride aqueous solution, and 4% paraformaldehyde solution injected into the heart, and the rats were decapitated. The wax-soaked tissues were embedded in an embedding machine (JB-P5, Wuhan Junjie Electronics Co., Ltd., Wuhan, China) after dehydration and wax leaching. The trimmed wax block was cooled to –20°C and then placed on a freezing table and sliced, using a paraffin slicer, into 4-μm-thick sections (RM2016, Shanghai Leica Instrument Co. Ltd., Shanghai, China). Paraffin sections were obtained of the coronal surface of the ischemic penumbra and the right motor cortex (Paxinos and Franklin, 2013). Sections were dewaxed with xylene, dehydrated with gradient alcohol, and rinsed with distilled water. Then, the sections were stained in hematoxylin for 5 minutes at room tempertature (Beyotime, Shanghai, China) and rinsed in tap water. The sections were then treated with ethanol hydrochloride for 30 seconds and rinsed in tap water. Sections were dehydrated in alcohol using a concentration gradient and placed in an alcohol eosin staining solution (Beyotime) for 3 minutes. The sections were dehydrated again and treated with xylene to induce transparency. Finally, drops of neutral resin were added to seal the sections. Pathological changes in the neurons were observed under a 200× fluorescence microscope (Nikon Eclipse C1; Nikon, Tokyo, Japan).

Enzyme-linked immunosorbent assay

Five rats in each group were decapitated under 4% isoflurane induced deep anesthesia after the neurological function evaluation was performed 7 days after modeling. The ischemic penumbra and motor cortex of the right cerebral cortex were quickly removed and frozen in liquid nitrogen. The samples analyzed by ELISA are obtained from the same source as samples analyzed by western blot assay. To each well of a 96-well plate, 100 µL of standard solution or sample solution was added, 50 µL each of Enzyme-labeled solution were added to each well except the blank well, and the reaction plate was mixed and incubated at 37°C for 60 minutes (maintain temperature and temperature during incubation), then the plate was washed five times.The color development reagent was added to every well, and the reaction was performed at 37°C in the dark for 10 minutes, after which 50 µL stop solution was added to each well and mixed well. The absorbance at 450 nm was measured with a microplate reader within 30 minutes. All reagents for the acetylcholine (Ach), IL-1β, TNF-α, and interleukin 6 (IL-6) ELISA kits were provided by Blue Gene, Shanghai, China. The results were analyzed using a microplate reader (Thermo-DENLEY DRAGON Wellscan MK 3, Helsinki, Finland).

Western blot assay

Five rats in each group were decapitated under 4% isoflurane-induced deep anesthesia after the neurological function evaluation 7 days after modeling. The ischemic penumbra and right motor cortex were quickly removed and frozen in liquid nitrogen. After grinding, a pre-chilled lysate solution (Cat# R0010, Solarbio (China), Beijing, China) was added to lyse the tissue. Fifty micrograms of total protein were separated by gel electrophoresis and electrotransferred to a polyvinylidene difluoride membrane. Protein extraction was performed using pre-cooled radioimmunoprecipitation assay protein extraction reagent containing added protease inhibitor cocktail. For analysis of phosphorylated proteins, a phosphatase inhibitor was also added to the lysis buffer. Approximately 40 mg of tissue was added to 400 µL radioimmunoprecipitation assay lysis buffer for homogenization. The lysate was incubated on ice for 1 hour and centrifuged at 4°C at 13,000 r/min for 10 minutes. The supernatant was taken for protein quantification and protein sample preparation. Protein quantification was performed using the bicinchoninic acid (BCA) method, following the instructions of the BCA protein quantification kit. First, an appropriate amount of BCA working solution was prepared according to the number of samples (reagent A:B = 50:1) and mixed well. BCA working solution was stable within 24 hours at room temperature; the protein standard product was completely dissolved, and 10 µL was diluted into 100 µL to make the final concentration of 0.5 mg/mL; the standard solution was added at volumes of 0, 1, 2, 4, 8, 12, 16, 20 µL to the standard wells of the 96-well plate, standard diluent was added to a total volume of 20 µL. The volume was adjusted to 20 µL using the standard diluent if the volume is less than 20 µL by BCA manual, and 200 µL of BCA working solution was added to each well and incubated at 37°C for 30 minutes. The absorbance at 570 nm was measured, and a standard curve was established to determine the protein concentrations. The membrane was blocked with 5% bovine serum albumin-TBST for 60 minutes. The membranes were incubated with the following primary antibodies at 4°C overnight: phosphorylated-Cx-43 (rabbit; 1:1000; Cat# ab30559, Abcam (China), Shanghai, China) and Cx43 (rabbit; 1:500; Cat# ab87645, Abcam (China)). The membranes were rinsed with Tris-buffered saline and polysorbate 20 buffer three times at room temperature, followed by incubation with secondary antibody (horseradish peroxidase-goat anti-rabbit IgG(H+L), 1:10,000, Cat# 111-035-003, Jackson, Lancaster, PA, USA) for 50 minutes at room temperature. Membranes were rinsed three times with Tris-buffered saline and polysorbate 20. The reaction was visualized using electrochemiluminescence (Cat# WBKLS0500, Millipore (China), Shanghai, China), using a color-developing solution in the dark. Glyceraldehyde 3-phosphate dehydrogenase was used as the reference protein. Each band was analyzed with ImageJ software ver.1.52 (National Institutes of Health, Bethesda, MD, USA).

Immunohistochemical staining

Five rats were anesthetized with 4% isoflurane 7 days after the induction of cerebral I/R injury and perfused with phosphate-buffered saline. The brains were post-fixed in 4% paraformaldehyde after being fully perfused. After being fixed with paraformaldehyde solution for 24 hours, the tissue was made into a paraffin block. Paraffin sections were dewaxed and subjected to antigen repair. The tissue section was placed in a repair box filled with citric acid antigen retrieval buffer (pH 6.0) for antigen retrieval in a microwave oven, subjected to high heat for 9 minutes until boiling, removed from heat for 7 minutes to keep warm, and then subjected to medium heat for 7 minutes. The slide was allowed to cool naturally, placed in phosphate-buffered saline (pH 7.4) under shaking conditions, and washed three times on a decolorizing shaker for 5 minutes each time. The goat anti-Cx43 polyclonal antibody (1:500; Cat# ab87645, Abcam (China)) was added to 5-µm slices laid flat in a wet box and incubated at 4°C overnight. After the slices were slightly dried, horseradish peroxidase-conjugated rabbit anti-goat IgG (1:200, Cat# GB23204, Servicebio, Wuhan, China) was added to cover the tissues and incubated for 50 minutes. After the slices were slightly dried, 3,3'-diaminobenzidine (Cat# G1211, Servicebio) was added for color development. The slices were differentiated for a few seconds with a hematoxylin differentiation solution to generate a blue color and rinsed with tap water. Dehydration and sealing were performed by successively moving the slices through an ethanol gradient: 75% alcohol for 5 minutes, 85% alcohol for 5 minutes, anhydrous ethanol I for 5 minutes, anhydrous ethanol II for 5 minutes, xylene I for 5 minutes (for dehydration and transparency). The slices were removed from xylene and dried slightly, and sealed with neutral balsam. Three slices were randomly selected, and photos were taken for each slice in each group at 200× magnification and analyzed using Image-Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis

The experimental data were analyzed and processed using SPSS 25.0 (IBM, Armonk, NY, USA). Data are expressed as the mean ± standard deviation (SD). One-way analysis of variance, followed by the least significant difference test, was used for group comparisons. $P < 0.05$ was considered significant.

Results

Ta-VNS decreases pathological changes in the penumbra and motor cortex of cerebral I/R model rats

The sizes and morphologies of neurons in the sham group were consistent, with a uniform and dense distribution. The structure was normal, and the cytoplasm was full. The nucleus was clear, and no vacuolar edema or degeneration was observed. No obvious nuclear shrinkage or nucleolysis was observed. A large number of cells were severely damaged with sparse distribution, and vacuolar edema and nucleolysis were observed in the I/R group. After 7 days of intervention, the number of cells featuring nuclear contraction and lysis decreased in the ta-VNS group, and the ta-VNS group was less damaged than the tn-VNS group (**Figure 3**).

Ta-VNS improves the neurological function of cerebral I/R model rats

As shown in **Figure 4**, the neurological deficit scores of cerebral I/R model rats increased significantly compared with those of the sham group ($P < 0.05$) before the intervention,

suggesting that the I/R model rats experienced functional neural damage. At 24 hours after I/R induction, no significant differences in neurological deficit scores were observed among the I/R, ta-VNS, and tn-VNS groups ($P > 0.05$). After 7 days of intervention, compared with those in the I/R group, the neurological deficit scores of rats in the ta-VNS and tn-VNS groups were significantly decreased ($P < 0.05$), and compared with those in the tn-VNS group, the neurological deficit scores of rats in the ta-VNS group significantly decreased ($P < 0.05$), indicating that ta-VNS intervention for 7 days significantly reduced neurological damage after I/R injury in rats.

Ta-VNS affects the levels of Ach, IL-1β, IL-6, and TNF-α in the penumbra and motor cortex of cerebral I/R model rats

At 7 days after I/R injury, compared with those in the sham group, the levels of TNF-α, IL-1β, and IL-6 in the penumbra and motor cortex of the I/R group significantly increased ($P < 0.01$), and the Ach level was significantly decreased ($P < 0.01$). Compared with those in the I/R group, the levels of TNF-α, IL-1β, and IL-6 were significantly reduced ($P < 0.01$), and the Ach level was significantly increased ($P < 0.05$) in the ischemic penumbra and motor cortex of the ta-VNS group. Compared with those in the I/R group, the levels of TNF-α, IL-1β, and IL-6 in the ischemic penumbra were significantly reduced in the tn-VNS group ($P < 0.01$), but no significant difference was observed for the motor cortex ($P > 0.05$; **Figure 5**).

Ta-VNS affects Cx43 expression and phosphorylation in the ischemic penumbra and motor cortex of cerebral I/R model rats

The western blot results showed that Cx43 phosphorylation in the ischemic penumbra and motor cortex of the I/R group increased significantly compared with those in the sham group ($P < 0.05$). The levels of Cx43 phosphorylation in the motor cortex and ischemic penumbra of the ta-VNS group were significantly decreased compared with those in the I/R group ($P < 0.05$). Compared with those in the I/R group, the levels of total Cx43 expression in the penumbra and motor cortex of the ta-VNS and tn-VNS groups did not change significantly ($P > 0.05$). The results from immunohistochemical staining showed that 7 days after cerebral I/R injury, compared with that in the sham group, the Cx43 immunopositivity in the motor cortex was significantly decreased in the I/R group ($P < 0.01$). Compared with that in the I/R group, the level of Cx43 immunopositivity in the motor cortex of the ta-VNS group increased significantly ($P < 0.05$). The Cx43 immunopositivity in the penumbra of the I/R group was significantly increased compared with that in the sham group ($P < 0.05$). Compared with that in the I/R group, the level of Cx43 immunopositivity in the penumbra of the ta-VNS group did not change significantly ($P > 0.05$; **Figures 6** and **7**).

Discussion

Ischemic stroke accounts for 80% of all strokes, and the key to successful treatment is timely thrombolysis to prevent irreversible, permanent damage (Hsu et al., 2017). Only 10% or fewer patients can be treated within the optimal time window, and contraindications to thrombolytic therapy can exist that are not conducive to its clinical application (Ma et al., 2019). Neuroprotection is an important auxiliary target of thrombolytic therapy during the ischemic stage, especially for the neuroprotection of the penumbra region (Moretti et al., 2015). Protecting the neurological function of the penumbra and motor cortex are highly significant for the rehabilitation of ischemic stroke. Studies have found that the inhibition of the inflammatory response and Cx43 phosphorylation after I/R play significant roles in reducing I/R injury to various organs (Cronin et al., 2008; Yuan et al., 2019), which contribute to the neuroprotection of the ischemic penumbra and promote the formation and stability of synapses during the rehabilitation period. Many studies have shown that ameliorated function is related to the recovery process, which may involve neuroprotection of the ischemic penumbra; therefore, the protection of neurons in the ischemic penumbra is important for the prognosis of ischemic stroke (Frost et al., 2003; Sutor and Hagerty, 2005). The protection of damaged neurons in the ischemic penumbra is of great significance for facilitating the recovery of patients' neurological function and reducing disability. Previous studies have shown that VNS significantly reduced the effects of acute ischemic injury (Ay et al., 2016). In our study, ta-VNS was used to treat the rat I/R model. The results showed that after 7 days of intervention, the neurological function score in the ta-VNS group was significantly reduced compared with that in the I/R group, indicating that ta-VNS effectively reduced nerve injury in I/R model rats and has a neuroprotective effect, which is consistent with the effects reported in other studies.

The inflammatory response is a normal defensive response mediated by the body's living tissue in reaction to various inflammatory factors and intended to restrict further injury to the host (Rock and Kono, 2008). The vagus nerve is involved in a complex neuroendocrine-immune network that maintains metabolic homeostasis and regulates various pathological processes in the brain (Mravec, 2010). In the I/R group, inflammatory factors significantly increased in the ischemic penumbra. After 7 days of ta-VNS intervention, the concentrations of inflammatory factors decreased significantly, whereas the levels of Ach significantly increased, indicating that the I/R inflammation model was successfully generated and that ta-VNS intervention could activate the cholinergic anti-inflammatory pathway in the ischemic penumbra, increasing the Ach contents, inhibiting the secretion of inflammatory factors, and reducing I/R damage. The activation of cholinergic receptors mediate neuronal protective effects through the regulation of immune cells by the cholinergic anti-inflammatory pathway. Ach produced during the inflammatory response of the vagus nerve is thought to inhibit the release of inflammatory cytokines. The animal experiments showed that the neuroprotective effects of VNS in an ischemic environment are closely associated with the activation of cholinergic anti-inflammatory pathways (Bernik et al., 2002), which is consistent with the results of our study.

Connexin, which is involved in the connections between cells, is an important factor in information transmission, substance metabolism, and energy transport between cells (Jiang et al., 2014). Connexins are widely expressed in astrocytes, neurons, and glial cells (Nakase et al., 2003). Cx43 is highly regulated by phosphorylation at over a dozen sites, likely by multiple kinases. One study found that Src activation promotes the formation of linkers, which involved the ERK-mediated phosphorylation of S279/282. In the presence of Src, the inhibitory effects of the proteasome were significant, and the GJ was quickly restored, which resulted in a significant change in the phosphorylation profile of Cx43 (Solan and Lampe, 2020). In addition, a study has shown that calcineurin may play a direct role in the regulation of the phosphorylation state of Cx43 (Li and Nagy, 2000). Many kinases regulate the phosphorylation of Cx43, and how ta-VNS regulates the phosphorylation of Cx43 in this study has not yet been clarified. The release of inflammatory factors was significantly inhibited after the application of a GJ inhibitory peptide, suggesting that the GJ, which is composed of connexins, plays a significant part in mediating inflammation (Reifler et al., 2015). Cx43 is the primary component protein of connexin channels in brain tissue, and its enhanced expression can accelerate intercellular signal transduction, aggravating damage after injury. Studies have shown that hypoxia and inflammation synergistically accelerate the activity of Cx43 hemichannels under ischemic conditions (Faigle et al., 2008), leading to the loss of astrocytes and neuronal death (Liang et al., 2020). One study showed that 1 hour after TBI,

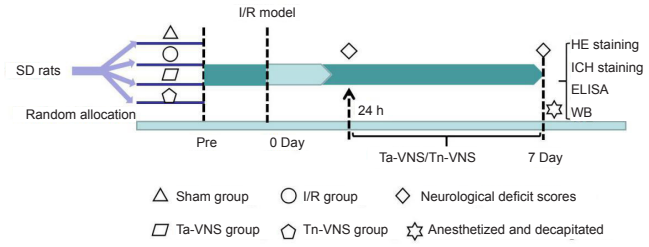

Figure 1 | **The procedure of the study.**
ELISA: Enzyme-linked immunosorbent assay; HE staining: hematoxylin and eosin staining; I/R: ischemia/reperfusion; ICH staining: immunohistochemical staining; SD: Sprague-Dawley; ta-VNS: transcutaneous auricular vagus nerve stimulation; tn-VNS: transcutaneous non-auricular vagus nerve stimulation; WB: western blot assay.

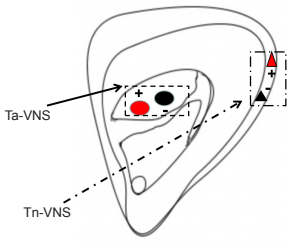

Figure 2 | **Positioning of ta-VNS and tn-VNS.**
The circles indicate transcutaneous auricular vagus nerve stimulation; The triangles indicate transcutaneous non-auricular vagus nerve stimulation. The red mark represents the positive electrode, and the black mark represents the negative electrode. ta-VNS: Transcutaneous auricular vagus nerve stimulation; tn-VNS: transcutaneous non-auricular vagus nerve stimulation.

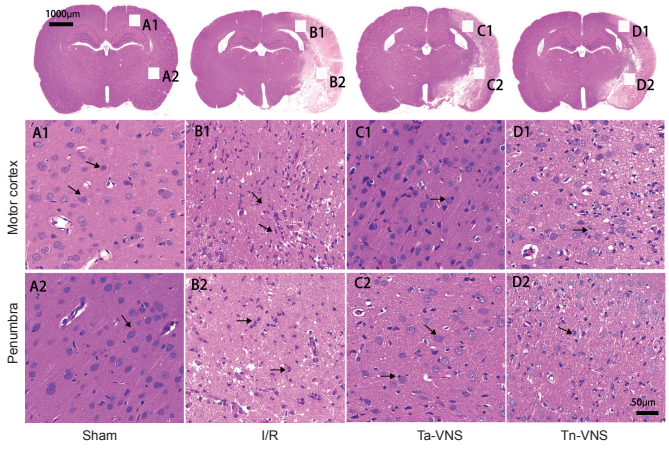

Figure 3 | **Effects of ta-VNS on pathological changes in the penumbra and motor cortex in the cerebral I/R rat model (hematoxylin and eosin staining).**
The size and morphology of neurons in the sham group were generally consistent, with a uniform and dense distribution in both the penumbra and motor cortex. Compared with the I/R group and tn-VNS group, the number of cells featuring nuclear contractions and lysis decreased in the ta-VNS group, especially in the motor cortex. Arrows indicate neurons. Scale bars: 1000 μm in the first row and 50 μm in the middle and last rows. I/R: Ischemia/reperfusion; ta-VNS: transcutaneous auricular vagus nerve stimulation; tn-VNS: transcutaneous non-auricular vagus nerve stimulation.

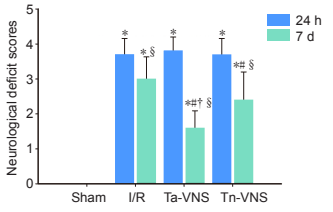

Figure 4 | **Effects of ta-VNS on the neurological deficit scores in cerebral I/R model rats**
The neurologic function score was measured 24 hours and 7 days after modeling. Data are expressed as the mean ± SD (n = 10). *P < 0.05, *vs.* sham group; #P < 0.05, *vs.* I/R group; †P < 0.05, *vs.* tn-VNS group; §P < 0.05, *vs.* 24 hours (one-way analysis of variance followed by the least significant difference test). I/R: Ischemia/reperfusion; ta-VNS: transcutaneous auricular vagus nerve stimulation; tn-VNS: transcutaneous non-auricular vagus nerve stimulation.

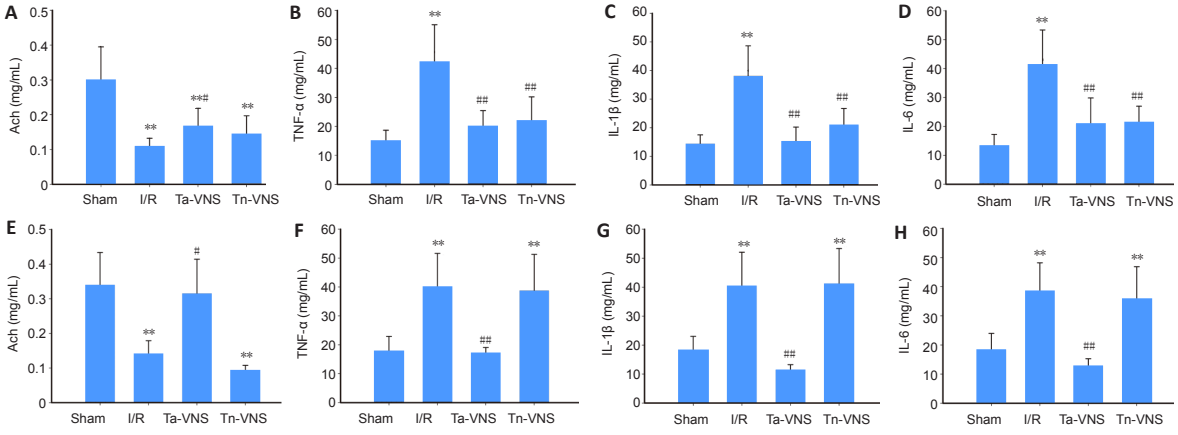

Figure 5 | **ta-VNS reduces the levels of acetylcholine and inflammatory factors in the ischemic penumbra and motor cortex in cerebral I/R model rats 7 days after injury.**
The quantitative results of enzyme-linked immunosorbent assays. (A–D) Levels of acetylcholine, TNF-α, IL-1β, and IL-6 in the ischemic penumbra. (E–H) Levels of acetylcholine, TNF-α, IL-1β, and IL-6 in the motor cortex. Data are expressed as the mean ± SD (n = 5). **P < 0.01, *vs.* sham group; #P < 0.05, ##P < 0.01, *vs.* I/R group (one-way analysis of variance followed by the least significant difference test). Ach: Acetylcholine; I/R: ischemia/reperfusion; ta-VNS: transcutaneous auricular vagus nerve stimulation; tn-VNS: transcutaneous non-auricular vagus nerve stimulation.

the expression of phosphorylated Cx43 in the ipsilateral hippocampus was significantly induced and remained at a high level until 24 hours after injury. At 6 hours after injury, the protein expression of phosphorylated Cx43 peaked, indicating that the phosphorylation level of Cx43 increases significantly after the occurrence of central nervous system injury (Ohsumi et al., 2010). Cx43 phosphorylation leads to the degradation of GJs and the opening of hemichannels in ischemic stroke, inducing the release of inflammatory factors and exacerbating damage (Cooper and Lampe, 2002). Proinflammatory cytokines reduce the expression of GJs between astrocytes, leading to the increased release of ATP, glutamate, and prostaglandins (Orellana et al., 2011; Avila-Muñoz and Arias, 2014). These molecules are toxic to adjacent cells and cause irreversible brain damage (Castellano et al., 2016). The results of the present study showed that after 7 days of intervention, the phosphorylation of Cx43 in the motor cortex and ischemic penumbra of the ta-VNS group was significantly inhibited,

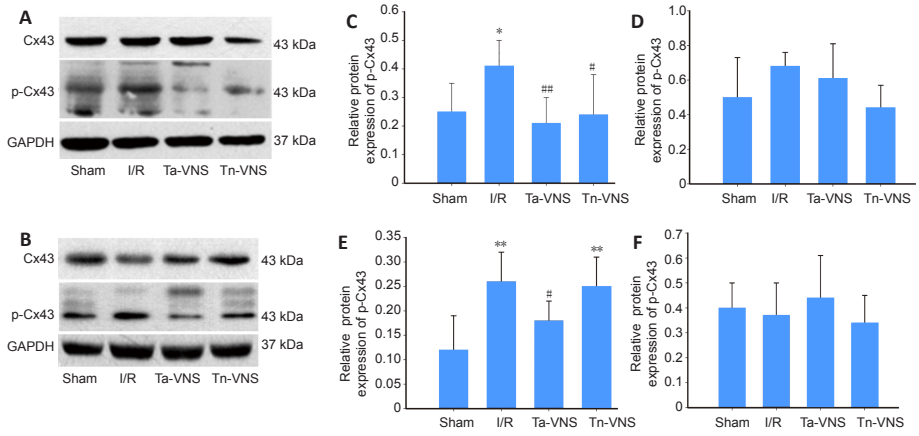

Figure 6 | Effects of ta-VNS on Cx43 and p-Cx43 protein expression levels in the ischemic penumbra and motor cortex of cerebral I/R model rats 7 days after modeling detected by western blot assay.

(A, B) Protein levels of Cx43 and p-Cx43 in the penumbra (A) and motor cortex (B) were detected by western blot assay. (C, D) Quantitative evaluation of p-Cx43 and Cx43 levels in the ischemic penumbra. (E, F) Quantitative evaluation of p-Cx43 and Cx43 levels in the motor cortex. The target protein expression was expressed as the optical density ratio to that for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The data are expressed as the mean ± SD (n = 5). *P < 0.05, **P < 0.01, *vs.* sham group; #P < 0.05, ##P < 0.01, *vs.* I/R group (one-way analysis of variance followed by the least significant difference test). Cx43: connexin protein 43; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; I/R: Ischemia/reperfusion; p-Cx43: phosphorylated connexin protein 43; ta-VNS: transcutaneous auricular vagus nerve stimulation; tn-VNS: transcutaneous non-auricular vagus nerve stimulation.

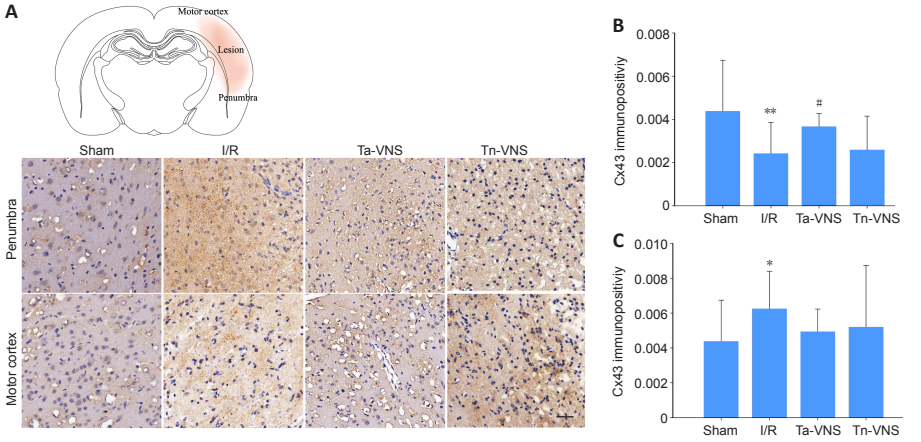

Figure 7 | Effect of ta-VNS on Cx43 immunopositivity in the ischemic penumbra and motor cortex of cerebral I/R model rats 7 days after modeling.

(A) Immunohistochemical staining for Cx43 in the penumbra and motor cortex. Compared with that in the I/R group, the Cx43 immunopositivity in the motor cortex of the ta-VNS group increased, whereas the Cx43 immunopositivity in the penumbra of the ta-VNS group did not change. The positive color was brownish-yellow. Scar bars: 50 μm. (B, C) Quantitative evaluation of Cx43 immunopositivity in the motor cortex (B) and ischemic penumbra (C). Data are expressed as the mean ± SD (n = 5). *P < 0.05, **P < 0.01, *vs.* sham group; #P < 0.05, *vs.* I/R group (one-way analysis of variance followed by the least significant difference test). Cx43: Connexin protein 43; I/R: ischemia/reperfusion; ta-VNS: transcutaneous auricular vagus nerve stimulation; tn-VNS: transcutaneous non-auricular vagus nerve stimulation.

the total level of Cx43 expression in the motor cortex of the ta-VNS group was increased compared with those in the I/R group. Although no significant differences were observed on the western blot analysis, the trends observed between the western blot and immunohistochemistry analyses were consistent, which may be due to the insufficient sample size included in the research group. These research results indicated that phosphorylated Cx43 plays a significant role in rat I/R injury, and ta-VNS inhibits the production of inflammatory factors by activating the cholinergic anti-inflammatory pathway, inhibiting the phosphorylation of Cx43 protein in the ischemic penumbra and motor cortex, improving ischemic neuronal plasticity. The findings of the present study showed that compared with that in the I/R group, ta-VNS treatment significantly increased the Ach contents in the penumbra and motor cortex while inhibiting inflammation, and the phosphorylation of Cx43 is involved in generating effects of ta-VNS improving the recovery of motor function after stroke. Ta-VNS inhibits the production of inflammatory factors by promoting the expression of Ach, which leads to the inhibition of Cx43 phosphorylation in the ischemic penumbra, protecting the neurons in the ischemic penumbra, and promoting the recovery of motor function in ischemic stroke rats.

In this study, we found that tn-VNS significantly activates Ach and inhibits the release of proinflammatory factors in the ischemic penumbra. The results of WB and immunohistochemistry experiments show that compared with the I/R group, p-Cx43 was significantly inhibited in the tn-VNS group but not in the motor cortex. We hypothesize that this outcome may be caused by the electrical stimulation applied to the ear edge of the rat during the experiment, which was partially transmitted to the distribution area of the vagus nerve of the concha, producing a therapeutic effect. However, other nerve conduction pathways in the ear edge may also be involved, which requires confirmation by other studies in the future. Ta-VNS and VNS are intervention methods based on the same underlying mechanism. The ta-VNS is the only superficial branch of the vagus nerve; therefore, the effects of ta-VNS are likely to be similar to the effects of VNS. The biggest difference is that ta-VNS is a non-invasive intervention method, which could be easily applied to patients and is more acceptable than VNS. In this experiment, we found that ta-VNS can result in good neuroprotective effects, providing research support for future clinical trials of ta-VNS for rehabilitation after ischemic stroke and as a possible complementary treatment for motor dysfunction.

Overall, our study demonstrated that ta-VNS reduced nerve injury in I/R model rats, increased the Ach contents, inhibited the release of TNF-α, IL-6, and IL-1β in the ischemic penumbra and motor cortex, and simultaneously inhibited Cx43 phosphorylation to promote neuronal function recovery. These results suggest that the cholinergic anti-inflammatory pathway is involved in the central anti-inflammatory effects of ta-VNS, ta-VNS plays a role in protecting the neurons after I/R injury. We conducted an exploratory study of ta-VNS to study the effects of ta-VNS on the inflammatory response surrounding the infarct focus and motor cortex in rats with cerebral infarction and the effects on Cx43 expression and phosphorylation; however, this was an *in vivo* study.

To understand the effects of ta-VNS on nerve cells more intuitively, *in vitro* experiments should be conducted in the future.

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