Possible mechanism underlying analgesic effect of Tuina in rats may involve piezo mechanosensitive channels within dorsal root ganglia axon


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Abstract

OBJECTIVE: To demonstrate the analgesic effect of Tuina mainly from mechanically sensitive ion channels in peripheral myelinated nerve fibers.

METHODS: A total of 40 healthy and pathogen-free adult male Sprague-Dawley rats were used in the study (weight: (220.0 ± 1.4) g, Shanghai Slac Laboratory Animal Co., Ltd., Shanghai, China; license No. Shanghai ICP 05033115). The rats were housed in cages with free access to water and food in a temperature-controlled room (22 ± 1 °C) and 12-h/12-h light-dark cycle. Thirty-two rats were randomly divided into five groups: naive, sham, chronic compression of dorsal root ganglion (CCD), Tuina (7 d) and Tuina (21 d). CCD rat model was established via unilateral DRG compression by “L” liked steel bar. Chinese Tuina treatment was accepted once per day. Behavior monitoring of paw withdrawal threshold (PWT) and paw withdrawal latency (PWL) were tested. The expression of Piezo1 and Piezo2 in myelinated nerve fiber were analyzed by immunohistochemistry and Western-blotting.

RESULTS: There was a high expression of Piezo2 and a low expression of Piezo1 in the naive and CCD groups. In contrast, the expression of Piezo2 was down regulated and Piezo1 was increased after a period of Tuina. There was significant difference (P ≤ 0.05) between the groups.

CONCLUSION: Our findings suggest that Tuina therapy can increase the expression of Piezo2 and decrease the expression of Piezo1 in the test rats. The different changes in the expressions of Piezo1 and Piezo2 may play an important role in alleviating CCD-induced allodynia and hyperalgesia.
INTRODUCTION

Chinese Tuina, one external therapy of Traditional Chinese Medicine (TCM), is a traditional method used for pain relief. Tuina has advantages in the treatment of acute and chronic pain, depression, chronic inflammation and mechanical injury. Tuina is based on the basic TCM theory and selects acupoints according to symptom pattern identification, which has obvious therapeutic advantage in clinical chronic common and non-communicable diseases such as motor system, nervous system, cerebrovascular system and other functional diseases. Tuina manipulation characterized by force-acupoint stimulation which directly acting on the subcutaneous tissues and nerve endings. It has a close relationship with mechanosensitive ion channels because mechanosensitive ion channels play a critical role in converting mechanical stimulus signals into bioelectrical signals that can be transmitted through nerve fibers and recognized by the brain. Mechanotransduction was the conversion of mechanical stimuli into biological signals. Particular attention was given to Piezo channels and their potential roles in various biological functions. Piezo1 and Piezo2 expressed in the dorsal root ganglion (DRG) neuron membrane played a direct role in perception intra-vertebral canal pressure and mechanical compression. Protrusion of lumbar intervertebral disc is a common and frequently-occurring disease. About 90% of lumbar cervical pain diseases, especially pain in waist and lower extremities was leaded by protrusion of lumbar intervertebral disc. After a period of Tuina therapy, pain in all patients were significantly alleviated or disappeared. Enable patients to return to work and improve the quality of life. Owing to unique and significant effect, attractive and reasonable price, convenience and little side effects, TCM Tuina is deeply favorable by a great deal of patients.

DRG is the primary afferent sensory neurons, which plays an important role in the formation of primary sensing. Mechanosensitive channels is activated by physical stimuli. GC Mccarter and DB Reichling performed patch clamp experiments on rats DRG neurons in culture, and reported the first mechanically-activated current in somatosensory neurons. Mechanosensitive channel activities have been detected by the patch-clamp electrophysiology in many cell types in mammals including DRG sensory neurons. However, the whole-cell current was observed in most dorsal root ganglion sensory neurons but not in non-sensory sympathetic ganglion neurons. Piezo channel is one of the mechanosensitive channels, which include Piezo1 and Piezo2. Piezo channels is widely distributed in various of body tissues, such as epithelial cell, pulmonary cells, sensory neuron cells, retina cells and so on. Piezo channels are composed of over 2000 residues which predicted that across the cell membrane about 30 to 40 times. They are the largest protein found in the membranes of cells. They can detect cellular stress induced by physical stimulation and convert it into bio-electricity signal which can be transmitted to the center nervous system for further information analysis and processing. The mechanosensitive channels plays a significant role in perception of the outside world. Piezo2 involved in mechanical allodynia illustrated by Piezo2 knockdown in vivo antisense strategy in mouse models of chronic neuropathic pain. Spinal canal stenosis and compression of spinal cord or nerve root are common symptoms in protrusion of lumbar intervertebral disc, which give rise to compression of DRG as well. In the circumstances, DRG mechanosensitive channels can be activated. Bradykinin and a selective Epac-1 agonist could upregulated Piezo2 currents in mouse somatosensory neurons, suggesting that Piezo2 may play a key role in hyperalgesia and allodynia. Piezo1 and Piezo2 constitutive knockout mice died embryonically or at birth which suggested that the Piezo1 or Piezo2 was crucial at early stages of life. We can safely draw the conclusion that Piezo channels play an significantly important role in our life. Chronic compression of the dorsal root ganglion (CCD) which was a classic animal neuropathic pain model was applied to imitate the clinical symptoms and detect analgesic effect of Tuina. Using this model could imitate the clinical symptoms of patients who suffering from protrusion of lumbar intervertebral disc. Thus, CCD rat model of neuropathic pain used in this study may provide a novel approach for investigating the molecular and physiological mechanism underlying the therapeutic effects of Tuina.

MATERIALS AND METHODS

Animals

Forty male Sprague-Dawley (SD) rats [age, 40-50 d; weight, (220.0 ± 1.4) g] were purchased from Experimental Animal Center of Fudan University (Shanghai, China) and housed in plastic cages at room temperature, and natural diurnal cycles were applied. All experiments were conducted in compliance with the Institutional Animal Care and Use Committee (Shanghai Municipal Commission of Health and Family Planning), and as few animals were used as possible in order to achieve statistical significance. The present study was approved by the Ethical Committee of the Yueyang Hospital of Integrated Traditional Chinese and Western Medicine (Shanghai 200437, China).

Rat pain models and surgical procedure

This research strictly complied with the Chinese Institutional Care Committee for the use of animals and the tests were performed in accordance with the Helsinki declaration. Great efforts have been made to minimize both the suffering and the number of animals that were used. Adult male SD rats were randomly di-
vided into naive groups, CCD groups, sham groups and CCD with Tuina groups. There were eight rats in each group. Tuina groups were subdivided into 7 d treatment group and the 21 d treatment group. Similarly, eight rats in each group. Animals was anesthetized by pentobarbital sodium (0.4 mL/100 g). The skin included the superficial fascia and deep fascia was incised along the spinous process and the muscle adhering to the vertebral plate and lumbar zygapophyseal joint needed blunt dissection. We could see the superior articular process after the two procedures that mentioned above. Then blunt separated the muscle tissues attached to the facet joints and exposed the fourth and fifth lumbar intervertebral foramen. Next step, we inserted two "L"-shaped stainless steel rod (0.6 mm diameter, 4 mm length) into the intervertebral foramen of the fourth lumbar vertebra and fifth lumbar vertebra. You would observe tail whip and ipsilateral lower limbs cramped which suggested that the inserting was success. The wound will be stitched layer by layer. Naive groups SD rats were doing nothing process. The surgical procedure of sham groups completely identical with the CCD groups except inserting "L"-shaped rod into the intervertebral foramen constantly. Penicillin intra-peritoneal injection was operated after the surgical to prevent from bacterial infection.

**Behavior examination**

Paw withdrawal threshold (PWT)\(^{16}\) and paw withdrawal latency (PWL)\(^{16}\) were used to monitor changes of pain behavior in rats. Their operating procedures were described as follows. We used Von Frey monofilaments to investigate SD rats paw withdrawal minimal response level of mechanical stimulation. Mechanical stimuli were applied to the plantar surface using a series of Von Frey monofilaments (BME-403) in an ascending order (6, 8, 10, 15, 20 g). Thermal stimulation reaction threshold was assessed by Hargreaves\(^{16}\) which theory was stimulating the center of the rats paw by the radiant heat emitted by tungsten halogen lamp to get the PWL by measure the radiant heat time from the beginning to appear paw withdrawal.

**Tuina procedure**

Hand manipulation maneuvers included clockwise pressing and rubbing, with moderate to strong pressure on the skin. Tuina was performed on the center of the gastrocnemius muscle\(^{17}\) for the indicated frequencies and durations. Massage intensity was 80% of the full potential (at this point, the rats exhibited sensory stimuli feeling without signs of pain and paw withdrawal). Prior to Tuina, rats were placed face down on a frame platform to adapt to the experimental environment and to minimize stress. The optimal application mode was 2 Hz for 15 min, consisting of 2 min cycles and 1 min intervals between cycles.\(^{18}\) Finger pressure recordings (units in Newton) were monitored using a pressure recording instrument (FingerTPS™, Pressure Profile Systems, Inc., Tokyo, Japan), as described in a previous study.\(^{19-22}\)

**Western blotting**

Western Blotting was a kind of immunochemical techniques which is used to detect a protein immobilized on a matrix. It was made up of four ingredients: extraction of protein samples, gel electrophoresis, electro-blotting, and detection of a specific antigen. Proteins which were extracted from tissues were resolved by gel electrophoresis which was called sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then the mixed liquid which included proteins and a buffer containing SDS prior was loaded onto a polyacrylamide gel. The proteins would be separated according to their molecular mass.\(^{23-25}\) Then, the separated proteins were transferred from the gel to a membrane by electro-blotting. Immersing the membrane into 5% non-fat milk which was in TTBS solution (the final volume is 20 mL) for 1 h at room temperature. The non-specific binding site should be blocked by bovine serum albumin. We detected the specific antigen immobilized on the membrane using primary and secondary antibodies and a chemiluminescent reagent. Finally, we exposed the protein side of the membrane to X-ray film.\(^{26-29}\)

**Immunofluorescence**

Neuron axons were incubated with Anti-200kD Neurofilament Heavy antibody-Neuronal Marker (ab8135; Abcam; 1: 1000; Shanghai, China) overnight at 4 °C. Then, Neuron axons were incubated with Piezo1 antibody (Sc-164318; Santa; 1: 1000) and Piezo2 antibody (Sc-84763; Santa; 1: 1000; Shanghai, China) respectively overnight at 4 °C. Cell nuclei were counterstained with DAPI prior to analysis under a confocal laser scanning microscope (Olympus, Tokyo, Japan).

**Chemical and reagents**

The following chemicals were used: Anti-200kD Neurofilament Heavy antibody-Neuronal Marker (ab8135; Abcam; 1: 1000; Shanghai, China), Piezo1 Antibody (Sc-164318; Santa; 1: 1000; Shanghai, China) and Piezo2 Antibody (Sc-84763; Santa; 1: 1000; Shanghai, China). Anti-Cytokeratin 8 antibody (ab9023; Abcam; 1: 1000; Shanghai, China). Both the dose and schedules were selected on previous report and on pilot experiments.\(^{27}\) All the experimental steps are in line with laboratory requirements.

**Data analysis**

Data were analyzed by Sigmaplot software (Systat software, Inc., New York, NY, USA). A two-way repeated measures analysis of variance was used to analyze between-group differences in the mechanical and thermal withdrawal latencies, t test was used to compare the differences in the distribution of Piezo1 and Piezo2 channels. Values in the text and figures were expressed as means ± standard error of mean (SEM). The significance level was set at P ≤ 0.05.
RESULTS

Analgesic effect of Tuina on CCD-induced allodynia
All rats woke up after the surgery and could walk correctly and safely which indicated that the surgery did not injure the motor nerve and did not impact the motor behavior. The CCD group rats pain threshold decreased deeply after the surgery (Figure 1). Compared to CCD and naive groups, the Tuina groups threshold value of the CCD-induced mechanical and thermal allodynia significantly increased after the rats were accepted 21 d Tuina treatment. The significant reduction in CCD-induced allodynia was observed at 1 d post-surgery, peaked at 7 d, and lasted no less than 21 d. We may safely draw the conclusion that Tuina treatment suppressed the CCD-induced allodynia including mechanical and thermal.

Mechanical sensitivity ion channels in peripheral nerve fibers
We used perfusion technique to study the dorsal root ganglia and sciatic nerve of rats for immunofluorescence experiments. Double labeling immunofluorescence assay demonstrated that the sciatic nerve expresses mechanically sensitive ion channels. Piezo2 was dyed green and Nefh was dyed red. After co-labeling, they become yellow light. Piezo mechanically sensitive ion channel was confirmed on the primary sensory center dorsal root ganglion and its myelinated peripheral sensory fibers (Figure 2). There are many acupoints around the sciatic nerve. It is reasonable to speculate that the mechanical sensitive ion channels on the sciatic nerve are the key to the perception of external mechanical stimulation (such as Tuina therapy).

Distribution patterns of mechanical sensitive ion channels Piezo1 and Piezo2 expressed at myelinated fiber of peripheral nerve
Piezo1 was dyed green fluorescence and Nefh was dyed red fluorescence. After co-labeling, they become yellow fluorescence. It was proved that Piezo1 was expressed in peripheral myelinated nerve fibers. However, its brightness is weaker than that of Piezo2, which proves that Piezo2 plays a major role in non-noxious stimulation.

Figure 1 Comparison of PWL and PWT in different groups
A: CCD-induced pain resulted in a suddenly decreasing of PWL in CCD group and CCD + Tuina group. The pain-induced by CCD could be suppressed by Tuina treatment from days 7 to 21 compared to CCD group (P ≤ 0.05); B: the PWT of CCD group and Tuina group declined sharply after CCD surgery. The analgesia effect of Tuina treatment began to work after Tuina treatment compared to CCD group from 7 to 21 d (P ≤ 0.05). CCD + Tuina group accepted Tuina therapy once a day until day 21. The others group don’t have intervention. The PWL and PWT of Tuina group and CCD group were inferior to naive group and sham group from beginning to end.

Figure 2 Mechanical sensitivity ion channels are expressed in peripheral nerve fibers by immunofluorescence of 200 μm
A: this is the expression of Piezo2 in sciatic nerve cross section (up) and vertical section (down) of naive rats, Scale bar, 200 μm; B: the full name of Nefh is Anti-200 kD Neurofilament Heavy antibody-Neuronal Marker (ab8135), which is the marker of peripheral myelinated afferent nerve fibers, Scale bar, 200 μm; C: Piezo2 is expressed in abundance in naive rat sciatic nerve, Scale bar, 200 μm; D: Piezo2 and Nefh merged into yellow respectively in the vertical section of rat’s thigh, Scale bar, 200 μm; E: Nefh which took red fluorescence color. F: Piezo2 which took green fluorescence color.
Immunofluorescence assay predicted that Piezo2 protein which were attached by red color and Nefh which were attached by green color overlapped together through which we could draw the conclusion that Piezos protein expressed at myelinated fiber of peripheral nerve. Piezos channels could perceive external mechanical stimulation and could be gated by it to control the entrance and exit of ions. Piezos channels were protein pores of cellular membranes and ion flow could change the trans-membrane voltage which was the basis of signal transfer between different cells. The Piezos channels played a significant role in converting the mechanical signals into electrical ones. Then, the electrical signals spread along the neural axon until arrived at the center nervous system for data processing and analysis.

Changes in the Piezos channels expression on the myelinated fiber of peripheral nerve
Piezo2 was highly expressed at the peripheral nerve myelinated fiber of naïve groups and CCD groups and decreased gradually when accepted Tuina treatment after CCD surgery. On the contrary, Piezo1 expression levels was low at the peripheral nerve myelinated fiber of naïve groups and CCD groups and increased gradually when accepted Tuina treatment after CCD surgery. The changing laws of Piezo1 and Piezo2 may play a significant role in analgesic effect of Tuina in curing prolapse of lumbar intervertebral disc (Figure 4).

Piezo1 and Piezo2 protein expression level after DRG surgery and Tuina
The changes in the contents of Piezo1 and Piezo2 after the Tuina treatment were observed by using the Western-Blotting technique. Piezo1 and Piezo2 were involved in the development of tactile allostrophy and hyperalgesia. After a period of Tuina treatment, the Piezo2 content decreased and the mechanical pain threshold increased. Piezo1 is mainly distributed in the dorsal root ganglion small neurons. After a period of Tuina treatment, it showed a gradual increase (Figure 5).

DISCUSSION
Neuropathic pain is a common clinical symptom which brings suffering to the vast majority of patients.40-52 A lot of researches have been carried out on neuropathic pain. Clinical practice has proved that Tuina of Traditional Chinese Medicine has a good analgesic effect.53-55 Prolapse of lumbar intervertebral disc was a common and frequently-occurring disease. Tuina had a significant curative effect in alleviating pain induced by prolapsus of lumbar intervertebral disc.21 However, the mechanism of the analgesic effect of Tuina was unclear. The chronic compression of dorsal root ganglion rat model has a good imitation of the symptoms of nerve root oppression of lumbar intervertebral disc.56-57 Piezos played a physiological role in mechanosensation in mammals. However, whether Piezos or fibers themselves sensed mechanical force was debated, and the molecular mechanism of mechanotransduction was unknown. Tuina manipulation as a physical stimuli activated the Piezos channels and then the membrane potential changed by charged ion flowing.58-61 The physical stimuli converted into electrochemical signals and delivered into the central nervous system for information processing and analysis.62-65 The therapeutic effects of Tuina has long been demonstrated by clinical practice for the treatment of pain, depression, chronic inflammation and mechanical injury.66-68 Based on the theory of peripheral sensory signaling, there exists an interacting network of sensory nerves in the skin and beneath the muscles.69-72 Various nerve pathways of the peripheral nervous system participate in distinct signal transductions, and it has previously been demonstrated that acupuncture may attenuate pain signaling by stimulating Aβ, Aδ and C-type nerve fibers.73-75 Tuina, which selectively stimulates mechanism channels, can activate and adjust the transmission of signals from one nerve cell to another, thus explaining its analgesic effects in clinical practice.76,77 In conclusion, our study demonstrated an analgesic effect of Tuina which was manipulated at the middle point of gastrocnemius muscle in a rat model of pain.
The analgesic effects of Tuina were associated with elevated pain thresholds.

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