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Effect and Mechanism of Electro-acupuncture on Neuralgia of Cervical Spondylotic Radiculopathy Based on the Neuron-gliocyte-chemokine Signaling Pathway

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Abstract

Cervical Spondylotic Radiculopathy (CSR) is the most common type of cervical spondylosis, which mainly presents as radioactive neuralgia. Neuralgia is closely related to gliocyte in the central nervous system. Therefore, to investigate the analgesic effect and mechanism of electro-acupuncture on neuralgia in rats with cervical spondylotic radiculopathy based on the neuron-gliocyte-chemokine signaling pathway. The models of rats with neuralgia in cervical spondylotic radiculopathy were established and the intervention of electro-acupuncture and LAA astrocyte inhibitor were carried out. The results found that the content of CGRP, AC, P in C6-C7 primary sensory neuron terminal of the spinal cord segment were significantly decreased in electro-acupuncture group compared with model group. Relative expression of RNA levels of PKC, VGCC, CCL2 and CXC3L1 in electro-acupuncture group were significantly decreased compared with model group. Westernblot detected that the expression of IL-1β, IL-6, IL-18, TNF-α, NMDA and AMPA proteins in LAA+electro+acupuncture group were obvioulsy decreased. It indicates that neuralgia is closely related to gliocyte in the central nervous system, and also proves that LAA+electro acupuncture can effectively relieve neuralgia caused by cervical spondylosis.

Keywords: Cervical spondylosis; Ligaments; Intervertebral discs; Cervical spondylotic radiculopathy; Radioactive radiculopathy

Introduction

Cervical spondylosis (CS) was mainly presented degeneration of intervertebral discs, vertebrae, joints, associated muscles, fascia, ligaments, and secondary pathological changes [1]. The complication of CS such as neuralgia, numbness, muscle atrophy, which seriously affect the life quality of patients [2]. Therefore, the prevention and treatment of CS appear to be particularly urgent. Cervical spondylotic radiculopathy (CSR) is the most common type of CS, accounting for about 60-71%. It also is a disease characterized by radioactive radiculopathy, numbness, decreased muscle strength, and even muscle atrophy [3-5].

At present, neuroinflammatory injury is the pathological basis of CSR, which is a kind of neuralgia. CSR belongs to the radiculopathic neuropathy in peripheral neuropathic pain, manifested as spontaneous pain, hyperalgesia and abnormal pain [6]. Modern medical treatment contain non-surgical treatment and surgical treatment. Non-surgical therapy mainly includes anti-inflammatory and analgesic treatment, dilatation of blood vessels, diuretic dehydration, nutritional nerves, etc. However, the efficacy is not exact, and the symptoms are easy to relapse. However, surgical therapy is risky and expensive [7]. Therefore, it is very important to find a green, safe and effective therapy for CSR. Early clinical observation on the treatment of cervical spondylosis by acupuncture proved that acupuncture has positive effects on cervical spondylosis, can effectively relieve chronic pain caused by cervical spondylosis, and significantly improve the life quality of patients. Studies have shown that acupuncture therapy for neuralgia caused by peripheral nerve injury is realized through the two-way pathway between gliocyte and neurons [8,9]. Acupuncture therapy has special advantages in the prevention and treatment of cervical spondylosis due to its unique theoretical system, such as wide indications, rapid early onset, stable long-term effect, multi-channel regulation, two-way balance regulation, psychosomatic co-treatment and high total effective rate, which can be widely and effectively used in the prevention and treatment of cervical spondylosis.

Materials and Methods

Material

48 healthy adult SD rats (SPF) (weight 200-220 g) were provided by Animal Experiment Center of Hunan University of Chinese Medicine. All rats were fed at a temperature of 24-26°C and the humidity of 50-70%. SDZ-H electro-acupuncture therapy apparatus (Hutuo); No.28 0.5 inch stainless steel needle (Hutuo); NO assay kit (A012-1) (Sigma); glutamic acid, ELISA kit (Thermo); MULTISKAN MK3microplate reader (Thermo); SK-1Micro oscillator (Jintan Medical instrument Factory); TGL-16B centrifugal machine (Anke).

Methods

Part I: Effects of electro-acupuncture on the excitability of traumatic sensory neurons in rats with neuralgia in cervical spondylotic radiculopathy

Preparation of rat model with neuralgia in cervical radiculopathy: Rats were anesthetized by intraperitoneal injection

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of 1% pentobarbital sodium (45 mg/kg). The spinous process of the second thoracic vertebra was marked and the center of the 7th cervical vertebra was used for routine skin preparation and disinfection. A median incision about 3.5 cm long was made on the posterior midline of the rats, which was gently separated to expose the left C6 and C7 spinal nerves, and the distal nerve roots of the C7 dorsal root ganglion were ligated with 6-0 silk thread, and then the incisions were sutured layer by layer. The sham operation group was performed the same surgery without nerve ligation.

Intervention of electro-acupuncture on neuralgia in rats with cervical spondylotic radiculopathy: All rats were fed adaptively for 1 week, and randomly divided into 4 groups (12 rats in each group). Control group: normal SD rats were fed with standard fodder. Model group: neuralgia in cervical spondylotic radiculopathy. After operation 2 weeks, the model was successfully bound with the electro-acupuncture group once a day, 20 min each time, for 14 consecutive days. Sham operation group: same operation procedure was performed as model group but no nerve ligation. After operation 2 weeks, the model was successfully bound with the electro-acupuncture group once a day, 20 min each time, for 14 consecutive days. Electro-acupuncture group: same operation procedure was performed as model group. After operation 2 weeks, the rats were bound to the plate in a prone position and treated with electro-acupuncture at Jiaji point, with dense wave frequency of 50 Hz and wave repacking frequency of 10 Hz, once a day, 20 min each time, for 14 consecutive days. After all rats being tested, they were fasted without water for 24 hours and 2% pentobarbital sodium was injected intraperitoneally for anesthesia. The cervical muscle tissue was separated, and the C7 spinal ganglions on the lesion side were taken. The corresponding spinal cord segment was labeled and the relevant indexes were detected.

Content of CGRP, adenylate cyclase and substance P: The levels of CGRP, adenylate cyclase (AC) and substance P in the primary sensory neurons of C6 and C7 spinal cord segments were detected by double immunofluorescence assay. The rat tissues were soaked with PBS and treated with 4% paraformaldehyde at room temperature for 10 min. The tissues were washed with PBS for 3 times, then 0.5%Txiton X-100 treated for 5 min. The tissues were washed with TBST for 3 times and sealed at room temperature for 1 h. Primary antibody Alexa Fluor 488 labeled goat anti-rat (0.1% BSA/TBST) were incubated with the tissues overnight at 4°C, then washed with 0.1% BSA/TBST for 3 times. Fluorescent Alexa Fluor 555 labeled donkey anti-rat was incubated for another 1 h. Fluorescence microscope was used to observe the imagines.

Expression of PKC, VGCC, CCL2 and CXC3L1: The expression levels of primary sensory nerve terminal protein kinase C (PKC), Ca^{2+} channel protein (VGCC), CCl2 and CXC3L1 were determined by RT-PCR. Tissue samples of 30-50 mg were taken, and 1 mL Trizol was added to homogenate for 3 times at 60 Hz, 30 s, 0 Hz and 10 s, and lysed for 5 min at room temperature. Chloroform of 1/5 volume of Trizol was added and vibrate vigorously for 15 s, emulsify the solution fully without phase separation. Total RNA was extracted after centrifuging 12,000 g at 4°C for 15 min. After computer amplification detection, $2^{-\Delta\Delta Ct}$ method to was used to calculate the relative expression.

Content of glutamic acid, prostaglandin and NO: The loading volume of the sample was 30 μL for NO content detection according to the instruction of NO kit assay. The absorbance was read at the wavelength of 550 nm immediately after the reaction using microplate analyzer. The detailed steps of glutamic acid and prostaglandin content detection are shown in the ELISA instruction manual, and the optical density (OD value) of each hole was measured sequentially at 450 nm with a microplate analyzer.

RNA levels of CGRP, AC and VGCC: The gene expressions of CGRP, AC and VGCC in dorsal root ganglion of spinal cord were detected by RT-PCR. The same methods as 4.2.4.

Part II: Effect of electro-acupuncture at cervical jiaji point on central gliocyte activation in neuralgia rats with cervical spondylotic radiculopathy

Preparation of rat model with intrathecal tube: Rats were anesthetized by intraperitoneal injection of 1% pentobarbital (50 mg/kg). The skin and subcutaneous fascia were cut longitudinally along the midline posterior to the neck of rats and the muscles were separated bluntly to both sides. Carefully remove the spinous process of the cervical spine with bone forceps. The lamina was opened and the PE-10 tube was inserted into the subarachnoid space through the cerebellar medulla cisterna until near the lumbar enlargement (L5-L6 segment). The 10 μL normal saline was slowly pushed into to prevent intraoperative blood clots from blocking the orifice microsyringe, and seal the outer orifice of the PE-10 tube. The exposed part of the skin was fixed on the neck, and the incision was sutured in layers, and local penicillin was used to fight infection.

All rats were fed adaptively for 1 week, and randomly divided into 6 groups (12 rats in each group). They were divided into control group, model group, sham operation group and electro-acupuncture group. The same methods as 4.2.2. LAA astrocyte inhibitor group (LAA group): rat model with intrathecal tube was injected LAA (5nmol) every day, and once a day for 4 weeks after surgery. LAA inhibitor + electro-acupuncture group: on the basis of LAA group treatment, electro-acupuncture was conducted for the model rats.

Expression of relative proteins in gliocyte: The rats were fasted without water for 24 h and were anesthetized by intraperitoneal injection of 1% pentobarbital sodium. The upper and lower 2 cm of spinal cord segments at C6 and C7 were fixed in 4% paraformaldehyde. After immune histochemical staining for 4 h, the relevant indicators were detected. Western blotting was used to detect the expression of IL-1β, IL-6, IL-18, TNF-α, NMDA, AMPA protein. The protein was extracted with RIPA lysate. The proteins were separated by SDS-polyacrylamide gel electrophoresis. After the protein was transferred and sealed, the primary antibody (IL-1β, IL-6, IL-18, TNF-α, NMDA, AMPA and GAPDH) was incubated at 1:1000 dilution for 2 h at room temperature. After TBST washing, HRP-labeled secondary antibodies were incubated at room temperature for 1 h. IL-1β, IL-6, IL-18, TNF-α, NMDA, AMPA protein bands in each group were scanned with Image J software and quantified with β-actin gray value.

Expressions of IL-1β, IL-6, IL-18, and TNF-α in the spinal dorsal horn: The spinal cord was transferred into 4% paraformaldehyde and fixed for 6 h at 4°C. 20% and 30% sucrose solution were added in turn. It is then dehydrated, transparent, paraffin impregnated and paraffin embedded. After hydration 100% alcohol-5 min, 95% alcohol-5 min, 85% alcohol-3 min, 75% alcohol-2 min. Washed 3 min × 2 times. 5% BSA was used to seal. Add properly diluted primary antibody and saved overnight. The tissue was washed with PBS for 3 times, and add the secondary antibody for incubation at 37°C. DAPI was used to red dye for 2 min, and the tissue was observed using fluorescence microscope.

Function of sensory neurons of spinal cord-thalamo-cortex: RT-PCR was used to detect the expression of inflammatory cytokine receptors such as IL-1β, IL-6, IL-18, TNF-α in the horizontal projection neurons of spinal dorsal horn, and the expressions of NMDA and AMPA receptors in spinal - thalamic-cortex. The same methods as 4.2.4.

Statistical analysis

Differences between treatment grooups were analyzed statistically using Student's t-test. A statistically significant difference was reported if $P < 0.05$. The data were expressed as mean ± standard deviation from at least there separate experiments.

Results

Content of CGRP, AC and P substance

Immunofluorescence double standard method was used to detect the content of CGRP, AC and substance P in C6-C7 primary sensory neuron terminal of the spinal cord segment, which were shown in Figure 1. CGRP is red fluorescence, AC is green fluorescence and substance P is pink fluorescence. Fluorescence intensity of CGRP, AC and substance P in model group were obviously higher than those in the control group, $^{**}P < 0.01$. but the electro-acupuncture group was significantly reduced compared with the model group, $^{**}P < 0.01$, and there is a significant statistically difference.

Expression of PKC, VGCC, CCL2 and CXC3L1

Relative expression of RNA levels of PKC, VGCC, CCL2 and CXC3L1 in model group were obviously higher than those in other groups. $^{**}P < 0.01$, $^{*}P < 0.05$ compared with control group. Relative expression of RNA levels of PKC, VGCC, CCL2 and CXC3L1 in electro-acupuncture group were significantly decreased compared with model group. $^{###}P < 0.001$ compared with model group. It was indicated in Figure 2.

Contents of glutamic acid, prostaglandin and NO

Contents of glutamic acid, prostaglandin and NO in model group were significantly higher than those in control and sham operation group, $^{***}P < 0.001$, and there is a significant statistically difference. However, contents of glutamic acid, prostaglandin and NO in electro-acupuncture group were decreased compared with model group, $^{#}P < 0.05$, and there is a significant statistically difference. There was no difference between the control group and the sham operation group, as shown in Figure 3.

RNA levels of CGRP, AC and VGCC

The gene expressions of CGRP, AC and VGCC protein in spinal dorsal root ganglion were detected by RT-PCR. Relative expression of RNA levels of CGRP, AC and VGCC in model group were much higher than that of other groups, $^{**}P<0.01$, $^{*}P<0.05$ compared with control group. However, the RNA expression of CGRP, AC and VGCC were decreased after using electro-acupuncture. There was a significant difference. $^{#}P<0.05$, $^{##}P<0.01$ compared with model group, as shown in Figure 4.

Expression of IL-1, IL-6, IL-18, TNF-α, NMDA and AMPA proteins on gliocyte membranes

IL-1β, IL-6, IL-18, TNF-α, NMDA and AMPA were highly expressed in the model group, which was significantly higher than those of the control group, $^{***}P < 0.001$. After being intervened by electro acupuncture, the expression of IL-6, IL-18, TNF-α, NMDA and AMPA proteins were slightly decreased. After using LAA, the protein expression in the model group decreased, but there was no statistical difference compared with the model group. LAA+electro+acupuncture group was conducted combined treatment of LAA and electro acupuncture. The results showed that the expression of various proteins decreased significantly, $^{###}P < 0.001$, and there is a statistical difference, as shown in Figure 5.

Expressions of IIL-1β, IL-6, IL-18, and TNF-α in the spinal dorsal horn

The expressions of IL-1β, IL-6, IL-18, and TNF-α in the spinal dorsal horn were detected by immunohistochemistry. The expression of IL-1β, IL-6, IL-18, and TNF-α were highest in the model group, and lowest in LAA+electro+acupuncture group, as shown in Figure 6. Positive rate % of IIL-1β, IL-6, IL-18, and TNF-α were showed in Figure 7. Positive rates of IL-1β, IL-6, IL-18, and TNF-α in the model group, Electro-acupuncture group, and LAA group, were much higher than that of control and sham operation group, $^{***}P < 0.001$, and there is a statistical difference. Positive rate IL-1β, IL-6, IL-18, and TNF-α in LAA-electro acupuncture were lower than that of model group, $^{##}P < 0.01$, and there was a significant differnce.

Function of sensory neurons of spinal cord-thalamo-cortex

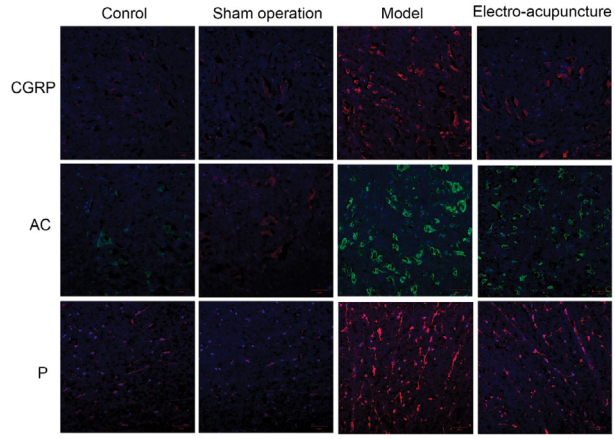

Figure 1: Content of CGRP, AC and substance P detection by immunofluorescence double standard method. $^{**}P < 0.01$ compared with model group.

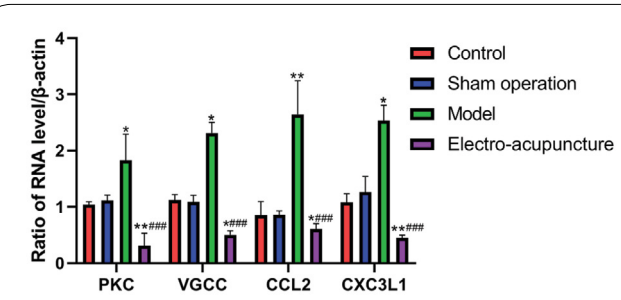

Figure 2: Relative expression of RNA levels of PKC, VGCC, CCL2 and CXC3L1 in different groups. $^{**}P < 0.01$, $^{*}P < 0.05$ compared with control group; $^{###}P < 0.001$ compared with model group.

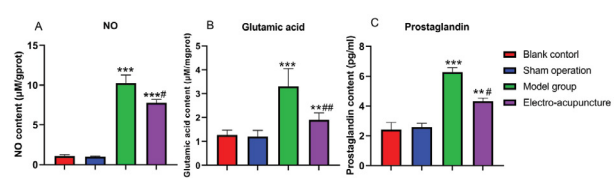

Figure 3: The contents of NO, glutamic acid and prostaglandin. **A)** NO content, **B)** Glutamic acid content and **C)** Prostaglandin content. $^{***}P < 0.001$, $^{**}P < 0.01$ compared with control group; $^{#}P < 0.05$, $^{##}P < 0.01$compared with model group.

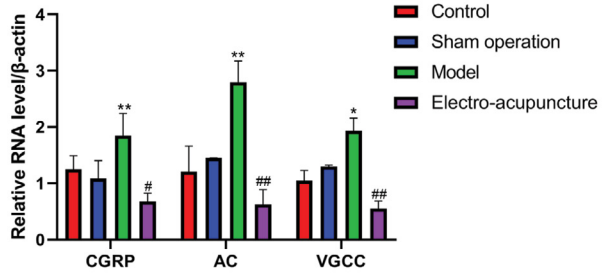

Figure 4: Relative expression of RNA levels of CGRP, AC and VGCC in different groups. $^{**}P$ <0.01, $^{*}P$<0.05 compared with control group; $^{\#}P$ <0.05, $^{\#\#}P$ <0.01 compared with model group.

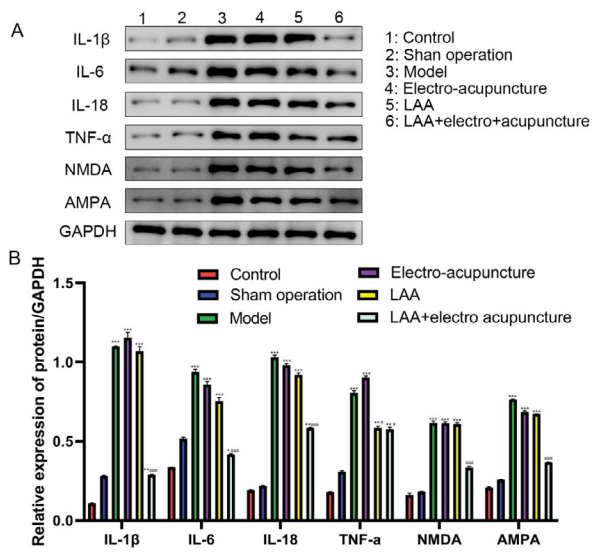

Figure 5: Expression of IL-1, IL-6, IL-18, TNF-α, NMDA and AMPA proteins on gliocyte membranes. **A)** grey-scale imaging and **B)** statistical analysis. ***P <0.001, compared with control group; $^{\#\#}$P <0.01 compared with model group.

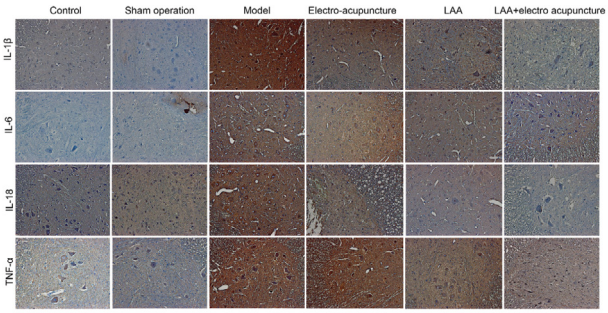

Figure 6: The expressions of IIL-1β, IL-6, IL-18, and TNF-α in the spinal dorsal horn were detected by immunohistochemistry.

The RNA expressions of inflammatory factor such as IL-1β, IL-6, IL-18, TNF-α, NMDA and AMPA receptors at the level of spinal cord-thalamo-cortex showed the same trend in different groups, as shown in Figure 8. The RNA expression of IL-1β, IL-6, IL-18, TNF-α, NMDA and AMPA in model, electro-acupuncture, LAA groups were obvously higher than that of control and sham operation groups, $^{***}P$ <0.001, $^{**}P$ <0.01; There was no significant difference between LAA-electro acupuncture group and control group, sham operation group, P >0.05. The RNA expression of IL-1β, IL-6, IL-18, TNF-α, NMDA and AMPA in LAA-electro acupuncture group were much lower than that of model group, $^{\#\#\#}P$<0.001, and had a statistical difference.

Discussion

A large number of researches have indicated that acupuncture has a certain analgesic effect. Xia YY, et al. [10] proved that electro-acupuncture can effectively fight against neuropathic pain. Liu W, et al. [11] found that electro-acupuncture may exert its analgesic effect by inhibiting p38 mitogen-activated protein kinase (MAPK) and extracellular regulatory protein kinase (ERK) on 4-5 spinal microglia cells. The analgesic effect of electro-acupuncture is closely related to the decreased expression levels of inflammatory response factors such as IL-1β, IL-6 and TNF-α. Wu H, et al. [12] found that electricity had a significant improvement effect on chronic nerve injury pain and allergy, and the inhibitory activity of astrocytes and microglia cells. The expression of inflammatory factors such as IL-1β, IL-6, IL-18 and TNF-α decreased, which is also consistent with the results of this study.

Studies on the treatment of neuralgia by acupuncture have found that gliocyte are closely related to neuralgia. Activated gliocyte can participate in the generation and maintenance of pain through the production of various inflammatory mediators and activation of various signaling pathways [13]. Electro-acupuncture can inhibit the activation of gliocyte. The focus of electro-acupuncture analgesic effect has shifted from neuron to neuron-glial cell-chemokine network mechanism. Gliocyte are widely distributed in the brain and spinal cord, accounting for more than 70% of the total number of cells in the central nervous system [14]. After nerve injury, gliocyte are activated

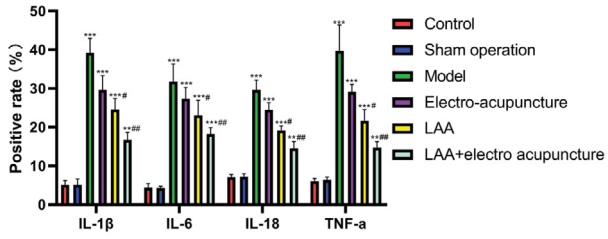

Figure 7: Positive rate of IL-1β, IL-6, IL-18, TNF-α. $^{***}P$ <0.001, $^{**}P$ <0.01 compared with the control group; $^{\#\#}P$ <0.01, $^{\#}P$ <0.05 compared with model group.

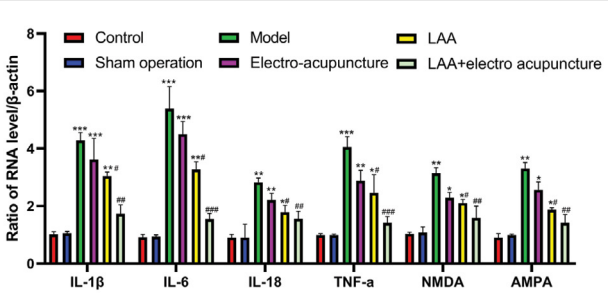

Figure 8: Relative expression of RNA levels of IL-1β, IL-6, IL-18, TNF-α, NMDA and AMPA in different groups. $^{***}P$ <0.001, $^{**}P$ <0.01, $^{*}P$ <0.05 compared with control group; $^{\#\#\#}P$ <0.001<$^{\#}P$ <0.05, $^{\#\#}P$<0.01 compared with model group.

by various signals, and their morphology, cell phenotype and cell function are obviously changed, which is beneficial to the isolation and phagocytosis of necrotic or apoptotic cells. At the same time, due to the production of a large number of inflammatory mediators, it can induce central sensitization and produce pathological pain [15]. Gliocyte trigger immune response through TLRs, and its family members include TLR2, TLR3 and TLR4, play an important role in nerve pain [16]. TLR2, TLR3, and TLR4 induce central sensitization by promoting gliocyte activation and the secretion of inflammatory mediators such as IL-1β, TNF-α, and IL-6. These inflammatory mediators directly enhance excitatory synaptic transmission and (or) weaken inhibitory synaptic efficacy, thereby change the polarization characteristics of harmful afferent neurons, and induce central sensitization and leading to the generation of chronic pain [17]. The chemokine CX3CL1, also known as Fractalkin, plays a role in inflammation and immunity by acting on chemical chemokines and adhesion molecules. Studies suggested that CX3CL1 binds to CX3CR1 on gliocyte, activates the p38-MAPK pathway, and induces gliocyte to activate and synthesize TNF-α, IL-1β, IL-6 and other pro-inflammatory cytokines, thereby triggering the neural immune response of CNS, inducing central sensitization and leading to the production of pathological pain [18].

Central sensitization refers to the amplification of the pain transmission response in the spinal cord and the spinal cord. Glutamate release increased in the presynaptic primary sensory afferent endings of the spinal dorsal horn when central sensitization occurred. Glutamate is the main excitatory neurotransmitter in the mammalian central nervous system [19]. On the one hand, peripheral nerve injury causes a significant increase in the secretion of primary afferent neuropeptides, such as calcitonin gene-related peptides CGRP and substance P. CGRP binds to receptors and causes increased glutamate release by activating adenylate cyclase (AC). Substances P activates neurokinin NK receptors, causing enhancive synthesis of prostaglandin and nitric oxide (NO), then retrograde acts on the primary sensory afferent endings to increase the release of glutamate [20]. On the other hand, increased concentration of endorphins in the spinal cord after nerve injury activates protein kinase C (PKC) and voltaged-gated Ca^{2+} channels (VGCC) at the distal end of primary sensory afferent to increase the release of glutamate and neuropeptides [21]. There are a large number of ionized glutamate receptors (NMDA receptor and AMPA receptor) in the membrane of spinal dorsal horn projection neurons [22]. At the same time, the released substance P acts on NK receptor and activates PKC. By closing potassium ion channels and phosphorylating NMDA receptor, the membrane of postsynaptic projection neurons is depolarized, and the voltage-dependent block of Mg^{2+} on NMDA receptor is removed, thus generating greater inward current [23]. All the above changes enhanced the amplification of the pain transmission response of spinal dorsal horn neurons, which is an important pathogenesis of neuralgia.

Conclusion

Acupuncture affects the change of neuron-gliocyte-chemokine signaling pathway of spinal dorsal horn by regulating the functional state of injured neurons, so as to regulate the central sensitization of neuropathic pain and achieve the effect of treating neuralgia of cervical spondylotic radiculopathy.

Acknowledgments

Not applicable.

Authors' Contributions

Each author has made an important scientific contribution to the study and has assisted with the drafting or revising of the manuscript.

Ethics, Consent and Permissions

Ethical approval was given by Hunan University of Traditional Chinese Medicine, Changsha, 410208.

Consent to Publish

All of the authors have consented to publish this research.

Interest of Conflict

All of the authors have no conflict of interest in this research.

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