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ORIGINAL RESEARCH

Exploring the Mechanism of Immediate Analgesia Induced by Tuina Intervention on Minor Chronic Constriction Injury in Rats Using LC-MS

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Purpose: This study aimed to investigate changes in metabolomic expression in the spinal dorsal horn (SDH) and thalamus during a Tuina session, aiming to elucidate the mechanism of immediate analgesia.

Methods: The rats were randomly divided into three groups: the Sham group, the Model group, and the Tuina group. A minor chronic constriction injury (minor CCI) model was established in both the Model group and the Tuina group. The therapeutic effect of Tuina was determined using the mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) tests. Differential metabolites of the SDH and thalamus were detected using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Bioinformatic analysis was performed using CV, PCA, Venn, and KEGG.

Results: The therapeutic effect of MWT and TWL after instant Tuina intervention was significant. The therapeutic effect of Tuina instant was significantly better compared to the Model group. In the Veen analysis, it was found that Tuina instantly regulates 10 differential metabolites in the SDH and 5 differential metabolites in the thalamus. In the KEGG enrichment analysis, we found that differential metabolites were enriched in 43 pathways in the thalamus and 70 pathways in the SDH.

Conclusion: Tuina therapy may have analgesic effects by metabolizing neurotransmitters such as 2-Picolinic Acid, 5-Hydroxy-Tryptophan Glutathione Betaine-aldehyde-chloride Leucine Lysine Methionine Sarcosine Succinic Acid Histidine Acetylcholine and 5-Hydroxyindoleacetic Acid through the cAMP pathway. It also affects pathways of neurodegeneration-multiple diseases, butanoate metabolism, tyrosine metabolism.

Keywords: neuropathic pain, tuina, neurotransmitters, thalamus, spinal dorsal horn, LC-MS

Introduction

Neuropathic pain (NP), as a chronic pain caused by disease or neurological damage,[1] such as acute posterior ganglionitis, epileptiform neuralgia, neuropathy, apoplexia, diabetic neuropathy, Inflammatory mediated, trauma.[2,3] The pain is divided into three categories: nociceptive pain, inflammatory pain and neuropathic pain,[4] and the neuropathic pain divided into central and peripheral two types. Peripherally-induced neuropathic pain is one of the most common types, which symptoms include spontaneous pain, for example burning, needling, tenderness, abnormal temporal summation, abnormal sensation, numbness, spontaneous pain hypersensitivity, hyperalgesia, allodynia, etc.[5–7] Epidemiological investigation and study finding that prevalence in the population is as high as 7–10%.[8–10] It brings serious damage to patients' physical and mental health. The first-line agents recommended for treatment are the calcium-channel-acting anticonvulsants pregabalin and gabapentin, tricyclic antidepressants, serotonin-norepinephrine reuptake inhibitors (duloxetine, venlafaxine), opioids and cannabinoids and non-drug therapy, such as interventional therapy, psychotherapy, surgery. Which are the effect is slow, so that patients have tolerance, dependent side effects and Non-drug therapy is

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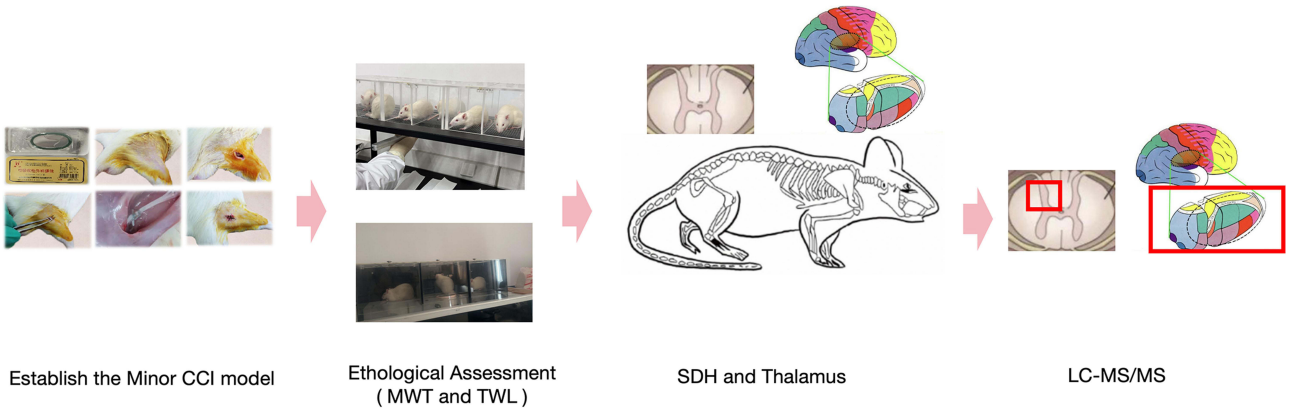
Graphical Abstract



expensive.[11–14] Tuina, as a complement and alternative therapy,[15] is one of the external treatment of traditional Chinese medicine, the unique advantages of the treatment of traditional Chinese medicine "arthralgia" gradually highlighted, with the "general rule is not painful" as the treatment principle, the manipulation of the meridians and acupoints, through dredging the meridians, Qi and blood circulation, to achieve the effect of pain relief.[16,17] A number of studies have shown that tuina treatment of peripheral nerve injury has better efficacy and has fewer side effects. A great number of clinical studies have found that the tuina can relieve pain effectively. Such as Yan H, etc[18] by meta-analysis finding that the safety and efficacy of tuina therapy were determined by the efficacy of tuina therapy in patients with sciatic nerve injury. Xiao B, etc[19] Visual analog scale score, upper limb index, electromyography and other indicators were used to evaluate the therapeutic effect of tuina on patients with brachial plexus injury, so as to provide therapeutic basis for tuina on peripheral nerve injury. Miao Z, etc[20] through meta analysis to determined that tuina is safety and effectivity for peripheral nerve injury caused by a lumbar disc herniation(LDH). A large number of basic experimental research findings the paining effect of tuina. For example, Xiao B, etc[21] findings that tuina can achieve analgesic effect by down-regulating β-endorphin and GABA of Brachial plexus injury in rats. Yao C, etc[22] research chronic compression of dorsal root ganglia (CCD) rats finding that the abirritation of tuina is mediated by regulation microRNA-547-3p through the Map4k4/NF-κB pahyway, to regulate inflammatory cytokines. Our team's preliminary experimental study using Three-Manipulation and Three-Acupoint to treat peripheral nerve injury has made good progress. The three manipulations are pointing manipulation, pulling manipulation and kneading manipulation. Pointing manipulation has the effect of warming the meridian and activating collaterals; Pulling manipulation can relieve paralysis and pain of limbs; The kneading manipulation can relieve muscle spasms, eliminate fatigue and reduce pain in the injured area. Use three can effectively relieve pain. The three Acupoints refers to Yinmen, Chengshan and Yanglingquan three points, the three Acupoints belong to the foot sun bladder meridian and foot Shaoyang gallbladder meridian, according to the sciatic nerve anatomy, Yinmen point in sciatic nerve trunk, Chengshan point in the tibial nerve and Yanglingquan point in the biceps femoris, gastrocnemius, tibial anterior muscle around. The preliminary study simulated NP in sciatic nerve injury (SNI), chronic constriction injury (CCI), chronic compression of the dorsal root ganglion (CCD) rats to detect the mechanism of NP in tuina repair.[23–25] But these researches mainly focused on the long-term analgesic effect of tuina. In clinical studies, tuina immediately can achieve analgesic effect.[26] However, tuina's immediate analgesic mechanism is not clear.

The pain signal is transmitted through myelinated A σ fibers and unmyelinated C fibers to the first-order neuronal spinal dorsal horn and passes through the medulla oblongata, pons and midbrain to the second-order neuronal thalamus, and then to the cerebral cortex, thus leading to the central sensitization.[27–29] A neurotransmitter is a chemical "messenger" molecule that transmits signals between synapses and performs its physiological function by specific binding to effectors or receptors on neural cloud cells. After peripheral nerve injury (PNI), nerve endings increase release neurotransmitters Causing central sensitization and pain.[30] Liquid chromatography tandem mass spectrometry (LC-

MS) can detect the compounds with high polarity and poor thermal stability and accurately quantify the substances. The method is simple, sensitive, accurate and can be used for the detection of amino acids and other neurotransmitters.[31] Previous studies of our research group Wang HR, etc.,[32] the minor chronic constriction injury (minor CCI) rats were used as a model to simulate the clinical peripheral nerve injury research, found that the therapy of three manipulations and three acupoints instant can achieve the analgesic effect. To test the scientific hypothesis of how tuina achieves immediate pain relief, in this study, metabolomics was used to detect the changes of relevant neurotransmitters in the pain transmission pathway of minor CCI model rats, so as to explore the analgesic initiation mechanism of tuina treatment instant of peripheral nerve injury.

Materials and Methods

Animals and Ethical Approval

Twenty-four male Sprague-Dawley (SD) rats with a body weight of about 200±10g (provided by Spafu Beijing Biotechnology Co., LTD.) were selected and raised in the animal house of Beijing University of Traditional Chinese Medicine, 4 rats/cage for 1 week, with free diet and water, ambient temperature of about 25±1°C, relative humidity of 50 ±5%, light for 12h, darkness for 12h (08:00/20:00). The experimental procedures carried out on animals were approved and reviewed by the Experimental Animal Ethics Sub-Committee, Academic Committee of Beijing University of Chinese Medicine (Approval number: BUCM-4-2022041502-2114). All animal experiments were conducted according to the Guidelines of the NIH for the welfare of laboratory animals.

Surgical Procedure

Modeling was started after 7 days of adaptive feeding. According to the results of behavioral tests, 24 rats were randomly divided into Tuina group (n=8), Model group (n=8), Sham group (n=8). The right limb of all rats was selected for modeling, and all rats were deprived of food and water 12h before and after modeling, and isoflurane was used for tracheal anesthesia before modeling. The rats were placed in prone position and fixed on the rats plate. The light hip femoral junction was shaved and disinfected with iodophor; A 0.5–1cm incision was cut along the path of the right sciatic nerve, thus the subcutaneous fascia and muscle layer were bluntly separated to expose the sciatic nerve; In the Model group and the Tuina group, the sciatic nerve was extracted with tissue forceps, and a write junction was ligated 7mm before the distal sciatic nerve bifurcation using a 4–0 absorbable surgical suture (made of cadmium). The total length of the knot is about 5–6mm. The strength of ligation should not affect the flow of the epineurium, and the knots can slide over the sciatic nerve. The whole process is appropriate to pull the nerve and cannot appear leg muscle fibrillation. The operation method of the Sham group was the same as that of the Model group, but the sciatic nerve was properly pulled without ligation.

Intervention Methods

All interventions began 1 week after modeling (After the model is established and stabilized).[33]

Tuina group: qualitative, timed and quantitative intervention was performed by one time tuina simulator, a rat was fixed on the information platform and the Tuina Manipulation Simulator was opened (self-developed machine, China Invention Patent No. ZL202320511277.5). Set the machine parameter to 4N the frequency was 60 times/min, 1min/point/method, a total of 9min/rat, and the stimulate method was used point, pull and kneading manipulations at Yinmen (BL37), Chengshan (BL57) and Yanglingquan (GB34), once a day.[23]

Model group and sham group: bind for 9 min with the same frequency as tuina group.

Behavior Tests

Behavioral tests were conducted before modeling, before intervention and after intervention.

Mechanical Withdrawal Threshold (MWT): Twenty minutes before the official test began, the animal was placed in a cage covered with a metal mesh. The testing or examination started when the rats' exploratory and grooming behavior were over, and the rats became calm and relaxed. Use an electronic Von Frey instrument (BIO-EVF5; Bioseb, USA), the

unit of measurement is g, avoid contact with metal wire, and the stimulation probe was applied to the same part of the bottom of the back foot. Step by step, when the rat showed foot shrinking and foot licking, the data was recorded and repeated for 3 times every 10 minutes.

Thermal Withdrawal Latency (TWL): Place the rats in the test cage with glass at the bottom 20 minutes before the formal test. The tests began when the exploring and grooming behavior stopped and the rats were static and relaxed. Using a thermal analgesia device (PL-200; Chengdu Techman Software Co., Ltd., China), set the parameter to 50% intensity and cut off 30 sec. Aim the infrared heat source at the same position on the sole surface of the rear paw and start stimulating. When the animals appeared foot shrinking and licking, the values were recorded and repeated for 3 times, each time at an interval of 10 min. If the animal stops recording due to walking or grooming, measure again at 10 min intervals.[34]

Behavioral results were analyzed using SPSS27.0 and the result expressed as mean ± standard deviation. One-way ANOVA was used for homogeneous and normal distribution, and LSD-t method was used for comparison between groups, $P< 0.05$ was statistically significant, $P< 0.01$ has significant significance.

LC-MS/MS

Sample Preparation

The spinal cord and thalamus were weighed 50±2.5mg in a 2mL centrifuge tube and the weight of each sample was recorded. The 70% methanol extraction solution 500μL pre-cooled at −20°C was put into a well-weighed sample and ground for 3 min. At 4°C, the extraction solution was centrifuged at 2500 r/min for 3 min and then at 12000r/min for 10 min. Take 300μL of supernatant and place it in a 1.5mL centrifuge tube. Refrigerated at −20°C for 30 min, centrifuged at 12,000 r/min again for 10 min at 4°C; Then 200μL supernatant was taken into sample preparation vial and stored at −20°C. Samples from different groups are mixed to form quality control samples (QC), and the same processing method is adopted. The QC samples also have the function of expressing the metabolic information of all samples. A quality control sample is inserted, while the 6 samples are tested and analyzed to ensure the accuracy and repeatability of the experiment.

UPLC Conditions

Instrument analysis platform: Ultra Performance Liquid Chromatography (UPLC); ExionLC™ AD and Tandem Mass Spectrometry (MS/MS; QTRAP® 6500+, SCIEX). Liquid phase conditions:

The chromatography was performed on Waters ACQUITY UPLC HSS T3 C18 column (1.8 μm 100 mm × 2.1 mm i.d.).

Mobile phase: positive and negative mode, positive mode A phase is ultra-pure water containing 0.1% formic acid; Negative mode B phase is acetonitrile containing 0.1% formic acid;

The flow rate was 350μL/min. Column temperature 40°C; The sample size was 2μL;

LC-MS mobile phase gradient shown in Table 1.

LC-MS/MS Conditions

- 3.1 Electrospray Ionization (ESI) temperature 550°C;
- 3.2 Positive ion mode mass spectrum voltage 5500V;
- 3.3 Mass spectrum voltage in negative ion mode −4500V;
- 3.4 Curtain Gas (CUR) 35psi.

Table 1 LC-MS Mobile Phase Gradient Table

Time (min)	Row (μL/min)	A%	B%
0	350	95	5
8.0	350	5	95
9.5	350	5	95
9.6	350	5	95
12.0	350	95	5

In Q-Trap 6500+, each ion is scanned according to the optimized Declustering Potential (DP) and Collision Energy (CE).

MWDB (Metware Database) is constructed based on standard products, and qualitative analysis of mass spectrometry data is carried out.

Data Quality Control

Using a mixed solution as a quality control (QC) sample, in the instrument analysis process, a quality control sample is usually inserted every 10 detection analysis samples. By overlapping and displaying the total ion flow (TIC) of the mass spectrometry detection analysis of the same quality control sample, the stability of the instrument during the project detection can be determined. The high stability of the instrument provides important guarantees for the repeatability and reliability of data.

Qualitative and Quantitative Principles

Quantification was performed using Multiple Reaction Monitoring (MRM) analysis using triple quadrupole mass spectrometry. In the MRM model, the four-pole first screens the precursor ions (parent ions) of the target substance, and excludes the ions corresponding to other molecular weight substances to preliminarily eliminate the interference. After ionization induced by the collision chamber, the precursor ions break to form a number of fragment ions, and then the fragment ions are filtered through the triple four-pole to select the required characteristic fragment ions, and eliminate the interference of non-target ions, so that the quantification is more accurate and the repeatability is better. After the mass spectrometry data of different samples were obtained, the chromatographic peaks of all target objects were integrated and quantitative analysis was performed by standard curves.

Data Processing

By using MultiQuant 3.0.3 software to process mass spectrometry data and referring to information such as peak shape and retention time, the peaks of the tested substance in each sample are comprehensively corrected to ensure the accuracy of qualitative and quantitative results.

Differential Metabolite Analysis

Multivariate statistical analysis methods include Principal Component Analysis (PCA) and Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA). If the fold change (FC) of univariate analysis is greater than 1.2 times or less than 0.833333333334 and P<0.05, then the difference is considered significant.

Bioinformatics Analysis

KEGG enrichment analysis: Pathway enrichment analysis was performed on the identified differential metabolites using Python software (Version 1.0.0), Fisher's exact test was used for enrichment analysis, and BH (Benjamin and Hochberg) was used to correct the P-value. When P<0.05, the pathway was considered significantly enriched.

Results

Changes of Hyperalgesia Symptoms and Chronic Abnormal Pain in Minor CCI Rats

MWT is performed 24 hours after tuina, and TWL can be performed after tuina (Conclusions from the preliminary experiment). MWT and TWL had no statistical significance between the Tuina group and the Model group before molding and intervention (Figure 1). After modeling (before intervention), the thresholds of Tuina group (P=0.00, P=0.01) and Model group (P=0.00, P=0.04) were significantly decreased compared with Sham group, indicated that minor CCI resulted in significant mechanical and thermal hyperalgesia in NP rats. After immediate intervention, the threshold value of tuina group increased significantly compared with the Model group (P=0.04, P=0.00). Indicated that the antinociceptive effect of tuina was relieved after 24h and the reverse heat hyperalgesia effect of tuina was relieved instant.

Bioinformatics Analysis of Thalamus and Spinal Dorsal Horn in Minor CCI Rats

The CV Analysis results of QC Samples

The Quality Control (QC) analysis mainly evaluates the stability of LC-MS. Coefficient of Variation (CV value) is the ratio of the standard deviation of the original data to the average of the original data, which can reflect the degree of data dispersion. As shown in the thalamus (Figure 2A) and SDH (Figure 2B), CV values of the three groups are all less than 0.3, indicating that the three groups of data are very stable and reliable and can be used for further experiments.

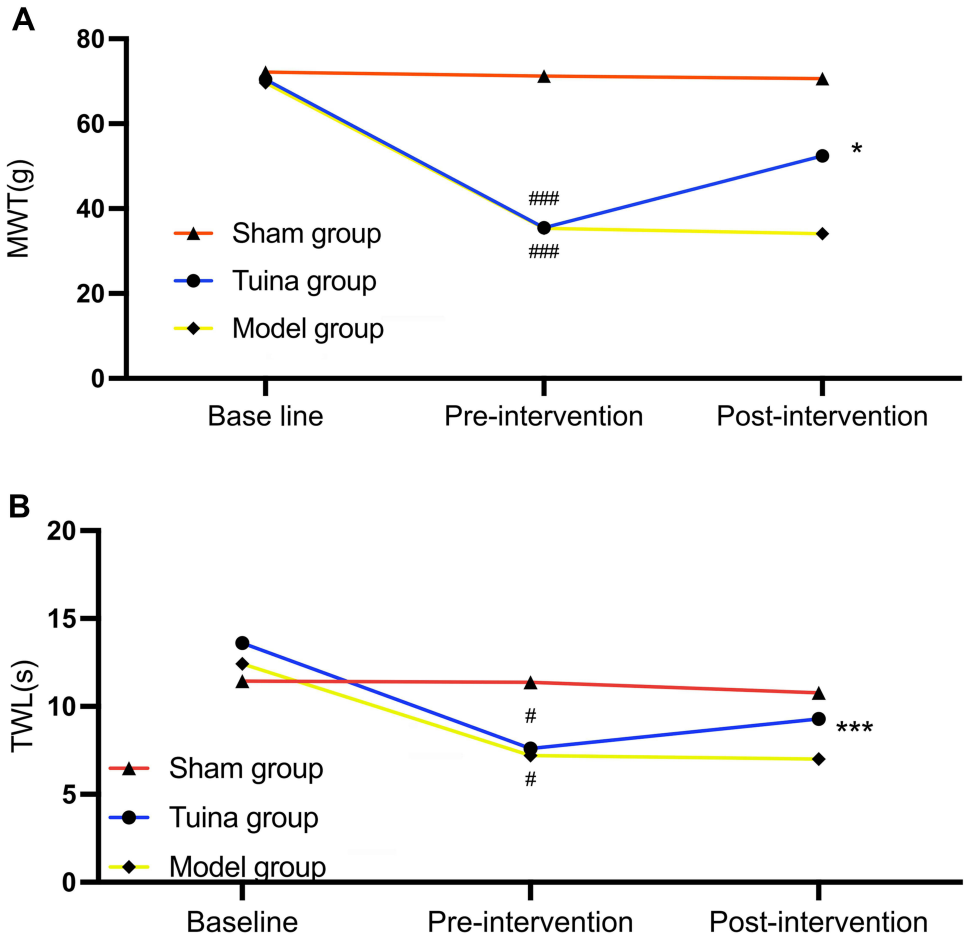


Figure 1 Result of MWT (**A**) and TWL (**B**), *P<0.05, ***P<0.001 compared with Model group, #P<0.05, ###P<0.001 compare with Sham group, Sham group (n=8), Tuina group (n=8), Model group (n=8).

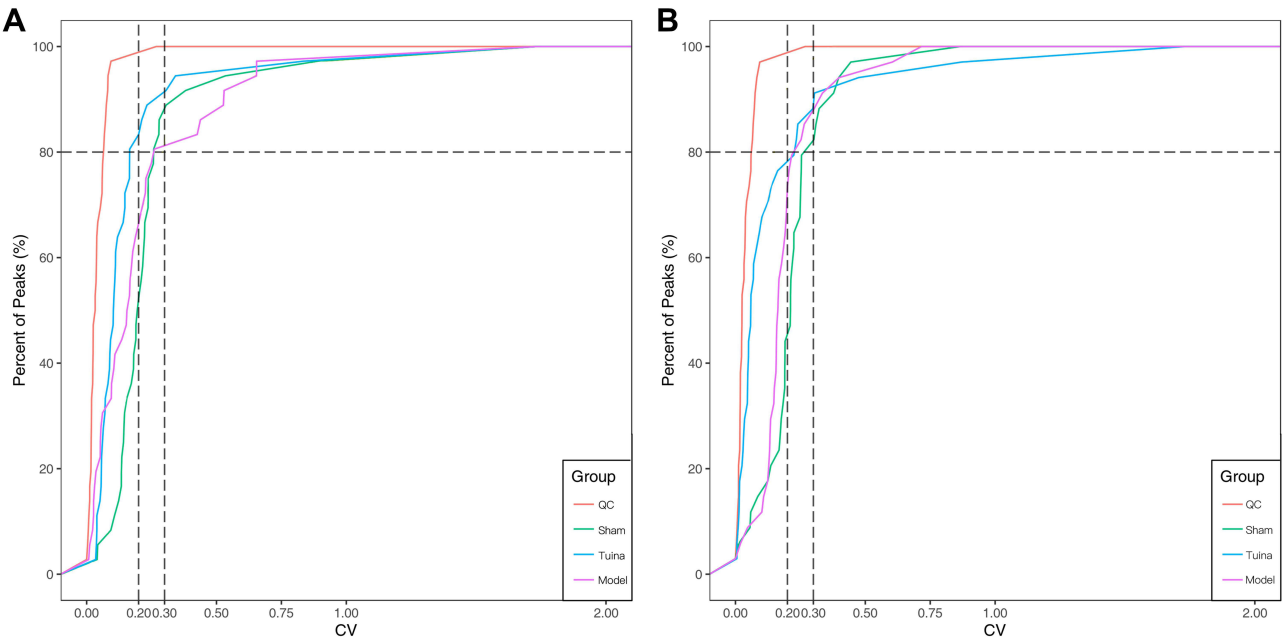


Figure 2 The horizontal coordinate represents the CV value of SDH (**A**) and thalamus (**B**), the vertical coordinate represents the proportion of the number of substances less than the corresponding CV value in the total number of substances, QC is the quality control sample (n=3).

The Result of PCA Analysis

The principal component analysis (PCA) results indicated the trend of metabolome separation among the groups, indicating the good aggregation and high similarity among the samples in each group. The good distribution among the groups indicated significant differences among the groups. As shown in Figure 3A and 3B, the samples of Sham group and Model group were obviously separated, minor CCI model could significantly change the metabolites of the thalamus and SDH in rats. The samples of Model group and Tuina group were obviously separated, indicating that tuina could change the metabolites of thalamus and SDH in rats.

The Result of Veen Analysis

The 36 neurotransmitters were detected in thalamus. The common and specific differential metabolites were identified by Veen analysis, there are 8 common differential neurotransmitters between the Tuina group vs Model group and the Sham group vs Model group (Figure 4A and 4B, Table 2), among which the three differential neurotransmitters Aspartic Acid, Succinic Acid and Kynurenic Acid show opposite trends, which proves that Tuina can change the expression of three neurotransmitters Aspartic Acid, Succinic Acid and Kynurenic Acid. There were 5 specific different neurotransmitters in Tuina group vs Model group (Table 3).

The 34 neurotransmitters were detected in SDH; there are 3 common differential neurotransmitters between the Model group vs Tuina group and the Sham group vs Model group (Figure 4C and 4D, Table 4), among which the one differential neurotransmitter Serine shows opposite trends, which proves that Tuina can change the expression of one neurotransmitter Serine. There were 10 specific different neurotransmitters in Tuina group vs Model group (Table 5).

The Result of KEGG Analysis

KEGG is a utility comprehensive database for genomic sequencing and other high-throughput experimental techniques generated from molecular datasets. A total of 43 pathways were detected by thalamus KEGG enrichment analysis, in which, the important 20 pathways included cAMP signaling pathway, pathways of neurodegeneration-multiple diseases, butanoate metabolism and so on. A total of 70 pathways were detected by SDH KEGG enrichment analysis, in which, the important 20 pathways including cAMP signaling pathway, tyrosine metabolism, tryptophan metabolism and so on (Figure 5).

Discussion

Tuina as a traditional therapy, in the clinical treatment of cervical spondylosis, lumbar disc herniation, diabetic and other diseases caused by neuropathic pain has a significant effect.[35–37] "Three-Manipulation and Three-Acupoint" a kind of manipulation of tuina. The research group's previous studies have shown that the Three-Manipulation and Three-Acupoint are effective in the treatment of peripheral nerve injury[38,39] and research that one-time Tuina could achieve analgesic effect, but the mechanism of analgesia remains to be explored. The modulation of neurotransmitters is an

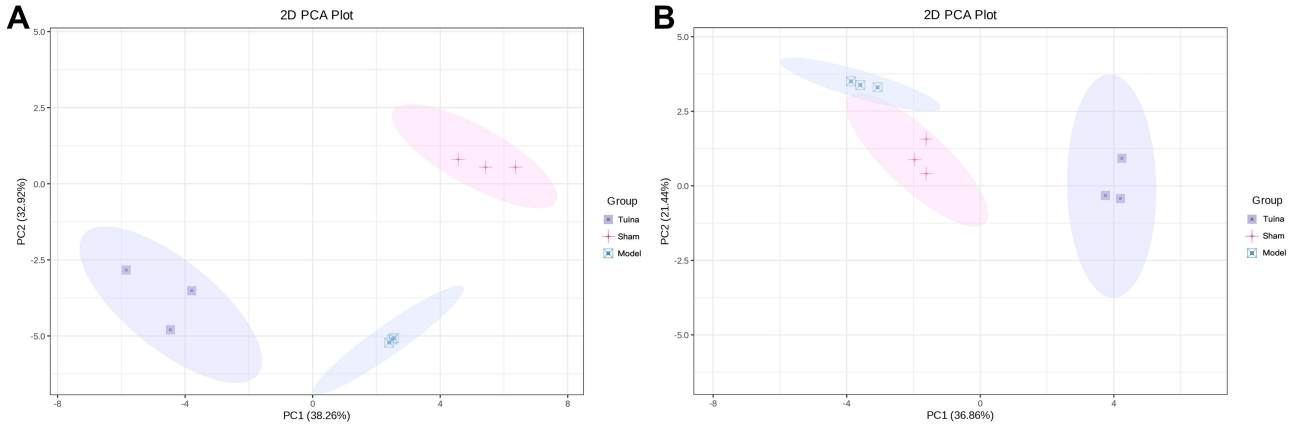


Figure 3 (**A**) SDH and (**B**) Thalamus PCA analysis; PC1 represents the first principal component; PC2 represents the second principal component; The distance of each coordinate point represents the degree of sample aggregation and dispersion, the closer the distance indicates the higher the similarity of the sample, and the farther the distance indicates the greater the difference of the sample.

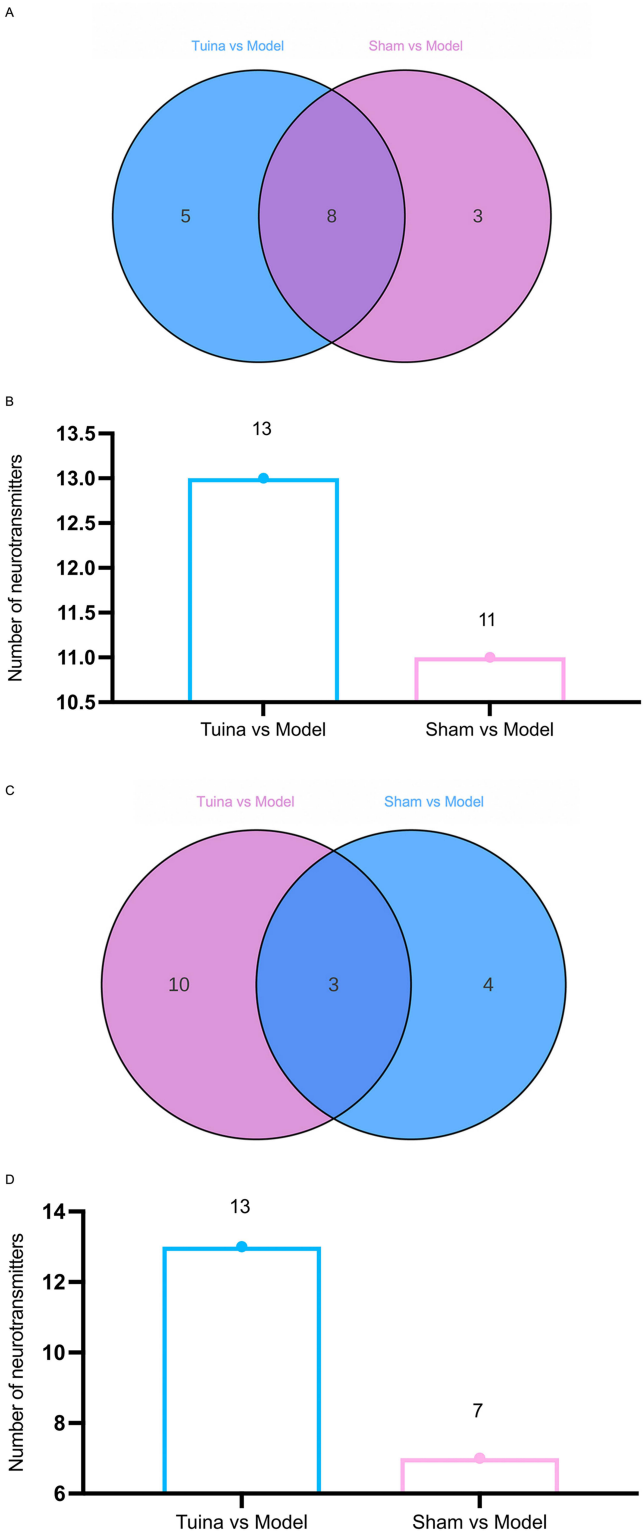


Figure 4 Veen analysis of thalamus (**A** and **B**) and SDH (**C** and **D**); Sham group (n=3), Model group (n=3), Tuina group (n=3); The overlapping part of the pie chart represents the number of metabolites shared by multiple metabolites, and the part that does not overlap represents the number of metabolites unique to that metabolite set, the number represents the corresponding metabolic number; The bar chart shows the number of metabolic sets.

Table 2 Common Differential Neurotransmitters Between the Tuina Group Vs Model Group and the Sham Operation Group Vs Model Group in Thalamus

Neurotransmitter Name	cpd ID	P	Fold_Change (Sham vs Model)	Type	Fold_Change (Tuina vs Model)	Type
Serotonin	C00780	<0.01	0.6897109233752748	down	0.7485724533482165	down
Tyramine	C00483	0.04	1.2546681413682417	up	1.3927694841653715	up
Thyroxine	C01829	0.01	1.5554088379958753	up	1.8019690249401858	up
3-hydroxybutyric acid	C01089	0.04	2.955611639379555	up	1.2955232198456792	up
Aspartic Acid	C00049	0.01	0.7724995730665765	down	0.8272578870937414	up
Betaine	C00318	0.02	2.040893184601702	up	2.2598340821925387	up
Kynurenic Acid	C01717	<0.01	0.4927881583851003	down	1.242637008743743	up
Succinic Acid	C00042	<0.01	1.5008163040835865	up	0.6091521485886062	down

Notes: cpd_ID: metabolite ID information in the KEGG database; P: probability value; P<0.05 indicated statistical significance; Fold_Change: difference multiple (Inf represents infinity, because Fold_Change = the average value of the experimental group/the average value of the control group, when the average value of the control group is 0, Fold_Change is infinity, represented by Inf); fold change ≥ 1.2 or a fold change ≤ 0.83333333333334 is considered to be significantly different; Type: the state of the differential metabolite.

Table 3 Specific Different Neurotransmitters in Tuina Group Vs Model Group in Thalamus

Neurotransmitter Name	cpd ID	P	Type	Fold_Change (Tuina vs Model)
2-Picolinic-Acid	-	0.04	up	1.2046824777095921
Acetylcholine	C01996	0.02	up	1.2878875427275418
Glutathione	C00051	0.01	down	0.7511218490923401
5-Hydroxyindoleacetic-Acid	-	0.01	down	1.2095214234436367
Betaine-aldehyde-chloride	-	0.04	up	1.3070826785599914

Notes: cpd_ID: metabolite ID information in the KEGG database; P: probability; P<0.05 indicated statistical significance; Fold_Change: difference multiple (Inf represents infinity, because Fold_Change = the average value of the experimental group/the average value of the control group, when the average value of the control group is 0, Fold_Change is infinity, represented by Inf); fold change ≥ 1.2 or a fold change ≤ 0.83333333333334 is considered to be significantly different; Type: the state of the differential metabolite.

Table 4 Common Differential Neurotransmitters Between the Model Group Vs Tuina Group and the Sham Operation Group Vs Model Group in SDH

Neurotransmitter Name	cpd ID	P	Fold_Change (Sham vs Model)	Type	Fold_Change (Tuina vs Model)	Type
Acetylcholine	C01996	0.04	0.5188632010196609	down	0.8110839612139344	down
Serine	C00065	<0.01	1.301378399597237	up	0.8329283105929113	down
Thyroxine	C01829	<0.01	0.7980319358315434	down	0.7410881809148744	down

Notes: cpd_ID: metabolite ID information in the KEGG database; P: probability; P<0.05 indicated statistical significance; Fold_Change: difference multiple (Inf represents infinity, because Fold_Change = the average value of the experimental group/the average value of the control group, when the average value of the control group is 0, Fold_Change is infinity, represented by Inf); fold change ≥ 1.2 or a fold change ≤ 0.83333333333334 is considered to be significantly different; Type: the state of the differential metabolite.

Table 5 Specific Different Neurotransmitters in Model Group Vs Tuina Group in SDH

Neurotransmitter Name	cpd ID	P	Fold_Change (Tuina vs Model)	Type
2-Picolinic-Acid	-	0.04	0.8286644795621051	up
5-Hydroxy-Tryptophan	-	0.02	1.3234855643786037	up
Glutathione	C00051	0.03	0.6971165088756972	down
Betaine-aldehyde-chloride	-	<0.01	0.725355550165078	down
Leucine	C00123	0.03	0.813986553696344	up
Lysine	C00047	0.02	0.8314526831248267	down
Methionine	C00073	0.01	0.798310608064606	down
Sarcosine	C00213	0.01	0.8272686871878683	up
Succinic-Acid	C00042	<0.01	0.6675562566077398	down
Histidine	C00135	0.01	0.8300366669291178	Down

Notes: cpd_ID: metabolite ID information in the KEGG database; P: probability; P<0.05 indicated statistical significance; Fold_Change: difference multiple (Inf represents infinity, because Fold_Change = the average value of the experimental group/the average value of the control group, when the average value of the control group is 0, Fold_Change is infinity, represented by Inf); fold change ≥ 1.2 or a fold change ≤ 0.83333333333334 is considered to be significantly different; Type: the state of the differential metabolite.

expanding field of pain medicine.[40] In this study, neurotransmitters were used as the starting point to explore the analgesic initiation mechanism of tuina on peripheral nerve injury.

The minor CCI model evolved from the classical CCI model, and its symptoms are more closely related to clinical pNP.[41] Therefore, we adopted minor CCI model to simulate clinical pNP. MWL and TWL are typical behavioral tests for assessing sensory recovery after sciatic nerve injury in rats.[42] Therefore, MWL and TWL tests, it was found that one time tuina could relieve the NP caused by peripheral nerve injury. After the modeling of minor CCI, the rats were observed to chew the lower limb of the modeling side and claudication, and the behavioral test result that Tuina group and the Model group had statistical significance compared with the Sham group, the result indicates that the model is created successfully. After immediate intervention of Three-Manipulation and Three-Acupoint, the test of MWT and TWL of Tuina group had statistical significance compared with the Model group, it shows that tuina can produce curative effect once.

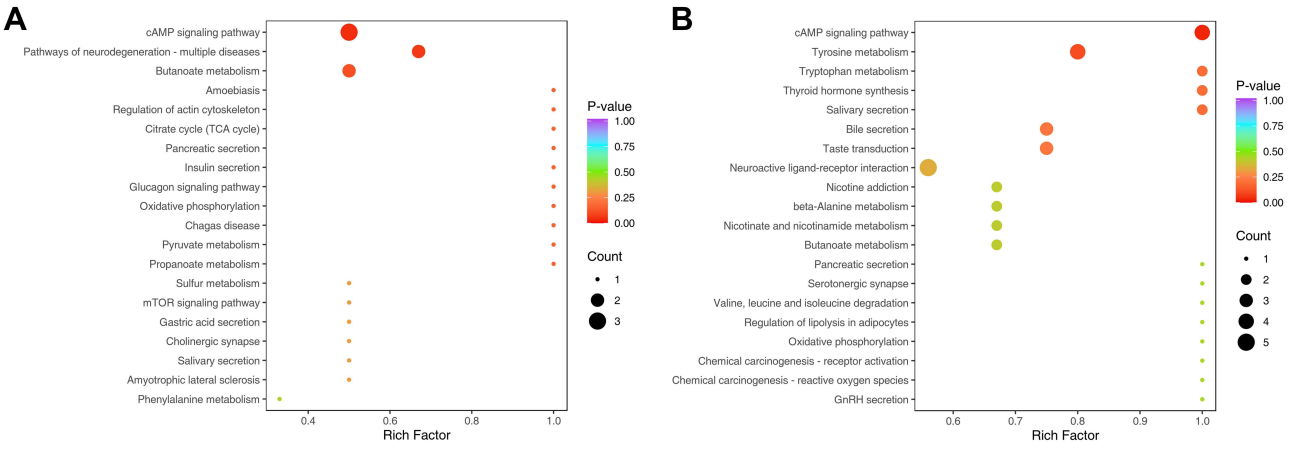


Figure 5 KEGG enriched analysis result of thalamus (**A**) and SDH (**B**). The horizontal coordinate represents the Rich factor corresponding to each path, the vertical coordinate is the path name, the color of the dot is P-value, and the red indicates the more significant enrichment. The size of the dots represents the number of differentiated metabolites enriched.

Metabolomics can reveal the metabolites of tuina's influence on NP from the outcome level, which is the most phenotypic technology. Metabolomics data is characterized by "high dimensional and massive", so it is necessary to combine univariate statistical analysis and multivariate statistical analysis and analyze the data from multiple angles according to the characteristics of the data, and finally accurately mine the differential metabolites. Differential metabolites were further screened by Fold Change (FC) analysis using univariate statistical analysis. The dorsal horn of the spinal cord and the thalamus are key areas for pain signaling. In this study, LC-MS/MS was used to detect neurotransmitter levels in the spinal dorsal horn and thalamus. The result of the analysis found that in SDH the 7 differential neurotransmitters are between the Sham group vs Model group, and the four neurotransmitters such as Epinephrine, 5-Methoxytryptamine-hydrochloride, Serotonin and 5-Hydroxyindoleacetic-Acid are related to NP. In thalamus, the 11 differential neurotransmitters are between the Sham group vs Model group, and the three neurotransmitters such as Arginine, Glycine and Serine are related to NP. Choi et al found that local injection of epinephrine agonists in patients with NP caused by shingles produced transient pain.[43] Strittmatter et al found that plasma Epinephrine levels were increased in patients with trigeminal neuralgia.[44] Sommer Claudia's study showed that peripheral nerve injury leads to an increase in Serotonin within the injured nerve.[45] Murai Nobuhito et al found that the level of Serotonin in the spinal cord of CCI model rats increased.[46] 5-Hydroxyindoleacetic-Acid is the main metabolites of Serotonin. Studies have shown that 5-hydroxyindoleacetic acid plays an important role in peripherally induced NP.[47] Rondon Lusliany et al found that Arginine supplementation helped prevent hyperalgesia.[48] Wallace et al showed that glycine antagonists showed analgesia in animal models of neuropathic pain,[49] and glycine transmission is important in central sensitization.[50] Wang Xiaoping found that the serine/threonine protein kinase mTOR could improve NP caused by spinal cord injury.[51] The above research is consistent with the results of this study. The study of 5-Methoxytryptamine-hydrochloride in NP was not found, but the presence of 5-Methoxytryptamine-hydrochloride in the intestinal metabolomics of depressed mice was found,[52] and NP was also associated with depression. In later studies, 5-Methoxytryptamine-hydrochloride was used as the entry point to verify the correlation of NP-induced depression model. Prove that in Epinephrine, 5-Methoxytryptamine-hydrochloride, Serotonin, 5-Hydroxyindoleacetic-Acid, Arginine, Glycine and Serine are related to NP.

In the SDH 13 differential neurotransmitters between the Tuina group vs Model group, the ten neurotransmitters such as 2-Picolinic-Acid, 5-Hydroxy-Tryptophan, Glutathione, Betaine-aldehyde-chloride, Leucine, Lysine, Methionine, Sarcosine, Succinic-Acid, Histidine related with Tuina analgesia. In thalamus, the 13 differential neurotransmitters between the Tuina group vs Model group, and the five neurotransmitters such as 2-Picolinic-Acid, Acetylcholine, Glutathione, 5-Hydroxyindoleacetic-Acid, Betaine-aldehyde-chloride related with Tuina analgesia. 2-Picolinic-Acid and Sarcosine are considered to be a neuroprotective factor, and its increased expression can alleviate neuroinflammation,[53,54] and NP is thought to be neuroinflammatory.[55] It was found that inducing the up-regulation of 5-Hydroxy-Tryptophan, Acetylcholine and down-regulation of Glutathione, Lysine, Succinic-Acid, Histidine, 5-Hydroxyindoleacetic-Acid could achieve the effect of NP analgesia;[56–62] Methionine can influence the change of NP by regulating excitatory neurotransmitters such as glutamate.[63] The above research results are consistent with the results of this study. Studies on the correlation between Betaine-aldehyde-chloride, Leucine and NP are still blank, and later studies can further clarify their correlation.

In the KEGG enriched pathways, we found that cAMP signaling pathway a mainly pathway in the immediate analgesic effect of tuina. The previous research indicated that Cyclic adenosine phosphate (cAMP) sensor, cAMP 1 directly activates the exchange protein Epac1, a guanine nucleotide exchange factor, which activates Rap1, a small GTP-binding protein of the GTase Ras family. Rap1 regulates PIEZO2-mediated electrical currents involved in mechanical pain abnormalities, this promotes the development of ectopic pain in animal models of chronic systolic injury.[64] Fangxia Xu et al found that Myr-NR2B9c can bind to the second PDZ domain of PSD-95 and separate the Ca2+ influx activated by NMDARs and activated by CaMKII, resulting in central sensitivities mediated by CREB, a reactive element of cAMP.[65] Some studies have found that cAMP changes significantly after peripheral nerve injury and tuina intervention can improve NP caused by peripheral nerve injury by modulating cAMP.[66–68] This study proved that tuina instant can analgesia through cAMP signaling pathways. In addition to cAMP, it can also be metabolized through Pathways of neurodegeneration-multiple diseases, butanoate metabolism, tyrosine metabolism and other pathways. It has the

characteristics of multi-channel and multi-target intervention, which is worth further exploration in this paper to provide a better theoretical basis for clinical promotion.

The main limitation of this study is the lack of validation of neurotransmitters and pathways. Further studies can further explore the mechanisms of neurotransmitters and pathways to further confirm the reliability of the results.

Conclusion

In our study, we found that tuina provides immediate pain relief, and pain relief is achieved by metabolizing 2-Picolinic-Acid, 5-Hydroxy-Tryptophan, Glutathione, Betaine-aldehyde-chloride, Leucine, Lysine, Methionine, Sarcosine, Succinic-Acid, Histidine, Acetylcholine and 5-Hydroxyindoleacetic-Acid neurotransmitters through the cAMP pathway, pathways of neurodegeneration-multiple diseases, butanoate metabolism, tyrosine metabolism. Our study sheds light on the mechanism of NP induced by minor CCI and confirms the uniqueness of non-pharmacological treatments for pain-related disorders.

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Disclosure

The authors report no conflicts of interest in this work.

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