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Original Article

Expression levels of CX3CL1 and CCL21 in the spinal cords of rats with neuropathic pain and correlation levels with JNK/MCP-1 signaling pathways

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Abstract: Objective: Neuropathic pain (NP) refers to pain caused by injuries or diseases of the somatosensory nervous system. CX3CL1 and CCL21 are activating factors presented in microglia. They have been shown to play important roles in the process of transmission of pain signals by sensory neurons. The current study hypothesized that expression levels of CX3CL1 and CCL21 play roles in the spinal cords of rats with NP via JNK/MCP-1 signaling pathways. Methods: Ninety SD rats were equally and randomly divided into the control group, model group, and intervention group. Results: At T1, the pain threshold of the rats showed insignificant differences between the intervention group and model group. At T2, T3, and T4, the pain threshold of the rats was higher and expression levels of CX3CL1, CCL21, JNK, and MCP-1 were lower in the intervention group than those of the model group. The pain threshold of rats in the intervention group appeared to be on the increase. In the model group, there were no significant differences in the pain threshold between T1 and T2. The pain threshold showed an upward trend at T3 and T4. In the intervention group, expression levels of CX3CL1, CCL21, JNK, and MCP-1 showed a downward trend. CX3CL1 and CCL21 participate in the development of NP via JNK/MCP-1 signaling pathways. Conclusion: Through inhibiting expression of CX3CL1 and CCL21, pain thresholds of rats can be elevated, achieving analgesic effects.

Keywords: CX3CL1, CCL21, NP, JNK/MCP-1 signaling pathway, pain threshold

Introduction

Neuropathic pain (NP) refers to pain caused by injuries or diseases of the somatosensory nervous system. This pain is chronic and commonly seen in all ages [1]. Current studies have pointed out that noxious stimuli, such as inflammation, injuries of tissues, and peripheral nerves, are the main causes of NP. When the central nervous system of the patient suffers such injuries, the spinal dorsal horn can be induced to generate Aβ fibers. These form abnormal synapses with nociceptive neurons and cause pain [2-4]. Current data shows that the incidence of NP has reached 9% [5]. A survey report by Rosenthal et al. showed that, in 2016, the number of new NP cases in the world had exceeded 6 million. Incidence rates have been increasing year by year [6]. In the face of the rising incidence of NP, investigators have been working tirelessly to prevent the occurrence of NP. However, no significant breakthroughs have been achieved yet. Treatment of NP in current clinical practice is still based on drugs. However, treatment is usually difficult and lasts for a long period, due to the chronic nature of NP [7]. Moreover, with increased drug resistance developed by long-term use of antibiotics, treatment outcomes have become increasingly unsatisfactory [8]. It has been reported that more than 42% of patients still present related symptoms after treatment of NP, with about 8% suffering worse conditions [9].

With the deepening of research in recent years, more and more research has begun to focus on targeted therapy of NP [10-12]. Of these targets, CX3CL1 and CCL21 are activating factors presented in microglia. They have been shown to play important roles in the process of transmission of pain signals by sensory neurons. CX3CL1 is involved in cellular migration and dif-

ferentiation in the central nervous system. It regulates intercellular transmission of information and has a strong protective effect on injured nerves [13]. One study revealed that neurons placed in a high concentration of glutamate solution would secrete a large amount of CCL21. Under a microscope, it was revealed that CCL21 neurons secrete many vesicles containing CCL21, indicating that CCL21 promotes the involvement of microglia in the occurrence and development of chronic pathological pain [14].

The c-Jun N-terminal kinase/monocyte chemoattractant protein 1 (JNK/MCP-1) pathway is a known mechanism important for the development of NP. JNK facilitates sensitization of central nerves by its activating effects in spinal astrocytes. Activation of JNK/MCP-1 pathways is the key to occurrence of NP. Inhibition of activation can effectively alleviate hyperalgesia [15]. However, there is little research studying the roles of CX3CL1 and CCL21 in NP. Studies concerning the correlation between CX3CL1, CCL21, and JNK/MCP-1 pathway have yet to be reported. Therefore, the present study observed expression levels of CX3CL1 and CCL21 in the ACC of rats with NP. This study further analyzed the effects of CX3CL1 and CCL21 on JNK/MCP-1 pathways, aiming to provide a theoretical foundation for future clinical diagnosis and treatment of NP.

Materials and methods

Experimental animals

A total of 90 8-week-old clean SD rats, weighing 180-250 g, were purchased from Kay Biological Technology (Shanghai) Co., Ltd. The rats were bred in an environment with natural light, a temperature of (24.00±2.00)°C, and humidity of (50.00±5.00)%. They were allowed free access to water. This animal experiment was approved by the Ethics Committee of The First Affiliated Hospital of Anhui Medical University and complied with the regulations of the International Association for the Study of Pain.

Methods

Modeling method: A total of 90 rats were randomly assigned a number from 1 to 90. Thirty numbers were randomly selected using a computer, while the corresponding rats were selected as the control group. The other 60 rats were subjected to modeling of NP. The modeling method of NP is described in the report by Gordon et al. [16]: To establish a rat model of peripheral nerve injury (spinal nerve ligation), rats were anesthetized with an intraperitoneal injection of 350 mg/kg 10% chloral hydrate (this anesthesia was also performed when sacrificing the rats), followed by sterilization. There were no animals exhibiting signs of peritonitis after the administration of 10% choral hydrate. A 3.5 cm horizontal incision was performed at the L3 level of the back of the rat to expose the L5/L6 articular process on the left side of the rat. Part of the L5 transverse process was removed until the left L4 spinal nerve was exposed. The distal end of the L5 dorsal root ganglion was ligated with a silk thread to form the sciatic nerve root. After the completion of ligation, the incision was sutured. One day later, pain thresholds of the two hind feet of all rats were determined. If the pain thresholds of the model rats were lower than those of the control group, a successful model was considered to have been established. All 60 model rats were then randomly divided into the model group (n=30) and intervention group (n=30). The model group was left untreated. The intervention group was subjected to one injection on the 5^{th}, 10^{th}, and 15^{th} day, respectively. The injection was performed as follows: Using a 5 μL micro-syringe, 2 μL of CX3CL1 antibody (purchased from Shanghai Qiaoyu Biotechnology Co., Ltd., QY-W0811R) and 1.5 μL of CCL21 antibody (purchased from Xiamen Yanke Biotechnology Co., Ltd., YKA-AP52875), diluted 1:100 with normal saline, were slowly injected into the anterior cingulate cortex, respectively. The injection time was more than 10 minutes. The interval between the two injections was more than 5 minutes. The injection site was sterilized with alcohol after the injections were completed. Paraffin was used for sealing and finally the skin was sutured.

Experimental method: Determination of pain thresholds: According to methods reported by Tamano et al. [17], a pain threshold detector (purchased from Shenzhen RWD Life Science, BIO-EVF3 VonFrey) was used to determine pain thresholds of the backs of rats.

Determination of expression levels of CX3CL1, CCL21, JNK, and MCP-1: After the behavioral

Table 1. Comparison of pain thresholds

	Intervention group (n=29)	Model group (n=29)	Control group (n=30)	F	P
T1	6.14±2.58	6.24±2.67	29.62±2.17[a,b]	207.813	<0.001
T2	12.84±3.58[c]	7.68±3.57[a,b,c]	30.55±1.82[a,b]	104.621	<0.001
T3	17.64±3.64[c,d]	9.61±2.66[a,c,d]	29.94±1.79[a,b]	93.582	<0.001
T4	21.53±3.24[c,d,e]	10.15±2.84[a,c,d,e]	30.24±2.01[a,b]	94.304	<0.001

Note: T1, T2, T3, and T4 represented, respectively, the time right after and on the 5th day, 10th day, and 15th day after the successful establishment of neuropathic pain model. Compared to the intervention group at the same period, [a]P<0.05; compared to the model group at the same period, [b]P<0.05; compared to the same group at T1, [c]P<0.05; compared to the same group at T2, [d]P<0.05; compared to the same group at T3, [e]P<0.05.

test and pain threshold determination tests were completed, the rats were anesthetized with an intraperitoneal injection of 350 mg/kg 10% chloral hydrate prior to being decapitated. The L3-L5 spinal cord segments were obtained. The spinal cord segments were then added with the lysis buffer, homogenized on ice, centrifuged for 10 minutes (4,000 rpm), and the supernatant was obtained. Contents of CX3CL1, CCL21, JNK, and MCP-1 in the supernatant were determined by ELISA. The CX3CL1 kit was purchased from Beijing Future Biotech Co., Ltd., GTX37183. The CCL21 kit was purchased from Shanghai Jingkang Bioengineering Co., Ltd., JK-(a)-6078. The JNK kit was purchased from Shanghai Jingkang Bioengineering Co., Ltd., JK-(a)-6326. The MCP-1 kit was purchased from Tecan (Shanghai) Trading Co., Ltd., BE45241. The DNM-9606 microplate reader was purchased from Beijing Perlong Medical Technology Co., Ltd. All measurements were performed in strict accordance with the instructions.

Indicators

Indicators included expression levels of CX3CL1, CCL21, JNK, and MCP-1 in the spinal cord tissues and the pain thresholds of the backs of the 3 groups of rats at T1, T2, T3, and T4, respectively. Correlation levels between CX3CL1, CCL21, JNK, and McP-1 were analyzed by selecting the time period data with the largest difference between the intervention group and the other two groups.

Statistical analysis

Data was analyzed and processed using SPSS 24.0 statistical software (China Shanghai YuChuang Network Technology Co., Ltd.). All results in this experiment are expressed as mean ± standard deviation. One-way ANOVA and LSD post-tests were used for comparisons among multiple groups, while repeated measurements ANOVA and Bonferroni's post-tests were used for comparisons among multiple time points. Pearson's testing was used for correlation analysis. P<0.05 indicates statistical significance.

Results

Modeling results

The NP model was successfully established in 58 out of 60 rats, with a success rate of 96.67%. Therefore, 29 model rats were randomly assigned to the intervention group and model group respectively, with 30 untreated rats in the control group.

Pain thresholds

At T1, there were no significant differences in pain threshold levels between the intervention group and model group (P>0.05). At T2, T3, and T4, pain threshold levels of the intervention group were higher than those of the model group and lower than those of the control group (P<0.05). Pain thresholds of the control group at T1, T2, T3, and T4 showed no significant differences (P>0.05). In the intervention group, T1 was the lowest, T2 began to rise, and T4 was the highest (all P<0.05). In the model group, there were no significant differences between T1 and T2 (P>0.05). T3 began to increase and T4 was the highest (P<0.05). See **Table 1**.

Expression levels of CX3CL1, CCL21, JNK, and MCP-1

Expression levels of CX3CL1, CCL21, JNK, and MCP-1 in the control group were lower than the other two groups at each time point (all P<0.05). At T1, there were no significant differ-

Table 2. Comparison of expression levels of CX3CL1 (pg/mL)

	Intervention group (n=29)	Model group (n=29)	Control group (n=30)	F	P
T1	254.62±12.53	259.14±13.62	34.62±5.24[a,b]	938.924	<0.001
T2	204.57±15.28[c]	246.15±16.57[a,c]	35.17±6.04[a,b]	564.213	<0.001
T3	159.87±12.66[c,d]	226.57±12.68[a,c,d]	34.87±5.92[a,b]	823.624	<0.001
T4	114.36±13.76[c,d,e]	207.62±15.37[a,c,d,e]	35.27±6.14[a,b]	522.017	<0.001

Note: T1, T2, T3, and T4 represented, respectively, the time right after and on the 5th day, 10th day, and 15th day after the successful establishment of neuropathic pain model. Compared to the intervention group at the same period, [a]P<0.05; compared to the model group at the same period, [b]P<0.05; compared to the same group at T1, [c]P<0.05; compared to the same group at T2, [d]P<0.05; compared to the same group at T3, [e]P<0.05.

Table 3. Comparison of expression levels of CCL21 (pg/mL)

	Intervention group (n=29)	Model group (n=29)	Control group (n=30)	F	P
T1	282.63±9.64	284.14±10.26	50.62±2.64[a,b]	1849.154	<0.001
T2	226.33±12.54[c]	269.63±15.84[a,c]	51.62±3.07[a,b]	669.927	<0.001
T3	167.17±15.07[c,d]	244.72±12.87[a,c,d]	50.94±2.87[a,b]	498.228	<0.001
T4	119.65±13.55[c,d,e]	217.69±16.52[a,c,d,e]	50.82±3.15[a,b]	316.640	<0.001

Note: T1, T2, T3, and T4 represented, respectively, the time right after and on the 5th day, 10th day, and 15th day after the successful establishment of neuropathic pain model. Compared to the intervention group at the same period, [a]P<0.05; compared to the model group at the same period, [b]P<0.05; compared to the same group at T1, [c]P<0.05; compared to the same group at T2, [d]P<0.05; compared to the same group at T3, [e]P<0.05.

Table 4. Comparison of expression levels of JNK (pg/mL)

	Intervention group (n=29)	Model group (n=29)	Control group (n=30)	F	P
T1	267.63±13.62	268.74±14.63	52.13±3.14[a,b]	576.625	<0.001
T2	214.87±12.37[c]	264.66±13.82[a]	53.22±3.24[a,b]	264.133	<0.001
T3	169.57±10.42[c,d]	260.14±10.57[a,c,d]	52.27±3.04[a,b]	214.608	<0.001
T4	135.65±8.66[c,d,e]	248.69±11.37[a,c,d,e]	52.69±3.15[a,b]	218.513	<0.001

Note: JNK: c-Jun N-terminal kinase. T1, T2, T3, and T4 represented, respectively, the time right after and on the 5th day, 10th day, and 15th day after the successful establishment of neuropathic pain model. Compared to the intervention group at the same period, [a]P<0.05; compared to the model group at the same period, [b]P<0.05; compared to the same group at T1, [c]P<0.05; compared to the same group at T2, [d]P<0.05; compared to the same group at T3, [e]P<0.05.

ences in expression levels of CX3CL1, CCL21, JNK, and MCP-1 between the intervention group and model group (P>0.05). At T2, T3, and T4, expression levels of CX3CL1, CCL21, JNK, and MCP-1 in the intervention group were lower than those of the model group (all P<0.001). Expression levels of CX3CL1, CCL21, JNK, and MCP-1 in the control group at T1, T2, T3, and T4 showed no significant differences (P>0.05). Expression levels of CX3CL1 in the intervention group were the highest at T1. Levels became lower at T2 and even lower at T3. Levels were the lowest at T4 (P<0.001). In the model group, there were no significant differences in expression levels of CX3CL1 between T1 and T2 (P>0.05). Expression levels of CX3CL1 were lower at T3 than at T2 and were the lowest at T4 (P<0.001). See **Tables 2**-**5**.

Correlation analysis of CX3CL1, CCL21, JNK, and MCP-1 in rats in the intervention group

According to the above results, correlation analysis of the T4 data of rats in the intervention group was finally conducted. Pearson's correlation analysis showed that CX3CL1 was positively correlated with JNK and MCP-1 (r=0.812, 0.951, P<0.05). CCL21 was positively correlated with JNK and MCP-1 (r=0.928, 0.807, P< 0.05). See **Figure 1**.

Discussion

Mechanisms of action of inflammatory factors involved in NP have been demonstrated. In the process of inflammation-induced nerve damage, levels of glial cell markers (OX-42 and GF-

Table 5. Comparison of expression levels of MCP-1 (pg/mL)

	Intervention group (n=29)	Model group (n=29)	Control group (n=30)	F	P
T1	242.67±16.53	243.86±15.14	12.33±2.14[a,b]	736.325	<0.001
T2	178.68±13.04[c]	238.94±14.86[a]	13.04±2.34[a,b]	725.033	<0.001
T3	134.30±12.55[c,d]	234.33±13.67[a]	12.67±2.04[a,b]	742.439	<0.001
T4	72.62±8.07[c,d,e]	218.69±16.34[a,c,d,e]	12.84±2.27[a,b]	773.324	<0.001

Note: MCP-1: monocyte chemoattractant protein 1. T1, T2, T3, and T4 represented, respectively, the time right after and on the 5th day, 10th day, and 15th day after the successful establishment of neuropathic pain model. Compared to the intervention group at the same period, [a]P<0.05; compared to the model group at the same period, [b]P<0.05; compared to the same group at T1, [c]P<0.05; compared to the same group at T2, [d]P<0.05; compared to the same group at T3, [e]P<0.05.

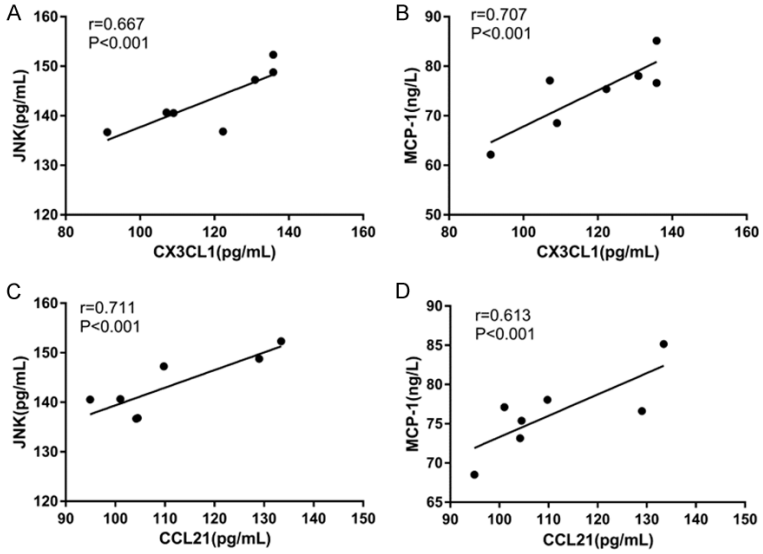

Figure 1. Correlation analysis of CX3CL1, CCL21, JNK, and MCP-1 in rats at T4 in the intervention group. A: Correlation analysis of CX3CL1 and JNK; B: Correlation analysis of CX3CL1 and MCP-1; C: Correlation analysis of CCL21 and JNK; D: Correlation analysis of CCL21 and MCP-1. JNK: c-Jun N-terminal kinase; MCP-1: monocyte chemoattractant protein 1.

AP) can be significantly increased, indicating that a large amount of glial cells are activated. This is one of the main causes of inflammatory response in patients [18, 19]. Glial cells are widely seen in the human brain and spinal cord, accounting for more than 70% of the total number of central neurons. Microglia accounts for about 5-10% of glial cells [20]. Microglia can release many cytokines and growth factors, including CX3CL1 and CCL21, after the central nervous system is injured [21]. CX3CL1 is a chemokine mainly present in the spinal cord and dorsal root ganglion neurons. It binds to CX3CR1 to activate microglia and induce pain. It can stimulate cells to secrete inflammatory mediators, promote inflammatory response, and have a strong regulatory effect on cell migration [22]. CCL21, a CC-type chemokine highly expressed in lymphoid organs, can promote the activation of T-cells, NK cells, and dendritic cells. It is closely related to biological effects, such as proliferation of smooth muscle cells and inflammatory response [23]. However, the mechanisms of action of CX3CL1 and CCL21 in NP remain unclear. In the present experiment, expression levels of CX3CL1 and CCL21, as well as their effects on JNK/MCP-1 pathways, were analyzed by establishing a NP rat model.

According to current results, expression levels of CX3CL1 and CCL21 at T1 in the intervention group were not significantly different from those in the model group. Both groups had significantly higher expression levels of CX3CL1 and CCL21, compared to the control group. Starting from T2, expression levels of CX3CL1 and CCL21 in the intervention group and model group began to decrease significantly. However, the intervention group had lower expression levels, compared to the model group, suggesting that CX3CL1 and CCL21 are involved in the process of NP. These were supposed to participate in the transmission of pain signals by activating microglia. When nerves are injured, CX3CL1 dissociates from the surface of neurons and is released into surrounding tissues [24]. There were no significant differences in pain thresholds between the intervention group and model group at T1. Starting from T2, pain thresholds of both groups began to rise. The intervention group had

significantly higher pain thresholds, compared to the model group, suggesting that CX3CL1 and CCL21 can increase the pain sensitivity of rats. Starting from T2, expression levels of CX3CL1 and CCL21 in the intervention group began to significantly decrease, suggesting that reduction of CX3CL1 and CCL21 can alleviate NP. When the central nervous system is injured, chemokines often become abnormal due to neural inflammation or neural degeneration. CX3CL1 and CCL21 can accelerate the activation of cells by promoting the secretion by microglia. This accelerates inflammation and the stress response [25]. It has been speculated that, by injecting CX3CL1 and CCL21 antibodies, the ability of CX3CL1 and CCL21 to activate microglia is inhibited. At this point, neurons can be repaired and regenerated and the disease begins to recover.

JNK, a member of the MAPK family, known as a stress-activated protease, is a small secreted protein that can orientate cell migration. It has been shown to play an important role in the development and progression of pain [26]. MCP-1, from the CC chemokine subfamily, exerts its biological effects through CCR2 receptors. It is involved in the formation and maintenance of chronic pain [27]. In the present study, JNK/MCP-1 was in a highly-expressed state in the model group. After injection of CX3CL1 and CCL21 antibodies in the intervention group, the JNK/MCP-1 signal began to decrease gradually. This suggests that administration of CX3CL1 and CCL21 antibodies can inhibit the signaling activity of JNK/MCP-1 in the spinal cord, achieving an analgesic effect. The reason may be that nerve injuries can activate phosphorylation of the transcription factor c-Jun by JNK to release MCP-1. However, further experimental research is necessary for verification [28]. It has been suggested that administration of CX3CL1 and CCL21 antibodies decreases expression of CX3CL1 and CCL21 and that the antioxidative stress activity in the central nervous system of rats is greatly enhanced. This inhibits the translocation of nuclear factors, such as AP-1 and NF-κB, and reduces the release of MCP-1. This has been suggested to relieve pain [29]. Kobayashi et al. reported that MCP-1 may be a downstream factor of JNK, supposing that CX3CL1 and CCL21 may cause sensitization of the central nervous system by upregulating expression of JNK/MCP-1 in the spinal cord, which aggravates pain [30]. However, further study and discussion is necessary for clarification of the detailed mechanisms of action.

In this experiment, the roles of CX3CL1 and CCL21 in NP, as well as their effects on JNK/MCP-1 signaling pathways, were examined by establishing a rat model of NP. However, the present study had certain limitations. The small sample size lead to no differences observed in positive rates of neurological behavior. Moreover, the rat model and the human body structure still have differences. Therefore, larger sample sizes and multi-center randomized clinical trials are necessary to verify current results.

In summary, CX3CL1 and CCL21 participate in the development of NP via JNK/MCP-1 signaling pathways. Downregulation of CX3CL1 and CCL21 expression can inhibit JNK/MCP-1 pathways, thereby elevating pain threshold levels of rats. This helps to achieve an analgesic effect. CX3CL1 and CCL21 should be considered potential targets for treatment of NP.

Disclosure of conflict of interest

None.

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