

Original Article

Intervention of Toutongning on early neuralgia in rats by inhibiting P2X3 and CGRP expression

Yunfang Chen^{1,2}, Ziliang Qian³

¹College of Chemistry and Molecular Engineering, Peking University Shenzhen Graduate School, Shenzhen, Guangdong Province, China; ²Department of Pain Management, The Eighth Affiliated Hospital, Sun Yat-sen University, Shenzhen, Guangdong Province, China; ³Department of Pain Management, Women and Children Health Institute Futian, Shenzhen, Guangdong Province, China

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Abstract: Objective: To investigate the effects of Toutongning on the expression level of purinergic receptor (P2X3) and calcitonin gene-related peptide (CGRP) of hyperalgesia-related neurons in rat model of early neuralgia and to study the therapeutic effects of Toutongning on neuralgia. Methods: Forty male SD rats of clean grade were randomly divided into normal group, sham operation group, spinal nerve ligation model group (SNL group) and Toutongning group (SNL+TG group), 10 rats in each group. In the SNL group, a rat model of neuralgia was established through the spinal nerve ligation. The rats in the SNL+TG group were treated with Toutongning based on the operation of SNL group. On the 7th day after modeling, the levels of P2X3 and CGRP in the L5 dorsal root ganglion at the operation side of the rats in each group were analyzed in histology and protein level through immunohistochemical and Western blot methods respectively. Results: The positive expression rates of P2X3 and CGRP in the SNL+TG group were 30% and 20% respectively, which were lower than those in the SNL group with statistical difference (both $P < 0.05$); in terms of the difference of them between the SNL+TG group and the normal group, sham operation group, there was no statistically significant difference (all $P > 0.05$). The expression levels of P2X3 and CGRP protein in the SNL+TG group were lower than those in the SNL group, and this difference was statistically significant (both $P < 0.05$); in terms of the difference of them between the SNL+TG group and the normal group, sham operation group, there was no statistically significant difference (all $P > 0.05$). Conclusion: The experimental results suggest that early neuralgia is associated with elevated levels of P2X3 and CGRP, and Toutongning can lower the levels of P2X3 receptor and CGRP, thus effectively improving early neuralgia.

Keywords: Neuralgia, spinal nerve ligation, Toutongning, P2X3 receptor, calcitonin gene-related peptide

Introduction

Neuralgia is a relatively common and frequently-occurring refractory disease in clinic, which is featured by complicated occurrence and development and regulated by pain-sensing neurons, hyperalgesia-related substances and others [1, 2]. Relevant data show that purinergic receptor (P2X3) is involved in the formation of a variety of neuralgias such as primary trigeminal neuralgia and transmission and modulation of pain signals [3]. It has also been found that calcitonin gene-related peptide (CGRP) plays an important role in the development of neuralgia [4]. P2X3 receptors are involved in the pathogenesis of neuralgia such as migraine. The possible pathogenesis is that in the course of neu-

ralgia such as migraine, P2X3 receptors can cause headache through its mediated ATP, and CGRP can continuously and selectively up-regulate the activity of P2X3 receptors to contribute to the transformation of P2X3 receptors. The role of P2X3 receptors in the onset of neuralgia such as migraine provides a new idea for the study on pathogenesis of neuralgia. At present, Toutongning capsule is a Chinese patent medicine made from gastrodia, saposchnikovia divaricata, polygonum multiflorum, etc. Recent study shows that Toutongning can be used to effectively treat and prevent neuralgia such as migraine with few complications, but the specific mechanism of pain relief is unclear [5]. Therefore, in this study, based on the rat model of neuralgia established by spinal nerve ligation

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(SNL), the effects of Toutongning on the expression of pain-producing factors P2X3 and CGRP in early neuralgia of SNL rats were analyzed in protein level to investigate the mechanism of Toutongning in relieving neuralgia.

Materials and methods

Experimental animals

Forty healthy male SD rats of clean grade (Shanghai SLAC Laboratory Animal Co, Ltd., China) were selected, weighting 210 ± 15 g. Those rats were raised with standard pellet feed and allowed to drink water freely. All experiments and operations were in line with the requirements of the Animal Experimentation Ethics Committee of The Eighth Affiliated Hospital, Sun Yat-sen University.

Reagents and instruments

Toutongning capsule (Shanxi Buchang Pharmaceutical Co., Ltd., China), rabbit anti P2X3 polyclonal antibody (Santa Cruz Biotechnology Inc, USA), rabbit anti CGRP monoclonal antibody (Promega, USA), SP kit (Sigma, USA), rabbit anti-mouse HRP-labeled secondary antibody (Sunbio, ROK), rabbit anti-goat HRP-labeled secondary antibody (ImmunoReagents Inc., USA), murine anti β -actin monoclonal antibody (Santa Cruz Biotechnology, USA), formalin (Zhongshan Kangnaixin Biomedical Technology Co., Ltd., China), chloral hydrate (Jinan Jiage Biological Technology Co., Ltd., China). Chemiluminescence apparatus (Shanghai Flash Spectrum Biological Technology Co., Ltd., China).

Grouping, modeling and treatment

Forty male SD rats were completely randomly divided into normal group, sham operation group, spinal nerve ligation model group (SNL group) and Toutongning group (SNL+TG group), 10 rats in each group. A rat model of neuralgia was established through the SNL based on references [6]. Specific steps of SNL: The skin in the L5-S1 of a rat back was incised to separate the muscle to the transverse process of L6; the transverse process was bit to expose the L5 spinal nerves and they were ligated with silk threads to suture muscle and skin. In the sham operation group, the nerves were not ligated,

while other operations were consistent with those in the SNL group. On the 1st day after modeling, the rats in the SNL group began to be treated with Toutongning capsule suspension from Toutongning Capsule (380 mg/kg) by continuous gavage for 7 days, once a day. The rats in the normal group, SNL group and sham operation group were given normal saline by continuous gavage for 7 days, once a day. The postoperative foot pain threshold of the rats under modeling was measured every other day after surgery. The results showed that all rats in the model group were successfully modeled [7].

Immunohistochemical method

On the 7th day after modeling, rats in each group were given anesthesia by intraperitoneal injection of 10% chloral hydrate (3.5 mL/kg). Cardiac perfusion was carried out with normal saline to create a surgical space to remove the L5 dorsal root ganglion (DRG) at the operation side of the rats, and then the isolated L5 DRG was placed in liquid nitrogen and stored in a refrigerator at -80°C for later use. The DRG was taken out and fixed with neutral formalin, followed by dehydration, investment and sectioning. At the time of detection, the paraffin sections were deparaffinized and rehydrated, incubated in 3% H_2O_2 for 5 minutes at room temperature, and washed twice in PBS (1X), 5 minutes each time. Then the specimen was blocked with 10% normal goat serum (diluted in PBS) and incubated for 10 minutes at room temperature. Rabbit anti P2X3 polyclonal antibody (working concentration of 1:500) and rabbit anti CGRP monoclonal antibody (working concentration of 1:50) were added dropwise, placed at 4°C overnight, and then washed in PBS (1X) three times, 5 minutes each time. Horseradish peroxidase was added to label the secondary antibody, incubated at 37°C for 20 minutes, and then washed in PBS (1X) 3 times, 5 minutes each time. And then color development was done with DAB for 5-10 minutes and counterstaining was done with hematoxylin. Positive P2X3 was indicated by brownish yellow particles in cytoplasm. Positive CGRP was indicated by brownish yellow particles in cell membrane or cytoplasm. Positive rate = The number of rats with positive immunohistochemistry results/10 * 100%.

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Table 1. Comparison of immunohistochemistry results in P2X3 (n, %)

Group	P2X3
Normal group (n=10)	2 (20.0)
Sham operation group (n=10)	3 (30.0)
SNL group (n=10)	9* (90.0)
SNL+TG group (n=10)	3 (30.0)

Note: Compared with SNL+TG group, *P<0.05. SNL, spinal nerve ligation; TG, Toutongning.

Table 2. Comparison of immunohistochemistry results in CGRP (n, %)

Group	CGRP
Normal group (n=10)	1 (10.0)
Sham operation group (n=10)	2 (20.0)
SNL group (n=10)	8* (80.0)
SNL+TG group (n=10)	2 (20.0)

Note: Compared with SNL+TG group, *P<0.05. CGRP, calcitonin gene-related peptide; SNL, spinal nerve ligation; TG, Toutongning.

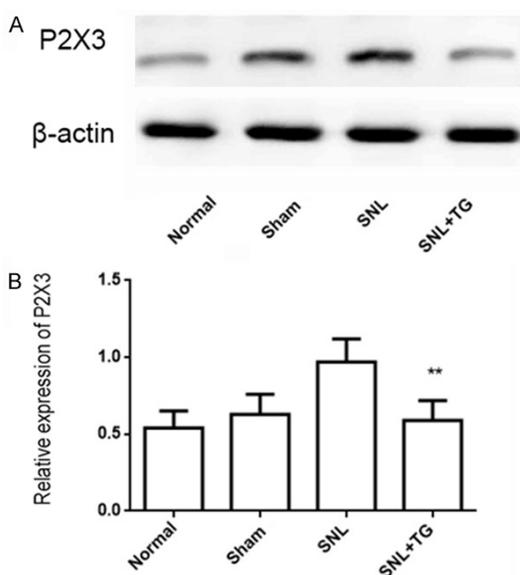


Figure 1. Comparison of P2X3 protein level between each group. A: Results of P2X3 protein detected by Western blot; B: Semi-quantitative analysis of P2X3 protein level. Compared with SNL group, **P<0.01. SNL, spinal nerve ligation; TG, Toutongning.

Western blot method

After being ground, the DRG specimen was centrifuged to take the supernatant as tissue protein solution, and the solution was stored in liquid nitrogen for later use. The BCA method

was adopted to detect the protein concentration. After SDS-PAGE electrophoresis and transfer membrane (PVDF membrane), the specimen was added with primary antibodies (rabbit anti P2X3 polyclonal antibody, rabbit anti CGRP monoclonal antibody, and murine anti β-actin monoclonal antibody), incubated at 4°C overnight, and then added with secondary antibodies (rabbit anti-mouse HRP-labeled secondary antibody and rabbit anti-goat HRP-labeled secondary antibody), and incubated at room temperature for 1 hour. After color development, Chemiluminescence apparatus was used for detection and photographing. The gray value of the objective protein was measured through the Image-pro plus (version 4.1) software. β-actin was an internal parameter.

Statistical analysis

SPSS 20.0 software was adopted for analysis. Measurement data of multiple groups were compared through one-way ANOVA, and the approximate F test Welch method was adopted if the variances were unequal. The comparison between two groups was performed with LSD test and enumeration data were analyzed by chi-square test. The result of P<0.05 is considered statistically significant.

Results

Immunohistochemistry results

The positive expression products of P2X3 and CGRP were brown particles. The positive rates of P2X3 and CGRP in the SNL+TG group were 30% and 20%, respectively, which were lower than those in the SNL group (P=0.022, P=0.025). The difference of the positive rates of them between the SNL+TG and the normal group, sham operation group was not statistically significant (all P>0.05). See **Tables 1, 2**.

Results of Western blot test

The protein expression levels of P2X3 and CGRP in the normal group, sham operation group, SNL group, and SNL+TG group were different with statistical significance (all P<0.01). The protein expression levels of P2X3 and CGRP in the SNL+TG group were lower than those in the SNL group (both P<0.01). The difference of protein expression levels of P2X3 and CGRP between the SNL+TG group and the

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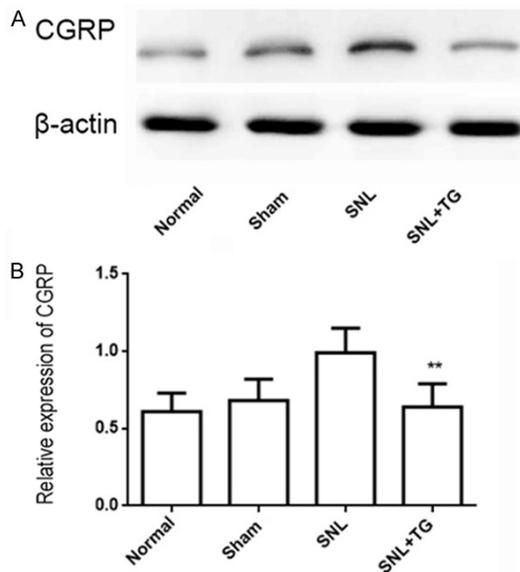


Figure 2. Comparison of CGRP protein level between each group. A: Results of CGRP protein detected by Western blot; B: Semi-quantitative analysis of CGRP protein level. Compared with SNL group, ** $P < 0.01$. CGRP, calcitonin gene-related peptide; SNL, spinal nerve ligation; TG, Toutongning.

normal group, sham operation group was not statistically significant (all $P > 0.05$). See **Figures 1, 2**.

Discussion

P2X3 receptor is a subtype of P receptor, which is specifically expressed on dorsal roots [8]. Relevant data show that ATP can activate P2X3 receptor to promote its formation of pain signals [9, 10]. Studies show that intrathecal application of P2X3 receptor antagonists with high selectivity and high affinity can effectively reduce tactile allodynia in rats with chronic sciatic nerve ligation or spinal nerve ligation [11, 12].

CGRP is a potent vasodilator peptide abundant in trigeminal ganglion. Some data indicate that synthesis and release of CGRP are often regulated by many factors such as capsaicin receptor (transient receptor potential vanilloid type-1) and intracellular Ca^{2+} concentration, and there is a difference in the release level of CGRP between different organs and tissues of a body [13, 14]. CGRP can increase the transcription and activity of P2X3 receptor, and promote the release of impulse signals of the P2X3 receptor to trigeminal nuclei of brainstem [15,

16]. P2X3 receptors, on the other hand, can lengthen and extend CGRP in a feedback way, thus triggering neuralgia [17, 18].

Toutongning capsule is a drug for various types of neuralgias such as migraine. Previous studies have confirmed that Toutongning capsule has good effects on inhibiting platelet aggregation and promoting blood circulation in patients, but its treatment mechanism is yet to be further investigated [19-21]. Studies show that the mechanism of Toutongning capsule for neuralgia may be closely related to the reduction of P2X3 mRNA overexpression of trigeminal ganglion for the reduction of pain information transmission [22-24]. Meantime, one study shows that electrical stimulation of trigeminal nerve can cause greatly increased CGRP content in the peripheral blood and increased meningeal blood flow of rats, and Toutongning can effectively inhibit the expression of CGRP, thus treating migraine [25]. In this study, the influence of intervention therapy with Toutongning on the expression of P2X3 and CGRP was examined from the histological level and protein expression level. The results of the study showed that the protein expression levels of P2X3 and CGRP in the SNL+TG group were lower than those in the SNL group, both after immunohistochemistry and Western blot test. But the difference of them between the SNL+TG group and the normal group, sham operation group was not statistically significant. It indicates that Toutongning capsule can significantly reduce the P2X3 and CGRP protein levels of DRG. The results of this study are consistent with those results of the above studies, which suggests that the intervention of early neuralgia with Toutongning capsule is related to the down-regulation of P2X3 and CGRP levels of DRG.

In summary, early neuralgia is associated with elevated levels of P2X3 and CGRP, and Toutongning can down regulate P2X3 and CGRP levels, thus effectively relieving early neuralgia. However, a more in-depth treatment mechanism is yet to be further investigated.

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Disclosure of conflict of interest

None.

Address correspondence to: Ziliang Qian, Department of Pain Management, Women and Children Health Institute Futian, No.2002 Jintian Road, Futian District, Shenzhen 518048, Guangdong Province, China. Tel: +86-0755-83816757; E-mail: qianziliang5b10@163.com

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