Botulinum toxin type A relieve neuropathic pain by suppressing the expression of CXCL13/CXCR5 and GAT-1 in chronic constriction injury rats

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Abstract

Botulinum toxin type A (BTX-A) was widely used to treat neuropathic pain in clinic. The underlying analgesic mechanism of BTX-A involves in axonal transport. The chemokine (C-X-C motif) ligand 13 (CXCL13) and GABA transporter 1 (GAT-1) played important roles in chronic pain. We established a chronic constriction injury (CCI) model. The pain behaviors of rats were measured by testing paw withdrawal thresholds (PWTs) and paw withdrawal latencies (PWLs). The level of proteins was measured by western blots. In our results, the CCI rats showed decrease of PWTs and PWLs, which were relieved by BTX-A. BTX-A reversed the over-expression of CXCL13 and GAT-1 in spinal cord, DRG, sciatic nerve and plantar in CCI rats and characterized in dose-dependent manner. The inhibition of BTX-A on proteins we examined didn't show significant trend among time points. The analgesic effect of BTX-A disappeared after the axon transport of sciatic nerve blocked by the colchicine. But the PWTs of the colchicine treated CCI rats were higher than non- colchicine-treated CCI rats. Colchicine decreased the levels of CXCL13 and GAT-1 in CCI rats. What's more, the proteins we examined peaked at the sciatic nerve in the non-colchicine group, but the phenomenon disappeared in the colchicine group. In conclusion, the BTX-A and colchicine relieve neuropathic pain and suppress the increase of CXCL13 and GAT-1. Colchicine prevents the analgesic effect of BTX-A by blocking axon transport. The axon transport may play roles in the peripheral mechanisms of neuropathic pain.

1. Introduction

Neuropathic pain, such as herpes zoster neuralgia, diabetic peripheral neuropathy, complex regional pain syndrome *etc.*, is characterized by severe pain, difficulty in effective control and long duration, and severely damage the patient's quality of life(Baba et al. 2020). At present, the commonly used drugs to treat neuropathic pain in clinical are ion channel inhibitors, such as pregabalin, gabapentin(Rodríguez et al. 2007). These drugs are derivatives of GABA, which achieve the aim of analgesia through inhibiting the release of certain neurotransmitters by regulating the calcium ion channels(Chincholkar 2020). Nerve block, pulsed radiofrequency and spinal cord electrical stimulation are also used to treat intractable neuropathic pain(Gerken et al. 2020; Liu et al. 2020). However, the treatment effect is still not good.

The refractory characteristic of neuropathic pain is related to its complex mechanism. At present, neuropathic pain are mainly caused by the increase and facilitation of ion channels on the cell membrane of painful neurons(Li et al. 2019b), abnormal nerve discharge(Tal and Eliav 1996), sympathetic sprouting(Zhang et al. 2004) and so on. If the pain cannot be effectively relieved, the central system will be centrally sensitized via the activation of glial cells in the DRG(Liang et al. 2010), spinal cord or nucleus of the spinal cord(Lim et al. 2017), the weak regulation of interneurons(Kim et al. 2012), and the decreased expression of opioid receptors(Custodio-Patsey et al. 2020). So far, the research on the mechanism of neuropathic pain is mainly at spinal cord level. However, the peripheral mechanisms of neuropathic pain are mainly at electrophysiological changes such as abnormal discharge of nerve fibers and activation of ion channels(Li et al. 2019a; Serra et al. 2012). Here we have a question that Is there

any other signal transmission between nerve fiber and neuron cell body except electrical signaling after nerve injury?

BTX-A is a macromolecular protein neurotoxin produced by Botox, and produces muscle relaxation paralysis mainly by inhibiting the release of acetylcholine from axon end. Recent research suggests that BTX-A may be a complementary therapy for postherpetic and trigeminal neuralgia(Chen and Chuang 2017). BTX-A applied to the plantar performed the effect of antinociceptive by SNAP-25 cleavage through retrograde axonal transport to spinal cord via sciatic nerve(Matak et al. 2012). BTX-A also can attenuate neuropathic pain by regulating the interaction between neuron and glial cell in spinal cord(Marinelli et al. 2012), inhibiting the release of neuroinflammatory molecules and so on(Zychowska et al. 2016). Based on this phenomenon, we supposed that whether there is axonal transport of pain-causing molecules between nerve fiber and neurons in peripheral nerve injury.

Our previous study has indicated that CXCL13/CXCR5 regulated morphine analgesia through p38, ERK and AKT signaling pathways in bone cancer pain rats(Bu et al. 2019). In previous studies, GAT-1 was increased after CCI injury and played an important role in the occurrence and development of neuropathic pain(Daemen et al. 2008). In our study, we suppose that the known pain-causing molecules CXCL13/CXCR5 and GAT-1 may be transported from the nerve injury site to the DRG neuron cell body or spinal cord through axon transport after nerve injured. Therefore, with the treatment of BTX-A for neuropathic pain, we explore that the axon transport mode between the nerve injury site and the neuron cell body using the cytokines CXCL13/CXCR5 and GAT-1 as research targets.

2. Experimental Procedures

2.1 Animal

The animals in this experiment were Specific pathogen-free (SPF) male Sprague-Dawley rats weighing 220-250 g, purchased from Zhengzhou University Experimental Animal Center, Zhengzhou, China. The rats were placed in a clean animal house, alternated between 12 h light and 12 h dark and were given enough Feed and drinking water. The animal room keeps constant temperature ($22^{\circ} \pm 0.5^{\circ}$) and constant humidity (40% ~ 60%). All animal procedures were performed in accordance with the guidelines of the National Institutes of Health Laboratory Animal Care and Use Guidelines and the IASP Pain Research Guidelines and approved by the Animal Care and Use Committee of Zhengzhou University. Rats were allowed to adapt for about 7 days before performing surgery and behavioral testing. In this experiment, all animals were randomly grouped.

2.2 CCI model

We established the CCI model according to the method of Bennet and Xie(1988)(Bennett and Xie 1988). Rats were anaesthetized with 10% Chloral hydrate (3~5 mL/ kg) by intraperitoneal injection. The left hind limb of each rat was shaved and disinfected with iodophor. The sciatic nerves on left side were exposed by making a skin incision and blunt dissecting through the connective tissue between the gluteus

superficialis and biceps femoris muscles. And then, the sciatic nerves were separated by a glass needle. Four ligatures are tied loosely around the sciatic nerve at 1mm intervals with 4-0 surgical wire, to just occlude but not arrest epineural blood flow. The incisions were closed with sutures and rats are placed to their cages. The sham surgery was implemented using a similar procedure without ligation.

2.3 Pain behavioral quantification

Mechanical paw withdrawal thresholds (PWTs) were carried out by a series of calibrated manual Von Frey's filaments (Stoelting, Kiel, WI, USA), ranging from 0.2 to 15 g. Rats were placed in opaque plastic cages with a wire mesh floor for 30 min to conform the environment before the test. A Von Frey filament was applied perpendicularly to the plantar surface of the hind paw for 5 s. Abrupt paw withdrawal, licking or shaking were considered as positive responses during the application of the stimulus or after removal of the filament. The time interval before the application of the next filament was at least 10 s. The "up-down" method was used to calculate mechanical paw sensitivity.

Thermal paw withdrawal latencies (PWLs) were carried out using the thermal plantar analgesia instrument (Ugo Basile, Varese, Italy). The I.R. intensity was adjusted to make an average paw withdrawal latency of 10-13 s in normal rats, and the cut-off time was set at 15 s in advance to prevent tissue damage. Rats were placed into the glass floored testing cages for 30min to acclimatize before the experiment. The infrared source was placed directly beneath the mid-plantar surface of the hind paw. The heat stimulation was repeated at least 3 times with an interval of about 10 min. The mean of the three latencies was used as the PWL. In our laboratory, the tests and the data analyzer were double-blinded.

2.4 Drugs

100 U/vial Botulinum Toxin Type A (BTX-A) (Heng-Li, catalog NO.20,190,425, Lanzhou China) was reconstituted with 1mL 0.9% saline solution (concentration of 0.1 U BTX-A/ μ L) laying in 4°C, and applied via i.pl. route into the ipsilateral hind paw. BTX-A was injected in a volume of 25 μ L with a 30- μ L microsyringe on the 7th day after CCI modeling. The control group was administered with an equal volume of 0.9% saline solution. No injection was made into the right hind paw. Colchicine was dissolved in 0.9% saline solution at 4°C, and administered at concentration 10 mmol/L. The Colchicine solution was used to disrupt axoplasmic transport of the sciatic nerves, and the method of application was performed according to previously standardized protocols (Dilley and Bove 2008).

2.5 Disruption of axoplasmic transport

The axoplasmic transport of the sciatic nerves was blocked by colchicine solution on the 7th day after CCI surgery according to the method described previously (Dilley and Bove 2008). The rats were treated with colchicine only in the last part of our experiment. Rats were anaesthetized with chloral hydrate. Then the left sciatic nerve with a 7-8 mm length was exposed by blunt dissection and carefully rid of the surrounding connective tissue. The nerve was wrapped around with a slice of absorbable gelatin sponge (5 mm x 5 mm x 10 mm) saturated in the colchicine, and a strip of parafilm (6 mm x 20 mm) was placed

under the nerve in advance to prevent leakage of the agent onto the surrounding tissue. The objects around the nerve were removed after 15 min, with the nerve rinsed with saline. The wound was sewed up using 4-0 surgical sutures. The same surgical procedure was executed except that the nerve was exposed to saline alone.

2.6 Western Blots

Rats were anaesthetized with chloral hydrate. The left L4-6 spinal cords, L4-6 DRG, proximal sciatic nerve, and plantar skin were removed and fully ground in RIPA lysis buffer according to the manufacturer's instruction (Beyotime, China). Total protein (30 ug) from each sample was denatured by boiling in 99 °C water before loading onto 10% SDS polyacrylamide gel. Electrophoresis of each gel was administered at 200 mA constant current for 90-120 min so that the proteins were subsequently electro-transferred to PVDF membranes. After being blocked with 5% non-fat milk for 2 h and incubated with rabbit anti-CXCL13 (1:1000, Abcam, UK), mouse anti-CXCR5 (1:200, Santa Cruz, USA), rabbit anti-GAT-1 (1:1000, Gene Tex, USA), rabbit anti-GAPDH (1:1000, Hangzhou Xianzhi, China) overnight at 4 °C, the membranes were incubated with HRP-conjugated goat anti-rabbit Ig G (1:5000, Boster, China), or HRP-conjugated goat anti-mouse anti-rabbit Ig G (1:5000, Boster, China) for 2 h. Proteins were detected by enhanced chemiluminescence (ECL) detection system (Beyotime, China) and visualized by exposure to Kodak film. The blot density analysis was quantified by densitometric scanning. The GAPDH was used as the loading control for the proteins in our experiment.

2.7 Statistical Method

Data are showed as mean $\pm$ SEM. The Graph-pad Prism software 7.0 was used for statistical analysis. The one-way ANOVA followed by post hoc Tukey's honestly significant difference (HSD) test or unpaired Student's *t* test was used to analyze western blot data. The behavioral data was analyzed by the two-way ANOVA with Bonferroni's multiple comparison test. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1 The expression of CXCL13, CXCR5 and GAT-1 proteins were increased in the spinal cord, DRG, sciatic nerve and plantar skin in CCI rats

The mechanical pain and thermal pain thresholds of rats at 1, 3, 5, 7, 10, 14 and 21 days after CCI surgery were carried out to determine the success of CCI model. As a result, CCI rats showed a significant decrease in PWT and PWL in the ipsilateral hind paw after CCI compared to the sham rats. The PWTs and PWLs of Ipsilateral were decreased significantly at 3th day after CCI surgery, and maintained from days 7 to 21 (Fig.1a, c). The PWTs and PWLs of the Contralateral showed no significant difference at the days we examined (Fig.1b, d).

Then, we measured the relative levels of CXCL13, CXCR5 and GAT-1 in the L4-6 spinal cord, L4-6 DRG, sciatic nerve and plantar skin of CCI rats by western blot. The expression of CXCL13, CXCR5 and GAT-1 showed a dramatic increase in L4-6 spinal cord, L4-6 DRG, sciatic nerve and plantar skin in CCI rats compared to those in sham rats ($P<0.05$) (Fig.2). These proteins were mostly increased from 3th day after surgery. But the CXCL13 in sciatic nerve and plantar skin, CXCR5 in spinal cord and sciatic nerve, and the GAT-1 in sciatic nerve were increased from 7th day after surgery (Fig.2g-k, o).

3.2 BTX-A relieved mechanical and thermal allodynia, decreased the over expression of CXCL13, CXCR5 and GAT-1 in CCI rats, and characterized in dose-dependent manner

To investigate the effects and characteristics of BTX-A, the CCI rats was administered with BTX-A at the doses of 4, 7 and 10 U/kg (n=6-8 each group) through i.pl. at the 7th day after CCI surgery. The CCI + NS group and sham group (n=6-8 each group) were given the same volume of normal saline. The results showed that the BTX-A of three doses all significantly relieved the mechanical and thermal hypersensitivity in some degree compared to the CCI + NS group, and the allodynia effect of the CCI + 10U/Kg BTX-A group showed significant differences compared to the CCI + 7U/Kg BTX-A group and the CCI + 4U/Kg BTX-A group (Fig3. a, c). It's obviously that the higher dose group had more significant change than the lower dose group. There were on significant differences in the data of the contralateral side in each group.

Western blots were used to detected the expression of CXCL13, CXCR5 and GAT-1 at the 14th day after medication. BTX-A decreased the over-expression of CXCL13, CXCR5 and GAT-1 in the spinal cord, DRG, sciatic nerve and plantar skin after CCI surgery, and the higher dose (7 and 10 U/kg) group had more reduction than the lower dose group (4 U/kg) (Fig.4). These data declared that BTX-A can change the expression of these proteins and relieve the hypersensitivity after CCI, and the effect of BTX-A is dose-dependent in a certain range.

3.3 The dose of 10 U/kg BTX-A has no effect on the mechanical hypersensitivity for sham rats. There was no significant trend among 10d, 14d, 21d about the inhibitory effect of BTX-A on CXCL13, CXCR5 and GAT-1

To investigate the effect of 10 U/kg BTX-A on the pain threshold of rats, and changes in the expression of the detected proteins, both of the CCI group and the sham group were given 10 U/Kg BTX-A or saline through plantar injection at 7th day after CCI surgery, and the proteins were tested at 10th, 14th and 21th day after CCI surgery. The pain measurement results showed that there was no significant difference between the sham group i.pl. with 10 U/Kg BTX-A or saline (Fig.5. a). BTX-A attenuated the mechanical hypersensitivity of the Ipsilateral significantly (Fig.5. a), and the mechanical hypersensitivity of the Contralateral indicated no significant between-group difference (Fig.5. b). Western blots were used to detected to expression of CXCL13, CXCR5 and GAT-1. The expression of CXCL13 and CXCR5 proteins in spinal cord, DRG, sciatic nerve and plantar skin reduced significantly by BTX-A treatment at 10th, 14th and 21th day after CCI surgery (Fig.6. d; Fig.7. a, b, c). The expression of GAT-1 in spinal cord, DRG and sciatic

nerve decreased significantly at 14th and 21th day after CCI surgery (Fig.6. b, c; Fig.7. a, b). The expression of GAT-1 in plantar skin decreased significantly at 10th, 14th and 21th day after CCI surgery (Fig.7. c).

3.4 After pre-treated with colchicine in sciatic nerves, the mechanical allodynia of CCI rats were significantly relieved

The purpose of this experiment was to indicate the analgesic effects and retrograde axon transport mechanism of BTX-A after blocking sciatic nerve axon transport. The axonal transport of the sciatic nerves was blocked by 10 mmol/L colchicine at 7th day after CCI surgery. And the 10U/Kg BTX-A was treated to rats through i.pl. at 7th day after CCI surgery. What interesting about the data in this table is that colchicine can significantly increase the pain threshold of rats. The PWTs of the CCI rats treated with colchicine were steadily maintained at 15 g above from days 10 to 21(Fig.8.a). the mechanical pain threshold on the contralateral did not show significantly difference between groups (Fig.8.b).

3.5 Colchicine blocked the retrograde axonal transport of BTX-A, and relieved the over-expression of CXCL13, CXCR5 and GAT-1 after CCI

To further clarify the mechanism of BTX-A, the sciatic nerves of rats were infiltrated by saline or 10mmol/L colchicine solution and the ipsilateral plantar were injected with saline or 10U/Kg BTX-A on 7th day after CCI surgery. Rats were anesthetized and the spinal cord, DRG, sciatic nerve and plantar of the ipsilateral were removed for western blots tests at 21th day after CCI. As the results showed, the expression of the proteins we tested in sham group was significantly lower than other groups, and the CCI-NS-10U/Kg BTX-A group had a significant difference compared with the CCI-NS-NS group. The expression of the proteins in the CCI groups treated with 10 mmol/L colchicine was significantly decreased compared with CCI-NS-NS group. And there was no significant difference between CCI-COL-NS group and CCI-COL-BTX-A group (Fig.9). The results suggested that the 10mmol/L colchicine inhibited the over-expression of CXCL13, CXCR5 and GAT-1 induced by CCI surgery. After the axonal transport of the sciatic nerve was blocked by 10 mmol/L colchicine, the BTX-A injected to the planta had no effect on the expression of CXCL13, CXCR5 and GAT-1.

3.6 Exploration for the peripheral mechanism of neuropathic pain about axon transport.

To further illustrate the mechanism of peripheral axonal transport for neuropathic pain, we designed the western blotting bands with the loading order of plantar, sciatic nerve, DRG and spinal cord. Rats were anesthetized and the spinal cord, DRG, sciatic nerve and plantar of the ipsilateral were removed for western blots tests at 21th day after CCI. These results suggested that the levels of CXCL13, CXCR5 and GAT-1 proteins peaked at the sciatic nerve in the CCI-NS-NS group (Fig.10. b, g, l, q), and the peak disappeared after the axonal transport of the sciatic nerve blocked by 10 Mm colchicine (Fig.10. d, i, n, s). According to these data, we supposed that the proteins we examined itself or the injury signal transported from the injury site to the cell body in DRG and spinal cord through retrograde axonal transport. The disappearance of the peak expression of the pain-causing factor at the sciatic nerve may be due to the

fact that colchicine blocked the transport of the pain-causing factor from the spinal cord and DRG to the injured site. What's more, the BTX-A may be inhibit the transport of CXCL13/CXCR5 and GAT-1 (Fig.10. c, h, m, r). The axonal transport may be a new mechanism of pain development. But the hypothesis needs to be studied in future.

4. Discussion

In our studies, BTX-A achieve analgesic effect by inhibiting the expression of CXCL13 / CXCR5 and GAT-1 in CCI rats and characterized in dose-dependent manner. The inhibitory effect of BTX-A on proteins we examined is not different among different time points. Colchicine prevents the analgesic effect of BTX-A by blocking axon transport, and decreased the levels of CXCL13/CXCR5 and GAT-1 in CCI rats. Peripheral axon transport mechanism may be play important role in neuropathic pain.

Chemokines is a family of small cytokines, or signaling proteins secreted by cells, with a molecular mass of between 8 and 10 KDa. The major role of chemokines is to act as a chemoattractant to guide the migration of cells. Cells are attracted to move through the gradient towards the higher concentration of chemokine. So, how is the gradient formed? It can be seen that the chemokines are transportable. The injury site of the CCI model in our experiment is the sciatic nerve. According to the chemotactic characteristics of chemokines, we can speculate that CXCL13 produced by the sciatic nerve can move to the proximal through retrograde axonal transport to form a concentration gradient to attract cells to the injury site. Previous study has indicated that CXCL12 and CXCR4 were transported from nerve cell bodies to spinal dorsal horn(Reaux-Le Goazigo et al. 2012). In our study, the expression of CXCL13/CXCR5 and GAT-1 peaked at the sciatic nerve in CCI group (Fig.10. b, g, l, q). But the phenomenon disappeared in the colchicine group (Fig.10. d, i, n, s). The colchicine blocked the axonal transport of sciatic nerve. So, the source of CXCL13/CXCR5 and GAT-1 in the sciatic nerve is not only from its own expression, but also from DRG or spinal cord.

CXCL13 was indicated to facilitate the occurrence and development of pain(Bu et al. 2019; Jiang et al. 2016). GABA is an inhibitory neurotransmitter, which plays an important role in nerve conduction function(Nagumo et al. 2020). The function of GAT-1 is to reuptake extracellular GABA to maintain the transmission of GABA signals. In pathophysiological pain states, GABA signaling can be blocked due to a decrease in extracellular GABA synthesis by GAD65 and increasing extracellular GABA reuptake via GAT-1(Ford et al. 2015). And previous study showed that the expression of GAT-1 increased in CCI models, and administration with GAT-1 inhibitor can relieve mechanical hypersensitivity(Daemen et al. 2008). Hui Du et al using RNA sequencing indicated that CXCL13 is increased in spinal cord in CCI rats(Du et al. 2018). It is consistent with our experimental results. In our studies, we examined the levels of CXCL13/CXCR5 and GAT-1 in the planter, sciatic nerve, DRG and spinal cord using western blots. And the proteins we examined were increased in the site mentioned above (Fig.2).

Previous study showed that BTX-A performed the antinociceptive actions by SNAP-25 cleavage via retrograde axonal transport through the planter, sciatic nerve, DRG and spinal cord[16] and prevented the

release of neurotransmitters such as acetylcholine and glutamate(Akaike et al. 2013). Recent researches have also revealed that BTX-A decreased the over-expression of the pronociceptive proteins IL-18, IL-6 and IL-1 β and increased the levels of antinociceptive proteins IL-10, IL-1RA in spinal cord and DRG in CCI rats(Zychowska et al. 2016). In our studies, BTX-A suppressed the over-expression of CXCL13/CXCR5 and GAT-1 in the planter, sciatic nerve, DRG and spinal cord in CCI rats to relieve mechanical pain and characterized in dose-dependent manner (Fig.3, 4). What's more, we speculate that the BTX-A may inhibit the axonal transport of the proteins we examined according to the results of Fig.10. c, h, m, r. But the speculation needs further proof in the future.

In our studies, the analgesic effect of BTX-A was related to the injection time. The analgesic effect of 21th day was better than that of 14th day (Fig.5. A). However, the levels of CXCL13/CXCR5 and GAT-1 didn't show a significant downward trend among 10th, 14th and 21th day (Fig.6, 7). The results indicated that the factors that affect the maintenance of neuropathic pain are complex. It's not determined by a single factor, but caused by multiple factors. Not only the rapid conduction of electrical signals(Alba-Delgado et al. 2021), but also some molecular signals(Myers and Shubayev 2011) via axonal transport to DRG or spinal cord to facilitate the development of neuropathic pain after peripheral nerve injury. Previous studies had suggested that tumor necrosis factor-alpha and protein kinase G transported from injury site to DRG to facilitating neuropathic pain(Myers and Shubayev 2011; Sung et al. 2006). Rory Curtis et al investigated the retrograde axonal transport of neurotrophins (NGF, BDNF et al) from the sciatic nerve to DRG and spinal neurons in normal rats or after nerve injury, and DRG neurons showed increased transport of all neurotrophins following crush injury to the sciatic nerve(Curtis et al. 1998). In our studies, the analgesic effect of BTX-A disappeared after colchicine (COL) blocked the axonal transport of the sciatic nerve, and the expression of CXCL13/CXCR5 and GAT-1 suggested no significant difference between CCI-COL-BTX-A group and CCI-COL-NS group (Fig.9). So, the BTX-A administered subcutaneously needs to rely on axonal transport to achieve analgesic effect. The axonal transport is indispensable in the mechanism of peripheral neuropathic pain.

What's more, we found that the colchicine showed an amazing analgesic effect. The PWTs of the colchicine-treated groups was significantly increased, and maintained at 15 g above from days 10 to 21(Fig.8.a). The overexpression of CXCL13/CXCR5 and GAT-1 were decreased significantly in CCI-COL-NS group compared with CCI-NS-NS group (Fig.9), but the degree of reduction was not as great as the CCI-NS-BTX-A group. The analgesic effect of colchicine was far superior to that of BTX-A, but the inhibitory effect on the expression of CXCL13, CXCR5 and GAT-1 was lower than BTX-A. Previous studies showed that colchicine applied to the proximal part of the injury site relieved the mechanical sensitivity, and the mechanically sensitive afferents exhibited impulse conduction blocks at the colchicine-treated site(Proske and Luff 1998). And colchicine can inhibit the levels of pro-inflammatory cytokines, such as IL-1 β , IL-6, and TNF- α (Shi et al. 2020). Based on these data, we speculated that the excellent analgesic effect of colchicine was due to blocking the conduction of electrical signals and reducing the levels of inflammatory cytokines. And we can anticipate that the performance of colchicine in related to the

concentration, the treatment methods and the infiltration time. But more studies need to be done in the future.

Conclusion

In conclusion, BTX-A and colchicine achieve analgesic effect by inhibiting the expression of CXCL13 / CXCR5 and GAT-1 in spinal cord, DRG, sciatic nerve and plantar in CCI rats and characterized in dose-dependent manner. Colchicine can antagonize the analgesic effect of BTX-A by blocking the axonal transport of sciatic nerve. We also explored the mechanism of axon transport, and speculated that the proteins we examined may not only cause changes through increased expression, but also through axonal transport. Peripheral axon transport mechanism may be a new mechanism of neuropathic pain and more relevant studies need to be done in the future.

5. Abbreviation

- BTX-A Botulinum toxin type A
- CCI Chronic constriction injury
- CXCL13 Chemokine (C-X-C motif) ligand 13
- CXCR5 C-X-C chemokine receptor type 5
- GAT-1 GABA transporter 1
- PWTs Mechanical paw withdrawal thresholds
- PWLs Thermal paw withdrawal latencies

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Figures

Figure 1

The mechanical paw withdrawal thresholds (PWTs) and thermal paw withdrawal latencies (PWLs) were significant decreased from day 3 after CCI surgery. This decrease last to day 21 after CCI modeling. d $P < 0.05$ VS. Sham; Two-way ANOVA; $n = 11$ rats each group

Figure 2

Chronic constriction injury of sciatic nerve upregulated the expression of CXCL13, CXCR5 and GAT-1 from day 3 or 7 in the spinal cord, DRG, sciatic nerve and planta skin by western blot. (a, e, i, m) The expression of CXCL13, CXCR5 and GAT-1 in the spinal cord; (b, f, j, n) The expression of these proteins in the DRG; (c, g, k, o) The expression of these proteins in the sciatic nerve; (d, h, l, p) The expression of these proteins in the planta skin. d $P < 0.05$ VS. sham; One-way ANOVA; $n = 3$ each group

Figure 3

The BTX-A relieved the mechanical and heat hyperalgesia significantly in rats, and characterized dose-dependent. From the 7th day, the PWTs and PWLs were increased in BTX-A-treated CCI rats. The PWTs and PWLs in BTX-A-10U/Kg-treated CCI rats were higher than those in the 4 and 7 U/kg BTX-A treated CCI rats. b $P < 0.05$ VS. CCI + NS group, Two-way ANOVA, $n=6$ each group; c $P < 0.05$ VS. CCI + 4 U/Kg BTX-A group, Two-way ANOVA, $n=6$ each group; d $P < 0.05$ VS. CCI + 7 U/Kg BTX-A group, Two-way ANOVA, $n=6$ each group

Figure 4

The BTX-A inhibited the over-expression of CXCL13, CXCR5 and GAT-1 in CCI rats, and the effect of BTX-A is dose-dependent. The BTX-A reversed the increased expression of CXCL13, CXCR5 and GAT-1 after CCI in the spinal cord (a, e, i, m), the DRG (b, f, j, n) and the sciatic nerve (c, g, k, o). (d, h, l, p) The increased expression of CXCL13, CXCR5 and GAT-1 after CCI in the planta skin was also reversed by BTX-A. a $P < 0.05$ VS Sham group, b $P < 0.05$ VS CCI+NS group, c $P < 0.05$ VS CCI+4 U/Kg BTX-A group, d $P < 0.05$ VS CCI+10 U/Kg BTX-A group; One-way ANOVA; n=3 each group

Figure 5

The BTX-A injected to the plantar area relieved the mechanical hyperalgesia of the Ipsilateral and the mechanical thresholds on the Contralateral side had no significant between-group difference. b $P < 0.05$ VS CCI+NS group, Two-way ANOVA, $n=6$ each group

Spinal cord

Figure 6

Western blot analysis showing changes in CXCL13, CXCR5 and GAT-1 levels of spinal cord at 10, 14 and 21d after surgery. Treatment with BTX-A prevented the increase in CXCL13, CXCR5 and GAT-1 proteins as graphed in (a, b, c, d). a $P < 0.05$ VS CCI+NS group, One-way ANOVA, $n=3$ each group

Figure 7

(a-c) Western blot data showing that the increased expression of CXCL13, CXCR5 and GAT-1 proteins after CCI surgery in the DRG, sciatic nerve and plantar skin was reversed by BTX-A. a $P < 0.05$ VS CCI+NS group, One-way ANOVA, $n = 3$ each group

Figure 8

The 10mmol/L colchicine (COL) significantly attenuated mechanical hypersensitivity after CCI surgery. The dose of BTX-A we used was 10U/Kg. b $P < 0.05$ VS CCI + NS + NS group, Two-way ANOVA, $n=6$ each group. c $P < 0.05$ VS CCI + NS + 10U/Kg BTX-A group, Two-way ANOVA, $n=6$ each group

Figure 9

The 10 U/Kg BTX-A injected to the planta had no effect on the expression of CXCL13, CXCR5 and GAT-1 after the sciatic nerve treated with 10 mmol/L colchicine. The colchicine (COL) relieved the over-expression of CXCL13, CXCR5 and GAT-1 after CCI surgery. a $P < 0.05$ VS Sham group, b $P < 0.05$ VS CCI-NS-NS group, c $P < 0.05$ VS CCI-NS-10U/Kg BTX-A group, One-way ANOVA, $n = 3$ each group

Figure 10

The western blotting bands with the loading order of plantar, sciatic nerve, DRG and spinal cord. There was no significant difference in sham group and CCI-NS-NS group. a $P < 0.05$ VS Sole of foot, b $P < 0.05$ VS Sciatic nerve, One-way ANOVA, n=3 each group