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Research article

IL-10 and CXCL2 in trigeminal ganglia in neuropathic pain

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ABSTRACT

Many trigeminal neuropathic pain patients suffer severe chronic pain. The neuropathic pain might be related with cross-excitation of the neighboring neurons and satellite glial cells (SGCs) in the sensory ganglia and increasing the pain signals from the peripheral tissue to the central nervous system. We induced trigeminal neuropathic pain by infraorbital nerve constriction injury (IONC) in Sprague-Dawley rats. We tested cytokine (CXCL2 and IL-10) levels in trigeminal ganglia (TGs) after trigeminal neuropathic pain induction, and the effect of direct injection of the anti-CXCL2 and recombinant IL-10 into TG. We found that IONC induced pain behavior. Additionally, IONC induced satellite glial cell activation in TG and cytokine levels of TGs were changed after IONC. CXCL2 levels increased on day 1 of neuropathic pain induction and decreased gradually, with IL-10 levels showing the opposite trend. Recombinant IL-10 or anti-CXCL2 injection into TG decreased pain behavior. Our results show that IL-10 or anti-CXCL2 are therapy options for neuropathic pain.

1. Introduction

Peripheral nerve injury induces neuropathic pain and neuronal hyperexcitation within sensory ganglia [1]. Although it has been reported that there are no synaptic contacts in sensory ganglia [2,3], depolarized sensory neuron somata can induce cross-excitation by activating neighboring neurons in the same ganglion [4], and this appears to be chemically mediated [5]. Some studies have reported that neurotransmitters such as substance P, calcitonin gene-related peptide (CGRP), and adenosine triphosphate are released from the somata of neurons in sensory ganglia [6–10], and go on to excite neurons [11,12]. The release of these neurotransmitters is reported to be increased in inflammatory and neuropathic pain conditions [7,13].

It has also been reported that cytokines are released from trigeminal ganglion (TG) glial-rich cultures [14,15]. The cytokines released may affect neighboring sensory neurons or the other satellite glial cells (SGCs) in the TGs. For instance, SGCs modulate the excitation of TG neurons through interleukin-1β (IL-1β) [16] and CGRP enhances communication between purinergic neurons and glial cells [17]. In some pain models, such as neuropathic pain and migraine, SGC activity increases cytokine release [18,19]. These reports showed that neuropathic pain might induce the cross-excitation of neighboring neurons and SGCs in the sensory ganglia, and increase pain signals from the peripheral tissue to the central nervous system.

One cytokine, chemokine (C-X-C motif) ligand 2 (CXCL2) also known as macrophage inflammatory protein 2-α belongs to the CXC chemokine family, along with growth-regulated protein β and Gro oncogene-2. CXCL2 is 90% identical in amino acid sequence to the related chemokine, CXCL1. This chemokine is reported to be secreted by monocytes and macrophages, and is chemotactic for polymorphonuclear leukocytes and hematopoietic stem cells [20–22]. It has been reported that CXCL2 and its receptor are up-regulated in neutrophils and macrophages that accumulate in injured sciatic nerves, and that this might elicit chronic neuroinflammation through neutrophil accumulation, leading to neuropathic pain [23].

IL-10 is a cytokine produced from type 2 helper T cells, activated B cells, monocytes, mast cells and keratinocytes. Its bioactivity varies widely, but it has a distinctly different feature from other cytokines of inhibitory activity. IL-10 mainly acts on monocyte line cells to suppress immune function, including the production of inflammatory cytokines

Abbreviations: GFAP, glial fibrillary acidic protein; CGRP, calcitonin gene-related peptide; CXCL2, chemokine (C-X-C motif) ligand 2; DRG, dorsal root ganglion; IONC, infraorbital nerve constriction; IL-1β, interleukin-1β; IL-10, Interleukin-10; SGCs, satellite glial cells; TG, trigeminal ganglion

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in an inhibitory manner, and against lymphocytes, indirectly through monocyte lineage cells [24,25]. It has been reported that dorsal root ganglion (DRG) IL-10 levels were significantly reduced 3 and 8 days following sciatic nerve constriction and partial sciatic ligation, and significantly increased 3 and 8 days following complete sciatic transection [26].

No studies have reported when CXCL2 and IL-10 levels increase in TG after trigeminal neuropathic pain model induction, or the effect of direct injection of related drugs of CXCL2 and IL-10 into TG on pain reduction. This study investigated the timing of changes in the levels of the cytokine CXCL2 and IL-10 in TG after trigeminal neuropathic pain induction, and the effect of direct injection of CXCL2 and IL-10 into TG.

2. Materials and methods

2.1. Animals

Sprague-Dawley male rats were used in this study (weighing 145–160 g, CLEA Japan, Osaka, Japan). The rats were kept in transparent polycarbonate cages (length: 38 cm, width: 33 cm, height: 17.5 cm) with paper bedding. The animal room temperature was maintained at 19–21 °C with a 12 h light/dark cycle (lights on at 06:00 and off at 18:00), and animals were fed ad libitum with a regular diet and allowed a free access to water. All experimental procedures were performed in accordance with the guidelines of National Institutes of Health Guide for the Care and Use of Laboratory Animals and International Association for the Study of Pain [27]. The animal protocols were approved by Tokushima University (T27-78, T29-57).

2.2. Infraorbital nerve constriction

After behavioral test training and baseline measuring, the rats underwent either infraorbital nerve constriction (IONC) or sham surgery of the infraorbital branch of the trigeminal nerve (maxillary nerve). The IONC surgery was performed intraorally as prescribed in previous studies [28,29]. The intraoral incision did not interfere with the tactile behavior testing because the incision was not in the testing field. Briefly, the rats were deeply anesthetized with medetomidine (Nippon Zenyaku Kogyo Co., Ltd, Fukushima, Japan) 0.375 mg/kg, midazolam (Sandoz K.K., Yamagata, Japan) 2 mg/kg and butorphanol (Meiji Seika Pharma Co., Ltd., Tokyo, Japan) 2.5 mg/kg. An incision was made in the hard palate and extended approximately 1 cm anteriorly toward the maxillary incisors, parallel to the lip. The nerve was exposed and two ligatures (4–0 silk suture) were tied loosely around the nerve. In the sham surgery, the nerve was exposed but not ligated. The wounds were adhered with mucosal adhesive (GLUture, World Precision Instruments, FL, USA).

2.3. Measuring mechanical sensitivity of the whisker pad area

The rats were lightly anesthetized with isoflurane (2.5% inhalation), and their hair was shaved from the bilateral orofacial region using clippers, followed by depilatory cream application, 1 day before the behavioral test [30,31]. Excess cream was washed off with water and removed with a moistened paper towel to minimize skin irritation. The rats had their snouts protruding from a holder (Durham Holders, 37100, Ugo Basile, Varese, Italy) that had a semicircular small hole (diameter: 7 cm, height: 2 cm). They could withdraw their snouts freely following application of mechanical stimuli applied to the whisker pad skin using an electronic von Frey hair pressure transducer (Model 1601C, IITC Instruments, Woodland Hills, CA, USA). Force was applied within the infraorbital nerve territory of the snout at the center of the whisker pad (i.e. between rows B and C of the vibrissae) until the animal withdrew its head. Stimuli were alternated between the ipsilateral and contralateral sides, with 1 min recovery between stimulations. The force (g) applied at the time of withdrawal was recorded. Five data points were recorded for each side, and the three median value after eliminating the highest and lowest value were averaged. Baseline behavioral testing was performed 1–2 days before the IONC surgery and the testing was repeated after IONC surgery.

2.4. Immunohistochemistry

The rats were deeply anesthetized with medetomidine 0.375 mg/kg, midazolam 2 mg/kg and butorphanol 2.5 mg/kg and the TGs were excised. The tissues were embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek USA Inc., Torrance, CA, USA) and stored at −80 °C until cryosectioning. Then, the ganglion tissues were sliced into 6–10 μm-thick sections in the horizontal plane along the long axis. The sections were thaw-mounted on a slide glass (Matsunami, Osaka, Japan) and dried for 2 h at 40–50 °C. The samples were then fixed in 4% paraformaldehyde for 30 min at room temperature. After rinsing with wash phosphate-buffered saline (PBS) and 1% polyxyethylene solution monolaurate (Tween20) (Wako Pure Chemical Industries. Ltd, Osaka, Japan) two times for 5 min each, and then PBS in Triton® X-100 (Nacalai, Kyoto, Japan) on ice for 10 min. The sections were blocked with 5% goat serum on ice for 30 min and then incubated in rabbit glial fibrillary acidic protein (GFAP) polyclonal antibody (1:500; Bioss antibodies, Boston, MA, USA) and/or Alexa Fluor 488 anti-rabbit IgG (1:200; Thermo Fisher Scientific, Waltham, MA, USA) in 0.01 M PBS for 1 h at room temperature. After rinsing with 0.01 M PBS, the sections were treated by a 100 diluted 4′, 6-Diamidino-2-phenylindole dihydrochloride (DAPI, 1:100, Nacalai Tesque, Inc, Kyoto, Japan) for 15 min. The sections were observed using a BZ-9000 system (Keyence, Osaka, Japan).

2.5. Analysis of cytokine levels in TGs

Cytokine levels in TGs were measured on days 1, 7 and 14 after the IONC surgery. Lytic buffer was prepared using 80 μl distilled water, 10 μl RIPA buffer and 10 μl sodium dodecyl sulphate. Excised TGs were washed in PBS, cut into small pieces, and homogenized in the lytic buffer on ice. Lysed tissues in the buffer were incubated for 30 min to 1 h on ice, and centrifuged at 10,000 × g for 10 min at 4 °C to remove cellular debris. The supernatant was collected and processed for cytokine measurement using Proteome Profiler Rat Cytokine Array Panel A (R&D systems, Minneapolis, USA) following the manufacturer's instructions. Briefly, tissue lysates were diluted and mixed with a cocktail of biotinylated antibodies. The samples/antibody mixture were incubated on a membrane with immobilized capture antibody (Rat Cytokine Array Panel A [R&D systems]). Streptavidin-horseradish peroxidase and chemiluminescent detection reagents were used to generate a signal at each spot corresponding to the amount of cytokine bound. The densitometric analysis of spots was performed using Chemi Doc systems (BioRad, Osaka, Japan). Background staining and spot sizes were analyzed and data from neuropathic pain TG tissue expressed as fold changes over control TG tissue. Simultaneous detection of 29 different rat cytokines and chemokines was achieved using the array kit.

2.6. Intra-TG administration of recombinant IL-10 and anti-CXCL2 to IONC model

Intra-TG drug administration was performed as described by Nuebert et al. [32], under deep anesthesia with i.p. medetomidine 0.375 mg/kg, midazolam 2 mg/kg and butorphanol 2.5 mg/kg. The facial hair was shaved with clippers. IONC surgery was performed with the same methods as previously written [28,29]. The injection needle was positioned at 10° lateral to the midline of the head. The tip of the needle was advanced approximately 22 mm along the infraorbital canal and subsequently through the foramen rotundum. Recombinant IL-10 (0.4 μg/100 g) in PBS, anti-CXCL2 (66 μg/100 g) in PBS or only PBS

(control) was injected into IONC side TG over 1 min (total volume was 18 μl), and the needle was slowly removed. The doses were decided in the previous study [33,34]. The drugs were injected on days 1, 2 and 3 after IONC. The mechanical sensitivity of the whisker pad area was measured before the surgery, after the IONC surgery, and after the injection of the drugs.

2.7. Statistical analysis

Data are expressed as means ± standard errors. Statistical analyses were performed using two-way analysis of variance (ANOVA) with repeated measures followed by Bonferroni's multiple-comparison test with software EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan). EZR is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria), and more precisely, is a modified version of R commander designed to add statistical functions frequently used biostatistics. A value of $p < 0.05$ was defined as statistically significant.

3. Results

3.1. Pain behavior after IONC

Before IONC, baseline mechanical stimulation behavioral testing was performed. Following IONC, the animals showed decreased withdrawal thresholds in the ipsilateral whisker pad area (Fig. 1, Table 1). The baseline withdrawal threshold from the mechanical stimulation was around 110 g before IONC surgery and the threshold of the ipsilateral side significantly dropped to around 50 g after IONC surgery. There were statistically significant differences between IONC and sham surgery sides on days 7 and 14 after surgery. Spontaneous pain behavior signs (scratching the face with the forepaw) were also observed. IONC sham surgery (exposure of the intraorbital nerve without constriction) did not result in a lowering of the withdrawal threshold (Fig. 1, Table 1).

3.2. IONC induces glial cell activation

The number of GFAP positive SGC numbers detected by immune histological assay were increased in the TG of IONC animals in comparison with sham surgery animals on days 1, 7 and 14 after surgery (Fig. 2). In IONC animals, GFAP signals were higher in the ipsilateral TG V2 area on days 1, 7 and 14 after surgery (Fig. 2). Whole ganglion picture showed that glial cells in V2 and V1 area were excited. In sham surgery animals, GFAP immunoreactivity showed no difference compared with baseline.

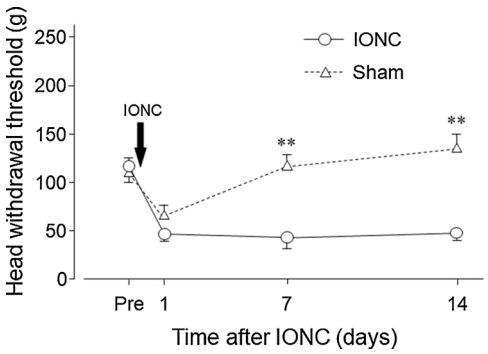

Fig. 1. Pain model with infraorbital branch of the trigeminal nerve chronic constriction injury. Head withdrawal thresholds (mean ± SEM) to mechanical stimuli on the ipsilateral side are significantly decreased compared with the contralateral sham surgery on days 7 and 14 after IONC surgery. **: $p < 0.01$ by two-way ANOVA with repeated measures followed by Bonferroni's multiple-comparison test. There are n = 5 in each group.

Table 1
Head withdrawal threshold.

Treatment	Time (days)			
	Pre	1	7	14
IONC	116.9 (12.7)	47.2 (3.4)	43.4 (7.5)**	48.2 (4.4)**
Sham surgery	110.9 (10.7)	65.3 (7.2)	116.7 (10.8)	134.8 (16.1)

Head withdrawal thresholds (g) (mean (SEM)) to mechanical stimuli.
There are n = 5 in each group.
**$p < 0.01$ in IONC vs sham surgeries.

3.3. IONC induces differential regulation of cytokines in TG

We analyzed the levels of 29 cytokines. IONC increased the 15 cytokines by at least 1.5-fold on day 1 after the surgery and 12 cytokines on day 14 after the surgery (Table 2). There were several types of expression patterns. Some cytokines (CXCL2, TNF-α, IL-1ra, IL-17, Il-3, CINC-2α/β and IL-4) were increased on day 1 after IONC and decreased by day 14 after the surgery. Another pattern type was increased levels only on day 14 after the surgery (IL-10 and MIP-3α) (Table 2, Fig. 3). Other cytokines showed high levels on days 1 and 14 after the surgery, or no change on days 1 and 14 after the surgery.

3.4. Behavioral change after intra-TG administration of recombinant IL-10 and anti-CXCL2 to IONC rats

Injection of recombinant IL-10 or anti-CXCL2 increased the threshold of tactile stimulation although the PBS injection did not increase the threshold (Fig. 4, Table 3). Pain was decreased by directly administering these drugs to TG. After injecting recombinant IL-10, the threshold was recovered and there was no statistical difference between IL-10 injected side and sham surgery side on days 2, 4 and 7 after IONC induction. After anti-CXCL2 injection, the threshold was recovered and there was no statistical difference between anti-CXCL2 injected side and sham surgery side on days 2 and 7 after IONC induction. There was statistical difference between only PBS injection and recombinant IL-10 injection (days 2, 4 and 7) or anti-CXCL2 injection (day 7).

4. Discussion

The main findings of this study were: (1) the induction of a trigeminal neuropathic pain model (IONC) reduced pain threshold to mechanical stimulation in orofacial region; (2) IONC induced SGC activation in TG; (3) cytokine levels of TGs were changed after IONC; (4) differential regulation of cytokines in TG with an increase in pro-inflammatory chemokine CXCL2 levels during the initial phase of neuropathic pain induction followed by a gradual decrease, whereas an increase in level of anti-inflammatory cytokine IL-10 towards the day 14 after IONC; and (5) in a therapeutic setting, recombinant IL-10 or anti-CXCL2 injection into TG decreased pain behavior in IONC model for several days.

It has been reported that peripheral nerve injury increases the excitation of primary sensory neurons and that this excitation is related to animal pain behavior [35]. It has also been reported that neurotransmitter release is increased within the sensory ganglia after neuronal hyperexcitation [6–10]. This neurotransmitter release is decreased by the application of botulinum toxin directly into the sensory ganglia accompanied with an alleviation in pain behavior [36].

Glial cell activation (assessed by GFAP staining) in TGs was observed after the induction of a neuropathic pain model in this study and this result is similar to the findings of other groups [37–41]. Activated SGCs increase mRNA expression of cytokines [18,19,42] and cytokine release, and this is related to the chronic pain state [18,19]. Also, it has been reported that stimulated SGCs release cytokines in vitro [15,17,18,43,44]. Temporomandibular joint inflammation also

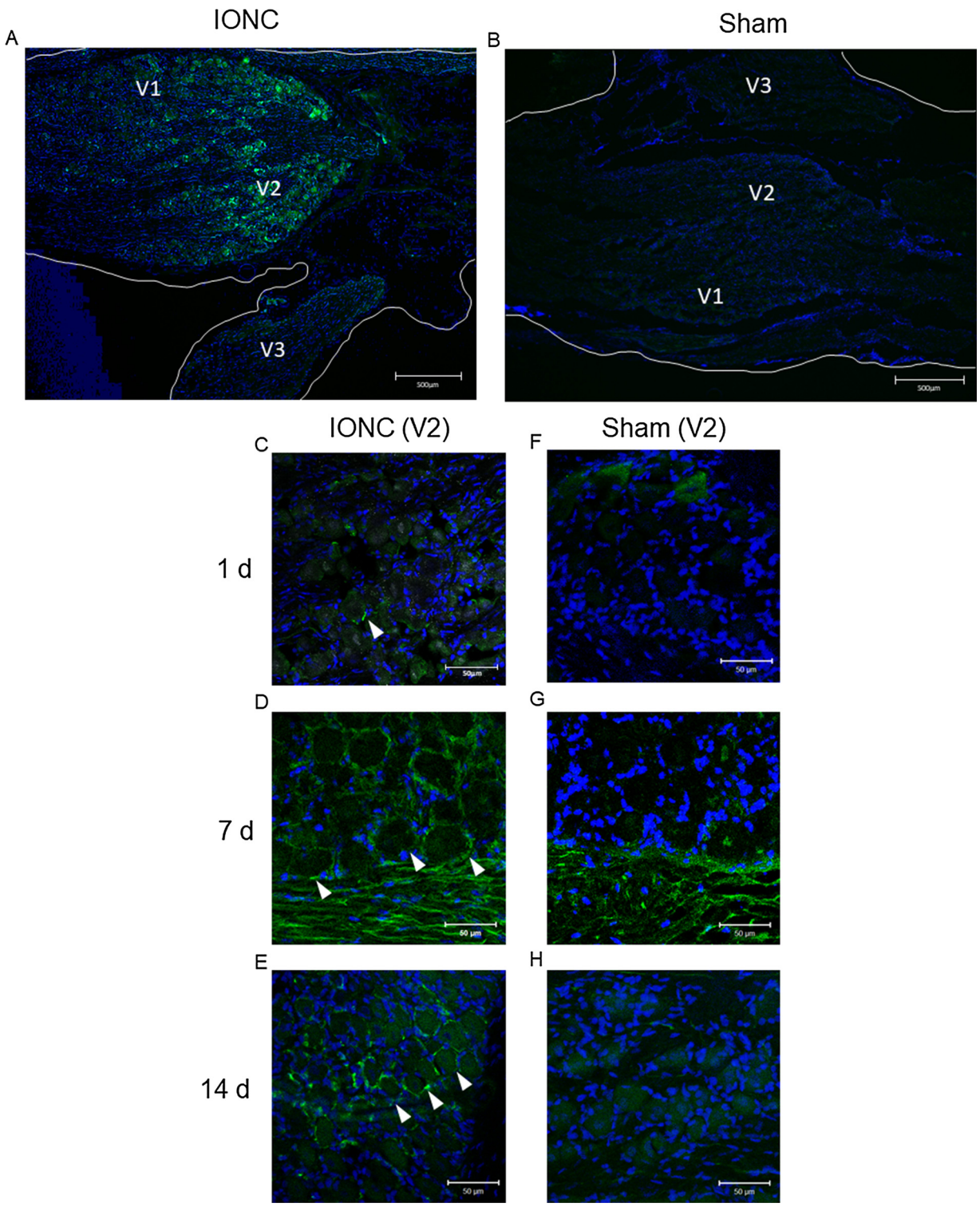

Fig. 2. GFAP staining of satellite glial cells in trigeminal ganglia. Numbers of GFAP positive satellite glial cells were increased in the ipsilateral TG of IONC animals on days 1, 7 and 14 after the surgery compared with in sham surgery animals. Green shows GFAP and blue shows DAPI. A, B: Low magnification view of TG on day 14. V1-V3 shows the branch of the trigeminal nerve. The scale bar shows 500 μm. C-H: Higher magnification view in ipsilateral TG V2 area (C-E) and sham surgery TG V2 area (F-H). The arrow heads show the samples of the neurons surrounding GFAP positive SGC. The scale bar shows 50 μm.

increases cytokines in TG [45]. In our study, TG cytokine levels were altered after induction of a trigeminal neuropathic pain model. We should consider the other immune cells increased the release of cytokines and this is the future study topic. Evidence from our study and previous studies, indicates that the cytokine release or expression in TG has different effects on nociception due to peripheral and central sensitization. It is also reported that a complex network of pro- and anti-inflammatory molecules plays a role in the communication between neurons and SGCs within the TG, and is crucially involved in the development of TG sensitization [46].

It has been shown that receptors of IL-10 and CXCL2 are expressed in rat DRG neurons, and are associated with the chronic pain state

Table 2
Cytokine array in trigeminal ganglia after infraorbital branch of the trigeminal nerve chronic constriction injury.

Rat cytokine antibody array	Day 1	Day 7 Fold change	Day 14
CXCL2 (CINC-3)	2.44	0.53	0.27
TNF-α	2.25	2.06	0.02
IL-1ra	1.97	0.69	1.21
IL-17	1.94	0.68	0.87
IL-3	1.93	0.56	0.94
CINC-2α/β	1.90	0.81	0.21
LIX	1.85	0.70	1.78
IL-2	1.82	0.69	2.17
MIG/CXCL9	1.73	0.43	2.39
GM-CSF	1.73	0.91	2.39
FRACTALKALINE	1.72	0.54	1.71
IP-10/CXCL10	1.64	0.79	0.66
IL-4	1.54	0.66	0.91
IL-6	1.53	0.54	2.41
TIMP-1	1.52	1.58	1.03
VEGF/VEGF-A/Vasculotropin	1.48	0.81	1.47
IL-1α	1.45	0.88	1.63
RANTES/CCL5	1.45	0.87	1.28
CNTF	1.38	0.99	1.45
MIP-1α/CCL-3	1.36	0.52	2.41
IL-1β	1.32	0.49	1.63
L-SELECTIN/CD62 L/LECAM-1	1.30	0.76	1.74
sICAM-1	1.27	1.07	1.06
IL-13	1.25	0.62	1.24
CINC-1	1.10	1.07	0.69
MIP-3α/CCL20	1.09	0.73	1.81
THYMUS CHEMOKINE/CXCL7	1.08	1.04	1.22
IFN-ϒ	0.57	1.02	0.79
IL-10	0.14	0.70	2.47

The data are represented IONC / Sham ganglia. CXCL2 (CINC-3), TNF-α, IL-1ra, Il-17, IL-3, CINC-2α/β and IL-4 were increased on day 1 after IONC and decreased on day 14 after the surgery. The other type was increased on day 14 after the surgery (IL-10 and, MIP-3α). Other cytokines showed high level on days 1 and 14 after the surgery, or non-increased level on days 1 and 14 after the surgery.

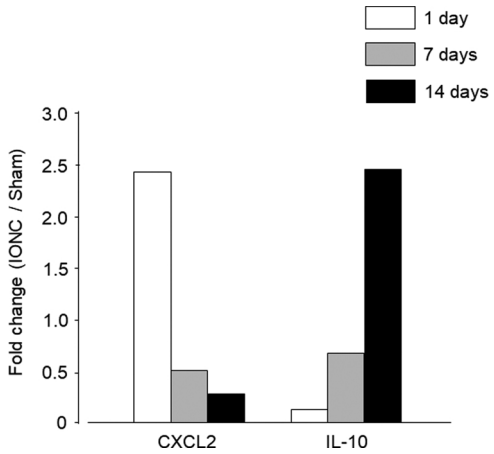

Fig. 3. The data are represented IONC / sham ganglia. CXCL2 was increased on day 1 after IONC and decreased on day 14 after the surgery. IL-10 was increased on day 14 after the surgery. There is n = 1 in each group.

[47,48]. Additionally, SGC activation has been reported to modulate the excitability of small-diameter TG neurons through IL1-β [16]. In our study, TG cytokine levels were changed after IONC. The pro-inflammatory chemokine CXCL2 was increased just after the nerve injury and decreased gradually. Conversely, the anti-inflammatory cytokine IL-10 increased gradually. Based on these findings, in a therapeutic setting the intraganglionar administration of IL-10 and anti-CXCL2 resulted in alleviation of pain symptoms in an IONC induced orofacial

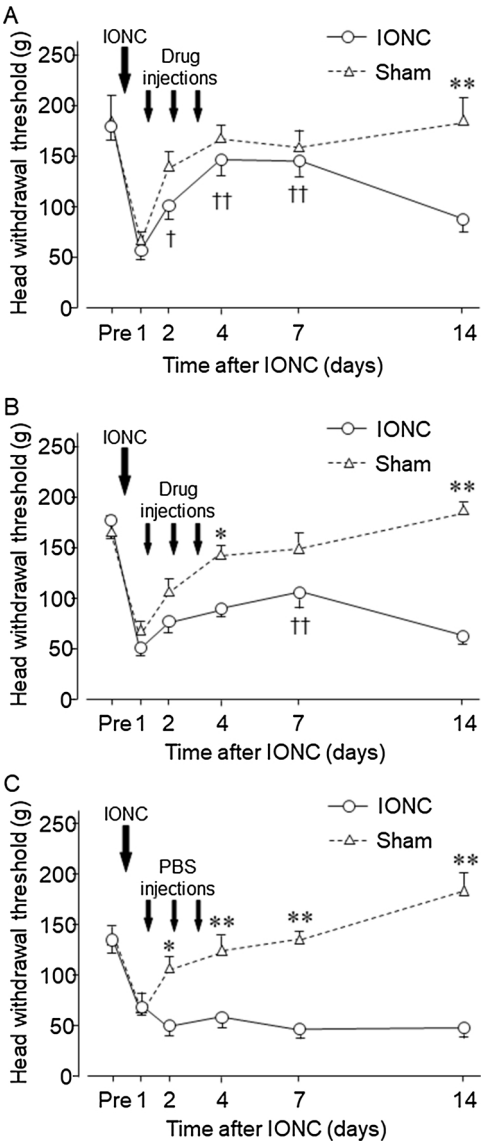

Fig. 4. Behavioral changes after intra-TG administration of recombinant IL-10, anti-CXCL2 or PBS to IONC rats. A: Injection of recombinant IL-10 increased the threshold of tactile stimulation. There was no statistical difference between IL-10 injected side and sham surgery side on days 2, 4 and 7 after the induction of IONC. There was statistical difference between only PBS injection and recombinant IL-10 injection on days 2, 4 and 7 after IONC induction. B: Injection of anti-CXCL2 increased the threshold of tactile stimulation slightly. There was no statistical difference between ant-CXCL2 injected side and sham surgery side on days 2 and 7 after the IONC induction. There was statistical difference between only PBS injection and anti-CXCL2 injection on day 7 after IONC induction. C: Injection of only PBS did not increase the threshold of tactile stimulation. There was statistical difference between PBS injected side and sham surgery side on days 2, 4, 7 and 14 after the IONC. The statistical analysis was done with two-way ANOVA with repeated measures followed by Bonferroni's multiple-comparison test. The data are shown with means ± SEM. * ; $p < 0.05$, ** ; $p < 0.01$ in drug injection side vs sham surgery side. † ; $p < 0.05$, †† ; $p < 0.01$ in drug injection vs PBS injection. There were n = 6 in each group.

pain model. Cytokines are considered as novel neuromodulators and using them therapeutically can be considered as novel therapy. The present study did not probe into the source of these cytokines at different time point from different cells including non-neuronal cells and immune cells. In addition, the involvement of various receptors including CXCL2 and IL-10 and change in there expression pattern

Table 3
Head withdrawal threshold.

Treatment	Time (days)					
	Pre	1	2	4	7	14
rIL-10	180.7 (14.2)	55.0 (7.0)	105.5 (17.0)†	148.8 (18.6)††	144.7 (15.3)††	89.7 (12.9)**
Sham surgery	184.1 (26.8)	66.3 (5.5)	141.4 (14.3)	166.6 (14.5)	158.9 (16.6)	186.9 (22.5)
Anti-CXCL2	177.6 (17.4)	52.2 (4.5)	77.5 (11.0)	89.7 (8.0)*	105.7 (14.9)††	61.8 (6.1)**
Sham surgery	165.0 (8.0)	67.2 (10.6)	107.9 (1.6)	142.8 (11.9)	149.1 (10.1)	184.4 (11.2)
PBS	133.7 (12.5)	69.5 (5.6)	50.8 (9.4)*	58.6 (11.1)**	43.3 (4.9)**	47.5 (7.1)**
Sham surgery	134.7 (13.5)	70.3 (13.6)	105.1 (13.3)	123.2 (17.5)	133.7 (9.7)	179.3 (21.3)

Head withdrawal thresholds (g) (mean (SEM)) to mechanical stimuli.
There were n = 6 in each group.
$^{*}p < 0.05$, $^{**}p < 0.01$ in drug injection side vs sham surgery side.
$^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ in drug injection vs PBS injection.

following IONC and intraganglionar injection needs to be ascertained in future.

5. Conclusion

We found that IONC induced animal pain behavior, induced SGC activation in TG, changed TG cytokine levels, and increased CXCL2 levels on day 1, after which they decreased gradually. IL-10 levels showed the opposite trend and recombinant IL-10 or anti-CXCL2 injection into TG decreased pain behavior. Our results show that IL-10 and anti-CXCL2 are therapy options for neuropathic pain.

Declaration of interest

None.

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