Pain-related mediators underlie incision-induced mechanical nociception in the dorsal root ganglia**

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Graphical Abstract

Abstract
Approximately 50–70% of patients experience incision-induced mechanical nociception after surgery. However, the mechanism underlying incision-induced mechanical nociception is still unclear. Interleukin-10 and brain-derived neurotrophic factor are important pain mediators, but whether interleukin-10 and brain-derived neurotrophic factor are involved in incision-induced mechanical nociception remains uncertain. In this study, forty rats were divided randomly into the incision surgery (n = 32) and sham surgery (n = 8) groups. Plantar incision on the central part of left hind paw was performed under anesthesia in rats from the surgery group. Rats in the sham surgery group received anesthesia, but not an incision. Von Frey test results showed that, compared with the sham surgery group, incision surgery decreased the withdrawal threshold of rats at 0.5, 3, 6 and 24 hours after incision. Immunofluorescence staining in the dorsal root ganglia of spinal cord (L3–5) showed that interleukin-10 and brain-derived neurotrophic factor were expressed mainly on small- and medium-sized neurons (diameter < 20 μm and 20–40 μm) and satellite cells in the dorsal root ganglia of the spinal cord (L3–5) in the sham surgery group. By contrast, in the surgery group, high expression of interleukin-10 and brain-derived neurotrophic factor appeared in large-sized neurons (diameter > 40 μm) at 6 and 24 hours after incision surgery, which corresponded to the decreased mechanical withdrawal threshold of rats in the surgery group. Our findings indicate that pain-related mediators induced by incision surgery in dorsal root ganglia of rats possibly underlie mechanical nociception in ipsilateral hind paws.
INTRODUCTION

Postoperative incision pain is a unique form of acute pain, which is obviously different from antigen-induced inflammatory pain, neuropathic pain and cancer-induced pain\[1-9\]. Postoperative incision pain includes evoked and non-evoked pain. Non-evoked pain is short lasting, moderate and ongoing pain at rest in patients. Evoked pain is severe, long lasting, and movement-related pain, which is usually induced by coughing, mobilization and ambulation\[1\]. Previous studies showed that incision surgery induced obvious changes at many parts in the nervous system\[10-13\]. Some scholars found that response threshold of primary afferent fibers to mechanical and thermal stimulus decreased after incision of the hind paw\[1, 14-19\]. By contrast, the response magnitude to suprathreshold stimuli, and spontaneous activity receptive field size increased\[1, 14-19\]. In the spinal cord, hind paw surgical incision dramatically up-regulated the expression of brain-derived neurotrophic factor (BDNF)\[20\] and extracellular signal-regulated kinase 1/2\[21\], and induced activation of glial cells\[22-23\]. Recently, Pogatzki-Zahn et al\[24\] detected changes in human brains after incision of the right forearm using functional MRI analysis. They found that incision increased activity of the somatosensory cortex, frontal cortex and limbic system. This evidence suggests that incision pain is a symptom involved in highly complex neural circuits. However, the mechanism underlying incision pain is still unclear\[25-27\]. In addition, analgesics including anti-inflammatory agents\[28-30\], N-methyl-D-aspartate (NMDA) receptor antagonists\[31-33\], spinal non-NMDA receptor antagonists\[31, 34\] and ionotropic purine receptor antagonists\[35\] exhibited poor treatment efficacy for incision-induced pain\[36-37\]. Even after ample pain treatment with these drugs and improved analgesic techniques, about 50–70% of patients experienced moderate to severe postoperative incision pain\[36-37\]. Thus, further investigation into postoperative incision pain is necessary.

Pain-related mediators play important roles in the pain process\[11, 38-40\]. These mediators include cytokines and chemokines [e.g., CXCCL5, interleukin-10 (IL-10)]\[41-43\], growth factors [e.g., BDNF, nerve growth factor]\[44-46\], purines/ATP [e.g., ATP]\[47\], protons (e.g., H+)\[48\], neuropeptides and peptide hormones (e.g., neurokinin-1, substance P\[49-50\], metalloproteinases (e.g., MMP9)\[51\], and lipid mediators (e.g., prostanoids)\[52-53\]. Previous studies showed that BDNF was closely involved in all types of pain, including incision pain, inflammatory pain, and neuropathic pain\[54-56\]. For example, Li et al\[22\] found that hind paw incision increased BDNF expression in the ipsilateral lumbar dorsal root ganglia and spinal cord. And intrathecal injection of BDNF antibody significantly inhibited the mechanical allodynia induced by incision. In visceral pain induced by intraperitoneal injection of acetic acid into Sprague-Dawley rats, pretreatment with anti-BDNF antibody obviously exacerbated the nocifensive response in males, but attenuated it in females\[40\].

IL-10 is also an important modulator of neuropathic and inflammatory pain\[58, 57-58\]. Jancálek et al\[57-58\] reported that unilateral chronic constriction injury of sciatic nerve increased IL-10 expression in large, medium and small neuronal bodies in lumbar and cervical dorsal root ganglia. Moreover, Ledeboer et al\[59-60\] found that intrathecal administration of IL-10 protein could attenuate neuropathic pain. Repeated intrathecal administration of plasmid DNA encoding IL-10 reversed mechanical allodynia after chronic constriction injury for more than 40 days\[61\]. These findings showed that IL-10 played very important roles in neuropathic pain and inflammatory pain. However, it is still unclear whether IL-10 is also involved in incision pain, especially mechanical allodynia. Plantar incision is a widely-used model of postoperative incision pain\[22, 62\]. To investigate effects of pain-related mediators in incision pain, we chose IL-10 and BDNF as representative pain mediators, and detected their expression changes in dorsal root ganglia of rats after plantar incision.

The goal of this study was to verify the following questions: (1) does incision surgery induce changes in pain-related mediators such as IL-10 and BDNF in the dorsal root ganglia, and (2) are the changes in pain mediators related to incision-induced mechanical nociception?

RESULTS

Quantitative analysis of experimental animals

Forty rats were divided randomly into the incision surgery
(n = 32) and sham surgery groups (n = 8). Rats in the incision surgery group were treated with isoflurane anesthesia and incision surgery on the plantar surface of the left hind paw was performed, while rats in the sham surgery group received only isoflurane anesthesia.

Rats in both groups underwent nociceptive testing at 0, 0.5, 1, 3, 6, 24 and 72 hours after surgery (n = 8 for von Frey test at each time point). All rats were killed immediately after von Frey test (n = 4 for immunofluorescence double staining at each time point). All 40 rats were used in the final analysis.

Plantar incision surgery decreased the withdrawal threshold of ipsilateral hind paws in rats
Withdrawal threshold to mechanical stimulus is commonly used to show the tolerance to this stimulus in rats[22]. Higher withdrawal threshold suggests higher tolerance to stimulus. In the study, we detected changes in withdrawal threshold in left hind paws to von Frey fiber stimulation in the sham surgery group and incision surgery group at 0, 0.5, 3, 6, 24, and 72 hours after incision or sham surgery. The von Frey test results showed that there was no significant difference in withdrawal threshold between the sham surgery group and incision surgery group immediately after plantar incision surgery of the hind paw and at 72 hours (Figure 1; P > 0.05).

However, the withdrawal threshold of the hind paw with incision surgery was significantly decreased at 0.5, 3, 6 and 24 hours after incision, compared with the sham surgery group (P < 0.05). These findings suggested that incision surgery decreased the tolerance to mechanical stimulus in postoperative hind paws of rats at these time points.

Plantar incision surgery induced expression shift of IL-10 in the ipsilateral dorsal root ganglia of rats
Dorsal root ganglion neurons can be divided into small (diameter < 20 μm), medium (diameter at 20–40 μm), and large (diameter > 40 μm)-sized cells according to their diameter. Immunofluorescence staining revealed that IL-10 was mainly detected in the small- and medium-sized neurons and satellite cells of dorsal root ganglia in the sham surgery group (Figure 2A). By contrast, IL-10 expression in the small- and medium-sized neurons of dorsal root ganglia was significantly up-regulated at 6 and 24 hours after incision surgery (Figure 2B, C). At 72 hours after incision surgery, IL-10 expression decreased to the level similar to that seen in the sham surgery group (Figure 2D).

Further quantitative detection of IL-10 expression showed that expression in large- and medium-sized neurons in the sham surgery group was significantly higher than that of the sham surgery group at 6 hours after incision (Figure 2E; P < 0.01, P < 0.05). IL-10 expression in the small-sized neurons did not show significant differences in comparison with the sham surgery group (Figure 2E). This evidence reveals that an expression profile shift of IL-10 from small- and medium-sized neurons to large-sized neurons in the dorsal root ganglia after incision surgery.

Figure 1 Effect of hind paw plantar incision surgery on the withdrawal threshold of ipsilateral hind paws in rats.
The withdrawal threshold of hind paw to mechanical stimulus was detected using nylon von Frey filaments.
*P < 0.05, vs. control. Quantitative data are expressed as mean ± SD (n = 8). Two-way analysis of variance followed by Bonferroni testing was used. Control: Sham surgery group; Incision: incision surgery group.

Plantar incision surgery induced expression shift of BDNF in the ipsilateral dorsal root ganglia of rats
BDNF was mainly detected in small- and medium-sized neurons and satellite cells of dorsal root ganglia in the sham surgery group (Figure 3A). At 6 and 24 hours after incision surgery, BDNF expression was significantly up-regulated in the small- and medium-sized neurons and satellite cells of dorsal root ganglia at 6 and 24 hours after incision surgery (Figure 3B, C). At the same time, strong expression of BDNF appeared in large-sized neurons (Figure 3B, C). At 72 hours after incision surgery, BDNF expression was decreased, which was similar to the sham surgery group (Figure 3D). Quantitative detection of BDNF expression showed that expression in the large- and medium-sized neurons in the incision surgery group at 6 hours after incision was significantly higher than that of the sham surgery group (Figure 2E; P < 0.01). These findings reveal an expression shift of BDNF in the dorsal root ganglia after incision surgery.
Figure 2  Plantar incision surgery of the hind paw induced an expression shift of interleukin-10 (IL-10) in rat ipsilateral dorsal root ganglia (DRG).

The expression shift of IL-10 was from small- and medium-sized neurons to large-sized neurons in the DRG after incision surgery.

(A–D) Immunofluorescence double staining of IL-10 (red) and glial fibrillary acidic protein (GFAP; green) at the DRG at 0, 6, 24, 72 hours after incision surgery. Scale bar: 40 µm (× 400).

(E) Higher magnification of double staining of IL-10 (red) and glial fibrillary acidic protein (GFAP; green) at the dorsal root ganglia at 6 hours after incision surgery (scale bar: 60 µm, × 600). (F) Average fluorescence intensity of IL-10 at 6 hours after incision surgery.

White arrows represent large-sized neurons (diameter > 40 µm). Blue arrows represent medium-sized neurons (diameter at 20–40 µm). Double blue arrow represents small-sized neurons (diameter < 20 µm).

Quantitative data are expressed as mean ± SD (n = 4). aP < 0.01, bP < 0.05, vs. control.

One-way analysis of variance followed by post hoc Dunnett test was used. Control: Sham surgery group; Incision: incision surgery group.

Figure 3  Plantar incision surgery of hind paw induced expression shift of brain-derived neurotrophic factor (BDNF) in the ipsilateral dorsal root ganglia (DRG) of rats.

(A–D) Immunofluorescence double staining of BDNF (red) and glial fibrillary acidic protein (GFAP; green) at the DRG at 0, 6, 24, and 72 hours after incision surgery (scale bar: 40 µm, × 400). (E) Higher magnification of double staining of BDNF (red) and glial fibrillary acidic protein (GFAP; green) at the DRG at 6 hours after incision surgery (scale bar: 60 µm, × 600).

(F) Average fluorescence intensity of BDNF at 6 hours after incision surgery (n = 8).

White arrows represent large-sized neurons (diameter > 40 µm). Blue arrows represent medium-sized neurons (diameter at 20–40 µm). Double blue arrows represent small-sized neurons (diameter < 20 µm).

*pP < 0.01, vs. control (sham surgery group). Quantitative data are expressed as mean ± SD (n = 4), one-way analysis of variance followed by post hoc Dunnett testing was used. Incision: Incision surgery group.
DISCUSSION

Our aim in this study was to observe changes in pain mediators, such as IL-10 and BDNF, in dorsal root ganglia after incision surgery and the relationship of pain mediators to incision pain. We found that incision surgery induced higher expression of IL-10 and BDNF in large-sized neurons of the dorsal root ganglia, which was obviously different from sham surgery. Increased expression of IL-10 and BDNF in the large-sized neurons of dorsal root ganglia closely corresponded to the lowered threshold to mechanical stimulus of the hind paw following plantar incision surgery.

Incision pain is greatly different from other kinds of pain[28-30]. Current pain-relief treatments including systemic opioids or non-steroidal anti-inflammatory drugs, decrease rest pain induced by incision surgery[25, 62-66]. However, these two groups of drugs had little effect on movement-evoked pain after incision surgery[25]. To determine the underlying mechanism and specificity of incision pain, we firstly detected the behaviors of rats at different time points after plantar incision surgery of the hind paw. We found that the mechanical withdrawal threshold of rats with plantar incision surgery decreased from 0.5 hour to 24 hours after hind paw incision surgery, and recovered to normal levels on day 3 after incision surgery. These observations were consistent with previous reports describing hind paw incision induced lower withdrawal threshold from 0.5 hours to 2 days after incision surgery[25]. This evidence suggested that our behavior test was objective and plantar incision surgery was successful. We then detected IL-10 and BDNF expressions in dorsal root ganglia after incision surgery. Interestingly, we found that IL-10 and BDNF were expressed mainly on small- and medium-sized neurons. However, at 6 and 24 hours after incision surgery, expressions of IL-10 and BDNF were obviously increased on large-sized neurons. These expression patterns corresponded to the decreased mechanical withdrawal threshold of rats with incision surgery. Previous studies showed that large-sized neurons in dorsal root ganglia were connected with A-β fibers, which transmitted the mechanical stimulus[69]. And central fibers of large-sized neurons in dorsal root ganglia projected mainly to the deep layers of spinal cord, which was involved in mechanical allodynia[69].

In addition, Geng et al[63] found that an increase in BDNF expression induced by nerve-ligated injury at the spinal dorsal horn was closely involved in mechanical allodynia. Thus it is reasonable to infer that up-regulated expression of IL-10 and BDNF in large-sized neurons of dorsal root ganglia is closely involved in movement-evoked pain after incision surgery. IL-10 and BDNF are derived from totally different families of pain-related factors. Based on the changes of expression pattern of IL-10 and BDNF in dorsal root ganglia after incision surgery, it is possible that (1) there is an expression pattern shift of all pain-related factors in dorsal root ganglia after incision surgery and (2) the expression pattern shift of pain-related factors underlies the specificity of incision pain.

It is recognized that surgery causes peripheral sensitization and central sensitization, which is hypothesized to contribute to acute postoperative pain[25]. Peripheral sensitization is characterized by lower response threshold, increased response magnitude to suprathreshold stimuli, higher spontaneous activity, and increased receptive field size[25]. Dorsal root ganglion neurons express many kinds of ion channels and receptors. These channels and receptors play roles in transducing noxious stimuli into electric impulses, propagating action potentials, and modulating synaptic transmission[67].

Previous studies showed that the calcium ion was a critical molecular player in the sensitization process. It could modulate the ion channels by activation of intracellular protein kinases that phosphorylate receptor proteins and voltage-gated ion channels. Interestingly, Geng et al[63] found that BDNF could activate the NR2B-containing NMDA receptors in rats with spinal nerve ligation. NR2B-containing NMDA receptors could modulate intracellular levels of calcium ions[63]. Thus it is possible that in our models, increased BDNF in large-sized neurons of dorsal root ganglia after incision surgery changes the level of calcium ion and makes large-sized neurons sensitized. In addition, recombinant rat IL-10 could down-regulate voltage gated sodium channels of in vitro dorsal root ganglion neurons[68]. Changes of voltage-gated sodium channels were related to abnormal spontaneous activity at dorsal root ganglion neurons[69]. Thus, high expression of IL-10 in large-sized neurons of dorsal root ganglia after incision surgery possibly plays a role in mechanical nociception by modulating sodium channels. However, the mechanism of action of BDNF and IL-10 in incision-induced mechanical nociception needs further investigation. In short, we found a shift of expression pattern of pain-related factors after incision surgery, which could indicate the underlying mechanism of movement-evoked pain after incision surgery.
MATERIALS AND METHODS

Design
A randomized, controlled animal experiment.

Time and setting
Experiments were performed at the Department of Neuroscience, Central South University, China from October 2011 to February 2013.

Materials
Sprague-Dawley male rats, aged 2 months, weighing 200–250 g, were purchased from Experimental Animal Center of Central South University, China [license No. SCXK (Xiang) 2009-0012]. Rats were kept under temperature-controlled environmental conditions and had free access to food and water. All protocols were approved by the Local Animal Ethics Committee, and conformed to the Guidelines for Animal Experiments of the Central South University and Guidance Suggestions for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of China[70].

Methods
Incision surgery
Incision surgery was performed as previously described by Yakima et al.[64]. Briefly, rats were anesthetized with a mixture of oxygen and 2% isoflurane. Under sterile conditions, a 1-cm long longitudinal incision in the plantar surface of the left hind paw was made using a scalpel blade, starting 0.5 cm from the edge of the heel. The plantar muscle was exposed and cut longitudinally with the scalpel blade. Then the skin was sutured closed using an everted mattress pattern, and topical triple antibiotic ointment was applied to the plantar hind paw. Rats in the sham surgery group received anesthesia with the mixture of oxygen and 2% isoflurane only.

Von Frey test
Von Frey test was performed using nylon von Frey filaments, as previously described[65]. The von Frey test was performed by two people who were blind to the experimental treatment. During the test, rats were first placed on wire mesh platforms for 20 minutes to adapt to the test environment. Then, fibers with different stiffness (0.008–300 g) were used to stimulate the plantar surface of the left hind paw. For incised rats, fibers were placed directly on the wound edge. For rats from the sham surgery group, fibers were placed on the center of the plantar surface of the left hind paw between the first set of foot pads and left in place for 5 seconds. Fibers for stimulation were used from low stiffness to a higher stiffness. When stimulation of a fiber did not induce a paw response in a tested rat, the next stiffest fiber in the series was used on the same paw. The same experimental procedure was repeated until the stimulation of a fiber induced a paw response. The mechanical strength of the fiber was recorded when the first withdrawal of the stimulated hind paw appeared. This test was repeated three times. The mean mechanical strength of the four tests was used as the withdrawal threshold of the rat.

Preparation of dorsal root ganglion and immunofluorescence staining
Animals were deeply anesthetized with 2% isoflurane inhalation and then transcardially perfused with 0.9% saline, followed by 4% paraformaldehyde in 0.01 mol/L PBS, pH 7.4. The dorsal root ganglia of lumbar segments (L3–5) were removed, post-fixed for 2 hours in 4% paraformaldehyde, and placed first in 20% sucrose for 4 hours and then 30% sucrose overnight. Sections were cut by constant low temperature micrometre. The thickness of sections was 30 μm. All sections were stored in 0.1 mol/L PBS at 4°C.

For immunofluorescence staining, sections were washed with 0.01 mol/L PBS three times and incubated in blocking solution (5% bovine serum albumin and 0.3% Triton X-100 in PBS) for 1 hour at room temperature. Then sections were incubated with primary antibodies [rabbit anti-BDNF polyclonal antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA); rabbit anti-IL-10 polyclonal antibody (1:200; Santa Cruz Biotechnology); mouse anti-GFAP monoclonal antibody (1:2 000; Chemicon, Philadelphia, PA, USA)] in PBS overnight at 4°C. After three washes with PBS, sections were incubated in corresponding goat anti-rabbit/mouse secondary monoclonal antibodies (1:200; Jackson ImmunoResearch Labotatories, Philadelphia, PA, USA), with fluorescence for 1 hour at room temperature. After washing three times, these sections were dried, and covered with mounting medium with DAPI (Vector, Philadelphia, PA, USA). Adjacent sections underwent the same procedure except without primary antibody incubation to serve as negative controls.

Detection of IL-10 and BDNF expression
Five sections from each animal (four rats for each group) were selected. Images of each section (one visual field/section) were captured under a 200 × magnification. Images were taken by Nikon Microscope (Tokyo, Japan). Profiles of the dorsal root ganglion neurons were divided into small- (< 20 μm), medium- (20–40 μm), and large- (>
40 µm) sized cells. Average fluorescence intensity of the positively labeled neurons and the background were detected with image J (NIH, Maryland, USA). The mean of relative average fluorescence intensity of a neuron was the difference between the average fluorescence intensity of the neuron and the average fluorescence intensity of the background. All measurements were performed by a person blind to the experimental design.

Statistical analysis

All analyses were performed using SPSS 13.0 software for Windows (SPSS, Chicago, IL, USA). Quantitative data are expressed as mean ± SD. Means of relative fluorescence intensity of the positive labeling neurons were analyzed using one-way analysis of variance followed by post hoc Dunnett testing. The data from nociceptive tests were analyzed using two-way analysis of variance followed by Bonferroni testing. P ≤ 0.05 was considered statistically significant.

Research background: The mechanism underlying incision-induced mechanical nociception is still unclear. Even after ample pain treatment with drugs and improved analgesic techniques, about 50–70% of patients experienced moderate to severe postoperative incision pain.

Research frontiers: Pain-related mediators play important roles in the pain process. Brain-derived neurotrophic factor, a classic modulator of pain, is closely involved in all kinds of pain including incision pain, inflammatory pain, and neuropathic pain. It is still unclear whether interleukin-10 is also involved in incision pain, especially mechanical allodynia induced by incision pain.

Clinical significance: Our data suggested that interleukin-10 and brain-derived neurotrophic factor were possible targets for prevention and treatment of incision-induced pain. Pain-related mediators of large-sized neurons at dorsal root ganglia should be the focus for treatment of incision-induced pain.

Academic terminology: Dorsal root ganglia are the nodules on a dorsal root of the spine that contains nerve cell bodies that carry signals from sensory organs toward the appropriate integration center.

Peer review: The present study investigated the changes of pain-related mediators at the dorsal root ganglia after incision surgery and the relationship of the pain mediators to incision-induced mechanical nociception. The data will be useful in exploring the new ways of prevention and treatment of incision-induced pain.

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