Tetsuhiro Ishikawa
Masayuki Miyagi
Seiji Ohtori
Yasuchika Aoki
Tomoyuki Ozawa
Hideo Doya
Tomoko Saito
Hideshige Moriya
Kazuhisa Takahashi

Characteristics of sensory DRG neurons innervating the lumbar facet joints in rats

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Tetsuhiro Ishikawa, Masayuki Miyagi, and Seiji Ohtori contributed equally to this work.

T. Ishikawa · M. Miyagi · S. Ohtori (✉)
Y. Aoki · T. Ozawa · H. Doya · T. Saito
H. Moriya · K. Takahashi
Department of Orthopaedic Surgery,
Graduate School of Medicine,
Chiba University, 1-8-1 Inohana,
Chuo-ku, Chiba 260-8670, Japan
E-mail: sohtori@faculty.chiba-u.jp
Tel.: +81-43-2262117
Fax: +81-43-2262116

Abstract The rat L5/6 facet joint, from which low-back pain can originate, is multisegmentally innervated from the L1 to L5 dorsal root ganglions (DRGs). Sensory fibers from the L1 and L2 DRGs are reported to non-segmentally innervate the paravertebral sympathetic trunks, whilst those from the L3 to L5 DRGs segmentally innervate the L5/6 facet joint. In the current study, characteristics of sensory DRG neurons innervating the L5/6 facet joint were investigated in rats, using a retrograde neurotransport method, lectin affinity- and immuno-histochemistry. We used four markers: (1) calcitonin gene-related peptide (CGRP) as a marker of small peptide containing neurons, (2) the glycoprotein binding the isolectin from *Griffonia simplicifolia* (IB4) or (3) the purinergic P2X₃ receptor for small, non-peptide containing neurons, and (4) neurofilament 200 (NF200) for small and large myelinated fibers. IB4-binding and CGRP and P2X₃ receptor containing neurons are typically involved in pain sensation, whereas NF200 is associated with pain and proprioception. Neurons innervating the L5/6 facet joints,

retrogradely-labeled with fluoro-gold (FG), were distributed throughout DRGs from L1 to L5. Of FG-labeled neurons, the ratios of NF200 immunoreactive (IR) neurons and CGRP-IR neurons were 37% and 35% respectively. The ratio of IB4-binding and P2X₃ receptor-IR neurons was 10%, significantly less than the ratio of CGRP-IR neurons to FG-labeled neurons. The ratios of IB4-binding and P2X₃ receptor-IR neurons were significantly higher, and that of CGRP-IR neurons was significantly less in L1 and L2 DRGs than those in L3, L4 or L5 DRGs. Under physiological conditions in rats, DRG neurons transmit several types of sensations, such as proprioception or nociception of the facet joint. Most neurons transmitting pain are CGRP-IR peptide-containing neurons. They may have a more significant role in pain sensation in the facets via peptidergic DRG neurons.

Keywords Sensory innervation · Lumbar facet joint · Calcitonin gene-related peptide · Isolectin B4 · Dorsal root ganglion · Low back pain

Introduction

Many studies have reported the lumbar facet joints as a possible source of low-back pain [18, 32]. Morpholog-

ically, the joint capsule is well innervated, receiving a nerve supply from the medial branches of the dorsal rami. Each medial branch segmentally innervates at least two or three facet joints. For example, the human

L4/5 facet joint is innervated by the medial branches of the dorsal rami from the L3 and L4 spinal nerves [7, 8, 11].

The L5/6 facet joint is reported to be multi-segmentally innervated by dorsal root ganglions (DRGs) from L1 to L5, and nerve fibers from L1 and L2 DRGs pass through the paravertebral sympathetic trunks [21, 23, 28, 29]. However, two types of neurons exist in the DRGs, and are related to pain and to position sense.

Dorsal root ganglion neurons can be divided into three categories that include large neurons, small neurons that contain neuropeptides, and small neurons that do not contain neuropeptides [27]. Large neurons stain for neurofilament 200 (NF200) and are involved in proprioception [25]. Sensory neurons involved in pain perception related to inflammatory pain are typically small, peptide-containing neurons immunoreactive for substance P (SP), calcitonin gene-related peptide (CGRP) and brain-derived neurotrophic factor (BDNF) [4, 27]. Small, non-peptide-containing neurons binding the isolectin B4 from *Griffonia simplicifolia* (IB4) and immunoreactive for the purinergic P2X₃ receptor may also be involved in various pain states, such as injured-nerve pain [17, 27].

The aim of this study was to determine the ratios of IB4-binding, CGRP-, P2X₃ receptor-, or NF200-labeled DRG neurons innervating the L5/6 facet joint using the retrograde neurotransport method and lectin affinity- and immuno-histochemistry. The L1 and L2 DRG neurons innervating the L5/6 facet joint through sympathetic trunks, and the L3, L4 and L5 DRG neurons innervating the L5/6 facet joint via other routes—and not through sympathetic trunks—are discussed separately.

Materials and methods

Retrograde FG labeling

Twenty-two male Sprague-Dawley (SD) rats weighing 250–300 g were used. The protocols for animal procedures in these experiments followed the 1996 revision of the National Institutes of Health guidelines for the Care and Use of Laboratory Animals, and received approval from the ethics committees of our institutions.

The rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and treated aseptically throughout the experiments. A midline dorsal longitudinal incision was made over the lumbar spine. The left L5/6 facet joint capsule was exposed under a microscope. A 26-gauge needle whose tip was filled with two fluoro-gold crystals (FG; Fluorochrome, Denver, CO, USA) was advanced into the facet joint. The hole was immediately sealed with cyanoacrylate to prevent leakage of the FG. Then the fascia and skin were closed.

Five days after the application of FG, the rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and perfused transcardially with 0.9% saline, followed by 500 ml of 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4). Bilateral DRGs from T13 to L6 levels were resected. The specimens were immersed in the same fixative solution overnight at 4°C. After storing in 0.01 M phosphate-buffered saline (PBS) containing 20% sucrose for 20 h at 4°C, each DRG was sectioned at 10 µm thickness on a cryostat, and mounted on poly-L-lysine-coated slides.

IB4-binding and immunohistochemistry for CGRP, P2X₃ receptors or NF200

Endogenous tissue peroxidase activity was quenched by soaking the sections for 30 min in 0.3% hydrogen peroxide solution in 0.01 M PBS. The specimens were then treated for 90 min in blocking solution, 0.01 M PBS containing 0.3% Triton X-100 and 3% skim milk, at room temperature. They were processed for IB4-binding and CGRP, P2X₃ receptor, or NF200 immunohistochemistry using biotin-labeled IB4 (1:1000; 1:1000; Chemicon, Temecula, CA, USA), rabbit antibody to CGRP (1:2000; Chemicon), guinea pig antibody to P2X₃ receptor (1:2000; Neuromic, Minneapolis, MN, USA), or mouse antibody to NF200 (1:1000; Chemicon) diluted with a blocking solution and incubated for 20 h at 4°C. After incubation with the labeled isolectin or each antibody, sections were incubated with streptavidin Alexa 594 (Texas red)(for IB4-binding; 1:400), goat anti-rabbit Alexa 488 (FITC) fluorescent antibody conjugate (for CGRP-immunoreactivity; 1:400; Molecular Probes Inc., Eugene, OR, USA), goat anti-guinea pig Alexa 594 (Texas red) fluorescent antibody conjugate (for P2X₃ receptor immunoreactivity; 1:400), or goat anti-mouse Alexa 488 (FITC) fluorescent antibody conjugate (for NF200-immunoreactivity; 1:400) respectively.

After each step, the sections were rinsed in 0.01 M PBS three times. The sections were observed with a fluorescence microscope. The number of FG-labeled neurons, and of FG-labeled and IB4-binding and CGRP-, P2X₃ receptor-, or NF200-immunoreactive (IR) neurons were counted.

Statistical analysis

The ratios of FG-labeled and IB4-binding and CGRP-, P2X₃ receptor-, or NF200-IR neurons in the DRG from T13 to L6 levels were compared using Welch's non-paired *t*-test. A *P* value of less than 0.05 was considered statistically significant.

Results

FG-labeled DRG neurons

Fluoro-gold-labeled DRG neurons; where FG was transported from the facet joint, labeled neurons were present in the left DRGs from L1 through L5 (Figs. 1, 2). No labeled neurons were observed in the bilateral T13 or L6 DRGs or in the contralateral DRGs from L1 through L5. Of the FG-labeled neurons, 66% were recognized in the L3, L4 and L5 DRGs, and the remaining 34% in the L1 and L2 DRGs.

FG-labeled IB4-binding and CGRP-, P2X₃ receptor-, or NF200-IR neurons

Fluoro-gold-labeled and IB4-binding and CGRP-, P2X₃ receptor-, or NF200-IR neurons were present in the left DRGs from L1 through L5. Figure 1 shows the FG-labeled and IB4-binding and CGRP-, P2X₃ receptor-, or NF200-IR neurons. Of all the FG-labeled neurons, the ratios of IB4-binding and CGRP-, P2X₃ receptor-, or NF200-IR neurons were $11 \pm 2\%$, $35 \pm 5\%$, $10 \pm 5\%$, and $37 \pm 6\%$ (mean $\pm$ SE) respectively. The ratios of CGRP- and NF200-IR neurons were significantly higher than those of IB4-binding and P2X₃ receptor-IR neurons ($P < 0.01$).

Fig. 1 Fluorescent photomicrographs show FG-labeled and IB4-binding and CGRP-, P2X₃ receptor-, or NF200-IR DRG neurons at the left L3 level. **a, c, e, g** showing the FG-labeled neurons innervating the L5/6 facet joint. **b, d, f, h** Labeled neurons in green or red show IB4-binding and CGRP-, P2X₃ receptor-, or NF200-IR DRG neurons. *Upper and lower* photomicrographs show the same sections. The *arrows* showing the same neurons as in Fig. 1a, c, e, g are FG-labeled IB4-binding or CGRP-, P2X₃ receptor-, or NF200-IR DRG neurons

Fig. 2 Distribution of FG-labeled DRG neurons innervating L5/6 facet joints. These neurons were observed from L1 to L5 DRG. There is no significant difference in number between each level

Figure 3 indicates ratios of IB4-binding and CGRP-, P2X₃ receptor-, or NF200-IR neurons to FG-labeled neurons at each level. There were no significant differences in the ratios of FG-labeled and NF200-IR neurons between each level. The ratios of CGRP-IR neurons to FG-labeled neurons in L1 and L2 DRGs were significantly lower than those in L3, L4 and L5 DRGs ($P < 0.05$). However, the ratios IB4-binding and P2X₃ receptor-IR neurons to FG-labeled neurons in L1 and L2 DRGs were higher than those in L3, L4 and L5 DRGs ($P < 0.01$).

Discussion

FG-labeled DRG neurons innervating rat L5/6 facet joints

Many investigators have reported that the lumbar facet joint is innervated by the dorsal ramus of spinal nerves

Fig. 3 Distribution of FG-labeled IB4-binding or CGRP-, P2X₃ receptor-, or NF200-IR DRG neurons at each level. The ratios of total CGRP- and NF200-IR neurons were significantly higher than those of total IB4-binding and P2X₃ receptor-IR neurons ($P < 0.01$). The ratios of CGRP-IR neurons to FG-labeled neurons in L1 and L2 DRGs were significantly lower than those in L3, L4 and L5 DRGs ($P < 0.05$). However, the ratios of IB4-binding and P2X₃ receptor-IR neurons to FG-labeled neurons in L1 and L2 DRGs were higher than those in L3, L4 and L5 DRGs ($P < 0.01$)

consisting of segmental spinal sensory fibers and post-ganglionic sympathetic fibers [7, 8, 11].

It has been reported that the rat L5/6 facet joint is innervated by DRGs from L1 to L5 levels. The L5/6 facet joint is innervated by two distinct nervous systems: innervation from corresponding and adjacent segments and from distant segments. In the latter innervation, sensory nerve fibers enter the paravertebral sympathetic trunks and reach L1 or L2 DRGs [21, 23, 28, 29]. The present study similarly demonstrated that the rat L5/6 facet joint was innervated by ipsilateral DRGs from L1 to L5 levels.

Characteristics of sensory DRG neurons innervating the facet joints

In the current study, FG-labeled neurons innervating facet joints were co-labeled with IB4-binding and CGRP- or P2X₃ receptor-IR neurons. In all of the FG-labeled neurons, the ratios of FG-labeled neurons with IB4-binding and CGRP- or P2X₃ receptor-IR were $11 \pm 2\%$, $35 \pm 5\%$, and $10 \pm 5\%$ (mean $\pm$ SE) respectively. On the other hand, some FG-labeled neurons were double-labeled with NF200-IR myelinated A-fiber neurons ($37 \pm 6\%$). The ratios of FG-labeled neurons labeled with CGRP-IR were significantly higher than those labeled with IB4-binding or P2X₃ receptor-IR ($P < 0.01$).

NF200 is a marker of small and large myelinated A-fiber neurons, which are involved in proprioception [25].

Electrophysiological studies have revealed that some neurons transmitting proprioception, such as position sense, innervate the facet joint in rabbits [32]. Our data is consistent with this previous report.

Nociceptive information is normally transmitted by small DRG neurons to the dorsal horn of the spinal cord. The small DRG neurons may be subclassified into two groups. One group, comprised of peptide-containing neurons (40% of total DRG neurons), contains CGRP [14, 17, 27]. CGRP-containing neurons terminate in lamina I, and the outer layer of lamina II in the spinal dorsal horn [17]. The other group, comprised of non-peptide-containing neurons (32% of total DRG neurons), lacks peptides, but expresses IB4-binding glycoprotein [27]. Adenosine-5'-triphosphate (ATP) is an important metabolite involved in energy transfer, and is also recognized as an extracellular neurotransmitter [1, 10]. ATP is released during tissue damage, and activates P2X₃ receptors, initiating nociceptive signals. Most P2X₃ receptor containing DRG neurons are co-labeled with IB4, and these neurons terminate at the inner part of lamina II in the spinal dorsal horn [9, 17].

It has also been reported that, in inflammatory models, SP and CGRP are increased in the dorsal root ganglia and the dorsal horn of the lumbar spinal cord [13, 19, 20]. SP- and CGRP-containing nerve fibers projecting to lamina I and the outer layer of lamina II are important for the transmission of inflammatory pain.

In the inner part of lamina II, there are interneurons that contain protein kinase C gamma (PKC) [15]. PKC-containing interneurons are related to the development of the neuropathic pain state produced by nerve injury [15]. Since PKC-containing interneurons receive input from non-peptide-containing neurons, lumbar facet lesions are unlikely to produce a PKC-related neuropathic pain state.

It has been reported that the ratio of IB4-binding neurons innervating skin (43%) is higher than that innervating bladder (29%) [5]. Muscle afferent neurons exhibited 22% CGRP-IR and 5% IB4-binding. On the other hand, cutaneous afferent neurons exhibited 26% CGRP-IR and 44% IB4-binding [2]. The ratios of IB4-binding and SP-IR DRG neurons innervating the lumbar disc were 0.6% and 44% respectively [24]. Taking into consideration these studies and the current study, we conclude that organs located in the center of the body are innervated by fewer IB4-binding DRG neurons.

CGRP-IR DRG neurons were subclassified into nerve growth factor (NGF)-dependent neurons (40% of total DRG neurons) [4, 5, 17, 30], which express the high-affinity NGF receptor tyrosine kinase A (trkA), [4, 17]. IB4-labeled DRG neurons are glial cell line-derived neurotrophic factor (GDNF)-dependent neurons (32% of total DRG neurons), [26, 31] which express the

NGF receptor [6, 26]. Aoki et al. [3] have reported that rat intervertebral discs are innervated mostly by CGRP-IR NGF-dependent small neurons, and that disc inflammation caused an increase in CGRP-IR neurons, but not IB4-binding neurons, suggesting that CGRP-IR NGF-dependent neurons have an overriding responsibility for discogenic pain. Furthermore, it has also been reported that NGF and trkA-expressing nerve fibers are present in painful lumbar discs in humans [14].

Human facet joints are innervated by NGF-dependent CGRP-IR DRG neurons, but few GDNF-dependent IB4-binding DRG neurons. Subcutaneous administration of NGF produces hyperalgesia in rats, and depression of NGF was found to decrease allodynia [12, 26]. On the other hand, IB-4 labeled neurons are

strongly correlated with production of injured nerve pain [27]. Facet joint pain may be mainly transmitted by CGRP-IR DRG neurons and closely related to inflammation.

Facet-joint pain is a well-recognized clinical problem for some patients. The present study suggests that NGF is a potential target for the relief of facet-joint pain. We have previously shown that CGRP increases in large DRG neurons, which transmit proprioception under physiological conditions, and increased facet-joint pain in rats [22].

These current findings contribute important information that may lead to the development of novel analgesic therapies for facet-joint pain, for example the use of anti-NGF antibodies.

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