Table 2. Distribution of the severity evaluated by the current JOA scoring system and finger-floor distance ($n = 355$)

Parameter	No.
Straight-leg raising (SLR) test	
Normal	183
30°–70°	130
<30°	42
Motor function	
Normal	182
Slight weakness (MMT good)	126
Severe weakness (MMT less than good)	47
Sensory function	
Normal	127
Slight disturbance	162
Severe disturbance	66
Bladder function	
Normal	315
Mild dysuria	36
Severe dysuria	4
Finger-to-floor distance (cm)	
to –15	1
–14 to –5	12
–4 to 4	69
5 to 14	73
15 to 24	69
25 to 34	43
35 to 44	25
45 to 54	30
55 to 64	6
65 to 74	4
Not measurable	14
Total number	355

JOA, Japanese Orthopaedic Association; MMT, manual muscle testing

specific. There was no marked difference in the distribution of the severity levels between the 451 patients who were initially recruited and the 355 who were finally analyzed.

Superficial validity

Superficial validity was checked in terms of the completion rate for filling out the questionnaire. Regarding the distribution of responses for each item, it was judged that none of the questions was too difficult to answer because the highest rate of nonrespose was 1.8%. With regard to deflection of an answer, the highest rate (78.3%) was concentrated on "yes" responses to question 1–14, although this was judged not to be inappropriate. Therefore, the distribution was not skewed, which would indicate "floor and ceiling" effects (Table 3).

Factor analysis

First, we tried to extract some observed variables from 25 items by the Maximum Likelihood Method. It was found that the eigenvalue was >1.0 for five items, and the accumulative contribution ratio until the fifth factor was 53.1% (Table 4).

Next, we performed orthogonal rotation by the direct oblimin method. The results are shown in Table 5. Each item was categorized into five factors: Four items (Q2-6, Q2-5, Q1-2, Q2-4) related to factor 1; seven items (Q2-8, Q2-7, Q2-11, Q1-13, Q2-9, Q2-10, Q2-1) related to factor 2; six items (Q1-9, Q1-6, Q2-3, Q1-8, Q1-5, Q1-4) related to factor 3; five items (Q1-10, Q2-4, Q1-12, Q1-14, Q2-2) to factor 4; and the last four items to factor 5. Although factor loading was <0.30 in three items (Q1-4 to factor 3, Q2-2 to factor 4, Q1-11 to factor 5), we adopted all of them for the reason that the question itself was important for the factor or the number of questions in each factor needed to be more than four.

Factor names were determined based on the commonality of the items that showed a large value on factor loading: factor 1, social function (four items); factor 2, mental health (seven items); factor 3, lumbar function (six items); factor 4, walking ability (five items); and factor 5, low back pain (four items).

Measurement scale

To establish a measurement scale for each factor, we determined the size of the coefficient for each item so the difference between the maximum factor scores and minimum factor scores was approximately 100 (Table 6). When a coefficient became a negative numerical value, we changed the coefficient to a positive numerical value by reversing the order of the answer choice. We adjusted the formula so the maximum for each factor score was 100 and the minimum was 0 (see Appendix 2).

Discussion

It is considered ideal for the outcome measure to evaluate patients from various perspectives, such as dysfunction, disability, handicap, and psychological problem. The outcome measure should be patient-oriented, and its reliability and validity should be confirmed by statistical analysis. However, the current JOA score does not include subjective evaluations and does not meet such requirements. We developed a new questionnaire, JOABPEQ, specifically to evaluate low back pain. It is patient-oriented and mainly based on recognizing problems with activities of daily living. We categorized 25 questions into five factors; each factor is then scored up to 100 points using the measurement scale. The factors are then evaluated separately. The point is to be aware that it is meaningless and inadequate to total

Table 3. Distribution of answers for each item in the questionnaire ($n = 451$)

Item	Choices for answer					No answer
	1	2	3	4	5	
Q1-1	336 (74.5%)	114 (25.3%)				1 (0.2%)
Q1-2	152 (33.7%)	297 (65.9%)				2 (0.4%)
Q1-3	302 (67.0%)	146 (32.4%)				3 (0.7%)
Q1-4	157 (34.8%)	291 (64.5%)				3 (0.7%)
Q1-5	242 (53.7%)	209 (46.3%)				0
Q1-6	167 (37.0%)	281 (62.3%)				3 (0.7%)
Q1-7	215 (47.7%)	236 (52.3%)				0
Q1-8	240 (53.2%)	208 (46.1%)				3 (0.7%)
Q1-9	272 (60.3%)	177 (39.2%)				2 (0.4%)
Q1-10	288 (63.9%)	160 (35.5%)				3 (0.7%)
Q1-11	158 (35.0%)	292 (64.7%)				1 (0.2%)
Q1-12	156 (34.6%)	286 (63.4%)				9 (2.0%)
Q1-13	116 (25.7%)	333 (73.8%)				2 (0.4%)
Q1-14	353 (78.3%)	90 (20.0%)				8 (1.8%)
Q2-1	4 (0.9%)	27 (6.0%)	155 (34.4%)	185 (41.0%)	79 (17.5%)	1 (0.2%)
Q2-2	103 (22.8%)	233 (51.7%)	113 (25.1%)			2 (0.4%)
Q2-3	126 (27.9%)	253 (56.1%)	67 (14.9%)			5 (1.1%)
Q2-4	181 (40.1%)	175 (38.8%)	95 (21.1%)			0
Q2-5	62 (13.7%)	111 (24.6%)	206 (45.7%)	48 (10.6%)	23 (5.1%)	1 (0.2%)
Q2-6	113 (25.1%)	124 (27.5%)	138 (30.6%)	50 (11.1%)	23 (5.1%)	3 (0.7%)
Q2-7	53 (11.8%)	66 (14.6%)	225 (49.9%)	72 (16.0%)	35 (7.8%)	0
Q2-8	52 (11.5%)	76 (16.9%)	224 (49.7%)	75 (16.6%)	23 (5.1%)	1 (0.2%)
Q2-9	11 (2.4%)	57 (12.6%)	190 (42.1%)	132 (29.3%)	60 (13.3%)	1 (0.6%)
Q2-10	64 (14.2%)	125 (27.7%)	114 (25.3%)	102 (22.6%)	45 (10.0%)	1 (0.2%)
Q2-11	48 (10.6%)	149 (33.0%)	141 (31.3%)	89 (19.7%)	23 (5.1%)	1 (0.2%)

Table 4. Results of factor analysis: eigenvalue of each item

Factor	Eigenvalue	Cumulative contribution rate (%)
1	**7.600**	**30.4**
2	**1.795**	**37.6**
3	**1.556**	**43.8**
4	**1.217**	**48.7**
5	**1.095**	**53.1**
6	0.996	57.0
7	0.942	60.8
8	0.893	64.4
9	0.783	67.5
10	0.756	70.5
11	0.728	73.4
12	0.680	76.2
13	0.656	78.8
14	0.643	81.4
15	0.617	83.8
16	0.584	86.2
17	0.505	88.2
18	0.482	90.1
19	0.433	91.9
20	0.427	93.6
21	0.387	95.1
22	0.361	96.6
23	0.320	97.8
24	0.302	99.0
25	0.239	100.0

Bold typeface indicates eigenvalues over 1.0

Table 5. Results of factor analysis: factor loading of each item

Item	Factors				
	1	2	3	4	5
Q2-6	**0.81**	0.04	0.04	−0.04	0.14
Q2-5	**0.71**	0.01	0.08	0.14	0.06
Q1-2	0.33	0.16	0.21	0.34	−0.21
Q2-8	0.07	**0.68**	0.08	−0.08	0.10
Q2-7	0.15	**0.62**	−0.07	0.12	0.15
Q2-11	−0.03	**0.62**	−0.12	−0.02	0.06
Q1-13	−0.04	**0.35**	0.08	−0.01	0.14
Q2-9	−0.23	**−0.52**	−0.11	0.05	0.05
Q2-10	0.06	**−0.55**	−0.06	−0.10	0.15
Q2-1	0.03	**−0.55**	−0.14	−0.11	−0.02
Q1-9	0.02	−0.07	**0.69**	−0.07	0.10
Q1-6	−0.01	0.12	**0.56**	0.08	−0.10
Q2-3	0.23	0.05	**0.56**	−0.04	0.07
Q1-8	−0.03	−0.09	**0.38**	0.15	0.31
Q1-5	0.00	0.09	0.32	0.03	−0.02
Q1-4	0.10	0.11	0.28	0.13	0.05
Q1-10	0.14	0.04	−0.04	**0.62**	0.03
Q2-4	**0.39**	0.05	−0.08	**0.61**	−0.13
Q1-12	−0.05	0.01	0.01	**0.46**	0.06
Q1-14	−0.07	0.06	0.20	0.34	0.13
Q2-2	0.30	−0.03	0.19	0.26	0.05
Q1-1	0.03	0.11	0.00	0.04	**0.46**
Q1-3	0.18	0.13	−0.05	0.13	**0.43**
Q1-7	0.07	0.06	0.20	0.01	**0.41**
Q1-11	0.10	0.04	0.16	0.25	0.28

Bold typeface indicates absolute value of the factor loading of more than 0.26

Table 6. Coefficient for each item of the formula for measurement scale

Item	1 Social function	2 Mental health	3 Lumbar function	4 Walking ability	5 Low back pain
Q1-1					20
Q1-2	2				
Q1-3					20
Q1-4			10		
Q1-5			10		
Q1-6			20		
Q1-7					20
Q1-8			10		
Q1-9			30		
Q1-10				30	
Q1-11					10
Q1-12				20	
Q1-13		3			
Q1-14				10	
Q2-1		−4			
Q2-2				10	
Q2-3			20		
Q2-4	4			30	
Q2-5	6				
Q2-6	10				
Q2-7		6			
Q2-8		6			
Q2-9		−3			
Q2-10		−3			
Q2-11		3			

the five factors' scores; rather, they should be treated by nonparametric analysis. The reliability of the questionnaire including 25 items for the JOABPEQ was confirmed in Part 2 of this project. The validity of the questionnaire was evaluated using factor analysis, and the measurement scale was established in Part 3 of this study. Further studies must be performed to confirm the responsiveness of the calculations of the severity score.

Conclusions

We confirmed the validity of the JOA Back Pain Evaluation Questionnaire (JOABPEQ) and established a measurement scale.

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Appendix 1. Items selected for the draft of a JOABPEQ document

With regard to your health condition during the last week, please choose the item number among the answers for the following questions that best applies as your condition varies depending on the day or time. Circle the item number when your condition is at its worst.

Q1-1 To alleviate low back pain, you often change your posture.
1) Yes
2) No

Q1-2 Because of low back pain, you do not do any routine housework these days.
1) No
2) Yes

Q1-3 Because of low back pain, you lie down more often than usual.
1) Yes
2) No

Q1-4 Because of low back pain, you sometimes ask someone to help you when you do something.
1) Yes
2) No

Q1-5 Because of low back pain, you refrain from bending forward or kneeling down.
1) Yes
2) No

Q1-6 Because of low back pain, you have difficulty standing up from a chair.
1) Yes
2) No

Q1-7 Your lower back aches most of the time.
1) Yes
2) No

Q1-8 Because of low back pain, turning over in bed is difficult.
1) Yes
2) No

Q1-9 Because of low back pain, you have difficulty putting on socks or stockings.
1) Yes
2) No

Q1-10 Because of low back pain, you walk only short distances.
1) Yes
2) No

Q1-11 Because of low back pain, you cannot sleep well. (If you take sleeping pills because of the pain, select "No.")
1) No
2) Yes

Q1-12 Because of low back pain, you stay seated most of the day.
1) Yes
2) No

Q1-13 Because of low back pain, you become irritated or angry at other persons more often than usual.
1) Yes
2) No

Q1-14 Because of low back pain, you go up stairs more slowly than usual.
1) Yes
2) No

Q2-1 How is your present health condition?
1) Excellent
2) Very good
3) Good
4) Fair
5) Poor

Q2-2 Do you have difficulty in climbing stairs?
1) I have great difficulty
2) I have some difficulty
3) I have no difficulty

Q2-3 Do you have difficulty with any one of the following motions: bending forward, kneeling, stooping?
1) I have great difficulty
2) I have some difficulty
3) I have no difficulty

Q2-4 Do you have difficulty walking more than 15 minutes?
1) I have great difficulty
2) I have some difficulty
3) I have no difficulty

Q2-5 Have you been unable to do your work or ordinary activities as well as you would like?
1) I have not been able to do them at all.
2) I have been unable to do them most of the time.
3) I have sometimes been unable to do them.
4) I have been able to do them most of the time.
5) I have always been able to do them.

Q2-6 Has your work routine been hindered because of the pain?
1) Greatly
2) Moderately
3) Slightly (somewhat)
4) Little (minimally)
5) Not at all

Q2-7 Have you been discouraged or depressed?
1) Always
2) Frequently
3) Sometimes
4) Rarely
5) Never

Q2-8 Do you feel exhausted?
1) Always
2) Frequently
3) Sometimes
4) Rarely
5) Never

Q2-9 Do you feel happy?
1) Always
2) Almost always
3) Sometimes
4) Rarely
5) Never

Q2-10 Do you think you are in reasonable health?
1) Yes (I am healthy)
2) Fairly (my health is better than average)
3) Not very much (my health is average)
4) Barely (my health is poor)
5) Not at all (my health is very poor)

Q2-11 Do you feel your health will get worse?
1) Very m uch so
2) A little bit at a time
3) Sometimes yes and sometimes no
4) Not very much
5) Not at all

Appendix 2. Measurement scale for JOABEPQ

Social life function
('Q1-2' × 2 + 'Q2-4' × 4 + 'Q2-5' × 6 + 'Q2-6' × 10 − 22) × 100 ÷ 74

Mental health
('Q1-13' × 3 + 'Q2-1' × 4 + 'Q2-7' × 6 + 'Q2-8' × 6 + 'Q2-9' × 3 + 'Q2-10' × 3 + 'Q2-11' × 3 − 28) × 100 ÷ 103

Lumbar function
('Q1-4' × 10 + 'Q1-5' × 10 + 'Q1-6' × 20 + 'Q1-8' × 10 + 'Q1-9' × 30 + 'Q2-3' × 20 − 100) × 100 ÷ 120

Walking ability
('Q1-10' × 30 + 'Q1-12' × 20 + 'Q1-14' × 10 + 'Q2-2' × 10 + 'Q2-4' × 30 − 100) × 100 ÷ 140

Low back pain
('Q1-1' × 20 + 'Q1-3' × 20 + 'Q1-7' × 20 + 'Q1-11' × 10 − 70) × 100 ÷ 70

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Contralateral Neuropathic Pain and Neuropathology in Dorsal Root Ganglion and Spinal Cord Following Hemilateral Nerve Injury in Rats

Satoshi Hatashita, MD, Miho Sekiguchi, MD, PhD, Hideo Kobayashi, MD, Shin-ichi Konno, MD, PhD, and Shin-ichi Kikuchi, MD, PhD

Study Design. The contralateral pain-related behavioral and immunohistochemical changes after hemilateral spinal nerve injury in rats were investigated.

Objectives. We evaluated the longitudinal changes in contralateral mechanical allodynia, expression of tumor necrosis factor (TNF)-α and glial fibrillary acidic protein (GFAP)-positive satellite cells in the contralateral dorsal root ganglion (DRG), and expression of astrocytes and microglia in the contralateral spinal dorsal horn after hemilateral spinal nerve injury in rats.

Summary of Background Data. In previous studies, hemilateral nerve injury has sometimes induced contralateral neuropathic pain. TNF-α expression and glial cell reactions in the DRG and spinal cord play an important role in the neuropathic pain state, and TNF-α is released from glial cells in the nervous system.

Methods. Adult male Sprague-Dawley rats were used. The spinal L5 nerve distal to the DRG was crushed once for 3 seconds. At days 2, 7, 14, and 21 after surgery, mechanical allodynia was determined in bilateral hind paws by the von Frey test. Expression of TNF-α and GFAP in bilateral L5 DRGs and expression of GFAP and ionized calcium-binding adaptor molecule-1 (Iba-1) in bilateral L5 spinal dorsal horns were studied using immunohistochemistry and immunoblotting.

Results. Mechanical withdrawal threshold of the ipsilateral hind paw was significantly decreased for 21 days. Conversely, mechanical withdrawal threshold of the contralateral hind paw was significantly decreased from 5 to 10 g for 7 days, and was <5 g at days 14 and 21. TNF-α expression and GFAP-positive satellite cells in the contralateral DRG significantly increased from day 7 to day 21. In the contralateral spinal dorsal horn, GFAP-positive astrocytes significantly increased for 21 days, but Iba-1 was not significant.

Conclusion. These results suggest that contralateral mechanical allodynia induced by hemilateral spinal nerve injury is associated with upregulation of satellite cells and TNF-α in the contralateral DRG. In addition, our results suggest that spinal astrocytes play an important role in these contralateral changes.

Key words: contralateral neuropathic pain, crush injury, tumor necrosis factor α, satellite cell, astrocyte.
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Responses in spinal cord and dorsal root ganglion (DRG) play important roles in neuropathic pain. The expression of proinflammatory cytokines and glial cells in the spinal cord and DRG have been implicated in neuropathic pain states.[1–4] Tumor necrosis factor (TNF)-α, one of the key proinflammatory cytokines, is a major factor in the initiation and maintenance of neuropathic pain states.[5] In peripheral nerve injury, TNF-α expression is upregulated in endoneurial macrophages and Schwann cells at injury sites, resulting in neuropathic pain.[6] TNF-α expression is upregulated in satellite cells of the DRG and astrocytes of the spinal cord after peripheral nerve injury.[4] Peripheral nerve injury and inflammation activate glial cells such as astrocytes, microglia, and satellite cells in spinal cord and DRG.[2–4] Activated microglia and astrocytes produce proinflammatory cytokines and upregulate other cytokines that are associated with nerve degeneration.[7–10] According to these reports, chemical and glial reactions are considered to be transmitted to the DRG and spinal dorsal horn through glial-cytokine interaction in neuropathic pain states.

Ligated hemilateral sciatic nerves have been shown to induce contralateral mechanical allodynia in a rat model, and this phenomenon has been called "mirror-image pain."[11] A small number of reports have described mirror-image pain in other neuropathic pain models.[12–14] In clinical situations, mirror-image pain has been documented in complex regional pain syndrome[15] and phantom pain.[16] Some cases of lumbar disc herniation have also reportedly involved contralateral neuropathic pain.[17–21] However, the mechanisms of mirror-image pain have not been well documented, and little has been reported on longitudinal changes in TNF-α expression and satellite cells in the contralateral DRG.

We hypothesized that the spread of chemical and glial reactions in the contralateral DRG and spinal cord might induce contralateral neuropathic pain, and that TNF-α would thus represent a key factor in the chemical pathogenesis of this phenomenon. The present study explored longitudinal changes in bilateral mechanical allodynia, expression and localization of TNF-α and glial cells in bilateral DRGs, and expression of glial cells in the bilat-

From the Department of Orthopaedic Surgery, Fukushima Medical University School of Medicine, Fukushima, Japan.
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Address correspondence and reprint requests to Satoshi Hatashita, MD, Department of Orthopaedic Surgery, Fukushima Medical University School of Medicine, 1-Hikarigaoka, Fukushima City, Fukushima 960-1295, Japan; E-mail: hatashi@fmu.ac.jp

eral spinal cord after hemilateral nerve injury using a rat crush injury model.

■ Materials and Methods

All experiments were approved by the Animal Studies Committee at Fukushima Medical University.

Animals and Anesthesia

A total of 132 male Sprague-Dawley rats (Japan SLC, Shizuoka, Japan) weighing 200 to 250 g were used in this study. Animals were housed in plastic cages at room temperature in a 12-hour light and dark cycle with *ad libitum* access to food and water.

Animals were anesthetized by intraperitoneal injection of 30 mg/kg sodium pentobarbital (Nembutal 50 mg/mL; Abbott Laboratories, North Chicago, MI).

Experimental Groups

Rats were divided into 2 groups: a crush injury group (n = 66); and a sham group (n = 66).

Surgical Procedures

Rats were placed into a prone position and an incision was made in the middle of the spine at the L4–L6 level. Using an operating microscope, the fascia was incised along the left side of the supraspinous ligament. The multifidus muscles were gently moved laterally to expose the facet joint between the fifth and sixth lumbar vertebrae. The L5 nerve root, DRG and spinal nerve were exposed by an L5/6 facetectomy. The spinal L5 nerve distal to the DRG (the site of the superior border of the L6 transverse process) was crushed once for 3 seconds, using smooth forceps (crush injury group).[22] In sham-operated animals (sham group), the same surgical procedure was performed except no crush injury was applied.

Behavioral Testing

Sensitivity to non-noxious mechanical stimuli was measured using the von Frey test. Baseline testing was performed before the start of the experiment to accommodate animals. Bilateral hind paw withdrawal responses to von Frey hair (Stoelting, Wood Dale, IL) stimulation of the plantar surface of the footpads was determined at postoperative days 2, 7, 14, and 21 (n = 10 for each group). For measurement, rats were placed in a transparent plastic box with a wire netting floor and allowed to acclimate for 15 minutes. The midplantar surface of the left hind paw was stimulated using von Frey filaments capable of exerting bending forces of 0.7, 1.2, 2.0, 3.6, 5.5, 8.5, 15.1, and 28.8 g. The force of stimulation that caused brisk withdrawal in 50% of trials was calculated using the up-down method as described by Chaplan *et al.*[23] Tests were started by filament of 3.6 g, with 1 trial for each filament and a total of 7 trials. Results are expressed as mean ± standard deviation of the 50% withdrawal threshold.

Immunohistochemistry

Animals were anesthetized using 99% diethyl ether (Wako PureChemical Industries, Osaka, Japan), perfused with fresh 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), and bilateral L5 DRGs and the L5 spinal cord were removed. DRGs and the spinal cord were postfixed briefly in 4% paraformaldehyde and subsequently embedded in paraffin. Two sections (6 μm) of DRGs and the spinal cord were cut from each sample and placed on slides.

Immunohistochemistry for DRGs

Bilateral L5 DRGs were stained using antibodies to TNF-α, NeuN (a marker for neurons), and glial fibrillary acidic protein (GFAP) (a marker for astrocytes) on postoperative days 2, 7, 14, and 21 (n = 5 for each group at each time point). Double-staining with TNF-α and either NeuN or GFAP antibodies was performed.

Sections were deparaffinized with xylene and rehydrated with 100% ethanol and then PBS, and pretreated with 0.1% trypsin at 37°C for 20 minutes to enhance immunoreactivity. After blocking with 2% normal donkey serum in PBS/0.3%Triton X-100 applied for 1 hour at room temperature, goat antirat TNF-α antibody (1:100; RD System, Minneapolis, MN) was applied overnight at 4°C, followed by incubation with donkey antigoat Alexa 555 (red) fluorescent antibody (1:200; Molecular Probes, Eugene, OR) for 1 hour at room temperature. Next, 2% normal rabbit serum in PBS/0.3%Triton X-100 was applied for 1 hour. Incubation with antibody to mouse anti-NeuN (1:200, MAB377; Chemicon, Billerica, MA) or mouse anti-GFAP (1:10; Progen, Heidelberg, Deutschland) was subsequently performed overnight at 4°C. Sections were rinsed in PBS and incubated for 1 hour at room temperature with goat antimouse Alexa Fluor 488 (green) (1:200; Molecular Probes). Fluorescent staining was analyzed using a BX50 fluorescent microscope (Olympus, Tokyo, Japan). Green-staining GFAP-positive satellite cells were counted at 200× magnification in each field using Axio Vision imaging software (Carl Zeiss, Gettingen, Germany). The ratio of GFAP-positive satellite cells was calculated (GFAP-positive encircled neurons/all neurons × 100 (%)).

Immunohistochemistry for Spinal Cord

Spinal cords were stained using GFAP (a marker for astrocytes) and ionized calcium-binding adaptor molecule-1 (Iba-1) (a marker for microglia) at postoperative days 2, 7, 14, and 21 (n = 6 for each group at each time point). Sections were deparaffinized and rehydrated (as above), and pretreated with 0.1% trypsin at 37°C for 20 minutes for Iba-1 (with no pretreatment for GFAP). After blocking with 2% normal goat serum in PBS/0.3%Triton X-100 applied for 1 hour at room temperature, mouse anti-GFAP (1:200; MAB3402; Chemicon) and rabbit anti-Iba-1 (1:200; Wako, Osaka, Japan) applied overnight at 4°C in 2% normal goat serum, followed by incubation with goat antimouse Alexa 488 (for GFAP) or goat antirabbit Alexa 488 (for Iba-1) fluorescent antibody (green) (1:200; Molecular Probes) for 1 hour at room temperature. For spinal cord, the area of GFAP- and Iba-1-positive cells per section was measured in the spinal dorsal horn using a computerized image analysis system (Image J version 1.33u; National Institute of Mental Health, Bethesda, MD).

Immunoblotting for TNF-α

Immunoblotting examinations were performed in the crush injury group (days 2, 7, 14, and 21) and sham group (day 7) (n = 3 for each group at each time point) to analyze TNF-α expression in bilateral DRGs. Preliminary immunoblotting examinations were performed in the sham group (n = 3 at each time point), revealing that TNF-α expression levels in bilateral DRGs at each time point did not vary significantly (data not shown). Therefore, in the sham group, we investigated TNF-α expression only at day 7 in bilateral DRGs.

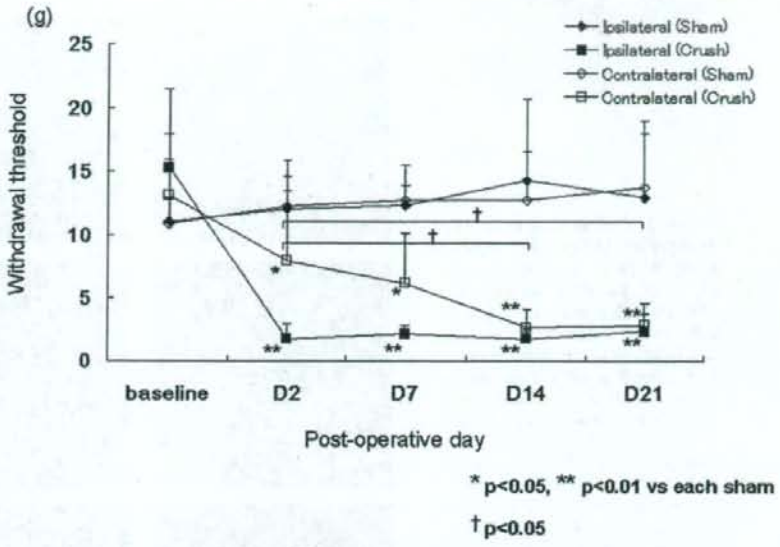

Figure 1. Changes in mechanical withdrawal threshold of bilateral hind paws in the crush injury and sham groups. Mechanical withdrawal thresholds were measured using von Frey filaments for 21 days after surgery. Measurements are given as mean ± SD (n = 10 for each group). $*P < 0.05$, $**P < 0.01$, compared with each sham group. $\dagger P < 0.05$.

Rats were decapitated rapidly under anesthesia, and bilateral L5 DRGs were removed and frozen in liquid nitrogen. Samples were homogenized in a lysis buffer (#9803; Cell Signaling, Danvers, MA), adding 10 μg/mL of leupeptin, 10 μg/mL of aprotinin, 10 μg/mL of trypsin inhibitor, and 100 μmol/L of phenylmethylsulfonyl fluoride. Protein concentrations of samples were measured using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL), then sodium dodecylsulfate (SDS) sample buffer comprising 62.5 mmol/L Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mmol/L DTT and 0.01% bromophenol blue was added. Protein samples were separated on 12.5% Tris-glycine SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad Laboratories, Hercules, CA, USA) for 1.5 hours at 10 to 40 mA, then transferred to polyvinylidene difluoride filter membranes (Millipore, Billerica, MA) for 1 hour at 15 V. Membranes were incubated overnight with diluted primary antibody in 5% nonfat milk, 1× TBS and 0.1% Tween-20 at 4°C with gentle shaking. Primary antibodies used included mouse antirat TNF-α monoclonal antibody (1:100; R&D Systems) and mouse anti-β-actin monoclonal antibody (1:5000; Sigma, St. Louis, MO). Those membranes were then incubated for 1 hour at room temperature using horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000; Bio-Rad Laboratories). Recombinant rat TNF-α (0.8 ng/lane; R&D Systems) was used as a positive control, and 20% bovine serum albumin was used as a negative control. As a control for these bands, we also analyzed TNF-α immunoreactivity in the left L5 DRG of a naïve rat (n = 1). Positive bands were visualized using an enhanced chemiluminescence system (Amersham Bioscience, Piscataway, NJ). Positive bands of immunoblots were analyzed by ratio against internal control β-actin using a computer-assisted imaging analysis system (Image J, version 1.38U; National Institute of Mental Health).

Statistical Analysis

Statistical significance for behavioral testing was analyzed using the Mann-Whitney *U* test and Student-Newmans-Keuls test. Statistical significance for immunoblotting was analyzed with the Mann-Whitney *U* test. Statistical significance for immunohistochemistry was analyzed with the Mann-Whitney *U* test and Student-Newmans-Keuls test. Values of $P < 0.05$ were considered significant. All measurements are reported as mean ± standard deviation (SD).

■ Results

Behavioral Test

In the crush injury group, mechanical withdrawal threshold of the ipsilateral (left) hind paw was significantly decreased to <5 g for 21 days compared with the sham group ($P < 0.01$). In the sham group, the mechanical withdrawal threshold of the bilateral hind paws was unchanged for 21 days compared with baseline (Figure 1). The mechanical withdrawal threshold of the contralateral (right) hind paw was significantly decreased from 5 to 10 g for 7 days ($P < 0.05$), and was <5 g at days 14 and 21 ($P < 0.01$) compared with the sham group. In the crush group, the mechanical withdrawal threshold of day 14 and 21 was significantly decreased, compared with that of day 2 ($P < 0.05$).

Immunohistochemical Analysis in DRGs

In the sham group, few TNF-α and GFAP positive cells were observed in bilateral L5 DRGs (Figures 2A, D). Conversely, in the crush injury group, TNF-α-positive cells were observed in the cytoplasm of both small and large neurons and around neurons (Figures 2B, C), colocalized with NeuN and GFAP (Figures 2G, H). GFAP -positive cells were observed in not only the ipsilateral (left) DRG, but also the contralateral (right) DRG (Figures 2E, F).

In the ipsilateral DRG, the ratio of GFAP-positive satellite cells in the crush injury group was significantly increased at each time point compared with the sham group ($P < 0.05$). Conversely, the ratio of GFAP-positive satellite cells in the contralateral DRG in the crush injury group significantly increased from

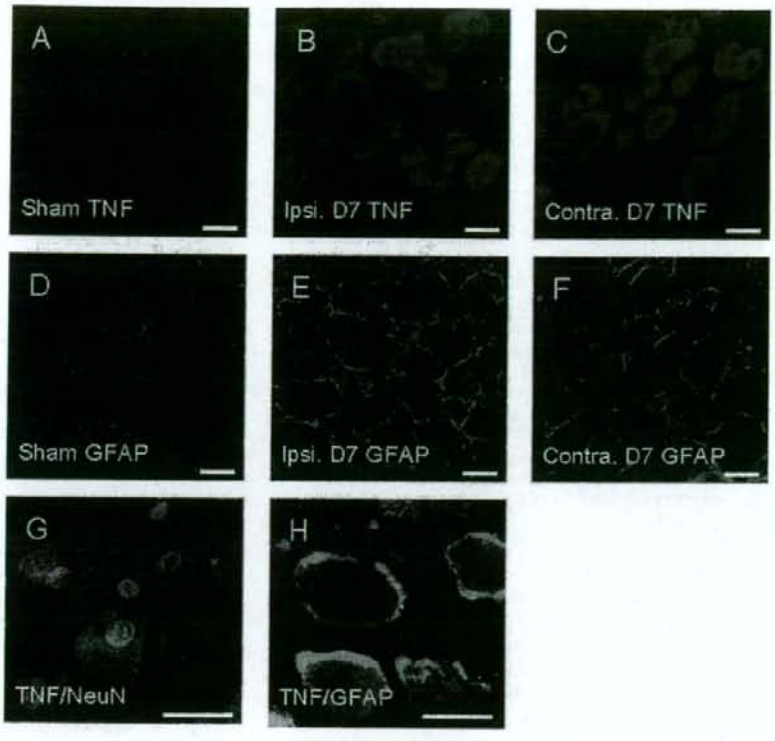

Figure 2. Immunofluorescence analysis of TNF-α- and GFAP-positive cells in bilateral L5 DRGs. In the sham group, few TNF-α-positive cells were seen in the ipsilateral (left) DRG **(A)** on day 7. In the crush injury group, TNF-α-positive cells (red) were observed in the cytoplasm of neurons and satellite cells in the ipsilateral (left) DRG **(B)** and contralateral (right) DRG **(C)** at day 7. In the sham group, few GFAP-positive satellite cells were seen in the ipsilateral (left) DRG **(D)** at day 7. In the crush injury group, GFAP-positive satellite cells (green) were observed in the ipsilateral (left) DRG **(E)** and contralateral (right) DRG **(F)** at day 7. Some TNF-α-positive cells were colocalized with both NeuN **(G)** and GFAP **(H)** (yellow). Scale bar = 20 μm.

day 7 to day 21, compared with the sham group ($P < 0.05$) (Figure 3).

Immunoblotting for TNF-α in DRGs

TNF-α bands in bilateral DRGs were detected at 26 kDa. In the ipsilateral (left) DRG, expression levels of TNF-α in the crush injury group were significantly increased for 21 days after surgery in comparison with the sham group. On the other hand, expression of TNF-α in the contralateral (right) DRG of the crush injury group was significantly increased from day 7 to day 21 after surgery in comparison with the sham group (Figure 4).

Immunohistochemical Analysis in Spinal Cord

In the sham group, few GFAP- or Iba-1-positive glial cells were observed in bilateral L5 spinal dorsal horns

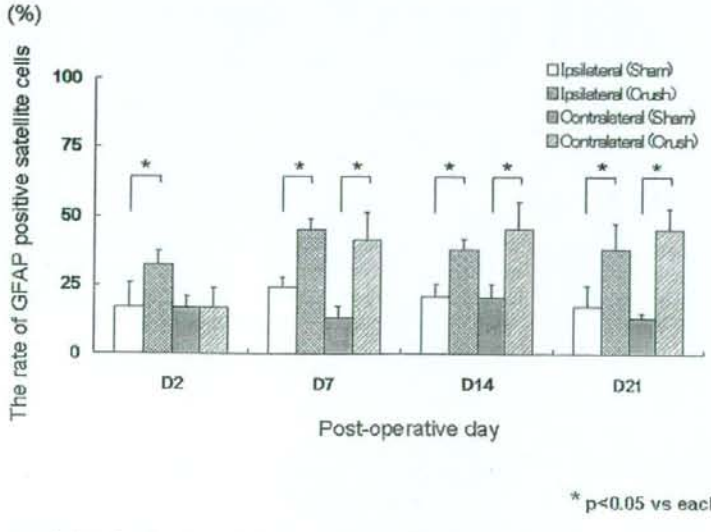

Figure 3. Ratio of GFAP-positive satellite cells in bilateral L5 DRGs in the crush injury and sham groups at each time point. Ratio of GFAP-positive satellite cells = GFAP positive encircled neurons/all neurons × 100 (%). Measurements are given as mean ± SD (n = 5 for each group at each time point). *$P < 0.05$, compared with each sham group.

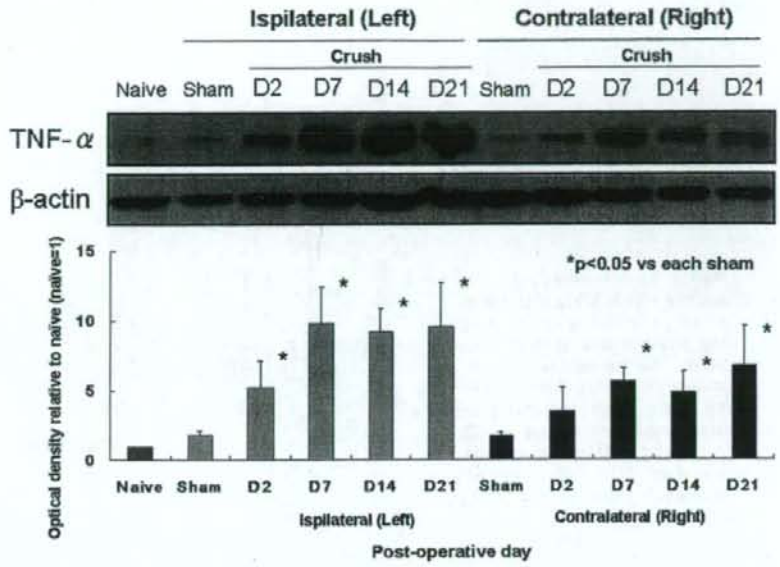

Figure 4. Expression of TNF-α proteins in bilateral DRGs in the crush injury group (days 2, 7, 14, and 21) and sham group (day 7). Expression levels were analyzed by immunoblotting using β-actin as an internal control. The ratio of expression levels was determined using Image J, and measurements were expressed using the ratio in naïve rat (n = 1) left L5 DRG considered as 1. Measurements are given as mean ± SD (n = 3 for each group at each time point). *$P < 0.05$, compared with each sham group.

(Figures 5A, D). In the crush injury group, hypertrophied GFAP-positive glial cells were expressed in both the ipsi- (left) and contralateral (right) spinal dorsal horn (Figures 5B, C). Conversely, in the crush injury group, numerous Iba-1-positive glia cells were expressed in the ipsilateral (left) spinal dorsal horn, but few Iba-1-positive glial cells were expressed in the contralateral (right) spinal dorsal horn (Figures 5E, F).

In the ipsilateral spinal dorsal horn, the percentage of GFAP-positive area in the crush injury group was significantly increased for 21 days after surgery compared with the sham group ($P < 0.05$), peaking at day 7 compared with the other days ($P < 0.05$). In the contralateral spinal dorsal horn, the percentage of GFAP-positive area was significantly increased for 21 days compared with the sham group ($P < 0.05$), and was significantly increased at days 7 and 14 compared with at days 2 and 21 ($P < 0.05$) (Figure 6). In addition, the percentage of Iba-1-positive area in the ipsilateral spinal dorsal horn of the crush injury group was significantly increased for 21 days compared with the sham group ($P < 0.05$), peaking at day 7 compared with the other days ($P < 0.05$), as seen with GFAP. In contrast, the percentage of Iba-1-positive area in the contralateral spinal dorsal horn was not significant for 21 days compared with the sham group (Figure 7).

■ Discussion

The present study demonstrated 3 major findings using a rat crush injury model. First, a hemilateral spinal nerve

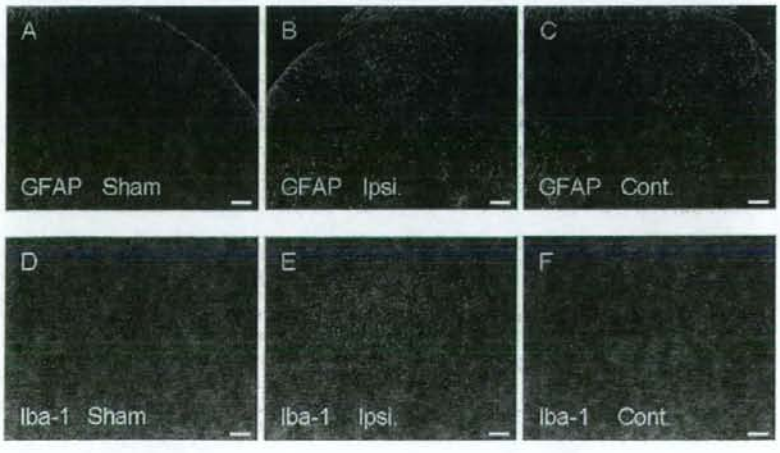

Figure 5. Immunofluorescence analysis of GFAP- and Iba-1-positive glia cells in bilateral L5 spinal dorsal horns at day 7. In the sham group, few GFAP-positive glia were seen in the contralateral (right) spinal dorsal horn **(A)**. In the crush injury group, hypertrophied and activated GFAP-positive glia cells (green) were observed in the ipsilateral (left) **(B)** and contralateral (right) spinal dorsal horn **(C)**. In the sham group, a few Iba-1-positive glia cells were seen in the contralateral (right) spinal dorsal horn **(D)**. In the crush injury group, numerous Iba-1-positive glia cells were observed in the ipsilateral (left) spinal dorsal horn **(E)**. Conversely, a few GFAP-positive glia cells were observed in the contralateral (right) spinal dorsal horn **(F)** in the crush injury group. Scale bar = 50 μm.

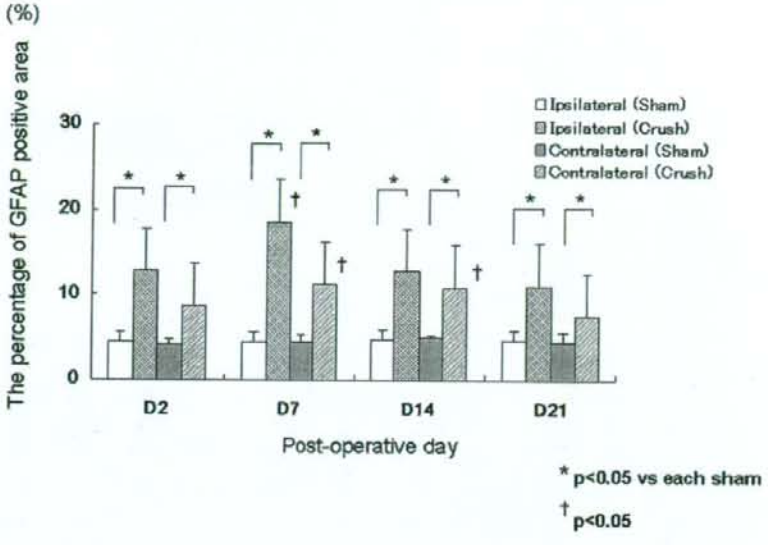

Figure 6. Percentage GFAP-positive area in bilateral L5 spinal dorsal horns in the crush injury and sham groups at each time point. The percentage GFAP-positive area was determined using Image J software. Measurements represent means ± SD (n = 6 for each group at each time point). *$P < 0.05$, compared with each sham group. †$P < 0.05$.

crush injury model induced contralateral mechanical allodynia, which gradually increased from early time points. Second, hemilateral spinal nerve injury induced increases in TNF-α expression and GFAP-positive satellite cells in the contralateral DRG from day 7. Third, hemilateral spinal nerve injury induced upregulation of astrocytes (not microglia) in the contralateral spinal dorsal horn from early time points. These results suggest that contralateral mechanical allodynia induced by hemilateral spinal nerve injury is associated with upregulation of satellite cells and TNF-α in the contralateral DRG, and that the spinal astrocytes play an important role in these contralateral changes.

We first explored bilateral mechanical allodynia for 21 days after surgery using a rat crush injury model, to evaluate how contralateral pain-related behaviors change over time. In the present study, mechanical allodynia on the contralateral side gradually increased from day 2 and peaked at days 14 and 21. Similarly, in the L5 nerve root chronic ligated model, contralateral mechanical allodynia gradually increases from early time points and peaks several days after surgery.[24,25] These findings suggest that some pathogeneses induce increasing contralateral mechanical allodynia for 14 days.

Secondly, we explored expression of TNF-α and GFAP in bilateral DRGs for 21 days after surgery, to evaluate whether hemilateral nerve injury induces pathologic changes in contralateral DRGs. In this study, expression of TNF-α- and GFAP-positive satellite cells in the contralateral DRG significantly increased from day

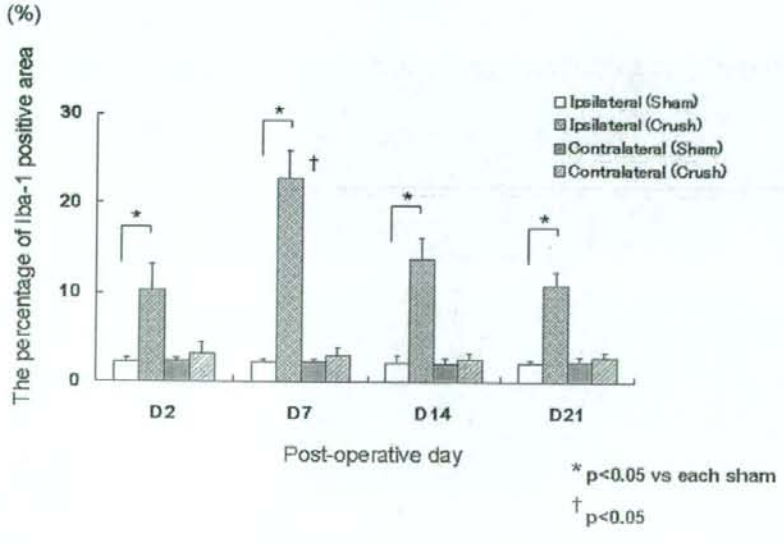

Figure 7. Percentage Iba-1-positive area in bilateral L5 spinal dorsal horns in the crush injury and sham groups at each time point. Percentage GFAP-positive area was determined using Image J software. Measurements represent means ± SD (n = 6 for each group at each time point). *$P < 0.05$, compared with each sham group. †$P < 0.05$.

7. These results suggest that the increase in TNF-α expression and GFAP-positive satellite cells in the contralateral DRG is associated with gradually increasing contralateral mechanical allodynia. Although much less is known about satellite cells in DRG, few studies have been reported. A recent study indicated that these cells in the sensory ganglion play important roles in nociception.[26] Injection of complete Freund's adjuvant (CFA) into the whisker pad area activates satellite cells in the trigeminal ganglion and induces increases in interleukin 1β (IL-1β)-positive satellite cells.[3] Peripheral nerve injury activates satellite cells in the ipsilateral DRG and induces an increase in TNF-α-positive satellite cells.[4] In the present study, the increase in TNF-α expression and GFAP-positive satellite cells showed the same tendency in bilateral DRGs. In addition, TNF-α was colocalized with GFAP-positive satellite cells. These findings thus suggest that the increase in GFAP-positive satellite cells is associated with increases in TNF-α expression in bilateral DRGs. Some pathologic analyses of contralateral DRGs have reported that expression of macrophages in the contralateral DRG increases as early as 14 days after surgery in a neuropathic pain model.[27,28] However, other pathologic findings have not previously been clearly reported. Our results offer the first finding of increased TNF-α expression and GFAP-positive satellite cells in the contralateral DRG. This finding is consistent with the time course of inducing contralateral mechanical allodynia. The present results suggest that the chemical and glial reactions in DRG might induce contralateral neuropathic pain.

Thirdly, we explored the expression of glial cells in bilateral spinal dorsal horns for 21 days after surgery, to evaluate whether the spinal cord represents one of the pathways inducing contralateral mechanical allodynia. In this study, astrocytes in the contralateral spinal dorsal horn were significantly expressed for 21 days after surgery compared with the sham group. In contrast, no significant difference in expression of microglia was apparent in the contralateral spinal dorsal horn for 21 days compared with the sham group. These results indicate that spinal astrocytes play an important role in inducing the contralateral mechanical allodynia, but spinal microglia do not. Astrocytes organize not only intercellular communications with surrounding neurons, but also widespread networks through gap junction in the nervous system.[29,30] In an inflammatory trigeminal model, activated spinal astrocytes exhibited hypertrophy and increased levels of connexin 43, an astrocyte gap junction protein.[31] Intrathecal administration of carbenoxolone, a gap junction decoupler, prevented mirror-image pain, but not ipsilateral mechanical allodynia.[32] According to these reports, we believe that excitation of activated astrocytes spreads from the ipsilateral to contralateral spinal dorsal horn through astrocyte gap junctions. Astrocytes also play an important role in initiating and regulating immune responses through the release of numerous proinflammatory cytokines, including TNF-α.[12,13] In sciatic nerve injury, TNF-α expression is upregulated in astrocytes of the ipsilateral spinal cord.[4] Intrathecal administration of proinflammatory cytokine antagonists [TNF-α, interleukin (IL)-1, IL-6] prevents mirror-image pain in a sciatic inflammatory neuropathy model.[33,34] In a ventral root transection model, TNF-α and TNF-α receptor 1 expression were upregulated in bilateral spinal dorsal horns, and colocalized with neurons, astrocytes, and microglia.[35] On the basis of these findings, expression of proinflammatory cytokines including TNF-α in spinal cord plays an important role in the pathogenesis of inducing contralateral mechanical allodynia, and activated contralateral spinal astrocytes might induce upregulation of TNF-α in the contralateral spinal dorsal horn. In the present study, activated astrocytes in the contralateral spinal dorsal horn peaked first at day 7, a finding consistent with increased TNF-α expression and GFAP-positive satellite cells in the contralateral DRG. Whether activated spinal astrocytes induce these changes in DRG of the same side remains unclear, but both antero- and retrograde axonal transport of TNF-α in the peripheral injured nerve has been demonstrated.[36,37] On the basis of these reports and our results, we believe that one of the mechanisms underlying mirror-image pain may be that upregulation of astrocytes transmit from the ipsilateral to the contralateral spinal cord through astrocyte gap junctions, and activated contralateral astrocytes upregulate proinflammatory cytokines, including TNF-α. Moreover, we believe that activation of TNF-α and satellite cells is induced from the contralateral dorsal horn to the contralateral DRG through axonal transport and/or some glial signal.

The present study explored bilateral mechanical allodynia until day 21 after surgery. In an L5 nerve root chronic ligated model, mechanical allodynia in bilateral hind paws returned to near baseline by day 42.[24] According to the present report, we believe that bilateral mechanical allodynia in this study gradually recovered after day 21. In this study, longitudinal changes to levels of proinflammatory cytokines in the contralateral spinal dorsal horn and interactions between spinal dorsal horn and DRG remain unclear. Additional studies are thus needed to elucidate these mechanisms.

In conclusion, this study provides new information on the upregulation of satellite cells and TNF-α in the contralateral DRG after hemilateral spinal nerve injury, and activated spinal astrocytes appear to play a significant role in the contralateral spread of neuropathic pain.

■ Key Points

- Hemilateral nerve injury induces contralateral mechanical allodynia, which increases gradually and peaks at days 14 and 21.
- In the contralateral DRG, TNF-α expression and GFAP-positive satellite cells increase from day 7 to day 21.

• In the contralateral spinal dorsal horn, hemilateral spinal nerve injury induces upregulation of astrocytes (not microglia) from early time points.

Acknowledgments

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Participation of 5-Hydroxytryptamine in Pain-Related Behavior Induced by Nucleus Pulposus Applied on the Nerve Root in Rats

Kinshi Kato, MD, Shin-ichi Kikuchi, MD, PhD, Shin-ichi Konno, MD, PhD and Miho Sekiguchi, MD, PhD

Study Design. The role of 5-hydroxytryptamine (5-HT) in sciatica in lumbar disc herniation (LDH) in rats was investigated.

Objective. We evaluated the effects of exogenous 5-HT applied on the nerve root on pain-related behavior, the release of endogenous 5-HT in plasma, and the expression of 5-HT2A receptors in dorsal root ganglion (DRG) in a rat LDH model.

Summary of Background Data. In previous studies, 5-HT2A receptor antagonists improved sciatica in patients with LDH and attenuated pain-related behavior induced by nucleus pulposus applied to the nerve root in rats.

Methods. Adult female Sprague-Dawley rats were divided into four experimental groups [control group; low-dose (10 μg) 5-HT-group; high-dose (30 μg) 5-HT-group; and autologous nucleus pulposus (NP) and saline group] and each drug was applied to the L5 nerve root. Von Frey tests were used for pain-behavior testing. To assess levels of endogenous 5-HT released in capillaries surrounding inflamed nerve roots, we measured 5-hydroxyindole acetic acid (5-HIAA), a metabolite of 5-HT. Expression of 5-HT2A receptors in the left L5 DRG was examined by immunohistochemical and immunoblotting analyses in the control and NP groups.

Results. Mechanical withdrawal thresholds of the high-dose 5-HT and the NP groups were significantly decreased after surgery compared with the control group and recovered after 14 days in the high-dose 5-HT group. 5-HIAA in plasma was increased by nucleus pulposus applied on the nerve root for 7 days after surgery. The expression of 5-HT2A receptors was enhanced in a time-dependent manner by nucleus pulposus.

Conclusion. Exogenous 5-HT to the nerve root induced pain-related behavior with short-lasting effects compared with the nucleus pulposus application. 5-HIAA content in plasma and expression of 5-HT2A receptors in DRG neurons increased early time points after the nucleus pulposus application. These results suggest that 5-HT plays a role in the early phase of the chemical pathogenesis of sciatica in LDH in rats.

Key words: lumbar disc herniation, sciatica, nucleus pulposus, dorsal root ganglion, serotonin, serotonin 2A receptor, 5-hydroxytryptamine (5-HT). **Spine 2008;33: 1330–1336**

Lumbar disc herniation (LDH) is a major cause of sciatica. The herniated disc induces sciatica by both mechanical and chemical means.[1–7] Several studies have demonstrated that various proinflammatory cytokines,[5–7] monoamine derived substances,[8] and other factors[9–12] play an important role in the chemical pathogenesis of sciatica. Serotonin (5-hydroxytryptamine [5-HT]), one of the monoamines, is an inflammatory mediator released from platelets in injured and inflamed tissues.[13–15] In the periphery tissues, 5-HT induces hyperalgesia in rats and humans. Several 5-HT receptor subtypes have been identified.[16] Among 5-HT receptor subtypes, 5-HT1A, 5-HT2A, and 5-HT3 receptors are involved in the sensitization of primary afferent fibers in the periphery.[17–21] In particular, 5-HT2A receptors are mainly expressed in small dorsal root ganglion (DRG) neurons and involved in the potentiation of inflammatory pain.[18–20]

In a clinical study, a selective 5-HT2A receptor antagonist was shown to improve sciatica in patients with LDH.[22] More recently, in an animal model, a selective 5-HT2A receptor antagonist was shown to attenuate pain-related behavior induced by nucleus pulposus applied to the nerve root.[8] Although 5-HT might be regarded as an important target for therapeutic intervention against sciatica in LDH, the pathophysiological importance of 5-HT in the chemical pathogenesis of sciatica is unclear. Moreover, it is still unknown how much and when 5-HT may affect pain-related behavior induced by nucleus pulposus applied to the nerve root in rats.

We hypothesized that nucleus pulposus applied to the nerve root might induce the immediate release of 5-HT from platelets in capillaries surrounding inflamed nerve roots and upregulation of the 5-HT2A receptors in DRG neurons. In this study, we compared the effect of nucleus pulposus applied to the nerve root with that of exogenous 5-HT on pain-related behavior. We also examined the release of endogenous 5-HT in plasma and the expression of 5-HT2A receptors in DRG in a rat model of LDH.

From the Department of Orthopaedic Surgery, Fukushima Medical University School of Medicine, Fukushima City, Japan.
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Address correspondence and reprint requests to Kinshi Kato, MD, Department of Orthopaedic Surgery, Fukushima Medical University School of Medicine, 1 Hikarigaoka, Fukushima City, Fukushima 960-1295, Japan; E-mail: kinshi@fmu.ac.jp

■ Materials and Methods

The experimental protocol was approved by the local animal ethics committee and conformed to Fukushima Medical University guidelines.

Animals and Anesthesia

A total of 141 adult female Sprague-Dawley rats (Japan SLC, Shizuoka, Japan) weighing 200–250 g were used. Rats were housed in plastic cages at 24°C ± 2°C in a 12-hour light–dark cycle with free access to food and water.

The animals were anesthetized by intra peritoneal injection of 30 mg/kg sodium pentobarbital (Nembutal 50 mg/m; Abbott Laboratories, North Chicago, IL).

Surgical Procedure

Rats were placed in a prone position and an incision was made at the spinal midline at level L4–L6. Using a surgical microscope, the thoracolumbar fascia was incised along the left side of the supraspinous ligament for approximately 20 mm. The paraspinalis muscles were gently moved laterally to expose the left L5–L6 facet joint. The L5 nerve root, DRG, and spinal nerve were exposed by L5/6 partial laminectomy with great care taken to avoid trauma to tissue. A 27-gauge needle connected to a microsyringe was inserted into the perineural space of the nerve root just distal to the DRG, and the substances described under "Experimental Groups" were slowly injected. All rat's paraspinalis muscles were sutured and the skin was closed with metal clips.

Experimental Groups

Rats were divided into 4 groups: a low-dose and high-dose 5-HT group, a nucleus pulposus (NP) plus saline group, and a control group. For the 5-HT group (n = 20 for behavioral testing), 10 μg and 30 μg of 5-HT (MP Biomedicals LLC, Irvine, CA) were dissolved in 100 μL of normal saline. Ten rats received 10 μg of 5-HT (low-dose 5-HT group) to the nerve root and the other 10 rats received 30 μg of the 5-HT (high-dose 5-HT group) to the nerve root. The doses of 5-HT were based on previous studies.[13,14,18] In the NP group (n = 10 for behavioral testing, n = 30 for high-performance liquid chromatography and immunoblotting, n = 20 for immunohistochemical examinations), rats received autologous NP, which was harvested from a coccygeal vertebral disc, to the DRG and 100 μL of normal saline to the nerve root at the same site as in the 5-HT group. In the control group (n = 60 for the same experiments as the NP group), rats received 100 μL of normal saline to the nerve root at the same site as in the 5-HT group.

Behavioral Test

Behavioral tests were performed in all groups (n = 10, each group) before and after surgery. All behavioral tests were performed by a technician who was unaware of the experimental groupings. Sensitivity to non-noxious mechanical stimuli was tested by the von Frey test. Baseline testing was performed before the start of the experiment to accommodate animals with normal responses.

The hind paw withdrawal response to von Frey hair (Stoelting, Wood Dale, IL) stimulation of the plantar surface of the footpads was determined at 2, 7, 14, and 21 days after surgery. The rat was placed individually in an acrylic cage with a mesh floor and was allowed to acclimate for 15 minutes, until cage exploration and major grooming activities ceased. The lateral-plantar surface of the operated hind paw, innervated by the L5 nerve,[23] was stimulated with 8 von Frey filaments (0.69, 1.20, 2.04, 3.63, 5.50, 8.51, 15.14, and 28.84 g) threaded under the mesh floor. The grams for von Frey hairs were based on the manufacturer's ratings. Stimulation was initiated with the 0.69-g filament. The filament was sequentially applied to the paw surface just until the filament bent, and was held for approximately 3 seconds. The response was considered positive if the hind limb indicated a lifting foot coupled with either licking or shaking of the foot as an escape response.

High-Performance Liquid Chromatography Analysis of Plasma 5-Hydroxyindole Acetic Acid

High-performance liquid chromatography (HPLC) examinations of plasma were performed in the NP and control groups (n = 5 in each group at 6 hours and 1, 7, 14, and 21 days after surgery). Rats were anesthetized with sodium pentobarbital. Before rats were killed by decapitation, blood samples (4.0 mL) were collected by heart puncture through a polyethylene tube (coated with heparin) and mixed with 1/10 volume of 1.5% disodium dihydrogen ethylenediamine tetraacetate dehydrate (EDTA-2Na). To obtain platelet-poor-plasma (PPP), the samples were centrifuged at 4000*g* for 10 minutes at 4°C. The supernatants (1500 μL) were stored at −20°C until assayed. 5-hydroxyindole acetic acid (5-HIAA), the metabolite of 5-HT, in PPP was measured by HPLC coupled with electrochemical detection. The lower detection limit of the method was 0.1 ng/mL.

Immunohistochemistry

Immnohistologic examinations were performed in the NP and control groups (n = 5 at 2, 7, 14, and 21 days after surgery). Rats were anesthetized using 99% diethyl ether (Wako Pure Chemical Industries, Osaka, Japan), and perfused with fresh 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS), and the L5 DRGs were removed. There were postfixed briefly in 4% paraformaldehyde and subsequently embedded in paraffin. Sections (6 μm) of DRGs were cut from each sample and placed on slides. Double-staining with 5-HT2A receptor and neuronal nuclei (NeuN) antibodies was performed as follows: Sections were deparaffinized with xylene and rehydrated with 100% ethanol. Nonspecific binding sites were blocked with 2% normal rabbit serum in PBS/Triton X-100 applied for 1 hour at room temperature. Rabbit antibody to 5-HT2A receptor (1:300; ImmunoStar Inc., Hudson, WI) was applied overnight at 4°C, followed by incubation with goat anti-rabbit Alexa 488 fluorescent antibody (1:200; Molecular Probes Inc., Eugene, OR) for 1 hour at room temperature. Next, 2% normal goat serum in PBS/0.3%Triton X-100 was applied for 1 hour. Subsequently, incubation with mouse antibody to NeuN (marker for neuron, 1:200; MAB377; Chemicon, Billerica, MA) was performed overnight at 4°C. Sections were rinsed in PBS and incubated for 1 hour at room temperature with goat anti-mouse Alexa 555 fluorescent antibody (1:200; Molecular Probes Inc.). After rinses, sections were mounted on microscope slides with VECTASHIELD® Mounting Medium with DAPI (H-1200, Vector, Burlingame, CA). DAPI (4′,6-diamidino-2-phenylindole) stains nuclei specifically, with little or no cytoplasmic labeling. Its blue fluorescence stands out in vivid contrast to green or red fluorescent probes of other structures. Fluorescent staining was analyzed using an Olympus Optical BX50 fluorescent microscope equipped with imaging software, Axio Vision (Carl Zeiss, Gettingen, Germany).

Immunoblotting for 5-HT2A Receptors

Immunoblotting examinations were performed in the NP and control groups (n = 5 at 2, 7, 14, and 21 days after surgery). Rats other than at 2 days after surgery were used after collecting blood samples for HPLC analysis. Rats were decapitated rapidly under anesthesia, and left L5 DRGs were removed and frozen in liquid nitrogen. Samples were homogenized in lysis buffer (#9803, Cell Signaling, Danvers, MA). The protein concentrations of samples were measured according to bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Twenty micrograms of total protein per lane were run on 10% Tris-glycine SDS-polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA) for 1.5 hours at 10 to 40 mA, and then transferred to polyvinylidene difluoride filter membranes (Millipore, Billerica, MA) for 1 hour at 15 V. The membranes were incubated with diluted primary antibody in 5% nonfat dry milk, 1X TBS, 0.1% Tween-20 at 4°C with gentle shaking, overnight. Primary antibodies used included 5HT2A receptor (1:150; Abcam Inc., Cambridge, MA) and mouse anti-β-actin (1:5000; Sigma, Saint Louis, MO). The membranes were incubated for 1 hour at room temperature with horseradish peroxidase conjugated secondary antibody (1:5000, Bio Rad). Positive bands were visualized using an enhanced chemiluminescence system (Amersham Bioscience, Piscataway, NJ). The positive bands of immunoblots were analyzed by ratio against internal control β-actin using an imaging analysis system (Image J, version 1.38u; National Institute of Mental Health, Bethesda, MD). As a positive control for these bands, we also analyzed 5-HT2A immunoreactivity in naïve rat (n = 1) brain lysate.

Statistical Analysis

All data were reported as mean ± SD. Statistical differences between any 2 groups were assessed using the Mann-Whitney *U*-test. Statistical differences among multiple groups were assessed using the Kruskal Wallis-H-test and the Bonferroni test. *P* values less than 0.05 were considered significant.

Results

Behavioral Test

Rats in all groups showed stable conditions before surgery in response to mechanical stimulation. In the NP group, the mechanical withdrawal thresholds were significantly decreased during the 21 days after surgery compared with the control group ($P < 0.01$). In the high-dose 5-HT group, mechanical thresholds were significantly decreased during the 7 days after surgery, in comparison with the control group ($P < 0.01$), and recovered after 14 days (Figure 1). There were no significant differences between the low-dose 5-HT group and control group during the 21 days after surgery. There were significant differences between the high-dose 5-HT group and the low-dose 5-HT group during the 7 days after surgery ($P < 0.05$ at day 2, $P < 0.01$ at day 7), and no significant differences after 14 days. There were no significant differences between the NP group and the high-dose 5-HT group during the 7 days after surgery, and significant differences after 14 days ($P < 0.01$ at day 14, $P < 0.05$ at day 21).

HPLC Analysis of Plasma 5-HIAA

At 6 hours, day 1, and day 7 after surgery, concentrations of plasma 5-HIAA in the NP group were significantly increased compared with the control group ($P < 0.05$ at 6 hours, day 1, $P < 0.01$ at day 7). At other time-points, there were no significant differences between the control and NP groups (Figure 2).

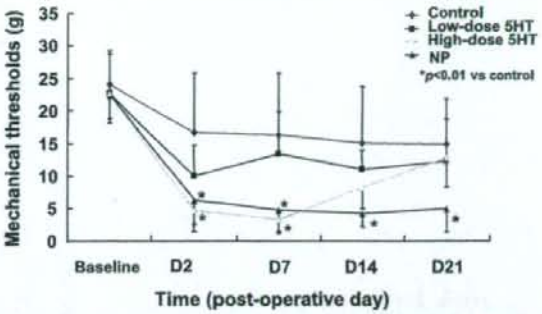

Figure 1. Changes in the mechanical withdrawal threshold of the foot pad in rats. Saline (control), 10 μg of 5-HT (low-dose 5HT), 30 μg of 5-HT (high-dose 5HT), and nucleus pulposus (NP) were administered into the left L5 DRG and mechanical withdrawal thresholds were measured using von Frey filaments. Data are means ± SD (n = 10 for each group). *$P < 0.01$, compared with the control group.

Immunohistochemical Analyses of 5-HT2A Receptors

5-HT2A receptors were expressed in the cytoplasm and beneath the cell membranes of neurons, colocalized with NeuN, in the L5 DRG (Figure 3A). In the control group, few 5-HT2A receptor-positive cells were seen during the 21 days after surgery (Figure 3B). On the other hand, in the NP group, some 5-HT2A receptor-positive cells were seen in the DRG during the 21 days after surgery (Figure 3C).

Immunoblotting for 5-HT2A Receptors

The expression of 5-HT2A receptors in the L5 DRGs in the NP and control groups at each time points were measured with enhanced chemiluminescence Western blots. 5-HT2A receptor bands in the DRGs were detected at 53 kDa. In the NP group, the expression level of 5-HT2A receptors was significantly increased during the 14 days after surgery in comparison with the control group ($P < 0.01$ at day 2 and 7, $P < 0.05$ at day 14). The expression level of 5-HT2A receptors in the NP group reached a maximum on day 7 and decreased in a time-dependent manner by day 21 (Figure 4).

Discussion

5-HT has recently been identified as a potential therapeutic target against sciatica in LDH. In the present study, using a rat LDH model, we demonstrated 3 novel findings. First, exogenous 5-HT applied to the nerve root induced short-lasting pain-related behavior compared with NP applied to the nerve root. Second, the release of endogenous 5-HT increased only at early time points. Third, 5-HT2A receptors were strongly expressed in DRG neurons at early time points and decreased in a time-dependent manner. These results suggest that 5-HT

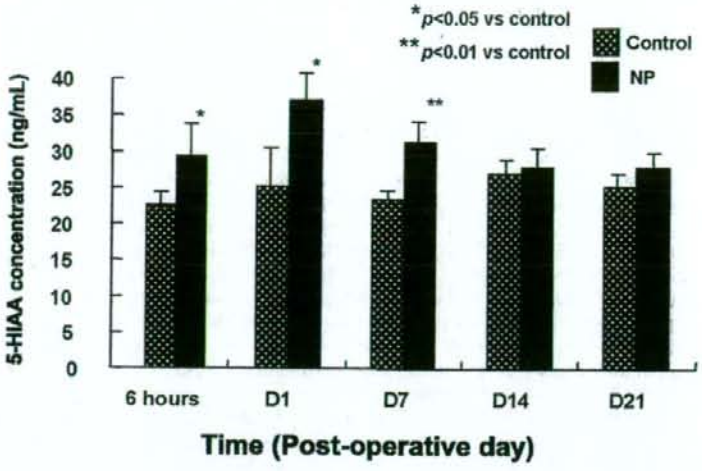

Figure 2. Plasma 5-HIAA concentrations after application of saline (control) and nucleus pulposus (NP) to the left L5 DRG. Data are means ± SD (n = 5 for each group). *$P < 0.05$, **$P < 0.01$, compared with the control group.

plays a role in the early phase of the chemical pathogenesis of sciatica in LDH.

We first evaluated whether exogenous 5-HT applied to the nerve root also induced the pain-related behavior observed after nerve roots had been exposed to herniated NP. It has been reported that intradermal injection of 5-HT into the rat hindpaw produces hyperalgesia in the paw *via* 5-HT1A, 5-HT2A, and 5-HT3 receptors.[17–21] In contrast, it has also been reported that intrathecally injected 5-HT shows antinociceptive effects in rat acute pain models *via* 5-HT1A, 5-HT1B, 5-HT2C, 5-HT3, and 5-HT4 receptors.[24–28] It has been widely accepted that the effects of 5-HT in the peripheral nerve system are totally opposite in the central nervous system, especially in the dorsal horn of the spinal cord, although there are slight discrepancies between reports. The blood-brain barrier, which 5-HT barely crosses, and the difference of 5-HT receptor subtypes have been implicated in the different effects of 5-HT on the peripheral and central nervous systems. The DRG has been reported to have a higher permeability compared with other nerve tissues, because the capillaries in the DRG are fenestrated and the blood-nerve barrier of nerve roots has not been sufficiently developed.[29,30] Moreover, there is a functional

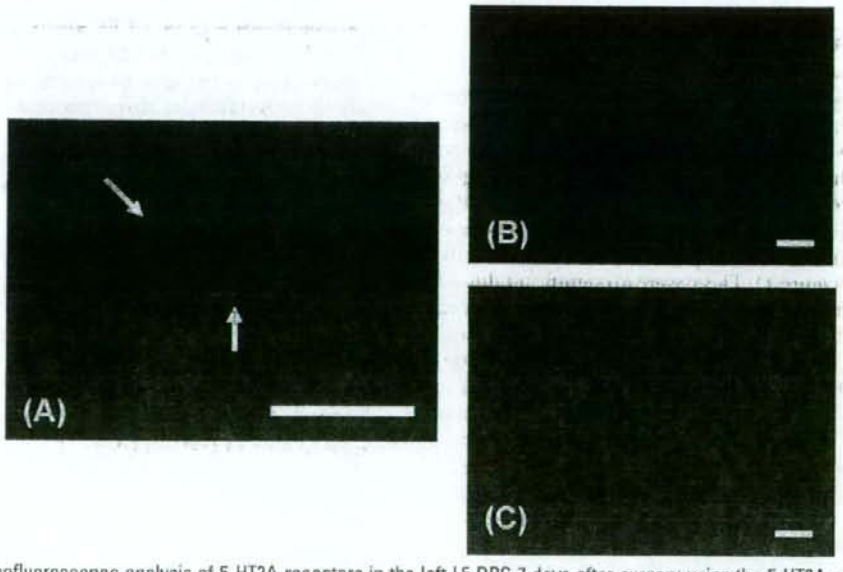

Figure 3. Immunofluorescence analysis of 5-HT2A receptors in the left L5 DRG 7 days after surgery using the 5-HT2A receptor antibody (green), NeuN (red), and DAPI (blue). 5-HT2A receptors were expressed in the cytoplasm and beneath the cell membranes (arrows) of neurons, colocalized with NeuN (**A**). In the control group, few 5-HT2A receptor-positive cells were seen in the L5 DRG (**B**). On the other hand, in the NP group, some 5-HT2A receptor-positive cells were seen in the L5 DRG (**C**). Scale bar = 25 μm.

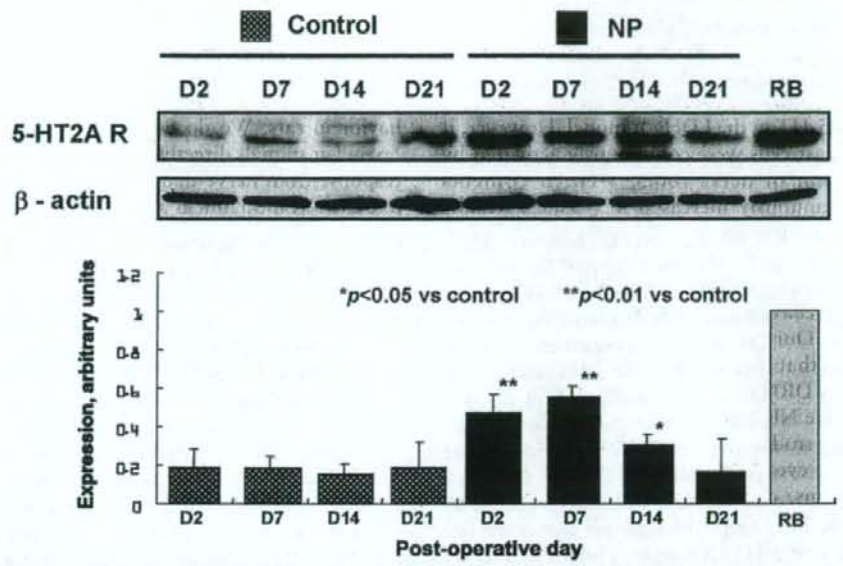

Figure 4. Expression of 5-HT2A receptor (5-HT2A R) proteins in left L5 DRGs in the control and NP groups. The expression levels were analyzed by immunoblotting using β-actin as an internal control. The ratio of the expression levels was determined by Image J (version 1.38u; National Institute of Mental Health, Bethesda, MD), and data were expressed with the ratio in naïve rat brain lysates (RB) considered a value of 1. Data are means ± SD (n = 5 for each group). $*P < 0.05$, $**P < 0.01$ compared with the control group.

pathway across the DRG capsule that allows access for epidurally applied macromolecules to the DRG endoneurial space.[31] Thus, before we attempted to apply the exogenous 5-HT to the nerve root, we could not determine whether the exogenous 5-HT induced the pain-related behavior or not. We preliminarily attempted to quantify the 5-HT contents in autologous NP, which was harvested (about 0.1 mg) from a coccygeal vertebral disc. However, HPLC determinations of 5-HT contents in the normal NP were barely detectable (data not shown). Thus, we believe that normal NP contains little or no 5-HT; the concentrations of 5-HT in the present study were based on previous studies.[13,14,18] Under pathologic conditions such as ischemia or thrombosis, extracellular 5-HT levels may reach as high as 100 μmol/L (42.5 μg/mL).[32] The concentrations of 5-HT applied in the present study (100 μg/mL and 300 μg/mL) were approximately two- to sevenfold higher than these values. However, we think that 5-HT applied to the nerve root in the present study might diffuse to surrounding tissue and be diluted. Thus, it might be possible that the actual concentration of 5-HT at the DRG endoneural space in the rat models was much lower than the concentration of 5-HT actually applied. Moreover, we preliminarily tried to analyze plasma 5-HIAA levels in the high-dose 5-HT group by HPLC. Plasma 5-HIAA levels in the high-dose 5-HT group were lower than in the NP group for all time points (data not shown). Therefore, we assume that the concentrations of 5-HT applied are pathophysiologically relevant in this regard, at least in this rat model.

Results from behavioral testing demonstrated that the higher dose of 5-HT applied to the nerve root induced pain-related behavior only at early time points but the lower dose of 5-HT did not. These results suggest that 5-HT surrounding the nerve root can induce pain-related behavior in rats, provided that the concentration of 5-HT is sufficient. Our results cannot clarify how 5-HT applied to the nerve root affect 5-HT receptors in the dorsal horn of the spinal cord. However, we believe that only a part of the 5-HT applied to the nerve root may penetrate into the DRG across the multilayered loose connective tissue and predominantly affect the DRG as the peripheral nervous system and not spinal cord. Further investigations are needed to evaluate the spinal antinociceptive effect of 5-HT in LDH model rats. In addition, results from behavioral testing also suggest that the effects of 5-HT related to pain-related behavior in LDH only last for a short period. We believe most of the endogenous 5-HT in LDH rat models is released from platelets and mast cells activated by the NP for the following reasons: (1) the prominent sources of peripheral 5-HT are platelets and mast cells in rats,[33,34] (2) NP provokes platelet activation in *in vitro* and *in vivo* experiments,[35,36] (3) autologous intervertebral disc applied to the nerve root induces nerve growth factor (NGF) produced by the inflammatory responses in the nerve root,[37] and (4) 5-HT released from mast cells is involved in NGF-mediated inflammation.[38]

Next, to evaluate whether the participation of endogenous 5-HT in pain-related behavior in the LDH rat model continues for a long period, we examined the concentration of the 5-HIAA in plasma for 21 days after surgery. Platelets and mast cells activated by the NP released 5-HT into the plasma in the capillary surrounding

inflamed tissue. Increased plasma 5-HT is reaccumlated rapidly by platelets[39] or rapidly metabolized to 5-HIAA by monoamine oxidase.[40] Thus, the concentration of 5-HT in plasma cannot be used as an index of the release of endogenous 5-HT in the LDH rat model. However, if platelets and mast cells were continuously activated by the inflammation of nerve roots, 5-HIAA contents should be continuously increased in plasma. Results from the HPLC measurements demonstrated that 5-HIAA contents in plasma increased for only 7 days. These results suggest that endogenous 5-HT plays a role in pain-related behavior only at early time points in the LDH rat model. Our results are consistent with a recent report showing that the level of 5-HT and 5-HIAA in nerve roots and DRG were unaffected on day 11 after application of the NP to lumbar nerve roots.[8]

In the present study, 5-HT2A receptors were strongly expressed in the cytoplasm and beneath the cell membranes of neurons, colocalized with NeuN, in the L5 DRG of the NP group, although we cannot exclude the possibility that 5-HT2A receptors might be expressed in the satellite cell cytoplasm. Among 5-HT receptor subtypes, 5-HT1A,[14,17] 5-HT2A,[18–20] and 5-HT3[21] receptors are involved in the sensitization of primary afferent fibers in the periphery. In particular, 5-HT2A receptors are mainly expressed in small DRG neurons and involved in the potentiation of inflammatory pain. 5-HT2A receptors are found on the axon of primary afferent neurons,[16,41] DRG,[16,19,42] dorsal horn of the spinal cord,[42] vascular smooth muscle cells, and platelets.[43] 5-HT binds to 5-HT2A receptors and activates adenyl cyclase to produce cyclic adenine monophosphate. It activates protein kinase A and closes K^+ channels, which depolarizes nociceptors and increases pain sensation.[14] We speculate that 5-HT contributes to pain-related behavior by binding to 5-HT2A receptors beneath the cell membranes of neurons.

Our immunoblotting results for 5-HT2A receptors demonstrated that the expression levels of 5-HT2A receptors were significantly higher compared with the control group for 14 days, reached a maximum on day 7, and decreased in a time-dependent manner by day 21. Our results suggest that 5-HT predominantly contributes to pain-related behavior at early time points *via* 5-HT2A receptors in the rat LDH model.

We hypothesized that NP applied on the nerve root would induce an immediate release of 5-HT from platelets and mast cells surrounding inflamed nerve roots and upregulation of the 5-HT2A receptors in DRG neurons. Previous studies have implied that the proinflammatory mediators, especially TNF-α, play a key role in the chemical pathogenesis of sciatica in LDH.[5–7] TNF-α is known to be produced and released from the chondrocyte-like cells of the NP.[5] In addition, TNF-α induces a procoagulant state by eliciting tissue factor production on the surface of vascular endothelium and monocytes, downregulating the protein C anticoagulant pathway and stimulating thrombin and fibrin formation.[44] Thus, TNF-α may contribute to the release of 5-HT indirectly by inducing intravascular coagulation.[45–47]

In the present study, we measured sensitivity to non-noxious mechanical stimuli to evaluate pain-related behavior in rats. We do not have a method to evaluate "pain" in animals directly. However, we can evaluate the response from nerve damage using pain-related behavior, such as mechanical allodynia and thermal hyperalgesia, in experimental studies. We believe that measuring the sensitivity to non-noxious mechanical stimuli of the food pad in behavior tests is one of the most valuable methods to evaluate pain-related behavior not only in a rat neuropathic pain model but also in a rat lumbar disc herniation model.[1,6,8] Although in the clinical situation, some patients with pain due to disc herniation have hypesthesia not allodynia in their foot, we rarely have patients with hyperalgesia-like severe sciatica due to disc herniation in the acute onset period of their symptoms. The time points of the experimental setting are different from the clinical situation. Therefore, we think that the pain-related behaviors such as paw allodynia in our rat models might indicate more severe and acute pathogenesis than that of common patients with pain due to disc herniation.

Results of the present study demonstrate that 5-HT plays a role in the early phase of the chemical pathogenesis of sciatica in LDH in rats. 5-HT may be an important target for therapeutic intervention against sciatica in LDH. We speculate that earlier administration of selective 5-HT2A receptor antagonists after the onset of sciatica in LDH may exert greater therapeutic effects. Further studies are required on the therapeutic effects of selective 5-HT2A receptor antagonists.

■ Conclusion

We demonstrated that exogenous 5-HT applied to the nerve root induced pain-related behavior and that effects only last for a short period when compared with NP. The release of endogenous 5-HT contents and the expression of 5-HT2A receptors increased at early time points in this LDH rat model. These results suggest that 5-HT may be involved in the early phase of the chemical pathogenesis of sciatica in LDH.

■ Key Points

- Exogenous 5-HT applied to the nerve root induces pain-related behavior that lasts for a shorter period compared with nucleus pulposus application.
- Nucleus pulposus applied to the nerve root induces the release of 5-HT and expression of 5-HT2A receptors in DRG neurons at early time points in a rat lumbar disc herniation model.
- 5-HT plays a role in the early phase of the chemical pathogenesis of sciatica in lumbar disc herniation.

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