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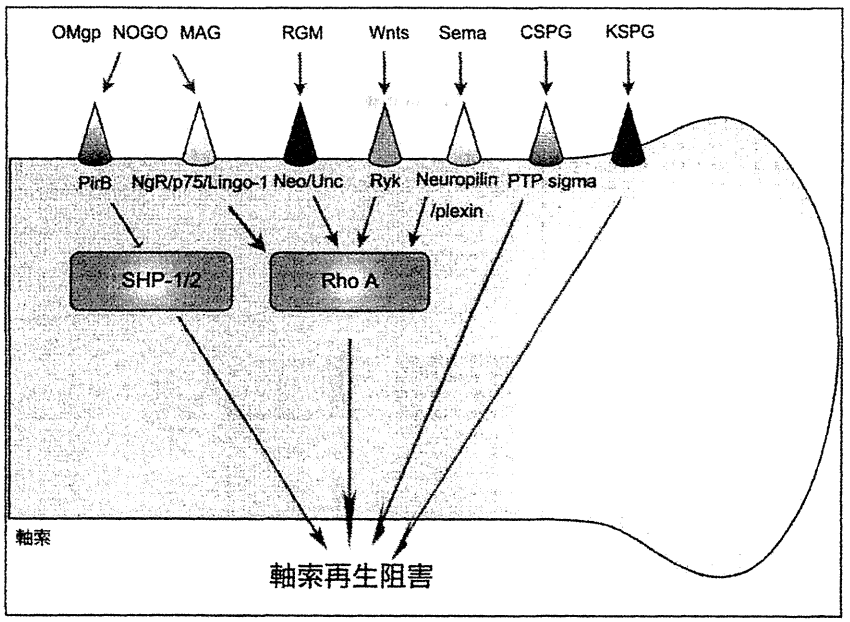
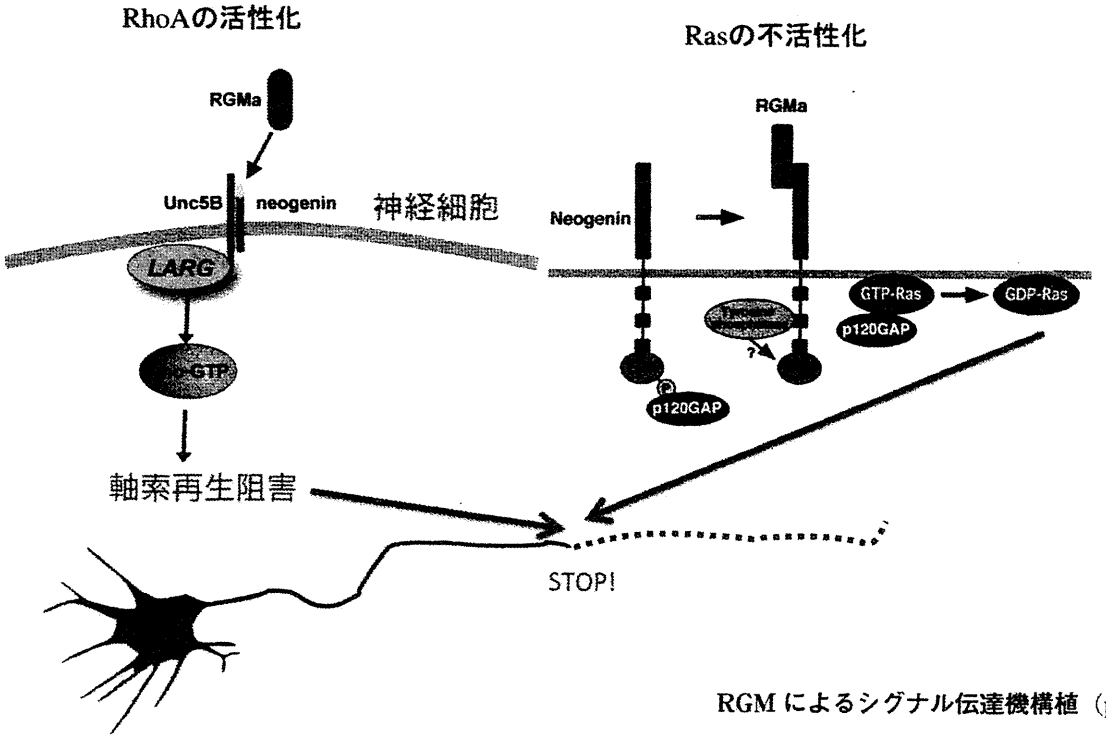
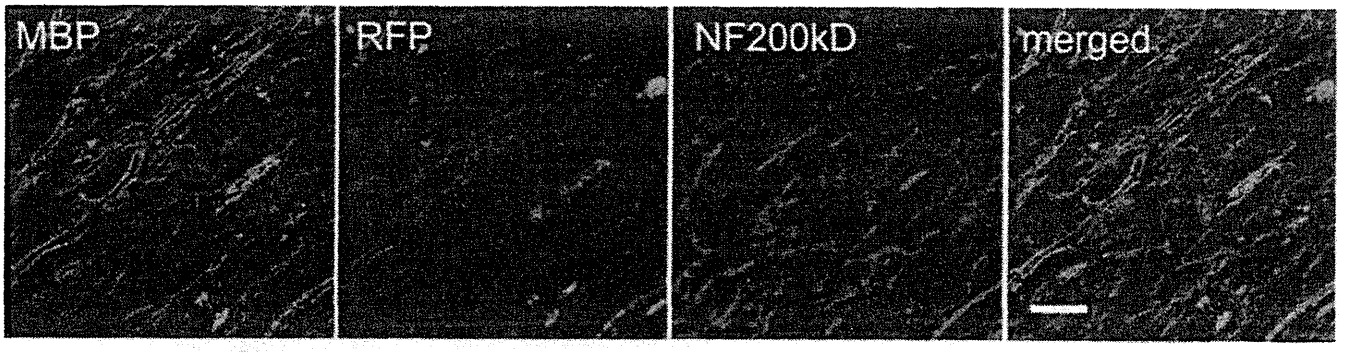


軸索再生阻害をもたらす複数のタンパク質（p.20）



RGM によるシグナル伝達機構植（p.20）



“安全な” iPS 細胞株由来ニューロスフェアの損傷脊髄への移植（p.37）

PAIN® 152 (2011) 1024–1032

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Changes in synaptic transmission of substantia gelatinosa neurons in a rat model of lumbar radicular pain revealed by in vivo patch-clamp recording

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Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

ARTICLE INFO

Article history:
Received 15 February 2010
Received in revised form 20 December 2010
Accepted 22 December 2010

Keywords:
Radicular pain
Nerve root injury
Substantia gelatinosa
Synaptic transmission
Patch clamp

ABSTRACT

Little is known about the pathophysiological mechanisms of radicular pain. We investigated changes in synaptic transmission of substantia gelatinosa (SG) neurons after an injury to the L5 nerve root using in vivo patch-clamp recording. A total of 141 SG neurons were recorded at L4 and L5 segmental levels of the spinal cord in root constriction rats and sham-operated control rats. At L4 and L5 segmental levels, SG neurons without a receptive field were observed only in root constriction rats, and the frequencies of spontaneous action potential firings in SG neurons were higher in the root constriction group than in the control group. At the L5 segmental level, the frequencies and amplitudes of spontaneous excitatory postsynaptic currents (EPSCs) as well as the proportion of multireceptive neurons among SG neurons was higher in the root constriction group than in the control group. At the L4 segmental level, the frequencies and amplitudes of spontaneous EPSCs were increased in the root constriction group, but the proportions of cell types did not change. The mean amplitudes of EPSCs evoked by mechanical stimuli at L4 and L5 segmental levels were larger in the root constriction group than in the control group. The results indicated that injuring the nerve root led to characteristic excitatory synaptic transmission in SG neurons at each segmental level and changed sensory processing in SG neurons at the segment to which the injured nerve projected. These changes could lead to spontaneous pain, mechanical allodynia, and hyperalgesia contributing to the pathogenesis of radicular pain.

1. Introduction

In clinical settings, radicular pain is commonly encountered as a symptom of neuropathic pain in patients with lumbar spinal canal stenosis or lumbar disc herniation. Radicular pain manifests as spontaneous pain and hyperalgesia that is felt in the gluteal region, thigh, leg, and foot. Radicular pain is difficult to relieve and develops into chronic neuropathic pain. To date, little is known about the pathophysiological mechanisms of radicular pain or why it develops into chronic neuropathic pain. Among many animal models of neuropathic pain, peripheral nerves are mostly injured at a site distal to the dorsal root ganglion (DRG) [2,16,33]. It is well known that neural hyperactivity of the DRG causes abnormal pain after a spinal nerve is injured at a site distal to the DRG [24]. However, most clinical causes of lumbar radicular pain in patients with lumbar canal stenosis or lumbar disc herniation result from an injury at a site proximal to the DRG. The causes of pain after a nerve root is injured may be different from those after a spinal nerve is injured because deafferentation occurs in the central portion of the nerve root in the former case [18]. An animal model of radicular pain in which a nerve root is ligated proximal to DRG was first reported [14] and later modified [17]. This model produces long-lasting mechanical allodynia and thermal hyperalgesia, which mimic clinical symptoms of lumbar radicular pain seen in patients with lumbar canal stenosis and lumbar disc herniation.

Radicular pain is thought to be caused by a series of changes in the sensory processing system, functional reorganization of sensory transmission, and the development of neural plasticity in the peripheral and central nervous systems. The substantia gelatinosa (SG; lamina II) [31] in the spinal cord dorsal horn receives primary afferent inputs from Aδ and C fibers, which predominantly convey nociceptive sensations [21,22,35]. The numerous γ-aminobutyric acid (GABA)ergic and glycinergic interneurons within the SG have an essential role in controlling pain [37,42]. Nociceptive information is thus modified and integrated in the SG, which

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doi:10.1016/j.pain.2010.12.039

consequently regulates the outputs of projection neurons located in lamina I and lamina VI–V [3,5], suggesting that the SG may be a therapeutic target for treating radicular pain.

In vivo patch-clamp recording from SG neurons is a useful method to analyze functional properties in synaptic transmission in response to naturally applied nonnoxious and noxious cutaneous stimuli [7,23].

The purpose of this study was to investigate changes in excitatory synaptic transmission of SG neurons after nerve root injury and to clarify the pathophysiological mechanisms of radicular pain using in vivo patch-clamp recording.

2. Materials and methods

2.1. Experimental animals

Six-week-old male Sprague–Dawley rats (n = 85) weighing 160 to 180 g at the beginning of the experiments were used. All experimental procedures were approved by the Animal Care and Use Committee of Sapporo Medical University School of Medicine, Sapporo, Japan, and Shinshu University, School of Medicine, Matsumoto, Japan. All efforts were made to minimize animal suffering and the number of animals used, and the experiments followed the ethical guidelines of the International Association for the Study of Pain [43].

2.2. Surgical procedures for producing the radicular pain model

Animals were randomly assigned to 2 different surgical groups: the root constriction group and the control group. The surgical procedure has been described in detail previously [17]. Briefly, after rats were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg), they were placed in the prone position, and a midline dorsal incision was made in the lower back to expose the laminas between the L4 and S1 vertebrae. The paraspinal muscles were retracted to expose the right L5–L6 facet joint. A right L5 hemilaminectomy and an L5–L6 partial facetectomy were performed. In the root constriction group, the right L5 spinal root was carefully exposed and tightly ligated extradurally with 8-0 nylon sutures proximal to the DRG. The subcutaneous tissue and skin were then closed with 4-0 nylon sutures. In the root constriction group, 53 rats (n = 10 for behavioral studies, n = 43 for patch-clamp recordings, as described below) underwent the operation. In the control group, 32 rats (n = 10 for behavioral studies, n = 22 for patch-clamp recordings, as described below) underwent the surgical procedure as described above without nerve root constriction.

2.3. Evaluation of mechanical hypersensitivity

The rats were placed in a Plexiglas chamber (IITC Life Science Inc, Woodland Hills, CA, USA), measuring 18 × 25 × 18 cm, above a wire mesh floor and were acclimatized to the environment for at least 20 minutes before the test. Mechanical stimuli were produced using a 3.8-g von Frey filament (Semmes–Weinstein Monofilaments, North Coast Medical Inc., San Jose, CA, USA) that was applied to the middle area between the foot pads on the plantar surface of the right and left hind paws. Each hind paw was probed consecutively by 10 stimulations alternating between the right and the left hind paw for each set. This was repeated 3 times at intervals of at least 10 minutes. Mechanical sensitivity was evaluated as the frequency of withdrawal responses. The mechanical withdrawal frequencies of each rat were expressed as the number of responses from the noninjured side subtracted from the number of responses from the injured side [17,27]. This procedure was performed 1 day before and 4, 7, 10, 13, 16, 19, 22, 25, and 28 days after surgery.

2.4. Evaluation of thermal hyperalgesia

The rats were placed in a Plexiglas chamber on a glass platform and allowed to acclimatize for at least 20 minutes before the test. A radiant heat source (Tail Flick Analgesia Meter, IITC Life Science Inc) was moved beneath the portion of the hind paw that was flush against the glass, and thermal stimulation was delivered to that site. Thermal hyperalgesia was evaluated as thermal withdrawal thresholds in response to noxious heat stimuli. The withdrawal latency was defined as the time from the onset of radiant heat to the withdrawal of the tested foot. A cut-off time of 10 seconds was set to prevent tissue damage. Each hind paw was tested 5 times with at least a 5-minute interval, alternating between the right and the left hind paw. The mean withdrawal latency was calculated from the last 4 measurements. Consequently, the thermal withdrawal latency of each rat was defined as the differential score, which was calculated by subtracting the withdrawal latency of the noninjured side from that of the injured side. This test was performed after evaluating mechanical sensitivity.

2.5. Estimation of spontaneous pain-related behaviors

The rats were placed in a Plexiglas chamber above a wire mesh floor and were allowed to acclimatize for 20 minutes before the test. Then, their behaviors were observed for 5 minutes. The degree of spontaneous behavior related to spontaneous pain was evaluated using a numerical scale as modified from a method described previously [1]. The scale used in this study was as follows: 0 = the paw of the operated side was pressed normally on the floor; 1 = the paw rested lightly on the floor; 2 = only the internal edge of the paw was pressed on the floor; 3 = only the heel was pressed on the floor, and the hind paw was in an inverted position; 4 = the whole paw was elevated; 5 = the animal licked the lesioned paw. The largest scale number was adopted to estimate the spontaneous pain-related behavior of the tested rat. This test was performed 11 to 15 days after surgery when mechanical hypersensitivity and thermal hyperalgesia had fully developed. Blinding procedures were used in these behavioral experiments.

2.6. Electrophysiological procedures

We performed in vivo patch-clamp recordings according to a previously described method [7,15,29,34]. The rats were used 11 to 15 days after surgery when mechanical hypersensitivity and thermal hyperalgesia had fully developed. The examiner was blinded to the 2 groups. The rats were artificially ventilated through a tracheotomy tube under anesthesia with intraperitoneal urethane (1.2 to 1.5 g/kg). Rectal temperature was maintained at 37 to 38°C with a heating pad. Laminectomy was performed from T13 to L3 levels, after which the rats were placed in a stereotaxic apparatus (Model ST-7, Narishige Group Co, Tokyo, Japan) and the spinal cord was carefully exposed. After removing the dura, the right L5 nerve root was observed microscopically to be a swelling with a darker color than the left L5 and the right L4 nerve roots. The pia-arachnoid membranes of the right L4 or L5 dorsal root entry zone were cut to create a window large enough to insert the patch electrode. The surface of the spinal cord was equilibrated with 95% O_2–5% CO_2 and irrigated with Krebs solution (in mM): 117 NaCl, 3.6 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 11 glucose, and 25 $NaHCO_3$ at 37.0 ± 0.5°C.

The patch electrodes were pulled from thin-walled borosilicate glass capillaries (outer diameter of 1.5 mm, TW150F-4, World Precision Instruments, Sarasota, FL, USA) using a puller (p-97, Sutter Instruments, Novato, CA, USA) and were filled with patch-pipette solutions composed of the following (in mM): 135 K-gluconate, 5 KCl, 0.5 $CaCl_2$, 2 $MgCl_2$, 5 EGTA, 5 ATP-Mg, 5 HEPES-KOH

(pH 7.2) for excitatory postsynaptic current (EPSC) recordings and current-clamp recordings, or 110 Cs_2SO_4, 0.5 $CaCl_2$, 2 $MgCl_2$, 5 EGTA, 5 ATP-Mg, 5 HEPES, 5 tetraethylammonium, CsOH (pH 7.2) for inhibitory postsynaptic current (IPSC) recordings. The recording electrode (resistance range, 8 to 12 MΩ) was advanced at an angle of 30° into the SG. After a gigaohm seal was formed with neurons at a depth of 50 to 150 μm from the surface of the spinal cord, the membrane patch was ruptured by negative pressure, and then the whole-cell patch-clamp recording was started. Recordings were made using a patch-clamp amplifier (Axopatch 200B, Axon Instruments, Union City, CA, USA). In the voltage-clamp mode, the holding potentials (V_H) were −70 and 0 mV, at which glycine-mediated and GABA-mediated IPSCs and glutamate-mediated EPSCs, respectively, were negligible [41]. Spontaneous excitatory postsynaptic potentials and action potentials (APs) were recorded in the current-clamp mode. Spontaneous and evoked EPSCs were recorded as described later. However, miniature EPSCs, which have been recorded from spontaneous EPSCs in the presence of 1 μM tetrodotoxin [6], were not recorded because it was difficult to measure miniature EPSCs correctly in the in vivo condition of the patch-clamp recordings. Whole-cell patch-clamp recordings were made from SG neurons at the L4 and L5 segments of the spinal cord in the root constriction and control groups. Data from neurons with resting membrane potentials more positive than −50 mV were excluded from this study.

After the electrophysiological experiment, some neurons that had been injected with biocytin during the recording were confirmed to be located in the SG as described previously [7,15,34]. The data were digitized with an A/D converter (Digidata 1322A, Axon Instruments), and analyzed with the Mini Analysis Program version 6.0.3 (Synaptosoft, Fort Lee, NJ, USA).

2.7. Stimuli to the receptive field

In the voltage-clamp mode, all SG neurons produce a large amplitude (>50 pA) barrage of EPSCs to nonnoxious or noxious stimuli such as a paintbrush or toothed forceps to the skin of the lumbar and pelvic regions or the hind paw [7,19,34]. The receptive field of a neuron was carefully determined after repeated stimuli using a paintbrush or toothed forceps to the skin of the abdominal wall, lumbar and pelvic regions, the hind paw, and the tail. We confirmed the 8 points at the edge of the receptive field by stimulating them and marking them with a felt pen. As the center of the 8 points was the most sensitive to stimuli, we defined the point as the stimulus location in the receptive field. If evoked EPSCs were not observed during the stimuli, that neuron was defined as one with no receptive field.

After determining the receptive field, the responses to nonnoxious stimuli were assessed with a puff of air (200 psi) through a pipette (outer diameter of 200 μm) applied repetitively to the center of the receptive field (duration of injection, 100 ms; frequency, 10 Hz; injection time, 10 seconds) using a pico-injector (PLI-100, Harvard Apparatus, Holliston, MA, USA), according to a previously described method [15]. The air-puff stimuli did not cause pain to the examiners. The noxious pinch stimuli were applied with toothed forceps that were firmly fixed on a rod. A weight (60 g) was placed on the forceps for 5 seconds, as referred to in previous reports [15,34]. SG neurons exhibited several response profiles during air-puff or pinch stimuli in the current-clamp mode [8,23]. We classified neurons into 4 types based on a classification schema reported previously [15]: (1) multireceptive type if they exhibited action potentials (APs) in response to nonnoxious stimuli and noxious stimuli, (2) nociceptive type if they responded only to noxious stimuli, (3) subthreshold type if they responded to nonnoxious stimuli and noxious stimuli with a small depolarization that failed to reach the AP threshold, and (4) light-touch type if they responded maximally to nonnoxious stimuli enough to reach the AP threshold. Light-touch type neurons were excluded in the present study because they may be located in lamina III or deeper. Spontaneous firing rates of APs and frequencies and amplitudes of spontaneous EPSCs were calculated for an average of 3 minutes when there were no stimuli in the receptive field. The air-puff and pinch stimuli were repeated 3 times to evaluate evoked activities of neurons. The mean frequencies and amplitudes of evoked EPSCs for each SG neuron during the stimulation were calculated by the values of the 3 records.

2.8. Statistical analysis

All numerical data are expressed as the mean ± SEM. The behavioral test data were analyzed with a 2-way repeated-measures analysis of variance. Changes in parameters and electrophysiological data between the 2 groups were compared with the unpaired Student's *t*-test and the Mann–Whitney *U* test. The ratios of neuron types with respect to response to air-puff (nonnoxious) and pinch (noxious) stimuli were analyzed by the chi-square test. $P < .05$ was considered statistically significant.

3. Results

3.1. Behavioral tests

The time courses of withdrawal frequencies in the root constriction group (n = 10) and the control group (n = 10) in response to mechanical stimuli using a 3.8-g von Frey filament are shown in Fig. 1A. The withdrawal frequencies in the root constriction group were increased significantly from 4 days after the nerve root injury and thereafter compared with those in the control group ($P < .05$). The differential score for noxious thermal stimuli was significantly lower in the root constriction group than in the control group from 4 days after the injury and thereafter ($P < .05$, Fig. 1B).

The scale number to evaluate the degree of spontaneous behavior related to spontaneous pain 11 to 15 days after the root constriction or sham surgery is shown in Fig. 1C. All rats in the control group showed normal positioning of the hind paw (n = 10 with scale 0), and so the median of the scale number was zero. All rats in the root constriction group exhibited abnormal positioning of the lesioned hind paw, and the median of the scale number was 2 ($P < .001$).

3.2. In vivo patch-clamp recordings

The recordings were usually obtained from SG neurons in a stable condition for 10 to 30 minutes, and stable recordings were maintained in several neurons for more than 3 hours. A total of 141 SG neurons was recorded in 65 animals. In the root constriction group, 57 SG neurons and 29 SG neurons were recorded at the L5 and the L4 segmental levels of the spinal cord, respectively. In the control group, 36 SG neurons and 19 SG neurons were recorded at the L5 and the L4 segmental levels, respectively. No significant differences in the mean depths of the recording sites were observed from the surface of the spinal cord, resting membrane potential, or input resistance of SG neurons between the 2 groups (Table 1). In the control group, no significant differences were observed between neurons at the L5 segmental level and those at the L4 segmental level.

3.3. Changes in spontaneous AP firing rates and SG neuron cell types at the L5 segmental level of the spinal cord

In the current-clamp mode, typical recording traces of spontaneous excitatory postsynaptic potentials and/or APs in SG neurons

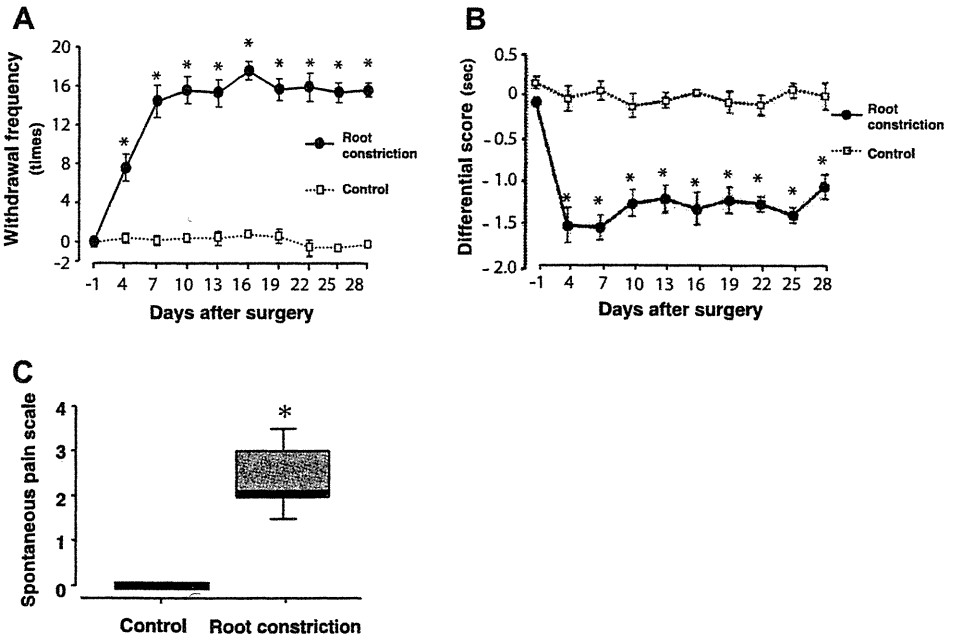

Fig. 1. The time course for evaluating mechanical hypersensitivity and thermal hyperalgesia in root constriction rats (n = 10) and control rats (n = 10). (A) Mechanical sensitivity was evaluated 30 times as the frequency of withdrawal in response to mechanical stimulation of 3.8 g applied to the middle area between the foot pads on the plantar surface of the right and left hind paws. The mechanical withdrawal frequencies for each rat were expressed as the number of responses from the noninjured side subtracted from the number of responses from the injured side. Root constriction rats (black circles) showed mechanical hypersensitivity from days 4 to 28 after the nerve root injury compared with control rats (white squares). (B) Thermal hyperalgesia was evaluated using the differential score. The differential score for each rat was the thermal withdrawal latency of the noninjured side subtracted from the thermal withdrawal latency of the injured side. The withdrawal latency was defined as the time from the onset of radiant heat to the withdrawal of the tested foot. The mean withdrawal latency was calculated from the last 4 measurements. The differential score was significantly lower in root constriction rats than that in control rats from day 4 and thereafter. Data are expressed as the mean ± SEM. *$P < .05$ compared with control rats; 2-way repeated-measures analysis of variance. (C) The degree of spontaneous pain-related behavior was evaluated using a numerical scale (see text) 11 to 15 days after nerve root constriction or sham surgery, when the electrophysiological experiment was conducted. The results are expressed as medians with 1st and 3rd quartiles, and 10th and 90th percentiles. All control rats (n = 10) showed normal positioning of the hind paw, so the median of the scale number was zero. All root constriction rats (n = 10) showed abnormal positioning of the lesioned hind paw, and the median of the scale number was 2. *$P < .001$ (Mann–Whitney U test).

Table 1
Cell locations and membrane properties of SG neurons.

Groups	Segment	Recording depth (μm)	RMP (mV)	IR (MΩ)
Control	L4	110 ± 6 (19)	−57.4 ± 1.2 (19)	302 ± 39 (9)
	L5	111 ± 5 (36)	−59.7 ± 1.8 (36)	277 ± 28 (11)
Root constriction	L4	109 ± 4 (29)	−59.2 ± 0.9 (29)	287 ± 26 (14)
	L5	109 ± 4 (57)	−58.2 ± 0.7 (57)	292 ± 19 (23)

Values expressed as the mean ± SEM, with the number of neurons in parentheses. Recording depth was determined as the distance between the electrode tip and the surface of the spinal cord. There were no significant differences in recording depth, RMP, or IR between SG neurons obtained from control rats and root constriction rats ($P > .05$). IR, input resistance; RMP, resting membrane potential; SG, substantia gelatinosa.

of the control and root constriction groups are shown in Fig. 2A. The incidence of APs in SG neurons in the root constriction group recorded from the L5 segmental level of the spinal cord was higher than that in the control group (34% vs 8%, $P < .05$, Fig. 2B). The mean frequency of AP firings was also significantly higher in the root constriction group than in the control group ($P < .01$) (Fig. 2C). Receptive fields were found in all SG neurons recorded from control rats. These fields were on the right lumbar and sacral regions, posterior or lateral aspect of the hind paw, and the plantar surface. In the root constriction group, SG neurons with no receptive field were seen and the proportion of these neurons was 16%. However, the receptive fields of the other SG neurons in the root constriction group were located in almost the same area as those in the control group.

Examples of cell types recorded in SG neurons are shown in Fig. 3A. The proportions of multireceptive, nociceptive, and subthreshold neurons in the control group were 18%, 33%, and 49%, respectively (Fig. 3B). In the root constriction group, with the exception of the neurons with no receptive field, the proportions of these neurons were 43%, 16%, and 41%, respectively. A significant difference in the proportions of the cell types was observed between the 2 groups ($P < .05$) (Fig. 3B).

3.4. Changes in spontaneous AP firing rates and SG neuron cell types at the L4 segmental level of the spinal cord

There was a tendency for an increased incidence of APs in SG neurons in the root constriction group recorded from the L4 segmental level of the spinal cord compared with that from the control group (21%, 6 of 28 vs 11%, 2 of 19, respectively), although no statistically significant difference was observed between the 2 groups ($P > .05$). The mean frequency of AP firings from the root constriction group was significantly higher than that from the control group (0.05 ± 0.02 Hz vs 0.01 ± 0.01 Hz, $P < .01$) (Fig. 4A). There was 1 SG neuron (4%) with no receptive fields in the rats in the root constriction group.

The proportions of multireceptive, nociceptive, and subthreshold neurons in the control group were 17%, 33%, and 50%, respectively (Fig. 4B). In the root constriction group, except for neurons with no receptive field, the proportions of these neurons were 15%, 30%, and 55%, respectively. No significant difference was observed between the 2 groups.

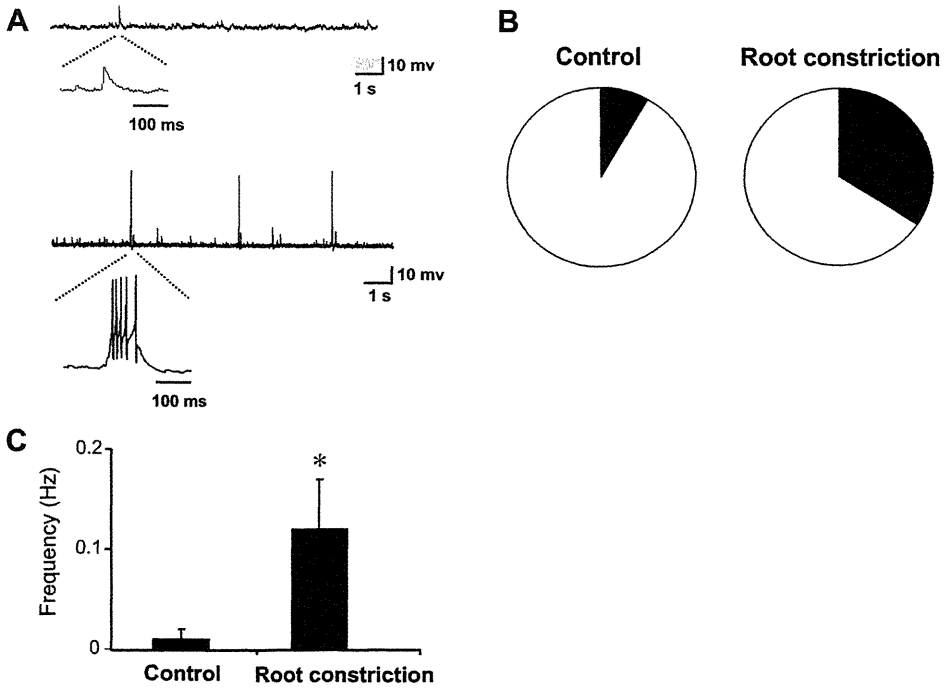

Fig. 2. Spontaneous activities in the substantia gelatinosa (SG) neurons at the L5 segmental level. (A) Typical recording traces of spontaneous excitatory postsynaptic potentials (EPSPs) and/or action potentials (APs) in SG neurons of control rats (upper panel) and root constriction rats (lower panel) recorded in the current-clamp mode. (B) Spontaneous AP firings were recorded in 34% (15 of 44) of the SG neurons in root constriction rats and in 8% (3 of 36) of control rats ($P < .05$; chi-square test). (C) The mean frequency of spontaneous AP firings obtained from SG neurons (n = 44) in root constriction rats was significantly higher than that from SG neurons (n = 36) in control rats (0.12 ± 0.05 and 0.01 ± 0.01 Hz, respectively, $P < .01$; Mann–Whitney U test). Error bars indicate SEM. *$P < .01$ compared with control rats.

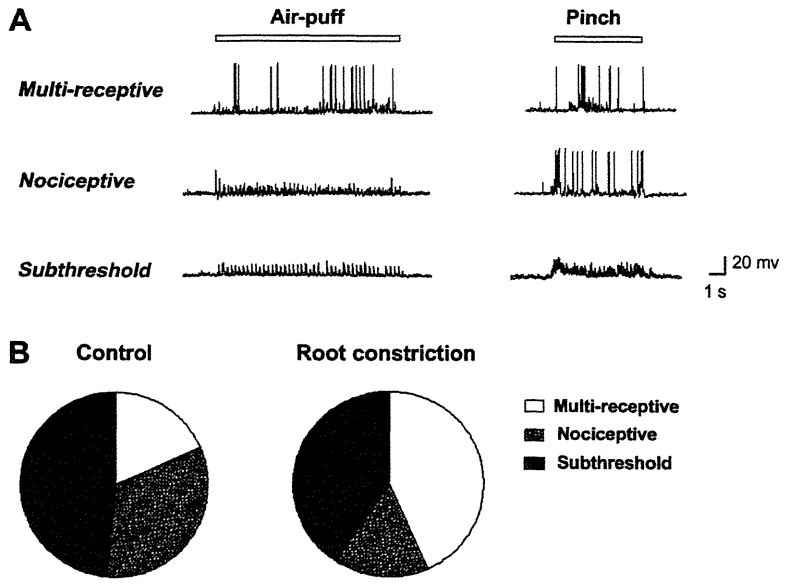

Fig. 3. Substantia gelatinosa (SG) neurons cell types at the L5 segmental level. (A) Examples of cell types recorded in SG neurons in the current-clamp mode. Multireceptive type showed action potentials (APs) in response to air-puff stimuli and pinch stimuli (upper panel). Nociceptive type showed APs only in response to pinch stimuli (middle panel). Subthreshold type responded to air-puff stimuli and pinch stimuli with a small depolarization that failed to reach the AP threshold (lower panel). (B) Pie graphs show the ratio of each cell type identified in recordings from the control and root constriction groups. The proportion of multireceptive neurons was higher in the root constriction group (43%, 16 of 37) than in the control group (18%, 6 of 33, $P < .05$; chi-square test).

3.5. EPSCs in SG neurons after a nerve root injury

All SG neurons exhibited spontaneous EPSCs and SG neurons that had a receptive field produced a large amplitude (>50 pA) barrage of EPSCs in response to air-puff or pinch stimuli to the skin of the gluteal region or hind paw at a holding potential (V_H) of −70 mV (Fig. 5). The mean frequencies and amplitudes of spontaneous EPSCs recorded at L4 and L5 segmental levels in SG neurons

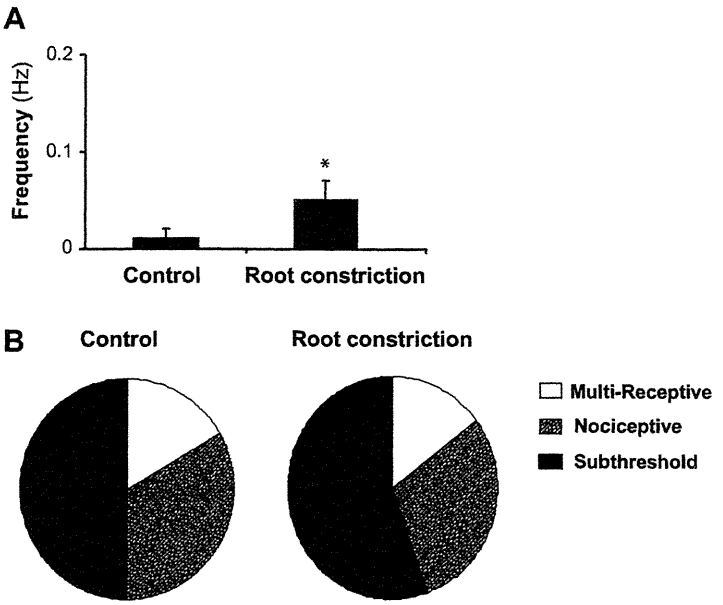

Fig. 4. The spontaneous action potential (AP) firings and substantia gelatinosa (SG) neuron cell types at the L4 segmental level. (A) The mean frequency of spontaneous AP firings obtained from SG neurons (n = 28) in the root constriction group was significantly higher than that from SG neurons (n = 19) in control rats (0.05 ± 0.02 and 0.01 ± 0.01 Hz, respectively, $P < .01$; Mann–Whitney U test). Error bars indicate SEM. *$P < .01$ compared with control rats. (B) Pie graphs show the ratio of each neural cell type in the control group and the root constriction group. No statistical differences were observed between the proportion of all 3 types of neurons in the 2 groups ($P > .05$; chi-square test).

in the root constriction group were higher and larger than those at the same segmental levels in the control group ($P < .05$) (Table 2).

Although the mean frequencies of EPSCs evoked by the air-puff stimuli in SG neurons at L4 and L5 segmental levels in the root constriction group were not significantly different from those in the control group, the mean amplitudes of EPSCs evoked by the air-puff stimuli in the root constriction group were significantly larger than those in the root constriction group ($P < .05$) (Table 2).

Similarly, the mean frequencies of EPSCs evoked by the pinch stimuli in SG neurons at L4 and L5 segmental levels in the root constriction group were not significantly different from those in the root control group, but the mean amplitudes of EPSCs evoked by the pinch stimuli in the root constriction group were significantly larger than those in the control group ($P < .05$) (Table 2).

3.6. Spontaneous IPSCs in SG neurons at the L5 segmental level of the spinal cord

All SG neurons tested exhibited spontaneous IPSCs at a holding potential (V_H) of 0 mV. Spontaneous IPSCs were recorded from 13 neurons in the root constriction group and from 16 neurons in the control group. The mean frequency of spontaneous IPSCs from the root constriction group was significantly lower than that from the control group (15 ± 1 vs 18 ± 1 Hz, $P < .05$). The mean amplitudes of spontaneous IPSCs in the root constriction group showed a tendency to decrease compared with those in the control group, but no significant difference was observed between the 2 groups (33 ± 5 vs 36 ± 4 pA, $P > .05$) (Fig. 6).

4. Discussion

The results of the present study can be summarized as follows. (1) Constriction of the L5 root resulted in spontaneous pain-related behavior, mechanical hypersensitivity, and thermal hyperalgesia that mimicked clinical symptoms of radicular pain seen in humans. (2) No receptive fields were seen in some SG neurons obtained from the root constriction group, and the incidences and frequencies of spontaneous AP firings in SG neurons at L4 and L5 segmental levels were higher in the root constriction group than those in the control group. (3) The frequencies and amplitudes of spontaneous EPSCs in the root constriction group at L4 and L5 segmental levels were higher and larger than those in the control group. (4) The proportion of multireceptive neurons at the L5 segmental level, but not at the L4 segmental level, in the root constriction group increased compared with the control group. (5) The frequencies of spontaneous IPSCs were lower in the root constriction group than in the control group.

4.1. Possible mechanisms of spontaneous pain after root constriction injury

Mechanical allodynia/hyperalgesia and thermal hyperalgesia develop during the first week after peripheral nerve injuries in scenarios such as tight ligation of a spinal nerve [16], chronic constriction injury of the sciatic nerve [2], and spared nerve injury models [28]. In the present study, mechanical allodynia/hyperalgesia and thermal hyperalgesia was seen 4 days after the root constriction injury and thereafter. Spontaneous pain-related behavior was also seen 11 to 15 days after the root injury when mechanical allodynia/hyperalgesia and thermal hyperalgesia had fully developed. Thus, pain-related behaviors after the root constriction injury as well as peripheral nerve injuries in the rats seem to be similar to clinical symptoms of radicular pain seen in humans.

Wallerian degeneration, which is anterograde degeneration, occurs in primary afferents of the L5 nerve root after the L5 root constriction injury [12], resulting in a decrease in the number of synapses between primary afferents and spinal dorsal horn neurons. This could explain why several SG neurons at the L5 segmental level did not have any receptive fields after L5 root constriction.

Wallerian degeneration could also influence dorsal root terminals and lead to an alteration in the properties of the neighboring uninjured afferents [39]. The rate of spontaneous APs firing

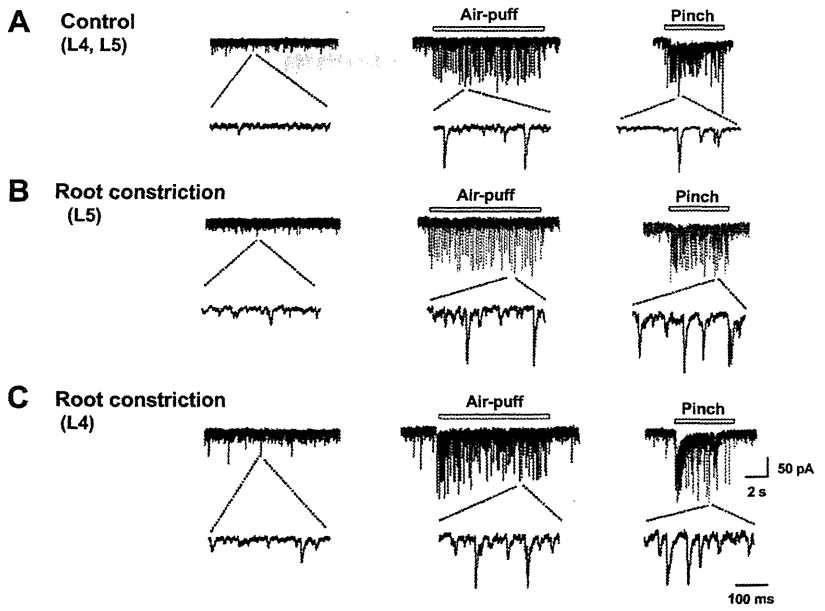

Fig. 5. Sample recording traces of excitatory postsynaptic currents (EPSCs) in substantia gelatinosa (SG) neurons at L4 and L5 segmental levels in the control group (A), SG neurons at the L5 segmental level in the root constriction group (B), and SG neurons at the L4 segmental level in the root constriction group (C). Spontaneous EPSCs (left panels) and evoked EPSCs in response to nonnoxious (air-puff) (middle panels) and noxious (pinch) stimuli (right panels). Recording were performed in the voltage-clamp mode ($V_H = -70$ mV). The frequencies and amplitudes of spontaneous EPSCs were significantly higher in the constriction group than in the control group ($P < .05$). The amplitudes of evoked EPSCs in response to the air-puff and pinch stimuli were significantly higher in the root constriction group than in the control group ($P < .05$), although the frequencies of evoked EPSCs were not significantly higher in the root constriction group than in the control group (see also Table 2).

Table 2
Frequencies and amplitudes of spontaneous and evoked EPSCs.

Groups	Spontaneous EPSCs			Evoked EPSCs			
	Segment	Frequencies (Hz)	Amplitudes (pA)	Air-puff stimuli		Pinch stimuli	
				Frequencies (Hz)	Amplitudes (pA)	Frequencies (Hz)	Amplitudes (pA)
Control (n = 16)	L4	8 ± 1	16 ± 1	20 ± 2	28 ± 2	26 ± 2	34 ± 3
Root constriction (n = 26)	L4	16 ± 2*	21 ± 1*	24 ± 2	38 ± 3*	29 ± 2	42 ± 2*
Control (n = 20)	L5	10 ± 1	17 ± 1	20 ± 3	27 ± 2	24 ± 2	31 ± 3
Root constriction (n = 21)	L5	16 ± 2*	20 ± 1*	25 ± 2	32 ± 3*	28 ± 2	42 ± 3*

Excitatory postsynaptic currents (EPSCs) in substantia gelatinosa (SG) neurons occurring spontaneously and in response to air-puff and pinch stimuli in the voltage-clamp mode ($V_H = -70$ mV). Values expressed as the mean ± SEM. N refers to the number of neurons.
* $P < 0.05$ compared with the control rats at the same segmental level.

increased in root constriction rats. It might be caused by changes in synaptic transmission of SG neurons after a nerve root had been injured. The number of neurons with spontaneous activity increases after transection of the nerve root [4]. The results of the present study showed that after the L5 dorsal root was injured, the spontaneous AP firing rate and the number of neurons generating spontaneous APs was higher in root constriction rats than in control rats, and that those at the L5 segmental level were higher than those at the L4 segmental level in root constriction rats. The injured nerve root might have affected not only the segment to which the dorsal root projected, but also the adjacent segment in the spinal cord, even if it was a single root injury. It is likely that such spontaneous AP firings contribute to occurrence of spontaneous pain seen in patients with lumbar radicular pain.

4.2. Possible mechanisms of allodynia/hyperalgesia after root constriction injury

SG neurons are heterogeneous in terms of morphological [9,40], immunohistochemical [36], functional [25], and electrophysiological properties [8,9]. In the present study, based on the responses to mechanical stimuli, the proportions of the types of SG neurons in the spinal cord at the level of the L5 segmental level, but not the L4 segmental level, varied after the nerve constriction injury [8,15]. At the L5 segmental level of the spinal cord, the proportion of multireceptive neurons in the root constriction group increased significantly, and the proportions of nociceptive and subthreshold neurons decreased, compared with those in the control group. These results suggest that some SG neurons, which responded only to noxious stimuli, began to respond to nonnoxious stimuli as well as noxious stimuli after the root constriction injury. Thus, the root constriction injury could produce functional changes in SG neurons in response to mechanical stimuli, possibly resulting in mechanical hyperalgesia and/or allodynia.

After a root constriction injury, collateral sproutings from primary afferents seem to form. Primary afferents may induce different types of fibers to cross, and inputs from the fibers that conveyed tactile sensation, such as Aβ fibers, might be amplified through interneurons [11]. Sensory processing in SG neurons at the segment to which the injured nerve projected was changed after the nerve root was ligated, which could have led to mechanical allodynia and hyperalgesia.

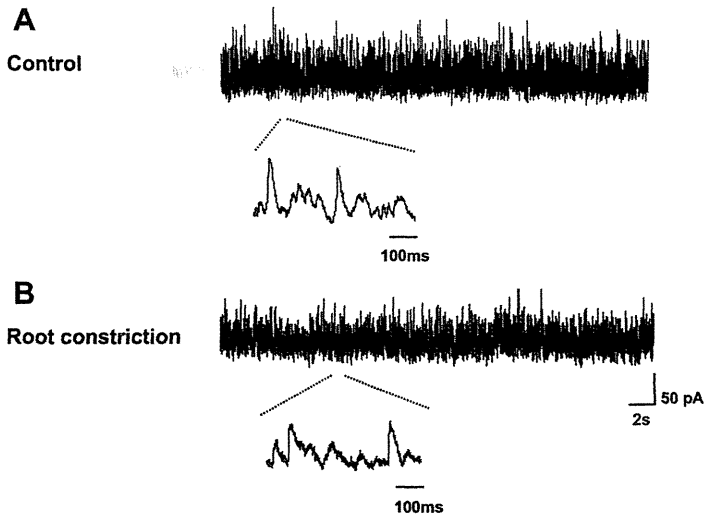

Fig. 6. Sample recording traces of spontaneous inhibitory postsynaptic currents (IPSCs) in substantia gelatinosa (SG) neurons at L5 segmental level in the control group (A) and in the root constriction group (B). The mean frequency of spontaneous IPSCs from the root constriction group was significantly lower than that from the control group (15 ± 1 vs 18 ± 1 Hz, $P < .05$). The mean amplitudes of spontaneous IPSCs from the root constriction group tended to be lower than those from the control group, but no significant difference was observed between the 2 groups (33 ± 5 vs 36 ± 4 pA, $P > .05$).

According to a previous study [13], Aδ-afferent-mediated and C-afferent-mediated EPSCs are produced by stimuli to the L5 nerve root in more than 70% of SG neurons at the L5 segmental level, whereas those EPSCs were recorded in more than 50% of SG neurons at the L4 segmental level. Several studies have shown that peripheral nerve injury alters excitatory synaptic transmission in SG neurons [20,26,32]. The results of the present study showed that the mean frequency and amplitude of spontaneous EPSCs obtained from SG neurons at L4 and L5 segmental levels in the root constriction group were significantly higher and larger than those in the control group. This result supports the finding that a nerve root projects through several segmental levels of the spinal cord, and we consider that the injured nerves may cause changes in SG neurons at several segmental levels of the spinal cord [30].

The mean amplitudes of EPSCs evoked by mechanical stimuli at the L5 segmental level in the root constriction group were significantly larger than those in the control group. Interestingly, the mean amplitude of EPSCs evoked by mechanical stimuli to the receptive field at the L4 segmental level in root constriction rats was significantly larger than those in control rats. The mean frequency of evoked EPSCs in the root constriction group was not higher than that in the control group at the L4 segmental level. These results suggest that postsynaptic changes may be observed in SG neurons, and that SG neurons are more sensitive to mechanical stimuli. As a result, allodynia and hypersensitivity occur in an area around the receptive field in which the injured nerve dominates.

4.3. The spinal cord as a target for treating radicular pain

Glycine and GABA are inhibitory transmitters, and glycinergic and GABAergic neurons are abundant in the SG and play an important role in modulating nociceptive transmission. GABAergic inhibition is markedly reduced in partial peripheral nerve injury, chronic constriction injury of the sciatic nerve, and spared nerve injury models [28]. This study demonstrated that the mean frequency was significantly lower and the amplitude was smaller for spontaneous IPSCs obtained from the L5 segmental level in root constriction rats than those in control rats. After a nerve root was injured, inhibitory synaptic transmission in SG neurons decreased. Consequently, this might be expected to amplify excitatory responses of SG neurons to afferent inputs.

The results of the present study indicated that injuring the nerve root led to characteristic excitatory changes in SG neurons at both the L5 and the L4 segmental levels and changed the properties of SG neurons. Central sensitization in SG neurons may contribute to the occurrence of radicular pain, so further study is needed to investigate how to prevent the excitatory synaptic reorganization in SG neurons, and this would be of value for the treatment of radicular pain.

It has been recently suggested that glial activation, enhanced interleukin-1β expression, and interleukin-6 expression in the superficial layer of the spinal dorsal horn including SG neurons occur after a nerve root is injured at a site proximal to the DRG [10,38]. Thus, further study is needed to reveal molecules that cause changes in the synaptic transmission of SG neurons after a root constriction injury.

Conflicts of interest statement

The authors have no conflicts of interest with regard to this study.

Acknowledgements

This work was supported in part by Grant-in-Aid for Scientific Research No. 21390432 to M.K. from the Ministry of Education, Science, Sports and Culture of Japan and a research grant to Y.T. from the Program for Developing the Supporting System for Upgrading Education and Research of Japan.

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Clin Orthop Relat Res (2011) 469:2568–2576
DOI 10.1007/s11999-011-1798-x

BASIC RESEARCH

Does Norepinephrine Influence Pain Behavior Mediated by Dorsal Root Ganglia?

A Pilot Study

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Received: 27 July 2010 / Accepted: 20 January 2011 / Published online: 11 February 2011

Abstract

Background Postganglionic neurons in the sympathetic nervous system reportedly are involved in lumbar radicular pain and release norepinephrine (NE), a neurotransmitter. Increased numbers of sympathetic nerve fibers have been found in dorsal root ganglion (DRG) neurons in a root constriction model. Whether this is a reasonable model for pain, however, is unclear

Questions/purposes We asked whether: (1) painful behaviors occurred in the root constriction model; (2) NE enhanced the excitability of DRG neurons in the root constriction model; and (3) which adrenoceptors were related to the mediation of the NE effects.

Methods The L5 root was sutured proximal to the DRG as the root constriction model. Behavioral tests were performed until 28 days after surgery. At 10 to 14 days after the root constriction, DRG neurons were quickly excised and digested with collagenase for electrophysiologic studies. Action potentials were recorded from single DRG neurons using a whole-cell patch clamp technique. NE (10 μmol/L) was directly applied to the DRG neurons. The adrenergic sensitivity was examined in combination with antagonists.

Results The rats with root constriction exhibited painful behavior. NE increased the excitability of DRG neurons in the root constriction model. The effects of NE were inhibited by pretreatment with an α-antagonist and α_2-antagonist but not an α_1-antagonist.

Conclusions Our observations suggest NE plays an important role in generating lumbar radicular pain mainly via α_2-adrenoceptors.

Clinical Relevance An α_2-antagonist may be an appropriate agent for trials to treat lumbar radicular pain.

One or more of the authors (TT, NT, and TY) have received funding from a research grant for the Grant-in-Aid for Science Research (B) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (Grant Number of 19390398) and the JOA-Subsidized Science Project Research 2007-8.
Each author certifies that his or her institution approved the animal protocol for this investigation and that all investigations were conducted in conformity with ethical principles of research.
This work was performed at Sapporo Medical University, Sapporo, Japan.

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Introduction

Lumbar radicular pain with lumbar disc herniation and lumbar spinal canal stenosis are common. Nonoperative treatment and surgery can alleviate lumbar radiculopathy, but 10% to 42% of patients continue to experience pain resistant to conventional treatment [2, 3, 41]. Intractable pain frequently is believed to be of neuropathic origin [18, 19], a complex pain state typically accompanied by tissue and nerve injury. Neuropathic pain is defined as pain arising as a direct consequence of a lesion or disease affecting the somatosensory system, commonly in lumbar radicular pain, diabetic neuropathy, and postherpetic neuralgia [47].

Animal models of neuropathic pain after peripheral nerve injury have been developed in the search for underlying mechanisms [4, 21]. Using such models, some

studies have documented that sympathetic nerve fibers sprout in the DRG [9, 10, 28, 34, 35, 52]. Sympathetic sprouting is a phenomenon in which sympathetic postganglionic axons extend around DRG neurons after peripheral nerve injury. Mizuno et al. reported sympathetic nerve fibers increased in corresponding DRG neurons in the root constriction model [30]. Sympathectomy alleviates pain behaviors such as mechanical allodynia and thermal hyperalgesia in animal models [20, 24, 30, 31, 37]. In clinical cases, lumbar sympathetic block has been performed to relieve chronic pain, including intractable lumbar radicular pain [6, 32, 46]. Therefore, the sympathetic nervous system currently is considered to be involved in lumbar radicular pain.

NE is the neurotransmitter that is released from postganglionic neurons in the sympathetic nervous system. In the abnormal somatosensory-sympathetic connection through sympathetic sprouting in the DRG, NE may influence the excitability of primary afferent DRG neurons. Some electrophysiologic studies suggest NE application induces hyperexcitability of DRG after a chronic constriction injury (CCI) of the sciatic nerve [50, 53]. Kirita et al. used whole-cell patch clamp recording as an intracellular recording technique to evaluate electrophysiologic properties of single DRG neurons and showed that root constriction increased the excitability of DRG neurons [23]. However, the effects of NE on DRG neurons in the root constriction model have not been clear.

We therefore asked whether: (1) painful behaviors occurred in rats with root constriction; (2) NE enhanced the excitability of DRG neurons in the root constriction model using whole-cell patch clamp recording; and (3) which adrenoceptors were related mainly to the mediation of the NE effects.

Materials and Methods

We created a root constriction model by suturing the L5 root proximal to the DRG. Fifty-one adult male Sprague-Dawley rats weighing 150 to 200 g at the beginning of the study were divided into two groups: (1) a root constriction group (n = 33), and (2) a sham group (n = 18) (Fig. 1). In the behavioral study, 16 rats (eight in each group) were used for 2 days before and on Days 3, 7, 10, 14, 21, and 28 after surgery. At each experimental day, we tested each hind paw with mechanical and thermal stimulations. In the electrophysiologic study, 35 rats (25 in the root constriction group

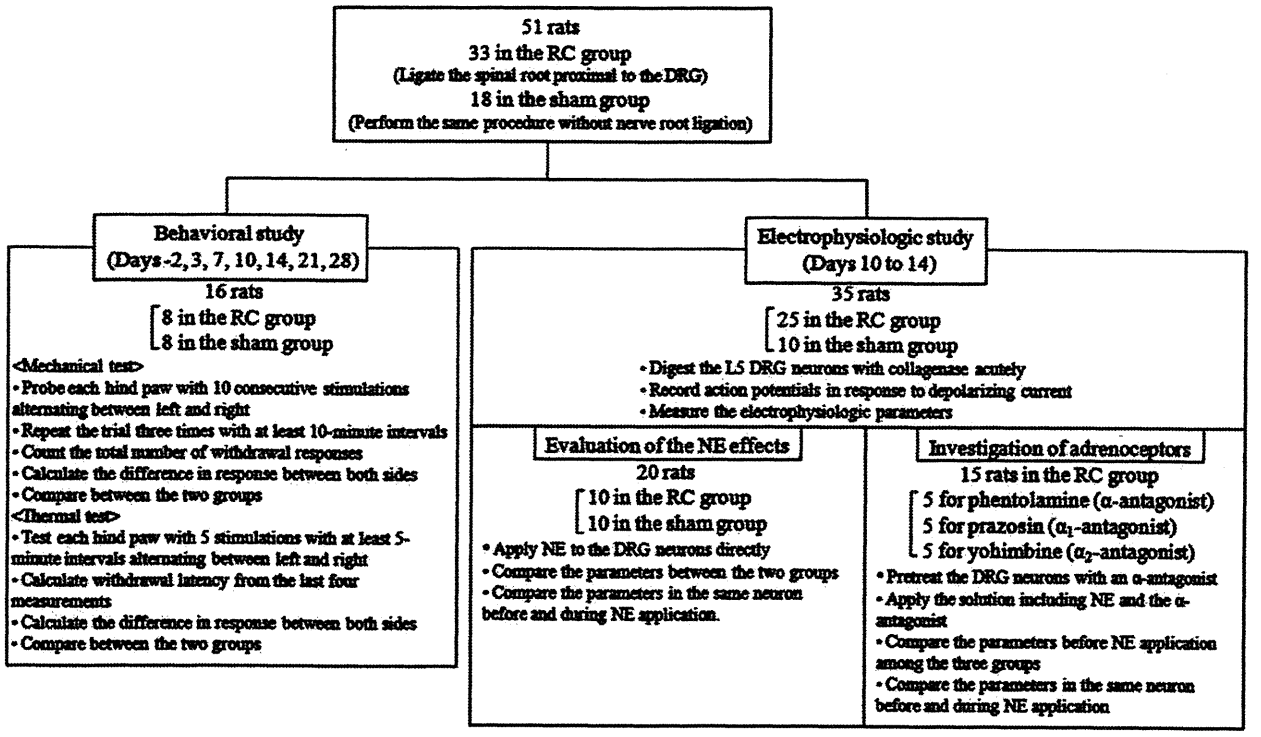

Fig. 1 The diagram of the study design shows the 51 rats were divided between the root constriction (RC) group (n = 33) and the sham group (n = 18). In the behavioral study, 16 rats (eight in each group) were used up to 28 days after surgery. In the electrophysiologic study, 35 rats (25 in the root constriction group and 10 in the sham group) were used on postoperative Days 10 to 14. Twenty rats (10 in each group) were used to evaluate the response to NE application. In addition, 15 rats in the root constriction group (five in each antagonist) were used to investigate adrenoceptor subtypes together with α-adrenergic antagonists.

and 10 in the sham group) were used on postoperative Days 10 to 14. The L5 DRG neurons were acutely digested with collagenase. Twenty rats (10 in each group) were used to evaluate the response to NE application. In addition, 15 rats in the root constriction group (five in each antagonist) were used to investigate adrenoceptor subtypes activated by NE application together with α-adrenergic antagonists: phentolamine (α-antagonist), prazosin (α_1-antagonist), and yohimbine (α_2-antagonist) (Fig. 1). All experimental protocols were approved by the Sapporo Medical University Animal Care and Use Committee.

Unilateral lumbar radiculopathy was induced as described previously [23]. Briefly, under sodium pentobarbital anesthesia (50 mg/kg, intraperitoneal), through a midline dorsal incision, we identified the left L5/L6 facet joint. A left L5 hemilaminectomy and L5/L6 partial facetectomy were performed. With a surgical microscope, we carefully exposed the L5 spinal root and DRG and the L5 spinal root was tightly ligated extradurally with 8-0 nylon suture proximal to the DRG as the root constriction group. The same surgical procedure without nerve root ligation was performed in a sham group.

An investigator (TI), blinded to the surgical protocols for each rat, performed behavioral tests using a previously described method [23]. For mechanical withdrawal response, we placed rats in a Plexiglas® chamber measuring 18 × 25 × 18 cm above a wire mesh floor that allowed full access to the hind paw. Before testing, behavioral accommodation was allowed for at least 20 minutes. We measured mechanical withdrawal response as the frequency of hind paw withdrawals elicited by a defined mechanical stimulus of 3.4 g using a calibrated nylon filament (Semmes-Weinstein Monofilaments; North Coast Medical Inc, San Jose, CA, USA). The mechanical stimulus was applied to the middle of the foot pad on the plantar surface of the hind paw. Each hind paw was probed with 10 consecutive tactile stimulations alternating between left and right. We repeated the trial three times in succession with at least a 10-minute interval, resulting in each foot receiving 30 mechanical stimulations. Mechanical sensitivity was assessed by counting the total number of withdrawal responses elicited for a total possible score of 30. The mechanical withdrawal frequency was expressed as the difference in responses between the two sides (= score on the constriction side - score on the contralateral side).

For thermal withdrawal response, we placed the rats in the Plexiglas® chamber on a glass platform. After accommodation, thermal withdrawal response was measured as the latency of hind paw withdrawals elicited by a radiant heat source (Tail Flick Analgesia Meter; ITC Inc, Woodland Hills, CA, USA), which was moved beneath a portion of the hind paw that could be seen in the glass. Thermal stimulation was delivered to the foot pad of the hind paw. Intensity of the heat stimulus was adjusted to elicit a quick withdrawal reflex at a latency of approximately 6 to 8 seconds in control rats and the intensity was kept constant at this setting throughout all experiments. A cutoff time of 10 seconds was set to prevent tissue damage. We tested each hind paw five times with at least 5-minute intervals alternating between left and right. Mean withdrawal latency was calculated from the last four measurements. We defined thermal withdrawal latency in each rat as the difference in latency between the two sides (= latency on contralateral side − latency on constriction side). For frequency and latency, positive scores indicated increased sensitivity on the constriction side.

At postoperative Days 10 to 14, 35 rats were deeply anesthetized with sodium pentobarbital (60 mg/kg intraperitoneal) and decapitated. Under a stereoscopic microscope, the left L5 DRG was quickly excised and placed in oxygenated normal Tyrode's solution consisting of (in mmol/L) NaCl 143, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, NaH_2PO_4 0.33, glucose 5.5, and HEPES 5.0 at a pH of 7.4 with NaOH. Dissected DRG was immersed in Ca^{2+}-free Tyrode's solution at room temperature (greater than 20 minutes) to remove extracellular Ca^{2+}. Tissue was digested with collagenase (8 mg/mL, Type II, 034-10533; Wako Pure Chemical Industries, Tokyo, Japan) for 90 minutes in a shaking incubator (37°C, 1.5 Hz) and rinsed with Ca^{2+}-free Tyrode's solution. The DRG neurons were dissociated in Ca^{2+}-free Tyrode's solution with a handmade pipette, which has a larger hole.

We recorded action potentials in response to depolarizing current using a whole-cell patch clamp apparatus (Axopatch 200B; Molecular Devices, Sunnyvale, CA, USA) at room temperature (22°–24°C). DRG neurons were placed in the recording chamber on the stage of an inverted microscope (IX-70; Olympus, Tokyo, Japan). Because small neurons may be involved in the transmission of nociceptive signals [43], we selected cells less than 40 μm in diameter, which corresponds to the size range of small neurons in rat DRG [13, 36, 44]. We used normal Tyrode's solution as an external solution. Electrode impedance was 3 to 5 MΩ when filled with solution, consisting of (in mmol/L) K-asparate 110, KCl 20, $MgCl_2$ 1.0, ATP-K_2 5.0, phosphocreatine-K_2 5.0, EGTA 5.0, and HEPES 5.0 at a pH of 7.4 with KOH. Electrode position was controlled by a three-dimensional hydraulic micromanipulator under the inverted microscope.

We used two protocols to evaluate the excitability of DRG neurons. In one protocol (short stimulation), depolarizing currents of 0.2 to 4.0 nA (0.5 ms duration) in increments of 0.2 nA were injected into a DRG neuron until an action potential (AP) was evoked. We then examined the threshold current, resting membrane potential (RMP), amplitude, after hyperpolarization (AHP),

threshold voltage, action potential duration 50 (APD50), and dV/dt max. The threshold current was defined as the minimum current required to evoke AP. RMP was taken 3 minutes after a stable recording was first obtained. We measured amplitude from the RMP to the peak. AHP was measured from the RMP to the valley peak. The threshold voltage was defined as the beginning of the upstroke of AP. APD50 was measured as the interval from AP onset to the point of 50% repolarization. The dV/dt max was the maximum rate of increase of AP.

In the other protocol (long stimulation), we used depolarizing currents of 0.01 to 0.39 nA (1000 ms duration) in increments of 0.02 nA. We confirmed the discharge pattern and examined the maximum number of spikes in each current (maximum spike count).

NE stock solution (10 mmol/L) was dissolved in distilled water with an equivalent amount of ascorbic acid and diluted with the external solution so that final concentration was 10 μmol/L. After baseline recording, we applied NE to DRG neurons by gravity through a valve connected to the chamber. Five minutes after NE application, data were recorded again.

We performed application of α-antagonists in the root constriction neurons. Phentolamine (α-antagonist), prazosin (α_1-antagonist), and yohimbine (α_2-antagonist) were used for this study. Fifteen DRG neurons (five in each antagonist) were used for recording. Phentolamine and yohimbine (10 mmol/L) were dissolved in distilled water. Prazosin (5 mmol/L) was made by dissolution in 10% ethanol in distilled water. Final concentration of all drugs was 10 μmol/L. DRG neurons had been pretreated with α-antagonists for 1 hour before recording. After electrophysiologic recording was performed in pretreatment with an antagonist, we applied a solution containing NE and the antagonist to DRG neurons.

Data were expressed as means ± standard error of mean and analyzed using SPSS 12.0 for Windows (IBM Corporation, Somers, NY, USA). Student's t-test was used to determine the differences in the behavioral data and the electrophysiologic parameters between the sham and the root constriction groups. We used the paired t-test to determine the differences in electrophysiologic parameters evoked by current injection in the same neuron before and during NE application. ANOVA was used to compare the root constriction groups without adrenergic antagonists.

Results

In the behavioral study, the rats with the root constriction showed mechanical allodynia and thermal hyperalgesia. The root constriction group exhibited an increase ($p = 0.002$) in tactile sensitivity from 7 days after surgery

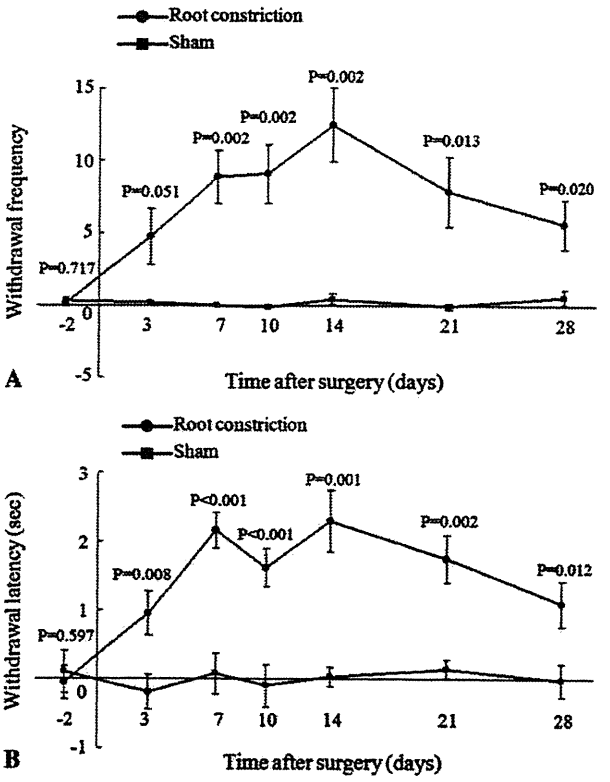

Fig. 2A–B The responses to (**A**) mechanical and (**B**) thermal stimulation of the hind paw in the root constriction and the sham groups were evaluated. The root constriction group showed mechanical allodynia from 7 days after surgery and thermal hyperalgesia from 3 days after surgery. Both of the changes were maintained up to 28 days after surgery.

as compared with the sham group, and the hypersensitivity was maintained until 28 days after surgery ($p_{10} = 0.002$, $p_{14} = 0.002$, $p_{21} = 0.013$, $p_{28} = 0.020$) (Fig. 2A). The withdrawal latency in the root constriction group decreased from 3 to 28 days after surgery as compared with that in the sham group ($p_3 = 0.008$, $p_7 < 0.001$, $p_{10} < 0.001$, $p_{14} = 0.001$, $p_{21} = 0.002$, $p_{28} = 0.012$) (Fig. 2B).

In the electrophysiologic study, NE enhanced the excitability of DRG neurons in the root constriction model. In the root constriction group, the mean threshold current was lower ($p = 0.038$) and the mean RMP was more depolarized ($p = 0.008$), compared with the sham group (Table 1). There were no major changes in the other parameters. With regard to discharge pattern, all the root constriction neurons showed a multiple spike pattern (Fig. 3), whereas seven of 10 sham neurons (70%) exhibited a single spike (Fig. 4). The maximum spike count of the root constriction group was greater ($p < 0.001$) than that of the sham group. In the root constriction group, NE application induced an increase ($p = 0.007$) in dV/dt max. The maximum spike count during NE stimulation was 15.9 ± 2.1, which was greater ($p = 0.006$) than the mean

Table 1. Effects of NE on electrophysiologic properties

Electrophysiologic parameter	Sham group		Root constriction group		p Value		
	Before NE	During NE	Before NE	During NE	Sham versus root constriction group (before NE)	Sham group Before versus during NE	Root constriction group Before versus during NE
Threshold current, nA	1.92 ± 0.12	1.80 ± 0.11	1.54 ± 0.12	1.46 ± 0.09	0.038	0.051	0.309
RMP, mV	−55.4 ± 1.4	−55.7 ± 1.4	−49.0 ± 1.6	−48.9 ± 1.5	0.008	0.632	0.948
Amplitude, mV	93.7 ± 4.4	96.2 ± 4.9	90.3 ± 5.3	96.1 ± 3.9	0.622	0.201	0.071
AHP, mV	8.9 ± 1.5	9.7 ± 1.4	9.8 ± 0.9	11.1 ± 1.3	0.647	0.659	0.647
Threshold voltage, mV	−5.7 ± 3.1	−7.0 ± 3.0	−9.8 ± 2.6	−10.7 ± 1.9	0.333	0.522	0.192
APD50, ms	3.4 ± 0.3	3.3 ± 0.3	4.6 ± 0.5	4.2 ± 0.5	0.072	0.475	0.602
dV/dt max, M/second	50.9 ± 7.8	56.8 ± 8.9	49.7 ± 8.6	63.0 ± 7.8	0.921	0.050	0.007
Max spike count	3.5 ± 1.4	3.1 ± 1.4	10.7 ± 1.8	15.9 ± 2.1	0.006	0.462	0.006

Values are means ± SEM; NE = norepinephrine; RMP = resting membrane potential; AHP = after hyperpolarization; APD50 = action potential duration at half width.

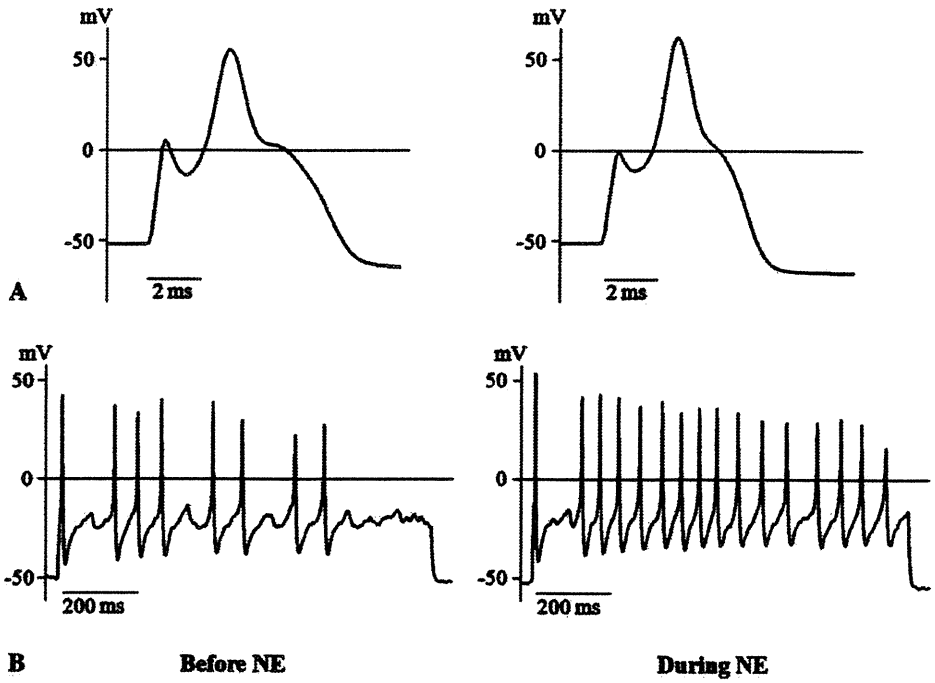

Fig. 3A–B Examples of action potentials generated from DRG neurons in the root constriction group are shown. NE enhanced the excitability of the root constriction neurons. In (**A**) short stimulation, NE induced the increase of dV/dt max, which represents the maximum rate of increase of action potential. In (**B**) long stimulation, the maximum spike count further increased during NE application.

of 10.7 ± 1.8 before NE stimulation (Fig. 3). In contrast, there were no changes in the sham group (Fig. 4).

As for the α-adrenergic antagonists, there were no differences among the three groups before NE stimulation. The effects of NE were inhibited by pretreatment with the α-antagonist phentolamine or the α_2-antagonist yohimbine. However, the α_1-antagonist prazosin failed to abolish the responses to NE. The dV/dt max and the maximum spike count increased from 30.3 ± 4.0 to 41.7 ± 3.2 ($p = 0.028$) and from 10.8 ± 2.2 to 17.8 ± 0.8 ($p = 0.014$), respectively (Fig. 5).

Discussion

We suspected NE plays an important role in regulating the excitability of DRG neurons in the root constriction model, because the sympathetic nervous system reportedly is associated with lumbar radicular pain. Therefore, we asked whether: (1) painful behaviors occurred in rats with root constriction; (2) NE enhanced the excitability of DRG neurons in the root constriction model; and (3) which adrenoceptors were related mainly to the mediation of the NE effects.

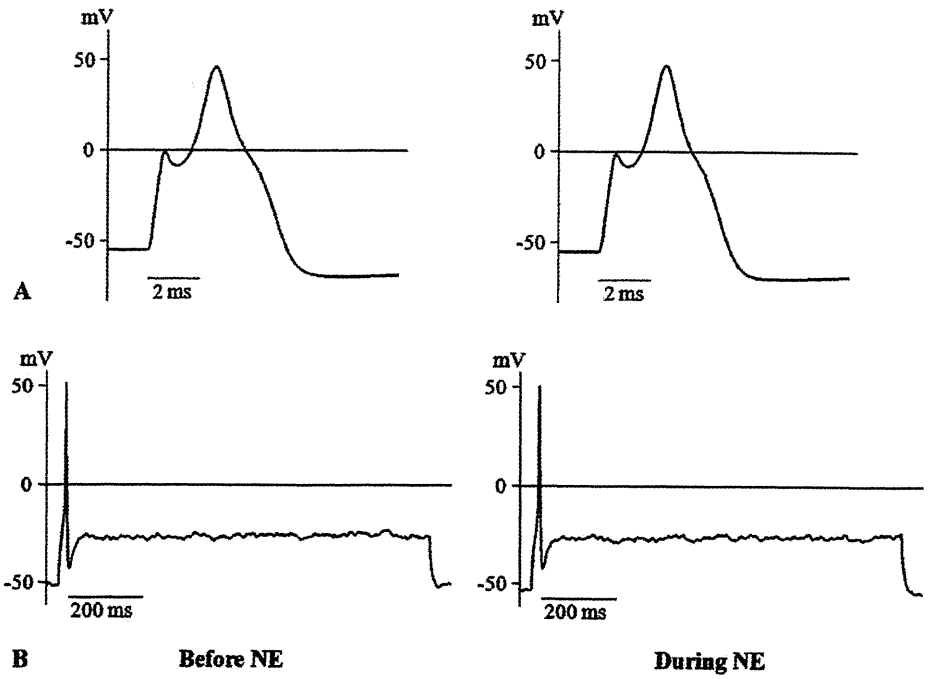

Fig. 4A–B Examples of action potentials generated from DRG neurons in the sham group are shown for (**A**) 2 ms and (**B**) 200 ms before and during NE application. There were no changes by applying NE.

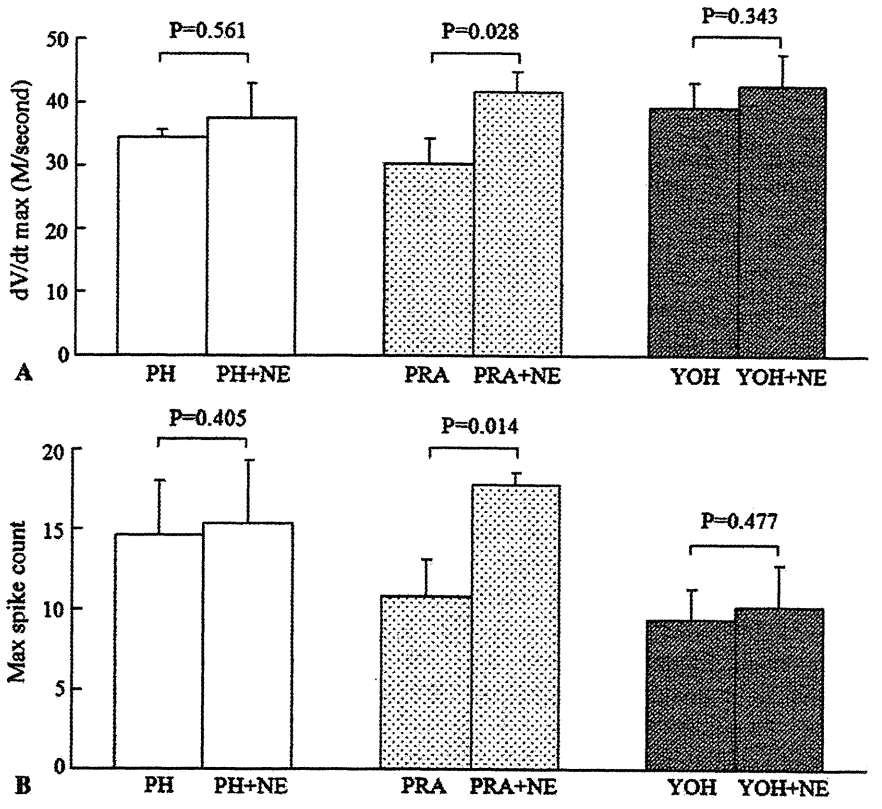

Fig. 5A–B The results of pretreatment with the α-adrenergic antagonists in the root constriction neurons are shown for (**A**) dV/dt max and (**B**) maximum spike count. The effects of NE were inhibited by the α-antagonist (phentolamine = PH) and the α_2-antagonist (yohimbine = YOH), not the α_1-antagonist (prazosin = PRA).

There were limitations in the experimental settings in this study. First, the root constriction model was created by ligating the spinal root with a nylon suture, which may not reflect spinal disorders such as lumbar disc herniation and lumbar spinal canal stenosis. However, suture of the spinal root is considered a reliable and reproducible procedure to induce a lumbar radiculopathy. Second, our electrophyiologic study was an in vitro study and the drugs were

administered directly to the DRG neurons. Adrenoceptors are said to exist systemically, not just in DRG. Accordingly, a specific method for delivering drugs to DRG neurons is needed to examine the effects of α_2-antagonists in humans. Third, the concentration of NE that reaches DRG neurons in our preparation is probably much lower than that in the perfusion solution yet is likely to be higher than that in the plasma of humans [12]. Fourth, we used collagenase to dissociate the excised DRG. Thus, in this model, communication with neighboring glial and DRG cells no longer is intact. However, in isolating the effects of single DRG neurons, this model provides certain advantages. To resolve these limitations, patch clamp recording in vivo should be performed to evaluate NE-induced activity. Fifth, we selected DRG neurons less than 40 μm in diameter for electrophysiologic studies. Although small neurons may be related to the transmission of the nociceptive signals including pain sensation, the function of the DRG neurons used in the current study was not clear.

Our observations confirm L5 root constriction results in behavioral signs of mechanical allodynia and thermal hyperalgesia. These pain behaviors were maintained for up to 28 days after surgery. Radiculopathy is induced by inflammation and mechanical compression. Epidural application of autologous nucleus pulposus has been used for an inflammatory radiculopathy model [45]. However, mechanical compression has been provided by suturing the nerve root or inserting a stainless steel rod in the spinal canal [14, 16, 48]. In the root constriction model, nerve deformation can be observed during surgery and a nylon suture which does not induce inflammation was used to ligate the L5 root. Accordingly, the responses to a root constriction model are presumed to be evoked by mechanical compression. These results are consistent with those of previous reports of lumbar root constriction models [14, 23, 30].

The current electrophysiologic analysis showed NE application induced further excitability of DRG neurons in the root constriction group using a whole-cell patch clamp recording technique, which resulted in larger dV/dt max and greater maximum spike count. Compared with the sham neurons, the root constriction neurons exhibited lower threshold current, more depolarized RMP, and greater maximum spike count, thus suggesting root constriction causes increased excitability of DRG neurons. These changes have been observed in different animal models of neuropathic pain including axotomy [1, 22, 49, 51], CCI [40, 44], spinal nerve ligation [27], and chronic compression of the DRG [26, 54]. In previous electrophysiologic studies, extracellular recording techniques have been used to evaluate the effects of NE on DRG neurons. Xie et al. found the spontaneous discharge rates of C-fibers after CCI were greater after application of NE [50]. Stimulation of the postganglionic sympathetic efferents reportedly augments sensory afferent discharge [11, 25, 29, 50]. The increase in dV/dt max indicates an increase in inward current, which may involve sodium ions. Honma et al. reported NE inhibited inward currents through voltage-dependent calcium channels and outward potassium currents in cultured DRG neurons in the CCI model [15]. Petersen et al. reported application of NE causes membrane depolarization in the responses of DRG after CCI [33]. These studies support the notion that excitation of DRG neurons is controlled by potassium channels. In our study, however, the RMP and APD50, which are parameters related to potassium channels, were not influenced by NE. Although this discrepancy may be the result of the differences in animal models and cell preparation, further studies are needed to clarify the current properties for NE-induced hyperexcitability of DRG neurons in the root constriction model.

We also observed that these excitatory effects of NE were inhibited by pretreatment with an α_2-antagonist but not an α_1-antagonist. These findings suggest the NE-induced hyperexcitability of DRG neurons was modulated by α_2-adrenoceptors. Chen et al. [7] and Xie et al. [50] reported the response to NE was blocked by α_2-antagonist yohimbine. Zhang et al. reported that clonidine, an α_2-agonist, enhanced the spontaneous activity of C- and Aδ-fibers [53]. The expression of adrenoceptors in DRG neurons are altered after nerve injury. Birder and Perl reported the number of L4 and L5 DRG neurons expressing α_{2A}-adrenoceptors increased sharply after sciatic nerve transection [5]. Using molecular biology techniques, they confirmed α_{2A}-adrenoceptor mRNA levels increased in DRG neurons of rats with SNL or axotomy [8, 38, 39]. Accordingly, DRG neurons in the root constriction model appeared to have an increased expression of α_2-adrenoceptors and responded more sensitively to the application of NE.

Our observations suggest NE enhanced the excitability of DRG neurons presumably through mediation of α_2-adrenoceptors. These findings indicate that NE may play a role in the development and persistence of lumbar radicular pain, leading to excitation of DRG neurons by activating α_2-adrenoceptors. However, intrathecal administration of NE has reversible antinociceptive effects by inhibiting Aδ- and C-fiber-mediated sensory transmission to substantia gelatinosa neurons in the spinal dorsal horn through the activation of α_2-adrenoceptors [17]. The presence of adrenoceptors in DRG and spinal dorsal horn neurons has been observed [42], thus suggesting a role for NE at presynaptic and postsynaptic sites in the modulation. Therefore, NE may have a different role in modulation of pain sensation depending on its release site. Our observations suggest the sympathetic nervous system plays an

important role in generating radicular pain and inhibiting the input through adrenoceptors and an α_2-antagonist could be a promising therapeutic agent for radicular pain. However, a more comprehensive understanding of the sympathetic nervous system is needed for treating lumbar radicular pain in humans.

Acknowledgments We thank Takehito Iwase for performing the behavioral studies and Dr. Takashi Kirita, Dr. Satoshi Mizuno, and Dr. Yoshinori Terashima for technical assistance related to the electrophysiologic experiments.

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