Changes in Properties of Spinal Dorsal Horn Neurons and Their Sensitivity to Morphine after Spinal Cord Injury in the Rat

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Background: To gain a better understanding of spinal cord injury (SCI)-induced central neuropathic pain, the authors investigated changes in properties of spinal dorsal horn neurons located rostrally and caudally to the lesion and their sensitivity to morphine in rats after SCI.

Methods: The right spinal cord of Sprague-Dawley rats was hemisected at the level of L2. At 10 to 14 days after the SCI, when mechanical hyperalgesia/allodynia had fully developed, spontaneous activity and evoked responses to mechanical stimuli of wide-dynamic-range (WDR) and high-threshold neurons rostral and caudal to the lesion were recorded. Effects of cumulative doses of systemic (0.1–3 mg/kg) and spinal (0.1–5 μg) administration of morphine on spontaneous activity and evoked responses to the stimuli of the neurons were evaluated.

Results: Spontaneous activity significantly increased in WDR neurons both rostral and caudal to the SCI site, but high-frequency background discharges with burst patterns were only observed in neurons rostral to the SCI site. Significant increases in responses to the mechanical stimuli were seen both in WDR and high-threshold neurons located both rostrally and caudally to the lesion. The responses to nonnoxious and noxious stimuli were significantly greater in caudal WDR neurons than in rostral WDR neurons. In contrast, the responses to pinch stimuli were significantly higher in rostral high-threshold neurons than those in caudal high-threshold neurons. Systemically administered morphine had a greater effect on responses to nonnoxious and noxious stimuli of rostral WDR neurons than those of caudal WDR neurons. Spinally administered morphine significantly suppressed responses of WDR neurons in SCI animals to nonnoxious stimuli compared with those in sham-operated control animals.

Conclusions: The findings suggest that changes in properties of spinal dorsal horn neurons after SCI are caused by different mechanisms, depending on the classification of the neurons and their segmental locations.

PATIENTS with spinal cord injury (SCI) suffer from various types of pain, including musculoskeletal, radicular, visceral, and neuropathic pain.[1] Central neuropathic pain syndrome, which is refractory to currently available modes of treatment and continues to present a significant challenge to physicians,[1] includes several categories and sources of pain. "At-level neuropathic pain" refers to pain in dermatomes near the SCI site and occurs from the time of injury or shortly thereafter.[2] "Below-level neuropathic pain" develops gradually after SCI and refers to pain in dermatomes in anesthetic areas supplying segments below the lesion level. "Above-level neuropathic pain" is seen in dermatomes rostral to the site of injury. Although little is known about the mechanisms of the onset of the central neuropathic pain, it is thought that different types of pain are caused by different pathophysiological mechanisms at different levels of the spinal cord, such as deafferentation and removal of tonic inhibition from descending systems.[2]

Although peripheral nerve injury-induced neuropathic pain in humans is usually associated with enhanced nociceptive responses to stimulation within regions near the distribution of spontaneous pain, a dissociation of spontaneous pain and enhancement of elicited nociception can present in patients with SCI-induced central neuropathic pain,[2] suggesting that components of the central neuropathic pain syndrome, spontaneous pain, and evoked pain result from different mechanisms in the central nervous system, including the spinal cord. The components of central neuropathic pain may thus have different sensitivities to analgesics and hence to modes of administration. One example is that associated with morphine. Indeed, recent clinical studies have shown that intravenous administration of morphine can reduce brush-evoked allodynia but not spontaneous pain or other stimuli-evoked pain (static mechanical and thermal hyperalgesia) in patients with SCI[3] and that intrathecal administration of morphine is more effective for suppression of SCI-induced pain.[4] Thus, it is possible that spinal neurons exert different pathophysiological properties in spontaneous activity and that the effectiveness of a drug varies depending on the classification of spinal neurons and their locations in the spinal cord.

The aim of the present study was to determine the characteristics of activity in spinal dorsal horn neurons located in the different segments rostral and caudal to the SCI site compared to the activity of sham-operated control animals. We also investigated the effects of systemic and spinal administration of morphine on activities of spinal neurons located in the spinal segments rostral and caudal to the site of SCI.

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Materials and Methods

Surgical Procedure

All of the protocols of this study were approved by the Animal Care and Use Committee of our institution. Efforts were made to minimize the number of animals used, and the experiments followed the ethical guidelines of the International Association for the Study of Pain. Adult male Sprague-Dawley rats (weighing 200–250 g) were housed in groups of four in $40 \times 60 \times 30$-cm plastic cages with soft bedding under a 12:12 h day: night cycle. The rats were under these conditions from 5–7 days before surgery to up to 3 weeks after surgery for behavioral experiments and up to 2 weeks after surgery for electrophysiological experiments.

SCI. The rats were anesthetized with halothane (3.5% initially and maintained with 2–2.5% in oxygen) delivered *via* a face mask and checked for absence of paw withdrawal to noxious pinch before commencing surgery. Rectal temperature was maintained at 36°–38°C using a thermo-controlled heating pad. A T13 small laminectomy was performed to expose the L2 spinal cord, and the spinal cord was hemisected on the right side between the L2 and L3 segments, between the two dorsal root entry zones of L2 and L3. To perform the spinal hemisectioning, the overlying L2 dorsal root and the spinal cord was cut using a No. 11 scalpel blade without cutting major dorsal vessel or vascular branches according to a previously described method[5] that was modified in the present study with respect to the level of hemisectioning. Sham surgery in which the surgical procedure was identical to that described above but without hemisectioning of the spinal cord was performed in control animals. The wound in each animal was closed in two layers and the animal received phosphate-buffered saline (2 ml subcutaneous) and penicillin G (0.5 mg/kg intraperitoneally). Each rat was allowed to recover for several hours in an oxygenated Perspex box under a heat lamp and was then placed back in the plastic cage.

Behavioral Study

Locomotor Function. Locomotor function was evaluated using the Basso, Beattie and Bresnahan (BBB) open-field locomotor test.[6] The BBB scale ranges from 0 (no hindpaw movement) to 21 (normal movement-coorganized gait with parallel paw placement). Scores from 0 to 7 indicate the return of isolated movements in the three joints (hip, knee, and ankle). Scores from 8 to 13 indicated the return of paw placement and coordinated movements with forelimbs. Scores from 14 to 21 show the return of toe clearance during stepping, predominant paw position, trunk stability, and tail position. BBB scores were measured 1 day before and 1, 2, 3, 5, 7, 10, 14, and 21 days after the SCI had been made.

Mechanical Stimuli. The following behavioral tests were performed 1 day before and 1, 2, 3, 5, 7, 10, 14,
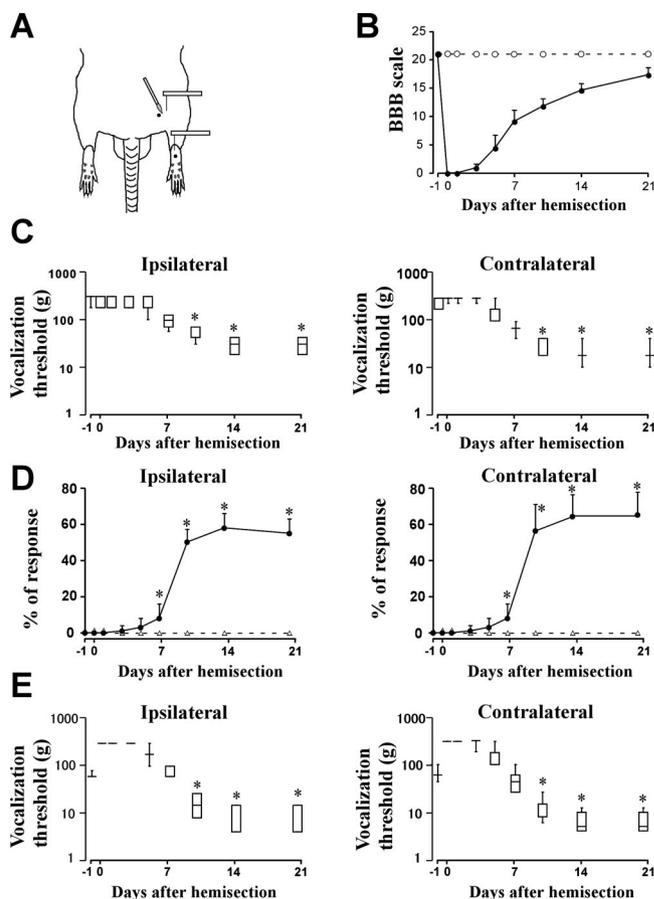

Fig. 1. Schematic representation of the site of application of mechanical stimuli in hairy skin of the hip and glabrous skin of the hindpaw (*A*). The time course of the Basso, Beattie and Bresnahan (BBB) scale on the ipsilateral (*closed circle*) and contralateral (*open circle*) sides to the SCI site at 1 day before and after the spinal cord injury (SCI) had been made (*B*). The vocalization threshold in response to von Frey hair stimulation in the hairy skin on the ipsilateral and contralateral sides to the SCI site (*C*). The frequency of response to brush stimulation in the hairy skin 1 day before and after the SCI had been made on the ipsilateral (*closed circle*) and contralateral (*open triangle*) sides to the SCI site (*D*). The vocalization threshold in response to von Frey hair stimulation in the glabrous skin on the ipsilateral and contralateral sides to the SCI site (*E*). The data are expressed as means ± SD (*B* and *D*) and as medians (*horizontal line*) with first and third quartiles (*boxes*), and 10th and 90th percentiles (*vertical lines*) (*C* and *E*). Data were obtained from 12 sham-operated rats and 12 SCI animals. *$P < 0.05$ *versus* prehemisection values (1 day before SCI had been made).

and 21 days after the SCI had been made. The sites of stimulation were glabrous skin of the hindpaws and shaved skin of the hip both on the ipsilateral and contralateral sides of the SCI (fig. 1A). The vocalization threshold to punctate mechanical stimulation to the shaved skin of the hip was determined using calibrated von Frey filaments (Stoeliting, Chicago, IL) that could produce graded pressure of 0.2 to 300 g according to a previously reported method.[7] Briefly, each von Frey filament was applied once, starting at the pressure of 0.2 g and continuing until vocalization had occurred or until a pressure of 300 g (cutoff value) had been reached, and

this procedure was repeated three times. The lowest force in the three tests that produced vocalization was considered as the vocalization threshold. During the examinations, the face, neck and upper body of the rat were gently wrapped with a soft cloth, and care was taken not to sensitize skin receptors by successively testing different parts of the body. Then, the shaved skin of the hip was briskly stroked with a camel hairbrush in a rostral-to-caudal direction on the ipsilateral and contralateral sides of the SCI according to a previously described method.[8] This test was repeated five times with intervals of 3 min between tests, and the response frequency was calculated from the results of the five tests. The responses of the animals to the brush stimulations were graded with scores of 0 to 3: 0 = no response, 1 = fine movements of the gluteal muscle caused by the stimulation, 2 = movements of the quadriceps or twisting of the hip to avoid the stimulation, 3 = vigorous efforts to escape from the stimulus, vocalization in response to the stimulation. Responses with grades of 2 and 3 were defined as withdrawal responses.

The rats were then placed individually on an elevated plastic mesh floor covered with a clear plastic cage top (20 × 25 × 15 cm) and allowed to acclimate. Withdrawal responses to punctate mechanical stimulation were determined using calibrated von Frey filaments applied from underneath the cage through openings (12 × 12 mm) in the plastic mesh floor to the glabrous skin of the hindpaw. The method of application of von Frey filaments was the same as that described above for assessment of the vocalization threshold of the shaved skin of the hip, and the lowest force of the three tests producing a withdrawal response was considered the withdrawal threshold. Because stereotyped brush stimulation by the paintbrush to glabrous skin from underneath the cage through the openings was hindered by the plastic mesh floor, the response to the brush stimulation was not evaluated in glabrous skin in the present study.

Spinal Cord Electrophysiology

Preparation. At 10 to 14 days after the SCI, anesthesia was induced with 3% of halothane in oxygen, and a cannula was inserted into the trachea. The left carotid artery and jugular vein were cannulated to allow for blood pressure monitoring and for drug and fluid administration, respectively. Under artificial ventilation, the skin overlying the site of the SCI was reopened and laminectomy was performed (vertebrae T12–L1) to expose segments L1–L5 of the spinal cord, and then the concentration of halothane was reduced to and maintained at 0.8%. At the level of anesthesia with 0.8–1.0% halothane, which has been used for anesthesia in rat experiments,[9,10] although rats have no spontaneous movements or evidence of tonic sympathetic nervous system, they could maintain motor reflex responses to nociceptive stimuli. The animals were subsequently par-

alyzed with pancuronium bromide (0.1 mg/kg) and placed in a stereotaxic apparatus. Under a binocular microscope with ×8 to ×40 magnification, the dura was cut and reflected. The superficial dorsal gray matter lateral to the dorsal root entry zone was discernible as a relatively translucent band under Lissauer's tract. The pia-arachnoid membrane was cut to make a window large enough to allow an electrode into the spinal cord. A small reservoir (50~100 μl) overlying the spinal cord was formed with dental impression material (Alflex®, Morita Co., Osaka, Japan) according to a previously described method,[11] and the reservoir was filled with phosphate-buffered saline (pH 7.4; Sigma-Aldrich Co., St. Louis, MO). Body temperature was recorded with a rectal probe and maintained at 37°C by an infrared heat lamp and the thermo-controlled heat pad.

Cell Characterization. In SCI animals, a tungsten microelectrode (10–12 MΩ, FHC Inc., Brunswick, ME) was advanced by a hydraulic micromanipulator into the dorsal horn of the spinal cord up to a depth of 1000 μm at a level rostral to the hemisectioning (L1 segment, 10–15 mm from the site of injury) or at a level caudal to the hemisectioning (L3–L4, 10–20 mm from the site of injury). In sham-operated animals, the tungsten electrode was advanced into the spinal cord of the corresponding segments in the same manner. The activity of a single neuron that had a receptive field (RF) on the shaved skin of the hindquarter or glabrous skin of the hindpaw was considered to be isolated when the spike was clearly distinguishable from the background neuronal noise and had uniform spike amplitude with a signal-to-noise ratio of at least 4:1. A neuron was classified as a wide-dynamic-range (WDR) neuron if responses were elicited by both low-intensity stimuli (light touch and brush) and high-intensity stimulus (pinch). It was confirmed that the firing frequency of the WDR neuron increased as the stimulus intensity increased, with maximum activation occurring only with presentation of the most intense stimuli. A neuron was classified as a high-threshold (HT) neuron if it did not respond to low-intensity stimuli (light touch and brush) but responded to high-intensity mechanical stimulus (pinch) in the same manner as that described above. A neuron was not defined as a WDR or HT neuron and was described as "unclassified" neuron if frequency of spontaneous activity was high (>10 Hz) and if responses were elicited by pinch in an RF of the hindquarter. This was because it could not be determined that the neuron did not respond to low-intensity stimuli.

After completion of classification of the neurons and obtaining stable baseline values for spontaneous activity over a period of 20–30 min, the low-threshold and high-threshold RF areas were carefully mapped on the shaved skin of the hindquarter by a procedure similar to that described previously.[12] The edge of the low-threshold RF was defined as those areas in which light touch

stimulation with the tip of a probe (a von Frey hair, 4 g) elicited a response 50% of the time. The edge of the high-threshold RF was determined as that area in which a response to high-intensity mechanical stimulation with a tungsten tip attached to a nylon filament (calibrated force of 25 g) evoked a response 50% of the time. This filament, with which stimulation produced pricking pain in examiners, was made according to a previously described method.[13]

Nonnoxious mechanical stimuli, such as brushing and punctate stimuli, were then applied in the most sensitive site of the RF. The responses to brush stimulation using a paintbrush were recorded three times at 1-min intervals. The responses to punctate mechanical stimuli using a 4-g von Frey hair were recorded for 5 s at 1-min intervals. Noxious stimuli were delivered by sustained application of the tungsten tip that was used to determine a high-threshold RF area as described above and an arterial clip that exerted a force of 250 g/mm^2 (#19–8080; Codman and Shurtleff Inc., Randolph, MA) for 5 s and 3 s, respectively, at 3-min intervals.

Pharmacological Studies. Two or three neurons were investigated in each animal, and physiologic characteristics were determined as described in "Cell Characterization." After determination of the physiologic characteristics of the last neuron examined on the experimental day, the following pharmacological trial was performed in the neuron. Stabilization of spontaneous activity and evoked responses were confirmed by at least three consistent predrug responses (<10% variation). These values were then averaged before drug administration to generate control values with which to compare the effects of drug administration on the subsequent spontaneous activity and evoked responses. Morphine sulfate (Sankyo Pharm Co., Tokyo, Japan) was dissolved in saline and was intravenously administered up to 3 mg/kg as cumulative doses through the intravenous catheter. The effects of morphine were then reversed by systemic administration of naloxone (1 mg/kg) after the final dose of morphine (3 mg/kg) had been administered. In a separate study, morphine was dissolved in 50 μl of phosphate-buffered saline and administered into the reservoir and applied directly onto the surface of the spinal cord (cumulative doses: 0.1, 0.25, 1, and 5 μg) according to a previously described method. The effects of morphine were then reversed by spinal administration of naloxone (50 μg) after the final dose of morphine (5 μg) had been spinally administered. The effect of morphine had been monitored over a period of 40 min after administration at each dose until maximal effect was elicited, and tests were carried out at 10-min intervals to determine the effects of morphine on the evoked responses.

Laminar locations of the recording sites were estimated from measurements of the depths of the elec-trodes from the surface of the cord. The animals were killed with an overdose of potassium chloride.

Data Analysis

The results are expressed as medians or means ± SD as appropriate. In the behavioral experiments, thresholds of withdrawal responses to von Frey hair stimulation and frequencies of withdrawal response to brush stimulation were compared using nonparametric analyses. Friedman's test for within-group and the Kruskal-Wallis test and Mann-Whitney rank sum tests for between-group comparisons were used. Multiple comparisons following Friedman's test and the Kruskal-Wallis test were performed using Dunnett's or Dunn's tests, respectively.

In the electrophysiological experiments, data obtained from animals in which systolic blood pressure decreased to less than 60 mmHg were excluded from analysis because activity of spinal neurons (RF size and responses to stimuli) is shown to decrease in association with such a decrease in blood pressure.[10] The outline of the RF mapped on the skin was transferred to tracing paper, digitized, and used to determine and analyze RF areas. Data were captured and analyzed by a CED 1401 interface (Cambridge Electronic Design Ltd., Cambrdige, UK) coupled to a Pentium computer with Spike 2 software (Cambridge Electronic Design, Ltd., Cambrdige, UK). Spontaneous firing rates were determined by averaging the activity over 60-s periods when there was no contact with the RF. To evaluate evoked activities of dorsal horn neurons, prestimulus spontaneous firing rates were subtracted from firing rates in response to nonnoxious and noxious stimuli.

Spontaneous and evoked firing rates in SCI animals and sham-operated animals were compared by one-way and two-way analyses of variance for repeated measures (analysis of variance) with Bonferroni's test for intragroup comparison. The effects of morphine are expressed as mean maximum percentage inhibition from the averaged predrug value for each neuron, and overall results for each dose are expressed as means ± SD. Statistical analysis of dose effects were performed by using a two-way analysis of variance with Bonferroni test. The ED_{50} values were determined by fitting the data points to a logistic equation that includes a parameter equivalent to the ED_{50}. Group differences in ED_{50} values were tested by one-way analysis of variance with the Fisher exact test for intragroup comparison. *P* values of < 0.05 were considered statistically significant.

Results

Behavioral Experiments

In the behavioral experiments, data were obtained from 12 SCI rats and 12 sham-operated rats. The BBB score was 0 in postoperative day 1 in all hemisected rats

Table 1. Classification of neurons and locations of the receptive fields in Spinal Cord Injury and sham-operated control animals

	Control			SCI		
	WDR	HT	Unclassified	WDR	HT	Unclassified
Rostral						
Hairy	22 (73%)	5 (63%)	0	25 (76%)	6 (67%)	8 (53%)
Glabrous	8 (27%)	3 (37%)	0	8 (24%)	3 (33%)	7 (47%)
Total	30	8	0	33	9	15
Caudal						
Hairy	15 (54%)	7 (58%)	0	14 (47%)	4 (67%)	5 (71%)
Glabrous	13 (46%)	5 (42%)	0	16 (53%)	2 (33%)	2 (29%)
Total	28	12	0	30	6	7

The numbers of classified neurons and locations of the receptive fields with percentages of the total numbers.

Caudal = neurons located caudal to the spinal cord injury lesion; glabrous = a receptive field of a neuron is on glabrous skin of the hindpaw; Hairy = a receptive field of a neuron is on hairy skin of the hindquarter; HT = high-threshold neurons; Rostral = neurons located rostral to the spinal cord injury lesion; SCI = spinal cord injury; Unclassified = neurons that are impossible to determine whether the neuron is a HT or a wide-dynamic-range neuron because of its high spontaneous activity ($>$10 Hz); WDR = wide-dynamic-range neurons.

but rapidly recovered to 11.8 ± 1.3 until on day 10 (fig. 1B). Sham-operated animals did not show any motor deficits during the experiments. The vocalization threshold to punctate stimulation using von Frey filaments in the hairy skin of the hindquarter (hip) significantly decreased from 7 days after the SCI and thereafter on both ipsilateral and contralateral sides ($P < 0.05$) (fig. 1C). In sham-operated animals, the vocalization threshold did not change during the experiments (data not shown). After the SCI had been made, the frequencies of withdrawal response on the ipsilateral and contralateral sides to the lesion to dynamic brush stimulation significantly increased on day 7 ($P < 0.05$) and reached to 50–60% on day 10 ($P < 0.001$), and the increased frequency was subsequently sustained for the duration of the examination (fig. 1D). The withdrawal threshold to punctate stimulation using von Frey filaments in the glabrous skin of the hindpaw significantly decreased on day 7 and thereafter after the SCI had been made ($P < 0.05$) (fig. 1E). In sham-operated animals, the withdrawal threshold did not change during the experiments (data not shown).

Electrophysiolgical Experiments

Data obtained from 3 sham-operated control animals and from two SCI animals in the electrophysiological experiments were excluded from analysis because of the low systolic blood pressure ($<$60 mmHg). As a result, activities of 178 neurons in 43 sham-operated control animals and 48 SCI animals were recorded. The systolic blood pressures in the sham-operated animals and SCI animals were 102 ± 21 and 107 ± 17 mmHg, respectively; there was no significant difference between the blood pressures in the two groups. The numbers of neurons observed, the types of cells and the locations of the receptive fields are shown in table 1. The spontaneous firing rates and firing rates of responses in the neurons examined to nonnoxious and noxious stimuli are shown in table 2. The effects of systemic morphine were investigated in 15 WDR neurons and 6 HT in sham-

Table 2. Physiology of neurons tested

	No. of neurons	Depth (μm)	SP (Hz)	BR (Hz)	VF 4 (Hz)	PP (Hz)	PI (Hz)
Control							
WDR							
Rostral	30	667 ± 276	1.1 ± 0.9	6.5 ± 3.1	6.8 ± 3.3	14.2 ± 3.8	30.1 ± 5.7
Caudal	28	781 ± 238	1.5 ± 1.3	8.4 ± 3.1	6.8 ± 3.0	13.0 ± 4.2	24.8 ± 6.9
HT							
Rostral	8	450 ± 235	1 ± 0.8	–	–	8.2 ± 5.6	20 ± 7.4
Caudal	12	487 ± 192	0.8 ± 0.3	–	–	10.7 ± 3.9	22.9 ± 13.3
SCI							
WDR							
Rostral	33	645 ± 284	$5.2 \pm 3.7*$	$9.9 \pm 3.3*$	8.1 ± 3.2	$19.3 \pm 8.6*$	$39.6 \pm 13.4*$
Caudal	30	735 ± 272	$4.8 \pm 3.3*$	$18.3 \pm 8.9*†$	$14.4 \pm 13.1*$	$33.9 \pm 12.8*†$	$77.7 \pm 40.4*†$
HT							
Rostral	9	509 ± 196	0.8 ± 0.6	–	–	$30.4 \pm 11.2*$	$73.5 \pm 22.8*†$
Caudal	6	552 ± 293	0.7 ± 0.3	–	–	$26.7 \pm 15.3*$	$40.7 \pm 15.4*$

Data are shown as mean $\pm$ SD.

BR = response to brush stimulation; Caudal = neurons located caudal to the spinal cord injury lesion; HT = high-threshold neurons; PP = response to pinprick stimulation; PI = response to pinch stimulation; Rostral = neurons located rostral to the spinal cord injury lesion; SCI = spinal cord injury; SP = spontaneous activity; VF4 = response to punctate stimulation using a 4-g von Frey filament; WDR = wide-dynamic-range neurons; – = not examined.

* $P < 0.01$ versus sham-operated control animals; † $P < 0.05$ versus neurons rostral to the lesion.

Fig. 2. Schematic representation of the hemisectioning site and recording sites for neurons located rostrally (10–15 mm) and caudally (10–20 mm) to the lesion (*A*). Patterns of spontaneous activity in "unclassified" neurons rostral and caudal to the spinal cord injury (SCI) site (*B-D*). The firing rates (upper panels) and raw spike examples are shown. *B*, a constant pattern of spontaneous activity with relatively constant high frequencies. *C*, a bursting pattern of spontaneous activity with a relatively short decreasing period. *D*, a bursting pattern of spontaneous activity with a relatively long silent period. Two of the 15 neurons located rostral to the lesion showed a "C" pattern of spontaneous activity and two neurons showed a "D" pattern of spontaneous activity. The other neurons located rostral to the lesion and all of the neurons located caudal to the lesion showed a "B" pattern of spontaneous activity.
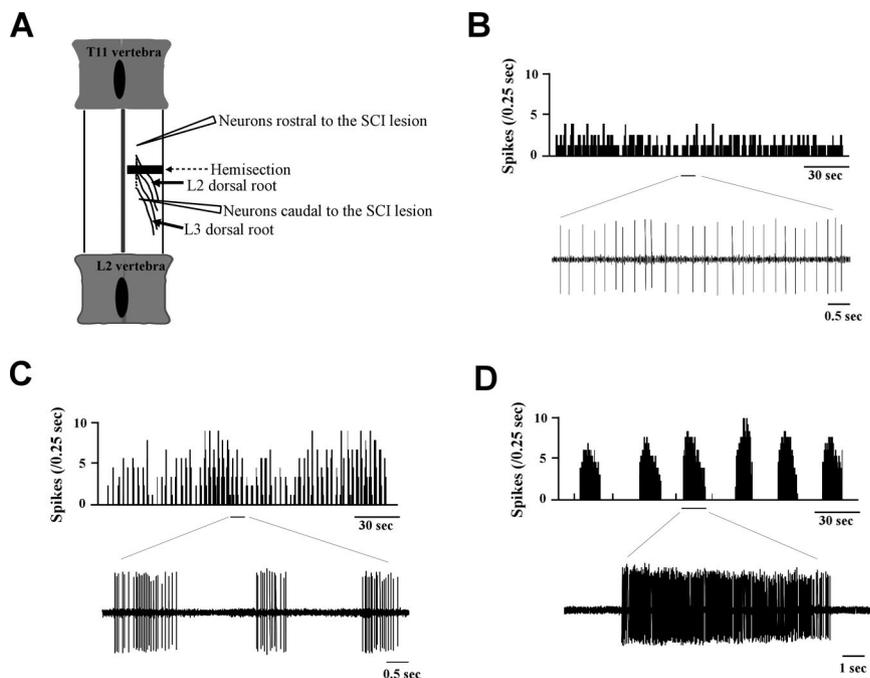
operated animals and 19 WDR neurons and 5 HT neurons in SCI animals. The effects of spinal morphine were examined in 16 WDR neurons and 5 HT neurons in sham-operated animals and 16 WDR neurons and 6 HT neurons in SCI animals.

Spontaneous Activity

Schematic representation of the hemisectioning site and recording sites for neurons located rostrally (10–15 mm) and caudally (10–20 mm) to the lesion are shown in figure 2A. In SCI animals, 26% (15 of 57) of the neurons rostral to the SCI site and 16% (7 of 43) of the neurons caudal to the SCI site showed spontaneous activity with high frequencies of 21.0 ± 7.1 Hz and 26.7 ± 12.5 Hz, respectively, these neurons being "unclassified" neurons, whereas spontaneous activity in neurons in sham-operated animals did not show such high-frequency firing rates (table 1). In these "unclassified" neurons, several patterns of the spontaneous firings were seen. In the majority of neurons, 73% (11 of 15) of the neurons rostral to the SCI site and 100% (7 of 7) of the neurons caudal to the SCI site, a relatively constant increase in spontaneous activity was seen (fig. 2B). Two of the remaining neurons located rostral to the SCI site showed burst activity with a short decreasing period (fig. 2C), and the other two neurons showed activity with a long silent period (fig. 2D). The "unclassified" neurons responded to pinch stimulation in the hindquarter. However, because accurate evaluation of neuronal activity was difficult because of the high-frequency firings, RF areas and evoked responses to various stimuli were not determined.

Spontaneous activity was significantly higher in the neurons both rostral and caudal to the SCI site in SCI animals than in sham-operated animals (table 2, $P < 0.01$). On the other hand, spontaneous firing rates in HT neurons rostral and caudal to the SCI site in SCI animals were not significantly higher than those in sham-operated animals (table 2, $P > 0.6$).

Locations and Sizes of Receptive Fields in WDR and HT Neurons

The most sensitive sites in and sizes of RF areas of WDR and HT neurons are shown in figures 3 and 4. Because the sizes of RFs on hairy skin of the hindquarter and on glabrous skin (plantar surface of the hindpaw) are greatly different, only the sizes of RF areas on the hairy skin were analyzed in this study (table 1). The locations of the RFs of WDR and HT neurons in SCI and sham-operated animals were similar in the present study. Both the sizes of RFs for low-threshold (4-g von Frey hair) stimulation and high-threshold (25-g tungsten tip) pinprick stimulation of WDR neurons both rostral and caudal to the SCI site in SCI animals were larger than those in sham-operated animals ($P < 0.01$). The sizes of RFs for the high-intensity stimulation of HT neurons located both rostrally and caudally in SCI animals were larger than those in sham-operated animals ($P < 0.05$).

Evoked Responses to Various Stimuli

The responses to dynamic nonnoxious stimuli applied by using a brush were significantly greater in WDR neurons located both rostral and caudal to the lesion in the SCI animals than in those in the sham-operated animals (table 2, $P < 0.01$). The responses to static innocuous stimuli applied by using a 4-g von Frey hair were also significantly greater in WDR neurons located caudal to the SCI site than in those in sham-operated animals,
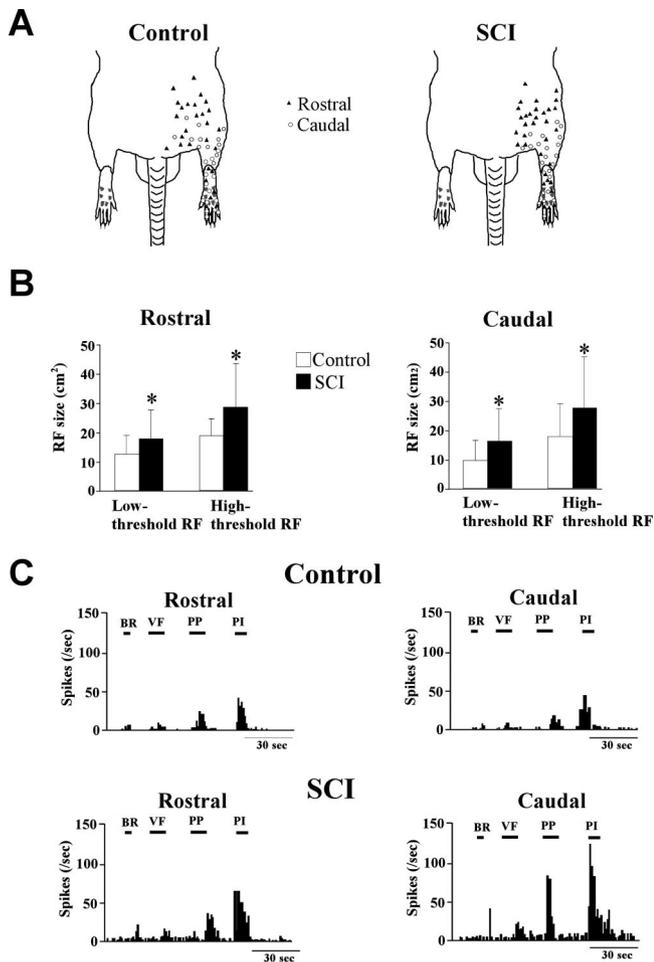
Fig. 3. Most sensitive sites (*A*) and mean sizes (*B*) of receptive fields (RFs) of wide-dynamic-range (WDR) neurons located rostral and caudal to the spinal cord injury (SCI) lesion. The data of RF sizes were obtained from 22 rostral WDR neurons and 15 caudal WDR neurons in sham-operated animals and 25 rostral WDR neurons and 14 caudal WDR neurons in SCI animals. Representative responses of WDR neurons located rostral and caudal to the SCI site to nonnoxious and noxious stimuli in control (sham-operated) animals and SCI animals (*C*). BR, brush stimulation; PI, pinch stimulation using an arterial clip with a force of 250 g/mm²; PP, pinprick stimulation using a 25-g tungsten tip; VF, stimulation using a 4-g von Frey hair. *$P < 0.01$ *versus* that in sham-operated control animals. Data are shown as means ± SD.

Fig. 4. Most sensitive sites (*A*) and mean sizes (*B*) of receptive fields of high-threshold (HT) neurons located rostral and caudal to the spinal cord injury (SCI) lesion. The data of receptive field (RF) sizes were obtained from five rostral HT neurons and seven caudal HT neurons in sham-operated animals and six rostral HT neurons and four caudal HT neurons in SCI animals. Representative responses to various stimuli of HT neurons located rostral and caudal to the SCI site in control (sham-operated) animals and SCI animals (*C*). BR, brush stimulation; PI, pinch stimulation using an arterial clip with a force of 250 g/mm²; PP, pinprick stimulation using a 25-g tungsten tip; VF, stimulation using a 4-g von Frey filament. * $P < 0.05$ *versus* that in sham-operated control animals Data are shown as means ± SD.

whereas there was no significant difference between the responses in WDR neurons located rostrally in the SCI and sham-operated animals ($P > 0.1$). Responses to noxious stimuli using the pinprick device and the arterial clip (pinching) were significantly greater in WDR neurons located both rostral and caudal to the SCI site in the SCI animals than in WDR neurons in the sham-operated animals ($P < 0.01$). The responses of caudally located WDR neurons to brush, pinprick, and pinch stimuli were significantly greater than the responses of WDR neurons located rostral to the SCI site ($P < 0.05$).

Responses to noxious stimuli (pinprick and pinching) were significantly greater those in HT neurons located rostral and caudal to the lesion in the SCI animals than in
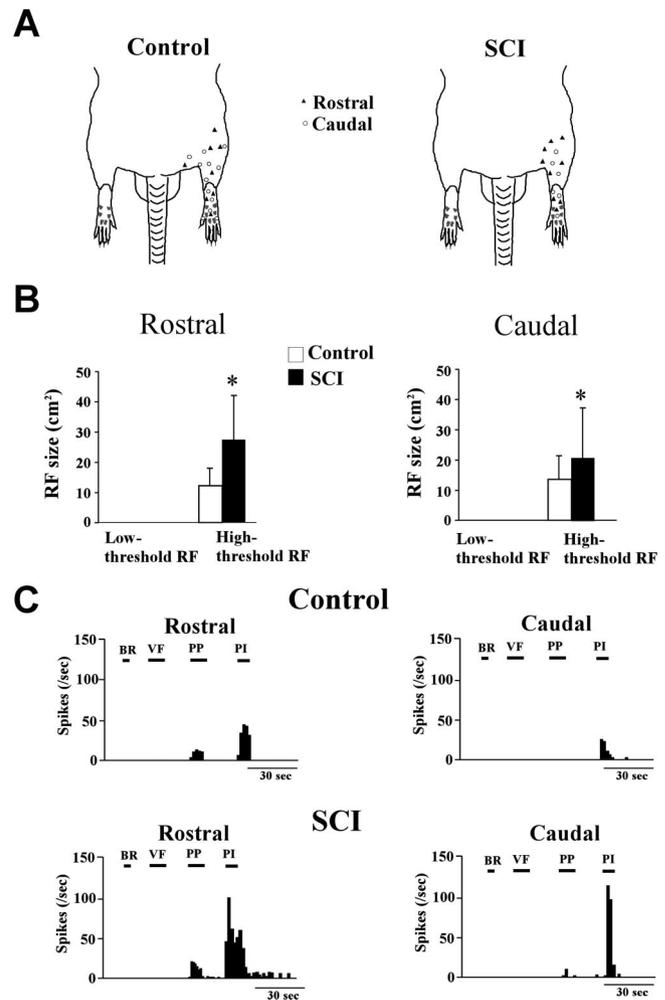
HT neurons in the sham-operated animals (table 2, $P < 0.01$). The mean values of responses to pinch stimuli were significantly higher in HT neurons located rostrally than those in HT neurons located caudally ($P < 0.05$).

Effects of Systemic and Spinal Administration of Morphine

Maximal effects of all doses of systemic morphine was observed 20–30 min after drug administration, and maximal effects were seen 10–20 min after spinal administration of morphine. Systemic and spinal administration of morphine did not significantly suppress the spontaneous activity in WDR neurons rostral or caudal to the SCI
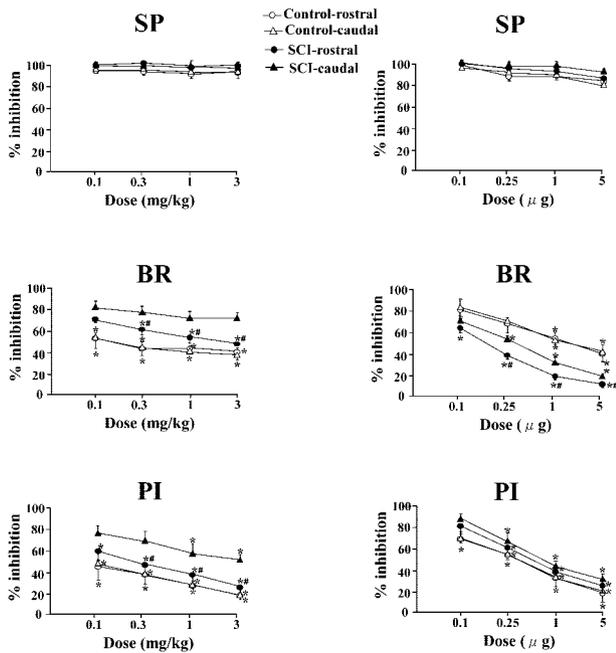
A. Systemic morphine

B. Spinal morphine

Fig. 5. Dose-effect curves of morphine treatments (*A*, systemic administration; *B*, spinal administration) of wide-dynamic-range (WDR) neurons located rostral and caudal to the spinal cord injury (SCI) site in sham-operated control animals and SCI animals. BR, evoked responses to brush stimulation; Control-caudal, neurons located caudally in sham-operated control animals; Control-rostral, neurons located rostrally in sham-operated control animals; PI, evoked responses to pinch stimulation; SCI-caudal, neurons located caudal to the lesion in SCI animals; SCI-rostral, neurons located rostral to the lesion in SCI animals; SP, spontaneous activity. *$P < 0.01$ *versus* that prior to administration of morphine. #$P < 0.05$ *versus* that in neurons caudal to the lesion in SCI animals. Data are shown as means ± SD.

site in the SCI animals or sham-operated control animals (fig. 5). Systemic morphine suppressed evoked responses to stimuli by a brush and pinching in a dose-dependent manner in neurons located rostrally and caudally in sham-operated control animals, and the suppression of responses to pinch stimulation was greater than the suppression of responses to brush stimulation (table 3). Systemic morphine also suppressed

brush stimulation-evoked and pinch stimulation-evoked responses in a dose-dependent manner in WDR neurons rostral to the SCI site in SCI animals. The responses to brush stimulation in WDR neurons caudal to the SCI were not significantly suppressed by systemic morphine at doses up to 3 mg/kg, whereas the responses to pinch stimulation were significantly suppressed but the degree of suppression was significantly less than that of responses in WDR neurons rostral to the SCI site. The maximal inhibitory effects of morphine were reversed by systemic administration of naloxone (1 mg/kg) to almost control values (data not shown).

On the other hand, spinal administration of morphine at doses up to 5 μg significantly suppressed brush stimulation-evoked responses in WDR neurons both rostral and caudal to the SCI site in SCI animals in a dose-dependent manner. The degrees of suppression in SCI animals were significantly higher than in sham-operated control animals (fig. 5, table 3). Spinal administration of morphine suppressed pinch stimulation-evoked responses in WDR neurons both rostral and caudal to the SCI site in a dose-dependent manner to degrees, similar to those seen in sham-operated animals. The maximal inhibitory effects of morphine were reversed by spinal administration of naloxone (5 μg) to almost control values (data not shown).

The responses in some HT neurons rostral and caudal to the SCI site slightly decreased, but the responses did not change, or increased, in other neurons after systemic (up to 3 mg/kg) and spinal (up to 5 μg) administration of morphine. Thus, morphine did not suppress the responses of HT neurons in SCI animals and sham-operated animals (data not shown).

Discussion

The results of the present study demonstrated that 1) spontaneous activity increased in WDR neurons both rostral and caudal to the SCI site after SCI, 2) evoked responses and RF sizes of WDR and HT neurons located both rostral and caudal to the SCI site increased, 3) WDR

Table 3. Effects of morphine treatment on activity in wide-dynamic-range neurons

	Control		SCI	
	Systemic (mg/kg)	Spinal (μg)	Systemic (mg/kg)	Spinal (μg)
Brush stimuli				
Rostral	0.22 (0.15–0.29)	3.4 (1.5–4.9)#	1.8 (1.3–2.4)*†#	0.14 (0.04–0.26)*†#
Caudal	0.23 (0.16–0.31)	3.6 (2.3–4.7)#	>3	0.45 (0.31–0.57)*
Pinch stimuli				
Rostral	0.15 (0.09–0.22)	0.36 (0.14–0.56)	0.17 (0.12–0.22)†	0.48 (0.27–0.69)
Caudal	0.16 (0.09–0.21)	0.37 (0.22–0.54)	2.1 (1.8–2.8)*	0.55 (0.26–0.81)

Values are ED_{50} (median effective dose 95% confidence interval).

Caudal = neurons located caudal to the spinal cord injury lesion; Rostral = neurons located rostral to the spinal cord injury lesion; SCI = spinal cord injury.

* $P < 0.01$ versus that in sham-operated control animals; † $P < 0.05$ versus that in neurons caudal to the SCI lesion; # $P < 0.05$ versus that in pinch stimuli; >3, unable to calculate because of its low suppression rate.

and HT neurons rostral to the SCI site still had RF areas in the below-level dermatomes, 4) the degrees of increases in response to mechanical stimuli of WDR and HT neurons located in spinal segments rostral and caudal to the SCI site were different, 5) the degrees of suppression of responses by spinal administration of morphine were greater in WDR neurons located both rostral and caudal to the SCI site than the degrees of suppression by systemic administration of morphine, and 6) HT neurons located both rostral and caudal to the SCI site were resistant to both systemic and spinal administration of morphine. The results suggest that SCI-induced central neuropathic pain is caused by different mechanisms, depending on the spinal segments rostral and caudal to SCI.

Changes in Spontaneous Activity in Spinal Neurons and Their Receptive Fields after SCI

Spinal hemisection in rats has been developed as an animal model of SCI, and it appears that this model is the equivalent of incomplete SCI in humans.[14] Hemisection of the spinal cord results in severe damage to both fibers and cells at the site of injury. The consequences for the directly injured segment are caused by direct cell death, but it is fiber interruption that in addition affects the function of adjacent spinal segments rostral and caudal to the site of injury. Areas rostral to the site of damage are deprived of propriospinal input from caudal segments and of input from branches of ascending tracts and primary afferent fibers. Segments caudal to the site of the damage will suffer from reduced propriospinal input from rostral segments, and, in addition, descending inhibitory controls of supraspinal origin are reduced. Because all of these interruptions could contribute to sensory disturbances, the properties of neurons proximal and distal to the site of SCI were examined in the present study.

Although increased spontaneous activity in spinal neurons has been observed in some animal models for SCI, such as an excitotoxic model,[15] spontaneous activity was not increased in other models, such as a photochemical model[16] and a contusion model.[17] Using a hemisection model, an increase in spontaneous activity was observed in a previous study,[18] but this increase was not seen in other studies.[14,19,20] The differences depend partly on the timing of the recordings after SCI, as it has been reported that spontaneous firing rates significantly increase immediately after hemisection of the spinal cord and remains high for several days and then gradually decrease to the basal level within the next several weeks.[20] The observation of increased spontaneous activity in WDR neurons located both rostral and caudal to the SCI site in the present study might have been a result of the fact that recordings were made 10–14 days after the hemisection had been made.

In the present study, bursting type of spontaneous activity of high frequencies for short periods alternating with silent and decreasing periods were seen only in neurons located rostral to the SCI site. In previous studies in which activity was recorded in spinal neurons caudal to the site of SCI, the bursting patterns were not seen.[17–20] However, early studies have shown that there is an altered background activity with burst in spinal neurons just rostral to the site of injury in paraplegic patients and spinal cats.[21,22] In addition, it has recently been reported that increased spontaneous activity with burst was observed in spinal neurons rostral to the site of contusion and transection of the spinal cord.[23] Thus, the bursting type of spontaneous activity might be characteristic of neurons rostral, but not caudal, to the site of injury after SCI. An abnormal pattern of spontaneous activity has been seen in spinal neurons after a peripheral nerve injury[24] and in afferents damaged by a constriction injury and transection,[25,26] suggesting that spontaneous hyperactivity of neurons rostral to the site of SCI is at least in part caused by similar patterns of excessive inputs from the neurons and dorsal roots damaged around the site of SCI. This hyperactivity might be the origin of chronic persistent or intermittent spontaneous pain perceived in segments around the site of SCI.

In the present study, spontaneous activity in HT neurons both rostral and caudal to the SCI site did not increase after the SCI. These results suggest that WDR neurons in the spinal cord both rostral and caudal to the site of SCI are involved in spontaneous pain in a body region that is thought to be located below the level of the SCI, that is, below-level pain. The dorsal horn projections of peripheral sensory nerves are arranged in a mediolateral and dorsoventral somatotopic pattern[27,28] and overlap between different nerves.[29] At the same level of the spinal cord, dorsal horn neurons receive primary afferent terminals of different nerves and different body surface regions, and sensory information from these surface regions is rearranged. Interestingly, in the present study, the distribution of RFs of spinal neurons in SCI animals was similar to that in sham-operated animals even though the spinal cord had been cut together with transection of the L2 dorsal root. Neurons rostral to the site of hemisectioning of the spinal cord at the level of L2 still had RFs on the plantar surface of the hindpaw that are dermatomes L3, L4 and L5.

After SCI, neuronal growth in the spinal dorsal horn can occur from spared unlesioned fibers as a result of the denervation of nearby target areas, although "collateral sprouting" is restricted and does not occur over larger distances.[30] Furthermore, afferent fibers terminate on both sides of the spinal dorsal horn,[31] and intraspinal neurons of the spinal dorsal horn project to neurons located on the contralateral side of the same segment and different segments of the dorsal horn.[32] Thus, the collateral sprouting and the relay of a primary afferent fiber and an intraspinal neuron of the dorsal horn may

explain the findings that the distribution of RFs after hemisectioning of the spinal cord was similar to that in sham-operated animals.

In the present study, the sizes of RFs of neurons rostral and caudal to the site of SCI in the SCI animals were larger than those in the sham-operated animals. Our results are in accordance with results of some previous studies in which recording was performed at a relatively early stage of the SCI.[33] In other previous studies in which recording was done at a late stage after SCI (4–8 weeks), no increases or only slight increases in RF sizes were observed.

Evoked Responses to Nonnoxious and Noxious Stimuli after SCI

The results of the present study showed dynamic changes in the properties of both WDR and HT neurons located both rostral and caudal to the site of SCI after hemisectioning of the spinal cord, supporting the notion of development of "central sensitization" of WDR and HT neurons as a result of hemisectioning. The responses to nonnoxious and noxious stimuli of WDR neurons caudal to the site of SCI were significantly greater than those of WDR neurons rostral to the site of SCI. The modulation of spinal nociceptive transmission by descending inhibitory systems is well established, and interruption of descending inhibitory neurons, including opioidergic, serotonergic, and noradrenergic fibers, by SCI confers release from inhibitory control on dorsal horn nociceptive circuitry,[34] possibly resulting in greater responsiveness of WDR neurons caudal to the SCI site than WDR neurons rostral to the SCI site.

In contrast, the responses to noxious stimuli of HT neurons located rostral to the SCI site were significantly greater than those of HT neurons located caudal to the site of SCI. Because HT neurons can functionally be converted to WDR neurons in persistent pain and after removal of descending inhibition,[35,36] some of the HT neurons caudal, but not rostral, to the SCI site could have been changed to and classified as WDR neurons. The conversion might result in different response properties of neurons classified as HT neurons rostral and caudal to the lesion, although the proportions of WDR neurons and the proportions of HT neurons rostral and caudal to the SCI site were not different in the present study. Another possibility is the influence of descending facilitation from supraspinal origin. It has been shown that descending facilitatory influence from the rostral ventromedial medulla and its surrounding areas participates in modulation of hyperexcitability in spinal dorsal horn neurons in persistent pain.[37] Stimulation of the descending facilitatory systems produce depolarizing potentials in neurons in spinal laminae I and II, mainly HT neurons.[38] Thus, if descending facilitation is enhanced after SCI, responses of HT neurons rostral to the SCI site could increase more greatly than those caudal to the SCI

site because of interruption of the influence of descending facilitation on neurons caudal to the SCI site. However, in general, low-intensity stimulation facilitates spinal nociceptive transmission, whereas high-intensity stimulation inhibits spinal nociceptive transmission.[37] Thus, the reason for the greater responsiveness of rostral HT neurons compared with caudal HT neurons is still unclear. The results of the present study suggest that different mechanisms of changes in the neural circuit in different spinal segments provide a basis for the increased responsiveness of WDR and HT neurons and hence a basis for behavioral responsiveness to noxious and nonnoxious stimuli (*i.e.*, the development of mechanical allodynia and hyperalgesia) after SCI.

On the other hand, it should again be pointed out that some of the neurons rostral to the SCI at the level of L2 still had RFs on the plantar surface of the hindpaw, which are thought to be dermatomes L3, L4, and L5, and that neurons located rostrally showed hyperexcitability in response to nonnoxious and noxious stimuli applied to the RF areas. These results suggest that neurons located in spinal segments both rostral and caudal to the site of SCI are responsible for allodynia and hyperalgesia in the below-level skin. Because the mechanisms of hyperexcitability in spinal neurons rostral and caudal to the site of SCI appear to be different, it is likely that such different mechanisms are involved in the occurrence of below-level pain.

Effects of Systemic and Spinal Morphine Administration on Neuronal Activity after SCI

In the present study spontaneous activity and evoked responses significantly increased in neurons located rostral and caudal to the site of SCI in SCI animals compared with those in sham-operated animals. The predrug values of the neurons examined were different in SCI animals and sham-operated control animals. However, because we aimed to compare the effects of morphine treatments on different types of neurons, that is, neurons rostral and caudal to the SCI site in SCI animals and sham-operated animals, the degrees of suppression of morphine were analyzed in the different groups of the neurons. The doses of systemic morphine tested (up to 3 mg/kg) were chosen because a higher dose (10 mg/kg) was shown in a previous behavioral study to produce motor depression.[39]

Systemic and spinal morphine did not suppress spontaneous activity in WDR neurons rostral or caudal to the SCI site in SCI animals. However, systemic administration of morphine had an effect on evoked responses of WDR neurons in SCI animals. The effects on responses to noxious stimuli in WDR neurons located caudal to the SCI site in SCI animals were less than those in WDR neurons located rostral to the site of SCI in SCI animals and those in WDR neurons located rostral and caudal to the site of SCI in sham-operated animals. Systemically

administered morphine produces its antinociceptive effect through a mechanism involving synergism of spinal and supraspinal opioid systems. Descending monoaminergic pathways participate in the production of antinociception by systematically administered morphine.[40] In spinal segments caudal to the lesion, descending inhibitory systems, including opioidergic, noradrenergic and serotonergic systems, were interrupted. As a result of that, systemic morphine may not have exerted suppressive effects on the responses to noxious stimuli in WDR neurons caudal to the lesion. On the other hand, with regard to responses to nonnoxious stimuli in the present study, WDR neurons rostral to the lesion were also relatively resistant to systemic morphine compared with WDR neurons in sham-operated control animals. There is ample evidence for the localization of μ-opioid receptors in small-diameter C-fiber afferents, but not large-diameter Aβ-afferents, and in a small population of postsynaptic sites.[41] Morphine mainly exerts presynaptic inhibitory effects on C-fiber-evoked activity in the spinal cord with minor effects on large-diameter fiber terminals. Furthermore, systemic morphine and microinjection of morphine within the nucleus raphes magnus suppress noxious, but not nonnoxious, stimulation-related responses in the spinal cord.[42] Thus, it is thought that morphine may be differentially effective against different types of response in WDR neurons to nonnoxious and noxious stimuli even at the same segments of the spinal cord relative to the injured site.

When morphine at doses up to 5 μg/μl was spinally administered, responses to noxious stimuli were suppressed in WDR neurons rostral and caudal to the SCI site in SCI animals. Thus, the relative ineffectiveness of systemic administration of morphine and the relative effectiveness of spinal administration of morphine in the present study suggests that a relatively high concentration of morphine in the spinal cord is required to produce the suppressive effects on activities of WDR neurons, consistent with the results of a recent behavioral study.[39] Furthermore, spinal morphine was more effective on the responses to nonnoxious stimuli in WDR neurons both rostral and caudal to the site of SCI in SCI animals than in sham-operated animals, suggesting that spinal administration of morphine is effective on allodynia after SCI.

On the other hand, neither systemic administration nor spinal administration of morphine suppressed responses to noxious stimuli of HT neurons located both rostral and caudal to the SCI site in SCI animals and sham-operated animals, and the activity of some HT neurons was increased by morphine administration, suggesting that HT neurons are resistant to morphine treatment even in a normal state and an SCI-induced pain state. It has been reported that activities of HT neurons were suppressed less by spinal administration of fentanyl than were WDR neurons.[43] Morphine (5 mg/kg) has also

been shown to cause various changes in C-evoked activity of units recorded within lamina II in rats, some units showing excitation, some showing inhibition, and some showing alterations in the timing and pattern of C-evoked activity.[44] Administration of high concentration of morphine results in suppression of C-fiber and pinch-evoked activity of spinal neurons, although a lower concentration of morphine elicited excitation. Furthermore, it has previously been shown that descending inhibition of HT neurons is less than that of WDR neurons.[45] Taken together with the results of the previous studies, the results of the present study suggest that there is still a difference between the sensitivities of HT neurons and WDR neurons to morphine after SCI and that HT neurons are not the target for morphine treatment in an SCI-induced pain state.

Clinical Implications

Previous studies have suggested that SCI-induced pain is resistant to conventional drug therapies, including opioid treatment, but more recent studies have shown the efficacy of opioids in different neuropathic pain states.[46] There have been no trials on the effects of morphine on above-level and below-level pain in a clinical setting, but the fact that intrathecal administration of morphine is more effective on at-level pain than on below-level pain in SCI patients suggests that at-level pain and above-level pain are more sensitive to morphine treatment than is below-level pain. However, the fact that systemic administration and spinal administration of morphine alone have little or no effect on spontaneous pain but decrease brush-induced allodynia in SCI patients[3] is consistent with our results on the effects of morphine on spontaneous activity and brush stimulation-evoked responses. Thus, to reduce spontaneous pain in SCI patients in a clinical setting, a combination of morphine with other drugs such as clonidine and gabapentin may be required.[46,47]

From the results of the present study, it appears that the changes in physiologic and pharmacological properties of spinal dorsal horn neurons are complicated, depending in part on the spinal segments rostral and caudal to the SCI lesion, classification of spinal neurons, types of neuronal activity such as spontaneous activity, responses to nonnoxious and noxious stimuli, and mode of drug administration. Furthermore, in a clinical setting, the type and degree of SCI is different in each SCI patient, possibly resulting in different types and locations of pain caused by the different mechanisms seen in the present study. Physical activities, posture, and physical therapy may change the severity and location of pain in SCI patients. A better understanding of mechanisms of SCI-induced pain is important because treatment could be directed against the specific pain mechanism.

Hemisectioning of the rat spinal cord results in mechanical allodynia/hyperalgesia and thermal hyperalgesia

in forelimbs and hindlimbs on both contralateral and ipsilateral sides to the SCI site.[14,18–20] Electrophysiological recordings were not performed on the contralateral side to the SCI site in this study, and it should thus be pointed out that mechanisms underlying the onset of central neuropathic pain on the contralateral side to the SCI might not be the same as those on the ipsilateral side. However, the time courses of development of hyperalgesia/allodynia were similar on the ipsilateral and contralateral sides to the hemisection, and similar mechanisms, as described below, have been postulated on both sides. The principal descending inhibitory systems, one of which is the serotonergic pathway, project both ipsilaterally and contralaterally at the segmental level of termination. It has been postulated that the key factor in the persistence of pain-related behavior that occurs concomitant with improved locomotor recovery after hemisection is the inadequate return of dorsal horn serotonin concentrations bilaterally and changes in serotonin transporter and receptor populations.[19] Other mechanisms that may be involved in bilateral nociceptive alterations include changes in primary afferents that terminate not only at and below but also above the level of innervation and changes in afferents that project bilaterally in the spinal cord.[48] Short-fiber multisegmental propriospinal pathways that can relay somatosensory information bilaterally and from below may also play a role.[49]

Although above-level pain occurs in SCI patients, below-level pain and at-level pain are more common than above-level pain,[1] suggesting that strategies for treatment should be focused on below-level pain and at-level pain. In the present study, recordings could not performed in neurons at the level of the SCI site because of adhesion to the connective tissue and bleeding as a result of rich vascularity. However, because the hyperexcitability in spinal neurons at the level of the lesion may contribute to the spontaneous pain and evoked pain in SCI patients, further study is needed to determine characteristics of spinal neurons located in the site of injury.

In summary, the electrophysiological evidence presented here supports the notion that different mechanisms depending on the spinal segments rostral and caudal to the SCI lesion contribute to the complexity of SCI pain and different sensitivities of SCI pain to drug treatment. The results provide mechanistic insights into the effectiveness of morphine application.

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