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Contribution of TRPV1 Receptor–Expressing Fibers to Spinal Ventral Root After-Discharges and Mechanical Hyperalgesia in a Spared Nerve Injury (SNI) Rat Model

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Abstract. Neuropathic pain induces allodynia and hyperalgesia. In the spared nerve injury (SNI) model, marked mechanical hyperalgesia is manifested as prolongation of the duration of paw withdrawal after pin stimulation. We have previously reported that spinal ventral root discharges (after-discharges) after cessation of noxious mechanical stimulation applied to the corresponding hindpaw were prolonged in anesthetized spinalized rats. Since these after-discharges occurred through transient receptor potential (TRP) V1–positive fibers, these fibers could contribute to mechanical hyperalgesia. Therefore, we examined whether selective deletion of TRPV1-positive fibers by resiniferatoxin, an ultrapotent TRPV1 agonist, would affect the behavioral changes and ventral root discharges in SNI rats. Mechanical allodynia in the von Frey test, mechanical hyperalgesia after pin stimulation, and enhancement of ventral root discharges, but not thermal hyperalgesia in the plantar test, appeared in Wistar rats with SNI. Mechanical hyperalgesia was abolished by treatment with resiniferatoxin, whereas mechanical allodynia was not affected. Moreover, resiniferatoxin eliminated after-discharges completely. These results show that TRPV1-positive fibers do not participate in the mechanical allodynia caused by sensitization of Aβ-fibers, but contribute to the enhancement of after-discharges and mechanical hyperalgesia following SNI. It is suggested that the mechanisms responsible for generating mechanical allodynia differ from those for prolongation of mechanical hyperalgesia.

Keywords: after-discharge, hyperalgesia, spared nerve injury model, spinal cord, transient receptor potential V1

Introduction

Allodynia (pain in response to innocuous stimuli) and hyperalgesia (increased sensitivity to noxious stimuli) are typical symptoms of neuropathic pain (1). These phenomena lower a patient's quality of life and most analgesics are insufficiently effective. Various animal models in which dominant hindlimb nerves are injured have been developed in order to assess neuropathic pain (2 – 4). Among them, the spared nerve injury (SNI) model is considered to closely mimic many features of clinical neuropathic pain because this model produces not only marked mechanical allodynia but also characteristic mechanical hyperalgesia, defined as prolongation of the duration of paw withdrawal behavior after pin stimulation (5).

We have previously reported that spinal ventral root after-discharges are evoked by application of noxious mechanical stimuli to the corresponding hindpaw (6, 7). These after-discharges are considered to be related to the above-mentioned mechanical hyperalgesia because these phenomena are prolonged after cessation of the noxious stimuli (5, 8). Such after-discharges are also observed in the spinal dorsal horn neurons (9, 10) and are simultaneously recorded from motor units after high intensity transcutaneous stimulation (11, 12). The after-discharges are generated in spinal dorsal horn neurons by plateau potentials (13, 14) mediated by voltage-dependent L-type

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Ca^{2+} channels (15, 16). Similarly, our previous study demonstrated that spinal ventral root after-discharges were depressed by application of L-type voltage-dependent Ca^{2+}-channel blockers to the spinal dorsal horn (7).

Furthermore, the after-discharges were abolished by resiniferatoxin (RTX) (6), an ultrapotent capsaicin analog (17), which, after a single injection, desensitizes transient receptor potential (TRP) V1−positive primary afferent fibers (18). TRPV1 is found mainly in small-to-medium-diameter primary afferents, particularly unmyelinated C- and myelinated Aδ-fibers (19, 20), which play an important role in nociception. If the mechanical hyperalgesia induced by SNI hypersensitizes these fibers, it would be anticipated that the mechanical hyperalgesia is affected by RTX in the same way as the after-discharges.

In this study, we examined whether selective deletion of TRPV1-positive fibers by RTX would affect the development of behavioral changes and the persistence of ventral root discharges after mechanical stimulation in SNI model rats.

Materials and Methods

All experimental protocols used here were approved by the Animal Care and Use Committee of Nagoya City University and were conducted in accordance with the guidelines of The Japanese Pharmacological Society.

Spared nerve injury model

The surgical procedure for producing the SNI model was originally described by Decosterd and Woolf (5). Male Wistar/ST rats (6-week-old; SLC, Shizuoka) were treated under isoflurane (2% – 4%) anesthesia. An incision was made into the skin on the lateral surface of the left thigh, followed by a section through the biceps femoris muscle to expose the sciatic nerves. The common peroneal and tibial nerves were then tightly ligated with 5-0 silk thread, sectioned distal to the ligation, and 2 − 4 mm of the distal nerve stump removed. The sural nerve was left intact, taking care not to stretch it. Sham controls involved exposure of the sciatic nerve and its branches without any lesion.

Tactile allodynia

Tactile allodynia was assessed by measuring hindpaw withdrawal responses to von Frey filaments (Touch-Test® Sensory Evaluators; North Coast Medical, Gilroy, CA, USA), with a pressure ranging from 2 to 60 g (2, 4, 6, 8, 10, 15, 26, and 60 g). Rats were placed in cages with wire-mesh floors. The 50% likelihood of a paw withdrawal response (50% threshold) was determined using the up–down method (21). Testing was initiated with the 8 g filament, and each filament was applied perpendicularly within the area innervated by the sural nerve on the lateral plantar surface (5) of the left hindpaw with sufficient force to cause slight bending of the filament, for about 3 s. If a positive response (lifting of the hindpaw) was elicited, the next weaker filament was used. If a negative response (absence of a hindpaw withdrawal) was elicited, the next stronger filament was used. This paradigm was continued until four measurements had been obtained after an initial change in behavior or until four consecutive negative (1.5 g) or five positive (80 g) scores had been obtained. The resulting scores were used to calculate the 50% threshold (22).

Response to a thermal stimulus

Rats were placed on a glass platform within a plastic chamber. After they had become habituated, the lateral plantar area was exposed to a beam of radiant heat through a transparent Perspex surface (Plantar Test; Ugo Basile, Comerio, Italy) (23). Paw withdrawal latency was measured to the nearest 0.1 s and determined as the average of two measurements per rat. The cut-off time was set to 30.0 s to avoid any tissue damage.

Response to a noxious mechanical stimulus

After the measurement of mechanical allodynia, a pin-prick test was performed using a safety pin. The lateral part of the paw was briefly stimulated at an intensity sufficient to indent, but not penetrate, the skin. The duration of paw withdrawal was recorded, with an arbitrary minimum time of 0.5 s (for a brief normal response) and a maximal cut-off of 15.0 s (8). Since the paw withdrawal behavior is often long-lasting, the pin-prick test was always preceded by the von Frey test to avoid any influence of the prolonged behavior evoked by the pin stimulus.

Electrophysiology protocols

After the behavioral study, the rats were anesthetized with α-chloralose (150 mg/kg, i.p.). Cannulae were then inserted into the trachea for artificial respiration (70 breaths/min, 1 ml / 100 g body weight, end-tidal CO_2 concentration about 5%). To produce spinalized rats, the vagus nerves were cut bilaterally in the cervical region to eliminate parasympathomimetic effects on the heart, and the spinal cord was transected at the level of the first cervical segment (C1) under lidocaine anesthesia (4%, 50 μl). A dorsal laminectomy was performed in the lumbo-sacral region of each rat. Both the ventral and dorsal roots below the sixth lumbar segment (L6) were cut distally at their points of exit from the vertebral column. The left fifth lumbar segmental (L5) ventral root was sectioned for recording, and the ipsilateral L5 dorsal root was left intact to receive peripheral signals. The

entire exposed surgical area was covered with liquid paraffin maintained at 36°C ± 0.5°C by radiant heat. Rectal temperature was maintained at 36°C ± 0.5°C. Heart rate was monitored with recording electrodes inserted into both forepaws.

The left plantar surface of a hindpaw was mechanically stimulated using von Frey filaments. Each stimulus was applied for 3 s to the most sensitive point where the largest number of discharges was observed. The ventral root discharges occurring during the 3 s of stimulation were defined as 'during-discharges' and those occurring during 60 s after the stimulation as 'after-discharges'. These responses were normalized by subtraction of the spontaneous activity measured before application of the stimuli. A pair of Ag–AgCl wire electrodes was used for recording. Multi-unit firing recorded from the left L5 whole ventral root was recorded on a digital recorder (sampling rate: 48 kHz, R-44; Roland, Shizuoka). The signals were amplified and analyzed using a PowerLab (ADInstruments, Colorado Springs, CO, USA) and Chart software.

Drugs

α-Chloralose was obtained from Tokyo Kasei (Tokyo); capsaicin, from Wako (Osaka); and RTX, from LKT Laboratories (St. Paul, MN, USA). α-Chloralose was dissolved in distilled water and administered intraperitoneally. Capsaicin and RTX were dissolved in a mixture of 10% ethanol (Wako) and 10% polyoxyethylene (20) sorbitan monooleate (Wako) in 0.9% w/v physiological saline. RTX was administered at 200 μg/kg subcutaneously (s.c.) 1 week after SNI surgery to desensitize TRPV1-positive primary afferent fibers. The desensitization of TRPV1-sensitive fibers was confirmed by lack of eye-scratching evoked by instillation of 1% capsaicin solution into the right eye (18).

Statistical analyses

All data were expressed as the mean ± S.E.M. In the behavioral study, Mann-Whitney's *U*-test was used for comparisons between two groups, and two-tailed non-parametric multiple comparisons with Bonferroni correction following the Kruskal–Wallis test were used for comparisons between the control and other groups. In the electrophysiological study, the *t*-test with Bonferroni correction following one-way analysis of variance (ANOVA) was used for multiple comparisons of the control and other groups (24). Differences at $P < 0.05$ (two-tailed) were considered to be significant.

Results

Mechanical allodynia in SNI model rats

All SNI rats developed marked hypersensitivity to innocuous von Frey filament stimulation of the hindpaw ipsilateral to the nerve injury. The 50% threshold showed a substantial decline starting 1 day after the surgery [before: 11.15 ± 1.61 g (N = 6); 1 day after: 2.37 ± 0.34 g (N = 6)] (Fig. 1), and this persisted for 7 days [7 days after: 1.54 ± 0.02 g (N = 6), $P < 0.05$, compared with the sham group]. There was no significant difference in the 50% threshold between sham [9.11 ± 0.95 g (N = 6)] and age-matched naïve [11.86 ± 2.00 g (N = 6)] rats 7 days after surgery. On the contralateral side, a significant decline of the 50% threshold was observed only 7 days after SNI surgery [naïve: 10.95 ± 1.34 g (N = 6); sham: 9.58 ± 0.52 g (N = 6); SNI: 6.78 ± 0.69 g (N = 6), $P < 0.05$, compared with the sham group].

Changes in ventral root discharges in SNI model rats

Ventral root discharges recorded in SNI and sham rats were compared (Fig. 2). In sham rats, low-intensity (10 g) mechanical stimulation of the hindpaw evoked only during-discharges, whereas high-intensity (60 g) stimulation evoked both during- and after-discharges (Fig. 2A). On the other hand, SNI rats showed during-discharges and after-discharges in response to low-intensity stimulation (10 g) (Fig. 2B). The discharge frequencies increased

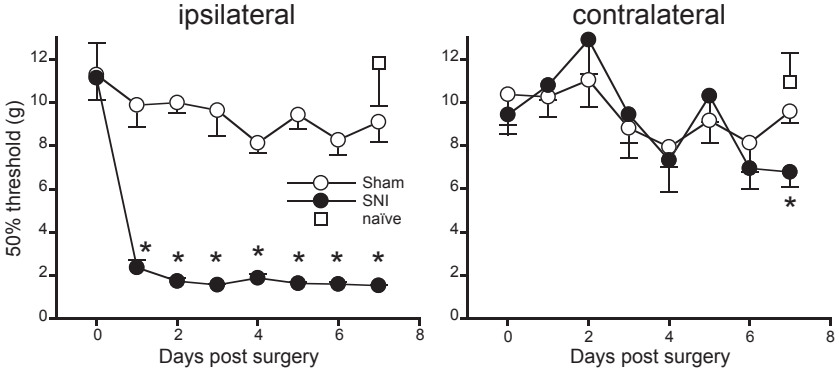

Fig. 1. Changes in the 50% thresholds of SNI rats. Age-matched naïve rats were also compared 7 days after surgery (just before measurement of ventral root discharges). Ipsilateral 50% thresholds were significantly reduced in SNI rats from 1 day after surgery. There were no changes in the threshold in sham and naïve rats. In the contralateral paw, the 50% threshold was slightly but significantly reduced only at 7 days after surgery. Each point represents the mean ± S.E.M. of 6 rats in each group. The statistical significance of differences was determined by Mann-Whitney's *U*-test. *$P < 0.05$ vs. sham.

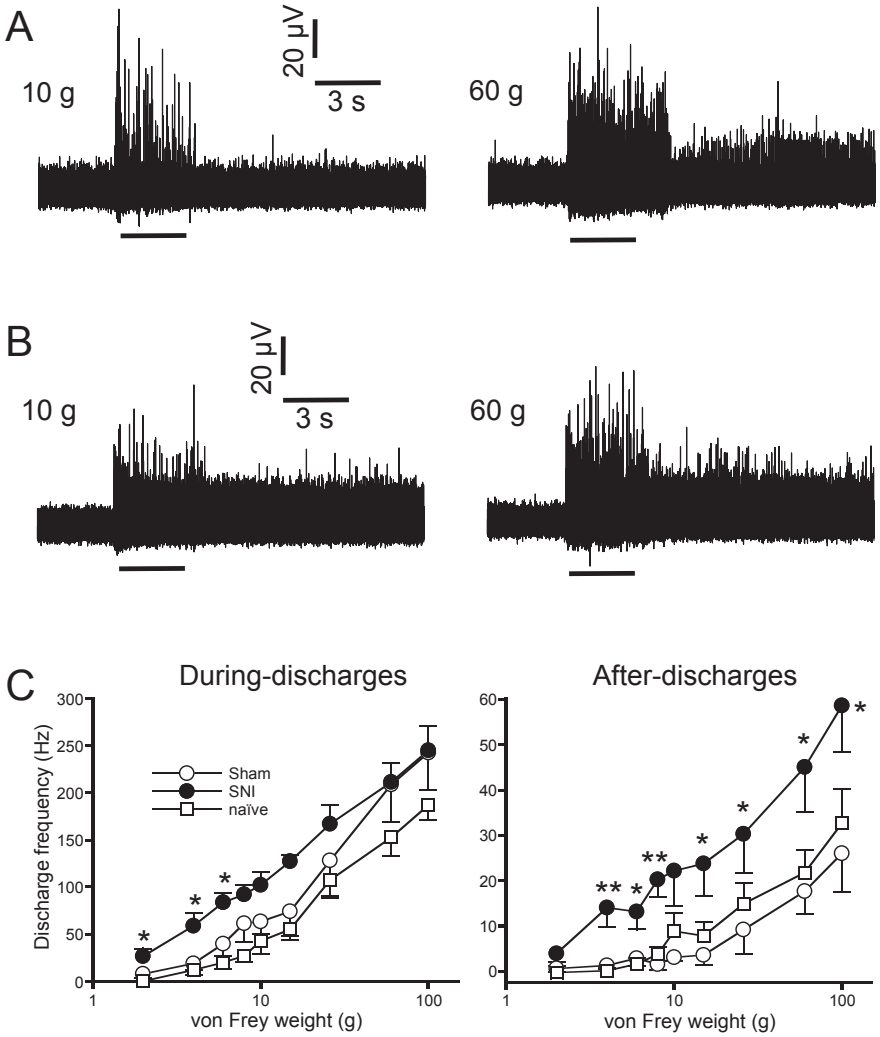

Fig. 2. Comparison of ventral root discharges recorded in a sham-treated rat (A) or a SNI model rat (B). Although low (10 g)-intensity mechanical stimulation of the hindpaw evoked during-discharges in both rats, after-discharges were only evoked in SNI model rats. High (60 g)-intensity stimulation evoked during- and after-discharges, and after-discharges occurred in both types of rats. Graphs in panel C show the stimulus–response recruitment curves of during- and after-discharges in sham, SNI, and naïve rats. In SNI rats, during-discharges were increased in response to low-intensity stimulation, and after-discharges were markedly enhanced in response to various intensities of stimulation with von Frey filaments. Each point represents the mean ± S.E.M. of 6 separate experiments. Ordinate: discharge frequency per second. Abscissa: stimulus intensity (grams). The statistical significance of differences between the sham and other groups was determined by the two-tailed multiple *t*-test with Bonferroni's correction following one-way analysis of variance (ANOVA) (2 comparisons in 3 groups). $*P < 0.05$, $**P < 0.01$ vs. sham.

in an intensity-dependent manner (Fig. 2C). A significant increase of during-discharges elicited by low-intensity stimulation under 6 g was observed in SNI rats [sham: 39.7 ± 12.8 Hz (N = 6); SNI: 83.5 ± 10.4 Hz (N = 6), $P < 0.05$, 6 g stimulation] (Fig. 2C). There was no significant difference in the during-discharges evoked by noxious stimuli between the sham and SNI groups [sham: 209.0 ± 40.2 Hz (N = 6); SNI: 211.7 ± 20.0 Hz (N = 6), 60 g stimulation]. Although after-discharges were not elicited by low-intensity stimulation in the sham and naïve groups, SNI rats showed after-discharges in response to low-intensity stimulation [sham: 1.2 ± 0.6 Hz (N = 6); SNI: 14.0 ± 4.2 Hz (N = 6), $P < 0.01$, 4 g stimulation]. The noxious stimuli elicited a further increase of after-discharges in SNI rats [sham: 17.6 ± 5.1 Hz (N = 6); SNI: 45.1 ± 9.9 Hz (N = 6), $P < 0.05$, 60 g stimulation] (Fig. 2C).

Effects of RTX on thermal responses of SNI model rats

No signs of severe discomfort were observed in the SNI surgery and RTX treatment groups. RTX-treated rats showed no eye-blinking reaction to 1% capsaicin solution throughout the experiment, indicating desensitization of TRPV1-sensitive fibers. The withdrawal latency upon application of thermal stimulation did not change after the SNI procedure (Fig. 3). However, RTX induced significant prolongation of the withdrawal latency in comparison with the vehicle-treated group on the 1st day after the treatment [SNI-RTX: 27.4 ± 2.6 s (N = 5); SNI-vehicle: 16.5 ± 0.8 s (N = 5), $P < 0.05$]. This prolongation was sustained for 7 days after RTX administration [SNI-RTX: 26.3 ± 1.4 s (N = 5); SNI-vehicle: 13.1 ± 1.3 s (N = 5), $P < 0.05$].

Effects of RTX on mechanical allodynia in SNI model rats

The decrease in the 50% threshold in the von Frey test

after SNI surgery was not affected by administration of RTX 1 week after the surgery (Fig. 4A). RTX treatment in the sham group decreased the 50% threshold slightly, and a significant difference relative to the RTX-treated SNI group was observed for 14 days after surgery [SNI-RTX: 1.5 ± 0.0 g (N = 5); sham-RTX: 10.1 ± 1.0 g (N = 5), $P < 0.05$]. On the contralateral side, SNI surgery and RTX treatment had no significant effect on the 50% threshold.

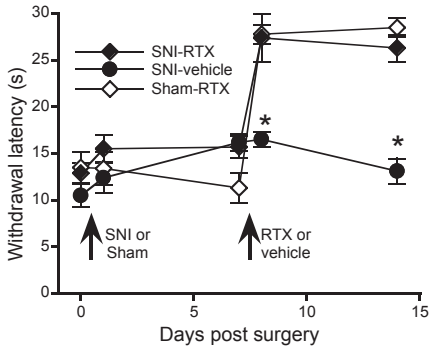

Fig. 3. Response to thermal stimuli in SNI rats. SNI produced no change in withdrawal latency. RTX treatment prolonged the withdrawal latency. Each datum represents the mean ± S.E.M. of 5 rats in each group. The statistical significance of differences was determined by two-tailed non-parametric comparison with Bonferroni's correction following the Kruskal-Wallis test (2 comparisons in 3 groups). *$P < 0.05$ vs. SNI-RTX.

Effects of RTX on response to noxious mechanical stimulation in SNI model rats

Quick withdrawal responses evoked by pin stimulation were observed in every group during the experimental period. Only in SNI rats was a prolonged paw withdrawal response to pin stimulation observed in the ipsilateral hindpaw 1 day after surgery. This behavior was not observed in the sham groups [SNI-RTX: 12.0 ± 1.7 s (N = 5); sham-RTX: 0.0 ± 0.0 s (N = 5), $P < 0.05$] (Fig. 4B). The RTX treatment drastically reduced the withdrawal duration in the pin-prick test [SNI-RTX: 0.3 ± 0.3 s (N = 5); SNI-vehicle: 8.2 ± 1.1 s (N = 5) in 8 days after SNI, $P < 0.05$]. This reduction persisted for 1 week after RTX treatment [SNI-RTX: 0.8 ± 0.6 s (N = 5); SNI-vehicle: 8.8 ± 0.9 s (N = 5) in 14 days after SNI, $P < 0.05$]. In the contralateral hindpaw, the paw withdrawal response did not occur in SNI rats and RTX did not influence this response.

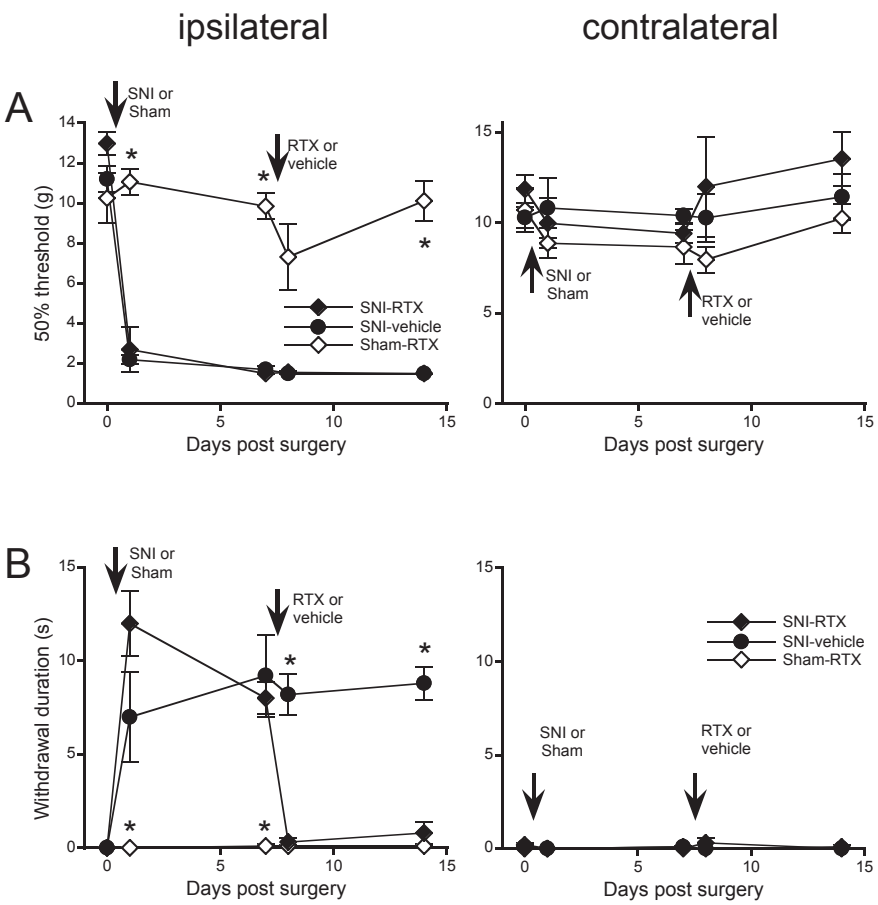

Fig. 4. Effect of RTX on 50% thresholds (A) and pin withdrawal duration (B) of SNI rats. A: The decreased ipsilateral 50% threshold was sustained for at least 14 days in SNI rats. Treatment with RTX at 7 days after surgery did not change the 50% threshold. There was no significant alteration of the threshold in the contralateral paw. B: SNI-induced mechanical hyperalgesia was reversed by RTX. Each datum represents the mean ± S.E.M. of 5 rats in each group. The statistical significance of differences was determined by two-tailed non-parametric comparison with Bonferroni's correction following the Kruskal-Wallis test (2 comparisons in 3 groups). *$P < 0.05$ vs. SNI-RTX.

Effects of RTX on ventral root discharges in SNI model rats

The after-discharges evoked by both low (10 g)- and high (60 g)-intensity mechanical stimulation were observed in vehicle-treated SNI rats, similarly to the results shown in Fig. 2 (Fig. 5A). During-discharges that were increased in SNI rats tended to be depressed by RTX, but not to a significant degree [SNI-RTX: 70.9 ± 12.3 Hz (N = 5); SNI-vehicle: 122.23 ± 33.6 Hz (N = 5), 10 g stimulation] (Fig. 5C). However, a marked depression of during-discharges was detected when high-intensity stimuli were applied [SNI-RTX: 108.2 ± 29.0 Hz (N = 5); SNI-vehicle: 208.1 ± 32.8 Hz (N = 5), 60 g stimulation]. The during-discharges in the RTX-treated sham group tended to be depressed at each stimulus intensity [30.3 ± 19.3 Hz (N = 5), 10 g stimulation; 88.6 ± 35.9 Hz (N = 5), 60 g stimulation] (Fig. 5C). On the other hand, the after-discharges in the SNI group were completely abolished by RTX administration [SNI-RTX: 1.8 ± 0.9 Hz (N = 5); SNI-vehicle: 69.5 ± 8.6 Hz (N = 5), $P < 0.01$, 60 g stimulation] (Fig. 5C). The after-discharges in the RTX-treated SNI group were similar to those in the RTX-treated sham group [0.3 ± 0.2 Hz (N = 5), 60 g stimulation] (Fig. 5C).

Discussion

In this study, SNI rats showed marked mechanical allodynia and prolongation of the duration of paw withdrawal after pin stimulation (mechanical hyperalgesia). The prolonged ventral root discharges after cessation of the stimulation were enhanced in SNI rats. The mechanical hyperalgesia was almost abolished by RTX treatment, whereas mechanical allodynia was not affected. Similarly to mechanical hyperalgesia, the enhanced after-discharges were also abolished by RTX treatment in SNI rats. These results suggest the contribution of TRPV1-positive fibers to the appearance of mechanical hyperalgesia and en-

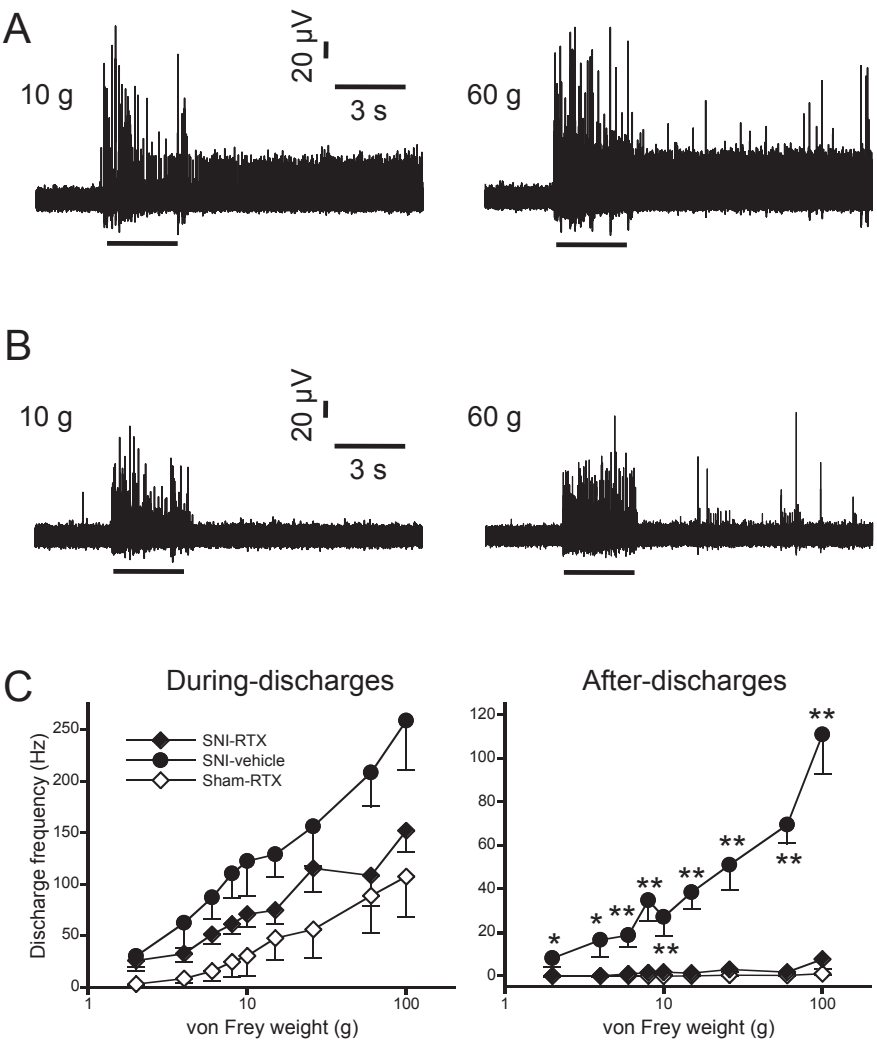

Fig. 5. Comparison of ventral root discharges recorded in a SNI-vehicle–treated rat (A) or a SNI-RTX–treated rat (B). Both of the after-discharges increased by low (10 g)- and high (60 g)-intensity mechanical stimulation in SNI were almost abolished by RTX. Graphs in panel C show stimulus–response recruitment curves of the during- and after-discharges in RTX-treated SNI model rats. Each point represents the mean ± S.E.M. of 5 separate experiments. Ordinate: discharge frequency per second. Abscissa: stimulus intensity (grams). The statistical significance of differences between the sham and other groups was determined by two-tailed multiple *t*-test with Bonferroni's correction following one-way analysis of variance (ANOVA) (2 comparisons in 3 groups). *$P < 0.05$, **$P < 0.01$ vs. SNI-RTX.

hanced ventral root after-discharges in SNI rats.

Low-intensity stimulation with von Frey filaments elicited robust mechanical allodynia and an increase of during-discharges in SNI model rats. It is considered that the transmission of touch sensation by Aβ-fibers is markedly perturbed in the SNI model. In fact, the levels of the β-catenin and menin proteins, which are involved in the formation of new synapses (25), and the number of neurons reacting to the Aβ-fiber stimulation (26) are increased in lamina II of the spinal dorsal horn of SNI rats. In dorsal root ganglia (DRG), macrophages form clusters around the cell bodies of A-fibers, but not small C-fibers, in the SNI model (27). These changes in spinal cord neurotransmission after SNI surgery may contribute to mechanical allodynia.

RTX is an ultrapotent capsaicin analog (17), a single injection of which desensitizes TRPV1-positive primary afferent fibers (18) by induction of cell death followed by persistent calcium influx (28). The activation of p38 in spinal microglia and the induction of mechanical allodynia are not prevented by RTX treatment before SNI surgery (29). Since RTX failed to induce any significant changes in mechanical allodynia or the increment of during-discharges, it is suggested that TRPV1-insensitive fibers participate in mechanical allodynia. A similar lack of depression of mechanical allodynia by RTX administration after surgery has also been shown in a spinal nerve ligation model (30). Our results and those of others suggest that TRPV1-positive fibers are dispensable for induction of mechanical allodynia.

SNI rats did not show any change in the latency of paw withdrawal in response to thermal stimulation, but the duration of withdrawal behavior was prolonged (data not shown), as reported in the original article (5). These results are consistent with a report that SNI did not reduce the expression of TRPV1 immunoreactivity within the territory innervated by the uninjured sural nerve in the spinal dorsal horn (31). From these results, it is considered that normal thermal sensitivity was maintained because of sparing of TRPV1-positive fibers in this SNI model. Treatment with RTX abolished the behavior evoked by thermal stimulus almost completely in both sham and SNI rats. In contrast, however, it has also been reported that thermal hyperalgesia occurs in the SNI model (32). It will be necessary to further validate the changes in heat-sensitive fibers that occur in the SNI model.

Mechanical hyperalgesia observed as prolongation of the duration of the reaction to pin stimulation was completely abolished by RTX. Enhancement of after-discharges in the SNI model was also depressed by RTX. As uninjured C-fibers are necessary for the induction and maintenance of hypersensitivity (33), it is considered that the activation of TRPV1-positive C-fibers in the uninjured sural nerve and/or spinal neurons receiving input from the sural nerve play an important role in mechanical hyperalgesia and generation of after-discharges in SNI rats. Furthermore, after-discharges were induced by lower-intensity stimuli in SNI rats than in naïve and sham rats. These after-discharges were also abolished by RTX. These results suggest that the activating threshold of TRPV1-positive fibers and related dorsal horn neurons are lowered by SNI. Since ventral root discharges were recorded in spinalized rats, it is suggested that the changes observed in this electrophysiological study occurred in primary afferent fibers and/or the dorsal horn, but not at supraspinal sites. In fact, N- and L-type voltage-dependent Ca^{2+} channels in the dorsal horn contribute to the generation of after-discharges in naïve rats (7). The participation of these channels in neuropathic pain models has been reported (34 – 36). On the basis of these results, a hypothesis can be proposed in which the after-discharges induced by low-intensity stimuli participate in the mechanical hyperalgesia caused by sensitization of TRPV1-positive fibers in SNI. The TRPV1-positive fibers abolished by RTX are heat-sensitive Aδ- and C-fibers (20). Although it is considered that TRPV1-negative Aδ- and C-fibers still remained after the treatment of RTX, the population of these fibers was not elucidated in this study.

In conclusion, TRPV1-positive primary afferent fibers contribute to the increment of ventral root after-discharges induced by application of mechanical stimuli in sham and SNI rats and that mechanical hyperalgesia is prolonged after cessation of stimulation in SNI, without contributing to mechanical allodynia. These results suggest that there is a difference in the mechanisms responsible for generating mechanical allodynia and prolonged mechanical hyperalgesia.

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