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# POSSIBLE INVOLVEMENT OF SYNTAXIN 1A DOWNREGULATION IN THE LATE PHASE OF ALLODYNIA INDUCED BY PERIPHERAL NERVE INJURY

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**Abstract—Syntaxin 1A is a membrane protein playing an integral role in exocytosis and membrane trafficking. The superficial dorsal horn (SDH) of the spinal cord, where nociceptive synaptic transmission is modulated, is rich in this protein. We recently reported that peripheral nerve ligation-induced nociceptive responses are considerably enhanced in syntaxin 1A-knockout mice [Takasusuki T, Fujiwara T, Yamaguchi S, Fukushima T, Akagawa K, Hori Y (2007) Eur J Neurosci 26:2179–2187]. On the basis of this earlier finding, we hypothesized that syntaxin 1A is involved in peripheral nerve injury-induced nociceptive plasticity. In this study, we examined this hypothesis by using nociceptive behavioral studies and tight-seal whole-cell recordings from neurons in the SDH of adult mouse spinal cord slices. Partial sciatic nerve ligation (PSNL) in adult male Institute of Cancer Research (ICR) mice increased the frequency of spontaneous miniature excitatory postsynaptic currents (mEPSCs). The amplitude of the mEPSCs did not exhibit any changes, suggesting that peripheral nerve injury is associated with increased synaptic release of excitatory neurotransmitters. Western blot and real-time quantitative reverse transcription-polymerase chain reaction analyses revealed that PSNL gradually decreased the expression level of syntaxin 1A in the spinal SDH. This downregulation of syntaxin 1A took several days to develop, whereas behavioral allodynia developed within one day after PSNL. Syntaxin 1A knockdown by intrathecal injection of an antisense oligodeoxynucleotide against the syntaxin 1A gene led to the gradual development of allodynia. These results indicate a possible involvement of syntaxin 1A downregulation in the late maintenance phase of peripheral nerve injury-induced allodynia. In addition, syntaxin 1A knockdown by ribonucleic acid interference enhanced the axonal elongation and sprouting of spinal dorsal horn neurons in culture, suggesting that PSNL-induced syntaxin 1A downregulation may result in the rearrangement of the synaptic connections between neurons in the spinal dorsal horn. Taken together, it is possible to conclude that syntaxin 1A might be involved in spinal nociceptive plasticity induced by peripheral nerve injury.**


**Key words: Syntaxin 1A, allodynia, spinal superficial dorsal horn, mEPSCs, antisense oligodeoxynucleotide, double-stranded RNA.**

Syntaxin 1A, belonging to the syntaxin family, is a membrane protein that is mainly expressed in neurons (Inoue and Akagawa, 1992, 1993; Inoue et al., 1992). It plays an integral role in exocytosis and membrane trafficking. Syntaxin 1A interacts with other proteins involved in the docking and fusion machinery of presynaptic vesicles, namely v-SNARE synaptobrevin (VAMP) and t-SNARE SNAP-25. Thus, it plays a crucial role in neurotransmitter release (Sudhof, 2004; Jahn and Scheller, 2006). In addition to its localization at the presynaptic terminal region, syntaxin 1A is distributed throughout the whole axonal membrane (Koh et al., 1993). Inhibition of syntaxin1A was found to increase neurite sprouting and neurite elongation in cultured dorsal root ganglion neurons and cultured retinal neurons (Yamaguchi et al., 1996), while overexpression of syntaxin1A by adenovirus was found to inhibit neurite extension in PC12 cells (Zhou et al., 2000). Syntaxin 1A is apparently involved in regulating neurite sprouting and elongation (Igarashi et al., 1996; Yamaguchi et al., 1996; Zhou et al., 2000; Kimura et al., 2003), although the precise role of syntaxin 1A in neurite outgrowth remains unclear.

Peripheral nerve injury or tissue inflammation often induces a state of abnormal pain known as neuropathic pain, including hyperalgesia (increased sensitivity to noxious stimuli) and allodynia (an abnormal state in which innocuous stimuli induce pain). One of the mechanisms underlying neuropathic pain is the sustained enhancement of synaptic transmission in the superficial dorsal horn (SDH) of the spinal cord (Randic et al., 1993; Liu and Sandkuehler, 1995, 1997; Sandkuehler and Liu, 1998; Moore et al., 2000; Sandkuhler, 2009), where nociceptive transmission is modulated (Willis and Coggeshall, 2004). Peripheral nerve injury may also cause sprouting of uninjured afferent fibers and synaptic rearrangement in the spinal dorsal horn, which in turn trigger neuropathic pain (Woolf et al., 1992, 1995; Shortland and Woolf, 1993; Koerber et al., 1994; Lekan et al., 1996); however, this notion has been challenged (Pubols and Bowen, 1988; McMahon et al., 1991; Bao et al., 2002; Hughes et al.,

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*Abbreviations:* antisense-ODN, antisense oligodeoxynucleotide; cDNA, complementary deoxyribonucleic acid; dsRNA, double-strand RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ICR mouse, Institute of Cancer Research mouse; mEPSCs, miniature excitatory postsynaptic currents; mRNA, messenger ribonucleic acid; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PSD-95, postsynaptic density-95; PSNL, partial sciatic nerve ligation; RNAi, ribonucleic acid interference; RT, reverse transcription; SDH, superficial dorsal horn.

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2003; Shehab et al., 2003; Costigan et al., 2009; Latremoliere and Woolf, 2009).

Immunohistochemical studies have revealed that the SDH of the spinal cord is rich in syntaxin 1A (Ruiz-Montasell et al., 1996; Aguado et al., 1999; Sun et al., 2002). Recently, we reported that excitatory synaptic transmission in the SDH is considerably enhanced after peripheral nerve injury in syntaxin 1A-knockout mice compared with wild-type mice. Furthermore, peripheral nerve injury has been observed to exacerbate allodynia in syntaxin 1A-knockout mice (Takasusuki et al., 2007).

Taking into account our previous observations in syntaxin 1A-knockout mice and the fact that syntaxin 1A is reported to be highly expressed in the SDH, we hypothesized that syntaxin 1A is involved in nociceptive plasticity induced by peripheral nerve injury. To test this hypothesis, we investigated whether sciatic nerve ligation induces the downregulation of syntaxin 1A expression in the spinal dorsal horn and whether syntaxin 1A knockdown by intrathecal administration of antisense oligodeoxynucleotides induces hyperalgesia. We also investigated the manner in which syntaxin 1A knockdown affects neurite sprouting in cultured neurons isolated from the spinal dorsal horn.

## EXPERIMENTAL PROCEDURES

### Animals

Electrophysiological and behavioral experiments were performed on male ICR mice aged between 6 and 8 weeks. For the culture experiments, fetuses were obtained from pregnant ICR mice on days 14 and 15 of gestation. All the animal experiments were approved by the institutional animal care and use committees. The care and handling of the animals were in accordance with the National Institutes of Health (NIH) guidelines on animal care and with the guidelines of the International Association for the Study of Pain (Zimmermann, 1983).

### Partial ligation of the sciatic nerve

The mice were maintained in a temperature-controlled room under a 12-h light–dark cycle. They were anesthetized with an i.p. injection of sodium pentobarbital (50 mg/kg body weight; Kyoritsu, Tokyo, Japan). The sciatic nerve was partially ligated under a binocular microscope according to methods described by Seltzer et al. (1990). The left common sciatic nerve was exposed at the level of the middle of the thigh by blunt dissection through the biceps femoris. The dorsum of the nerve was carefully freed from the adhering tissues. An 8–0 silicon-treated silk suture was inserted into the nerve and tightly ligated so that the dorsal one-third to one-half of the nerve thickness was trapped in the ligature. In the sham-operated control mice, the sciatic nerve was exposed but not ligated. We performed sciatic nerve ligation and sham operations on the left hind limb; withdrawal threshold measurement, electrophysiological recordings, and biochemical analysis were performed ipsilaterally to surgery, and comparisons between nerve-ligated and sham-operated mice were made.

### Behavioral experiments

To assess the effects of sciatic nerve ligation or intrathecal injection of antisense oligodeoxynucleotides on the nociceptive behavior, the withdrawal threshold on mechanical stimulation was determined by using an electronic von Frey device (Model 1601; IITC Life Science, Woodland Hills, CA, USA). The use of the electronic von Frey device has been validated for investigations on nociceptive behaviors (Moller et al., 1998; Cunha et al., 2004). The probe of the electronic von Frey device was manually applied with the force increasing at a rate of 1.6–8.1 g/s. Each trial of stimuli was composed of 10 applications of mechanical stimulation at approximately 10-s intervals; each trial was repeated three times at approximately 3-min intervals.

First, we carried out nociceptive behavior experiments and constructed the averaged time course of changes in withdrawal threshold after the nerve ligation, which is depicted in the graph in Fig. 1. We then performed further biochemical and electrophysiological experiments on animals that exhibited mechanical allodynia after partial sciatic nerve ligation (PSNL). Animals whose withdrawal thresholds fell outside the range of the mean±standard deviation of the experimental groups were excluded from further analysis. All behavioral experiments were performed under double blind conditions.

In some additional experiments, withdrawal response was measured by conventional von Frey filaments. Mechanical stimulation was applied to the plantar surface of the hind limb ipsilaterally to PSNL using von Frey filaments (Stoelting, IL, USA) of 0.07 or 0.16 g force. The mechanical sensitivity of the hind limb was determined by the number of positive withdrawal responses to 10 repetitive von Frey stimuli according to the protocol outlined by Schwartz et al. (2009). Measurements were made before and at 7, 14, and 21 days after the partial ligation of the sciatic nerve.

### Electrophysiological experiments

Mice were anesthetized with halothane (Takeda, Osaka, Japan), and the lumbosacral spinal cord was removed. Transverse slices of the L4–L6 segments were cut with a microslicer (Dosaka EM, Kyoto, Japan) at 4 °C and immersed in Krebs solution containing the following chemicals (in mM): NaCl, 113; KCl, 3; $NaH_2PO_4$, 25; $CaCl_2$, 2; $MgCl_2$, 1; and D-glucose, 11. The pH was maintained at 7.4 by bubbling 95% $O_2$ and 5% $CO_2$ through the solution.

After incubation in the Krebs solution for 1 h at 37 °C, the spinal slices were mounted onto a recording chamber on a microscope stage (Axioskop FS; Carl Zeiss MicroImaging GmbH, Göttingen, Germany) and continuously perfused with the Krebs solution. Conventional tight-seal whole-cell recordings of neurons visually localized to the SDH were obtained by using an infrared differential interference contrast (IR-DIC) optical device and a charge-coupled device (CCD) video camera (IR-CCD 2741; Hamamatsu Photonics K.K., Hamamatsu City, Japan), as described previously (Iwata et al., 2007).

Patch pipettes were prepared from thin-walled borosilicate glass capillaries pulled with a micropipette puller (P-97; Sutter Instrument Co., Novato, CA, USA). The pipettes were then filled with a solution containing the following chemicals (in mM): potassium gluconate, 123; KCl, 14; sodium gluconate, 2; ethylene glycol tetraacetic acid (EGTA), 1; and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10. The pH of the solution was neutralized to 7.4 with KOH. The direct-current resistance of the filled pipettes was 5–10 MΩ. The membrane potential value was corrected for the junction potential. Fast and slow capacitances were neutralized. The series resistance was compensated by 60%. The access resistance (11–20 MΩ) was continuously monitored, and the data were discarded when the series resistance showed a change of more than 10%.

Spontaneously occurring miniature excitatory postsynaptic currents (mEPSCs) were recorded at a holding potential of −70 mV in the presence of tetrodotoxin (0.5 μM; Sankyo, Tokyo, Japan), strychnine (1 μM; Sigma-Aldrich, St. Louis, MO, USA), and bicuculline methobromide (10 μM; Biomol International, Plymouth, PA, USA). The mEPSCs thus recorded were lost in the presence of 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX, 5 μM; data not shown; Tocris Cookson Inc., Ellisville, MO, USA); therefore, these mEPSCs were considered to be mediated by the non-*N*-methyl-D-aspartate (non-NMDA) subtypes of glutamate receptors. The mEPSCs were recorded in the voltage-clamp mode