

## Long-Term Potentiation of Excitatory Synaptic Strength in Spinothalamic Tract Neurons of the Rat Spinal Cord

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Spinal dorsal horn nociceptive neurons have been shown to undergo long-term synaptic plasticity, including long-term potentiation (LTP) and long-term depression (LTD). Here, we focused on the spinothalamic tract (STT) neurons that are the main nociceptive neurons projecting from the spinal cord to the thalamus. Optical technique using fluorescent dye has made it possible to identify the STT neurons in the spinal cord. Evoked fast mono-synaptic, excitatory postsynaptic currents (eEPSCs) were measured in the STT neurons. Time-based tetanic stimulation (TBS) was employed to induce long-term potentiation (LTP) in the STT neurons. Coincident stimulation of both pre- and postsynaptic neurons using TBS showed immediate and persistent increase in AMPA receptor-mediated EPSCs. LTP can also be induced by postsynaptic spiking together with pharmacological stimulation using chemical NMDA. TBS-induced LTP observed in STT neurons was blocked by internal BAPTA, or  $\text{Ni}^{2+}$ , a T-type VOCC blocker. However, LTP was intact in the presence of L-type VOCC blocker. These results suggest that long-term plastic change of STT neurons requires NMDA receptor activation and postsynaptic calcium but is differentially sensitive to T-type VOCCs.

**Key Words:** Long-term potentiation, NMDA receptor, Spinothalamic tract neurons, T-type VOCC


### INTRODUCTION

After the initial study of synaptic plasticity in the hippocampus and cerebellum as a mechanism for the Hebb process underlying learning and memory, a rapid advancement has taken place in the study of the central nervous system in general [1]. Long-term potentiation (LTP) and long-term depression (LTD) are the electrophysiological terms used for explaining the neuronal plasticity observed due to the change in activity-dependent synaptic plasticity. The term LTP was much more generally used following the report in 1973 by Bliss and Lomo, which the synaptic strength maintained a potentiated state for hours by giving tetanic stimulation to the perforant pathway of the dentate gyrus in the mammalian hippocampus, while LTD was used when the synaptic strength was weakened over a long period of time. Long-term synaptic plasticity, such as LTP or LTD, was then understood as the synaptic mechanism for memory storage or forgetting in many brain regions, including

the neocortex, midbrain, hippocampus, striatum, and spinal cord [2]. Long-term synaptic plasticity at the spinal cord level, ruling out the fact that synaptic plasticity is neurokinin (NK) receptor activation-dependent [3], is similar to that of the LTP or LTD observed in the hippocampus in terms of induction mechanism, NMDA receptor dependence, and duration of the increased reaction pattern [4-6]. Distinct from the many interneurons in the spinal cord, spinothalamic tract (STT) neurons, which are projection neurons, have been studied in various aspects for its direct relationship to pain and its mechanism. However, most studies have used extracellular recording methods *in vivo* and some have used immunochemistry to confirm the characteristics of STT neurons in the spinal cord [7]. Since it is difficult to distinguish the STT neurons from the other neurons in the spinal cord *in vitro*, the synaptic plasticity and general characteristics of these neurons have not yet been studied. In this study, using *in vitro* spinal cord slice preparations, STT neurons were isolated and examined under a cell-to-cell condition. By examining the presence and direction (LTP or LTD) of synaptic plasticity in the spinal cord, changes in synaptic plasticity of projection neurons and its related mechanisms in terms of cell physiology are further discussed.

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**ABBREVIATIONS:** ACSF, artificial cerebrospinal fluid; AMPA,  $\alpha$ -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; EPSC, excitatory postsynaptic current; LTD, long-term depression; LTP, long-term potentiation; NK, neurokinin; NMDA, N-methyl-D-aspartate; STT, spinothalamic tract; TBS, time-based tetanic stimulation; TTX, tetrodotoxin; VOCC, voltage-operated calcium channel.

## METHODS

### *Fluorescent dye for labeling STT neurons in vivo*

A fluorescent-labeling method was employed to find spinothalamic tract (STT) neurons in lumbar enlargement of the spinal cord, fluorescent dye ( $\Delta^9$  Di-I) was injected into ventral posterior lateral (VPL) nucleus of the thalamus. Postnatal 3~4 day old pups (Sprague-Dawley) were anaesthetized with Entobar, pentobarbital sodium (50 mg/ml, 40 mg/kg) and placed in a stereotaxic frame. For VPL injection, a glass pipette with Di-I was lowered stereotaxically using coordinates from Paxinos and Watson. A small volume of Di-I (1  $\mu$ l, 25 mg/0.5 ml EtOH) was injected from the pipette. After ~2 weeks, the rats were used for experimental purposes.

### *Slice preparation for electrophysiology*

Transverse slices of the spinal cord (350  $\mu$ m) from postnatal 16~19 rats using a vibratome (Campden, 765, U.K.) were dissected and placed immediately into cold (2.5°C) modified ACSF composed of the following (in mM): 110 choline chloride, 2.5 KCl, 7 MgCl<sub>2</sub>, 0.5 CaCl<sub>2</sub>, 2.4 Na-pyruvate, 1.3 Na-ascorbic acid, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 25 NaHCO<sub>3</sub>, and 20 glucose, equilibrated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> (pH 7.4, 305 $\pm$ 5 mmol/kg). After cutting, slices were incubated for 15 min at 32°C and then for up to 5 hr at 25°C in ACSF. All experiments were performed under protocols approved by the Animal Care and Use Committee of Seoul National University School of Medicine for rats.

### *Electrophysiology*

Whole-cell patch-clamp recordings from spinal cord slices were carried out at 30~32°C. The recording chamber was continuously perfused with artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 2.5 KCl, 1.3 MgCl<sub>2</sub>, 2.5 CaCl<sub>2</sub>, 1 NaH<sub>2</sub>PO<sub>4</sub>, 26.2 NaHCO<sub>3</sub>, and 10 glucose, equilibrated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> (pH 7.4, 305 $\pm$ 5 mmol/kg). The bath solution also contained both 1  $\mu$ M strychnine and 10  $\mu$ M bicuculline to block the glycine receptors and GABA<sub>A</sub> receptors, respectively. The pipette solution contained (in mM): 136 K-gluconate, 10 NaCl, 1 MgCl<sub>2</sub>, 1 CaCl<sub>2</sub>, 0.5 EGTA, 2 Mg-ATP, 0.1 Na-GTP, and 10 HEPES (pH 7.3, 285 $\pm$ 5 mmol/kg). Patch pipettes were pulled from borosilicate glass (4~5 M $\Omega$ ) using a horizontal puller (Sutter Instruments, Novato, CA). Dorsal horn neurons in lamina I-II region in lumbosacral enlargement of the spinal cord were visually identified using differential interference contrast (DIC). Then, retrogradely labeled STT neurons using fluorescent dye ( $\Delta^9$  Di-I) were detected by ADC software (TILL ACD Communication) using a monochromator (Polychrome II, TILL Photonics). Signals were recorded with an EPC8 (HEKA Elektronik)-patch clamp amplifier filtered at 2 kHz and sampled at 10 kHz. Data were acquired 3 min after achieving the whole-cell configuration. Series resistance (Rs) of recordings ranged between 10 and 15 M $\Omega$ . Cells were rejected from analysis if Rs changed by more than 15%. Choline chloride, NaCl, KCl, MgCl<sub>2</sub>, CaCl<sub>2</sub>, Na-pyruvate, Na-ascorbic acid, NaH<sub>2</sub>PO<sub>4</sub>, NaHCO<sub>3</sub>, glucose, EGTA, Mg-ATP, Na-GTP, NiCl<sub>2</sub>, BAPTA, strychnine, HEPES, nimodipine, and L-703,606 were purchased from Sigma (St. Louis, MO, USA). TTX, CNQX, substance P, and bicuculline were purchased from Tocris (Ellisville,

MO, USA). Di-I was purchased from Invitrogen (Carlsbad, CA, USA).

### *AMPA receptor-mediated excitatory postsynaptic currents (EPSCs)*

For measurement of the excitatory synaptic transmission, evoked EPSCs were recorded at a holding potential ( $V_h$ ) of -65 mV to access AMPA receptor (AMPA)-mediated responses. Dorsal root entry zone (DREZ) was stimulated with a theta glass electrode. In case of need, experiments were performed in the presence of the selective AMPAR antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10  $\mu$ M). To test whether spinothalamic tract neurons showed substance P receptor-mediated responses, whole-cell voltage-clamp recordings of the identified STT neurons were performed in the presence of substance P (1~2  $\mu$ M).

### *Long-term potentiation (LTP) in spinal cord*

Synaptic responses were evoked by a 100  $\mu$ sec current pulse to DREZ with a bipolar stimulating electrode. Stable baseline responses were collected every 30 sec by using a stimulation intensity (10~30  $\mu$ A) yielding 50~60% of the maximal eEPSC amplitude. LTP was induced by time-based electrical stimulation. The relative timing between the presynaptic and postsynaptic spiking was determined according to the pre- and postsynaptic spiking. Repetitive postsynaptic spiking (1~5 Hz, 0.1 ms width, 100 pulses) within a time window of presynaptic activation (the peak of evoked excitatory postsynaptic potential) was applied simultaneously. To explore whether spike-timing dependent LTP require postsynaptic calcium, the role of VOCC was examined in the presence of L-type VOCC blocker (nimodipine, 10  $\mu$ M), T/R-type VOCC blocker (NiCl<sub>2</sub>, 100  $\mu$ M), or BAPTA (10 mM, in the pipette).

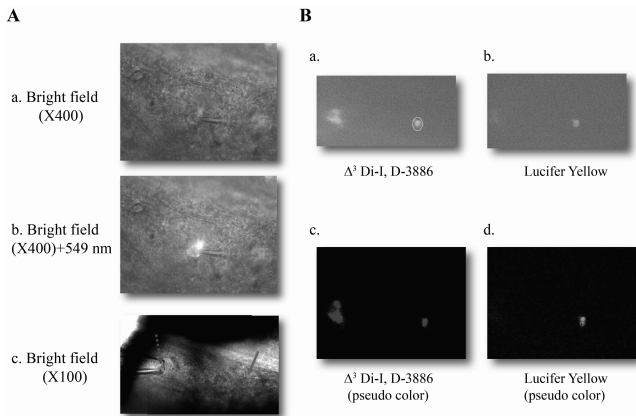
### *Statistical analysis*

All group data are shown as mean $\pm$ SEM (the standard error of the mean). Significance was determined by a paired Student's *t*-test.

## RESULTS

### *Identification of STT projection neurons in rat in vitro spinal cord slices*

To identify the projection neurons in bright field among many neurons in the dorsal horn of spinal cord, a fluorescent-labeling method was employed (Fig. 1). Fluorescent dye ( $\Delta^9$  Di-I) was excited at 549 nm using a Xenon Short ARC lamp. Afterwards, with an emission filter for 564 nm, the projection neurons were able to be distinguished and identified as shown in Fig. 1Ab. By placing the stimulating electrode near the recording electrode (Fig. 1Ac), or by stimulating either the dorsal horn, dorsal root entry zone, or the Lissaur's tract, the STT neurons were examined for fast excitatory mono synaptic currents (Fig. 2A). Lucifer yellow (1 mM) was added in the recording solution for some experiments (Fig. 1Bd).



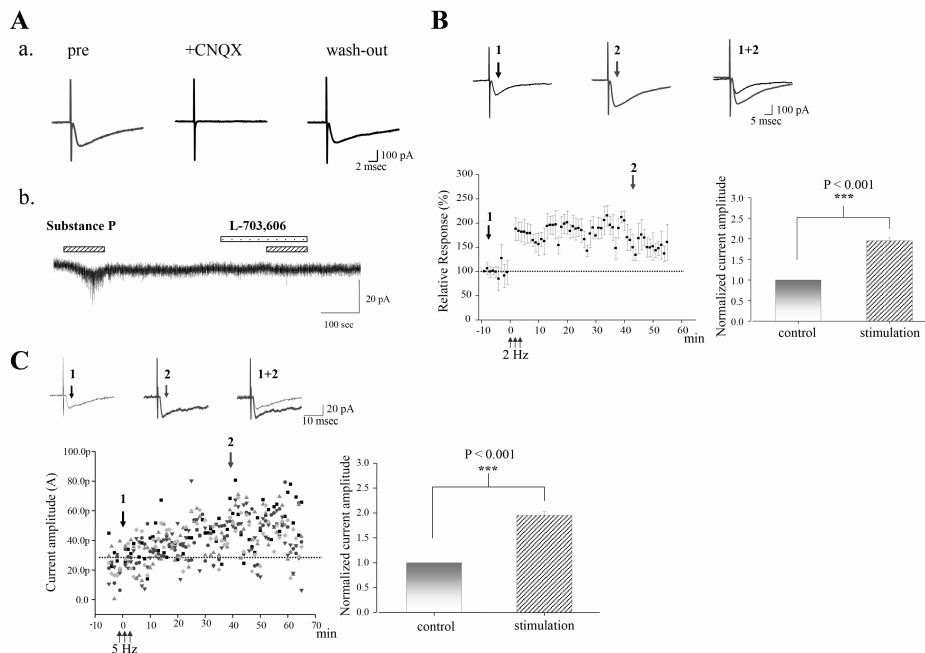
**Fig. 1.** Spinothalamic tract neurons (STT) identified in rat spinal cord slices. (Aa, Ac) An example of recorded STT neuron in the bright field (magnification  $\times 400$ ,  $\times 100$ , respectively), dashed line-stimulation electrode, solid line-recording electrode. (Ab) Labeling of neurons using fluorescent dye ( $\Delta^9$  Di-I, Abs.  $-549$  nm, Em.  $-564$  nm). (Ba, Bc) An example of STT neurons stained by lipophilic tracer (Abs.  $-549$  nm, Em.  $-564$  nm). (Bb) STT neuron was highlighted by lucifer yellow (Abs.  $-428$  nm, Em.  $-536$  nm) in the recording pipette. (Bc, Bd) Pseudo color for Di-I, lucifer yellow, respectively.

### Properties of fast excitatory monosynaptic currents of STT neurons

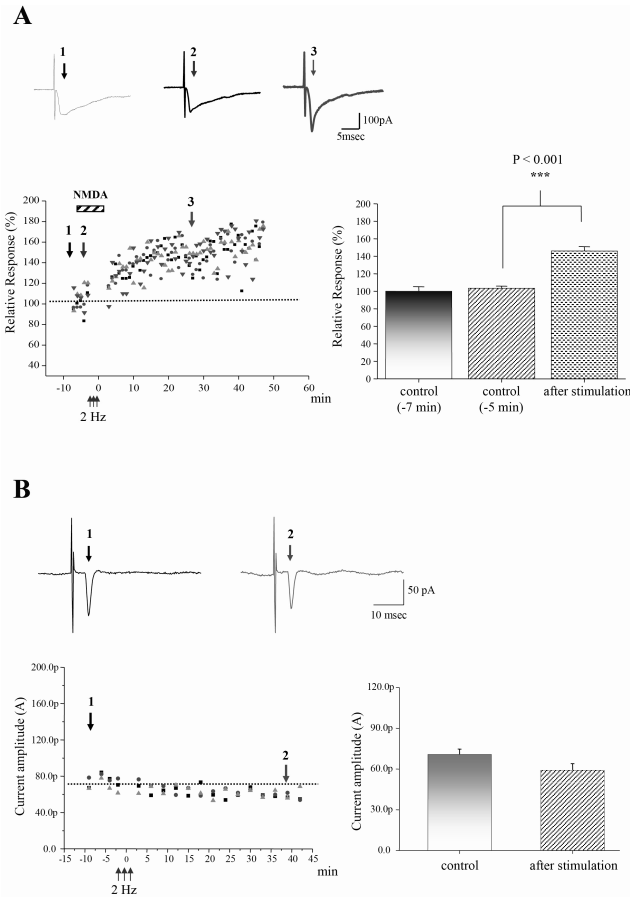
To confirm the properties of the electrical stimulation-evoked excitatory postsynaptic currents (eEPSCs) recorded at membrane holding potential ( $-65$  mV), CNQX, a non-NMDA receptor antagonist, was applied. The eEPSC was blocked by the AMPA receptor antagonist,  $10 \mu\text{M}$  CNQX (Fig. 2Aa), thus confirming that the eEPSC was an excitatory current mediated by glutamate which is the neurotransmitter in excitatory synapses. Also, in some experiments bath application of substance P ( $1 \mu\text{M}$ ) were given to confirm the inward current which is carried by a NK1 receptor (Fig. 2Ab). Substance P-mediated inward current was inhibited by L-703,606 ( $10 \mu\text{M}$ ), a NK1 receptor antagonist (Fig. 2Ab).

### Long-term synaptic plasticity of STT neurons

To confirm whether long-term synaptic changes were induced in STT neurons, spike timing-based electrical stimulation (TBS) protocol was performed as described with slight modifications [8]. After examining the relative timing, which is the time after the stimulation of the pre-synapse to the peak of the evoked EPSC or evoked EPSP of the postsynaptic cell, by inducing the formation of repeti-

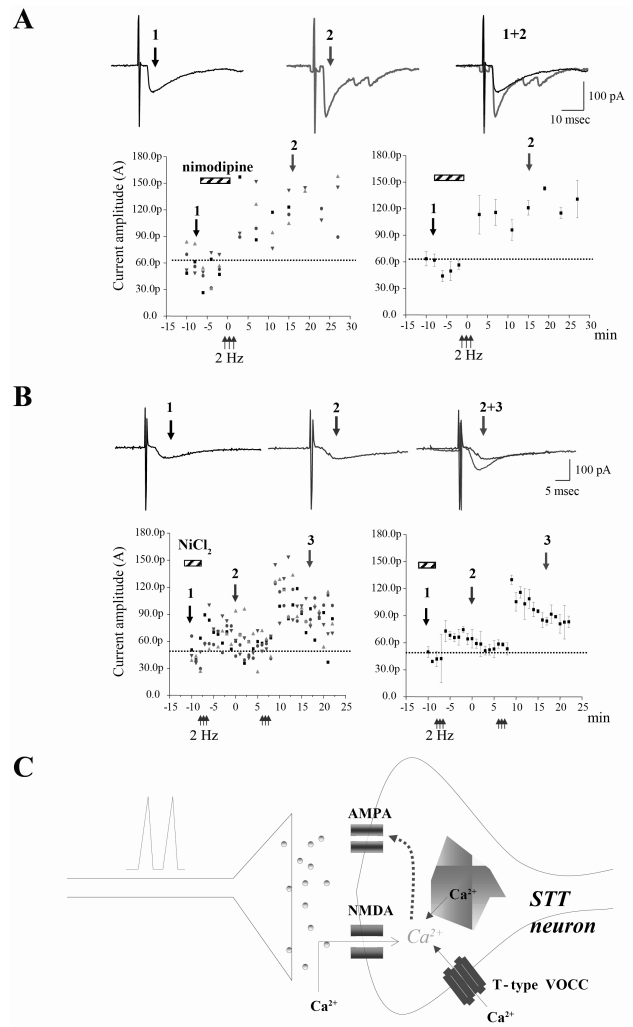


**Fig. 2.** LTP is induced by time-based electrical stimulation (TBS) in STT neurons. (A) Fast monosynaptic, excitatory postsynaptic currents recorded from an identified STT neuron. (Aa) CNQX ( $10 \mu\text{M}$ ), AMPA receptor antagonist, completely abolished the evoked postsynaptic current, indicating AMPA receptor-mediated excitatory postsynaptic current (EPSC). (Ab) STT neurons showed inward current in response to bath application of substance P ( $2 \mu\text{M}$ ) in the presence of TTX ( $0.5 \mu\text{M}$ ), Na channel blocker. The effect of substance P was inhibited by L-703,606, NK1 receptor antagonist. (B) LTP is induced by time-based electrical stimulation (TBS). Averaged time course of the change in evoked EPSC (eEPSC) by low-frequency TBS ( $2$  Hz,  $n=33$ ). Error bars indicate the standard error of the mean. Note that  $2$  Hz TBS can induce LTP in most recorded STT neurons ( $33$  out of  $36$  neurons). Bar graphs represent the normalized current amplitude (mean $\pm$ sem). \*\*\* $p < 0.001$  at time point 2 when compared to the time point 1 (before LTP induction). (C) LTP is also induced by different low-frequency TBS ( $5$  Hz,  $n=4$ , different shapes indicate different STT neurons). Note that this  $5$  Hz TBS can induce LTD in a subset of STT neurons ( $4$  out of  $8$  neurons, data not shown). Bar graphs represent the normalized current amplitude (mean $\pm$ sem). \*\*\* $p < 0.001$  at time point 2 when compared to the time point 1 (before LTP induction).



**Fig. 3.** TBS-induced LTP in STT neurons requires NMDA receptor activation and postsynaptic calcium. (A) Original raw traces before (left arrow at time point 1), during bath application of NMDA (middle arrow at time point 2), and after (right arrow at time point 3). LTP is induced by 2 Hz postsynaptic stimulation in the presence of chemical NMDA (100 nM) without presynaptic stimulation. When NMDA alone was applied to acute slices in the presence of test stimulation, evoked EPSC shows no effect on the responses. Different shapes indicate different STT neurons (n=4). Bar graphs represent the normalized current amplitude (mean±sem). \*\*\*p < 0.001 at time point 3 when compared to the time point 2 (before LTP induction). (B) Original raw traces before (left arrow at time point 1) and after LTP (right arrow at time point 2). LTP is impaired by internal BAPTA (10 mM), Ca<sup>2+</sup> chelator. Different shapes indicate different STT neurons (n=3). Bar graphs represent the normalized current amplitude (mean±sem).

tive postsynaptic spiking within the 10 msec time window of the presynaptic activation, LTP of synaptic plasticity was successfully induced in the recorded cells. The raw trace examples of the eEPSC before and after the 2 Hz conditional stimulus of the presynaptic cell are shown in Fig. 2B. The current at 47 min after the TBS was 169.6±16.6%, an increase of approx. 70% of the current before the TBS (100%). As time passed, the evoked EPSC amplitude was increased and maintained for 60 min. However, it is important to mention that 3 out of 36 recorded neurons showed long-term synaptic depression after the 2 Hz stimulus (data not shown). Thus, it is believed that coincident 2 Hz low-frequency synaptic stimulation of both pre- and postsynaptic neurons using TBS is an effective protocol to



**Fig. 4.** TBS-induced LTP in the STT neurons requires T-type voltage-operated calcium channel (VOCC), but not L-type VOCC. (A) TBS-induced LTP in the STT neurons is not affected by L-type VOCC blocker, nimodipine (10 μM). Original raw traces before (left arrow at time point 1) and after LTP (right arrow at time point 2). When nimodipine alone was applied to acute slices in the presence of test stimulation, evoked EPSC shows no effect on the responses. Different shapes indicate different STT neurons (n=4). Right, averaged time course of the changes represent the normalized current amplitude (mean±sem). (B) Original raw traces before (left arrow at time point 1), during bath application of NiCl<sub>2</sub> (middle arrow at time point 2), and after (right arrow at time point 3). LTP is impaired by the application of NiCl<sub>2</sub> (100 μM), T/R type VOCC blocker. When NiCl<sub>2</sub> alone was applied to acute slices in the presence of test stimulation, evoked EPSC shows no effect on the responses. Right, averaged time course of the changes represent the normalized current amplitude (mean±sem). (C) NMDA receptor and T-type VOCC, but not L-type VOCC in TBS-induced LTP in the STT neurons. Spike-timing dependent TBS-LTP in the STT neurons requires postsynaptic calcium but are differentially sensitive to T-type VOCC blocker, NiCl<sub>2</sub>, indicating the role of T-type VOCC in LTP of STT neurons.

induce LTP in STT neurons. We also tested different types of stimulation frequencies. When the stimulation frequency was increased to 5 Hz TBS, in 4 out of 8 neurons LTP was

observed (Fig. 2C). In comparison with before the TBS, the 5 Hz TBS significantly increased the synaptic current from  $21.1 \pm 5.3$  pA to  $61.7 \pm 2.2$  pA ( $p < 0.001$ ) (Fig. 2C, bar graph).

### NMDA receptor-dependent LTP

To determine whether LTP observed in STT neurons requires NMDA receptor activation which is associated with long-term changes of synaptic strength in neurons of the cerebral cortex, hippocampus, and striatum, we bath-applied NMDA receptor selective agonist, NMDA ( $100 \mu\text{M}$ ) without the presynaptic stimulation, and only stimulating the postsynaptic STT neurons at 2 Hz. Four out of 5 neurons showed a time-dependent increase in synaptic plasticity (Fig. 3A). While the amplitude of the eEPSC was not altered significantly before the stimulation with the bath application of NMDA, the eEPSC at 28 min significantly increased approximately 45% under the same condition after stimulation. The increase of fast monosynaptic currents lasted for 40 min.

### Calcium-dependent LTP

To study the role of postsynaptic calcium in association to LTP in STT neurons, 10 mM BAPTA was included in the internal solution of the recording electrode. The synaptic currents before and after LTP, as shown in Fig. 3B, did not significantly change for over 40 min. Although it did appear to have a decreasing characteristic, it was not significant statistically. Such long-term synaptic plasticity appears to have a calcium dependency, thus the role of the calcium channels directly related to the influx of calcium were then examined. First, the L-type calcium channel, which is known to be related to LTP in the hippocampus and widely in the central nervous system, was examined. The specific blocker for the L-type calcium channel, nimodipine ( $10 \mu\text{M}$ ), was applied to the bath solution and TBS was given to STT neurons, showing LTP of synaptic strength (Fig. 4A) resulting in a significant increase ( $195.7 \pm 9.8\%$  of baseline at  $t=15$  min,  $n=4$ ). Next, the T-type calcium channel was examined by adding the specific blocker  $\text{Ni}^{2+}$  to the bath and applying the TBS. TBS-LTP was impaired in the presence of  $\text{Ni}^{2+}$ . However, TBS after washout of  $\text{NiCl}_2$  induced a 63% increase in synaptic current ( $n=4$ , Fig. 4B).

## DISCUSSION

Peripheral tissue and nerve injuries induce peripheral hypersensitivity and/or central sensitization of the spinal cord dorsal horn neurons associated with plastic changes mediating pain as hyperalgesia or allodynia [9-11]. Hyperalgesia is clinically defined as the increase in sensitivity of pain in a pathophysiological condition to an already painful stimulus, and allodynia as the pain provoked by a normally painless stimulus [12,13]. These types of physiological pain are closely associated with changes in synaptic transmission in nociceptive neurons such as STT neurons. Indeed, STT neurons can convey the pain signals ascending from the periphery to various regions of the central nervous system, which also includes many other signals such as onset, intensity, and duration of the noxious input [14,15]. This form of neural activation pattern from the periphery via STT neurons ascends to the cortex through the thalamus,

resulting in activity-dependent plasticity. To understand the mechanism underlying pain signaling, many researchers have investigated the phenomenon of central sensitization in terms of synaptic plasticity [16-19]. The purpose of this study is identifying the projection STT neuron *in vitro* in the spinal cord dorsal horn and recording the electrophysiological characteristics of these neurons. To conclude, here we observed that 1) LTP in STT neurons was induced by spike timing-dependent electrical stimulation in STT neurons, 2) the recorded STT neurons expressed NK1 receptors on the membrane, which is consistent with previous studies in which NK1 receptors are involved in the sensitization of STT neurons after peripheral injury, 3) LTP observed in STT neurons were closely related to the NMDA receptor activation and increased in postsynaptic calcium, and 4) the high-voltage-activated L-type calcium channel, known to be involved in LTP in the hippocampus and the central nervous system in general, was not crucial for the induction of LTP in STT neurons. However, the low-voltage-activated T-type calcium channel showed an essential role for the induction of synaptic plasticity (Fig. 4C). So far, many studies have reported on LTP at the spinal cord level in relation to the plasticity of spinal cord neurons [16-18]. However, most reports are observations of signal transduction from the peripheral to the spinal cord, which has limitations on physiologically explaining in what way synaptic plasticity influences at the supraspinal level. Therefore, in the study of long-term synaptic plasticity in STT neurons which ascends sensory information, especially pain information, will help us understand better on hypersensitivity, antinociception, as well as the analgesic mechanism which is associated with the central plasticity mechanism. The present study focuses attention on the possible cellular mechanisms with implications for pain processing and central sensitization in conjunction with long-term plasticity of STT neurons.

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