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Role of the spinal cord NR2B-containing NMDA receptors in the development of neuropathic pain

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ABSTRACT

Activation of *N*-methyl-D-aspartate (NMDA) receptors in the spinal dorsal horn has been shown to be essential for the initiation of central sensitization and the hyperexcitability of dorsal horn neurons in chronic pain. However, whether the spinal NR2B-containing NMDA (NMDA-2B) receptors are involved still remains largely unclear. Using behavioral test and *in vivo* extracellular electrophysiological recording in L5 spinal nerve-ligated (SNL) neuropathic rats, we investigate the roles of spinal cord NMDA-2B receptors in the development of neuropathic pain. Our study showed that intrathecal (*i.t.*) injection of Ro 25-6981, a selective NMDA-2B receptor antagonist, had a dose-dependent anti-allodynic effect without causing motor dysfunction. Furthermore, *i.t.* application of another NMDA-2B receptor antagonist ifenprodil prior to SNL also significantly inhibited the mechanical allodynia but not the thermal hyperalgesia. These data suggest that NMDA-2B receptors at the spinal cord level play an important role in the development of neuropathic pain, especially at the early stage following nerve injury. In addition, spinal administration of Ro 25-6981 not only had a dose-dependent inhibitory effect on the C-fiber responses of dorsal horn wide dynamic range (WDR) neurons in both normal and SNL rats, but also significantly inhibited the long-term potentiation (LTP) in the C-fiber responses of WDR neurons induced by high-frequency stimulation (HFS) applied to the sciatic nerve. These results indicate that activation of the dorsal horn NMDA-2B receptors may be crucial for the spinal nociceptive synaptic transmission and for the development of long-lasting spinal hyperexcitability following nerve injury. In conclusion, the spinal cord NMDA-2B receptors play a role in the development of central sensitization and neuropathic pain via the induction of LTP in dorsal horn nociceptive synaptic transmission. Therefore, the spinal cord NMDA-2B receptor is likely to be a target for clinical pain therapy.

Introduction

The mechanisms underlying the development of neuropathic pain are still not fully understood. Early studies have shown that noxious stimulation or nerve injury may lead to a long-lasting increase in synaptic efficacy such as long-term potentiation (LTP) or central sensitization in the spinal dorsal horn, which in turn causes an amplification of the response to sensory inputs (Randic et al., 1993; Rygh et al., 1999; Sandkuhler and Liu, 1998; Svendsen et al., 2000). It is now accepted that central sensitization is an important mechanism underlying neuropathic pain (Campbell and Meyer, 2006; Sandkuhler, 2000, 2007; Woolf, 2007). Substantial evidence has shown that the molecular mechanisms of synaptic plasticity between spinal central sensitization and hippocampal LTP are strikingly similar (Ji et al., 2003), and activation of *N*-methyl-D-aspartate (NMDA) receptors in the spinal dorsal horn is essential for the initiation of central sensitization (Bleakman et al., 2006; Chizh and Headley, 2005; Salter, 2005; Woolf, 2007) and the hyperexcitability of spinal dorsal neurons in nerve injury-induced neuropathic pain (Sotgiu and Biella, 2000).

The NMDA receptor is an ionotropic glutamate receptor that plays key role in excitatory synaptic transmission. Functional NMDA receptors are heteromeric complexes including the essential NR1 subunit and one or more of the NR2A-D subunits (Meguro et al., 1992; Monyer et al., 1992; Paoletti and Neyton, 2007). It is found that the NR2B subunit has a relatively restricted distribution in nociceptive transmission and pain regulatory pathways such as in the forebrain (Laurie et al., 1997) and in the superficial dorsal horn of the spinal cord (Boyce et al., 1999; Nagy et al., 2004). This kind of distribution suggests that NR2B subunit may play a role in pain transmission and in the development of chronic pain. In support of this notion, Malmberg et al. (2003) reported that spinal administration of the selective NR2B antagonist Conantokin G produced potent analgesic effects in several pain models. Tan et al. (2005) found that intrathecal administration of

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small interfering RNAs against the NR2B subunit reduced the pain responses induced by peripheral inflammation. Recently, Pedersen and Gjerstad (2008) demonstrated that in intact rats, spinal administration of the NMDA-2B receptor antagonist Ro 25-6981 not only showed a clear antinociceptive effect on spinal dorsal horn neurons, but also attenuated the magnitude of spinal cord LTP. These studies suggest that NR2B subunits distributed in the spinal cord may play an important role in the formation of central sensitization and persistent pain. In contrast, a supraspinal site of action for NR2B subunit antagonists has also been proposed (Chizh et al., 2001; Nakazato et al., 2005). Mice overexpressing NMDA-2B receptors in the forebrain exhibited enhanced pain behavior compared with control animals (Wei et al., 2001), and a selective upregulation of NMDA-2B receptors in the anterior cingulate cortex has been shown to contribute to behavioral sensitization caused by inflammation (Wu et al., 2005). Therefore, whether the spinal cord NMDA-2B receptors contribute to the development of central sensitization and neuropathic pain following nerve injury is still largely unclear.

In the present study, we showed that spinal administration of the selective NMDA-2B receptor antagonists not only alleviated neuropathic pain without causing motor dysfunction, but also inhibited the noxious stimulation-evoked activities and the induction of LTP in dorsal horn WDR neurons. Our results suggest that activation of spinal cord NMDA-2B receptors are involved in the development of central sensitization and neuropathic pain via the induction of LTP in dorsal horn nociceptive synaptic transmission.

Materials and methods

Animals

Male Sprague–Dawley rats weighing 180–200 g at the beginning of the experiment were provided by the Department of Experimental Animal Sciences, Peking University Health Science Center. The rats were housed in separated cages with free access to food and water. The room temperature was kept at 24±1 °C under natural light–dark cycle. All animal experimental procedures were conducted in accordance with the guidelines of the International Association for the Study of Pain (Zimmermann, 1983) and were approved by the Animal Care and Use Committee of our university.

Spinal nerve ligation (SNL)

Under general anesthesia with chloral hydrate (0.3 g kg^{-1}, i.p.), the left L5 spinal nerves distal to the dorsal root ganglia were tightly ligated with 4-0 silk sutures as described earlier by Kim and Chung (1992). In control animals, sham surgery with identical procedure except for ligation of the L5 spinal nerves was received. Six days after surgery, behavioral testing or electrophysiological recording were performed. Any rats exhibiting motor deficiency or lack of tactile allodynia were excluded from the study.

Behavioral studies

Implantation of intrathecal catheter

Under chloral hydrate (0.3 g kg^{-1}, i.p.) anesthesia, implantation of intrathecal cannula was performed following the method of Storkson et al. (1996). Briefly, a PE-10 polyethylene catheter was implanted between the L5 and L6 vertebrae to reach the lumber enlargement of the spinal cord. The outer part of the catheter was plugged and fixed onto the skin on closure of the wound. All surgical procedures were performed under sterile conditions. Rats showing neurological deficits after the catheter implantation were euthanized. Drugs or vehicle were intrathecally injected via the implanted catheter in a 20-μl volume of solution followed by 10 μl of normal saline (NS) for flushing. Each injection lasted at least 5 min. After an injection, the needle remained in situ for 2 min before being withdrawn.

Assessment of mechanical allodynia

Mechanical allodynia, as a behavioral measure of neuropathic pain, was assessed by measuring the 50% paw withdrawal threshold (PWT) as described in our previous reports (Sun et al., 2005; Xing et al., 2007). The 50% PWT in response to a series of *von* Frey filaments (Stoelting, Wood Dale, IL, USA) was determined by the Up and Down method (Chaplan et al., 1994) and was calculated as previously described (Dixon, 1980). The anti-allodynic effects of antagonists were presented as 50% PWT in grams or as percentages of the maximum possible effect (%MPE) following the formula described by Obata et al. (2001), where %MPE = (post-drug PWT value − pre-drug PWT value) × 100/(15 g cut-off value − pre-drug PWT value).

Assessment of thermal hyperalgesia

Thermal hyperalgesia of the hind paws was tested as described by Hargreaves et al. (1988). Rats were allowed to acclimate for a minimum of 30 min within acrylic enclosures on a clear glass plate maintained at 30 °C. A radiant heat source was focused onto the plantar surface of the hind paw. Measurements of paw withdrawal latency (PWL) were taken by a timer that was started by the activation of the heat source and stopped when withdrawal of the paw was detected with a photodetector. A maximal cut-off time of 30 s was used to prevent unnecessary tissue damage. Three measurements of PWL were taken for each hind paw and were averaged as the result of each test session. The hind paw was tested alternately with greater than 5 min intervals between consecutive tests.

Assessment of motor function

Inclined-plate testing was used for the assessment of motor function. The rat was placed crosswise to the long axis of an inclined plate. The initial angle of the inclined plate was 50°. The angle was then adjusted in 5-degree increments. The maximum angle of the plate on which the rat maintained its body position for 5 s without falling was determined according to the method reported by Rivlin and Tator (1977).

Measurement of drug effects

The first behavioral experiment examined the time course of anti-allodynic effects and the dose–response effects of NR2B subunit antagonist Ro 25-6981 on neuropathic pain. Ro 25-6981 at the doses of 10, 30, 100, 300 or 1000 μg in a 20-μl volume of solution, or vehicle (normal saline or 5% DMSO) in an equal volume, was intrathecally administrated to the SNL rats. 50% PWT was measured just before drug injection, and measured again at 30, 60, 90 and 120 min after drug injection. The anti-allodynic effect of Ro 25-6981 was calculated as %MPE for each dose at each time point. The inclined-plate testing was performed at 60 min before and 150 min after drug injection to assess the effect of Ro 25-6981 on motor function.

The second experiment determined the antinociceptive effect of pre-treatment with ifenprodil, another NR2B subunit antagonist, on the development of neuropathic pain. Ifenprodil at the dose of 20 μg in a 20-μl volume of solution, or vehicle in an equal volume, was intrathecally delivered to rats 20 min prior to SNL operation, and repeated at the end of the first day and then twice a day in the following 2 days after SNL surgery. The 50% PWT to *von* Frey filaments and the PWL to radiant heat were then measured at day 3, 7, 14 and 28 after SNL.

Electrophysiological studies

Surgery

The rat was initially anesthetized with urethane (1.2–1.5 g kg^{-1}, i.p.). The trachea was cannulated to allow mechanical ventilation

with room air. A catheter was inserted into the right jugular vein for continuous infusion of Tyrode's solution [in mmol l^{-1}: NaCl 137, KCl 2.7, $CaCl_2$ 1.4, $MgCl_2$ 1.0, $NaHCO_3$ 6.0, NaH_2PO_4 2.1, D-(+)-glucose 6.5; pH 7.4] at a rate of 1.0–1.5 ml h^{-1}. The rectal temperature was maintained at 36.5–37.5 °C via a feedback-controlled under-body heating pad. A pair of bipolar silver hook electrode was placed under the sciatic nerve immediately proximal to the trifurcation for electrical stimulation. The vertebral column was rigidly fixed in the frame with two clamps. The lumbar enlargement of the spinal cord was exposed by laminectomy at the vertebrae T13 and L1 and the dura covering lumbosacral spinal segments was carefully removed. A small well was built with 3% agar on the dorsal spinal cord at the recording segment to allow application of drugs or vehicles (Kelly and Chapman, 2002; Liu et al., 2007). The exposed spinal tissue was covered with warm (37 °C) saline solution.

After surgery, the animal was artificially ventilated with a small animal ventilator and paralyzed with curare (2.0 mg kg^{-1}, i.v.), and continuous anesthesia and paralysis were maintained with urethane (0.10–0.15 g kg^{-1} h^{-1}) and curare (0.20 mg kg^{-1} h^{-1}) during the whole experiment. The depth of anesthesia was monitored by examination of pupillary size and reflexes. The physiological condition of the animal was monitored by recording the electrocardiogram (330–460 beats/min), end-expiratory CO_2 (3.5–4.5%), and rectal temperature (36.5–37.5 °C), and was maintained within the range indicated (Liu et al., 2007).

Extracellular recording

Single-unit extracellular recordings were made from the lumbar dorsal horn neurons within 1200 μm of the dorsal surface of the spinal cord with 2–5 MΩ parylene-coated tungsten microelectrodes (Friedrick Haer & Co., Bowdoinham, ME, USA). The microelectrode was inserted perpendicularly into the dorsal horn from a point about midway between the midline and the medial edge of the dorsal root entry zone. During electrode advancement, electrical pulses (0.5 Hz, 0.3-ms pulse width, 0.4 mA) were applied to the ipsilateral sciatic nerve as search stimuli so that a neuron with no spontaneous firing could be identified. Once a single unit was identified, the receptive field and response characteristics were determined by a range of mechanical stimuli of varying intensities, including brushing or touching the skin with a cotton brush, light pressure with a probe, and pinching a fold of skin with toothed forceps. A neuron responding to innocuous tactile stimuli, light pressure, and noxious pinch in a graded manner was identified as a WDR neuron and was selected for further investigation (Liu et al., 2007; Zhang et al., 2001). The recorded signals were amplified with an AC pre-amplifier, filtered with a passing bandwidth 500–1000 Hz, displayed on an oscilloscope, and fed to a Pentium computer via a CED 1401 interface for off-line analysis using the Spike 2 software (Cambridge Electronic Design, Cambridge, UK). Spikes appearing 45–300 ms after stimulus were defined as C-fiber responses, i.e. responses in the WDR neurons evoked by C-fiber activation (Liu et al., 2007). Single cell recording was ensured on the basis of amplitude and shape of the action potentials. In the following electrophysiological studies, only one cell was studied in each animal, and each animal received only one dose of Ro 25-6981 or vehicle.

Measurement of drug effects

The first part of the electrophysiological experiment was designed to investigate the effects of spinal application of selective NR2B subunit antagonist Ro 25-6981 on the activities of WDR neuron in both normal and SNL rats. In this experiment, a train of 10 stimuli (0.5 Hz, 0.5-ms pulse width, with a pulse current of 2×C-fiber response threshold) used as test stimulus was applied repeatedly to the sciatic nerve at 5-min interval, and post-stimulus histograms from the responses of WDR neurons were constructed. After three stable control responses were recorded, Ro 25-6981 at the doses of 30, 100, or 300 μg in a 20-μl volume of solution, or the equal volume of vehicle, was applied topically to the dorsal surface of the spinal cord, and the post-drug responses evoked by the same test stimulus as above were measured at 5-min intervals for up to 60 min. In the present study, only C-fiber responses were examined and analyzed as described in our previous report (Liu et al., 2007). All of the C-fiber responses values were expressed as percentages of the mean response value of three pre-drug consecutive trains of test stimuli. Cells showing variation of less than 20% were selected for further experiments.

The second part of the experiment was designed to study effects of spinal administration of Ro 25-6981 on the induction of LTP of the C-fiber responses in dorsal horn WDR neurons. In this experiment, a 2-ms rectangular pulse with a pulse current of 2×C-fiber response threshold given every 5 min was used as the test stimulus. After three stable C-fiber responses to the test stimulus were recorded, Ro 25-6981 at the dose of 100 μg in a 20-μl volume of solution (based on the above dose–response observation), or equal volume of normal saline, was applied topically to the dorsal surface of the spinal cord, and the post-drug responses were monitored for 20 min under the same test stimulus as above. Throughout this experiment, the mean value of four post-drug C-fiber responses to the test stimulus was served as the baseline of the cell responsiveness for the subsequent experiment. A high-frequency stimulation (HFS) conditioning (20 trains of 2-s duration, 100 Hz, 0.5-ms rectangular pulses, 10-s intervals between trains, pulse current 6 times the current for the C-fiber threshold previously found with 2-ms pulses) (Svendsen et al., 1999) was delivered to the sciatic nerve for the induction of LTP of the C-fiber responses. After HFS conditioning, the same test stimulus as described above was delivered again to the sciatic nerve every 5 min and the recording of the C-fiber responses was continued for another 2 h after HFS application. The C-fiber responses value following each test stimulus was normalized and then expressed as a percentage of the mean baseline value.

At the end of each experiment, the recording site was marked by electrolytic lesion (20.0 μA positive depolarizing DC current for 20 s), and the animal was subsequently euthanized with an overdose of pentobarbital sodium. The spinal cord was fixed in 4 °C 4% paraformaldehyde overnight, and sectioned into 20 μm-thick transverse sections on a cryostat and stained with cresyl violet. The recording site was then identified and plotted on a schematic representation of the lumbar spinal cord.

Chemical preparation and application

Ro 25-6981, (R-(R*,S*)-a-(4-hydroxyphenyl)-b-methyl-4-(phenylmethyl)-1-piperidine propanol (Tocris Cookson, Saint Louis, MO, USA) was dissolved in sterile 0.9% saline solution at concentrations of 0.5, 1.5, and 5 μg $μl^{-1}$, or dissolved in sterile 5% dimethyl sulfoxide (DMSO) with 95% saline solution at concentrations of 15 and 50 μg $μl^{-1}$, and given at the doses of 10, 30, 100, 300 and 1000 μg in behavioral experiments, or 30, 100 and 300 μg in electrophysiological recordings. Ifenprodil (Sigma-Aldrich, Saint Louis, MO, USA) was dissolved in sterile DMSO in stock solution, and diluted to the working concentration of 1.0 μg $μl^{-1}$ with 9% Tween-80 and 91% saline solution, and given at the dose of 20 μg in behavioral experiments.

Statistical analysis

All data were expressed as mean±S.E.M. One or two way analysis of variance (ANOVA) followed by Bonferroni *post-hoc* test was used for multiple comparison. *F* values with their associated Degrees of Freedom (treatment, time, interaction and residual) were expressed as *F*(d*f* of treatment, time, interaction/residual)=*F*

values (treatment, time, interaction) in two-way ANOVA, and F(df of treatment/residual)=F values in one-way ANOVA. Two-tailed unpaired Student's t-test was used for the comparison of the mean values between two groups. Area under the time-course curve (AUC) values during the analysis time was used to measure the summed effects of Ro 25-6981 at different doses as described by Honda et al. (2006). Differences with $p<0.05$ were considered statistically significant.

Results

Effects of i.t. Ro 25-6981 on the mechanical allodynia and the motor function in SNL rats

To study whether spinal cord NMDA-2B receptors play a role in the development of neuropathic pain, we first investigated the effect of *i.t.* Ro 25-6981, a selective NR2B antagonist, on the mechanical allodynia

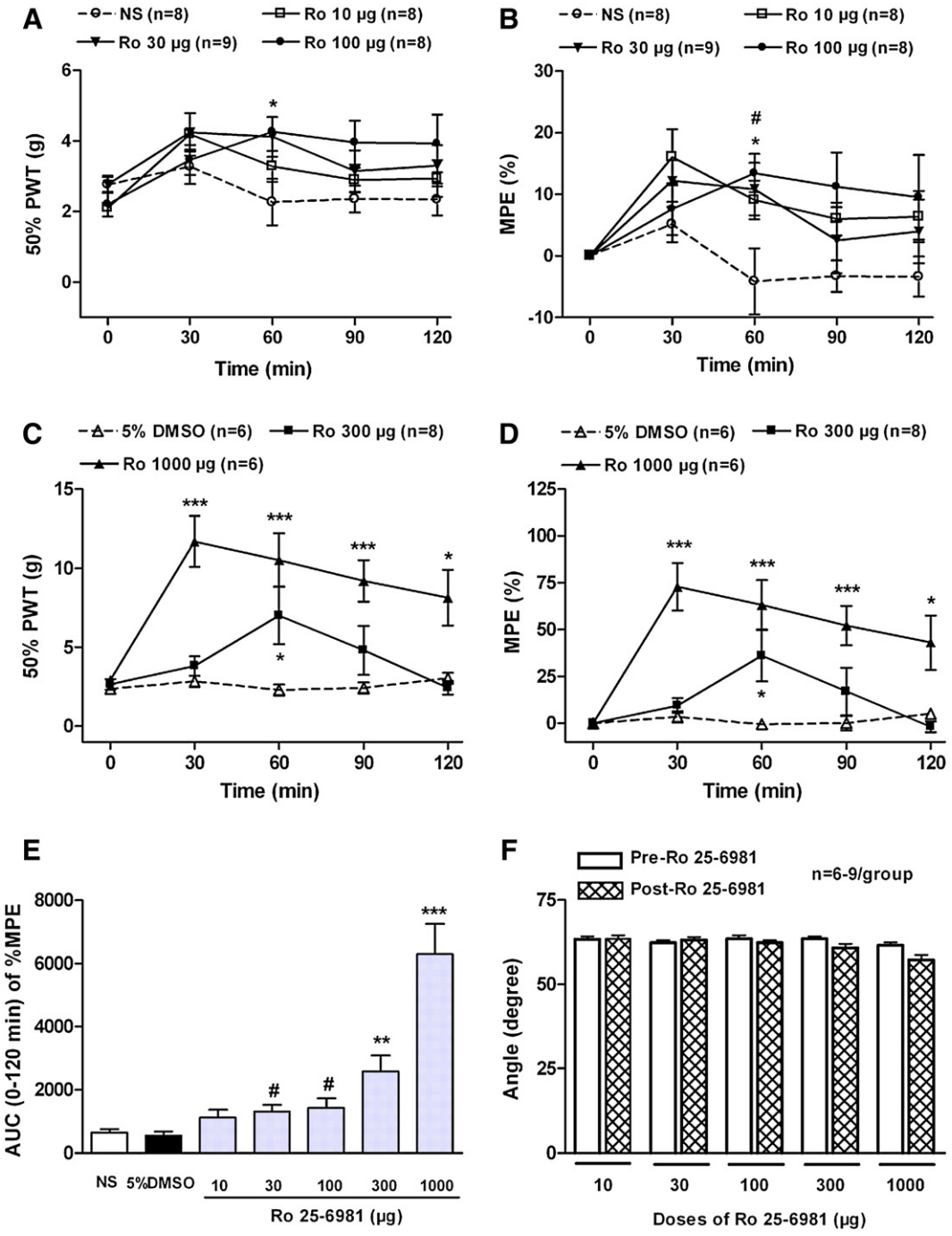

Fig. 1. Effects of *i.t.* application of Ro 25-6981 (Ro) on the mechanical allodynia and the motor function in neuropathic pain rats induced by L5 spinal nerve ligation. (A), (C) Effects of Ro 25-6981 at 10, 30, 100 μg (A) and 300, 1000 μg (C) on 50% PWT. (B), (D) Effects of Ro 25-6981 at 10, 30, 100 μg (B) and 300, 1000 μg (D) on percentage of the maximum possible effect (%MPE). $^{*/\#}p<0.05$, $^{***}p<0.001$ as compared with NS or 5% DMSO control group, two-way ANOVA followed by Bonferroni *post-hoc* test, $n=6$–9. (E) AUC (0–120 min of the analysis time) of %MPE in (B) and (D). $^{\#}p<0.05$ as compared with the NS control group; $^{**}p<0.01$, $^{***}p<0.001$ as compared with the 5% DMSO control group, one-way ANOVA followed by Bonferroni *post-hoc* test, $n=6$–9. (F) Effects of Ro 25-6981 on the motor function of rats measured by the angle of the inclined-plate that rat begins to fall (see Materials and methods for detail). Note that spinal administration of Ro 25-6981 at doses of 10, 30, 100, 300 and 1000 μg had no significant damage on the motor function of rats ($p>0.05$, two-tailed unpaired Student's t-test, pre-drug injection versus post-drug injection, $n=6$–9). Data are expressed as mean±S.E.M. NS: normal saline.

of SNL rats. The behavioral test showed that, at the doses of 10 and 30 μg, Ro 25-6981 had no obvious effect on the 50% PWT of the ipsilateral hind paw as compared with that in NS control animals at any time point observed ($p>0.05$, two-way ANOVA, $F(3, 4, 12/140)=(4.19, 4.10, 1.08)$, $n=8–9$, Fig. 1A), although the maximum possible effect (MPE) of the 50% PWT was increased to 10.86±4.27% from the NS control of −4.15±5.36% at the time point of 60 min after injection at 30 μg ($p<0.05$, two-way ANOVA, $F(3, 4, 12/145)=(5.95, 3.88, 0.95)$, $n=9$, Fig. 1B). While at the dose of 100 μg, Ro 25-6981 significantly increased the 50% PWT and the MPE of 50% PWT to 4.26±0.22 g and 13.43±3.08% from the NS control of 2.26±0.66 g and −4.15±5.36% at the time point of 60 min after injection, respectively (two-way ANOVA, $p<0.05$, $F(3, 4, 12/140)=(4.19, 4.10, 1.08)$, and $p<0.05$, $F(3, 4, 12/145)=(5.95, 3.88, 0.95)$, respectively, $n=8$, Figs. 1A and B). As the dose increased, the inhibitory effect of Ro 25-6981 on the mechanical allodynia was increased gradually. At 1000 μg, Ro 25-6981 increased both of the 50% PWT and the MPE of 50% PWT from 30 min after injection and lasted 120 min of our observation. The 50% PWT and the MPE of 50% PWT were markedly increased to 10.52±1.69 g and 63.17±13.13% from the vehicle control of 2.30±0.36 g and −0.59±1.85% at 60 min after injection, respectively (two-way ANOVA, $p<0.001$, $F(2, 4, 8/85)=(35.53, 5.95, 3.06)$, and $p<0.001$, $F(2, 4, 8/85)=(33.88, 6.72, 3.46)$, respectively, $n=6$, Figs. 1C and D). As summarized in area under the time-course curve (AUC) values of MPE, the AUC (0–120 min of the analysis time) was increased to 1312±227 ($p<0.05$), 1424±312 ($p<0.05$) and 2582±506 ($p<0.01$), 6291±957 ($p<0.001$) from the vehicle control of 648±109 and 562±121 after Ro 25-6981 injection at 30, 100 and 300, 1000 μg, respectively (one-way ANOVA, $F(6/46)=21$, $n=6–9$, Fig. 1E).

To assess whether Ro 25-6981 had side-effects during the effective dose of anti-allodynia, we also examined the effect of Ro 25-6981 on the motor function of rats at each of the aforementioned dose. The results showed that spinal administration of Ro 25-6981 at each dose of 10, 30, 100, 300 and 1000 μg, had no obvious motor dysfunction in rats with neuropathic pain ($p>0.05$, two-tailed unpaired Student's *t*-test, pre-drug injection versus post-drug injection, $n=6–9$, Fig. 1F).

These results suggested that intrathecal injection of NR2B antagonist Ro 25-6981 had a dose-dependent anti-allodynic effect without motor dysfunction.

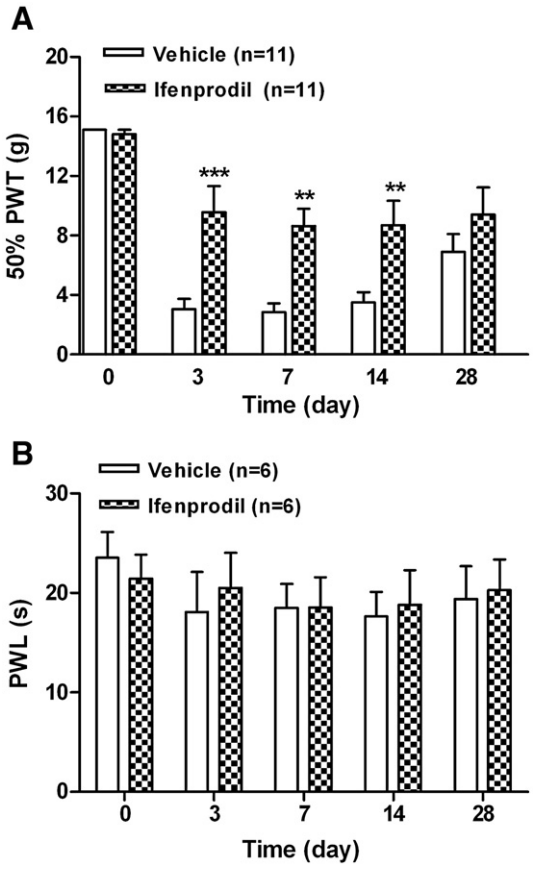

Fig. 2. Effects of pre-treatment with *i.t.* application of ifenprodil on the mechanical allodynia (A) and the thermal hyperalgesia (B) in SNL rats. (A) Effects of ifenprodil on 50% PWT to *von* Frey filaments. (B) Effects of ifenprodil on PWL to radiant heat. Note that pre-treatment with ifenprodil had significant inhibitory effect on mechanical allodynia, but no obvious effect on thermal hyperalgesia in SNL rats. $^{**}p<0.01$, $^{***}p<0.001$ as compared with the vehicle control group, two-tailed unpaired Student's *t*-test, $n=6–11$. Data are expressed as mean±S.E.M.

Effects of pre-treatment with i.t. ifenprodil on the mechanical allodynia and the thermal hyperalgesia in SNL rats

Although Ro 25-6981 has a highly potent and selective antagonistic effect on NMDA-2B receptors (Fischer et al., 1997), its effect was short-lasting (only about 2 h) as demonstrated above. To further investigate whether spinal cord NMDA-2B receptors contribute to the development of neuropathic pain at the early initiation stage or in the maintenance stage, we also examined the effects of pre-treatment with *i.t.* ifenprodil, another selective antagonist of NMDA-2B receptors that has a longer effect than Ro 25-6981 (see Discussion), on the mechanical allodynia and the thermal hyperalgesia in SNL rats. Pre-treatment with *i.t* ifenprodil 20 μg prior to SNL significantly inhibited the mechanical allodynia but not the thermal hyperalgesia in SNL rats. The 50% PWT to *von* Frey filaments was increased to 9.58±1.73 ($p<0.001$), 8.64±1.16 ($p<0.01$), and 8.70±1.62 g ($p<0.01$) from the vehicle control of 3.08±0.66, 2.85±0.59, and 3.51±0.68 g at day 3, 7, and 14 after SNL, respectively (two-tailed unpaired Student's *t*-test, vehicle versus ifenprodil, $n=11$, Fig. 2A). While the PWL to radiant heat showed no significant change at any time point after SNL ($p>0.05$, two-tailed unpaired Student's *t*-test, vehicle versus ifenprodil, $n=6$, Fig. 2B).

These behavioral and pharmacological tests suggested that the spinal cord NMDA-2B receptors play an important role in the development of neuropathic pain, especially at the early stage following nerve injury.

Effects of spinal administration of Ro 25-6981 on C-fiber responses of dorsal horn WDR neurons

To further elucidate that the potential mechanisms of spinal cord NMDA-2B receptors contribute to the development of neuropathic pain following nerve injury, we first examined the effects of spinal administration of Ro 25-6981 on C-fiber responses of dorsal horn WDR neurons, because the C-fiber responses of WDR neurons are usually regarded as related to nociception (Liu et al., 2007;Rygh et al., 2000). As shown in Figs. 3 and 4, either in normal or SNL rats, Ro 25-6981 at 30 μg did not significantly change the C-fiber responses of WDR neurons as compared with those in NS group at any time point observed (two-way ANOVA, $p>0.05$, $F(4, 15, 60/400)=(366.5, 15.12, 7.81)$, $n=7$ in normal rats, and $p>0.05$, $F(4, 15, 60/416)=(116.9, 7.80, 2.92)$, $n=5$ in SNL rats, respectively, Figs. 4A and B). While at 100 and 300 μg, Ro 25-6981 significantly inhibited the C-fiber responses of WDR neurons, and the maximal inhibition was observed at 20–50 min after Ro 25-6981 application. The C-fiber responses at 20–50 min after application of 100 and 300 μg Ro 25-6981 was reduced to 54.84±0.91% ($p<0.001$, $n=6$) and 18.87±1.78% ($p<0.001$, $n=5$) from the vehicle control of 102.40±1.80% and 104.00±1.76% in normal rats (two-tailed unpaired Student's *t*-test, vehicle versus Ro 25-6981, Fig. 4A), and to 48.97±2.16% ($p<0.001$, $n=7$) and 37.31±2.23% ($p<0.001$, $n=7$) from the vehicle control of 100.10±1.10% and 101.80±1.76% in SNL rats (two-tailed unpaired Student's *t*-test, vehicle versus Ro 25-6981, Fig. 4B), respectively. As

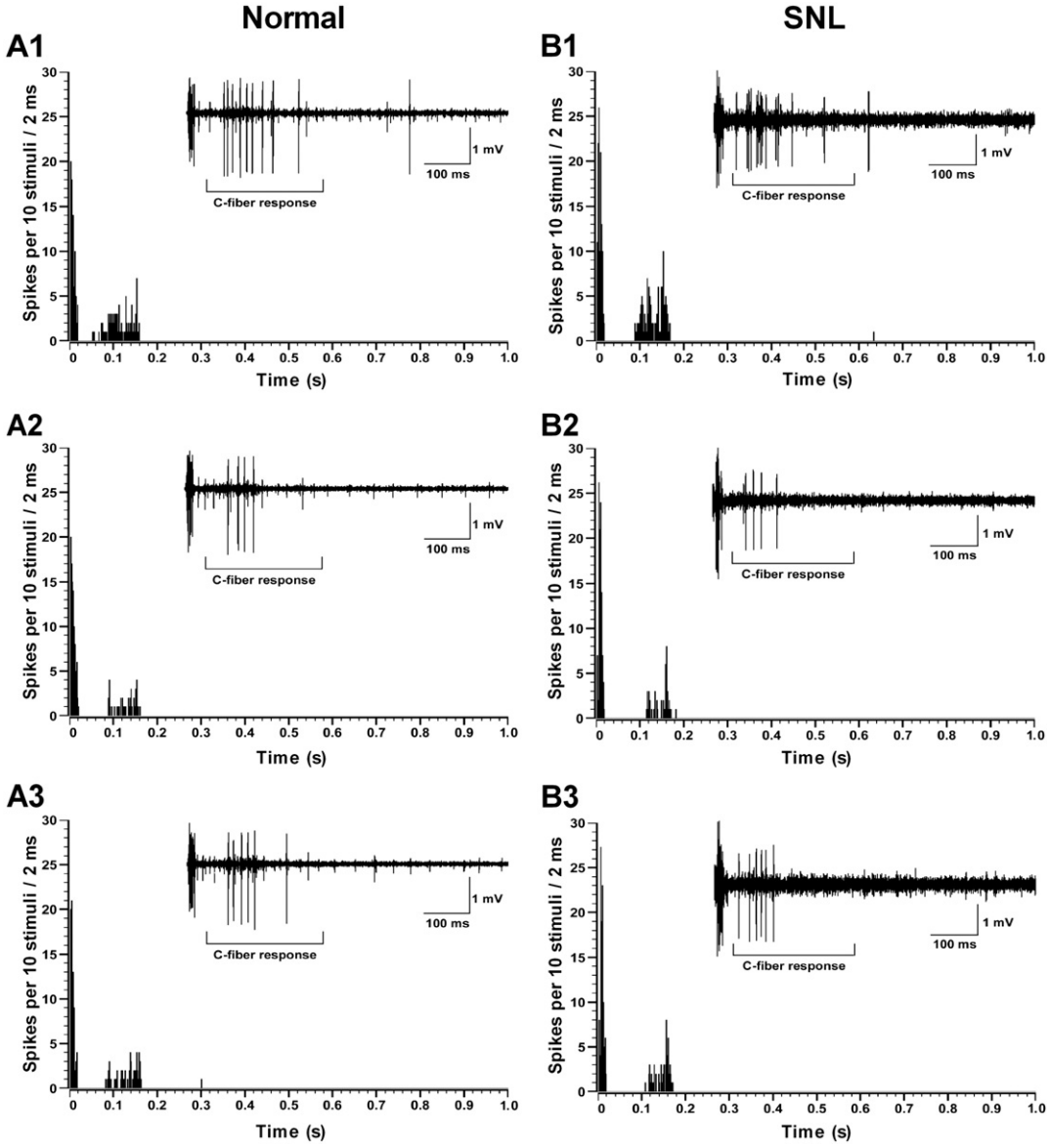

Fig. 3. Examples showing the inhibitory effects of Ro 25-6981 (300 μg) on the C-fiber responses of dorsal horn WDR neurons in normal (A1–A3) and SNL (B1–B3) rats. Panels illustrate the post-stimulus histogram of the electrically evoked neuronal responses in a dorsal horn WDR neuron at the time point of 15 min before Ro 25-6981 application (A1 and B1), 40 min (A2 and B2) and 60 min (A3 and B3) after Ro 25-6981 application, respectively. Inset shows the original recordings of the first electrically evoked neuronal responses corresponding to each time point respectively.

summarized in AUC (−15–60 min) of C-fiber responses, after application of Ro 25-6981 at 100.0 and 300.0 μg, the AUC (−15–60 min of the analysis time) was decreased to 5166±283 ($p<0.001$, $n=6$) and 2854±412 ($p<0.001$, $n=5$) from the vehicle control of 7532±161 and 7665±68 in normal rats (one-way ANOVA, $F(4/25)=68.44$, Fig. 4C), and to 4813±356 ($p<0.001$, $n=7$) and 4098±723 ($p<0.001$, $n=7$) from the vehicle control of 7372±187 and 7579±184 in SNL rats (one-way ANOVA, $F(4/26)=14.80$, Fig. 4D), respectively. However, no significant difference in the AUC (−15–60 min of the analysis time) of C-fiber responses was observed after Ro 25-6981 application between the normal and SNL rats ($p>0.05$, two-tailed unpaired Student's t-test, $n=5–8$, Fig. 4E). Two representative examples illustrating the inhibitory effects of Ro 25-6981 (at 300 μg) on C-fiber responses of dorsal horn WDR neurons in normal and SNL rats were shown in Fig. 3. These results suggested that the spinal cord NMDA-2B receptors are likely to be involved in the spinal nociceptive synaptic transmission by activating the dorsal horn WDR neurons, which are usually regarded as related to nociception.

Effects of spinal administration of Ro 25-6981 on the induction of LTP of C-fiber responses in dorsal horn WDR neurons

As the use-dependent LTP in spinal nociceptive systems are widely considered a cellular and synaptic model of injury-induced central sensitization (Rygh et al., 2002, 2005; Sandkuhler, 2000), we further examined whether spinal cord NMDA-2B receptors contribute to the induction of LTP of C-fiber responses in dorsal horn WDR neurons. Our results showed that spinal administration of NR2B antagonist Ro 25-6981 significantly inhibited the dorsal horn LTP induced by high-frequency stimulation (HFS) applied to the sciatic nerve. As shown in Fig. 5, after spinal application of NS, HFS induced a prolonged increase (177.1±2.2% of the baseline, $n=5$, Fig. 5A) in the C-fiber responses of WDR neurons, and this enhancement lasted more than 2 h until experiment termination. This result suggests that a consistent LTP in the C-fiber responses of dorsal horn WDR neurons can be induced by HFS when NS applied. However, after spinal application of Ro 25-6981 at 100 μg,

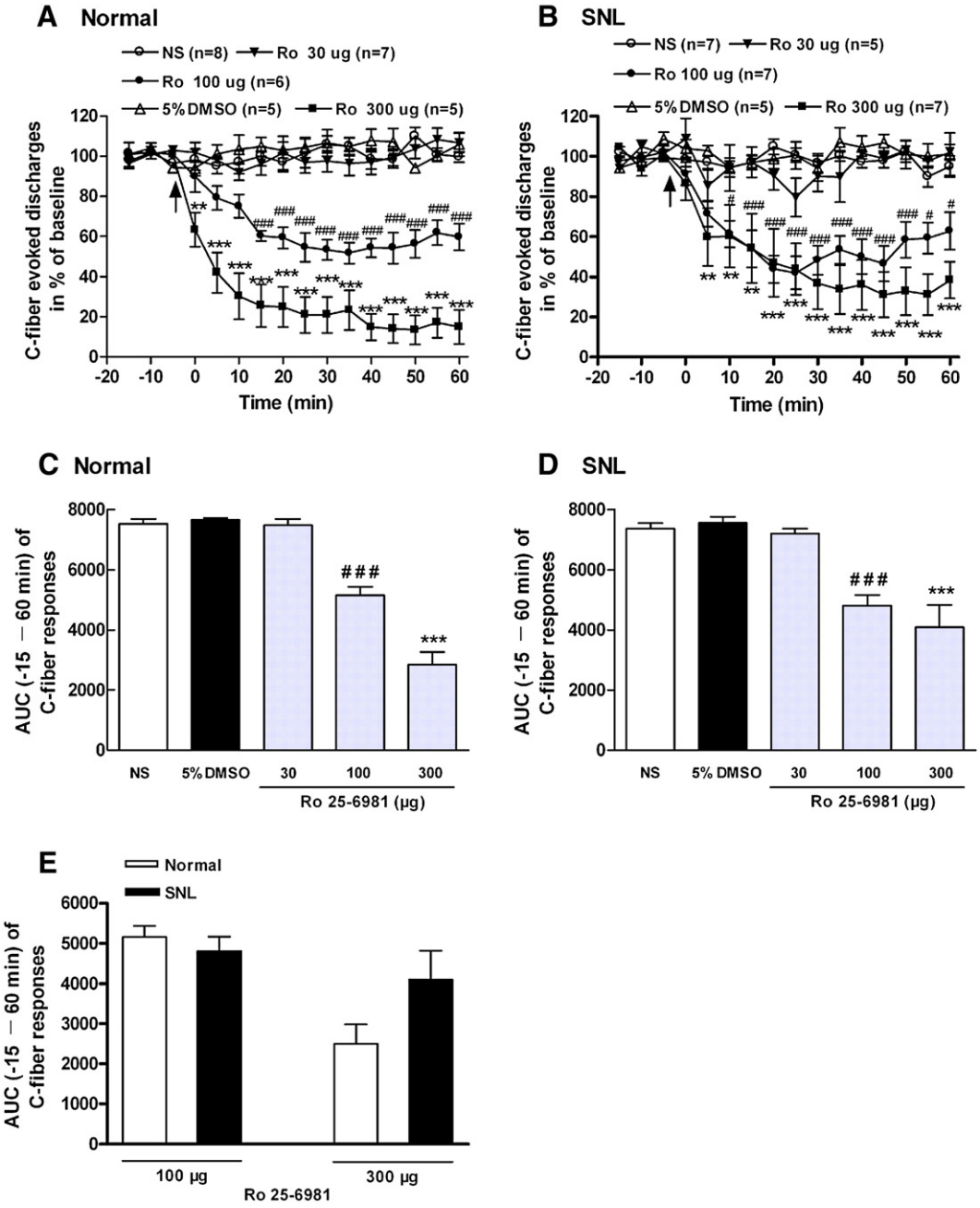

Fig. 4. Effects of spinal application of Ro 25-6981 on the C-fiber responses of dorsal horn WDR neurons. (A) and (C) in normal rats, Ro 25-6981 attenuated the C-fiber responses of WDR neurons significantly at 100 and 300 μg although no obvious effect was observed at 30 μg. (C) AUC (−15–60 min of the analysis time) of the C-fiber responses in (A). (B) and (D) in SNL rats. Similar inhibitory effects of Ro 25-6981 on the C-fiber responses of WDR neurons were also observed in SNL rats. $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ as compared with NS control group. $^{**}p<0.01$, $^{***}p<0.001$ as compared with 5% DMSO control group, one or two-way ANOVA followed by Bonferroni *post-hoc* test, $n=5$–8. Ro 25-6981 or vehicle was delivered at the time point as arrowhead shows. (E) Comparison of the AUC (−15–60 min of the analysis time) of the C-fiber responses after application of Ro 25-6981 at 100 and 300 μg between normal (C) and SNL (D) rats. No significant difference was observed between the normal and SNL rats ($p>0.05$, two-tailed unpaired Student's *t*-test, $n=5$–8). Data are expressed as mean±S.E.M.

the HFS induced only 97.2±2.3% of the baseline in C-fiber responses ($n=5$, Fig. 5B). The C-fiber responses in the Ro 25-6981+HFS group were significantly lower than those in NS+HFS group ($p<0.001$, two-tailed unpaired Student's *t*-test between the NS+HFS group versus the Ro 25-6981+HFS group at 0–120 min after HFS, $n=5$, Fig. 5C). These suggest that the LTP in C-fiber responses of dorsal horn WDR neurons were significantly inhibited by spinal administration of Ro 25-6981, and the spinal cord NMDA-2B receptors contribute to the induction of LTP of C-fiber responses in dorsal horn WDR neurons.

Discussion

In the present study, we found for the first time to our knowledge that spinal administration of the selective NMDA-2B receptor antagonists not only inhibited the noxious stimulation-evoked activities and the induction of LTP in dorsal horn WDR neurons but also alleviated neuropathic pain without motor dysfunction. These data indicate that activation of spinal cord NMDA-2B receptors is involved in the development of central sensitization and neuropathic pain via the induction of LTP in dorsal horn nociceptive synaptic transmission.

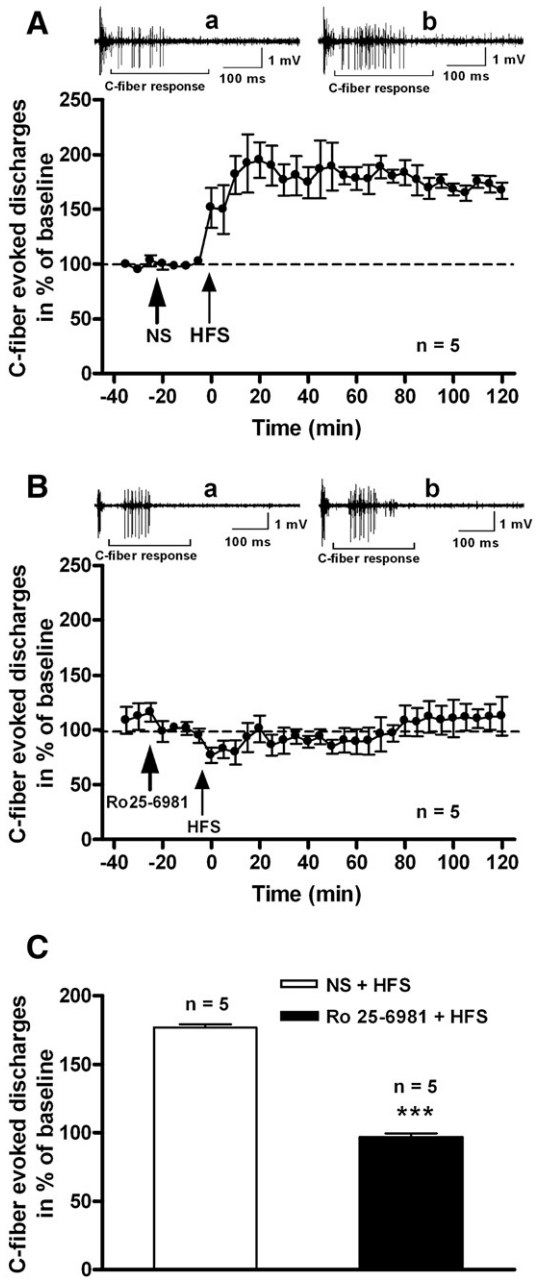

Fig. 5. Effects of spinal application of Ro 25-6981 on the induction of LTP of C-fiber responses in dorsal horn WDR neurons. The C-fiber responses were recorded before and after spinal application of NS (A) or Ro 25-6981 at the dose of 100 μg (B) in combination with high frequency stimulation (HFS) to the sciatic nerve. Two individual original discharges recorded in an animal are shown in insets in each panel respectively. Trace in (a) is an example of a recording before HFS, trace in (b) was recorded 60 min after HFS. Note that Ro 25-6981 significantly blocked the induction of LTP of C-fiber responses in dorsal horn WDR neurons after HFS application. (C) Comparison of the C-fiber responses at 0–120 min after HFS application between the Ro 25-6981 and the NS control group. ***$p<0.001$ as compared with NS group, two-tailed unpaired Student's *t*-test, $n=5$. Data are expressed as mean±S.E.M. NS: normal saline.

Role of the spinal cord NMDA-2B receptors in the development of neuropathic pain

It is well accepted that activation of NMDA receptors in the spinal dorsal horn play crucial roles in the development and maintenance of neuropathic pain following nerve injury (Bennett et al., 2000; Bleakman et al., 2006; Chizh and Headley, 2005; Gao et al., 2005; Woolf and Thompson, 1991). However, whether the spinal cord NMDA-2B receptors are involved still remains largely unclear (Chizh et al., 2001; Malmberg et al., 2003; Tan et al., 2005). Although previous behavioral studies have shown that systematical application of the selective NMDA-2B receptors antagonists ifenprodil, Ro 25-6981 and CP 101,606 produce analgesia in animals with persistent inflammatory or neuropathic pain (Bernardi et al., 1996; Boyce et al., 1999; Taniguchi et al., 1997), the exact site of action of these inhibitors were difficult to determine. In the present study, we demonstrated that *i.t.* injection of Ro 25-6981 had a dose-dependent anti-allodynic effect in neuropathic pain rats. We believe that Ro 25-6981 exerted its analgesic effect via acting on the spinal cord NMDA-2B receptors. Several lines of evidence support this notion. First, Ro 25-6981 is a highly potent and selective antagonist of NMDA-2B receptors (Fischer et al., 1997) showing a relatively low affinity to alpha 1 adrenergic as well as serotonergic binding sites (Chenard et al., 1991; Mutel et al., 1998). It is also found that Ro 25-6981 is 5000 folds more selective for the heteromeric NR1/NR2B receptors than the NR1/NR2A receptors, 50 folds less potent for the voltage-gated sodium channels and several hundred folds less potent in blocking the calcium channels in comparison with its potency in blocking NMDA-2B receptors (Fischer et al., 1997). Second, Ro 25-6981 was topically applied to the spinal cord by intrathecal injection in our behavioral experiments, so its action site was at the spinal cord. Third, the NR2B subunit had a relatively restricted distribution in the superficial laminae of the rat spinal cord, an area considered to be important in nociceptive transmission and processing (Boyce et al., 1999; Nagy et al., 2004). Finally, Tan et al. (2005) reported that knockdown of the NR2B subunit in the spinal cord via intrathecal administration of small interfering RNAs abolished formalin-induced pain behavior in rats. Taken together, our results provide strong evidence that the spinal cord NMDA-2B receptors play an important role in the development of nerve injury-induced neuropathic pain.

To further investigate whether spinal cord NMDA-2B receptors contribute to the development of neuropathic pain at the early initiation stage or in the maintenance stage, we examined the effects of pre-treatment with *i.t.* ifenprodil, another antagonist of NMDA-2B receptors, on the mechanical allodynia and the thermal hyperalgesia in SNL rats. We used ifenprodil instead of Ro 25-6981 in this part of the experiment, because the antagonistic effect of Ro 25-6981 on NMDA-2B receptors was short-lasting (only about 2 h) as demonstrated in our behavioral tests. Our results showed that pre-treatment with ifenprodil by intrathecal injection at pre-SNL and the following 2 days significantly inhibited the mechanical allodynia but not the thermal hyperalgesia in SNL rats. These findings are consistent with several previous reports that pre-treatment with NMDA receptor antagonist memantine, MK801 or ketamine for 3–8 days pre- and post-operation of SNL- or sciatic nerve chronic constriction injury (CCI)-surgery, reduced the development of mechanical allodynia in SNL rats (Carlton and Hargett, 1995; Smith et al., 1994), or thermal hyperalgesia in CCI model (Eisenberg et al., 1995; Mao et al., 1993). Preemptive administration of intrathecal ketamine or systemic memantine prior to SNL injury attenuated mechanical allodynia from 6 h to 2 weeks in SNL rats (Burton et al., 1999; Carlton and Hargett, 1995), or delayed its development for 3 days in CCI model (Hartrick et al., 1998). The precise reason for the present finding that pre-treatment with ifenprodil only inhibited the mechanical allodynia but not the thermal hyperalgesia is not fully clear, so whether Ro 25-6981 and other NR2B antagonists have the same profile and whether NR2B subunit is selective for mechanical allodynia need further investigation. Several lines of evidence support the notion that the causing of nerve injury-evoked mechanical allodynia and the thermal injury-evoked hyperalgesia is different (Bolay and Moskowitz, 2002; Cervero and Laird, 1996), and A-fiber sprouting in the spinal cord is one of the central mechanisms accounting for the development of mechanical allodynia (Bolay and Moskowitz, 2002). Peripheral nerve injury induces sprouting of Aβ-fiber terminals from the deeper laminae to the superficial laminae of the spinal cord. As a possible correlate,

NR2B subunit localized in the superficial laminae of the spinal cord may be preferential for mediating the mechanical allodynia (Minami et al., 2001). Our present data suggested that blockade of the spinal cord NR2B subunit before or at the early stage of nerve injury could inhibit the occurrence and development of neuropathic pain. Therefore, the spinal cord NMDA-2B receptors play pivotal roles in the development of neuropathic pain, especially at the early stage following nerve injury.

Role of the spinal cord NMDA-2B receptors in activation of dorsal horn WDR neurons

To further elucidate the potential mechanisms of spinal cord NMDA-2B receptors underlying the development of nerve injury-induced neuropathic pain, we examined the effects of spinal administration of Ro 25-6981 on C-fiber responses of dorsal horn WDR neurons, because the C-fiber responses of WDR neurons are usually regarded as related to nociception (Liu et al., 2007; Rygh et al., 2000). Our results showed that either in normal or in SNL rats, Ro 25-6981 significantly inhibited the C-fiber responses of WDR neurons in a dose-dependent manner. In our present experiment, Ro 25-6981 was topically applied to the dorsal surface of the spinal cord, so the inhibitory effects of Ro 25-6981 on the activities of WDR neurons were directly via blockade of the NMDA-2B receptors at the spinal cord level.

Extensive studies reveal that the dorsal horn WDR neurons have a great ability to code noxious and innocuous stimuli (Rygh et al., 2005; Suzuki et al., 2002). Electrophysiological investigation of sensory synaptic responses between primary afferent fibers and dorsal horn neurons provide strong evidence that glutamate is the principal fast excitatory transmitter, and synaptic responses are mediated by postsynaptic glutamate receptors (Yoshimura and Jessell, 1990), in which the NMDA receptors play an important role in synaptic transmission, including nociceptive transmission in the spinal dorsal horn (Dingledine et al., 1999). Recently, it is reported that NMDA-2B receptors accumulate on the synaptic site and are responsible for the unique properties of synaptic function and plasticity (Miwa et al., 2008). The NMDA receptor 2B subunit-mediated synaptic transmission is increased in the superficial dorsal horn of peripheral nerve-injured neuropathic mice (Iwata et al., 2007), and Fyn kinase-mediated phosphorylation of NMDA receptor 2B subunit at Tyr1472 is essential for maintenance of neuropathic pain (Abe et al., 2005). Together with previous reports, the present study suggests that the spinal cord NMDA-2B receptors are likely to be involved in the spinal nociceptive synaptic transmission by activating the dorsal horn WDR neurons, which are usually regarded as related to nociception (Liu et al., 2007; Rygh et al., 2000).

Role of the spinal cord NMDA-2B receptors in the induction of LTP of dorsal horn nociceptive synaptic transmission

Several studies have shown that LTP-like phenomena can be induced not only in the brain but also in the spinal cord. Electrical high-frequency peripheral nerve stimulation, natural noxious stimulation, or acute nerve injury evokes LTP of C-fiber-evoked potentials in the spinal dorsal horn (Liu and Sandkuhler, 1997, 1998; Sandkuhler and Liu, 1998), or a long-term increase of the excitability of WDR neurons in the spinal cord (Rygh et al., 1999; Svendsen et al., 1997, 1998, 2000). It is well accepted that sensitization of both peripheral nociceptors and the spinal dorsal horn neurons is responsible for the abnormal pain sensation (Dubner, 1991; Sandkuhler, 2000; Woolf, 1983; Woolf and Salter, 2000; Zimmermann, 2001), and LTP in the nociceptive pathways may be a cellular or synaptic mechanism underlying the central sensitization and the abnormal pain sensation (Ji et al., 2003; Sandkuhler, 2000). This is supported by accumulative evidence that spinal LTP and injury induced central sensitization share signal transduction pathways, time course, and pharmacological profiles, which make this use-dependent LTP an attractive model of injury-induced central sensitization and neuropathic pain (Ji et al., 2003; Rygh et al., 2002, 2005; Sandkuhler, 2000). In the present study, we demonstrate that spinal administration of NR2B antagonist Ro 25-6981 significantly inhibit the LTP in C-fiber responses of dorsal horn WDR neurons induced by high-frequency stimulation (HFS) to the sciatic nerve. Our results are in line with the previous data reported by Pedersen and Gjerstad (2008) and support the notion that the expression of LTP in the dorsal horn neurons may be dependent on the NMDA-2B receptors. Together with our present behavioral test, we suggest that spinal cord NMDA-2B receptors contribute to the induction of LTP of C-fiber responses in dorsal horn WDR neurons, and this LTP is likely to be an underlying mechanism of injury induced central sensitization and neuropathic pain.

In summary, our present study suggests that: (1) spinal cord NMDA-2B receptors play an important role in the development of neuropathic pain, especially at the early stage of nerve injury. (2) Activation of the spinal cord NMDA-2B receptors may be crucial for the dorsal horn nociceptive synaptic transmission and for the development of long-lasting spinal hyperexcitability following nerve injury. The underlying mechanism of the spinal cord NMDA-2B receptors that contribute to the development of central sensitization and neuropathic pain is likely via the induction of LTP in dorsal horn nociceptive synaptic transmission.

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