

# Intrathecal Injection of the $\sigma_1$ Receptor Antagonist BD1047 Blocks Both Mechanical Allodynia and Increases in Spinal NR1 Expression during the Induction Phase of Rodent Neuropathic Pain

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**Background:** Selective blockade of spinal  $\sigma_1$  receptors (Sig-1R) suppresses nociceptive behaviors in the mouse formalin test. The current study was designed to verify whether intrathecal Sig-1R antagonists can also suppress chronic neuropathic pain.

**Methods:** Neuropathic pain was produced by chronic constriction injury (CCI) of the right sciatic nerve in rats. The Sig-1R antagonist BD1047 was administered intrathecally twice daily from postoperative days 0 to 5 (induction phase of neuropathic pain) or from days 15 to 20 (maintenance phase). Western blot and immunohistochemistry were performed to determine changes in Sig-1R expression and to examine the effect of BD1047 on *N*-methyl-D-aspartate receptor subunit 1 expression and phosphorylation in spinal cord dorsal horn from neuropathic rats.

**Results:** BD1047 administered on postoperative days 0–5 significantly attenuated CCI-induced mechanical allodynia, but not thermal hyperalgesia, and this suppression was blocked by intrathecal administration of the Sig-1R agonist PRE084. In contrast, BD1047 treatment during the maintenance phase of neuropathic pain had no effect on mechanical allodynia. Sig-1R expression significantly increased in the ipsilateral spinal cord dorsal horn from days 1 to 3 after CCI. Importantly, BD1047 (30 nmol) administered intrathecally during the induction, but not the maintenance phase, blocked the CCI-induced increase in *N*-methyl-D-aspartate receptor subunit 1 expression and phosphorylation.

**Conclusions:** These results demonstrate that spinal Sig-1Rs play a critical role in both the induction of mechanical allodynia and the activation of spinal *N*-methyl-D-aspartate recep-

tors in CCI rats and suggest a potential therapeutic role for the use of Sig-1R antagonists in the clinical management of neuropathic pain.

THE  $\sigma_1$  receptor (Sig-1R) plays an important role in a variety of cellular functions and biologic processes.<sup>1,2</sup> It is well recognized that Sig-1Rs are present throughout the central nervous system and are highly expressed in the olfactory bulb, certain cortical areas, the amygdala, the hypothalamus, the periaqueductal gray, and the dorsal horn of the spinal cord.<sup>3</sup> Recently, it has been shown that the Sig-1R has a pronociceptive effect in formalin-induced pain using Sig-1R knockout mice.<sup>4</sup> Further support for a pronociceptive role for this receptor comes from a study demonstrating that systemic administration of the putative Sig-1R antagonist haloperidol markedly reduces nociceptive behaviors in both phases of the formalin test.<sup>5</sup> In addition, a recent study from our laboratories reported that intrathecal pretreatment with the Sig-1R antagonist BD-1047 or BMY-14802 reduces nociceptive behaviors and spinal Fos expression associated with the formalin test.<sup>6</sup> Although a role for spinal cord Sig-1Rs has been established for acute pain using the formalin test, it is currently not known whether Sig-1Rs contribute to the induction or maintenance of chronic pain conditions.

Peripheral neuropathic pain, which results from damage or dysfunction of peripheral nerves, is one of the most challenging chronic pain conditions to treat. The development of neuropathic pain, characterized by hyperalgesia and allodynia, is associated with a variety of pathophysiologic changes, including peripheral and central sensitization.<sup>7,8</sup> Central sensitization at the spinal cord level is closely mediated by activation of the *N*-methyl-D-aspartate (NMDA) receptor, which has many similarities to the long-term potentiation phenomenon observed in the hippocampus. Recently, a number of preclinical studies have demonstrated that selective agonists for the Sig-1R positively modify higher-ordered brain functions, including learning, memory, cognition, and mood.<sup>9,10</sup> In this regard, Monnet *et al.*<sup>11,12</sup> demonstrated that activation of Sig-1R in rat hippocampus potentiated glutamate-induced intracellular  $Ca^{2+}$  influx through NMDA receptors, which was mediated *via* a protein kinase C-dependent mechanism. Based on the similarities between the hippocampus and the spinal cord, we speculated that the activation of spinal Sig-1Rs

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that occurs during central sensitization (or chronic pain) might be associated with altered expression or function of NMDA receptors in this region.

*N*-Methyl-D-aspartate receptors are heteromers composed of three different subunits (NR1, NR2, and NR3), with the NR1 subunit encoded by one gene, alternatively spliced to give eight variants, whereas the four NR2 subunits, NR2A-D, are each encoded by separate genes.<sup>13</sup> Although it is currently not clear what functions and pathophysiological processes the NMDA receptor NR3A subunit is involved in, the NR1 subunit is considered an essential component of all functional NMDA receptors.<sup>14,15</sup> Moreover, increased phosphorylation of NR1 (pNR1), which occurs *via* intracellular signaling pathways, has been recognized as a major mechanism contributing to the regulation of NMDA receptor function.<sup>16</sup> Recently, several reports, including a report from our laboratories, have indicated that peripheral nerve injury produces a time-dependent up-regulation of NR1 subunits in the dorsal horn.<sup>17,18</sup> In addition, it was shown that there is a significant increase in the number of pNR1-immunoreactive neurons in the spinal cord dorsal horn in a variety of rodent neuropathic pain models.<sup>18-20</sup> In this regard, we hypothesized that the analgesic effects of intrathecally administered Sig-1R antagonists might be associated with the potential suppression of the enhanced NR1 or pNR1 expression in the spinal cord of neuropathic rats.

Therefore, the current study was designed to determine (1) whether intrathecal administration of the Sig-1R antagonist BD1047, given during the induction phase of neuropathic pain (days 0-5 after chronic constriction injury [CCI] surgery) *versus* the maintenance phase (days 15-20 after CCI surgery), could suppress CCI-induced mechanical allodynia and/or thermal hyperalgesia in a rodent model of neuropathic pain; (2) whether peripheral nerve injury alters Sig-1R expression in the spinal cord dorsal horn and the time course over which changes in expression occur; and (3) whether intrathecal BD1047 injection modulates the CCI-induced increased expression of the NMDA receptor NR1 subunit and its phosphorylated form in the spinal dorsal horn of CCI rats.

## Materials and Methods

### Animals

Experiments were performed on male Sprague-Dawley rats weighing 200-250 g. All experimental animals were obtained from the Laboratory Animal Center of Seoul National University, Seoul, Republic of Korea. They were housed in colony cages with free access to food and water and maintained in temperature- and light-controlled rooms (24° ± 2°C, 12-12 h light-dark cycle with lights on at 07:00) for at least 1 week before the study. All of the methods used in the current study were approved by the Animal Care and Use Committee at Seoul

National University and conform to National Institutes of Health guidelines (publication No. 86-23, revised 1985).

### Intrathecal Catheter Placement and Neuropathic Surgery

An intrathecal catheter was implanted 7 days before sciatic nerve surgery for spinal injection of drugs. This was performed by carefully threading a piece of polyethylene tubing (PE-10) down the subarachnoid space to the level of the lumbosacral cord as previously described.<sup>21-23</sup> Because placement of an intrathecal catheter does not require major surgery and does not produce neurologic deficits, and because injected drugs can diffuse as far as 2.5 cm from the catheter tip,<sup>24</sup> the indwelling catheter method has been also used successfully for long-term repeated spinal cord treatment for as long as 33 days after catheterization.<sup>25</sup> During isoflurane anesthesia, the catheter was advanced 8 cm from the cisterna magna to the level of the lumbar enlargement through an incision in the atlanto-occipital membrane. The intrathecal catheter was externalized to the back of the head and sutured to the musculature and skin at the incision site. Each rat that received an intrathecal catheter was given soluble cephadrine (25 mg/kg Safdin; Daehan New Pharm Corp., Seoul, Korea) by intramuscular injection at the end of the surgical procedure to prevent the development of subsequent infection. Rats showing any signs of neuromuscular dysfunction and/or hemorrhage from the subarachnoid space after the intrathecal catheterization procedure were killed immediately.

A CCI of the common sciatic nerve was performed according to the method described by Bennett and Xie.<sup>26</sup> Briefly, rats were anesthetized with 3% isoflurane in a mixture of nitrous oxide-oxygen gas. The right sciatic nerve was exposed at the mid-thigh level, and four loose ligatures of 4-0 chromic gut were placed around the dissected nerve with a 1.0- to 1.5-mm interval between each ligature. Sham surgery (*n* = 5/group for Western blot assays and for immunohistochemistry experiments) consisted of exposing the sciatic nerve in the same manner, but without ligating the nerve. During recovery, animals were housed in clear plastic cages with a thick layer of sawdust bedding.

### Drug Treatment

BD1047 dihydrobromide (*N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(dimethylamino)ethylamine dihydrobromide) is a selective antagonist for the Sig-1R and has a greater affinity for the  $\sigma_1$  than for the  $\sigma_2$  receptor. Moreover, BD1047 has a binding profile similar to that of BD1063, but with higher affinity (approximately 10-fold) at both  $\sigma_1$  and  $\sigma_2$  sites.<sup>27</sup> BD1047 (3, 10, 30, and 100 nmol; Tocris, Avonmouth, United Kingdom) was dissolved in 10  $\mu$ l phosphate-buffered saline and injected intrathecally into CCI-induced neuropathic rats (*n* = 7/group). Control animals were injected with 10  $\mu$ l

phosphate-buffered saline (vehicle;  $n = 7$ ). Rats were briefly anesthetized with 3% isoflurane in a mixed nitrous oxide-oxygen gas before intrathecal drug injection to prevent any handling-induced stress. Then, while the rats were under anesthesia, the drug was slowly infused. Animals awoke immediately after the intrathecal injection procedure and were freely moving within 45 s after injection. Intrathecal treatments with BD1047 were performed twice a day, either on postoperative days 0-5 (induction period) or on postoperative days 15-20 (maintenance period). In a separate experiment, the Sig-1R agonist PRE084 (3 and 10 nmol; Tocris) was administered intrathecally twice a day on postoperative days 6-8 after BD1047 pretreatment (days 0-5) to determine whether agonist treatment restored the CCI-induced mechanical allodynia that was significantly reduced in BD1047-pretreated animals. PRE084 hydrochloride (2-(4-morpholinethyl) 1-phenylcyclo-hexanecarboxylate hydrochloride) is a selective Sig-1R agonist and has a very high affinity for this receptor subtype ( $K_i$  values are 2.2 for the  $\sigma_1$  receptor and 13,091 nM for the  $\sigma_2$  receptor).<sup>28</sup> There were six animals in each group including the vehicle groups for the intrathecal PRE084 experiments.

#### Behavior Assessments

Behavioral tests were performed 1 day before CCI surgery on all animals to obtain normal baseline values of withdrawal response to mechanical and heat stimuli. Animals were randomly assigned to each drug-treatment group, and all subsequent behavioral testing was performed blindly.

The number of paw withdrawal responses to normally innocuous mechanical stimuli was measured by using a von Frey filament with a force of 2.0 g (North Coast Medical, Morgan Hill, CA). Rats were placed on a metal mesh grid under a plastic chamber, and the von Frey filament was applied from underneath the metal mesh flooring to each hind paw. The von Frey filament was applied 10 times to each hind paw, and the number of paw withdrawal responses out of 10 was then counted. The results of mechanical behavioral testing in each experimental animal were expressed as a percent withdrawal response frequency (PWF), which represented the percentage of paw withdrawals out of the maximum of 10 as previously described.<sup>18,22</sup>

To assess nociceptive responses to heat stimuli, we measured paw withdrawal response latency (PWL) by using the plantar paw-flick latency test as previously described by Hargreaves *et al.*<sup>29</sup> Briefly, rats were placed in a plastic chamber with a glass floor and were allowed to acclimatize for 10 min before testing. A radiant heat source was positioned under the glass floor beneath the hind paw to be tested, and withdrawal latency was measured using a plantar analgesia meter (IITC Life Science Inc., Woodland Hills, CA). The test was duplicated

in the ipsilateral hind paw of each animal, and the mean withdrawal latency was calculated. Cutoff time in the absence of a response was set at 20 s.

#### Western Blot Assay for $\sigma_1$ Receptor

The spinal cords were collected from CCI ( $n = 30$ ) and sham animals ( $n = 30$ ) at six different time points (days 0, 1, 3, 7, 14, and 21;  $n = 5$  rats/time point) after CCI or sham surgery. Rats were first deeply anesthetized with 5% isoflurane in a mixture of nitrous oxide-oxygen gas, and next we verified the location of the L4-L6 spinal cord segments by identifying the attachment site of each lumbar spinal nerve in the anesthetized rats.<sup>20</sup> Then the L4-L6 spinal cord segments were immediately extracted by pressure expulsion with air into an ice-cooled and saline-filled glass dish and were snap-frozen in liquid nitrogen.<sup>30</sup> Next, the spinal cord segments were separated into left (contralateral) and right (ipsilateral) halves under a neurosurgical microscope (World Precision Instruments Inc., Sarasota, FL). The spinal cord was subsequently further subdivided into dorsal and ventral halves by cutting straight across from the central canal laterally to a midpoint in the white matter. The separated ipsilateral and contralateral dorsal quadrants of each spinal cord were then used for Western blot analysis.

Spinal dorsal horn segments were homogenized in buffer containing 1 M Tris (pH 7.5), 1% NP-40, 0.5 M EDTA (pH 7.5), 50 mM EGTA, 1 M dithiothreitol, 1 M benzanidine, and 0.1 M PMSF. The total amount of protein in each sample was determined using the Bradford assay before loading on polyacrylamide gels. Spinal cord homogenates (20  $\mu$ g protein) were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose. After the blots had been washed with TBST (10 mM Tris-HCl [pH 7.6], 150 mM NaCl, and 0.05% Tween-20), the membranes were blocked with 5% skim milk for 1 h and incubated with the appropriate primary antibody for Sig-1R (1:500; Santa Cruz Biotechnology Inc., Santa Cruz, CA) and  $\beta$ -actin (loading control; Sigma, St. Louis, MO). The membranes were then washed, the primary antibodies were detected using goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase, and the bands were subsequently visualized with enhanced chemiluminescence (Amersham Pharmacia Biotech, Uppsala, Sweden). The positive pixel area of specific bands was then measured with a computer-assisted imaging analysis system (Metamorph; Universal Imaging Co., West Chester, PA) and normalized against corresponding loading control bands. Normalization was performed by calculating the percent of positive pixel area of the Sig-1R from those of the loading control in each experiment. Normalized data from before nerve injury (0 day) was set as 100%, and the results are shown as the percent change from the pre-CCI condition at each time point.



### *Immunohistochemistry and Imaging Analysis for NR1 and pNR1 Expression*

In a separate experiment, rats (total  $n = 30$ ) were killed at the endpoint of drug treatment (either 5 or 20 days after sham or CCI surgery), and the L4–L6 segments of the spinal cord were removed for NR1 and pNR1 immunohistochemistry ( $n = 5/\text{group}$  at 5 or 20 days). Animals were deeply anesthetized with 5% isoflurane and then perfused transcardially with calcium-free Tyrode solution, followed by a fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH 6.9). The spinal cord was removed immediately, postfixed at 4°C overnight in the same fixative, and then cryoprotected in 30% sucrose in phosphate-buffered saline (pH 7.4) for 48 h. Frozen serial frontal (cross) sections (40  $\mu\text{m}$ ) were cut through the lumbar L4–L6 spinal cord using a cryostat (Microm, Walldorf, Germany). After elimination of endogenous peroxidase activity with 0.3% hydrogen peroxide and preblocking with 3% normal goat serum in phosphate-buffered saline, the free-floating sections were incubated in rabbit anti-NR1 antibody (1:1,000; Upstate, Lake Placid, NY) or in rabbit anti-pNR1 antibody (1:1,000; Upstate) at 4°C for 48 h, respectively. The pNR1 antibody used is selective for NR1 phosphorylated on Ser896. The antibody-specificity information available from Upstate Biotechnology states that the anti-phospho-NR1 Ser896 is specific for NR1 phosphorylated on serine 896, and it also recognizes NR1 that is dually phosphorylated on serine residues 896 and 897. Anti-phospho-NR1 Ser896 does not recognize the subunit if only residue 897 is phosphorylated. With regard to the NR1 antibody, this antibody was raised against a region (834–864) common to all NR1 isoforms, and therefore it recognizes NR1 and cross-reacts with all NR1 isoforms. To determine immunohistochemical method specificity, some sections were processed in an identical fashion, but without primary antibodies. The sections were subsequently processed using the avidin-biotin-peroxidase procedure previously described.<sup>18</sup> Finally, visualization was performed using 3,3'-diaminobenzidine (Sigma) with 0.2% nickel chloride intensification.

The sections were examined under a bright-field microscope (Zeiss Axioscope; Hallbergmoos, Germany) at  $\times 100$  to localize NR1- or pNR1-immunoreactive (ir) neurons. For quantitative analysis of NR1- or pNR1-ir neurons, five spinal cord sections from the L4–L6 lumbar spinal cord segments were randomly selected from each animal and subsequently scanned. Individual sections were digitized with 4,096 gray levels using a cooled charge-coupled device camera (Micromax Kodak 1317; Princeton Instruments, Tucson, AZ) connected to a computer-assisted image analysis system (Metamorph). To maintain a constant threshold for each image and to compensate for subtle variability of the immunostaining, we only counted neurons that were at least 70% darker

than the average gray level of each image after background subtraction and shading correction. The microscope illumination and data acquisition settings were fixed throughout the entire analysis procedure. The average number of NR1- or pNR1-ir neurons per section from each animal was obtained, and these values were averaged across each group and presented as group data. The expression of NR1 or pNR1 was quantified in the following three dorsal horn regions: (1) the superficial dorsal horn (laminae I and II), (2) the nucleus proprius (laminae III and IV), and (3) the neck region (laminae V and VI). These regions were identified based on cytoarchitectonic criteria as defined by Abbadie and Besson.<sup>31</sup> All NR1 or pNR1 quantitation procedures described above were performed blindly with regard to the experimental condition of each animal.

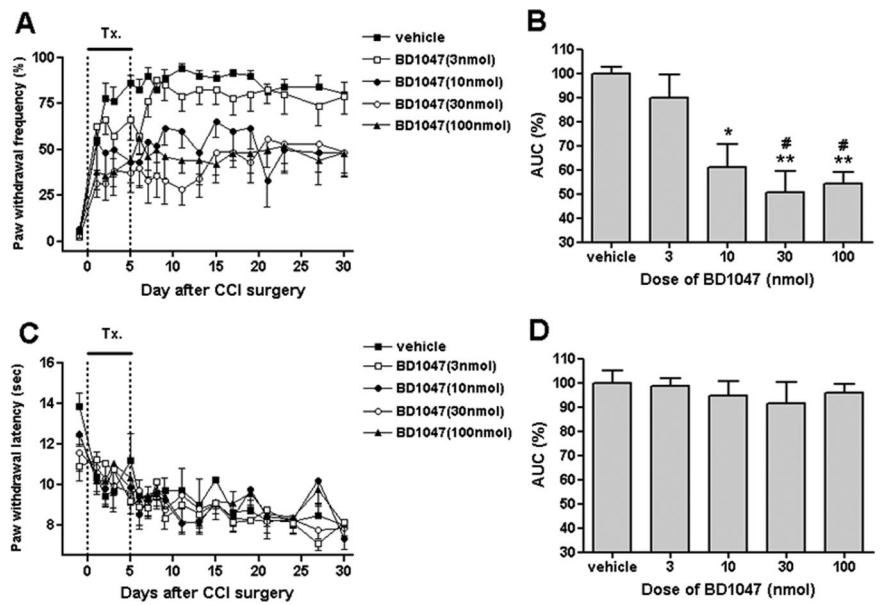
### *Western Blot Assay for NR1 and pNR1 Expression*

The general procedures for the Western blot assay including the spinal cord collection from CCI animals were identical to previously described Western blot methods for Sig-1R expression, except for the application of primary antibodies. The same antibodies used in the immunohistochemistry experiments to detect NR1 and pNR1 expression were also used for the Western blot assays. Rats (total  $n = 16$ ) were killed at the endpoint of drug treatment (either 5 or 20 days after CCI surgery), and the L4–L6 segments of the spinal cord were removed ( $n = 4/\text{group}$  at 5 and 20 days). Normalized data of vehicle-treated animals at each time point were set as 100%.

### *Statistical Analysis*

All values are expressed as mean  $\pm$  SEM. Statistical analysis was performed using Prism 4.0 (Graph Pad Software, San Diego, CA). We first performed the Kolmogorov-Smirnov test of normality to determine whether the data represented a gaussian distribution. To determine whether there were significant effects of drug treatment on PWF (%) and PWL (seconds), we calculated the time course data as the area under curve (%). Either a Kruskal-Wallis test or a Mann-Whitney test was then used to determine differences in the area under curve (%) from the PWF or PWL data and in the data obtained from immunohistochemical and Western blot assays of spinal cord NR1 and pNR1 expression. *Post hoc* analysis after a Kruskal-Wallis test was performed using a Dunn test to determine the *P* value. Two-way analysis of variance followed by a *post hoc* Bonferroni comparison test was also used to determine overall differences and the *P* value at each experimental time point in PWF, PWL, and the expression of spinal Sig-1R in Western blot assays.  $P < 0.05$  was considered statistically significant.

**Fig. 1.** Graphs illustrating the effect of intrathecal treatment with the  $\sigma_1$  receptor antagonist BD1047 (administered from days 0 to 5 after surgery) on the development of mechanical allodynia (A and B) and thermal hyperalgesia (C and D) in rats with chronic constriction injury (CCI)-induced neuropathic pain (n = 7 in each BD1047- or vehicle-treated group). Repeated daily treatment with BD1047 (10, 30, 100 nmol) blocked the increase in paw withdrawal frequency (%) that occurred in vehicle-treated CCI rats (A), and the area under curve (AUC) data analysis showed a strong antiallodynic effect of BD1047 (B) (\* P < 0.05, \*\* P < 0.01 as compared with that of the vehicle-treated group; # P < 0.05 as compared with the low dose of BD1047 [3 nmol]-treated group). However, the decrease in paw withdrawal latency (seconds) to heat stimuli (thermal hyperalgesia) and the AUC (%) in CCI rats was unaffected by repeated intrathecal injection with even the highest dose of BD1047 (C and D).



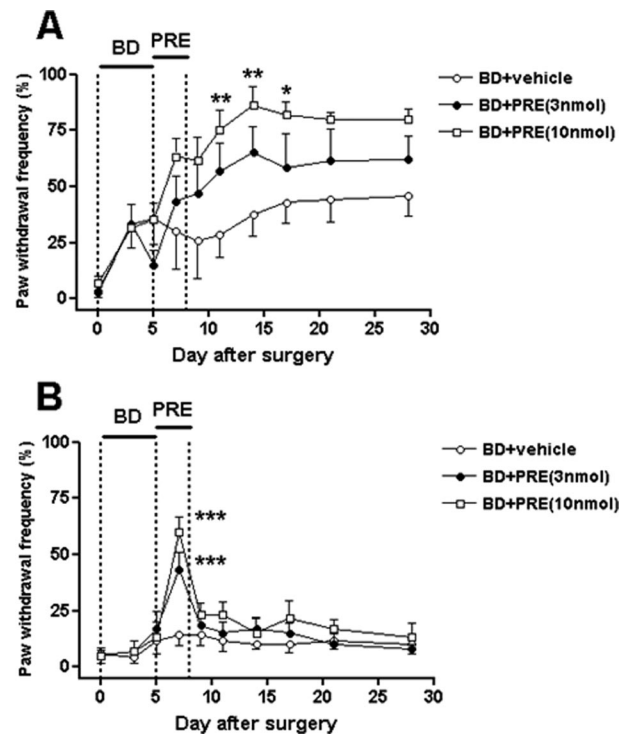
**Results**

*Effect of Intrathecal BD1047 on the Induction Phase of Neuropathic Pain Behaviors*

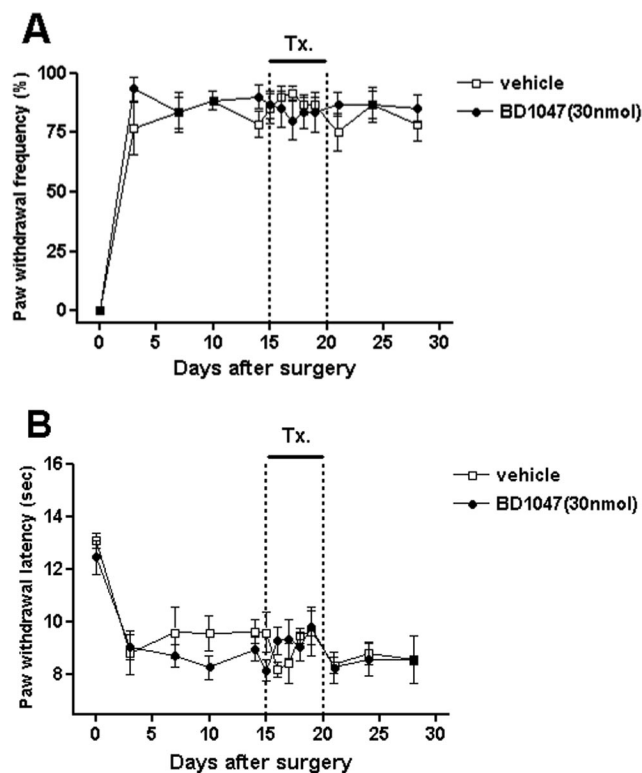
The effects of intrathecal administration of the Sig-1 antagonist BD1047 on the early induction of mechanical allodynia in CCI rats are illustrated in figures 1A and B, and the effects on the early induction of thermal hyperalgesia in CCI rats are illustrated in figures 1C and D. Repeated daily (from days 0 to 5), intrathecal treatment with BD1047 (10, 30, or 100 nmol) dramatically reduced the increase in PWF (%) as compared with vehicle-treated CCI rats (fig. 1A). After termination of repeated BD1047 injection (10, 30, or 100 nmol) on day 5, this suppressive effect of BD1047 on mechanical allodynia was sustained throughout the 30-day experimental period after CCI surgery. In addition, area-under-the-curve data analysis showed a significant analgesic effect of BD1047 on the development of mechanical allodynia in neuropathic rats (fig. 1B; \* P < 0.05 and \*\* P < 0.01 as compared with the vehicle group; # P < 0.05 as compared with the low dose of BD1047 (3 nmol)-treated group). On the other hand, the decrease in PWL (seconds) to heat stimuli (thermal hyperalgesia) and the associated area under curve (%) in CCI rats were not influenced by repeated intrathecal treatment with BD1047 (at doses from 3 to 100 nmol) throughout the 30-day testing period (figs. 1C and D).

In addition, we found that intrathecal administration of the Sig-1 agonist PRE084 on postoperative days 6–8 blocked the suppressive effect of BD1047 treatment on the ipsilateral mechanical allodynia induced by CCI (fig. 2A; \* P < 0.05 and \*\* P < 0.01 as compared with control vehicle-treated animals pretreated with BD1047 [30 nmol, BD + vehicle]). It is important to note that vehicle-vehicle-treated CCI animals (those that did not receive BD1047 or PRE084) developed mechanical allodynia that was almost identical to the mechanical

allodynia of the vehicle-pretreated rats illustrated in figure 1A (data not shown). Interestingly, intrathecal PRE084 treatment produced a temporary mechanical



**Fig. 2.** Graphs illustrating that intrathecal injection of the  $\sigma_1$  receptor agonist PRE084 (PRE; on days 6–8 after surgery) blocked the suppressive effect of intrathecal BD1047 (BD; 30 nmol) treatment (twice daily for 5 days after chronic constriction injury) on mechanical allodynia induced by chronic constriction injury (n = 6 in each drug-treated group). Intrathecal PRE084 injected on days 6–8 after chronic constriction injury restored mechanical allodynia in the ipsilateral hind paw that was suppressed by intrathecal BD1047 injection on days 0–5 (A). In addition, intrathecal PRE084 treatment produced a temporary mechanical allodynia in the contralateral hind paw on postoperative day 7 (B). \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001 as compared with those of BD1047-vehicle treated animals.



**Fig. 3.** Graphs illustrating the effect of intrathecal administration of the  $\sigma_1$  receptor antagonist BD1047 (administered on days 15–20 after surgery) on the maintenance phase of mechanical allodynia (**A**) and thermal hyperalgesia (**B**) in chronic constriction injury rats. Daily intrathecal treatment with BD1047 (30 nmol;  $n = 7$ ) neither suppressed the chronic constriction injury–induced increase in paw withdrawal frequency (%) to mechanical stimuli (**A**) nor reversed the chronic constriction injury–induced decrease in paw withdrawal latency (seconds) as compared with the vehicle-treated group (**B**;  $n = 7$ ).

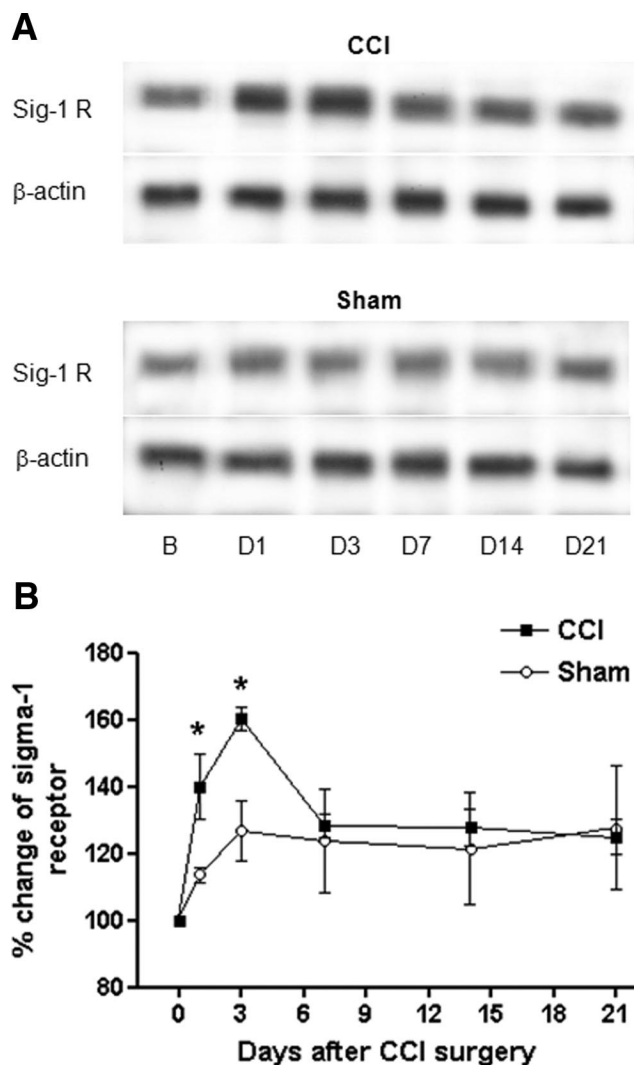
allodynia on postoperative day 7 in the hind paw contralateral to nerve injury (fig. 2B; \*\*\*  $P < 0.001$  as compared with those of BD + vehicle-treated rats).

#### Effect of Intrathecal BD1047 on the Maintenance Phase of Neuropathic Pain Behaviors

The effect of intrathecal administration of the Sig-1R antagonist BD1047 on the maintenance phase of mechanical allodynia established in CCI rats is illustrated in figure 3A, and the effect on the maintenance phase of thermal hyperalgesia established in CCI rats is illustrated in figure 3B. Daily treatment (from days 15 to 20, twice a day) with BD1047 (30 nmol) neither suppressed the CCI-induced increase in PWF (%) to mechanical stimuli (fig. 3A) nor reversed the CCI-induced decrease in PWL (seconds) as compared with those in the vehicle-treated group (fig. 3B).

#### Time Course of CCI-induced Changes in $\sigma_1$ Receptor Expression in the Dorsal Horn of Neuropathic Rats

After peripheral nerve injury, the expression of Sig-1Rs in the ipsilateral spinal cord dorsal horn was significantly



**Fig. 4.** Western blot analysis illustrating the change in  $\sigma_1$  receptor (Sig-1R) expression in the ipsilateral spinal cord dorsal horn at various time points after chronic constriction injury (CCI) versus sham rats (upper panel in **A** and also **B**). After nerve injury ( $n = 5$  at each time point in the CCI or sham groups), Sig-1R expression was significantly increased by postoperative day 1 (D1) and reached a peak level by postoperative day 3 (D3) when compared with Sig-1R expression in sham surgery animals ( $n = 5$ ; lower panel in **A**). Sig-1R expression in the ipsilateral dorsal horn decreased to levels seen in sham-treated animals by 7 days after injury (D7), and there were no statistical differences between CCI and sham animals at the 7-day time point. B = before (a day before surgery). \*  $P < 0.05$  as compared with those of sham surgery rats.

increased from day 1 to day 3 after CCI (figs. 4A and B; \*  $P < 0.05$  as compared with that of sham surgery group). The CCI-induced increase in Sig-1R expression on immunoblots peaked at 3 days after surgery. By 7 days after CCI, Sig-1R expression in the ipsilateral dorsal horn was restored to normal pre-CCI values, and no statistical significance was evident when compared with Sig-1R expression in the dorsal horn of sham animals (figs. 4A and B). Moreover, there was no significant difference in the expression of Sig-1R in the contralateral spinal cord dorsal horn between sham and CCI rats at any time point



measured during the 30-day testing period (data not shown).

*Effect of Intrathecal BD1047 on Spinal NR1 and pNR1 Expression in Neuropathic Rats*

Rats were killed on day 5 (vehicle and BD1047, n = 5) or day 20 (vehicle and BD1047, n = 5) after CCI surgery to determine the effect of daily intrathecal injection of BD1047 (30 nmol) on NR1 and pNR1 expression in the spinal cord dorsal horn when administered during the induction (days 0–5; figs. 5 and 6) or the maintenance phase (days 15–20; fig. 7) of neuropathic pain development. Sham surgery animals (n = 5/group) were killed at the same time points for comparison. The number of NR1-ir neurons was significantly increased in all regions of spinal dorsal horn on days 5 and 20 after CCI surgery as compared with that of the sham surgery control group (figs. 5A and 7A; \*  $P < 0.05$  and \*\*  $P < 0.01$ ). The number of pNR1-ir neurons was also increased in the nucleus proprius (laminae III and IV) and in the neck region (laminae V and VI) on days 5 and 20 after CCI, respectively (figs. 5B and 7B; \*  $P < 0.05$  and \*\*  $P < 0.01$  as compared with that of sham surgery animals).

Intrathecal treatment with BD1047 during the induction period significantly reduced the CCI-induced increase in the number of NR1-ir neurons (fig. 5A and figs. 6A and B) in all regions of ipsilateral spinal dorsal horn and in the number of pNR1-ir neurons (fig. 5B and figs. 6C and D) in the nucleus proprius and the neck region (#  $P < 0.05$  and ##  $P < 0.01$  as compared with those of vehicle-treated animals). In contrast, daily intrathecal injection of BD1047 during the maintenance phase had no effect on the CCI-induced increase in the number of NR1 and pNR1-ir neurons in any of the dorsal horn regions (figs. 7A and B). In addition, the number of NR1 and pNR1-ir neurons in dorsal horn located contralateral to the CCI surgery side was similar to that counted in sham surgery animals. Moreover, the number of NR1 and pNR1-ir neurons in the contralateral dorsal horn was not modified by intrathecal BD1047 treatment, whether it was administered during the induction period or during the maintenance period (data not shown).

Western blot analysis confirmed the effects of intrathecal administration of the Sig-1R antagonist BD1047 (30 nmol) on NMDA receptor NR1 expression (fig. 8A) and pNR1 (fig. 8B) during the induction period. Therefore, the relative pixel area (%) of NR1 and pNR1 expression was significantly reduced by BD1047 treatment during the induction period (days 0–5) as compared with rats of the vehicle-treated CCI group (fig. 8C; \*  $P < 0.05$ ). Conversely, BD1047 treatment during the maintenance period (days 15–20) did not affect the expression of NR1 and pNR1 (fig. 8D), which is similar to the immunocytochemical data.

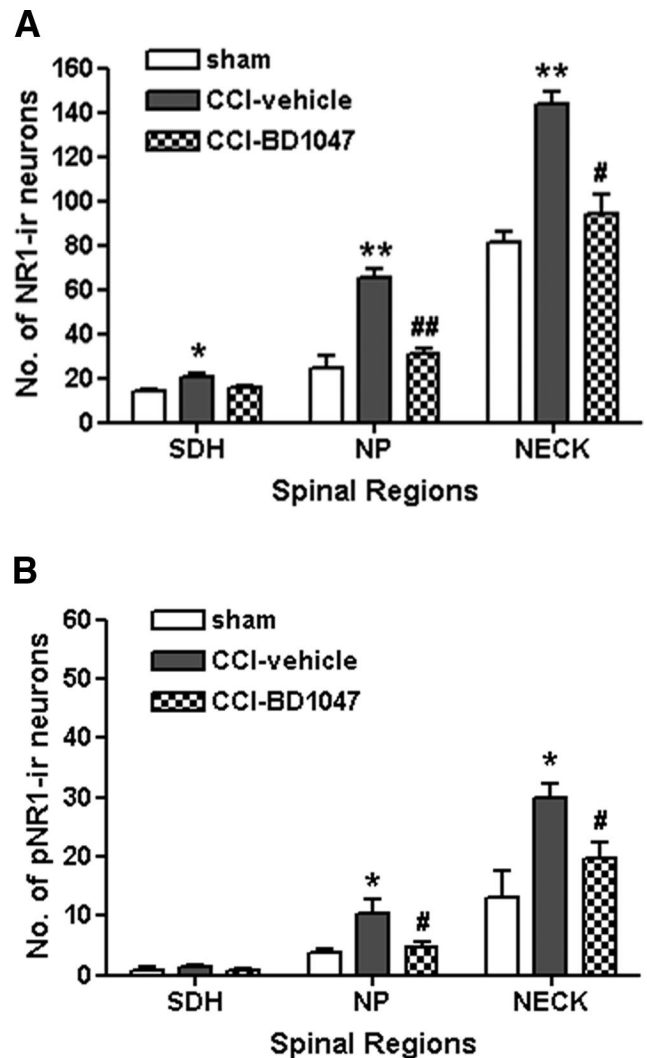
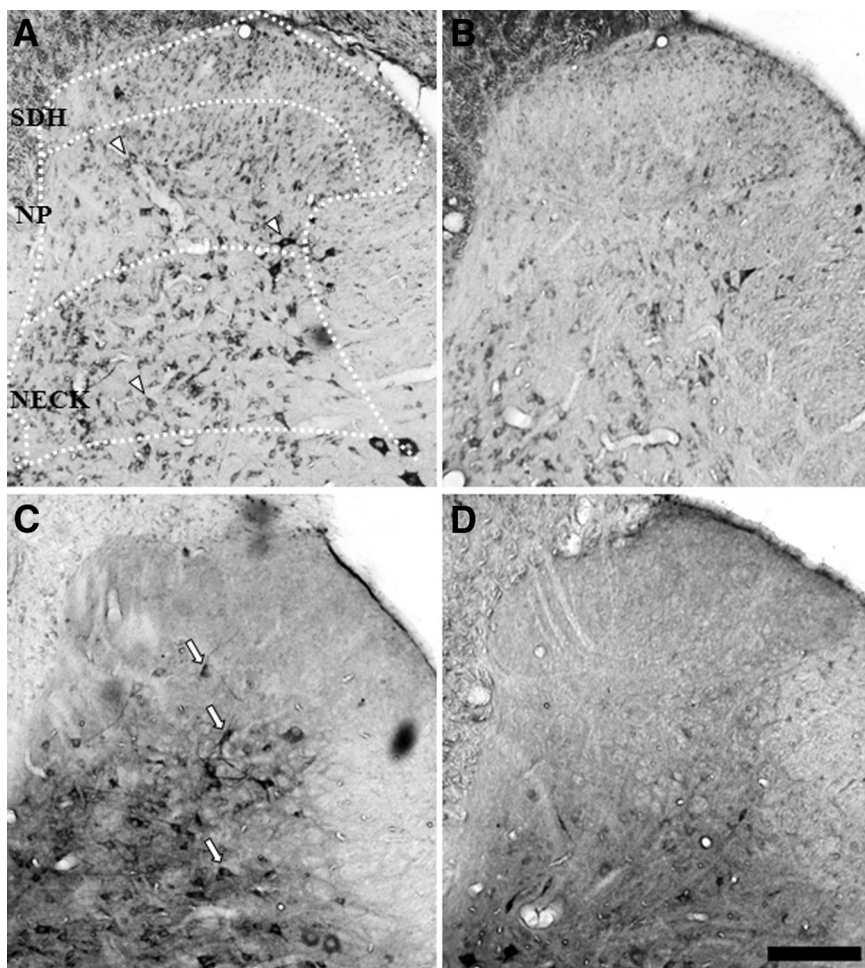


Fig. 5. Graphs illustrating the effect of intrathecal administration of the  $\sigma_1$  receptor antagonist BD1047 (30 nmol; n = 5) from days 0 to 5 after surgery on the chronic constriction injury (CCI)-induced increase in N-methyl-D-aspartate receptor NR1 expression (A) and NR1 phosphorylation (pNR1) (B) in each region of the dorsal horn. The number of NR1-immunoreactive (ir) and pNR1-ir neurons in spinal dorsal horn was significantly increased on day 5 after CCI surgery (\*  $P < 0.05$ , \*\*  $P < 0.01$  as compared with that of sham surgery animals [n = 5]). Intrathecal daily injection of BD1047 during induction phase of neuropathic pain significantly blocked the CCI-induced increase in the number of NR1-ir neurons (A) and pNR1-ir neurons (B) in the nucleus proprius and in the neck region of the ipsilateral spinal cord dorsal horn (#  $P < 0.05$ , ##  $P < 0.01$  as compared with vehicle-treated CCI animals [n = 5]). SDH = superficial dorsal horn (laminae I and II); NP = nucleus proprius (laminae III and IV); NECK = neck of dorsal horn (laminae V and VI).

**Discussion**

There are several important observations that came out of this study. First, daily intrathecal treatment with the specific Sig-1R antagonist BD1047 dose-dependently suppressed mechanical allodynia in neuropathic rats, but only if administered during the induction phase (days 0–5 after CCI) of neuropathic pain. This treatment was ineffective if administered during the maintenance phase

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**Fig. 6.** Photomicrographs of representative L4–L6 spinal cord sections illustrating *N*-methyl-D-aspartate receptor NR1-immunoreactive (ir) neurons (*A* and *B*) and phosphorylated NR1 (pNR1)-ir neurons (*C* and *D*) in the ipsilateral dorsal horn of chronic constriction injury rats 5 days after surgery. The number of NR1-ir neurons present in the vehicle-treated neuropathic rats (*A*) was significantly decreased after administration of the  $\sigma_1$  receptor antagonist BD1047 (30 nmol) to neuropathic animals (*B*). Similarly, the number of pNR1-ir neurons in vehicle-treated neuropathic rats (*C*) was also significantly decreased after intrathecal daily injection of BD1047 (30 nmol) (*D*). SDH = superficial dorsal horn (laminae I and II); NP = nucleus proprius (laminae III and IV); NECK = neck of dorsal horn (laminae V and VI). *White arrowhead* = representative NR1-ir neurons; *white arrow* = representative pNR1-ir neurons. *Scale bar* = 200  $\mu$ m.

(days 15–20 after CCI). This antiallodynic effect of BD1047 was reversed by subsequent intrathecal administration (days 6–8 after CCI) of the specific Sig-1R agonist PRE084. Second, chronic peripheral nerve injury induced an early up-regulation (days 1–3 after CCI) of Sig-1R expression primarily within the ipsilateral spinal cord dorsal horn, and then spinal Sig-1R expression returned to normal levels by day 7 after surgery. Finally, intrathecal BD1047 administration given during the induction phase of neuropathic pain was found to prevent the CCI-induced early increase in the expression of both the NMDA receptor NR1 subunit and its phosphorylated form in the spinal cord dorsal horn. By contrast, administration of BD1047 during the maintenance phase of neuropathic pain had no effect on the CCI-induced increases in NR1 expression and pNR1 expression in the ipsilateral dorsal horn. These findings demonstrate that the activation of spinal Sig-1R plays a critical role in the induction, but not the maintenance, of CCI-induced mechanical allodynia in a rat model of neuropathic pain.

Recently, it has been suggested that neurosteroids including pregnenolone, dehydroepiandrosterone, and progesterone are endogenous ligands for the Sig-1R (pregnenolone and dehydroepiandrosterone are agonists and progesterone is an antagonist).<sup>32</sup> These neuro-

steroids seem to bind allosterically to the Sig-1R. Moreover, it has been reported that the concentration of pregnenolone in the spinal cord is significantly increased in a rodent nerve injury neuropathic pain model<sup>33</sup> and in a diabetes mellitus neuropathic pain model.<sup>34</sup> These studies suggest that the activation of Sig-1Rs resulting from increases in the concentration of endogenous neurosteroids might be involved in the development of nerve injury-induced neuropathic pain, particularly mechanical allodynia based on the current findings.

Although several possible mechanisms that potentially underlie peripheral nerve injury-induced neuropathic pain have been suggested,<sup>7,8</sup> they have not yet been fully evaluated, and this contributes to the difficulty in developing an effective treatment for this abnormal pain condition. A recent study from our laboratories has reported that spinal cord Sig-1Rs may be a potential target for the modulation of acute, inflammatory nociception based on experiments using the mouse formalin model.<sup>6</sup> Sig-1Rs are highly expressed throughout the central nervous system, including the olfactory bulb, amygdala, hippocampus, hypothalamus, periaqueductal gray, and dorsal horn of the spinal cord.<sup>3</sup> Based on this wide distribution, it is perhaps not surprising that a number of preclinical studies have demonstrated that selective ago-



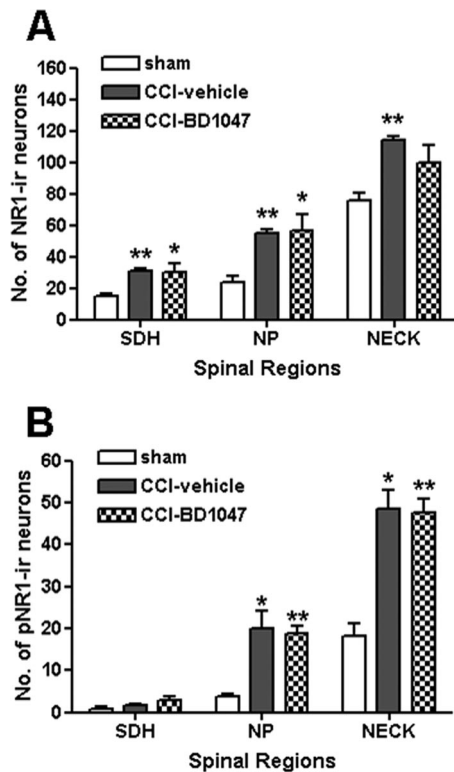


Fig. 7. Graphs illustrating the effect of intrathecal administration of the  $\sigma_1$  receptor antagonist BD1047 (30 nmol; n = 5) on days 15–20 after chronic constriction injury (CCI) surgery on the CCI-induced increase in the number of neurons expressing the N-methyl-D-aspartate receptor NR1 subunit (A) and the number of neurons expressing phosphorylated NR1 (pNR1) (B) in each region of the dorsal horn. The number of NR1-immunoreactive (ir) and pNR1-ir neurons in spinal dorsal horn was significantly increased on day 20 after CCI surgery (\*  $P < 0.05$ , \*\*  $P < 0.01$  as compared with that of sham surgery animals [n = 5]). Daily intrathecal injection of BD1047 during the maintenance phase of neuropathic pain did not affect the number of NR1 and pNR1-ir neurons in any of the regions of dorsal horn. SDH = superficial dorsal horn (laminae I and II); NP = nucleus proprius (laminae III and IV); NECK = neck of dorsal horn (laminae V and VI).

nists for Sig-1R positively modify higher-ordered brain functions, including learning, memory, cognition, and mood.<sup>9,10</sup> In particular, a study by Monnet *et al.*<sup>11,12</sup> reported that the activation of Sig-1R in the rat hippocampus evoked the potentiation of intracellular calcium influx *via* a protein kinase C-dependent mechanism and that this influx was associated with long-term potentiation. Because long-term potentiation represents one of the mechanisms that underlies the transition from acute to chronic pain and because an increase in intracellular calcium concentration also plays a major role in the development of central sensitization, particularly that associated with NMDA receptor activation, we speculated that activation of spinal Sig-1Rs might also be associated with development of chronic neuropathic pain.

One of the major findings of this study is that intrathecal treatment with BD1047 during the induction phase of neuropathic pain development produces a potent

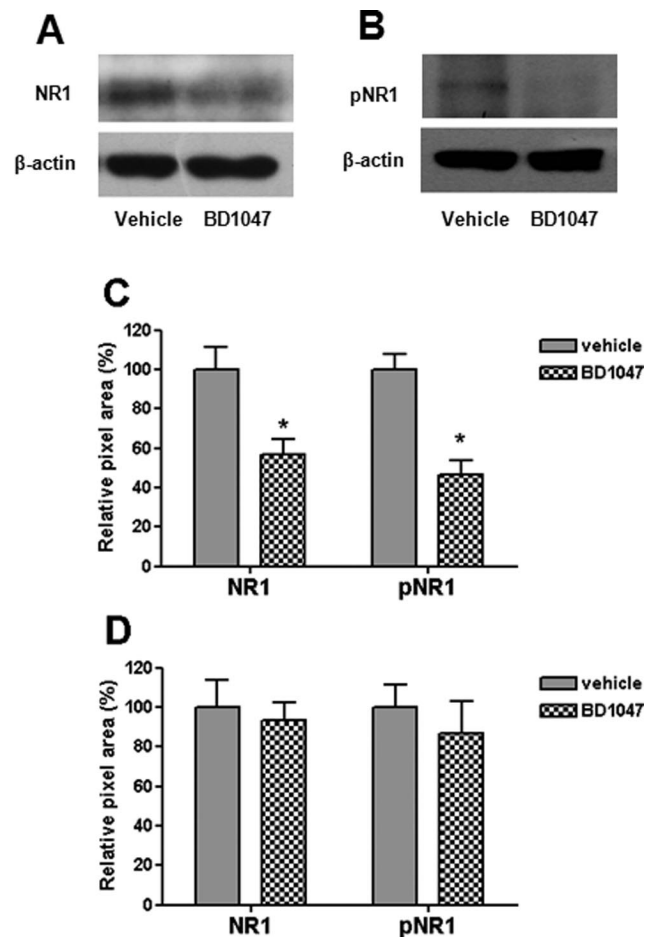


Fig. 8. Western blot analysis illustrating the effect of intrathecal administration of the  $\sigma_1$  receptor antagonist BD1047 (30 nmol; n = 4) on days 0–5 or on days 15–20 after surgery on N-methyl-D-aspartate receptor NR1 expression and NR1 phosphorylation (pNR1). The representative blots for vehicle or BD1047 treatment during the induction period (days 0–5) are shown for NR1 in A and for pNR1 in B. The relative pixel area (%) of NR1 and pNR1 expression was dramatically and significantly reduced by BD1047 treatment during the induction period (days 0–5) as compared with those of vehicle-treated chronic constriction injury rats (C; n = 4; \*  $P < 0.05$ ), whereas BD1047 treatment during the maintenance period (days 15–20) did not affect NR1 and pNR1 expression in the spinal cord dorsal horn (D; n = 4 for each group).

antiallodynic effect in CCI rats. Interestingly, there was also a corresponding up-regulation of spinal cord Sig-1Rs during the induction phase, but not the maintenance phase, of neuropathic pain. These results imply that the activation of dorsal horn Sig-1R can trigger, but cannot maintain, the mechanical allodynia induced by CCI in this rodent model of neuropathic pain. Because of this antiallodynic effect of Sig-1R antagonists, we further suggest that Sig-1R antagonist administration might also be useful as a preemptive or initiative analgesic, which can modulate the establishment of a chronic pain state. Although the results of preemptive analgesia are still controversial with respect to reducing postoperative pain in a clinical setting,<sup>35</sup> there is now convincing evidence demonstrating that preemptive analgesia can prevent

the development of central sensitization in a variety of experimental models.<sup>36,37</sup> It is our contention that early administration of Sig-1R antagonists may also prevent the development of central sensitization and thus serve as a preemptive analgesic by preventing sensitization of the nervous system to subsequent stimuli that could amplify pain. Preemptive or initiatory analgesia in the spinal cord has not been widely tested in neuropathic pain patients, because it is almost impossible to administer drug treatment preemptively in these patients because of the unpredictable clinical evolution of neuropathic pain.<sup>38</sup> However, the ability to alter neuropathic pain expression in situations in which the possibility for the development of this condition is high would be of considerable clinical value. Therefore, patients who experience direct nerve trauma or cancer patients who are treated with either radiation or chemotherapeutic agents known to cause neuropathic pain are ideal candidates for preemptive analgesic treatment with Sig-1R antagonists. Although it is impossible to predict which patients with peripheral nerve injury or being treated with cancer chemotherapeutic drugs will develop chronic neuropathic pain, our results would suggest that there is a critical time window during the induction phase in which preemptive analgesic treatment would be effective.

On the other hand, potential side effects (*e.g.*, memory deficits and/or depression) have been reported after systemic administration of BD1047, and these apparently stem from interference with the important role of Sig-1Rs in learning and memory processes, responses to stress or depression, psychoses, and addiction.<sup>39,40</sup> Although intrathecal treatment with Sig-1R blockers may have great therapeutic potential to treat pain-related conditions without many potential side effects, any type of systemic treatment would require clearly defined preclinical experiments to prevent or reduce potential side effects. In addition, it is important to note that a previous study from our laboratories has determined that intrathecal application of BD1047 does not affect normal motor coordination or function.<sup>6</sup> Based on this previous finding, we administered BD1047 intrathecally in the current study and could rule out any unexpected side effects related to motor function. Nonetheless, we believe that future studies be conducted to further elucidate the details of the pharmacologic mechanisms of action of BD1047.

It is now well documented that activation of spinal cord NMDA receptors plays an important role in the induction and maintenance of central sensitization and in the generation of pain hypersensitivity at the level of the spinal cord.<sup>41</sup> In addition, pNR1 *via* several protein kinases (*e.g.*, protein kinase C, protein kinase A, calcium/calmodulin-dependent protein kinase II) has also been recognized as a major mechanism involved in the regulation of NMDA receptor function.<sup>16</sup> In this regard, we have shown in the current study that early intrathecal treatment with a  $\sigma_1$  antagonist prevented the nerve injury-induced increase in

both NR1 and pNR1 expression in the spinal cord dorsal horn, particularly in laminae III-IV and V-VI. Conversely, intrathecal injection of BD1047 during the maintenance phase of neuropathic pain did not alter the CCI-induced increase in spinal cord NR1 and pNR1 previously established during the induction phase. These results may be associated with the nerve injury-induced early up-regulation of Sig-1R levels in the spinal cord dorsal horn. We further suggest that this increase in Sig-1R expression and activity may influence NMDA receptor function at both the transcriptional (*e.g.*, the modulation of gene transcription) and posttranslational (*e.g.*, the activation of protein kinase *via* intracellular signaling) levels as it relates to spinal cord plasticity. This is based on our finding that BD1047 inhibits the early CCI-induced increase in both NR1 and pNR1 expression in the dorsal horn as well as on our previous work.<sup>42</sup> Although our studies suggest that Sig-1Rs activation may ultimately affect NMDA receptor expression and function, the precise mechanisms by which this occurs need to be further investigated. Recently, we have demonstrated that the direct activation of spinal Sig-1R by the select Sig-1R agonist PRE084 or carbetapentane facilitates transiently expressed pain *via* a protein kinase C-dependent mechanism and an enhanced phosphorylation of spinal NMDA receptors.<sup>42</sup> In this regard, we hypothesize that the transient mechanical allodynia observed in the contralateral hind paw in the current study is due to the pharmacologic action of PRE084 on protein kinase C activity in the contralateral spinal cord dorsal horn. Therefore, it is conceivable that blockade of Sig-1R activation at the spinal cord level alters the abnormal neuronal signaling induced by the development of chronic pain conditions. This modulation could in turn reduce the increases in NMDA receptor function by further modulating both NR1 expression and its phosphorylation in the dorsal horn of CCI rats.

The current study demonstrates that early treatment with BD1047 is effective in preventing the induction of mechanical allodynia, but not thermal hyperalgesia, in CCI rats. This result highlights that fact that different mechanisms underlie the sensory disorders associated with neuropathic pain. This concept is supported by previous studies showing that different receptor systems are implicated in thermal hyperalgesia *versus* mechanically evoked pain-related behavior.<sup>43-45</sup> Our findings ultimately suggest that activation of spinal Sig-1Rs specifically contributes to mechanical allodynia, but not thermal hyperalgesia, in chronic neuropathic rats. Therefore, it is unlikely that spinal Sig-1Rs are involved in activation of the transient receptor potential vanilloid 1 receptor or the sympathetic nervous system in CCI animals, because activation of the transient receptor potential vanilloid 1 receptor and the sympathetic nervous system are known to be two important factors that underlie the induction of thermal hyperalgesia in neuropathic rats.<sup>18,46-48</sup>

In conclusion, the current study demonstrates that (1) the activation of spinal Sig-1Rs plays a critical role in the

induction of mechanical allodynia in neuropathic pain; (2) nerve injury induces an early up-regulation of Sig-1R expression in the dorsal horn, and this increased expression is correlated with the induction of mechanical allodynia; and (3) the antiallodynic effect of an intrathecally administered  $\sigma_1$  antagonist is closely associated with the early blockade of NMDA receptor NR1 subunit expression and phosphorylation in the spinal cord dorsal horn of neuropathic rats. These results suggest a potential therapeutic use for Sig-1R antagonists in the clinical management of neuropathic pain, but administration of Sig-1R antagonists must be done during the induction phase of neuropathic pain for the treatment to be most effective.

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