

Intravenous Administration of Substance P Attenuates Mechanical Allodynia Following Nerve Injury by Regulating Neuropathic Pain-Related Factors

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Abstract

This study aimed to investigate the analgesic effect of substance P (SP) in an animal model of neuropathic pain. An experimental model of neuropathic pain, the chronic constriction injury (CCI) model, was established using ICR mice. An intravenous (i.v.) injection of SP (1 nmole/kg) was administered to the mice to examine the analgesic effects of systemic SP on neuropathic pain. Behavioral testing and immunostaining was performed following treatment of the CCI model with SP. SP attenuated mechanical allodynia in a time-dependent manner, beginning at 1 h following administration, peaking at 1 day post-injection, and decaying by 3 days post-injection. The second injection of SP also increased the threshold of mechanical allodynia, with the effects peaking on day 1 and decaying by day 3. A reduction in phospho-ERK and glial fibrillary acidic protein (GFAP) accompanied the attenuation of mechanical allodynia. We have shown for the first time that i.v. administration of substance P attenuated mechanical allodynia in the maintenance phase of neuropathic pain using von Frey's test, and simultaneously reduced levels of phospho-ERK and GFAP, which are representative biochemical markers of neuropathic pain. Importantly, glial cells in the dorsal horn of the spinal cord (L4-L5) of SP-treated CCI mice, expressed the anti-inflammatory cytokine, IL-10, which was not seen in vehicle saline-treated mice. Thus, i.v. administration of substance P may be beneficial for improving the treatment of patients with neuropathic pain, since it decreases the activity of nociceptive factors and increases the expression of anti-nociceptive factors.

Key Words: Mechanical allodynia, GFAP, IL-10, Neuropathic pain, Substance P, Glial inactivation

INTRODUCTION

Neuropathic pain, characterized by spontaneous pain, allodynia, and hyperalgesia, is still a clinical challenge. Damage to any part of the nervous system due to insults such as trauma, infection, ischemia, or metabolic derangement can induce neuropathic pain. The mechanisms of neuropathic pain are partially understood, and it is now regarded as a neuro-inflammatory disorder caused by complex interactions between neurons and activated glia in the nervous system (Austin and Moalem-Taylor, 2010; Bradesi, 2010). Activated astrocytes in the dorsal horn of the spinal cord are particularly implicated in

the development and maintenance of neuropathic pain (Mika *et al.*, 2013). Therefore, pharmacologic inhibition of activated astrocytes may be a novel drug target for the treatment of neuropathic pain (Takeda *et al.*, 2004; Gao and Ji, 2010).

Substance P (SP), an 11-amino-acid neuropeptide released from sensory neurons in the brain and spinal cord, has been considered one of the main transmitters of nociceptive impulses and inflammatory pain (Sahbaie *et al.*, 2009; Li *et al.*, 2012). We recently reported that systemic administration of SP contributes to accelerated wound healing in the eyes (Hong *et al.*, 2009) and spinal cord (Jiang *et al.*, 2013), which is related to immunomodulation in the lesions. In addition, supraspinal

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and intramuscular injection of SP attenuates mechanical allodynia in carrageen-induced inflammatory pain (Parenti *et al.*, 2012) and acid-induced chronic muscular pain (Lin *et al.*, 2012). However, there have been no reports demonstrating the anti-nociceptive effects of systemically administered SP on neuropathic pain.

We hypothesize that intravenously (i.v.) administered SP may decrease neuropathic pain activation factors and glial activation, especially astrocytic activation, in the spinal dorsal horn of mice with neuropathic pain following sciatic nerve injury. We also hypothesize that mechanical allodynia will consequently be attenuated.

This study aimed to evaluate pain-related behavioral changes after i.v. administration with SP and to examine the possible mechanism of immunomodulation in the spinal cord, using a murine model of chronic constriction injury (CCI), with mechanisms that closely resemble those underlying inflammation during neuropathic pain (Kim *et al.*, 1997; Vissers *et al.*, 2006; Lee *et al.*, 2010).

MATERIALS AND METHODS

Animals and ethical approval

The official permission number for these animal experiments from the Institutional Animal Care and Use Committee of Seoul National University (Seoul, Korea) is SNU-110125-8. All experiments were performed on male ICR mice (Orient Bio Inc., Seoul, Korea) weighing 25-30 g. Five mice were housed per cage and had *ad libitum* access to food and water. The cages were covered with soft bedding and maintained on a 12:12-h light-dark cycle (7 am/7 pm) at a constant temperature (23°C) and humidity (50%). All experimental procedures followed the ethical guidelines for the use of animals in research of the International Association for the Study of Pain (IASP) and the Institutional Animal Care and Use Committee of Seoul National University. The mice were acclimatized for at least 3 days before any behavioral tests were performed. All behavioral tests were performed under double-blind conditions.

The chronic constriction injury (CCI) model and SP administration

The CCI model was established in ICR mice as previously described (Lee *et al.*, 2013). Briefly, mice with mechanical thresholds more than 2.0 g were anesthetized using 3% isoflurane. An incision was made in the left hind limb at the level of the middle thigh, and a section was made through the biceps femoris. The muscle was retracted and the common sciatic nerve was exposed. Proximal to the trifurcation of the sciatic nerve, the nerve was freed from the adhering muscle and 4 loose ligatures of 6-0 chromic gut (W812, Ethicon Inc., Somerville, NJ, USA) were tied about 0.5 mm apart. Following nerve ligation, the muscle and skin were closed separately using 6-0 black silk (W802, Ethicon Inc.). The mice with mechanical thresholds less than 1.0 g on the von Frey's test (described below) were selected and injected i.v. with 0.2, 1, or 2 nmol/kg of SP (Sigma-Aldrich, St. Louis, MO, USA) on day 14 following the nerve injury. Simultaneously, the control group received i.v. saline. RP 67580 (Tocris bioscience, Bristol, UK) was used to antagonize the Neurokinin 1 (NK-1) receptor, and it was injected i.v. at a dose of 1 μ mol/kg.

Behavioral tests

To assess mechanical allodynia, the plantar surface of the left hind paw was poked with a von Frey monofilament (Stoelting Co., Wood Dale, IL, USA). The animals were housed in transparent Plexiglass boxes (5×10×5 cm³) placed on an elevated floor of metal mesh that allowed the von Frey filaments to be applied to the left hind paw from below. At least 30 min after habituation, the von Frey monofilaments were applied perpendicular to the whole plantar surface. Each filament was tested five times at intervals of more than 5 s before the filaments were changed. The von Frey monofilaments have varying degrees of stiffness that exhibit a constant level of force when they are pressed until bent. We used grams of force (g) because the contact area was not uniform owing to the elasticity of the skin. Prior to the rotarod test, the mice were trained for 2 days. The rotarod (Panlab, Barcelona, Spain), consisting of a non-slippery plastic rod (30 mm in diameter) and four lines (50 mm wide), was set to run mode (16 rpm) for over 1 min. The habituated mice were subjected to running on the rod for at least 1 min. The test was performed 3 times at 0 h, 1 h, 4.5 h, 1 day, and 3 days after the administration of SP and saline. MK-801 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), an antagonist of the *N*-methyl-D-aspartate (NMDA) receptor, was used as a positive control and was injected i.v. at a dose of 45 μ mol/kg.

Histological analyses

Immunohistochemical staining for phospho-ERK (Cell Signaling, Danvers, MA, USA) at the L4-L5 level of the spinal cord was performed as previously described (Lee *et al.*, 2013). Immunofluorescence staining using anti-GFAP (Abcam, Cambridge, MA, USA), anti-interleukin (IL)-10 (MBL, Woburn, MA, USA), and anti-tumour necrosis factor alpha (TNF α ; Santa Cruz Biotechnology, Inc.) was carried out as described previously (Jiang *et al.*, 2012). The number of phospho-ERK-immunoreactive (IR) neurons was determined by assessing the total number of stained and unstained neuronal profiles from 5 sections at 100- μ m intervals from the L4-L5 level of the spinal cords of ICR mice with CCI using the NIH Image 1.62 program. Images were obtained from immunostained slides of sections using a Leica CTR 4000 fluorescence microscope or a Zeiss LSM 510 META confocal microscope (Carl Zeiss, Jena, Germany) and quantified using Image-Pro Plus 4.5 (Media Cybernetics Inc. Jenoptik, AG, Rockville, MD, USA). All histological tests were performed under double-blind conditions.

Statistical analysis

Data are expressed as means \pm standard errors of the mean (SEMs). The differences between the groups were analyzed using a one-way analysis of variance (ANOVA). Differences were considered significant at $p < 0.05$.

RESULTS

Intravenous treatment with SP attenuated mechanical allodynia in a dose-dependent manner

To determine the anti-nociceptive effect of systemically administered SP on neuropathic pain, SP was injected i.v. to mice with mechanical allodynia at the maintenance phase of neuropathic pain 14 days after nerve injury. Injection (i.v.) of SP at doses of 0.2, 1, and 2 nmole/kg induced significant

anti-nociceptive effects compared to saline. Injection (i.v.) treatment with SP attenuated mechanical allodynia, with the effects beginning at 1 h, peaking at day 1, and decaying by day 3 (Fig. 1A). SP (1 nmole/kg) was administered again 3 days after the first injection to determine whether SP has anti-nociceptive effects. Interestingly, the second injection of SP also increased the threshold of mechanical allodynia, with the effect peaking at day 1 and decaying by day 3 (Fig. 1B). The second dose of SP notably had an enhancing effect on mechanical allodynia. This result suggests that single i.v. injection of SP every 3 d is sufficient to treat neuropathic pain. The rotarod test was used to investigate the potential neurotoxic effects of i.v. treatment with SP at the selected dose (1 nmole/kg). The motor coordination of CCI mice was impaired 1 h after the treatment of mice with MK-801 (Fig. 1C), which evokes motor dysfunction (Lee *et al.*, 2011), however, no change was observed in either the saline (control) or SP treated groups. As shown in Fig. 1C, the time spent on the rod was not different between the mice treated i.v. with SP (1 nmole/kg) and the controls, indicating that the reduction in behavioral responses after the administration of SP may not be because of an effect of SP on motor coordination. Together, these data indicate that i.v. administration of SP significantly increases the mechanical threshold in the CCI experimental model of chronic neuropathic pain, and this effect was not related to impairments of normal motor function.

Intravenous administration of SP decreases the expression of phospho-ERK in the spinal cord

We analyzed the expression of phospho-ERK, a major molecular indicator of the maintenance phase of neuropathic pain, in the dorsal horn at the L4-L5 level of the spinal cord (Ma and Quirion, 2005). In the CCI model, phospho-ERK was detected at a high density in lamina I-II, as shown by the dotted line. Phospho-ERK expression was significantly decreased 1 h and 1 d following the administration of SP (Fig. 2A, 2B). The expression of phospho-ERK is consistent with the behavioral changes associated with mechanical allodynia over time. In more detail, the quantitative analysis from 5 independent experiments showed an inverse correlation between phospho-ERK and the mechanical threshold (Fig. 2B).

Attenuation of mechanical allodynia by SP is dependent on the activation of the NK-1 receptor

A possible role for the NK-1 receptor in the reduction of mechanical allodynia in the CCI model has previously been found (Muto *et al.*, 2012). To investigate this role for the NK-1 receptor further, an NK-1 receptor antagonist (RP 67580) was administered in addition to SP to the CCI model to determine whether the anti-nociceptive effects of SP are linked to NK-1 receptors. The administration of the NK-1 receptor antagonist alone did not attenuate mechanical allodynia. Blocking of the NK-1 receptor with RP 67580 completely eliminated the anti-nociceptive effects of SP (Fig. 3). These results suggest that the anti-nociceptive effects of SP are dependent upon NK-1 receptor activation and downstream signaling.

Glial inactivation is involved in the SP-induced attenuation of mechanical allodynia

Current data strongly suggest that glial activation in the dorsal horn of the spinal cord is closely associated with the development and maintenance of chronic pain (Milligan and

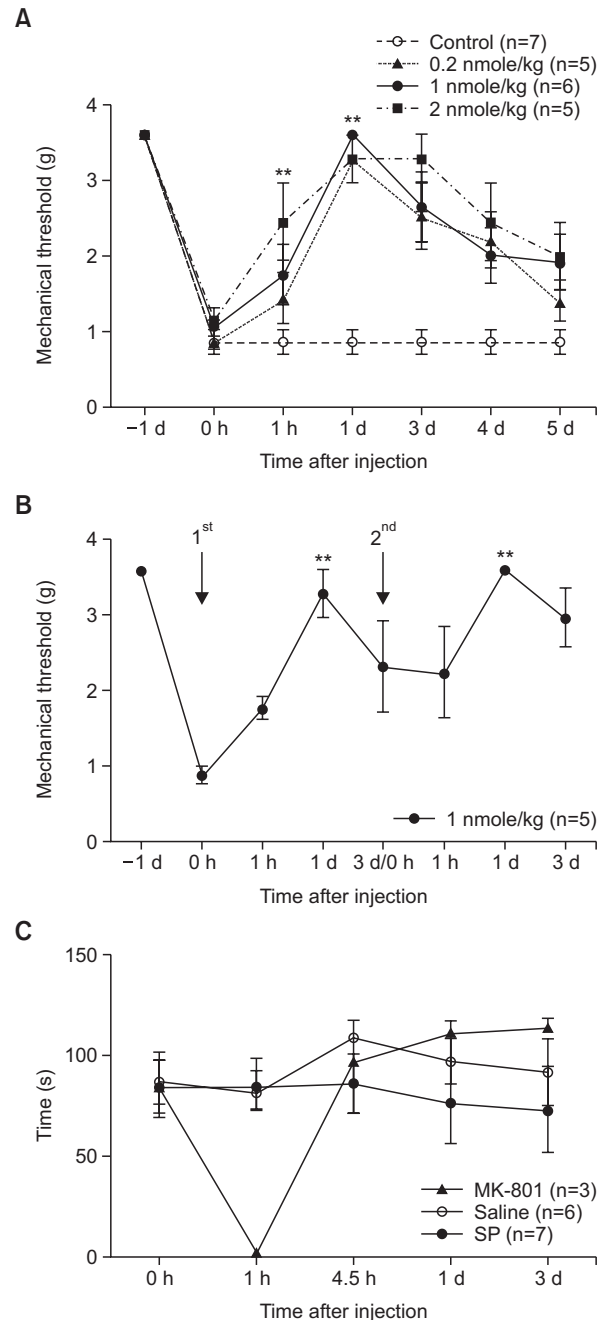


Fig. 1. Anti-nociceptive effects of intravenous administration of SP. (A) Intravenous (i.v.) administration of SP at doses of 0.2, 1, and 2 nmole/kg induced anti-nociceptive effects compared to saline, with the effect beginning at 1 h, peaking at 1 d, and decaying 3 d after injection. (B) The enhancing effects of i.v. administration of SP in the attenuation of mechanical allodynia. The second dose of SP also showed anti-nociceptive effects with the effect peaking at 1 d and decaying by 3 d after injection. (C) The motor coordination of CCI mice was not altered following the i.v. administration of SP (1 nmol/kg). MK-801, an NMDA receptor antagonist, was used as a positive control and it was injected i.v. at a dose of 45 μ mol/kg. Data are shown as means \pm SEM. ** $p < 0.01$.

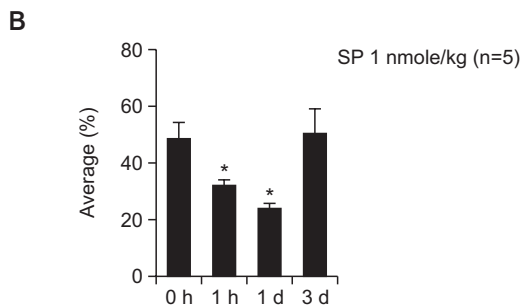
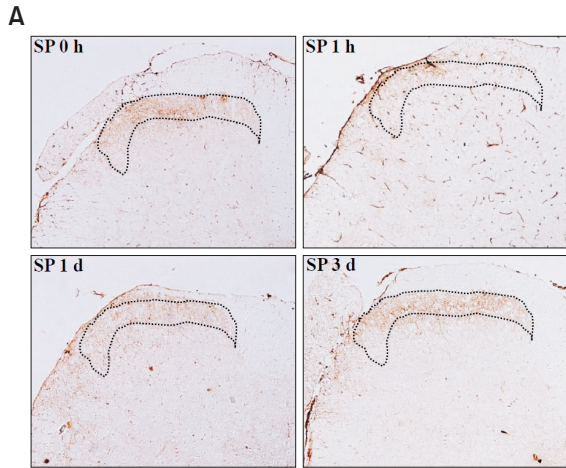


Fig. 2. The expression of phospho-ERK in the spinal cord is decreased by the intravenous administration of SP: (A) Immunohistochemical analysis of phospho-ERK in the lamina I-II at the L4-L5 level of the spinal cord, as indicated by the dotted line, showed reduced immunoreactivity following the i.v. administration of SP intravenously in the neuropathic pain model. The expression was decreased at 1 h after injection, remained low for 1 d post-injection, and increased again 3 d post-injection. Scale bar: 50 μ m. (B) The statistical summary of the immunohistochemically positive stained area from five independent results of immunocytochemistry ($*p < 0.05$). Mean percentages (%) of phospho-ERK-immunoreactive (IR) neurons relative to the total number of neurons at the L4-L5 level of the spinal cord. Data are presented as means \pm SEM.

Watkins, 2009). In addition, the modulation of neuroinflammation in neuropathic pain has emerged as an important potential therapeutic target (Ledebor *et al.*, 2007; Shimizu *et al.*, 2009; Shibata *et al.*, 2011). Since astrocytes enhance neuropathic pain, we examined astrocyte activation in SP-induced attenuation of mechanical allodynia by performing immunofluorescence staining for GFAP. In CCI mice, glial activation in the dorsal horn of the spinal cord was detected, and correlated with the maintenance of neuropathic pain. However, a significant reduction in GFAP was observed in SP-treated CCI mice (Fig. 4A). The quantitative analyses of 5 independent experimental sets showed identical kinetics to the anti-nociceptive effects (Fig. 4B). Additionally, we analyzed the expression of cytokines involved in neuroinflammation in the dorsal horn of the spinal cord. Double-immunofluorescence staining with GFAP showed increased expression of the anti-inflammatory cytokine IL-10 in reactive astrocytes and a reduction in the pro-inflammatory cytokine TNF- α at the same sites (Fig. 4C).

Collectively, i.v. administration of SP attenuated mechanical allodynia in our experimental neuropathic pain model. The an-

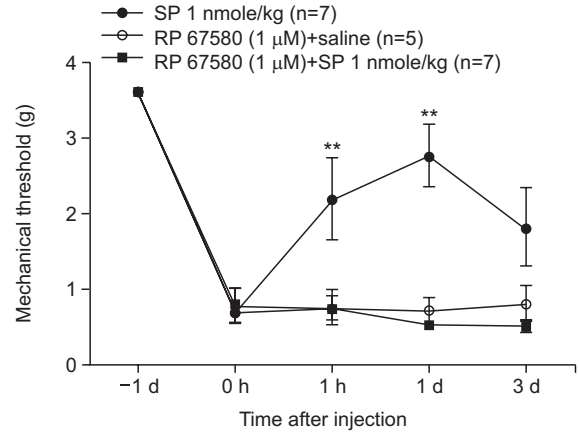


Fig. 3. NK-1 receptor-dependent anti-nociceptive effects of intravenous SP. The i.v. administration of the NK-1 receptor antagonist, RP 67580, with SP completely eliminated the anti-nociceptive effects of SP in the CCI model. Data are presented as means \pm SEM. $**p < 0.01$.

ti-nociceptive effects of SP were totally dependent upon NK-1 receptor activation and were accompanied by a reduction of phospho-ERK at the L4-L5 level of the spinal cord along with astrocytic inactivation, a reduction in TNF α , and an increase in IL-10.

DISCUSSION

SP, a member of the tachykinin family, is produced in the sensory neurons and plays an essential role in pain transmission in the spinal cord (Nichols *et al.*, 1999; Gao *et al.*, 2003). When nociceptive stimuli activate a sensory neuron, SP is released in the periphery (Gao *et al.*, 2003; Chen *et al.*, 2006) and the spinal cord (Gao *et al.*, 2003). The $T_{1/2}$ of SP in microsomes pooled from mouse liver is 5.6 min (Pailleux *et al.*, 2013). Intrathecal (*i.t.*) administration of SP induces hyperalgesia and pain-related behaviors (Nakayama *et al.*, 2010), while selective NK-1 receptor antagonists prevent the effects of SP on hyperalgesia at the level of the spine (Traub, 1996). Nonetheless, substantial data show that SP also has anti-inflammatory and anti-nociceptive effects (Holden *et al.*, 2009; Lin *et al.*, 2012; Parenti *et al.*, 2012). We first reported on the anti-inflammatory roles of SP in a rat model of spinal cord injury (Jiang *et al.*, 2012, 2013).

In this study, we demonstrated that i.v. administration of SP attenuates mechanical allodynia by reducing phospho-ERK levels at the L4-L5 level of the spinal cord and inactivating glia, in particular astrocytes as indicated by a reduction of GFAP, in the spinal cord after CCI in mice. Considering the major effects of SP on pain transmission, we examined pain-related behaviors in mice following the administration of SP. There were no pain-associated symptoms or behaviors detected 1 h following the administration of SP. Mice did not show any strange behaviors such as inactivity, hunched posture, writhing, or any pain-related behaviors after treatment with SP. A previous study indicated that SP induced itching behaviors, which are associated with the NK1 receptor (Andoh *et al.*, 1998). It is generally assumed, that painful and itching sensations are antagonistic to each other. In other words, pain can

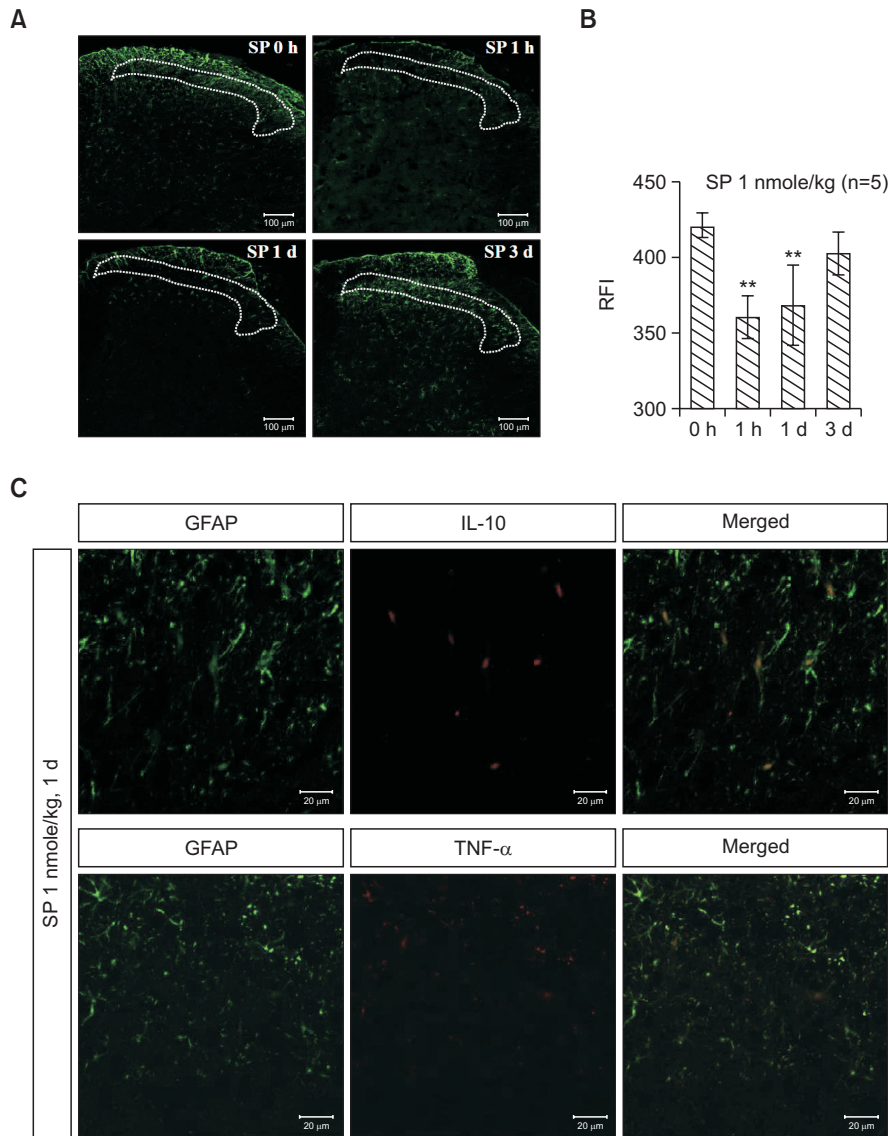


Fig. 4. Glial inactivation in the antinociceptive effects of intravenous SP. (A) A reduction in GFAP immunoreactivity was observed in the CCI mice injected i.v. with SP. GFAP immunoreactivity decreased 1 h after injection, remained low 1 d post-injection, and increased again 3 d post-injection. Scale bar: 100 μ m. (B) Quantitative analysis demonstrating identical kinetics to the analgesic effects of SP. Gray-value means from the region of interest were converted to relative fluorescence intensity (RFI) units using the formula: $y=1-\log_{10}(255/x)$, where y is densitometry and x is the gray value. The statistical summary of the immunohistochemically positive stained area from five independent results of immunocytochemistry (** $p<0.01$). Data are presented as means \pm SEM. (C) Double immunofluorescence staining in the dorsal horn of the spinal cord shows increased expression of the anti-inflammatory cytokine IL-10 and a reduction in the pro-inflammatory cytokine TNF α in reactive astrocytes. Scale bar: 20 μ m.

suppress itching, and itching can relieve pain. In this regard, it could be that itching alleviated the neuropathic pain. In an attempt to answer this question, we examined whether any itching behaviors were induced following the administration of SP. However, we did not find any itching behaviors in mice following treatment with SP. These results suggest that i.v. SP has only anti-nociceptive effects. It is known that nerve injury-induced ERK phosphorylation occurs sequentially, first in neurons (several minutes to several hours after the lesion) and second in microglia and astrocytes (several weeks after the lesion) in the ipsilateral dorsal horn. In addition, all phospho-ERK-positive cells co-localized with the astrocytic

marker GFAP, indicating that all of these cells represent activated astrocytes. Moreover, a phospho-ERK inhibitor, U0126, is known to inhibit CCI-induced mechanical allodynia (Ma and Quirion, 2005). Thus, substance P may attenuate mechanical allodynia by reducing phospho-ERK levels at the L4-L5 level of the spinal cord, and glial inactivation may result from phospho-ERK inhibition. The anti-nociceptive effects of SP were enhanced by repeated administration of SP. This suggests that only single i.v. injection of SP every 3 d is needed to treat neuropathic pain. Surprisingly, the antinociceptive effects of SP were reversed when an NK-1 receptor antagonist, RP 67580, was co-administered. Consistent with our data, an

NK-1 receptor antagonist (L-703606) injected into the lateral hypothalamus-induced antinociception following treatment with carbachol, suggesting that SP could be involved in the activation of the descending anti-nociceptive pathway and induction of analgesia through noradrenergic-mediated descending inhibition after binding to its receptor, the NK1 receptor (Holden *et al.*, 2009; Muto *et al.*, 2012). However, most studies have suggested that the anti-nociceptive effects of SP, especially substance P (1-7), are attributed to its binding to the NK1 receptor resulting in activation of co-localized opioid receptors (Dong and Yu, 2005; Komatsu *et al.*, 2009). Recent studies have suggested that the reduction of the capsaicin-induced nociceptive response by substance P (1-7) is because of an inhibition of ERK phosphorylation, induced by activation of the capsaicin-mediated transient receptor potential vanilloid 1 (TRPV1) (Komatsu *et al.*, 2011). In addition, another study has shown that upregulation and increased sensitization of spinal TRPV1 is involved in the development and/or maintenance of mechanical allodynia in the rat CCI model (Kanai *et al.*, 2005). Furthermore, concurrent with our results, the curcuminoid KMS4034 showed anti-nociceptive effects via modulation of TRPV1 in the CCI model (Lee *et al.*, 2013) and curcumin induced anti-nociceptive effects via inhibition of astrocytic activity, as indicated by a reduction of GFAP in the spinal dorsal horn and ERK signaling pathways (Ji *et al.*, 2013). Collectively, these results suggest that substance P may induce anti-nociception via modulation of TRPV1 and/or activation of opioid receptors following NK1 binding.

Recent studies have indicated that the activation of glia may be a main contributory factor in pathological and chronic pain mechanisms (Milligan and Watkins, 2009). Reactive astrocytes secrete many signaling molecules, which can have protective and/or pathological functions. Systemic treatment with SP plays a protective role in chronic neuropathic pain by regulating glial activation, which results in anti-nociceptive and anti-inflammatory actions.

In conclusion, while SP is considered to display excitatory effects and promote nociception in the spinal cord, systemic treatment with SP shows anti-nociceptive properties. Interestingly, among patients with diabetes, one of the clinical groups at risk for neuropathic pain, showed lower serum levels of SP than healthy volunteers (Wang *et al.*, 2012). In addition, patients with chronic lower back pain have significantly reduced salivary and plasma levels of immunoreactive SP compared to healthy volunteers (Parris *et al.*, 1990). Injection (i.v.) of SP affects neither motor activity nor inflammation-induced edema, as determined using the rotarod (Fig. 1C) and formalin (data not shown) tests, respectively. We have demonstrated for the first time that i.v. administration of substance P attenuates mechanical allodynia in the maintenance phase of neuropathic pain using the von Frey test and simultaneously reduces phospho-ERK levels at the L4-L5 level of the spinal cord. Moreover, GFAP was reduced, especially from astrocytes in the dorsal horn at the L4-L5 level of the spinal cord, IL-10 expression was increased, and TNF- α expression was reduced. These findings suggest that SP may represent a good drug candidate for use in patients suffering from neuropathic pain owing to its long effect on activators of neuropathic pain, ~3 d, while it also increases inhibitory neuropathic pain factors. However, further investigations should be performed to determine its exact mode of action and long-term effects.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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