

Intrathecal Gabapentin Increases Interleukin-10 Expression and Inhibits Pro-Inflammatory Cytokine in a Rat Model of Neuropathic Pain

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We examined the possible anti-inflammatory mechanisms of gabapentin in the attenuation of neuropathic pain and the interaction between the anti-allodynic effects of gabapentin and interleukin-10 (IL-10) expression in a rat model of neuropathic pain. The anti-allodynic effect of intrathecal gabapentin was examined over a 7-day period. The anti-allodynic effects of IL-10 was measured, and the effects of anti-IL-10 antibody on the gabapentin were assessed. On day 7, the concentrations of pro-inflammatory cytokines and IL-10 were measured. Gabapentin produced an anti-allodynic effect over the 7-day period, reducing the expression of pro-inflammatory cytokines but increasing the expression of IL-10 (TNF- α , 316.0 \pm 69.7 pg/mL vs 88.8 \pm 24.4 pg/mL; IL-1 β , 1,212.9 \pm 104.5 vs 577.4 \pm 97.1 pg/mL; IL-6, 254.0 \pm 64.8 pg/mL vs 125.5 \pm 44.1 pg/mL; IL-10, 532.1 \pm 78.7 pg/mL vs 918.9 \pm 63.1 pg/mL). The suppressive effect of gabapentin on pro-inflammatory cytokine expression was partially blocked by the anti-IL-10 antibody. Expression of pro-inflammatory cytokines was significantly attenuated by daily injections of IL-10. The anti-allodynic effects of gabapentin may be caused by upregulation of IL-10 expression in the spinal cord, which leads to inhibition of the expression of pro-inflammatory cytokines in the spinal cords.

Key Words: Allodynia; Gabapentin; Interleukin-10; Intrathecal; Pro-inflammatory Cytokines

INTRODUCTION

Peripheral nerve injury may lead to a chronic pain condition called neuropathic pain, which is characterized by dysesthesia (unpleasant, abnormal sensations), hyperalgesia (increased sensitivity to a normally painful stimulus) and allodynia (pain due to a normally painless stimulus) (1). Because the underlying mechanisms of neuropathic pain are poorly understood, treatments are frequently unsatisfactory. In a rat model, unilateral ligation of the L5 and L6 spinal nerves has been shown to produce some signs that are indicative of neuropathic pain (2, 3). Of the several experimental animal models available, signs of mechanical allodynia are most evident in the nerve ligation model (4).

The intrathecal administration of cytokine antagonists or the interleukin-1 (IL-1) receptor antagonist (IL-1ra) have been found to decrease central cytokine production and glial activation, and to inhibit allodynia in neuropathic pain situations (5). Neuroinflammation and the expression of pro-inflammatory cytokines are related to pain transmission and the development of inflam-

matory and neuropathic pain (6). It has been shown that pro-inflammatory cytokines and chemokines modulate, either directly or indirectly, mechanical allodynia and thermal hyperalgesia (7). Additionally, activated glial cells in the spinal cord can release pro-inflammatory cytokines, which initiate a signal transduction cascade and cause the release of excitatory amino acids and promote pain transmission (8).

IL-10 is a potent anti-inflammatory cytokine. It is produced by monocytes and macrophages and inhibits the synthesis of pro-inflammatory cytokines by activated macrophages (9). It has already been reported that IL-10 possesses anti-allodynic or anti-hyperalgesic effects by inhibiting the release of the pro-inflammatory cytokines TNF- α and IL-1 β by peritoneal macrophages (10).

Meanwhile, gabapentin is a 3-alkylated analogue of gamma-amino butyric acid with anticonvulsant effects (11). These effects are due to modulation of $\alpha_2\delta$ calcium-channel subunits, which inhibits glutamate release and increases the threshold for neuronal excitation (12). Gabapentin also has a well established role in the treatment of chronic pain (13, 14), especially

neuropathic pain (15), such as diabetic neuropathy (13) and postherpetic neuralgia (14, 16). Analgesic effects of gabapentin are not affected by opioid antagonists and repeated administration of gabapentin does not lead to analgesic tolerance (17). However, the mechanisms underlying the analgesic effects of gabapentin are not fully understood and possible interactions between gabapentin and IL-10 have not yet been reported.

In our present study, we examined the possible anti-inflammatory mechanisms of gabapentin in the attenuation of neuropathic pain. This is the first direct investigation of the interaction between IL-10 expression and the anti-allodynic effect of gabapentin in a rat model of neuropathic pain.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats, weighing 200-250 g, were housed individually in plastic cages with soft bedding at room temperature and maintained in a 12:12 hr light:dark cycle with free access to food and water. All animal experiments conformed to the guidelines of, and were approved by, the institutional animal care and use committee at Asan Institute for Life Science, Seoul, Korea (IACUC No. 2012-13-233).

Surgical procedure: L5/6 spinal nerve ligation

All surgical procedures were performed under inhalational anesthesia with sevoflurane in 100% oxygen, which was induced at 6% and maintained at 3%. Neuropathic pain was induced as described previously (2). Briefly, rats were anesthetized and placed under a microsurgical apparatus in a prone position. A midline incision was made in the back, and the left paraspinal muscles were separated from the spinous processes at the L4-S2 levels. The left L6 transverse process was carefully removed and the L4/5 spinal nerves were identified. The left L5 nerve was tightly ligated with a 6-0 silk thread. The left L6 spinal nerve, located just caudal and medial to the sacroiliac junction, was tightly ligated with a silk thread.

Intrathecal catheter implantation

The rats were chronically implanted with catheters for intrathecal drug administration as previously described (18). Briefly, under sevoflurane anesthesia, the rats were placed in a stereotaxic head holder and the occipital muscles were separated from their attachment points and retracted caudally to expose the cisternal membrane at the base of the skull. Intrathecal catheters (PE-10 polyethylene tubing, Becton Dickinson, Sparks, MD, USA) were passed caudally from the cisterna magna to the spinal cord level of the lumbar enlargement and then externalized through the skin. Proper intrathecal location was confirmed by a temporary motor block of both hind limbs after the injection of 10 μ L 2% lidocaine, followed by saline. Only animals with no evidence

of neurological deficit after the operation were included in the study. At least a 5-day recovery period was allowed before the animals were used in experiments. All drugs were injected through the intrathecal catheter using a microinjection syringe (Microliter™ #702, Hamilton, Reno, NV, USA) over a 60-s interval in a volume of 10 μ L, followed by a 10 μ L flush. The drugs were given in a blinded fashion.

Drugs

Gabapentin (molecular weight 171.24, lot number G154), rat IL-10 (I9154), and goat anti-IL-10 antibody (I5020) were purchased from Sigma, (St Louis, MO, USA). Gabapentin was dissolved in 0.9% saline. The drug dosages were selected according to our previous study (19) in which the ED₅₀ of intrathecal gabapentin was measured at 10.4 μ g (95% confidence interval, 5.3-20.4) in spinal nerve ligation (SNL) rats. Hence, in our present study we selected a 30 μ g gabapentin dose, which produces about 60% of the maximum possible effect (19). IL-10 (0.1, 0.3, and 1.0 μ g in 10 μ L of saline) was injected daily 1 hr before the behavior test to measure the anti-allodynic effect of IL-10 in the neuropathic pain rat model. Anti-IL-10 antibody (10 μ g in 10 μ L of saline) was intrathecally injected daily at 1 h before the intrathecal administration of gabapentin and behavioral testing to investigate its effects on the anti-allodynic effects of gabapentin. The doses for IL-10 or anti-IL-10 antibody were determined based on a previous study (20). Neither IL-10 nor anti-IL-10 antibody produced any observable abnormal neurological behavior at these doses (data not shown).

Behavioral assessments (mechanical allodynia threshold)

For each rat, the left hind paw withdrawal threshold to the von Frey filament test was determined before surgery (baseline, day 0) and once daily 30 min after intrathecal drug administration for 7 days post-surgery. All the behavioral tests were performed between 9 a.m. and 2 p.m. by an examiner blinded to the treatment groups. To quantify the mechanical sensitivity of the foot, the threshold of foot withdrawal in response to normally innocuous mechanical stimuli was determined using the von Frey filaments (North Coast Medical, Morgan Hill, CA, USA) and the "up and down" method (21). A series of eight calibrated von Frey filaments (0.4, 0.6, 1.0, 2.0, 4.0, 6.0, 8.0, and 15.0 g) were applied serially to the paw in ascending order of strength. Each rat was placed in a transparent plastic dome with a metal mesh floor, which allowed access to the plantar surface of the hind paw. Following habituation for 30 min to this environment, the von Frey filament was pressed perpendicular to the plantar surface of the left hind paw with sufficient force to cause slight buckling and was held for approximately 6 sec. A positive response was noted if the left hind paw was sharply withdrawn. Flinching immediately on removal of the filament was also considered a positive response. Each trial was repeated twice at approximately 2

min intervals, and the mean value was used as the force required to produce withdrawal responses.

Spinal cord sample preparation and measurement of cytokine proteins

On day 7, at the end of all experiments, all rats were sacrificed by exsanguination under sevoflurane anesthesia. A laminectomy was then performed at the lower edge of the 12th thoracic vertebra and the left L5-S3 segment of the spinal cord was removed immediately and stored at -80°C until used for cytokine measurement. The frozen spinal cord segments were homogenized in ice-cold RIPA buffer (150 mM NaCl, 1.0% IGEPAL[®] CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris, pH 8.0; lot number R0278, Sigma) with a protease inhibitor cocktail (104 mM AEBSF, 80 μM aprotinin, 4 mM bestatin, 1.4 mM E-64, 2 mM leupeptin, 1.5 mM pepstatin A; lot number P8340, Sigma) and centrifuged at 13,000 rpm for 20 min at 4°C . The supernatants were used for analysis of TNF- α , IL-1 β , IL-6, and IL-10. Commercially available enzyme-linked-immunosorbent assay (ELISA) kits were used for assessing cytokine proteins (R&D Systems, Minneapolis, MN, USA; sensitivity: 5 pg/mL). Bradford protein assay was used to measure total protein concentration in the tissue supernatant. ELISA microplates were analyzed using a Victor3 V multilabel counter (1420, PerkinElmer, Boston, MA, USA) and data were standardized as picograms of TNF- α , IL-1 β , IL-6, and IL-10 per 200 μg of total supernatant protein. The concentration of each target cytokine was determined based on an appropriate set of internal standard curves using recombinant rat cytokines.

Experimental groups

Experimental groups were established as follows:

1. Sham ($n = 6$) \times 4 (for behavioral assessments and ELISA of TNF α , IL-1 β , IL-6, and IL-10)
2. SNL group ($n = 6$) \times 4 (for behavioral assessments and ELISA of TNF α , IL-1 β , IL-6, and IL-10)
3. SNL + gabapentin 30 μg daily intrathecal injections ($n = 6$) \times 4 (for behavioral assessments and ELISA of TNF α , IL-1 β , IL-6, and IL-10)
4. SNL + IL-10 (0.1 μg) daily intrathecal injections ($n = 6$) \times 3 (for behavioral assessments and ELISA of TNF α , IL-1 β , and IL-6)
5. SNL + IL-10 (0.3 μg) daily intrathecal injections ($n = 6$) \times 3 (for behavioral assessments and ELISA of TNF α , IL-1 β , and IL-6)
6. SNL + IL-10 (1.0 μg) daily intrathecal injections ($n = 6$) \times 3 (for behavioral assessments and ELISA of TNF α , IL-1 β , and IL-6)
7. Sham + anti-IL-10 antibody (10 μg) daily intrathecal injections ($n = 6$) for behavioral assessments
8. SNL + gabapentin 30 μg + anti-IL-10 (10 μg) daily intrathe-

cal injections ($n = 6$) \times 3 (for behavioral assessments and ELISA of TNF α , IL-1 β , and IL-6)

9. SNL + IL-10 (single large dose, 7.0 μg) on day 7 ($n = 6$) for behavioral assessments

Statistical analysis

All behavioral data were expressed as the mean \pm standard error of the mean (SEM). Statistical analysis was carried out using one-way analysis of variance (ANOVA) and the Kruskal-Wallis one-way ANOVA on ranks in comparison to the control group at the same time point between groups and followed by a Tukey's test for multiple comparisons. The significance of the differences in the responses of treatment groups in comparison to the preoperative baseline value of each group was determined using one-way repeated-measures ANOVA followed by a Tukey's test for multiple comparisons. Statistical evaluation was performed with SigmaPlot[®] software version 11 (Systat Software, San Jose, CA, USA). *P* values of < 0.05 were considered statistically significant.

RESULTS

Overview of rat usage

In our present study, 180 rats were prepared in total. However, 24 rats were excluded due to no allodynic production, neurological deficit after intrathecal catheter implantation, obstructed catheters, or catheter loss. Among the 156 rats, 30 rats were used for sham group, and 126 rats were prepared for SNL and drug administration. The mechanical allodynia threshold was measured for all animals and 144 rats were subsequently sacrificed for the tissue cytokine protein assay. All rats remained healthy and continued to gain weight throughout the experimental period. No motor dysfunction was observed in the rats that received experimental drugs.

Effects of intrathecally administered gabapentin on the mechanical allodynia threshold

As shown in Fig. 1, the mechanical allodynia threshold in the SNL group, as measured by von Frey filaments, was decreased to just under 4.0 g by the 2nd day after SNL. However, the intrathecal administration of gabapentin (30 μg) produced a significant anti-allodynic effect over the 7 day period.

Effects of intrathecally administered gabapentin on pro-inflammatory cytokine expression and the IL-10 protein levels

As shown in Fig. 2A-C, protein levels of the pro-inflammatory cytokines TNF- α , IL-1 β , and IL-6 on day 7 were significantly increased after SNL, and this effect was significantly attenuated by intrathecal administration of gabapentin (TNF- α , 316.0 ± 69.7 pg/mL vs 88.8 ± 24.4 pg/mL, $P < 0.05$; IL-1 β , $1,212.9 \pm 104.5$ vs

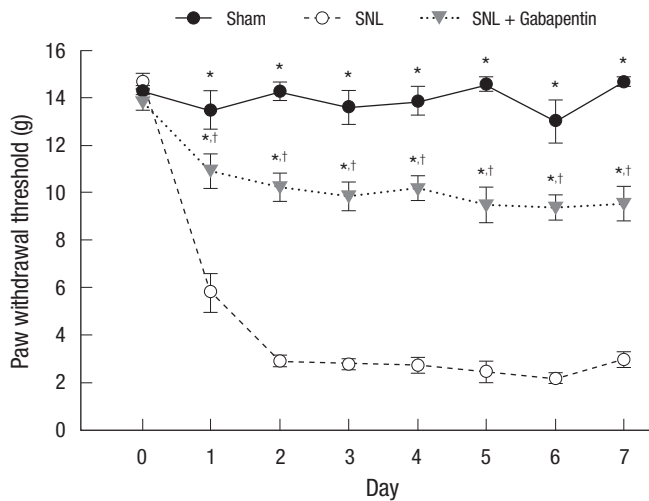


Fig. 1. Time course of paw withdrawal thresholds after spinal nerve ligation and with or without daily intrathecal injections of gabapentin (30 µg) over a 7-day period (6 rats per group). All data points are expressed as the mean ± SEM. **P* < 0.05 vs SNL group, †*P* < 0.05 vs Sham group.

577.4 ± 97.1 pg/mL, *P* < 0.05; IL-6, 254.0 ± 64.8 pg/mL vs 125.5 ± 44.1 pg/mL, *P* < 0.05). In the sham group, no significant changes were seen on day 7 in the concentrations of TNF-α, IL-1β, and IL-6 with administration of gabapentin. As shown in Fig. 2D, IL-10 protein was significantly increased with administration of gabapentin (918.9 ± 63.1 pg/mL) compared to the SNL alone group (532.1 ± 78.7 pg/mL, *P* < 0.05). In the sham group, administration of gabapentin did not cause significant changes in the concentration of IL-10 protein.

The role of IL-10 in the anti-allodynic effect in SNL rats

As shown in Fig. 3, daily intrathecal injections of IL-10 (0.1, 0.3, and 1.0 µg) for 7 days after SNL produced an anti-allodynic effect in a dose-dependent manner throughout the 7 day treatment period. A single large dose injection of IL-10 (7 µg) on day 7 did not produce an anti-allodynic effect.

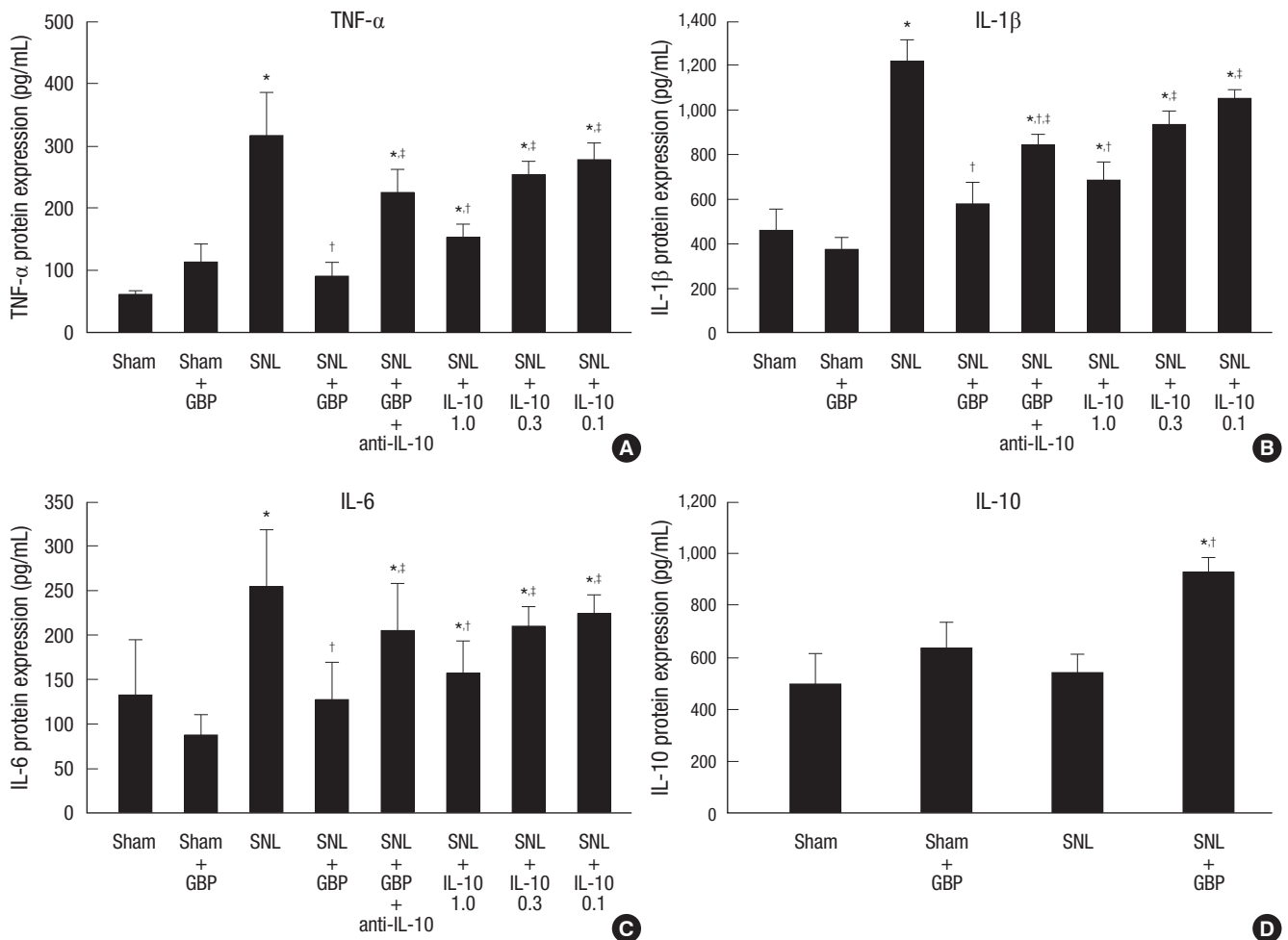


Fig. 2. Intrathecally injected gabapentin reduces the expression of TNF-α (A), IL-1β (B), and IL-6 (C) but increases the expression of IL-10 (D) in the dorsal horn in SNL rats (6 rats per group). The anti-allodynic effect of gabapentin on the expression of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 are inhibited by daily injections of the anti-IL-10 antibody (10 µg). Protein expression of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 on day 7 are significantly attenuated by IL-10 (1 µg) daily injections compared with the SNL alone group. All data points are expressed as the mean ± SEM. **P* < 0.05 vs sham group; †*P* < 0.05 vs SNL group; ‡*P* < 0.05 vs SNL + gabapentin group. SNL, spinal nerve ligation; Ab, antibody.

The role of anti-IL-10 antibodies and gabapentin in anti-allodynia in SNL rats

Daily intrathecal injections of anti-IL-10 antibody (10 µg) had an inhibitory effect on the anti-allodynic effects of gabapentin while intrathecal injections of anti-IL-10 antibody (10 µg) alone had neither allodynic nor anti-allodynic effects in the sham group of rats (Fig. 4). These data demonstrate that gabapentin beneficially modulates IL-10 expression, which not only counteracts the pro-inflammatory response but also facilitates anti-allodynia.

Effects of IL-10 and anti-IL-10 antibodies on pro-inflammatory cytokine expression

As shown in Fig. 2A-C, the protein expression of the pro-inflammatory cytokines TNF- α , IL-1 β , and IL-6 on day 7 were significantly reduced with daily IL-10 (1.0 µg) injections compared with the SNL alone group (TNF- α , 316.0 \pm 69.7 pg/mL vs 151.6 \pm 23.1 pg/mL, $P < 0.05$; IL-1 β , 1,212.9 \pm 104.5 vs 685.2 \pm 85.2 pg/mL, $P < 0.05$; IL-6, 254.0 \pm 64.8 pg/mL vs 155.3 \pm 38.7 pg/mL, $P < 0.05$). Daily administration of low dose IL-10 (0.1 and 0.3 µg) produced no significant differences compared with the SNL alone group. In contrast, daily anti-IL-10 antibody injections blocked the intrathecal gabapentin-induced decrease in the levels of the pro-inflammatory cytokines TNF- α , IL-1 β , and IL-6 (TNF- α , 88.8 \pm 24.4 pg/mL vs 225.2 \pm 36.2 pg/mL, $P < 0.05$; IL-1 β , 577.4 \pm 97.1 vs 841.7 \pm 51.5 pg/mL, $P < 0.05$; IL-6, 125.5 \pm 44.1 pg/mL vs 205.4 \pm 51.9 pg/mL, $P < 0.05$).

DISCUSSION

In the present study, intrathecally administered gabapentin showed an anti-allodynic effect in an SNL rat model of neuropathic pain. This effect could be explained, in part, by a gabapentin-induced reduction in the expression of the SNL-induced spinal pro-inflammatory cytokines TNF- α , IL-1 β , and IL-6. We observed increased expression of IL-10 protein with the intrathecal administration of gabapentin in a rat model of SNL. Intrathecal IL-10 administration not only had an anti-allodynic effect but also inhibited pro-inflammatory cytokine production in the SNL rat spinal cord, both of which were blocked by daily intrathecal injections of anti-IL-10 antibody. It has previously been reported that both mRNA and protein expression of the pro-inflammatory cytokines TNF- α , IL-1 β , and IL-6 are increased during neuropathic pain (7, 22, 23). Pro-inflammatory cytokines are released as a consequence of spinal glial cell activation in neuropathic pain (24). Taken together, we suggest from our current data and previous findings that intrathecally administered gabapentin promotes an advantageous control of neuroinflammation.

IL-10 was first reported to be a cytokine synthesis inhibitory factor (25). Later it was also described to attenuate nociception in many animal models through the inhibition of pro-inflammatory cytokines and spinal glial cell activation (26). Intrathecal IL-10 gene therapy has been shown to have a therapeutic effect in a rat model of neuropathic pain (27). Our present results support the idea that an increase in IL-10 expression in the spinal cord may be a mechanism by which intrathecally admin-

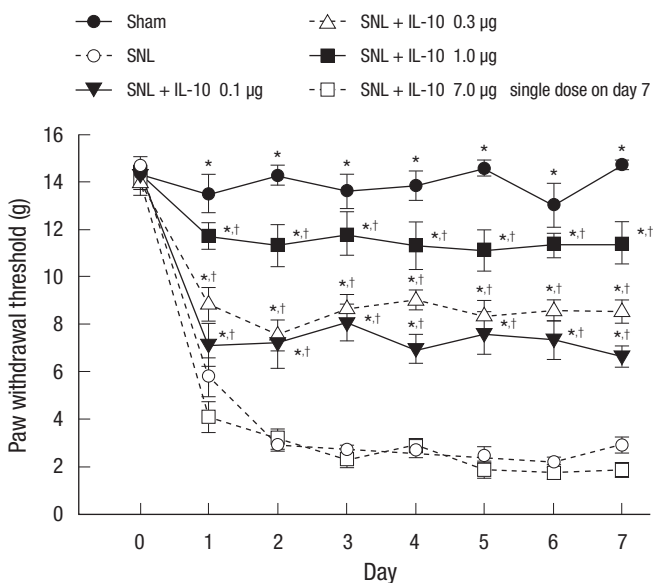


Fig. 3. Time course of paw withdrawal thresholds over the 7 day period of daily injections of various doses (0.1, 0.3, or 1 µg) of IL-10 in SNL rats (6 rats per group). A single large dose injection of IL-10 (7 µg) on day 7 does not produce an anti-allodynic effect. * $P < 0.05$ vs SNL group, $^{\dagger}P < 0.05$ vs sham group. SNL, spinal nerve ligation.

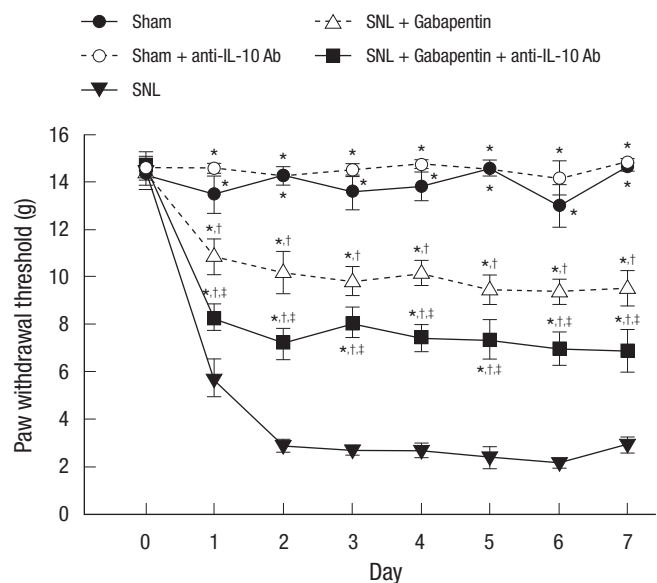


Fig. 4. Time course of paw withdrawal thresholds over the 7 day period of daily injections of anti-IL-10 antibody (10 µg) with intrathecally administered gabapentin (30 µg). * $P < 0.05$ vs SNL group, $^{\dagger}P < 0.05$ vs sham group. $^{\ddagger}P < 0.05$ vs SNL + gabapentin group. SNL, spinal nerve ligation; Ab, antibody.

istered gabapentin inhibits pro-inflammatory cytokine production and attenuates the mechanical allodynia induced by SNL. In our present analyses, daily IL-10 intrathecal injections (0.1, 0.3 and 1.0 μg) attenuated mechanical allodynia in a dose-dependent manner. However, a single large dose injection of IL-10 (7 μg , equivalent to seven daily doses of 1 μg) on day 7 failed to produce anti-allodynic effect, suggesting that both the timing and dose of IL-10 are important factors in inhibiting inflammatory responses. The anti-allodynic effect produced by daily injections of 1 μg IL-10 instead of a single injection of 7 μg IL-10 on day 7 in SNL rats strongly imply that it is the inhibiting effect of IL-10 on microglia activation and cytokine production that is responsible for the early phase of neuroinflammation induced by SNL. Accordingly, a neutralizing anti-IL-10 antibody was found to counteract the anti-allodynic effect of gabapentin in SNL rats.

Intracerebroventricular IL-10 administration has been shown to inhibit the lipopolysaccharide-induced production of TNF- α in the brain (28). It has been demonstrated that the degree of neuroinflammation induced by neuropathy in conjunction with chronic morphine administration in nerve-injured rats is higher than that induced by neuropathy alone (5). This suggests that neuroinflammation is the common mechanism in both neuropathy-induced and chronic morphine-induced glial activation. In our present study, daily anti-IL-10 antibody injections produced significant pro-inflammatory cytokine expression and attenuated the anti-allodynic effect of gabapentin in the SNL rats on day 7. Conversely, on day 7, daily IL-10 (1 μg) injections produced a significant reduction in pro-inflammatory cytokine expression compared with SNL alone rats. Taken together, the increased pro-inflammatory cytokine expression observed in SNL rats contributes to the development of allodynia induced by SNL (29) and inhibition of pro-inflammatory cytokine expression can potentiate the anti-allodynic effects of gabapentin. Based on current data, intrathecally administered gabapentin enhances IL-10 expression and counteracts SNL-induced neuroinflammation, and may be responsible for preventing neuropathic pain and inhibiting SNL-induced pro-inflammatory cytokine expression in the rat spinal cord. Further studies will be needed to elucidate the underlying mechanism of action of intrathecal gabapentin and the precise mechanism by which gabapentin induces the upregulation of IL-10 expression.

In conclusion, our present results demonstrate that intrathecally administered gabapentin attenuates mechanical allodynia in SNL rats. The effects of gabapentin may be due, at least in part, to the upregulation of anti-inflammatory cytokine IL-10 expression in the spinal cord, which leads to inhibition of the expression of pro-inflammatory cytokines TNF- α , IL-1 β , and IL-6 in SNL rat spinal cords. Consequently, our present report provides an explanation for the manner by which intrathecally administered gabapentin beneficially modulates IL-10 expression to

counter a pro-inflammatory response triggered by SNL.

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The authors declare no conflict of interest.

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