Spinal Mechanism Underlying the Antiallodynic Effect of Gabapentin Studied in the Mouse Spinal Nerve Ligation Model

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Abstract. We studied the antiallodynic effect of gabapentin (GBP) in the mouse model of neuropathic pain, aiming at clarifying the underlying mechanism. The L5 spinal nerve ligation induced tactile allodynia, an increase of CD11b expression, and an increase in the protein expression level of the voltage-dependent Ca2+ channel α2δ-1 subunit in the spinal dorsal horn on the injured side. The chronic intrathecal administration of GBP (100 μg/body per day) as well as ω-conotoxin MVIIA, an N-type Ca2+-channel blocker, completely suppressed allodynia, but did not attenuate the CD11b expression. The antiallodynic effect of GBP lasted for several days after the termination of the drug, while that of ω-conotoxin MVIIA disappeared immediately after the termination. GBP suppressed the elevation of the protein level of the α2δ-1 subunit in the spinal dorsal horn, although it did not affect its mRNA level in the L5 DRG. These results suggest that GBP inhibits the development of allodynia by suppressing the up-regulation of N-type Ca2+ channels, through normalization of the protein level of the α2δ-1 subunit at the primary afferent nerve terminal via the inhibition of its anterograde transport. In addition, we propose that the nerve injury enhances the expression level of α2δ-1 in the downstream of the activation of microglia.

Keywords: neuropathic pain, gabapentin, microglia, voltage-dependent Ca2+ channel, spinal cord

Introduction

Neuropathic pain, one of the causes of inveterate chronic pain such as post-herpetic neuralgia or diabetic neuropathy, involves structural alteration of the sensory system. The therapeutic management of neuropathic pain is an important issue, since the chronic pain impairs the quality of life of patients. However, many existing analgesics like non-steroidal anti-inflammatory drugs or opioids, which have been rendered effective against acute pain, appear to show relatively low capacity against chronic pain (1). The lack of an established valid treatment against chronic pain has motivated the clarification of molecular mechanisms underlying the development of neuropathic pain.

Various nerve-injury pain models in animals (2, 3) have been used for the development of effective therapeumatic medication or the explication of the mechanism of pain. In those nerve-injury models, microglia showed hypertrophy in 24 h after injury and started to proliferate in several days (4). The neuropathic pain was induced by the intrathecal injection of the activated microglia (4) and was suppressed by inhibiting the activation of microglia in the spinal cord (5). Therefore, we assume that the spinal microglia plays an essential role in the development of neuropathic pain.

Voltage-dependent Ca2+ channels are heteromeric complex consisting of the pore-forming α subunit, β subunit, and α2δ subunit. The α2δ subunit plays an essential role in the membrane trafficking and functional expression of the Ca2+ channels (6, 7). Among the four subtypes of the α2δ subunit family, α2δ-1 is ubiquitously expressed, while α2δ-2 is expressed mainly in brain and heart (8). In the spinal nerve ligation model, the protein level of the α2δ-1 subunit has been reported to be significantly higher in the spinal dorsal horn and dorsal root ganglion (DRG) on the injured side compared with the non-injured side (9). In addition, it has been reported that
Therefore, α2δ-1 may play an important role in the development of neuropathic pain. Gabapentin (GBP), an anticonvulsant, is known for its pain relief effect in neuropathic pain models and is considered a valid treatment in neuropathic pain patients (8, 11). Although GBP is originally a synthetic analogue to GABA, it does not have a direct effect on GABA receptors (8). GBP is a high affinity ligand for the α2δ-1 subunit (8, 12). Recently, Hendrich et al. suggested that GBP alters the function of neuronal Ca2+ channels through the impairment of the trafficking of α2δ-1 to the cell membrane (13). However, the detailed mechanism linking the development of neuropathic pain and the antiallodynic effect of GBP has not been clearly shown. In the present study, we produced a mouse neuropathic pain model by the ligation of the L5 spinal nerve and investigated the antiallodynic effect of GBP with respect to the mechanism underlying the development of neuropathic pain.

Materials and Methods

Animals

The present study was approved by the Animal Care and Use Committee of Toho University and was carried out in accordance with the Guideline “Principles for the Care and Use of Laboratory Animals Approved by The Japanese Pharmacological Society”. Male C57BL/6J mice (Charles River Laboratory, Tsukuba) weighing 19 – 24 g (6 – 7-week-old) were used. Animals were housed with a 12-h light/dark cycle (lights on at 7:00 A.M., lights off at 7:00 P.M.) at a continuous room temperature of 23°C ± 3°C and humidity of 55% ± 10% with free access to standard laboratory diet and tap water.

Neuropathic pain model

The left L5 spinal nerve of mice was tightly ligated with 8-0 silk suture under isoflurane (2%) anesthesia. In sham-operated mice, the nerve was exposed without ligation. To assess the tactile allodynia, calibrated von Frey filaments (0.008 – 1.4 g; North Coast Medical, Morgan Hill, CA, USA) were applied to the plantar surface of the hind paw. The withdrawal threshold was estimated as the smallest fiber size which evoked at least three withdrawal responses during five consecutive applications with the same fiber (14).

Intrathecal catheterization

As previously described by Yaksh and Rudy (15), intrathecal catheters were implanted in mice. A PE-5 catheter of 25-mm length (dead volume of 5 μl) was sterilized with 70% ethanol and filled with sterile saline. Under isoflurane (2%) anesthesia, the catheter was inserted through an incision in the atlanto-occipital membrane of the cisterna magna so that the tip of the catheter is placed at the lumbar enlargement. The catheter was sealed with a piece of stainless steel wire, and it was externalized behind the head for a single dose and then subcutaneously internalized for continuous administration with an osmotic minipump. After catheter implantation, mice were carefully monitored to determine whether they displayed signs of motor dysfunction (forelimb or hind limb paralysis). Only animals with no neurologic deficit after the operation were used in the experiments. After a recovery period of 6 days, animals were subjected to the examination of withdrawal thresholds for tactile stimulation and a temporary motor block of both hindlimbs after injection of 1% lidocaine for validation of the intrathecal cannulation. After those tests, the animals were subjected to the intrathecal administration of the drug.

Drug administration

Drug solutions were prepared in sterile saline before injection. For single administration, gabapentin (Toronto Research Chemicals Inc., Ontario, Canada) was injected subcutaneously or intrathecally 14 days after the spinal nerve ligation. For continuous administration, GBP or ω-conotoxin MVIIA (Peptide Institute Inc., Osaka) was injected via an osmotic minipump (Model 1007D; Alzet, Palo Alto, CA, USA) either subcutaneously or intrathecally immediately before the ligation. In some experiments, continuous administration of GBP was started 14 days after the ligation. Continuous administration of the drug was terminated by surgically removing the pump 7 days after the insertion.

Western blotting

Three days after spinal nerve ligation, the ipsilateral and contralateral dorsal areas were dissected from the L4 to L6 of the spinal cord. Each sample was homogenized in 50 mM Tris buffer, pH 7.5, containing 0.5% Triton X-100, 150 mM NaCl, 1 mM EDTA, protease inhibitors, and phosphatase inhibitors. The homogenate was centrifuged at 10,000 × g for 10 min at 4°C and the supernatant was mixed with an SDS sample buffer. Samples were separated on a 10% SDS-PAGE gel and transferred to a polyvinylidene fluoride membrane (Clearblot P; Atto, Tokyo). The blotting membranes were then blocked with 5% non-fat BSA in 25 mM Tris-HCl buffer (pH 7.5) with 0.1% Tween 20 for 1 h at room temperature and incubated overnight at 4°C with anti-α2δ-1 antibody (Alomone Labs, Jerusalem, Israel; 1:500) or anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:5,000; Chemicon International, Inc., Temecula, CA, USA) diluted in Can...
Get Signal-1 (Toyobo, Osaka). The blotting membranes were incubated for 2 h at room temperature with horse-radish peroxidase (HRP)-conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ, USA); diluted 1:25,000 in Can Get Signal-2 (Toyobo); developed in ECL solution (Amersham Biosciences) for 5 min; and exposed onto hyperfilms (Amersham Biosciences).

Polymerase chain reaction

Samples were immediately stored in an RNAlater stabilizing solution (Ambion, Austin, TX, USA) and kept at 4°C until RNA extraction. Total RNA for reverse transcription polymerase chain reaction (RT-PCR) was extracted with the RNeasy mini kit (Qiagen, Valencia, CA, USA). The total RNA was heat-denatured at 65°C for 5 min and reverse-transcribed into cDNA using Superscript III (Invitrogen, Carlsbad, CA, USA). Quantitative real-time RT-PCR (qPCR) reactions were performed using TaqMan gene expression assays (Applied Biosystems, Appl.) and the appropriate primer sets (custom synthesized by Sigma Genosys, Ishikari) for the voltage-dependent Ca²⁺ channel α₁ subunit. The condition for PCR amplification was as follows: the initial denaturation reaction at 94°C for 3 min and reverse-transcribed into cDNA using Superscript III (Invitrogen, Carlsbad, CA, USA). The total RNA was heat-denatured at 65°C for 5 min and reverse-transcribed into cDNA using Superscript III (Invitrogen, Carlsbad, CA, USA). Quantitative real-time RT-PCR (qPCR) reactions were performed using TaqMan gene expression assays (Applied Biosystems, Foster City, CA, USA). The following TaqMan primers and probes were used: Mm00486607_m1 (α₁δ), Mm00432142_m1 (C1q), and Mm00446968_m1 (Hprt1). The cycle threshold values were calculated automatically by a 7500 Software (Applied Biosystems). The amount of each mRNA was normalized to that of the co-amplified hypoxanthine guanine phosphoribosyl transferase (HPRT).

The semi-quantitative RT-PCR was performed using ExTaq (Takara, Shiga) and the appropriate primer sets (custom synthesized by Sigma Genosys, Ishikari) for the voltage-dependent Ca²⁺ channel α₁ subunit. The condition for PCR amplification was as follows: the initial denaturation reaction at 94°C for 3 min was followed by 40 cycles of denaturation (94°C for 30 s), annealing (56°C for 30 s), and extension (72°C for 1 min). The final extension was prolonged to 10 min. The sequences of primers for mouse α₁ were as follows: 5'-ACG-ACC-TCT-AAA-GAC-CAT-CAA-GC-3' and 5'-CCA-CAG-CGA-AGA-TGA-ACA-TGA-ACA-3' for Ca₅.1 α₁ (P/Q-type), 5'-TGG-CTC-CTT-CTT-CAT-GCT-CAA-CCT-GG-3' and 5'-TCG-GCT-CTT-GGT-GGC-AGC-TCT-CT-3' for Ca₅.2.1 α₁ (N-type), and 5'-AGA-TGG-GAG-GCC-ATC-ACA-TT-3' and 5'-CAA-ATC-CAA-CAA-AGT-CTG-GC-3' for HPRT. The size of target amplicons was confirmed by electrophoresis of PCR products on 2% agarose gels in TAE buffer. Densitometric analysis was carried out using ImageJ software (http://rsb.info.nih.gov/ij/).

Immunohistochemistry

Animals were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused transcardially with 20 mL of 0.2% heparin in phosphate-buffered saline (PBS, pH 7.2) followed by 20 ml of 4% paraformalde-hyde phosphate buffer solution (Wako, Osaka) on day 3 or 7 after the spinal nerve ligation. The L5 spinal cord was removed and postfixed at 4°C for 2 h and then transferred to 20% sucrose in PBS for 24 h. The spinal cord was horizontally sectioned with a freezing microtome at a thickness of 60 μm. For the detection of microglia, floating sections were incubated with rat anti-mouse CD11b monoclonal antibody (R&D Systems, Minneapolis, MN, USA) at a dilution of 1:1,000 containing 2% normal donkey serum (NDS) for 2 days at 4°C. After washing, the sections were incubated with goat anti-rat secondary antibody conjugated to Alexa Fluor 555 (Invitrogen, 1:2,000) containing 2% NDS for 2 h at room temperature.

For the detection of the α₁δ-1 subunit, sections underwent heat-induced antigen retrieval (10 mM citrate buffer, pH 6.0, 0.05% Tween 20, 95°C for 10 min) before blocking with 10% NDS in the presence of 0.1% Triton X-100 in PBS for 1 h (16). To detect α₁δ-1, sections were incubated with the mouse monoclonal anti-α₁δ-1 antibody (Sigma-Aldrich, St. Louis, MO, USA; 1:100) in PBS containing 5% NDS and 0.05% Triton X-100 for 48 h at 4°C. After washing with PBS containing 0.1% Triton X-100, sections were incubated with biotinylated goat anti-mouse IgG (Bethyl Laboratories, Montgomery, TX, USA; 1:500) for 2 h and streptavidin-Alexa Fluor 488 (Invitrogen, 1:500) for 1 h at room temperature. Then these labeled sections were treated with Gel Mount (Biomeda, Foster City, CA, USA) and coverslipped. Fluorescent images were obtained with a laser scanning confocal microscope (LSM-510 META or LSM-510 Pascal, from Carl Zeiss, Jena, Germany or A1Rsi Ti-E, from Nikon, Tokyo) and analyzed with LSM Image Browser (Carl Zeiss) or NIS-Elements (Nikon).

Statistical analyses

All data are presented as the mean ± S.E.M. The Stat-Light software package (Yukms, Tokyo) was used for analysis of data. Student’s t-test was employed for comparisons between 2 animal groups, and Dunnett’s multiple comparison test was used for comparison of 3 or more groups. A P-value of less than 0.05 was considered to be statistically significant.

Results

Expression of hypersensitive pain reaction to tactile stimuli and increase of CD11b expression induced by spinal nerve ligation

We produced a neuropathic pain model by tightly ligating the L5 spinal nerve on the left side in mice. After the ligation, we observed a decrease of the threshold for
tactile stimuli (allodynia) at the hind paw of the affected side. The threshold reached the minimum level a week after ligation and maintained that level for several weeks, and then it recovered in approximately 10 weeks (Fig. 1A). In contrast, no change of the threshold for pain sensation was observed in the untreated hind paw of the ligated animal or the sham operated animals (Fig. 1B).

Next, we estimated the CD11b expression in the spinal L5 section under immunofluorescence microscopy and detected an increase of CD11b immunofluorescence in the dorsal and ventral horns on the ligated side (Fig. 1C). It is generally known that L5 spinal nerve injury causes the up-regulation of CD11b expression in both the dorsal and ventral horns because the L5 spinal nerve ligation also damages not only primary afferent neurons but also nerve axons from motor neurons (17). Therefore, these results suggest that immunohistochemical experiments were performed in L5 segments of the spinal cord.

**Effects of subcutaneous administration of GBP on the induction of allodynia and increase of CD11b expression induced by spinal nerve ligation**

The allodynia reaction to tactile stimuli was stable for 14 days after the spinal nerve ligation. A subcutaneous bolus administration of GBP at 50 mg/kg on the 14th day of the spinal nerve ligation showed transient pain relief effects (Fig. 2A). Ninety minutes after the administration, the pain threshold recovered to the pre-ligation level. However, a slight effect was also observed on the non-ligated side. In addition, with a subcutaneous con-
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Continuous administration of GBP (1.2 mg/body per day, the maximum possible dosage for GBP solubility) for 7 days, a significant rise of the pain threshold was observed, which disappeared within 24 h after the termination of the administration (Fig. 2B). In contrast, when the chronic subcutaneous infusion of GBP was started immediately prior to the ligation of the spinal nerve, it effectively suppressed the development of hyperalgesia induced by the ligation (Fig. 2C). Continuous infusion of GBP raised the threshold for the pain response to a significantly higher level, compared to the control group, even 7 days after the ligation. Interestingly, after the termination of the GBP administration, the pain threshold continued to be significantly higher than that of the saline control for several days. In the dorsal horn on the ligated side, microglia were transformed to the activated phenotype that is characterized by hypertrophied soma bearing short and thick processes (Fig. 3A). In contrast, in the dorsal horn on the non-ligated side, microglia have a small soma bearing thin and branched processes and are homogeneously distributed. The intensity of CD11b immunofluorescence in the dorsal horn on the ligated side was enhanced to approximately 1.7- and 1.9-fold of the non-ligated side when measured 3 and 7 days after ligation, respectively (Fig. 3: A and B). However, the CD11b expression in the spinal dorsal horn was not attenuated by the continuous administration of GBP (1.2 mg/body per day), given in the same protocol as shown in Fig. 2C, when examined 3 and 7 days after the ligation (Fig. 3: A and B).

**Effect of GBP on the α2δ-1 expression in the DRG and the spinal dorsal horn**

Since GBP is known as a ligand for the voltage-dependent Ca2+ channel α2δ subunit, we examined the effects of the continuous administration of GBP on the expression level of the α2δ-1 subunit in the dorsal horn of L5 spinal cord. The expression of the α2δ-1 subunit in the L5 spinal dorsal horn, examined by immunohistological analysis 3 days after spinal nerve ligation, was increased in superficial layers of the dorsal horn on the ligated side. In contrast, the continuous intrathecal administration of GBP (100 μg/body per day) suppressed the increase of α2δ-1 immunofluorescence (Fig. 4: A and B). Western blot analysis also showed that α2δ-1 subunit protein expression was enhanced by approximately 60% as compared to the non-ligated side (Fig. 5A). GBP reversed the enhanced expression of α2δ-1 subunit protein in the L5 spinal dorsal horn (Fig. 5A). In accordance with these results, the mRNA level of the α2δ-1 subunit was significantly elevated in the L5 DRG on the ligated side (Fig. 5B). However, in L5 spinal dorsal horn, the mRNA level of the α2δ-1 subunit was unchanged between the ligated

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Fig. 2. Effect of subcutaneous administration of gabapentin (GBP) on the mechanical allodynia induced by spinal ligation in mice. A: GBP (50 mg/kg) was subcutaneously injected 14 days after the spinal nerve ligation. Each point represents the mean ± S.E.M. of 5 mice. B: Subcutaneous infusion of GBP (1.2 mg/body per day) via an osmotic minipump was started on the 14th day after the spinal nerve ligation and continued for 7 days. The horizontal arrow indicates the duration of GBP infusion. Each point represents the mean ± S.E.M. of 4 – 5 mice. C: Subcutaneous infusion of GBP (1.2 mg/body per day) via an osmotic minipump was started just before the spinal nerve ligation and continued for 7 days. The horizontal arrow indicates the duration of GBP perfusion. Each point represents the mean ± S.E.M. of 7 mice.*P < 0.05, **P < 0.01, compared with the saline control group (Student’s t-test).
and non-ligated side (Fig. 5C). These results indicate that the ligation of L5 spinal nerve promoted the expression of the $\alpha_2/\delta-1$ subunit in L5 DRG neurons and their downstream presynaptic nerve terminals of the primary afferent neurons in the dorsal horn without changing the expression level in the spinal interneurons. In contrast, GBP did not affect the mRNA levels of the $\alpha_2/\delta-1$ subunit in the L5 DRG and dorsal horn on the ligated side at all (Fig. 5: B and C), thus suggesting that GBP alters the transport of the $\alpha_2/\delta-1$ subunit from the DRG to the primary afferent nerve terminals rather than its transcription.

We also studied the mRNA levels of voltage-dependent Ca$^{2+}$ channel $\alpha_1$ subunits in the L5 DRG. The mRNA levels of $\alpha_1$ subunits of the N-type and P/Q-type Ca$^{2+}$ channels in the L5 DRG were not significantly different between the ligated and contralateral sides (Fig. 5D).

**Effect of intrathecal administration of GBP on the tactile allodynia induced by spinal nerve ligation**

In order to specify the target of GBP, we examined effects of the intrathecal bolus administration of GBP. When the allodynia response was examined 14 days after the spinal nerve ligation, the intrathecal administration of GBP (10 – 100 $\mu$g/body) exhibited dose-dependent pain relief effects, which peaked at 90 – 120 min after the administration (Fig. 6A). However, it did not cause any effects on the pain threshold of the non-ligated side (data not shown). Next, the chronic intrathecal administration of GBP (100 $\mu$g/body per day), which started immediately prior to the spinal nerve ligation and continued for 7 days, suppressed the pain expression almost completely. The antiallodynic effect of the intrathecal administration of GBP was more prominent than that of the subcutaneous administration (compare Figs. 2C and 6B). After the termination of the administration, the pain threshold
continued to be significantly higher than that of the saline control for 5 days (Fig. 6B).

**Effect of the intrathecal administration of ω-conotoxin MVIIA on tactile allodynia induced by spinal nerve ligation**

Based on the fact that GBP suppressed the elevated expression of the α2/δ-1 subunit induced by nerve ligation in the spinal dorsal horn, we paid attention to the voltage-dependent N-type Ca2+ channel that is expressed in the nerve terminal of primary afferent neurons. Hence, we examined the effects of intrathecal continuous administration of ω-conotoxin MVIIA (0.08 or 0.24 μg/body per day), an N-type Ca2+-channel blocker, on the expression process of the tactile allosthesia following the spinal nerve ligation. The 7-day long continuous administration of ω-conotoxin MVIIA, started immediately prior to the ligation, was found to exert a dose-dependent suppressive effect on the allodynia response. The continuous administration of ω-conotoxin MVIIA at 0.24 μg/body per day almost completely suppressed the allodynia response (Fig. 7). On the other hand, in the group treated with 0.08 μg/body per day, a gradual decline of the pain threshold was observed, although it strongly suppressed the pain expression process following the ligation. After termination of the continuous administration of ω-conotoxin MVIIA, the threshold for the allodynia response promptly receded to the same levels as that in the control group within 1 day even in the 0.24 μg/body per day–treatment group, which was in sharp contrast to the sustaining effect of GBP (Fig. 6B).

GBP and ω-conotoxin MVIIA exert antiallodynic effect under significant increase of CD11b expression

Even though the intrathecal continuous administration of ω-conotoxin MVIIA (0.24 μg/body per day) completely suppressed the allodynia response, the ligation-induced increase of CD11b expression in the spinal dorsal horn was not affected at all (Fig. 8: A and B). Similarly, the intrathecal continuous administration of GBP (100 μg/body per day) also completely suppressed the allodynia response without significant effect on CD11b expression.

The activation of microglia was also evaluated as the increase of the mRNA level of microglial marker (complement component 1q, C1q) (18). The spinal nerve ligation induced the increase of the mRNA level of C1q in the dorsal horn and L5 DRG on the ligated side. However, the continuous administration of GBP exhibited no effects on the mRNA level of C1q (Fig. 8: C and D).

**Discussion**

GBP has been reported to exert analgesic effects in neuropathic pain models (8, 9, 19), and its efficacy has been shown in clinical chronic pain (8, 20). GBP has been shown to modify central sensitization at the spinal cord level (8). Unlike most other anticonvulsants, GBP does not exhibit any Na+ channel–blocking effect nor any inhibitory effect on the ectopic firing in peripheral nerves in rat neuropathic pain models (8, 21). GBP is considered to exert the analgesic effect through its binding to the Ca2+ channel α2/δ-1 subunit because the antiallodynic effect was completely lost in the knock-in mice expressing a mutated α2/δ-1 subunit that does not bind to GBP or pregabalin (22). The α2/δ-1 subunit has been shown to enhance the Ca2+-channel current when co-expressed...
with the pore forming \( \alpha_1 \) subunit (6) and to be involved in the membrane trafficking of the \( \alpha_1 \) subunit (7, 23). An increase in the expression of the \( \alpha_2/\delta-1 \) subunit in the spinal cord and DRG after nerve injury (9, 16) implies the involvement of the voltage-dependent Ca\(^{2+} \) channel of the primary afferent nerve terminal in the development of tactile allodynia. However, the role of voltage-dependent Ca\(^{2+} \) channels in the activation of microglia and the development of allodynia has not been clearly shown. We therefore elucidated the role of the Ca\(^{2+} \) channel through the investigation of the spinal mechanism for the antiallodynic effects of GBP.

We established a mouse L5 spinal nerve ligation model and quantified the tactile allodynia induced by the nerve injury. In this model, the pain response reached the peak 7 days after the ligation. We confirmed that the increase of CD11b expression and the morphological change in microglia coincides with the expression of the pain response in the spinal dorsal horn on the ligated side. By use of this model, we investigated the effect of GBP on the development of the neuropathic pain.

In this study, GBP administrated prior to the nerve injury strongly suppressed the initial process of pain development in the mouse L5 spinal ligation model. However, when administrated after the pain response was established, GBP exhibited only a weak analgesic effect. The
continuous intrathecal administration of GBP was more effective compared to subcutaneous or intraperitoneal administration. Interestingly, we found that the continuous administration of GBP continued to alleviate neuropathic pain for several days after the termination of the administration, which was in contrast to a single dose of GBP that exhibited a transient analgesic effect and disappeared in a few hours. We demonstrated that GBP suppressed the increase in the protein expression level of the α₂δ-1 subunit in the superficial spinal dorsal horn on the injured side that receives the primary afferent nerve input. In addition, we showed that the mRNA level of the α₂δ-1 subunit was increased in the L5 DRG, but not in the dorsal horn, on the injured side, and that GBP exhibited no effect on the mRNA levels of the α₂δ-1 subunit. We therefore assume that the elevation of the α₂δ-1 content in the dorsal horn reflects an increase in the α₂δ-1 level in the primary afferent nerve terminal. These results indicate that GBP exerts inhibitory effects on the developmental process of tactile allodynia that involves the membrane trafficking or the axonal transport of the α₂δ-1 subunit from L5 DRG to the primary afferent nerve terminal. Bauer et al. have reported that spinal nerve ligation induced the accumulation of the α₂δ-1 subunit protein at the ligation site and the presynaptic terminals in the spinal dorsal horn by anterograde trafficking from the DRG to both peripheral and central nerve terminals, which was inhibited by the chronic treatment with pregabalin (16). Our results indicate that GBP normalizes the α₂δ-1 protein level in the spinal dorsal horn by inhibiting the transport of the α₂δ-1 subunit from the DRG to the primary afferent nerve terminal. In accordance with these results, the antiallodynic effect of GBP sustained several days after the termination of the administration, indicating that several days were required for the recovery of the transport of the α₂δ-1 subunit from the DRG to the primary afferent nerve terminal.

Fig. 6. Effect of the intrathecal administration of GBP on the mechanical allodynia induced by spinal nerve ligation in mice. A: GBP (10, 30, and 100 μg/body) was intrathecally injected 14 days after spinal nerve ligation. Each point represents the mean ± S.E.M. of 5 mice. *P < 0.05, **P < 0.01, compared with the saline control (Dunnnett’s multiple comparison test). B: Intrathecal infusion of GBP (100 μg/body per day) via an osmotic minipump was started just before spinal nerve ligation and continued for 7 days. The horizontal arrow indicates the duration of GBP infusion. Each point represents the mean ± S.E.M. of 5–6 mice. ***P < 0.001, compared with the saline control group (Student’s t-test).

Fig. 7. Effect of intrathecal infusion of the N-type Ca²⁺-channel inhibitor ω-conotoxin MVIIA on the mechanical allodynia induced by spinal nerve ligation in mice. Intrathecal infusion of ω-conotoxin MVIIA via an osmotic minipump was started just before spinal nerve ligation and continued for 7 days. The horizontal arrow indicates the duration of ω-conotoxin MVIIA infusion. Each point represents the mean ± S.E.M. of 4 mice. **P < 0.01, compared with the saline control group (Dunnnett’s multiple comparison test).
nerve terminal.

The intrathecally administered drug may reach not only the spinal cord but also the supraspinal region via cerebrospinal fluid. It has been shown that a supraspinal mechanism, such as the activation of the descending noradrenergic neuron, is involved in the analgesic effect of GBP in the neuropathic pain model (24). We do not exclude the possibility that GBP alters the expression level of the α2/δ-1 subunit in the supraspinal neurons. Such a possibility would be elucidated in future studies.

N-type Ca\(^{2+}\) channels are expressed mainly in the surface layer (laminae I and II) of the spinal dorsal horn and DRG and are involved in the release of neurotransmitters such as glutamate, CGRP, and substance P from

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**Fig. 8.** Effects of intrathecal infusion of GBP and ω-conotoxin MVIIA on the increase of CD11b expression induced by spinal nerve ligation in the spinal dorsal horn. Intrathecal infusion of GBP (100 μg/body per day) or ω-conotoxin MVIIA (0.24 μg/body per day) via an osmotic minipump was started just before spinal nerve ligation and continued for 3 or 7 days. A: The CD11b expression in the L5 spinal dorsal horn of the GBP- or ω-conotoxin MVIIA–treated group examined 7 days after spinal nerve ligation. B: The CD11b expression in the ligated side (ipsi) was quantified as the relative change of the fluorescent intensity in comparison with the non-ligated side (contra). Each bar represents the mean ± S.E.M. of sections from 4 mice. **P < 0.01, compared with the non-ligated side (Student’s t-test). C, D: Real-time RT-PCR analysis of the mRNA level of complement component 1q (C1q) in the L5 DRG (C) and L5 spinal cord (D) examined 3 days after the spinal nerve ligation. The mRNA level of C1q mRNA was normalized to that of HPRT. Each bar represents the mean ± S.E.M. of 6 mice. *P < 0.05, **P < 0.01, compared with the non-ligated side (Student’s t-test).
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the primary afferent nerve terminal (25). The protein expression of N-type Ca\(^{2+}\) channel has been reported to be increased after a nerve injury in the DRG and the surface layer of the spinal dorsal horn (25). In addition, the expression of neuropathic pain was impaired in N-type Ca\(^{2+}\) channel–knock-out mice (25, 26). In the post-operative pain model, \(\omega\)-conotoxin MVIIA, an N-type Ca\(^{2+}\)-channel blocker, but not other blockers (L-, T-, and P/Q-type Ca\(^{2+}\)-channel blocker), significantly attenuated the post-incision induced allodynic response (8, 27). Thus, we hypothesized that the \(\alpha_2/\delta-1\) subunit consisting of the N-type Ca\(^{2+}\) channel could be the final target of the antiallodynic effect of GBP and studied the analgesic effect by inhibiting the N-type Ca\(^{2+}\)-channel activity at the spinal cord level in the early phase of the development of neuropathic pain. The intrathecal infusion of \(\omega\)-conotoxin MVIIA was found to strongly inhibit the development of tactile allodynia, thus indicating that N-type Ca\(^{2+}\) channels are largely responsible for the allodynia induced by nerve injury.

However, the analgesic effect of \(\omega\)-conotoxin MVIIA disappeared immediately after termination of the administration, which was in contrast to GBP that exhibited a sustained antiallodynic effect for several days after termination of the administration. This may be because \(\omega\)-conotoxin MVIIA blocks the N-type Ca\(^{2+}\) channel, but does not affect the number of functional N-type Ca\(^{2+}\) channels.

Since the time course of the activation of microglia matches that of the expression of neuropathic pain (28), the activation of microglia in the spinal dorsal horn is considered to be an essential factor in the initial stages of the pain development. Accordingly, we examined the effect of continuous administration of GBP on the increase of CD11b expression induced by spinal nerve ligation. As a result, although GBP showed no effect on the CD11b expression in the spinal cord, it completely suppressed the neuropathic pain. It has been reported that the administration of bupivacaine, a Na\(^+-\)channel blocker, inhibited nerve conduction and the activation of microglia induced by nerve-injury (29). In contrast, we have clearly shown that although the N-type Ca\(^{2+}\)-channel blocker suppressed neuropathic pain, it did not suppress either the CD11b expression or the nerve conduction. Therefore, we conclude that the enhanced N-type Ca\(^{2+}\)-channel activity in the primary afferent nerve terminal is not responsible for the activation of microglia.

Based on our findings, we conclude that 1) spinal nerve injury induces the activation of microglia, which somehow gives rise to the elevation of the expression of \(\alpha_2/\delta-1\) and the N-type Ca\(^{2+}\)-channel activity in the primary afferent nerve terminal in the spinal dorsal horn, and then causes the neuropathic pain; 2) GBP suppresses the development of allodynia, when administered immediately after the nerve injury, through the suppression of the up-regulation of voltage-dependent N-type Ca\(^{2+}\) channels by normalizing the expression level of the \(\alpha_2/\delta-1\) subunit at the primary afferent nerve terminal in the spinal dorsal horn via inhibition of its axonal transport (Fig. 9). Further studies are to be carried out for clarifying the mechanisms linking the activation of microglia and the elevation of the protein expression level of the \(\alpha_2/\delta-1\) subunit.

The mRNA level of the \(\alpha_2/\delta-1\) subunit was elevated in the DRG, but not spinal dorsal horn, on the ligated side. However, the protein expression level of the \(\alpha_2/\delta-1\) subunit was elevated in the spinal dorsal horn. The mRNA level of C1q, a marker gene of the activated microglia, was elevated in both the DRG and spinal dorsal horn on the ligated side, which was consistent with the proliferation and hypertrophy of microglia observed with immunofluorescent staining with anti-CD11b antibody. The continuous administration of GBP suppressed the expression level of \(\alpha_2/\delta-1\) subunit protein, but not mRNA, in the spinal dorsal horn. In sharp contrast, GBP exhibited no effects on the activation of microglia evaluated as the C1q mRNA level and the anti-CD11b immunofluorescent staining. These results indicate that GBP prevents the transport of the \(\alpha_2/\delta-1\) subunit from the DRG to the primary afferent nerve terminals without affecting the activation of microglia.
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