The Novel Gabapentinoid Mirogabalin Prevents Upregulation of $\alpha_2\delta$-1 Subunit of Voltage-Gated Calcium Channels in Spinal Dorsal Horn in a Rat Model of Spinal Nerve Ligation

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ABSTRACT

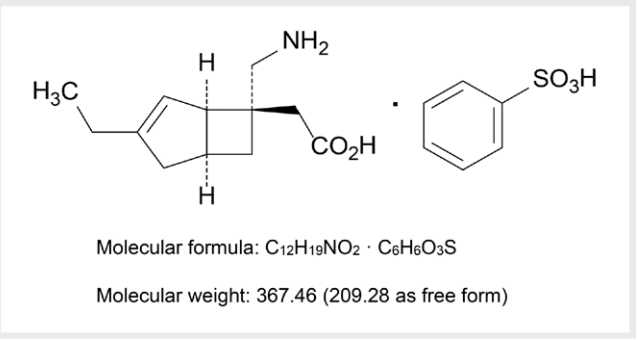
Gabapentinoids are specific ligands for the $\alpha_2\delta$-1 subunit of voltage-gated calcium channels. This class of drugs, including gabapentin and pregabalin, exert various pharmacological effects and are widely used for the treatment of epilepsy, anxiety, and chronic pain. The mechanism of action of gabapentinoids involves both direct modulation of calcium channel kinetics and inhibition of channel trafficking and expression, which contribute to the above pharmacological effects. In the present study, we investigated the effects of mirogabalin, a novel potent gabapentinoid, on expression levels of the $\alpha_2\delta$-1 subunit in the spinal dorsal horn in a rat model of spinal nerve ligation (SNL) as an experimental animal model for peripheral neuropathic pain. The neuropathic pain state was induced by SNL in male Sprague – Dawley rats. After the development of mechanical hypersensitivity, the animals received 10 mg/kg mirogabalin or vehicle orally for 5 consecutive days and were subjected to immunohistochemical analysis of $\alpha_2\delta$-1 subunit expression in the spinal cord. In the SNL model rats, expression of the $\alpha_2\delta$-1 subunit significantly increased in the spinal dorsal horn at the ipsilateral side of nerve injury, while mirogabalin inhibited this increase. In conclusion, the $\alpha_2\delta$-1 subunit was upregulated in the spinal dorsal horn of SNL model rats, and repeated administration of mirogabalin inhibited this upregulation. The inhibitory effect of mirogabalin on upregulation of the $\alpha_2\delta$-1 subunit after nerve injury is considered to contribute to its analgesic effects in peripheral neuropathic pain.

Introduction

Voltage-gated calcium channels (VGCCs) consist of four subunits: the pore-forming α_1 subunit and three auxiliary $\alpha_2\delta$, β, and γ subunits [1]. The $\alpha_2\delta$ subunit has four distinct isoforms (i. e., $\alpha_2\delta$-1, -2, -3, and -4), with the $\alpha_2\delta$-1 subunit in particular playing important roles in several neurological disorders [2, 3]. For instance, the upregulation of $\alpha_2\delta$-1 mRNA and protein has been reported in various experimental animal models of neuropathic pain [4–15], anxiety [16], and epilepsy [17]. Transgenic mice with overexpression of the $\alpha_2\delta$-1 subunit in neurons have been reported to show mechanical and thermal hypersensitivity [18] or epileptic seizures [19]. The $\alpha_2\delta$-1 subunit is the molecular target for gabapentinoids such as gabapentin and pregabalin [20, 21], and pharmacological studies with $\alpha_2\delta$ mutant mice have demonstrated that the analgesic, anxiolytic, and anticonvulsant effects of gabapentinoids are mediated via their specific binding to the $\alpha_2\delta$-1 subunit rather than the $\alpha_2\delta$-2 subunit [22–25]. Although the mechanism of action of gabapentinoids remains to be completely elucidated, it involves both direct modulation of calcium channel kinetics and inhibition of channel trafficking and expression, which result in the inhibition of calcium ion influx and excitatory synaptic transmission at synaptic endings [26–28]. Furthermore, recent studies have suggested the additional mechanism of action of gabapentinoids based on novel roles of the $\alpha_2\delta$-1 subunit, such as interaction with throm-

bospondins and NMDA receptors, not VGCCs [29–31]. Mirogabalin {[(1*R*,5*S*,6*S*)-6-(aminomethyl)-3-ethylbicyclo[3.2.0]hept-3-en-6-yl]acetic acid} is a newly synthesized gabapentinoid [32], which has been approved for the treatment of neuropathic pain in Japan and other Asian countries [25, 33, 34]. Three pivotal phase 3 clinical trials of mirogabalin demonstrated its efficacy and safety in patients with postherpetic neuralgia [35, 36], diabetic peripheral neuropathic pain [37, 38], and central neuropathic pain after spinal cord injury [39]. We previously reported that mirogabalin possessed potent and selective binding affinity for $\alpha_2\delta$-1 subunit [32] and inhibited N-type calcium channel currents [40], and it showed more potent and sustained analgesic effects than pregabalin in experimental animal models of peripheral and central neuropathic pain [32, 41] and fibromyalgia [42]. We also reported that analgesic doses of mirogabalin alleviated anxiety-like behaviors and cognitive impairments in chronic pain models of neuropathic pain and fibromyalgia [43–45].

Here, to obtain further information on the mechanism of action of mirogabalin, we investigated its inhibitory effects on upregulation of the $\alpha_2\delta$-1 subunit of VGCCs in the spinal dorsal horn in a rat model of spinal nerve ligation (SNL) as an experimental animal model for peripheral neuropathic pain.



▶ **Fig. 1** Chemical structure of mirogabalin besylate.

Materials and Methods

Test compounds

Mirogabalin besylate (DS-5565, CAS number: 1138245–21–2, PubChem CID: 81689826) was synthesized by Daiichi Sankyo Co., Ltd. (Tokyo, Japan). The test compound was dissolved in JP-grade distilled water (Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) and administered at an oral dose of 10 mg/2 mL/kg (expressed as free form). The control groups (SNL model control and sham control) were administered with an equal amount of JP-grade distilled water. The dosing protocol of the test compound (dose level, volume, route, frequency, and period) was determined based on our previous study [32]. The chemical structure of mirogabalin besylate is shown in ▶ **Fig. 1**.

Experimental animals

The male Slc:SD rats (Japan SLC, Inc., Shizuoka, Japan) used in this study were 6 weeks old at the time of surgery. The animals were housed under conditions of regulated temperature (23 ± 2 °C) and relative humidity (55 ± 10 %) in a room with a 12-h day/night cycle (lights on 07:00–19:00 h). A standard laboratory diet (FR-2; Funabashi Farm Co., Ltd., Chiba, Japan) and tap water were available *ad libitum*. All experimental procedures were carried out in compliance with the Basic Guidelines for the Use of Experimental Animals in Institutions under the Jurisdiction of the Ministry of Health, Labour and Welfare (Notification No. 0601001 of the Science Bureau, Japanese Ministry of Health, Labour and Welfare, June 1, 2006) and the Guidelines of the Institutional Animal Care and Use Committee of Daiichi Sankyo Co., Ltd.

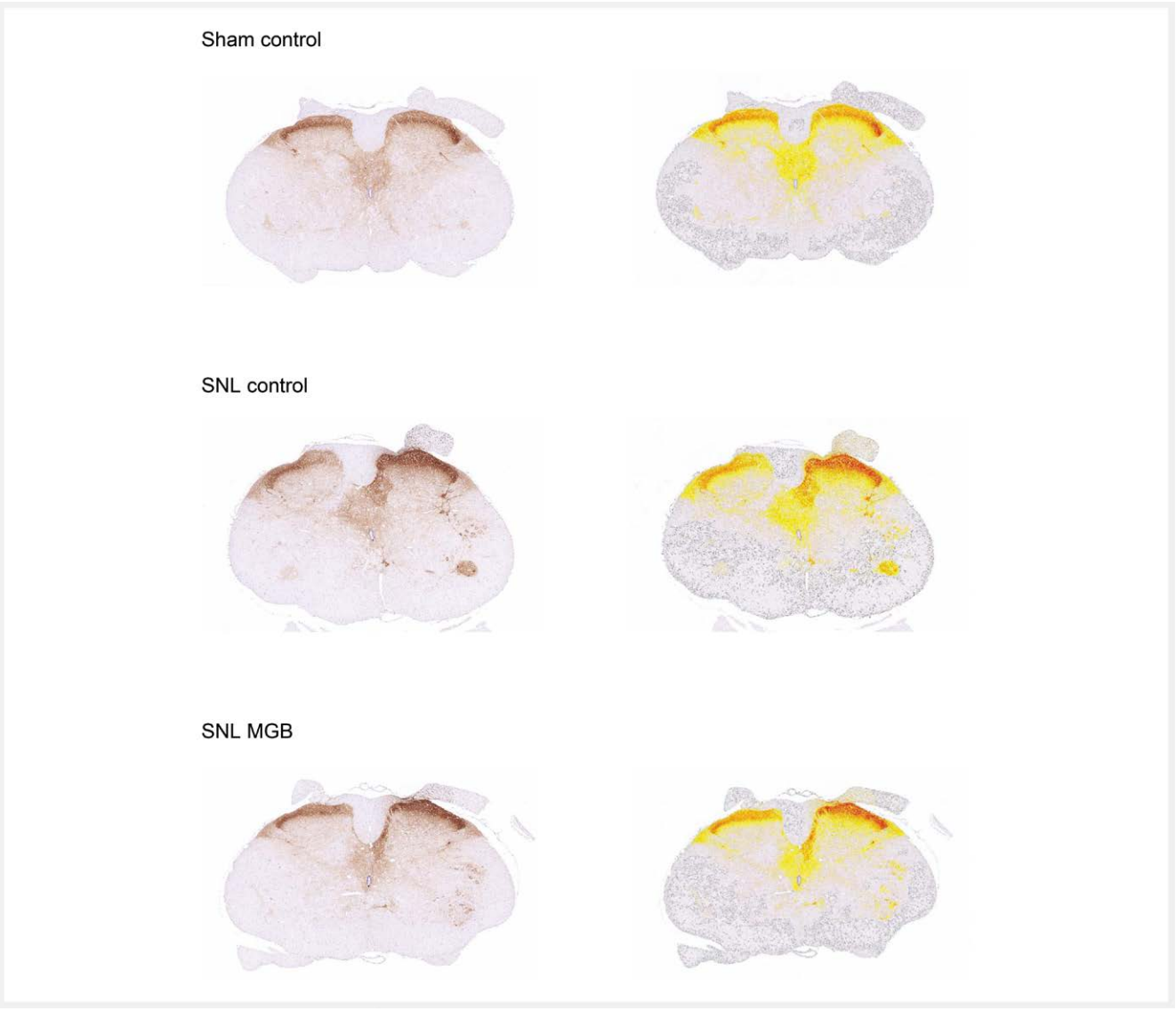
Experimental design

The SNL model was prepared in accordance with the method of Kim and Chung [46], with minor modifications. Briefly, under 1.5–2.0 % isoflurane anesthesia (Pfizer Japan Inc., Tokyo, Japan), the animals were placed in a prone position. The skin on the left lower back was incised and the transverse process of the lumbar vertebrae (L6) was exposed and removed. The left L5 and L6 spinal nerves were isolated and tightly ligated with 6–0 surgical threads. The surgical area was sutured and 5 mg/kg enrofloxacin (Kyoritsu Seiyaku Corp., Tokyo, Japan) was subcutaneously injected for postoperative infection control. The animals received subcutaneous injection of 0.05 mg/kg buprenorphine hydrochloride (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) for 2 days after surgery. Sham operation was conducted in the same manner, except that the L5 and L6 spinal nerves were not ligated.

Two weeks after SNL surgery, the development of mechanical hypersensitivity (i. e., neuropathic pain) was confirmed using the von Frey test. In brief, the plantar region of the left hind paw was stimulated with von Frey filaments (North Coast Medical Inc., Gilroy, CA, USA), and the paw withdrawal threshold was measured as described in our previous reports [42, 44, 45]. SNL model rats with a paw withdrawal threshold of 4 g or lower were selected and randomly assigned to two treatment groups of seven animals each. The SNL rats received the test compound (mirogabalin at 10 mg/2 mL/kg) or vehicle (JP-grade distilled water at 2 mL/kg) orally for 5 consecutive days (twice-daily from day 1 to day 4, and once-daily on day 5). As a normal control group, four sham-operated rats received the vehicle in the same manner. After the last administration on day 5, the animals were subjected to immunohistochemical analysis. Because the potent and sustained analgesic effects of mirogabalin in neuropathic pain model rats have already been confirmed under the above dosing protocol [32], we focused on changes in expression levels of the $\alpha_2\delta$-1 subunit of VGCCs in the spinal dorsal horn in the present study.

Immunohistochemistry

Under combined anesthesia involving the intraperitoneal injection of 0.3 mg/kg medetomidine hydrochloride (Kyoritsu Seiyaku Corp.), 4 mg/kg midazolam (Maruishi Pharmaceutical Co., Ltd., Osaka, Japan), and 5 mg/kg butorphanol tartrate (Meiji Seika Pharma Co., Ltd., Tokyo, Japan), the animals were transcardially perfused with heparinized saline solution (2000 units/L; heparin sodium injection, Mochida Pharmaceutical Co., Ltd., Tokyo, Japan; and JP-grade normal saline, Otsuka Pharmaceutical Factory, Inc.), followed by 4 % paraformaldehyde phosphate buffer solution (4 % PFA; Fujifilm Wako Pure Chemical Corporation, Osaka, Japan). Following



▶ **Fig. 2** Typical examples of original-scanned (left) and pseudo-colored (right) images of the spinal cord sections.; Top: Sham control (animal No. 2), Middle: SNL control (animal No. 5), Bottom: SNL mirogabalin (animal No. 12).

perfusion, the lumbar spinal cord at the L5 level was removed and post-fixed in 4 % PFA at room temperature overnight. The 4 % PFA-fixed spinal cord tissues were routinely processed and embedded in paraffin. Tissue sections of 3 µm thickness were prepared and immunohistochemistry was performed using PT Link 100 Pretreatment Module and Autostainer Link 48 (Dako/Agilent, Santa Clara, CA, USA). Sections were heated at 60 °C for 60 min, and then heat-induced antigen retrieval was carried out using Target Retrieval Solution High pH (Dako/Agilent) at 97 °C for 20 min. After endogenous peroxidase and protein blocking, the spinal cord sections were incubated with anti-$\alpha_2\delta$-1 antibody [1:500 dilution, CACNA2D1 monoclonal antibody (20 A), Invitrogen MA3–921; Thermo Fisher Scientific, Waltham, MA, USA] at room temperature for 30 min. The primary antibody was localized by the application of peroxidase-labeled polymer-conjugated secondary antibody (EnVision + HRP-mouse; Dako/Agilent) and visualized using a substrate-chromogen system (DAB + chromogen; Dako/Agilent). The sections were then counterstained using hematoxylin (FLEX Hematoxylin; Dako/Agilent).

Image analysis

Slides were digitally scanned using a virtual slide scanner (NanoZoomer S360; Hamamatsu Photonics K.K., Shizuoka, Japan) and analyzed with digital imaging analysis software (HALO, version 2.2.1870; Indica Labs Inc., Albuquerque, NM, USA). The area quantification module was used for the automated analysis of scanned sections. To determine the $\alpha_2\delta$-1-positive staining intensity, the thresholds were set based on the optical density as follows: weak (≥ 0.116), moderate (≥ 0.234), and strong (≥ 0.546). The areas of $\alpha_2\delta$-1-positive signals were determined for each staining intensity and the ratio between the ipsilateral and contralateral sides of SNL (i. e., Ipsi/Contra ratio) was calculated.

Statistical analysis

Summarized data are presented as the mean ± standard error. Statistical comparisons (sham control vs. SNL control, SNL control vs. SNL mirogabalin) were performed using the *F*-test, followed by Aspin – Welch's or Student's *t*-test. Two-tailed *P* values of less than 0.05 were considered as statistically significant. Microsoft Excel for Microsoft 365 (Microsoft Japan Co., Ltd., Tokyo, Japan) was used for these analyses.

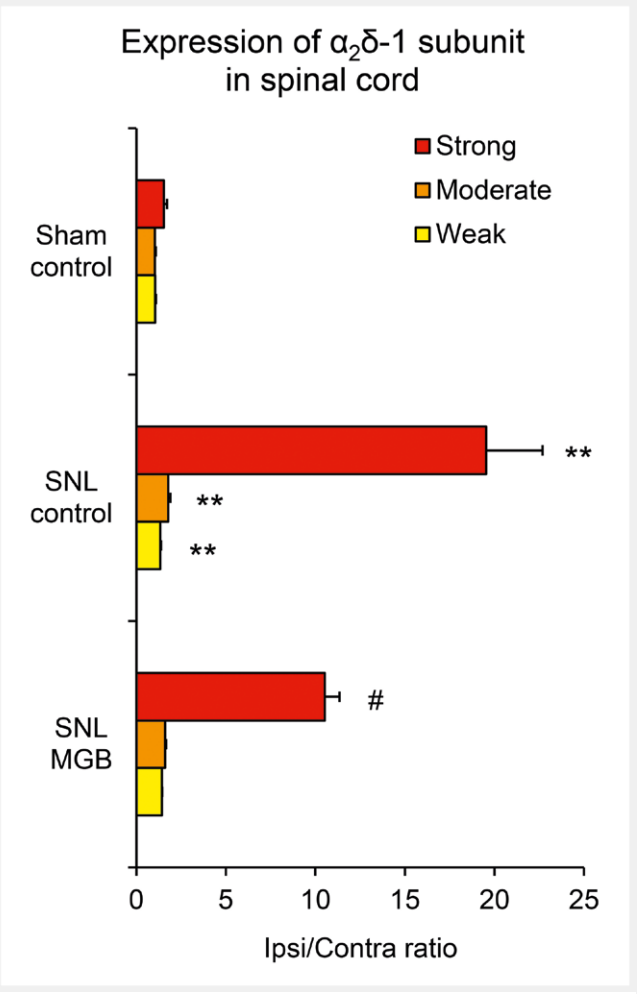
Results

Representative images of the spinal cord sections are presented in ▶ **Fig. 2**, and the results of statistical analysis are illustrated in ▶ **Fig. 3**. The $\alpha_2\delta$-1-positive signals were predominantly observed in the spinal dorsal horn. In particular, strong $\alpha_2\delta$-1-positive signals were observed at the highest density in the superficial layers of the spinal dorsal horn (▶ **Fig. 2**).

In the sham control group, the Ipsi/Contra ratio of the $\alpha_2\delta$-1-positive area was approximately 1 at all signal strengths (1.05 ± 0.04 for weak, 1.04 ± 0.04 for moderate, 1.53 ± 0.18 for strong), indicating no differences in expression of the $\alpha_2\delta$-1 subunit in the spinal dorsal horn between the ipsilateral and contralateral sides of sham surgery (yellow, orange, and red bars of the sham control group in ▶ **Fig. 3**).

The Ipsi/Contra ratio of the $\alpha_2\delta$-1-positive area in the SNL control group was significantly higher than that in the sham control group for all signal strengths: weak ($P = 0.0066$ by Student's *t*-test, yellow bars in ▶ **Fig. 3**), moderate ($P = 0.0013$ by Aspin – Welch's *t*-test, orange bars in ▶ **Fig. 3**), and strong ($P = 0.0018$ by Aspin–Welch's *t*-test, red bars in ▶ **Fig. 3**). This indicated increased expression of the $\alpha_2\delta$-1 subunit in the spinal dorsal horn at the ipsilateral side of SNL surgery.

In the comparison between the SNL control group and the SNL mirogabalin group, there were no significant differences in the Ipsi/Contra ratio of the $\alpha_2\delta$-1-positive area for weak signals ($P = 0.1534$ by Aspin – Welch's *t*-test, yellow bars in ▶ **Fig. 3**) and moderate signals ($P = 0.2814$ by Aspin – Welch's *t*-test, orange bars in ▶ **Fig. 3**). Meanwhile, for strong signals, the Ipsi/Contra ratio of the $\alpha_2\delta$-1-positive area in the SNL mirogabalin group was significantly lower than that in the SNL control group ($P = 0.0383$ by Aspin – Welch's *t*-test, red bars in ▶ **Fig. 3**). This indicated that mirogabalin inhibited the increase in expression of the $\alpha_2\delta$-1 subunit in the spinal dorsal horn of SNL model rats.



▶ **Fig. 3** Effects of mirogabalin on expression of the $\alpha_2\delta$-1 subunit of VGCCs in the spinal cord of SNL model rats.; Mirogabalin besylate (10 mg/2 mL/kg as free form) was administered orally for 5 consecutive days (twice-daily from day 1 to day 4, and once-daily on day 5), while the control groups received JP-grade distilled water (2 mL/kg) in the same manner. After the last administration on day 5, the animals were subjected to immunohistochemical analysis. Data are represented as the mean ± standard error (n = 4 for the sham control, n = 7 for the SNL control and SNL mirogabalin). $^{**}P < 0.01$ compared with the sham control group (Aspin–Welch's or Student's *t*-test). $^{\#}P < 0.05$ compared with the SNL control group (Aspin–Welch's *t*-test).

Discussion and Conclusions

The SNL model is regarded as one of the most validated experimental animal models for peripheral neuropathic pain and is widely used for pharmacological evaluations of analgesics and pathophysiological studies of peripheral neuropathic pain [47, 48]. Although the SNL model requires more extensive and complicated surgical techniques, it has some advantages over other nerve injury models such as chronic constriction injury (CCI) and partial sciatic nerve ligation (PSL) [49, 50]. For example, the surgical procedure of SNL is stereotyped (i. e., tight ligation of the same spinal nerves in each animal), and the intra- and inter-experimental variability due to differences in the numbers and types of injured nerve fibers can be lower than in the other models [46, 49, 50]. Furthermore, the levels of injured and uninjured spinal segments are completely separated in the SNL model. Therefore, injured spinal nerves among the three spinal nerves contributing to the sciatic nerve (i. e., L4, L5, and L6) and their corresponding levels of the dorsal root ganglia and spinal segments are more distinct in the SNL model than in the other models [46, 49, 50].

In the present study, the distribution of $\alpha_2\delta$-1 protein in the spinal cord was clearly determined using immunohistochemistry and imaging analysis. Strong $\alpha_2\delta$-1-positive signals were detected at the highest density in the superficial layers of the spinal dorsal horn, which are known as the projection sites of the primary afferent fibers [51], consistent with previous reports [3, 8, 9, 26].

In SNL model rats, expression of the $\alpha_2\delta$-1 subunit markedly increased in the spinal dorsal horn at the ipsilateral side of nerve injury. These findings are consistent with previous reports on the SNL model [6–10], and similar changes have been reported in other unilateral sciatic nerve injury models such as PSL [5] and CCI [10]. Increases in the $\alpha_2\delta$-1 subunit in the spinal cord have also been reported in various experimental animal models for peripheral and central neuropathic pain including diabetes [10], chemotherapy-induced peripheral neuropathy [11], post-spinal cord injury [12, 13], post-stroke [14], and multiple sclerosis [15]. Not limited to neuropathic pain models, upregulation of the $\alpha_2\delta$-1 subunit in the brain has been reported in experimental animal models of innate anxiety [16] and post-traumatic epilepsy [17]. In addition, transgenic mice overexpressing the neuronal $\alpha_2\delta$-1 subunit have been reported to show mechanical and thermal hypersensitivity [18] or epileptic seizures [19], without physical neuronal damage. Taking these findings together, it is apparent that the $\alpha_2\delta$-1 subunit plays dominant roles in the development and maintenance of various neurological disorders.

In the present study, repeated administration of mirogabalin significantly inhibited the increased expression of $\alpha_2\delta$-1 subunit in the spinal dorsal horn at the ipsilateral side of nerve injury. This finding parallels previous studies on the classical gabapentinoids, gabapentin and pregabalin [8, 9, 13]. Although the mechanism of action of gabapentinoids is not fully understood, these drugs modulate and inhibit not only calcium channel function but also channel trafficking and expression, resulting in the inhibition of calcium ion influx and excitatory synaptic transmission at synaptic endings [26–28]. The results of the present study demonstrate the latter, the inhibitory effect of mirogabalin on the trafficking and expression of the $\alpha_2\delta$-1 subunit of VGCCs. In addition, recent studies have proposed novel roles of the $\alpha_2\delta$-1 subunit, such as interaction with thrombospondins and NMDA receptors, not VGCCs [29–31]. These VGCC-independent pathophysiological roles of $\alpha_2\delta$-1 subunit might also be involved in the mechanism of action of gabapentinoids including mirogabalin.

In our previous study using the same dosing protocol, repeated administration of mirogabalin enhanced its analgesic effects without an increase in drug exposure in rats with streptozotocin-induced diabetes, a typical experimental animal model for peripheral neuropathic pain. In particular, at 12 h after 4 consecutive days of oral administration of 10 mg/kg mirogabalin (i. e., before the last administration of mirogabalin on day 5), significant analgesic effects were still observed, despite undetectable plasma levels of mirogabalin [32]. These notable findings can be explained by the inhibitory effect of mirogabalin on the trafficking and expression of the $\alpha_2\delta$-1 subunit in the spinal dorsal horn. Meanwhile, the single oral administration of mirogabalin showed acute analgesic effects, which emerged 1 or 2 h after administration and disappeared within a day [32, 41, 42]. The acute analgesic effects of mirogabalin appear to be mediated by its acute inhibition of calcium channel function. Therefore, mirogabalin can modulate both the function of upregulated $\alpha_2\delta$-1 subunit and the process of $\alpha_2\delta$-1 subunit upregulation in a state reflecting neuropathic pain.

In conclusion, the $\alpha_2\delta$-1 subunit was upregulated in the spinal dorsal horn of SNL model rats, and repeated administration of mirogabalin inhibited this upregulation. The inhibitory effect of mirogabalin on upregulation of the $\alpha_2\delta$-1 subunit after nerve injury is considered to contribute at least in part to its analgesic effects in peripheral neuropathic pain.

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Conflicts of Interest

YD, NK, KK, and YK are employees of Daiichi Sankyo Co., Ltd., and HU, YS, KI, and YO are employees of Daiichi Sankyo RD Novare Co., Ltd. This work was sponsored by Daiichi Sankyo Co., Ltd.

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