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Original

Effects of Peripheral Nerve Injury on the Kinetics of Electrically Evoked GABA Receptor-mediated Currents in GABAergic and Non-GABAergic Neurons of the Spinal Dorsal Horn

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Summary

The neurotransmitters and neuromodulators implicated in the processing of sensory and nociceptive information, γ-aminobutyric acid (GABA) is of importance in the spinal dorsal horn (SDH) neurons. Decrease in GABAergic inhibitory synaptic transmission plays an important role in mechanisms of neuropathic pain (NeP). We hypothesized that functional and structural changes in GABA receptors of SDH may occur after peripheral injury (PNI). In the present study, using whole-cell patch-clamp recording, we characterized the GABA receptor-mediated currents (I_{GABA}) and GABA receptor-mediated inhibitory postsynaptic currents (GABA-IPSCs) recorded from SDH neurons, and investigated the effects of the sciatic nerve ligation on these currents in the glutamate decarboxylase (GAD) 67 green fluorescent protein (GAD67-GFP) knock-in mice. Furthermore, using the quantitative real-time PCR analysis, we evaluated alterations of the expression level of the GABA receptor subunits by the sciatic nerve ligation. We observed changes in I_{GABA} and GABA-IPSCs in both GFP-negative and positive neurons, and alterations of the expression level of the $GABA_A$ receptor subunits, in SDH after the sciatic nerve ligation. Our results suggest that influences on the function and structure of GABAergic neuron in SDH by PNI may play an important role in the mechanism of NeP.

Key Words: neuropathic pain, peripheral nerve injury, GABA, glutamate decarboxylase, green fluorescent protein

Introduction

There are various types of chronic pain associated with diseases. It is well known that neuropathic pain (NeP) has been getting attention from both clinicians and basic researchers due to its intractable feature. NeP is defined as 'pain caused by a lesion or disease of the somatosensory nervous system' by International Association for Study of Pain in 1994. NeP should not be indicates as a single disease, but rather should be recognized as a pathological condition involved in many patients with chronic pain. Varied lesions or diseases can develop NeP and its mechanisms have been still unclear. If there is a lesion or disease in any types of the nociceptive pathways from the pe-

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ripheral nerves to the spinal cord and the brain, patients will complain of NeP. The pathological mechanisms include abnormal sensitivity of the somatosensory nervous system and functional impairment in the descending pain modulatory system.

Peripheral nerve injury (PNI), which is one of NeP, often induces a state of abnormal pain, including hyperalgesia and allodynia. It is well known that PNI-induced NeP has been associated with neural plastic changes in the spinal dorsal horn (SDH). Possible mechanisms for NeP including primary afferent fiber sprouting, synaptic rearrangement, and loss of inhibitory interneurons in SDH, among others. Especially, SDH is one of the important regions and SDH neurons of different cell types play specific roles in pain processing.

Among the neurotransmitters and neuromodulators implicated in the processing of sensory and nociceptive information, γ-aminobutyric acid (GABA) is of importance in SDH neurons, and GABAergic neurons are distributed in a high concentration in SDH neurons[1-5]. The distribution of $GABA_A$ receptors in SDH has also been reported[6-11]. The $GABA_A$ receptors regulates neuronal excitability and information integration in the central nervous system.

In the previous reports, it was reported that the decrease in GABAergic inhibitory synaptic transmission must play an important role in the mechanisms of NeP[12-17]. However, its detailed mechanisms have been still unclear. Therefore, we hypothesized that functional and structural changes in GABA receptors of SDH may occur after PNI.

In the present study, we characterized the GABA receptor-mediated currents (I_{GABA}) and GABA receptor-mediated inhibitory postsynaptic currents (GABA-IPSCs) recorded from SDH neurons and investigated the effects of PNI on these currents in the glutamate decarboxylase (GAD) 67 green fluorescent protein (GAD67-GFP) knock-in mice.

Furthermore, we evaluated alterations of the expression level of the GABA receptor subunits after PNI.

Materials and Methods

1. Animals

The experiments were performed on GAD 67 green fluorescent protein (GFP) (Δneo) mice, which express GFP under the control of the endogenous GAD67 gene promoter[18]. In the present study, these transgenic mice were referred to as GAD67-GFP knock-in mice. Male heterozygous mice were crossed with Institute of Cancer Research (ICR) wild-type mice to obtain the experimental mice. All animal experiments were approved by the institutional animal care and use committees at Dokkyo Medical University. Care and use of the animals were in accordance with the National Institutes of Health guidelines on animal care and with the guidelines of the International Association for the Study of Pain[19].

2. Partial ligation of the sciatic nerve

All mice were maintained in a temperature-controlled room under a 12 h/12 h light/dark cycle. A partial sciatic nerve injury was produced by tight ligation with a 9-0 silk suture around about half the diameter of the sciatic nerve according to Selzer et al[20] (ligation mouse). In the sham-operated control mice, the sciatic nerve was exposed but not ligated (sham-operated control mouse).

To assess effects of the sciatic nerve ligation, we measured the frequency of withdrawal responses to 10 repetitive stimuli with von Frey filament (Stoeling, Wooddale, IL) of 1.6 g force. Behavioral assessments were performed starting 1 day before nerve ligation or sham operation. Sciatic nerve ligation increased the frequency of withdrawal responses, and this increase developed within 1 day after nerve ligation and persisted throughout the experimental period.

3. Preparation of the spinal cord slices

The spinal cord was removed from mice under ketamine/xylazine anesthesia on post-surgical day 7 to 10. Transverse spinal cord slices (350 μm) were cut by a vibratome (DTK-1500, Dosaka, Japan). Slices were transferred to a recording chamber, which was continually superfused (2-3 mL/min) with an artificial cerebral spinal fluid of the following composition (mM): NaCl 113, KCl 3, $NaHCO_3$ 25, NaH_2PO_4 1, $CaCl_2$ 2, $MgCl_2$ 1, D-glucose 11, equilibrated with 95%O_2-5%CO_2 at 4℃. When necessary, $MgCl_2$ was excluded to make a nominally Mg^{2+}-free solution.

4. Whole-cell patch-clamp recordings

For the electrophysiological experiments, we used a fixed-stage upright microscope (BX50WI; Olympus, Tokyo) equipped with a confocal laser scanning system (FluoView 500; Olympus), infrared differential interference contrast (IR-DIC) optics, and a CCD video camera (IR-CCD 2400; Hamamatsu Photonics, Hamamatsu, Japan). After incubation for 1 h in modified Krebs solution at 37℃, the spinal slices were mounted into the recording chamber on the microscope stage and continuously perfused with Krebs solution [equilibrated with 95% O_2-5% CO_2, containing (in mM) NaCl 113, KCl 3, $NaHCO_3$ 25, NaH_2PO_4 1, $CaCl_2$ 2, $MgCl_2$ 1, and d-glucose 11; pH 7.4]. After the identification of GFP-negative or positive neurons in SDH by using the confocal laser scanning system described by Fukushima et al[21], conventional tight-seal whole-cell recordings were obtained from neurons under IR-DIC optics. The current evoked by a GABA puff application (I_{GABA}, 100 mM, 0.3-1.0 sec) was recorded.

Inhibitory postsynaptic currents mediated by $GABA_A$ receptors evoked by extracellular stimulation with an electrical pulse (GABA-IPSCs) was also recorded. All recordings were made in the presence of tetrodotoxin (TTX: 0.5 μM) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX: 10 μM). Recordings in voltage-clamp mode used an Axopatch 200B patch-clamp amplifier (Axon Instruments, US). Data were sampled at a rate of 10.0 kHz through a Digidata 1440 interface (Axon Instruments, US). pCLAMP software (Axon Instruments, US) was used to analyze the data.

5. Quantitative real-time PCR analysis

To identify GFP-negative or positive neurons, the neurons were aspirated into another pipets after whole-cell recordings, according to a preciously described protocol[22]. The neurons were then ejected into thin-walled autoclaved PCR tubes by applying a gentle positive pressure, and immediately frozen and stored at −80℃ until use. On the following day, lysis was performed using IGEPAL. CA-630 (Sigma-Aldrich, US) at room temperature for 5 min followed by reverse transcription (RT) with Superscript II (Life Technologies, US).

After RT reaction, preamplification of cDNA samples was performed using the TaqMan PreAmp Matter Mix (Applied Biosystem, US) according to the manufacturer's directions using the recommended program for 14 cycles. Then preamplified cDNA was utilized for real-time PCR using TaqMan Fast Universal Master Mix and TaqMan Gene Expression Assay (Applied Biosystems). The reaction was performed on Eppendorf Real Plex 2 (Eppendorf, Germany). Samples were assayed in triplicates. Each particular gene was regarded as present in individual single-cell samples when its fluorescence intensity exceeded a predetermined threshold value in <45 cycles of PCR. Following RT, cDNA was subjected to real-time PCR to analyze the expression profile of mRNAs for $GABA_A$ receptor subunits α1, α2, α3, α5 and δ. Housekeeping gene GAPDH was used as an internal control. We calculated relative quantification of the $GABA_A$ receptor subunit genes using the 2-dd CT method.

6. Statistical analysis

Results are expressed as mean ± SEM. Statistical analyses were carried out using a two-way analysis of variance (ANOVA) followed by a simple effects test and by post hoc multiple comparisons using Tukey's test. The χ^2 test with Yate's correction was also used when appropriate. A value of $p < 0.05$ was considered as statistically significant.

Results

1. GABA evoked inward current

Fig. 1 shows a sample of GABA evoked inward current in SDH neuron. Membranes currents evoked by a puff application of GABA were recorded. GABA (100 μM) was applied with a short pressure puff. At the holding potential of −70 mV. GABA elicited an inward current. Recordings were made in the presence of TTX (0.5 μM) and CNQX (10 μM). Fig. 1a, 1b and 1c show the currents before, during, and after perfusion of GABA receptor blocker bicuculine, respectively. The current was abolished by bicuculine, reversibly.

2. Changes in the reversal potential of the GABA receptor-mediated currents by the sciatic nerve ligation

Fig. 2a and 2b show each sample of I_{GABA} in response to step depolarizations from the holding potential (−70 mV) in ligation mouse and sham-operated control

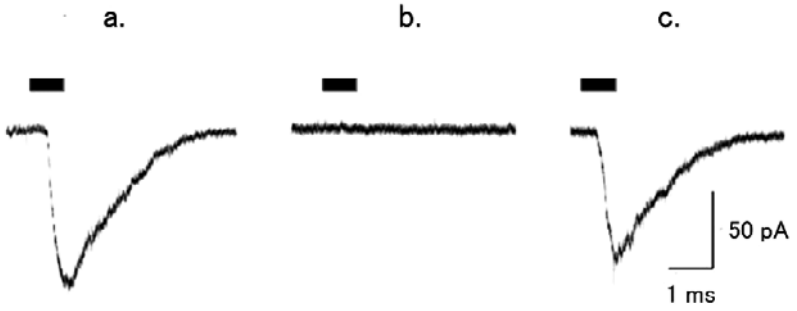

Figure 1 The puff application of GABA evokes and inward current in the spinal dorsal horn neuron.
a, **b** and **c** show the currents before, during and after perfusion of bicuculine, respectively. A black bar indicates GABA (100 μM) application with a short pressure.

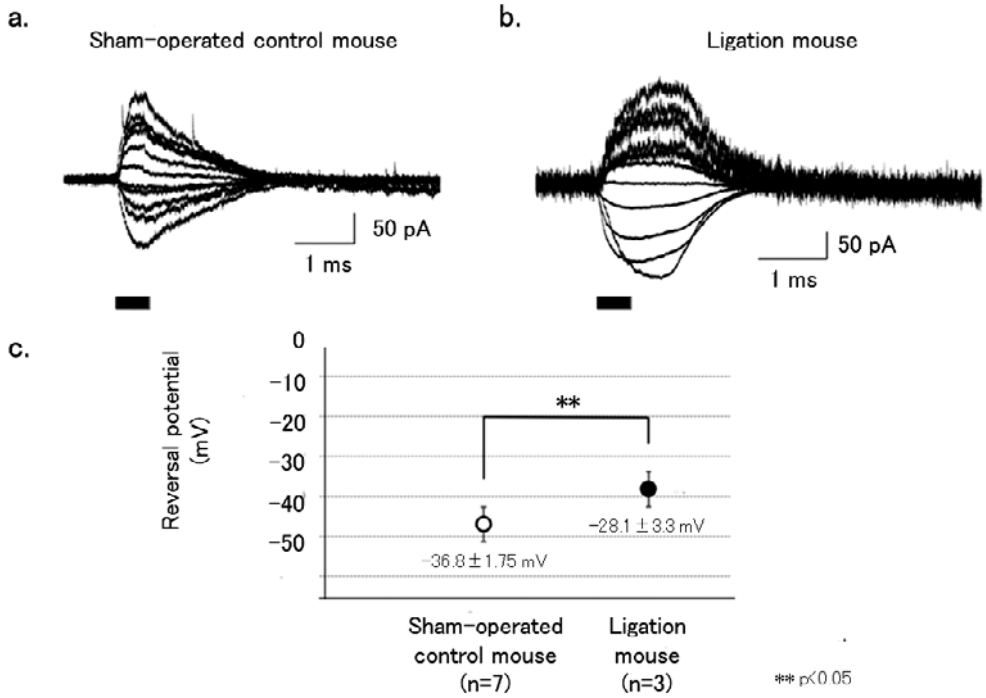

Figure 2 Changes in the reversal potential of the GABA receptor-mediated currents by the sciatic nerve ligation.
a and **b** show each sample of GABA receptor-mediated currents (I_{GABA}) in response to step depolarizations from the holding potential (− 70 mV) in ligation mouse and sham-operated control mouse, respectively. A black bar indicates GABA (100 μM) application with a short pressure. **c** shows the reversal potential of the spinal dorsal horn neuron in ligation mouse and sham-operated control mouse.

mouse, respectively. 10 mV steps of voltage were applied between −70 mV to + 30 mV. The reversal potential of SDH neurons in ligation mouse (n = 3, −28.1 ± 3.3 mV) was significantly larger than that in sham-operated control mouse (n = 7, −36.8 ± 1.75 mV) (Fig. 2c: $p < 0.05$).

3. Effects of the sciatic nerve ligation on I_{GABA} in GFP-negative and positive neurons of the spinal dorsal horn

I_{GABA} by GABA puff application were recorded in both GFP-negative and positive neurons. In GFP-negative neurons, the amplitude of I_{GABA} was decreased by the sciatic nerve ligation (Fig. 3a). On the other hand, in GFP-positive neurons, the amplitude of I_{GABA} was increased by the sciatic nerve ligation (Fig. 3b).

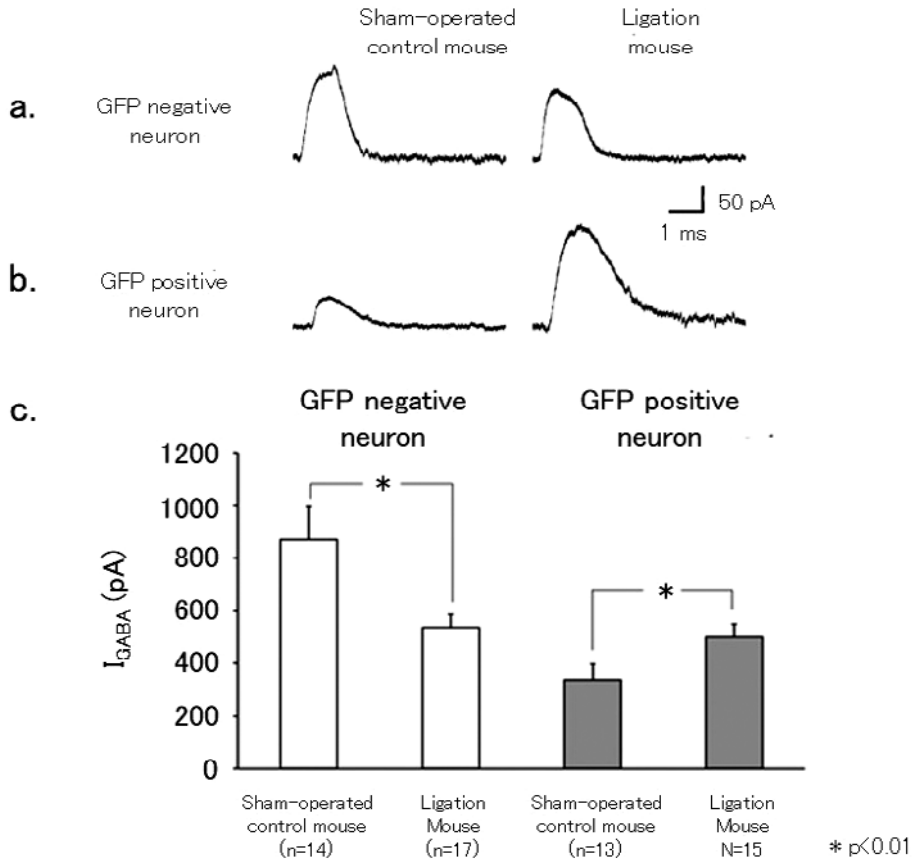

Figure 3 Effects of the sciatic nerve ligation on GABA receptor-mediated currents in GFP-negative and positive neurons of the spinal dorsal horn.

a and **b** show each sample of representative recordings of GABA receptor-mediated current (I_{GABA}) in GFP-negative and positive neurons of both sham-operated control mouse and ligation mouse, respectively. **c** shows influences on in the amplitude of I_{GABA} by the sciatic nerve ligation in GFP-negative and positive neurons of the spinal dorsal horn.

Fig. 3c shows differences in the amplitude of I_{GABA} between sham-operated control mouse and ligation mouse in both GFP-negative and positive neurons. The amplitude of I_{GABA} was significantly decreased in GFP-negative neurons ($p < 0.01$) and significantly increased in GFP-positive neurons ($p < 0.01$) by the sciatic nerve ligation.

4. Effects of peripheral nerve injury on GABA-IPSCs in GABAergic and non-GABAergic neurons of the mouse spinal dorsal horn

GABA-IPSCs evoked by electrical stimulation were recorded. The recordings were made at the holding potential of 0 mV. GABA-IPSCs were pharmacologically isolated in the presence of the glycine receptor blocker (strychnine), the non-N-methyl-D-aspartate receptor (NMDA) receptor blocker (CNQX), and the NMDA receptor blocker (2-amino-5-phosphonovalerate: APV). The decay was fitted by a double exponential function. The fitted functions are superimposed (thin line) with their time constants (slow and fast components of GAB-IPSCs) (Fig. 4a: GFP-negative neuron and Fig. 4b: GFP-positive neuron).

Fig. 4c shows effects of peripheral nerve injury on GABA-IPSCs in GABAergic and non-GABAergic neurons of the SDH. The sciatic nerve ligation did not affect the amplitude of the GABA-IPSCs, but shortened their decay over time in GFP-negative neurons (τ_{fast}: $p < 0.05$, τ_{slow}: $p < 0.05$). On the other hand, in GFP-positive neurons, the sciatic nerve ligation did not affect the amplitude of the GABA-IPSCs, but prolonged their decay over time (τ_{fast}: no statistical change, τ_{slow}: $p < 0.01$).

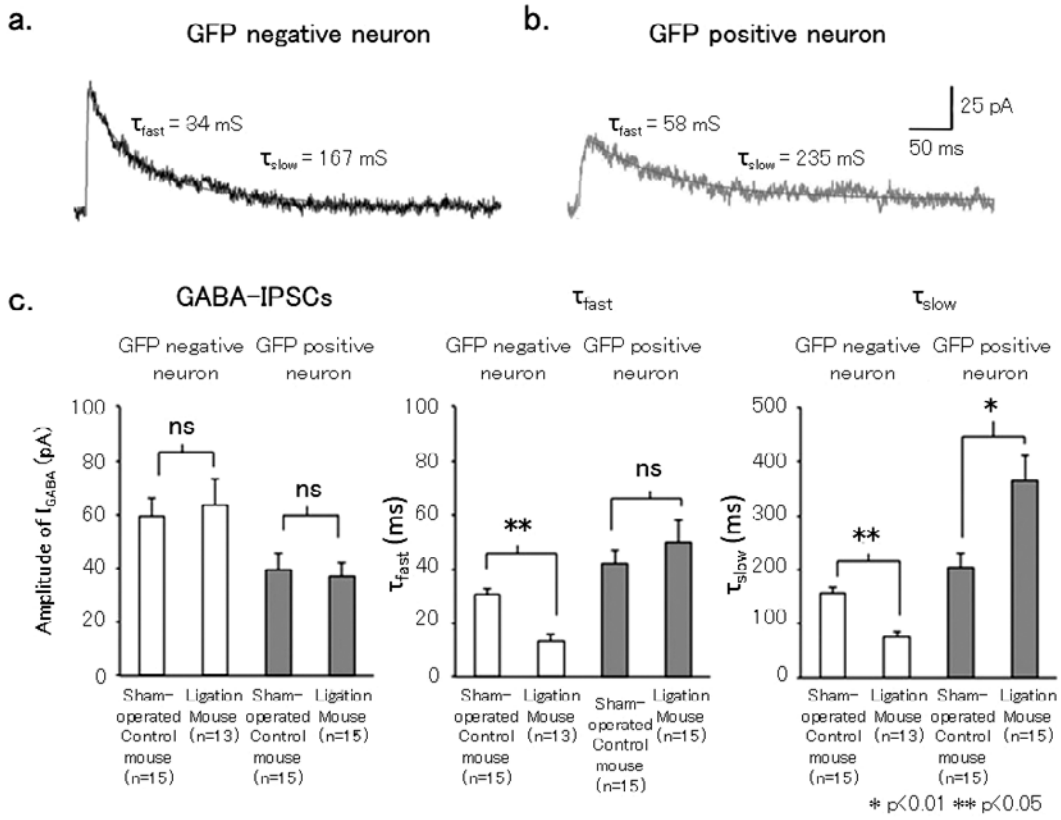

Figure 4 Effects of the sciatic nerve ligation on GABA receptor-mediated inhibitory postsynaptic currents in GFP-negative and positive neurons of the spinal dorsal horn.

a and **b** show each sample of GABA receptor mediated inhibitory postsynaptic currents (GABA-IPSCs) evoked by electrical stimulation in GFP-negative and positive neurons of the spinal dorsal horn, respectively. The decay was fitted by a double exponential function. The fitted functions are superimposed (thin line) with their time constants (slow and fast components of GABA-IPSCs). **c** shows influences on the amplitude of I_{GABA}, τ_{fast} and τ_{slow} by the sciatic nerve ligation in GFP-negative and positive neurons of the spinal dorsal horn.

5. Effect of nerve ligation on the expression level of the $GABA_A$ receptor subunits

Fig. 5 shows differences in the expression level of the $GABA_A$ receptor subunits, α1, α2, α3, α5 and δ in SDH neurons between sham-operated mouse and ligation mouse. The quantitative RT-PCR analysis indicated that the sciatic nerve ligation increased the expression of α3 ($p < 0.01$) and α5 ($p < 0.05$), but decreased that of α1 ($p < 0.05$) and δ($p < 0.05$) of the GABA receptor subunits in SDH. There was no significant difference in α2 of the GABA receptor subunit in the spinal dorsal horn between sham-operated mouse and ligation mouse.

Discussion

In the present study, firstly, we observed the effect of the sciatic nerve ligation on I_{GABA} and GABA-IPSCs in both GFP-negative and positive neurons of SDH using whole-cell recordings. In the previous study using GAD67-GFP knock-in mice, Fukushima et al.[23] demonstrated a correlation of GFP fluorescence with expressions of GAD65 and GAD67mRNAs and endogenous GABA. And also, they described that GFP-negative and positive neurons indicated excitatory and inhibitory neurons in GAD67-GFP knock-in mice, respectively. Therefore, for recordings GABA-IPSCs in our study, GAD67-GFP knock-in mice were used to distinguish both excitatory and inhibitory neurons.

In GFP-negative (excitatory) neurons, the sciatic nerve ligation significantly decreased the amplitude of I_{GABA} and shortened the rate of the decay time of GABA-IPSCs. These results suggest that GABAerigc inhibitory influence in excitatory neurons may be attenuated by the sciatic nerve ligation. On the other

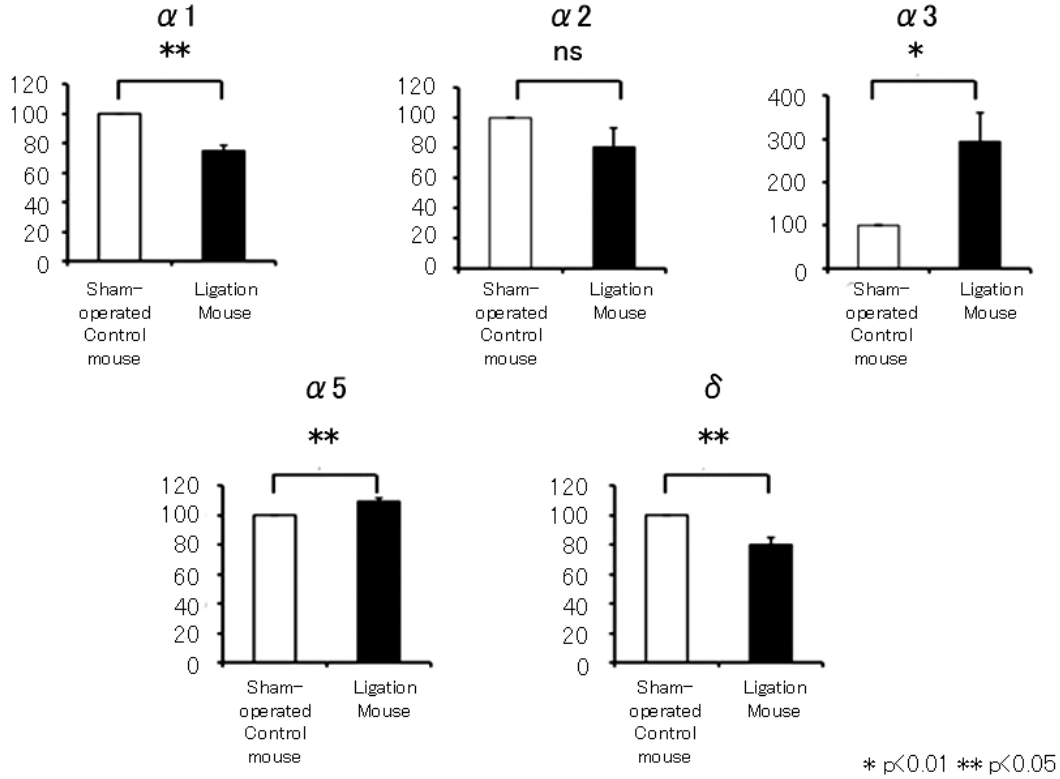

Figure 5 Effect of the sciatic nerve ligation on the expression level of $GABA_A$ receptor subunits.
The quantitative RT-PCR analysis indicated that the sciatic nerve ligation increased the expression of α3 and α5, but decreased that of α1 and δ of GABA receptor subunits in the spinal dorsal horn.

hand, in GFP-positive (inhibitory) neurons, the sciatic nerve ligation increased the amplitude of I_{GABA} and prolonged the rate of the decay time of GABA-IPSCs. These results suggest that GABAerigc inhibitory influence in inhibitory neurons may be enhanced by the sciatic nerve ligation. These phenomena, which we observed in excitatory and inhibitory neurons, may result in progress of NeP.

There are many reports[24-28], hypothesized that one of the factors underlying the enhanced pain sensitivity observed in NeP states is the loss of activity of the inhibitory neurotransmitter GABA in the spinal cord. Moore et al.[28] showed that the incidence, magnitude and duration of primary afferent evoked GABA-mediated inhibitory postsynaptic currents in lamina II neurons is reduced after PNI, presumably as a consequence of attenuated presynaptic GABA release. Our electrophysiological data, including both I_{GABA} and GABA-IPSCs, can support and extend the previous reports.

Secondly, we evaluated the expression level of the $GABA_A$ receptor subunits in SDH neurons using the quantitative RT-PCR analysis, and obtained results that the sciatic nerve ligation increased α3 and α5 GABA receptor subunits and decreased α1 and δ of GABA receptor subunits. Those results may suggest that GABAerigc inhibitory influence may be modulated by changes in GABA receptor subunits.

The previous reports using immunohistochemical, in situ hybridization, and functional studies demonstrated the expression and localization of some GABA receptor subunits in SDH[29-32]. In particular, the α5 GABA receptor subunits are expressed extrasynaptically in neurons of the superficial dorsal horn, such as laminae I and II[33,34]. Our results about the increase of α5 GABA receptor subunit in SDH may be interesting, as there are some reports suggesting that α5 GABA receptor subunit in SDH have antinociceptive and pronociceptive roles in healthy and chronic pain[12-17]. For example, De la Luz-Cuellar et al.[12] showed that intrathecal administration of L-655,708 (α5 $GABA_A$ receptor inverse agonist) decreases pain threshold in naïve rats. Furthermore, Delgado-Lezama et al.[35] described a possibility of α5 GABA receptor subunit, as a valid pharma-

cological target to treat chronic pain states. These results by the quantitative RT-PCR analysis can support those previous data and the possibility to develop a novel drug for NeP.

Our results in the present study, including functional and structural changes in GABAergic neurons of SDH by the sciatic nerve ligation may play an important role in developing NeP. One of the limitations in this study is that we only observed influences by the sciatic nerve ligation about both changes in I_{GABA} and GABA-IPSCs in GFP-negative and positive neurons, and expression level of the $GABA_A$ receptor subunits in SDH, separately. Therefore, in the next study, to confirm our theory and to establish suitable pharmacotherapy for NeP, the expression level of the $GABA_A$ receptor subunits in both inhibitory and excitatory SDH neurons after PNI must be evaluated by using single-cell real-time PCR.

Conclusion

In the present study, we observed changes in I_{GABA} and GABA-IPSCs in GFP-negative and positive neurons, and alterations of the expression level of the $GABA_A$ receptor subunits, in SDH after the sciatic nerve ligation. These results suggest that influences on function and structure of GABAergic neurons in SDH by PNI may play an important role in the mechanism of NeP.

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