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Original Article

Intrathecal Administration of the α1 Adrenergic Antagonist Phentolamine Upregulates Spinal GLT-1 and Improves Mirror Image Pain in SNI Model Rats

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Mirror image pain (MIP) is a type of extraterritorial pain that results in contralateral pain or allodynia. Glutamate transporter-1 (GLT-1) is expressed in astrocytes and plays a role in maintaining low glutamate levels in the synaptic cleft. Previous studies have shown that GLT-1 dysfunction induces neuropathic pain. Our previous study revealed bilateral GLT-1 downregulation in the spinal cord of a spared nerve injury (SNI) rat. We hypothesized that spinal GLT-1 is involved in the mechanism of MIP. We also previously demonstrated noradrenergic GLT-1 regulation. Therefore, this study aimed to investigate the effect of an α1 adrenergic antagonist on the development of MIP. Rats were subjected to SNI. Changes in pain behavior and GLT-1 protein levels in the SNI rat spinal cords were then examined by intrathecal administration of the α1 adrenergic antagonist phentolamine, followed by von Frey test and western blotting. SNI resulted in the development of MIP and bilateral downregulation of GLT-1 protein in the rat spinal cord. Intrathecal phentolamine increased contralateral GLT-1 protein levels and partially ameliorated the 50% paw withdrawal threshold in the contralateral hind paw. Spinal GLT-1 upregulation by intrathecal phentolamine ameliorates MIP. GLT-1 plays a role in the development of MIPs.

Key words: alpha adrenergic receptor, glutamate transporter-1, mirror image pain, neuropathic pain, spared nerve injury

Extraterritorial pain is a phenomenon in which pain or allodynia occurs at a different site of the body from where the injury or inflammation exists. Mirror image pain (MIP) is a type of extraterritorial pain that occurs on the contralateral side. Some clinical MIP cases have been described in the literature [1]. Experimental MIP studies with neuropathic, inflammatory, and cancer pain models have suggested a variety of mechanisms, such as gap junctions, activated glia, and various cytokines/receptors. However, there is no established treatment for MIPs.

Glutamate is a major excitatory neurotransmitter that regulates higher brain functions, such as memory and learning, in the central nervous system [2]. Glutamate from presynaptic terminals binds to its receptors, such as ion channel-type glutamate receptors of the N-methyl-D-aspartate, kainate, and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid types and metabolic regulatory glutamate receptors [3].

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However, 90% of glutamate is speculated to remain unbound to the receptors and is removed by specific transporters. Glutamate transporter-1 (GLT-1), also known as slc1a2 and EAAT2, is expressed exclusively in astrocytes and plays a role in maintaining low glutamate levels in the synaptic cleft [4-5]. Dysfunction of GLT-1 results in excessive extracellular glutamate, which eventually causes neural cytotoxicity [6], neurodegenerative disorders [7], traumatic neuronal injury [8], inflammatory pain [9], and neuropathic pain [9]. Previous studies on neuropathic pain models reported changes in the expression and activity of GLT-1 and other glutamate transporters, such as glutamate-aspartate transporter and excitatory amino acid carrier 1 [10]. Among these transporters, GLT-1 made the greatest contribution to neuropathic pain in most cases [10].

In our previous study, we showed downregulation of GLT-1 in rat primary astrocytes via α1 adrenoceptors by noradrenaline [11], which is one of the major neurotransmitters in the descending inhibitory pathway [12]. Downregulation of GLT-1 in the spinal astrocytes is involved in the development of neuropathic pain. However, the relation between MIP and GLT-1 expression in the spinal cord is still ambiguous. The aim of the present study was to examine the involvement of GLT-1 and noradrenaline receptors in MIP in a rat pain model.

Materials and Methods

Primary culture of astrocytes from the rat whole spinal cord. Primary astrocytes were isolated from the thoracic and lumbar part of the whole spinal cord of 5- to 7-week-old rats, as described previously [11]. Briefly, the dissected spinal cord was cut into small pieces and trypsinized at 37°C for 30 min. Mixed primary cells were maintained in Dulbecco's modified Eagle's medium supplemented with GlutaMAX (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% heat-inactivated fetal bovine serum (BioWest, Nuaillé, France), 100 U/mL penicillin, and 100 mg/mL streptomycin (Wako Pure Chemical Industries, Osaka, Japan). After a few weeks of culture, the mixed glial cells were shaken in culture flasks (TRP, Trasadingen, Switzerland) at 240 rpm for 6 h, and the supernatant containing microglia and oligodendrocytes was discarded. After a few weeks of additional culture, the cells were plated onto appropriate plates for experiments. All cultures were maintained in an incubator containing 5% CO_2 at 37°C.

Immunocytochemistry. Primary astrocytes were plated onto chamber slides (Nunc Lab-Tek chamber slides; Thermo Fisher Scientific) coated with 0.1% gelatin (Merck Millipore, Burlington, MA, USA). Cells were fixed with methanol for 5 min at 4°C, followed by blocking in 10% normal goat serum for 1 h. Cells were then incubated with an Alexa Fluor 488-conjugated anti-glial fibrillary acidic protein (GFAP) antibody (1 : 200; Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. Coverslips were placed on the slides with ProLong Gold Antifade Mountant with DAPI (40, 6-diamidino-2- phenylindole; Thermo Fisher Scientific). Images were captured using a fluorescent microscope equipped with a 20x Plan Fluor objective lens (BZ-X700; Keyence, Osaka).

In vitro drug treatment. Primary astrocytes were serum-starved for 12 h and incubated with phentolamine (Novartis Pharma, Tokyo), silodosin (Wako Pure Chemical Industries), or BMY7378 (Abcam, Cambridge, UK) at concentrations of 0, 10, 30, or 90 μM with 0.3 μM noradrenaline (Daiichi-Sankyo, Tokyo, Japan). After 12 h of incubation, the cells were harvested and lysed with QIAzol reagent (Qiagen, Hilden, Germany) for subsequent analysis.

Animal model. This study was approved by the Animal Care and Use Committee of the Okayama University Medical School, Japan. Animals were treated in accordance with the ethical guidelines for the investigation of experimental pain in conscious animals issued by the International Association for the Study of Pain [13]. Five-week-old male Sprague-Dawley rats (35 rats; CLEA Japan) were used in this study. The animals were housed individually in cages under a 12 h/12 h light/dark cycle with free access to food and water. Inhalation anesthesia with 1-1.5% isoflurane in 100% oxygen was administered during the surgery. Spared nerve injury (SNI) of the sciatic nerve was performed as described previously [14], with modifications. Briefly, the left tibial nerve was exposed at the mid-thigh level, ligated with 6-0 silk thread, and cut, while the common peroneal and sural nerves remained intact. After appropriate hemostasis was confirmed, the wound was closed with two layers. All procedures were performed under aseptic conditions.

Behavioral assessment. Mechanical hypersensitivity was assessed as the hind paw withdrawal threshold (PWT) with von Frey filaments (Touch-Test Sensory

Evaluator; North Coast Medical, Morgan Hill, CA, USA) before surgery and at 1, 3, 4, 7, and 10 days after surgery. Mechanical stimuli were applied to the plantar aspect of each hind paw with one of a series of nine von Frey filaments (0.4, 0.6, 1.0, 1.4, 2.0, 4.0, 6.0, 8.0, and 15.0 g). Each trial was started with a von Frey force of 2.0 g. On the basis of the response pattern and the force of the final filament, the 50% PWT was determined using Dixon's up-down method [15] and calculated using the formula described by Chaplan *et al.* [16]. If the strongest filament did not elicit a response, the 50% PWT was recorded as 15.0 g. Rats with less than 10 g of preoperative 50% PWT were excluded. We defined MIP as less than 5 g of 50% PWT on day 3 after SNI on the contralateral side. Rats with MIP were used for the behavioral study.

Intrathecal catheterization and drug administration. After SNI was performed, a polyethylene catheter (SP-8; Natsume, Tokyo, Japan) was inserted from the interspace between L5 and L6 and passed cranially inward so that the tip of the catheter located near the L4 and L5 dorsal root ganglions [17]. The other side of the catheter was exteriorized between the ears. Phentolamine (Novartis Pharma) (100 μg for n=6 rats; 200 μg for n=7) or the same volume of saline (n=6) was administered into the subarachnoid space daily for one week from day 3 after catheterization.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR). For the *in vivo* study, rats were sacrificed by decapitation under deep anesthesia. The L5 spinal cord was dissected out, separated bilaterally, and dipped immediately in RNAprotect Tissue Reagent (Qiagen). Total RNA was extracted using an RNeasy Lipid Tissue Mini Kit (Qiagen) according to the manufacturer's instructions. For the *in vitro* study, total RNA was extracted from the cell lysate using the ethanol precipitation method. cDNA was reverse-transcribed from 1 μg of total RNA using a QuantiTect Reverse Transcription Kit (Qiagen). Genomic DNA was removed using the gDNA wipeout buffer included in the kit. Primer sequences are summarized in Table 1. The GLT-1 primer pair was designed to amplify both GLT-1 transcript variants 1 and 2. Quantitative PCR analysis was performed with TB Green Premix Ex Taq II (Takara-Bio, Shiga, Japan) at an annealing temperature of 60°C (StepOnePlus; Applied Biosystems, Waltham, MA, USA). The absolute copy number of each target cDNA in the samples was determined against the corresponding standard curve. The expression of GLT-1 in each sample was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). PCR specificity was confirmed using melting curve analysis, gel electrophoresis, and DNA sequencing.

Western blotting. The rats were deeply anesthetized with isoflurane and decapitated. We attempted to perform western blotting with the spinal dorsal horn of rats, but the sample was too small and the results were unreliable, so we used the spinal cord instead. The L5 spinal cord was excised and lysed using a standard radio-immunoprecipitation assay buffer. The total protein concentration was measured using a BCA protein assay kit (Pierce, Rockford, IL, USA). Proteins were denatured and reduced by boiling in sodium dodecyl sulfate- and β-mercaptoethanol-containing buffers. They were then separated on gradient polyacrylamide gels (Any kD precast gel; Bio-Rad, Hercules, CA, USA) using electrophoresis and transferred onto nitrocellulose membranes. The following primary antibodies were used: anti-GLT-1 antibody (1 : 1000, #GLT11-A; Alpha Diagnostic International, San Antonio, TX, USA)

Table 1 Primer pairs for quantitative PCR

Target	Primer sequence (5′ > 3′)	PCR product size (bp)	GenBank Accession No.
GLT-1	Forward: ATTGGTGCAGCCAGTATTCC Reverse: CCAAAAGAATCGCCCACTAC	158	Transcript variant 1: NM_017215.3 Transcript variant 2: NM_001035233.1
GAPDH	Forward: GACAACTTTGGCATCGTGGA Reverse: ATGCAGGGATGATGTTCTGG	133	NM_017008.4

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GLT-1, Glutamate transporter-1.

and anti-GAPDH antibody (1 : 10000, #G8795; Sigma Aldrich, St. Louis, MO, USA) for 2 h at room temperature. The following secondary antibodies were used: horseradish peroxidase-conjugated anti-rabbit IgG (1 : 5000, #W401B; Promega, Madison, WI, USA) and horseradish peroxidase-conjugated anti-mouse IgM (1 : 5000, #sc-2064; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were imaged using an enhanced chemiluminescence procedure. Densitometry was performed using Image Lab (Bio-Rad, Hercules, CA, USA), and protein levels were expressed as values relative to the controls.

Statistical analysis. Data are shown as mean or mean ± standard error of the mean (SEM). Data from quantitative PCR were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's and Bonferroni's multiple comparison tests. Behavioral assessment data were analyzed using two-way ANOVA followed by Dunnett's multiple comparison test. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using Prism software (version 5.0; GraphPad Software, San Diego, CA, USA).

Results

Changes in GLT-1 mRNA expression in rat primary astrocytes stimulated with noradrenaline and its antagonist. To examine the involvement of noradrenaline in *GLT-1* expression, we cultured astrocytes from the rat spinal cord. To confirm that the cells were appropriate, astrocyte-enriched primary cultures were immunostained for GFAP, a marker of astrocytes. As previously described, most cells were GFAP-immunoreactive (Fig. 1).

Our previous study showed that noradrenaline suppressed *GLT-1* mRNA expression through the α1 adrenergic receptor in the RNB cell line [11]. Next, therefore, we examined the effect of noradrenaline on *GLT-1* mRNA expression in rat primary astrocytes. We found that the primary cultured astrocytes constitutively expressed *GLT-1* mRNA. *GLT-1* mRNA expression was suppressed by noradrenaline after 12 h of incubation. *GLT-1* downregulation was blocked by phentolamine, an α1 adrenergic antagonist (Fig. 2A).

α1 adrenoceptors have three subtypes: α1A, α1B, and α1D. To the best of our knowledge, there has been no study about the expression of α1 adrenoceptors subtypes in spinal astrocytes. A previous study reported

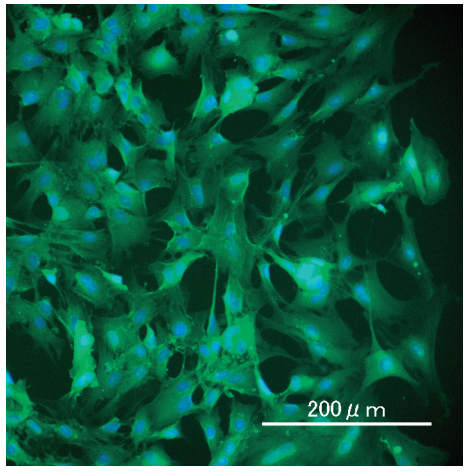

Fig. 1 Fluorescent immunocytochemistry in astrocyte-enriched primary culture. GFAP immunoreactivity is shown in green. Nuclei stained with DAPI are shown in blue.

that the predominant subtype in spinal astrocytes was α1A [18], whereas another study reported that α1B was absent in brain astrocytes [19]. Thus, we examined the involvement of α1 adrenoceptors subtypes α1A and α1D in noradrenaline-induced *GLT-1* mRNA downregulation using subtype-selective antagonists. Contrary to the almost complete recovery of *GLT-1* mRNA expression by the non-selective α1 adrenergic antagonist phentolamine, each selective α1 adrenergic antagonist partially restored *GLT-1* mRNA expression (Fig. 2B).

Changes in GLT-1 protein expression in the spinal cord of SNI model rats. We previously reported a decrease in *GLT-1* mRNA expression in the spinal cord of rats with SNI. In our present study, in order to examine the changes in *GLT-1* protein levels, we performed western blotting of the rat L5 spinal cord one week after surgery. Consistent with the mRNA changes reported in the previous study, protein levels decreased on both the ipsilateral and contralateral sides to the same extent as in naïve rats (Fig. 3A, B). The changes in GLT-1 protein levels were consistent with those in *GLT-1* mRNA expression levels in our previous study.

Changes in GLT-1 protein by single intrathecal phentolamine administration in the spinal cord of SNI rats. An *in vitro* study indicated that non-specific blockade of α1 adrenergic receptor was necessary to restore GLT-1 expression in astrocytes. We administered 200 μg of phentolamine intrathecally one week after SNI and examined GLT-1 levels in the L5 spinal cord 6 h and 24 h after the injection. There were no

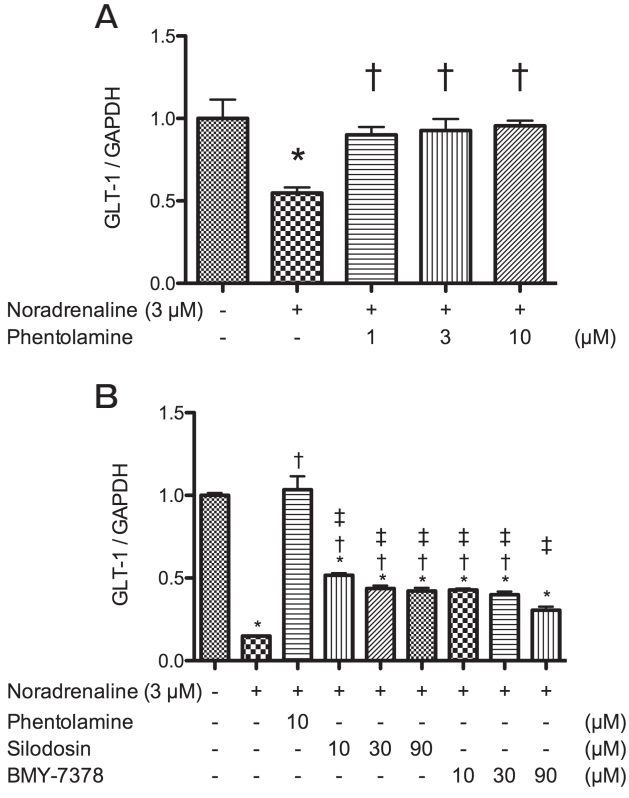

Fig. 2 Changes in *GLT-1* expression in primary astrocytes incubated with noradrenaline. Drugs were added at the indicated concentrations. *GLT-1* expression was quantified using quantitative PCR. GAPDH was used as an endogenous control. Values are expressed relative to GAPDH (n=3 per group). Statistical analysis was performed by one-way ANOVA and Bonferroni's multiple comparison test. Data are expressed as the mean ± SEM. (**A**) $^{*}P<0.001$ compared with the control group. $^{\dagger}P<0.05$ compared with the noradrenaline group. (**B**) $^{*}P<0.001$ compared with the control group. $^{\dagger}P<0.001$ compared with the noradrenaline group. $^{\ddagger}P<0.001$ compared with the noradrenaline and phentolamine groups.

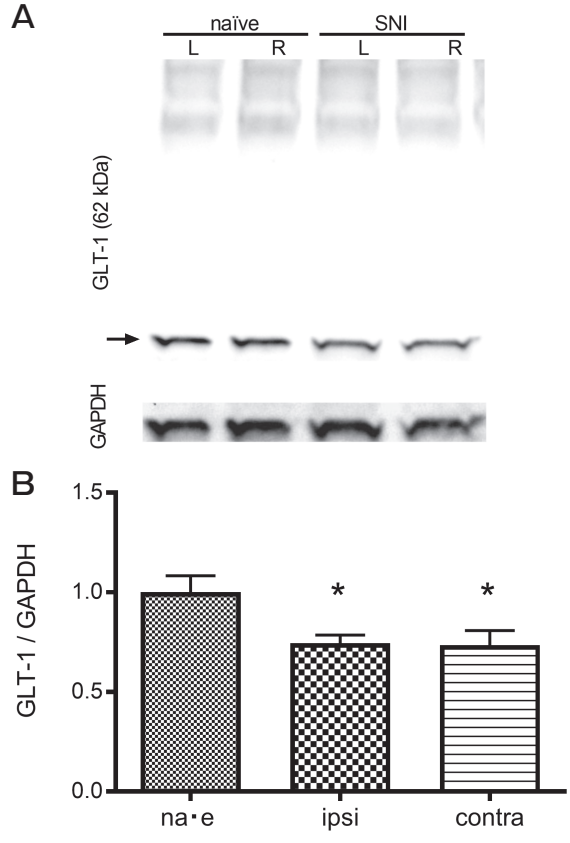

Fig. 3 Changes in GLT-1 protein levels after SNI in the L5 spinal cord. (**A**) Western blotting of GLT-1 in the spinal cords. GAPDH was used as a loading control. (**B**) Densitometry of the immunoreactive bands. Values are expressed as relative to the naïve rats (n=4 per group). Statistical analysis was performed by one-way ANOVA and Dunnett's multiple comparison test. Data are expressed as the means ± SEM. $^{*}P<0.05$ compared with the control.

significant changes on the ipsilateral side compared to the control group. In contrast, GLT-1 levels on the contralateral side increased significantly 24 h after the injection (Fig. 4C, D). Although the dose of phentolamine was higher than that in a previous study [20], the rats showed no motor or behavioral abnormalities after administration.

Changes in pain behavior by repeated intrathecal phentolamine injection in the rats with SNI. We next examined the effect of intrathecal phentolamine on pain behavior in the bilateral hind paw after SNI. Phentolamine has a half-life of 19 min in the blood; however, data on the pharmacokinetics of phentolamine in cerebrospinal fluid are not available. We administered phentolamine repeatedly based on a previous study which showed that the cardiovascular effect of intrathecal phentolamine lasted for approximately 30 min [20]. An intrathecal catheter was implanted at the same time as the SNI. Saline or 100 μg or 200 μg phentolamine was administered through the catheter daily for one week from day 3 after SNI. Changes in 50% PWT were examined using the von Frey test. Consistent with the results of a previous report, modified SNI caused MIP [21-22]. A bilateral decrease in 50% PWT was established on day 3, and it did not recover during the observation period in the saline group. There was no difference in pain behavior in the

100 μg group compared to the saline group. In the 200 μg group, 50% PWT on the ipsilateral side was similar to that in the other groups; however, the 50% PWT on the contralateral side was partially but significantly increased after the drug had been injected 7 times (Fig. 5).

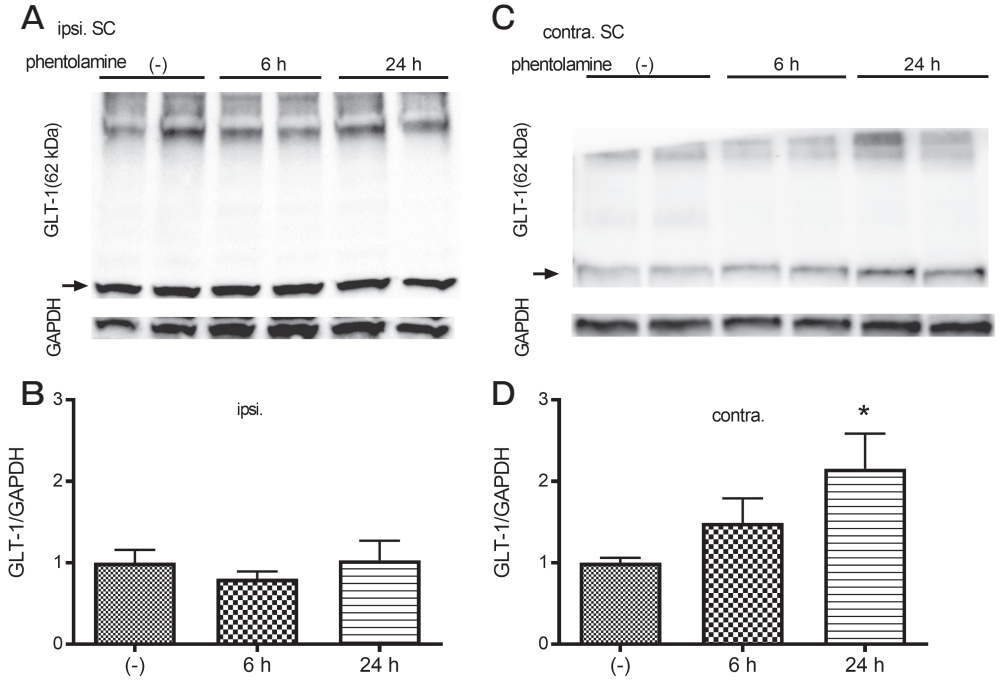

Fig. 4 Changes in GLT-1 protein levels in the L5 spinal cord after intrathecal phentolamine injection in rats with SNI. (**A**) GLT-1 western blot in the spinal cord 6 or 24 h after phentolamine injection. GAPDH was used as a loading control. Two representative samples at each time point are shown. (**B**) Densitometry of the immunoreactive bands. Values are expressed as relative to the control (n=4 per group). Statistical analysis was performed by one-way ANOVA and Dunnett's multiple comparison test. Data are expressed as the means ± SEM. $^{*}P<0.05$ compared with the control. SC, spinal cord.

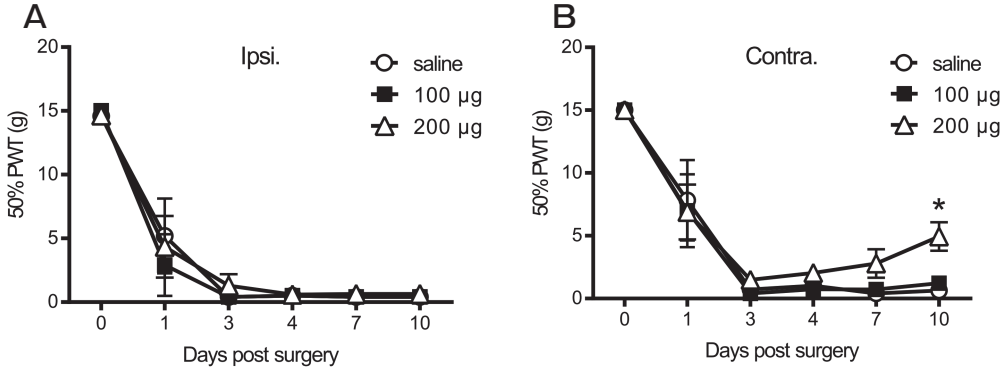

Fig. 5 Changes in the pain behavior of rats with SNI following repeated intrathecal phentolamine injection. The mechanical sensitivity of the hind paw was determined as 50% PWT in the ipsilateral hind paw (**A**) and the contralateral hind paw (**B**). Statistical analysis was performed using two-way ANOVA and Dunnett's multiple comparison test. Data are expressed as the mean ± SEM. Saline group: n=6; phentolamine 100 μg group: n=6; phentolamine 200 μg group: n=7. $^{*}P<0.05$, compared with the saline group of the same period. PWT, paw withdrawal threshold; SNI, spared nerve injury.

Discussion

In this study, we showed the effect of the α1 adrenergic receptor antagonist phentolamine on spinal GLT-1 levels and MIP in SNI model rats. Phentolamine restored GLT-1 in the contralateral spinal cord of SNI model rats and partially reversed MIP without ameliorating ipsilateral hyperalgesia. We also showed phentolamine blockade of noradrenaline-induced downregulation of *GLT-1* mRNA in cultured astrocytes. These results suggest that spinal GLT-1 dysfunction is one of the etiologies of MIP.

Regarding MIP, previous studies have proposed a variety of mechanisms to account for this phenomenon. At the cerebral level, the local GABAergic system and activated astrocytes in the somatosensory cortex are involved in MIP [23]. At the spinal level, the suggested mechanisms include Ca^{2+} oscillation [24] transmitted from one side to the other through gap junction connexin 43 [25] and glial activation [26]. Various signals such as interleukin-1β [27], tumor necrosis factor-α, nerve growth factor [28], cannabinoid receptor 2 [29], 5-hydroxytryptamine 3, and acid-sensing ion channel 3 [30] may play roles in the periphery, in addition to the central nervous system. The role of GLT-1 has not been suggested as an etiological factor in MIP; however, accumulating evidence shows the role of GLT-1 in the development of neuropathic pain. Spinal cord-specific GLT-1 knockout mice showed enhanced neuropathic pain caused by nerve injury [31]. Ceftriaxone, a potent activator of *GLT-1* transcription [32], alleviated tactile hyperalgesia in nerve-damaged wild-type mice [31]. However, ceftriaxone did not exert its analgesic effect in spinal cord-specific GLT-1 knockout mice [31]. GLT-1 in the locus coeruleus plays an essential role in the regulation of the descending inhibitory pathway by affecting spinal noradrenaline release [33]. In previous studies, the relationship between GLT-1 and neuropathic pain has been discussed. However, no previous studies have focused on the contralateral GLT-1. It is thus an important contribution of the present study that it highlights the role of GLT-1 in the development of MIP.

Noradrenaline and adrenergic receptors play major roles in descending inhibitory pathways and are important targets of clinically essential analgesic agents. For example, dexmedetomidine, a highly selective α2-adrenergic agonist, acts on the descending inhibitory pathways in the locus coeruleus to achieve its sedative action and analgesic effect on neuropathic pain [34]. Antidepressants used to treat chronic pain are known to enhance the noradrenergic inhibitory pathway. Contrary to these analgesic characteristics, the present results suggest that noradrenaline is involved in potentiating pain signals. A similar phenomenon was also reported clinically: chronic pain was associated with a dysregulation in the descending inhibitory pathways [35]. Unlike transient noradrenergic inhibition, excess chronic noradrenaline in the spinal cord might paradoxically enhance pain through GLT-1 regulation. In an *in vitro* assay, the addition of noradrenaline to the medium of primary astrocytes transiently increased the radio-isotope-labeled glutamine uptake [36]. By contrast, Kurita *et al.* reported that noradrenaline incubation decreased *GLT-1* mRNA expression via αl adrenoceptors in a dose-dependent manner [11]. Indeed, pain behavior gradually developed after SNI despite the increased concentration of noradrenaline in the bilateral spinal dorsal horn in their report [11]. Classically, the conflicting results on pain modulation by spinal noradrenaline are understood as different responses of dorsal and ventral neurons and α1/2 receptors in the spinal cord [37]. However, recent molecular tools have revealed that a selective population of astrocytes has a distinct role in noradrenergic descending inhibition. Kohro *et al.* showed that intrathecal noradrenaline administration caused mechanical pain hypersensitivity via α1A adrenoceptors on Hes5 transcription repressor-positive astrocytes located in the superficial laminae of the dorsal horn [38]. The details of noradrenergic pain modulation remain to be clarified.

Targeting α1 adrenergic receptor and GLT-1 for the treatment of MIP has some limitations. First, such research requires selective spinal cord treatment. We administered phentolamine intrathecally to act only on the spinal cord. However, in a previous study, upregulation of GLT-1 in the periaqueductal gray matter resulted in insufficient suppression of descending pain, leading to neuropathic pain [31]. Taken together, these results suggest that both upregulation of GLT-1 in the periaqueductal gray matter and its downregulation in the spinal cord may be necessary to improve pain behavior. Second, an α1 adrenergic receptor subtype non-specific treatment is required. The present study showed that GLT-1 downregulation was mediated by redundant signaling pathways, that is, α1A and α1D, which resulted in selective antagonism and partial res-

toration of *GLT-1* mRNA expression. The implications of such redundancy in this pathway are unclear. In a previous study, ceftriaxone was shown to induce GLT-1 upregulation via nuclear factor (NF)-κB activation in primary human fetal astrocytes [39]. Both α1A and α1D adrenoceptors activate the Gq protein, but there has been no study on the signaling pathway downstream of Gq and GLT-1 transcriptional induction. In this study, we did not explore additional mechanisms, so further studies will be needed to yield any findings regarding this signaling. Third, MIP did not completely recover after intrathecal phentolamine administration. As mentioned above, a variety of substances may contribute to MIP in the spinal cord and somatosensory cortex [23].

Repeated intrathecal injection of phentolamine failed to reverse the pain behavior on the ipsilateral side. Due to the fact that the pathology of neuropathic pain is complicated and glutamate dysregulation does not appear in itself to be the key, the intervention by α1 adrenergic receptors/GLT-1 alone was not sufficient to improve ipsilateral pain. Phentolamine also failed to increase GLT-1 protein levels in the ipsilateral spinal cord. We could not identify the reason for this phenomenon in the present study; however, several modulators would be expected to be involved in the regulation of GLT-1 in this pain model. For the development of a treatment for MIP, further studies on the detailed signaling pathways in astrocytes are required. Nonetheless, the present study adds a new finding regarding the mechanism of MIP.

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