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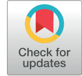
Norepinephrine-induced downregulation of *GLT-1* mRNA in rat astrocytes



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ABSTRACT

Aim of the research: Glutamate transporter-1 (GLT-1; also known as excitatory amino acid transporter 2) plays an important role in the maintenance of glutamate homeostasis in the synaptic cleft. Downregulation of GLT-1 in the spinal cord has been reported in chronic pain models, which suggests that GLT-1 is involved in the development of chronic pain. However, the mechanism by which GLT-1 is downregulated in the spinal cord is still unknown. We hypothesized that norepinephrine is involved in the regulation of GLT-1. The aim of this study was to investigate the effect of norepinephrine on *GLT-1* expression in cultured astrocytes.
Methods: This study involved both *in vivo* and *in vitro* experiments. We first validated changes in *GLT-1* mRNA expression in the spinal cord of rats with spared nerve injury (SNI) using real-time RT-PCR. Next, cultured primary astrocytes from the rat spinal cord were stimulated with norepinephrine, and *GLT-1* mRNA was subsequently quantitated. RNB cells, an astrocytic cell line, were also stimulated with norepinephrine and other α-adrenoceptor agonists.
Results: SNI resulted in bilateral downregulation of *GLT-1* in rat spinal cord. The *in vitro* study showed that norepinephrine and phenylephrine dose-dependently downregulated *GLT-1* in primary astrocytes and RNB cells. Furthermore, the effect of norepinephrine was reversed by an α-adrenoceptor antagonist.
Conclusion: Norepinephrine downregulates *GLT-1* mRNA expression in astrocytes via the α_1-adrenoceptor. Our results provide new insight into the mechanisms involved in downregulation of GLT-1 in the chronic pain models.

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1. Introduction

Glutamate is the primary excitatory neurotransmitter in the central nervous system and plays important roles in higher brain functions, such as learning, memory, and pain transmission [1]. However, excessive glutamate in pathological conditions has excitotoxic effects [2]. Astrocytes play a key role in homeostasis and the metabolism of glutamate in the synaptic cleft [3] by regulating glutamate-glutamine metabolism [4]. They predominantly express glutamate transporter-1 (GLT-1; also known as excitatory amino acid transporter 2). GLT-1 on astrocytes re-uptakes 90% of the excess glutamate released into the synaptic cleft and maintains glutamate concentrations at low levels. GLT-1 dysfunction is associated with various neurological disorders, such as Amyotrophic lateral sclerosis, Alzheimer's disease, epilepsy, and cerebral ischemia [5].

Downregulation of GLT-1 in the spinal cord has also been reported in chronic pain models. GLT-1 in the spinal dorsal horn is decreased after partial sciatic nerve injury [6], unilateral cervical contusion [7], and spinal nerve ligation [8]. Restoration of GLT-1 by adenoviral-mediated gene transfer and administration of

Abbreviations: ANOVA, analysis of variance; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's Modified Eagle's Medium; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFAP, anti-glial fibrillary acidic protein; GLT-1, glutamate transporter-1; PCR, polymerase chain reaction; PWT, paw withdrawal threshold; SEM, standard error of the mean; SNI, spared nerve injury.

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ceftriaxone, which induces GLT-1 [9], reduces the neuropathic pain behavior observed in neuropathic pain models [10,11]. These reports suggest that downregulation of GLT-1 is involved in the development of chronic pain. However, the mechanism by which GLT-1 becomes downregulated in spinal astrocytes is not understood.

Several studies have suggested a relationship between GLT-1 and norepinephrine. A series of reports have shown that GLT-1 in the locus coeruleus affects the function of norepinephrinergic descending inhibitory pathways [12–14]. Norepinephrine transiently potentiates glutamate uptake in cultured astrocytes [15]. Thus, the aim of the present study was to investigate the effect of norepinephrine on *GLT-1* expression in cultured spinal astrocytes and in the RNB cell line. This study began with validation of *GLT-1* expression in the rat pain model, followed by an *in vitro* study.

2. Materials & methods

2.1. Animal model

This study was approved by the Animal Care and Use Committee of 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 [16]. Ten-week-old male Sprague-Dawley rats (total 43 rats, CLEA Japan) were used for this study. The animals were housed in cages individually under a 12–12 h light-dark cycle with free access to food and water. Before surgery the rats were placed under anesthesia with sodium pentobarbital (40 mg/kg intraperitoneally). Additional inhalation anesthesia with 1–1.5% isoflurane in 100% oxygen was administered as needed. Spared nerve injury (SNI) of the sciatic nerve was performed as described previously [17], with modification. 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 in 2 layers. All procedures were performed under aseptic conditions.

2.2. Behavioral assessment

Mechanical hypersensitivity was assessed as the hind paw withdrawal threshold (PWT) with von Frey filaments (Touch-Test Sensory Evaluator, North Coast Medical) before, and 1, 3, 7, 10, and 14 days after surgery. Mechanical stimuli were applied to the lateral 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 [18], and calculated using the formula described by Chaplan et al. [19]. If the strongest filament did not elicit a response, the PWT was recorded as 15.0 g.

2.3. Primary culture of astrocytes from rat spinal cord

Primary astrocytes were isolated as described previously [20]. The spinal cord was obtained from 10-week-old rats that were deeply anesthetized and sacrificed by decapitation. The thoracic and lumber spinal cord were dissected and dipped immediately in cold phosphate buffered saline. The meninges and blood vessels were removed. The spinal cord was cut into small pieces and centrifuged at 1000 × rotations per minute (rpm) for 1 min. The precipitated cells were resuspended in 0.25% trypsin (Thermo Fisher Scientific), and incubated at 120 × rpm for 30 min in a 37 °C water bath with pipetting every 10 min. The cells were filtered using a 100 μm cell-strainer (BD Falcon). The culture medium (Dulbecco's Modified Eagle's Medium (DMEM)), supplemented with GlutaMAX (Thermo Fisher Scientific) containing 10% heat-inactivated fetal bovine serum (FBS) (BioWest), 100 U/mL penicillin, and 100 mg/mL streptomycin (Wako Pure Chemical Industries), was added to the filtered solution, and centrifuged at 800 × rpm for 6 min. The cells were resuspended and added to culture flasks (TPP T75 flask, Sigma-Aldrich). All cultures were maintained in an incubator containing 5% CO_2 at 37 °C for 7 days. The medium was changed every 2 days for the first week. After one week of culture, mixed glial cells were shaken in incubator at 37 °C and 240 × rpm for 6 h. Detached cells, consisting of microglia and oligodendrocytes, were removed, and attached cells were maintained as astrocyte-enriched cultures. After one week of additional culture, cells were plated onto appropriate plates for experiments.

2.4. Culturing of the RNB cell line

The RNB astrocytic cell line was obtained from the Japanese Collection of Research Bioresources (# IFO50491). The cells were thawed and maintained in DMEM (Thermo Fisher Scientific) supplemented with 10% heat-inactivated FBS (BioWest), 100 U/mL penicillin, and 100 mg/mL streptomycin (Wako), in an incubator containing 5% CO_2 at 37 °C. Before the experiments were performed, cells were serum-starved in DMEM without antibiotics for 24 h.

2.5. Immunocytochemistry

Primary astrocytes were plated onto chamber slides (Lab-Tek chamber slides, Nunk) coated with 0.1% gelatin (Merck Millipore). Cells were fixed with 4% paraformaldehyde for 25 min at 4 °C followed by blocking in 10% normal goat serum for 1 h. Cells were incubated with an AlexaFluor 488-conjugated anti-glial fibrillary acidic protein (GFAP) antibody (1:200, Cell Signaling Technology) overnight at 4 °C. Coverslips were placed on the slides with ProLong Gold Antifade Mountant with DAPI (4′,6-diamidino-2-phenylindole; Thermo Fisher Scientific). Images were captured using a fluorescent microscope (EVOS-FL, Thermo Fisher Scientific) equipped with a 20× Plan Fluor objective lens.

2.6. Drug treatment

Cells were incubated with norepinephrine (Daiichi-Sankyo), phenylephrine hydrochloride (Kowa Pharmaceutical), or dexmedetomidine hydrochloride (Maruishi Pharmaceutical) at concentrations of 0, 0.1, 1, or 10 μM for 12 h. RNB cells were also incubated with phentolamine mesylate (Novartis) at concentrations of 0, 10, 30, or 90 μM with or without norepinephrine for 12 h.

2.7. Quantitative reverse transcription-polymerase chain reaction (PCR) for GLT-1

For the *in vivo* study, rats were sacrificed by decapitation under deep anesthesia. The L4–5 spinal cord was dissected out, separated bilaterally, and dipped immediately in RNAlater (Qiagen). Total RNA was extracted from individual spinal cords with an RNeasy Lipid Tissue Mini Kit (Qiagen) according to the manufacturer's manual. For the *in vitro* study, the cells were harvested and lysed with QIAzol reagent (Qiagen), and total RNA was extracted using the standard ethanol precipitation method. cDNA was reverse-transcribed from 1 μg of total RNA with a QuantiTect Reverse Transcription Kit (Qiagen). Genomic DNA was degraded using the gDNA wipeout buffer included in the kit. Primer sequences are

summarized in Table 1. The primer pair was designed to amplify both *GLT-1* transcript variants, 1 and 2. Quantitative PCR analysis was performed with a Light Cycler (Roche Diagnostics) with SYBR Premix Ex Taq II (Takara-Bio). 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 the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). PCR specificity was confirmed using gel electrophoresis and DNA sequencing.

2.8. Norepinephrine quantification in the spinal dorsal horn

Rats were sacrificed by decapitation under deep anesthesia. The bilateral L4–5 spinal dorsal quadrants were removed rapidly and homogenized in 10 μl/mg of 0.01 N hydrochloric acid containing 0.15 mM EDTA and 4 mM sodium metabisulfite. After centrifugation, the supernatants were collected and norepinephrine concentration was measured by enzyme-linked immunosorbent assay (ELISA) (ImmuSmol) according to the manufacture's instruction.

2.9. Statistical analysis

Data are shown as mean or mean ± standard error of the mean (SEM). Behavioral assessment was analyzed using a one-way analysis of variance (ANOVA) followed by a Dunnett's multiple comparison test. Quantitative PCR and ELISA data were analyzed using a one-way ANOVA followed by Tukey's and Sidak's multiple comparison tests. Differences with a P-value < 0.05 were considered statistically significant. All statistical analyses were performed using Prism software (version 6.0. GraphPad Software).

3. Results

3.1. Pain behavior after SNI

Before the surgery rats did not show any mechanical hypersensitivity. On the day after surgery, the 50% PWT was decreased transiently on the ipsilateral side, presumably because of the direct surgical injury of the nerves. This exacerbation was reversed on day 3; however, the 50% PWT significantly decreased over time after day 7 (Fig. 1A). On the contralateral side, the 50% PWT was no different from the ipsilateral side at baseline (Fig. 1B). Although the decrease in the 50% PWT was not as marked as on the ipsilateral side, the 50% PWT was decreased significantly on day 10 compared to baseline.

3.2. Norepinephrine content in the spinal dorsal horn

Norepinephrine concentration in the spinal dorsal horn was measured 7 and 14 days after surgery and normalized to naïve rats. Although the difference was not statistically significant, norepinephrine tended to peak on day 7 and declined to day 14 (Table 2). This trend was observed bilaterally.

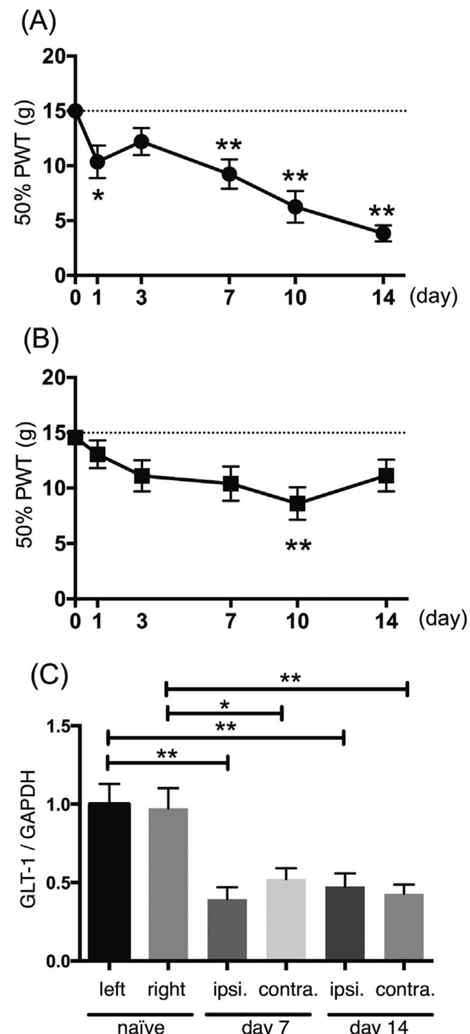

Fig. 1. Changes in pain behavior and *GLT-1* expression in the spinal cord of rats with SNI. Mechanical sensitivity of the hind paw was determined as the 50% PWT in the ipsilateral hind paw (A) and the contralateral hind paw (B). Expression of *GLT-1* was quantitated using quantitative PCR 7 and 14 days after SNI (C). GAPDH was used as the endogenous control. The bar chart shows the expression levels relative to those of the left side of the naïve rats. Data are expressed as mean ± SEM. Naïve group: n = 8; SNI day 7 group: n = 7; SNI day 14 group: n = 12. *P < 0.05 and **P < 0.01 compared with the control.
GAPDH: glyceraldehyde 3-phosphate dehydrogenase, GLT-1: glutamate transporter-1, PWT: paw withdrawal threshold, SNI: spared nerve injury.

Table 2
Changes in norepinephrine content in the spinal dorsal horn after SNI.

	Naïve	Day 7	Day 14
Ipsilateral (%)	100.0 ± 14.9	130.5 ± 10.8	114.7 ± 14.8
Contralateral (%)	100.0 ± 9.1	127.9 ± 10.4	86.9 ± 8.5

Data are relative to naïve (mean ± SEM, n = 4 in each group).

Table 1
Primer pairs for quantitative PCR.

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

3.3. *GLT-1* mRNA expression in the spinal cord after SNI surgery

Previous studies have reported downregulation of *GLT-1* in the spinal cord in some pain models [6–8]. We examined changes in *GLT-1* expression in rats with SNI on days 7 and 14. Naïve rats were used as controls. SNI caused bilateral downregulation of *GLT-1* mRNA in the spinal cord, even after unilateral surgery (Fig. 1C). There were no significant differences in the expression of *GLT-1* mRNA after SNI surgery between the ipsilateral and contralateral sides.

3.4. *GLT-1* mRNA expression in rat primary astrocytes and an astrocytic cell-line stimulated with norepinephrine

To explore the mechanism involved in the bilateral downregulation of *GLT-1* in the animal model, we cultured astrocytes from the rat spinal cord. Astrocyte-enriched primary cultures were immunostained for GFAP, a marker of astrocytes. Most cells were GFAP-positive (Fig. 2A), as previously described [20]. The cells constitutively expressed *GLT-1* under regular culture conditions. After the treatment of cell cultures with norepinephrine, *GLT-1* expression was significantly reduced in comparison to baseline conditions at a concentration of 10 μM (Fig. 2B). To further examine this phenomenon, we used a rat astrocytic cell line, namely RNB cells [21], as a surrogate of primary astrocytes. RNB cells also constitutively expressed *GLT-1* under regular culture conditions. The expression of *GLT-1* in RNB cells decreased significantly after treatment with norepinephrine, in a similar manner to that observed in the primary cells (Fig. 3A). Although norepinephrine binds to both α- and β-adrenoceptors, it shows much greater affinity for α-adrenoceptors than β-adrenoceptors. Thus, we treated RNB cultures with phenylephrine (a selective α1-adrenoceptor agonist) and dexmedetomidine (a selective α2-adrenoceptor agonist) at the same concentrations as those used for norepinephrine. Phenylephrine dose-dependently suppressed the expression of *GLT-1* (Fig. 3B). The relative expression of *GLT-1* at 1 and 10 μM was 49.1 ± 4.4%, and 18.9 ± 1.9% of the control,

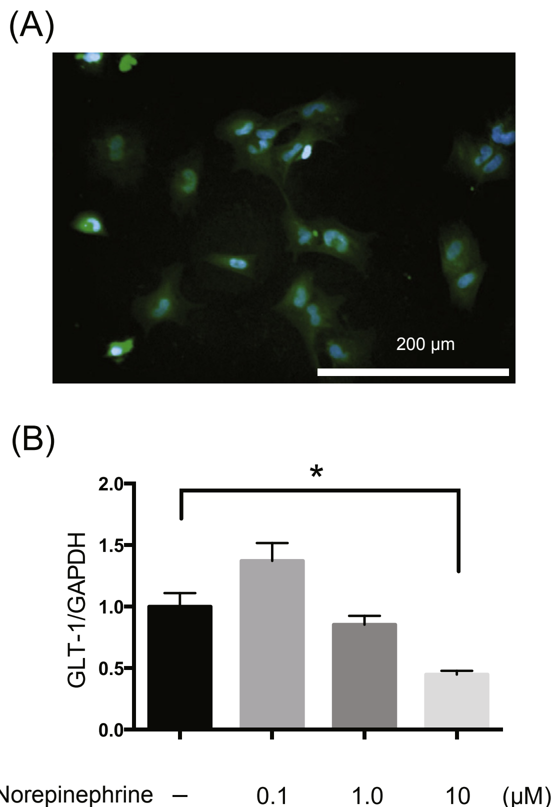

Fig. 2. GFAP immune-reactivity in astrocyte-enriched primary cultures and *GLT-1* expression in cells incubated with norepinephrine. (A) GFAP immuno-reactivity is shown in green. The nuclei of the cells are shown in blue following DAPI staining. (B) Changes in *GLT-1* expression in astrocyte-enriched primary cultures. Norepinephrine was added at the indicated concentrations for 12 h. *GLT-1* expression was quantitated using quantitative PCR. GAPDH was used as an endogenous control. Bar chart shows the expression levels relative to those of the control. Data are expressed as mean ± SEM (n = 3 per group). *P < 0.05 compared with the control. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

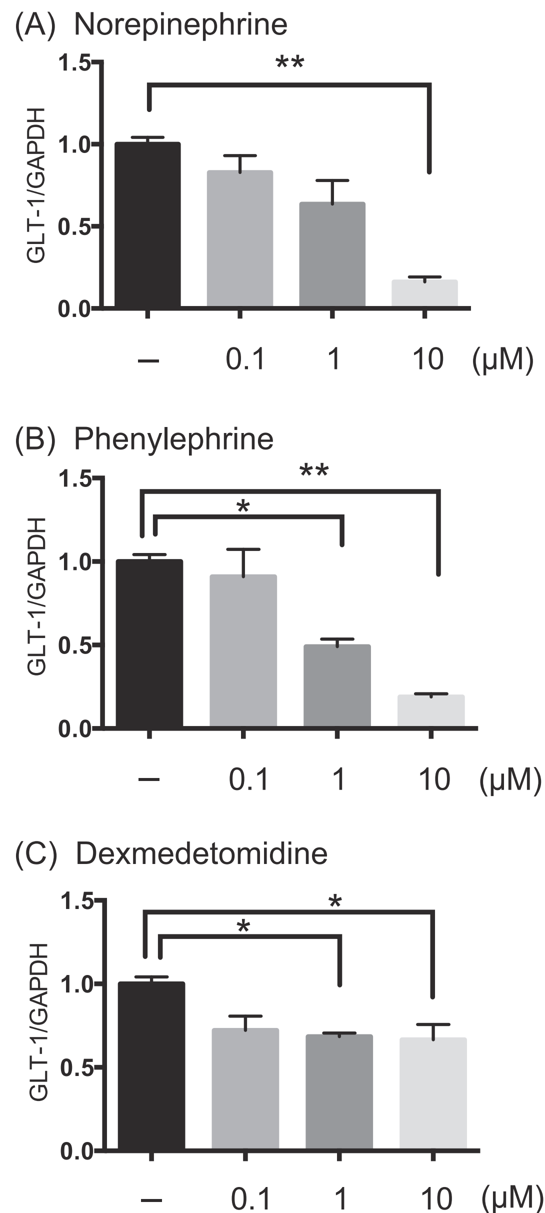

Fig. 3. *GLT-1* expression in RNB cells incubated with α-adrenoceptor agonists. RNB cells were stimulated with α-adrenoceptor agonists. Norepinephrine (A), phenylephrine (B), and dexmedetomidine (C) were added at the indicated concentrations for 12 h. *GLT-1* expression was quantitated using quantitative PCR. GAPDH was used as an endogenous control. Bar chart shows the expression levels relative to those of the control. Data are expressed as mean ± SEM (n = 4 per group). *P < 0.05 and **P < 0.01 compared with the control.

respectively. Dexmedetomidine suppressed *GLT-1* expression to a lesser extent than phenylephrine; however, no dose-dependent effect was observed (Fig. 3C). The relative expression of *GLT-1* at 1 and 10 μM was 68.4 ± 2.2% and 66.6 ± 9.1% of the control, respectively. Next, we investigated the effect of phentolamine, an α-adrenoceptor antagonist, on the norepinephrine-induced downregulation of *GLT-1*. *GLT-1* expression was suppressed by 3 μM of norepinephrine. Phentolamine blocked the norepinephrine-induced downregulation of *GLT-1* at 90 μM (Fig. 4).

4. Discussion

In this study, we sought to investigate the mechanism involved in the bilateral downregulation of *GLT-1* mRNA observed in the spinal cord of rats with unilateral nerve injury. Using primary astrocytes from the rat spinal cord and an astrocytic cell-line, we demonstrated that norepinephrine downregulates the expression of *GLT-1* mRNA via the α_1-adrenoceptor. To our knowledge, this association has not been reported previously.

Previous studies have assessed the expression of *GLT-1* in the ipsilateral spinal dorsal horn in pain models [6,7]. The expression of *GLT-1* decreased to an equivalent extent bilaterally in the spinal cord in the present study. This result raises questions about the mechanism by which unilateral nerve injury affects the contralateral spinal cord. Several studies have attempted to address this. Pro-inflammatory cytokines are involved in the development of mirror-image pain in the sciatic inflammatory neuropathy model [22]. Ca^{2+}-oscillations spread in the spinal cord through gap junctions [23]. Bilateral activation of spinal cord astrocytes has been reported following spinal nerve transection [24]. We hypothesized that the contralateral side of the spinal cord is affected partly via activation of the norepinephrinergic descending inhibitory pathway, since norepinephrinergic descending inhibition is activated in various pain models [25], and norepinephrine concentrations in the spinal dorsal horn show no laterality after unilateral spinal nerve ligation [26]. The norepinephrine-induced suppression of *GLT-1* mRNA in the present study indirectly supports our hypothesis. The mechanism of sustained *GLT-1* suppression observed on day 14, when norepinephrine returned to the baseline, remains unknown and should be addressed in future study. Bilateral downregulation of *GLT-1* is predicted to cause glutamate spillover on both sides of the spinal cord. This prediction is in line with the 50% PWT decrease in the contralateral hind paw observed here. The phenomenon of mirror-image pain is often reported both clinically [27] and experimentally [22].

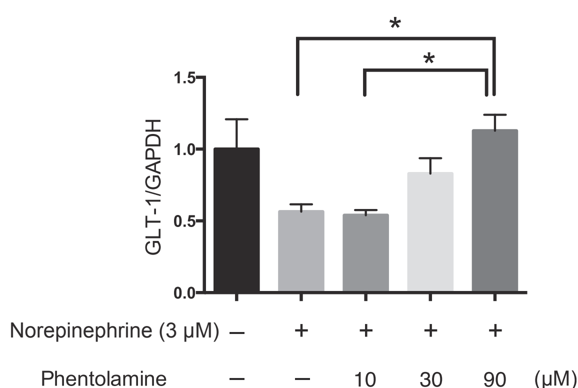

Fig. 4. *GLT-1* expression in RNB cells incubated with norepinephrine and phentolamine. RNB cells were incubated with norepinephrine (3 μM) and phentolamine (0, 10, 30, 90 μM) for 12 h. *GLT-1* expression was quantitated using quantitative PCR. GAPDH was used as an endogenous control. Data are expressed mean ± SEM (n = 4 per group). *P < 0.05 compared with the control.

α- and β-adrenoceptors are expressed in astrocytes [28], and play diverse roles, such as the induction of Ca^{2+} elevation [29] and antigen presentation [30]. With regard to glutamate homeostasis, some studies have reported the involvement of astrocytic adrenoceptors. Glutamate uptake is increased by α_1-adrenergic activation and is not affected by α_2-adrenergic stimulation in primary astroglial cultures [15]. This effect is mediated by α_{1B}-adrenoceptors [31]. Glutamate reuptake is increased by the α_1-adrenergic agonist phenylephrine in the frontal cortex of rats [32]. Thus, the norepinephrine-induced increase in glutamate uptake has been consistently reported. On the other hand, our results suggest that norepinephrine might inhibit glutamate uptake in astrocytes. Although the present study on *GLT-1* expression was not in line with previous studies on GLT-1 function, these seemingly conflicting results may reflect the different time-points used in the experiments. Glutamate uptake was measured 15 s to 10 min after adrenergic stimulation in previous studies, whereas we quantitated *GLT-1* mRNA 12 h after adrenergic stimulation. Taken together, the current evidence suggests that norepinephrine transiently increases glutamate uptake; however, prolonged adrenergic stimulation in turn suppresses the expression of *GLT-1*.

We further studied the subtypes of α-adrenoceptors involved in the regulation of *GLT-1*. The relationship between norepinephrine and α_2-adrenoceptors has been well described, especially in the locus coeruleus, and is utilized clinically by sedative and analgesic agents. On the other hand, our findings suggest that the effect of norepinephrine on *GLT-1* expression is more likely to be mediated by α_1-adrenoceptors than α_2-adrenoceptors. Dexmedetomidine, a highly selective α_2-agonist with an α_{2A} to α_1 ratio of 1300:1 [33], showed weaker effects on the expression of *GLT-1* in RNB cell cultures in the present study. Given the selectivity of dexmedetomidine, α_2-adrenergic stimulation might therefore play a role in the regulation of *GLT-1*.

Norepinephrine is one of the neurotransmitters of the descending inhibitory pathways projecting to the spinal dorsal horn [34], and plays a role in the suppression of pain transduction. As patients with established chronic pain are reported to have dysfunctional descending inhibitory pathways [34], this system is considered a target for the treatment of chronic pain. The relationship between norepinephrine and GLT-1 has been demonstrated in previous studies [12–14], and here. *GLT-1* upregulation by ceftriaxone has been shown to reverse pain behavior in neuropathic pain models [9]. These clinical and experimental reports suggest GLT-1 may represent a target for the treatment of chronic pain.

This study has several limitations. First, we only quantitated *GLT-1* mRNA expression. Changes in GLT-1 protein level, membrane localization, and glutamate uptake need to be validated. Second, although we obtained the primary cells from the rat, comparable to those used in the behavioral experiment, the gap between *in vitro* and *in vivo* models should be bridged by replicating the *in vitro* findings in the animal models.

In conclusion, we demonstrated that norepinephrine downregulates *GLT-1* mRNA via the α_1-adrenoceptor in cultured astrocytes. Our findings provide new insight into the mechanisms involved in downregulation of GLT-1 in the chronic pain models.

Disclosure

All authors declare no conflict of interest regarding the publication of this paper.

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Transparency document

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