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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.

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

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