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# Valproate prevents dysregulation of spinal glutamate and reduces the development of hypersensitivity in rats after peripheral nerve injury

**Masaru Yoshizumi**, **James. C. Eisenach**, and **Ken-ichiro Hayashida**
Department of Anesthesiology, Wake Forest University School of Medicine, Medical center blvd. Winston-Salem, NC 27157, USA

## Abstract

The present study examined whether the histone deacetylase inhibitor valproate prevents down-regulation of glutamate transporters in the primary cultured astrocytes and in the spinal cord after L5-L6 spinal nerve ligation (SNL), and whether this action of valproate on spinal glutamate transporters prevents spinal glutamate dysregulation and development of hypersensitivity after SNL. In cultured astrocytes, valproate prevented down-regulation of glutamate transporter-1 (GLT-1) and glutamate-aspartate transporter (GLAST) in a concentration dependent manner. Repeated oral administration of valproate reduced the development of hypersensitivity and prevented the down-regulation of spinal GLT-1 and GLAST expression in rats after SNL, but did not affect mechanical nociception and expression of those transporters in normal rats. Valproate's effects on hypersensitivity and spinal GLT-1 expression in SNL rats were blocked by intrathecal administration of the selective GLT-1 blocker dihydrokainic acid or the GLT-1 selective small interfering RNA (siRNA). Extracellular glutamate concentration in the spinal cord, measured by microdialysis, was increased in animals with SNL or after GLT-1 selective siRNA treatment, and valproate prevented the SNL-induced glutamate increase. These results suggest that valproate reduces the development of chronic pain after nerve injury in part via preventing down-regulation of glutamate transporters, especially GLT-1, to maintain normal extracellular glutamate concentrations in the spinal cord.

### Keywords

Valproate; Glutamate transporter; chronic pain; Astrocyte; Spinal cord

## Introduction

The preeminent excitatory neurotransmitter glutamate underlies normal physiology and pathophysiology in the central nervous system. Extracellular concentrations of glutamate are



**Corresponding authors:** Ken-ichiro Hayashida, DVM, PhD Department of Anesthesiology, Wake Forest University School of Medicine, Medical Center Boulevard, Winston-Salem, NC 27157, USA Tel: +1-336-716-2743, Fax: +1-336-716-6744, khayashi@wakehealth.edu.



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regulated by two types of astroglial glutamate transporters, predominantly by glutamate transporter-1 (GLT-1) and, to a lesser extent, by glutamate-aspartate transporter (GLAST).[19, 20] Impairment or down-regulation of astroglial glutamate transporters results in elevation of extracellular glutamate, contributing to many neurological diseases including amyotrophic lateral sclerosis, epilepsy, stroke, and chronic pain.[3, 4, 9, 21]

Clinical studies have demonstrated that an antiepileptic drug valproate is effective in various painful conditions such as migraine headaches,[14] diabetic neuropathy,[16] and post-herpetic neuralgia.[17] Valproate is known to act by several mechanisms to reduce pain, including inhibition of voltage-gated sodium channels, increasing γ-aminobutyric acid (GABA) levels via enhancing GABA synthesis and inhibiting GABA degradation, and direct stimulation of $GABA_A$ receptors.[6] In addition to those classic actions, valproate also inhibits histone deacetylase (HDAC).[8] Histone deacetylation by HDACs has been recognized as an important mechanism in regulation of gene transcription responsible for the induction and maintenance of chronic pain, including proinflammatory cytokine production and neuroglia plasticity in the central nervous system. Previous studies demonstrated that direct inhibition of HDAC by valproate activated gene transcription of glutamate transporters in cultured glial cells[1, 18] and reduced hypersensitivity in mice after peripheral inflammation.[2] Similarly, we recently observed in rats after L5-L6 spinal nerve ligation (SNL) that repeated oral administration of valproate starting from 3 weeks after surgery restored down-regulated expression of GLT-1 and GLAST in the spinal cord and reduced chronic hypersensitivity after this nerve injury.[13] These results suggest that increase in expression of glutamate transporters is an important mechanism of valproate action in the spinal cord to reduce established chronic pain.

The current study examined whether valproate, in addition to its actions in established pathology, also prevents down-regulation of glutamate transporters in the primary cultured astrocytes *in vitro* and in the spinal cord after SNL *in vivo*, and whether this action of valproate on glutamate transporters, especially on GLT-1, prevents hypersensitivity and elevation of extracellular glutamate concentration in the spinal cord after SNL. We also examined whether elevation of extracellular glutamate concentration by knock-down of GLT-1 with the selective small interfering RNA (siRNA) induces hypersensitivity in normal rats.

## Methods

### Animals

Male (5 weeks old at arrival) and pregnant female Sprague-Dawley rats from Harlan Industries (Indianapolis, IN), housed under a 12-h light-dark cycle with food and water *ad libitum*, were used. All experiments were approved by Animal Care and Use Committee at Wake Forest University (Winston Salem, NC).

### Astrocyte Culture

Primary astrocyte cultures were prepared from the cerebral cortices of neonatal rats between postnatal days 1 and 2 as previously reported with minor modifications.[11] Cerebral cortices were mechanically dissociated in ice-cold Hank's buffered salt solution (HBSS, pH = 7.2) by fire-polished glass pipettes and centrifuged at 300×G for 5 min. Cells were re-dissociated in ice-cold HBSS and the procedure was repeated two times using smaller pipette tip diameters. Cells were seeded onto T-50 flasks and incubated in Dulbecco's modified Eagle's medium, containing 10% fetal bovine serum, 100 units/ml penicillin/streptomycin, and 2 mM L-glutamine, with or without sodium valproate (Sigma Chemical CO., St Louis, MO), at 37°C and 5% $CO_2$ for up to 2 weeks.

## Surgical Preparations

**Spinal Nerve Ligation—**L5-L6 spinal nerve ligation (SNL) was performed as previously described.[15] Briefly, under anesthesia with 2% isoflurane in oxygen, the right L6 transverse process was removed and the right L5 and L6 spinal nerves were tightly ligated using 5–0 silk suture.

**Intrathecal Catheterization—**Animals were anesthetized with 2% isoflurane, and intrathecal catheterization was performed as previously described.[24] A small puncture was made in the atlanto-occipital membrane of the cistern magnum, and a polyethylene catheter (ReCathCo LLC, Allison Park, PA), 7.5 cm, was inserted so that the caudal tip reached the lumbar enlargement of the spinal cord. Animals were allowed at least 5 days to recover from the surgery.

## Behavioral Testing

The person conducting behavioral testing was blinded to the treatments.

**von Frey test—**Hypersensitivity to light touch following SNL was assessed using calibrated von Frey filaments (Stoelting, Wood Dale, IL) applied to the plantar surface of the hind paw ipsilateral to surgery. Filaments were applied to the bending point for 5 seconds, and a brisk paw withdrawal was considered a positive response. Withdrawal threshold was determined using an up-down statistical method.[5]

**Randall-Selitto test—**Nociceptive mechanical thresholds in normal rats were measured with the Randall-Selitto test using an analgesimeter (Ugo Basile, Comerio, Italy). The test was performed by applying pressure to the hind paw. When the animal withdrew the paw or vocalized, the pedal was immediately released and the nociceptive threshold was read on a scale. A cutoff of 250 g was used to avoid potential tissue injury.

## Drugs and siRNA treatments

Sodium valproate was dissolved with vehicle (0.5% carboxymethylcellulose solution) and orally administered twice a day by a feeding tube (300 mg/5 mL/kg) from day 0 to day 21, and SNL surgery was performed at day 1. The dose of valproate was determined from our previous study[13] and behavioral tests were performed at the morning 1 hr prior to the first administration during day 0 to day 22. For pharmacological blockade of spinal GLT-1, dihydrokainic acid (DHK, 10 μg/10 μL/rat, Tocris Bioscience, Ellisville, MO) was dissolved in saline and intrathecally injected, followed by 10 μL saline, in valproate-treated SNL rats at day 14, and withdrawal threshold was tested 1 hr after injection. For knock-down of spinal GLT-1, an siRNA mixture for rat GLT-1 (SMARTpool #M-091209-02, Thermo Fisher Scientific Inc., Rockford, IL) or a non-targeting siRNA pool (#D-001206-14, Thermo Fisher Scientific Inc.) was dissolved in double distilled water, diluted with the transfection reagent (i-Fect; Neuromics, Edina, MN) to achieve a final concentration of 0.17 nmol/10 μl, and intrathecally injected for 5 days in normal or valproate treated SNL rats from day 17 to day 21. On day 22, rats were anesthetized for microdialysis in the spinal cord or sacrificed by $CO_2$ exposure to obtain the lumbar spinal dorsal horn for Western blotting.

## Western Blotting

Western blotting for GLT-1 and GLAST was performed as we previously reported.[13] The lumbar spinal dorsal horn tissues ipsilateral to SNL or cultured astrocytes were homogenized, lysed, and centrifuged for 10 min at 4°C at 1000G. Protein content in each supernatant was measured using a standard Bradford method. Samples (25 μg protein) were placed on the 10-20% gradient gels (Criterion Tris-HCl Gel; Bio-Rad, Hercules, CA), run at

100V for 1 hr, and transferred to PVDF membrane (Bio-Rad). The membrane was blocked with 1% bovine serum albumin in Tris-buffer saline containing 0.1% Tween 20 (TBST), and incubated overnight at 4°C with a guinea pig anti-GLT-1 (1:1000; Millipore, Temecula, CA), a rabbit anti-GLAST (1:1000; Cell Signaling, Danvers, MA), or a rabbit anti-α-tubulin (1:1000; Cell Signaling). After washing with TBST, the membrane was incubated for 1 hr at room temperature with a corresponding HRP-conjugated secondary antibody (1:5000; anti-guinea pig or 1:1000; anti-rabbit, Santa Cruz, Santa Cruz, CA), treated for 1 min with the West Pico chemiluminescence (Thermo Fisher Scientific INC.), and exposed to X-ray film (Kodak BioMax film, Sigma). The density of each specific band was measured using a computer-assisted imaging analysis system (Sigma Scan Pro 5 software, Systat Software Inc., Chicago, IL).

### Microdialysis for Glutamate Measurement

Microdialysis in the spinal cord was performed as we previously reported. [12] Animals were anesthetized with 2% isoflurane, and then maintained with 1.25-1.5% isoflurane during the study. A heating blanket was used to maintain rectal temperature 36.5 ± 0.5°C and the right jugular vein was cannulated for saline infusion (1.2 ml/kg/hr). The L3-L6 level of spinal cord was exposed by a T13-L1 laminectomy. A microdialysis probe (OD = 0.22 mm, ID = 0.20 mm, length = 1 mm, CX-I-8-01, EICOM CO., Kyoto, Japan) was inserted from just lateral to the right dorsal root and perfused with Ringer's solution (1.0 μL/min) 1 hr prior to the study. Then, a microdialysate sample was collected for 1 hr and kept at −20°C until assayed for glutamate. G lutamate content in the microdialysates was measured by a high pressure liquid chromatography (HPLC) system with electrochemical detection (HTEC-500, EICOM CO.).

### Statistical Analyses

Data are presented as mean ± SE. Behavioral data were by Shapiro-Wilke test for normality then followed by one-way or two-way repeated-measures analysis of variance (ANOVA), using SigmaPlot software (Chicago, IL). Other data in Western blotting and microdialysis experiments were analyzed by one-way or two-way ANOVA. $P<0.05$ was considered significant.

## Results

### Valproate prevents down-regulation of glial glutamate transporters in cultured astrocytes

Primary cultured astrocytes were used to examine direct effect of valproate on the expression of astroglial glutamate transporters. Primary astrocytes showed a reduction in the expression of GLT-1 and GLAST in a time-dependent manner in culture (Fig. 1A, B and C), consistent with previous observations.[7, 22] In astrocytes cultured for 2 weeks, valproate treatment concentration-dependently prevented the downregulation of GLT-1 and GLAST compared to the non-treatment.

### Valproate reduces SNL-induced hypersensitivity and prevents down-regulation of spinal glutamate transporters after SNL

All animals tolerated repeated oral administration of vehicle or valproate with normal weight gain and no change in spontaneous behavior, including grooming and exploration activity (data not shown). In both vehicle and valproate treated animals, withdrawal threshold values in the paw ipsilateral to SNL significantly decreased 3 days after surgery (Day 4) and remained lower than pre-surgery during the experiment (Fig. 2A). Valproate treated animals showed significantly higher post-surgical withdrawal threshold values in the

paw ipsilateral to SNL compared to vehicle treated animals. In normal rats, neither vehicle nor valproate affected withdrawal threshold values in the hind paw (Fig. 2B).

Fig. 3A depicts representative Western blotting images of GLT-1 and GLAST in the ipsilateral spinal dorsal horn from normal and SNL rats treated with vehicle or valproate. Quantitatively, vehicle treated SNL animals showed significantly lower expression of GLT-1 and GLAST in the spinal dorsal horn compared to vehicle-treated normal animals (Fig. 3B and C). Valproate treated SNL animals showed significantly higher expression of GLT-1 and GLAST in the spinal dorsal horn compared to vehicle-treated SNL rats. However, valproate treatment did not affect expression of GLT-1 and GLAST in the spinal dorsal horn in normal animals.

### Blockade or knockdown of spinal GLT-1 reduces the anti-hypersensitivity effect of valproate

In valproate treated SNL rats, intrathecal administration of the selective GLT-1 blocker DHK significantly reduced withdrawal threshold in the paw ipsilateral to surgery compared to pre-drug and post-saline (Fig. 4A), consistent with our previous observation.[13] Intrathecal treatment with a GLT-1 selective siRNA (GLT-1 siRNA) time-dependently reduced withdrawal threshold in the paw ipsilateral to surgery compared to non-targeting siRNA (control) in valproate treated SNL rats (Fig. 4B). Intrathecal GLT-1siRNA treatment slightly but significantly reduced withdrawal threshold (124 ± 3 g) in the paw compared to pre-treatment value (135 ± 4 g) in normal animals (Fig. 4C) Fig. 5A depicts representative Western blotting images of GLT-1 and GLAST in the ipsilateral spinal dorsal horn from normal animals with or without intrathecal GLT-1siRNA treatment and valproate treated SNL animals with intrathecal GLT-1siRNA or non-targeting siRNA treatment. Quantitatively, in both normal and valproate treated SNL animals, spinal GLT siRNA treatment did not alter GLAST but reduced GLT-1 expression in the spinal dorsal horn compared to non-treatment or non-targeting siRNA treatment (Fig. 5B and C).

### Valproate reduces the increase in extracellular glutamate concentration in the spinal dorsal horn after SNL

As shown in Fig. 6, vehicle-treated SNL animals and spinal GLT siRNA treated normal animals showed significantly higher glutamate concentrations in microdialysates from the spinal cord compared to non-treated normal animals. In SNL animals, valproate treatment significantly reduced glutamate concentrations in microdialysates from the spinal cord compared to vehicle treatment.

## Discussion

Epigenetic changes, especially histone deacetylation by HDACs, are recognized as key mechanisms in resultant neuro-inflammation and neuroglia plasticity in the spinal cord after inflammation and nerve injury. One major consequence of HDAC activation is down-regulation of glutamate transporters in spinal astrocytes, which results in elevation of extracellular glutamate level and contributes, in part, to chronic pain after nerve injury.[4, 21] The current study supports previous observations in rodents which indicate anti-hypersensitivity effects of HDAC inhibitors, including valproate, on expression of glutamate transporters and hypersensitivity after inflammation and nerve injury,[2, 10, 13, 23] and extends these observations by showing that preventive effects of valproate on the glutamate regulation in the spinal cord and the development of hypersensitivity after nerve injury.

Because of its direct actions to inhibit sodium channels and to enhance GABAergic pathways, most laboratory studies have focused on the acute mechanisms of action to study

analgesia from valproate. However, in clinical practice, patients with chronic pain are treated with valproate at least for a few weeks. Previous studies demonstrated that valproate activates transcription of glutamate transporters in cultured glial cells[1, 18] and chronic treatment of valproate increases glutamate transporters in the spinal cord in rats after peripheral nerve injury and in the hippocampus in normal and seizure-induced rats.[10, 13, 23] Similarly, in the current study valproate prevented down-regulation of glutamate transporters over time in cultured astrocytes *in vitro* and in the spinal cord after nerve injury *in vivo*. In the current study, however, 3 week treatment of valproate failed to affect the spinal expression of glutamate transporters and mechanical nociception in normal rats. This lack of effect of valproate in normal rats may due to the length of treatment (3 weeks) in the current study, shorter than the 3 month treatment previously reported to increase glutamate transporters in the hippocampus.[10] Consistent with its effects on the expression of spinal glutamate transporters, valproate prevented the increase in extracellular glutamate concentration in the spinal cord and reduced the development of hypersensitivity after nerve injury, and the latter effect of valproate was abolished by the pharmacological blockade or knock-down of GLT-1 in the spinal cord. These results suggest that GLT-1 plays an important role in the preventive effects of chronic valproate treatment on the extracellular glutamate concentration in the spinal cord and hypersensitivity after nerve injury.

Various changes in gene and protein expression in pain pathways have been demonstrated to induce and maintain chronic pain after inflammation and nerve injury. This indicates that one single mechanism does not underlie chronic pain. In the current study, valproate reduced the SNL-induced increase in extracellular glutamate concentration in the spinal cord to near normal levels, but hypersensitivity after SNL still partially remained, consistent with the observation in normal rats that selective knock-down of spinal GLT-1 resulted in a minor reduction in mechanical withdrawal threshold compared to its relatively large effect on extracellular glutamate concentration in the spinal cord. These results indicate that increase in extracellular glutamate level is an important but not sole factor which contributes to the hypersensitivity in the spinal cord after peripheral nerve injury.

In summary, the current study demonstrated that chronic treatment of valproate inhibits the development of chronic pain after peripheral nerve injury in part via preventing down-regulation of glutamate transporters, especially GLT-1, to maintain extracellular glutamate concentration in the spinal cord. Given clinical availability and established safety profiles, perioperative use of valproate should be tested to prevent chronic pain after surgery.

## Acknowledgments

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## Perspective

This study demonstrates that valproate prevents the down-regulation of glutamate transporters in the spinal cord which contributes in part to the development of chronic pain after nerve injury. Given clinical availability and established safety profiles, perioperative use of valproate should be tested to prevent chronic pain after surgery.

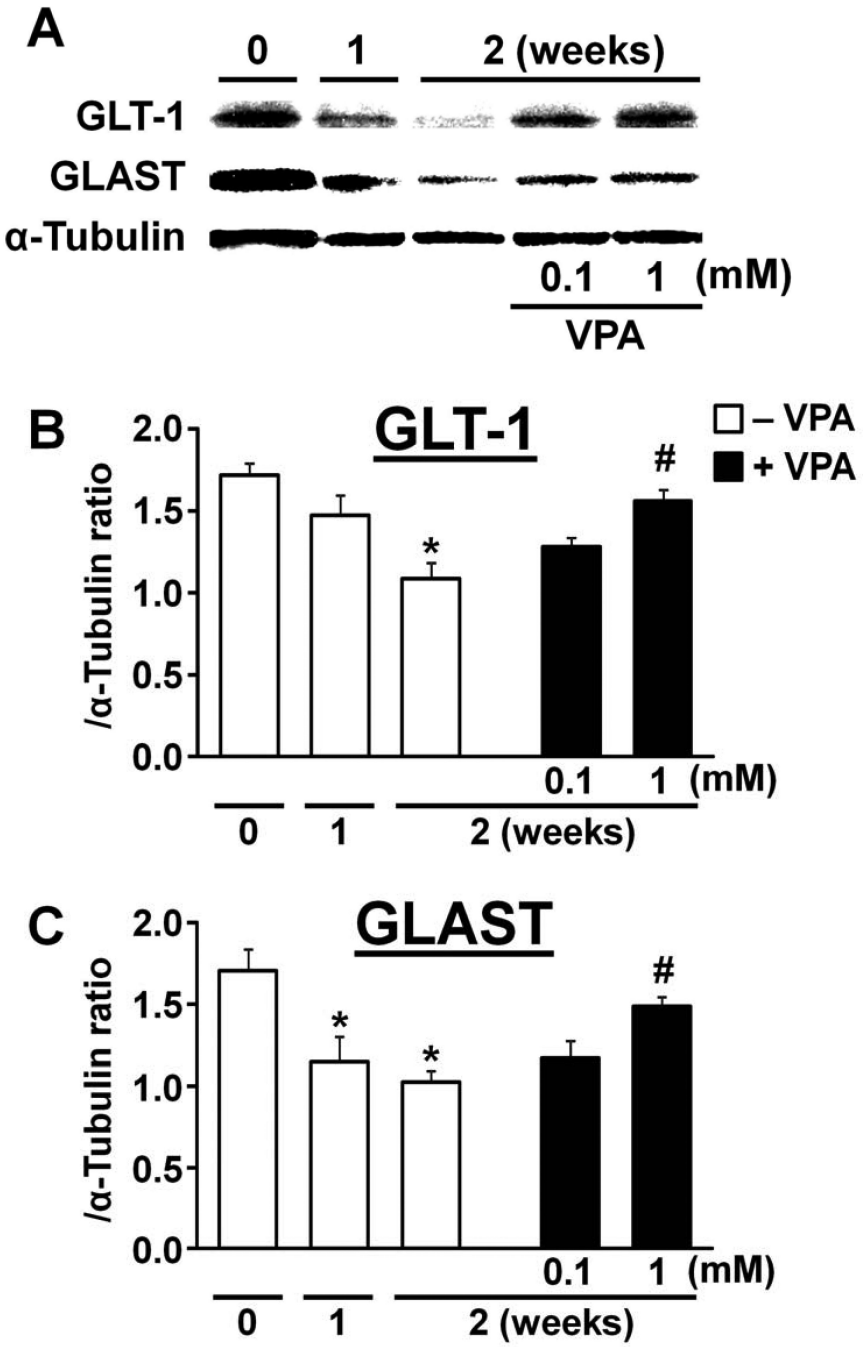


**Fig. 1.**

Valproate (VPA) prevented down-regulation of glutamate transporters in primary cultured astrocytes. (A) Representative western blotting images of GLT-1 and GLAST on astrocytes cultured for 2 weeks without or with VPA (0.1-1 mM). (B and C) Quantification of GLT-1 (B) and GLAST (C) expression in primary cultured astrocytes (n=5 in each group). *P<0.05 vs. week 0. #P<0.05 vs. without VPA at 2 weeks.

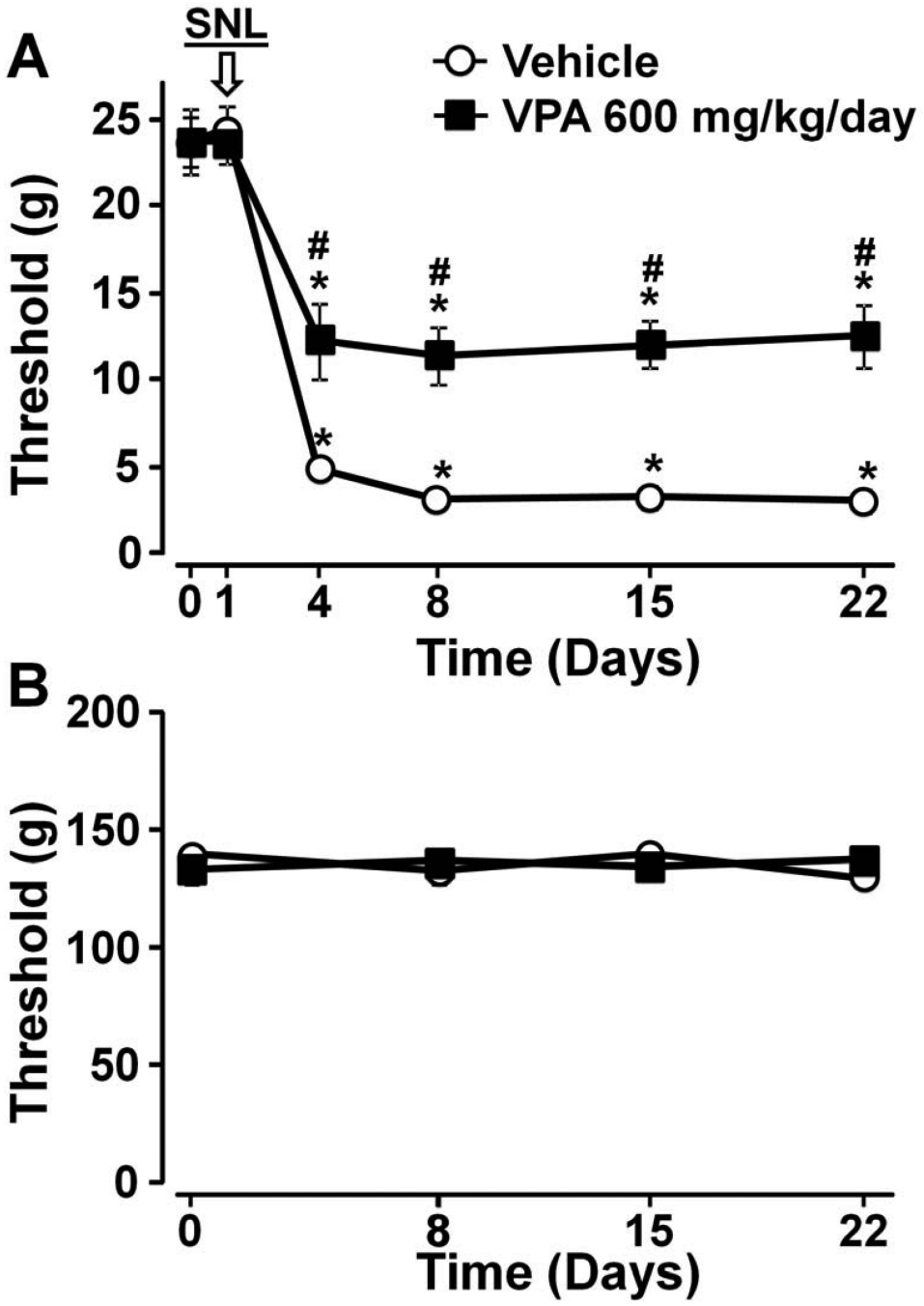


**Fig. 2.**

Repeated oral administration of valproate (VPA) prevented the development of hypersensitivity after SNL. Rats received oral administration of vehicle (n=8) or VPA (300 mg/kg, n=10) twice a day from day 0 to day 21 with (A) or without (B) SNL surgery at day 1. Each behavioral testing was performed at the morning 1 hr prior to the first administration. *P<0.05 vs. Day0. #P<0.05 vs. vehicle.

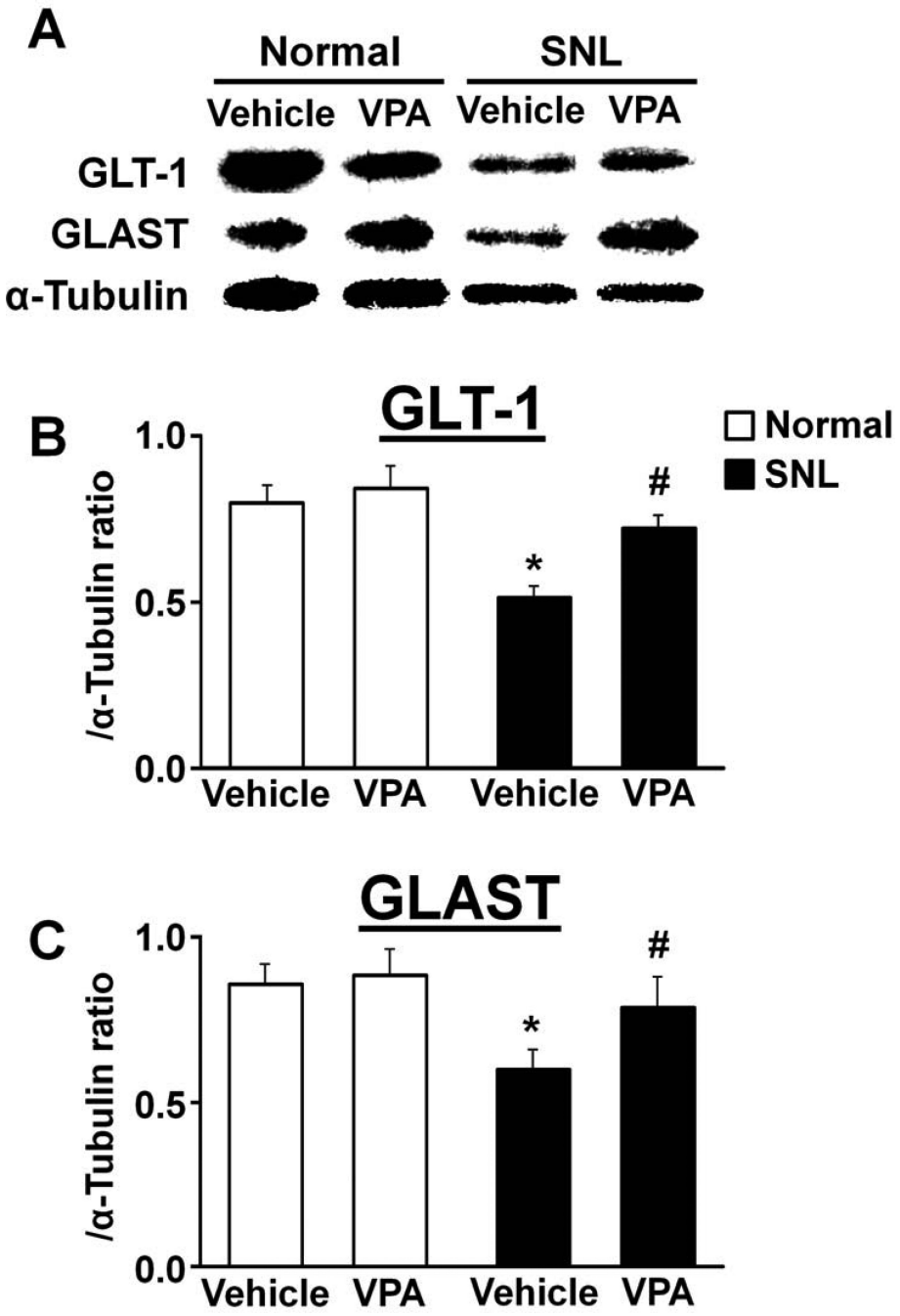


**Fig. 3.**

Valproate (VPA) prevented down-regulation of GLT-1 and GLAST in the spinal dorsal horn after SNL. (A) Representative western blotting images of GLT-1 and GLAST in the ipsilateral spinal dorsal horn from SNL or normal rats treated with vehicle or VPA for 22 days. (B and C) Quantification of GLT-1 (B) and GLAST (C) expression in the ipsilateral spinal dorsal horn (n=8 in each group). *P<0.05 vs. vehicle-treated normal. #P<0.05 vs. vehicle-treated SNL.

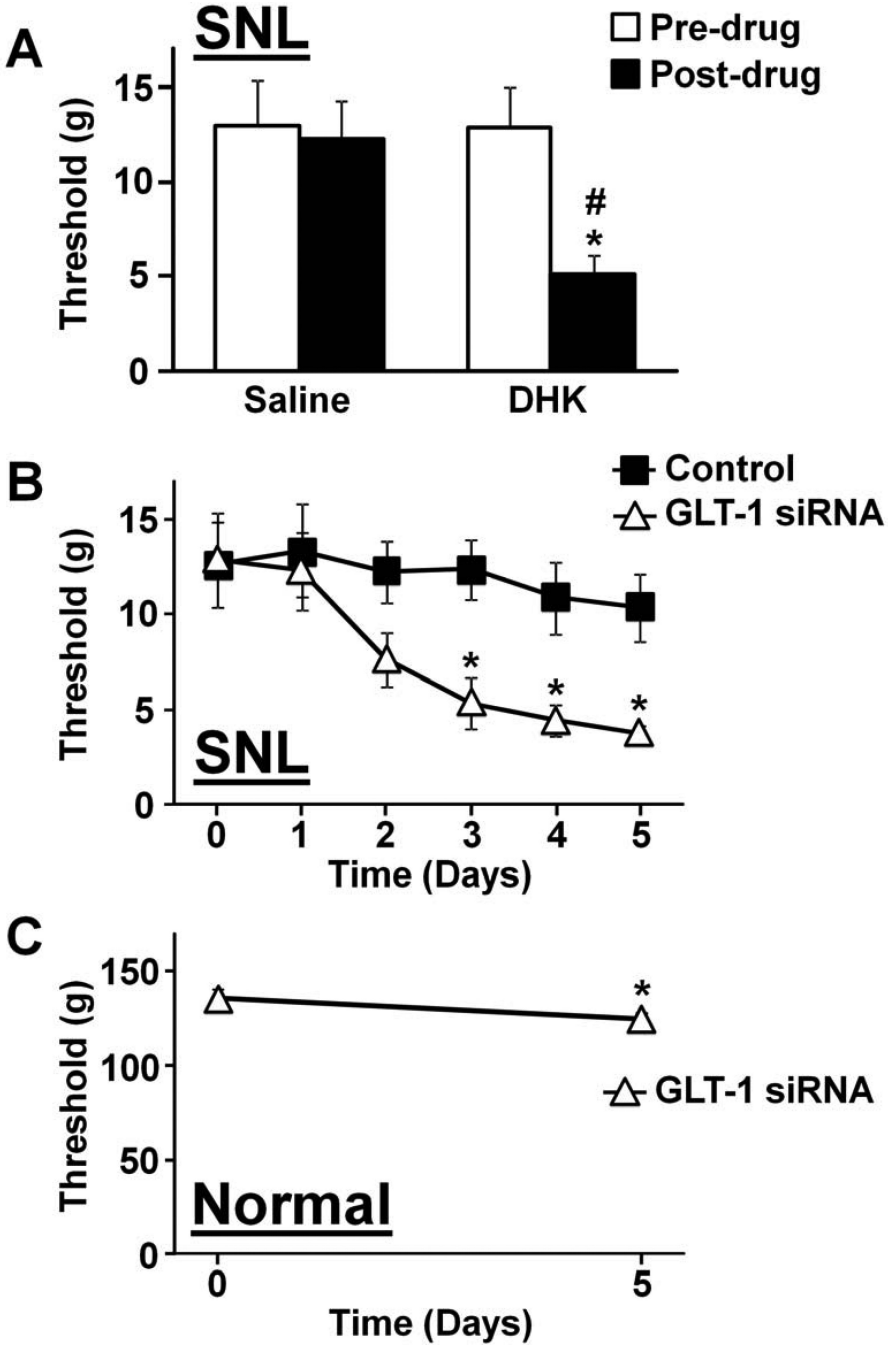


**Fig. 4.**

Blockade or knockdown of spinal GLT-1 reduced antihypersensitivity effect of valproate (VPA) in SNL rats. (A) VPA-treated SNL rats (n=7 in each group) were received an intrathecal injection of saline or dihydrokainic acid (DHK, 10 μg/rat) at 14 days after beginning of VPA treatment and behavioral testing was performed at 1 hr after injection. *P<0.05 vs. saline. #P<0.05 vs. pre-drug. (B) VPA-treated SNL rats were received intrathecal injections of non-targeting (control, 0.17 nmol/rat, n=7) or GLT-1 selective siRNA (GLT-1 siRNA, 0.17 nmol/rat, n=7) for 5 consecutive days starting from 17 days after beginning of VPA treatment and each behavioral testing was performed at the morning 1 hr prior to the first VPA administration. *P<0.05 vs. control. (C) Normal rats were

received intrathecal injections of GLT-1 siRNA (2 μg/rat, n=10) for 5 consecutive days and Randall-Selitto testing was performed after 5 days. *P<0.05 vs. day 0.

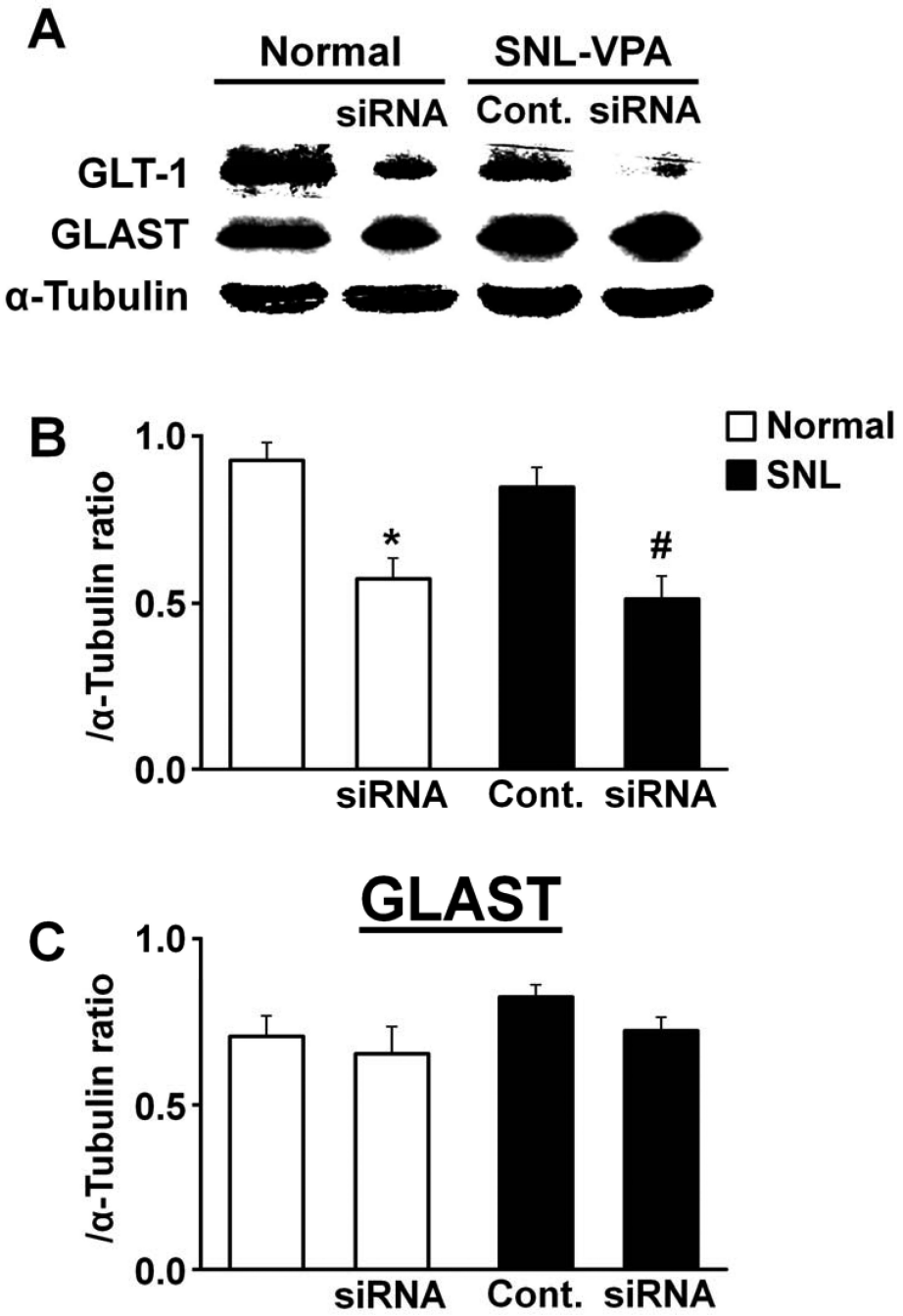


**Fig. 5.**

Spinal treatment with GLT-1 selective siRNA reversed the effect of valproate (VPA) on GLT-1 expression in the spinal dorsal horn after SNL. (A) Representative western blotting images of GLT-1 and GLAST in the ipsilateral spinal dorsal horn from normal rats with or without intrathecal injections of GLT-1 selective siRNA (siRNA, 0.17 nmol/rat) for 5 days, or VPA-treated SNL rats with intrathecal injections of non-targeting (cont.) or GLT-1 selective siRNA (siRNA, 0.17 nmol/rat) for 5 days starting from 17 days after beginning of VPA treatment. (B and C) Quantification of GLT-1 (B, n=8 in each group) and GLAST (C, n=8 in each group) expression in the ipsilateral spinal dorsal horn. *P<0.05 vs. non-treated normal. #P<0.05 vs. cont..

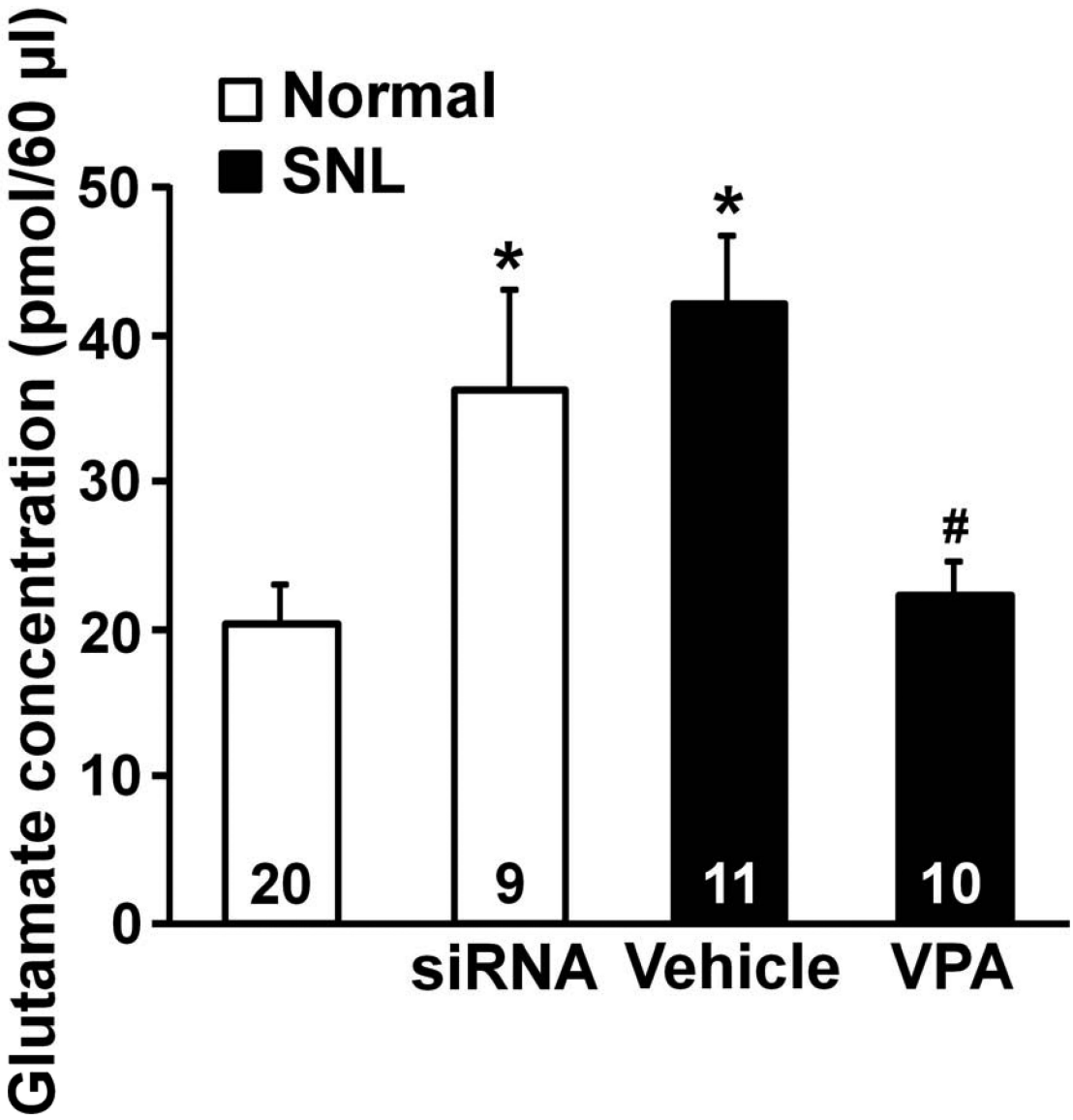


**Fig. 6.**

Basal glutamate release in the lumbar spinal dorsal horn. Glutamate concentration in microdialysate from the lumbar spinal dorsal horn was measured in normal rats with or without GLT-1 selective siRNA (siRNA, 0.17 nmol/rat) for 5 days and in SNL rats treated with vehicle or valproate (VPA) for 22 days. n=9-20. *P<0.05 vs. non-treated normal. #P<0.05 vs. vehicle.