Published in final edited form as:
Neurosci Lett. 2011 September 8; 502(1): 52–55. doi:10.1016/j.neulet.2011.07.023.

Up-regulation of spinal glutamate transporters contributes to anti-hypersensitive effects of valproate in rats after peripheral nerve injury

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Abstract

Valproate produces analgesia in animals and humans, however, its mechanisms of action are yet unknown. The present study examined effects of repeated administration of valproate on behavioral hypersensitivity and expression of glutamate transporter-1 (GLT-1) and glutamate-aspartate transporter (GLAST) in the spinal dorsal horn in rats after L5–L6 spinal nerve ligation (SNL). SNL significantly reduced mechanical withdrawal threshold and expression of GLT-1 and GLAST in the spinal dorsal horn. Repeated oral administration of valproate reduced hypersensitivity, restored down-regulated expression of GLT-1 and GLAST in the spinal dorsal horn, and enhanced analgesia from the glutamate transporter activator riluzole. This analgesia from valproate was blocked by the selective GLT-1 blocker dihydrokainic acid (DHK). These data suggest that valproate restores down-regulated expression of glutamate transporters in the spinal cord to presumably reduce glutamate signaling and to reduce hypersensitivity after nerve injury, and that combination of valproate with riluzole produces enhanced analgesia which relies on the spinal glutamate transporters.

Keywords

valproate; neuropathic pain; glutamate transporter; dihydrokainic acid; riluzole

Introduction

As an important excitatory neurotransmitter in the central nervous system, glutamate plays key roles not only in physiological but also in pathological conditions, including stroke, epilepsy, and chronic pain. Extracellular glutamate concentration is predominantly regulated by glutamate transporters, which are classified into five subtypes [6]. Glutamate-aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1) are expressed in astrocytes and the others are enriched in neurons and retina. Among those glutamate transporters, GLT-1 plays a predominant role in the regulation of extracellular glutamate in the spinal cord and brain [3, 19, 20].

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Clinical studies have shown that the antiepileptic drug valproate reduces the frequency of migraine headaches [13] and reduces pain from diabetic neuropathy and post-herpetic neuralgia [15, 16]. Valproate has several mechanisms of action [21], including inhibition of voltage-gated sodium channels via prolonging repolarization, increasing GABA concentration via enhancing glutamic acid decarboxylase and inhibiting GABA degradation enzymes, and activation of GABAA receptors via a direct interaction, thereby inhibiting pain pathways. Based on these data, most laboratory studies have focused on the acute effects of valproate to study its mechanisms of action. In clinical practice, however, patients with chronic pain are treated with valproate at least for a few weeks, while mechanisms of chronic valproate treatment are not fully understood.

Down-regulation of GLAST and GLT-1 in spinal astrocytes occurs in rats after peripheral nerve injury and is felt to contribute to the maintenance of hypersensitivity after injury [24, 26]. Blockade of spinal glutamate transporters induces hypersensitivity to mechanical and thermal stimuli in normal rats [25], whereas gene-transfer of glutamate transporters in the spinal cord reduces hypersensitivity in rats after peripheral nerve injury and inflammation [17]. Valproate activates transcription of GLT-1 and GLAST in cultured glial cells [1, 18]. Chronic treatment of valproate increased expression of GLT-1 and/or GLAST in the hippocampus in normal and seizure-induced rats [11, 22]. We therefore hypothesized that chronic treatment of valproate restores down-regulated glutamate transporters in the spinal cord and by this mechanism reduces hypersensitivity after peripheral nerve injury. The present study examined the effects of repeated oral administration of valproate on hypersensitivity and expression of GLT-1 and GLAST in the spinal dorsal horn in rats after L5–L6 spinal nerve ligation (SNL). We also examined effects of GLT activator and blocker on hypersensitivity in valproate treated SNL rats.

Materials and Methods

The study was performed in Sprague-Dawley rats (200–300g) from Harlan Industries (Indianapolis, IN, USA), housed under a 12-hours light-dark cycle with food and water *ad libitum*. All procedures were performed under Wake Forest University Animal Care and Use Committee approval and guidelines on the ethical use of animals (Winston-Salem, NC, USA). L5–L6 SNL was performed as previously described [14]. Animals were anesthetized with 2% isoflurane, and the right L5 and L6 spinal nerves were tightly ligated using 5–0 silk suture. Two weeks after SNL, intrathecal catheterization was performed as previously described [27]. A small puncture was made in the atlanto-occipital membrane of the cisterna magnum and a polyethylene catheter (ReCathCo LLC, Allison Park, PA, USA), 7.5 cm, was inserted so that the caudal tip reached the lumbar enlargement of the spinal cord. Hypersensitivity to light touch was tested by means of calibrated von Frey filaments (Stoelting,Wood Dale, IL, USA) applied to the plantar surface of the hind paw and withdrawal threshold was determined using an up-down statistical method [5]. The person conducting behavioral testing was blinded to the treatments.

Single dose studies were performed in rats 3 weeks after SNL. Sodium valproate (Sigma, St Louis, MO) dissolved with 0.5% carboxymethyl cellulose was administered by a feeding tube (5 mL/kg) and behavioral testing was performed at 2 hours following administration. For repeated dose studies, vehicle or valproate was administered twice a day (morning and evening) for 10 days starting 3 weeks after SNL and each behavioral testing was performed at the morning 1hour prior to the first administration. At 10 days after beginning of treatment, animals were decapitated after exposure to CO_2 and the spinal cord was quickly removed for the Western blotting experiment. The L4–L6 level of lumbar spinal dorsal horn was 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 (25μg protein)

were placed on the gel (7.5% resolving gel; Bio-Rad, Hercules, CA), run at 100 V for 1 hour, and transferred to nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5 % bovine albumin serum in tris-buffer saline containing tween 20, and incubated overnight at 4°C with a guinea pig anti-GLAST (1:1000; Millipore, Temecula, CA), guinea pig anti-GLT-1 (1:1000; Millipore), or a rabbit α-tubulin (1:1000; Cell Signaling, Danvers, MA). The membrane was incubated for 1 hour at room temperature with a corresponding HRP-conjugated secondary antibody (1:5000; Santa Cruz, Santa Cruz, CA), treated for 1 min with the West Pico chemilulescence (ThermoFisher Scientific, Rockford, IL), and exposed to X-ray film (Kodak BioMax film, Sigma-Aldrich). 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).

In a separate experiment, animals received an intrathecal injection of the selective GLT-1blocker dihydrokainic acid (DHK, 10 μg/rat followed by 10 μL saline, Tocris bioscience, Ellisville, MO) or saline (20 μL) at 10 days after beginning vehicle or valproate treatment, and behavioral testing was performed 1 hour after injection. Then, vehicle or valproate treatment continued for 5 days in animals which received saline injection. At 15 days after beginning of vehicle or valproate treatment, all animals received an intrathecal injection of the glutamate transporter activator riluzole (10 μg/rat, Sigma) followed by 10 μL saline and behavioral testing was performed 1 hour after injection. DHK and riluzole were dissolved in saline.

Data were expressed as means ± S.E. Behavioral data were analyzed by one way or two way repeated measures analysis of variance (ANOVA). The differences in the GLT-1/ α-tubulin and GLAST/ α-tubulin ratio were analyzed by one way ANOVA. $P < 0.05$ was considered significant.

Results

All animals showed less than 4 g of withdrawal threshold values in the paw ipsilateral to surgery at 3 weeks after surgery. Valproate dose-dependently increased withdrawal thresholds in the paw ipsilateral to SNL compared to pre-drug and vehicle (Fig.1A). No animal showed apparent sedation or motor dysfunction by a single administration of any dose of valproate. Based on this result, we chose twice a day administration of 300 mg/kg valproate for the subsequent experiments. Repeated administration of valproate (300mg/kg, twice a day) significantly increased withdrawal threshold values in the paw ipsilateral to SNL at 4 days after beginning of treatment and this valproate effect sustained during the study (Fig.1B, $p<0.05$). Animals appeared normal during the 10 days of vehicle and valproate treatments, with normal grooming, response to handling, and weight gain.

Figure 2A depicts representative immunoblotting images of GLT-1 and GLAST in the ipsilateral spinal dorsal horn from normal and vehicle or valproate treated SNL rats. SNL significantly reduced expressions of GLT-1 and GLAST in the ipsilateral spinal dorsal horn of both vehicle and valproate treated rats compared to the normal rats (Fig. 2 B and C, $p<0.05$). Valproate treated SNL rats showed significantly higher expression of GLT-1 and GLAST in the spinal dorsal horn ipsilateral to surgery compared to the vehicle treated rats ($p<0.05$).

In vehicle treated SNL rats, intrathecal administration of the selective GLT-1 blocker DHK did not alter withdrawal thresholds in the paw ipsilateral to surgery (Fig. 3). However, in valproate treated rats, DHK significantly reduced withdrawal thresholds in the paw ipsilateral to surgery compared to the pre-drug and saline ($p<0.05$). Intrathecal administration of the glutamate transporter activator riluzole did not alter withdrawal

thresholds in vehicle treated SNL rats (Fig. 4, $p=0.17$), but significantly increased them in valproate treated SNL rats compared to the pre-drug value ($p<0.05$).

Discussion

The current study demonstrates that repeated treatment of valproate can restore spinal glutamate regulatory mechanisms to reduce hypersensitivity in rats after peripheral nerve injury. Various changes in gene and protein expression in pain pathways have been demonstrated to play important roles in the spinal cord to induce and maintain chronic pain after peripheral inflammation and nerve injury. As such, histone acetylation, catalyzed by histone acetyltransferase and removed by histone deacetylases (HDACs), has been recognized as an important mechanism in regulation of gene transcription [23]. Recent study has shown that valproate reduced hypersensitivity in mice after peripheral inflammation via a direct inhibition of class 2 histone deacetylase (HDAC), which contributes to the induction and maintenance of inflammatory pain [2]. In cultured glial cells, valproate induce hyperacetylation in histone via inhibition of HDACs activity and thereby activates transcription of GLT-1 and GLAST genes [1, 18], consistent with *in vivo* studies that chronic treatment with valproate up-regulates GLT-1 and/or GLAST expression in rat hippocampus [11, 22]. The current study supports these observations by demonstrating that repeated oral administration of valproate increased expression of GLT-1 and GLAST in the spinal dorsal horn after peripheral nerve injury.

Recent studies have demonstrated in rats with chronic pain that gene transfer of GLT-1 or the antibiotic, ceftriaxone, up-regulates GLT-1 expression in the spinal dorsal horn and produced analgesia, which is blocked by DHK or GLT-1 antisense oligodeoxynucleotide treatment [9, 12, 17]. Consistent with those observations, the antihypersensitive effect of valproate was strongly reduced by DHK in the current study. These results suggest that restoring down-regulated glutamate transporters, especially GLT-1, in the spinal cord reduces hypersensitivity after peripheral nerve injury. In the current study, valproate increased expression of both GLT-1 and GLAST. Although GLT-1 plays the predominant role in the regulation of extracellular glutamate in the spinal cord and brain [3, 19, 20], GLAST may also have some influence on hypersensitivity after peripheral nerve injury and blockade of both GLT-1 and GLAST might completely block valproate effect. However, since blockade of both GLT-1 and GLAST in the spinal cord itself induces severe hypersensitivity [25], we did not test a non-selective glutamate transporter blocker in the current study. Further study will be required to clarify this point.

Riluzole is a neuroprotective drug approved for amyotrophic lateral sclerosis [4] and laboratory studies have shown that riluzole activates glutamate transporters to enhance glutamate uptake [7, 8]. Based on these results, one would expect that spinally administered riluzole should reduce hypersensitivity after nerve injury via reducing extracellular glutamate level in the spinal cord. However, previous study showed lack of effect of spinally administered riluzole on hypersensitivity in rats after spinal cord injury [10]. The current study also confirms this observation by demonstrating that intrathecal injection of riluzole failed to produce analgesia in vehicle treated SNL rats. Interestingly, the same dose of riluzole produced analgesia in SNL rats treated with valproate, which up-regulates glutamate transporters in the spinal cord. These results suggest that riluzole depends on the degree of glutamate transporter expression in the spinal cord to reduce hypersensitivity after nerve injury and that pretreatment with valproate can enhance analgesia from riluzole.

Conclusions

The current study demonstrated that valproate increases glutamate transporters in the spinal cord and reduces hypersensitivity after nerve injury. This study also demonstrated that combination of valproate with riluzole can produced enhanced analgesia which relies on the expression of spinal glutamate transporters. Established safety profiles, clinical availability, and enhanced analgesia observed in the current study suggest that this combination should be tested in chronic pain patients.

Acknowledgments

This work was supported by grants DA27690 to KH and NS59574 to JE from the National Institutes of Health, Bethesda, Maryland.

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Highlights

Peripheral nerve injury resulted in down-regulation of spinal glutamate transporters, which is associated with behavioral hypersensitivity. Valproate restores spinal glutamate transporters to alleviate neuropathic pain. Valproate also enhanced analgesia from the glutamate transporter activator riluzole.

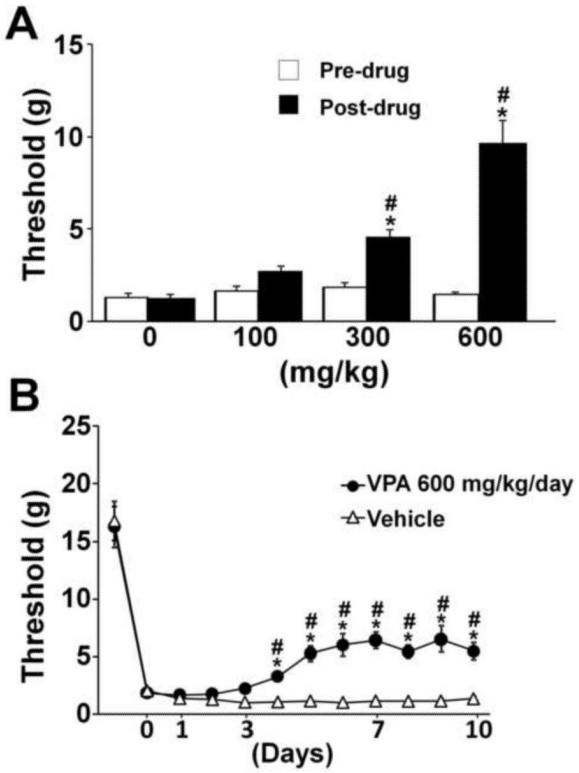

Figure 1. Effects of single and repeated oral administration of valproate on hypersensitivity in SNL rats

(A) Withdrawal threshold values in the paw ipsilateral to SNL surgery were measured at 2 hours following oral valproate administration (VPA, 0–600 mg/kg, n=9–10) # $p<0.05$ versus pre-drug. *$p<0.05$ versus 0 mg/kg. (B) Animals received oral administration of vehicle (n=8) or VPA (300 mg/kg, n=8) twice a day started from 3weeks after SNL and behavior testing was performed at the morning 1 hour prior to the first administration. #$p<0.05$ versus Day0. *$p<0.05$ versus vehicle.

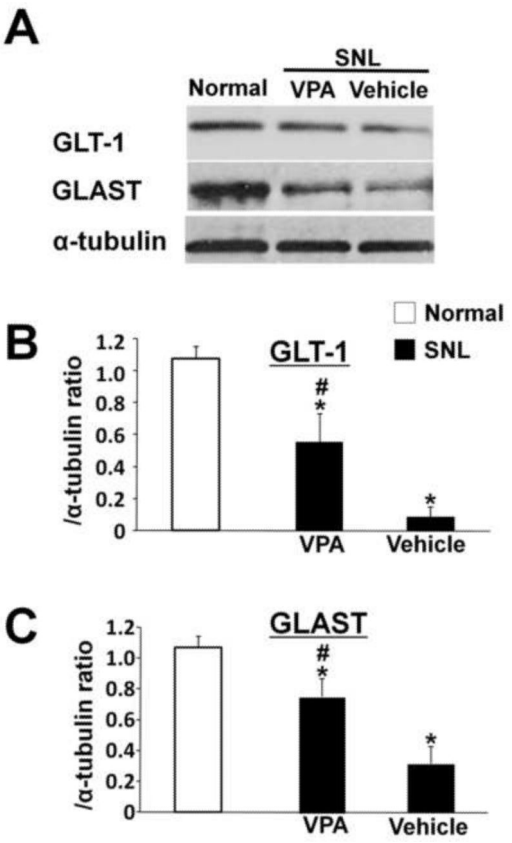

Figure 2.

Valproate (VPA) restored glutamate transporters in the spinal dorsal horn of SNL rats. (A) Representative western blotting images of GLT-1 and GLAST in the ipsilateral spinal dorsal horn from normal and vehicle or VPA treated SNL rats. (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. *$p<0.05$ versus normal. #$p<0.05$ versus vehicle.

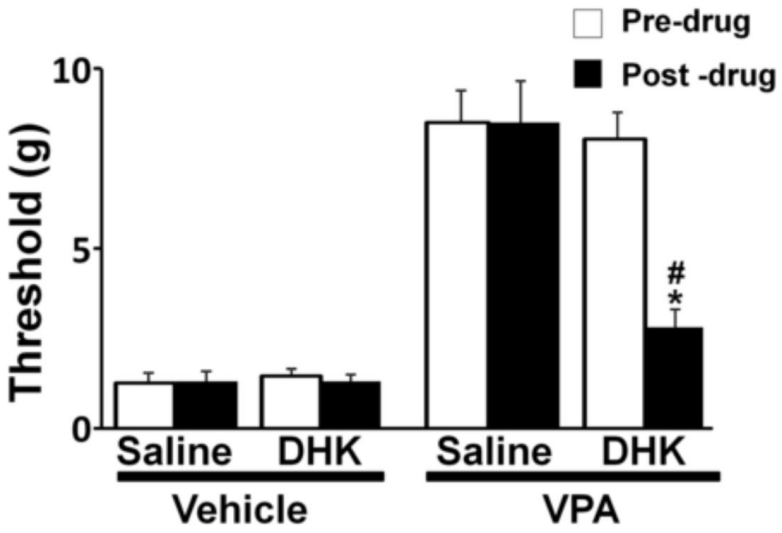

Figure 3.

Blockade of spinal glutamate transporters reduced antihypersensitivity effect of valproate (VPA) in SNL rats. Animals (n=8 in each group) were treated with oral vehicle or VPA for 10 days and withdrawal threshold values in the paw ipsilateral to SNL surgery were measured at 1 hour following intrathecal injection of saline or DHK (10μg/rat). #$p<0.05$ versus pre-drug. *$p<0.05$ versus saline.

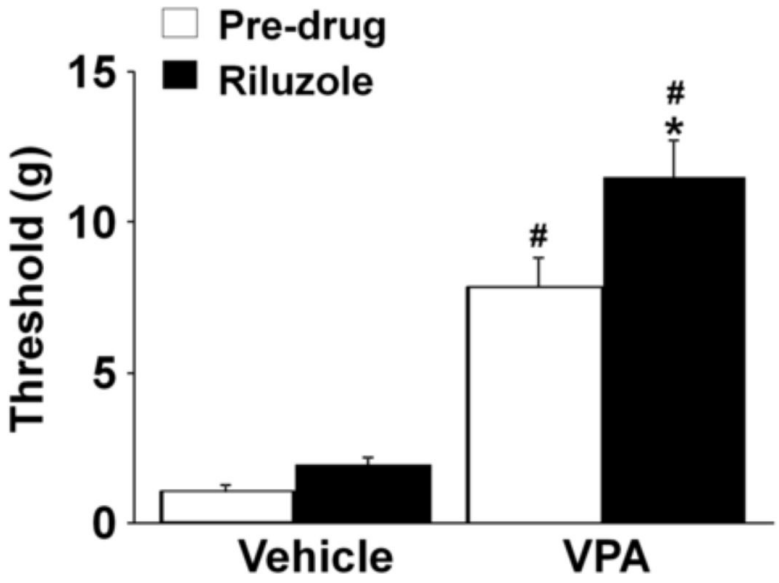

Figure 4.

Intrathecal Riluzole produced enhanced analgesia in valproate (VPA) -treated SNL rats. Animals (n=8 in each group) were treated with oral vehicle or VPA for 15 days and withdrawal threshold values in the paw ipsilateral to SNL surgery were measured at 1 hour following intrathecal injection of saline or riluzole (10μg/rat). *p <0.05 versus pre-drug. #p<0.05 versus vehicle.