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RESEARCH ARTICLE

# In vivo microdialysis of glutamate in ventroposterolateral nucleus of thalamus following electrolytic lesion of spinothalamic tract in rats

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**Abstract** Central pain is one of the most important complications after spinal cord injury (SCI), and thereby, its treatment raises many challenges. After SCI, in a cascade of molecular events, a marked increase in glutamate at the injury site results in secondary changes which may impact on supraspinal regions, mainly ventroposterolateral (VPL). There is little information about the changes in glutamate metabolism in the VPL and whether it contributes to SCI-related central pain. The present study was performed to evaluate glutamate release in the VPL following electrolytic lesion of spinothalamic tract (STT). A laminectomy was performed at spinal segments of T9–T10 in male rats, and then, unilateral electrolytic lesions were made in the STT. Glutamate concentrations in ipsilateral VPL dialysate were measured by HPLC method at days 3, 7, 14, 21 and 28 post-injury. Tactile pain and motor activity were also examined. Glutamate levels were significantly increased in ipsilateral VPL of spinal-cord-injured rats 2 weeks after SCI and remained high up to day 28 post-surgery. The STT lesions had no marked effect on our measures of motor activity, but there was a significant decrease in paw withdrawal threshold in the hind paws at day 14 post-SCI. These findings suggest that an increased release of glutamate in VPL plays a role in secondary pathologic changes, leading to neuronal hyperexcitation and neuropathic pain after SCI.

**Keywords** Central pain · Spinal cord injury · Glutamate · VPL · Microdialysis

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## Introduction

Central pain is one of the major problems following CNS damage including spinal cord injury (Beric 2003). Allodynia, hyperalgesia and spontaneous pain are the main characteristics of this type of pain (Greenspan et al. 2004). Epidemiological studies have shown that 70 % of spinal cord injury (SCI) patients suffer from chronic neuropathic pain (Siddall et al. 2002). Pain arises from damaged tissue at the site of injury or surrounding regions, which leads to sensory loss (Tasker et al. 1992). Pain appears shortly after the injury, but is sometimes delayed for weeks or months (Tasker et al. 1991). In spite of substantial research in the field, the underlying mechanism of SCI-related central pain is not yet fully understood. Several cascades of molecular events occur following SCI that result in physiological changes including hyperexcitability and hyperactivity of post-synaptic neurons, leading to spontaneous or evoked pain (Yezierski 2000). A number of studies have found a reduction in GABAergic tone in the spinal dorsal horn (Gwak and

Hulsebosch 2011) and a simultaneous release of excitatory amino acids, particularly glutamate, in and around the site of injury (McAdoo et al. 1999) that triggers secondary pathological changes including neuronal hyperexcitability, giving rise to chronic pain (Loubser and Donovan 1991). It is believed that prolonged changes in neuronal excitability are not limited to spinal cord and a great portion of persistent pain results from structural and functional adaptive changes in brain regions that are directly or indirectly involved in pain-related signals (Bhangoo and Swanson 2012). Persistent excitation of neuronal and glial NMDARs at the injury or even remote sites occurs through dramatic glutamate release (Zhuo 2007). The thalamus is the structure in the CNS known to mediate some aspects of pain perception. We recently demonstrated that electrolytic lesions of the spinothalamic tract and interruption of pain afferents to the thalamus lead to prominent activation of spinal and thalamic glial cells and a significant increase in glutamate release at the site of injury. We showed a correlation between this glial cell activity and the development of central pain in rats (Naseri et al. 2012; Saghaei et al. 2013). Considering the excitatory effects of glutamate on glial cells in releasing proinflammatory cytokines (Gwak and Hulsebosch 2005) and also the crucial role of astrocytes in glutamate clearance from synaptic spaces, investigation of possible alterations in glutamate release at remote regions could provide valuable information about the role of glutamate in the cellular events related to central pain. In this study, we have focused on alterations of glutamate release in the ventroposterolateral (VPL) following electrolytic lesion of spinothalamic tract using microdialysis.

## Materials and methods

### Animals

Male *Sprague–Dawley* rats weighing 200–230 g were used. Rats were kept on a 12-h light/dark cycle, housed two to three per cage and received food and water freely. Animals were divided into two groups: sham and SCI groups. All experimental procedures were approved by the ethical committee of the Neuroscience Research Center of Shahid Beheshti University of Medical Sciences (Tehran, Iran).

### Spinothalamic tract lesion

Spinothalamic tract lesions were made by a slightly modified version of a method described Wang and Thompson (Naseri et al. 2012; Wang and Thompson 2008). After the induction of anesthesia with ketamine/xylazine (80:5 mg/kg, i.p.), a laminectomy was performed on T9–T10 vertebrae. The dura was lifted with fine forceps and opened with iris scissors. A tungsten microelectrode (1 MΩ impedance) was stereotaxically inserted 0.5–0.7 mm lateral to midline and 1.6–1.9 mm deep into the right ventrolateral funiculus, corresponding to the location of the STT. A constant current pulse (300 μA, 90 s) was passed between the microelectrode and a wire electrode placed on the muscle in order to destroy the nearby axons. During the first 10 s of lesion induction, the current pulse was ramped up from 0 to 300 μA and maintained at that level for 80 s (total time = 90 s). All animals received physiological saline (s.c.) and penicillin G (i.m.) to prevent electrolyte imbalance and infection, respectively. The sham group received the same surgical protocol, but no lesion.

### Behavioral assessment

Behavioral tests including assessment of motor activity and nociceptive responses were performed 3, 7, 14, 21 and 28 days after surgery in all groups, allowing the time course of the effects of STT lesions on behavioral performance to be determined. Totally, 65 animals (35 rats in sham and 30 rats in SCI groups) were examined in motor activity and nociceptive response tests during 4-week post-surgery period.

#### *Open field test*

Motor activity was assessed using a 60 × 60 cm arena and 40-cm-height black wooden box (Naseri et al. 2012). Animals were habituated in the room for 30 min and then placed in one corner of the box and allowed to move freely for 5 min. Each trial was recorded by a camera placed right above the box hanging from the ceiling. After each trial, the arena was thoroughly cleaned. The experiments were conducted under artificial laboratory illumination. Data were obtained using EthoVision software (version 7), a video tracking system for automation of behavioral experiments (Noldus Information Technology, the Netherlands). The behavior of each rat was evaluated off-line as distance moved during the trial.

#### *Mechanical pain*

Mechanical nociception was assessed using the method described by Ren (1999) with calibrated von Frey's hairs (Stoelting, Wood Dale, IL, USA). Filaments were applied in ascending manner to the dorsal surface of the hind paws based on studies confirming that the dorsal approach more reliably indicates nociceptive responses than the plantar surface (Ren 1999). Stimulus–response curves were

prepared by plotting the paw withdrawal threshold (PWT) versus days post-surgery. PWT was defined as the force (in grams) at which the animal withdrew to three of five stimuli delivered. PWT was assessed blindly by an uninformed observer. Mechanical pain was evaluated at days 3, 7, 14, 21 and 28 post-surgeries in SCI, after laminectomy in sham and 3 days after implanting the probe in control intact groups.

## Microdialysis experiments

### *Microdialysis probes and implantation*

Microdialysis probes can be obtained commercially; however, such probes are rather expensive and customization to the experimental needs (e.g., varying dialysis surface area and shape) is sometimes a problem. For these reasons, we made the probes in our laboratory by the method described by Horn and Engelmann (2001). A concentric probe consisted of an inlet, outlet and a 5-mm shaft and a polysulfone dialysis hollow fiber (cutoff: 1,000 kDa) at the tip. Under ketamine/xylazine (80:5 mg/kg, i.p.) anesthesia, rats were placed into a stereotaxic frame and a guide cannula inserted unilaterally on the right according to stereotaxic coordinates corresponding to the VPL nucleus (bregma, −2.52 mm; lateral, 3.4 mm; vertical, 4.5–5 mm) from Paxinos and Watson (2005). After implanting and fixing the probe, the rats were isolated in individual cages. Guide cannulae were implanted 3 days before the microdialysis experiments.

### *Relative recovery of microdialysis probe*

For the detection of relative recovery, the probe was connected to the perfusion system and submerged in a polypropylene tube (1.5 ml) containing glutamate solution [(1,000 ng/ml) in artificial cerebrospinal fluid (ACSF)] and then perfused with ACSF solution. Sampling was carried out at a flow rate of 2 μl/min. Samples were collected from the outlet tubing and stored at −20 °C in Eppendorf tubes prior to analysis. Extraction fraction was expressed in percentages.

### *In vivo microdialysis*

Microdialysis experiments were conducted by inserting a microdialysis probe into the VPL through guide cannulae at 3, 7, 14, 21 and 28 days after injury for SCI rats, intact and sham control animals in the absence of anesthesia. Animals were allowed to move freely during the dialysis session. The probes were perfused with filtered (through a 0.2 μm pore size membrane) artificial cerebrospinal fluid (ACSF; 1.3 mM $CaCl_2 \cdot 2H_2O$, 2.6 mM KCl, 0.9 mM $MgCl_2$, 21.0 mM $NaHCO_3$, 2.5 mM $Na_2HPO_4 \cdot 7H_2O$, 125.0 mM NaCl, prepared in sterile $H_2O$, pH 7.4) (Hains and Waxman 2006) at a rate of 2 μl/min by a microinjection pump (Stoelting). After 1 h of stabilization, dialysis samples were collected at 30-min intervals and stored at −20 °C in Eppendorf tubes before analysis. Totally, 46 microdialysate samples (20 rats for sham, 22 for SCI and 4 in intact control groups) were analyzed in this study. The exact localization of the probes in VPL was examined by postmortem histological analysis in hematoxylin- and eosine-stained brain sections.

## Glutamate detection

### *Preparation of 0.1 M sodium tetraborate*

0.168 g boric acid and 0.745 g KCl were dissolved in 100 ml distilled water. 50 ml potassium tetraborate plus 26.4 ml NaOH 0.1 M were topped up to 200 ml with distilled water. pH of prepared sodium tetraborate was adjusted to 9.3 by adding phosphoric acid.

### *Derivatization procedure*

The derivatizing solution was made according to Donzanti and Yamamoto (1988). For preparing stock solutions of the OPA/β-ME derivatizing reagent, 27 mg O-phthaldialdehyde (OPA) was dissolved in 1 ml ethanol. β-mercaptoethanol (β-ME), 5 μl, and tetraborate buffer (0.1 M sodium tetraborate, pH 9.3), 9 ml, were then added. The solution is stable for up to 5 days when protected from light and stored in a refrigerator. To obtain fresh working derivatizing reagent daily, 1 ml of stock OPA/β-ME was diluted with 3 ml of tetraborate buffer.

Fifteen microliters of the working derivatizing reagent was added to 50 μl microdialysis perfusate, and after 1-min mixing, 25 μl was injected to the HPLC system. Glutamate was detected and measured by fluorescence detection (wavelengths 340 nm/475 nm, excitation/emission). Retention time and peak area were calculated automatically by computer and LC-solution software.

### *Standard curve (linearity test)*

Glutamate standards were prepared with 5 different concentrations freshly. After derivatizing, each concentration was injected to the HPLC system 3 times to yield a linear standard curve before analyzing microdialysate samples.

### *Accuracy test*

The calibration of amino acid responses on repeated injections of the standard with the linearity curve of 5

concentration points showed an intraday accuracy above 86.8 %. The interday measures showed accuracy above 85.7 %.

*Mobile phase*

Mobile phase contained disodium hydrogen phosphate solution 100 mM (76.5 %), acetonitrile (3.5 %), and HPLC-grade methanol (%20), adjusted to pH 6.7 by adding phosphoric acid. Mobile phases were filtered through a 0.22-μm hydrophilic polypropylene membrane filters (GH Polypro, Pall Corporation, Michigan, USA) and degassed in an ultrasonic bath (Sturdy Industrial Co, Taipei County, Taiwan) before circulation on the HPLC system.

Statistical analysis

Behavioral data were analyzed by two-way ANOVA with Bonferroni post hoc test, and glutamate values were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison post hoc tests, using the GraphPad Prism 5 Demo. $P$ values less than 0.05 were considered significant. All data are expressed as mean ± SEM.

## Results

Behavioral results

Three paralyzed animals were excluded from the study. The extent of the lesion measured by postmortem histology was not significantly different between groups.

*Motor activity*

Animals with STT electrolytic lesions had no significant difference in locomotor activity to the sham animals. There was no marked difference between the distance moved by SCI and by sham rats within 5 min (Fig. 1). This result shows that the motor pathways were left intact during electrolytic lesion of STT.

*Tactile allodynia*

Electrolytic injury of STT led to tactile allodynia in the SCI groups. Generally, to evaluate the development of neuropathic pain by SCI, tactile allodynia was tested by measuring paw withdrawal threshold in response to the application of von Frey's filaments. The PWT of the lesioned rats significantly decreased in the hind paws both ipsilateral (Fig. 2a) and contralateral (Fig. 2b) to the lesion. Paw withdrawal threshold became significantly lower in the lesion

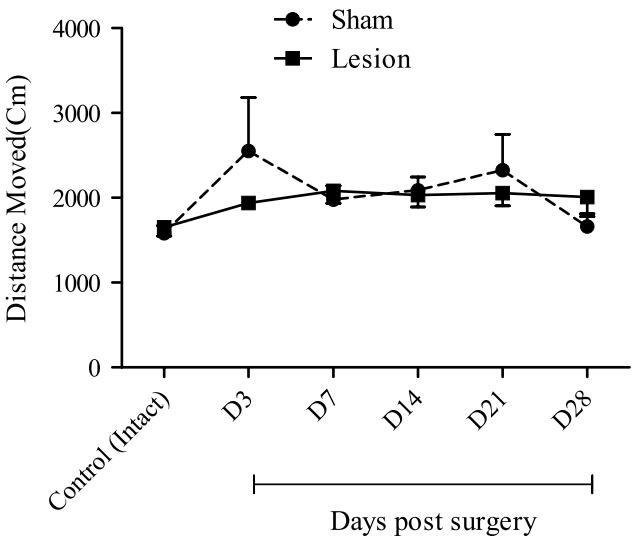


**Fig. 1** Motor activity in the lateral STT electrolytic injured rats. Distance moved by sham and SCI rats showed no significant differences post-surgery. All data are presented as mean ± SEM. (two-way ANOVA followed by Bonferroni post hoc test). $n = 6$. (*Filled circle* sham, *Filled square* SCI)

group compared to the sham group from day 14 onwards. Mean mechanical nociceptive thresholds in ipsilateral hind paw of SCI and sham rats were 20.5 ± 2.5 and 43 ± 7 g, respectively ($P < 0.05$, $F = 25.97$), and in contralateral hind paw were 22.2 ± 2 g in SCI rats compared to 48.7 ± 5.3 in sham rats ($F = 23.78$, $P < 0.01$) at day 14.

Glutamate analysis

*Relative recovery of the probe*

Determination of microdialysate glutamate removed by the perfusion medium containing known concentration of glutamate showed a probe relative recovery of about 20 %, which was obtained by HPLC analysis.

*In vivo glutamate analysis*

Glutamate release in the ipsilateral ventroposterolateral nucleus (VPL) of the thalamus was increased after electrolytic lesions in the STT. At 3 days after SCI, glutamate release was reduced compared to sham group ($P < 0.05$), but at day 14 after SCI, it was significantly increased compared to control intact ($P < 0.001$) and sham ($P < 0.01$) and remained high throughout the 4 week period of the experiment (Fig. 3). There was no significant difference between control intact and sham groups. Glutamate release in the ipsilateral VPL at 14 days was 495 ± 48 ng/ml after SCI, compared with 135.4 ± 7 ng/ml in control intact animals, a significant increase ($P < 0.001$).

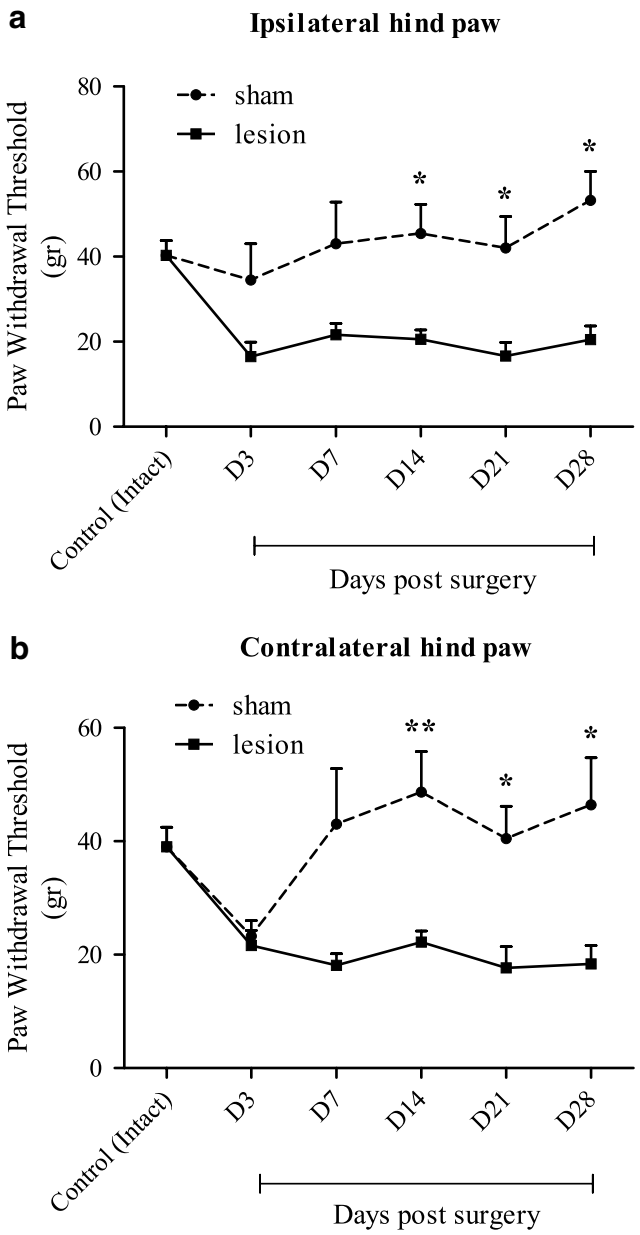


**Fig. 2** Tactile allodynia in the lateral STT lesioned rats. The SCI rats showed a significant increase in responses to von Frey's stimulation. Paw withdrawal threshold significantly decreased 14 days after surgery compared to sham groups in both ipsilateral (**a**) and contralateral (**b**) hind paws. This decrease was maintained for at least 28 days post-lesion. Data are presented as mean ± SEM. $n = 6$. (*$P < 0.05$, **$P < 0.01$, two-way ANOVA followed by Bonferroni post hoc test). (*Filled circle* sham, *Filled square* SCI)

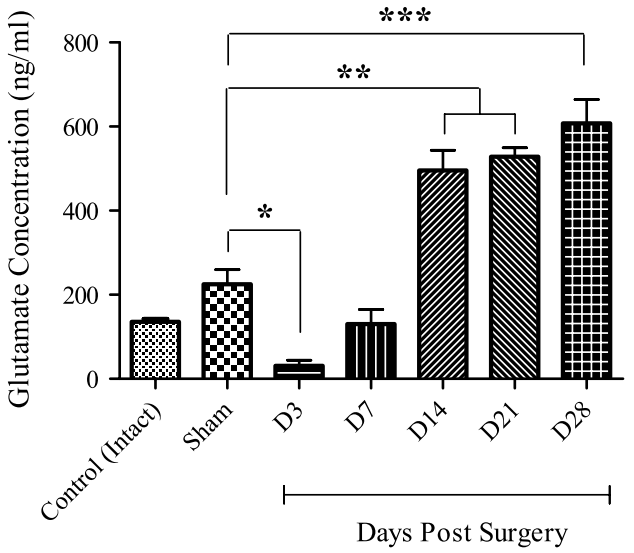


**Fig. 3** Effect of unilateral STT lesion on glutamate release in the right VPL. Glutamate release showed a decrease 3 days after SCI ($P < 0.05$, one-way ANOVA) but subsequently increased and was significantly higher in SCI group than in sham group ($P < 0.01$) at day 14. Data are presented as mean ± SEM. $n = 3$–6. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, one-way ANOVA followed by Tukey's post hoc test)

## Discussion

In the present study, we show that glutamate levels in the VPL are significantly increased 14 days after SCI and remained so for another 2 weeks throughout the experiment. It seems likely that pain following spinal lesions not only results from cellular and molecular events at the spinal lesion sites but also results from the reactive consequences of spinal injury on supraspinal neurons and glial cells in structures associated with pain perception. The thalamus plays a potentially important role in central pain pathophysiology and other types of chronic pain (Lenz et al. 2000). Lesions limited to ventral caudal part of the thalamus have been shown to lead to central burning pain (Kim et al. 2007). Several studies on human and non-human models have reported the impact of SCI on the functional activity of thalamic neurons (Siddall et al. 2003; Hains et al. 2006; Morrow et al. 2000).

Ventroposterolateral neurons respond to somatosensory afferent inputs with prolonged burst discharges after SCI (Siddall et al. 2003). Nociceptive afferent pathways to the thalamus are eventually sent to cerebral cortex via thalamofugal pathways, an important component of the pain system (Waxman and Hains 2006). Gwak et al. (2010) reported that spinal hemisection leads to ipsilateral VPL neuronal hyperexcitability. A possible explanation is that strong impulses resulting directly as a consequence of spinal traumatic lesions led to STT fiber activation and then release of pain generator substances such as excitatory amino acids (EAAs) and neuropeptides in the thalamus, which in turn result in neuronal hyperactivity via intracellular calcium increase through the activation of NMDARs. Therefore, intra- and intercellular biochemical cascades activated by NMDARs induce VPL neuronal hyperexcitability and pain (Gwak et al. 2010; Silva et al. 2001). A similar

extrapolation can be made for the present findings. It has been demonstrated that glutamate increases several fold after SCI at the lesion site and 5 mm distant from it (McAdoo et al. 1999). Samandari et al. (2012) demonstrated that peak of glutamate release at the lesion site occurs 40 min after SCI. Injured neurons in the SCI site, in addition to glutamate, release chemokines CCL2 and CX3CL1 capable of activating CCR2 and CX3CR1 on spinal microglial cells, which can subsequently produce proinflammatory cytokines TNF-a, IL-6 and IL-1β. TNF-a increases spontaneous excitatory postsynaptic currents by activating AMPA and NMDA receptors, which leads to sensitization of dorsal horn neurones and neuropathic pain (Gao and Ji 2010). These conditions may occur in supraspinal regions after SCI. It has been reported that glutamate excitotoxicity after SCI results in the production of chemokine CCL21, which is a potent microglial activator, in remote regions such as thalamus (Zhao et al. 2007). Therefore, the increase in VPL glutamate observed in our results could have happened through glutamate release from activated glial cells. In addition, cytokines released by activated microglial cells activate the JNK signaling pathway in spinal astrocytes, which leads to inhibition of astrocytic GLT1, and as a result an increase in glutamate level (Gao and Ji 2010) and hyperexcitation of neuronal membrane. As a consequence, activity generated by non-noxious stimulus becomes perceived as painful (allodynia). Spinal astrocytes express glutamate transporters GLT1 and GLAST, which clear extracellular and synaptic glutamate (Tawfik et al. 2006). Spinal injury downregulates these transporters after a temporary upregulation (4 days), which results in transient decrease and then prolonged increase in the synaptic glutamate and excitatory neurotransmission at the site of injury (Olsen et al. 2010; Ji et al. 2006). In our experiments, a significant early decline in glutamate ($P < 0.05$) was soon reversed and remained high throughout the 4-week experimental period. These findings are consistent with other reports on spinal cord (Sung et al. 2003) and show that glutamate release is changed even in remote sites to injury several weeks post-SCI. Supramaximal electrical stimulation of STT led to chemokine CCL21 release in the VPL that in turn excited glial cell activity (especially astrocytes) and eventually led to changes in synaptic glutamate uptake (Zhao et al. 2007). In addition, it has been reported that pathological conditions not only reduce the electrochemical gradient of glutamate uptake but may also reverse glutamate transporter function to increase extracellular glutamate (non-vesicular release) (Longuemare et al. 1996). Moreover, mice with thoracic contusion SCI express different levels of GLT1 in various anatomical locations (Lepore et al. 2011). As a result, changes in glutamate transporter expression in the thalamic VPL could explain the alterations glutamate release observed in our experiments.

In the present study, we destroyed the right STT electrolytically. We did not observe significant difference in motor activity between SCI and sham rats. Direct observations failed to show any change in walking or rearing behavior (limping and foot dragging) or uneven gait.

Based on our findings, mechanical allodynia occurred in both hind paws after SCI. Several studies have found similar mechanical allodynia in hind paws after SCI. Wang and Thompson (2008) reported that tactile allodynia occurred in the hind paws 3 days after STT lesions. Consistent with this, the tactile allodynia in the hind paws observed at day 3 in our study was not different in experimental and sham groups, but became statistically significant 11 days later, and was maintained for at least 4 weeks. Wang and Thompson (2008) showed also a decrease in paw withdrawal threshold at day 3 after surgery, which recovered by day 7 post-surgery in sham animals. Mechanical allodynia has also been reported in sham rats 3 days after STT injury that disappeared by 7 days post-surgery (Naseri et al. 2012). Moderate reductions in head withdrawal threshold 3 days after developing trigeminal neuropathic pain in a sham group have been reported (Wei et al. 2008), which were blocked completely by application of local anesthetic in the surrounding tissues. Therefore, the initial tactile allodynia we observed in the sham group could be attributed to the response of the tissue to the surgical procedure alone 1–3 days after SCI caused by input from local tissue (e.g., incision, laminectomy and inflammation). We observed allodynia in both hind paws, which is also reported in many rodent models of central pain (e.g., Quiton et al. 2010; Zhang et al. 2011). Cerebral cortex plays a prominent role in pain perception; therefore, atypical activity following central pain could be caused of bilateral pain (Masri et al. 2009).

In conclusion, in spinal-cord-injured rats, an immediate increase in glutamate release at the site of injury is followed by delayed changes in glutamate concentration at supraspinal level in the VPL. This may be one of the mechanisms that lead to neuronal hyperexcitation and post-injury sustained neuropathic pain. Obviously, the results would be much stronger by the study of the effects of glutamate receptor antagonists and transporters. We are going to do this to provide more evidences that the increased responses to pain are produced by elevated glutamate.

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**Conflict of interest** The authors declare that there are no competing financial interests.

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