Spinal glutamate uptake is critical for maintaining normal sensory transmission in rat spinal cord

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Abstract

Glutamate is a major excitatory neurotransmitter in primary afferent terminals and is critical for normal spinal excitatory synaptic transmission. However, little is known about the regulation of synaptically released glutamate in the spinal cord under physiologic conditions. The sodium-dependent, high-affinity glutamate transporters are the primary mechanism for the clearance of synaptically released glutamate. In the present study, we found that intrathecal injection of glutamate transporter blockers DL-threo-β-benzyloxyaspartate (TBOA) and dihydrokainate produced significant and dose-dependent spontaneous nociceptive behaviors, such as licking, shaking, and caudally directed biting, phenomena similar to the behaviors caused by intrathecal glutamate receptor agonists. Intrathecal TBOA also led to remarkable hypersensitivity in response to thermal and mechanical stimuli. These behavioral responses could be significantly blocked by intrathecal injection of the NMDA receptor antagonists MK-801 and AP-5, the non-NMDA receptor antagonist CNQX or the nitric oxide synthase inhibitor L-NAME. In vivo microdialysis analysis showed short-term elevation of extracellular glutamate concentration in the spinal cord after intrathecal injection of TBOA. Furthermore, topical application of TBOA on the dorsal surface of the spinal cord resulted in a significant elevation of extracellular glutamate concentration demonstrated by in vivo glutamate voltammetry. The present study indicates that defective spinal glutamate uptake caused by inhibition of glutamate transporters leads to excessive glutamate accumulation in the spinal cord. The latter results in persistent over-activation of synaptic glutamate receptors, producing spontaneous nociceptive behaviors and sensory hypersensitivity. Our results suggest that glutamate uptake through spinal glutamate transporters is critical for maintaining normal sensory transmission under physiologic conditions.
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1. Introduction

Glutamate acts as the major excitatory neurotransmitter at central synapses and has been implicated in excitatory synaptic transmission, synaptic plasticity, and neuronal development (Mayer and Westbrook, 1987). Glutamate is released at the presynaptic membrane, diffuses through the synaptic cleft, and acts on glutamate receptors, including *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate, and metabotropic glutamate receptors, at the postsynaptic membrane. Unlike some other neurotransmitters, no enzymes have been

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discovered that metabolize the extracellular glutamate. Nonetheless, the glutamate concentration in synaptic cleft is tightly controlled under physiologic conditions, as excessive or prolonged activation of glutamate receptors can cause excitotoxicity and neuronal death (Choi, 1992). The clearance of glutamate from the synaptic cleft is solely dependent on the high affinity, sodium-dependent glutamate transporters that surround excitatory synapses (Danbolt, 2001; Tao et al., 2004b). The genes for glutamate transporters have been cloned, and at least five subtypes have been identified: glutamate/aspartate transporter (GLAST) (Storck et al., 1992; Tanaka, 1993), glutamate transporter-1 (GLT-1) (Pines et al., 1992), excitatory amino acid carrier (EAAC) 1 (Kanai and Hediger, 1992), excitatory amino-acid transporter (EAAT) 4 (Fairman et al., 1995), and EAAT5 (Arriza et al., 1997).

In the dorsal root ganglion, the small and medium neurons contain abundant glutamate. The central projections of these neurons are thinly myelinated or unmyelinated high-threshold Aδ- and C-fibers that terminate predominantly in the superficial dorsal horn of spinal cord (Dickenson et al., 1997; Dubner and Basbaum, 1997). These afferent terminals release glutamate as a major excitatory neurotransmitter critical for spinal sensory transmission and for generation and maintenance of spinal states of pain hypersensitivity via activation of glutamate receptors (Malmberg and Yaksh, 1995; Sluka and Willis, 1998; Vetter et al., 2001; Yoshimura and Jessell, 1990). In the spinal cord, three glutamate transporters, GLAST, GLT-1, and EAAC1, have been reported and they are concentrated on the superficial dorsal horn (Rothstein et al., 1994; Tao et al., 2004a,b). EAAC1 is also expressed in small- and medium-sized dorsal root ganglion neurons (Tao et al., 2004a,b). Although much effort and progress have been made in studying the roles of glutamate receptors and subsequent intracellular signals in spinal excitatory synaptic transmission and sensitization, little is known about the action of glutamate transporters in the mechanisms of sensory synaptic transmission and pain hypersensitivity at the spinal cord level. The purpose of this study was to determine whether the spinal glutamate transporters are critical for maintaining normal sensory synaptic transmission. We first examined whether the blockade of spinal glutamate transporters produced excitatory behavioral responses, including spontaneous pain behaviors and hypersensitivity, in response to thermal and mechanical stimulation. We then defined whether these behavioral responses were dependent on the activation of glutamate receptors. Finally, we observed whether the blockage of spinal glutamate transporters resulted in the increases in the concentration of endogenous extracellular glutamate in the spinal cord of in vivo rats.

2. Methods

2.1. Animal preparation

Male Sprague-Dawley rats (250–300 g) were housed individually in cages on a standard 12–12 h light-dark cycle. Water and food were available ad libitum until rats were transported to the laboratory approximately 1 h before the experiments. The experiments were carried out with the approval of the Animal Care and Use Committee at the Johns Hopkins University and were consistent with the ethical guidelines of the National Institutes of Health and the International Association for the Study of Pain. All efforts were made to minimize the number of animals used and their suffering.

The rats were implanted with an intrathecal catheter under halothane anesthesia. A polyethylene-10 catheter was inserted into the subarachnoid space at the rostral level of the spinal cord lumbar enlargement through an incision at the atlanto-occipital membrane according to the method described previously (Tao and Johns, 2002; Tao et al., 2000; Zhang et al., 2003). The animals were allowed to recover for a week before being used experimentally. Rats showing neurologic deficits postoperatively were excluded from the study. After the experiments, location of the intrathecal catheter was confirmed and lumbar enlargement segments were harvested for histochemical staining as described below. All drugs used below were dissolved in 0.9% physiologic saline. Animal behavioral tests described below were performed by a single experimenter who was blinded to the treatment groups.

2.2. Spontaneous pain behaviors

The rats were placed individually in an open Plexiglas chamber ($34 \times 30 \times 30$ cm^3) for 1 h before actual experimental sessions. To observe whether blockade of glutamate transporters produced spontaneous nociceptive behaviors (such as hindpaw elevation, licking, shaking, caudally directed biting and scratching), saline (control; 10 μl, $n=10$), one of three doses of DL-threo-β-benzyloxyaspartate (TBOA) (Tocris, Ellisville, MO, USA) (1, 5, and 10 μg/10 μl; $n=10$ each dose), or one of three doses of dihydrokainate (DHK) (Tocris) (5, 10, and 20 mM/10 μl; $n=10$ each dose) was injected intrathecally followed by a flush of 10 μl of 0.9% physiologic saline. Immediately after injection, each rat was returned to the Plexiglas chamber. The amount of time spent exhibiting spontaneous nociceptive behaviors was recorded by stop-watch during a 10-min period. To determine whether these spontaneous nociceptive behaviors were dependent on glutamate receptors and their downstream signaling, saline (10 μl, $n=10$), dizocilpine maleate (MK-801) (RBI, Natick, MA, USA) (10 nmol/10 μl; $n=10$), D-(−)-2-amino-5-phosphonopentanoic acid (AP-5) (Tocris) (10 nmol/10 μl; $n=7$), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (RBI) (20 nmol/10 μl; $n=10$), N^G-Nitro-L-arginine-methyl ester (L-NAME) (Alexis, Carlsbad, CA) (1000 μg/10 μl, $n=10$), or N^G-Nitro-D-arginine-methyl ester (D-NAME) (Alexis) (1000 μg/10 μl, $n=10$) was administered intrathecally 10 min prior to the intrathecal injection of TBOA (10 μg/10 μl). The duration of spontaneous nociceptive behaviors was recorded as described above. To observe whether these antagonists/inhibitors given alone affect locomotor functions, saline (10 μl; $n=5$), MK-801 (10 nmol/10 μl; $n=5$), AP-5 (10 nmol/10 μl; $n=7$), CNQX (20 nmol/10 μl; $n=5$), or

L-NAME (1000 µg/10 µl, $n=5$) was injected intrathecally. Ten minutes later, locomotor functions including placing, grasping, and righting reflexes were examined according to our previous work (Tao et al., 2000; Zhang et al., 2003). The doses of these antagonists/inhibitors were based on previous studies (Begon et al., 2001; Nozaki-Taguchi and Yaksh, 2002; Osborne and Coderre, 1999).

To exclude the possibility that spontaneous pain behaviors were due to spinal cord injury caused by the presence of an intrathecal catheter, we intrathecally injected TBOA or DHK through direct lumbar puncture in rats (De la Calle and Paino, 2002; Mestre et al., 1994). Briefly, with the rat under halothane anesthesia, the skin of the back was incised along the spinous processes at the L2 to L5 level, and the wound was sutured. On the second day, after extraction of the suture, saline (10 µl; $n=6$), TBOA (10 µg/10µl; $n=7$), or DHK (20 mM/10 µl; $n=6$) was intrathecally administered to the freely moving rat through a lumbar puncture between L4 and L5 using a 25-gauge stainless steel needle attached to a glass microsyringe. A quick flick of the rat's tail confirmed the accurate intrathecal position of the needle. After injection, rats were placed into the chamber and the spontaneous behaviors were observed as described above.

2.3. Hypersensitivity in response to thermal and mechanical stimulation

Rats were tested for thermal hypersensitivity by a method described previously (Tao and Johns, 2002; Zhang et al., 2003). Behavioral measurements were made 1 day before TBOA injection (baseline) and 20 min after intrathecal TBOA injection [0 (saline), 1, 5, and 10 µg/10 µl; $n=7$ each dose]. Each rat was placed in a Plexiglas chamber on a glass plate above a light box. A beam of radiant heat from the apparatus (Model 336 Analgesia Meter, IITC Inc./Life Science Instruments, Woodland Hills, CA) was applied through a hole in the light box to the middle of the plantar surface of each hindpaw through the glass plate. When the rat lifted its foot, the light beam was turned off. The length of time between the start of the light beam and the foot lift was defined as the paw withdrawal latency. Each trial was repeated five times at 5 min intervals for each side. A cut-off time of 20 s was used to avoid tissue damage to the paw. To determine whether the thermal hypersensitivity was dependent on glutamate receptors, saline (10 µl, $n=5$), AP-5 (10 nmol/10 µl; $n=6$), or CNQX (20 nmol/ 10 µl; $n=5$) was administered intrathecally 10 min before the intrathecal injection of TBOA (10 µg/10 µl). Behavioral measurements as described above were made 1 day before these agents were injected (baseline) and 20 min after intrathecal TBOA injection.

Rats were tested for mechanical hypersensitivity according to the methods previously described (Zhang et al., 2003). Behavioral measurements were made 1 day before TBOA injection (baseline) and 20 min after intrathecal TBOA injection [0 (saline), 1, 5, and 10 µg/10 µl; $n=8$]. A single trial of mechanical stimuli consisted of eight applications of a calibrated von Frey filament (8.01 mN, Stoelting, Wood Dale, IL) within a 2–3 s period. Each trial was repeated 10 times at 3-min intervals on each hindpaw. The number of times that the rat withdrew its paw in each of these 10 trials was expressed as a percent response frequency. This percentage was used as an indication of the amount of hindpaw withdrawal. To determine whether the mechanical hypersensitivity was dependent

on glutamate receptors, saline (10 µl, $n=5$), AP-5 (10 nmol/10 µl; $n=6$), or CNQX (20 nmol/10 µl; $n=5$) was administered intrathecally 10 min before the intrathecal injection of TBOA (10 µg/10 µl). Behavioral measurements as described above were made 1 day before the injection of these agents (baseline) and 20 min after intrathecal TBOA injection.

2.4. In vivo microdialysis assay

The in vivo intrathecal microdialysis technique that we used has been described previously (Marsala et al., 1995). Rats under halothane anesthesia were implanted with a triple dialysis catheter (Marsil Scientific, San Diego, CA) into the subarachnoid space through an incision at the atlanto-occipital membrane. The total length of the catheter was 9 cm, including an active dialysis fiber length of 4 cm (200 µm inner diameter, 300 µm outer diameter, 11 kDa cut off). The active site was positioned to span lumbar enlargement segments. The dialysis fiber was connected to the outer lumens of the polyethylene tubing via 1 cm lengths of fused silica tubing used as stents, with methacrylate adhesive on the outside. Fine gauge wire lengthwise inside the dialysis fiber prevented crimping. The center lumen of the polyethylene tubing was used as an injection line. All three lumens were heat-fused to short lengths of single lumen polyethylene tubing (PE-10) to permit connection. This tubing was tunneled through the subcutaneous space, externalized over the frontal bones, and plugged with short lengths of wire. After the incision was closed, sterile normal saline was then flushed through the catheter, and rats were placed in a warm chamber to recover consciousness. Rats showing motor weakness or signs of paralysis upon recovery from anesthesia were immediately sacrificed.

Dialysis experiments were conducted after 2 or 3 days' recovery from surgery. The dialysis inflow and outflow tubing was connected to a two-channel fluid swivel. The effluent was routed to a programmable, refrigerated fraction collector (Eicom, Kyoto, Japan), while the infusate was delivered by syringe drivers at 10 µl/min (Harvard Apparatus, South Natick, MA). Artificial cerebral spinal fluid [aCSF (mM): 151 NaCl, 2.6 KCl, 0.9 $MgCl_2$, 1.3 $CaCl_2$, 2.5 $NaHPO_4$, and 21 $NaHCO_3$], bubbled with 5% CO_2 in air, was pumped at a rate of 10 µl/min for a 30-min stabilization period prior to sample collection (Malmberg and Yaksh, 1995). Fractions were then collected at 10-min intervals into refrigerated plastic microcentrifuge tubes and frozen at $-20\,°C$ immediately. Saline ($n=4$) or one of three doses of TBOA (1, 5, and 10 µg/ 10 µl; $n=5$ each dose) was given intrathecally via the center lumen.

Glutamate in the spinal perfusate was detected by reversed-phase, high-performance liquid chromatography with fluorometric detection following pre-column derivatization with *o*-phthalalde-hyde. Chromatography was performed on a reversed-phase C-18 column (MetaChem Polaris; 5 µm$\times$25 cm; MetaChem Technol-ogies, Torrance, CA), using a pH sodium acetate methanol gradient. Methionine sulfone was added to each sample as an internal standard. External standards, containing 40, 400, and 4000 pmol/20 µl of glutamate, were run at the beginning and end of each sample group. Sensitivity was 5–10 pmol/50 µl. Glutamate peak heights were initially normalized to the methionine sulphone peak and then quantified based on a linear relationship between peak height and amounts of corresponding standards.

Data are expressed as percentages of the means of two to three baseline fractions.

2.5. Real time in vivo measurement of glutamate using ceramic-based multisite microelectrodes

To exclude the possibility that glutamate release demonstrated by in vivo microdialysis assay was due to spinal cord injury caused by the implantation of intrathecal microdialysis catheter, we used the ceramic-based multisite microelectrode for in vivo measurement of the extracellular glutamate concentration, as described previously (Binns et al., 2004a,b; Burmeister et al., 2002). Calibration of commercially available glutamate voltammetry electrodes (Quanteon LLC, Nicholasville, KY) involved placement of the working and reference electrodes in a stirred 40-ml solution of 0.1 M phosphate buffered saline (PBS; 37 °C, pH 7.4). To perform electrochemical measurements using the FAST-16 system, a voltage of $+0.7$ V was applied versus the Ag/AgCl reference electrode. The current generated by H_2O_2 oxidation on the surface of the working electrode, corresponded to the concentration of glutamate in solution. Aliquots of 20 mM of ascorbic acid and glutamate were sequentially added to the PBS solution to create final concentrations as follows: 250 μM ascorbic acid, 20, 40, 60, and 80 μM glutamate.

Calibration tests resulted in selectivity ratios of glutamate over ascorbic acid of at least 50:1 for the electrodes used in these studies. The linearity of the slope created by sequential glutamate additions was quantified by Pearson correlation coefficient (R^2) values; 0.990 or greater was considered acceptable. Limit-of-detection values produced by the electrodes below 2.5 μM were deemed sufficient to utilize an electrode for in vivo studies.

Briefly, rats ($n=4$) were anesthetized with chloral hydrate, and placed in a stereotaxic frame. The spinous processes from T12-L1 were removed, exposing the dorsal region of spinal cord. A miniature 200-μm diameter Ag/AgCl reference electrode was implanted at a spinal cord site proximal from the recording area. The microelectrode and micropipette were inserted vertically into the dorsal aspect of the spinal cord about 2 mm at the L5-L6 level. At least 20 min of basal activity was recorded. After topical application of TBOA at 10 μg/10 μl on the dorsal surface of the spinal cord, the resulting glutamate signals were measured for at least 20 min at the multisite microelectrodes, using constant potential amperometry carried out using the FAST-16 (Quanteon, LLC, Nicholasville, KY) recording system ($+0.7$ V vs Ag/AgCl). The data were analyzed by the integrated WinFAST-16 software (Center for Sensor Tech., UK). After the experiments were done, 1% Evans Blue was injected through the glass micropipette adjoining the glutamate voltammetry electrode, and the animals were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. Approximate location of electrode tip within spinal cord was examined.

2.6. TdT-Mediated dUTP nick end labeling (TUNEL) and cresyl violet histochemical staining

Normal rats ($n=4$) and the rats after behavioral tests described above were deeply anesthetized and perfused transcardially with 100 ml of 0.01 M phosphate-buffered 0.9% saline (PBS, pH 7.4), followed by 300 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The lumbar enlargement segments and thymus (as a positive control) were harvested, postfixed at 4 °C for 4 h, and cryoprotected in 30% sucrose overnight. The transverse sections (25 μm) were cut on a cryostat, and two sets of sections were collected. TUNEL histochemical staining was performed on one set of sections using an in situ cell death detection kit (Roche Molecular Biochemical, IN). Briefly, the sections were incubated with proteinase K solution (20 μg/ml) for 20 min at room temperature, and then with a TUNEL reaction mixture composed of terminal deoxynucleotidyl transferase (TdT) at 37 °C in a humidified chamber. TdT enzyme-incorporated fluorescein was detected with coverter-alkaline phosphatase (AP), consisting of sheep anti-fluorescein antibody conjugated with AP. The signal was detected using nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate as color substrates. Another set of sections was stained with cresyl violet. The sections were rinsed in distilled water and incubated for 30 min in a solution of 0.2% cresyl violet (cresyl violet acetate; Sigma, St Louis, MO) in acetate buffer, washed in distilled water, dehydrated through a graded series of ethanol, and coverslipped.

2.7. Statistical analysis

The data were statistically assessed by an analysis of variance. Intergroup differences were analyzed by the Newman–Keuls test. Data were assessed as mean $\pm$ SEM. Significance was set at $P<0.05$.

3. Results

3.1. Glutamate receptor-mediated spontaneous nociceptive behaviors evoked by the glutamate transporter blockers

The intrathecal administration of TBOA, a potent, non-transportable blocker of glutamate transporters, evoked spontaneous nociceptive behaviors characterized by hind-paw elevation, licking, shaking, and caudally directed biting and scratching in rats both with and without intrathecal catheter implantation (Fig. 1A). As shown in Fig. 1A, the time spent on pain-related behavior was dose-related. Occasionally, TBOA at the highest dose produced agitation and vocalization. These pain-related behaviors usually appeared immediately after TBOA injection and completely disappeared 9–10 min after injection of TBOA at the doses as indicated (Fig. 1A). As expected, no pain-related behaviors were observed after intrathecal saline injections (Control) (Fig. 1A). To further confirm glutamate transporter blocker-evoked nociceptive behaviors, we also employed another potent, non-transportable blocker of glutamate transporters (a relatively selective blocker for GLT-1), DHK. We found that intrathecal injection of DHK produced pain behaviors similar to those produced by TBOA in the rats with and without catheter implantation (Fig. 1B).

Furthermore, we found that TBOA-evoked nociceptive behaviors were mediated by glutamate receptors and their downstream signaling. As shown in Fig. 1C, TBOA-induced spontaneous nociceptive behaviors were completely
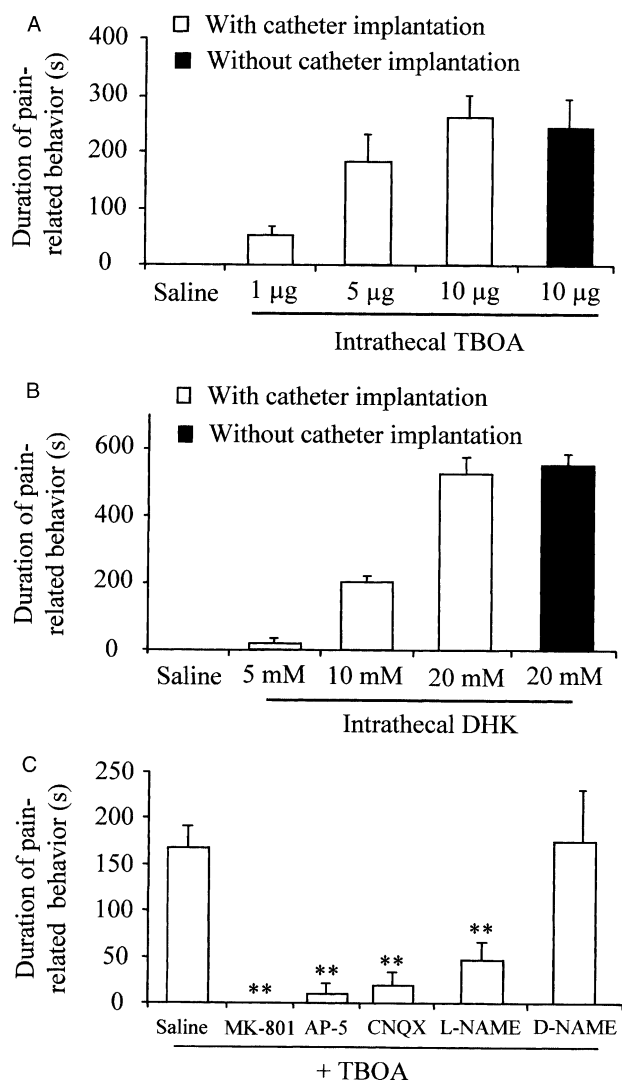
Fig. 1. Glutamate receptor-mediated spontaneous nociceptive behaviors produced by the glutamate transporter blockers. A: Intrathecal TBOA, a glutamate transporter blocker, produced remarkable spontaneous nociceptive behaviors in rats with and without catheter implantation. B: Intrathecal DHK, another glutamate transporter blocker, produced remarkable spontaneous nociceptive behaviors in rats with and without catheter implantation. C: Effects of the glutamate receptor antagonists (MK-801, AP-5, and CNQX) and an NOS inhibitor (L-NAME) on spontaneous nociceptive behaviors evoked by intrathecal TBOA. D-NAME is an inactive isomer of L-NAME. $^{**}P < 0.01$ compared to the group treated with TBOA and saline.

Table 1
Effects of intrathecal injection of MK-801, AP-5, CNQX, and L-NAME, respectively, on locomotor functions

Agents	Placing	Grasping	Righting
Saline	5 (0)	5 (0)	5 (0)
MK-801 10 nmol	5 (0)	5 (0)	5 (0)
AP-5 10 nmol	5 (0)	5 (0)	5 (0)
CNQX 20 nmol	5 (0)	5 (0)	5 (0)
L-NAME 100 µg	5 (0)	5 (0)	5 (0)

Mean (SEM) change in locomotor tests, $N = 5.5$ trials.

pain-associated behaviors in the rats treated with TBOA and L-NAME was decreased by 72% compared to the rats treated with TBOA alone ($P < 0.01$). Intrathecal injection of MK-801, AP-5, CNQX, or L-NAME alone at the doses indicated above did not affect locomotor functions, including placing, grasping, and righting reflexes (Table 1). We also did not observe any significant difference in general behaviors, including spontaneous activity, between the saline-treated group and the groups treated with MK-801, AP-5, CNQX, or L-NAME alone.

3.2. Glutamate receptor-mediated thermal and mechanical hypersensitivity induced by the glutamate transporter blocker

Next, we examined whether TBOA produced hypersensitivity in response to thermal and mechanical stimulation. Twenty minutes after intrathecal injection of TBOA, paw withdrawal latencies in response to heat stimulation in either left or right hindpaw were significantly and dose-dependently decreased. In the left hindpaw, the paw withdrawal latencies at 5 and 10 µg/10 µl TBOA were reduced by 25.5% ($P < 0.05$) and 36.9% ($P < 0.01$) compared to corresponding baseline latencies, respectively (Fig. 2A). In the right hindpaw, the paw withdrawal latencies at the two higher doses of TBOA were reduced by 30% ($P < 0.05$) and 40% ($P < 0.01$) compared to corresponding baseline latencies, respectively (Fig. 2B). Paw withdrawal latency was not significantly changed at 1 µg/10 µl TBOA in both hindpaws. Consistently, the paw withdrawal frequencies in response to mechanical stimulation were significantly increased 20 min after intrathecal injection of TBOA. Following the two higher doses of TBOA injection, the paw withdrawal frequencies were increased by 2.5-times ($P < 0.01$) and 4.1-times ($P < 0.01$) of corresponding baseline frequencies, respectively, in left hindpaw (Fig. 2C), and by 2.6-times ($P < 0.01$) and 3.3-times ($P < 0.01$) of corresponding baseline frequencies, respectively, in right hindpaw (Fig. 2D). TBOA at the lowest dose did not produce significant increases in paw withdrawal frequencies compared to baseline frequencies in either hindpaw (Fig. 2C and D).

We found that the decreases in paw withdrawal latencies following intrathecal injection of TBOA at the dose of 10 µg

abolished by pretreatment with MK-801 (an NMDA receptor antagonist), and significantly blocked by pre-administration of AP-5 (another NMDA receptor antagonist) and CNQX (a non-NMDA receptor antagonist). The durations of pain-related behaviors in the rats treated with TBOA and AP-5 or CNQX were reduced by 94.1 and 88.7%, respectively, compared to the rats treated with TBOA alone ($P < 0.01$). In addition, L-NAME (a nitric oxide synthase (NOS) inhibitor), but not D-NAME, also markedly reduced nociceptive behaviors induced by TBOA (Fig. 1C). The duration of
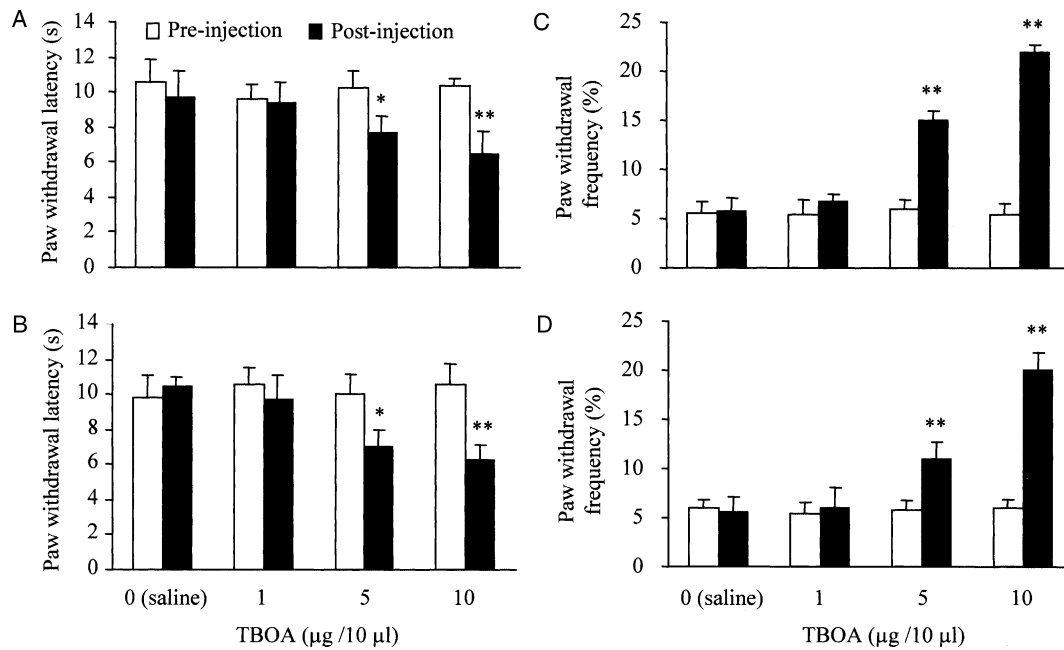
Fig. 2. TBOA-evoked hypersensitivity in response to thermal (A and B) and mechanical (C and D) stimuli in both left (A and C) and right (B and D) hindpaws. [*]$P<0.05$ and [**]$P<0.01$ vs corresponding baseline.

could be significantly attenuated by pre-treatment with AP-5 at the dose of 10 nmol, or CNQX at the dose of 20 nmol, in either left or right hindpaw (Fig. 3A and B). Similarly, TBOA-induced increases in the paw withdrawal frequencies could be markedly blocked by pre-administration of AP-5 and CNQX in both hindpaws (Fig. 3C and D).

3.3. Elevated concentration of spinal cord extracellular glutamate produced by TBOA

Basal level of glutamate was 107 pmol/50 µl. The intrathecal injection of TBOA at two higher doses (5 and 10 µg/10 µl) produced a significant, but short-term increase
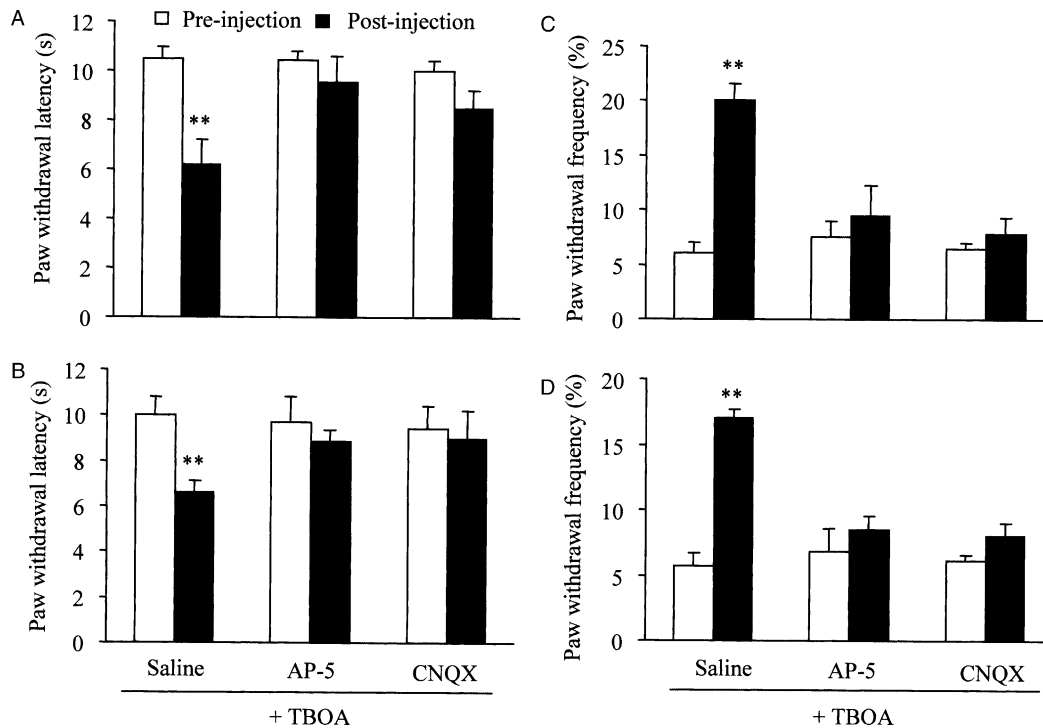

Fig. 3. Effects of glutamate receptor antagonists AP-5 and CNQX on thermal (A and B) and mechanical (C and D) hypersensitivity in left (A and C) and right (B and D) hindpaws evoked by TBOA. [**]$P<0.01$ vs corresponding baseline.

A TBOA
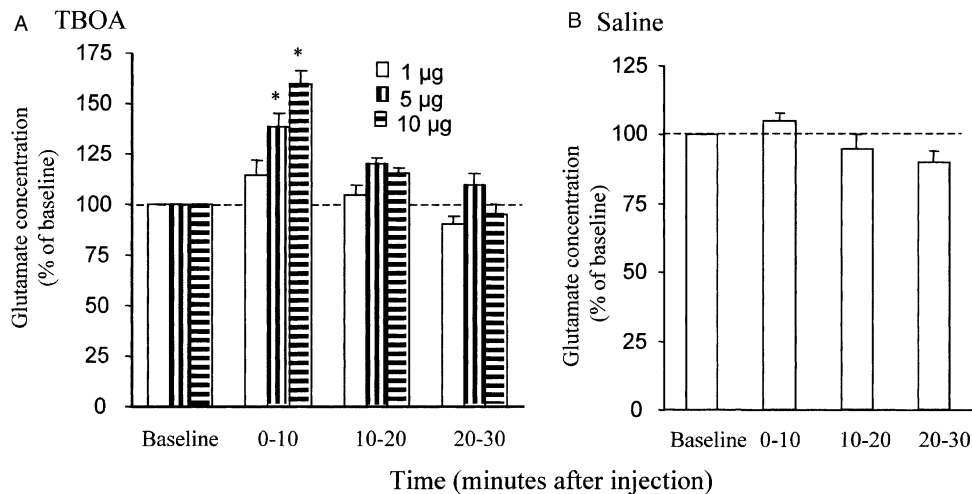

B Saline

Time (minutes after injection)

Fig. 4. The concentration of glutamate in dialysate determined from samples collected at 10-min intervals for a total of 30 min. Glutamate levels after intrathecal injection of one of three doses of TBOA (1, 5, and 10 µg/10 µl) (A) or saline (B) were measured. Data are expressed as percentage of the baseline fractions. $^*P < 0.05$ vs corresponding baseline.

in the release of spinal cord glutamate as demonstrated by in vivo intrathecal microdialysis (Fig. 4A). Glutamate concentration was increased by $139 \pm 7\%$ and $160 \pm 6\%$, respectively, of the baseline ($P < 0.05$). The significantly elevated concentration of glutamate was detected only in the fractions collected during the first 10 min of dialysis. Intrathecal administration of TBOA at a low dose (1 µg/10 µl) did not produce a remarkable increase in glutamate concentration, although a tendency of glutamate

concentration to increase was observed ($P > 0.05$, Fig. 4A). As expected, intrathecal saline did not result in significant changes in the level of glutamate compared to the baseline ($P > 0.05$, Fig. 4B). Furthermore, topical administration of TBOA (10 µg/10 µl) resulted in an immediate marked increase in glutamate concentration measured by voltametric methods (Fig. 5A). Topical TBOA resulted in an increase in extracellular glutamate concentration ($n = 4$), and the elevated extracellular glutamate dissipated within

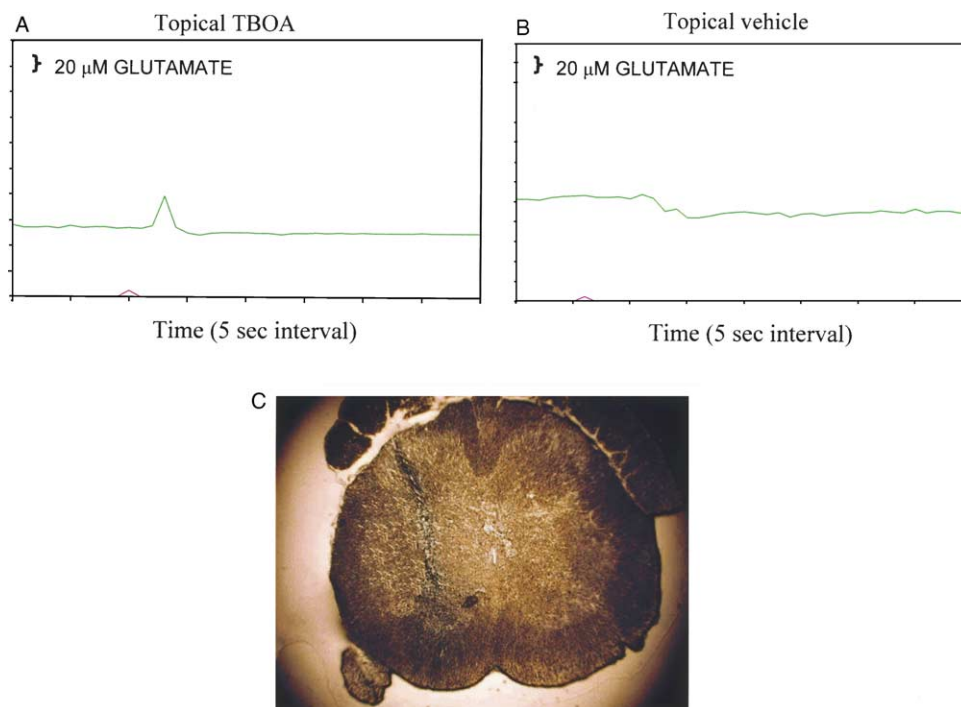A Topical TBOA

B Topical vehicle

C

Fig. 5. Representative depiction of the effect of topical treatment of TBOA on spinal extracellular glutamate concentration. A: Topical application of TBOA (10 µg/10 µl) on the dorsal surface of the L5-6 spinal cord (indicated by the tic mark on the X-axis) resulted in an increase in extracellular glutamate concentration. B: the vehicle (saline, 10 µl) had no stimulatory effect. C: The placement site of the micropipette is revealed after Evans blue injection through the adjoining glass micropipette.

5 s after topical TBOA administration. No further elevation in glutamate concentration was observed within 20 min after topical TBOA administration. In contrast, topical vehicle produced a small reduction in baseline concentration 5–15 s after administration, indicative of dilution of basal extracellular glutamate concentration (Fig. 5B).

3.4. Normal morphologic structure in spinal cord after intrathecal catheter implantation

To further exclude the possibility that the presence of an intrathecal catheter causes spinal injury to evoke glutamate release, we examined the spinal cord morphology after chronic intrathecal catheter implantation. Cresyl-violet staining showed that spinal cord neurons did not have any pathologic changes in rats with intrathecal catheter implantation compared to normal rats (Fig. 6A). Neuronal cell architecture appeared normal in both dorsal and ventral horns (Fig. 6A). We counted the number of cresyl-violet-stained cells in the spinal cord from normal rats ($n=4$) and rats with intrathecal catheter ($n=6$). No significant
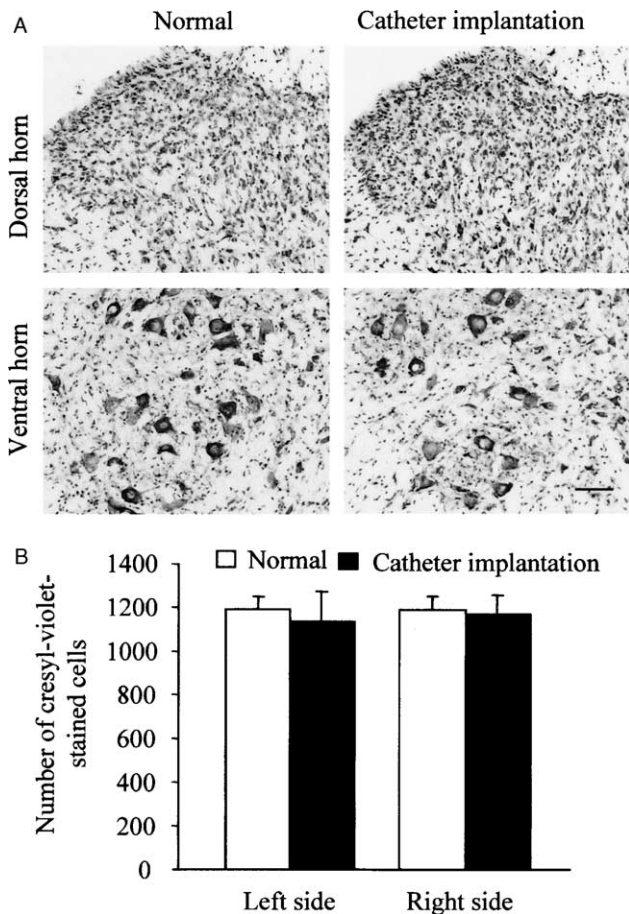

Fig. 6. Cresyl-violet staining in spinal cord of normal rats and rats with catheter implantation. A: Representative photograph showing neuronal cell architecture in both dorsal and ventral horns. Scale bar: 200 μm. B: Average number of cresyl-violet-stained cells on the left and right sides of spinal cord grey matter in normal rats and rats with implanted catheters.
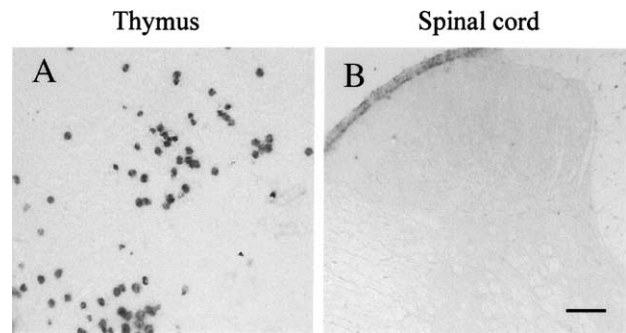

Fig. 7. Representative photograph showing TUNEL-positive cells in thymus and spinal dorsal horn. Scale bar: 200 μm.

differences were observed in number of stained cells of spinal cord between these two groups (Fig. 6B). Furthermore, we examined whether chronic catheter implantation caused neuronal damage in the spinal cord using TUNEL histochemistry. Since, under normal conditions, thymus expresses many apoptosis-positive cells (Denis et al., 1998), we used thymus as a positive control. As shown in Fig. 6A, many thymus cells were positive for TUNEL. However, we did not find any TUNEL-positive cells in either white or grey matter of the spinal cord of rats with intrathecal catheter implantation (Fig. 7B).

4. Discussion

It is well known that glutamate is a major excitatory synaptic transmitter in primary afferent fibers and is critical for spinal excitatory synaptic transmission (Malmberg and Yaksh, 1995; Sluka and Willis, 1998; Vetter et al., 2001; Yoshimura and Jessell, 1990). However, little is known about the role of regulating synaptically released glutamate in spinal excitatory synaptic transmission under physiologic conditions. In the present study, we have made a novel discovery that the intrathecal injection of TBOA, a potent, non-transportable blocker of glutamate transporters, produces significant spontaneous nociceptive behaviors, and thermal and mechanical hypersensitivity. We also found that intrathecal injection of DHK, another blocker of glutamate transporters, evoked similar spontaneous pain-related behaviors. These findings are consistent with a recent report that showed an increase in spontaneous activity and responses of wide dynamic range neurons to both innocuous mechanical (brush, pressure) and noxious mechanical (pinch) stimuli after topical L-trans-pyrrolidine-2,4-dicarboxylic acid (PDC), a glutamate transporter blocker (Weng et al., 2004a,b). Unlike DHK and PDC, TBOA does not act as an agonist or antagonist at glutamate receptors (Jabaudon et al., 1999; Minami et al., 2001; Phillis et al., 2000; Shimamoto et al., 1998). Thus, spontaneous pain-related behaviors and sensory hypersensitivity evoked by the glutamate transporter blockers used in the present study cannot be explained completely by direct interaction

between the glutamate transporter blockers and the glutamate receptors. Interestingly, these spontaneous pain-related behaviors are very similar to the behaviors caused by glutamate receptor agonists, such as glutamate, NMDA, or AMPA, when given intrathecally (Aanonsen and Wilcox, 1987; Brambilla et al., 1996; Kontinen and Meert, 2002). It seems that spontaneous nociceptive behaviors produced by glutamate transporter blockers might be due to an elevation of extracellular glutamate, resulting from the blockade of glutamate reuptake in the spinal cord. Indeed, using ceramic-based, multisite microelectrodes, we found a remarkable increase in extracellular glutamate concentration in the spinal cord after topical application of TBOA. This is consistent with the previous studies showing that topical treatment of PDC or TBOA induced a rapid increase in basal glutamate in brain (Isaacson and Nicoll, 1993; Pomerleau et al., 2003). Moreover, our microdialysis experiments demonstrated that glutamate concentration was significantly elevated during the first 10 min after intrathecal TBOA injection. This short-term increase in extracellular glutamate concentration parallels the onset and duration of remarkable spontaneous, pain-related behaviors evoked by intrathecal TBOA. However, we also detected thermal and mechanical hypersensitivity 20 min after intrathecal injection of two higher doses of TBOA, while the increase of spinal cord glutamate release could not be detected in dialysis fractions, even at the highest dose of TBOA used. In addition, with the glutamate voltametry microelectrode study, no elevation of glutamate concentration was observable after the early transient peak for the 20-min duration of the study. It is possible that the TBOA-evoked release in spinal cord glutamate was too small to be detected in the subarachnoid space past 10 min by the current microdialysis or voltametry systems. However, the acute stimulation of glutamate receptors by this transiently elevated concentration of glutamate in the spinal cord might lead to central sensitization of spinal cord neurons mediated by activation of glutamate receptors. This hypothesis was further supported by the results of co-injecting glutamate receptor antagonists with TBOA. Pre-administration of AP-5 or CNQX not only significantly blocked TBOA-induced spontaneous nociceptive behaviors, but also attenuated TBOA-evoked thermal and mechanical hypersensitivity. In addition, MK-801, another glutamate receptor antagonist, completely abolished TBOA-induced, pain-related behaviors. Nitric oxide is one of the downstream signals of the NMDA receptor pathway (Meller and Gebhart, 1993; Snyder, 1992). We found that L-NAME (but not D-NAME), a non-selective NOS inhibitor, significantly reduced pain-related behaviors induced by TBOA. These findings suggest that glutamate receptors (at least NMDA and non-NMDA receptors) and their downstream signals might mediate production of these pain-related behaviors and sensory hypersensitivity, and require for full expression of the responses to excessive glutamate in the spinal cord.

Three glutamate transporters, EAAC1, GLT-1, and GLAST, are expressed in the spinal cord, particularly in the superficial dorsal horn (Rothstein et al., 1994; Tao et al., 2004a,b). EAAC1 is also expressed in small- and medium-sized dorsal root ganglion neurons (Tao et al., 2004a,b). Under electron microscopy, EAAC1 is localized in pre- and post-synaptic membranes, and axonal and dendritic membranes at non-synaptic sites in the superficial dorsal horn, whereas GLAST and GLT-1 are distributed exclusively in spinal cord glial cells (Tao et al., 2004b). An in vitro glutamate uptake assay demonstrated that TBOA significantly blocks glutamate uptake activity through inhibition of EAAC1, GLAST, and GLT-1, and that DHK mainly affects glutamate uptake activity of GLT-1 (Jabaudon et al., 1999; Phillis et al., 2000; Shimamoto et al., 1998). Under physiological conditions, glutamate receptors (particularly NMDA receptors) are tonically activated by ambient glutamate in central neurons (Sah et al., 1989). It is very likely that defective glutamate uptake of spinal neuronal and glial glutamate transporters, caused by glutamate transporter blockers, produces excessive accumulation of extracellular glutamate, which results in persistent over-activation of synaptic glutamate receptors and their downstream signaling and leads to spontaneous nociceptive behaviors and sensory hypersensitivity. Thus, glutamate uptake through spinal glutamate transporters is critical for maintaining normal sensory transmissions in the spinal cord.

It should be noted that, in most experiments (particularly behavioral studies) of the present study, glutamate transporter blockers and glutamate receptor antagonists were given intrathecally through an inserted catheter in the subarachnoid space. The insertion of the spinal catheter might cause spinal cord injury. It is reported that spinal cord injury results in elevated levels of extracellular glutamate in spinal cord (McAdoo et al., 1999; Xu et al., 1998). Therefore, it is possible that spontaneous pain-related behaviors and sensory hypersensitivity evoked by glutamate transporter blockers in the present study might be attributed to spinal cord injury caused by chronic implantation of intrathecal catheter. To exclude this possibility, we first examined the action of intrathecal TBOA or DHK in rats without chronic catheter implantation. Our data showed that direct intrathecal injection of TBOA or DHK through a lumbar puncture led to spontaneous nociceptive behaviors similar to intrathecal injection of TBOA and DHK through a catheter. This finding indicates that glutamate transporter blocker-evoked spontaneous pain-related behaviors in rats with implanted intrathecal catheters cannot be completely explained by spinal cord injury caused by the insertion of the catheter. Second, we demonstrated that topical application of TBOA could induce the increase of extracellular glutamate concentration in vivo detected by ceramic-based multisite microelectrodes. This indicates that spinal glutamate release detected by intrathecal dialysis catheter is attributed to inhibition of glutamate uptake rather than to

the dialysis catheter-caused spinal cord injury. Third, we directly examined whether insertion of the spinal catheter produced spinal cord injury. We allowed all rats to recover for one week after intrathecal catheter implantation and excluded from the experiments any that showed neurologic deficits postoperatively. Cresyl-violet staining showed normal cell architecture in both dorsal and ventral horns in the rats with intrathecal catheter implantation. Quantitative analysis revealed that total number of cresyl-violet-stained cells in the grey matter of the spinal cord of rats with intrathecal catheter was similar to that in normal rats. Finally, we examined whether chronic implantation of intrathecal catheter caused cell death in the spinal cord using TUNEL histochemistry. No TUNEL-positive cells were observed in either grey or white matter of the spinal cord of rats with intrathecal catheter. Thus, glutamate transporter blocker-evoked behavioral responses in the present study cannot be explained by spinal cord damage.

In summary, the present study demonstrated for the first time that blockade of spinal glutamate transporters produced significant spontaneous nociceptive behaviors and thermal and mechanical hypersensitivity. These behavioral responses could be significantly reduced by glutamate receptor antagonists. The blockage of spinal glutamate transporters also led to the elevated concentration of extracellular glutamate in the spinal cord. The present results suggest that spinal glutamate transporters might play a critical role in spinal excitatory synaptic neurotransmission under physiologic conditions.

Acknowledgements

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