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Spinal glial glutamate transporters downregulate in rats with taxol-induced hyperalgesia

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Abstract

Changes in the expression of glial glutamate transporters (GLAST and GLT-1) were examined in the spinal cord of rats with chemotherapy (taxol)-induced mechanical hyperalgesia. Immunohistochemical studies show that the expression of both GLAST and GLT-1 in the L4–L5 spinal dorsal horn is decreased by 24% ($P < 0.001$) and 23% ($P < 0.001$), respectively, in rats with taxol-induced hyperalgesia as compared with those in control rats. These changes were further confirmed using an enzyme-linked immunosorbent assay that confirmed downregulation of GLAST by 36% ($P < 0.05$) and GLT-1 by 18% ($P < 0.05$) in the L4–L5 spinal cord of taxol-treated rats. These data indicate that downregulation of glutamate transporters may contribute to the development of hyperalgesia induced by taxol and suggest that glutamate transporters may be a new target for treatment of pain.

Keywords: Glutamate transporters; GLAST; GLT-1; Astrocytes; Hyperalgesia; Pain; Chemotherapy; Paclitaxel

Painful peripheral neuropathy produced by paclitaxel (taxol) is one of the major dose-limiting factors for chemotherapy in cancer patients [7,9,15,21]. Rats treated with taxol develop mechanical and thermal hyperalgesia [4,8,14] and also show increased spontaneous activities and prolonged afferent-evoked after discharges in dorsal horn wide dynamic range neurons [4]. Mechanisms underlying taxol-induced hyperalgesia are not fully understood. Taxol-induced hyperalgesia in rats is partially or completely reversed by ethosuximide (a relatively selective T-type calcium channel blocker) [8,14], knockdown of the transient receptor potential vanilloid 4 [1] or beta1 integrin gene expression in sensory nerves [6].

Ample evidence has demonstrated that activation of glial cells and their subsequent release of proinflammatory cytokines contributes to the development of neuropathic pain [23]. However, plasticity of glial glutamate uptake in hyperalgesia has attracted very limited attention. Glutamate released from presynaptic terminals within the synaptic cleft is cleared passively by diffusion and actively by glutamate transporters [5]. Two glial transporters, the L-glutamate-L-aspartate transporter (GLAST, also called EAAT1) and glutamate transporter 1 (GLT-1, also called EAAT2) exist in the spinal cord [10,12,16,18,20]. Glutamate transport in glial cells plays a key role in maintaining homeostasis of extracellar glutamate concentrations [5]. The function of glutamate transporters can be regulated by changes in the number expressed on the cell surface and by alterations in the efficacy of transporter activity [11]. In this study, changes in expression of glial glutamate transporters (GLAST and GLT-1) in taxol-induced hyperalgesic rats have been investigated.

Twenty-four adult male Sprague–Dawley rats weighing 175–220 g were used. All experiments were conducted with approval of the Institutional Animal Care and Use Committee at the MD Anderson Cancer Center and were in complete

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compliance with the National Institutes of Health Guidelines for Use and Care of Laboratory Animals.

Behavioral studies: Taxol (Sigma) was dissolved in 5% DMSO (vehicle) and stored in a 1 mg/ml stock solution. It was then diluted in saline to 1.0 ml final volume and injected at a dose of 1.0 mg/kg intraperitoneally on four alternate days for a final cumulative dose of 4 mg/kg. Animals injected with vehicle (1.5% DMSO) alone in the same protocol provided the comparison control group.

Behavioral tests were performed daily to evaluate changes in the mechanical sensitivity of the rats [25]. Briefly, the animals were placed on a wire mesh, loosely restrained under a plexiglass cage (12 cm × 20 cm × 15 cm) and allowed to accommodate for at least 10 min. A series of von Frey monofilaments with a range of bending forces from 0.6 to 14.6 g were applied from below through the mesh onto the mid plantar side of each hind paw. Each paw was stimulated five times for each von Frey monofilament. Mechanical thresholds were defined based on the least bending force that induced paw withdrawal responses in more than 50% of all 10 trials [25]. All rats had developed mechanical hyperalgesia prior to advancing into the immunohistochemistry and enzyme-linked immunosorbent assay (ELISA) protocols.

Immunocytochemistry: Five taxol-treated and five control rats were deeply anesthetized with pentobarbital (60 mg/kg, i.p.) and perfused intracardially with 300 ml heparinized saline solution (4 °C) followed by 500 ml of a solution of 4% paraformaldehyde and 0.1% picric acid in 0.1 M phosphate-buffered saline (PBS) (4 °C). Spinal segments L4 and L5 were then removed and post-fixed for 24 h at 4 °C in the same fixative and cryoprotected for 24 h at 4 °C in 30% sucrose in 0.1 M phosphate buffer.

Serial transverse sections, 30-μm thick, were cut on a freezing microtome (−20 °C) and collected in 0.1 M PBS (pH 7.4) and processed while free-floating. The sections were rinsed in 0.1 M PBS three times (10 min each) and then incubated in 10% normal goat serum plus 0.3% Triton X-100 for 1 h at room temperature (20 °C). The sections were then incubated for 36 h at 4 °C in guinea pig anti-GLAST (1:2000) or anti-GLT-1 (1:2000) (Chemicon Int., USA) in 2% normal goat serum. The sections were washed three times in 0.1 M PBS (10 min each) and then incubated for 2 h at room temperature with the corresponding FITC-conjugated secondary antibody (1:500, Jackson Immuno labs, USA). After rinsing in 0.1 M PBS, the sections were mounted onto gelatin-coated slides, air-dried, and cover-slipped with DPX mounting medium. To examine specificity of the secondary antibodies, control sections were processed with the primary antibodies omitted. Sections taken from taxol treated and control rats were placed in different wells of the same plate during all immunochemical procedures. Six nonadjacent sections from the L4 and L5 segments were selected randomly, and the distribution of glutamate transporters in the spinal cord dorsal horn was recorded with a digital camera (CoolSnap cf Photometrics, Rober scientific, USA). Immunostaining densities of the dorsal horn were measured and averaged across six spinal sections [20]. The density of white matter was used as a reference point to ensure equal staining between sections. Immunostaining density (arbitrary units) for each lamina was expressed as mean ± S.E.M. The immunohistochemical data were analyzed for statistical significance with the Student's *t*-test between the normal- and taxol-treated rats. *P*-values of less than 0.05 were considered significant.

ELISA: Seven taxol-treated hyperalgesic rats and seven vehicle-treated rats were deeply anesthetized with pentobarbital (60 mg/kg, i.p.). The L4–L5 spinal segments were removed and rapidly frozen in liquid nitrogen and stored at −80 °C. Frozen tissues were later homogenized in ice-cold lysis buffer containing 50 mM Tris–HCl (pH 7.5), 5 mM EDTA, 1% SDS, 1% NP-40, 10% Protease Inhibitor Cocktail (Complete mini, Roche, Germany) supplemented with 1 mM phenylmethyl sulfonyl fluoride. Homogenates were then centrifuged at 14000 × *g* for 30 min at 4 °C and the supernatant was collected. Protein concentration of the homogenate was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). All materials used for the following ELISA were purchased from Alpha Diagnostic Int., USA. One hundred microliters of diluted tissue sample (50 μg/ml) and diluted standard peptides (GLAST or GLT-1) at different concentration (10–500 ng/ml) was dispensed onto each microwell that was then kept overnight at 4 °C. The microwells were then incubated with blocking agents for 4 h and air-dried. Diluted anti-GLAST (1:3000) and anti-GLT-1 (1:3000) solutions were applied into the wells in 100 μl aliquots and incubated at room temperature (22 °C) for 30 min. The microwells were then washed three times. Ab-enzyme conjugate (1:1000, 100 μl) was then dispensed into each well and incubated for 30 min at room temperature. The microwells were then washed four times. TMB substrate solution (100 μl) was then added into each well and incubated for 20 min, followed by addition of 100 μl/well of stop solution. The absorbance at 450 nm was read on a MRX II microplate reader (Dynex, UK).

Statistics: Data were presented as mean ± S.E. Comparisons of data between control and taxol-treated group were made using Mann–Whitney *U* test. $P < 0.05$ was considered significant.

Rats receiving i.p. injection of paclitaxel did not show any significant visible morbidity or measurable loss of body weight compared to vehicle-treated control rats consistent with previous reports using this chemotherapy regimen [8,14]. Additionally, no animals showed any signs of impaired/excessive grooming, abnormal motor behavior or autotomy.

Hind paw mechanical withdrawal thresholds were essentially identical in vehicle-treated ($n = 12$) and taxol-treated ($n = 12$) groups at the outset of the study (vehicle rats: 12.35 ± 0.65 g versus taxol rats: 12.91 ± 0.61 g). All taxol-treated rats developed mechanical hypersensitivity at day 5 of chemotherapy, as indicated by a significant drop ($P < 0.05$) in the 50% mechanical withdrawal threshold (5.84 ± 1.80 g).

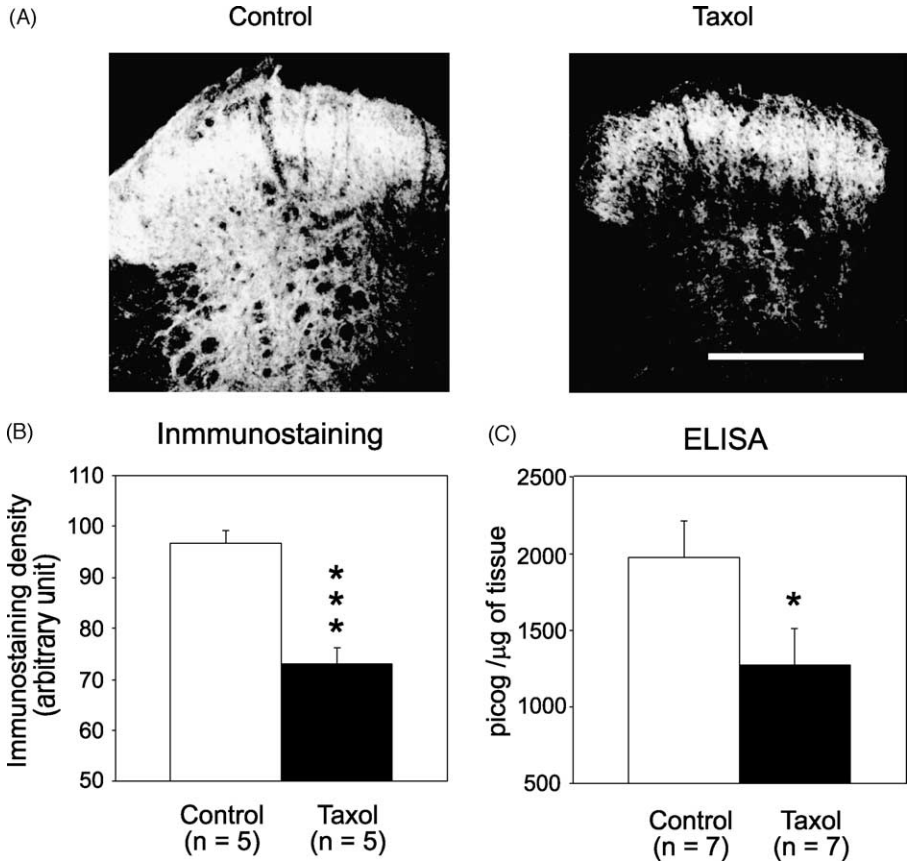

Fig. 1. Decreased expression of GLAST in the spinal cord in rats with taxol-induced mechanical hyperalgesia. (A) Representative pictures taken from the L5 spinal dorsal horn of control (left) and taxol-treated (right) rats. Note GLAST is mainly distributed in the laminae I and II. Scale bar, 300 μm. (B) Bar graphs show mean (±S.E.) immunostaining density of GLAST measured from L4 and L5 spinal dorsal horn taken from taxol-treated rats ($N = 5$) is significantly lower than that in control rats ($N = 5$). (C) Bar graphs show mean (±S.E.) concentrations of GLAST protein (picogram/per microgram of tissue) in L4 and L5 spinal cord taken from taxol-treated rats ($N = 7$) are significantly lower than those in control rats ($N = 7$). $^{*}P < 0.05$, $^{***}P < 0.001$.

In contrast, thresholds remained unchanged from baseline at 11.65 ± 1.03 g in vehicle-treated rats. This effect persisted through the rest of observation period (up to day 15 of chemotherapy) such that the 50% withdrawal threshold on the day of euthanization for immunohistochemistry or ELISA was significant lower in taxol-treated rats (3.34 ± 0.84 g) than that in vehicle-treated rats (12.24 ± 1.01 g) ($p < 0.001$).

Expression of GLAST and GLT-1 in the dorsal horn of taxol-induced mechanical hyperalgesia and vehicle-treated rats was examined using immunohistochemistry. In the normal rats ($n = 5$), GLAST was expressed at the highest levels in laminae I and II in the spinal dorsal horn, while GLT-1 showed a more generalized pattern of expression throughout the dorsal horn (Figs. 1A and 2A). While topographic distributions of GLAST and GLT-1 in the dorsal horn of taxol-treated rats ($n = 5$) were similar to those in normal rats, the immunoreactivity of GLAST and GLT-1 measured in the L4 and L5 segment dorsal horns in taxol-treated rats was significantly reduced by 24% ($P < 0.001$) and 23% ($P < 0.001$), respectively, in comparison with those in control groups (Figs. 1B and 2B).

Expressions of glial glutamate transporters (GLAST and GLT-1) in taxol-treated ($n = 7$) and normal rats ($n = 7$) were further verified using ELISA (Figs. 1C and 2C). The expression of GLAST in the L4 to L5 spinal segments was significantly reduced by 36% ($P < 0.05$) in taxol-treated rats as compared with that in control rats. Meanwhile, the expression of GLT-1 analyzed from the same tissue also showed a significant reduction of 18% ($P < 0.05$) in taxol-treated rats in comparison with that in control rats.

The major finding in this study is that the expression of both glial glutamate transporters (GLAST and GLT-1) in the spinal cord is downregulated during taxol-induced mechanical hyperalgesia in rats.

The homeostasis of extracellular glutamate is maintained by a family of glutamate transporters in the plasma membrane of both glial cells and neurons since glutamate is not metabolized extracellularly [5,11]. GLT-1 gene knockout mice develop seizures and most died by 6 weeks [17] while GLAST knockout animals show increasing susceptibility to drug-induced seizures [22]. Blockade of glial glutamate transporters in neocortex significantly increases the amplitude and duration of epileptiform discharges [3] and the

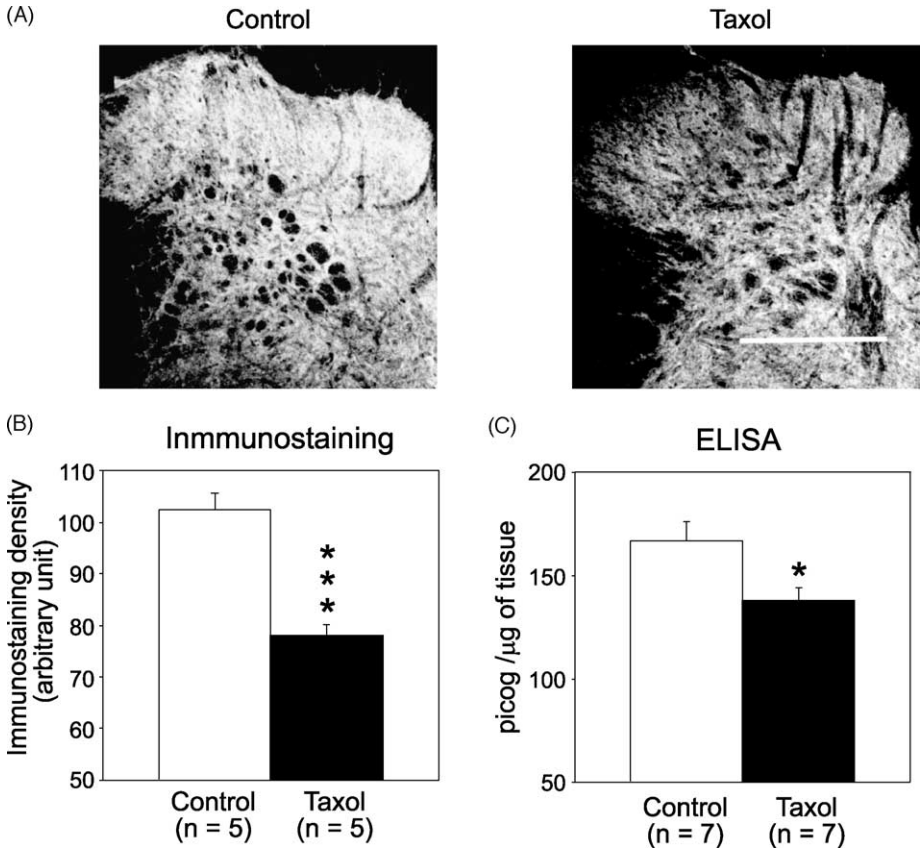

Fig. 2. Decreased expression of GLT-1 in the spinal cord in rats with taxol-induced mechanical hyperalgesia. (A) Representative pictures taken from the L5 spinal dorsal horn of control (left) and taxol-treated (right) rats. Note GLT-1 is distributed in the entire dorsal horn. Scale bar, 300 μm. (B) Bar graphs show the mean (±S.E.) immunostaining density of GLT-1 measured from L4 and L5 spinal dorsal horn taken from taxol-treated rats ($N = 5$) is significantly lower than that in control rats ($N = 5$). (C) Bar graphs show mean (±S.E.) concentrations of GLT-1 (picogram/per microgram of tissue) in L4 and L5 spinal cord taken from taxol-treated rats ($n = 7$) are significantly lower than those in control rats ($n = 7$). $^{*}P < 0.05$, $^{***}P < 0.001$.

amplitude [19] and the duration [2,13] of afferent-evoked EPSCs and EPSPs in cerebellar neurons [2,13,19]. We have recently demonstrated that inhibition of glutamate uptake in the rat spinal cord results in mechanical allodynia and hyperalgesia, increased acute responses and after-discharges of WDR neurons in the dorsal horn to peripheral mechanical stimulation [24]. Such functional changes are similar to those found in dorsal horn WDR neurons in rats with taxol-induced mechanical hyperalgesia [4]. Thus, it is conceivable that the downregulation of GLAST and GLT-1 expressions in taxol-treated rats results in impairment of glutamate uptake that would lead to increased spontaneous activities and prolonged afferent-evoked afterdischarges in dorsal horn neurons and hyperalgesia. Our results are consistent with previous studies showing that the decreased expression of glutamate transporters in the spinal dorsal horn in the later phase of hyperalgesia induced by chronic nerve constriction injury [16] and hyperalgesia induced by chronic morphine treatment [12]. It would be of great interest to further investigate whether a treatment that changes nociceptive behavior also affects the expression of glial glutamate transporters.

In conclusion, changes in glutamate transporters in hyperalgesic states found in this study suggest that correction of dysfunctional glutamate transporters may be a new avenue for treatment of pain.

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