GLT-1 overexpression attenuates bladder nociception and local/cross-organ sensitization of bladder nociception


GLT-1 overexpression attenuates bladder nociception and local/cross-organ sensitization of bladder nociception. Am J Physiol Renal Physiol 300: F1353–F1359, 2011. First published March 23, 2011; doi:10.1152/ajprenal.00009.2011.—Glutamatergic pathways mediate transmission of pain. Strategies to reduce glutamatergic neurotransmission may have beneficial effects to mitigate nociception. Recent work revealed that overexpression of the astrocytic glutamate transporter (GLT-1) by transgenic or pharmacologic approaches produced a diminished visceral nociceptive response to colonic distension. The purpose of this study was to determine the effect of GLT-1 overexpression on the visceralomotor response to bladder distension. Increased glutamate uptake activity produced by 1-wk ceftriaxone (CTX) treatment attenuated 60–64% the visceromotor response to graded bladder distension compared with vehicle-treated mice. One-hour pretreatment with selective GLT-1 antagonist dihydrokainate reversed the blunted visceralomotor response to bladder distension produced by 1-wk CTX treatment. Compared with CTX receiving intracolonic TNBS showed no enhanced visceromotor response to graded bladder distension produced a 75–138% increase in visceromotor response compared with animals receiving intracolonic vehicle. In marked contrast, animals treated with 1-wk CTX + intracolonic TNBS showed no enhanced visceromotor response compared with the 1-wk vehicle + intracolonic vehicle group. The study suggests that GLT-1 overexpression attenuates the visceromotor response to bladder distension and both local irritant-induced and cross-organ-sensitized visceromotor response to bladder distension.

VISCERAL PAIN IS A LEADING cause of visits to the physician. For example, the National Institutes of Health estimates that up to 1 million people in the United States suffer from visceral pain associated with painful bladder syndrome or interstitial cystitis (28). Current hypotheses suggest that bladder irritation and inflammation lead to release of chemical messengers that sensitize centrally projecting primary afferents that utilize predominantly glutamate as the neurotransmitter (23).

Glutamate is normally rapidly cleared from the synaptic cleft by high-affinity, sodium-dependent glutamate transporters located in both neurons and glia. The glial glutamate transporter GLT-1 is the quantitatively dominant glutamate transporter in the mammalian central nervous system and plays a major role in terminating synaptic transmission and protecting neurons from glutamate neurotoxicity (24). Recently, the innovative approach of reducing extracellular glutamate by overexpressing the predominant glutamate transporter GLT-1 was found effective in animal models of both visceral (14) and neuropathic (9, 15) pain. Specifically, ceftriaxone (CTX) is a β-lactam antibiotic that when administered to rodents produces GLT-1 overexpression, also linked with neuroprotective effects in vitro and in vivo in diseases such as amyotrophic lateral sclerosis and epilepsy (20, 22), and has reached Phase III clinical trials for the treatment of amyotrophic lateral sclerosis (ClinicalTrials.gov identifier NCT00349622). The putative mechanism utilized by CTX to overexpress GLT-1 is nuclear translocation of p65 and activation of NF-kB that binds to the GLT-1 gene promoter region (12). Thus, it is hypothesized that this novel approach to decrease glutamate extracellular levels by upregulating glutamate transporter function may be beneficial in the relief of bladder pain.

Among the issues observed clinically and receiving increasing experimental support is viscerovisceral referral of pain and cross-organ sensitization. For example, cross-organ sensitization of lumbosacral spinal neurons receiving input from the bladder occurs after colonic inflammation (19). Moreover, inflammation of the colon sensitizes the response of the bladder to noxious stimulation (2, 11, 17, 27), and colon irritation increased the resting firing rate and the firing rate in response to distension of bladder afferents (25). Thus, colonic irritation or inflammation results in enhanced bladder nociception and altered urodynamic function (18), all responses mediated significantly by the principle excitatory neurotransmitter glutamate (4). Thus, strategies to attenuate glutamatergic primary afferent neurotransmission may decrease referred pain.

This study will explore the effect of augmenting GLT-1 expression by 1-wk CTX treatment (14) on the visceralomotor response to bladder distension. In addition, the effect of GLT-1 overexpression on both local irritant-induced and cross-organ sensitization (colon to bladder) of the visceralomotor response to bladder distension will be examined. The results reveal that pharmacological enhancement of GLT-1 expression and activity blunts the murine visceromotor response to bladder distension and both local irritant-induced and colon-to-bladder sensitization of the visceromotor response to bladder distension.

METHODS

Animals. Two-month-old female FVB/N mice (20–25 g) were used for the experiments. Animals were housed singly on wood shavings with ad libitum access to food and water. The 12:12-h light-dark cycle was maintained. Before experiments, animals were fasted for 12 h to

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limit the fecal content of the colon; access to water was maintained. Estrus cycle status was not controlled in these studies. Experiments were approved by Institutional Animal Care and Use Committee from The Ohio State University and followed the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain.

**Drug administration.** CTX (200 mg/kg) was prepared in saline and administered intraperitoneally (ip) at 10:00 for 7 consecutive days; the control group received 0.9% saline ip. This dosing regime of CTX selectively enhances GLT-1 expression in rodents (20). In some experiments, the selective GLT-1 antagonist dihydrokainate (DHK) was administered at an effective dose (10 mg/kg ip) (13) 1 h before urinary bladder distension; the control group received 0.9% saline ip. In the study of colon-to-bladder sensitization of bladder nociception, intracolonic trinitrobenzene sulfonic acid (TNBS; 35 mg/ml; 50 μl) (25) was administered 1 h before bladder distension in 1-wk vehicle and 1-wk CTX-treated cohorts, lightly anesthetized with isoflurane; the control group received the same volume of intracolonic vehicle (50% ethanol). Intracolonic TNBS treatment was recently shown effective to sensitize urinary bladder afferents to mechanical and chemical stimuli (23). Another set of experiments examined the effect of GLT-1 overexpression on augmented visceromotor response to bladder distension produced by local irritation by intravesicular acrolein. Animals were administered acrolein (0.4 mM, 100 μl) intravesically 1 h before bladder distension was performed; the control group was pretreated with intravesicular 0.9% saline.

**Surgery.** Wires for electromyographic recording were surgically implanted as previously described (14). Briefly, animals were anesthetized with ip ketamine (37.5 mg/kg; Hospira, Lake Forest, IL) and xylazine (10 mg/kg; Bayer, Shawnee Mission, KS). The abdomen of the animal was shaved and a surgical incision (1 cm in length) was made lateral to the abdominal midline. The external oblique abdominal musculature was identified and electrode wires (Teflon-coated stainless steel wire; Cooner Wire Sales, Chatsworth, CA) were sewn to the neck muscles. The incisions were then closed using 6-0 nylon suture. The wounds were cleaned with saline and allowed to heal by second intention. The animals were allowed to recover a minimum of 72 h. Mice that developed motor weakness were euthanized before, during, and after distension was easily monitored by the tube for 60 min before beginning recordings. The behavior of the mice before, during, and after distension was easily monitored by partial retraction of the fabric.

**Bladder distension and EMG recordings.** Visceromotor responses were quantified from the EMG signals due to urinary bladder distension-evoked musculature contractions as previously described (16). Graded intensities of urinary bladder distension (0.05, 0.1, 0.15, and 0.2 ml normal saline) were used to compare the differences between 1-wk vehicle and 1-wk CTX-treated animals. Preliminary experiments determined that these volume distensions corresponded to 15, 30, 45, and 60 mmHg pressure, respectively. Contractions of the external oblique muscle (visceromotor responses) were recorded 10 s before bladder distension to establish baseline activity and 20 s during distension (response = increase above baseline), and 10 s after termination bladder distension. The EMG signal was then normalized as change over baseline using Spike2 data-acquisition software.

**Western blotting.** To determine whether increased GLT-1 expression occurred after 1-wk CTX compared with 1-wk vehicle, mice were euthanized after these treatments and the lumbosacral region (L5 to S2) was excised. The spinal cord was homogenized using a hand-held pestle in a lysis buffer containing a commercial mixture of phosphatase inhibitors and proteinase inhibitors. The samples (40 μl; 80 μg) were loaded onto wools and run in a 10% SDS-PAGE gel and then electroblotted onto nitrocellulose membrane using a mini gel and mini transblot apparatus (Bio-Rad Laboratories, 170–3935). The membranes were then incubated with primary antibodies consisting of primary rabbit anti-GLT-1 (1:3,000; Santa Cruz Biotechnology) COOH-terminal antibody or the primary rabbit antibody β-actin (1:1,000; Santa Cruz Biotechnology) in 1% milk-PBST buffer overnight. The membranes were then rinsed and placed in blocking buffer (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.1% Tween 20, and 15% nonfat milk) at room temperature for 1 h. Afterwards, the membranes were exposed to the secondary antibody goat anti-rabbit IgG with horseradish peroxidase (HRP) in 1% milk-PBST buffer (1:6,000 dilution) for 1.5 h. LuminoGl chemiluminescent substrates were used to detect HRP antibody signal with X-ray film exposure. The optical densities of protein blots were analyzed by standardizing the optical density of EAAT2 against the optical density of the β-actin protein.

**Glutamate uptake assay.** Lumbosacral sections (L5-S2) of spinal cord were dissected from animals and minced into small pieces. Tissue from each mouse was homogenized 10 strokes by a rotor-driven homogenizer in 3-ml ice-cold tissue buffer containing 1× complete protease inhibitors (0.2 M sucrose, and 0.05 M Tris (pH 7.4). Cell debris was removed by 1,000 g centrifugation for 10 min at 4°C, and the synaptosomal fraction in the supernatant was separated into two tubes, one with 2 ml and the other one with 1 ml of tissue buffer. Synaptosomes were then spun down at 16,000 g for 10 min at 4°C. For each animal, uptake was measured in triplicate: control group, DHK (GLT-1 antagonist)-treated group, and sodium-free control group. Synaptosomes were resuspended in 1.5 ml Krebs buffer (pH 7.4) for first two groups, or in 1.5-ml Na-free Krebs buffer (120 mM choline CI, 5 mM KCl, 1 mM MgSO4, 1 mM KH2PO4, 25 mM Tris-HCl, 0.55 mM d-glucose, 2 mM CaCl2, pH 7.4). Two-hundred microliters of synaptosome suspension were loaded into clean eppendorf tubes and preincubated in 37°C for 10 min. DHK (1 mM) was added into the DHK group. To initiate the reaction, 0.1 mM unlabeled glutamate and 0.05 μM tritium-labeled glutamate (Perkin Elmer; 1 μCi/ml) were added into each tube and tubes were incubated in 37°C for 10 min. Five hundred microliters of ice-cold tissue buffer were added to stop the reaction and tubes were placed on ice. Synaptosome suspension was exposed to vacuum filtration by using filter paper precoated in 0.2% polyethylene solution. Synaptosomes retained by the filter paper were then washed three times by 4 ml PBS buffer and the filter paper was transferred to scintillation vials with 3 ml scintillation buffer and 0.1 N NaOH. Filter paper that received 0.05 μM tritium-labeled glutamate without synaptosome incubation was used as negative control. Tritium-labeled glutamate inside the synaptosome was measured in scintillation counter (Beckmann). GLT-1-mediated glutamate uptake was calculated by subtracting the uptake in the DHK-treated group from the normal control group. Sodium-depen-
dent glutamate uptake was calculated by subtracting the uptake in the Na-free negative group from the normal control group.

**Statistical analysis.** The Student’s *t*-test was used to determine significant differences in the glutamate uptake assay. Regarding the visceromotor response to urinary bladder distension, the elicited EMG signal was plotted as area under the curve for the 20-s distension and compared with the 10-s baseline before and after distension as previously described (16). The quantitative data in each study were expressed as means ± SE. A random block design with a one-way ANOVA with post hoc least significant difference analysis was used for comparison of treatment groups; data were considered statistically significantly different if *P* < 0.05.

**RESULTS**

Enhancement of GLT-1 expression and glutamate uptake activity blunts the visceromotor response to bladder distension. GLT-1 expression was enhanced 20% and glutamate uptake activity enhanced 113% in lumbosacral spinal cord after 1-wk CTX (200 mg/kg daily) treatment (Fig. 1, *A* and *B*). The visceromotor response to bladder distension was compared in 1-wk vehicle vs. 1-wk CTX-treated animals (Fig. 2A). One-week CTX treatment resulted in a 60–64% decrease in the visceromotor response at each of the three highest distension volumes (*P* < 0.05). A representative depiction of the blunted visceromotor response caused by 1-wk CTX is presented in Fig. 2B. Thus, similar to previous findings studying murine colorectal distension after GLT-1 overexpression (14), bladder distension-induced visceromotor response was also mitigated by GLT-1 overexpression.

Selective GLT-1 antagonist DHK reverses 1-wk CTX-induced mitigation of visceromotor response caused by bladder distension. To verify that 1-wk CTX induced a decrease in the visceromotor response due to increased expression of GLT-1, a selective antagonist of the GLT-1 transporter, DHK, was used to assess whether enhanced GLT-1 activity might mediate the diminished visceromotor response produced by 1-wk CTX treatment. DHK (10 mg/kg) was administered 1 h before urinary bladder distension in animals treated with either 1-wk vehicle or CTX. One-week CTX-treated cohorts showed a (62–73%) reduction in the visceromotor response to graded bladder distension (Fig. 3). DHK pretreatment reversed the CTX-mediated significant reduction in visceromotor response to bladder distension (Fig. 3).

Effect of GLT-1 overexpression on local acrolein-induced changes in the visceromotor response to bladder distension. The effect of GLT-1 overexpression on local irritant-enhanced visceromotor response to bladder distension was studied. Intravesical acrolein, administered 24 h before the measurement of the visceromotor response to bladder distension, produced a 30–83% increase in the response (Fig. 4, *A* and *B*). GLT-1 overexpression produced by daily CTX treatment for 1 wk completely attenuated this response. Thus, pharmacologic enhancement of glutamate uptake also reduces the enhanced effect of local bladder irritation on evoked visceral nociception.

Cross-organ sensitization: effect of increased GLT-1 expression on sensitization of bladder nociception. Emerging evidence suggests that colon irritation can alter neuronal activity of afferents serving other visceral organs, such as the bladder (5). To determine whether increased expression of GLT-1 can mitigate cross-organ sensitization resulting in enhanced visceromotor response to bladder distension, intracolonic TNBS (35 mg/ml; 100 μl) (24) was administered 1 h before the visceromotor response to graded bladder distension volumes was elicited in 1-wk CTX and 1-wk vehicle-treated cohorts. Mice receiving 1-wk vehicle + intracolonic TNBS had a 75 to 138% increase in the visceromotor response to graded bladder distension compared with animals receiving 1-wk vehicle + intracolonic vehicle (Fig. 5, *A* and *B*). In marked contrast, animals treated with 1-wk CTX + intracolonic TNBS showed no enhanced visceromotor response compared with the 1-wk vehicle + intracolonic vehicle group. The data suggest that increased glutamate uptake via enhanced expression of GLT-1 by 1-wk CTX attenuates sensitized visceromotor response to bladder distension produced by colonic irritation.

**DISCUSSION**

The principal findings of this study were that enhanced glutamate uptake by pharmacologically augmented GLT-1 expression 1) reduced the visceromotor response to urinary bladder distension by 2) effects reversible by the selective GLT-1 antagonist DHK, 3) enhanced visceromotor response caused by local bladder irritation was significantly attenuated by enhanced glutamate uptake, and 4) cross-organ sensitization (colon to bladder) of bladder visceromotor response to distension was also reduced by enhanced glutamate uptake. These
findings expand on earlier observations showing that GLT-1 overexpression mitigates the visceromotor response to colorectal distension (14). This finding together with recent reports showing that transfection (15) and pharmacologic (9) approaches to enhance GLT-1 expression reduce somatic pain suggest that strategies to reduce extracellular glutamate may provide new avenues for pain therapeutics.

Recent studies disclosed that the mechanisms of visceral and somatic chronic pain have points in common and divergence (23). Among the similarities is the role of glutamate receptor-mediated activation of second-order spinal neurons. Noxious stimuli evoke long-term increases in the excitability of dorsal horn neurons; glutamate, acting primarily at N-methyl-D-aspartate (NMDA) and amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors, contributes to the development of this phenomenon (8). NMDA and AMPA antagonists also attenuate the visceromotor response to colonic distension (23). However, these agents have severe limitations as therapeutic agents due to the wide distribution of glutamate receptors throughout the central nervous system.

The overexpression of GLT-1 after systemic CTX is well-characterized to occur preferentially in astrocytes throughout the neuroaxis (20). Thus, spinal cord and supraspinal regions show enhanced expression after 1-wk CTX (20). The anatomical site(s) of the beneficial effect of GLT-1 overexpression is unclear; however, a recent study using a rat neuropathic pain model revealed that intrathecal CTX was effective as both a preventative or therapeutic approach to relieve thermal hyperalgesia and to a lessor extent mechanical allodynia (9). Moreover, CTX is a likely site of action of enhanced GLT-1 expression to mitigate visceral pain. Enhanced glutamate uptake activity in spinal sites involved in visceral nociception is correlated with attenuated

Fig. 2. A: visceromotor responses to graded bladder distension in female mice treated with 1-wk CTX (200 mg/kg daily) were compared with mice treated with 1-wk Veh. CTX-treated animals showed a diminished visceromotor response compared with littermates treated with Veh. *P < 0.05. B: raw representative electromyographic recordings elicited after graded bladder distension in 1-wk Veh vs. 1-wk CTX-treated female mice.

Fig. 3. Effect of selective glutamate transporter (GLT-1) antagonist dihydrokainate (DHK) on the blunted visceromotor response to graded bladder distension caused by GLT-1 overexpression. CTX (200 mg/kg daily) or Veh were each administered in 2 separate groups of animals for 7 days. One hour before graded bladder distension, one 1-wk Veh (●) and 1-wk CTX group (○) was treated with DHK (10 mg/kg ip). One-week CTX + Veh (○) produced a significantly reduced visceromotor response to bladder distension compared with the 1-wk Veh + Veh group (●) with the 0.1- and 0.15-ml volumes. DHK pretreatment 1 h before the study reversed the significantly blunted responses. *P < 0.01 compared with 1-wk Veh + 1-h Veh group.
visceromotor response in this report. Regarding potential peripheral sites of action, there are emerging data suggesting that dorsal root ganglion (DRG) may be a site of action of glutamatergic mechanisms mediating the nociceptive response (10), and this intriguing possibility is worthy of further exploration. GLT-1 does appear in the cytoplasm of DRG cells (1), but its role in modulating glutamate homeostasis in the DRG and whether 1-wk CTX is effective in its overexpression are unanswered questions. A recent study also suggested that the locus coeruleus may be a site of action of a glutamate transporter activator to activate descending inhibition mitigating neuropathic pain (7). Further work will be necessary to ascertain the possible contribution of supraspinal or peripheral systems in mediating the apparent anti-nociceptive actions of GLT-1 overexpression. Transgenic animals or 1-wk CTX-treated animals overexpressing GLT-1 show no motor or behavioral abnormalities and no changes in respiratory function (6, 20). Thus, the present report supports the novel and potential translational approach of glutamate transporter overexpression to reduce glutamatergic neurotransmission and mitigate nociception.

New findings of this study include 1) acute colon irritation produced enhanced visceromotor response to bladder distension (colon to bladder) and 2) colon-to-bladder cross-organ sensitization was attenuated by preemptive GLT-1 overexpression. Multiple mechanisms have been advanced to explain cross-organ sensitization; these mechanisms can be conveniently categorized as either central or peripheral. Irritant-induced sensitization of afferent nerve endings (peripheral and/or central) serving multiple visceral organs or development of central sensitization within second-order neurons or higher in the neuroaxis are among proposed mechanisms of cross-organ sensitization (3). Given the importance of glutamate as the predominant excitatory neurotransmitter of visceral afferents (23), the present report suggests that reduction of extracellular glutamate significantly reduces glutamatergic transmission mediating cross-organ sensitization. Moreover, the findings of the present study suggest that attenuation of glutamate...
matergic transmission mitigates colon irritation-enhanced visceromotor response to graded bladder distension (Fig. 5, A and B).

In addition, alteration in the visceromotor response to bladder distension caused by local bladder irritation was attenuated by GLT-1 overexpression (Fig. 4, A and B). Thus, the strategy of reducing extracellular glutamate by GLT-1 overexpression appears effective to mitigate enhanced bladder nociception due to locally activated bladder sensory mechanisms (6).

The reversal of the effect of 1-wk CTX by DHK treatment was not complete at the 0.1- and 0.15-ml distension volumes, in contrast to the effect of DHK on CTX-blunted visceromotor response to colon distension (14). Potential explanations include 1) the optimal time of DHK administration was missed with regard to reversal of CTX mitigation of the visceromotor response to bladder distension, 2) response variability due to different phases of the estrus cycle present within the cohorts, or 3) other effects produced by CTX (i.e., effects on gastrointestinal flora) (26) may mediate its reduction of the visceromotor response to bladder distension.

The exact mechanism of GLT-1 overexpression responsible for the blunted visceral pain response awaits further exploration. However, reduced activation of spinal glutamate receptors in GLT-1-overexpressing animals during urinary bladder distension that results in a reduced activation of second-order spinal neurons is a leading possibility. Decreased activation of second messenger pathways (PKA, PKC, PIP2, NO/GC/PKC, ERK, p38) downstream from glutamate receptors may also be involved (21).

This report extends work showing the effectiveness of enhanced glutamate uptake to mitigate the visceromotor response.
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to bladder distension to two additional visceral nociception models (bladder distension and colonic-bladder cross-organ sensitization). An important unexplored question surrounds the therapeutic application of this approach (i.e., does GLT-1 upregulation mitigate previously established cross-organ sensitization of bladder nociception and function). A recent study suggests therapeutic effectiveness of GLT-1 overexpression in an animal model of somatic nociception (9). Success of this approach fuels optimism that strategies to enhance GLT-1 activity may lead to improved mechanistic-based therapeutic options for treating visceral hyperalgesic disorders and bladder hyperreflexia.

GRANTS
This work was supported by National Institutes of Health Grant DK071839 to R. L. Stephens and the National Natural Science Foundation of China (81070298/H0307).

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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