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Upregulation of GLT-1 by Treatment With Ceftriaxone Alleviates Radicular Pain by Reducing Spinal Astrocyte Activation and Neuronal Hyperexcitability

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Cervical nerve root injury commonly leads to radicular pain. Normal sensation relies on regulation of extracellular glutamate in the spinal cord by glutamate transporters. The goal of this study was to define the temporal response of spinal glutamate transporters (glial glutamate transporter 1 [GLT-1], glutamate-aspartate transporter [GLAST], and excitatory amino acid carrier 1) following nerve root compressions that do or do not produce sensitivity in the rat and to evaluate the role of glutamate uptake in radicular pain by using ceftriaxone to upregulate GLT-1. Compression was applied to the C7 nerve root. Spinal glutamate transporter expression was evaluated at days 1 and 7. In a separate study, rats underwent a painful root compression and were treated with ceftriaxone or the vehicle saline. Glial glutamate transporter expression, astrocytic activation (glial fibrillary acidic protein [GFAP]), and neuronal excitability were assessed at day 7. Both studies measured behavioral sensitivity for 7 days after injury. Spinal GLT-1 significantly decreased ($P < 0.04$) and spinal GLAST significantly increased ($P = 0.036$) at day 7 after a root injury that also produced sensitivity to both mechanical and thermal stimuli. Within 1 day after ceftriaxone treatment (day 2), mechanical allodynia began to decrease; both mechanical allodynia and thermal hyperalgesia were attenuated ($P < 0.006$) by day 7. Ceftriaxone also reduced ($P < 0.024$) spinal GFAP and GLAST expression, and neuronal hyperexcitability in the spinal dorsal horn, restoring the proportion of spinal neurons classified as wide dynamic range to that of normal. These findings suggest that nerve root-mediated pain is maintained jointly by spinal astrocytic reactivity and neuronal hyperexcitability and that these spinal modifications are associated with reduced glutamate uptake by GLT-1.

Key words: radiculopathy; pain; ceftriaxone; glutamate transporter; neuronal hyperexcitability

The cervical nerve root is a common source of neck pain because of its susceptibility to injury from foraminal impingement, disc herniation, and/or foraminal stenosis (Wainner and Gill, 2000; Abbed and Coumans, 2007). Glutamate is a primary neurotransmitter in pain signaling, and its concentration is tightly regulated by glutamate transporters (Anderson and Swanson, 2000; Gegelashvili et al., 2000). The rat has three spinal glutamate transporters: glial glutamate transporter 1 (GLT-1), glutamate-aspartate transporter (GLAST), and excitatory amino acid carrier 1 (EAAC1) (Tao et al., 2005). Because GLT-1 removes as much as 90% of extracellular glutamate in the central nervous system (Danbolt, 2001; Holmseth et al., 2012), normal glutamate uptake by this transporter is essential for maintaining the proper extracellular glutamate concentration (Rothstein et al., 1996; Sung et al., 2003). Upregulation of GLT-1 with ceftriaxone restores the normal concentration of spinal glutamate and prevents the development of behavioral sensitivity after a sciatic nerve ligation in the rat (Rothstein et al., 2005; Inquimbert et al., 2012). Although glutamate transporters are implicated in pain, it is not known whether transient neural tissue injury in the central nervous system also mediates spinal glutamate transporter expression or whether modulation of spinal GLT-1 is sufficient to alleviate nerve root-mediated pain.

Both neurons and astrocytes are activated in the spinal cord by glutamate signaling, and activation of these cells is associated with pain (Weng et al., 2006; Gao and Ji, 2010). Because astrocytic activation is induced only by *painful* transient nerve root compression (Nicholson et al., 2012), spinal astrocytic activation via elevated glutamate signaling is hypothesized to contribute to nerve-root-mediated pain. Increased spinal glutamate enhances neuronal excitability (Cata et al., 2006; Nguyen et al., 2009).

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However, the excitability of neurons in the dorsal horn after a painful nerve root compression has not been defined, and it is unknown whether pharmacologic modulation of spinal glutamate uptake by GLT-1 mediates the spinal neuronal hyperexcitability and/or astrocytic activation that develops after a painful root injury (Hubbard and Winkelstein, 2005; Terashima et al., 2011).

These studies test the hypothesis that spinal glutamate transporters contribute to behavioral sensitivity following a transient cervical nerve root compression. Two separate studies were performed. In the first study, the temporal spinal expression of GLT-1, GLAST, and EAAC1 was evaluated following root compressions of different durations that do (15 min) and do not (3 min) elicit mechanical allodynia (Rothman et al., 2010). Glutamate transporter expression was assessed at days 1 and 7, time points relevant to the establishment and persistence of mechanical allodynia (Hubbard and Winkelstein, 2005). Because pain is multidimensional (Jensen et al., 2011), thermal hyperalgesia was also evaluated at these times to provide a complete picture of nerve root-mediated pain. Based on findings from that first study showing that spinal GLT-1 decreases with painful injury, a second study administered ceftriaxone daily after a painful root compression to determine whether upregulating GLT-1 can alleviate behavioral sensitivity. Spinal glial fibrillary acidic protein (GFAP) and GLAST were evaluated at day 7 after ceftriaxone treatment to evaluate whether restoring GLT-1 also restores spinal astrocytic activity (GFAP) and/or the expression of this glial glutamate transporter (GLAST). The effects of ceftriaxone on dorsal horn neuronal excitability also were evaluated by measuring the frequency of evoked action potentials at day 7 after treatment.

MATERIALS AND METHODS

Male Holzman rats (Harlan Sprague-Dawley; Indianapolis, IN; 275–375 g) were housed under USDA- and AAALAC-compliant conditions with a 12–12-hr light–dark cycle and free access to food and water. All studies were IACUC approved and were carried out under the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain (Zimmerman, 1983).

Temporal Spinal Glutamate Transporter Expression After Nerve Root Compression

Surgical procedures. Compression of the right C7 dorsal nerve root was performed with animals under isofluorane inhalation anesthesia (4% for induction, 2% for maintenance) using procedures previously described (Hubbard et al., 2008; Rothman et al., 2010; Nicholson et al., 2012). With the rat in a prone position, a midline incision was made through the muscular tissue over the cervical spine to expose the C6 and C7 vertebrae. A C6–C7 hemilaminectomy and partial facetectomy on the right exposed the C7 nerve root, and a 10gf microvascular clip (World Precision Instruments, Sarasota, FL) was placed around the nerve root through a small incision in the dura (Hubbard et al., 2008; Rothman et al., 2010; Nicholson et al., 2012). Sham procedures were also included, in which the C7 nerve root was exposed, but no compression was applied. Each rat underwent one of three procedures (n = 14/group): a single compression applied for either 3 min or 15 min or sham exposure. After the surgical procedure, wounds were closed using 3-0 polyester sutures and surgical staples. Rats were under anesthesia for no more than 75 min and were allowed to recover in room air and monitored throughout the recovery period.

Behavioral assessments. Bilateral behavioral sensitivity was evaluated by measuring mechanical allodynia and thermal hyperalgesia prior to (baseline) and up to postinjury day 7. To quantify mechanical allodynia, rats were acclimated for 20 min prior to testing. Then, a 4.0gf von Frey filament (Stoelting Co., Wood Dale, IL) was applied to the plantar surface of each forepaw 10 times for three rounds with a 10-min rest period in between (Hubbard and Winkelstein, 2005). The total number of paw withdrawals (from among 30) was summed across the three rounds of testing for the ipsilateral and contralateral forepaws separately.

Thermal hyperalgesia was measured by applying a radiant heat source to the forepaw using a commercially available device (UC San Diego) and established methods (Hargreaves et al., 1988; Dirig et al., 1997). After a 20-min acclimation period, the thermal stimulus was applied to the plantar surface of each forepaw. The amount of time that lapsed between when the stimulus was first applied and when the forepaw was withdrawn was recorded as the withdrawal latency. On each day of testing, the withdrawal latency was measured three times, with a 10-min rest between all measurement rounds. The average withdrawal latency for each forepaw across the three rounds was recorded. On each day when thermal hyperalgesia was evaluated, mechanical allodynia was measured first.

Differences between injury groups (group) over time (time point) for each behavioral assessment were determined by separate two-way repeated-measures ANOVAs with Bonferroni-corrected post hoc tests for the ipsilateral and contralateral forepaws. All statistical analysis was performed in JMP10 (SAS Institute, Cary, NC).

Immunohistochemistry. To assess the temporal profile of spinal glutamate transporters, C7 spinal cord tissue was harvested at day 1 (n = 7/group) and day 7 (n = 7/group). Matched spinal cord sections were harvested from normal, naïve rats (n = 2) and were included as controls. Rats were anesthetized with an intraperitoneal injection of 65 mg/kg pentobarbital, then transcardially perfused with 200 ml Dulbecco's phosphate-buffered saline (PBS; Mediatech, Manassas, VA), followed by 300 ml 4% paraformaldehyde (Sigma, St. Louis, MO). The C7 spinal cord was removed and postfixed overnight. The samples were then transferred to 30% sucrose for cryoprotection and embedded in OCT media, then stored at −80°C (Sakura Finetek USA, Torrance, CA). Each spinal cord tissue was axially sectioned (14 μm) and thaw-mounted onto slides.

Sections were labeled for the glial glutamate transporters GLT-1 and GLAST or the neuronal glutamate transporter EAAC1. Each slide was labeled for only one glutamate transporter. Sections were blocked in 5% normal goat serum (Vector Laboratories, Burlingame, CA) with 0.3% Triton X-100 (Bio-

Rad Laboratories, Hercules, CA), then incubated overnight at 4°C in rabbit anti-GLT-1 (1:1,000; Abcam; Cambridge, MA), rabbit anti-GLAST (1:1,000; Abcam), or rabbit anti-EAAC1 (1:1,000; Alpha Diagnostics). The slides were then fluorescently labeled with goat anti-rabbit Alexa Fluor 546 (1:1,000; Invitrogen, Carlsbad, CA), and the ipsilateral and contralateral dorsal horn was digitally imaged at ×200 from three to six sections per slide.

To quantify the expression of each glutamate transporter in the superficial dorsal horn, images were cropped around laminae I–II, and quantitative densitometry was used to measure the percentage positive pixels as a measure of positive labeling (Abbadie et al., 1996; Romero-Sandoval et al., 2008; Rothman et al., 2010; Nicholson et al., 2012). The same threshold was used for all images for each labeled protein and was determined based on immunolabeling in normal tissue. Results are reported relative to the expression of each marker measured in normal tissue. Transporter expression in the ipsilateral and contralateral dorsal horns were evaluated separately. A two-way ANOVA with Bonferroni-corrected post hoc tests was used to test for differences in each glutamate transporter expression over time (time point) for each injury group (group).

Treatment With Ceftriaxone After Painful Nerve Root Compression

Ceftriaxone administration. Rats underwent a 15-min compression to the nerve root or sham exposure as described above. Starting on postinjury day 1, rats were randomly assigned to receive either ceftriaxone or saline. The treatment group (injury + ceftriaxone; n = 16) received a 40-μl intrathecal injection of 10 μg ceftriaxone (Wockhardt, Parsippany, NJ) dissolved in saline in the space between L4 and L5. In the vehicle treatment group (injury + saline; n = 15), rats received a 40-μl intrathecal injection of saline. The same vehicle treatment was administered to rats that underwent sham procedures (sham + saline; n = 15). Both the treatment and the vehicle were administered via lumbar puncture on days 1–6 immediately following behavioral assessments.

Behavioral assessments and immunohistochemistry. A subgroup of rats from each treatment group was evaluated for behavioral sensitivity and immunohistochemical analysis as described above (injury + ceftriaxone, n = 8; injury + saline, n = 7; sham + saline, n = 7). Bilateral mechanical allodynia was evaluated daily at baseline and on postoperative days 1–7, and bilateral thermal hyperalgesia was evaluated at postoperative days 1 and 7. Two-way, repeated-measures ANOVAs with post hoc Bonferroni correction tested for differences between each treatment group (group) in each behavioral assessment over time (time point) for the ipsilateral and contralateral forepaws separately.

The C7 spinal cord was harvested at day 7 and labeled for the glial glutamate transporters GLT-1 or GLAST as described above. In addition, spinal GFAP expression was also evaluated as a marker of activated astrocytes. For labeling with GFAP, sections were blocked in 5% normal goat serum with 0.3% Triton X-100, then incubated overnight at 4°C in mouse anti-GFAP (1:500; Millipore, Bellerica, MA). The slides were then fluorescently labeled with goat anti-mouse Alexa Fluor 546 (1:1,000; Invitrogen). The ipsilateral and the contralateral dorsal horn was digitally imaged at ×200 from three to six sections per slide, then cropped over the superficial dorsal horn (laminae I–II). Positive labeling was quantified by using quantitative densitometry. Results are reported relative to the expression of each marker measured in normal tissue. Contralateral and ipsilateral transporter expression was evaluated separately. For each marker (GLT-1, GLAST, GFAP), a one-way ANOVA tested for differences between injury groups.

Spinal electrophysiological recordings. In a separate group of rats, electrophysiological recordings were made in the spinal cord at day 7 (n = 8/group). Bilateral mechanical allodynia was also assessed at day 7. The methods for testing mechanical sensitivity described above were adapted such that, in addition to the 4.0gf filament, mechanical allodynia was also measured for stimulation by the 1.4gf and 10.0gf filaments in order to match the mechanical stimuli that were applied to the forepaw during the extracellular recording sessions. A *t*-test compared the number of paw withdrawals elicited by the 4.0gf filament between each group used for the electrophysiological study and their matched group in the immunohistochemistry study to ensure that the two studies used comparable conditions. For statistical analysis, a one-way ANOVA tested for differences in the number of paw withdrawals between groups that were elicited on day 7 by each filament (1.4, 4.0, 10.0gf) for the ipsilateral and contralateral forepaws separately.

To record extracellular voltage recordings in the spinal cord, rats were anesthetized with 45 mg/kg pentobarbital via i.p. injection, and adequate anesthesia was confirmed by a hind paw pinch. Additional doses of pentobarbital (1–5 mg/kg i.p.) were administered approximately every 40–50 min, or as needed. The cervical spine was re-exposed via a dorsal, midline incision, and any scar tissue that had formed over the right C6/C7 spinal cord from the initial surgery was carefully removed. The remaining bone at C6 and C7 on the left side was removed by a laminectomy to expose the spinal cord fully at those levels, and the overlying dura was removed. The rat was placed on a stereotactic frame using bilateral ear bars and a clamp on the spinous process of T2. Mineral oil was applied to the spinal cord to maintain hydration. A thoracotomy was performed to minimize spinal cord motion associated with normal breathing, and respiration was maintained by mechanical ventilation via a midcervical tracheotomy (40–50 cycles/min; Harvard Small Animal Ventilator model 683; Harvard Apparatus, Holliston, MA; Crosby et al., 2013). Expired CO_2 concentration was continuously monitored (Capnogard; Novametrix Medical Systems, Wallingford, CT), and the core body temperature was maintained at 35–37°C using a heat plate and a rectal probe (TCAT-2DF; Physitemp Instruments, Clifton, NJ).

Extracellular spinal cord recordings were acquired using a glass-insulated tungsten probe (<1 μm tip; FHC, Bowdoin, ME) inserted vertically into the dorsal spinal cord, proximal to the site where the C7 nerve root exits the spinal cord. Recordings were made in the ipsilateral and contralateral dorsal horn. Mechanical stimuli were applied to the ipsilateral forepaw to record neurons in the ipsilateral spinal cord, and contralateral neurons were recorded during stimuli applied to the contralateral forepaw. The signal was amplified with a gain of 3,000

(ExAmp-20KB; Kation Scientific, Minneapolis, MN), processed with a 60-Hz noise eliminator (Hum Bug; Quest Scientific, North Vancouver, British Columbia, Canada), and digitally stored at 25 kHz (MK1401; CED; Cambridge, United Kingdom). While slowly advancing the probe through the deep laminae (400–1,000 μm below the pial surface), we searched for mechanoreceptive neurons innervating the forepaw by lightly brushing the plantar surface of the forepaw (Hains et al., 2003; Quinn et al., 2010; Crosby et al., 2013). Once a neuron had been identified, a sequence of six mechanical stimuli was applied to the forepaw: 1) 10 light brush strokes with a brush applied over 10 sec; 2–5) a series of four von Frey filaments (1.4, 4.0, 10.0, 26.0gf), each applied five times for 1 sec, with a 1-sec rest between application; and 6) a 10-sec, 60gf pinch by a microvascular clip (Roboz, Gaithersburg, MD; Quinn et al., 2010). There was 60 sec of rest between the applications of each of the different stimuli.

Voltage recordings were spike-sorted in Spike2 (CED) to count the number of action potentials evoked by each stimulus for individual neurons. For the brush stimulus, the number of action potentials was summed over the period of the light brushing. For each von Frey filament application, the number of action potentials was summed over both the stimulation period and the 1-sec rest period that immediately followed. For both the brush and the von Frey filament stimuli, the baseline number of spikes occurring in the 10-sec period prior to the first stimulation was subtracted from the spike counts during stimulation in order to identify only the spikes evoked by those stimuli (Hains et al., 2003). For the 60gf pinch, the number of spikes was summed over the 5-sec period between 3–8 sec after the clip was applied in order to consider only those spikes evoked by the pinch and to exclude the spikes evoked by the application and removal of the clip (Quinn et al., 2010). The number of spikes evoked by the clip stimulus was determined by subtracting the baseline number of spikes that occurred in the 5-sec window prior to the first stimulation from the spike count.

Neurons were classified as either wide dynamic range (WDR) or low-threshold mechanoreceptive (LTM) neurons by comparing the spike rate (spikes/sec) evoked by the light brushing and the 60gf clip stimuli (Laird and Bennett, 1993; Hains et al., 2003; Saito et al., 2008). Neurons that responded maximally to the light brush were identified as LTM, and those that responded in a graded manner were identified as WDR (Woolf and Fitzgerald, 1983; Hains et al., 2003).

For statistical analysis, the number of spikes counted in the electrophysiological study was log-transformed because of a positive skew in the distribution of data (Quinn et al., 2010). Separate mixed-effect one-way ANOVAs with Tukey HSD post hoc tests compared differences in the number of action potentials that were evoked by each filament between groups; neurons were nested within rats, and rats were nested in groups. A mixed-effect one-way ANOVA with the same levels of nesting tested for differences between groups for the depth at which the neurons were recorded. The distribution of neurons identified as WDR and LTM was compared between groups using Pearson's χ^2 tests. All electrophysiology data are expressed as mean ± SEM.

RESULTS

Behavioral Sensitivity After Nerve Root Compressions for 3 Min or 15 Min

Both mechanical allodynia and thermal hyperalgesia in the ipsilateral forepaw were significantly different between the 15- and the 3-min compression groups and sham over time (group × time point interaction term; mechanical allodynia, $P < 0.001$; thermal hyperalgesia, $P = 0.013$; Fig. 1). There was no difference in the contralateral paw between groups or relative to baseline. Specifically, at both day 1 and day 7 after root compression, mechanical and thermal sensitivity was established only after the 15-min nerve root compression (Fig. 1A,B). Within 1 day after the applied compression, the number of paw withdrawals in response to filament stimulation increased after a 15-min compression (vs. sham, $P = 0.003$; vs. 3 min, $P = 0.008$) and remained significantly greater than both the 3-min compression and sham exposure groups at day 7 (vs. sham, $P < 0.001$; vs. 3 min, $P = 0.002$; Fig. 1A). Similarly, the withdrawal latency was significantly decreased at day 1 and day 7 after a 15-min compression compared with the sham (day 1, $P = 0.032$; day 7, $P = 0.015$) and 3-min compression groups (day 1, $P = 0.046$; day 7, $P = 0.009$; Fig. 1B). Mechanical allodynia and thermal hyperalgesia after a 3-min compression did not differ from responses after sham procedures at either time point. In the contralateral forepaw, no differences were observed in mechanical allodynia (Fig. 1C) or thermal hyperalgesia (Fig. 1D) between any of the groups at any time.

Temporal Spinal Glutamate Transporter Expression

The spinal expression of GLT-1 in the ipsilateral dorsal horn had decreased significantly only at day 7 following the 15-min compression (Fig. 2). All groups exhibited GLT-1 expression that was comparable to normal levels at day 1, but, by day 7, the expression of GLT-1 in the ipsilateral superficial dorsal horn after the 15-min compression (0.79 ± 0.28) had significantly decreased compared with the expression in normal, naïve tissue ($P = 0.022$; Fig. 2). Furthermore, this decrease in GLT-1 was also significant when compared with sham and a 3-min compression at day 7 (sham, $P = 0.039$; 3 min, $P = 0.010$) and also with the GLT-1 expression after a 15-min compression at day 1 ($P = 0.025$). The GLT-1 expression on the side contralateral to the nerve root injury was not different between any group and normal levels at either time point (Fig. 2).

The expression of GLAST in the ipsilateral dorsal horn had increased in all injury groups at day 1, but this increase remained at day 7 only after a 15-min compression (Fig. 3). GLAST expression had increased significantly by 20–30% over normal levels on day 1 after all of the surgical procedures, including sham (sham, $P = 0.020$; 3 min, $P = 0.011$; 15 min, $P = 0.025$; Fig. 3). At day 7, GLAST expression in the ipsilateral dorsal horn

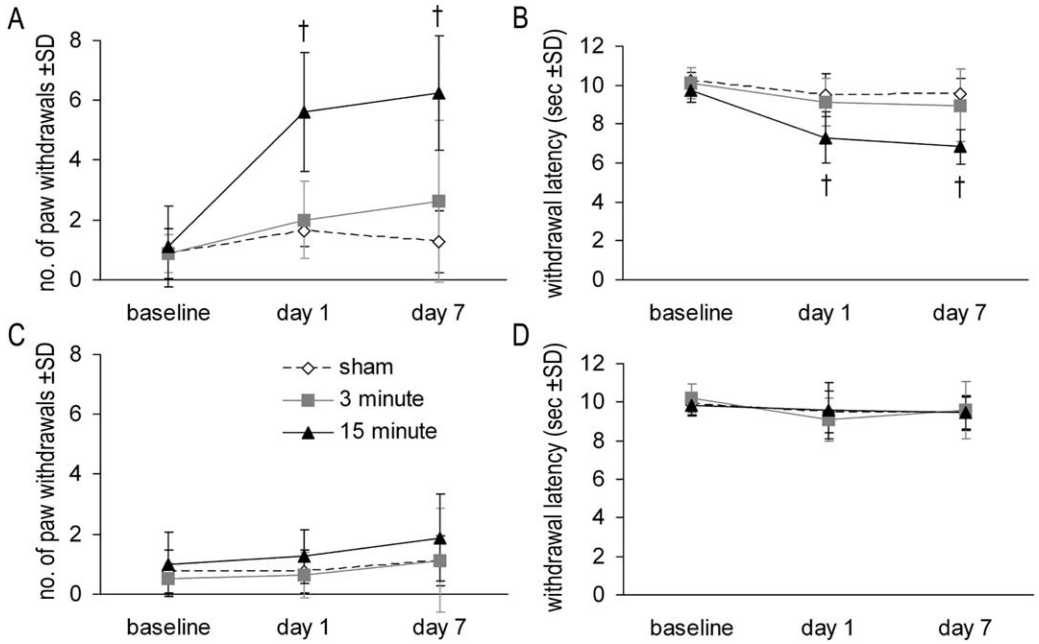

Fig. 1. Mechanical allodynia and thermal hyperalgesia in the forepaws after root compression. **A:** In the ipsilateral forepaw, a 15-min compression elicits significantly more paw withdrawals in response to a 4.0gf von Frey filament than a 3-min compression or sham treatment (†$P < 0.009$). **B:** Similarly, the ipsilateral withdrawal threshold to a thermal stimulus after a 15-min compression significantly decreases compared with a 3-min compression or sham treatment (†$P < 0.047$). There are no changes in contralateral mechanical allodynia (**C**) or thermal hyperalgesia (**D**).

after a 15-min compression remained significantly elevated over normal ($P = 0.036$) and significantly decreased after sham, but not in the 3-min group, between day 1 and day 7 ($P = 0.024$). No differences were observed in the expression of GLAST in the contralateral dorsal horn between any injury group at day 1 or day 7 (Fig. 3). Similarly, no significant differences in the expression of EAAC1 in the ipsilateral and contralateral dorsal horns were observed between any groups at either time point (data not shown).

Spinal GLT-1 Upregulation by Ceftriaxone

The expression of GLT-1 in the ipsilateral dorsal horn at day 7 after a painful nerve root compression increased after the ceftriaxone treatment (Fig. 4). Spinal GLT-1 expression in the ipsilateral dorsal horn decreased following a painful nerve root compression treated with saline (injury + saline) compared with the group treated with ceftriaxone (injury + ceftriaxone; $P = 0.012$) and sham procedures (sham + saline; $P < 0.001$). The expression of GLT-1 was not significantly different between the sham + saline and the injury + ceftriaxone groups. In the contralateral dorsal horn, the expression of GLT-1 was unchanged among all of the groups.

Behavioral Sensitivity After Ceftriaxone Treatment

Ceftriaxone abolished both mechanical allodynia and thermal hyperalgesia that developed in the ipsilateral forepaw after a painful nerve root compression (Figs. 5A,B). At day 1, the number of paw withdrawals elicited by the von Frey filament significantly increased in both groups that had received a nerve root compression compared with sham + saline (vs. injury + saline, $P < 0.001$; vs. injury + ceftriaxone, $P < 0.001$; Fig. 5A). However, by day 3 (2 days after the start of treatment), the number of paw withdrawals in the treatment group (injury + ceftriaxone) returned to sham levels and remained significantly lower than the number of paw withdrawals in the vehicle-treated group (injury + saline) for the remainder of the study ($P < 0.012$; Fig. 5A). Sham + vehicle did not vary from baseline at any postinjury time point. The number of withdrawals in the contralateral forepaw did not differ from baseline for any group, and no differences were detected between groups at any time point (Fig. 5C).

As with mechanical allodynia, thermal hyperalgesia in the ipsilateral forepaw after the nerve root compression treated with ceftriaxone had decreased by day 7 (Fig. 5B). At day 1, the withdrawal latency was significantly shorter after a nerve root compression (injury + saline, $P = 0.041$; injury + ceftriaxone, $P = 0.048$) compared with sham (sham + saline; Fig. 5B), but, by day 7, the withdrawal latency in the group receiving ceftriaxone treatment (injury + ceftriaxone, 10.1 ± 3.2 sec) was significantly greater than the latency for the vehicle-treated group (injury + saline, 5.8 ± 0.7 sec; $P = 0.005$) and was not different from that of the sham + saline (9.4 ± 1.1 sec; Fig. 5B). The withdrawal latency in the sham + saline group did not vary from baseline at either of the postoperative time points. Thermal hyperalgesia in the contralateral paw was unchanged from baseline for all groups (Fig. 5D)

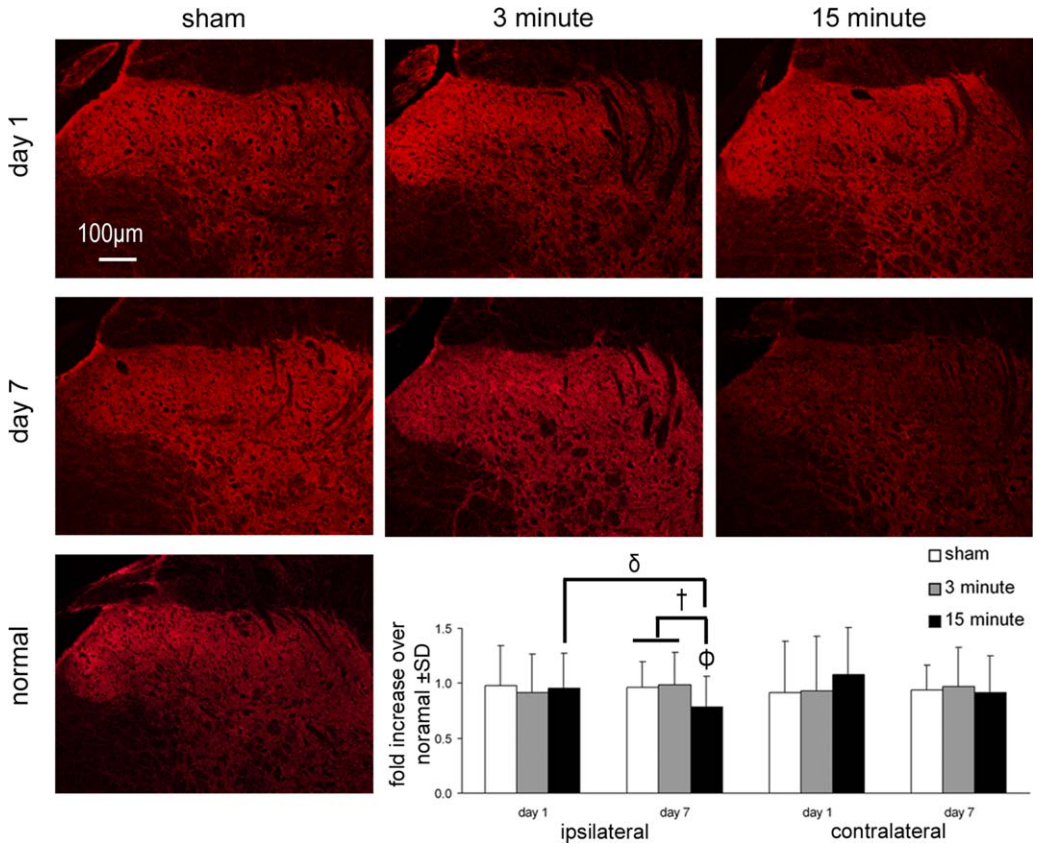

Fig. 2. GLT-1 expression in the superficial laminae of the dorsal horn. GLT-1 expression decreases only at day 7 after a 15-min compression compared with expression levels in normal tissue ($\Phi P = 0.022$) and in the sham and 3-min-compression groups at day 7 ($\dagger P < 0.040$). GLT-1 expression is significantly decreased from day 1 to day 7 in the 15-min group ($\delta P = 0.025$). The expression of GLT-1 in the contralateral dorsal horn does not change over time or between groups. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Effect of Ceftriaxone on Spinal GLAST and GFAP Expression

Ceftriaxone treatment modulated the expression of spinal GFAP and GLAST (Fig. 6). In particular, GFAP expression in the ipsilateral dorsal horn was significantly elevated after a nerve root compression with vehicle treatment (injury + saline) over both injury with ceftriaxone treatment (injury + ceftriaxone; $P = 0.035$) and sham (sham + saline; $P = 0.010$; Fig. 6). Likewise, this same relationship was evident in the contralateral dorsal horn ($P < 0.017$). Notably, the ceftriaxone treatment returned GFAP expression to sham levels on both sides of the spinal cord (Fig. 6). The expression of GLAST in the ipsilateral spinal cord is normally elevated at day 7 after a nerve root injury (Fig. 3), but was significantly reduced in the ceftriaxone treatment group (injury + ceftriaxone) compared with vehicle (injury + saline; $P = 0.023$) and was not different from sham levels (Fig. 6).

Mechanically Evoked Action Potentials in the Spinal Cord After Ceftriaxone Treatment

As with the behavioral sensitivity and spinal GFAP and GLAST responses (Figs. 5, 6), the ceftriaxone treatment reduced the neuronal hyperexcitability that develops in the ipsilateral spinal dorsal horn after a nerve root compression (Fig. 7). As was observed in the rats used to characterize the spinal glutamate transporter expression (Fig. 1A), the number of paw withdrawals elicited by the 4.0gf filament was significantly elevated in the vehicle-treated group (injury + saline) over both the ceftriaxone treatment group (injury + ceftriaxone; $P < 0.001$) and the sham exposure (sham + saline; $P = 0.001$; data not shown). Likewise, for testing with the 10.0gf filament, a significant increase in the number of ipsilateral paw withdrawals was observed in the injury + saline group compared with both the injury + ceftriaxone ($P = 0.006$) and the sham + saline ($P < 0.001$; data not shown) groups. There were no differences in the number of paw withdrawals elicited by the 1.4gf filament (data not shown). No differences were observed in the contralateral forepaw between any groups for testing with any filament (data not shown). In total, 199 neurons were recorded for all three groups, at an average depth of 648 ± 138 μm. The average depths of neurons recorded for each group were not significantly different from each other.

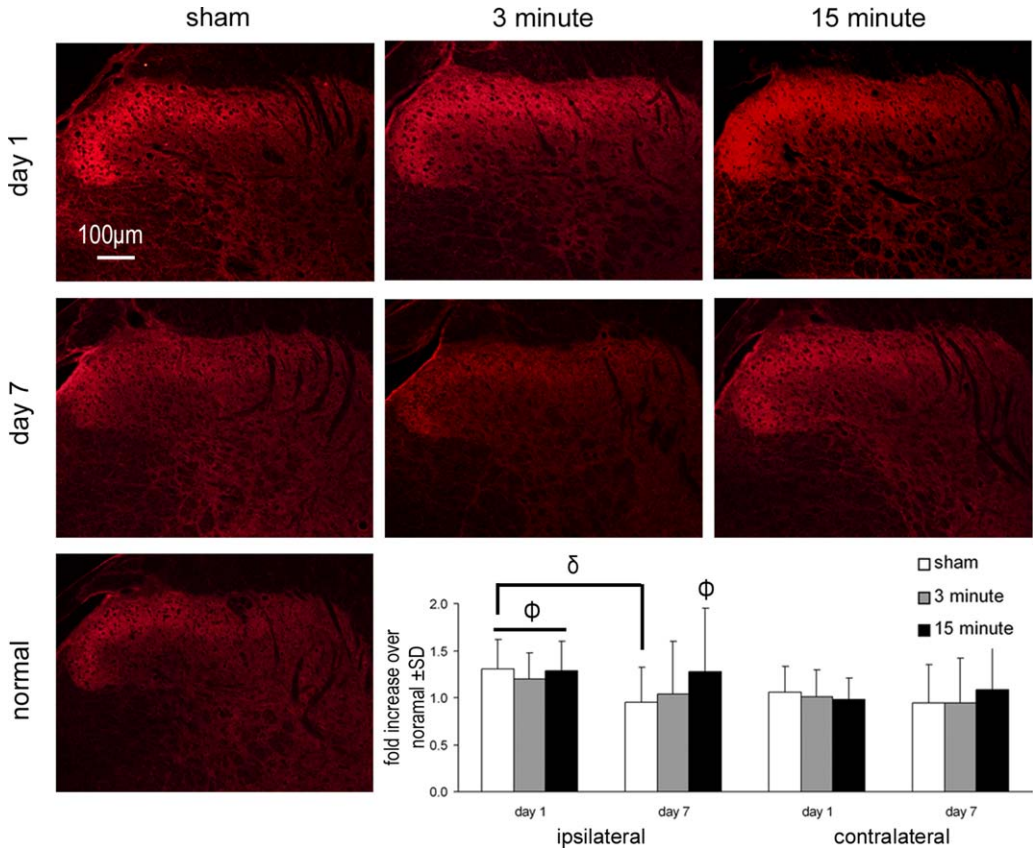

Fig. 3. Spinal GLAST expression after nerve root compressions. At day 1, GLAST significantly increases over normal levels in the ipsilateral dorsal horn following sham, 3 min compression, and 15 min compression ($\Phi P < 0.026$). GLAST remains significantly elevated over normal at day 7 only after a 15-min compression ($\Phi P = 0.036$). GLAST expression significantly decreases from day 1 to day 7 after sham treatment ($\delta P = 0.024$). No differences in the contralateral GLAST expression are observed between any groups or over time. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Similarly to the behavioral response, the number of action potentials evoked by the 4.0, 10.0, and 26.0gf filament increased significantly in the injury + saline group compared with sham procedures (sham + saline; $P < 0.002$) and also the ceftriaxone treatment (injury + ceftriaxone; $P < 0.013$; Fig. 7A). On average, the number of spikes evoked in the injury + saline group was two to three times greater than the number of spikes evoked in any other group. For example, for stimulation by the 4.0gf filament, the number of evoked spikes in the injury + saline group was 32.5 ± 5.7, which was significantly greater than the number of spikes evoked by that filament for the sham + saline (8.0 ± 1.1; $P = 0.001$) and the injury + ceftriaxone (11.4 ± 1.6; $P = 0.005$) groups. Although the number of spikes elicited by the 1.4gf was elevated in the injury + saline group, this increase was not significant. Similarly, the small increase in the number of spikes in the contralateral dorsal horn that was evoked in the injuruy + saline group by stimulation using the 10.0 and 26.0gf filaments was not significant compared with sham + saline or injury + ceftriaxone groups (Fig. 7B). In addition, the proportion of WDR neurons identified in the ipsilateral spinal cord that were recorded in the injury + saline group (91%) was significantly greater than the proportion of WDR neurons recorded in ipsilateral dorsal horn of either the sham + saline (60%; $P = 0.004$) or the injury + ceftriaxone (64%; $P = 0.008$) group (Fig. 7C). The proportions of WDR neurons in the contralateral dorsal horn (63–72%) did not differ between any of the groups (Fig. 7C).

DISCUSSION

The glutamate transporter GLT-1 decreased in the dorsal horn after a painful nerve root compression (Figs. 1, 2) and ceftriaxone treatment restored its expression while abolishing behavioral sensitivity and the spinal astrocytic activation, GLAST upregulation, and neuronal hyperexcitability that normally develop (Figs. 3–7). Although this is the first study demonstrating that daily intrathecal lumbar injection of ceftriaxone in the rat upregulates GLT-1 in the cervical region, it is not the first to show that pharmacological agents delivered by lumbar puncture can mediate cellular outcomes in the cervical

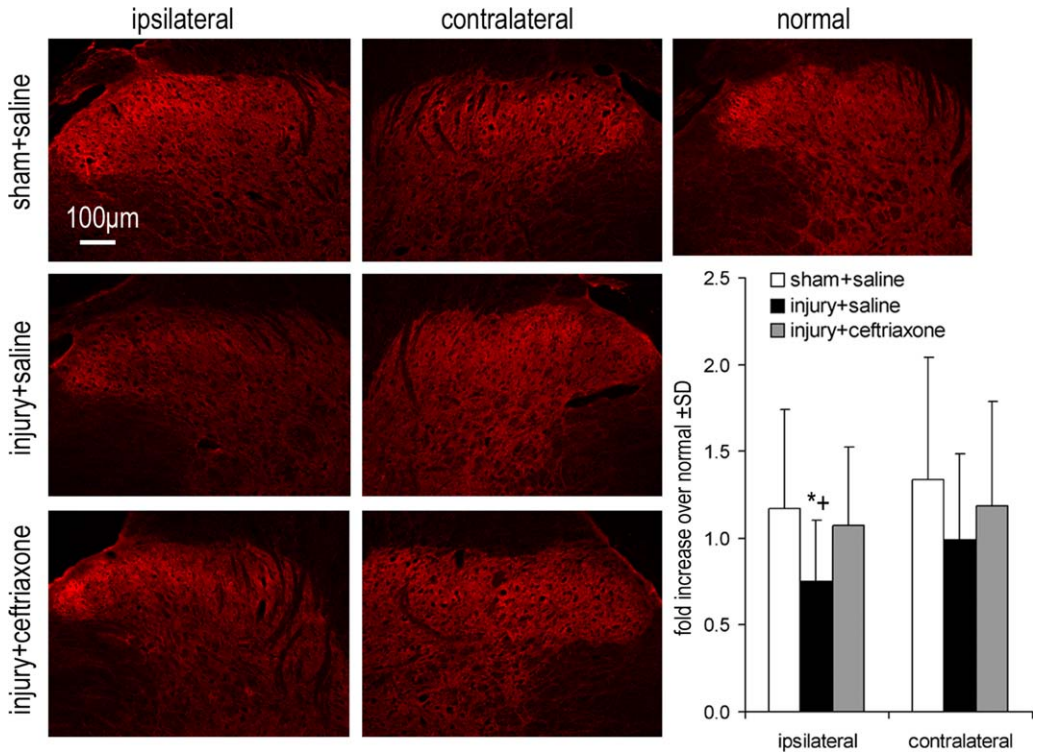

Fig. 4. Spinal GLT-1 in the superficial dorsal horn after a nerve root compression treated with ceftriaxone (injury + ceftriaxone) or the vehicle (injury + saline) or sham procedures (sham + saline). At day 7, GLT-1 in the ipsilateral dorsal horn is unchanged compared with sham + saline after a nerve root compression treated with ceftriaxone (injury + ceftriaxone) but is significantly downregulated in injury + saline compared with sham + saline ($*P < 0.001$) and injury + ceftriaxone ($+P = 0.012$). Expression of GLT-1 in the contralateral dorsal horn is not different between groups. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

spinal cord. In this same painful injury model, cervical spinal inflammation was attenuated by cytokine antagonists delivered via lumbar puncture (Rothman and Winkelstein, 2010). Daily intrathecal ceftriaxone injections have been shown to increase GLT-1 expression in the lumbar cord without affecting hindpaw sensitivity in normal rats (Hu et al., 2010). Therefore, behavioral sensitivity in the hind paw likely was not affected by the ceftriaxone treatment or the delivery method in the current study.

Thermal hyperalgesia, downregulation of spinal GLT-1, and a sustained increase in spinal GLAST expression were observed only after the same 15-min root compression that also elicits mechanical allodynia (Figs. 1–3; Hubbard and Winkelstein, 2005; Rothman et al., 2010). Although sensitized myelinated Aδ-fibers mediate mechanical allodynia, thermal hyperalgesia is mediated by unmyelinated C-fibers (Boulais and Misery, 2008; Jensen et al., 2011). The presence of mechanical allodynia and thermal hyperalgesia after a 15-min compression (Fig. 1) indicates that mechanical trauma to the root likely impairs normal signaling along both its myelinated and its unmyelinated fiber populations (Boulais and Misery, 2008; Jensen et al., 2011). Indeed, extensive axonal damage, including swelling and loss of axonal transport, develops in both of these axon populations after a painful root compression (Hubbard and Winkelstein, 2008; Chang and Winkelstein, 2011; Nicholson et al., 2011). Such damage to the primary afferents likely contributes directly to the downregulation of GLT-1 observed in our study (Fig. 2). Astrocytes require presynaptic neuronal signaling in order to express GLT-1 (Yang et al., 2009; Ghosh et al., 2011). The afferent damage that extends to the primary synapse in the dorsal horn after a root compression (Kobayashi et al., 2008) provides a mechanism to impair the normal afferent signaling to spinal astrocytes and to lead to the downregulation of spinal GLT-1 (Fig. 2). At day 1 after injury, when GLT-1 is unchanged (Fig. 2), the primary afferents also retain their normal morphology (Hubbard and Winkelstein, 2008); the similar temporal profiles of GLT-1 downregulation and afferent degeneration further support the idea that the primary afferents contribute to reducing spinal GLT-1 after painful nerve root injury.

Unlike GLT-1, GLAST expression does not require neuronal signaling but is rapidly upregulated when extracellular glutamate is elevated (Gegelashvili et al., 2000; Yang et al., 2009). The downregulation of GLT-1 after a painful trauma (Fig. 2) likely increases extracellular glutamate in the dorsal horn and thereby increases GLAST. Interestingly, GLAST had increased at day 1 in all groups (Fig. 3). This early upregulation of spinal GLAST may indicate that the surgical procedures alone are sufficient to increase spinal glutamate but that normal glutamate

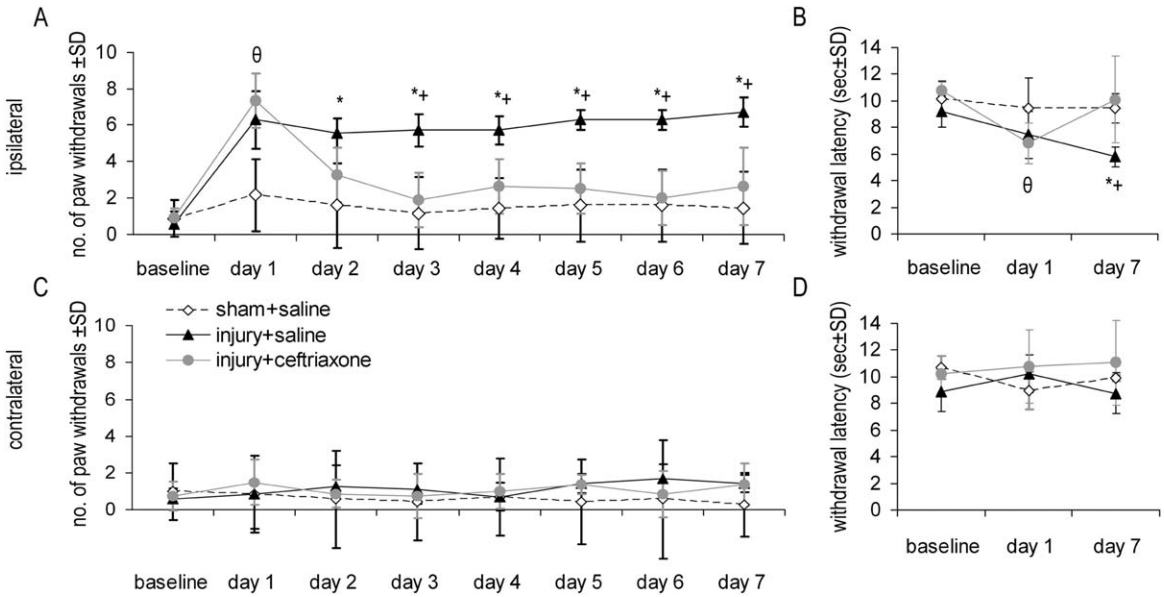

Fig. 5. Mechanical allodynia and thermal hyperalgesia for daily ceftriaxone treatments starting on day 1. Prior to treatment on day 1, there is a significant increase in the number of paw withdrawals (**A**) and a significant decrease in the paw withdrawal threshold (**B**) in the ipsilateral forepaw of both of the groups undergoing root compression (injury + ceftriaxone; injury + saline) compared with sham + saline ($\theta P < 0.049$). The number of paw withdrawals in the injury + saline group is significantly elevated over sham for all postoperative time points ($\star P < 0.001$). However, starting on day 3, the number of withdrawals after a root compression treated with ceftriaxone (injury + ceftriaxone) significantly decrease compared with injury + saline ($+P < 0.012$). Similarly, at day 7, the thermal withdrawal latency is significantly greater after injury + ceftriaxone ($+P = 0.005$) and sham + saline ($\star P = 0.018$) compared with injury + saline. Contralateral mechanical allodynia (**C**) and thermal hyperalgesia (**D**) are unchanged.

clearance after the nonpainful procedures (sham, 3 min compression) prevented the development of further imbalances in the glutamatergic system. Even though GLAST remained elevated after the painful compression, it likely was unable to compensate for the loss of GLT-1, which dominates glutamate uptake in the central nervous system (Danbolt, 2001; Holmseth et al., 2012). EAAC1 was unchanged in the present study. Although shifts in EAAC1 could occur within hours after the injury (Vera-Porocarrero et al., 2002), modified spinal EAAC1 expression does not appear to play a significant role in maintaining nerve-root-mediated pain.

This is the first study to demonstrate that daily ceftriaxone injection abolishes radicular pain and associated spinal astrocyte and neuronal activation (Figs. 4–7). Spinal astrocyte activation is initiated *only* when behavioral sensitivity also develops, indicating that reactive astrocytes have substantial contributions to the persistence of behavioral sensitivity (Rothman et al., 2010; Nicholson et al., 2012). Ceftriaxone decreases GFAP promoter activity and suppresses GFAP upregulation in vitro (Bachetti et al., 2010; Yamada and Jinno, 2011), suggesting that ceftriaxone might have reduced behavioral sensitivity here by both downregulating GFAP and upregulating GLT-1 (Figs. 4–6). However, ceftriaxone activates the GLT-1 promoter by promoting nuclear factor-κB (NF-κB) signaling and decreases GFAP promoter activity by reducing NF-κB activity (Bachetti et al., 2010; Ghosh et al., 2011). These divergent actions of ceftriaxone on NF-κB preclude any direct conclusions regarding the relationship between ceftriaxone and these two astrocytic responses but do suggest that the reduced astrocyte reactivity observed here (Fig. 6) is likely a consequence of GLT-1 upregulation (Fig. 4) rather than a direct effect of ceftriaxone. Certainly, in addition to its wide use as a GLT-1 promoter, ceftriaxone also reduces GFAP upregulation after neural tissue damage (Bachetti et al., 2010; Ramos et al., 2010; Yamada and Jinno, 2011). The current study did not explicitly determine in which cells downregulation of GFAP and upregulation of GLT-1 after ceftriaxone treatment occur or whether they are in the same cell types. Microglial upregulation of GLT-1 could contribute to increased expression of this transporter after ceftriaxone treatment. Interestingly, decreased spinal expression of GLT-1 after a painful sciatic nerve ligation has been associated with decreased astrocytic GLT-1 but increased microglial GLT-1 (Xin et al., 2009). These opposing GLT-1 expression responses suggest that GLT-1 is differentially regulated between microglia and astrocytes in response to neural trauma. In fact, the glutamate transporter EAAT1 (homologous to GLAST in the rat) is differentially expressed by activated astrocytes and microglia within the first week after focal cerebral ischemia in humans (Beschorner et al., 2007), further supporting separate roles for astrocytes and microglia in taking up glutamate following neural injury. It is possible that

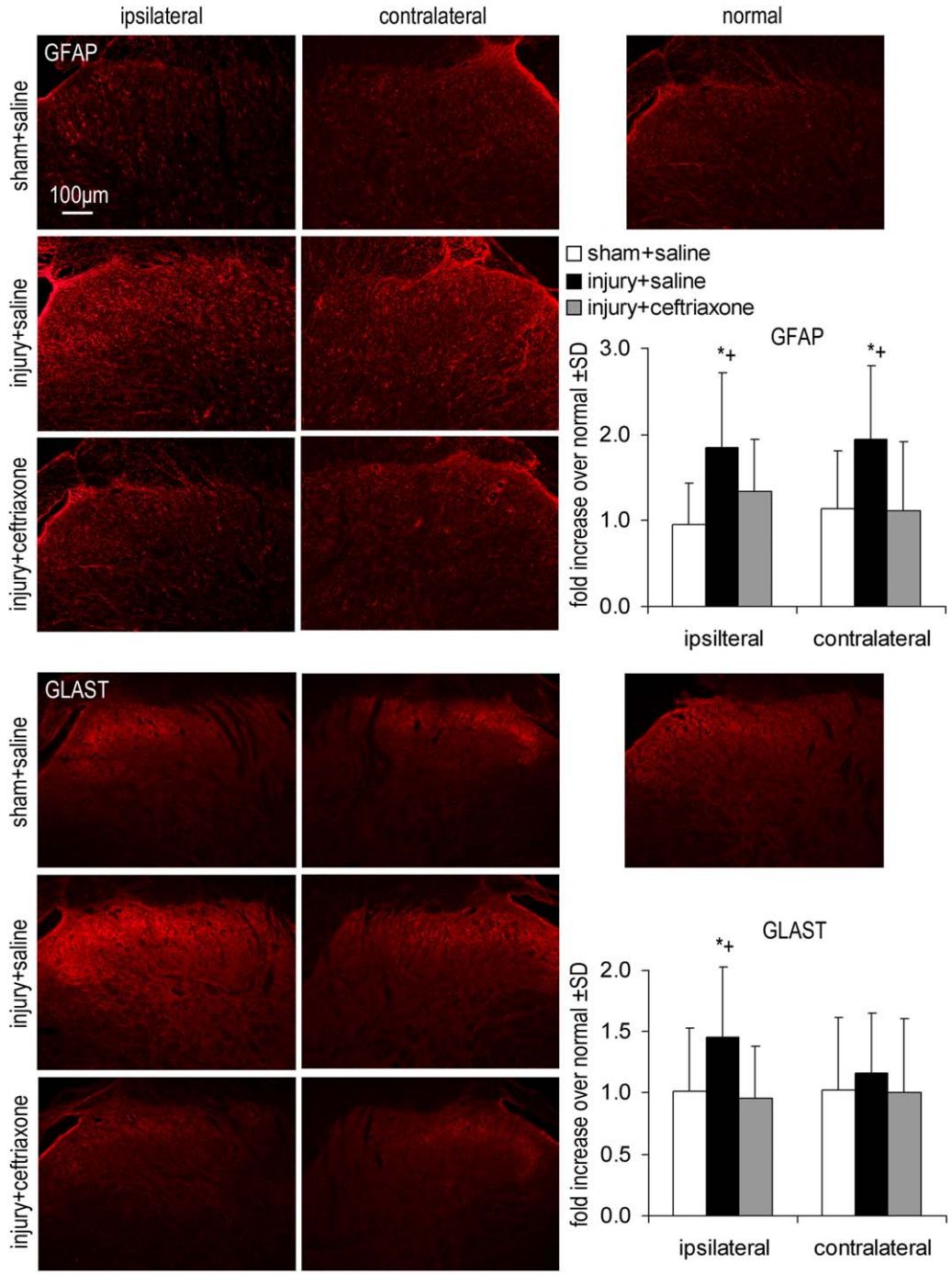

Fig. 6. Spinal GFAP and GLAST at day 7 after root compression treated with ceftriaxone (injury + ceftriaxone) and saline vehicle (injury + saline) as well as sham treatment (sham + saline). Bilateral spinal GFAP in injury + saline is significantly elevated over both sham + saline ($\star P < 0.01$) and injury + ceftriaxone ($+P = 0.035$). GLAST increases in the ipsilateral dorsal horn only after injury + saline compared with injury + ceftriaxone ($+P = 0.023$) and sham + saline ($\star P = 0.039$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

ceftriaxone enhances microglial GLT-1 and contributes to the overall increase in spinal expression of this transporter that was observed here, while also downregulating the expression of GFAP in astrocytes (Figs. 4, 6).

Like astrocytes, microglia are also activated in the spinal dorsal horn following a painful root compression in the rat (Colburn et al., 1999; Tao et al., 2005; Rothman and Winkelstein, 2007). Although ceftriaxone has been

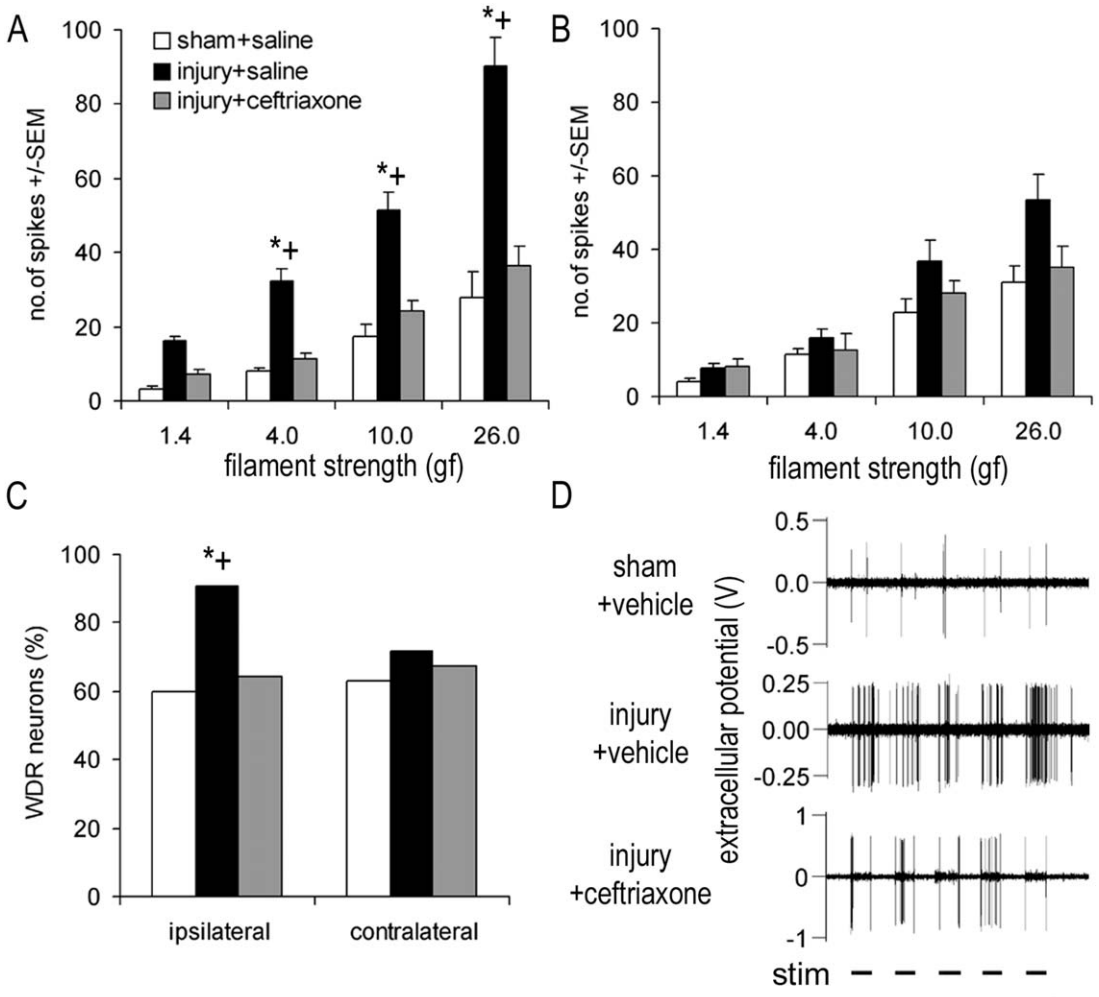

Fig. 7. Ceftriaxone reduces neuronal hyperexcitability and the proportion of WDR neurons in the spinal cord at day 7 after injury (injury + ceftriaxone) compared with vehicle (injury + saline) and sham (sham + saline). **A:** The number of spikes evoked in the ipsilateral spinal cord by the 4.0gf, 10.0gf, and 26.0gf filaments to the paw is significantly elevated in injury + saline compared with sham + saline ($\star P < 0.005$) and injury + ceftriaxone ($+P < 0.013$). **B:** Contralateral neuronal excitability is not different between any groups. **C:** The percentage of WDR neurons after injury + saline increases only in the ipsilateral spinal cord compared with sham + saline ($\star P = 0.004$) and injury + ceftriaxone ($+P = 0.008$). **D:** Representative extracellular potentials recorded in the ipsilateral spinal cord for each group during application of the 4.0gf filament stimulus (stim).

shown to reduce microglial activation in the hypoglossal nucleus after a nerve transection (Yamada and Jinno, 2011), no study has identified a mechanism by which ceftriaxone modulates microglial GLT-1 expression. Therefore, it cannot be determined whether ceftriaxone alleviates radicular pain exclusively by acting on astrocytes or also by modulating microglial activation or GLT-1 expression. Furthermore, because both astrocytes and microglia also express GLAST (Danbolt, 2001; Tao et al., 2005; Beschorner et al., 2007), and there is evidence to suggest that distinct subpopulations of astrocytes expression GLT-1 and GLAST (Perego et al., 2000), identifying the cellular source of GLAST would also further define the roles of these immune cells in taking up extracellular glutamate in the spinal cord. Additional studies evaluating the relationship among GLT-1, GLAST, astrocytes, microglia, and ceftriaxone are needed to clarify the mechanism(s) by which ceftriaxone alleviates radicular pain.

GLAST expression and neuronal hyperexcitability are both mediated, in part, by activation of glutamate receptors (Aronica et al, 2003; Jourdain et al., 2007; Tilleux and Hermans, 2007; Ren and Dubner, 2008). Therefore, the reduction in spinal GLAST and neuronal hyperexcitability after painful root compression treated with ceftriaxone (Figs. 6, 7) both indicate reduced spinal glutamate signaling (Aronica et al, 2003; Tilleux and Hermans, 2007). Ceftriaxone has no effect on GLAST in either normal rats or brain-injured neonate pups (Rothstein et al., 2005; Mimura et al., 2011), indicating that the downregulation of GLAST observed here (Fig. 6) likely

was not directly attributable to ceftriaxone. Excitatory signaling in the rat brain after cocaine administration is also reduced with ceftriaxone (Knackstedt et al., 2010; Trantham-Davidson et al., 2012). Although that study, along with our results (Fig. 7), indicates that ceftriaxone can alter neuronal excitability, it is not clear whether neuronal signaling is directly modulated by ceftriaxone or by GLT-1 regulation of extracellular glutamate in the spinal cord. Inhibition of spinal GLAST in the rat reduces excitatory neuronal signaling, which opposes the notion that glutamate transporter inhibition would increase excitatory signaling (Niederberger et al., 2006). Although it is not clear how GLAST regulates excitatory circuits, its decreased expression after ceftriaxone treatment may also contribute to reducing spinal neuronal hyperexcitability (Figs. 6, 7).

Ceftriaxone decreased the proportion of WDR neurons in the spinal cord after injury compared with the saline treatment (Fig. 7). The increase in WDR neurons after a painful root compression implies a functional reorganization of afferent fibers in the dorsal horn (Okamoto et al., 2001; Kohno et al., 2002; Baba et al., 2003; Keller et al., 2007). Specifically, the shift from LTM to WDR neurons suggests that high-threshold Aδ- and C-fibers form synapses with neurons that normally synapse only with low-threshold Aβ fibers (Okamoto et al., 2001; Keller et al., 2007; Basbaum et al., 2009). Alternatively, the phenotypic shift could indicate enlarged WDR neuron receptive fields after injury (Hanai et al., 1996; Suzuki et al., 2000; Kondo et al., 2002), increasing the likelihood of finding this type of neuron using the search protocol in this study. Increased receptive field size is also attributed to central reorganization of afferents in the spinal cord after nerve injury (Suzuki et al., 2000; Kondo et al, 2002). Whether or not the phenotypic shift observed here reflects an increase in the number, or receptive field size, of WDR neurons, it does indicate spinal plasticity after a painful nerve root injury (Suzuki et al., 2000; Kohno et al., 2003; Keller et al., 2007). Enhanced excitatory signaling is also thought to be responsible for such reorganization (Kohno et al., 2003; Keller et al., 2007). Intrathecal injection of ceftriaxone, which has been shown to reduce glutamate signaling in the spinal cord (Inquimbert et al., 2012), restored the distribution of WDR neurons after injury (Fig. 7). These two effects of ceftriaxone indicate that excitatory, rather than inhibitory, circuits contribute, at least in part, to spinal reorganization after a painful root injury (Okamoto et al., 2001; Baba et al., 2003; Kohno et al., 2003).

Unlike the case for ceftriaxone's effects on behavioral sensitivity and GLT-1 expression (Rothstein et al., 2005; Hu et al., 2010), few studies report effects associated with ceftriaxone such as spinal inflammation, neuronal signaling, or the expression of other glutamate transporters in pain (Ramos et al., 2010; Verma et al., 2010; Mimura et al., 2011; Trantham-Davidson et al, 2012). Results of this study help to define the effects of ceftriaxone by evaluating astrocyte activation, GLAST expression, and neuronal hyperexcitability in a model of painful nerve root compression (Figs. 6, 7). Although we did not measure glutamate in the spinal cord, ceftriaxone specifically increases the expression of the membrane-bound, dimer form of GLT-1, which is the only functionally active form of GLT-1, and also increases GLT-1 activity even when the transporter's expression is unchanged in the rat (Haugeto et al., 1996; Sung et al., 2003; Lipski et al., 2007; Ramos et al., 2010). Therefore, upregulation of GLT-1 with ceftriaxone treatment (Fig. 4) suggests that glutamate uptake also increased. Ceftriaxone has also been shown to promote antioxidant protection by inducing the cystine/glutamine antiporter system (Lewerenz et al., 2009), so it cannot be concluded whether the behavioral, glial, or neuronal responses after ceftriaxone treatment following a painful root compression are attributable solely to increased GLT-1 expression. Further studies are needed to determine the mechanism(s) by which ceftriaxone alleviates the spinal inflammation, neuronal hyperexcitability, and increased GLAST expression associated with nerve-root-mediated pain.

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