

Simulated Whiplash Modulates Expression of the Glutamatergic System in the Spinal Cord Suggesting Spinal Plasticity Is Associated with Painful Dynamic Cervical Facet Loading

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Abstract

The cervical facet joint and its capsule have been reported to be injured during whiplash scenarios and are a common source of chronic neck pain from whiplash. Both the metabotropic glutamate receptor 5 (mGluR5) and the excitatory amino acid carrier 1 (EAAC1) have pivotal roles in chronic pain. In this study, spinal mGluR5 and EAAC1 were quantified following painful facet joint distraction in a rat model of facet-mediated painful loading and were evaluated for their correlation with the severity of capsule loading. Rats underwent either a dynamic C6/C7 joint distraction simulating loading experienced during whiplash (distraction; $n = 12$) or no distraction (sham; $n = 6$) to serve as control. The severity of capsular loading was quantified using strain metrics, and mechanical allodynia was assessed after surgery. Spinal cord tissue was harvested at day 7 and the expression of mGluR5 and EAAC1 were quantified using Western blot analysis. Mechanical allodynia following distraction was significantly ($p < 0.001$) higher than sham. Spinal expression of mGluR5 was also significantly ($p < 0.05$) greater following distraction relative to sham. However, spinal EAAC1 was significantly ($p = 0.0003$) reduced compared to sham. Further, spinal mGluR5 expression was significantly positively correlated to capsule strain ($p < 0.02$) and mechanical allodynia ($p < 0.02$). Spinal EAAC1 expression was significantly negatively related to one of the strain metrics ($p < 0.003$) and mechanical allodynia at day 7 ($p = 0.03$). These results suggest that the spinal glutamatergic system may potentiate the persistent behavioral hypersensitivity that is produced following dynamic whiplash-like joint loading; chronic whiplash pain may be alleviated by blocking mGluR5 expression and/or enhancing glutamate transport through the neuronal transporter EAAC1.

Key words: excitatory amino acid carrier 1; facet; glutamate; metabotropic glutamate receptor 5; pain

Introduction

WHIPLASH-RELATED INJURY is a leading cause of injury responsible for emergency room visits in the United States (Quinlan et al., 2004). Most whiplash patients develop chronic neck pain, with annual costs reaching as much as \$61 billion, including litigation and lost work time (Freeman et al., 1999; Kasch et al., 2001; Riddle and Schappert, 2007). Clinical and biomechanical studies have identified the cervical facet joint as the most common source of injury and neck pain (Aprill and Bogduk, 1992; Barnsley et al., 1995; Bogduk and Marsland, 1988; Luan et al., 2000; Ono et al., 1997; Panjabi, 1998; Panjabi et al., 1998b; Yoganandan et al., 1998). Up to 62% of neck pain patients report that local anesthetic blocks to the facet can alleviate or even abolish neck pain (Aprill and

Bogduk, 1992; Barnsley et al., 1994, 1995), which indicates that the nerve fibers that innervate the facet joint and its capsule play a role in nociception and may contribute to chronic neck pain following whiplash-related facet joint injuries in the neck. Although the exact mechanisms of neck pain remain largely unexplored, studies using human cadaveric models have hypothesized that excessive stretching of the facet joint and its capsular ligament is one of the major causes of pain and/or injury during whiplash (Deng et al., 2000; Panjabi et al., 1998c,d; Pearson et al., 2004; Sundararajan et al., 2004). However, while clinical and cadaveric work provide preliminary evidence suggesting that the cervical facet capsular ligament may be at mechanical risk for generating neck pain, the underlying physiologic factors that relate local joint injury and biomechanics with the cellular mechanisms driving

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persistent pain are not well understood, especially for pain arising from whiplash injury.

Glutamate is a principal neurotransmitter and is released by primary afferent terminals that synapse in the spinal dorsal horn (Broman et al., 1993; Valtchanoff et al., 1994). In addition, in response to tissue-damaging stimuli, glutamate has been shown to be substantially increased in the superficial dorsal horn of the spinal cord (al-Ghoul et al., 1993; Besson and Chaouch, 1987; Dingleline and McBain, 1999; He et al., 2000; Miyoshi et al., 2006). Further, inflammation can also enhance glutamate in the periphery and at the site of injury in both humans and rats (Nordlind et al., 1993; Omote et al., 1998) and direct injection of glutamate in the skin induces mechanical and thermal hyperalgesia in the rat (Carlton et al., 1995), suggesting a role for glutamate in pain. Despite its important involvement in nociceptive processing, little is known about the role of the glutamatergic system, such as the metabotropic glutamate receptors (mGluRs) and the neuronal glutamate transporter, in joint or whiplash-related pain.

Although there are two classes of glutamate receptors, the mGluRs have been shown to play a pivotal role in nociceptive processing (Bordi and Ugolini, 2000; Jesse et al., 2008; Miyoshi et al., 2006). They are G protein-coupled receptors that can initiate downstream intracellular signaling cascade by regulating potassium and sodium channels and activating several protein kinases, leading to long-term molecular effects of nociceptive modulation (Byrnes et al., 2009; Mills et al., 2002; Satow et al., 2008). The mGluRs are classified into groups I, II, and III based on their sequence homology, agonist pharmacology, and signaling pathways (Miyoshi et al., 2006; Satow et al., 2008). In particular, group I receptors (mGluR1 and mGluR5) have been shown to enhance behavioral responses to noxious stimulation; both intrathecal and intraplantar administration of these receptor agonists provoke thermal and mechanical hypersensitivity in both mice and rats (Bhave et al., 2001; Dogrul et al., 2000; Hama, 2003; Karim et al., 2001). In the spinal cord, mGluR5 has also been reported to increase excitability of primary afferents and to modulate nociceptive neurotransmission in a rat model of inflammatory pain (Pitcher et al., 2007). While these studies strongly implicate group I glutamate receptors in nociceptive transmission, it remains to be seen whether mGluR5 plays a role in pain induced by joint injury.

Glutamate transporters that are bound to the membranes of both neurons and glial cells are the major regulators responsible for the clearance of glutamate from the synaptic cleft, maintaining cellular homeostasis and preventing neuronal apoptosis (Dingleline and McBain, 1999; Rimaniol et al., 2001; Vera-Portocarrero et al., 2002; Ye and Sontheimer, 1996). At least five subtypes of membrane glutamate transporters have been identified (Chaudhry et al., 1995; Gegelashvili et al., 1997; Kanai et al., 1993). The excitatory amino acid transporters (EAATs) limit the extracellular concentration of glutamate and prevent overstimulation of glutamate receptors. EAAT1 and EAAT2 are localized in astrocytes (Rothstein et al., 1994). EAAT3, also known as excitatory amino acid carrier 1 (EAAC1), is abundantly expressed in neurons (Ginsberg et al., 1995; He et al., 2000). EAAC1 has also been localized on primary astrocytes in cultures from rat brain cortex (Miralles et al., 2001) and is shown to be neuroprotective in cell cultures of hippocampal and striatal neurons (Brustovetsky et al., 2004). EAAC1 has been reported to be down-regulated after

painful peripheral nerve injury in the rat (Sung et al., 2003; Wang et al., 2006). Additionally, Mao et al. (2002) demonstrated the involvement of spinal EAAC1 in the abnormal pain sensitivity in a rat model of chronic morphine administration. Together, these studies suggest a potentially profound effect of EAAC1 in the sensation of pain. Although a growing body of evidence has identified the involvement of both mGluR5 and EAAC1 in the cellular mechanisms of pain, no work has elucidated their roles in facet-mediated neck pain or investigated if they relate to injury severity.

In an effort to understand the mechanisms of painful facet joint injury, our laboratory previously developed an *in vivo* rat model of quasistatic facet joint distraction that induced tensile loading in the facet capsular ligament comparable to its loading in painful neck injury (Lee et al., 2004a,b). In that model, the magnitude of distraction across the C6/C7 facet joint produced different mean strains in the facet capsular ligament and different patterns of behavioral hypersensitivity that depended on the magnitude of joint distraction (Lee and Winkelstein, 2009; Lee et al., 2004a). Distractions that induced strains of $27.7 \pm 11.9\%$ were sufficient to elicit persistent behavioral hypersensitivity indicative of clinical pain symptoms (Lee et al., 2004a). Moreover, in separate studies using that injury model, mRNA for proinflammatory cytokines (tumor necrosis factor α [TNF α], interleukin [IL]-6) and substance P mRNA and protein level were modulated in the dorsal root ganglion (DRG) and spinal cord at day 7 in direct relationship to mechanical loading of the joint and behavioral outcomes (Lee and Winkelstein, 2009; Lee et al., 2008). Those findings suggest an association between joint loading and inflammation in facet-mediated painful joint injury; however, it remains to be seen whether there is any plasticity in the spinal glutamatergic system, such as persistent modifications of the glutamate receptor and transporter, in mechanical facet injury and if those modifications relate to loading severity.

The goal of this study was to examine aspects of the spinal glutamate response following facet joint loading-induced behavioral sensitivity in a rat model simulating painful whiplash injury. Behavioral hypersensitivity is hypothesized to be produced following injurious loading across the cervical facet joint in association with modification in spinal glutamate activity, particularly mGluR5 and EAAC1. To test that hypothesis, mechanical allodynia was assessed as a measure of pain symptoms in the forepaws following a painful facet joint distraction that simulates the capsular loading scenario induced by whiplash (Dong et al., 2008) and spinal expression of mGluR5 and EAAC1 were quantified at day 7 using Western blot. It was further hypothesized that the magnitude of joint loading correlates with both the behavioral outcomes and the spinal mGluR5 and EAAC1 responses. To that end, linear regression models were developed to test whether there is a quantitative relationship between two common metrics of strain induced in the facet capsular ligament and the behavioral, mGluR5, and EAAC1 responses that are induced.

Methods

Male Holtzman rats weighing 375–450 g were housed under U.S. Department of Agriculture and Association for Assessment and Accreditation of Laboratory Animal Care compliant conditions with free access to food and water. All experimental procedures were approved by the University of

Pennsylvania Institutional Animal Care and Use Committee and carried out under the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain (Zimmermann, 1983).

An injury model in the rat was used to test the hypothesis that a transient dynamic facet joint distraction can elicit persistent behavioral hypersensitivity and sustained spinal modifications of mGluR5 and EAAC1. Rats ($n = 12$) received a distraction of the facet joints at the C6/C7 spinal level, to magnitudes between 0.2 and 0.7 mm since distractions of 0.30 ± 0.21 mm have been shown to produce sustained mechanical allodynia (Dong et al., 2008). Distractions were applied at 0.2–0.4 mm ($n = 5$), 0.4–0.6 mm ($n = 5$), and ≥ 0.6 mm ($n = 2$). Distraction was applied across the bilateral C6/C7 joints at a rate of 15 mm/s, inducing a local strain rate (500%/s) of tension across the facet joint and its capsule comparable to the loading rate of that tissue during whiplash (Panjabi et al., 1998a; Stemper et al., 2005; Sundararajan et al., 2004; Yoganandan et al., 1998). After 30 s of distraction, the C6/C7 facet joint was also unloaded at the same rate (15 mm/s); this trapezoidal loading–unloading paradigm was identical to that used for a facet-mediated pain model in quasistatic loading conditions (Lee et al., 2004a,b). Sham procedures were also performed using a separate group of rats ($n = 6$) that underwent surgery but had no facet distraction applied, in order to serve as controls for the surgical procedures. Rats were survived and monitored for behavioral sensitivity for 7 days following surgery at which point spinal cord tissue was harvested at the C6 cervical level to probe for the expression of mGluR5 and EAAC1 protein using Western blot. Because the applied joint distraction magnitudes ranged by as much as 0.5 mm, the applied strain in the facet capsule also varied. Accordingly, in order to determine if the local ligament loading severity affects the resulting behavioral hypersensitivity and each of the spinal mGluR5 and EAAC1 responses, linear regressions were individually performed between strain metrics—maximum tensile strain in the rostral–caudal direction and maximum principal strain—and total mechanical allodynia, and between those strain metrics and each of mGluR5 and EAAC1 expression. Additional regression analyses were performed to determine whether mechanical allodynia at day 7 after distraction was correlated with each of mGluR5 and EAAC1 expression. The significance of those correlations was also tested.

Surgical procedures and analysis

All procedures were performed under inhalation anesthesia (4% isoflurane for induction, 2.5% for maintenance) and were modified from previously described procedures for a facet-mediated pain model in the rat (Dong et al., 2008; Lee et al., 2004a,b). Briefly, rats were placed in a prone position and a skin incision was made to bilaterally isolate the C6/C7 facet capsules. Each of the C6 and C7 vertebrae was attached to microforceps coupled with a loading device which uses a stepper motor (Danaher Precision Systems, Salem, NH) to apply controlled distraction across the C6/C7 facet capsule via moving the C6 microforceps while C7 remained fixed (Dong et al., 2008). A linear variable differential transducer (LVDT) (MicroStrain Inc., Williston, VT) with a 24 mm stroke and $5.7 \mu\text{m}$ resolution was attached to the C7 microforceps to continuously record the microforceps displacement (Fig. 1).

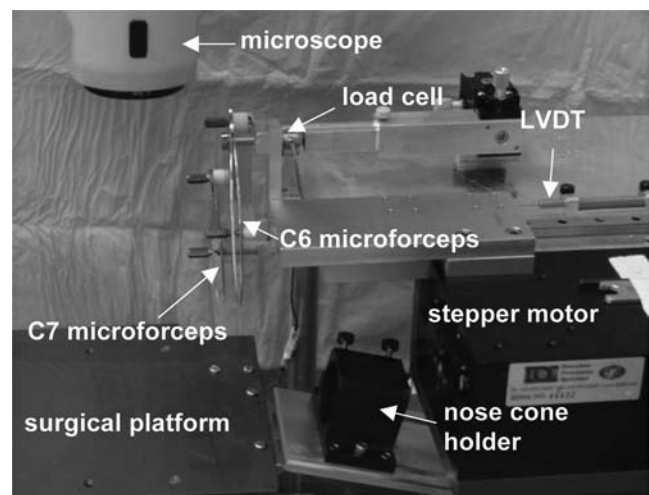


FIG. 1. Customized facet joint loading device showing the distraction device and set up including the microforceps, motor, LVDT, load cell, and nose cone holder for anesthesia delivery. A surgical microscope was mounted above the device to acquire image data throughout loading while the microforceps coupled to the C6 vertebra was displaced using the motor automated by a LABVIEW program; the microforceps coupled to the C7 vertebra remained stationary.

For this study, C6/C7 facet joint distractions (distraction) were imposed to induce a painful capsule ligament loading. Sham procedures involved attachment of both the C6 and C7 vertebrae to the loading device but had no distraction (0 mm) applied across the joint.

In order to quantify the facet joint and capsular ligament loading mechanics *in vivo* and to quantify the severity of capsule loading that was applied for each distraction, imaging and standard measurements of joint mechanics were synchronized and acquired at 500 Hz during each applied joint distraction. A load cell (Interface Inc., Scottsdale, AZ; 0.02 N resolution) was attached to the C7 microforceps and monitored the tensile force generated across the C6/C7 joint during distraction. The motion of the C6 vertebra relative to C7 was tracked using polystyrene microspheres (SpheroTech, Inc., Libertyville, IL; diameter = 0.17 ± 0.01 mm) that were affixed to the lamina of each vertebra (Fig. 2). Additional tracking particles were also placed in a grid covering the right C6/C7 facet capsule that spanned from the origin to insertion of the ligament (Fig. 2). These microspheres were placed directly on the surface of lamina and capsule without slipping due to their hydrophilic properties, and were monitored using high-speed imaging (Phantom v4.3 CCD camera; Vision Research, Inc., Wayne, NJ; 50 pixels/mm resolution). The severity of loading applied across the facet joint capsule in each case was measured by estimating the strain in the capsule; the maximum tensile strain in the direction across the joint (in the rostral–caudal direction) and the maximum principal strain in the capsule were quantified to provide relevant mechanical metrics for loading to this joint. Both strain measurements were measured for the C6/C7 right facet capsule joint, using LS-DYNA software (Livermore Software Technology Corp., Livermore, CA) according to methods previously described for *in vivo* studies of this joint and other biomechanical studies of soft tissues (Cavanaugh et al., 1996; Dong et al., 2008;

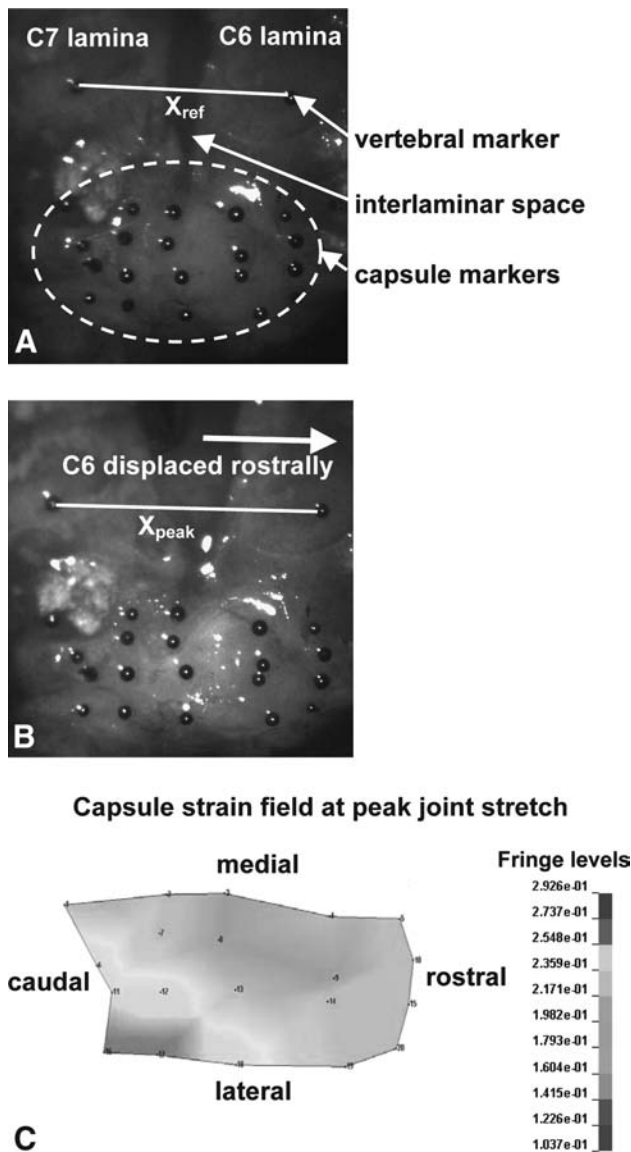


FIG. 2. Representative images showing the facet joint and capsule as well as the associated strain field. (A) The C6 and C7 vertebrae were marked by vertebral markers and their initial separation length (x_{ref}) defined the joint reference condition. (B) At the peak of capsule stretch (x_{peak}) C6 was distracted away from C7. Vertebral distraction was calculated as the vector difference between these separations and the maximum tensile strain (not shown) and maximum principal strain in the capsule was calculated using the capsule markers. (C) The maximum principal strain field associated with the images (A, B) is shown.

Hardy et al., 2008; Lee et al., 2004b; Winkelstein and Santos, 2000) (Fig. 2).

Behavioral assessments

Behavioral sensitivity was assessed in each rat after the surgical procedures by measuring bilateral mechanical allodynia in the forepaws on post-operative days 1, 3, 5, and 7, using von Frey filaments of two strengths (2 and 4 g; Stoelting Co., Wood Dale, IL). Rats were also assessed prior to surgery

to provide baseline measurements and to serve as a control unoperated response. Behavioral testing was performed with each filament applied separately to each forepaw for three rounds of 10 stimulations each. Each round of stimulation was separated by at least 10 min to allow for an adequate rest period. For each von Frey filament, the number of paw withdrawals was taken as the total number of positive responses counted for each rat for each forepaw. A paired *t*-test was used to compare the responses between the left and right paws for each rat to test for symmetry in the behavioral sensitivity response following the bilateral loading. A repeated measures ANOVA with *post hoc* Bonferroni correction was used to statistically compare temporal allodynia between the distraction and sham groups. In order to provide a quantitative measure of cumulative sensitivity for each rat following joint loading, total allodynia was also calculated as the sum of all paw withdrawals from all of the post-operative days 1, 3, 5, and 7. This measure of cumulative sensitivity was employed as the measure of behavioral response for the linear regression against maximum tensile and principal strains in the capsule. All statistical tests were performed using SYSTAT (SYSTAT Software Inc., Richmond, CA), with significance at $p < 0.05$ for all tests.

Tissue harvest and Western blot analysis

Whole spinal cord tissue at the C6 cervical spinal level was harvested on day 7 to evaluate the relative expression of mGluR5 and EAAC1. Normal rats ($n = 2$) were also used as controls for context of the normal protein response. After behavioral testing on day 7, all rats were transcardially perfused with 250 mL of phosphate-buffered saline (pH 7.4) and spinal cord tissue was rapidly removed. Following tissue harvest, spinal cord samples were homogenized using lysis buffer containing 50 mM Tris HCl (pH 8.0), 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, and protease and phosphatase inhibitors (Sigma-Aldrich Corp., St. Louis, MO). Protein samples (50 μ g) were heated at 95–100°C in preparation for electrophoresis and were loaded on a Tris-HCl ready polyacrylamide gel (BioRad Laboratories, Hercules, CA). Protein was transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA) and was blocked for 30 min with 5% dry-milk blocking reagent (Invitrogen Corp., Carlsbad, CA) in 0.1% Tween-20 Tris-buffered saline. The membrane was incubated overnight at 4°C with the rabbit polyclonal antibodies anti-mGluR5 (1:500; Chemicon International Inc., Billerica, MA) or anti-EAAC1 (1:200; Alpha Diagnostic International, San Antonio, TX). The membrane was washed in Tris-buffered saline with 0.1% Tween-20 buffer three times for 10 min each followed by a 1 h incubation at room temperature with horseradish peroxidase-conjugated anti-rabbit (1:10,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The membrane was exposed to HyBlot CL autoradiography film through a chemiluminescent reaction using the SuperSignal West Pico detection kit (Pierce Biotechnology, Inc., Rockford, IL). The film was developed using an X-OMAT processor (2000A; Eastman Kodak, Rochester, NY). To evaluate the amount of protein loaded for each sample, the membrane was stripped with stripping buffer (Pierce Biotechnology Inc.) at room temperature for 15 min before reprobing for actin (1:200; Santa Cruz Biotechnology Inc.).

Quantitative analysis was performed using Image J (National Institutes of Health, Bethesda, MD) to measure the density of immunoreactive bands for each lane. The amount of mGluR5 and EAAC1 was normalized separately against the relative amount of actin for each sample. Equal loading for each sample was further confirmed by staining the PVDF membranes with Coomassie Brilliant-Blue R-250 (BioRad) solution for 10 min followed by destaining with 25% acetic acid for three rounds of 10 min wash (Lindl et al., 2007). The membrane was also scanned and analyzed by Image J to confirm that the difference in protein expression was not due to unequal protein loading between samples. There was no difference detected in mGluR5 and EAAC1 expression between normal and sham groups, so protein expression of mGluR5 and EAAC1 was reported as a fold-increase over sham. A two-tailed *t*-test assuming equal variances was used to compare spinal protein responses between groups (distraction vs. sham). Significance was set at $p < 0.05$.

Statistical analysis

Linear regression models were used to determine if the degree of behavioral sensitivity (measured by cumulative mechanical allodynia) and spinal mGluR5 and EAAC1 expression were sensitive to the severity of applied facet joint distraction, and if behavioral hypersensitivity at day 7 was related to the corresponding modifications of spinal mGluR5 and EAAC1 expression. For these analyses, the tensile strain in the rostral-caudal direction and the maximum principal strain in the facet capsule were individually used for each distraction (Fig. 2). As such, the maximum tensile strain and the maximum principal strain in the capsule were separately taken as the independent variables for plots against total mechanical allodynia using both of the von Frey filaments (2 and 4 g, separately), and each of mGluR5 or EAAC1 protein levels, in separate analyses. For correlation analyses between mGluR5 and EAAC1 expression and mechanical allodynia, the number of paw withdrawals measured on day 7 was used since that time point corresponds to the day of tissue harvest and protein assessments of mGluR5 and EAAC1. The strain components for sham were taken as 0 since this group received 0 mm facet joint distraction. The significance of correlation for each regression was tested using an *F*-test and defined at $p < 0.05$ for all regressions.

Results

No gross mechanical tissue rupture was observed in the capsular ligament during loading for any joint distraction. In addition, careful examination of the facet capsule was also performed under a surgical microscope at the time of tissue harvest and did not reveal any evidence of gross tissue rupture or ligament damage. After surgery, all rats showed normal head mobility, consistent weight gain and normal grooming, indicating that there were no adverse effects of either the surgical or joint loading procedures.

Mean applied vertebral distraction in the distraction group was 0.47 ± 0.17 mm, with an average force generated across the joint of 4.04 ± 0.82 N. The average rate of applied distraction across the C6/C7 joint was measured to be 14.5 ± 0.5 mm/s, with a corresponding applied strain rate of $483 \pm 16.7\%/s$. The corresponding average maximum tensile strain in the rostral-caudal direction was $16 \pm 7\%$ and the

average maximum principal strain in the capsule was $24 \pm 10\%$. Since mechanical allodynia was not significantly different between the left and right paws for any of the rats for either filament strength, the average responses between the two forepaws were used for comparisons and reporting results for each rat. Mechanical allodynia induced following distraction was significantly ($p < 0.001$) greater than the number of paw withdrawals elicited by sham throughout the entire post-operative time for testing with both the 4 and 2 g von Frey filaments (Fig. 3). Although allodynia after distraction was immediate and exhibited a significant ($p < 0.0001$, 4 g; $p < 0.001$, 2 g) sustained elevation above baseline responses for all time points, the behavioral responses following sham procedures remained at baseline levels at all times and were not significantly different ($p = 0.38$) from baseline at any time point for testing with either filament. Further, allodynia after distraction was significantly greater ($p < 0.0001$) than sham at each post-operative day and for testing with each filament (Fig. 3).

Mirroring the trends observed for the behavioral outcomes, protein responses in the spinal cord also exhibited significant differences in spinal mGluR5 and EAAC1 at day 7 for the distraction group relative to sham. The expression of spinal mGluR5 was 1.4-fold greater after distraction compared to sham, and that increase was significant ($p = 0.04$; Fig. 4A). In

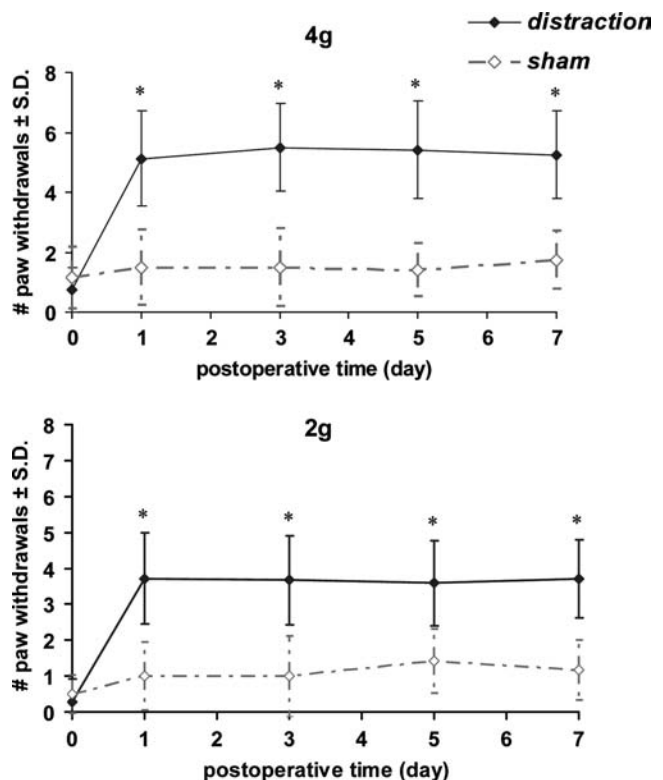


FIG. 3. Average forepaw mechanical allodynia as measured by the total number of paw withdrawals on each day for distraction and sham. Higher numbers of withdrawals indicated increased sensitivity. Distraction produced a significantly ($p < 0.001$) elevated and sustained response compared to sham for each day during the post-operative testing period, for testing with both the 4 and 2 g von Frey filaments. Asterisk (*) indicates significant increase over sham ($p < 0.001$).

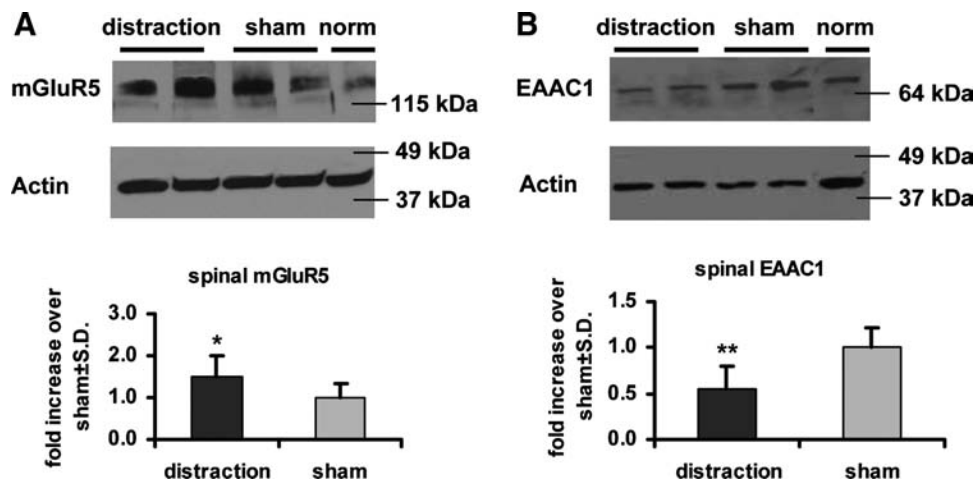


FIG. 4. (A) Spinal metabotropic glutamate receptor 5 (mGluR5) and (B) excitatory amino acid carrier 1 (EAAC1) expression was significantly modified following facet joint distraction compared to sham. Bands were detected at expected locations based on to their molecular weight. Quantitative analysis using normalization to actin for each specimen showed that mGluR5 was significantly ($*p < 0.01$) increased in distraction compared to sham. EAAC1 levels were significantly ($**p < 0.001$) reduced following distraction compared to sham levels. Data are shown as average \pm standard deviation.

contrast, distraction induced a significant ($p = 0.0003$) reduction of spinal EAAC1 expression compared to sham (Fig. 4B). In fact, after a facet joint distraction that induced significant mechanical allodynia, spinal EAAC1 protein was down-regulated by approximately 44% relative to sham levels (Fig. 4B).

Using the maximum tensile strain and the maximum principal strain established in each ligament as proxies for quantifying the loading severity for each rat, significant correlations were found with the behavioral and spinal outcomes (Fig. 5). Results were similar between the maximum tensile strain and the maximum principal strain in the capsule, yielding the same trends of correlation overall. Total mechanical allodynia was significantly ($p < 0.01$) positively correlated with the maximum tensile strain (Fig. 5A, C) and maximum principal strain (Fig. 5B, D) in the capsule, for both the 4 and 2 g von Frey filaments. The greater the strain sustained by the capsule during loading, the greater the number of cumulative post-operative paw withdrawals were produced in the forepaws (Fig. 5A–D). Similarly, spinal mGluR5 expression at day 7 also exhibited a significant ($p < 0.05$) positive linear correlation with each of the maximum tensile and maximum principal strains in the capsule for distraction (Fig. 5E, F). In contrast, while EAAC1 expression in the spinal cord at day 7 exhibited a slight negative but insignificant correlation with maximum principal strain (Fig. 5H), it was significantly ($p < 0.001$) negatively correlated with the tensile strain (Fig. 5G). Further, spinal mGluR5 expression showed a significant ($p < 0.05$) positive correlation with behavioral changes at day 7 that was stronger than the EAAC1 relationship to allodynia (Fig. 6). EAAC1 expression was only significantly ($p = 0.03$) negatively correlated with mechanical allodynia when responses were tested using a 4 g filament, but not for the 2 g (Fig. 6C, D).

Discussion

Findings from this study support the hypothesis that persistent behavioral hypersensitivity produced by dynamic fa-

cet joint distraction is potentiated, at least in part, by glutamate activities in the spinal cord. In particular, mGluR5 is up-regulated while the neuronal glutamate transporter EAAC1 is down-regulated after painful facet joint distraction (Figs. 4, 5). Further, total mechanical allodynia and spinal expression of mGluR5 were both significantly related to the magnitude of applied strain (Fig. 5A–F), suggesting that the severity of the mechanical insult to the facet capsular ligament may directly modulate the mechanisms responsible for the production of pain. The negative correlation between spinal EAAC1 expression and capsular strain was only significant when using the maximum tensile strain and not for the maximum principal strain metric (Fig. 5G, H). This discrepancy suggests that maximum tensile strain, which is a more general representation of overall average strain in the capsule across the entire surface, may provide a more meaningful and sensitive characterization of the painful loading to the capsule as a whole. Because of the sensitivity of neuronal glutamate transporter expression levels in relation to the mechanical strain experienced by the facet capsule during painful loading (Fig. 5G, H), it is possible that the nociceptors in the capsule may detect the specific magnitude and/or relative intensity of injury. However, the differential correlations observed between the tensile and maximum principal strain in the capsule and mGluR5 and EAAC1 expression in the spinal cord (Fig. 5E–H) suggest that the glutamate receptor and transporter may act together to potentiate and maintain behavioral hypersensitivity. This is further supported by the fact that the behavioral responses at day 7 following joint loading were significantly correlated with both mGluR5 and EAAC1 expression in the spinal cord (Fig. 6). In fact, the mGluR5 expression was positively correlated to the behavioral sensitivity while EAAC1 expression was negatively correlated (Fig. 6), further supporting that these modifications in the spinal glutamatergic system may work cooperatively in the maintenance of behavioral hypersensitivity in this model.

The mechanical profile of the facet capsular ligament distraction used in this *in vivo* model of pain simulates the loading that this joint experiences in whiplash exposures.

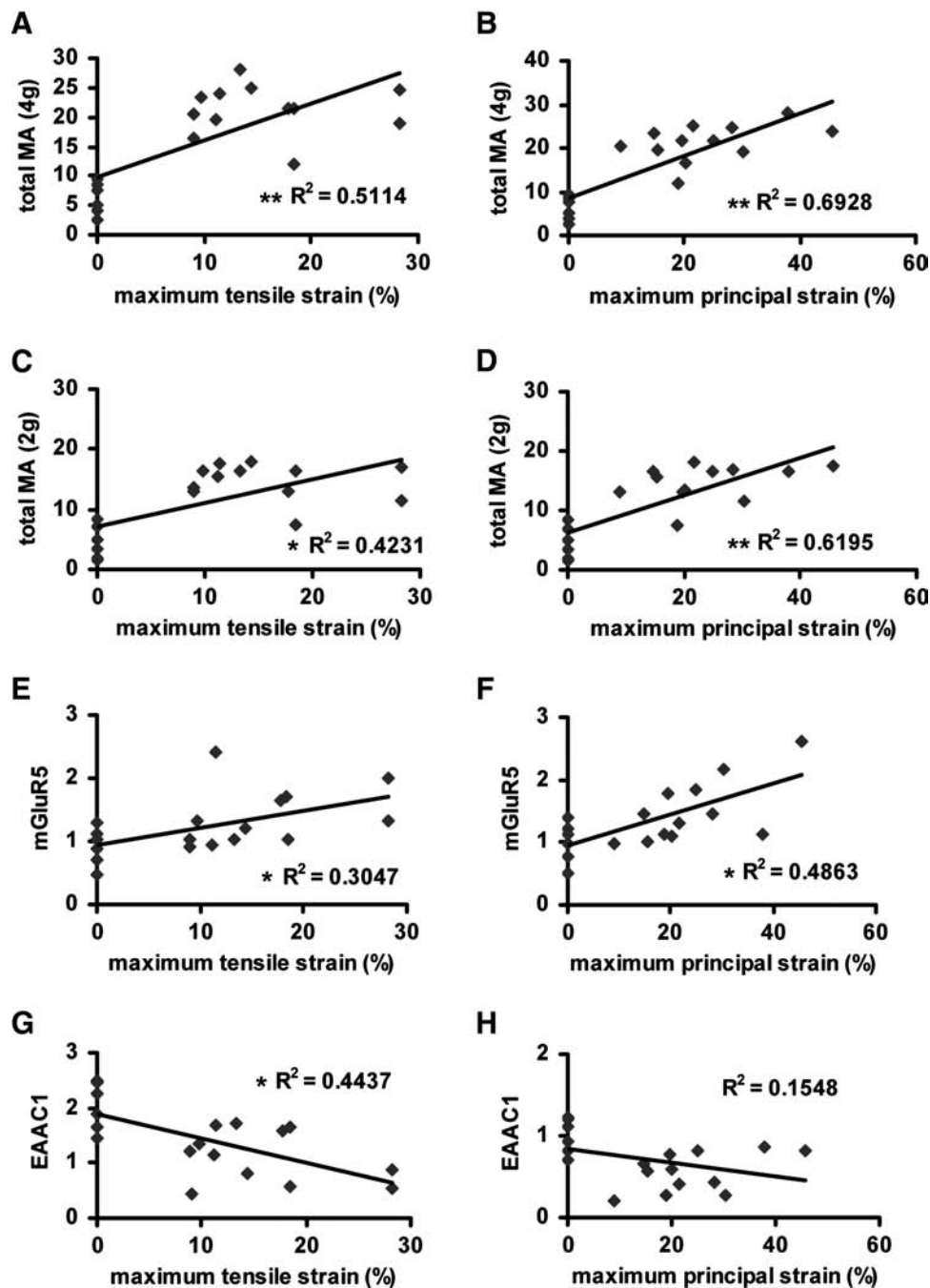


FIG. 5. Regression plots showing relationships between the strain metrics (maximum tensile strain and maximum principal strain) in the capsule and each of the behavioral and spinal outcomes. Both strain metrics were positively correlated with total mechanical allodynia (MA) detected using both the (A, B) 4 g and the (C, D) 2 g von Frey filaments. (E, F) Both strain metrics were also positively related to spinal metabotropic glutamate receptor 5 (mGluR5) expression. (G) Maximum tensile strain was significantly negatively correlated to excitatory amino acid carrier 1 (EAAC1), (H) but maximum principal strain was not significantly correlated to spinal EAAC1 expression. The coefficient of determination (R^2) for each correlation is displayed on each plot to illustrate the goodness of fit and significant relationships are indicated by asterisks (* $p < 0.05$; ** $p < 0.001$).

Specifically, the rate of joint loading (483%/s) is comparable to that reported in whiplash (150–1000%/s) (Deng et al., 2000; Lu et al., 2005; Luan et al., 2000; Pearson et al., 2004); the corresponding tensile strain ($16 \pm 7\%$) and maximum principal strain ($24 \pm 10\%$) in the capsule are also within the range of strains sustained by the capsule in lower cervical spinal levels (13–40%) during such impacts (Ito et al., 2004; Panjabi

et al., 1998c; Pearson et al., 2004; Stemper et al., 2005; Yoganandan et al., 2002). Although this study simulates the rate of whiplash loading to the facet joint, it also holds the distraction for 30 s before unloading. While this overall loading profile for the joint is not the same as that sustained during whiplash, it is consistent with other physiologic *in vivo* studies in this and other species and provides insight into relationships

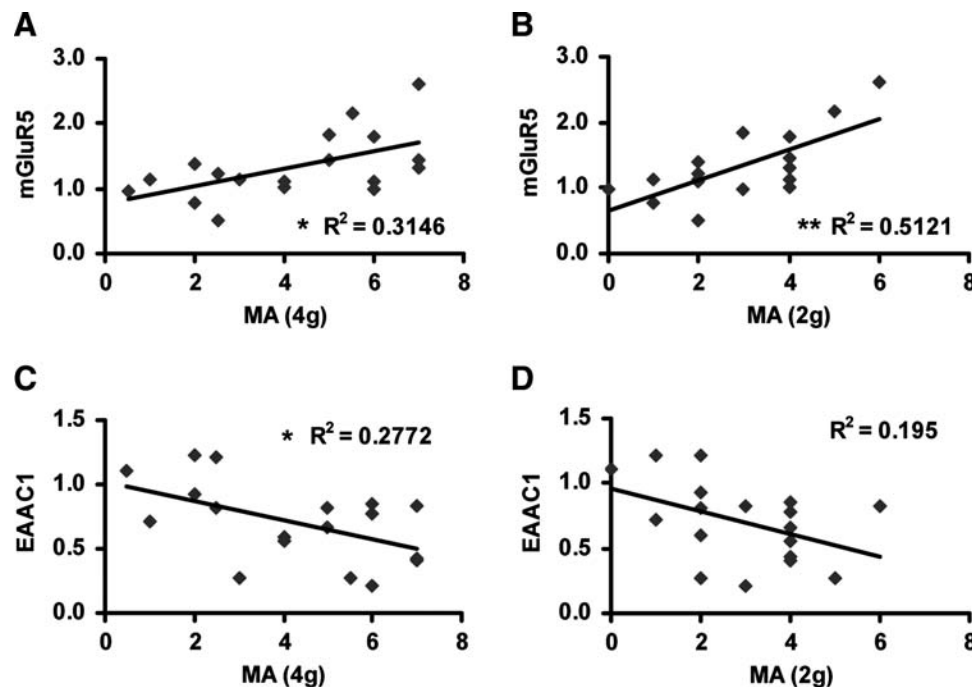


FIG. 6. Regression plots showing relationships between mechanical allodynia (MA) and each of spinal metabotropic glutamate receptor 5 (mGluR5) and excitatory amino acid carrier 1 (EAAC1) expression at day 7. MA values shown here represent the number of paw withdrawals for each rat on day 7. The level of mGluR5 expression was positively correlated with allodynia measured at day 7 for testing with both (A) the 4g and (B) the 2g von Frey filaments. In contrast, spinal EAAC1 expression was negatively correlated with allodynia at day 7 for both (C) the 4g and (D) the 2g filament. The coefficient of determination (R^2) for each correlation is shown to illustrate the goodness of fit and significant relationships are indicated by asterisks ($*p < 0.03$; $**p = 0.001$).

between joint mechanics and nociception. Behavioral sensitivity was assessed only in the forepaws (Fig. 3); whiplash patients commonly report mechanical sensitivity along the back of the neck and shoulders (Barnsley et al., 1994; Kasch et al., 2001; Mayou and Radanov, 1996). However, distraction of this same cervical facet joint has previously shown mechanical hyperalgesia in the both the shoulders and the forepaws, validating the use of forepaw sensitivity (Lee and Winkelstein, 2009; Lee et al., 2008). In that rat model, comparable maximum principal strains ($27.7 \pm 11.9\%$) were reported in the facet capsule when distraction was applied quasistatically (3%/s) in a rat model that provoked behavioral hypersensitivity that was sustained for 7 days after loading (Lee et al., 2004a). Interestingly, in that work, a physiological strain of 8.1% was not sufficient to initiate even a transient behavioral response (Lee and Winkelstein, 2009; Lee et al., 2004a). In the current study, the maximum tensile strain ranged from 9% to 28% while the maximum principal strain ranged from 9% to 46%, with no distractions inducing strains in the physiologic range (Panjabi et al., 1998c; Pearson et al., 2004) (Fig. 5). While capsular strains lower than 20% did produce heightened behavioral sensitivity compared to sham in the current study, the spinal mGluR5 expression at these low strains was comparable between distraction and sham (Fig. 5E, F). This finding implies that strains lower than 20% might not be sufficient to produce mGluR5 modifications in the central nervous system, despite an associated elevation in mechanical allodynia. As suggested by the regressions showing significant positive correlations between capsular strains (maximum tensile and principal strains) and the total

mechanical allodynia (Fig. 5A–D), a physiologic strain may exist in which pain symptoms are not produced by dynamic joint loading. Therefore, there may be a critical strain threshold for eliciting persistent behavioral hypersensitivity and associated sustained nociceptive responses in this facet injury model.

Physiological studies have highlighted the cooperative interactions between the glutamatergic system and inflammatory responses contributing to pain transmission (Balosso et al., 2009; Li et al., 2009; Ren and Dubner, 2008; Ye and Sontheimer, 1996). We have previously shown that distraction across the C6/C7 facet joint modulates spinal cytokine (TNF- α , IL1- β , IL-6) mRNA in accordance with mechanical allodynia (Lee et al., 2008). Since cytokines have a known role in facilitating pain transmission through glutamate modulation, including the activation of glutamate receptors in the brain and the increased release of glutamate in the superficial dorsal horn (Li et al., 2009; Sama et al., 2008; Uçeyler et al., 2009), it is not surprising to find that persistent behavioral sensitivity in this model is linked to the glutamate receptor and transporter (Figs. 5, 6). Further, elevation in extracellular glutamate has also been reported in both cultured hippocampal microglia and astrocytes and rat models of N-methyl-D-aspartate (NMDA)-induced pain (Bezzi et al., 1998; Svensson et al., 2003). Since spinal glial expression has been shown to be graded according to the magnitude of facet capsule distraction in quasistatic facet joint loading (Lee et al., 2004a), the correlation of glutamate activity with capsular strain observed in this dynamic joint loading model (Fig. 5E–H) is not unexpected.

It has been postulated that mGluR5 contributes to both inflammatory and neuropathic pain (Dolan et al., 2003; Fisher andCoderre, 1996; Mills et al., 2002; Miyoshi et al., 2006; Osikowicz et al., 2008; Pitcher et al., 2007). In particular, the expression of mGluR5 is upregulated after complete Freund's adjuvant injection in both the sheep and rat (Dolan et al., 2003; Pitcher et al., 2007). Additional studies that block mGluR5 activation using a glutamate receptor antagonist report decreased firing rate of nociceptive neurons in the ventral posterior lateral nucleus of the thalamus of the rat in response to pressure stimuli, and reduced allodynia after chronic constriction injury to the sciatic nerve in the mouse (Bordi and Ugolini, 2000; Osikowicz et al., 2008). This collection of behavioral studies suggests a mechanistic role of mGluR5 in pain. Consistent with those findings, results from the current study demonstrate significant upregulation of spinal mGluR5 at day 7 following painful facet joint loading compared to sham controls (Fig. 4A), which further substantiate the role of mGluR5 in the maintenance of joint-mediated pain. The sustained response of mGluR5 in the spinal cord has been shown to involve the downstream protein kinase C (PKC) pathway or an ionotropic receptor (i.e., NMDA) (Byrnes et al., 2009; Karim et al., 2001; Mills et al., 2001; Xu et al., 2007). However, since this study only examined spinal mGluR5 expression at day 7, the exact mechanisms by which mGluR5 contributes to pain remains undefined. It is plausible that this glutamate receptor works cooperatively with other downstream activities to cause persistent sensitivity. Further, the changes in mGluR5 expression between distracted and sham were small but significant (Fig. 4A); it is possible if only the superficial regions of the dorsal horn were probed, stronger fold-differences or more cell-specific relationships could have been detected between these groups. Therefore, future studies investigating the PKC pathway and NMDA receptor, in conjunction with the spinal mGluR5 at earlier time points and within specific regions of the spinal cord, are necessary to elucidate the temporal contributions of mGluR5-triggered cascades to the persistence of pain in this facet-mediated pain model.

In association with mechanical allodynia and increased spinal mGluR5, this study also demonstrated a down-regulation of spinal EAAC1 at day 7 after painful facet joint distraction (Figs. 3–5). EAATs are thought to be rapidly up-regulated in response to the high concentrations of extracellular glutamate in the spinal cord that can be due to injury or perceived injury in the central nervous system (Liu et al., 1991; McAdoo et al., 1999; Vera-Portocarrero et al., 2002). Chronic sciatic nerve constriction in the rat can elevate EAAC1 expression in the spinal dorsal horn for up to 4 days after the initial injury, but this elevation is reversed and even decreased by days 7 and 14 (Sung et al., 2003). Similarly, at day 7 after a peripheral nerve injury, spinal EAAC1 expression has also been shown to be down-regulated (Shashidharan et al., 1997), indicating a decreased transport of extracellular glutamate at day 7 post-injury. Those studies suggest that EAAC1 may respond differently at different time points, and that the neuronal glutamate transporter may play a different role than scavenging extracellular glutamate at times later than the initial injury. This is further supported by the fact that EAAC1 expression is unchanged in primary astrocyte cultures despite lowered glutamate uptake, in response to oxidative stress (Miralles et al., 2001). Taken

together with the upregulation of spinal mGluR5 observed in the current study (Fig. 4), it is possible that the primary afferents from the facet joint that may synapse in the spinal cord may be sensitized through the activation of mGluR5 due to the lack of neuronal glutamate uptake by EAAC1, contributing to persistent pain symptoms. This is in accordance with previous findings suggesting that the cellular stress response is activated in neurons that innervate the capsule following painful joint loading (Dong et al., 2008). However, it still remains to be seen whether the expression of EAAC1 is localized to nociceptive afferents, particularly A β and C fibers, in the spinal cord. Identifying the localization of EAAC1 expression is an important first step to understand if neuronal glutamate transporter may have a role in abnormal firing of nociceptive afferents in the spinal cord leading to pain associated with facet joint injury.

Although this study did not specifically localize the expression of mGluR5 in the spinal dorsal horn, histological studies have shown that mGluR5 is mostly located in the superficial laminae I and II (Alvarez et al., 2000; Hudson et al., 2002; Valerio et al., 1997), where the first level of nociceptive processing occurs (Mantyh and Hunt, 2004). Therefore, it is conceivable that the increase in mGluR5 regulates nociceptive transmission, causing spinal plasticity. Using a goat model of mechanical facet joint injury, Lu et al. (2005) found that capsular strains of 10% are sufficient to activate nociceptors in the facet capsule, while higher capsular strains (~40%) can actually induce saturation of the nociceptive discharge. In the current study, given that the strains in the capsular ligament were 16% and 24%, it is plausible that nociceptors in the facet capsule may be activated by strain, leading to physiological sequelae for the maintenance of pain. Biomechanical studies using cadaveric specimens have reported minor ligament ruptures in the facet capsule at strains ranging from 35% to 65% (Siegmund et al., 2001; Winkelstein et al., 2000), implying that capsule integrity is actually retained for loading that induces strains measured in this study (Fig. 2). Since intact facet capsules are necessary to transmit any nociceptive information in the facet distraction model (Lee et al., 2008; Winkelstein and Santos, 2008), the activation of the innervating nociceptive fibers in the facet capsule (Cavanaugh et al., 1997) may trigger physiological cascades in the nervous system and in the DRG (Dong et al., 2008). In parallel with the physiologic response in the DRG, the increased level of mGluR5 and decreased scavenging of glutamate (as evidenced by reduced EAAC1 expression) in the spinal cord, may lead to persistent behavioral hypersensitivity.

In conclusion, this study is the first to detect modifications in the glutamatergic system in the spinal cord for whiplash-related facet joint injury that produces pain, and demonstrates an opposite trend in expression of spinal mGluR5 and EAAC1 in relation to tissue loading severity (Fig. 5). These results suggest the possibility that spinal plasticity occurs via the glutamate receptor and transporter regulatory systems after painful facet capsule distraction. Future work is necessary to fully understand the temporal contributions and the mechanisms of the glutamate receptor and transporter to the induction and maintenance of pain. Nonetheless, this work presents a potential therapeutic intervention in which reversing abnormal glutamate activities by blocking mGluR5 and enhancing EAAC1, could attenuate or abolish facet-mediated neck pain following whiplash-like joint loading.

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Author Disclosure Statement

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