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Spinal upregulation of glutamate transporter GLT-1 by ceftriaxone: therapeutic efficacy in a range of experimental nervous system disorders

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Abstract

Glutamate neurotransmission is highly regulated, largely by glutamate transporters. In the spinal cord, the glutamate transporter GLT-1 is primarily responsible for glutamate clearance. Downregulation of GLT-1 can occur in activated astrocytes, and is associated with increased extracellular glutamate and neuroexcitation. Among other conditions, astrocyte activation occurs following repeated opioids and in models of chronic pain. If GLT-1 downregulation occurs in these states, GLT-1 could be a pharmacological target for improving opioid efficacy and controlling chronic pain. The present studies explored whether daily intrathecal treatment of rats with ceftriaxone, a β -lactam antibiotic that upregulates GLT-1 expression, could prevent development of hyperalgesia and allodynia following repeated morphine, reverse pain arising from central or peripheral neuropathy, and reduce glial activation in these models. Ceftriaxone pre-treatment attenuated the development of hyperalgesia and allodynia in response to repeated morphine, and prevented associated astrocyte activation. In a model of multiple sclerosis (experimental autoimmune encephalomyelitis; EAE), ceftriaxone reversed tactile allodynia and halted the progression of motor weakness and paralysis. Similarly, ceftriaxone reversed tactile allodynia induced by chronic constriction nerve injury (CCI). EAE and CCI each significantly reduced the expression of membrane-bound, dimerized GLT-1 protein in lumbar spinal cord, an effect normalized by ceftriaxone. Lastly, ceftriaxone normalized CCI- and EAE-induced astrocyte activation in lumbar spinal cord. Together, these data indicate that increasing spinal GLT-1 expression attenuates opioid-induced paradoxical pain, alleviates neuropathic pain, and suppresses associated glial activation. GLT-1 therefore may be a therapeutic target that could improve available treatment options for patients with chronic pain.

Keywords

opioid; spinal cord; multiple sclerosis; astrocyte; allodynia; hyperalgesia

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Dedication: The authors wish to dedicate this work to Dr. Evan Sloane, who lost his life to cancer prior to the publication of this work.

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The excitatory neurotransmitter glutamate is implicated in creating and maintaining spinal sensitization, and associated hypersensitivity to nociceptive stimuli (Coderre, 1993). Additionally, there is significant interaction between opioid and glutamatergic signaling, and glutamate receptor antagonists can reverse opioid-induced “paradoxical” pain (King et al., 2005). Upon binding to post-synaptic NMDA, AMPA, or kainate receptors, glutamate causes depolarization of the post-synaptic membrane and influx of cations including Ca^{2+} . Given that Ca^{2+} influx alters functioning of the post-synaptic neuron and can cause excitotoxicity if not properly regulated, the maintenance of normal extracellular levels of glutamate is crucial (Danbolt, 2001). High affinity, Na^{+} -dependent glutamate transporters provide the major mechanism for extracellular glutamate uptake and homeostasis (Danbolt, 2001). Five such transporters have been cloned and characterized, including GLT-1.

Each glutamate transporter exhibits a unique pattern of regional localization in the central nervous system (CNS). In the normal adult rat spinal cord, the primary route of glutamate clearance is GLT-1. Early studies showed that GLT-1 is primarily expressed by astrocytes, though several groups have documented GLT-1 protein expression in neuronal cell bodies and axon terminals in various brain regions but not, to date, in the spinal cord (Rothstein et al., 1994; Yamada et al., 1998; Schmitt et al., 2002; Chen et al., 2004; Furness et al., 2008; Melone et al., 2009). Spinal astrocytes are activated by a variety of conditions including repeated opioid administration and chronic pain (Smith et al., 1983; Garrison et al., 1991; Song and Zhao, 2001). As activated astrocytes can exhibit reduced GLT-1 expression and glutamate uptake, impaired glial glutamate uptake in spinal cord could contribute to neuroexcitability in subjects with chronic pain or exhibiting opioid-induced paradoxical pain (Liao and Chen, 2001; Zhang et al., 2009). Importantly, glutamate receptor activation on cultured microglia and astrocytes, and exposure of spinal cord *in situ* to abnormally elevated levels of glutamate, have been shown to regulate release of pro-inflammatory cytokines implicated in central sensitization (Aronica et al., 2005; Taylor et al., 2005; Kawasaki et al., 2008; Liu et al., 2008). Therefore, downregulation of spinal GLT-1 expression, and associated elevated levels of extracellular glutamate, may be a common mechanism undermining opioid efficacy, and underlying the development and maintenance of central sensitization.

Ceftriaxone is an FDA approved β -lactam antibiotic recently discovered to upregulate GLT-1 expression in the CNS, by increasing transcription of the *GLT1* gene (Rothstein et al., 2005). If downregulation of GLT-1 were to be of central importance to pain dysregulation, then treatment with ceftriaxone would be predicted to resolve both opioid-induced paradoxical pain and pathological pain states. Thus, the first study tested whether pre-treatment with intrathecal ceftriaxone would upregulate spinal membrane-bound GLT-1 in naïve rats and consequently block the development of morphine-induced hyperalgesia and allodynia. Additionally, daily intrathecal treatment with ceftriaxone was tested for its ability to reverse established tactile allodynia in rat models of central and peripheral neuropathic pain: an experimental autoimmune encephalomyelitis (EAE) rat model of multiple sclerosis (Storch et al., 1998), and the chronic constriction injury (CCI) model of peripheral nerve injury-induced pain (Bennett and Xie, 1988). Ceftriaxone was also tested in the EAE model to define whether it could halt the progression of motor weakness and paralysis. Finally, given that astrocytes and microglia themselves express glutamate receptors and respond to abnormally elevated extracellular glutamate with release of pro-inflammatory cytokines (Aronica et al., 2005; Taylor et al., 2005), the effect of ceftriaxone treatment on glial activation was analyzed in all three models.

EXPERIMENTAL PROCEDURES

Subjects

Pathogen-free adult male Sprague Dawley rats (325–350 g; Harlan Laboratories) were used in Experiments 1, 2 and 4. Pathogen-free adult male Dark Agouti rats (250–300 g; Harlan Laboratories) were used in Experiment 3. In each case, rats were housed two per cage in a temperature-controlled environment ($23 \pm 2^\circ\text{C}$) with a 12 h light/dark cycle (lights on at 7:00 AM), and with standard rat chow and water available *ad libitum*. All procedures occurred in the light phase. All rats were allowed 1 week of acclimation to the colony room before experimentation. The Institutional Animal Care and Use Committee of the University of Colorado at Boulder approved all procedures.

Drugs

The drug ceftriaxone was a gift from Roche. Ceftriaxone was dissolved in sterile endotoxin-free 0.9% saline (Abbot laboratories) at 60 mg/ml and stored at -20°C . Ceftriaxone was delivered via intrathecal injection for all studies, so as to specifically define the spinal cord as its site of action. For intrathecal injections, the 60 $\mu\text{g}/\mu\text{l}$ ceftriaxone stock was diluted with saline to 30 $\mu\text{g}/\mu\text{l}$, and 5 μl were injected for a total dose of 150 μg (227 nmol). This dose was chosen based on preliminary dosing studies to determine a dose tolerated by the rats that would cause significant upregulation of spinal GLT-1 protein expression in naïve rats. The 30 $\mu\text{g}/\mu\text{l}$ ceftriaxone solution was made fresh immediately before injections. Morphine sulfate was dissolved in saline at 5 $\mu\text{g}/\mu\text{l}$ and stored at 4°C until the time of injections. 2 μl were used for each injection for a total dose of 10 μg , as has been used in our prior repeated intrathecal morphine study (Johnston et al., 2004).

Chronic intrathecal catheter surgery and intrathecal drug administration

Lumbosacral intrathecal catheters were constructed and implanted by lumbar approach under isoflurane anesthesia as described previously (Milligan et al., 1999). The indwelling catheters were used to microinject ceftriaxone, morphine or saline into the cerebrospinal fluid (CSF) space surrounding the lumbosacral spinal cord. All catheter placements were verified by visual inspection upon euthanasia. To perform each injection, an injector consisting of PE-10 tubing attached to a 30G needle stripped of its hub was used. The injector was prefilled with 30 μl total, consisting of either ceftriaxone (5 μl), morphine (2 μl) or saline vehicle (volume matching either ceftriaxone or morphine volume, as appropriate for each experiment), and a void volume of saline such that the total volume was 30 μl . This total volume was chosen so as to ensure the drug being injected would completely traverse the catheter and flush into the CSF. The rat was loosely restrained, the injector was connected to the rat's catheter, and the injection proceeded as 15 μl over 30 sec, followed by a 3 min pause, then the remaining 15 μl over an additional 30 sec. This ensured that the first 15 μl entering the CSF would be saline left in the catheter from the prior injection, and normal CSF flow during the 3 min pause would flush this saline away before the drug would be flushed from the catheter during the second half of the injection. For all experiments, injections were performed each day after behavior or motor testing, except as noted otherwise.

Induction of experimental autoimmune encephalomyelitis (EAE)

EAE was induced as described previously (Sloane et al., 2009). Recombinant protein corresponding to the N-terminal sequence of rat myelin oligodendrocyte glycoprotein (MOG; amino acids 1–125) was expressed in *Escherichia coli* and purified to homogeneity by Ni-chelate chromatography using a 6-His tag. The purified protein was dissolved in 6 M urea and dialyzed against 10 mM sodium acetate buffer (NaAc; pH 3.0) then stored at

–80°C. EAE was induced via injection of 10 or 8.75 µg MOG in 0.01 M NaAc (pH 3.0) emulsified in incomplete Freund's adjuvant (Sigma, St. Louis, MO) in a 1:1 ratio of MOG (10 or 8.75 µg in 50 µl): incomplete Freund's adjuvant (50 µl). Rats were briefly anaesthetized with isoflurane and given a single intra-dermal injection (100 µl total volume) into the dorsal skin just rostral to the base of the tail. Rats were monitored daily for body weight changes, motor symptoms, and sensory symptoms (von Frey testing, see below). During periods of muscle paralysis, the rats' bladders were expressed manually to help prevent bladder infections. Any rat that developed a bladder infection was treated with Baytril (Bayer HealthCare, Morristown, NJ) at a dose of 1.5 mg via subcutaneous injection, twice on the first day and once daily for two more days.

EAE motor scoring

Motor deficits were scored on a scale from 0 to 7 based on the degree of ascending paralysis. The scores correspond to the following deficits: 0, no symptom expression; 1, partial tail paralysis; 2, full tail paralysis; 3, hindlimb weakness (unsteady gait while walking); 4, partial hindlimb paralysis (no weight bearing but observable movement of the limb); 5, full hindlimb paralysis; 6, complete paralysis of lower body (rat is unable to hold his belly up from the ground); 7, euthanasia due to disease progression. Motor scores were recorded during the first few hours of the light cycle.

Chronic constriction injury (CCI)

CCI was created at mid-thigh level of the left sciatic nerve (Bennett and Xie, 1988). Four ligatures of sterile surgical chromic gut sutures (cuticular 4-0, chromic gut; Ethicon, Somerville, NJ) were loosely tied around the sciatic nerve under isoflurane anesthesia. The sciatic nerves of sham-operated rats were identically exposed but not ligated. All rats undergoing CCI or sham surgery received an indwelling intrathecal catheter within the same surgery session.

von Frey test for tactile allodynia

Rats were habituated to the wire grill testing apparatus for 1 h per day on 4 consecutive days. The von Frey test (Chaplan et al., 1994) was performed within the sciatic innervation area of the plantar hindpaw as described previously (Milligan et al., 2000), using calibrated Semmes-Weinstein monofilaments (Stoelting, Wood Dale, IL). For testing Sprague Dawley rats, the von Frey hairs used range from 0.407 – 15.136 g, with $\log \text{ stiffness} = \log_{10}(\text{milligrams} \times 10)$. This range of stimuli produces a logarithmically graded slope (Chaplan et al., 1994). The von Frey hairs were applied randomly to the left and right hindpaws to determine the stimulus intensity required to elicit a paw withdrawal response. For testing Dark Agouti rats, the range of von Frey hairs was extended to include hairs applying 0.07 and 0.16 g of pressure, and testing began with two presentations of the 0.07 g hair and proceeded sequentially through hairs of increasing force until three consecutive responses at a given level of stimulation were observed (Sloane et al., 2009). For all experiments, von Frey testing was performed blind with respect to drug administration. The raw behavior data were used to calculate the absolute thresholds by fitting a Gaussian integral psychometric function using a maximum-likelihood fitting method, which allows for parametric statistical analyses (Harvey, 1986; Treutwein and Strasburger, 1999; Milligan et al., 2000). For the CCI model, absolute thresholds are presented separately for the ipsilateral and contralateral hindpaws, given the unilateral injury. For the EAE and repeated morphine model, absolute thresholds are presented as the average of the left and right hindpaws, given that neither model includes unilateral manipulations.

Hargreaves test for thermal hyperalgesia

Thresholds for behavioral response to heat stimuli applied to the tail were assessed using the Hargreaves test (Hargreaves et al., 1988). Rats were habituated to the testing apparatus for 1 h per day on 4 consecutive days. Withdrawal latencies were calculated as the median of three consecutive withdrawal latencies measured on the tail. Voltage to the heat source was adjusted to yield baseline latencies ranging from 8 to 12 s, and a cut-off time of 20 s was imposed to avoid tissue damage. Behavior testing was performed blind with respect to drug administration.

Spinal cord tissue subcellular fractionation

Lumbar spinal cord tissue (L4 – L6) was harvested via hydraulic extrusion with ice cold saline immediately after decapitation at the specified time points, flash frozen and stored at -80°C . The tissue was later processed using a Compartmental Protein Extraction Kit (BioChain, Hayward, CA) in order to produce fractions enriched for membrane or cytoplasmic proteins.

Western blotting

Western blot analyses were performed on spinal cord tissue that was fractionated as described above, or that was harvested fresh, at the specified time points, in 50 mM Tris buffer containing 0.5% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1% SDS, and protease inhibitors. After extraction, proteins were subjected to NuPAGE Bis-Tris (4–12%) gel electrophoresis under reducing conditions (Invitrogen, Carlsbad, CA) and then transferred to nitrocellulose membranes electrophoretically. Nonspecific binding sites on the membrane were blocked with 5% non-fat milk or 5% bovine serum albumin in TBS containing 0.1% Tween 20 (TBS-T) for 1 hr at $22-24^{\circ}\text{C}$. Membranes were subsequently incubated with primary antibodies in TBS-T buffer overnight at 4°C . The membranes were then washed with TBS-T, and probed with appropriate secondary antibodies labeled with horseradish peroxidase for 1 h at $22-24^{\circ}\text{C}$ and detected with SuperSignal West Femto or Pico chemiluminescent reagents (Pierce, Rockford, IL). Membranes were then stripped with a ReBlot Western Blot Recycling Kit (Millipore, Billerica, MA) and reprobed with an antibody against a designated loading control protein. Primary antibodies and dilution ratios used were: GLT-1 1:5,000 (Millipore, Billerica, MA), glial fibrillary acidic protein (GFAP) 1:10,000 (Cell Signaling, Danvers, MA), and CD11b 1:1,000 (Abcam, Cambridge, MA). Primary antibodies used against loading control proteins and dilution ratios used were: GAPDH 1:4,000 for whole cord homogenates (Cell Signaling, Danvers, MA), and $\text{Na}^{+}/\text{K}^{+}$ ATPase 1:1,000 for membrane fractions (Cell Signaling, Danvers, MA). Secondary antibodies used were: goat anti-rabbit IgG, HRP-tagged (Cell Signaling, Danvers, MA); goat anti-mouse IgG, HRP-tagged (Cell Signaling, Danvers, MA); and goat anti-guinea pig IgG, HRP-tagged (Santa Cruz Biotechnology, Santa Cruz, CA). Bands were quantified using the Quantity One software (Bio-Rad, Hercules, CA).

Experiment 1: Effect of intrathecal ceftriaxone treatment on spinal GLT-1 expression in naïve rats

Systemic ceftriaxone treatment has been previously shown to upregulate the GLT-1 monomer in whole CNS tissue in rats (Rothstein et al., 2005). However, GLT-1 is a membrane-bound transporter and is only functional as an oligomer (Haugeto et al., 1996). Haugeto et al. demonstrated that in rat CNS, GLT-1 monomers exhibit no glutamate transport activity, and instead GLT-1 dimers or trimers or both are the functionally active form (1996). Therefore, the first question addressed here was whether ceftriaxone treatment would upregulate expression of GLT-1 in membrane protein fractions in naïve rats, and also whether this upregulation would be observed as a change in the expression of GLT-1

monomers or oligomers. Ceftriaxone was administered via intrathecal injection, so as to define the spinal cord as the site of therapeutic action in Experiments 2–4 (below). In order to measure whether 1 week of daily intrathecal ceftriaxone treatment (150 µg per day) would upregulate spinal GLT-1 in naïve rats (naïve + saline, $n = 5$; naïve + ceftriaxone, $n = 5$), the rats were first implanted with an indwelling intrathecal catheter. The rats then had a 7 day recovery period, and on the second day of this time, each rat's catheter was flushed with 30 µl of sterile saline to clear the catheter in advance of intrathecal injections. After the post-surgical recovery period, the rats received 7 daily intrathecal injections with saline or ceftriaxone (150 µg per day). 24 hr after the last intrathecal injection, the rats were anesthetized with isoflurane, decapitated, and the spinal cords removed via hydraulic extrusion with ice cold saline. Lumbar spinal cord tissue (L4 – L6) was dissected out. From each rat, the caudal 0.5 cm of this tissue was homogenized whole and the rest was subjected to subcellular fractionation to produce separate membrane-bound or cytoplasmic protein fractions.

To test the duration of ceftriaxone-induced upregulation of spinal GLT-1 in the absence of continued treatment, a separate group of rats received 7 days of daily intrathecal injections of ceftriaxone or saline, exactly as above (naïve + saline, $n = 6$; naïve + ceftriaxone, $n = 5$). These rats, however, were maintained for 1 week following the last intrathecal injection, and received no further treatment during this time. The rats were then anesthetized with isoflurane, decapitated, and the spinal cords removed via hydraulic extrusion with ice cold saline. Lumbar spinal cord tissue (L4 – L6) was treated as above.

Experiment 2: Effect of intrathecal ceftriaxone pre-treatment on repeated intrathecal morphine-induced thermal hyperalgesia, tactile allodynia and glial activation

Using the same dosing regimen as in Experiment 1 to upregulate spinal GLT-1, a separate study examined whether ceftriaxone pre-treatment would affect thermal hyperalgesia and tactile allodynia induced by repeated morphine administration (saline-saline, $n = 7$; saline-morphine, $n = 13$; ceftriaxone-saline, $n = 7$; ceftriaxone-morphine, $n = 12$). This model was chosen because of previous data showing that rats treated with repeated morphine exhibit hyperalgesia, allodynia, and spinal astrocyte activation (Song and Zhao, 2001; Johnston et al., 2004). The study was designed with ceftriaxone treatment ceasing before morphine injections began, because ceftriaxone inhibits the function of the multiple-drug resistance pump P-glycoprotein (P-gp), and morphine is known to be transported out of CNS capillary endothelium via P-gp (Letrent et al., 1999). Thus cessation of ceftriaxone was aimed at avoiding elevated levels of intrathecal morphine due to P-gp inhibition, which would confound the results by exaggerating the development of morphine-induced “paradoxical” pain in the ceftriaxone-treated group, an effect opposite to the results obtained (see below). The rats were first implanted with an indwelling intrathecal catheter. The rats then had a 7 day recovery period, and on the second day of this time, each rat's catheter was flushed with 30 µl of sterile saline to clear the catheter in advance of behavioral testing and intrathecal injections. After the post-surgical recovery period, the rats received 7 days of daily intrathecal injection with saline or ceftriaxone (150 µg per day). The next day, the rats underwent baseline von Frey testing for each hindpaw, followed by baseline Hargreaves testing of the tail. Each rat was then injected with saline or morphine (10 µg). The saline or morphine injections were repeated once daily, for a total of 5 days of injections. 24 hr following the last saline or morphine injection, rats underwent von Frey testing of each hindpaw followed by Hargreaves testing of the tail. Immediately after this behavior testing, the rats were anesthetized with isoflurane, decapitated, and the spinal cords removed via hydraulic extrusion with ice cold saline. Lumbar spinal cord tissue (L4 – L6) was treated as above.

Experiment 3: Effect of intrathecal ceftriaxone treatment on EAE-induced muscle paralysis, tactile allodynia, GLT-1 downregulation and glial activation

The goal of Experiment 3 was to examine whether daily intrathecal ceftriaxone treatment would slow the progression of paralysis and/or reverse central neuropathic pain (tactile allodynia) caused by EAE, and whether any positive motor or behavior effects would be mirrored by normalization of GLT-1 downregulation and/or glial activation. The EAE model was chosen as a model of central neuropathy with associated astrocyte activation in the spinal cord (Smith et al., 1983). Two doses of MOG were used: 10 µg per rat (EAE + saline, $n = 10$; EAE + ceftriaxone, $n = 8$), for analysis of motor paralysis; and 8.75 µg per rat (EAE + saline, $n = 6$; EAE + ceftriaxone, $n = 5$), for analysis of tactile allodynia. For the higher dose of MOG, rats first received an indwelling intrathecal catheter. The rats then had a 7 day recovery period, and on the second day of this time each rat's catheter was flushed with 30 µl of sterile saline to clear the catheter in advance of motor scoring and intrathecal injections. After the post-surgical recovery period, MOG was injected. The lower dose of MOG was used to allow an unconfounded examination of allodynia by choosing a dose of MOG that minimizes impact on hindpaw motor function. For the lower dose of MOG, on day 1 of the study, baseline von Frey responses were recorded for each hindpaw. The next day, each rat was implanted with an indwelling intrathecal catheter. The rats then had a 7 day recovery period, and on the second day of this time each rat's catheter was flushed with 30 µl of sterile saline to clear the catheter in advance of behavior testing, motor scoring, and intrathecal injections. After the post-surgical recovery period, the rats again underwent von Frey testing, and MOG was injected two days later. Monitoring of body weight, motor score, and tactile sensitivity (for lower dose of MOG) began the day after MOG injection. On the first day that any individual rat showed a motor score greater than 0, daily intrathecal injections began for that rat. On any given day, weight, motor score, and von Frey response thresholds were measured before injections were done. For each dose of MOG, the study ended one month post-MOG injection, which allowed for analysis of the effects of ceftriaxone while limiting the time the rats lived with muscle weakness and/or paralysis. At the end of each study, immediately following the last von Frey test point or motor scoring as shown on the graphs in Fig. 4, the rats were anesthetized with isoflurane, decapitated, and the spinal cords removed via hydraulic extrusion with ice cold saline. Three naïve rats were included at this point in the study. Lumbar spinal cord tissue (L4 – L6) was harvested from all of the EAE rats and the naïve rats. L4 – L6 tissue was treated as above. For Western blot analyses of GLT-1, GFAP and CD11b, tissue samples from rats injected with both doses of MOG were run on the same gels and analyzed together.

Experiment 4: Effect of intrathecal ceftriaxone treatment on CCI-induced tactile allodynia, GLT-1 downregulation and glial activation

This experiment examined whether daily intrathecal ceftriaxone treatment would reverse tactile allodynia caused by CCI, and whether any positive behavioral effects would be mirrored by normalization of dimerized, membrane-bound GLT-1 downregulation and/or glial activation. This model was chosen as a model of peripheral neuropathy with associated spinal astrocyte activation (Garrison et al., 1991). Three experimental groups were used: sham rats treated with saline ($n = 6$), CCI rats treated with saline ($n = 11$), and CCI rats treated with ceftriaxone (150 µg per day; $n = 14$). Having observed in Experiment 2 that this dose of ceftriaxone did not alter von Frey responses in non-neuropathic animals, a sham group treated with ceftriaxone was not included in order to minimize animal use. At the start of the experiment, baseline von Frey measures were recorded for each hindpaw. Each rat then underwent a single surgery session that included sham or CCI surgery and implantation of an indwelling intrathecal catheter. On day 7 post-surgery, each rat's catheter was flushed with 30 µl of sterile saline to clear the catheter in advance of behavior testing and intrathecal injections. On day 11 post-surgery, von Frey measures were again recorded for each

hindpaw. Daily intrathecal injections began on day 12 post-surgery, and continued daily thereafter. On any day when rats underwent behavior testing and received intrathecal injections, the behavior testing was done first, followed by injections. Von Frey responses were again measured on days 18, 25 and 32 post-surgery. Immediately following the von Frey testing on day 32 post-surgery, the rats were anesthetized with isoflurane, decapitated, and the spinal cords removed via hydraulic extrusion with ice cold saline. Lumbar spinal cord tissue (L4–L6) was treated as above.

Statistical analyses

For the repeated morphine model, Hargreaves data and von Frey data were analyzed by one-way ANOVA followed by Tukey's post-hoc test. For the CCI and EAE models, time-courses of von Frey behavior data were analyzed by two-way ANOVA with repeated measures, followed by Bonferroni post-hoc tests. For the EAE model, motor deficit scores were analyzed using a non-parametric Wilcoxon rank sum test. Western blot data were analyzed by t-test when comparing two groups, or one-way ANOVA followed by Tukey's post-hoc test when comparing three or more groups.

RESULTS

Experiment 1: Daily intrathecal ceftriaxone upregulates spinal membrane-bound, dimerized GLT-1 in naïve rats

Spinal GLT-1 upregulation in naïve rats—This experiment tested the ability of 7 days of daily intrathecal ceftriaxone injections to upregulate GLT-1 expression in a normal spinal cord. In cytoplasmic protein fractions, the GLT-1 antibody labeled a band at ~ 65 kDa, consistent with the known molecular weight of GLT-1, but no GLT-1 dimer or trimer bands were observed. In membrane protein fractions, while the GLT-1 antibody did label a band at ~ 65kDa, it also labeled a band at ~130 kDa, or twice the molecular weight of the single band observed in cytoplasmic protein fractions. No GLT-1 trimer bands were observed in membrane protein fractions. In this and all further experiments, we quantified the dimer and monomer forms of GLT-1 in membrane fractions, and the GLT-1 monomer in cytoplasmic fractions. Given that GLT-1 is only functional as a membrane-bound oligomer (Haugeto et al., 1996; Danbolt, 2001), we focused on membrane-bound, dimerized GLT-1 expression, but also analyzed membrane-bound and cytoplasmic monomer expression so to allow comparison to prior literature where the monomer was the focus of study. One week of once daily intrathecal ceftriaxone treatments caused a 77% increase in membrane-bound, dimerized GLT-1 protein expression in the lumbar spinal cord (Fig. 1A; $p < 0.05$). There was no difference between the two groups when analyzing GLT-1 monomer in membrane or cytoplasmic fractions.

To investigate how long the upregulation of membrane-bound, dimerized GLT-1 would persist in the absence of continued treatment, a second group of naïve rats received 7 days of daily intrathecal injection with ceftriaxone (150 µg) or saline, followed by 7 days without treatment. Western blot analysis showed that by 7 days after the last intrathecal injection, there was no longer any difference in expression of membrane-bound, dimerized GLT-1 between the two groups (Fig. 1B). In these rats, there was also no difference between the two groups when analyzing GLT-1 monomer in membrane or cytoplasmic fractions.

Experiment 2: Daily intrathecal ceftriaxone pre-treatment attenuates thermal hyperalgesia, tactile allodynia and astrocyte activation induced by repeated morphine

Hyperalgesia and allodynia—Rats received daily intrathecal injection with ceftriaxone (150 µg) or saline for 7 days, to cause upregulation of spinal GLT-1 expression as in Experiment 1, above. The next day, the rats started 5 days of daily intrathecal injection with

morphine (10 µg) or saline in the absence of further ceftriaxone treatment, to determine if ceftriaxone pre-treatment would prevent thermal hyperalgesia and tactile allodynia caused by repeated morphine. Baseline Hargreaves and von Frey testing, performed on the day after the last ceftriaxone (or saline) injection and just before the first morphine (or saline) injection, revealed no differences between the groups in either test, indicating that ceftriaxone pre-treatment did not alter normal thermal and tactile sensation. Behavior testing 24 hours after the last morphine or saline injection showed that rats pre-treated with 7 days of intrathecal saline before daily morphine injections (saline-morphine rats) exhibited significantly shorter tail flick latencies than saline-saline rats, indicative of thermal hyperalgesia (Fig. 2A; $p < 0.05$). Similarly, saline-morphine rats exhibited significantly lower absolute paw withdrawal thresholds than saline-saline rats, indicative of tactile allodynia (Fig. 2B; $p < 0.05$). For both behavioral tests, responses of ceftriaxone-morphine rats were not significantly different from those of saline-saline rats. However, while a strong trend was apparent for ceftriaxone-morphine rat thresholds to differ from saline-morphine rats (Fig. 2A and 2B), this did not reach statistical significance.

Spinal protein changes—24 hours after the last morphine or saline injection, immediately following the final Hargreaves and von Frey testing point, lumbar spinal cord tissue was collected and analyzed by Western blot for GLT-1 and CD11b protein expression in membrane fractions, GLT-1 protein expression in cytoplasmic fractions, and GFAP protein expression in whole cord homogenates. As in Experiment 1, GLT-1 analysis revealed that cytoplasmic fractions contained GLT-1 monomer only, while membrane fractions contained GLT-1 monomer and dimer but not trimer. There was no difference between the groups when analyzing expression of CD11b, membrane-bound, dimerized GLT-1, or GLT-1 monomer in membrane or cytoplasmic fractions at this time-point (1 day after morphine; 7 days after the last intrathecal ceftriaxone injection, in agreement with the results of Experiment 1). In contrast, GFAP analysis showed a 19% increase in saline-morphine rats, but only a 1% increase in ceftriaxone-morphine rats, as compared with saline-saline rats (Fig. 3). Thus 5 days of repeated morphine injections caused significant astrocyte activation, which was prevented by 7-day pre-treatment with ceftriaxone, ending prior to the start of morphine (Fig. 3; $p < 0.05$).

Experiment 3: Intrathecal ceftriaxone treatment prevents the progression of paralysis and reverses tactile allodynia in the EAE model, and reverses GLT-1 downregulation and glial activation

Motor paralysis with high dose of MOG (10 µg)—To generate central neuropathy, the first group of rats was injected with MOG at a dose of 10 µg per rat (Fig. 4A). Daily intrathecal injections of ceftriaxone (or saline) began at the onset of motor symptoms, to allow a normal autoimmune response to develop before initiating treatment. The average time post-MOG injection until ceftriaxone (or saline) injections began was 8.0 ± 0.2 days. Ceftriaxone-treated rats exhibited an initial peak of motor symptoms after 4 days of treatment (average score = 2.4) but no further progression of paralysis (Fig. 4A). Saline-treated rats, however, exhibited a relapsing-remitting pattern of motor paralysis, and had significantly higher motor deficit scores than ceftriaxone-treated rats on days 14, 15, 19, and 21 of treatment, corresponding to the 2nd and 3rd peaks of motor symptoms (Fig. 4A; $p < 0.05$ or 0.01).

Motor function and tactile allodynia with lower dose of MOG (8.75 µg)—To allow analysis of allodynia unconfounded by hindpaw paralysis, a separate group of rats was injected with MOG at a lower dose (8.75 µg per rat) that would minimize impact on hindpaw motor function. Daily intrathecal injections of ceftriaxone (or saline) again began at the onset of motor symptoms. At this dose of MOG, the average time post-MOG injection

until ceftriaxone (or saline) injections began was 15.2 ± 0.3 days. For all rats regardless of treatment, the average motor score stayed between 2 and 3 for the duration of the study, indicating minimal impact on hindpaw function (Fig. 4B). Baseline von Frey testing performed two days before MOG injection indicated the average paw withdrawal threshold for all rats was 4.91 ± 0.34 g, with no difference between the groups. The rats exhibited a gradual decline in withdrawal thresholds over time, and this tactile allodynia was maximal before the onset of motor symptoms, when daily intrathecal injections began (Fig. 4C). After 9 days of injections, there was a significant difference in absolute withdrawal thresholds between ceftriaxone- and saline-treated rats (Fig. 4C; $p < 0.05$). This significant alleviation of tactile allodynia was also observed on days 11, 12, 13, 14 and 16 of treatment (Fig. 4C; $p < 0.05$ or 0.001).

Spinal protein changes—GLT-1 dimer and monomer, GFAP and CD11b protein levels were measured in lumbar spinal cord tissue from these rats, to assess the effect of ceftriaxone on GLT-1 expression and glial activation. Lumbar spinal cord tissue samples from naïve rats and rats injected with either dose of MOG were analyzed together. As in Experiments 1 and 2, GLT-1 analysis revealed that cytoplasmic fractions contained GLT-1 monomer only, while membrane fractions contained GLT-1 monomer and dimer but not trimer. As compared with naïve rats, membrane-bound, dimerized GLT-1 protein expression was reduced by 51% in saline-treated EAE rats (Fig. 5A; $p < 0.05$). This decrease was significantly reversed in EAE rats treated with ceftriaxone (Fig. 5A; $p < 0.05$). Expression of GLT-1 monomer in cytoplasmic fractions was not different between the groups. In contrast, there was a 43% decrease in membrane-bound GLT-1 monomer expression in saline-treated EAE rats, as compared with naïve rats, which was not reversed by ceftriaxone treatment (Fig. 5B; $p < 0.01$ and 0.05). Analysis of spinal GFAP protein expression indicated that EAE caused a significant 46% increase, which was reduced by 62% following ceftriaxone treatment (Fig. 5C; $p < 0.05$). Analysis of CD11b expression in membrane fractions revealed a 170% increase in saline-treated EAE rats (Fig. 5D; $p < 0.05$). CD11b expression in ceftriaxone-treated EAE rats was 108% increased over that of naïve rats, but was not significantly different from either the naïve group or the saline-treated EAE group (Fig. 5D).

Experiment 4: Intrathecal ceftriaxone treatment alleviates tactile allodynia in the CCI model, and normalizes GLT-1 downregulation and astrocyte activation

Allodynia—Baseline von Frey testing before CCI or sham surgery revealed no significant differences between the groups (Fig. 6A and 6B). At 11 days post-surgery, robust tactile allodynia was observed in both the ipsilateral and contralateral hindpaws of CCI rats. Daily intrathecal injections of ceftriaxone (or saline) began 12 days post-surgery, and after 20 days of treatment (32 days post-surgery) there was a significant attenuation of tactile allodynia in both the ipsilateral and contralateral hindpaws of ceftriaxone-treated rats (Fig. 6A and 6B; $p < 0.001$).

Spinal protein changes—Western blots were used to measure changes in spinal GLT-1 expression caused by CCI and ceftriaxone. As in Experiment 1, 2 and 3, GLT-1 analysis revealed that cytoplasmic fractions contained GLT-1 monomer only, while membrane fractions contained GLT-1 monomer and dimer but not trimer. There was no difference between the groups when analyzing GLT-1 monomer expression in membrane fractions. Analysis of GLT-1 dimer expression in membrane fractions revealed a significant downregulation in saline-treated CCI rats as compared with sham rats at 32 days after CCI surgery, which was normalized by treatment with ceftriaxone (Fig. 7A; $p < 0.05$). As measured in cytoplasmic protein fractions, CCI caused a significant 57% decrease in expression of GLT-1 monomer, which was not affected by treatment with ceftriaxone (Fig. 7B; $p < 0.01$).

Western blots were also used to measure GFAP protein in whole cord homogenates and CD11b protein in membrane fractions from the CCI and sham rats that had been tested for allodynia, above, as indicators of glial activation. Saline-treated CCI rats had a 65% increase in GFAP protein expression, as compared with sham rats, at 32 days after CCI surgery (Fig. 7C; $p < 0.05$). In contrast, GFAP protein expression in ceftriaxone-treated CCI rats was increased by only 17% as compared with sham rats, representing a significant 75% reduction as a result of treatment (Fig. 7C; $p < 0.05$). Analysis of CD11b expression revealed no significant difference between the groups at this time post-surgery (day 32).

DISCUSSION

The present studies explored the effect of ceftriaxone, a drug reported to upregulate the glutamate transporter GLT-1 (Rothstein et al., 2005), on three mechanistically distinct rat models of spinal neuroinflammation. This was done to define whether upregulating membrane-bound GLT-1 improves pain control and/or paralysis of diverse etiologies. The three models studied were repeated morphine, central neuropathy (EAE), and peripheral neuropathy (CCI). Ceftriaxone was delivered intrathecally to implicate the spinal cord as its likely site of action. Pre-treatment with ceftriaxone partially blocked the later development of repeated morphine-induced tactile allodynia and thermal hyperalgesia. Both peripheral neuropathy and central neuropathy caused downregulation of membrane-bound, dimerized GLT-1 in lumbar spinal cord, with this GLT-1 downregulation resolved by ceftriaxone treatment. In these same animals, ceftriaxone suppressed neuropathic pain (tactile allodynia) arising both from peripheral neuropathy and from central neuropathy, and prevented the progressive development of paralysis caused by EAE. Lastly, ceftriaxone treatment blocked elevated spinal GFAP expression induced by repeated morphine, reversed elevated spinal GFAP expression in the EAE and CCI models, and partially reduced elevated spinal CD11b expression in the EAE model. Thus, across diverse paradigms and endpoints, intrathecal ceftriaxone had therapeutic effects.

Prior studies on GLT-1 modulation in various nervous system pathologies, and in response to treatment with ceftriaxone, have analyzed expression of the GLT-1 monomer band in whole tissue samples. However, GLT-1 is a membrane-bound transporter. Further, Haugeto et al. (1996) showed that GLT-1 is only functional when it forms homomultimers, although their data was unable to resolve whether GLT-1 dimers or trimers or both constitute the active form. We therefore argue that measuring GLT-1 oligomers in membrane fractions is the most informative way to assess expression of GLT-1 using Western blots. With the recent advent of compartmental protein extraction kits, tissue samples can now be readily processed to prepare proteome fractions enriched for the various subcellular compartments. Our Western blots for GLT-1 in all membrane fraction samples, across two different rat strains, always showed a GLT-1 dimer and monomer band, but no trimer band. The monomer band observed in membrane fractions may be due to incomplete subcellular fractionation, as there was GAPDH detectable in membrane fractions. The dimer band observed likely does not reflect an artifact of tissue or sample processing given that all Western blots were run under reducing conditions, and the dimer was observed only in membrane but not cytoplasmic fractions, despite both fraction types being processed identically. As no prior study examined whether ceftriaxone upregulates membrane-bound GLT-1, the present study provides important new information on the actions of ceftriaxone in this regard.

The first model used here was repeated intrathecal morphine. Prior studies have shown that systemic treatment with ceftriaxone limits the development of tolerance to repeated morphine injections as well as alleviating naloxone-induced morphine withdrawal (Wang et al., 2008; Rawls et al., 2009). The present data show that intrathecal ceftriaxone pre-

treatment partially blocked the development of paradoxical pain caused by repeated morphine, and completely prevented associated astrocyte activation. It is striking that there was no change in spinal GLT-1 expression induced by morphine at the end of the study. This may reflect that the rats received only 5 days of morphine injections and tissue collection was delayed until 24 hr after the last morphine injection. Others have found that spinal GLT-1 expression is initially upregulated, before ultimately being downregulated, in response to nervous system injury (Sung et al., 2003). This biphasic change in GLT-1 expression is thought to reflect an initial compensatory neuroprotective mechanism that is ultimately lost. It is possible that spinal GLT-1 expression in the morphine-treated rats was transitioning from a state of being upregulated to one of downregulation, and with continued daily morphine injections a separation between the groups might have developed. The discrepancy of behavioral differences between the four groups without differences in spinal GLT-1 expression suggests that ceftriaxone might be able to modulate GLT-1 function independent of GLT-1 protein expression, a possibility supported by prior work showing that ceftriaxone increased GLT-1 activity without increasing GLT-1 mRNA or protein levels in a rat model of stroke (Thone-Reineke et al., 2008).

In the EAE model, the present data show that downregulation of membrane-bound, dimerized GLT-1 persists through at least one month post-MOG immunization. The studies presented here utilized intrathecal delivery of ceftriaxone to avoid the confound of peripheral or supraspinal effects, and are the first to demonstrate the ability of intrathecal ceftriaxone treatment to reverse sensory symptoms and halt the progression of motor paralysis caused by EAE. Further, Western blot analyses of lumbar spinal cord tissue from these animals provide the first evidence that ceftriaxone reverses astrocyte activation in the EAE model, and partially affects microglial/monocyte activation as well.

It has previously been observed that spinal expression of the GLT-1 monomer in whole tissue homogenates is reduced by 50% in rats with MOG-induced EAE at two weeks after immunization with MOG (Ohgoh et al., 2002). As discussed above, interpretation of these results is problematic without analysis of membrane-bound GLT-1 expression. In the one prior study using ceftriaxone to treat motor symptoms caused by EAE, ceftriaxone was administered systemically (200 mg/kg) to mice with MOG-induced chronic EAE (Melzer et al., 2008). Treatment started the first day that a motor score above 0 was observed, and ceftriaxone-treated EAE mice had significantly lower motor scores than vehicle-treated EAE mice at 50 days post-MOG injection. In that study, however, there was no difference between ceftriaxone-treated and vehicle-treated EAE mice, when analyzing GLT-1 monomer in whole spinal cord homogenates. Our data confirm that EAE causes spinal GLT-1 monomer expression to decrease by ~50%, specifically in membrane fractions. This reduction was not reversed by ceftriaxone, in agreement with Melzer et al. (2008). However, ceftriaxone did reverse the reduction of membrane-bound, dimerized GLT-1, and did alleviate EAE-induced pain, paralysis, and glial activation. These data underscore the importance of measuring oligomerized membrane-bound GLT-1.

The present study is the first to demonstrate that CCI causes significant reduction of spinal expression of membrane-bound, dimerized GLT-1 protein, and that ceftriaxone treatment normalizes this reduction. Additionally, our data are the first to show that ceftriaxone treatment suppresses spinal astrocyte activation caused by CCI. Others have shown that spinal expression of the GLT-1 monomer in whole cord homogenates is reduced by approximately 50% two weeks post-CCI (Sung et al., 2003; Hu et al., 2009). Our results confirm that CCI reduces lumbar GLT-1 monomer expression by ~50%, but this was observed in functionally irrelevant cytoplasmic fractions only and was not affected by ceftriaxone. As in the EAE model, the ability of ceftriaxone to attenuate neuropathic pain and glial activation in the CCI model correlates with the normalization of membrane-bound,

dimerized GLT-1 protein, again highlighting the importance of measuring this form of GLT-1.

During the preparation of this manuscript, Hu et al. (2009) reported that intrathecal ceftriaxone treatment (100 µg per day) reversed CCI-induced thermal hyperalgesia and tactile allodynia. Their data showed that 2 days of treatment, beginning at 9 days post-surgery, produced a small but significant suppression of CCI-induced allodynia, whereas we found a much longer treatment period (20 days) was necessary. It is, however, difficult to compare the two data sets given the numerous procedural differences, including the use in the Hu et al. study of daily anesthesia and lumbar puncture, undefined time of testing relative to daily injections, and markedly higher (40 g) baseline thresholds suggestive of markedly different testing methodologies.

The ability of ceftriaxone to normalize or prevent astrocyte activation in all three models is novel and especially interesting. Though our experiments did not explore the mechanism by which ceftriaxone alters astrocyte activation, this question merits future investigation given that ceftriaxone has exhibited therapeutic efficacy in a range of CNS disorders, as shown here and by others. Rasmussen et al. (2010) have demonstrated that repeated systemic ceftriaxone treatment produced a dose-dependent decrease in extracellular glutamate levels in the nucleus accumbens of the rat, mediated specifically by GLT-1 transporters. It would be of value to know if the ceftriaxone-induced reduction in astrocyte activation observed here were due to a GLT-1-mediated decrease in extracellular glutamate levels as well.

Another unexplored question pertains to cell type-specific spinal GLT-1 localization in the models used here, and thus ceftriaxone's cellular site of action. Early studies showed that GLT-1 is primarily localized to astrocytes, and GLT-1 has been historically referred to as an astrocytic glutamate transporter (Rothstein et al., 1994). However, several studies have documented neuronal GLT-1 expression in various brain regions, both in cell bodies and axon terminals (Schmitt et al., 2002; Chen et al., 2004; Furness et al., 2008; Melone et al., 2009). To date, immunohistochemistry has indicated that GLT-1 is expressed by growing axons in the developing spinal cord, but solely by astrocytes in the adult spinal cord (Yamada et al., 1998; Schmitt et al., 2002). It is possible that using different immunohistochemical techniques GLT-1 expression might be revealed in spinal cell types other than astrocytes, or that it may be expressed *de novo* beyond astrocytes under conditions of pathology. To our knowledge, this latter issue has been explored only in a model of partial sciatic nerve ligation, wherein spinal microglia but not neurons exhibited *de novo* GLT-1 expression after nerve injury (Xin et al., 2009). The question of whether GLT-1 expression near synapses is limited to astrocytes or also found in axon terminals requires analysis by electron microscopy to determine with certainty whether GLT-1 molecules are inserted into astrocytic or neuronal membranes. Indeed, electron microscopy is the sole technique that has been used to document GLT-1 expression in axon terminals in various brain regions (Chen et al., 2004; Furness et al., 2008; Melone et al., 2009). Interestingly, the only report on ultrastructural localization of GLT-1 in the spinal cord showed by electron microscopy that GLT-1 was not present in axon terminals in the adult mouse spinal cord (Yamada et al., 1998).

In conclusion, upregulating GLT-1 expression in lumbar spinal cord with ceftriaxone attenuates opioid-induced pain and astrocyte activation, reverses established neuropathic pain, prevents the worsening of paralysis in a rat model of multiple sclerosis, and normalizes glial activation associated with chronic nervous system disease. These data show that GLT-1 is a promising therapeutic target for treating chronic pain, and likely for reversing glial activation in a range of other CNS disorders.

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LIST OF ABBREVIATIONS

GLT-1	glutamate transporter (EAAT2)
EAE	experimental autoimmune encephalomyelitis
CCI	chronic constriction injury
CNS	central nervous system
CSF	cerebrospinal fluid
MOG	myelin oligodendrocyte glycoprotein
TBS-T	tris-buffered saline with tween-20
P-gp	permeability-glycoprotein
GFAP	glial fibrillary acidic protein

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A. 24 hours after last ceftriaxone injection **B. 1 week after last ceftriaxone injection** **Figure 1.**

Ceftriaxone upregulates spinal expression of membrane-bound, dimerized GLT-1 in naïve rats. Naïve rats treated with intrathecal ceftriaxone for 7 days (150 µg daily) exhibited a 77% increase in expression of membrane-bound, dimerized GLT-1 protein (A), as compared with saline-treated rats (naïve + saline, $n = 5$; naïve + ceftriaxone, $n = 5$). In a second group of rats, 7 days of daily intrathecal injection with ceftriaxone (150 µg daily) or saline was followed by 7 days without treatment (B). Western blot analysis with samples from these animals indicated that at one week after the final ceftriaxone (or saline) injection, expression of membrane-bound, dimerized GLT-1 was not different between the two groups (naïve + saline, $n = 6$; naïve + ceftriaxone, $n = 5$). Data are presented as mean $\pm$ SEM. * $p < 0.05$ naïve + saline vs. naïve + ceftriaxone. Western blots shown are examples of each treatment group.

Figure 2.

Ceftriaxone partially blocks repeated morphine-induced thermal hyperalgesia and tactile allodynia. Rats pre-treated with saline before morphine injections exhibited thermal hyperalgesia as compared with rats pre-treated with saline before saline injections (**A**), and this hyperalgesia was not present in rats pre-treated with ceftriaxone before saline or morphine injections. Similarly, rats pre-treated with saline before morphine injections exhibited tactile allodynia as compared with rats pre-treated with saline before saline injections (**B**), and this allodynia was not present in rats pre-treated with ceftriaxone before saline or morphine injections. (Saline-saline, $n = 7$; saline-morphine, $n = 13$; ceftriaxone-saline, $n = 7$; ceftriaxone-morphine, $n = 12$.) Data are presented as mean $\pm$ SEM. * $p < 0.05$ saline-morphine vs. saline-saline rats.

GFAP protein

Figure 3.

Ceftriaxone prevents repeated morphine-induced spinal astrocyte activation. Rats pre-treated with saline before 5 d of daily intrathecal morphine injection exhibited significant upregulation of GFAP protein, as compared with saline-saline rats. In contrast, ceftriaxone pre-treatment blocked this morphine-induced GFAP upregulation. (Saline-saline, $n = 7$; saline-morphine, $n = 13$; ceftriaxone-saline, $n = 7$; ceftriaxone-morphine, $n = 12$.) Data are presented as mean $\pm$ SEM. * $p < 0.05$ saline-morphine rats vs. saline-saline rats and vs. ceftriaxone-morphine rats. S-S = saline-saline; S-M = saline-morphine; C-S = ceftriaxone-saline; C-M = ceftriaxone-morphine. Western blots shown are examples of each treatment group.

Figure 4.

Ceftriaxone prevents the progression of paralysis and reverses tactile allodynia in the EAE model. After injection of 10 µg MOG per rat, saline-treated rats exhibited a relapsing-remitting pattern of motor paralysis, with 3 peaks of symptom expression (A). Ceftriaxone-treated rats exhibited an initial peak of motor symptoms but no further progression of paralysis, resulting in a significant difference between the treatment groups during the 2nd and 3rd peaks of motor symptoms (10 µg MOG + saline, $n = 10$; 10 µg MOG + ceftriaxone, $n = 8$). After injection of 8.75 µg MOG per rat, the average motor score stayed between 2 and 3 for the duration of the study, regardless of treatment (B). At the onset of motor symptoms, all rats exhibited stable tactile allodynia (C), which was reversed after 9 d of daily intrathecal treatment with ceftriaxone ($p < 0.001$; interaction of drug and time,

$F_{(22,198)} = 8.351$; 8.75 μg MOG + saline, $n = 6$; 8.75 μg MOG + ceftriaxone, $n = 5$). Data are presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ceftriaxone vs. saline treatment. BL = baseline before MOG injection.

Figure 5.

EAE-induced down-regulation of membrane-bound, dimerized GLT-1 and up-regulation of GFAP in the spinal cord are normalized by treatment with ceftriaxone. A significant decrease in membrane-bound, dimerized GLT-1 protein expression was observed in saline-treated EAE rats as compared with naïve rats, which was reversed by ceftriaxone treatment (A). Also in membrane protein fractions, saline-treated EAE rats exhibited a significant decrease in GLT-1 monomer expression as compared with naïve rats, but this was not affected by treatment with ceftriaxone (B). As compared with naïve rats, saline-treated EAE rats expressed significantly increased spinal GFAP protein, which was reduced by treatment with ceftriaxone (C). As compared with naïve rats, saline-treated EAE rats expressed significantly increased spinal CD11b protein (D). In ceftriaxone-treated EAE rats, spinal CD11b expression was not significantly different from naïve rats or from saline-treated EAE rats. (Naïve, $n = 3$; EAE + saline, $n = 16$; EAE + ceftriaxone, $n = 13$.) Data are presented as mean $\pm$ SEM. * $p < 0.05$, *** $p < 0.001$, EAE + saline vs. naïve and vs. EAE + ceftriaxone. x $p < 0.05$, naïve vs. EAE + ceftriaxone, xx $p < 0.01$, naïve vs. EAE + saline. + $p < 0.05$, naïve vs. EAE + saline. Western blots shown are examples of each treatment group.

A. Tactile allodynia – ipsilateral hindpaw

B. Tactile allodynia – contralateral hindpaw

Figure 6.

Tactile allodynia induced by CCI is attenuated by treatment with ceftriaxone. Daily intrathecal treatment with saline or ceftriaxone (150 μ g) began 12 d post-surgery, and after 20 d of treatment, ceftriaxone alleviated tactile allodynia in both the ipsilateral (**A**) and contralateral (**B**) hindpaw ($p < 0.001$; interaction of drug and time: $F_{(8,112)} = 4.483$ for ipsilateral hindpaw, $F_{(8,112)} = 7.941$ for contralateral hindpaw; sham + saline, $n = 6$; CCI + saline, $n = 11$; CCI + ceftriaxone, $n = 14$). Data are presented as mean $\pm$ SEM. Open circles, sham + saline; open squares, CCI + saline; closed triangles, CCI + ceftriaxone. *** $p < 0.001$, CCI + ceftriaxone vs. CCI + saline. BL = baseline before surgery.

Figure 7.

CCI-induced down-regulation of membrane-bound, dimerized GLT-1 and up-regulation of GFAP in the spinal cord are normalized by treatment with ceftriaxone. There was a significant decrease in membrane-bound, dimerized GLT-1 protein expression in saline-treated CCI rats that was reversed in CCI rats treated with ceftriaxone (A). As compared with sham rats, saline-treated CCI rats expressed significantly increased spinal GFAP protein, which was alleviated by treatment with ceftriaxone (C). (Sham + saline, $n = 6$; CCI + saline, $n = 11$; CCI + ceftriaxone, $n = 14$.) Data are presented as mean $\pm$ SEM. * $p < 0.05$, CCI + saline vs. sham + saline and vs. CCI + ceftriaxone; ** $p < 0.01$, sham + saline vs. CCI + saline and vs. CCI + ceftriaxone. Western blots shown are examples of each treatment group.