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ALTERED SPINAL ARACHIDONIC ACID TURNOVER AFTER PERIPHERAL NERVE INJURY REGULATES REGIONAL GLUTAMATE CONCENTRATION AND NEUROPATHIC PAIN BEHAVIORS IN RATS

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Abstract

Spinal glutamate transporters (GT) have been implicated in the mechanisms of neuropathic pain; however, how spinal GT uptake activity is regulated remains unclear. Here we show that alteration of spinal arachidonic acid (AA) turnover after peripheral nerve injury regulated regional GT uptake activity and glutamate homeostasis. Chronic constriction nerve injury (CCI) in rats significantly reduced spinal GT uptake activity (³H-glutamate uptake) with an associated increase in extracellular AA and glutamate concentration from spinal microdialysates on postoperative day 8. AACOCF3 (a cytosolic phospholipase A2 inhibitor, 30 μg) given intrathecally twice a day for postoperative day 1 – 7 reversed this CCI-induced spinal AA production, prevented the reduced spinal GT uptake activity and increased extracellular glutamate concentration. Conversely, alteration of spinal AA metabolism by diclofenac (a cyclooxygenase 1/2 inhibitor, 200 μg) further reduced spinal GT uptake activity and increased extracellular glutamate concentration in CCI rats. GT uptake activity was also attenuated when AA (10 or 100 nM) was directly added into spinal samples of naïve rats in an *in vitro* ³H-glutamate uptake assay, indicating a direct inhibitory effect of AA on GT uptake activity. Consistent with these findings, AACOCF3 reduced the development of both thermal hyperalgesia and mechanical allodynia, whereas diclofenac exacerbated thermal hyperalgesia, in CCI rats. Thus, spinal AA turnover may serve as a regulator in CCI-induced changes in regional GT uptake activity, glutamate homeostasis, and neuropathic pain behaviors. These data suggest that regulating spinal AA turnover may be a useful approach to improving the clinical management of neuropathic pain.

Keywords

Glutamate transporter; neuropathic pain; nerve injury; phospholipase A2; Arachidonic acid; glutamate uptake; microdialysis; High pressure liquid chromatography; Gas chromatography

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Introduction

Peripheral nerve injury can result in signs and symptoms of neuropathic pain such as allodynia, hyperalgesia, and spontaneous pain (Wall et al. 1974;Devor 1983;Woolf and Mannion 1999;Zimmermann 2001). Primary sensory afferents release glutamate into synaptic clefts within the spinal cord dorsal horn when stimulated by physical and/or chemical sources (al Ghouli et al. 1993). While glutamate plays a key role in a number of physiological functions (Maragakis and Rothstein 2004), excessive and sustained excitation of glutamate receptors has been implicated in the mechanisms of neurotoxicity and neuropathic pain (Yamamoto and Yaksh 1992;Mao et al. 1992a;Mao et al. 1992b;Mao et al. 1995). Regional glutamate homeostasis is maintained mainly through a high capacity glutamate transporter (GT) system that exists perisynaptically with neuronal and glial cells (Robinson and Dowd 1997;Semba and Wakuta 1998;Danbolt 2001;Kanai and Hediger 2003). The activity of GT directly influences synaptic glutamate turnover through removing glutamate from synaptic cleft (Maragakis and Rothstein 2004), and may play a pivotal role in a variety of neurodegenerative diseases (Lievens et al. 2000;Trotti et al. 2001;Urban et al. 2003).

In a previous study (Sung et al. 2003), we have shown that peripheral nerve injury resulted in an initial increase in spinal GT expression followed by a progressive downregulation of spinal GT expression mediated through a mitogen-activated protein kinase pathway, which contributed to the development and maintenance of neuropathic pain behaviors in rats. However, it remains unclear as to how GT activity is regulated by endogenous regulators in relation to the pathogenesis of neuropathic pain.

Arachidonic acid (AA), produced from membranes of both neuronal and glial cells in response to pathological insults (Danbolt 2001), is a cell membrane lipid product and a potent inhibitor of GT activity in *in vitro* studies (Volterra et al. 1994;Trotti et al. 1995;Manzoni and Mennini 1997;Dorandeu et al. 1998). The inhibitory effect of AA on GT activity is more potent in the cerebellum and spinal cord than in the hippocampus, which is likely due to its direct inhibitory action on GT (Dorandeu et al. 1998). Furthermore, the AA metabolic pathway has been well delineated, which begins with activation of phospholipase A2 (PLA2) resulting in the enzymatic hydrolysis of AA from lipid membranes and is followed by the downstream AA metabolism via several enzymatic pathways including cyclooxygenase 1 and 2 (COX-1/2) (Raja et al. 1988;Sawin et al. 1997;Kirchheiner et al. 2003;Farooqui and Horrocks 2004). If AA were an active endogenous regulator of GT activity, it would be expected that regulation of regional AA turnover through inhibition of either PLA2 or COX-1/2 should alter GT uptake activity and regional glutamate concentration with a concurrent change in neuropathic pain behaviors following peripheral nerve injury. This hypothesis was examined in the present study by utilizing a rat model of chronic constriction nerve injury (CCI) and a combination of spinal ³H-glutamate uptake assay, intrathecal microdialysis, regional glutamate and AA assay by high performance liquid chromatography (HPLC) and gas chromatography (GC), and behavioral testing.

Materials and Methods

Animal surgery and drugs

Adult male Sprague Dawley rats weighing 275–325 gm (Charles River Laboratories, Wilmington, MA) were used. The animal room was artificially lighted from 7:00 A.M. to 7:00 P.M. The surgical procedure was performed aseptically under pentobarbital anesthesia (50 mg/kg, intraperitoneally). CCI rats were produced by loosely ligating one common sciatic nerve according to the method of Bennett and Xie (Bennett and Xie 1988). Briefly, on one side the rat's sciatic nerve was exposed in the mid-thigh, and four loose ligatures using 4.0 chromic gut sutures were made around the dissected sciatic nerve with a 1.0–1.5 mm interval between each

ligature. Skin wound was closed with wound clips. Sham rats were made following the same surgical procedure except for nerve ligation. The general experimental protocol was approved through our Institutional Animal Care and Use Committee.

A cytosolic PLA₂ inhibitor, AACOCF₃ (Lucas et al. 2005), and a COX-1/2 inhibitor, diclofenac (Kirchheiner et al. 2003), were used to regulate the AA metabolism. The final dose for either AACOCF₃ (30 µg) or diclofenac (200 µg, (Pinardi et al. 2003)) used in this study was determined after a pilot dose-response experiment for each agent. We first examined the effect of each agent (30, 60 µg for AACOCF₃ and 200, 400 µg for diclofenac) using a small number (3) of rats. We did not find further increase in the effects of each agent with a higher dose. To avoid possible neurological toxicity such as gait and sensory function, we chose to use AACOCF₃ (30 µg) and diclofenac (200 µg) in the experiments. Both agents were purchased from Sigma (St. Louis, MO). AACOCF₃ was dissolved in 100% DMSO and diclofenac in 5% DMSO and 95% artificial CSF. The 100% and 5% DMSO solutions were used as vehicles for the AACOCF₃ and the diclofenac, respectively. Vehicles contained the same solution as that for AACOCF₃ or diclofenac. Each agent (10 µl volume) was injected intrathecally (i.t.) using a microsyringe injector followed by a saline flush (10 µl). The injections were given twice a day at 9:00 AM and 5:00 PM beginning within 1 min after the operation. Doses were divided into two injections each day for the drugs to be distributed more constantly.

Behavioral tests

Animals were habituated to the test environment for 60 min per day for two consecutive days before baseline testing. All animals were tested for thermal hyperalgesia and mechanical allodynia. For testing thermal hyperalgesia, the plantar surface of a rat's hindpaw was exposed to a beam of radiant heat through a transparent perplex glass surface (Hargreaves et al. 1988). The withdrawal latency was averaged from at least two trials separated by a 2-min interval, and the cutoff was set at 25 sec to avoid tissue damage. For examining mechanical allodynia, each rat was placed on a metal mesh floor and covered with a plastic box (15×15×18 cm). Each test began after a 30 min period of habituation. The mechanical stimulation resulting from the bending force of a von Frey filament was applied to the plantar surface of each hindpaw. Each trial consisted of five applications of a von Frey filament given every 4 sec, and the cutoff force was 20 gm. Brisk foot withdrawals (at least three times of five applications) in response to von Frey filament stimulation were considered positive. Depending on the initial response, subsequent filaments were applied in the order of either descending or ascending force to determine the threshold force (Tal and Bennett 1994; Mao et al. 1997).

Construction and placement of a microdialysis catheter

Intrathecal loop dialysis catheters were constructed from hollow fibers (200 µm ID, 216 µm OD, 18,000 M.W. cut-off; Spectra/Por, Part No:132295, www.spectrapor.com) according to the modified method described by Hua et al. (1999). A Nichrome wire (50 µm thick, Part No: 7620, A-M systems, www.a-msystems.com) was then passed through the fiber. The fiber was then bent in the middle to form a "U"-shaped loop. Both ends of this fiber were attached to the two side holes of the triple lumen tubing of 8 cm long (Part No; E100-0353, Spectranetics, 1-800-231-0978, www.spectranetics.com) by a 1 cm-long polycarbonate tubing connector (Part No; 2000021; Polymicro Tech., Inc. 1-602-375-4100, www.polymicro.com). The three holes at the other end of the triple lumen tubing were connected to PE-10 tubing (5 cm in length) by the above-mentioned polycarbonate tubing connector with cyanoacrylate.

To place a microdialysis catheter of which each triple lumen connected to a PE-10 tubing (Yaksh and Rudy 1976), rats were anesthetized with pentobarbital as described above. The animals were then placed in a stereotaxic head holder with the head slightly flexed forward. After the cisternal membrane was exposed and cut open, the end of the U-shaped loop of the

catheter was inserted and passed caudally to the lumbar intrathecal space (about 9 cm from the incision site for the size of rats used in the study). The other end of the dialysis catheter was then externalized and secured on the neck. Of the three external tubing holes, the median hole (a PE 10 catheter) was used as a drug injection while the other holes at both sides were reserved for microdialysis study – one end for infusing the artificial cerebrospinal solution and the other end for collecting the infused solution.

All animals were allowed to recover for three days prior to the experiments. Those rats exhibiting postoperative neurological deficits or poor grooming were excluded from the experiments. We only observed fewer than 10 % animals with neurological changes after the catheter insertion with the size of rats described above. We did not experience blockage of the intrathecal catheter for microperfusion and drug injection when the experiments were conducted under 8 days after the implantation.

Intrathecal microdialysis

To initiate dialysis, one of the externalized PE-10 connections was attached to a piece of PE-10 tubing (inflow, 30 cm in length) and the other arm to another PE-10 tubing (outflow, 25 cm in length) in an awake animal gently placed in a container. A syringe pump (Harvard Compact Infusion Pump, Model 975 with a 5 ml plastic syringe) was connected and the dialysis catheter was perfused with artificial cerebrospinal fluid (ACSF) at a rate of 10 $\mu\text{l}/\text{min}$. ACSF contained (mM) Na^+ 151.1, K^+ 2.6, Mg^{2+} 0.9, Ca^{2+} 1.3, Cl^- 122.7, HCO_3^- 21.0, HPO_4^- 2.5 and dextrose 3.5. ACSF was bubbled with 95% O_2 /5% CO_2 before each experiment to adjust the final pH to 7.2. The dialysis procedure typically began with a 30-min washout period followed by two collection periods of 10 min each. The single median PE-10 catheter was used only for intrathecal drug injection in order to avoid potential interference with the dialysis.

Spinal glutamate concentration assay (HPLC)

Glutamate concentration from the intrathecal microdialysates was analyzed using HPLC according to a modified method (Hua et al. 1999). The analysis was accomplished by using the phenylisothiocyanate (PITC) derivatization procedure (Shimadzu HPLC, SCL 10A-VP) with Waters AccQ•Taq amino acid analysis column (3.9 \times 150 mm, 4 μm particle size, silica base bonded with C18) and a UV detector (254 nm, Shimadzu, SPD-10A VP). A volume of 40 μl microdialysates was vacuum-dried after being mixed with 4 μl methionine sulfone (0.2 mM in 0.1 M HCl) as an internal standard. The mixtures were dried again after adding a coupling buffer [20 μl , methanol:water:triethylamine (TEA), 2:2:1 in volume ratio]. The coupled amino acids were derivatized with PITC (Molnar-Perl I 1994). The derivatization reagent (20 μl , methanol:water:TEA:PITC, 7:1:1:1 in volume ratio, freshly made on each day of assay) was reacted with the coupled amino acids for 20 minutes at room temperature. Excessive PITC was removed through the vacuum-dry process. Derivatized amino acid samples were then reconstituted with 70 μl of 0.05 M sodium acetate buffer before being injected (25 μl of reconstituents) by an autosampler (Shimadzu, SIL-10AF).

The solvent system consisted of two eluents: A) an aqueous buffer and B) 60% acetonitrile in water (Bidlemeier et al. 1984). A typical aqueous buffer was 0.14 M sodium acetate containing 0.5 ml/L TEA and titrated to pH 6.35 with glacial acetic acid. A gradient, which was run for the separation, consisted of 10% B eluent traversing to 51% B eluent in 10 min. After this step, a washing step was programmed to 100% B eluent so that any residual sample components were cleaned from the column. The accuracy of the measurement was expressed as the ratio of glutamate concentration determined by standard curves to the known concentration of glutamate together with methionine sulfone added as an internal standard. Standard curves were made daily by injections of 1 to 10 μM of L-glutamic acid (M.W. 147.1) from Sigma. These curves, with Correlation Coefficients of 0.990 or greater, were linear within

the range between 0.1 and 10 μM of glutamate concentrations. For a given of experiment, the measurement quality control was made using five replicates of quality control samples in which glutamate concentrations ranged from 0.1 to 10 μM . Between days, the measurement quality control was made once a week by using freshly prepared control samples of same concentrations as above. Glutamate Ratio over the methionine sulfone as an internal standard was measured and expressed in the Y-axis in figures.

Spinal glutamate uptake assay

A spinal synaptosome (GT) preparation was used to assess glutamate uptake activity according to a previously published method (Mitrovic et al. 1999; Azbill et al. 2000; Mu et al. 2000). In brief, animals were sacrificed under pentobarbital after microdialysis. Fresh tissue samples from L4 and L5 spinal dorsal horns were removed as quickly as possible by laminectomy. Dorsal horns ipsilateral and contralateral to CCI were homogenized separately in an ice-cold buffer solution (pH 7.2) containing 0.32 M sucrose plus (in g/ml) 4 pepstatin, 5 aprotinin, 20 trypsin inhibitor (# T1426, Sigma, St. Louis, MO), 4 leupeptin, and (in mM) 0.2 PMSF (phenylmethanesulfonyl fluoride), 2 EDTA, 2 EGTA, and 20 HEPES. The homogenates were centrifuged at 1,500 rpm for 10 min at 4°C and the supernatant was collected. The remaining pellets were resuspended by using the same buffer solution and recentrifuged as above. Both supernatants were combined and again centrifuged at 13,000 rpm for 10 min at 4°C. The so-obtained synaptosomal pellets, which contained both neuronal and glial GT (Mitrovic et al. 1999; Azbill et al. 2000; Mu et al. 2000), were suspended in 2 ml of Locke's buffer containing (in mM) 154 NaHCO_3 , 5.6 glucose, 5 HEPES, pH 7.2, and saturated with 95% O_2 /5% CO_2 . The protein concentration of final synaptosome pellets was measured by the Bradford method and was adjusted to 200 $\mu\text{g/ml}$ in Locke's buffer.

Glutamate uptake activity was determined by incubating the synaptosome preparation (in 100 μg protein content) with 0.4 μl [^3H]L-glutamate (Perkin-Elmer, Boston, MA) at 37°C for 5 min. The reaction was terminated by filtering synaptosomes through a Whatman (Maidstone, UK) GF/C 2.4 cm filter that was presoaked in the same buffer solution. The filter was then washed three times with ice-cold Locke's buffer (2 ml) and transferred to a vial containing scintillation mixture (10 ml; Fisher Scientific, Houston, TX). Radioactivity in the final samples was measured by a liquid scintillation counter (Bio-Rad). The basal uptake activity in counts per minute (cpm) was measured in the absence of any sample.

The unit of cpm measured by the instrument was converted to the mole concentration of radioactive glutamate or ^3H -glutamate uptaken to GT by using the following conversion units:

$$\begin{aligned} &\text{Number of mole of } ^3\text{Hglutamate in the reaction (mmole/g)} \\ &= (\text{Sample Radioactivity (cpm)} - \text{basal uptake activity (cpm)}) \times \text{Efficiency of the} \\ &\text{liquid scintillation counter} \times \\ &\frac{\text{Ci}}{2.22 \times 10^{12} \text{ dpm}} \times \frac{1}{\text{stock con'c of } ^3\text{Hglutamate}} \times \frac{1}{\text{synaptosome protein (g)}} \end{aligned}$$

Note: the efficiency of the liquid scintillation counter = 0.612 dpm/cpm; 1 Ci = 2.22×10^{12} disintegrations per minute (dpm); stock concentration of the ^3H -glutamate by the manufacturer = 52.0 Ci/mmol; the synaptosome included in each reaction = 10^{-4}g .

Spinal AA concentration assay (GC)

Arachidonic acid in spinal microdialysates was analyzed according to a previously described method (Pound et al. 2001). A volume of 40 μl microdialysate with 2 μg tricosanoic acid as an internal standard was mixed with 1.5 ml BF_3 /Methanol (Product No: 49370, Pierce Co. Rockford, IL) and 2 ml 100% hexane (Sigma, St. Louis, MO). The mixture in the screw capped-glass tube filled with Nitrogen gas was heated to 100°C for 60 minutes in a heat-block. The tube was cooled to room temperature, 1.0 ml H_2O was added and the contents mixed with a

vortex for 1 minute. Two phases were separated; the top hexane phase was placed in a new tube for an extraction to be repeated once again. The hexane phase was dried under nitrogen gas in a fume hood and redissolved with 40 μ l of hexane. The redissolved sample was transferred to a GC tube and sealed with crimper. Arachidonic acid methyl esters were quantified with GC/MS by using an HP-5890 Series II gas chromatograph equipped with a Supelcowax SP-10 capillary column (Supelco, Bellefonte, PA, USA). The temperatures of the injector and detector were 260°C and 280°C, respectively. The oven program was maintained initially at 150°C for 2 min, ramped to 200°C at 10°C/min and held for 4 min, ramped again at 5°C/min to 240°C, held for 3 min, and finally ramped to 270°C at 10°C/min and maintained for 5 min. The carrier gas-flow rate was held constantly at 0.8 mL/min throughout the process. The total ion was monitored and encompassing mass ranged from 50 to 550 atomic mass units. The fatty acid (AA) mass was determined by comparing various areas of analyzed fatty acids to that of a fixed concentration of internal standard tricosanoic acid. Tricosanoic acid was an internal standard and the ratio of AA over tricosanoic acid was expressed in the Y-axis.

For the AA analysis by GC, the internal standard, tricosanoic acid, and AA were quantified by the peak area analysis. The detector response was linear, with correlation coefficients of 0.990 or greater within the sample concentration ranges for all standards. The accuracy of the method was expressed as the ratio of AA measured by the GC to the known concentration of the AA standard from Sigma. The assay precision was assessed by calculating the standard deviation of repeated measurements as a percentage of the mean value. Within-day and between-day quality controls were made using five replicates of quality control samples and freshly prepared control samples once a week, respectively, as described for HPLC.

Statistical data analysis

Data from both thermal hyperalgesia and mechanical allodynia tests were analyzed by using nonparametric Friedman Repeated Measures ANOVA on Ranks across various time points to detect overall differences in each groups followed by the Dunnett's method. For comparisons between each group, Mann-Whitney Rank Sum Test was used. For glutamate uptake and dialysis data analysis, either Kruskal-Wallis one-way ANOVA on Ranks followed by the Dunn's method or Student's t-test was used according to the normality test. For all data analysis, differences were set as statistically significant at the level of $\alpha=0.05$.

Results

Decreased spinal GT uptake following CCI: effect of AACOCF3 and diclofenac

Spinal GT uptake activity was significantly reduced within the ipsilateral spinal cord dorsal horn of CCI rats (n=5) as compared to the sham control (n=5), when examined on postoperative day 8 (Fig. 1, $P < 0.01$). In addition, our pilot time course experiments showed a statistically significant change of GT uptake activity on postoperative day 8, which was consistent with the behavioral change on that day after CCI (see below). In our previous study (Sung et al. 2003), we reported that the GT expression showed a biphasic pattern in CCI rats with an initial GT upregulation on postoperative days 1 and 4 and followed by GT downregulation on postoperative days 7 and 14. In the present study, we selected the samples on postoperative day 8 for assays because the GT downregulation was stable at postoperative day 8, which was similar to that on postoperative day 14. There were also no differences in pain behaviors between postoperative days 8 and 14. Thus, the data analysis was focused on the ipsilateral spinal cord dorsal horn on postoperative day 8 following CCI.

The GT uptake activity within the ipsilateral spinal cord dorsal horn of CCI rats (n=7) treated with the PLA2 inhibitor AACOCF3 (30 μ g, i.t., twice daily for postoperative day 1–7) was nearly seven folds as high as in those CCI rats (n=5) treated with a vehicle, when examined

on postoperative day 8 (Fig. 1, $P < 0.05$). The GT uptake activity also was significantly higher in those CCI rats treated with AACOCF3 than in sham-operated rats ($n=5$, $P < 0.05$), while AACOCF3 alone did not significantly change GT uptake activity in sham-operated rats ($n=8$). In contrast, the GT uptake activity within the ipsilateral spinal cord dorsal horn was further reduced in CCI rats ($n=6$) treated with diclofenac (a COX-1/2 inhibitor, 200 μg , i.t., twice daily for postoperative day 1–7) as compared to both sham-operated rats ($n=5$) and CCI rats ($n=5$) treated with a vehicle (Fig. 1, $P < 0.05$). These results indicate that AACOCF3 effectively prevented, while diclofenac further exacerbated, the reduced spinal GT uptake activity following CCI.

Increased spinal glutamate concentration following CCI: effect of AACOCF3 and diclofenac

Spinal microdialysates were assayed using HPLC to measure regional glutamate concentration. On postoperative day 8, there was a significant increase (102%) in regional glutamate concentration in CCI rats ($n=6$) over that of sham rats ($n=3$) in the absence of overt peripheral stimulation (Fig. 2a,b, $P < 0.05$). Consistent with the effect of AACOCF3 on spinal GT uptake activity, the same AACOCF3 treatment (30 μg , i.t., twice daily for postoperative day 1–7) in these CCI rats ($n=4$) resulted in a reduction of regional glutamate concentration in microdialysates by 35% as compared to CCI rats ($n=3$) treated with a vehicle, when also examined on postoperative day 8 (Fig. 2a, $P < 0.05$). Furthermore, regional glutamate concentration in the spinal microdialysates was no longer statistically different between CCI rats ($n=4$) treated with AACOCF3 and sham-operated rats ($n=6$) (Fig. 2a, $P > 0.05$).

Similar to the effect of diclofenac on spinal GT uptake activity in CCI rats, CCI rats ($n=4$) treated with diclofenac (200 μg , i.t., twice daily for postoperative day 1–7) showed an over 200% and 400% increase in regional glutamate concentration as compared to the vehicle-treated CCI rats ($n=6$) and sham rats ($n=3$), respectively, when examined on postoperative day 8 (Fig. 2a,b, $P < 0.05$). Diclofenac alone did not alter the basal spinal glutamate concentration in sham-operated rats. Overall, these results indicate that inhibition of PLA2 with AACOCF3 significantly reduced, whereas inhibition of COX-1/2 with diclofenac dramatically increased, spinal regional glutamate concentration after CCI.

Increased spinal AA concentration following CCI: effect of AACOCF3 and diclofenac

Spinal microdialysates also were assayed using the GC method to measure spinal regional AA concentration. When examined on postoperative day 8, there was a significant increase (more than 4-folds) in spinal regional AA concentration in CCI rats ($n=4$) over that of sham rats ($n=3$) (Fig. 3, $P < 0.05$). Consistent with the role of AACOCF3 as a cytosolic PLA2 inhibitor, the treatment with AACOCF3 (30 μg , i.t., twice daily for postoperative day 1–7) in CCI rats ($n=3$) resulted in a substantial reduction of regional AA concentration by nearly 80% as compared to CCI rats treated with a vehicle ($n=3$), when both examined on postoperative day 8 (Fig. 3, $P < 0.05$). When compared with both sham and vehicle-treated groups, the AACOCF3 treatment effectively prevented the increase in regional AA production following CCI (Fig. 3).

While there was a mild decrease in the spinal regional AA concentration in diclofenac-treated CCI rats, the AA concentration was not statistically different between CCI rats (each $n=4$) treated with either a vehicle or diclofenac (Fig. 3, $P > 0.05$). Thus, regional AA concentration in the spinal microdialysates from CCI rats treated with diclofenac (200 μg , i.t., twice daily for postoperative day 1–7) remained elevated as compared to both sham rats and AACOCF3-treated CCI rats (Fig. 3, $P < 0.05$). However, the diclofenac treatment did not further increase regional AA concentration in CCI rats as compared to CCI rats without receiving the diclofenac treatment (Fig. 3, $P > 0.05$). Thus, inhibition of PLA2 with AACOCF3 effectively prevented the increased AA production after CCI, although regional AA concentration remained unchanged in CCI rats after the diclofenac treatment.

Taken together with the data from measuring spinal GT uptake activity and regional glutamate concentration, these results indicated an overall correlation between the increased regional AA concentration, the decreased GT uptake, and the increased regional glutamate concentration after CCI.

Inhibition of *in vitro* GT uptake by exogenous AA

In order to confirm the effect of AA on spinal GT uptake activity, lumbar spinal samples of naïve rats were taken and processed to perform the ³H-glutamate uptake assay in the presence and absence of exogenous AA. Compared with the vehicle group (n=3), exogenous AA at the concentration of either 10 or 100 nM (each n=6) significantly reduced GT uptake activity (Fig. 4, each P< 0.01), supporting an inhibitory effect of AA on spinal GT uptake activity. The data also indicated that the effect of AA on spinal GT uptake was saturated at the concentration of 10 nM AA, since there were no differences in the reduction of GT uptake activity between two doses of exogenous AA.

Effect of AACOCF3 and diclofenac on thermal hyperalgesia

Thermal hyperalgesia developed over time on the hindpaw ipsilateral to CCI as compared to the sham control. While sham operation did not change the baseline thermal nociceptive response over time (Fig. 5a, ANOVA, P> 0.05, n=6), the ipsilateral hindpaw withdrawal latency to radiant heat was progressively reduced in CCI rats (n=8) and was reliably different from that of sham rats on postoperative day 8 (Fig. 5a, Mann-Whitney, P< 0.05). The same AACOCF3 treatment regimen mentioned above (30 µg, i.t.) attenuated the development of thermal hyperalgesia over the experimental period in CCI rats (Fig. 5a, ANOVA, P< 0.05, n=8), whereas the same diclofenac treatment (200 µg, i.t.) exacerbated the development of thermal hyperalgesia during the same experimental period after CCI (Fig. 5a, ANOVA, P< 0.05, n=8).

Effect of AACOCF3 and diclofenac on mechanical allodynia

Mechanical allodynia also developed over time on the hindpaw ipsilateral to CCI as compared to the sham control. While sham operation did not change the baseline mechanical nociceptive responses over time against the preoperative (PRE) day (Fig. 5b, ANOVA, Postoperative day (N) 1: P=0.905; N4: P=0.730; N8: P=0.571, n=6), the ipsilateral hindpaw withdrawal threshold to von Frey filament stimulation was progressively reduced in CCI rats (n=8) beginning on postoperative day 1 and was reliably different from that of sham rats on postoperative day 8 (Fig. 5b, Mann-Whitney, P< 0.05). The AACOCF3 treatment regimen mentioned above (30 µg, i.t.) effectively attenuated the development of mechanical allodynia over the experimental period in CCI rats (Fig. 5b, ANOVA, P< 0.05, n=8), whereas the diclofenac treatment (200 µg, i.t.) had a minimal effect on mechanical allodynia after CCI (Fig. 5b, n=8).

Discussion

The present data demonstrate that 1) CCI significantly reduced spinal GT uptake activity with an associated increase in spinal AA and glutamate concentration when examined on postoperative day 8; 2) reduction of spinal AA production by AACOCF3 (a cytosolic PLA2 inhibitor) prevented the reduced spinal GT uptake activity and increased extracellular glutamate concentration in CCI rats; 3) alteration of spinal AA metabolism by diclofenac (a COX-1/2 inhibitor) further reduced spinal GT uptake activity and increased extracellular glutamate concentration in CCI rats; 4) exogenous AA directly inhibited *in vitro* GT uptake activity in naïve rats; and 5) consistent with these findings, AACOCF3 attenuated the development of both thermal hyperalgesia and mechanical allodynia, whereas diclofenac exacerbated thermal hyperalgesia, in CCI rats. Thus, spinal AA turnover may serve as a

regulator in CCI-induced changes in regional GT uptake activity, glutamate homeostasis, and neuropathic pain behaviors.

Several methodological issues should be considered with regard to the data interpretation. In the present study, we selected the samples on postoperative day 8 for assays because the GT downregulation was stable at postoperative day 8, which was similar to that on postoperative day 14 (Sung et al. 2003). There were also no differences in pain behaviors between postoperative days 8 and 14. Thus, the data analysis was focused on the ipsilateral spinal cord dorsal horn on postoperative day 8 following CCI.

In this study, two critical sites of the AA metabolic pathway (PLA2 and COX-1/2) were regulated by AACOCF3 and diclofenac, respectively. However, changes of spinal glutamate homeostasis through regulation of AA metabolism in an *in vivo* system could be influenced by a number of factors other than AA itself. For instance, changes in spinal glutamate concentration in microdialysates would be a net result of glutamate release and reuptake and the former can be mediated at least in part through spinal prostaglandins that are clearly regulated by both PLA2 and COX-1/2 activity. We consider that the present findings primarily reflect the effect of AA on spinal GT uptake activity for several reasons: 1) there was an overall correlation between changes in AA production, spinal GT uptake activity, and regional glutamate concentration in CCI rats treated with either a vehicle, AACOCF3, or diclofenac; 2) AACOCF3 resulted in the decreased regional glutamate concentration associated with an increased spinal GT uptake activity; 3) diclofenac, which decreases the production of prostaglandins, dramatically increased regional glutamate concentration in association with a worsening reduction of spinal GT uptake activity in CCI rats; and 4) exogenous AA directly inhibited spinal GT uptake activity in an *in vitro* ³H-glutamate uptake assay, supporting a specific inhibitory effect of AA on spinal GT uptake activity. The AA concentrations used in the *in vitro* experiment were chosen based on the AA concentration used for the intracerebrovascular or intrathecal injections (Okubo et al. 1984; Okuyama et al. 1985; Lucas et al. 2005). Besides using exogenous AA, the effect of endogenous AA on the GT uptake activity from spinal cord samples was indirectly assessed in our experiments using agents that modulate the production of endogenous AA.

Cytosolic PLA2 (cPLA2) is expressed within the spinal cord dorsal horn (Mabuchi et al. 2004; Lucas et al. 2005) and the main active enzyme in the production of AA, contributing to the development of inflammatory pain (Bazan 1998; Gil et al. 2002; Pompeia et al. 2003). Kuwata et al. (1998) observed that cytosolic PLA2 is required for cytokine-induced expression of type IIa secretory PLA2 (sPLA2). While sPLA2 also enhanced excitatory neurotransmission in rat's Substantia Gelatinosa (Yue et al. 2005) and inhibition of sPLA2 reduced the response to von Frey hair stimulation after the carrageenan injection (Yeo et al. 2004), it has been suggested that certain metabolites produced by the cPLA2-dependent pathway are essential for the subsequent induction of type II sPLA inhibitor (Morioka et al. 2002) that there is a functional crosstalk between cPLA2 and sPLA2 enzymes. In this study, AACOCF3 was used as a PLA2 inhibitor because it selectively blocks the activity of cPLA2 (Lucas et al. 2005).

AA has been shown to contribute to neuronal toxicity through the production of free radicals as well as to inflammatory pain via its metabolic products including prostaglandins at both peripheral and central sites (Malmberg and Yaksh 1992; Katsuki and Okuda 1995; Samad et al. 2001; Farooqui et al. 2004). In addition, previous studies have shown that AA inhibits both *in vitro* and *in vivo* GT uptake thereby enhancing glutamate availability in the synaptic space (Volterra et al. 1992; Trotti et al. 1995; Zerangue et al. 1995; Manzoni and Mennini 1997; Breukel et al. 1997; Kawahara et al. 2001; Binns et al. 2005), which is consistent with our result on the *in vitro* GT uptake activity using exogenous AA. Our findings indicate that spinal AA played an important role in regulating spinal GT uptake activity, regional glutamate

homeostasis and neuropathic pain behaviors after peripheral nerve injury. This is supported by an increased spinal AA production following CCI through the PLA2 activity because AACOCF3 substantially reduced spinal AA production and increased GT uptake activity in CCI rats. This is consistent with a previous report (Lucas et al. 2005) that the cPLA2 inhibitor AACOCF3 administered intrathecally prevented thermal hyperalgesia induced by peripheral inflammation.

It should also be noted that glutamate itself may stimulate AA production through glutamate receptor-mediated changes in cytosolic Ca⁺⁺ concentration (Oomagari et al. 1991; Zerangue et al. 1995; White et al. 2000). However, since AACOCF3 inhibited spinal AA production with a resultant increase in GT uptake activity and a decrease in regional glutamate concentration in CCI rats, this glutamate-regulated feedback mechanism of AA metabolism, if present in an *in vivo* setting, is unlikely to have made a substantial contribution to the outcome in our experiment.

Prostaglandins administered intrathecally have shown to result in mechanical allodynia mediated through glutamate receptors (Minami et al. 1994; Minami et al. 2001). Prostaglandins also contribute to the development and maintenance of neuropathic pain behaviors, which is mediated at least in part through prostaglandin-regulated glutamate release from primary afferents (Syriatowicz et al. 1999; Ma et al. 2002; De et al. 2003; Hefferan et al. 2003; Zhu and Eisenach 2003; Broom et al. 2004; Chiechio et al. 2004; Hefferan and Loomis 2004; Muja and DeVries 2004; Schafers et al. 2004). The present data indicate that inhibition of COX-1/2 with diclofenac, a potent water-soluble agent and clinically available non-steroid anti-inflammatory drug (Scholer et al. 1986; Kirchheiner et al. 2003), played a significant role in the mechanisms of neuropathic pain. Diclofenac is likely to modulate the AA metabolism, because diclofenac did increase regional glutamate concentration by several folds with an associated decrease in spinal GT uptake activity in CCI rats. Should the observed change in regional glutamate concentration be due to an altered glutamate release through the effect of prostaglandins, there would have been a decrease in regional glutamate concentration in the spinal microdialysates from CCI rats treated with diclofenac. Thus, the data from diclofenac-treated CCI rats is in agreement with the previous *in vitro* data indicating that it is the AA but not prostaglandins that regulates GT uptake activity (Danbolt 2001).

Of interest to note is that spinal AA concentration from CCI rats treated with diclofenac was not statistically different from CCI rats receiving a vehicle treatment. That is, diclofenac did not further increase regional AA concentration although diclofenac substantially increased regional glutamate concentration and decreased spinal GT uptake activity. This observation is likely due to the fact that AA is a substrate of multiple metabolic pathways including those mediated by lipoxygenase. In addition, our *in vitro* assay indicated that the inhibitory effect of AA on GT uptake activity was saturated at a dose range close to the physiological AA concentration (Rychkov et al. 2005). Thus, it is possible that alteration of the AA metabolism through inhibition of its major metabolic pathway (COX-1/2) by diclofenac enhanced the inhibitory effect of AA on spinal GT uptake activity, leading to an overall increase in glutamate concentration in spinal microdialysates from CCI rats.

Our previous study has shown that the expression of spinal glial and neuronal GT after peripheral nerve injury was regulated through the cellular mechanisms involving mitogen-activated protein kinases (Sung et al. 2003). The present findings indicate that modulation of GT uptake activity itself through an AA-mediated endogenous regulatory mechanism may also play a crucial role in the mechanisms of neuropathic pain behaviors in rats. Consistently, AACOCF3 and diclofenac prevented and exacerbated the development of thermal hyperalgesia, respectively. AACOCF3 also prevented the development of mechanical allodynia in CCI rats, whereas diclofenac at the present dose moderately reduced mechanical

allodynia. The differential effects of diclofenac on the mechanical allodynia and the thermal hyperalgesia may be explained by separate pathways to develop the allodynia and the hyperalgesia (Ossipov et al. 2000). Nonetheless, the present findings suggest that regulating spinal AA turnover may be a useful approach to improving the medical management of neuropathic pain.

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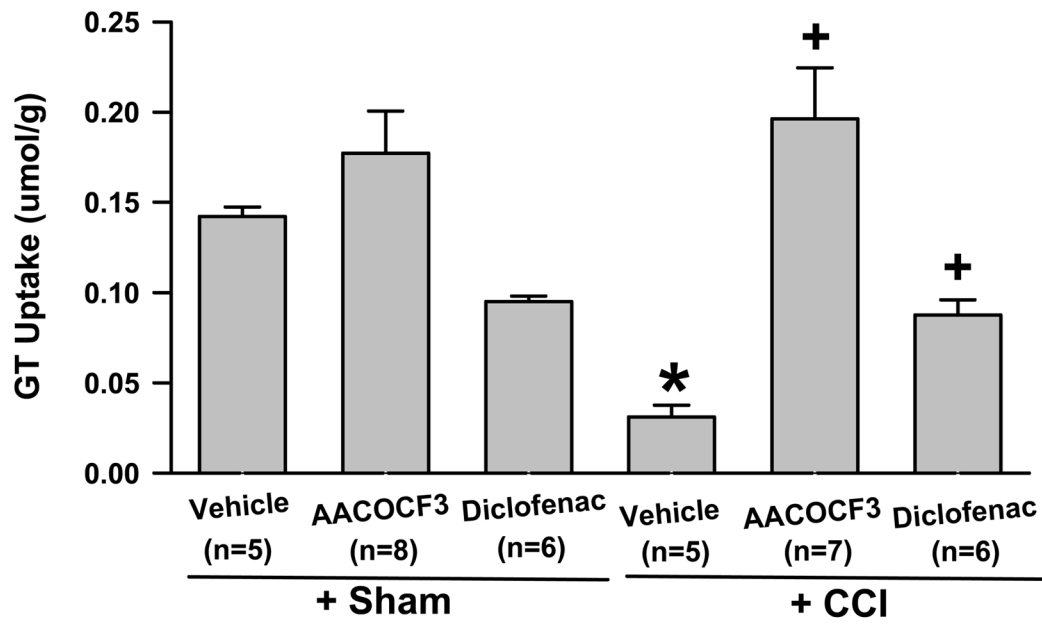


Fig. 1. Changes of spinal GT uptake activity following CCI

The GT uptake activity from the spinal dorsal horn homogenates ipsilateral to CCI was reduced when examined on postoperative day 8. AACOCF3 (a cytosolic PLA2 inhibitor, 30 μ g) and diclofenac (a COX-1/2 inhibitor, 200 μ g), given intrathecally twice a day for postoperative day 1 – 7, enhanced and further reduced spinal GT uptake activity, expressed as mean and S.E. (standard error) respectively. GT uptake was analyzed by converting the cpm unit into the mole concentration of radioactive 3H-glutamate per 100 μ g of proteins. * $P < 0.05$, as compared to the sham group; + $P < 0.05$, as compared to both sham and CCI+vehicle groups. cpm: counts per minute;

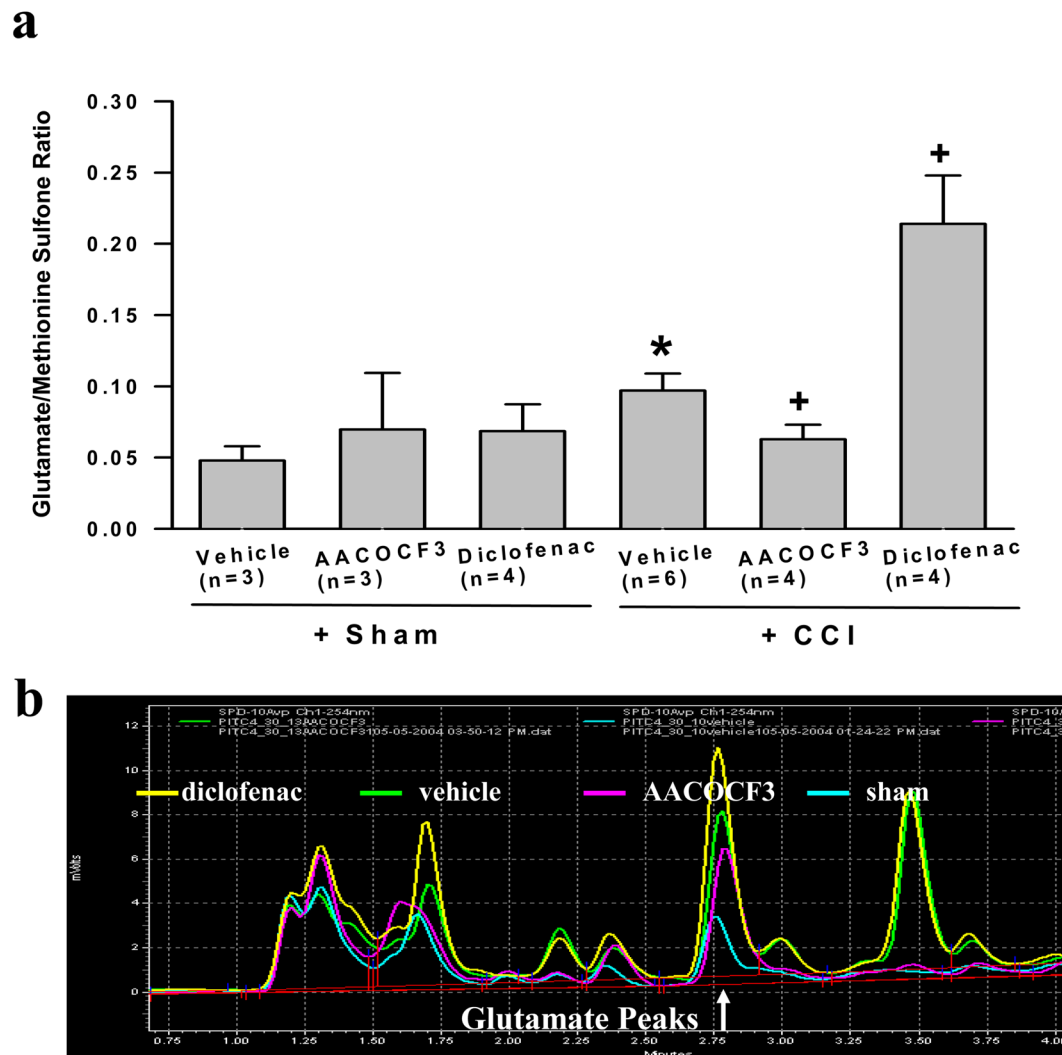


Fig. 2. Changes of spinal glutamate concentration following CCI

The regional glutamate concentration from spinal microdialysates was increased when examined on postoperative day 8 in CCI rats. **A:** AACOCF3 (30 μ g) and diclofenac (200 μ g), given intrathecally twice a day for postoperative day 1 – 7, reduced and further increased regional glutamate concentration, expressed as mean and S.E., respectively. * $P < 0.05$, as compared to the sham group; + $P < 0.05$, as compared to both sham and CCI+vehicle groups. Methionine sulfone is an internal standard and the ratio of glutamate over methionine sulfone was expressed in the Y-axis. **B:** Representative peaks of glutamate concentration over an internal standard (methionine sulfone) as assayed by HPLC. The glutamate peaks from several samples groups, as labeled, were superimposed to provide a direct comparison.

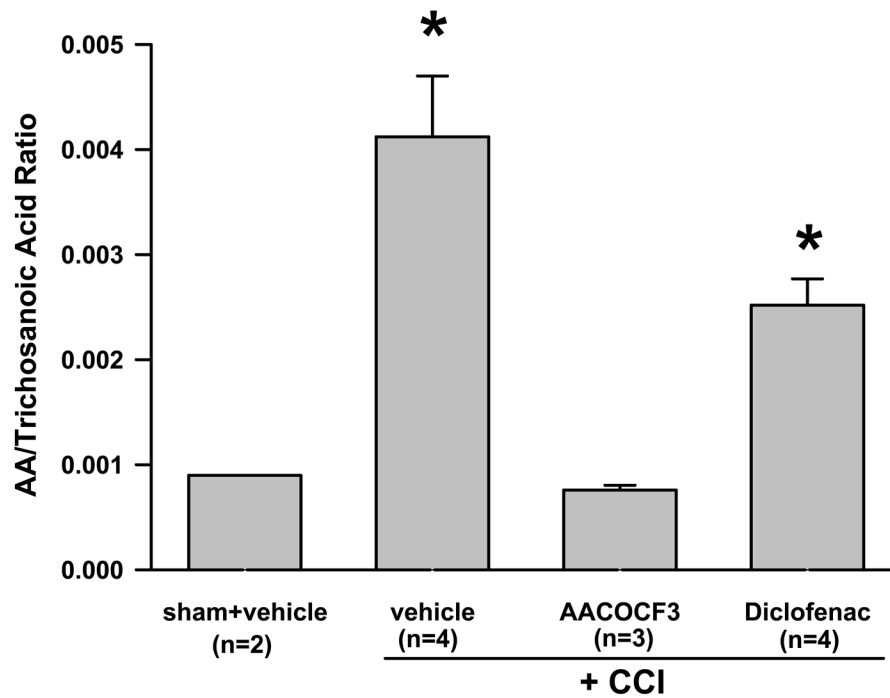


Fig. 3. Changes of spinal AA concentration following CCI

The regional AA concentration from spinal microdialysates was increased when examined on postoperative day 8 in CCI rats. AACOCF3 (30 μ g) given intrathecally twice a day for postoperative day 1 – 7 reduced the regional AA concentration. The spinal AA concentration, expressed as mean and S.E., remained elevated in CCI rats treated with diclofenac (200 μ g) for postoperative day 1–7. * $P < 0.05$, as compared to the sham group. Trichosanoic acid is an internal standard and the ratio of AA over trichosanoic acid was expressed in the Y-axis.

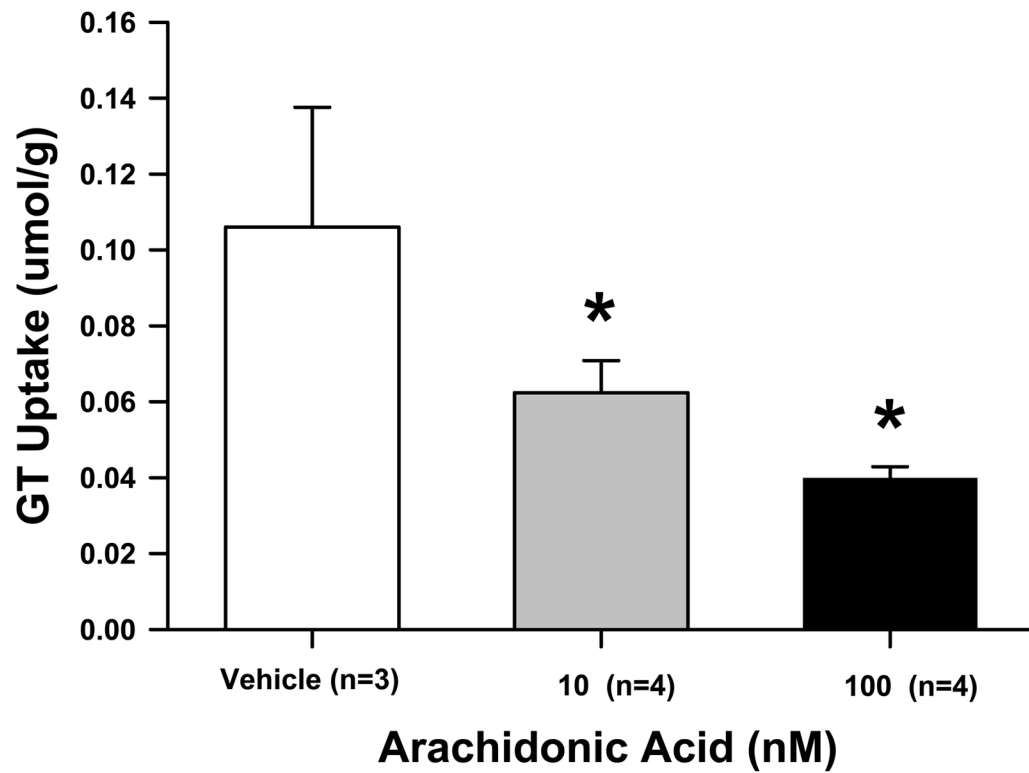


Fig. 4. Inhibition of GT uptake activity by exogenous AA

Addition of exogenous AA (10 or 100 nM) into the synaptosome preparation obtained from the lumbar spinal segments of naïve rats attenuated GT uptake activity, expressed as mean and S.E. See Figure 1 legend for the description of the GT uptake unit. *P <0.05, as compared to the vehicle control.

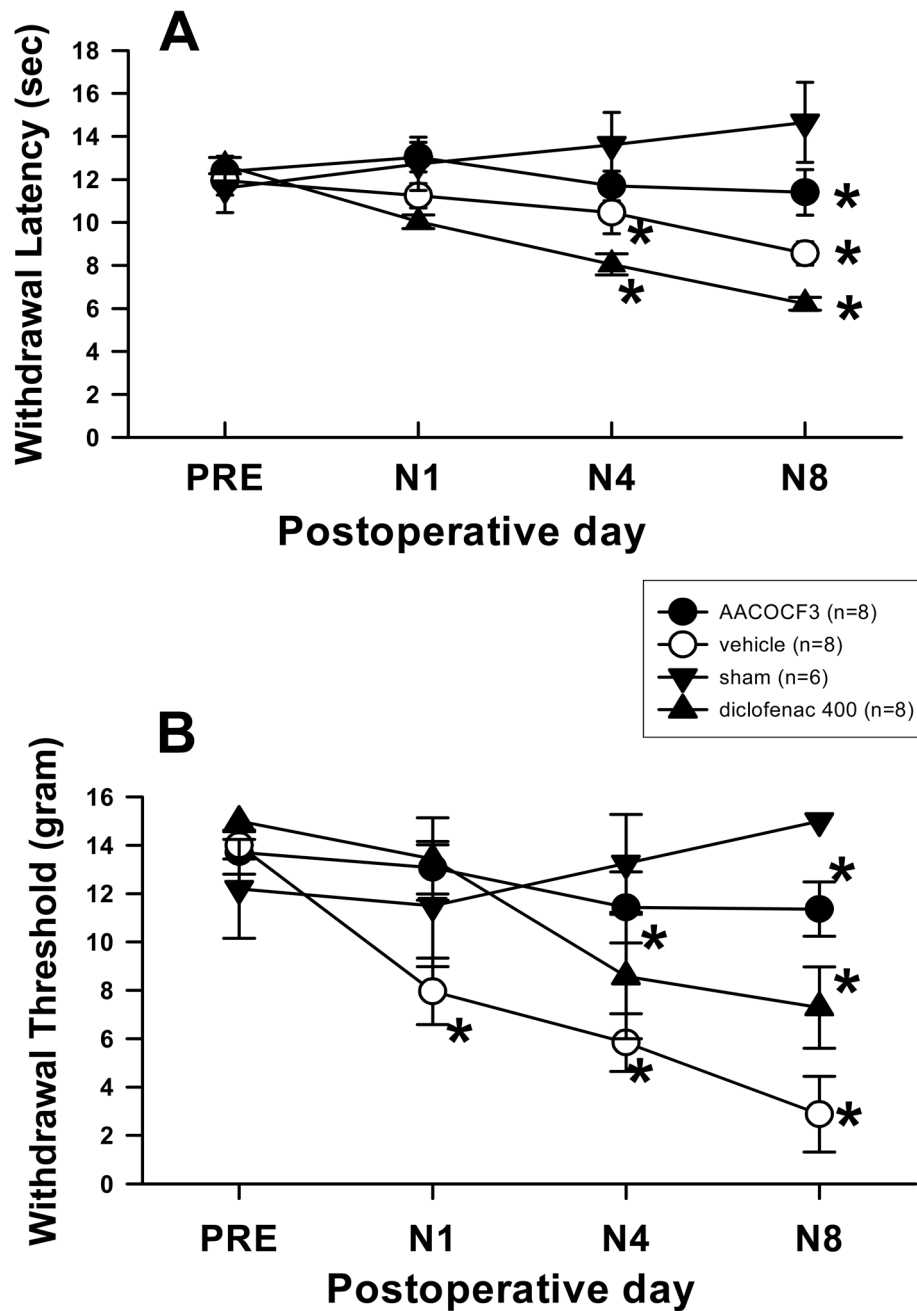


Fig. 5. Effect of AACOCF3 and diclofenac on the development of neuropathic pain behaviors in CCI rats

Thermal hyperalgesia (A) and mechanical allodynia (B) developed over time on the hindpaw ipsilateral to CCI as compared to the sham group. AACOCF3 (30 μ g) and diclofenac (200 μ g), given intrathecally twice a day for postoperative day 1 – 7, prevented and exacerbated the development of thermal hyperalgesia (A), respectively, over the experimental period in CCI rats. AACOCF3 also prevented the development of