Intrathecal Injection of Metabotropic Glutamate Receptor Subtype 3 and 5 Agonist/Antagonist Attenuates Bone Cancer Pain by Inhibition of Spinal Astrocyte Activation in a Mouse Model

Bing-xu Ren, M.D., Ph.D.,* Xiao-ping Gu, M.D., Ph.D.,† Ya-guo Zheng, M.D.,* Cheng-long Liu, M.D.,* Dan Wang, M.D.,* Yu-e Sun, M.D.,* Zheng-liang Ma, M.D., Ph.D.‡

ABSTRACT

Background: Astrocytes and metabotropic glutamate receptors play important roles in nociceptive processing. However, their roles in bone cancer pain were not well understood. This study sought to investigate whether selective mGluR3 and mGluR5 agonist or antagonist develop antinoceptive effects on bone cancer pain by inhibition of spinal astrocyte activation.

Methods: C3H/HeNCrIv mice were inoculated into the intramedullary space of the femur with sarcoma NCTC 2472 cells to induce bone cancer pain. Quantitative real-time reverse transcription-polymerase chain reaction and Western blot experiments examined messenger RNA and protein expression of spinal glial fibrillary acidic protein, mGluR3, and mGluR5. The authors further investigated effects of intrathecal treatment with the mGluR3 agonist (APDC), the mGluR3 antagonist (LY341495), the mGluR5 agonist (CHPG), or the mGluR5 antagonist (MTEP) on nociceptive behaviors and spinal astrocyte activation associated with bone cancer pain.

Results: Inoculation of sarcoma cells, but not α-MEM solution, induced progressive bone cancer pain and resulted in up-regulation of glial fibrillary acidic protein, mGluR3, and mGluR5 expression on days 10, 14, and 21 postinoculation. Intrathecal administration of APDC and MTEP attenuated bone cancer-evoked spontaneous pain, mechanical allodynia, thermal hyperalgesia, and reduced spinal glial fibrillary acidic protein expression. However, treatment with LY341495 and CHPG induced thermal hyperalgesia and spinal glial fibrillary acidic protein expression in control mice.

Conclusions: Spinal mGluR3 activation or mGluR5 inhibition reduced bone cancer pain. Inhibition of spinal astrocyte activation may contribute to the analgesic effects. These findings may lead to novel strategies for the treatment of bone cancer pain.

What We Already Know about This Topic

• Metabotropic glutamate receptors (mGluRs) play an important role in some forms of hypersensitivity, but whether they are central to that from bone cancer has not been investigated

What This Article Tells Us That Is New

• In mice with cancer of the femur, spinal injection of mGluR3 agonists and -5 subtype antagonists inhibited spontaneous behaviors thought to reflect pain as well as hypersensitivity to mechanical stimuli and astrocyte activation

BONE cancer pain is a severe, chronic pain that has a strong effect on the cancer patient’s quality of life, but no breakthrough regarding the mechanisms and therapeutics of bone cancer pain has yet been achieved.1–3 Therefore, novel and more efficacious therapies are urgently needed for improvement of patient quality of life.

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system and exerts its action through multiple ionotropic and metabotropic receptors. Metabotropic glutamate receptors (mGluRs) are expressed abundantly in spinal cord and have been shown to play important roles in modulation of nociceptive transmission.4,5 Eight mGluRs have been cloned so far and they can be sub-
divided into three groups based on sequence identity, pharmacologic profile, and signal transduction.\(^6\)\(^–\)\(^8\) Group I mGluRs (mGluR1 and mGluR5) are mainly coupled to phospholipase C (PLC), whereas group II (mGluR2 and mGluR3) and group III receptors (mGluR4, 5, 6, 7, and 8) are negatively coupled to adenylylate cyclase.\(^9\)

There have been a number of studies suggesting that activation of mGluR3 may be of analgesic effects. Antinociceptive actions of mGluR3 agonists have been demonstrated in the periphery, spinal cord, and amygdale.\(^10\)\(^–\)\(^13\) However, some evidence has shown that activation of mGluR3 could promote nociception.\(^14\)\(^,\)\(^15\) The role of mGluR3 in bone cancer pain has not been investigated. Metabotropic glutamate receptor subtype 5 is expressed both presynaptically and postsynaptically in the superficial spinal dorsal horn.\(^16\) Intrathecal administration of the mGluR5 antagonist produced antinociceptive effects in the formalin pain model,\(^17\) and spinal administration of anti mGluR5 IgG antibodies reduced cold hyperalgesia in the neuropathic pain model.\(^11\) Some studies also demonstrated that systemic administration of mGluR5 antagonist attenuated nociceptive behaviors in neuropathic pain models.\(^18\)\(^,\)\(^19\) However, whether spinal mGluR5 is involved in cancer-induced pain has not been investigated.

In addition to being expressed in neurons, mGluRs are expressed in astrocytes. Most of the reports showed the presence of mGluR3 and mGluR5 in astrocytes, whereas a few studies suggested the presence of other receptor subtypes.\(^20\)\(^–\)\(^22\) Metabotropic glutamate receptors were also involved in regulating astrocyte proliferation: activation of mGluR3 could promote astrocyte proliferation, but activation of mGluR5 had the opposite influence on astrocyte proliferation.\(^23\) A number of studies suggested that regulation of mGluR subtype expression in astrocytes might represent a general mechanism for modulation of glial function and for changes in glial-neuronal communication in pathologic conditions.\(^24\)\(^–\)\(^28\) Several studies have exposed the critical importance of astrocytes to pain perception and modulation. Spinal astrocytes participated in the initiation and maintenance of persistent pain induced by tissue inflammation, nerve injury, and bone cancer.\(^29\)\(^–\)\(^33\) Despite these advances, little is known about the role of mGluR3 and mGluR5 regulating spinal astrocyte activation during the development of bone cancer pain.

Therefore, in this study we detected the expression of spinal glial fibrillary acidic protein (GFAP), mGluR3, and mGluR5 in a mouse model of bone cancer pain, and further examined whether mGluR3 and mGluR5 agonist or antagonist attenuated bone cancer pain by inhibition of spinal astrocyte activation.

**Materials and Methods**

**Animals**

The current experiments were approved by the Medical College of Nanjing University Animal Care and Use Committee (Nanjing, China) and were in accordance with the ethical guidelines of the National Institutes of Health. Experiments were performed on male C3H/HeNCrlVr mice (20–25g; Weitong Lihua Laboratory Animal Technology Co., Ltd., Beijing, China; SCXK JING 2000–0009). Mice were housed in a temperature-controlled (21 ± 1°C) room with a 12-h light/dark cycle and given food and water ad libitum.

**Intrathecal Catheter**

The mice were anesthetized with intraperitoneal injection of 50 mg/kg pentobarbital sodium (1% in normal saline) and a polyethylene intrathecal catheter with an ID of 0.25 mm and an OD of 0.5 mm (Anlai software corporation, Ningbo, China) was inserted 5 mm cephalad into the mouse lumbar subarachnoid space at the L4–L5 intervertebrae, with the tip of the catheter located near the lumbar enlargement of the spinal cord to administer the drugs intrathecally, according to the modification of a method described previously.\(^19\) The catheter was tunneled subcutaneously and externalized through the skin in the neck region. The volume of dead space of the intrathecal catheter was 5 μl. To avoid occlusion of the catheter, 5 μl normal saline was injected via a catheter on alternate days. After the end of the experiment, the effects of intrathecal lidocaine (2%, 2 μl) were examined. Only animals that had shown complete paralysis of the tail and bilateral hind legs after intrathecal lidocaine were analyzed. Intrathecal drug administration was accomplished using a microinjection syringe connected to an intrathecal catheter. Drugs were injected manually over a 10-s period in a single injection volume of 5 μl followed by a flush of physiologic saline (5 μl). In addition, the previous and our preliminary studies showed that intrathecal catheter implantation did not induce spinal cord injury or result in signs of neurologic deficit.\(^19\)

**Cell Culture and Implantation**

Sarcoma NCTC 2472 cells (American Type Culture Collection, 20, 87, 787) were maintained in NCTC 135 media (Sigma–Aldrich, St. Louis, MO) containing 10% horse sera (Gibco, Carlsbad, CA) and passaged weekly according to American Type Culture Collection recommendations. Implantation of sarcoma cells was performed after intrathecal catheterization on the same day as previously described by Schwei et al.\(^34\) A superficial incision was made with eye scissors in the skin overlying the left patella. The patellar ligament was then cut, exposing the condyles of the distal femur. A 30-gauge needle was used to perforate the bone cortex and a light depression was made using a dental burr, then a 25-μl microsyringe was used to inject a volume of 20 μl α-minimum essential medium (α-MEM) containing no or 10^5 NCTC 2472 cells into the medullary cavity of the distal femur, corresponding to control mice or tumor-bearing mice. Afterward, the injection hole was sealed with bone wax, followed by copious irrigation with normal saline. The wound was then closed.
**Drugs**

The mGluR3 receptor agonist (2R,4R)-4-aminopyrrolidine-2,4-dicarboxylate (APDC), mGluR5 receptor antagonist(2S)-2-amino-2-[(1S,2S)-2-carboxycloprop-1-yl]-3-(xan th-9-yl) propanoic acid (LY341495), mGluR5 receptor agonist(RS)-2-chloro-5-hydroxyphenylglycine (CHPG), and mGluR5 receptor antagonist 3-[(2-methyl-1,3-thiazol-4-yl) e-thynyl] pyridine hydrochloride (MTEP) were purchased from Tocris Cookson Ltd (Ellisville, MO). APDC and CHPG were dissolved in 1.1 eq NaOH. LY341495 and MTEP were dissolved in DMSO. The fluorocotic solution for intrathecal injection was prepared as follows: 8 mg DL-fluorocitic acid barium salt (Sigma-Aldrich) was dissolved in 1 ml 0.1 M HCL. Two to three drops of 0.1 M Na2SO4 were added to precipitate the Ba2+. Two milliliters of 0.1 M NaHPO4 was added, and the suspension was centrifuged at 1,000 g for 5 min. The supernatant was diluted with 0.9% NaCl to the final concentration, and the pH was adjusted to 7.4. Appropriate vehicle of dilute NaOH in 0.9% saline (vehicle 1) or DMSO in 0.9% saline (vehicle 2) was used as control treatment for each drug that was dissolved by the solvent. APDC (150 nmol), LY341495 (15 nmol), CHPG (300 μmol), MTEP (150 nmol), morphine (5 μmol), fluorocitrato (0.75 μmol), or vehicles (1 and 2) were respectively injected through the intrathecal catheter once daily on days 14–20.

**Assessment of Bone Cancer Pain**

All tests were performed during the light phase. Before each test, mice were allowed to habituate for at least 30 min. All behavioral responses were measured by an experimenter who was blind to the treatment groups.

**Spontaneous Lifting Behavior**

The mice were housed in individual Plexiglass compartments (10 cm × 10 cm × 15 cm) for 30 min and observed during 2 min to count the number of spontaneous flinches of the left hind limb. Every lift of the left hind limb not related to walking or grooming was considered to be one flinch. Data were presented as mean ± SD.

**Mechanical Allodynia Test**

Mechanical allodynia was assessed by von Frey filaments (Stoelting, Wood Dale, IL) applied to the hind paw according to our previous study. Mechanical threshold was measured using a set of von Frey filaments (0.16 g, 0.4 g, 0.6 g, 1.0 g, 1.4 g, and 2.0 g). Each mouse was tested five times per stimulus strength. The lowest von Frey filament that had three or more positive responses was regarded as the paw withdrawal mechanical threshold (PWMT). Data were presented as mean ± SD.

**Thermal Hyperalgesia Test**

Thermal hyperalgesia was assessed with the paw withdrawal thermal latency (PWTL) to radiant heat according to our previous study. There were five trials per mouse and 5-min intervals between trials. The mean PWTL was obtained from the latter three stimuli. Data were presented as mean ± SD.

**Quantitative Real Time Reverse Transcription-Polymerase Chain Reaction Technique**

The L3–L5 spinal cord segments of sacrificed mice were dissected and frozen in liquid nitrogen and stored at −70°C. Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA). RNA concentration was measured using a NanoDrop ND-1000 Spectrometer (NanoDrop Technologies, Wilmington, DE). The purity of the RNA was assessed by the ratio of absorbance at 260 nm and 280 nm. RNA was stored in aliquots at −70°C. RNA samples were reversely transcribed using the Reverse Transcription-Polymerase Chain Reaction Kit (Takara, Dalian, China), and quantitative real-time reverse transcription-polymerase chain reaction was performed using the SYBR PrimeScript Quantitative Real Time Reverse Transcription-Polymerase Chain Reaction Kit (Takara) according to the manufacturer’s protocol. Information about the quantitative real-time reverse transcription-polymerase chain reaction was obtained from the SYBR PrimeScript Quantitative Real Time Reverse Transcription-Polymerase Chain Reaction Kit (Takara) according to the manufacturer’s protocol. Information about the quantitative real-time reverse transcription-polymerase chain reaction analysis primers: mGluR3 primers (upstream primer, 5’ CAGGAGTCAT-TGGCGGT TCG 3’, and downstream primer, 5’TGGCACTTGGTGGAGGCGTAG3’), mGluR5 primers (upstream primer, 5’ CAGTCGAGTGGAC GTATGG 3’, and downstream primer, 5’ GCCC AATGACT CCCAC T3’), GFAP primers (upstream primer, 5’ GTGGTATCGGTCT AAGTTTGC 3’, and downstream primer, 5’ CATGGCGCTTCTCCGTG 3’ and β-actin primers (upstream primer, 5’ CTGGTCCTCTGTCGCTGCTTG 3’, and downstream primer, 5’ ATGCTA CGCAGATTTC 3’). Reaction mix was aliquoted to the wells on a real-time polymerase chain reaction plate. Each sample was made in duplicate. A volume of 2 μl complementary DNA was added to each well. A no-template control sample contained water instead of complementary DNA. Quantitative real-time reverse transcription-polymerase chain reaction was run on ABI PRISM 7500 (Applied Biosystems, Carlsbad, CA) using standard conditions. Expression of mGluR3, mGluR5, and GFAP was normalized to RNA loading for each sample using the reference gene β-actin as an internal standard. The quantity of messenger RNA (mRNA) was presented as mean ± SD.

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given as $2^{-\Delta \Delta ct}$. $\Delta ct$ and $\Delta \Delta ct$ were calculated as follows: $\Delta ct = ct$ (gene of interest) – $ct$ (reference gene); $\Delta \Delta ct = \Delta ct$ (group of interest) – $\Delta ct$ (control group). Data were presented as mean ± SD.

**Western Blotting**

The mice were deeply anesthetized with pentobarbital sodium (1% in normal saline) and sacrificed by decapitation. The spinal cord L3–L5 segments were removed rapidly and stored in liquid nitrogen. Tissue samples were homogenized in 1 ml 0.1M phosphate buffered saline (PBS). The homogenate was transferred to a fresh tube and spun at 13,000 rpm for 5 min at 4°C. The supernatant was removed and the pellet washed twice in ice-cold PBS and spun at 13,000 rpm for 5 min at 4°C. PBS was aspirated, and the pellet resuspended in ice-cold lysis buffer (1% Nonidet P-40; 0.5% sodium deoxycholate, 0.1% sodium lauryl sulfate in PBS) with freshly added protease inhibitors (Roche Diagnostics, Shanghai, China), and incubated for 30 min on ice. Tubes were centrifuged at 13,000 rpm for 20 min at 4°C and supernatants containing the protein lysates collected and aliquots stored at −70°C. The protein concentration was determined using BCA method according to kit’s instructions (Kaiji Biotechnology, Nanjing, China). Protein lysates (100 μg) and a protein molecular weight marker, PageRular Prestained Standard (10 –170kDa, Multiscience Biotechnology, Hangzhou, China) were separated on SDS-PAGE (8%), and run for 90 min at 120 V. Proteins were transferred onto polyvinylidene fluoride membranes (Life Technologies, Carlsbad, CA) at 200 mA for 2 h using wet blotting system. Membranes were blocked in PBS/5% skim milk/0.1% Tween 20 for 2 h at room temperature, followed by overnight incubation at 4°C with anti mGluR3 (goat affinity purified polyclonal antibody; 1:500; Santa Cruz Biotechnology, Santa Cruz, CA), anti mGluR5 (rabbit affinity purified polyclonal antibody; 1:500; Abcam, Hong Kong, China), anti-GFAP (goat affinity purified polyclonal antibody; 1:1000; Santa Cruz Biotechnology) or anti-β-actin (rabbit affinity purified polyclonal antibody; 1:1000;Cell Signaling, Danvers, MA) diluted in blocking buffer. Immunoblots were then incubated for 2 h at room temperature with horseradish-peroxidase linked secondary antibody (Cell Signaling) diluted 1:5,000 in blocking buffer. Immunoblots were developed by enhanced chemiluminescence (ECL) (Multiscience Biotechnology) and visualized on Kodak BIOMAX MR X-ray film (Eastman Kodak, Rochester, NY). Immunoreactivity was quantified using densiometric analysis on Gel-Pro analyzer 4.0 software (Media Cybernetics, Silver Spring, MD). Data were presented as mean ± SD.

**Assessing the Extent of Bone Destruction and Spinal Astrocyte Activation**

Animals were radiologically assessed using standard x-ray film (TMG/RA; Eastman Kodak, Rochester, NY) at day 0 (normal mice) and at day 14 and 21 after sarcoma cells or culture medium (α-MEM) implantation; the spinal cords and the ipsilateral femurs were processed for immunohistochemical analysis. Serial frozen spinal cord sections, 60 μm-thick, were cut on a sliding microtome, collected in PBS, and processed as free-floating sections. Free-floating sections were incubated in a blocking solution containing 5% normal goat serum in Tris buffered saline-Tween (Tris buffered saline and 0.2% Triton X-100), followed by incubation with a goat polyclonal antiserum recognizing glial fibrillary acidic protein (GFAP;1:2500 working dilution; Santa Cruz Biotechnology) to label astrocytes. Spinal cord sections were incubated overnight at 4°C with primary antibodies, followed by incubation with rabbit antigen secondary antibodies (1:200 working dilution; Cell Signaling) conjugated to Alexa 488. Sections were mounted on glass slides, air-dried and coveredslipped using Aquamount (Fisher Scientific, Ottawa, Canada). Images were taken at 250× magnification using the Leica TCS SP2 multiphoton confocal microscope (Leica Microsystems, Wetzlar, Germany). The femurs were demineralized in 10% EDTA for 3 weeks and processed for routine paraffin histology to visualize the extent of tumor infiltration and destruction of the bone.

**Assessing the Effects of the Intrathecal Injection of Drugs or Vehicles on Tumor Growth**

At day 21 after surgery, we measured treated groups of mice’s bilateral maximum thigh circumference and used the ratio of maximum thigh circumference (ipsi/contra) to reflect the relative size of the tumor. Data were presented as mean ± SD.

**Statistical Analysis**

All data are expressed as mean ± SD. Changes in the number of flinches, PWTL, quantitative real-time reverse transcription-polymerase chain reaction, and Western blot data after inoculation were compared with the basal value using a two-way ANOVA for repeated measures followed by Bonferroni correction for between-group comparisons. Nonparametric Kruskal-Wallis test and Nemenyi test were used to make statistical comparisons of groups followed by Bonferroni correction for between-group comparisons. Changes in PWMT were compared with the basal values using the non-parametric Friedman test for repeated measures followed by q test for between-group comparisons. The number of flinches, PWTL, quantitative real-time reverse transcription-polymerase chain reaction, and Western blot data from the mice that were treated with drugs or vehicles were analyzed using a one-way ANOVA for overall differences among groups followed by Bonferroni correction for between-group comparisons. Nonparametric Kruskal-Wallis H test and Nemenyi test were used to make statistical comparisons of the PWMT data from the mice that were treated with drugs or vehicles. The nature of significance testing was 2-tailed test. Statistical analysis was performed using SPSS 16.0 software (IBM Corporation, Armonk, NY). A P value less than 0.05 was considered significant.
Results

Bone Cancer-induced Pain Behaviors over Time

Before the surgery (day 0), the baseline number of spontaneous flinches, PWMT, and PWTL were similar in all groups of mice ($P = 0.961, 0.854,$ and $0.935,$ respectively), and there was no statistical difference between left and right hind limb. At day 4 and day 7 after surgery, both tumor-bearing and sham group mice displayed incremental spontaneous lifting behavior of the ipsilateral hind limb to the surgery, and the number of spontaneous flinches of sham group mice recovered to the baseline level at day 10. However, tumor-bearing mice showed the number of spontaneous flinches gradually increased over time and the number was $13.50 \pm 2.09$ during a 2-min period at day 21 (Fig. 1A). The ipsilateral hind paw to the surgery of both tumor-bearing and sham group mice displayed the decrease of PWMT to von Frey filaments stimulation at day 4 and day 7. The PWMT of sham group mice recovered to the baseline level at day 10 and tumor-bearing mice then showed a decrease of PWMT ($P = 0.002$), which gradually decreased over time until day 21, when the value was $0.58 \pm 0.23g$ (Fig. 1, B). The ipsilateral hind limb of both tumor and sham group mice displayed a decrease in PWTL to radiant heat stimulation at day 4 and day 7. The PWTL of sham group mice on the whole recovered to the baseline level at day 10. Nevertheless, the PWTL of the tumor-bearing mice showed a profound decrease at day 10 ($P = 0.001$) and gradually decreased along with the development of cancer pain. At day 21, the value was $6.36 \pm 0.59$ s in tumor-bearing mice (Fig. 1, C). In addition, the contralateral limb of all groups of mice showed no change in pain behaviors, and no statistical differences in the number of spontaneous flinches, PWMT, and PWTL were observed at day 4 and day 7 between the tumor-bearing group and sham group.

Changes in the Expression of Spinal GFAP and mGluRs mRNA in Response to Bone Cancer Pain

Relative quantification of mGluR3, mGluR5, and GFAP mRNA extracted from the lumbar spinal cord from healthy control animals (day 0) confirmed that they are constitutively expressed in mice spinal cord. Compared with healthy control animals (day 0), the level of GFAP (Fig. 2, A), mGluR3 (Fig. 2, B) and mGluR5 (Fig. 2, C) mRNA of tumor-bearing mice gradually increased over time. At day 21, a significant up-regulation in level of spinal GFAP ($3.13 \pm 0.39$), mGluR3 ($3.23 \pm 0.32$), and mGluR5 ($3.19 \pm 0.34$) mRNA was detected of tumor-bearing mice compared with those of sham group mice ($P = 0.002, 0.015,$ and $0.001,$ respectively). However, only at day 4 and day 7, a up-regulation in the level of spinal GFAP ($1.26 \pm 0.39; 1.62 \pm 0.41$), mGluR3 ($1.31 \pm 0.13; 1.87 \pm 0.26$), and mGluR5 ($1.48 \pm 0.15; 1.77 \pm 0.17$) mRNA was detected in the sham group. At day 10, the level of GFAP, mGluR3, and mGluR5 mRNA expression of sham group recovered to the level before surgery.

Changes in the Expression of Spinal GFAP and mGluRs Protein in Response to Bone Cancer Pain

Western blot analyses was carried out to characterize the expression of spinal mGluRs and GFAP protein. The GFAP, mGluR3, mGluR5, and β-actin antibody labeled a band at 50 kDa, 110 kDa, 150 kDa, and 45k Da, respectively (Fig. 3, A and B). Results were expressed as integrated optical density ratio (interest protein vs. β-actin) relative to day 0 (Fig. 3, C, D, and E). Similar results with mRNA analyses were observed. The levels of expression of GFAP and mGluRs subtype protein in the spinal cord from tumor-bearing mice increased gradually over time compared with healthy control animals (day 0). At day 21, the values were $2.86 \pm 0.58,$ $0.58,$ $0.59,$ and $0.58,$ respectively.
At day 4 and day 7 after surgery, the levels of expression of GFAP and mGluRs subtype protein have no statistical difference between tumor-bearing mice and sham group mice. However, at day 10 the levels of expression of GFAP and mGluRs subtype protein in spinal cord from sham group mice have recovered to the baseline level before surgery.

**Changes in Morphology of Bone Destruction and Spinal Astrocyte Activation**

Hematoxylin-eosin staining of normal (fig. 4, A-0), 14 days sham (fig. 4, A-1), 14 days tumor-bearing (fig. 4, A-2), and 21 days tumor-bearing (fig. 4, A-3) femora, showing the replacement of the darkly stained marrow cells with the more lightly stained sarcoma cells that have induced bone destruction and grown through the bone in 14 days and 21 days tumor-bearing mice. Compared with normal (fig. 4, B-0) and 14 days sham (fig. 4, B-1), radiographs of the femur showing the loss of bone caused by tumor growth in 14 days (fig. 4, B-2) and 21 days tumor-bearing mice (fig. 4, B-3). Compared with normal (fig. 4, C-0) and 14 days sham (fig. 4, C-1), confocal images showing the increase in the astrocyte marker GFAP in L4 spinal cord sections that were ipsilateral to the femur with cancer in 14 days (fig. 4C-2) and 21 days tumor-bearing mice (fig. 4, C-3).

**Effects of Intrathecal Injection of mGluR3 and 5 Agonists or Antagonist on Nociceptive Behaviors Induced by the Bone Cancer Pain**

We examined whether activation or inhibition of spinal mGluR3 and 5 could regulate bone cancer-related pain behaviors (fig. 5, A, B, and C). All drugs and vehicles in a volume of 5 μl were respectively injected through the intrathecal catheter once daily on the days 14–20 after inoculation. APDC (150 nmol/day), MTEP (150 nmol/day), morphine (5 μmol/day), and fluorocitrate (0.75 nmol/day) reduced bone cancer related pain behaviors of the ipsilateral hind limb to the surgery. Intrathecal APDC MTEP, morphine, or fluorocitrate had no effects on the three pain behaviors of the contralateral hind limb of tumor-bearing mice and those of the bilateral hind limb of sham mice. The vehicles also did not affect the three pain behaviors of bilateral hind limb in tumor-bearing and sham group mice. LY 341495 or CHPG by intrathecal route resulted in thermal hyperalgesia in the contralateral hind paws of tumor-bearing mice and bilateral hind paws of sham group mice, but did not affect spontaneous flinches and mechanical allodynia.

**Effects of the Intrathecal Injection of mGluR3 and 5 Agonists or Antagonist on the Expression of Spinal GFAP mRNA and Protein in Response to Bone Cancer Pain**

Compared with the groups of tumor-bearing mice received vehicles, morphine (5 μmol/day), LY341495 (15 nmol/day) or CHPG (300 μmol/day) by intrathecal route (once daily on days 14–20 after inoculation), fluorocitrate (0.75 nmol/day), APDC (150 nmol/day) and MTEP (150 nmol/day) down-regulated the expression of spinal GFAP mRNA and protein. Morphine, LY341495 and CHPG up-regulated the expression of spinal GFAP mRNA and protein in sham group mice. However, vehicles APDC and MTEP had no significant effects on the expression of spinal GFAP mRNA and protein in sham mice (fig. 6, A, B, and C).

**Changes of Tumor Growth during the Treatment**

At day 21 after surgery, compared with the groups of tumor-bearing mice received vehicles, the ratio of maximum thigh circumference (ipsi/contra) of morphine (5 μmol/day), LY341495 (15 nmol/day) or CHPG (300 μmol/day) by intrathecal route (once daily on days 14–20 after inoculation), fluorocitrate (0.75 nmol/day), APDC (150 nmol/day) and MTEP (150 nmol/day) treated tumor-bearing mice have no statistical difference (P = 0.873) (fig. 7). Intrathecal injection of drugs also has no significant effects on the ratio of maximum thigh circumference (ipsi/contra) of sham group mice.
mice compared with that of vehicle-treated sham group mice (P = 0.921) (fig. 7).

**Discussion**

**Bone Cancer-related Pain Behaviors in Sarcoma-implanted Mice**

In past years, animal models for bone cancer pain have been established. Animal models provide pivotal systems for preclinical study of pain. This study demonstrated that sarcoma cells inoculation of the left femur of male C3H/HeNcrlVr mice produced progressive spontaneous flinches, mechanical and thermal hyperalgesia, and bone destruction, indicating the successful establishment of a model of bone cancer pain. These findings were similar with our previous study and the fact that patients with various kinds of bone cancer destruction suffer progressively from spontaneous and evoked pain at the end of their lives. In our study, at day 4 and 7 after surgery, the PWMT and PWTL were decreased and the number of spontaneous flinches were increased both in tumor-bearing and sham group mice. But there was no statistical difference between tumor-bearing and sham mice at these two time points. Ten days after surgery, the pain behavior level of sham group mice returned to baseline, but that of tumor-bearing mice showed progressive increase. These data suggested that changes in pain behavior at day 4 and 7 after surgery might be due to gonarthrotomy rather than a cause of cancer pain.

**Up-regulation of Metabotropic Glutamate Receptor Subtypes 3 and 5 in Spinal Cord in a Mouse Model of Bone Cancer Pain**

This study is the first report of altered spinal mGluR3 and mGluR5 mRNA and protein expression in a model of bone cancer pain. Here we demonstrated alterations in spinal mGluR subtypes both in tumor-bearing mice and sham group mice early (at day 4 and 7), indicating that up-regulation of mGluR3 and mGluR5 expression may be a common phenomenon that occurs in pain states. This study found that in tumor-bearing mice, spinal mGluR3 and mGluR5 expression gradually increased and was maintained at a high level, indicating mGluR3 and mGluR5 may play important roles in the maintenance of pain states.

**Bone Cancer Pain Induces Glial Activation**

Our work also showed that spinal GFAP mRNA and protein expression were rapidly increased in parallel to pain behaviors. Confocal images showed there was an increase in the astrocyte marker GFAP in spinal sections that were ipsilateral to the femur with cancer. Glial fibrillary acidic protein, as a specific marker of astrocytes, is often used to detect tissue activation of astrocytes. Astrocyte activation has been observed at the lumbar spinal cord in various rodent models of pain, including bone cancer pain models, inflammatory pain models, and neuropathic pain models. Activated astrocytes release a host of pronociceptive mediators, such as reactive oxygen species, proinflammatory cytokines, prostaglandins, and NO, all of which facilitate pain process-
ing by enhancing either presynaptic release of neurotransmitters or postsynaptic excitability. It has been demonstrated that blocking the activation of spinal cord astrocytes with fluorocitrate, a glial metabolic inhibitor, impeded the exaggerated pain induced by peripheral tissue inflammation, nerve injury, and spinal cord impairment. In this study, we used fluorocitrate as a positive control drug. The results showed fluorocitrate relieved bone cancer pain and inhibited the expression of spinal GFAP. Accordingly, the activation of spinal astrocyte may contribute to the development and maintenance of bone cancer pain. However, both tumor-bearing mice and sham group mice early (at day 4 and day 7) spinal astrocytes were activated, indicating that astrocyte activation is also a common phenomenon which occurs in pain states, rather than a phenomenon unique to cancer pain. But the extent and stage of astrocyte activation may be different, which requires further studies.

Central mGluR3 Activation or mGluR5 Inhibition Induces Antinociception and Reduces Bone Cancer Pain-induced Astrocytes Activation

We observed that the repeated administration of the selective mGluR3 agonist (APDC) or mGluR5 antagonist (MTEP) not only attenuated the development of spontaneous flinching, mechanical allodynia, and thermal hyperalgesia of the ipsilateral hind limb as well as spinal astrocyte activation in tumor bearing mice. In addition, intrathecal injection of APDC or MTEP did not affect any of these three pain measures in the contralateral hind paw of tumor-bearing mice or bilateral hind paws of sham mice.
In addition, intrathecal administration of APDC and MTEP did not induce spinal astrocyte activation in sham group mice. Our results also indicated that activation of mGluR3 relieved bone cancer pain, although there were some conflicting reports regarding activation of mGluR3 being analgesic. Therefore, up-regulation of mGluR3 in the spinal cord during the development of bone cancer pain may be one of the endogenous analgesia mechanisms. Activation of the endogenous analgesic system of the body may be a new strategy in the future. In agreement with previous studies, we found mGluR5 inhibition induced antinociception in the model of bone cancer pain. In this study, we also found that the selective mGluR3 antagonist...
(LY341495) or mGluR5 agonist (CHPG) had no significant effects on the nociceptive responses of the ipsilateral hind paws of tumor-bearing mice and spinal astrocyte activation induced by bone cancer pain, but could induce thermal hyperalgesia of the contralateral hind paws of tumor-bearing mice and bilateral hind paws of sham group mice. In addition, intrathecal injection of LY341495 and CHPG induced activation of spinal astrocytes in the sham group of mice. Therefore, the treatment to bone cancer pain requires a mix of methods.

**References**

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**Fig. 7.** Effects of intrathecal administration of APDC (mGluR3 agonist; 150 nmol/5 μl), LY 341495 (mGluR3 antagonist; 15 nmol/5 μl), CHPG (mGluR5 agonist; 300 μmol/5 μl), MTEP (mGluR5 antagonist; 150 nmol/5 μl), morphine (5 μmol/5 μl), fluorocitrate (0.75 nmol/5 μl), Vehicle 1 (dilute NaOH in 0.9% saline; 5 μl) and Vehicle 2 (dilute DMSO in 0.9% saline; 5 μl) on tumor growth. The ratio of maximum thigh circumference (ipsi/contra) of mice indicated the size of the tumor. Animals were treated once daily injections on days 14–20 after surgery with the drugs or vehicles. Data were presented as mean ± SD. If the two groups were marked with different letters (a, b), it showed the data were of statistical difference between these two groups (P < 0.05).

**Effects of the Intrathecal Injection of Drugs or Vehicles on Tumor Growth**

The spinal therapies were given chronically for 7 days; it was possible that the therapies directly or indirectly affected tumor growth in this model, and the extent of tumor burden might also affect the degree of astrocyte activation. Hence, we examined the effects of the intrathecal injection of drugs or vehicles on tumor growth. The results suggested the therapies have not significant effects on the size of tumor [the ratio of maximum thigh circumference (ipsi/contra)].

**Conclusions**

In conclusion, this study demonstrated that bone cancer pain induced an increase in mGluR3 and mGluR5 expression in spinal cord. The spinal up-regulation of these receptors may indicate enhanced signaling serving to modulate ongoing sensory input from the tumor-bearing limb. In addition, spinal astrocyte activation may be contributing to the initiation and maintenance of persistent hyperalgesia. The central mGluR3 activation or mGluR5 inhibition relieved hypersensitivity without overt behavioral side effects, and the antihypersensitivity effects were parallel with inhibition of spinal astrocyte activation. However, the relief was a minor degree. The reason may be that mGluRs activation was only one aspect of the complex mechanisms of bone cancer pain. Therefore, the treatment to bone cancer pain requires a mix of methods.


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