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Reduction of Bone Cancer Pain by Activation of Spinal Cannabinoid Receptor 1 and Its Expression in the Superficial Dorsal Horn of the Spinal Cord in a Murine Model of Bone Cancer Pain

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Background: **Bone cancer pain has a strong impact on the quality of life of patients, but it is difficult to treat. Therefore, development of a novel strategy for the treatment of bone cancer pain is needed for improvement of patient quality of life. This study examined whether selective spinal cannabinoid receptor 1 (CB1) activation alleviates bone cancer pain and also examined the spinal expression of CB1.**

Methods: **A bone cancer pain model was made by implantation of sarcoma cells into the intramedullary space of the mouse femur. In behavioral experiments, the authors examined the effects of activation of spinal CB1 and inhibition of metabolism of endocannabinoid on bone cancer-related pain behaviors. Immunohistochemical experiments examined the distribution and localization of CB1 in the superficial dorsal horn of the spinal cord using specific antibodies.**

Results: **Spinal CB1 activation by exogenous administration of a CB1 agonist arachidonyl-2-chloroethylamide reduced bone cancer–related pain behaviors, including behaviors related to spontaneous pain and movement-evoked pain. In immunohistochemical experiments, although μ-opioid receptor 1 expression was reduced in the superficial dorsal horn ipsilateral to the site of implantation of sarcoma cells, CB1 expression was preserved. In addition, CB1 was mainly expressed in the axon terminals, but not in the dendritic process in the superficial dorsal horn.**

Conclusion: **Spinal CB1 activation reduced bone cancer–related pain behavior. Presynaptic inhibition may contribute to the analgesic effects of spinal CB1 activation. These findings may lead to novel strategies for the treatment of bone cancer pain.**

AS advances in cancer detection and treatment have increased the life expectancy of cancer patients, more attention to improving patient quality of life is needed. Among several types of cancer pain, bone cancer pain is often debilitating, difficult to treat, and insufficiently relieved.[1] Although opioids are used for the treatment of advanced bone cancer pain, according to the guidelines of the World Health Organization's "analgesic ladder," patients often require higher doses of morphine, which produce diverse and disabling side effects such as sedation, somnolence, and constipation that may diminish quality of life.[2,3] Animal studies using a mouse model of bone cancer pain have also shown that bone cancer pain is resistant to morphine compared to inflammation pain.[4,5] Therefore, development of a novel strategy for the treatment of bone cancer pain is needed for improvement of patient quality of life.

There has been recent increasing interest in the use of cannabinergic system-based drugs for the management of pain. Endocannabinoids, of which the two most well-investigated are anandamide and 2-arachidonoyl glycerol, are synthesized on demand and serve several important physiologic functions, including nociception.[6] After their release, anandamide and 2-arachidonoyl glycerol are removed from the extracellular space and are then hydrolyzed by fatty acid amide hydrolase[7] and degraded by monoacylglycerol lipase.[8] Cannabinoids exert their effects *via* activation of type I cannabinoid receptor (CB1) and type II cannabinoid receptor (CB2), which are coupled to $G_{i/o}$ protein, and activation of CB1 and CB2 inhibits adenylyl cyclase.[9,10] CB1 is extensively distributed in the peripheral and central nervous systems, including the spinal cord, whereas CB2 is expressed primarily in peripheral tissues, immune cells, and glial cells in the central nervous system. In the spinal cord, CB1 is expressed in the superficial dorsal horn,[11] which plays an important role in the processing of nociceptive transmission from the periphery to the central nervous system.[12,13] The role of spinal CB1 in nociceptive transmission has been extensively studied. Activation of spinal CB1 has been shown to inhibit glutamatergic excitatory postsynaptic currents in spinal cord slices of naïve rats[14] and c-fiber–evoked neuronal response of the dorsal horn in naïve rats,[15] neuropathic rats,[16] and rats with inflammation.[17] It has also been shown that spinal CB1 activation reduced vincristine-induced allodynia[18] and inflammation-induced mechanical hypersensitivity.[19] However, the role of spinal CB1 in bone cancer pain has not been elucidated. Therefore, we focused on spinal CB1 to develop a novel strategy for the treatment of bone cancer pain.

In this study, we used a preclinical model of bone cancer pain involving injection of osteolytic sarcoma cells into the intramedullary space of the mouse femur.[20] We examined whether selective spinal CB1 activation

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alleviated bone cancer pain. We also examined the spinal expression of CB1 using a specific antibody.

Materials and Methods

Animals

The present experiments were approved by the Sapporo Medical University Animal Care Committee, Sapporo, Hokkaido, Japan and were in accordance with the ethical guidelines of the National Institutes of Health. Experiments were conducted in adult male C3H/HeJ mice (20–25 g; Japan SLC, Hamamatsu, Japan) and CB1-deficient mice of C57BL/6J strain (20–25 g). The mice were housed in a temperature-controlled (21 ± 1°C) room with a 12-h light/dark cycle and given free access to food and water.

Drugs

The CB1 agonist arachidonyl-2-chloroethylamide (ACEA), CB1 antagonist *N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (AM251), and CB2 antagonist 6-Iodo-2-methyl-1-[2-(4-morpholinyl) ethyl]-1H-indol-3-yl](4-methoxyphenyl)methanone (AM630) were purchased from Tocris Cockson Inc. (Ellisville, MS). The monoacylglycerol lipase inhibitor [1,1′-biphenyl]-3-yl-carbamic acid (URB602) and fatty acid amide hydrolase inhibitor 3′-(aminocarboxyl)[1,1′-biphenyl]-3-yl-cyclohexylcarbamate (URB597) were purchased from Cayman Chemical (Ann Arbor, MI). All drugs were dissolved in dimethylsulfoxide and diluted in physiologic saline.

Intrathecal Catheter

Under general anesthesia (3% isoflurane in oxygen), a polyurethane intrathecal catheter with an inner diameter of 0.35 mm and an outer diameter of 0.84 mm (R-ITC, Pittsburgh, PA) 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.[21] The catheter was tunneled subcutaneously and externalized through the skin in the neck region. The volume of dead space of the intrathecal catheter was 2 μl. To avoid occlusion of the catheter, 2 μl of normal saline was injected *via* a catheter on alternate days until the end of the experiment. After the end of the experiment, the effects of intrathecal lidocaine (2%, 2 μl) was 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 (Hamilton, Reno, NV) connected to an intrathecal catheter under brief general anesthesia (isoflurane in oxygen). Drugs were administered manually over a 10-s period in a single injection volume of 2 μl followed by a flush of physiologic saline (3 μl). After discontinuance of general anesthesia, mice fully recovered within 2–3 min. Our preliminary study showed that the effect of 2 μl of 2% lidocaine is limited in the lower limbs but that 4 μl of 2% lidocaine produced paralysis of upper limbs and respiratory depression, suggesting that intrathecally administered drugs at a dose of 4 μl had supraspinal effects. Therefore, we used the dose of 2 μl of the drugs in the behavioral study. In addition, our preliminary study also showed that intrathecal catheter implantation did not significantly increase the spinal expression of the microglia marker Iba-1, which was increased by inflammation and nerve injury.

Bone Cancer Model

Implantation of sarcoma cells was performed after intrathecal catheterization on the same day as previously described.[22] Murine sarcoma cells (NCTC 2472; ATCC, Rockville, MD) were maintained in NCTC 135 media containing 10% horse sera (HyClone, Logan, UT) and passaged weekly according to ATCC recommendations. A superficial incision was made in the skin overlying the left patella. The patellar ligament was then cut, exposing the condyles of the distal femur. A 0.5-mm depression was then made using a half-round burr in a pneumatic dental high-speed handpiece to facilitate mechanical retention of the amalgam plug. Then, either 20 μl of α-minimum essential medium (sham-implanted mice) or 20 μl of medium containing 1×10^5 sarcoma cells (sarcoma-implanted mice) was injected using a 29-gauge needle and a 0.25-ml syringe. To prevent leakage of cells outside the bone, the injection site was closed with dental-grade amalgam, followed by copious irrigation with filtered water. The wound was then closed.

The following experiments were conducted at day 14 after sarcoma implantation; it has been shown that there is maximal exhibition of cancer-related pain behaviors and maximal change in expression of neurochemical markers of peripheral and central sensitization at this time.[23] Each animal was used in only one experiment.

Assessment of Bone Cancer Pain

Ongoing and movement-evoked pain behaviors were analyzed as described previously.[22] Quantification of spontaneous flinches was used for assessment of ongoing pain. Limb use during spontaneous ambulation and weight-bearing during spontaneous standing were also observed for assessment of movement-evoked pain. Mice were placed in a clear plastic box and allowed to habituate for 30 min. The behavioral assessments were then performed. Limb use during spontaneous ambulation was assigned scores on a scale of 0–4: 0, complete lack of limb use; 1, relative lack of use of the limb in locomotor activity; 2, limping and guarding behavior; 3, substantial limping; 4, normal use. The number of spontaneous flinches was recorded during a 2-min observation period. Flinches

were defined as holding of the hind paw aloft while not ambulatory. In addition, weight-bearing during standing was scored on a scale of 0–3: 0, no weight-bearing (the hind paw on the injected side is always lifted and never touches the floor); 1, touch weight-bearing (the hind paw on the injected side is lifted from the floor but occasionally touches it); 2, partial weight-bearing (partial support of body weight with the hind limb on the injected side); 3, full weight-bearing (full support of 100% of body weight with both hind limbs).

Effects of ACEA, AM251, URB597, and URB602

After recording basal values of the number of spontaneous flinches, limb use score and weight-bearing score, sarcoma-implanted mice received intrathecal vehicle, ACEA (1, 0.3, and 0.1 nmol/2 μl), URB597 (3.0, 1.5, and 0.3 nmol/2 μl), URB602 (1.7, 0.4, and 0.17 nmol/2 μl), AM251 (0.06 nmol/2 μl), and AM630 (10 nmol/2 μl). Behavioral measurement was carried out 10, 20, 30, 40, 50, and 60 min after drug administration. In some sarcoma-implanted mice, a mixture of AM251 and ACEA (AM251 0.06 nmol + ACEA 1 nmol/2 μl) or a mixture of AM630 and ACEA (AM630 10 nmol + ACEA 1 nmol/2 μl) was intrathecally administered. The observer was blind to the type of treatment.

Evaluation of Catalepsy and Motor Impairment

To evaluate the contribution of motor impairment and catalepsy to the effects of ACEA on pain-related behavior, catalepsy and motor reflex were tested. The bar test was used to determine the level of catalepsy.[24] Each mouse was placed with its fore limbs on a metal bar (diameter of 1 cm) positioned 5 cm above and parallel to the counter top. The hind paws of the mouse rested on the counter top. The time each mouse spent in this position in 2 consecutive trials (60 s each) was recorded and then averaged. Catalepsy was defined as an increase in the time that mice were motionless on the bar. For evaluation of motor reflex, righting and placing-stepping tests were used.[25] The placing-stepping reflex was tested by placing the rostral aspect of the hind paws on the edge of a table and was quantified as the seconds in which the animals put the paws up and forward into a position to walk. The righting test consists of placing the animal prone and recording the ability to right itself. The test was assigned scores on a scale of 0–3: 0, normal (an immediate and coordinated twisting of the body to an upright position); 1, mild (ability to completely right, but slowly); 2, moderate (ability to right the forelimbs slowly followed by the hind limbs with more difficulty); 3, severe impairment (inability to right in 20 s). Each test was performed before and 20, 40, 60, and 90 min after intrathecal administration of 1.0 nmol ACEA. There is a possibility of the bar test causing bone fracture in sarcoma-implanted mice; therefore, these tests were performed using naïve mice.

Dorsal Root Rhizotomy

The effect of dorsal root rhizotomy on spinal CB1 expression was examined in naïve mice. Under general anesthesia (3% isoflurane in oxygen) and aseptic precautions, the left L4 dorsal root of the naïve mice was identified and cut after exposure by hemilaminectomy. The incision was closed in layers. Seven days after rhizotomy, mice were perfused, and the spinal cord innervated by the L4 nerve was processed for immunohistochemical analysis as described in the Immunohistochemistry section.

Cholera Toxin β Subunit Labeling

To label myelinated afferents in the lumbar spinal cord, transganglionic tracing with cholera toxin β subunit (CTb) was carried out in the sarcoma-implanted mice. The sarcoma-implanted mice received CTb injection at day 11 after sarcoma implantation. Under general anesthesia (3% isoflurane in oxygen) and aseptic precautions, the left L4 spinal nerve was exposed, and 0.5 μl of 1% CTb (List Biologic Laboratories, Inc., Campbell, CA) was injected into the nerve with a microinjection syringe (Hamilton). Three days after injection, mice were perfused, and the spinal cord innervated by the L4 nerve was processed for immunohistochemical analysis as described in the Immunohistochemistry section.

Immunohistochemistry

We used polyclonal antibodies raised against the following molecules: mouse CB1 (0.1 μg/ml, guinea pig)[26]; mouse protein kinase Cγ (PKCγ; 1.0 μg/ml, rabbit)[27]; mouse synaptophysin (1.0 μg/ml, rabbit)[28]; mouse microtubule-associated protein-2 (1.0 μg/ml, goat)[29]; calcitonin gene-related peptide (CGRP; 1:8000, C8198, rabbit; Sigma, St. Louis, MO); CTb (1:10000, #703, goat; List Biologic Laboratories). We also used biotinylated isolectin B4 (IB4; 1:100, L3759; Sigma). In addition, we developed an antibody to mouse μ-opioid receptor 1 (MOR1). A fragment of complementary DNA encoding the *N*-terminal 39 amino acids of mouse MOR1 (GenBank accession number U26915) was subcloned into *Bam*HI/*Eco*RI site of the pGEX4T-2 plasmid vector (Amersham Biosciences, Bucks, United Kingdom) to be expressed as glutathione *S*-transferase fusion protein. The fusion protein was emulsified with Freund's complete in the first immunization and with incomplete adjuvant in the subsequent immunization (DIFCO, Detroit, MI) and injected subcutaneously into female rabbits at intervals of 2 weeks. Two weeks after the sixth injection, MOR1-specific antibody was collected by affinity purification using glutathione *S*-transferase–free polypeptides coupled to cyanogen bromide-activated Sepharose 4B (Amersham Biosciences). Glutathione *S*-transferase–free peptides were prepared by in-column thrombin digestion of glutathione *S*-transferase fusion proteins

bound to glutathione-Sepharose 4B media. Mice were deeply anesthetized with 50 mg/kg ketamine intraperitoneally and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer. The spinal cord innervated by the L3 or L4 (for rhizotomy and CTb experiments) dorsal root for sarcoma-implanted mice and naive mice was removed. Our preliminary study using a retrograde neuronal tracer (Fluoro-Gold; Fluorochrome, Denver, CO) showed that the neurons innervating the distal femur originated from L2/3/4 dorsal root ganglions (DRGs). The spinal cord was immersed in 4% paraformaldehyde in phosphate buffer for 2 h for postfixation and then cryoprotected in 25% sucrose in phosphate-buffered saline (PBS) overnight at 4°C. The samples were placed in Tissue-Tek embedding medium (Sakura Finetechnical Co., Tokyo, Japan) and rapidly frozen. Frozen sections of spinal cord were cut at 50 μm by using a sliding cryostat (Sakura). The following procedure was done in a free-floating state. The tissue sections were washed in PBS and incubated for 1 h at room temperature in a blocking solution consisting of 10% normal donkey serum and 0.2% TritonX-100 (Sigma) in PBS (PBS-t). Sections were then incubated with anti-CB1 antibody or a mixture of primary antibodies overnight. After rinses with PBS-t, the sections were incubated with Alexa Fluor 488-, Alexa 597-, and Alexa 647-labeled species-specific secondary antibodies at a dilution of 1:500 (Invitrogen, Carlsbad, CA) in PBS-t for 2 h at room temperature. Images were taken with a confocal laser scanning microscope (DIGITAL ECLIPSE C1; Nikkon, Tokyo, Japan). In all cases, images were a single stack and were acquired with line-by-line sequential scanning to prevent bleed-through and cross-excitation of fluorophores.

Western Blot Analysis

The mice were deeply anesthetized with ketamine and sacrificed by decapitation. The bilateral halves of the spinal cord innervated by L2-4 dorsal roots were rapidly removed. Samples were homogenized in the presence of 0.01-M PBS and protease inhibitor cocktail (Sigma) on ice. The crude homogenates were centrifuged at 15,000*g* for 20 min at 4°C. Supernatants of the homogenates were collected, and protein concentrations were determined using the DC protein assay (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin. Equal amounts of protein (0.2 mg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4%) and transferred to nitrocellulose membranes (Millipore, Billerica, MA). Membranes were then incubated with guinea pig anti-CB1 antibody (1 μg/ml) in PBS containing 10% skim milk overnight at 4°C. The concentrations of actin, a housekeeping protein, were also measured using rabbit anti-actin antibody (1:5,000; Sigma; Cat. No. A2066). Immunoreaction was visualized with an enhanced chemilunescence plus chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ).

Quantitative Immunohistochemical Analysis

Changes in CB1 and MOR1 staining were quantified using gray scales (0-black to 255-white). Analyses were performed on four randomly selected sections from each animal. Images of four randomly chosen sections from each animal were imported into Win Roof 5.6.2 software (Mitani Corporation, Fukui, Japan). The relative intensity of gray level was determined by dividing the gray level in the ipsilateral side to sarcoma implantation by the gray level in the contralateral side in the sarcoma-implanted mice. In naïve mice, the relative intensity of gray level was determined by dividing the gray level in the left dorsal half of the spinal cord by the gray level in the right side. The average of the relative intensity was calculated to determine the value for each animal. Analysis of colocalization of CB1 with synaptophysin was performed on four randomly selected from each animals. An assistant who was unaware of the treatment group of sections performed all measurements.

Statistical Analysis

The scores for limb use and weight-bearing are expressed as median with first and third quartiles, and minimum and maximum values. The number of flinches, the data for bar test, and placing-stepping test are expressed as means ± SD. Changes in limb use score and weight-bearing score were compared to the basal values using the nonparametric Friedman's test for repeated measures followed by Bonferoni test within a single group and were analyzed using the Kruskall-Wallis test followed by Bonferoni test for between-group comparisons. Changes in number of flinches were compared to the basal value using a one-way analysis of variance for repeated measures followed by Dunnett test within a single group and were analyzed using a one-way analysis of variance followed by Dunnett test for between-group comparisons. The data for bar test and placing-stepping test were compared to the basal value using a one-way analysis of variance for repeated measures followed by Dunnett test within a single group. For immunohistochemical experiments, the relative intensity of gray scale was compared using the Mann–Whitney U test. For Western blot analysis, the intensity of bands was determined using an image densitometer (NIH Image 1.63; National Institutes of Health, Bethesda, MD) and was normalized to the intensity of β-actin. The relative intensity of bands was compared using the Mann–Whitney U test. $P < 0.05$ was considered to be statistically significant. Statistical analysis was performed

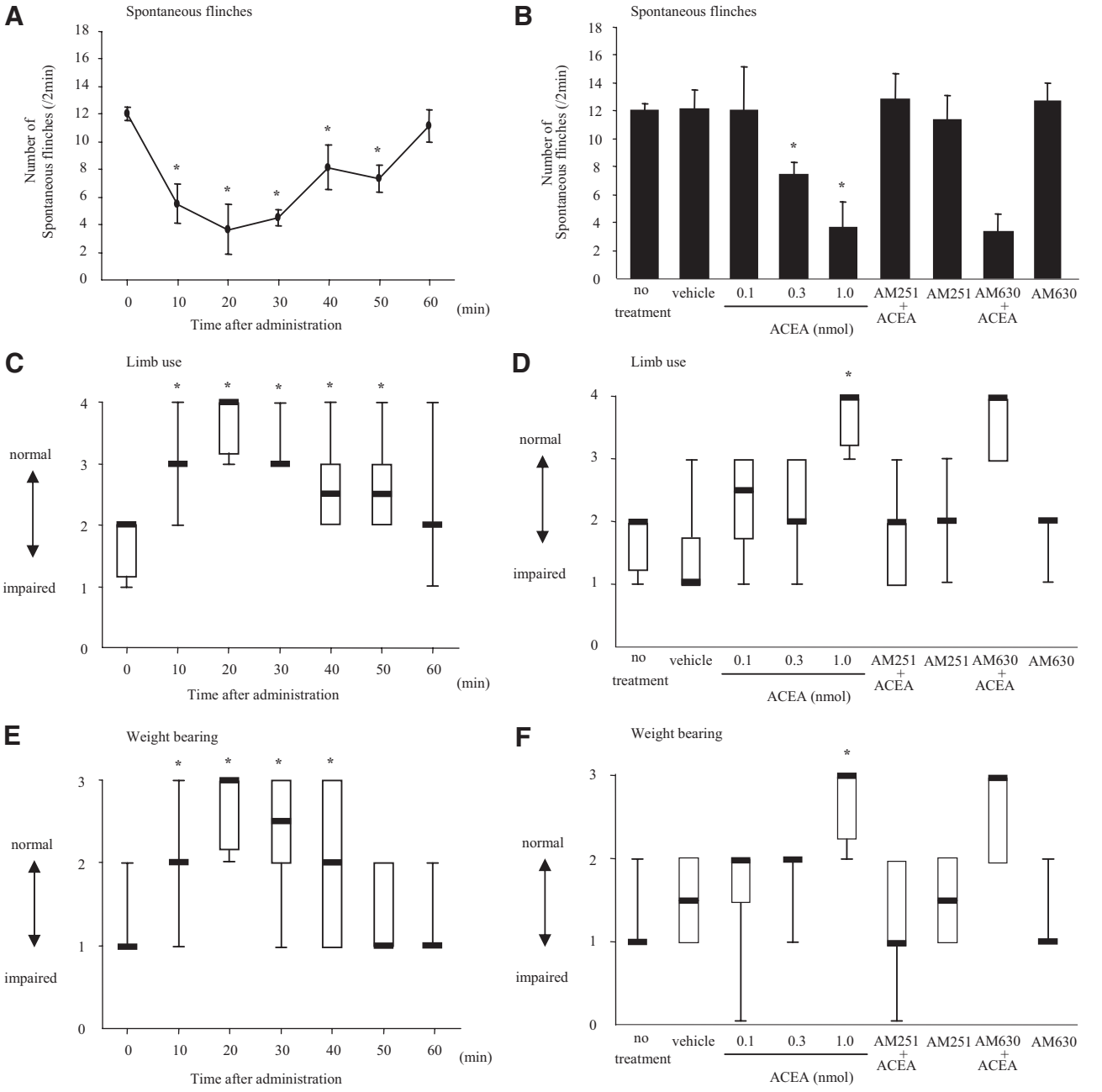

Fig. 1. Analgesic effects of intrathecal arachidonyl-2-chloroethylamide (ACEA) on bone cancer–related pain behavior. (*A*, *C*, and *E*) Time courses of analgesic effects of intrathecal ACEA at the dose of 1.0 nmol. (*B*, *D*, and *F*) Dose-response relationships of ACEA and the effects of AM251 (0.06 nmol) or AM630 (10 nmol) on ACEA (1 nmol)–induced analgesic effects. (*A* and *B*) Effects of ACEA on spontaneous flinches. (*C* and *D*) Effects of ACEA on limb use. (*E* and *F*) Effects of ACEA on weight-bearing. Data of flinches are presented as means ± SD. Data of limb use score and weight-bearing score are presented as medians with first and third quartiles, and minimum and maximum values. * $P < 0.05$ *versus* values at 0 min (before drug administration) in *A–C*. * $P < 0.05$ *versus* vehicle in *D–F*. $n = 6$ in no-treatment group; $n = 7$ in vehicle group; $n = 8$ in the remaining groups.

using Statview 5.0 software (Abacus Concepts, Berkeley, CA) and NP Multi (Nagata T, Tokyo, Japan).

Results

Development of Bone Cancer–related Pain Behaviors in Sarcoma-implanted Mice

Before sarcoma implantation, the mice exhibited no detectable pain-related behavior. At day 14 after sarcoma implantation, the mice with an intrathecal catheter exhibited spontaneous flinches (12 ± 1 flinches over a 2-min observation period), impaired limb use during spontaneous ambulation (limb use score, 2), and impaired weight-bearing during spontaneous standing (weight-bearing score, 1). These behavioral scores were similar to those in our previous study using sarcoma-implanted mice without an intrathecal catheter,[22] suggesting that implantation of the intrathecal catheter did not affect the development of bone

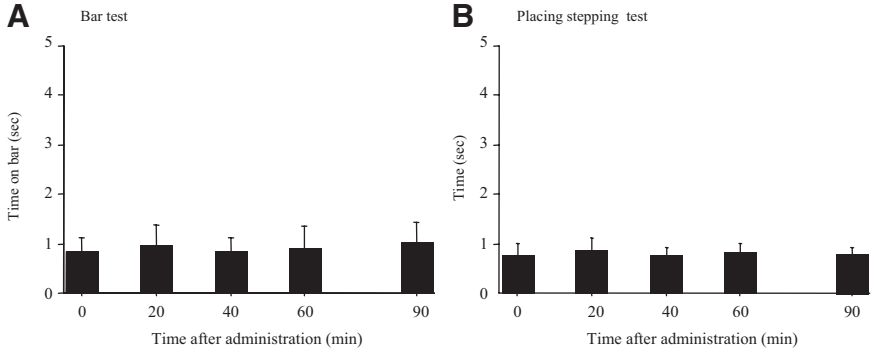

Fig. 2. Effects of intrathecal arachidonyl-2-chloroethylamide (ACEA) at the dose of 1.0 nmol on the bar test. (*A* and *B*) Bar test and the placing stepping test, respectively. The bar test and the placing stepping test were performed using naïve mice. Data are presented as means ± SD; $n = 6$.

cancer-related pain behavior. On the other hand, sham-implanted mice did not show any detectable pain-related behavior.

Intrathecal ACEA Reduced Bone Cancer-related Pain Behaviors

We examined whether activation of spinal CB1 reduced bone cancer–related pain behavior. Intrathecal ACEA at 1 nmol significantly ($P < 0.05$) reduced the number of flinches and increased limb use score and weight-bearing score compared to the basal values (fig. 1, A–F). Peak effects were observed 20 min after intrathecal administration, and the effect of ACEA disappeared within 60 min after administration (fig. 1, A, C, and E). The number of flinches was significantly ($P < 0.05$) reduced from 12 to 4 (flinches over a 2-min observation period) 20 min after intrathecal administration. Limb use score and weight-bearing score were significantly ($P < 0.05$) improved from 2 to 4 and from 1 to 3, respectively, 20 min after administration. The effect of ACEA at 1.0 nmol was completely inhibited by simultaneous administration of AM251 at 0.06 nmol but not AM630 at 10 nmol, which did not affect any of the three bone cancer–related pain behaviors (fig. 1, B, D, and F). ACEA at 0.3 nmol significantly ($P < 0.05$) reduced the number of flinches but did not improve limb use score or weight-bearing score (fig. 1, B, D, and F). ACEA at 0.1 nmol did not affect bone cancer-related pain behavior. The vehicle also did not have any effect on bone cancer–related pain behavior (fig. 1, B, D, and F).

Intrathecal ACEA did not Induce Catalepsy and Motor Impairment

Before intrathecal administration of ACEA, the time that mice spent on the bar was 0.8 ± 0.3 s. Intrathecal ACEA at 1.0 nmol did not increase the time spent on the bar throughout the observation periods (0.9 ± 0.4, 0.8 ± 0.3, 0.9 ± 0.4, and 1.0 ± 0.4 s at 20, 40, 60, and 90 min after administration, respectively), compared to that before intrathecal administration (fig. 2A). Intrathecal ACEA also did not significantly impair placing-stepping reflex (fig. 2B) and righting reflex (normal at all time-points), compared to that before intrathecal administration. These results suggest that the effects of ACEA on pain-related behavior are not due to a cataleptic effect and motor impairment.

Intrathecal URB597 and URB602 did not Reduce Bone Cancer–related Pain Behaviors

Next, we intrathecally administered URB597 and URB602 to examine whether inhibition of the metabolism of two endogenous cannabinoids, anandamide and 2-arachidonoyl glycerol, reduced bone cancer–related pain behavior (fig. 3). Neither intrathecal URB597 (0.3, 1.5, and 3.0 nmol/2 μl) nor URB602 (0.17, 0.4, and 1.7 nmol/2 μl) altered bone cancer–related pain behavior. According to the manufacturer's protocol, both 1.7 nmol/2 μl of URB597 and 3.0 nmol/2 μl URB602 are the maximal doses that can be dissolved in 50% dimethylsulfoxide. The vehicle, 2 μl of 50% dimethylsulfoxide, also did not alter bone cancer–related pain behavior.

Specificity of Anti-CB1 and Anti-MOR Antibodies

We used affinity-purified primary antibodies raised against the mouse CB1 receptor and the mouse MOR1. Figure 4 shows the specificity of anti-CB1 and anti-MOR1 antibodies. By Western blot analysis, MOR1 antibody recognized a major band at 50 kDa in the spinal cord of the wild-type mouse (fig. 4C). MOR1 antibody yielded strong labeling in the superficial dorsal horn of the spinal cord of the wild-type mouse (fig. 4A). Preabsorption of MOR antibody (addition of 50 μg/ml antigen peptide) completely abolished the characteristic staining in the spinal cord (fig. 4B). CB1 antibody yielded strong labeling in the spinal cord of the wild-type mouse (fig. 4D), but no specific staining was found in the spinal cord of the CB1-deficient mouse (fig. 4E), as shown in the hippocampus in our precious study.[26] In addition, our previous study showed that this CB1 antibody detected a single band corresponding to expected molecular weight in Western blot.[30] Therefore, these antibodies were considered suitable for subsequent immunohistochemical experiments.

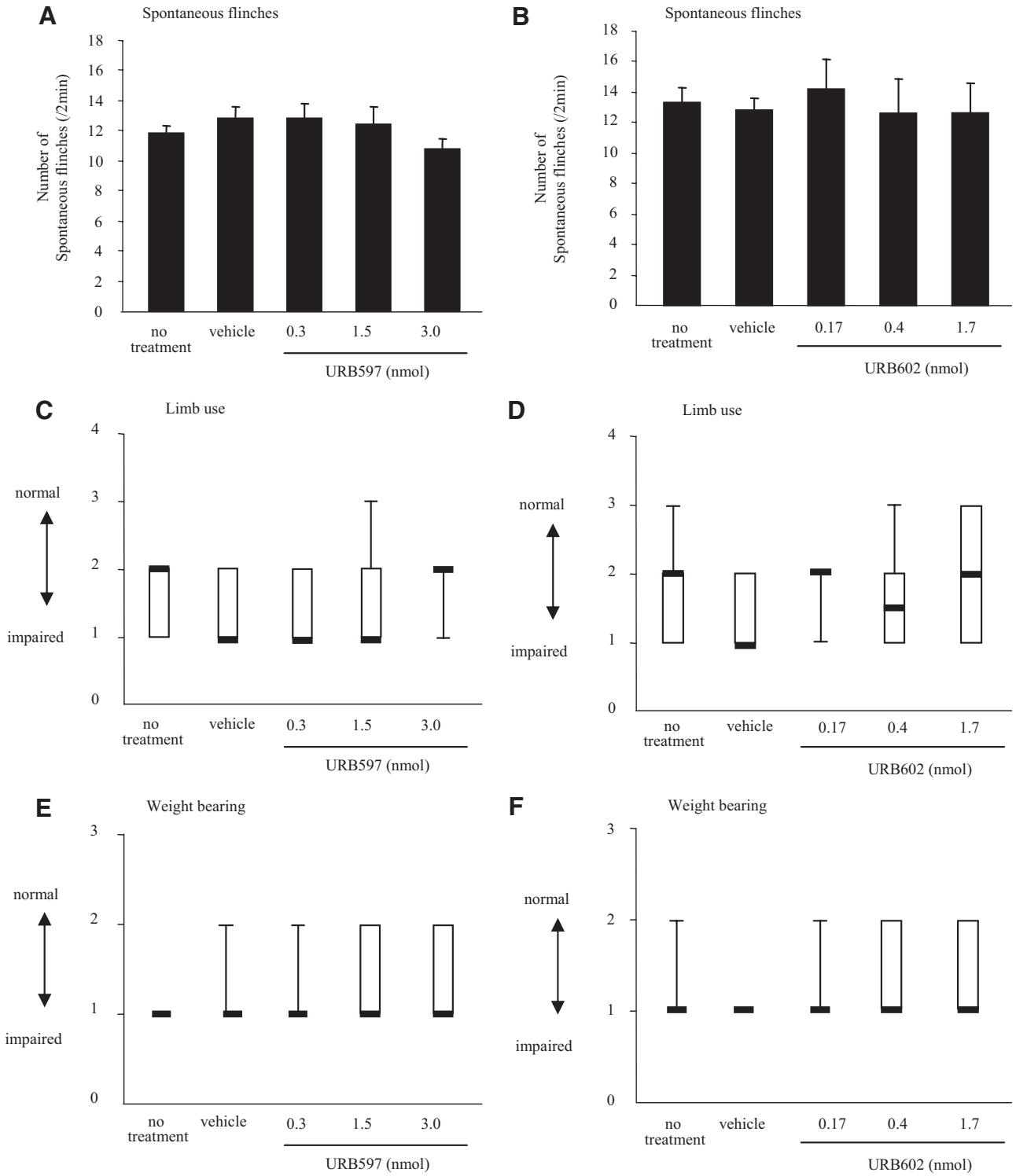

Fig. 3. Analgesic effects of intrathecal URB597 (*A*, *C*, *E*) and URB602 (*B*, *D*, *F*) on bone cancer–related pain behavior. (*A* and *B*) Effects on spontaneous flinches. (*C* and *D*) Effects on limb use. (*E* and *F*) Effects on weight-bearing. Data of flinches are presented as means ± SD. Data of limb use score and weight-bearing score are represented as medians with first and third quartiles, and minimum and maximum values. $n = 6$ in no-treatment group; $n = 7$ in vehicle group; $n = 8$ in the remaining groups.

Laminal Distribution of CB1 in the Spinal Cord of Naïve Mice

We characterized spinal CB1 distribution in the naïve mice using a specific CB1 antibody. Intense CB1 immunoreactivity (ir) was found in the superficial layer and deep layer in the dorsal horn, in the dorsolateral funiculus, and around the central canal of the lumbar spinal cord (fig. 4D). Faint CB1-ir was detected in the entire gray matter of the spinal cord. Immunofluorescence at high magnifications visualized CB1 as tiny puncta in the

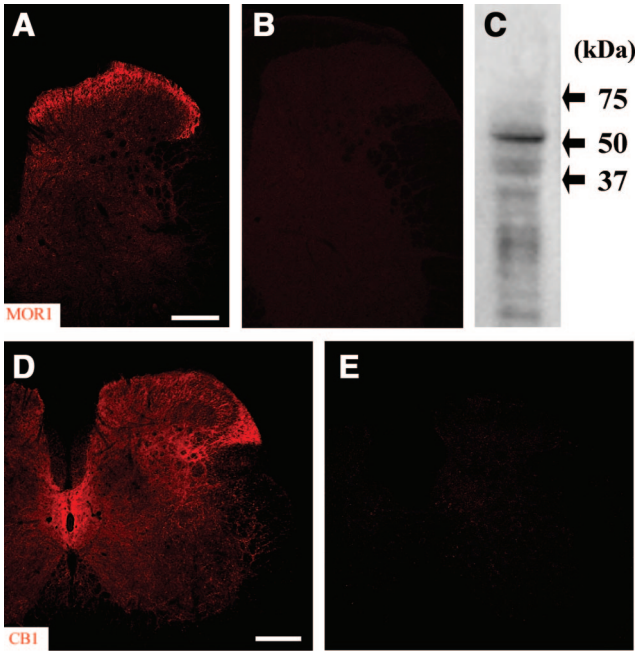

Fig. 4. Specificities of anti–μ-opioid receptor 1 (MOR1) antibody (*A–C*) and anti-cannabinoid receptor 1 (CB1) antibody (*D* and *E*). (*A* and *B*) Immunostaining in the dorsal horn of a wild-type mouse by anti-MOR1 antibody and antigen-preabsorbed anti-MOR1 antibody (1 μg/ml anti-MOR1 antibody + 50 μg/ml antigen peptide), respectively. (*C*) Immunoblot detection of anti-MOR1 from mouse lumbar spinal cord extract with anti-MOR1 antibody. (*D* and *E*) Immunostaining in the dorsal horn of a wild-type mouse and CB1-deficient mouse, respectively, by anti-CB1 antibody. *Scale bar* = 200 μm.

dorsal horn (fig. 5A). No CB1-ir was observed in the cell body. In the superficial dorsal horn, CB1-ir showed a double layer, which was the outer part and inner part of the superficial layer (fig. 4D and fig. 5A). These layers were separated by a band of minimal staining. The mouse superficial dorsal horn, but not the rat superficial dorsal horn, is divided into the three layers by the distribution of the three distinct molecular markers, CGRP, IB4, and PKCγ.[31,32] The area labeled by CGRP-ir corresponds to lamina I and the outer part of lamina II (IIo), and the area labeled by IB4-positive reactivity and PKCγ-ir corresponds to the dorsal part of lamina II inner (dIIi) and the ventral part of lamina II (vIIi), respectively. The superficial and deeper bands of the CB1-ir double layer showed dense laminal overlap with CGRP-ir and PKCγ-ir, respectively, whereas CB1-ir was faint in the IB4-positive region (fig. 5, B–D). These observations indicate that CB1 is mainly expressed in lamina I-IIo and vIIi rather than lamina dIIi in the superficial dorsal horn.

Recently, Agarwal *et al.*[19] showed that CB1-ir is little altered in the dorsal horn of DRG-specific CB1 knockout mice. Consistent with their study, CB1-ir in the dorsal horn ipsilateral to dorsal root rhizotomy was roughly comparable to that contralateral to rhizotomy, whereas CGRP-ir was dramatically decreased in the dorsal horn ipsilateral to rhizotomy (fig. 5E).

Unilateral Sarcoma Implantation into the Femur did not Change CB1 Expression in the Superficial Dorsal Horn. At day 14 after sarcoma implantation, CB1-ir in the superficial dorsal horn ipsilateral to the site of sarcoma implantation showed a laminal distribution similar to that contralateral to the site of sarcoma implantation and to that in the naïve mice (fig. 6). These observations indicate that unilateral sarcoma implantation did not change the laminar distribution of CB1 in the superficial dorsal horn of the spinal cord.

Next, we examined whether unilateral sarcoma implantation altered CB1 expression level in the superficial dorsal horn of the spinal cord. Western blot analysis showed that CB1 protein levels in the half of the spinal cord ipsilateral to the site of implantation in sarcoma-implanted mice were comparable to those contralateral to the site of implantation in sarcoma-implanted mice and those in the spinal cord in naïve mice at days 7 and 14 after sarcoma implantation (fig. 7A). Furthermore, we compared the relative intensity of CB1-ir in each lamina in sarcoma-implanted mice with that in naive mice at day 14 after sarcoma implantation (fig. 7, B and C). Specific laminal regions were determined by double staining of CB1 with CGRP, IB4, or PKCγ. The relative intensity of CB1-ir in sarcoma-implanted mice and naïve mice was approximately 1.0 in lamina I-IIo, lamina vIIi, and lamina I-II, and there was no significant difference in the relative intensity of CB1-ir between sarcoma-implanted mice and naïve mice (fig. 7, B and C). On the other hand, the relative intensity of MOR1 in sarcoma-implanted mice was 0.85 and was significantly lower ($P < 0.05$) than that in naïve mice (fig. 7, B and C), similar to the results of our previous study in which the relative intensity of MOR1-ir was compared in sarcoma-implanted mice and sham-operated mice.[5] These results suggest that unilateral sarcoma implantation decreases MOR1 expression in the superficial dorsal horn of the spinal cord ipsilateral to the site of implantation compared to that contralateral to the site of implantation, whereas CB1 expression is not changed.

Localization of CB1 in the Superficial Dorsal Horn. We examined whether CB1 was expressed on primary afferent terminals in a bone cancer pain state. We used three markers of primary afferent neuron, including CGRP (a marker of peptidergic nociceptive primary afferent neurons), IB4 (a marker of nonpeptidergic unmyelinated primary afferent neurons), and CTb (a marker of myelinated primary afferent neurons). Few CB1-ir were overlapped with CGRP-ir, IB4 and CTb-ir in the superficial dorsal horn ipsilateral to sarcoma implantation in sarcoma-implanted mice (fig. 8, A and B). These results suggest that CB1 is expressed weakly in terminals of primary afferent neurons and strongly in spinal interneurons and/or descending fibers at day 14 after sarcoma implantation, similar to that in naïve mice.

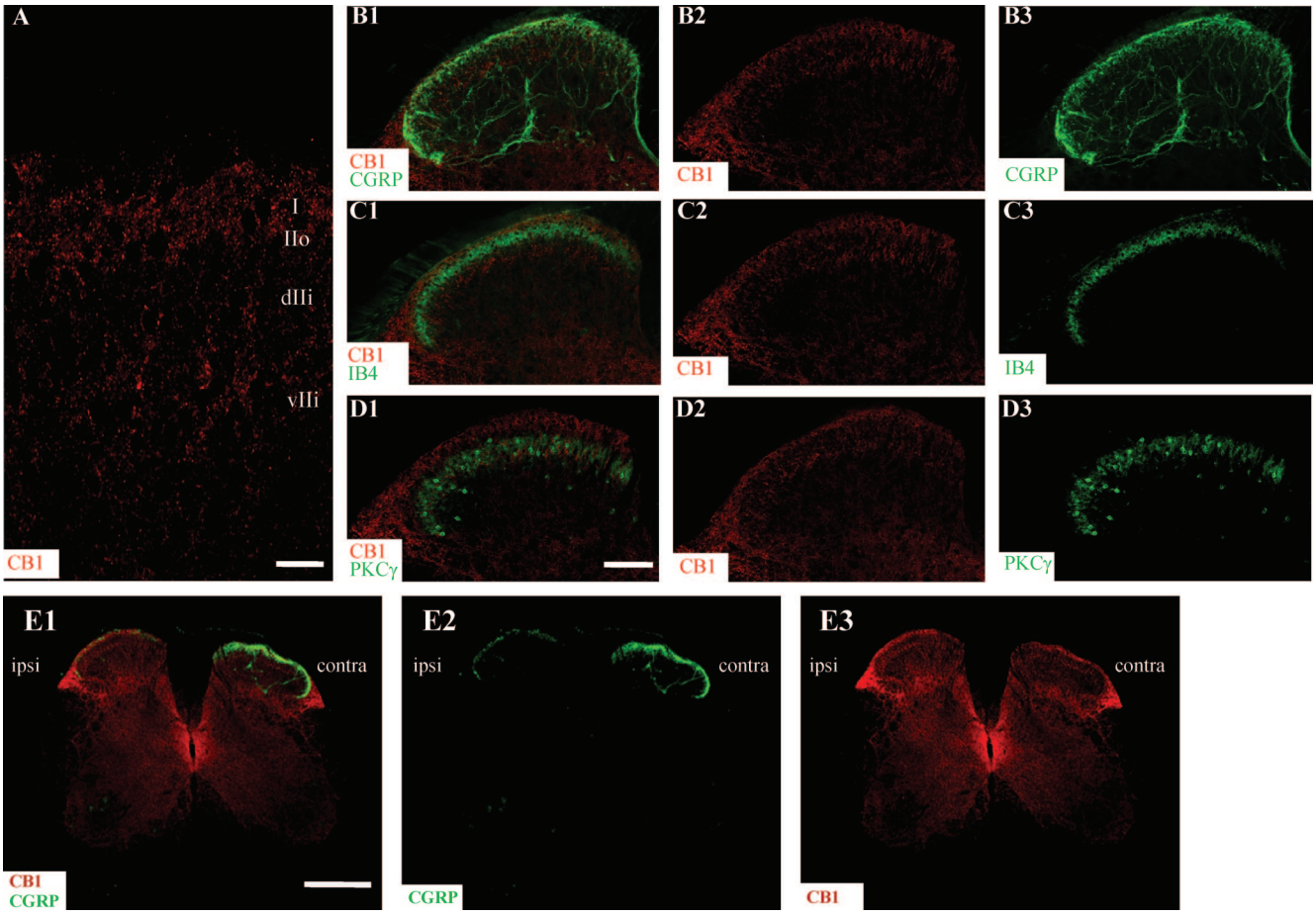

Fig. 5. Laminal distribution of cannabinoid receptor 1 (CB1) in the superficial dorsal horn of the spinal cord of naïve mice. (*A*) Distribution of CB1 in the superficial dorsal horn. (*B1–B3*, *C1–C3*, and *D1–D3*) Double immunostaining for CB1 (*red*) with calcitonin gene-related peptide (CGRP) (*green*), isolectin B4 (IB4) (*green*), and protein kinase Cγ (PKCγ) (*green*), respectively. (*E1–E3*) Effects of dorsal root rhizotomy on expression of CB1 (*red*) and CGRP (*green*) in the spinal cord. contra = contralateral to the site of rhizotomy; Ipsi = ipsilateral to the site of rhizotomy. *Scale bar* = 20 μm in *A*, 100 μm in *B1-B3*, *C1–C3*, and *D1–D3*, and 400 μm in *E1–E3*.

Next, we examined prelocalization or postlocalization of CB1-ir in the superficial dorsal horn of the spinal cord (fig. 9). Many puncta of CB1-ir were overlapped with synaptophysin-ir, which is a marker of the axon terminal; 70.3 ± 3.6% and 71.5 ± 4.6% of the puncta of CB1-ir were overlapped with synaptophysin-ir in laminae I-IIo and vIIi, respectively (n = 4). On the other hand, few puncta of CB1-ir were overlapped with microtubule-associated protein-2-ir, which is a marker of dendrite. In addition, some puncta of CB1-ir was preferentially distributed on the surface of microtubule-associated protein-2–positive elements in the sarcoma-implanted mice. These indicated that CB1 was at least expressed within the axon terminals rather than dendritic process in lamina I, IIo, and vIIi. In addition, localization of CB1 in lamina I, IIo, and vIIi in the naïve mice is comparable to that in sarcoma-implanted mice (data not shown).

Discussion

The major findings of this study were: (1) that spinal CB1 activation by exogenously administered CB1 agonist reduced bone cancer–related pain behaviors, including behaviors related to spontaneous pain and movement-evoked pain; (2) that CB1 expression but not MOR expression was preserved in the superficial dorsal horn ipsilateral to the site of implantation of sarcoma cells, and (3) that CB1 was mainly expressed in the axon terminals but not in the dendritic process in the superficial dorsal horn.

Alleviation of Bone Cancer Pain by Spinal CB1 Activation

We found five reports on analgesic effects of cannabinoids on bone cancer-related pain behavior. In four of those studies, the effects on mechanical hyperalgesia were examined using von Frey filaments and deep tissue hyperalgesia.[33–36] In the other study, the effects on movement-evoked pain were examined.[37] In clinical settings, pain originating from bone cancer is commonly divided into two categories, ongoing pain and movement-evoked pain.[2,38] Therefore, we examined the effect on spontaneous flinches as assessment of ongoing pain and the effect on limb use during spontaneous

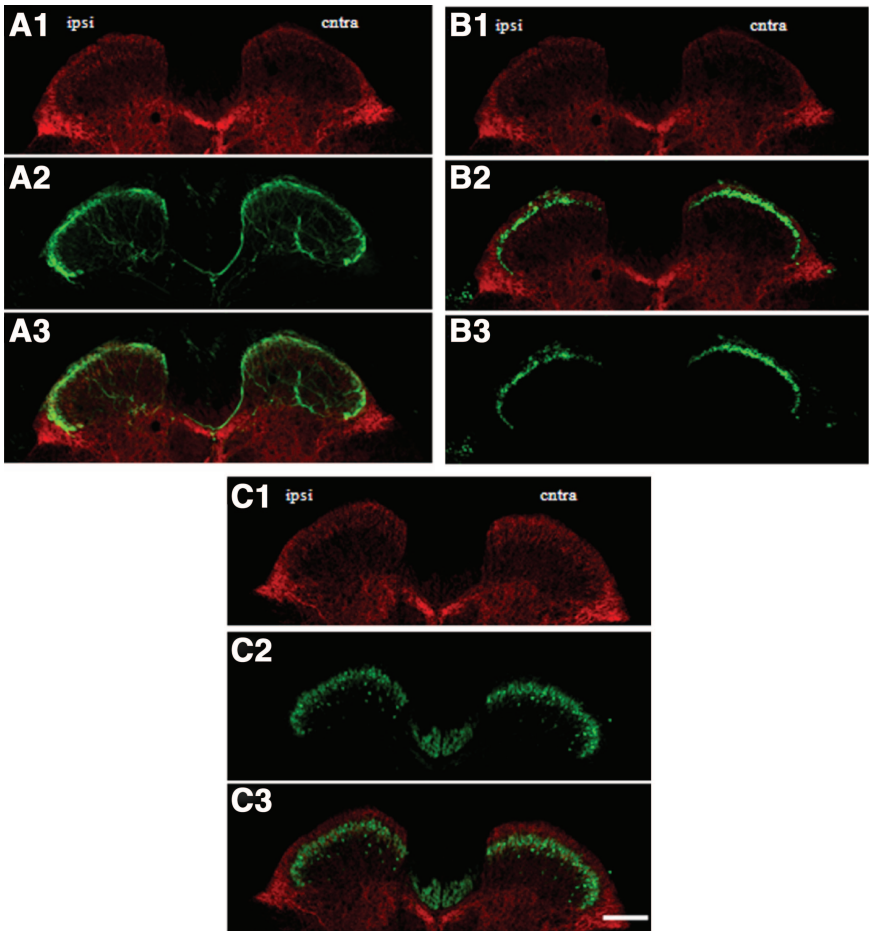

Fig. 6. Laminal distribution of cannabinoid receptor 1 (CB1) in the superficial dorsal horn of the spinal cord of sarcoma-implanted mice. (*A1–A3*, *B1–B3*, and *C1–C3*) Double immunostaining for CB1 (*red*) with calcitonin gene-related peptide (CGRP) (*green*), isolectin B4 (IB4) (*green*), and protein kinase Cγ (PKCγ) (*green*), respectively. contra = contralateral to the site of sarcoma implantation; ipsi = ipsilateral to the site of sarcoma implantation. *Scale bar* = 200 μm.

ambulation and weight-bearing during spontaneous standing as assessment of movement-evoked pain rather than evoked hyperalgesia, as previously reported.[22]

A previous study showed that systemic administration of WIN55,212–2, a nonselective CB1/CB2 agonist, reduced bone cancer pain assessed by limb use and weight-bearing in the same model as that used in this study.[37] However, it is generally thought that systemically administered cannabinoid-related drugs act at peripheral, spinal, and supraspinal sites, producing their pharmacological effects; therefore, the role of spinal CB1 in bone cancer pain is unclear. In this study, by using the cannabinoid agonist ACEA, which has extremely high selectivity for CB1,[39] we showed for the first time that spinal CB1 activation reduced bone cancer pain, including spontaneous pain and movement-evoked pain. The effect of intrathecal ACEA was reversed by simultaneous administration of AM251, a selective CB1 antagonist, but not AM630, a selective CB2 antagonist, further confirming that intrathecal ACEA acts *via* the CB1. In addition to spinal CB1 activation by an exogenously administered CB1 agonist, endogenous cannabinoids accumulated by their metabolic inhibitors can activate CB1 in the spinal cord.[40–42] Both URB597 and URB602 enhanced stress-induced analgesia by foot shock through CB1 activation in rats, although spinal inhibition of CB1 *per se* did not alter stress-induced analgesia.[40] In both nerve injury rats and naïve rats, spinal administration of URB597 attenuated evoked responses of spinal neurons through CB1 activation and elevated levels of endocannabinoids, although spinal inhibition of CB1 *per se* did not alter spinal neuronal activity.[42] These findings suggest that increasing accumulation of endocannabinoids by their metabolic inhibitors, but not tonically released endocannabinoids, results in antinociception at the level of the spinal cord. Therefore, we expected that intrathecal URB597 and URB602 would reduce bone cancer–related pain behavior through CB1 activation. However, neither intrathecal URB597 nor URB602 affected bone cancer–related pain behavior. In addition, intrathecal AM251 alone at a dose that completely reversed the effect of ACEA at 1 nmol did not affect bone cancer–related pain behavior, suggesting that nociceptive input by bone cancer does not activate the spinal endocannabinoid system involving CB1, at least at day 14 after sarcoma implantation. We used URB597 at a dose of 3.0 nmol/2 μl and URB602 at a dose of 1.7 nmol/2 μl, which are the highest concentrations according to the manufacturer's protocol. These doses are higher than those used in enhancement of stress-induced analgesia.[40] Therefore, accumulation of endocannabinoids by pharmacological inhibition of their

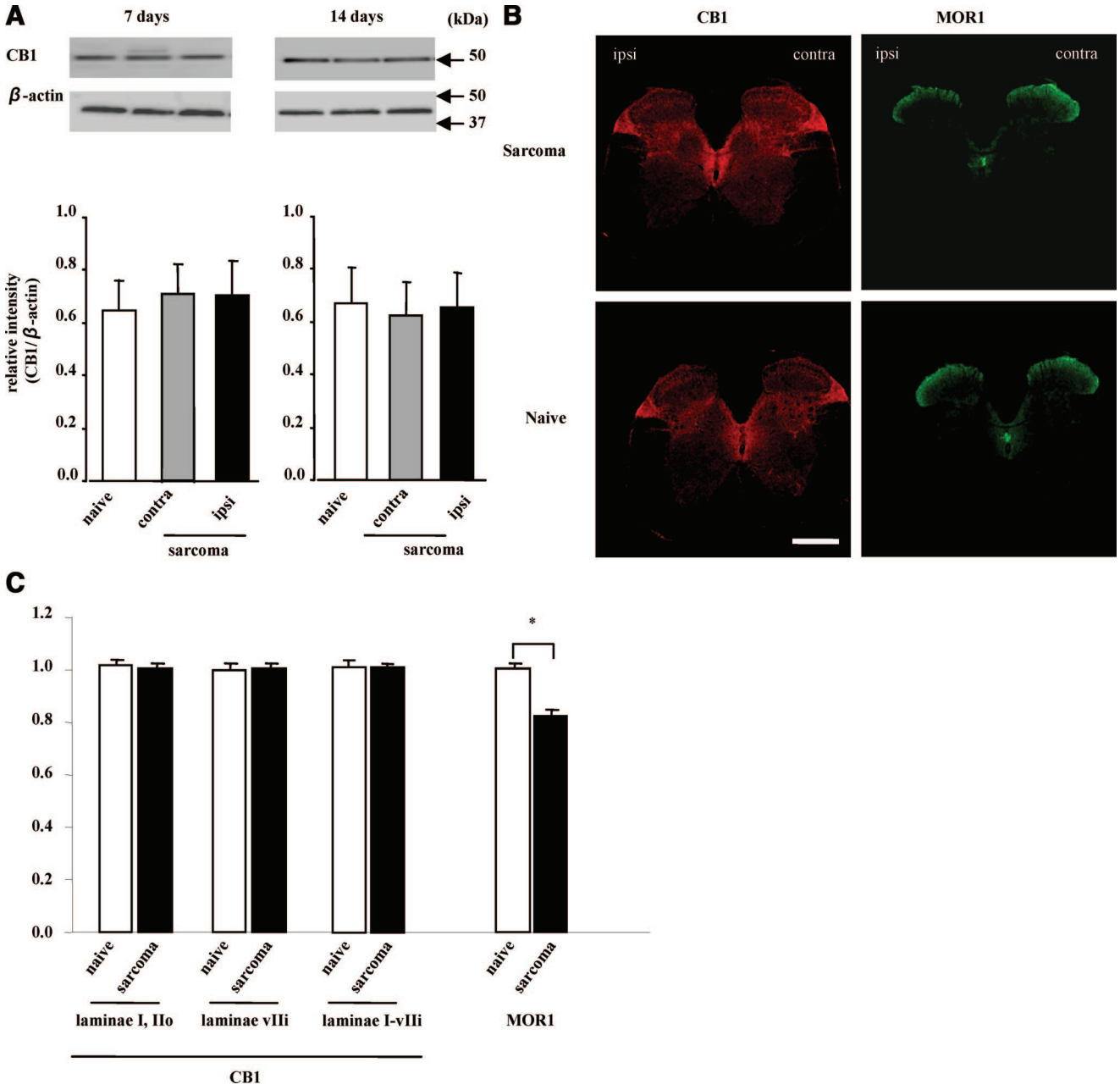

Fig. 7. Changes in spinal cannabinoid receptor 1 (CB1) and μ-opioid receptor 1 (MOR1) expression in sarcoma-implanted mice. (*A*) Immunoblot of CB1 at day 7 and day 14 after sarcoma implantation. (*Upper panel*) Representive bands of CB1 and β-actin; (*lower panel*) statistical summary of the results of densitometric analysis of immunoblot analysis. CB1 expression at (*left panels*) day 7 and (*right panels*) day 14. (*B*) Immunostaining of CB1 and MOR1 in the spinal cord of naïve mice and sarcoma-implanted mice at day 14 after sarcoma implantation. (*C*) Statistical summary of the results of relative intensity of gray level at day 14 after sarcoma implantation. contra = contralateral to the site of sarcoma implantation; ipsi = ipsilateral to the site of sarcoma implantation; Naïve = naïve mice; Sarcoma = sarcoma-implanted mice. *Scale bar* = 400 μm. Values of histograms are presented as means ± SD. * $P < 0.05$ *versus* naive mice. $n = 6$ in each group (naïve mice and sarcoma-implanted mice).

metabolic enzymes may be insufficient for reduction of bone cancer–related pain behavior. However, we cannot exclude the possibility that the doses of URB597 and URB602 used in our study were not effective for enhancing endocannabinoid concentrations in the spinal cord due to their solubility.

Recently, it has been reported that CB2 expression is induced in the spinal cord in peripheral nerve injury–induced pain models.[43,44] In a bone cancer pain model, CB2 messenger RNA level was increased in the dorsal root ganglion but not in the spinal cord.[37] However, analgesic effects of CB2 activation on bone cancer pain have been conflicting.[33–36] One study has shown that a peripheral nonselective cannabinoid receptor agonist, WIN 55,212–2, attenuated bone cancer–induced hyperalgesia *via* activation of both CB1 and CB2,[34] whereas other studies have shown that CB2 is not involved in the antihyperalgesic effects of systemic nonselective canna-

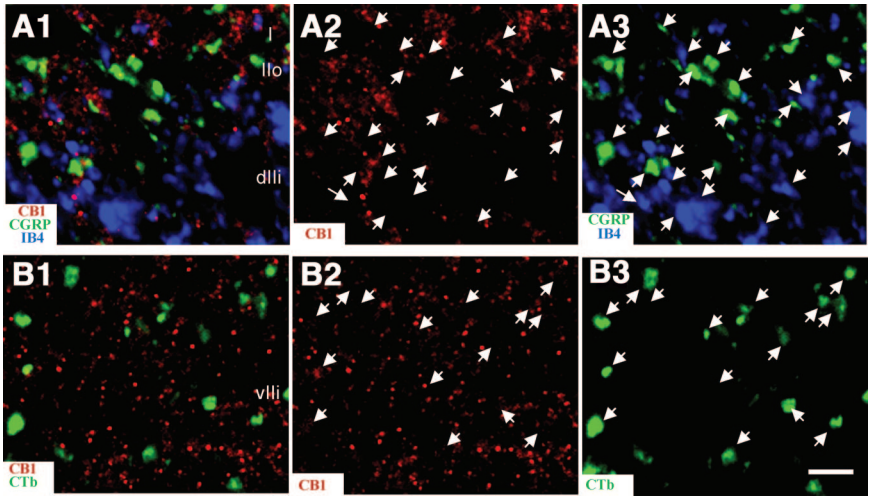

Fig. 8. Colocalization of cannabinoid receptor 1 (CB1) with markers of primary afferents in the superficial dorsal horn of the spinal cord of sarcoma-implanted mice. (*A1–A3*) Colocalization of CB1 with calcitonin gene-related peptide (CGRP) (*green*) and isolectin B4 (IB4) (*blue*) in lamina I and IIo. (*B1–B3*) colocalization of CB1 with cholera toxin β subunit (CTb) (*green*) in lamina vIIi. *Arrows* = CGRP-immunoreactive (ir), IB4-positive, and CTb-ir. *Scale bar* = 5 μm.

binoid receptor agonists.[33,36] In addition, it has been shown that the antihyperalgesic effect of local URB597 on bone cancer–induced hyperalgesia is inhibited by CB1 inhibition.[35] Thus, the role of CB2 in bone cancer pain requires further study.

Expression of CB1 in the Superficial Dorsal Horn of the Spinal Cord in Naïve Mice

Studies have shown CB1 expression in the rat spinal cord, whereas there have been no published studies showing the distribution and localization of CB1 in the mouse spinal cord. The expression pattern of some molecules in the nervous system is different among species. For example, in primary afferents neurons, transient receptor potential vanillaid subfamily 1 is expressed in few IB4-positive neurons in mice,[22,45] whereas more than 50% of TRPV1-positive neurons are IB4-positive in rats.[46] Although PKCγ-ir is overlapped with the IB4-positive area in the superficial dorsal horn of the rat spinal cord, PKCγ-ir is rarely overlapped with the IB4-positive area and is found just ventral to the IB4-positive area with a distinct segregation in the mouse spinal cord.[31,32] Accordingly, the mouse superficial dorsal horn, but not the rat superficial dorsal horn, is divided into the three layers by the distribution of the three distinct molecular markers, CGRP, IB4, and PKCγ.[31,32] Therefore, we examined the expression of CB1 in the dorsal horn of the mouse spinal cord using a specific CB1 antibody we developed. The specificity of our CB1 antibody was confirmed by the absence of labeling in the spinal cord of CB1-deficient mice. We found that CB1 is expressed in the superficial dorsal horn, deep layer of

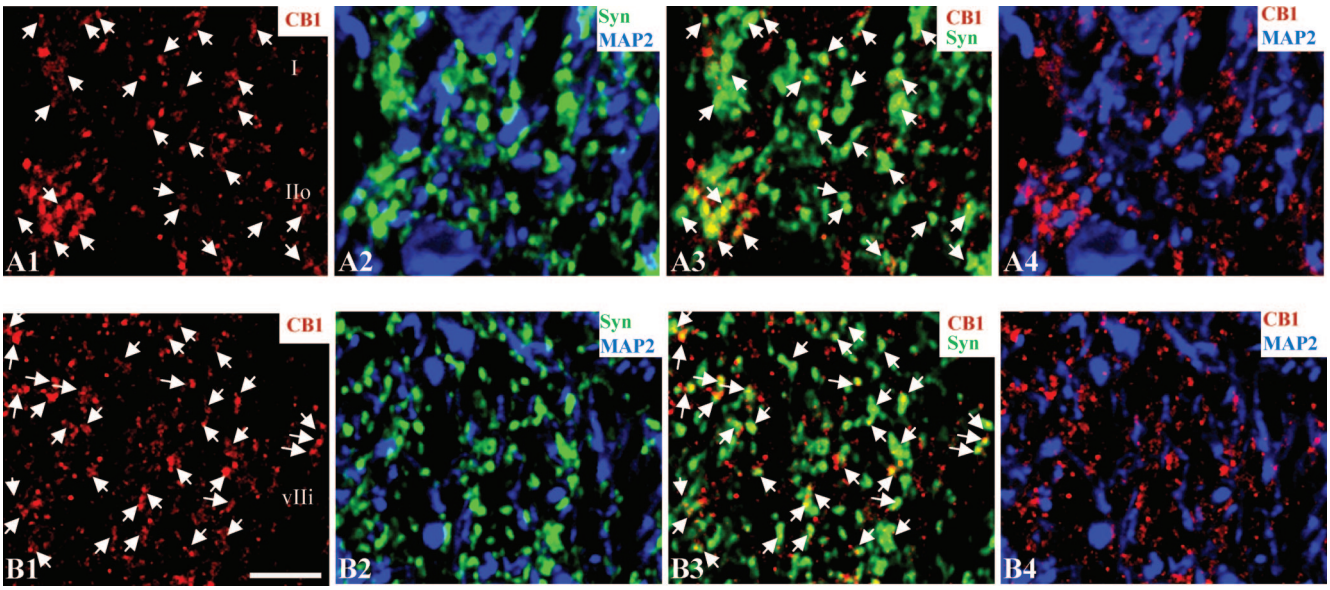

Fig. 9. Localization of cannabinoid receptor 1 (CB1) in the superficial dorsal horn of the spinal cord of sarcoma-implanted mice. (*A1–A4*) CB1 localization in lamina I and IIo. (*B1–B4*) CB1 localization in lamina vIIi. *Arrows* = typical overlap of CB1-immunoreactive (ir) with synaptophysin-ir. *Red* = CB1; *green* = synaptophysin (Syn); *blue* = microtubule-associated protein-2 (MAP2). *Scale bar* = 5 μm. Syn.

the dorsal horn, dorsolateral funiculus, and around the central canal as previously reported in the rat spinal cord.[11] In the superficial dorsal horn, CB1 was expressed in lamina I-IIo and vIIi but not in lamina dIIi. CB1 expression was sparse in the IB4-positive area (lamina dIIi). Since myelinated primary afferents labeled by cholera toxin β subunit are projected to lamina vIIi,[31] CB1 is mainly expressed in the specific lamina in the superficial dorsal horn, where CGRP-positive primary afferents and myelinated primary afferents are projected. Interestingly, the laminar distribution of CB1 in the superficial dorsal horn is the same as the area where primary afferents innervating to bone are projected, because CGRP-positive and myelinated but not IB4-positive primary afferents are innervated to bone marrow, mineralized bone, and periosteum.[47]

There are some conflicting reports regarding CB1 expression in primary afferent terminals in the superficial dorsal horn. In our study, we observed that CB1-ir in the dorsal horn ipsilateral to dorsal root rhizotomy was roughly comparable to that contralateral to rhizotomy. Consistent with our results, Agarwal *et al.*[19] showed that CB1-ir is little altered in the dorsal horn of DRG-specific CB1 knockout mice. These results collectively suggest that CB1 is mainly expressed in spinal interneurons and/or descending fibers rather than terminals of primary afferent neurons in the superficial dorsal horn.

Cb1 Expression and Localization in Sarcoma-implanted Mice

We previously reported downregulation of MOR1 in the superficial dorsal horn ipsilateral to the site of sarcoma implantation in sarcoma-implanted mice, which resulted from decreased expression of MOR1 in primary afferent terminals but not spinal interneurons.[5] It is likely that sarcoma-induced peripheral nerve injury reduces MOR1 expression of primary afferent neurons because the pathogenesis of bone cancer pain is believed to involve at least nerve injury and inflammation. Previous studies showed that peripheral nerve injury induced upregulation of CB1 in the dorsal horn of the spinal cord ipsilateral to nerve injury.[48,49] In the current study, we found that MOR1 expression in the superficial dorsal horn ipsilateral to the site of sarcoma implantation was decreased compared to that contralateral but that CB1 expression in the superficial dorsal horn ipsilateral to the site of sarcoma implantation was comparable to that contralateral to the site of sarcoma implantation in sarcoma-implanted mice. A recent study has shown that bone cancer does not change the expression level of CB1 messengerRNA in the DRG and spinal cord,[37] supporting our results.

We found that CB1-ir was overlapped with synaptophysin-ir but not microtubule-associated protein-2-ir in both naïve and sarcoma-implanted mice, indicating that CB1 is expressed in the presynaptic terminals. In addition, we observed that few CB1-ir were overlapped with markers of primary afferents. These results suggest that activation of spinal CB1 reduces bone cancer pain through presynaptic inhibition of spinal interneurons and/or descending fibers. A previous study also showed that spinal CB1 activation evoked presynaptic but not postsynaptic inhibition of excitatory postsynaptic currents in substantia gelatinosa neurons of rat spinal cord slices.[14] However, some CB1-ir did not overlap with synaptophysin-ir. In many regions of the brain, CB1 is localized not only in terminals but also in the nonterminal portion of axons.[26] Therefore, precise subcellular localization should be determined by immunoelectron microscopy in a future study. In addition, because intrathecally administered drugs can act directly on the DRG neurons, it is necessary to examine the expression of CB1 in the DRG neurons in future study.

Conclusion

The results of our study demonstrate that spinal CB1 activation by an exogenously administered CB1 agonist reduced bone cancer–related pain behaviors, including behaviors related to spontaneous pain and movement-evoked pain. Presynaptic inhibition of spinal neurons and/or descending fibers may contribute to spinal CB1 activation–induced analgesia. In addition, MOR expression in the superficial dorsal horn ipsilateral to the site of implantation of sarcoma cells was decreased compared to that contralateral to the site of implantation, whereas CB1 expression in the superficial dorsal horn was preserved. The findings of this study may lead to novel strategies for the treatment of bone cancer pain.

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