

Behavioral and neurochemical analysis of ongoing bone cancer pain in rats

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Abstract

Cancer-induced bone pain is described as dull, aching ongoing pain. Ongoing bone cancer pain was characterized after intratibial injection of breast cancer cells in rats. Cancer produced time-dependent bone remodeling and tactile hypersensitivity but no spontaneous flinching. Conditioned place preference (CPP) and enhanced dopamine (DA) release in the nucleus accumbens (NAc) shell was observed after peripheral nerve block (PNB) selectively in tumor-bearing rats revealing nociceptive-driven ongoing pain. Oral diclofenac reversed tumor-induced tactile hypersensitivity but did not block PNB-induced CPP or NAc DA release. Tumor-induced tactile hypersensitivity, and PNB-induced CPP and NAc DA release, was blocked by prior subcutaneous implantation of a morphine pellet. In sham rats, morphine produced a modest but sustained increase in NAc DA release. In contrast, morphine produced a transient 5-fold higher NAc DA release in tumor bearing rats compared with sham morphine rats. The possibility that this increased NAc DA release reflected the reward of pain relief was tested by irreversible blockade of rostral anterior cingulate cortex (rACC) μ -opioid receptors (MORs). The rACC MOR blockade prevented the morphine-induced transient increased NAc DA release in tumor bearing rats but did not affect morphine-induced effects in sham-operated animals. Consistent with clinical experience, ongoing cancer pain was controlled by morphine but not by a dose of diclofenac that reversed evoked hypersensitivity. Additionally, the intrinsic reward of morphine can be dissociated from the reward of relief of cancer pain by blockade of rACC MOR. This approach allows mechanistic and therapeutic assessment of ongoing cancer pain with likely translation relevance.

Keywords: Cancer bone pain, Ongoing pain, Conditioned place preference, Rostral anterior cingulate cortex, Morphine, Diclofenac, Dopamine release

1. Introduction

Pain is the most feared consequence of cancer and can impact patients' lives more than the cancer itself.^{29,34,52,55} Despite improvements in cancer prevention and detection, pain is often the first sign of cancer, with an estimated 70% to 75% of advanced stage cancer patients presenting with skeletal metastases.^{9,26,37,43} Cancer metastasis to the bone is associated with persistent pain that increases in intensity over time.^{7,30,48} Current treatments follow the World Health Organization (WHO) analgesic ladder for cancer pain management suggesting nonsteroidal anti-inflammatory drugs (NSAIDs) for mild-to-moderate pain and opioids for moderate-to-severe pain.^{1,33,48} However, estimates indicate as many as 50% to 80% of cancer patients worldwide receive inadequate pain management.^{5,11,43} Moreover, opioid

doses required for these patients are associated with adverse side effects further diminishing quality of life.^{32,35,36,40,46} Development of improved nonopioid therapies is dependent on increased understanding of mechanisms driving cancer pain and its relief.

Knowledge of cancer-induced bone pain (CIBP) has been greatly advanced by seminal work using a mouse model of cancer metastasis to the bone indicating inflammatory, neuropathic, and mechanical components of CIBP.^{12,17,18,30} Multiple disease-related factors have been identified to directly activate and/or sensitize nociceptive afferent fibers within the bone microenvironment.^{20,30} However, compounds targeting mechanisms that have displayed efficacy on the outcome measures used in preclinical models of inflammation, cancer, and neuropathic pain have not shown efficacy in human studies.^{31,45,47,51,56} One contributing factor may be the challenge of assessment of mechanisms underlying "stimulus-independent" or ongoing pain, a primary complaint of patients in the clinical setting.^{31,45,47,51,56} We have developed an approach that allows the assessment of motivational drive to seek relief from the aversive state of ongoing pain.^{24,25,41,42,49} Ongoing pain can be unmasked through behavioral assessment of conditioned place preference (CPP) to a context paired with pain relief.^{25,38,41,42} In addition, pain relief in rats with incisional injury evoked dopamine (DA) release within the nucleus accumbens (NAc) shell, suggesting that pain relief is rewarding,^{38,39} consistent with human imaging studies.²⁻⁴ This study determined if relief of ongoing CIBP in rats could be achieved by peripheral nerve block (PNB) of sensory input from the tumor-bearing tibia by evaluating

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behavior and NAc shell DA release. In accordance with the recommendations of the WHO analgesic ladder for cancer pain, we also assessed the relative effectiveness of an NSAID, diclofenac, and an opioid, morphine, in the acute treatment of ongoing bone cancer pain. Further analysis was conducted to delineate the analgesic and innate rewarding aspects of morphine administration through blockade of μ -opioid receptors in the rostral anterior cingulate cortex (rACC), as the rACC is implicated in the affective component of pain.^{21,22,49,50}

2. Material and methods

2.1. Animals

Female Fisher F344/NHsd rats (Harlan Laboratories Inc, Indianapolis, IN) weighing 140 to 200 g were used for all experiments, which were performed in accordance with the guidelines set forth by the National Institutes of Health and the Institutional Animal Care and Use Committee of the University of Arizona. Rats were housed 3 to a pan with food and water ad libitum on a 12-hour dark/light circadian cycle.

2.2. Cell line

Mammary adenocarcinoma cells of histocompatible 13762 MATBIII (CRL-1666, ATCC) rats were maintained in McCoy's 5A media (CellGro, Manassas, VA) with 10% fetal bovine serum and 2% penicillin/streptomycin at 37°C in a 5% CO₂ atmosphere. Cells were harvested for use in this study between passages 10 and 25.

2.3. Surgical procedures

2.3.1. Intratibial surgery

Rats were anaesthetized (intraperitoneal [i.p.] ketamine/xylazine, 80/12 mg/kg; Western Medical Supply, Arcadia, CA; Sigma, St Louis, MO). After the pinch test to confirm that the rats had no reflexive response, the right rear hindlimb was shaved to expose the skin over the femoral-tibial joint, which was cleaned 3 times with 70% EtOH and betadine. A rostral-caudal incision was then made exposing the patellar tendon. A hole was drilled with a 1.0-mm tip diameter microdrill bit (Stoelting, Wood Dale, IL) through the tibial epiphyseal plate and into the intramedullary space. A 5-cm, 28-gauge guide cannula (Plastics One, Roanoke, VA) connected by Tygon tubing (Cole-Parmer, Vernon Hills, IL) to a 10- μ L syringe (Hamilton, Reno, NV) was used to inject either 1×10^5 cells per 5 μ L of MATBIII cells or 5 μ L of serum-free McCoy's 5A media into the tibia. Before injection, confirmation of proper drill placement was confirmed using radiograph imaging (Faxitron, Tucson, AZ). The drilled hole was then sealed with bone cement (Simplex P, Kalamazoo, MI), the overlying skin was closed with 5-0 vicryl sutures (Ethicon, Somerville, NJ), and a surgical staple was used for additional support. Staples were removed 7 days after surgery. All rats received an injection of gentamicin (1 mg/mL subcutaneously [s.c.]) on completion of the surgery.

2.3.2. Intracranial NAc cannulation for microdialysis

Surgical intracranial cannulation of the NAc shell was performed in anesthetized rats (i.p. ketamine/xylazine, 80/12 mg/kg; Western Medical Supply/Sigma) as previously described.³⁸ Because of the smaller size of Fisher rats compared with other strains, new coordinates were verified based on previous coordinates obtained

from the rat brain atlas.⁴⁴ The rat skull was exposed and a single guide cannula (EICOM, San Diego, CA) was implanted into the left NAc shell (AP: +1.5 mm, ML: -1.0, DV, dura: -4.7 mm) and secured to the skull with acrylic resin. To ensure the cannula remained clear of particulates, a stainless steel dummy was inserted into the guide cannula and held in place with a corresponding screw cap (EICOM). Rats received a combination bolus of gentamicin (1 mg/mL, s.c.) and saline (1.5 mL) on completion of the surgery. Cannulated rats were housed individually and allowed to recover for 5 days before any additional surgical procedures. Cannula placement was verified using injections of ink as previously described.³⁸ Any animals with misplaced cannulas were removed from the data analysis.

2.3.3. Intracranial ACC cannulation for drug administration

Stereotaxic rostral ACC surgeries were conducted on anesthetized rats (i.p. ketamine/xylazine, 80/12 mg/kg; Western Medical Supply/Sigma). ACC cannulation occurred in conjunction with NAc cannulation to reduce the amount of surgeries performed on each animal. Coordinates for the rostral ACC were adapted from the rat brain atlas and adjusted to account for the smaller size of the Fischer rats. The manipulator swivel base was rotated and locked at 90°. The vertical alignment was positioned at +25° angle, and two 26-gauge guide cannulas separated by 1.2 mm (Plastics One) were directed towards the rostral ACC (AP: +3.7 mm from bregma, ML: \pm 0.6 mm, DV, skull: -2.7 mm). The cannula was cemented in place with acrylic resin, held by 2 screws anchored to the skull. Rats received a dual injection of gentamicin (1 mg/mL, s.c.) and saline (1.5 mL) on completion of the surgery. Cannula placement was verified using injections of ink. Any animals with misplaced cannulas were removed from the data analysis.

2.4. Drug administration

2.4.1. Saphenous lidocaine

Rats were anaesthetized with a 2% isoflurane O₂ mixture. To produce an effective PNB, lidocaine (Roxane Laboratories, Columbus, OH) was administered over the saphenous nerve in a single s.c. injection (4% wt/vol, 350 μ L). Equivolume saline was given as a vehicle control. The saphenous nerve was chosen because it is the primary innervation of the tibia in rats.^{19,23}

2.4.2. Diclofenac

Diclofenac (Sigma-Aldrich) was brought up in a solution of PEG400/saline (10:90 vol/vol) and administered systemically (30 mg/kg, p.o.), with PEG400/saline given as a vehicle.

2.4.3. Morphine

A single morphine sulfate (75 mg) or placebo pellet, generously provided by the National Institute on Drug Abuse Drug Supply Program, was implanted (s.c.) on the contralateral pelvic region of the rat. The animals were anesthetized with a 2% isoflurane O₂ mixture, and the region was shaved and cleaned 3 times with 70% EtOH and betadine. A small incision was made with surgical scissors and a pocket created between the skin and muscle membrane. The pellet was placed into the pocket and the incision closed with a surgical staple.

2.4.4. Beta-funaltrexamine

Beta-funaltrexamine (β -FNA) (Tocris, Minneapolis, MN), a selective irreversible μ -opioid receptor antagonist, was solubilized in

saline for a final concentration of 3 $\mu\text{g}/\mu\text{L}$. The compound was sonicated for several minutes and kept in 52°C water bath to maintain its soluble state. Before drug administration, rats were anaesthetized with a 2% isoflurane O₂ mixture. Using a Quintessential Stereotaxic Injector (Stoelting), a bilateral microinjection (1 μL per hemisphere) of β -FNA was administered over a 5-minute period to allow for diffusion of the drug. Rats received β -FNA 20 hours before the start of the morphine hourly microdialysis study.

2.5. Behavioral testing

All behavioral testing was performed by an experimenter blinded to the treatment groups.

2.5.1. Tactile allodynia

Paw withdrawal thresholds were determined in response to probing with calibrated von Frey filaments (Stoelting) with spaced increments ranging from 0.5 to 15 g. All animals were allowed to acclimate in suspended wire mesh cages for 30 minutes before the start of the study, and each filament was applied to the middle of the plantar surface of the paw using the “up and down” method and analyzed using a Dixon nonparametric test.

2.5.2. Limb use

Limb use was assessed as previously described.²⁷ The animal was placed in an empty pan and observed while walking. Usage of the treated limb was rated on the following scale: 0 = complete lack of use, 1 = partial nonuse, 2 = limping and guarding, 3 = limping, and 4 = normal walking.

2.5.3. Conditioned place preference

Conditioned place preference to pain relief provided by PNB has been previously used to reveal underlying mechanisms of ongoing pain in several other pain models.^{38,41,42} We determined whether saphenous nerve block by lidocaine produces CPP in rats with tumor-bearing tibias as previous studies have demonstrated that the tibia is predominantly innervated by saphenous nerve.^{16,23} We performed a single-trial CPP protocol on days 11 through 13 postintra-tibial surgery. The 3-chamber CPP apparatus consists of 2 conditioning chambers with distinct tactile, visual, and olfactory cues, connected by a smaller neutral chamber that was brightly lit. Unlike previous protocols, animals for this study were not handled before the start of this experiment to minimize the potential to produce pain by inadvertent movement of the hindlimb or damaging the cancer-bearing limb. White noise was played to provide background noise and block out any extraneous sounds. On the first day (D11, preconditioning) of the experiment, rats were introduced to the neutral chamber and allowed to explore all 3 chambers for 15 minutes. Baseline time spent in the chambers was measured using ANY-maze tracking software (Stoelting). Exclusion criteria for rats were spending <20% or >80% time in a chamber. Rats were assigned treatment–chamber pairings using a counterbalanced design for the following day. Care was taken so that group means for the morning (vehicle) and afternoon (drug) chamber pairings were not significantly different (unbiased CPP design). On the second day (D12, conditioning), rats were lightly anesthetized with isoflurane and given a vehicle treatment of saphenous saline followed by immediate (<2 minutes) confinement into the appropriate pairing chamber for 30 minutes, following which they were returned to their home cage. Four hours later, rats were lightly anesthetized

with isoflurane and treated with saphenous lidocaine followed by immediate (<2 minutes) confinement to the opposite pairing chamber for 30 minutes. All rats awoke within 1 minute of isoflurane removal. On the final day (D13, testing), rats were once again allowed to freely explore the apparatus for 15 minutes. Time spent in the chamber was recorded by ANY-maze. Preference for the lidocaine-paired chamber was calculated as difference scores, subtracting the baseline time from the testing time (test – baseline). Preference is indicated by a positive score.

2.5.3.1. Conditioned place preference with diclofenac

As diclofenac was given systemically, the time course of the pain-alleviating effect likely has a slow onset that would be difficult to associate with the paired context. Therefore, the effects of systemic administration of diclofenac were determined by assessing the ability to block lidocaine-induced CPP at a time that peak reversal of cancer-induced tactile hypersensitivity is observed (45–75 minutes after diclofenac administration). On conditioning day (day 12 postsurgery), all rats were treated in the morning with a vehicle control (1 mg/kg, PEG400/saline, 10:90 v/v, p.o.) 45 minutes before saphenous saline injection. For the afternoon treatment, rats were given systemic diclofenac (30 mg/kg, p.o.) 45 minutes before saphenous lidocaine administration. If ongoing pain was effectively treated with diclofenac, saphenous lidocaine would no longer be expected to produce CPP in tumor-bearing rats.

2.5.3.2. Conditioned place preference with morphine

After preconditioning baselines (D11 postintra-tibial surgery), cancer- and sham-treated rats were assigned treatment–chamber pairings and then implanted with either a morphine (75 mg) or placebo pellet. The following day (conditioning day), rats received saphenous saline in the morning, followed 4 hours later by saphenous lidocaine for the afternoon treatment session. This corresponded to 20 to 24 hours after the pellet implant. As described above, if the tumor-induced ongoing pain is controlled by the morphine infusion, cancer-treated rats would not be expected to display CPP to the lidocaine-paired chamber.

2.6. Neurochemical analysis

2.6.1. In vivo microdialysis

After 12-day postintra-tibial and 17-day postintra-cranial surgery, rats were used for microdialysis. Throughout the experiment, rats were awake and freely moving, as previously described.³⁸ A microdialysis probe (AZ-8-02; EICOM) was inserted into the NAc guide cannula, with a 2-mm semipermeable membrane projecting past the end of the guide cannula. The probe was perfused with artificial cerebral spinal fluid (147.0 mM NaCl, 2.8 mM KCl, 1.2 mM MgCl₂, and 1.2 mM CaCl₂) at a flow rate of 0.8 $\mu\text{L}/\text{min}$ set with a syringe pump drive (BASi, West Lafayette, IN). After a 90-minute waste flush, two 30-minute baseline fractions were collected. Rats then followed a 3-hour time course with fractionations taken every 30 minutes. All dialysates were collected in 1.5 mL amber Eppendorf vials containing 1.5 μL of 40 \times antioxidant solution (6.0 mM L-cysteine, 2.0 mM oxalic acid, and 1.3% [vol/vol] glacial acetic acid). During the experiment, vials were placed in a 4°C micro-sampler (Univentor, Malta) timed for 30-minute intervals. Drug treatments included saphenous lidocaine, diclofenac (30 mg/kg), and their corresponding vehicles, respectively. In the last treatment cohort, a morphine sulfate pellet or placebo pellet was administered 24 hours before baseline collection, after which rats received either a saphenous

lidocaine or saline bolus injection followed by the 3-hour time course. On completion of all drug treatment time courses, rats received an injection of cocaine (20 mg/kg, i.p.), generously donated by the National Institutes on Drug Abuse Drug Supply Program, and two 30-minute fractions were collected. Rats were humanely killed at the conclusion of the study, and their brains were harvested for verification of proper cannulation placement. Any rats that failed to be cannulated in the NAc shell were subsequently removed from the study.

2.6.2. Morphine hourly study

After the initial 90-minute washout and two 30-minute baseline collections, rats were implanted with either a morphine sulfate pellet or a placebo pellet. Fractionations were then collected every hour for 16 hours at a flow rate of 0.8 $\mu\text{L}/\text{min}$, with 1 hour of cocaine treatment (20 mg/kg, i.p.) concluding the experiment. Circadian 12-hour dark/light cycles were adhered to for the duration of the study.

2.6.3. Quantification of dopamine release

As previously described by Navratilova et al.,³⁸ dialysate fractions were analyzed using Agilent 1100 HPLC system with an MD-150 column (Thermo Scientific, Waltham, MA) with a Coulochem III 5014B electrochemical detector. A 5020 guard cell, used to minimize background noise, was set to 350 mV (electrode 1: -150 mV and electrode 2: 250 mV). A standard curve was previously obtained from 7 serial dilutions of DA (2.5-160 pg in 20 μL artificial cerebral spinal fluid plus antioxidant mixture), along with the limit of detection and limit of quantification, limit of detection = 3.3 (SDr/S) and limit of quantification = 10 (SDr/S), respectively. MD-TM mobile phase (Fisher Scientific, Waltham, MA) was optimized for perfusate analysis at a final concentration of 9% acetonitrile. Chromatograms were integrated by persons blinded to the study. Area under the curve was converted to picograms per microliter, and treatment fractionations were compared with baseline averages to obtain percent change from the baseline. Cocaine was used as a positive control, and data from rats that failed to produce a DA increase in response to cocaine treatment were excluded. Data points were analyzed by JFlashCalc to determine the total area under the curve of percent change.

2.7. Radiograph analysis

Confirmation of tumor-induced bone remodeling was determined by radiograph imaging (Faxitron). Animals that failed to display bone loss were removed from the study. Radiographs were evaluated by an observer blinded to the treatment. On completion of all behavioral (day 13) and microdialysis testing (day 12), final radiographs were performed and rats were humanely killed following IACUC protocol and the American Veterinary Medical Association guidelines of CO₂ administration.⁸

2.8. Statistical analysis

Baseline-evoked and ongoing pain responses were compared with postadministration values by 2-way analysis of variance (ANOVA) followed by post hoc analysis using Bonferroni's multiple comparisons test using GraphPad Prism 5. A probability level of 0.05 was used to establish significance. For CPP, the effects of treatment on cancer vs sham and conditioning chamber were analyzed by 2-way ANOVA followed by post hoc analysis between preconditioning (BL) vs postconditioning (testing) values within each treatment group using Bonferroni's

t tests. Analysis of variance on the difference scores calculated as (postconditioning) - (preconditioning) time spent in the drug-paired chamber was performed to determine differences between treatment groups with GraphPad Prism 5. Microdialysis results were analyzed by ANOVA with post hoc Tukey's multiple comparison tests between treatment groups. Results are expressed as mean \pm SEM.

3. Results

3.1. Cancer-induced bone remodeling, tactile hypersensitivity, and impaired limb use

Tibia radiographs taken at days 0, 7, 10, and 13 postintra-tibial surgery demonstrate time-dependent bone loss and bone remodeling in cancer-treated rats at day 7 postinjection of MATBIII cells. Osteolytic lesions were developed at the proximal and distal ends of the tibia by day 10 (Fig. 1A, arrows), with expansion of osteolytic lesions and fractures observed along the cortical shaft and at the proximal end of the tibia by day 13 (Fig. 1A, arrows). Sham-operated rats had no detectable bone loss postsurgery.

Injection of MATBIII cancer cells into the tibia intramedullary space produced time-dependent expression of hypersensitivity to evoked stimuli with von Frey filaments. Tumor-bearing rats displayed tactile hypersensitivity beginning day 6 postintra-tibial surgery, with paw withdrawal thresholds continuing to decrease through day 13 (Fig. 1B; **P* < 0.001 vs presurgery thresholds). No significant alterations in response thresholds were observed in sham-treated rats at any time point (Fig. 1B). Similar time-dependent impaired limb use was observed (Fig. 1C; **P* < 0.001 vs presurgery thresholds). Impaired limb use was observed on days 10 and 13, with many of the cancer-treated rats displaying both limping and guarding behaviors (score of 2) and partial nonuse (score of 1) of the tumor-bearing hindlimb. Sham-operated rats displayed no impaired limb use throughout the duration of the study (Fig. 1C). No flinching or guarding behaviors were observed in the tumor-bearing rats in the absence of ambulation.

3.2. Saphenous nerve block reverses cancer-induced tactile hypersensitivity and impaired limb use

Twelve days after postintra-tibial injection of MATBIII cells, PNB was produced by injection of lidocaine administration (4% wt/vol, 350 μL , s.c.) around the saphenous nerve in the tumor-bearing limb. Lidocaine increased tumor-induced impaired limb use from partial nonuse and limping and guarding (scores of 1 and 2) to limping (scores of 2 and 3, 4 being normal use). The peak effect was observed 30 minutes after lidocaine administration and gradually reversed to baseline levels 180 minutes after administration (Fig. 2A; **P* < 0.05 vs D12). No change in limb use was observed in sham rats given the PNB (Fig. 2A). Saphenous lidocaine administration also blocked cancer-induced tactile hypersensitivity (Fig. 2B, C). The peak effect occurred between 30 and 90 minutes after lidocaine administration, with reversal in withdrawal thresholds of tumor-bearing rats returning to baseline by 120 minutes after injection (Fig. 2B; **P* < 0.001 vs D12). No changes in withdrawal thresholds were observed in sham-operated rats in response to saphenous lidocaine. No motor impairment was detected in rats that received the PNB, consistent with studies that indicate the saphenous nerve is exclusively sensory in function.⁵⁸ Ipsilateral administration of saphenous lidocaine reversed tumor-induced tactile hypersensitivity within 15 minutes (Fig. 2C; **P* < 0.001 vs BL). In contrast,

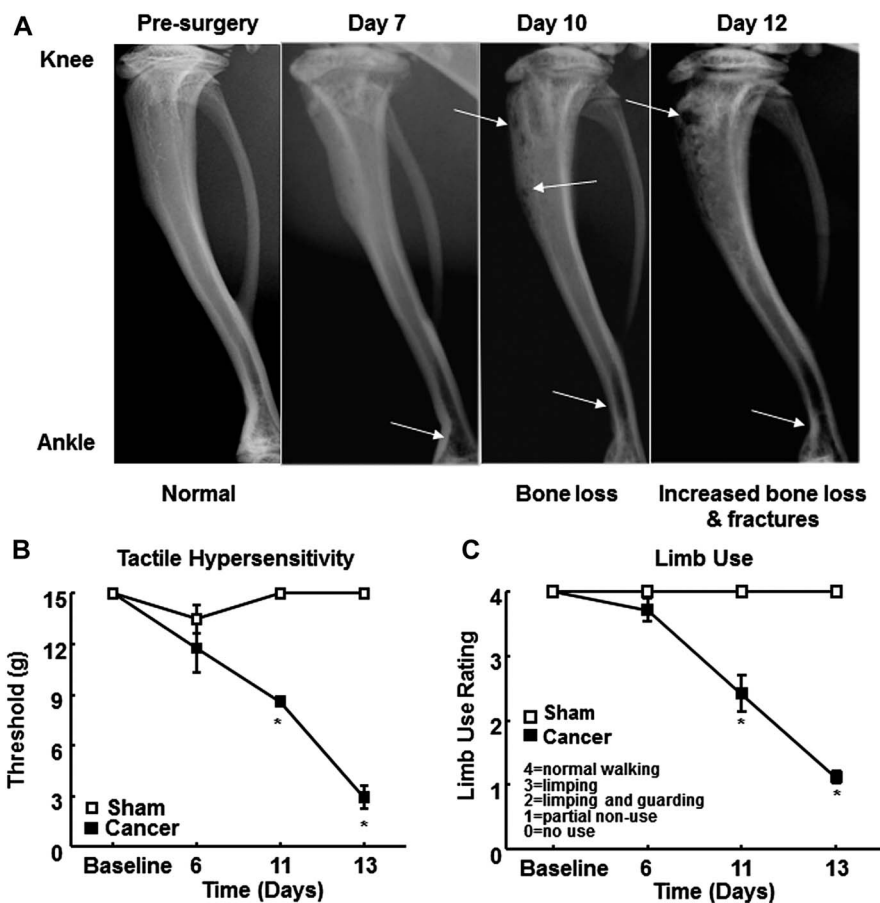


Figure 1. Injection of MATBill cells into the tibia produces time-dependent pain behaviors and bone remodeling. (A), Representative X-ray images demonstrating time-dependent tumor-induced bone loss. Bone loss, as indicated by pits (arrows) and increased dark area at the distal (ankle) portion of the tibia, increases, with development of full cortical bone loss indicating development of fractures at the day 12 time point. (B), Tumor-induced hypersensitivity to evoked stimuli measured as tactile hypersensitivity is observed within 6 days after intratibial cancer cell injection. Withdrawal threshold decreases with disease progression, $*P < 0.05$ vs BL ($n = 7$). (C), Tumor-induced impairment of limb use is observed within 6 days after surgery and is decreased with disease progression, $*P < 0.05$ vs BL ($n = 7$). All graphs show mean \pm SEM.

contralateral administration of saphenous lidocaine did not alter cancer-induced tactile hypersensitivity, indicating that the effects of lidocaine remain localized to the area of administration (Fig. 2C).

3.3. Cancer-induced ongoing pain is dependent on peripheral input

The role of sensory input from the tumor-bearing tibia on tumor-induced ongoing pain was determined by assessing whether saphenous nerve block produces CPP 12 days after intratibial surgery. Preconditioning time spent in saline vs lidocaine-paired chambers did not differ in sham- or tumor-bearing rats ($P > 0.05$); therefore, data were pooled together for graphical representation (Fig. 3A). Cancer-bearing rats displayed CPP for the chamber paired with saphenous lidocaine, as exemplified by the significant increase in time spent in the lidocaine-paired chamber compared with preconditioning times (Fig. 3A; $*P < 0.001$ vs BL). Sham rats did not alter time spent in either saline- or lidocaine-paired chambers (Fig. 3A). Comparison of difference scores verified that cancer-bearing rats showed increased time spent in the lidocaine-paired chamber compared with the sham controls (Fig. 3B; $*P < 0.001$).

Saphenous lidocaine, but not saline, induced a significant increase in DA release from the NAc shell in the tumor-bearing

rats (Fig. 3C; $*P < 0.001$ vs sham/saline). Lidocaine or saline administration to the saphenous nerve failed to alter DA release in the NAc shell of sham-operated rats (Fig. 3C). After the experiment, microdialysis probe placement was verified (Fig. 3D). Any rats with incorrect placement ($< 12.5\%$) were removed from the study. Similarly, any rats that failed to elicit an increase in DA in response to cocaine administration were also excluded from the study.

3.4. Diclofenac blocks cancer-induced tactile hypersensitivity but not ongoing pain

The ability of diclofenac (30 mg/kg, p.o.) to block tumor-induced bone pain was determined 12 days after intratibial surgery. Diclofenac fully reversed cancer-induced tactile hypersensitivity, with the peak effect observed at 60 minutes after administration (Fig. 4A; $*P < 0.001$ vs D12). Withdrawal thresholds returned to baseline levels at 90 minutes after administration. Sham-operated rats had no change in withdrawal thresholds in response to systemic diclofenac (Fig. 4A).

To determine whether diclofenac blocks ongoing cancer bone pain, diclofenac was given as a pretreatment 45 minutes before saphenous lidocaine to assess the ability to block saphenous lidocaine-induced CPP, indicating blockade of ongoing CIBP. Preconditioning time spent in the saphenous saline vs

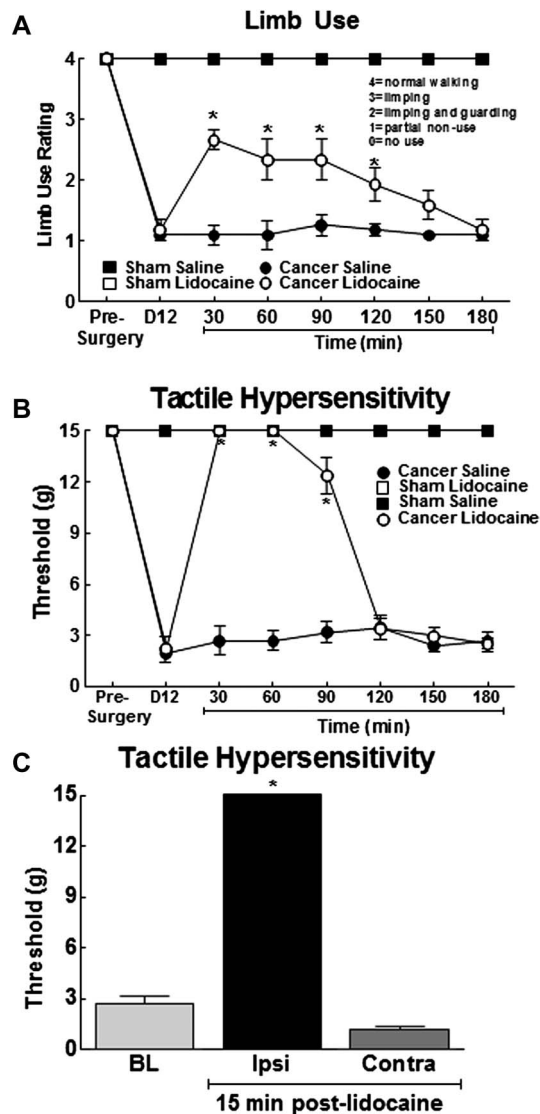


Figure 2. Blockade of peripheral afferent input blocks cancer-induced tactile hypersensitivity. (A), Blockade of tumor-induced impaired limb use by administration of lidocaine (350 μ L, 4% wt/vol) to the saphenous nerve 12 days after surgery. Maximal blockade was observed 30 to 90 minutes after administration, $*P < 0.05$ vs D12 ($n = 6-7$). (B), Blockade of tactile hypersensitivity by administration of lidocaine to the saphenous nerve 12 days after surgery. Maximum blockade of tactile hypersensitivity was observed 30 to 90 minutes after administration, $*P < 0.05$ vs D12 ($n = 7$). (C), Blockade of tactile hypersensitivity is because of local and not systemic effects of peripheral nerve block. No change in withdrawal threshold of the ipsilateral limb after nerve block of the contralateral limb is observed, $*P < 0.05$ vs BL ($n = 6-8$). All graphs show mean \pm SEM.

lidocaine-paired chambers did not differ irrespective of sham or cancer treatment ($P > 0.05$); therefore, data were pooled for graphical representation. Saphenous lidocaine produced robust CPP in cancer rats treated with vehicle (p.o.) 45 minutes earlier (Fig. 4B; $*P < 0.001$ vs BL). Tumor-bearing rats treated with systemic diclofenac demonstrated a similar increase in time spent in the chamber paired with saphenous lidocaine (Fig. 4B; $*P < 0.001$ vs BL). Sham rats displayed no preference for either conditioning chamber (Fig. 4B). Difference scores confirm that the diclofenac and vehicle-treated tumor-bearing rats demonstrated equivalent increased time spent in the lidocaine-paired chamber compared with sham controls, indicating that ongoing pain failed to be controlled by diclofenac (Fig. 4C; $*P < 0.001$ vs sham/vehicle).

Tumor- and sham-operated rats were treated with systemic administration of either vehicle or diclofenac (30 mg/kg, p.o.), and microdialysis perfusate samples were collected from the NAc shell for 3 hours. Diclofenac failed to alter DA release, regardless of cancer or sham treatment (Fig. 4D).

3.5. Morphine infusion blocks cancer-induced tactile hypersensitivity and ongoing pain

Morphine (75 mg) or placebo pellets were implanted (s.c.) 12 days after surgery, 20 to 24 hours before assessing tactile hypersensitivity or impaired limb use. Morphine treatment reversed tactile hypersensitivity in tumor-bearing rats (Fig. 5A; $*P < 0.001$ vs cancer pre-morphine). Impaired limb use was also attenuated in cancer-bearing rats, from partial nonuse, limping and guarding behaviors (scores of 1 and 2, respectively) to limping (score of 3) (Fig. 5B; $*P < 0.05$ vs cancer pre-morphine). Morphine infusion did not alter responses on either behavioral measure in sham-treated rats (Fig. 5A, B). Placebo pellets had no effect in either cancer- or sham-operated rats.

Saphenous nerve block was used to assess ongoing CIBP in rats pretreated 24 hours earlier with placebo or morphine pellets. Conditioned place preference difference scores demonstrated that cancer-treated rats receiving placebo pellets spent significantly more time in the chamber paired with the saphenous nerve block (Fig. 5C; $*P < 0.01$ vs sham/placebo). In contrast, tumor-bearing rats pretreated with a morphine pellet did not show significant CPP to saphenous nerve block (Fig. 5C; $P > 0.05$ vs sham/placebo). Saphenous nerve block did not alter time spent in the lidocaine-paired chamber in sham rats, irrespective of morphine or placebo treatment (Fig. 5C).

Tumor-bearing rats treated with placebo pellets demonstrated robust release of DA in response to saphenous lidocaine (Fig. 5D; $*P < 0.05$ vs sham/placebo). Morphine-treated cancer rats, however, showed no change in DA levels when administered saphenous lidocaine (Fig. 5D). Saphenous saline did not produce DA release within the NAc in any of the treatment groups (data not shown).

3.6. Delineating analgesia from innate reward in morphine-treated rats

As morphine is intrinsically rewarding,⁵⁷ we determined whether implantation of the morphine pellet altered DA release in the NAc across 16-hour postpellet implant in cancer- or sham-treated rats. After baseline collection, rats were implanted with either morphine sulfate (75 mg) or placebo pellets. Hourly fractionations were collected for 16-hour postpellet implantation. The morphine pellet produced an initial increase in NAc DA release that was evident by fraction 1 and sustained through fraction 5 in cancer-treated rats and in fractions 2 through 7 in sham-treated rats (Fig. 5E; $*P < 0.0001$ vs BL).

Cancer rats with continuous morphine infusion displayed a 5.5-fold greater DA release at the peak time point (2 hours) compared with sham rats provided the same treatment. We hypothesized that the cancer cohort might be experiencing a combined influence of both analgesia and innate reward brought on by the morphine. To study these possibilities, β -FNA, an irreversible μ -opioid antagonist, was injected into the ACC (1 μ L bilateral, 3 μ g/ μ L) 20 hours before the start of the hourly morphine microdialysis. Cancer rats pretreated with β -FNA displayed attenuated DA release, mirroring that of sham rats with onboard morphine (Fig. 5F; $*P < 0.01$). Sham rats pretreated with β -FNA had no change in DA release when morphine was

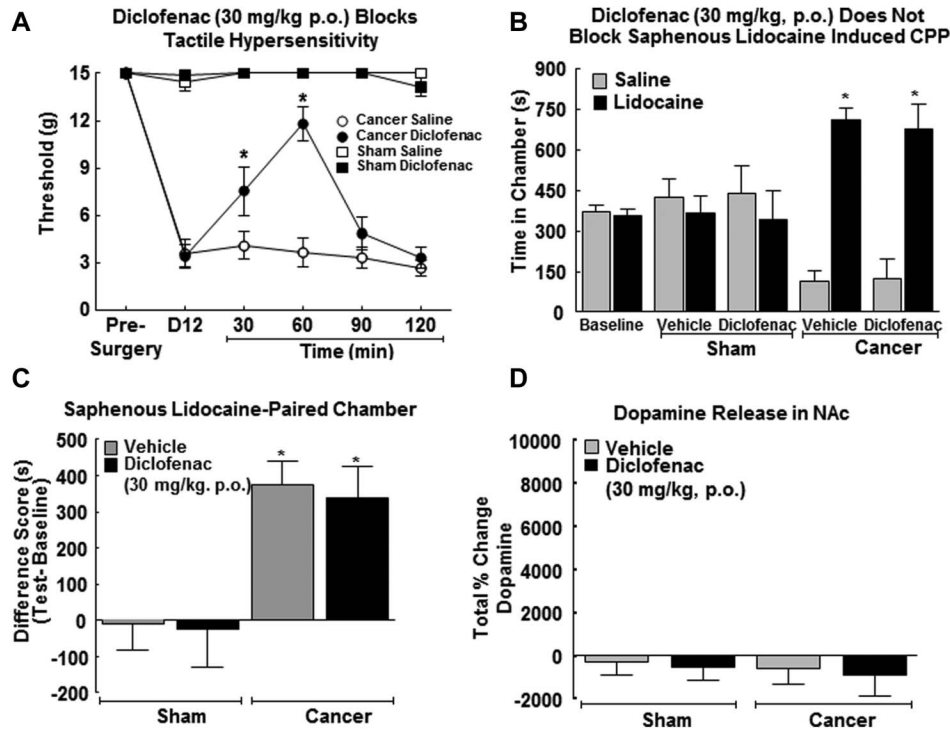


Figure 4. Diclofenac blocks referred tactile hypersensitivity but not ongoing pain. (A), Systemic administration of diclofenac (30 mg/kg, p.o.) reverses tactile hypersensitivity with peak effect at 60 minutes, $*P < 0.05$ ($n = 7-9$). (B), Pretreatment on day 12 with diclofenac failed to prevent conditioned place preference for the saphenous lidocaine in cancer-bearing rats, $*P < 0.05$. (C), Difference scores demonstrate that both vehicle- and diclofenac-treated cancer rats spend significantly more time in the chamber paired with the peripheral nerve block, $*P < 0.05$ ($n = 7-14$). (D), Neither cancer- nor sham-operated rats show changes in dopamine levels in response to diclofenac treatment ($n = 5-9$). All graphs show mean \pm SEM.

NSAIDs and limited evaluation of whether patients receive improved pain relief with the coadministration of NSAIDs with opioids.^{32,35} Our data demonstrate that acute diclofenac administration at a dose that blocks tumor-induced tactile hypersensitivity failed to induce CPP or DA release within the NAc shell, suggesting that it is not effective in alleviating the tumor-induced ongoing bone pain in this model at the time point tested. In contrast, morphine treatment across a period of 20 to 24 hours effectively alleviated the tumor-induced ongoing pain. This is consistent with reports from patients in which treatment with morphine or other opioids, such as fentanyl, effectively alleviates tumor-induced ongoing pain in some, but not all patients, whereas NSAIDs are not sufficient to block moderate-to-severe cancer bone pain.^{15,33,35,48} Our data indicate that, at the time point tested, tumor-induced ongoing bone pain is resistant to NSAIDs but can be controlled by morphine, consistent with patients with bone metastases reporting moderate-to-severe CIBP. Whether combined administration of diclofenac and morphine would offer benefit in producing opioid-sparing actions in this model has yet to be determined.

The demonstration of CPP to saphenous nerve block suggests that alleviation of the tumor-induced ongoing pain aversiveness is rewarding, as previously demonstrated in rats with incisional pain.³⁸ Supporting this, saphenous nerve block in tumor-bearing rats elicited DA release within the NAc shell. These observations are consistent with previous findings by our laboratory wherein activation of the mesolimbic reward-valuation circuitry occurs in response to manipulations that produce pain relief in rat models of hindpaw incision,^{38,59} nerve injury,⁵⁹ and cephalic pain.¹⁰ These data indicate that activation of the mesolimbic reward valuation circuitry occurs in response

to pain relief,³⁹ which is consistent with observations within the clinical setting that fMRI readings from the NAc change in response to pain onset and offset³ and during placebo analgesia.⁵⁴ Time course analysis of DA release within the NAc shell after morphine pellet administration demonstrated a transient increase in DA efflux in cancer-bearing rats compared with their sham counterparts. An irreversible μ -opioid receptor antagonist, β -FNA, blocked this enhanced DA release administered within the rACC. Previous studies demonstrated that lesions of the rACC blocked CPP to pain relief in rats with experimental neuropathic pain, consistent with preclinical and clinical observations that the ACC is required to mediate the affective component of pain processing.^{21,22,49,50} Blockade of the enhanced morphine-induced DA release by the β -FNA injection into the rACC suggests that the difference in DA release between the tumor-bearing and sham rats is likely because of the reward of pain relief rather than the inherent rewarding properties of the opioid. These observations are consistent with the hypothesis that different neural pathways mediate relief of pain and innate reward, as previously proposed.^{13,14}

We have characterized a measure of tumor-induced ongoing bone pain that can be used to explore mechanisms driving the persistent ongoing pain described by patients with cancer pain. Although morphine controls this ongoing pain, up to an estimated 43% of cancer patients worldwide fail to receive adequate pain relief.^{6,34,35,48} Moreover, doses required to produce adequate pain relief in these patients are commonly associated with adverse side effects further diminishing the quality of life in these patients.^{32,35,36,40,46} A better understanding of mechanisms underlying CIBP is necessary for the development of improved therapies with diminished side effects. We have characterized

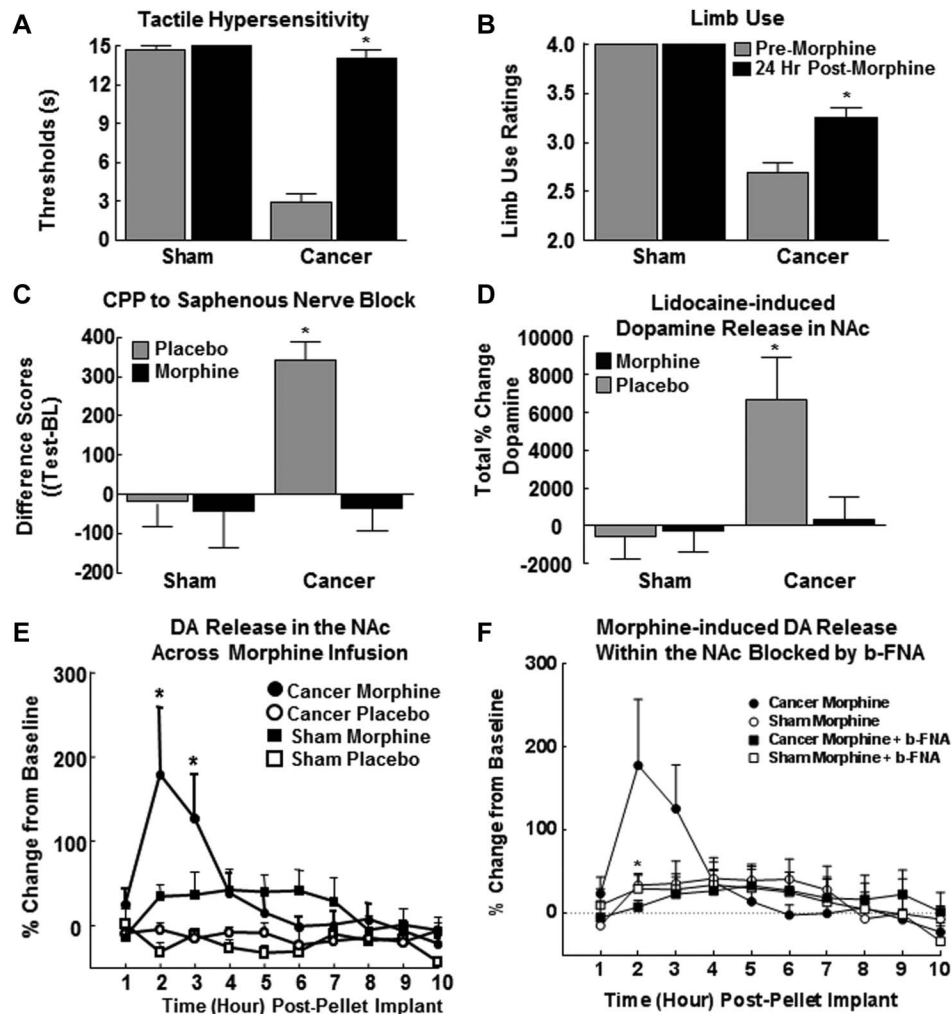


Figure 5. Morphine pellet administration blocks referred pain, impaired limb use, and ongoing pain. Morphine treatment across 20 to 24 hours by implantation (s.c.) of slow release morphine pellets blocked (A) tactile allodynia and (B) impaired limb use tested 12 days after sham or cancer surgery, $*P < 0.01$ vs pre-morphine ($n = 11-13$). (C), Difference scores demonstrate that saphenous lidocaine increased time spent in the lidocaine-paired chamber in cancer rats treated with placebo pellets compared with sham-treated rats. Morphine treatment blocked the saphenous lidocaine-induced increase in the time spent in the lidocaine-paired chamber, $*P < 0.01$ vs sham placebo ($n = 8-13$). (D), Cancer-bearing rats with placebo pellets demonstrate significantly increased levels of dopamine when administered saphenous lidocaine. Morphine blocked saphenous lidocaine-induced dopamine release, with levels equivalent to sham-treated rats, $*P < 0.05$ vs sham/placebo ($n = 5-11$). (E), Cancer-bearing rats treated with morphine display a 5.5-fold increase in DA release at its peak effect vs sham rats with onboard morphine, $*P < 0.0001$ ($n = 6-13$). (F), Beta-funaltrexamine administered into the ACC reverses analgesia produced by morphine administration but fails to affect innate reward, $*P < 0.01$ ($n = 5-9$). All graphs show mean \pm SEM.

a preclinical model of CIBP that includes behavioral and biochemical measures of relief of ongoing pain not evoked by the experimenter. This can be used to determine mechanisms driving tumor-induced persistent pain. Moreover, used in conjunction with measures of other tumor-induced pain, such as movement-evoked breakthrough pain, important mechanistic differences may be determined that can influence drug development for more comprehensive pain management in these patients.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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