A rat model of bone inflammation-induced pain by intra-tibial complete Freund's adjuvant injection

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ABSTRACT

In prior studies, models of inflammatory pain were produced through injecting complete Freund's adjuvant (CFA) or capsaicin directly into either the deep somatic tissue or the animal's hind paw. In contrast, bone cancer-induced pain (BCIP) was simulated through injecting tumor cells into the cavity of the femur or the tibia. It has been reported that, due to differences in afferent innervation, the same stimulus to various tissue types might result in differing patterns of pain response. Hence, the aim of this study is to establish a rat model of bone inflammation-induced pain (BIIP) by injecting CFA into the tibial cavity, the same site involved in the BCIP model. The differences in body weight, bone histology, mechanical allodynia, thermal hyperalgesia, and the pain relieving effects of Celebrex on this model of BIIP were evaluated. The results showed that there was evidence of significant inflammation seen in the bone marrow two days after intra-tibial CFA injection, including nuclear condensation and fragmentation, massive neutrophilic granulocytes, and prominent fibrinous exudates. Fourteen days after injection, marked fibrosis of the bone was detected by histological staining. After unilateral CFA injection, behavioral studies showed mechanical allodynia to von Frey hair stimulation, but no thermal hyperalgesia was observed. Celebrex showed significant anti-allodynic effects on the BIIP model. The results demonstrated that CFA is an effective agent for inducing bone inflammation and subsequent pain-related behavior in rat models, and, thus, provides a practical and valuable contrast for BCIP research.

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In addition to the neuropathic component, tumor-induced inflammation is considered to be another important ingredient in development of cancer-induced pain (CIP) [2,13,29]. Previous research on inflammatory pain and CIP has provided a basis for understanding the particular neurochemical and behavioral aspects of CIP [10,24,26]. In one example, protein kinase Cγ, calcitonin gene-related peptide, substance P and its receptor in the spinal cord were all significantly increased in complete Freund's adjuvant (CFA)-induced inflammatory pain in mice. Notably, rather than displaying these neurochemical changes, a mouse model of sarcoma bone cancer-induced pain (BCIP) displayed massive astrocyte hypertrophy and increased neuronal expression of c-Fos and dynorphin [26]. Behaviorally, the morphine dose required to block bone cancer-related pain is significantly higher than that required to block inflammation-related pain of comparable magnitude both in clinical and animal experiments [24]. All of these suggest that there is a fundamental difference in the mechanisms underlying BCIP and inflammatory pain. This distinction elucidates some of the complex mechanisms involved in CIP.

In these studies, inflammatory pain was produced through injecting CFA or capsaicin directly into either the animal's hind paw or deep somatic tissue. In contrast, in the BCIP model tumor cells were injected into the cavity of the femur or tibia [10,16,24,26,27]. It has been reported that even the same stimulus may result in a different pattern of response when applied to different tissues. This difference might be related to differences in the anatomy of spinal pathways and the biochemical and physiological differences between skin and deep tissues [22,23]. In one example, injection of capsaicin into muscles or joints resulted in a long-lasting (1–4 weeks) bilateral mechanical allodynia with a simultaneous thermal hyperalgesia, whereas capsaicin injected into the superficial skin resulted in a secondary mechanical allodynia and thermal hyperalgesia lasting only about 3 h [27]. Therefore, we concluded that if it is possible to study the inflammatory process specifically in the intra-medullary location of bone cancer, it could further facilitate our understanding of the mechanisms of BCIP.

Here, we describe a simple, easily established, new model of BIIP in rats by injecting CFA into the medullary cavity of the tibia, as previously described [14]. The body weight, bone histology and the pain-related behavioral changes, as well as the relieving

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effects on pain-related behavior by Celebrex (a non-steroidal anti-inflammatory drug) were evaluated in the present study. Our prior data showed that the rat model of BIIP was an appropriate inflammatory contrast for BCIP research.

Female Wistar rats (150–170 g, from Shanghai Laboratory Animal Center, China Academy Sciences, Shanghai) were kept under controlled conditions ($23 \pm 0.5\,°C$, 12 h alternating light–dark cycle, free food and water ad libitum). The animals were divided into groups randomly and all experiments were carried out with double blind methods. All tests were carried out in a temperature-controlled room ($23 \pm 0.5\,°C$) from 8 a.m. to 12 a.m. to avoid behavioral variation by circadian rhythm. The experimental procedures were approved by Animal Care and Use Committee of Fudan University, and were consistent with the NIH's Guide for the Care and Use of Laboratory Animals and the Ethical Issues of the IASP [31].

According to the surgical procedure for intra-tibial injection of tumor cells [14], CFA (Sigma, St. Louis, USA; 1 mg/ml), a common inflammatory agent used for chronic inflammatory pain research [9,11], was injected into the tibial cavity as follows: The rats were anesthetized with chloral hydrate (i.p. 400 mg/kg). The skin overlying the patella was disinfected with 70% (v/v) ethanol after hair shaving. A 23-gauge needle punctured directly through the skin, aimed at the inner side of intercondylar eminence, pierced 1 cm below the knee joint into the medullary cavity of tibia, and then removed and replaced with a 25 or 50 μl microinjection syringe. CFA (10 μl, 20 μl or 30 μl) or normal saline (vehicle group) was slowly injected into the tibial cavity. The syringe was removed 1 min later to prevent CFA or normal saline from leaking out along the injection track. All animals were allowed to recover from the surgery for 1 day prior to any experimentation.

According to the up-and-down method as described by Dixon [4], mechanical allodynia was measured as the hind paw withdrawal response to von Frey filament stimulation. After acclimation for 30 min in a plastic cage (26 cm × 20 cm × 14 cm) with a mesh floor covered with transparent plastic boxes, an ascending series of von Frey filaments with logarithmic incremental stiffness (0.40, 0.60, 1.4, 2.0, 4.0, 6.0, 8.0, and 15.0 g) (Stoelting, Wood Dale, IL, USA) were applied perpendicular to the mid-plantar surface (avoiding the less sensitive tori) of each hind paw. Each von Frey filament was held about 2–3 s, with a 10 min interval between each application. A trial began with the application of the 2.0 g von Frey filament. The positive response was defined as a withdrawal of hind paw upon the stimulus. Whenever a positive response to a stimulus occurred, the next lower von Frey filament was applied, and whenever a negative response occurred, the next higher filament was applied. The testing consisted of five more stimuli after the first change in response occurred, and the pattern of response was converted to a 50% von Frey threshold using the method described.

By using IITC Model 390 Paw Stimulator Analgesia Meter (Life Science Instruments, USA) the paw withdrawal latency (PWL) to radiant heat was examined as previously described [7]. After an adaptation period of 30 min in a clear plastic cage upon an elevated floor of window glass, radiant heat was applied to the plantar surface of each paw until the rat lifted its paw from the glass. The intensity of radiant heat was adjusted to elicit the response around 12 s in normal rat and the heat was maintained at a constant intensity. A cut-off of 20 s was imposed to avoid tissue damage. The time from onset of radiant heat application to withdrawal of the rat's hind paw was defined as the PWL. Both hind paws were tested independently with a 10-min interval between trials. The average of the three trials was then determined.

To assess the bone inflammation induced by CFA injection, rats were anesthetized with chloral hydrate and transcardially perfused with 250 ml of 0.9% normal saline followed by 250 ml of 4% paraformaldehyde. The tibial bones were removed and decalcified
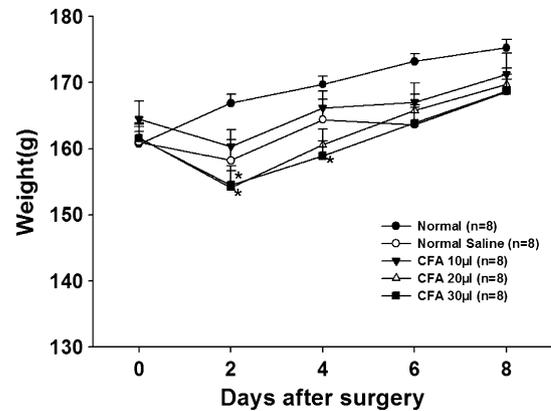

Fig. 1. Changes of body weight after the rats received intra-tibial CFA injection. Data are expressed as mean ± SEM. *$P < 0.05$ vs. normal rats.

in decalcifying solution for 24 h. The bones were rinsed, dehydrated, embedded in paraffin, cut into 7 μm cross-sections using a rotary microtome (Reichert-Jung 820, Cambridge Instruments GmbH, Germany), and stained with hematoxylin and eosin.

Celebrex (Pfizer, USA) is a highly specific cyclooxygenase-2 (COX-2) inhibitor with little or no residual COX-1 activity. As previously described [13], Celebrex was suspended in 0.5% methyl cellulose (MC) and administered twice daily in divided doses (20 mg/kg per day) by orogastric gavage in a volume of 1 ml, at 7:00 and 19:00 every day. Vehicle rats received 0.5% MC in comparable volumes.

Data were presented as mean ± SEM and were analyzed for statistical significance by one-way analysis of variance (ANOVA) followed by Shapiro–Wilk' test, using STATA 7.0 statistical software (Stata Corporation, College Station, TX). $P < 0.05$ was considered statistically significant.

Only on days 2 post-surgical injection in the 20-μl CFA group and on days 2 and 4 post-surgical injection in the 30-μl CFA group, a decrease in body weight was observed. There were no statistically significant differences in body weight among the normal rats, normal saline-injected rats and rats injected with differing doses of CFA (Fig. 1).

To evaluate intra-tibial CFA-induced inflammation, the proximal ends of the tibial bones of the rats were removed for histological examination. Hematoxylin and eosin staining showed that two days after intra-tibial CFA injection, significant nuclear condensation and fragmentation, massive invasion of neutrophilic granulocytes, as well as prominent fibrinous exudate were observed in the bone marrow (Fig. 2A–D). Normal saline-injected bone showed no such changes. However, there was marked fibrosis instead of the inflammatory cells in the bone marrow 14 days after CFA injection (Fig. 2E and F).

To examine the development of mechanical allodynia induced by intra-tibial CFA injection, the paw mechanical withdrawal threshold was detected using the von Frey test. Rats injected with CFA displayed a profound decrease in mechanical threshold to von Frey filament stimulation, on both the ipsilateral (Fig. 3A) and contralateral (Fig. 3B) hind paws. There were no significant differences in mechanical threshold among the rats receiving different doses of CFA. In contrast, no significant differences in mechanical threshold were observed between normal saline-injected rats and normal rats.

To examine whether thermal hyperalgesia exists in this rat model of bone inflammation, PWL was measured by Hargreaves' test, as described above. CFA-injected rats showed no significant change in PWL to radiant heat stimulation on either hind paw during the entire experiment (Fig. 3C and D).
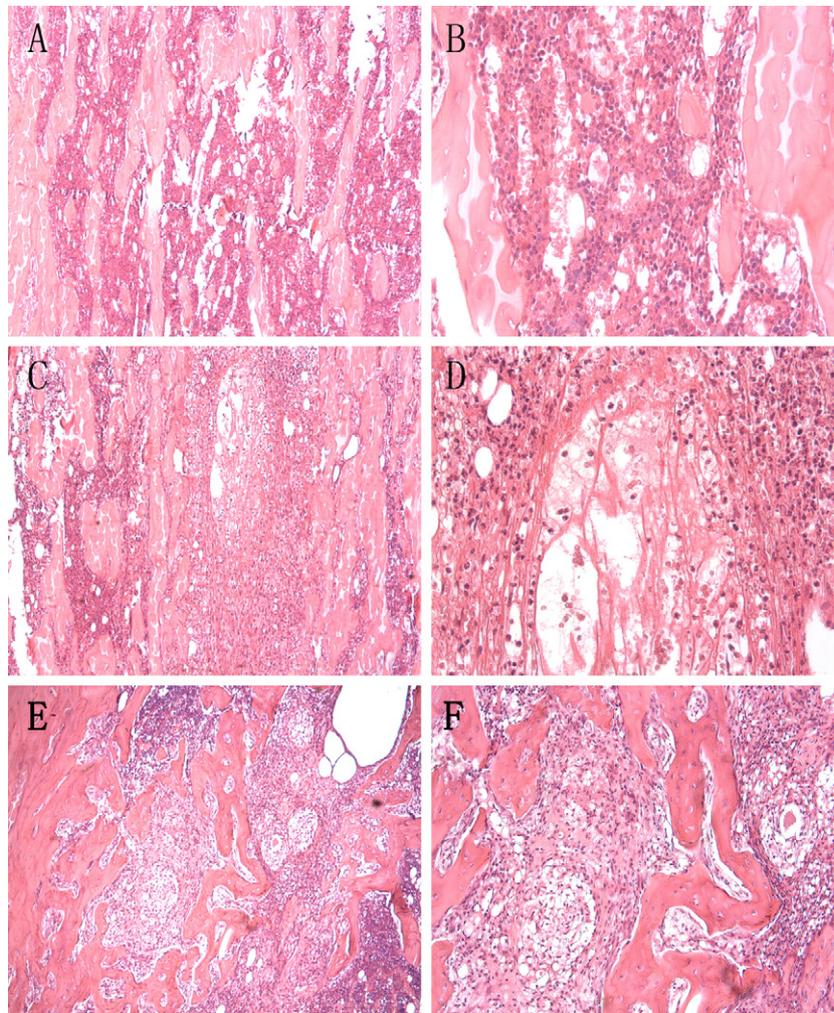
Fig. 2. Histology of tibial bone inflammation. The sections (7 μm) were taken from tibial bone stained by hematoxylin–eosin. (A and B) Bone injected with normal saline; (C and D) inflammatory cell infiltration in bone marrow two days after CFA injection; (E and F) bone marrow fibrosis 14 days after CFA injection. (A, C and E) 100× and (B, D and F) 400×.

Celebrex, a selective COX-2 inhibitor, is widely used for the treatment of conditions characterized by pain or inflammation, such as rheumatoid arthritis and osteoarthritis, and has been proved effectively by both clinical and animal researches [6,19,21]. In this study, we evaluated the palliative effects of Celebrex on mechanical allodynia in CFA-induced bone inflammation in rats. The CFA-injected rats displayed a marked increase in mechanical threshold after treatment with Celebrex (20 mg/kg per day) as compared to treatment with MC. No effect was seen on mechanical threshold in normal rats treated with Celebrex (Fig. 4).

In the present study, CFA was injected into the medullary cavity of the tibia via bone puncture to establish a rat BIIP model. In accordance to a previously described surgical procedure for tibial injection of tumor cells [14], in this study, modified bone puncture was carefully performed to minimize damage to the knee joint. There were no significant differences on body weight or basal behavioral responses found between the control rats and the normal saline-injected rats, even shortly after intra-tibial injection, demonstrating that the function of knee joint remained intact (Figs. 1 and 3). The bone puncture procedure is a feasible and ethically acceptable method for intra-tibial injection.

CFA is a type of water-in-oil emulsion containing killed dried *Mycobacterium butyricum* and has the capability of causing inflammation. It is commonly used in the establishment of animal models of peripheral inflammatory pain following injection into the plan-

tar foot, the ankle joint or the temporomandibular joint [5,8,19]. In this study, intra-tibial CFA injection successfully induced bone inflammation, as indicated by significant nuclear condensation and fragmentation, massive invasion of neutrophilic granulocytes and prominent fibrinous exudates (Fig. 2A–D) at two days, and significant fibrosis, instead of inflammatory cells (Fig. 2E and F), at 14 days after CFA injection. Further pain-related behavioral tests showed that, beginning at two days after CFA injection, the rats displayed significant mechanical allodynia but no detectable thermal hyperalgesia on both sides of the hind paws (Fig. 3). This distinction may be due to the activation of high-threshold mechanonociceptors (HTM), which are only sensitive to mechanical stimulation, as no activation of polymodal nociceptors (POLY), which are sensitive to mechanical and chemical and/or thermal stimulation in present rat model. The central mechanisms which contribute to mechanical allodynia induced by bone inflammation need to be investigated further [28]. The present research suggests that intra-tibial CFA injection does induce BIIP in rats. The fact that this injection produced an effective BIIP model was further demonstrated by the anti-allodynic effect of Celebrex.

Previous research has demonstrated that the different afferent spinal innervations of superficial and deep tissues consequently result in distinct behavioral responses despite receiving the same stimulation [22,23,27]. For example, cutaneous nociceptors send dense neuronal projections to laminas I and II in the spinal cord,
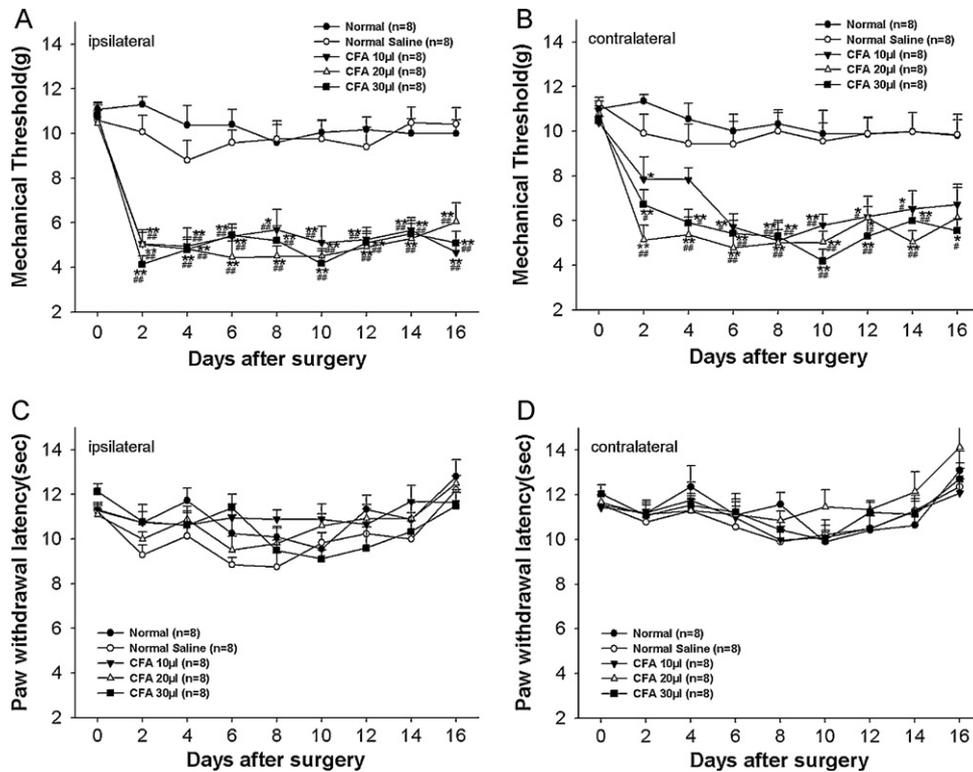
Fig. 3. Changes of pain-related behavior after the rats received intra-tibial CFA (10 μl, 20 μl, 30 μl) injection. (A) and (B) show mechanical response thresholds to von Frey filament stimulation on the ipsilateral and contralateral hind paws, respectively; (C) and (D) show thermal hyperalgesia on the ipsilateral and contralateral hind paws, respectively. Data are expressed as mean ± SEM. *$P < 0.05$, **$P < 0.01$ vs. normal rats. #$P < 0.05$, ##$P < 0.01$ vs. normal saline-injected rats.

whereas joint and muscle afferent neurons project to laminas I, V and II [3,12,17,30]. Therefore, the injection of capsaicin into muscles or joints resulted in a different pattern of behavior than injection of capsaicin into the superficial skin [27]. In our research, BIIP was induced by injection with CFA into the tibial cavity, the same site in which we injected tumor cells in the BCIP model in a prior study. Therefore, the present model of CFA-induced BIIP is a better control model for BCIP than those of previous studies because they might share the same neural pathway despite the different methods of stimulation. Furthermore, this study showed that the pain-related behavior is quite similar to bilateral mechanical allodynia, but no thermal hyperalgesia seen in the bone cancer rat models, as we described previously [14].

However, there were different responses observed between the BIIP and BCIP models to Celebrex treatment. In this study, treatment with Celebrex (20 mg/kg per day), started on the second day after CFA injection, had a significant analgesic effect on the BIIP model

(Fig. 4). It has been reported that the development and maintenance of BCIP were partially attributable to inflammatory activity, which is provoked by cytokines and prostaglandins produced by the cancer cells [13,16]. NSAIDs have been demonstrated as effective agents in relieving mild CIP [15,18]. However, both our prior study and previous reports have shown that Celebrex does not significantly improve bone cancer-related pain behavior in either rats or mice [14,16,20,25]. These results suggest that the underlying mechanism of CIP is distinct from that of inflammatory pain [10,24,26].

It has been reported that many factors contribute to the development and maintenance of BCIP, such as nerve injury by tumor growth, increased osteoclast activity, as well as pro-inflammatory factors produced by the tumor cells [1,13,29]. CIP generates a unique set of neurochemical changes in the spinal cord and sensory neurons that are distinct from peripheral inflammatory pain [10,26]. Consequently, the differing response to Celebrex further
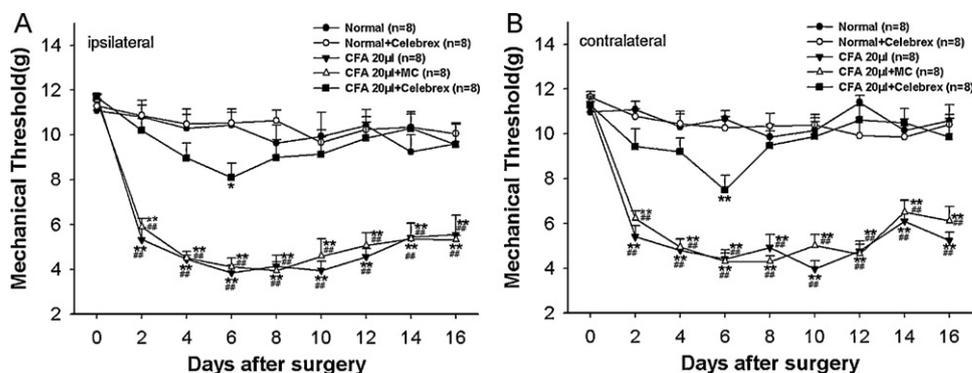

Fig. 4. Alleviating effects of Celebrex on mechanical allodynia with von Frey filament stimulation of the ipsilateral (A) and contralateral (B) hind paws in rats that received intra-tibial CFA injections. Data are expressed as mean ± SEM. *$P < 0.05$, **$P < 0.01$ vs. normal rats. #$P < 0.05$, ##$P < 0.01$ vs. normal saline-injected rats.

demonstrates the distinct intrinsic mechanisms behind BCIP and BIIP. Further investigations of the different intrinsic mechanisms in these two chronic pain conditions are needed to understand the unique mechanisms of BCIP.

The present study describes the development of a practical and valuable BIIP rat model by intra-tibial CFA injection. In this BIIP rat model, intra-tibial CFA injection induced significant inflammation in the bone marrow and bilateral mechanical allodynia, which was alleviated by Celebrex. The differing response to Celebrex between BCIP and BIIP further confirms the distinct mechanisms. Further investigations are required to fully understand these mechanisms.

Acknowledgements

References

[1] D.M. Cain, P.W. Wacnik, L. Eikmeier, A. Beitz, G.L. Wilcox, D.A. Simone, Functional interactions between tumor and peripheral nerve in a model of cancer pain in the mouse, Pain Med. 2 (2001) 15–23.

[2] D.M. Cain, P.W. Wacnik, M. Turner, G. Wendelschafer-Crabb, W.R. Kennedy, G.L. Wilcox, D.A. Simone, Functional interactions between tumor and peripheral nerve: changes in excitability and morphology of primary afferent fibers in a murine model of cancer pain, J. Neurosci. 21 (2001) 9367–9376.

[3] A.D. Craig, B. Heppelmann, H.G. Schaible, The projection of the medial and posterior articular nerves of the cat's knee to the spinal cord, J. Comp. Neurol. 276 (1988) 279–288.

[4] W.J. Dixon, Efficient analysis of experimental observations, Annu. Rev. Pharmacol. Toxicol. 20 (1980) 441–462.

[5] F.G. Garrett, P.L. Durham, Differential expression of connexins in trigeminal ganglion neurons and satellite glial cells in response to chronic or acute joint inflammation, Neuron Glia Biol. (2009) 1–12.

[6] M.M. Goldenberg, Celecoxib, a selective cyclooxygenase-2 inhibitor for the treatment of rheumatoid arthritis and osteoarthritis, Clin. Ther. 21 (1999) 1497–1513 (discussion 1427–1428).

[7] K. Hargreaves, R. Dubner, F. Brown, C. Flores, J. Joris, A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia, Pain 32 (1988) 77–88.

[8] Y. Hong, Y. Liu, J.G. Chabot, A. Fournier, R. Quirion, Upregulation of adrenomedullin in the spinal cord and dorsal root ganglia in the early phase of CFA-induced inflammation in rats, Pain 146 (2009) 105–113.

[9] P.W. Howorth, S.R. Thornton, V. O'Brien, W.D. Smith, N. Nikiforova, A.G. Teschemacher, A.E. Pickering, Retrograde viral vector-mediated inhibition of pontospinal noradrenergic neurons causes hyperalgesia in rats, J. Neurosci. 29 (2009) 12855–12864.

[10] L.J. Kehl, D.T. Hamamoto, P.W. Wacnik, D.L. Croft, B.D. Norsted, G.L. Wilcox, D.A. Simone, A cannabinoid agonist differentially attenuates deep tissue hyperalgesia in animal models of cancer and inflammatory muscle pain, Pain 103 (2003) 175–186.

[11] H.E. Krug, S. Frizelle, P. McGarraugh, M.L. Mahowald, Pain behavior measures to quantitate joint pain and response to neurotoxin treatment in murine models of arthritis, Pain Med. 10 (2009) 1218–1228.

[12] L.J. Ling, T. Honda, Y. Shimada, N. Ozaki, Y. Shiraishi, Y. Sugiura, Central projection of unmyelinated (C) primary afferent fibers from gastrocnemius muscle in the guinea pig, J. Comp. Neurol. 461 (2003) 140–150.

[13] P.W. Mantyh, D.R. Clohisy, M. Koltzenburg, S.P. Hunt, Molecular mechanisms of cancer pain, Nat. Rev. Cancer 2 (2002) 201–209.

[14] Q.L. Mao-Ying, J. Zhao, Z.Q. Dong, J. Wang, J. Yu, M.F. Yan, Y.Q. Zhang, G.C. Wu, Y.Q. Wang, A rat model of bone cancer pain induced by intra-tibia inoculation of Walker 256 mammary gland carcinoma cells, Biochem. Biophys. Res. Commun. 345 (2006) 1292–1298.

[15] E. McNicol, S. Strassels, L. Goudas, J. Lau, D. Carr, Nonsteroidal anti-inflammatory drugs, alone or combined with opioids, for cancer pain: a systematic review, J. Clin. Oncol. 22 (2004) 1975–1992.

[16] S.J. Medhurst, K. Walker, M. Bowes, B.L. Kidd, M. Glatt, M. Muller, M. Hattenberger, J. Vaxelaire, T. O'Reilly, G. Wotherspoon, J. Winter, J. Green, L. Urban, A rat model of bone cancer pain, Pain 96 (2002) 129–140.

[17] S. Mense, A.D. Craig Jr., Spinal and supraspinal terminations of primary afferent fibers from the gastrocnemius-soleus muscle in the cat, Neuroscience 26 (1988) 1023–1035.

[18] S. Mercadante, Malignant bone pain: pathophysiology and treatment, Pain 69 (1997) 1–18.

[19] W.L. Mi, Q.L. Mao-Ying, Q. Liu, X.W. Wang, Y.Q. Wang, G.C. Wu, Synergistic anti-hyperalgesia of electroacupuncture and low dose of celecoxib in monoarthritic rats: involvement of the cyclooxygenase activity in the spinal cord, Brain Res. Bull. 77 (2008) 98–104.

[20] M.E. Mouedden, T.F. Meert, Pharmacological evaluation of opioid and non-opioid analgesics in a murine bone cancer model of pain, Pharmacol. Biochem. Behav. 86 (2007) 458–467.

[21] M. Noguchi, A. Kimoto, S. Kobayashi, T. Yoshino, K. Miyata, M. Sasamata, Effect of celecoxib, a cyclooxygenase-2 inhibitor, on the pathophysiology of adjuvant arthritis in rat, Eur. J. Pharmacol. 513 (2005) 229–235.

[22] C. O'Brien, C.J. Woolf, M. Fitzgerald, R.M. Lindsay, C. Molander, Differences in the chemical expression of rat primary afferent neurons which innervate skin, muscle or joint, Neuroscience 32 (1989) 493–502.

[23] M.B. Plenderleith, P.J. Snow, The plant lectin Bandeiraea simplicifolia I-B4 identifies a subpopulation of small diameter primary sensory neurones which innervate the skin in the rat, Neurosci. Lett. 159 (1993) 17–20.

[24] M.A. Sabino, N.M. Luger, D.B. Mach, S.D. Rogers, M.J. Schwei, P.W. Mantyh, Different tumors in bone each give rise to a distinct pattern of skeletal destruction, bone cancer-related pain behaviors and neurochemical changes in the central nervous system, Int. J. Cancer 104 (2003) 550–558.

[25] O. Saito, T. Aoe, T. Yamamoto, Analgesic effects of nonsteroidal antiinflammatory drugs, acetaminophen, and morphine in a mouse model of bone cancer pain, J. Anesth. 19 (2005) 218–224.

[26] M.J. Schwei, P. Honore, S.D. Rogers, J.L. Salak-Johnson, M.P. Finke, M.L. Ramnaraine, D.R. Clohisy, P.W. Mantyh, Neurochemical and cellular reorganization of the spinal cord in a murine model of bone cancer pain, J. Neurosci. 19 (1999) 10886–10897.

[27] K.A. Sluka, Stimulation of deep somatic tissue with capsaicin produces long-lasting mechanical allodynia and heat hypoalgesia that depends on early activation of the cAMP pathway, J. Neurosci. 22 (2002) 5687–5693.

[28] Y. Takeuchi, J.L. Zeredo, R. Fujiyama, T. Amagasa, K. Toda, Effects of experimentally induced inflammation on temporomandibular joint nociceptors in rats, Neurosci. Lett. 354 (2004) 172–174.

[29] P.W. Wacnik, L.J. Eikmeier, T.R. Ruggles, M.L. Ramnaraine, B.K. Walcheck, A.J. Beitz, G.L. Wilcox, Functional interactions between tumor and peripheral nerve: morphology, algogen identification, and behavioral characterization of a new murine model of cancer pain, J. Neurosci. 21 (2001) 9355–9366.

[30] W.D.C.R. Willis, Sensory Mechanisms of the Spinal Cord, Plenum, New York, 1991.

[31] M. Zimmermann, Ethical guidelines for investigations of experimental pain in conscious animals, Pain 16 (1983) 109–110.