Regular Article

Activation of spinal chemokine receptor CXCR3 mediates bone cancer pain through an Akt-ERK crosstalk pathway in rats

Xue-Hai Guan a, Qiao-Chu Fu a, Dai Shi a, Hui-Lian Bu a, Zhen-Peng Song a, Bing-Rui Xiong a, Bin Shu a, Hong-Bing Xiang a, Bing Xu b, Anne Manyande c, Fei Cao d, Yu-Ke Tian a,⁎

a Department of Anesthesiology and Pain Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1095 Jiefang Road, Wuhan 430030, PR China
b Department of neurology, Liuzhou Traditional Chinese Medical Hospital, the Third Affiliated Hospital of Guangxi University of Chinese Medicine, 32 Jiefang West Road, Liuzhou 545001, PR China
c School of Psychology, Social Work and Human Sciences, University of West London, London, UK
d Department of Neuroscience, Baylor College of Medicine, Houston, TX 77030, USA

A R T I C L E  I N F O

Article history:
Received 16 June 2014
Revised 30 August 2014
Accepted 12 September 2014
Available online 2 October 2014

Keywords:
Bone cancer
Pain
CXCR3
ERK
Akt

A B S T R A C T

Previously, we showed that activation of the spinal CXCL9, 10/CXCR3 pathway mediated bone cancer pain (BCP) in rats. However, the cellular mechanism involved is poorly understood. Here, we found that the activated CXCR3 was co-localized with either neurons, microglia, and astrocytes in the spinal cord, or non-peptidergic-, peptidergic-, and A-type neurons in the dorsal root ganglion. The inoculation of Walker-256 mammary gland carcinoma cells into the rat's tibia induced a time-dependent phosphorylation of Akt and extracellular signal-regulated kinase (ERK1/2) in the spinal cord, and CXCR3 was necessary for the phosphorylation of Akt and ERK 1/2. Meanwhile, CXCR3 was co-localized with either pAkt or pERK1/2. Blockage of either Akt or ERK1/2 prevented or reversed the mechanical allodynia in BCP rats. Furthermore, there was cross-activation between PI3K/Akt and Raf/MEK/ERK pathway under the BCP condition. Our results demonstrated that the activation of spinal chemokine receptor CXCR3 mediated BCP through Akt and ERK 1/2 kinase, and also indicated a crosstalk between PI3K/Akt and Raf/MEK/ERK signaling pathways under the BCP condition.

Crown Copyright © 2014 Published by Elsevier Inc. All rights reserved.

Introduction

Bone cancer pain (BCP) is one of the most common pain syndromes encountered in patients suffering from primary or metastatic bone cancer of the breast, lung or prostate (Goblirsch et al., 2005; van den Beuken-van Everdingen et al., 2007). Clinically, BCP is still intractable since response to current pharmacological intervention remains poor. Understanding the detailed molecular mechanisms may explore new targets for the treatment of this complex problem.

Akt, a serine/threonine protein kinase, which is a critical downstream target of phosphatidylinositol 3-kinase (PI3K), plays an important role in regulating cellular functions and results from extracellular stimuli. There is growing evidence that activation of Akt regulates cell survival and apoptosis (Rane et al., 2003; Yang et al., 2011). Akt is also widely expressed in the spinal cord, particularly in laminae I-IV of the spinal dorsal horn and mediates various hyperalgesia (Honeth et al., 2006; Kim et al., 2012; Pereira et al., 2011; Pezet et al., 2008; Sun et al., 2006; Xu et al., 2007, 2011, 2014).

Equally, extracellular signal-regulated kinase (ERK), a member of mitogen-activated protein kinases (MAPKs) family, can transduce a large number of extracellular information into intracellular responses. ERK signaling pathway has been reported to play an important role in synaptic and neuronal plasticity (Adams and Sweatt, 2002), and is involved in the modulation of peripheral and central sensitization induced by noxious stimuli and nociceptive information (Cao et al., 2008; Guan et al., 2010; Ji et al., 1999; Kawasaki et al., 2004; Liu et al., 2011; Ruan et al., 2010; Song et al., 2005).

Recently, our results from a previous study showed that activation of CXCR3 contributed to the development and maintenance of BCP (Bu et al., 2014). There are nonetheless, more than 20 chemokine receptors identified, which are classified into four subfamilies: C, CC, CXC, and CXC3. CXCR3 belong to the CXC subfamily and most of them have been shown to belong to the superfamil of rhodopsin-like seven transmembrane guanosine-binding protein coupled receptor (GPCRs) (Murphy et al., 2000; Thelen, 2001). However, the detailed downstream molecular mechanism of CXCR3 remains unclear. MEK and PI3K are also known to be regulated by GPCRs, including chemokine receptor (Vlahakis et al., 2002). It is further reported that the up-regulation of CXCR3 is able to activate Akt and ERK (Aksoy et al., 2006; Willox et al., 2010), and there might be crosstalk between PI3K/Akt and Raf/MEK/ERK (Guan et al., 2010; Hawes et al., 1996; Hong et al.,...
Thus, it is possible that spinal Akt and ERK, and crosstalk between Akt and ERK, may be the downstream effectors of CXCR3 that contribute to the modulation of BCP. We provide strong evidence to support this hypothesis.

Materials and methods

Animals

Female Wistar rats (180–200 g, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, PR China) were
maintained individually at a temperature of 22 ± 1 °C in a 12-h light/dark cycle, with ad libitum access to diet and water. All experimental protocols were approved by the Animal care and Use Committee of the university and were in accordance with the guidelines published in the National Institutes of Health Guide for Care and Use of Laboratory Animals (revised 2011).

**Drug application**

AMG487, a special antagonist of CXCR3, was acquired from Amgen (USA), and was dissolved in 20% of 2-hydroxypropyl-β-cyclodextrin (HPβCD), as previously described (Walser et al., 2006). Wortmannin, an inhibitor of PI3K, and U0126, a MEK inhibitor were purchased from Sigma (St. Louis, MO) and Selleck, respectively, and were dissolved in 5% DMSO. The dosage of drugs is based on the results of preliminary experiments. The usage and dose for each drug are presented in the results section and figure legends.

**Intrathecal catheters and drug administration**

Under pentobarbital sodium (50 mg/kg, i.p.) anesthesia, implantation of intrathecal cannula was performed as described previously (Yaksh and Rudy, 1976). The position of the catheter was determined by intrathecal injection of 1% lidocaine (5 μl). Rats showing neurological deficits were excluded from the experiment. AMG487, wortmannin, U0126, or vehicle was intrathecally injected via the implanted catheter in a 10-μl volume followed by 10-μl sterile saline for flushing, lasting at least 3 min.

**Preparation of carcinoma cells**

Walker 256 rat mammary gland carcinoma cells were obtained from the Institute of Cancer Research of Chinese Academy of Medical Science and Peking Union Medical College (China). The cells (500 μl, 2 × 10^7) were inoculated into the abdominal cavity of female rats. After 7 days, carcinoma cells were extracted from the ascites, rinsed in D-Hank’s solution and centrifugation at 600 rpm for 5 min at 4 °C (4 cycles). Then the pellet was re-suspended in D-Hank’s solution and the concentration was adjusted to 4 × 10^5/ml using a hemocytometer. The cell suspension was maintained on ice until inoculation.

**Bone cancer pain model**

The procedure was carried out as previously described (Cao et al., 2010; Liu et al., 2012b). Briefly, under anesthesia with pentobarbital sodium (50 mg/kg, i.p.), rats were placed in supine position. The right leg was shaved and the skin was disinfected with 7% iodine. The top half of the tibia was exposed with minimum damage to the muscle or blood vessels. A 23-gauge needle was inserted into the intramedullary canal of tibia, 5 mm distal to the epiphyseal growth plate below the knee joint. Then the needle was removed and replaced with a long thin blunt needle attached to 10 μl Hamilton syringe containing the cells or vehicle (D-Hank’s solution) to be injected. Walker 256 carcinoma cells (10 μl, 4 × 10^4), or vehicle was injected into the bone cavity slowly. The injected site was immediately closed with bone wax after the injection. The incision was sutured using 3-0 silk thread and dusted with benzyl penicillin antibiotic powder. Rats were placed on a heated pad until they had regained consciousness and then returned to their home cages.

**Fig. 2.** Time-dependent up-regulation and cellular localization of CXCR3 in ipsilateral DRG in BCP rats. The expression of CXCR3 was assayed at sham (day 15), day 5, day 10 and day 15 time-points after carcinoma cells inoculation. (A–C) The time course of CXCR3 following carcinoma cells inoculation and the quantitative data for the CXCR3-positive cells in the DRG (detected by immunofluorescence). ⁎ P < 0.05, ⁎⁎ P < 0.01, compared with the sham group. n = 4 rats in each group. Double immunofluorescence of CXCR3 (D–F, red) was performed with cell-specific markers: isolectin B4 (IB4, D, green) for non-peptidergic neurons, neurofilament-200 (NF200, E, green) for A-type neurons and CGRP (F, green) for peptidergic neurons in the DRG at day 15 after modeling. CXCR3 was co-localized with non-peptidergic (D, yellow), A-type (E, yellow) and peptidergic neurons (F, yellow). Scale bar = 100 μm.
versed the bilateral mechanical allodynia in BCP rats. The behavior tests were conducted at 0, 0.25, 0.5, 1, 2, and 4 h after AMG487 injection. (A) Chronic blockage of CXCR3 in the tip of BCP-cont-vehicle group. # P < 0.05, compared with sham-ipsi-vehicle group. (B) Acute blockage of CXCR3 reversed the bilateral mechanical allodynia in BCP rats. *P < 0.05, compared with sham-ipsi-vehicle group, #: P < 0.05, compared with BCP-ipsi-vehicle group, & P < 0.05, compared with BCP-cont-vehicle group. n = 8 rats in each group. ipsi: ipsilateral; cont: contralateral.

The mechanical paw withdrawal threshold

Animals were placed in individual plastic observation boxes with a metal mesh floor and allowed to acclimate for 30 min. Behavioral tests used to measure mechanical allodynia were carried out.

The mechanical paw withdrawal threshold (PWT) was determined by using von Frey filaments (1-, 1.4-, 2-, 4-, 6-, 8-, 10- and 15-g bending force; Stoelting, Wood Dale, IL), starting with 1 g and ending with 15 g in ascending order, as previously reported (Pogatzki and Raja, 2003). The investigator was trained to apply the tip of filament perpendicular to the plantar surface, with sufficient force to cause slight bending against the paw. The duration of each stimulus was approximately 1 s. Each monofilament was applied 5 times with a 10-s interval. Quick withdrawal or paw flinching was considered a positive response. The paw withdrawal frequency (PWF) to each monofilament was calculated from five applications. PWT was considered the force at which PWF ≥ 60%; 15 g was recorded as the PWT if PWF < 60% to all filaments (Xu et al., 2014).

The individual investigators responsible for behavioral testing were blinded to the experimental situation.

Immunohistochemistry

Under deep anesthesia with sodium pentobarbital (60 mg/kg, i.p.), the rats were perfused transcardially with 200 ml saline followed by 200 ml 4% ice-cold paraformaldehyde in 0.1 mol/l phosphate buffer saline (PBS). The spinal cord and DRG of L2-L5 were removed (Niiyama et al., 2007; Xu et al., 2013; Zhang et al., 2005) and post-fixed in 4% paraformaldehyde for 24 h, and subsequently allowed to equilibrate in 30% sucrose in PBS overnight at 4 °C. Twenty-μm (for the spinal cord) and 10 μm (for the DRG) transverse series sections were cryostat cut and every five sections were collected in PBS. After washing with PBS, the tissue sections were penetrated with 0.3% TritonX-100 at room temperature (RT) for 15 min and blocked with 5% bovine serum albumin for 1 h at RT, then incubated for 48 h at 4 °C with a mixture of primary antibodies against CXCR3 (1:50; Santa Cruz), β-actin (1:200; Cell signaling Technology, CST), or pERK1/2 (1:200; CST), and anti-neuronal nuclei (NeuN; neuronal marker, 1:200; Chemi-Con, Temecula, CA), anti-IBA1 (IBA1; microglia marker, 1:300; Abcam, Cambridge, UK) or anti-glia fibrillary acidic protein (GFAP; astrocytes marker, 1:400; CST), and isolectin B4 (FITC conjugated, IB4; a marker for non-peptidergic nociceptors, 20 μg/ml; Sigma), anti-Calcitonin Gene Related Peptide (CGRP, a marker for peptidergic nociceptors, 1:50; Abcam) or anti-neurofilament-200 (NF200; a marker for myelinated A-fibers, 1:200; Sigma). Except the IB4-treated DRG sections which were treated with CY3-conjugated Immunoglobulin G (1:500, Abcam), all the other sections were treated with a mixture of CY3- and Alexa Fluor 488-conjugated Immunoglobulin C (1:100, Abbkine) for 2 h at RT. Nonspecific staining was determined by excluding the primary antibodies. Sections were rinsed, mounted and cover-slipped with 50% glycerol and stored at −20 °C in the dark. Images were captured using a fluorescence microscope (DM2500, Leica, Germany).

The quantification of the immunofluorescence staining in DRG was performed by counting the number of CXCR3-immunoreactive (IR) neurons per section. The assessor was blind to the experimental design. Every fifth section was picked from a series of consecutive DRG sections and 5 sections were counted per DRG. The proportion of CXCR3-expressed neurons was determined by counting the neuronal profiles that showed distinctive labeling in the DRG sections. For the spinal cord, the area of CXCR3 per section was measured in the spinal dorsal horn by using a computerized image analysis system (NIH Image).

Briefly, to identify positive stained structures, a density threshold was set above the background level firstly, and the area occupied by these structures was defined as positive area. Five spinal sections of each rat were selected randomly. An average percentage of CXCR3-IR neurons relative to the total number of neuron cells or positive area of CXCR3-IR relative to the total area of the spinal dorsal horn was calculated for each rat across the different tissue sections, and the mean ± standard error of the mean (S.E.M.) across rats was determined (Ji et al., 2002b; Obata et al., 2004; Xu et al., 2014).

Western blot analysis

The whole spinal cord at L2-L5 segments was quickly removed and stored in liquid nitrogen. Tissue samples were homogenized in lysis buffer containing (in mill moles): sucrose, 250.0 mM; Tris, 20.0 mM; Na2VO4, 0.03 mM; MgCl2, 2.0 mM; EDTA, 2.0 mM; EGTA, 2.0 mM; phenylmethylsulfonyl fluoride, 2.0 mM; dithiothreitol, 1.0 mM; protease inhibitor cocktail, 0.02% (v/v); and pH 7.4. The homogenates were centrifuged at 10,000 × g for 30 min at 4 °C. The supernatant was collected and protein concentration was measured according to the Bradford method using bovine serum albumin as standard. The protein samples were stored at −80 °C. Protein samples were dissolved in sample buffer.
Results

Time-dependent up-regulation and cellular localization of CXCR3 in the spinal cord and DRG in BCP rats

Our previous data of microarray analysis showed that CXCR3 mRNA expression increased in the spinal cord of BCP rats (unpublished). In order to determine whether CXCR3 was activated under BCP, we detected the time course and gauged the protein levels of CXCR3 in the spinal cord and dorsal root ganglion (DRG) by Western Blot analysis and immunohistochemistry. As shown in Figs. 1A–F and 2A–C, the protein level of CXCR3 in the ipsilateral spinal cord and DRG increased significantly in a time-dependent manner, starting at day 5, remained at peak level until day 15 (the last test day) after modeling, whereas the levels remained low in sham rats. The protein level CXCR3 in the contralateral spinal cord increased too, which is consistent with our previous results (Bu et al., 2014) (data not shown).

To identify the cell types of activated CXCR3 in the spinal cord and DRG, double immunofluorescence of CXCR3 was performed with either cell-specific markers: neuronal nuclei (NeuN) for neurons, glial fibrillary acidic protein (GFAP) for astrocytes and IB1 for microglia in the spinal cord, or isoleucin B4 (IB4) for non-peptidergic neurons, calcitonin gene related peptide (CGRP) for peptidergic neurons, and neurofilament-200 (NF200) for A-type neurons in the DRG at day 15 after modeling. In the spinal cord, CXCR3 was predominantly distributed in the spinal dorsal horn ipsilateral on the inoculation side, and was co-localized with neurons (Fig. 1I), astrocytes (Fig. 1L) and microglia (Fig. 1O). Furthermore, in DRG, CXCR3 was co-localized with non-peptidergic (IB4)- (Fig. 2D), A- (Fig. 2.E), and peptidergic (CGRP)-type (Fig. 2F) neurons.

Inhibition of spinal CXCR3 alleviated the mechanical allodynia in BCP rats

CXCR3 was activated in the spinal cord and DRG under the BCP condition. Therefore, in theory, if CXCR3 takes part in mediating the pain development, inhibition of CXCR3 would attenuate pain behaviors. To detect this possibility, CXCR3 antagonist, AMG487 was used. For chronic treatment, AMG487 (20 μg/10 μl), or vehicle (20% HP/CD, 10 μl) was administered intrathecally (i.t.) once daily from postoperative day 1 to day 15. As shown in Fig. 3A, inoculation of Walker 256 mammary gland carcinoma cells into the rat tibia induced bilateral mechanical allodynia, which was consistent with the results of Mao-Ying et al. (2006). The intrathecal treatment of AMG487 prevented the down-regulation of the bilateral mechanical paw withdrawal threshold (PWT) significantly. For acute treatment, AMG487 (i.t, 20 μg/10 μl) was administered at day 15 and PWT was conducted at 0-, 0.25-, 0.5-, 1-, 2-, and 4-h after the AMG487 injection. The post-treatment of

Statistics

All data are expressed as mean ± S.E.M. Statistical analysis between two samples was performed using Student’s t-test. Statistical comparison of more than two groups was performed using one-way analysis of variance (ANOVA) followed by a Tukey test. Data from the behavior test were analyzed via two-way ANOVA. “Time” was treated as “within subjects” factor and “treatment” was treated as a “between” subject factor. A value of P < 0.05 was considered significant.
AMG487 reversed the bilateral PWT at 0.5 h time point and lasted at least 4 h (Fig. 3B). The administration of AMG487 had no effect on bilateral PWT in sham rats (the contralateral data is not shown), and neither did vehicle on BCP rats.

The activation of spinal Akt induced by BCP was dependent on CXCR3

To determine whether spinal Akt was activated under BCP condition, we detected the time course and gauged the protein level of pAkt, by Western Blot analysis and immunohistochemistry. As shown in Figs. 4A–C, the protein level of spinal pAkt was dramatically increased in a time-dependent manner, from day 5 to day 15 after modeling, whereas the levels were lower in sham rats, and predominantly distributed in the superficial dorsal horn ipsilateral on the inoculation side. The total Akt did not change significantly. Since activation of CXCR3 was able to stimulate ERK (Willox et al., 2010), we therefore posed the question as to whether the activation of ERK under BCP condition was dependent on CXCR3. To answer this question, the co-localization between CXCR3 and pERK1/2 was assayed by immunofluorescence at day 15 after modeling, and the pERK1/2 protein level was detected on post-operative day 15 after the treatment of AMG487 (20 μg/10 μl, i.t., once daily from postoperative day 1 to day 15). As shown in Fig. 6F, CXCR3 was co-localized with pERK1/2 in the ipsilateral spinal cord. AMG487 inhibited the up-regulation of pERK1/2 significantly, compared with the vehicle group (P < 0.05), and had no effect on total ERK1/2 levels (Fig. 6G).

Inhibition of spinal Akt alleviated the mechanical allodynia in BCP rats

While the activation of spinal Akt was dependent on CXCR3, and inhibition of spinal CXCR3 alleviated mechanical allodynia in BCP rats. Therefore, in theory, if pAkt is involved in mediating the pain development, inhibition of pAkt may attenuate pain behaviors too. To examine this possibility, wortmannin, the inhibitor of PI3K (the main upstream of Akt) was used. For chronic treatment, wortmannin (5 μg/10 μl), or vehicle (5% DMSO, 10 μl) was administered intrathecally (i.t.) once daily from postoperative day 1 to day 15. As shown in Fig. 5A, wortmannin prevented the down-regulation of the bilateral PWT significantly. For acute treatment, wortmannin (i.t, 5 μg/10 μl) was administered postoperatively at day 15. The behavior tests were conducted at 0-, 0.25-, 0.5-, 1-, 2-, and 4-h after wortmannin injection. The post-treatment of wortmannin reversed the bilateral PWT at 1 h time point, and lasted at least 4 h (Fig. 5B). However the administration of wortmannin did not have any effect on bilateral PWT in sham rats (the contralateral data is not shown), and neither did vehicle on BCP rats.

The activation of spinal ERK1/2 induced by BCP was dependent on CXCR3

To determine whether spinal ERK1/2 was activated on BCP status, we detected the time course and evaluated the protein level of pERK1/2, by Western Blot analysis and immunohistochemistry. As shown in Figs. 6A–C, the protein level of spinal pERK1/2 was increased significantly in a time-dependent manner, from day 5 to day 15 after modeling, whereas the levels were lower in sham rats, and predominantly distributed in the superficial dorsal horn ipsilateral on the inoculation side. While the activation of spinal Akt was dependent on CXCR3, and inhibition of spinal CXCR3 alleviated bone cancer pain-related behaviors, and likewise, if pERK1/2 is involved in mediating the pain development, it is plausible to conclude that inhibiting the activation of Akt would attenuate pain behaviors too. To test this assumption, U0126, the inhibitor of MEK (the upstream of ERK) was employed. For chronic treatment, U0126 (4 μg/10 μl), or vehicle (5% DMSO, 10 μl, i.t., once daily from postoperative day 1 to day 15) was administered. Compared with BCP-ipsi-vehicle group or BCP-cont-vehicle group, U0126 prevented the down-regulation of the bilateral PWT (Fig. 7A). For acute treatment, U0126 (i.t, 4 μg/10 μl) was administered at day 15. The behavior tests were conducted at 0-, 0.25-, 0.5-, 1-, 2-, and 4-h after U0126 injection. The post-treatment of U0126 reversed the bilateral PWT at 1 h time point and lasted at least 4 h (Fig. 7B), and had no effect on bilateral PWT in
The vehicle group.

Akt and Raf/MEK/ERK pathways (Yang et al., 2011). To further investigate the cross-talk between Akt and ERK1/2 under the BCP condition, we examined the pAkt or pERK1/2 protein level after treatment with U0126 (MEK inhibitor, 5 μg/10 μL, once a day, from postoperative day 1 to day 15) or wortmannin (an irreversible PI3K inhibitor, 5 μg/10 μL, once daily from postoperative day 1 to day 15). Wortmannin inhibited the activation of both Akt and ERK1/2 (Figs. 8A, D), which means that the activation of Akt is dependent on PI3K, and to maintain activation of Raf/MEK/ERK requires the PI3K activity under BCP condition. U0126 inhibited the activation of both ERK1/2 and Akt (Figs. 8B, C), which suggests that the activation of ERK1/2 is dependent on MEK, and to maintain activation of PI3K/Akt requires the activity of MEK under BCP status.

Discussion

The principal findings of this study are: (1) the inoculation of Walker-256 rat mammary gland carcinoma cells into the tibia produced mechanical allodynia, accompanied by a time-dependent increase of CXCR3, pAkt and pERK1/2 in the ipsilateral spinal cord and/or DRG. (2) inhibition of spinal CXCR3 alleviated the mechanical allodynia and the activation of Akt and ERK1/2. (3) inhibition of spinal Akt and ERK1/2 alleviated the mechanical allodynia. (4) there was cross-talk between Akt and ERK1/2 under the BCP condition. These results together demonstrate that activation of Akt and ERK1/2 and their cross-talk in the spinal cord are important mechanisms underlying the contribution of CXCR3 signaling to the development of BCP in rats. This study suggests that CXCR3/Akt and CXCR3/ERK signaling pathway may be a potential target for the prevention and reversion of BCP.

More recent studies have reported that CXCR3 belongs to the CXC subfamily, and is involved in multiple physiological and pathological functions, such as cell migration, cell proliferation, metastasis, multiple sclerosis, cancer, chronic inflammation, immune dysfunction, and pain modulation (Breser et al., 2013; Lee et al., 2012a; Lo et al., 2010; Murakami et al., 2013; Wu et al., 2012). Furthermore, the continued expression of neuronal chemokine/receptors appears to correlate with changes in chronic nociceptive behavior (Bhangoo et al., 2007). In this study, CXCR3 was up-regulated in a time-dependent manner in the ipsilateral (Figs. 1A–F) and contralateral (data is not shown) spinal cord under the BCP condition, and was markedly expressed in laminae I–V of the spinal dorsal horn (Figs. 1B–E), the important site for processing nociceptive information, which is consistent with our previous results (Bu et al., 2014). The CXCR3 receptor was also expressed in the ipsilateral DRG (Figs. 2A–C), in a time-dependent manner. According to Bhangoo et al. (2007), administration of CCR2 receptor antagonist attenuated the nociceptive behaviors induced by the induction of focal demyelination of the sciatic nerve. More recently, Schmitz et al. (2013) showed that, CXCR3 deficiency reduced encephalomyelitis-evoked hyperalgesia. In order to explore the effect of CXCR3 receptor on the pain modulation, we inhibited the activation of spinal CXCR3 with the inhibitor AMG487, and then investigated the PWT in BCP rats. Both chronic and acute treatment with AMG487 alleviated the ipsi- and contralateral mechanical allodynia in BCP rats (Fig. 3), without changing the bone destruction process (data is not shown), which is consistent with our previous results (Bu et al., 2014). Chemokines are released locally and their effects are limited to local tissues. Because of this characteristic, and that intrathecal drug delivery could be a better target for pain therapy with less off-target side effects, we did not evaluate the effects of the systemic treatment with AMG487. In addition, most drugs targeting chemokine pathways were originally developed for autoimmune and inflammatory diseases such as rheumatoid arthritis, psoriasis, multiple sclerosis and asthma (Wells et al., 2006), and tested for safety, it would be therefore, worthwhile to make use of the inhibitors of CXCR3 to treat BCP.

The CXCR3 were co-localized with peptidergic (CGRP), non-peptidergic (IB4) and A-type (NF200) neurons in the DRG (Figs. 2D–F). These neurons were activated and the nociceptive information from the tibia was transduced to the neurons in the spinal cord by the axons. The CXCR3 receptors were co-localized with the neurons, astrocytes and microglia in the spinal cord (Figs. 1G–O), which supports the concept that glial cells and neuron cells express similar receptors and ion channels actively interact and contribute to neurofunction (Eulnerburg and Gomez, 2010; Jarvis, 2010; Porter and McCarthy,
ERK1/2, p38 and JNK) and PI3K/Akt, which result in elevation of intracellular calcium leads to the activation of signaling pathways such as MAPKs (Taniguchi and Ishii, 1997). It was already well documented that astrocytes and microglia were activated and proliferated under BCP conditions (Bu et al., 2014; Honore et al., 2000; Liu et al., 2013; Wang et al., 2012b; Zhang et al., 2005), however the relationship between these cells requires further study. Since intrathecal administration of minocycline a microglia inhibitor, prevented BCP (Bu et al., 2014), we had reason to believe that microglia may be one of the initial sources of CXCR3. Recent studies have shown that activation of chemokine receptors induced an increase in cytosolic calcium, \([\text{Ca}^{2+}]_i\) (Willcox et al., 2010). In T cells and renal mesangial cells, CXCR3 agonists increased \([\text{Ca}^{2+}]_i\) (Korniejewska et al., 2011). We therefore, have reason to speculate that the activation of CXCR3 in the neurons may increase the concentration of cytosolic calcium and then the activated neurons generate action potentials and propagate it to the supraspinal system.

Some authors have suggested that MEK and PI3K are regulated by GPCRs, including chemokine receptor (Korniejewska et al., 2011; Vlahakis et al., 2002). While others report that activation of the receptors leads to the activation of signaling pathways such as MAPKS (ERK1/2, p38 and JNK) and PI3K/Akt, which result in elevation of intracellular calcium, DNA synthesis and cell proliferation or chemotaxis both in physiological cells (human airway epithelial cells and type II pneumocyte) as well as cancer cells (glioma cells) (Aksoy et al., 2006; Ji et al., 2008; Maru et al., 2008). Especially, the activation of CXCR3, has been found to activate Akt or ERK (Aksoy et al., 2006; Korniejewska et al., 2011; Shahabuddin et al., 2006; Willox et al., 2010). In addition stimulation of CXCR3 with IFN-γ-inducible, T cell α-chemoattractant has been demonstrated to enhance thymidine incorporation and cell proliferation through activation of p-38 and ERK 1/2 (Aksoy et al., 2006). Nevertheless, CXCR3-alt has also been shown to elicit signaling such as ERK1/2 and Akt phosphorylation (Ehlerth et al., 2004; Korniejewska et al., 2011). In our study, both spinal ERK 1/2 and Akt were up-regulated in a time-dependent manner in BCP rats, and were distributed markedly in the superficial laminae of the spinal dorsal horn (Figs. 4A–C, 6A–C), the key location for the modulation of nociceptive information. CXCR3 was co-localized with pAkt and pERK 1/2 respectively (Figs. 4F, 6F). Furthermore, the activation of both ERK1/2 and Akt were inhibited by AMG487, the CXCR3 inhibitor, which suggests that their activation was dependent on CXCR3 under BCP status (Figs. 4G, 6G). As mentioned above, the activation of CXCR3 in the neurons may induce an increase in the concentration of cytosolic calcium. Moreover, the increase of \([\text{Ca}^{2+}]_i\) is essential for Akt and ERK activation (Lever et al., 2003). We can therefore, speculate that the activation of CXCR3 could activate Akt and ERK by increasing the cytosolic calcium. If the activation of CXCR3 resulted in the phosphorylation of ERK1/2 and Akt, it would ultimately lead to the increased synthesis and release of pro-nociceptive mediators. These mediators diffuse and bind to the receptors on the dorsal horn neurons, which results in hypersensitivity and spontaneous firing, a characteristic of central sensitization. Spinal central sensitization is the key mechanism of BCP (Wang et al., 2012a; Yanagisawa et al., 2010).

Substantial research has highlighted how Akt is expressed in the spinal cord, particularly in laminae I-IV and DRG (Kim et al., 2012; Ma et al., 2006; Pereira et al., 2011; Pezet et al., 2008; Sun et al., 2007, 2006; Xu et al., 2007, 2011, 2014). Our results show that the protein level of pAkt was increased in a time-dependent manner, and was localized in laminae I-IV, the key site for processing nociceptive information (Figs. 4A–C). More recent studies indicate that the activation of Akt substantially contributes to the central sensitization induced by various kinds of stimuli, such as capsaicin, carrageenan, brain derived neurotrophic factor, formalin, incisinal and neuropathic pain (Distrutti et al., 2010; Duan et al., 2012; Guedes et al., 2008; Kim et al., 2012; Sun et al., 2007, 2006; Xu et al., 2007, 2011, 2014; X. Zhang et al., 2014). The BCP-related increase of pAkt in the spinal cord was prevented by chronic treatment with PI3K inhibitor and wortmannin without affecting the total Akt level (Fig. 8A). But there is evidence that inhibition of PI3K/Akt pathway attenuates the pain-related behaviors induced by the above stimuli (Distrutti et al., 2010; Duan et al., 2012; Guedes et al., 2008; Kim et al., 2012; Sun et al., 2007, 2006; Xu et al., 2007, 2011, 2014). In our study, both chronic and acute treatment with wortmannin, the inhibitor of PI3K, attenuated the mechanical allodynia in BCP rats (Fig. 5). Therefore, it is reasonable to assume that the activation of Akt may mediate the pain behaviors in BCP rats. Akt is the pivotal downstream of PI3K and mediates many of its functions through phosphorylation and regulation of transcription factors, including nuclear-factor-kappa B, GSK3, cAMP response element binding protein (CREB) and mammalian target of rapamycin (mTOR) (Kay et al., 2013; Shih et al., 2012; Xu et al., 2011). Whether Akt mediates BCP through the above mentioned transcription factors remains to be seen.

Unsurprisingly, ERK is distributed in the superficial spinal dorsal cord (Bu et al., 1999, 2002a; Ruan et al., 2010). Our result show that the protein level of PERK1/2 was increased in a time-dependent manner and was localized in laminae I-IV, the key site for processing nociceptive information (Figs. 6A–C), which is consistent with other previous research (Wang et al., 2011; Wang et al., 2012b). Further evidence suggest that ERK 1/2 transduce extracellular stimuli into intracellular post-translational and transcriptional responses (Obata and Noguchi, 2004). An increasing body of literature has also shown that ERK in the spinal dorsal horn contributes to hypersensitivity induced by numerous
stimulus, such as formalin, complete Freund’s adjuvant, capsaicin, ephrinBs, chronic constrictive injury of the sciatic nerve and so on (Berta et al., 2013; Cruz et al., 2005; Ji et al., 1999, 2002a; Lee et al., 2012b; L Liu et al., 2012; Ruan et al., 2010; Svensson et al., 2006; Wang et al., 2013; Yu et al., 2012; J. Zhang et al., 2014). The BCP-related increase of pERK1/2 in the spinal cord was prevented by chronic treatment with MEK inhibitor and U0126 without affecting the total ERK1/2 level (Fig. 8C). At the same time, the chronic or acute treatment with U0126 attenuated the mechanical allodynia in BCP rats (Fig. 7). In addition to an inflammatory role (Ji et al., 1999, 2002a; Karim et al., 2001; Pang et al., 2008; Polgar et al., 2007; Sammons et al., 2000; Tillu et al., 2012; J. Zhang et al., 2014) and neuropathic pain (Cheng et al., 2003; Ciruela et al., 2003; Ma and Quirion, 2002; Peng et al., 2009; Son and Kwon, 2010; Song et al., 2005; Yang et al., 2004; Zhuang et al., 2005), our data illustrates that ERK activation in the spinal cord plays a role in BCP. Therefore, it is reasonable to assume that the high levels of pERK1/2 play an important role in the pain behaviors in BCP rats. Significant research has shown that ERK activation in the dorsal horn neurons contributes to the development of the central sensitization induced by inflammation or nerve injury, through the transcriptional regulation of several downstream genes, for example, NK-1 and prodynorphin, Ets-like transcription factor, c-fos and CREB, which contribute to persistent pain (Ji et al., 2002a; Kawasaki et al., 2004; Kominato et al., 2003; Ma and Quirion, 2005; Song et al., 2005; Xin et al., 2006). Thus, we can speculate that the activation of ERK1/2 can play its role in BCP by regulating transcriptions of these gene too, although this was not tested in this study.

PI3K/Akt and Raf/MEK/ERK pathways have been shown to crosstalk (Guan et al., 2010; Hawes et al., 1996; Hong et al., 2013; Huang et al., 2013; Moelling et al., 2002; Welsh et al., 1994; Wennstrom and Downward, 1999; Yang et al., 2011; Yu et al., 2012), including cross-inhibition and cross-activation. There are reports that Akt positively regulates Raf phosphorylation and ERK1/2 activation in prostate cancer cells in response to androgen depletion (Hong et al., 2013) and that PI3K inhibitor LY294002 inhibits the increase of pERK1/2 and Raf-1 in glucose-regulated protein 75 (Grp75) overexpression of cells under glucose deprivation condition (Yang et al., 2011). In our study, PI3K inhibitor and wortmannin, inhibited not only the activation of Akt, but also ERK1/2 (Figs. 8A, D), which means that the activation of Akt is dependent on the activation of PI3K, and the continuous activation of Raf/MEK/ERK required the activity of PI3K/Akt signaling pathway at the level of PI3K under BCP condition. However, A3 adenosine receptor has been shown to stimulate the activation of PI3K/Akt pathway, leading to the reduction of basal levels of ERK1/2 which, in turn, inhibits cell proliferation (Merighi et al., 2006). On the other hand, MEK inhibitor U0126 dramatically inhibited Akt phosphorylation (Niba et al., 2013; Sun et al., 2008; Yang et al., 2011). But from biochemical experiments and in silico analysis, the pAkt level was remarkably up-regulated by MEK inhibitor in the BRAF mutation case (Won et al., 2012). Also, U0126 inhibited the activation of both ERK1/2 and Akt (Figs. 8A, B, C), which could mean that the activation of ERK1/2 is dependent on MEK, and the continuous activation of PI3K/Akt required the activity of Raf/MEK/ERK pathway at the level of MEK. Our findings are in agreement with previous results that expression of pAkt and pERK1/2 in flouxetine-treated neural stem cells were blocked by both PI3K inhibitor (LY294002) and MEK inhibitor (PD98059) (Huang et al., 2013) and that PI3K siRNA or wortmannin reduced ERK phosphorylation. MEK inhibitor (PD98059) also prevented the phosphorylation of Akt in MSTO-211H cells (Niba et al., 2013). Thus, this observation added a new element to the phenomenon of crosstalk between PI3K/Akt and Raf/MEK/ERK. Whether cross-inhibition or cross-activation would occur between these pathways is dependent on different conditions.
But we believe that cross-activation plays a key role in modulating pain information (Guan et al., 2010; Yu et al., 2012).

In conclusion, this study has demonstrated that activation of spinal chemokine receptor CXCR3 mediates BCP through Akt and ERK pathways in rats. Moreover, these data provide the evidence of crosstalk between PI3K/Akt and Raf/MEK/ERK pathways at the level of PI3K and MEK as an underlying mechanism of interaction under the BCP condition. Our future research will elucidate the mechanism of this cross-activation.

Conflicts of interest statement

The authors declare there are no conflicts of interests.

Acknowledgments

We are grateful to Amgen for kindly providing AMG487 to us. This study was supported by National Natural Science Foundation of China (Nos. 81371250; 81400832; 81400917).

References
