Up-regulation of PKCγ subunits of rACC neurons contributes to the development of pain sensitivity in bone cancer rats

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Abstract

To explore the role of PKCγ subunits of rostral anterior cingulate cortex (rACC) neurons in the development of bone cancer pain in rats. Healthy female Sprague-Dawley rats were randomly divided into five groups: blank control group (naive group), sham operation group (sham group), bone cancer pain group (BCP group), BCP plus empty lentiviral vector group (vehicle group) and BCP plus PKCγ/shRNA recombinant lentiviral vector group (PKCγ group). The BCP group, vehicle group and PKCγ group received a 10 μl intra-tibial injection of MADB-106 rat mammary carcinoma cell suspension (4.6×10 8 cell/ml). In comparison, the sham group received a 10 μl intra-tibial injection of saline. The mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) were assessed on pre-operation day 0 (baseline) and days 3, 7, 14 and 21 after intra-tibial injection, respectively. To downregulate the PKCγ subunits of rACC neurons, the PKCγ group received a 10 μl bilateral rACC injection of shRNA/PKCγ recombinant lentivirus (1.25×10 9 TU/ml) on the day 7 after intra-tibial injection, whereas the vehicle group received an injection of the same dose of empty lentiviral vector. Western blotting, immunohistochemical and immunofluorescence analysis were performed to detect the different expression of PKCγ subunits in rACC neurons among these groups on postoperative days 7 or 21. No significant difference in the baseline of MWT and TWL was found among these five groups ($P > 0.05$). However, compared with the naive group and sham group, the rats with bone cancer (BCP group, vehicle group and PKCγ group) demonstrated marked mechanical allodynia and thermal hyperalgesia that was evoked starting on postoperative day 7 following intra-tibial injection of carcinoma cells ($P < 0.05$). Meanwhile, the western blotting analysis also confirmed that the expression of PKCγ in rACC neurons was significantly increased in the BCP model groups ($P < 0.05$). However, from postoperative days 14-21, the injection of shRNA/PKCγ recombinant lentivirus in the PKCγ group alleviated mechanical allodynia and thermal hyperalgesia ($P < 0.05$).The present study indicates that up-regulation of PKCγ subunits of rACC neurons in bone cancer pain rats contributes to the development of bone cancer pain.

Background

The pathogenesis of bone cancer pain remains unknown, and there has been no effective treatment [1,2]. The anterior cingulate cortex (ACC) is an essential part of the cerebral cortex; in particular, the rostral ACC (rACC) is associated with pain perception and regulation [3-5]. Under the persistent action of noxious stimulation, neurons or synapses in the rACC, in terms of their structure and function, undergo long-term changes, collectively known as neuroplasticity. As a critical signalling molecule in cells, PKCγ plays a vital role in neuronal proliferation, differentiation, synapse formation, transmitter release, and long-term potentiation (LTP) of neuron excitability [6,7]. Previous studies have suggested that PKCγ is involved in the processing of peripheral pain signals and plays an essential role in the treatment of noxious stimulation in the dorsal horn of the spinal cord [6,8-10]. Do PKCγ subunits of rACC neurons play an essential role in the formation of bone cancer pain? In this study, the role of the PKCγ subunit of rACC neurons in the development of bone cancer pain was studied in a bone cancer pain (BCP) rat model. The present results show that intra-tibial injection of mammary carcinoma cells leads to mechanical allodynia

and thermal hyperalgesia as well as the up-regulation of the PKCγ subunit of rACC neurons. Notably, the silencing of the PKCγ subunit of rACC neurons via bilateral rACC injection of shRNA/PKCγ recombinant lentivirus alleviates mechanical allodynia and thermal hyperalgesia. This finding indicates that the PKCγ subunits of rACC neurons contribute to the development of bone cancer pain.

Methods

Animals and grouping

Healthy adult female SD rats, weighing 180-200 g, were provided by the Experimental Animal Center of Shandong University (Jinan, China). All animal procedures were carried out in line with the recommendation of the Principles of Laboratory [11]. The number of animals used was kept as small as possible, and animal suffering was minimized to the lowest degree according to the ethics committee of the International Association for the Study of Pain (IASP) [12]. The study was approved by the ethics committee for Animal Care and Use Committees of the Experimental Animal Center of the Second Hospital of Shandong University (Jinan, China) before the start of the experiments (Permit number: KYLL-2017 (LW) 017). All rats were maintained in the following identical conditions: a controlled temperature of 22 °C, a 12-hour light/dark cycle and *ad libitum* access to food and water. One week later, the rats were randomly divided into five groups (n=10/group): the blank control group (naive group), sham operation group (sham group), bone cancer pain model group (BCP group), BCP plus empty lentiviral vector group (vehicle group) and BCP plus PKCγ/shRNA recombinant lentiviral vector group (PKCγ group). Naive group: healthy rats without any treatment. Sham group: unilateral intra-tibial injection of normal saline. BCP model -treated groups (BCP group, vehicle group and PKCγ group): unilateral 10 μl intra-tibial injection of MADB-106 rat mammary carcinoma cells (cell density 4.6×10^8 cell/ml) (from Cancer Institute of Concord Medical University of Chinese Academy of Medical Sciences).

Preparation of MADB-106 rat mammary carcinoma cells

MADB-106 rat mammary carcinoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM); supplemented with 10% foetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin; and cultured at 37 °C in a humidified atmosphere of 5% CO_2. The cells were then passaged hebdomadally in terms of ATCC guidelines. For treatment, the cells were disengaged by scouring and then centrifuged at 900 rpm for 3 minutes. The pill was suspended in Hank's balanced salt solution. Cells in the logarithmic growth phase were selected for experiments and then used for intra-tibial injection.

Establishment of the rat BCP model

The bone cancer pain model of rats was established as previously described [4]. The rats were anaesthetized with an intraperitoneal injection of 10% chloral hydrate (300 mg/kg). Superficial incisions were made in the skin overlying the patella to expose the tibial head with minimal damage. A 23-gauge needle was inserted into the medullary cavity of the tibia, and 10 μl MADB-106 rat mammary carcinoma cell suspension (4.6×10^8 cells/ml) was slowly injected into the tibial cavity through the needle. The

injection site was closed with bone wax immediately after the syringe was removed to prevent the cell suspension from leaking out. The wound was sutured to avoid leaving a dead space and was disinfected with iodophors to prevent infection. The initial treatment of the vehicle group and PKCγ group was the same as that of the BCP group. In the sham group, unilateral intra-tibial injection of normal saline was used. No experimental procedures were performed in the naive group.

Construction of lentiviral vectors expressing PKCγ/shRNA

The lentiviral vectors expressing PKCγ/shRNA (LV-PKCγ/shRNA recombinant lentivirus) were packaged using the PKCγ interference sequence TGAATGTGCACCGACGCTG, the plasmid pLVTHM (Shanghai Gene Chem Gene Co., Ltd, Shanghai, China) and the lentiviral packaging plasmid. The PKCγ interference sequence was cloned into the lentiviral vector pLVTHM-GFP (Shanghai Gene Chem Gene Co., Ltd, Shanghai, China). Moreover, the lentiviral vector pLVTHM-GFP and packaged plasmids were co-transfected into 293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The final titre of PKCγ/shRNA recombinant lentivirus was 1.25×10^9 TU/ml.

PKCγ/shRNA recombinant lentivirus administration into the rACC

7 days After treated with intra-tibial injection, rats were implanted with stainless steel cannulas for intra-rACC drug infusions. For the microinjection studies, rats were anaesthetized with intraperitoneal chloral hydrate (300 mg/kg) and were firmly fastened into a brain stereotactic apparatus with the lambda and bregma at the horizontal level. A 30-gauge stainless steel cannula with a 33-gauge stainless steel stylet plug was bilaterally implanted 0.5 mm above the rACC injection site [2.6 mm anterior to bregma, 0.6 mm lateral from the midline, and 2.5 mm beneath the surface of the skull] in-line with the atlas of Paxinos and Watson [13]. A 10 μl Hamilton syringe with PE-10 tubing was linked to the cannula that extended 0.5 mm over the tip of the guide cannula. The cannula was fixed with denture cement, and all surgical procedures were performed under sterile conditions. Before and at the end of the experiment, the brains were sectioned for cresyl violet staining to verify the cannula position and injection site. The rats were monitored daily after surgery for signs of motor deficiency or infection. In the PKCγ group, 10 μl shRNA/PKCγ recombinant lentivirus (1.25×10^9 TU/ml) was injected into the bilateral rACC over 5 minutes. In the vehicle group, the same dose of empty recombinant lentivirus was injected. No experimental procedures were performed in the naive, sham and BCP groups.

Assessments of Pain-related behaviours

Before the baseline trial, The rats had a natural appearance and level of activity and ate regularly and were acclimated to the testing environment. The experimental rats were placed in a plastic cage (10×10×15 cm) with a Plantar von-Frey TM Dynamic Plantar Stimulator (Stoelting, USA) at the bottom, and the cage was placed on a wire mesh plate for the experimental operation and observation. After 15 minutes of acclimation, mechanical allodynia was measured as the hind paw withdrawal response to von Frey hair stimulation according to the up-down method. An ascending series of von Frey hairs with logarithmically incremental stiffness (1.0, 2.0, 4.0, 6.0, 8.0, 15.0 and 20.0 g) were applied perpendicularly

to the mid-plantar surface (avoiding the less sensitive tori) of each hind paw. The stimulus lasted for ten seconds, and the interval between each measurement was 10 minutes. The minimum stimulus that caused rat paw withdrawal was defined as the MWT.

Rats were placed under a cage on a glass plate that was elevated to allow manoeuvring of a radiant heat source from below. Controlled radiant heat stimuli were applied to the plantar surface of the hind-paw (BME-410A bolometer, Institute of Biomedical Engineering, Chinese Academy of Medical Sciences). The time from the onset of radiant heat application to the withdrawal of the hind paw was defined as the TWL. The glass plate was kept dry and clean during the measurement. Both hind paws were tested independently with a 5 minutes interval between trials so that pain could be restored to normal. A blocking time of 20 seconds was imposed on the stimulus duration to prevent tissue damage. The paw of each rat was tested three times, and the average value was taken. Both MWT and TWL were commonly used as the index to assess mechanical allodynia and thermal hyperalgesia and were measured during 3 weeks: pre-operative day 0 (baseline) and on days 3, 7, 14 and 21 following the intra-tibial injection.

Western blotting analysis

After the behavioural tests, rats in all groups were anaesthetized with an overdose of chloral hydrate before the perfusion of 100 ml of phosphate-buffered saline (PBS) through the ascending aorta and then rapidly sacrificed by decapitation. On days 7 or 21 after intra-tibial injection, the rACC tissues were immediately removed and frozen in liquid nitrogen, washed with cold PBS containing 2 mM EDTA and lysed with denaturing SDS-PAGE sample buffer using standard methods. Protein lysates were separated and transferred onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA). The membranes were blocked and then incubated with rabbit polyclonal anti-PKCγ antibody (dilution at 1:300; Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C overnight. After the membranes were washed, they were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) antibody (dilution at 1:5,000; Santa Cruz) at room temperature for 2 hours. Western blotting was performed to detect the expression of PKCγ in rACC tissues.

Immunohistochemical analysis

To further verify the expression level of PKCγ in neurons after lentiviral vector injection, immunohistochemical analysis was performed. On the postoperative day 21, rats were deeply anaesthetized with an overdose of chloral hydrate and perfused transcardially with 100 ml of PBS, followed by 250 ml of ice-cold 4% paraformaldehyde. The rACC sections were removed and fixed at 4 °C for 5 hours and then transferred to 30% sucrose/PBS for 24 hours. rACC sections (20 mm) were incubated for 2 hours at room temperature in a blocking solution (3% normal goat serum) and then incubated for 48 hours at 4 ℃ with rabbit polyclonal anti-PKCγ antibody (dilution of 1:500; Santa Cruz). Following incubation, the tissue sections were washed and incubated for 3 hours at room temperature in the secondary antibody solution HRP-conjugated goat anti-rabbit IgG antibody (dilution of 1:2,000; Santa

Cruz). The rACC sections were analysed using an LSM confocal imaging system (Carl Zeiss Japan, Tokyo, Japan).

Immunofluorescence analysis

On the postoperative day 21, rats were deeply anaesthetized with an overdose of chloral hydrate and perfused transcardially with 100 ml of PBS, followed by 250 ml of ice-cold 4% paraformaldehyde. The rACC tissues were subsequently cut into7 μm sections on a cryostat, and all sections were prefixed with acetone, blocked with goat serum at 37 ℃ and incubated overnight at 4 ℃. Following this, the tissue sections were washed and incubated for 2 hours in a dark room. The stained sections were scanned and images were subsequently captured using an inverted fluorescent microscope (Nikon Corporation, Tokyo, Japan).

Statistical analysis

Data are shown as the mean ± standard deviation (SD) and were analysed using SPSS 23.0 software (IBM SPSS, Armonk, NY, USA). Data from the pain-related behavioural assessments were analysed using a two-way repeated ANOVA (Analysis of Variance) to detect the difference among the groups; whereas One-way ANOVA followed by Student-Newman-Keuls (SNK) post hoc test was used to compare MWT and TWL at different time points and the differences in the numbers of PKCγ-immune-positive cells and protein expression levels of PKCγ among the groups. A P value of less than 0.05 (two-tailed) was considered to indicate a statistically significant difference.

Results

Rats with bone cancer exhibit increased mechanical allodynia and thermal hyperalgesia

Comparison of the baseline in MWT and TWL, no significant differences in pain-related behavioural tests were demonstrated among these groups ($P > 0.05$). Furthermore, No significant differences in behavioural tests were measured among the naive and sham groups in the period of time examined ($P > 0.05$). However, following the intra-tibial injection, rats with bone cancer (the groups BCP, vehicle and PKCγ) demonstrated significantly decreased MWT and TWL on the postoperative day 7 compared with the naive and sham groups ($P < 0.05$). Moreover, the reduction of MWT and TWL in groups BCP and vehicle was not suspended until postoperative day 21 (Figure 1, 2). This suggests that the rats with the intra-tibial injection of mammary carcinoma cells consistently develop mechanical allodynia and thermal hyperalgesia.

Intra-tibial injection of mammary carcinoma cells specifically increases PKCγ protein expression levels in the rACC

Rats were sacrificed and then the rACC tissues were removed. The PKCγ protein expression in rACC neurons following the development of mechanical pain and thermal hyperalgesia was assessed on the day 7 after intra-tibial injection. As outlined in graphs, the expressions of PKCγ protein among the groups

were examined by western blotting. Compared with naive and sham groups, the expression levels of PKCγ protein in the rats with bone cancer were significantly increased on the postoperative day 7 ($P < 0.05$) (Figure 3). Intra-tibial injection of mammary carcinoma cells in the BCP group resulted in a 84.0 % increment in PKCγ protein expression levels, as compared with the naive groups ($P < 0.05$) (Figure 3).

Bilateral intra-rACC injection of LV-PKCγ/shRNA reduces mechanical allodynia and thermal hyperalgesia in a BCP model

The downregulation of PKCγ gene in the rACC was investigated whether it could moderate bone cancer pain. Therefore, pain-related behaviours of the rats were observed for 14 days following the injection of the lentiviral vectors. The rats exhibited a natural appearance and level of activity and ate regularly following bilateral intra-rACC injection of the LV-PKCγ/shRNA. Remarkably, all rats with bone cancer demonstrated increased mechanical allodynia and thermal hyperalgesia at day 7 following mammary carcinoma cell inoculation; whereas bilateral intra-rACC injection of LV-PKCγ/shRNA improved tactile allodynia and thermal hyperalgesia in the PKCγ group from postoperative days 14-21 ($P < 0.05$). On the contrary, intra-rACC administration of empty lentiviral vectors had no notable effect on tactile allodynia or thermal hyperalgesia in the rats with bone cancer ($P > 0.05$) (Figure1, 2). This phenomenon indicates that mechanical allodynia and thermal hyperalgesia of rats in PKCγ group alleviate.

Bilateral intra-rACC injection of LV-PKCγ/shRNA decreases PKCγ protein expression levels in the rACC

The expression level of PKCγ protein in rACC neurons was evaluated following bilateral intra-rACC injection of LV-shRNA/PKCγ. Hence, rats were killed and then rACC tissues were removed on day 21 after intra-tibial injection. As shown in the graphs, the expressions of PKCγ protein among the groups were examined by western blotting. Compared with the BCP and vehicle groups, the expression level of PKCγ protein in the rats with LV-PKCγ/shRNA injection was significantly decreased ($P < 0.05$) (Figure 4). Consistent with the immunohistochemical and immunofluorescence results, intra-rACC injection of LV-PKCγ/shRNA resulted in a 12.8% reduction in PKCγ protein expression levels, as compared with the BCP group ($P < 0.05$) (Figure 4, 5).

Accurate location of PKCγ low expression within the rACC following intra-rACC administration of LV-PKCγ/shRNA

LV-PKCγ/shRNA was investigated whether it decreases the PKCγ protein expression within the bilateral rACC areas. bilateral rACC tissues were removed on day 21 after intra-tibial injection. PKCγ-positive neurons in the rACC neurons were assessed by semi-quantitative analysis of immunohistochemical staining and immunofluorescence staining. The immunoreactivity of PKCγ protein was found predominantly in superficial laminae of the rACC. Furthermore, the number of PKCγ-positive neurons in rats with intra-rACC administration was lower than that in BCP and vehicle groups ($P < 0.05$) (Figure 5). In the BCP, vehicle and PKCγ groups, the number of positive neurons of PKCγ within the bilateral rACC was 218.5, 213.5 and 181.5, respectively (Figure 5). In Immunofluorescence staining, photomicrographs demonstrated protein expression of PKCγ in the rACC neurons at day 14 following intra-rACC injection of

LV-PKCγ/shRNA or empty LV. The image illustrates neurons that project to the PKCγ-IP cells located in the rACC. Note that GFP+ cells are located bilaterally, which demonstrates that PKCγ proteins in neurons existed in ipsilateral as well as the contralateral cortex (Figure 6 F). Immunofluorescence graphs and quantification of the PKCγ-IP cells demonstrated that intra-rACC transduction of LV-PKCγ/shRNA significantly decreased PKCγ protein expression levels in the rACC neurons, as compared with the BCP or Vehicle group ($P < 0.05$) (Figure 6). The results not only confirmed that rACC-administered LV-PKCγ/shRNA infection decreased the PKCγ protein expression but also suggesting that lentivirus microinjection was accurately positioned in rACC area.

Discussion

The present study found that the mechanical allodynia and thermal hyperalgesia developed following intra-tibial injection of mammary carcinoma cells, and bilateral rACC microinjection of PKCγ/shRNA recombinant lentivirus alleviated the sensitization of mechanical and thermal pain. The results indicate that up-regulation of PKC subunit of rACC neurons with bone cancer pain rats contributes to the development of bone cancer pain.

Bone cancer pain is one of the symptoms in terminal cancer patients, which has been described as a deep, burning-like chronic pain and has intense inflammatory and neuropathic components [1]. So far, although there are treatments such as opioid, diphosphonate, radiotherapy, chemotherapy and surgery for relieving cancer pain, it has been reported that many cancer patients have inadequate and undermanaged pain control [14,15]. Therefore, in order to solve this problem, the pathophysiological causes of bone cancer pain need to be further concerned. A large number of cancer pain animal models have been performed to examine the mechanisms that underlie tumour-evoked pain and hyperalgesia. Using models in which mammary carcinoma cells are implanted into the tibial bone, researchers have begun to clarify the pathophysiological processes by which cancer produces pain [16]. In the present study, we discovered that the hind paw mechanical withdrawal threshold and thermal withdrawal latency gradually declined by the infusions of mammary carcinoma cells in bone from postoperative days 7-21. This suggests that bone cancer caused both induction and maintenance of the cancer-induced persistent nociception, which was pathologically and physiologically meet to the intended clinical situation.

The present study showed that intra-rACC injection of LV-PKCγ/shRNA alleviates mechanical allodynia and heat hyperalgesia in bone cancer rats. This suggests that rACC neurons play an essential role in the development of bone cancer pain. Some studies have also shown that the excitability of neurons in the supraspinal cord, such as the ACC, and the enhancement of synaptic transmission play essential roles in the development of chronic pain [17]. The ACC is a considerably large structure of the limbic system that reflects affective and motivational aspects of pain. ACC, especially the rACC, transmits and regulates the nociceptive information [18]. Imaging studies have also reported an increased ACC activity under noxious stimulation and chronic pain conditions [19]. Furthermore, we already have known that the efferent nerves from the ACC area innervate the grey matter around the midbrain aqueduct and the involvement of the rostral loop [20]. Studies have also demonstrated that spinal nociception is regulated by descending

modulation from supraspinal structures, including neurons in the ACC and insular cortex [21]. These findings suggested that neuronal activity in the ACC may affect spinal nociception through descending modulatory systems. All of these results further demonstrate that enhanced nerve excitability of rACC region play a vital role in both the induction and maintenance of the bone cancer-induced mechanical and heat hyperalgesia.

According to the structural and functional characteristics of different subtypes, PKCs can be divided into conventional (α, β □/□, γ), novel (δ, ε, η, θ) and atypical (ζ, λ, ι, μ) forms [22]. The PKC family has a wide range of functions. When the cell membrane receptor coupled to phospholipase C is activated, DAG is produced, followed by activation of PKC, thus exerting a biological effect. Previous studies demonstrated that PKCs might be necessary in the processing of nociceptive information in chronic hyperalgesia. PKCγ activates the protein kinase system in neurons, thereby changes the phosphorylation state of the substrate and considers to be a central molecular integrator of nociceptive signalling. In particular, increasing evidence suggests that PKCγ is highly involved in central sensitization [23] as well as synaptic remodelling of neurons and long-term potentiation. Many studies have also indicated that some enhanced processes of reactivities, such as hyperalgesia, may be related to central sensitization. The molecular mechanisms underlying PKCγ-mediated pain hypersensitivity have been examined in recent studies. PKCγ in the trigeminal nucleus caudalis participated in the pathogenesis of chronic migraine [24]. Spinal protein kinase C was also involved in the induction and maintenance of both persistent spontaneous inching reflex and contralateral heat hyperalgesia in rats [25]. Huang also found that CCR5/PKCγ signalling pathway may contribute to the maintenance of BCP in rats [8]. Nevertheless, findings in rACC regions related to PKCγ-mediated pain have not been well established. In this present study, The PKCγ expression in the rACC was measured and found that BCP surgery markedly upregulated the expression of this protein, suggesting that increased PKCγ expression participated in inflammatory and neuropathic pain formation. Subsequently, we investigated whether PKCγ in the rACC played a critical role in the development of BCP in rats. The immunohistochemical staining, immunofluorescence staining, as well as western blotting also showed that the number of PKCγ immunoreactive neurons in rACC was significantly decreased following rats suffered from the injection of LV-PKCγ/shRNA. Therefore, these results suggest that BCP activated PKCγ in rACC neurons. We also found that the inhibition of PKCγ by LV-PKCγ/shRNA injection increased the hind paw TWL and MWT. However, the activation of PKCγ by BCP establishment reduced the paw TWL and MWT. It indicates that when PKCγ is silenced, or its function is inhibited, the hyperexcitability of rACC neurons can be no longer induced. Malmberg found that acute pain in PKCγ knockout mice was not affected significantly, while chronic pain was weakened, which is consistent with the present study [10]. The antiallodynic effects of PKCγ antagonists have also been reported in other animal models of chronic pain [8].

As universally used gene delivery systems, recombinant lentiviral vectors is capable of infecting the intermitotic cells and mitotic cells. Once a virus binds to a cell, its genes can be incorporated into the genomes of cells as a stable component of cytogenetics that can be passed on to its offspring during cell division. Meanwhile, the pathogenic genes of lentivirus have been deleted, so recombinant lentiviral vectors are used to express small interfering RNA (short interference RNA, siRNA) [26]. One way to deliver

siRNA in vivo is to clone siRNA sequences into plasmid vectors as shRNA (short hairpin RNA). Viral delivery of shRNA expression cassettes allows efficient transduction in brain tissues. In our previous study, We successfully transfected LV-GluN2B/shRNA in rACC neurons and relieved the debilitating pain of bone cancer by selectively decreasing GluN2B expression levels in the rACC [27]. To further explore whether the PKC subunit of rACC neurons play a significant role in bone cancer pain, we injected PKCγ/shRNA recombinant lentiviral vectors into the bilateral rACC to silence the PKCγ subunits of rACC neurons after establish the BCP model. Following intra-rACC administration of LV-PKCγ/shRNA, western blotting, immunofluorescence as well as immunohistochemical staining demonstrated a marked reduction in protein expression levels of PKCγ in the rACC. Recombinant lentiviral vectors can play the role of down-regulating the expression of target gene safely and persistently. Compared with Protein inhibitor, lentiviral vectors have the advantages of robust targeting and high specificity and are not limited by the half-life of the drug. The lentiviral vector can maintain a stable “blood concentration” by integrating the host gene into the target gene, which provides an experimental basis for the specific long-term down-regulation of PKCγ expression in rACC. In our study, we found that the analgesic effect did not diminish until postoperative day 21, which may be related to RNA interference inhibiting the function of the PKCγ gene and long-term impact on the expression of PKCγ protein. This experiment also showed hyperalgesia on postoperative day 7, and the level of pain was not notably reduced until postoperative day 14. This phenomenon was since that a week was needed for the target gene of the recombinant lentiviral vector to integrate into the target cell genome and to be regularly expressed. This result further supports the hypothesis that RNAi mediated gene silencing is a potential therapeutic tool for the treatment of various nervous diseases. In our study, the pain sensitivity was significantly reduced after administration of the PKCγ/shRNA recombinant lentivirus. It proved that up-regulation of the PKCγ does play a role in the development of bone cancer pain, whereas the degree of pain sensitivity in rats did not fully return to normal. This result may be related to both the interference of our target sequence, which could only partially downregulate the expression of PKCγ and other mechanisms of bone cancer pain. Therefore, a follow-up study should exclude more affecting factors and obtain more accurate results, and we need to screen out more representative genes that affect the development of bone cancer pain.

Conclusions

In summary, this is the first study to show that mechanical allodynia and thermal hyperalgesia develop following intra-tibial injection of mammary carcinoma cells injection accompanied by enhancement of PKCγ expression in the rACC region whereas the silencing of PKCγ protein in rACC neurons by bilateral rACC injection of shRNA/PKC recombinant lentivirus reversed mechanical and thermal hyperalgesia. These results are significant and suggest that PKCγ subunits in rACC neurons contribute to the development of bone cancer pain.

List Of Abbreviations

ACC: Anterior cingulate cortex, rACC: Rostral Anterior cingulate cortex, LTP: Long-term potentiation, MWT: Mechanical withdrawal threshold, TWL: Thermal withdrawal latency, LV: lentiviral vector, IP: immuno-positive, siRNA: short interference RNA, shRNA: short hairpin RNA.

Declarations

Ethics approval and consent to participate

All experimental procedures and animal handling were performed according to both the Guiding Principles for the Care and Use of Laboratory Animals. The protocol was approved by the committee on the Ethics of Animal Experiments of the Shandong University.

Consent for publication

Not applicable

Availability of data and material

The datasets generated and analyzed during the current study are not publicly available due to copyright issues, but are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Meng She, Hao Feng, Gongming Wang, and Kailin Li submitted Ethics application, participated in provision of teaching sessions, collected data and prepared manuscript. Zequn Feng, Ruoyi Wang and Guanghui Cheng carried out compilation of data and performed statistical analysis. Hao Feng and Xiaohui Li designed study, assisted with ethics application, participated in design and delivery of teaching sessions and supervised preparation of manuscript writing. All authors read and approved the final manuscript.

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Authors' information

Not applicable.

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Tables

Due to technical limitations, tables are only available as a download in the supplemental files section.

Figures

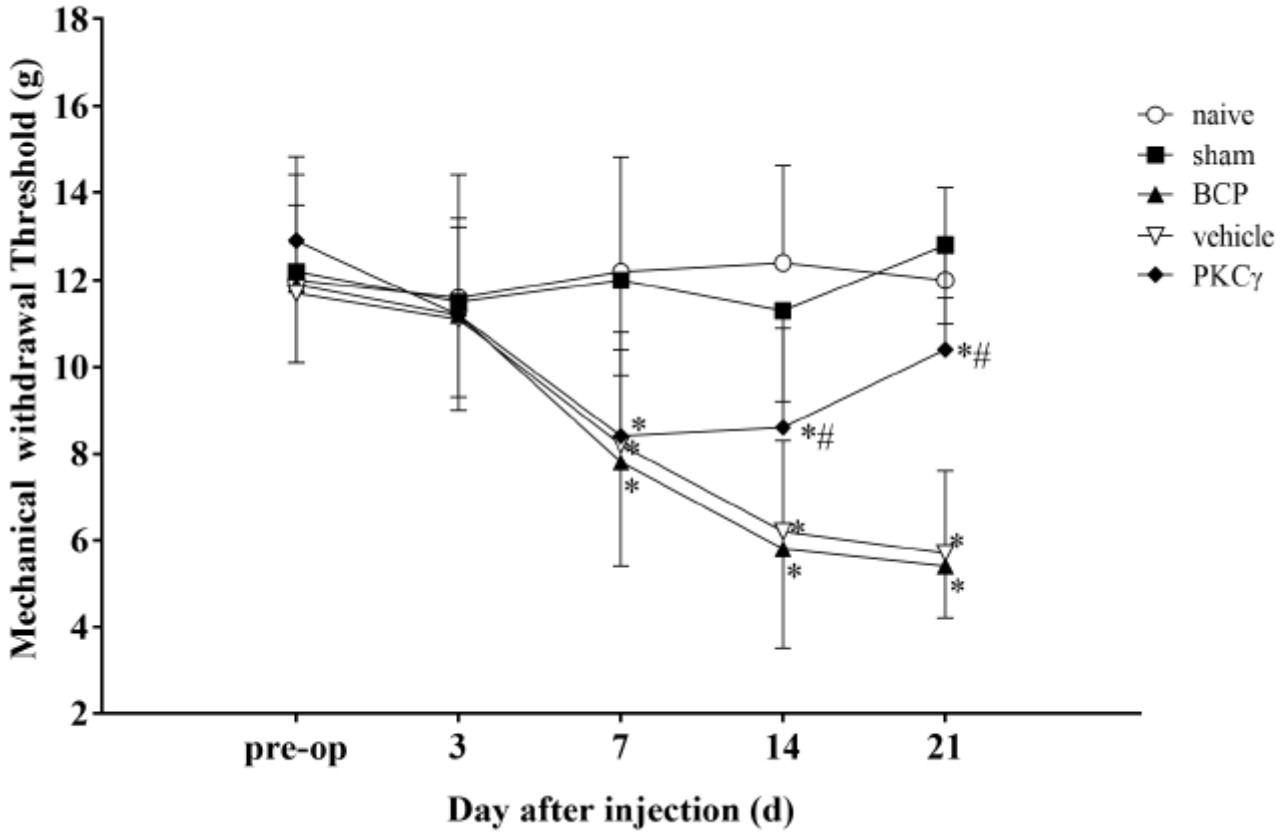

Figure 1

Mechanical allodynia after intra-tibial injection and rACC microinjection of lentiviral vectors. The rats subjected to the bone cancer showed a significant reduction in MWT ($P < 0.05$). The rats treated with LV-PKCγ/shRNA showed a significant increase in MWT compared with the groups BCP and vehicle ($P < 0.05$). The MWT was measured pre-operation day 0 (baseline) and on days 3, 7, 14 and 21 after intra-tibial injection. Furthermore, “pre-op” is the time point of the baseline of MWT before rats with intra-tibial injection, and “3, 7, 14 and 21” is the time point of 3, 7, 14 and 21 days after intra-tibial injection. Significance was defined as *$P < 0.05$, compared with the naive or sham group; #$P < 0.05$, compared with the BCP or vehicle group. Data are presented as mean±SD for at least 6 rats per group.

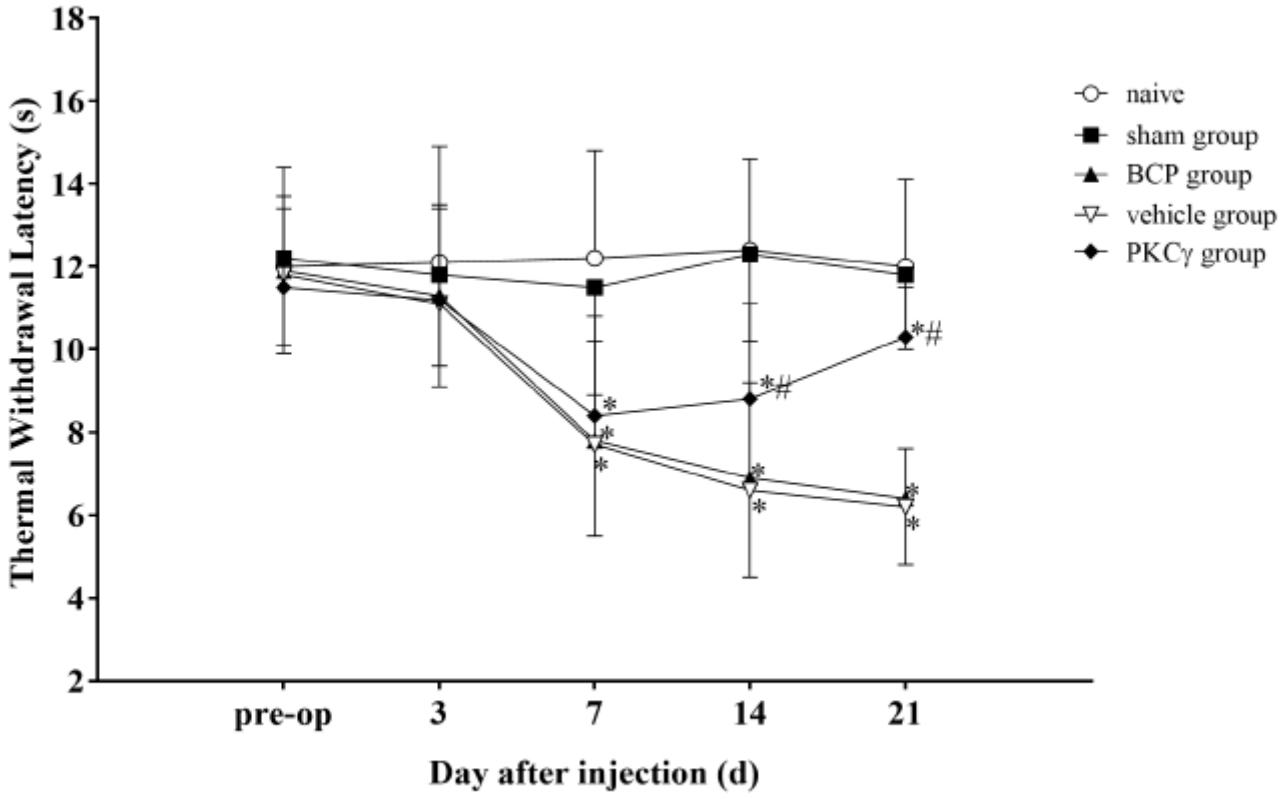

Figure 2

Thermal hyperalgesia after intra-tibial injection and rACC microinjection of lentiviral vectors. The rats subjected to the bone cancer showed a significant reduction in TWL ($P < 0.05$). The rats treated with LV-PKCγ/shRNA showed a significant increase in TWL compared with groups BCP and vehicle ($P < 0.05$). The TWL was measured pre-operation day 0 (baseline) and on days 3, 7, 14 and 21 after intra-tibial injection. “pre-op” is the time point of the baseline of TWL before intra-tibial injection in rats, “3,7,14 and 21” is the time point of 3, 7, 14 and 21 days after intra-tibial injection. Significance was defined as *$P < 0.05$, compared with the naive or sham group; #$P < 0.05$, compared with the BCP or vehicle group. Data are presented as mean±SD for at least 6 rats per group.

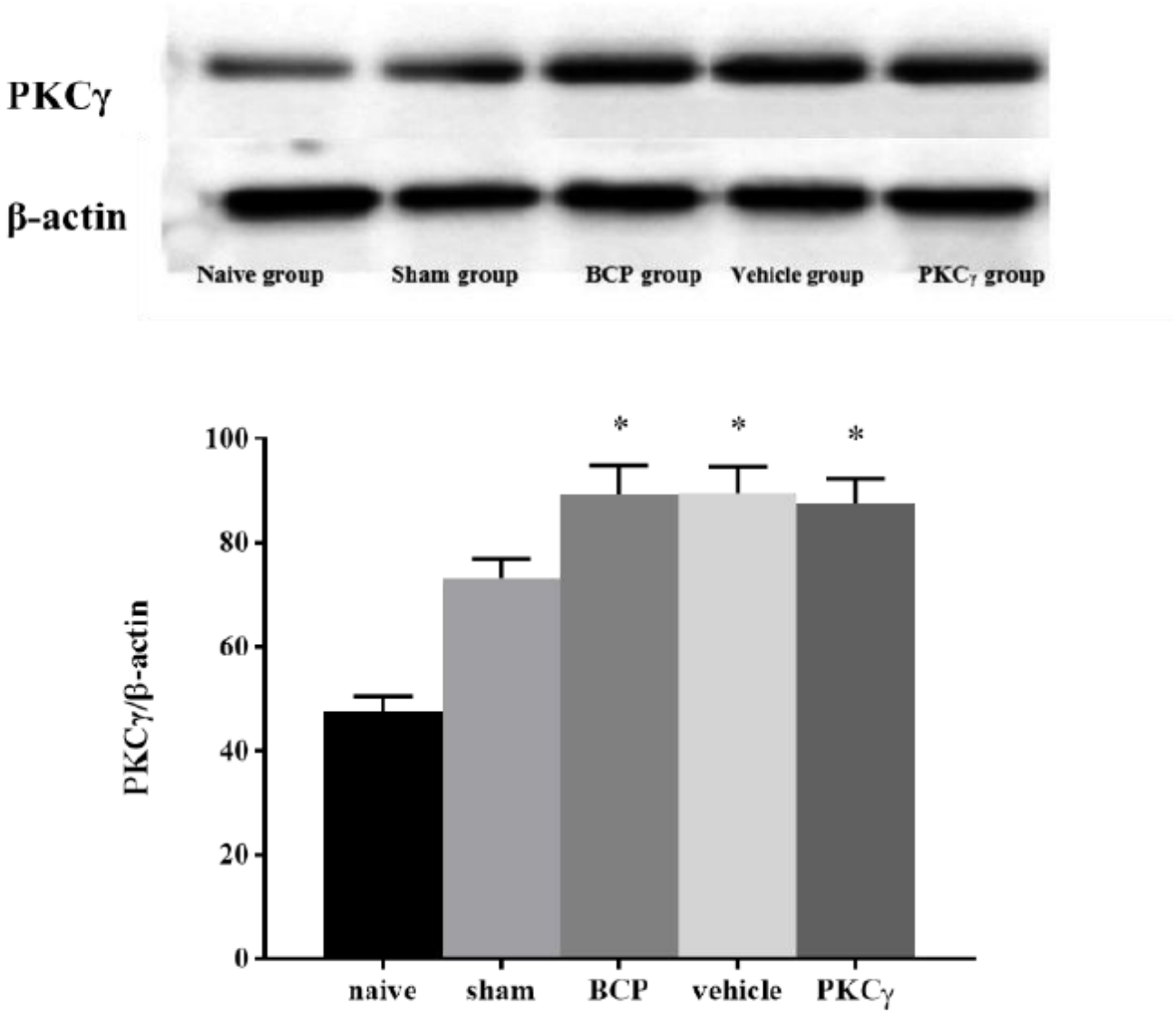

Figure 3

PKCγ protein expression in the rACC neurons was assessed 7 days after intra-tibial injection. Western blot analysis showed that PKCγ protein was upregulated in the rats with bone cancer (BCP group, vehicle group and PKCγ group), 7 days after intra-tibial injection ($P < 0.05$). Lane 1, naive group; lane 2, sham group; lane 3, BCP group; lane 4, vehicle group; lane 5, PKCγ group. Significance was defined as $*P < 0.05$, compared with naive or sham group. Data are presented as mean±SD for 3 rats per group.

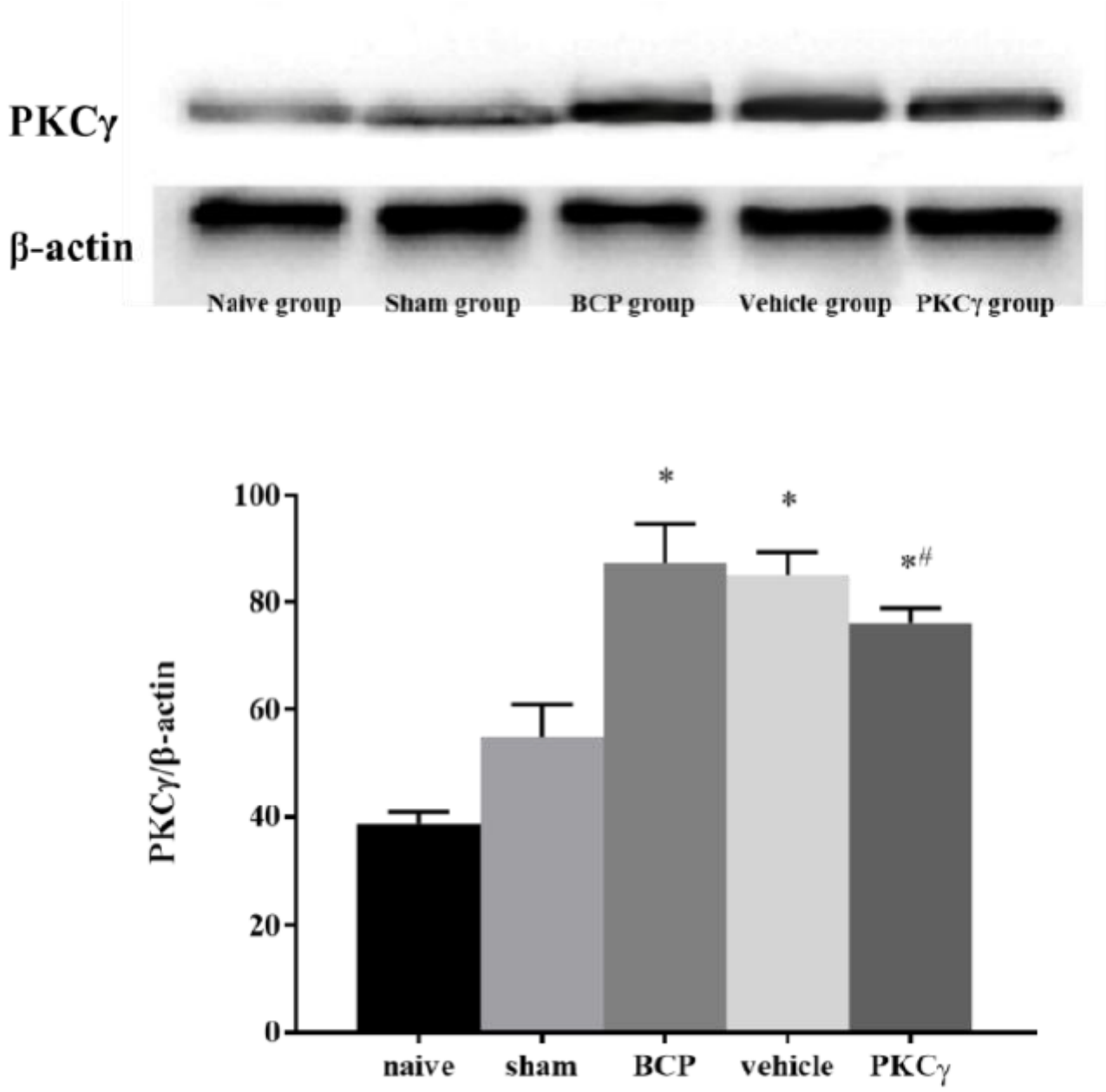

Figure 4

PKCγ protein expression in the rACC neurons was assessed 21 days after intra-tibial injection. Western blot analysis showed that PKCγ protein was upregulated in the rats with bone cancer (BCP group, vehicle group and PKCγ group), 21 days after intra-tibial injection ($P < 0.05$), however, PKCγ protein was downregulated by LV-PKCγ/shRNA, 21 days after intra-tibial injection compared with the naive and sham groups ($P < 0.05$). Lane 1, naive group; lane 2, sham group; lane 3, BCP group; lane 4, vehicle group; and lane 5, PKCγ group. Significance was defined as *$P < 0.05$ compared with the naive or sham group; #$P < 0.05$, compared with the BCP or vehicle group. Data are presented as mean±SD for 3 rats per group.

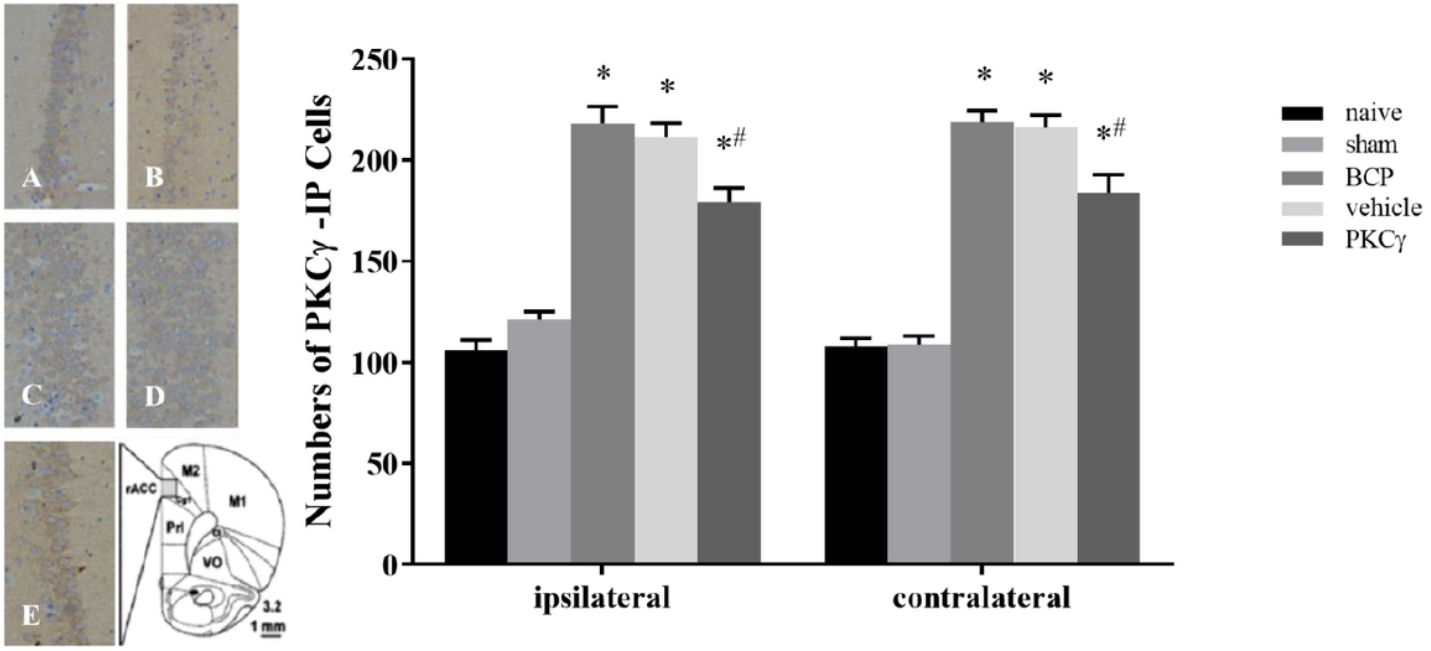

Figure 5

PKCγ immunohistochemistry staining in the rACC neurons after bilateral rACC microinjection. Immunoreactivity of PKCγ was found predominantly in the superficial laminae of the rACC sections. PKCγ immunoreactivity was increased in the rat with intra-tibial injection (C, D and E) when compared with the naive or sham groups (A or B) ($P < 0.05$). PKCγ immunoreactivity was decreased significantly in the PKCγ group (E) compared with group BCP or vehicle (C or D) ($P < 0.05$). Rats treated with LV-PKCγ/shRNA showed a more significant decrease in the number of cells with active PKCγ (staining intensity) than the vehicle and BCP groups (C and D) ($P < 0.05$). Tissues were collected 21 days after rat with intra-tibial injection. Immunohistochemistry graph A. naive group B. sham group C. BCP group D. vehicle group E. PKCγ group (A, B, C, D and E ×200). Significance was defined as *$P < 0.05$, compared with the naive or sham group; #$P < 0.05$, compared with the BCP or vehicle group. Data are presented as mean±SD for 3 rats per group.

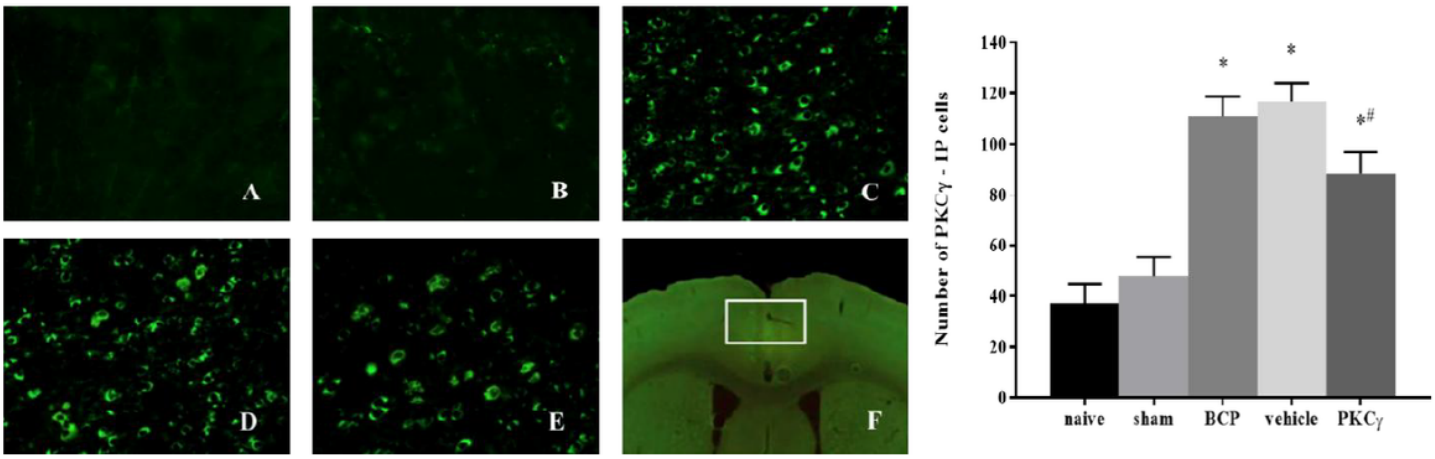

Figure 6

Immunofluorescence staining images of PKCγ protein in rACC neurons 7 days after transduction with lentiviral vectors. Photomicrographs demonstrating protein expression of PKCγ in the rACC neurons at day 7 following intra rACC injection of LV PKCγ/shRNA or empty LV. (A, B, C, D and E×200). Image illustrates neurons that project to the PKCγ-IP cells located in the rACC. Note that GFP+ cells are located bilaterally, which demonstrates that PKCγ proteins in neurons were existed in ipsilateral as well as contralateral cortex (F×20). Quantification of the PKCγ IP cells demonstrated that intra-rACC transduction of LV-PKCγ/shRNA significantly decreased PKCγ protein expression levels in the rACC neurons, as compared with the BCP or Vehicle group ($P < 0.05$). Immunofluorescence image A. naive group B. sham group C. BCP group D. vehicle group E. PKCγ group. Significance was defined as *$P < 0.05$, compared with naive or sham group; #$P < 0.05$, compared with BCP or PKCγ group. Data are presented as mean±SD for 3 rats per group.

Supplementary Files

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