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

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Abstract

Background: To explore the role of PKCγ subunits in rostral anterior cingulate cortex (rACC) neurons in bone cancer pain (BCP) development in rats.

Methods: Healthy female Sprague-Dawley rats were randomly divided into five groups: the blank control (naive), sham operation (sham), BCP, BCP plus empty lentiviral vectors (vehicle) and BCP plus PKCγ/shRNA recombinant lentiviral vectors (LV-PKCγ/shRNA) (PKCγ) groups. The BCP, vehicle and PKCγ groups were intra-tibially injected with an MADB-106 rat mammary carcinoma cell suspension (10 μl, 4.6×106 cells/ml), whereas the sham group was intra-tibially injected with saline (10 μl). The mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) were evaluated on preoperative day 0 (baseline) and days 3, 7, 14 and 21 after intra-tibial implantation. To downregulate PKCγ subunits in rACC neurons, the PKCγ group received a 10 μl bilateral rACC microinjection of shRNA/PKCγ recombinant lentivirus (1.25×109 TU/ml) on day 10 after intra-tibial implantation. The GFP expression of LV-shRNA/PKCγ was examined by microscopy. The vehicle group was injected with an equal dose of empty lentiviral vectors. Western blot, and immunohistochemical approaches were performed to assess the differential expression of PKCγ subunits in rACC neurons among these groups on postoperative days 7 or 21.

Results: The baseline MWT and TWL values did not significantly differ among these five groups ($P > 0.05$). However, compared with the naive and sham groups, the tumour-bearing rats (BCP , vehicle and PKCγ groups) demonstrated marked mechanical allodynia and thermal hyperalgesia starting on postoperative day 7 following intra-tibial implantation of carcinoma cells ($P < 0.05$). Western blot on postoperative day 7 confirmed a significant increase in PKCγ expression in rACC neurons in the tumour-bearing rats ($P < 0.05$). However, from postoperative days 14-21, the LV-shRNA/PKCγ microinjection alleviated mechanical allodynia and thermal hyperalgesia in the PKCγ group ($P < 0.05$). Furthermore, western blot, and histological examination on postoperative day 21 indicated that PKCγ expression in bilateral rACC neurons was significantly lower in the PKCγ group than in the BCP and vehicle groups ($P < 0.05$).

Conclusion: Upregulation of PKCγ subunits in rACC neurons of rats with bone cancer contributes to BCP development.

Background

The pathogenesis of bone cancer pain (BCP) remains unknown, and no effective treatment has been identified [1,2]. The anterior cingulate cortex (ACC) is an essential part of the cerebral cortex; specifically, the rostral ACC (rACC) is associated with pain perception and regulation [3–5]. Under the persistent action of noxious stimulation, the structure and function of neurons or synapses in the rACC undergo long-term changes, collectively known as neuroplasticity. As a critical signalling molecule in cells, PKCγ has a vital role in neuronal proliferation, differentiation, synapse formation, transmitter release, and long-term

potentiation (LTP) of neuronal excitability [6,7]. Previous studies have suggested that PKCγ is involved in the processing of peripheral pain signals and is essential to the response to noxious stimulation in the dorsal horn of the spinal cord [6,8–10]. Are PKCγ subunits in rACC neurons indispensable to the formation of BCP? In this study, the role of the PKCγ subunits in rACC neurons in the development of BCP was studied in a BCP rat model. Intra-tibial implantation of mammary carcinoma cells led to mechanical allodynia and thermal hyperalgesia in concert with overexpression of PKCγ subunits in rACC neurons. Notably, silencing the expression of the PKCγ subunits in rACC neurons via bilateral rACC microinjection of LV-shRNA/PKCγ alleviated mechanical allodynia and thermal hyperalgesia. This finding indicates that PKCγ subunits in rACC neurons result in the development of BCP.

Methods

Animals and grouping

Healthy adult female SD rats weighing 180–200 g were provided by the Experimental Animal Centre of Shandong University (Jinan, China). All animal procedures described were executed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals [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 committees of the animal care and use committees of the Experimental Animal Centre 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 under 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 assigned to five groups (n = 10 rats/group): the blank control (naive), sham operation (sham), tumour-bearing rats (BCP), tumour-bearing rats injected with empty lentiviral vectors group (vehicle) and tumour-bearing rats injected with LV-PKCγ/shRNA (PKCγ) groups. Rat in the groups were treated as follows. Naive group: healthy rats without any treatment. Sham group: unilateral intra-tibial injection of normal saline. tumour-bearing rats (BCP, vehicle and PKCγ groups): unilateral intra-tibial implantation of MADB–106 rat mammary carcinoma cells (10 μl, cell density = 4.6×10^6 cells/ml) (from the Cancer Institute of Concord Medical University of the Chinese Academy of Medical Sciences). The experiments were performed in blind manner.

Experimental design

The experiment was designed and administrated to study the function of PKCγ gene in tumour-bearing rats and explore its potential mechanisms with it. Before the establishment of the tumour-bearing rats, rat were acclimatized beginning on day −7, and the rats were then induced by implantation of saline or tumour cells on day 0. The intra-rACC cannulation was implanted on day 7. Lentivirus microinjection was administrated on postoperative day 10. Fluorography was conducted on day 14. Western blot analysis was performed on days 7 and 21. Immunohistochemistry was performed on day 21. The results of the

behavioural tests (MWT and TWL) on days 0, 3, 7, 14 and 21 manifested the bone cancer-induced pain and that the one at day 0 was regarded as the baseline values *(Fig. 1).*

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 containing of 5% CO_2. The cells were then passaged hebdomadally in terms of ATCC guidelines. For treatment, the cells were disengaged by scraping and were then centrifuged at 900 rpm for 3 minutes. The pallet was suspended in Hank's balanced salt solution. Cells in the logarithmic growth phase were selected for experiments and were then used for intra-tibial implantation.

Establishment of the tumour-bearing rats

The tumour-bearing rats were established as previously described [4]. Rats were anaesthetized with an intraperitoneal injection of 10% chloral hydrate (300 mg/kg). A minimal skin incision was made overlying the patella to expose the tibial head. A 23-gauge needle was drilled into the medullary cavity of the tibia, and 10 μl of the MADB–106 rat mammary carcinoma cell suspension (4.6×10^6 cells/ml) was slowly injected into the tibial cavity through the needle. To prevent leakage of the cell suspension, the injection site was closed with bone wax immediately and thoroughly irrigated with sterile saline. The wound was sutured to avoid leaving an open area and was disinfected with iodophors to prevent infection. The initial treatment of the vehicle and PKCγ groups was the same as that of the BCP group. The sham group underwent a unilateral intra-tibial implantation of normal saline alone. No experimental procedures were performed in the naive group.

Construction of lentiviral vectors expressing PKCγ/shRNA

Lentiviral vectors expressing PKCγ/shRNA (LV-PKCγ/shRNA recombinant lentivirus) were packaged using the PKCγ interference sequence TGAATGTGCACCGACGCTG, pLVTHM-GFP plasmid (Shanghai Gene Chem Gene Co., Ltd, Shanghai, China) and 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 transducing units (TU)/ml.

Administration of PKCγ/shRNA recombinant lentivirus into the rACC

Seven days after treated with intra-tibial implantation, stainless steel cannulas were implanted in the rats for intra-rACC drug infusions. For the microinjection studies, rats were anaesthetized via intraperitoneal injection of chloral hydrate (300 mg/kg) and were firmly fastened into a brain stereotactic apparatus with the lambda and bregma in the same horizontal plane. A 30-gauge stainless steel cannula with a 33-gauge stainless steel stylet plug was bilaterally implanted 0.5 mm above the rACC cannulation site [2.6 mm anterior to the bregma, 0.6 mm lateral from the midline, and 2.5 mm beneath the surface of the skull] in accordance with the atlas of Paxinos and Watson [13]. A 10 μl Hamilton syringe with PE–10 tubing was connected to the cannula and extended 0.5 mm above the tip of the guide cannula. The cannula was fixed with denture cement, and all surgical procedures were performed under sterile conditions. Prior to and at the end of the experiment, the brains were sectioned for cresyl violet staining to verify the cannula position and cannulation site. The rats were monitored daily after surgery for signs of motor deficiency or infection. Three 3 days after cannula implantation, 10 μl of shRNA/PKCγ recombinant lentivirus (1.25×10^9 TU/ml) was injected into the bilateral rACC of in the PKCγ group over 5 minutes. Rats in the vehicle group were injected with the same dose of empty recombinant lentivirus. No experimental procedures were performed in the naive, sham and BCP groups. To confirm the presence of the LV-shRNA/PKCγ in rACC neurons, GFP expression was verified by microscopy on day 4 after LV-shRNA/PKCγ administration.

Assessments of pain-related behaviours

Before the baseline trial, the rats had a natural appearance and activity level and were observed to eat regularly. The rats and were acclimated to the testing environment for 7 days. 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 experimentation 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-minute interval between trials so that the pain level could be restored to normal. To prevent tissue damage, the maximum duration of stimulation was 20 seconds. The paw of each rat was tested three times, and the values were averaged. Both the MWT and TWL are commonly used as indexes to assess mechanical allodynia and thermal hyperalgesia, respectively, and were measured here

over 3 weeks: on pre-operative day 0 (baseline) and on days 3, 7, 14 and 21 following the intra-tibial implantation.

Western blot analysis

After the behavioural tests, rats were anaesthetized with an overdose of chloral hydrate before the perfusion of 100 ml of phosphate-buffered saline (PBS) through the ascending aorta and were then rapidly sacrificed by decapitation. On days 7 and 21 after intra-tibial implantation, 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 to polyvinylidene fluoride membranes (Millipore, Bedford, MA). Membranes were blocked and were then incubated with a rabbit polyclonal anti-PKCγ antibody (diluted 1:300; Santa Cruz Biotechnology, CA) at 4 °C overnight. After the membranes were washed, they were incubated with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) antibody (diluted 1:5,000; Santa Cruz Biotechnology, CA) at room temperature for 2 hours. Western blot 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 microinjection, immunohistochemical analysis was performed. On 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. rACC sections were removed and fixed at 4 °C for 5 hours and were 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 °C with a rabbit polyclonal anti-PKCγ antibody (diluted 1:500; Santa Cruz Biotechnology, CA). Following incubation, the tissue sections were washed and incubated for 3 hours at room temperature in a solution containing the HRP-conjugated goat anti-rabbit IgG secondary antibody (diluted 1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA). rACC sections were analysed using an LSM confocal imaging system (Carl Zeiss Japan, 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 measures analysis of variance (ANOVA) to assess differences among the groups; whereas one-way ANOVA followed by the Student-Newman-Keuls (SNK) post hoc test was used to compare the 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 (two-tailed) of less than 0.05 was considered to indicate a statistically significant difference.

Results

Tumour-bearing rats exhibit increased mechanical allodynia and thermal hyperalgesia

Comparison of the baseline in MWT and TWL, values revealed no significant difference among these groups ($P > 0.05$). Furthermore, no significant differences in the behavioural tests results on postoperative day 3 were found among the naive and sham groups ($P > 0.05$). However, following the intra-tibial implantation, the progressive decrease of MWT and TWL in the tumour-bearing rats (the BCP, vehicle and PKCγ groups) began by postoperative day 7 which is significantly less than the values observed in the naive and sham groups, and persisted thereafter throughout the study. ($P < 0.05$). This result suggests that the rats with the intra-tibially implanted with mammary carcinoma cells consistently develop mechanical allodynia and thermal hyperalgesia *(Fig. 2).*

BCP increases PKCγ protein expression levels specifically in the rACC

PKCγ protein expression in rACC neurons after the development of mechanical pain and thermal hyperalgesia was assessed on day 7 after intra-tibial implantation. Compared rats in the naive and sham groups, western blot showed that rats with bone cancer (the BCP, vehicle and PKCγ groups) exhibited significantly increased PKCγ protein expression levels on the postoperative day 7. Intra-tibial implantation of mammary carcinoma cells in the BCP group resulted in an 84.0 % increase in the PKCγ protein band density compared with that in the naive group ($P < 0.05$) *(Fig. 4).*

Confirmation of LV-shRNA/PKCγ transduction in bilateral rACC neurons

The rat was then sacrificed and rACC tissues were removed on day 4 after LV-shRNA/PKCγ administration. Histological examination of brain sections revealed robust and bilateral expression of GFP in the rACC neurons. Therefore, the presence of LV-shRNA/PKCγ in bilateral rACC neurons was confirmed *(Fig. 3).*

Bilateral intra-rACC microinjection of LV-PKCγ/shRNA attenuated mechanical allodynia and thermal hyperalgesia

The rats exhibited a natural appearance and activity level and were observed to eat regularly following bilateral intra-rACC microinjection. The pain-related behaviours of the rats were observed for 11 successive days following the injection of the lentiviral vectors. Remarkably, bilateral intra-rACC microinjection of LV-PKCγ/shRNA attenuated mechanical allodynia and thermal hyperalgesia in the PKCγ group from postoperative days 14 to 21 ($P < 0.05$). In comparison, intra-rACC administration of empty lentiviral vectors did not appreciably affect on mechanical allodynia or thermal hyperalgesia in the vehicle group ($P > 0.05$). This phenomenon indicates that downregulation of PKCγ in bilateral rACC neurons results in the relieve of mechanical allodynia and thermal hyperalgesia *(Fig. 2).*

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

For this assay, rats were sacrificed and rACC tissues were removed on day 21 after intra-tibial implantation. The PKCγ protein expression level in rACC neurons was evaluated following bilateral intra-rACC microinjection of LV-shRNA/PKCγ. As shown in the western blot, bilateral intra-rACC microinjection of LV-PKCγ/shRNA significantly decreased the PKCγ protein expression level in PKCγ group compared with the BCP and vehicle groups ($P < 0.05$). Consistent with the histological examination results, intra-rACC microinjection of LV-PKCγ/shRNA resulted in a 12.8% reduction in the PKCγ protein band density compared with that in the BCP group ($P < 0.05$) *(Fig. 4).*

Bilateral intra-rACC microinjection of LV-PKCγ/shRNA decreases PKCγ immunopositive reactions in the rACC

PKCγ-immunopositive reactions (IRs) in the rACC neurons were evaluated by histological examination on day 21 after intra-tibial implantation. Immunohistochemical analysis by microscopy showed that PKCγ-IRs in the rACC were significantly increased in tumour-bearing rats (BCP, vehicle and PKCγ groups) but not in the sham or naive group. Interestingly, cancer-induced pain increased PKCγ-IRs in both sides of rACC neurons. However, rats treated with LV-PKCγ/shRNA showed a more significant decrease in the integrated optical density (IOD) of PKCγ than rats in vehicle and BCP groups ($P < 0.05$). These results indicate that the expression of PKCγ was significantly higher in tumour-bearing rats than in control groups, but pretreatment with LV-PKCγ/shRNA partially blocked the upregulation of PKCγ *(Fig. 5).*

Discussion

In this study, we have demonstrated that mechanical allodynia and thermal hyperalgesia developed following intra-tibial implantation of mammary carcinoma cells, and that bilateral rACC administration of PKCγ/shRNA recombinant lentivirus alleviated the sensitization to mechanical and thermal pain. Together, these results provide experimental support for the concept that overexpression of PKCγ subunit

of rACC excitatory neurons in tumour-bearing rats can effectively intensify the development and maintenance of BCP.

BCP, a symptom in terminal cancer patients, is described as a chronic, deep, burning pain with overlapping but features of both intense inflammatory and neuropathic components. [1]. Currently, many cancer patients have inadequate and undermanaged pain control, although treatments such as opioids, diphosphonates, radiotherapy, chemotherapy and surgery can relieve cancer pain [14,15]. Therefore, the pathophysiological causes of BCP need further investigation. Many animal models of cancer-pain, such as the model in which mammary carcinoma cells are implanted into the tibial bone, have been established to examine the mechanisms that underlie tumour-induced pain, [16]. Our present behavioural results revealed that the hind paw MWT and TWL gradually declined following the implantation of mammary carcinoma cells in the bone, suggesting that bone cancer caused both the induction and maintenance of the cancer-induced persistent nociception, a state that was pathologically and physiologically consistent with the intended clinical situation.

Some studies have also shown that the neuroexcitability in regions of the supraspinal cord, such as the ACC, and the enhancement of synaptic transmission is critically implicated in the processing of chronic pain perception and modulation [17]. The ACC, especially the rACC, transmits and regulates the nociceptive information [18]. In addition, brain imaging studies have also reported increased ACC activity under noxious stimulation and chronic pain conditions [19]. Furthermore, the efferent nerves from the ACC area innervate the grey matter around the midbrain aqueduct and involve the rostral loop [20]. Studies have also demonstrated that spinal nociception is regulated by descending modulation from supraspinal structures, including neurons in the ACC [21]. These findings suggest that neuronal activity in the rACC may affect spinal nociception through descending modulatory systems. In our present study, histological examination and the robust GFP expression indicated marked increases in PKCγ expression in the rACC not only on the side with tumour implantation but also on the contralateral side. This pattern can be attributed to the neuroanatomical foundation of neural system. The intralaminar thalamic nuclei receive a noxious stimulus from the spinal cord and send ascending projections to the bilateral rACC via the spinoreticular tract.[22]. This interpretation is supported by our and other laboratory's results showing that enhanced neuroexcitability in the bilateral ACC leads to the maintenance of mechanical hypersensitivity induced by bone tumour [23, 24]. Subsequently, mechanical allodynia and thermal hyperalgesia were observed in bone cancer rats. Collectively, these observation may thus be supportive of pivotal role for the enhanced neuroexcitability of bilateral rACC regions in the sensory component of the bone cancer-induced mechanical and thermal hyperalgesia. A pressing question that follows these observations is how the rACC neurons regulates the sensitization to mechanical and thermal pain.

The contribution of our study is the identification of a contribution of PKCγ protein in allodynia regulation. 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 [25]. Previous studies demonstrated that PKCs might be necessary for the processing of nociceptive information in chronic hyperalgesia. PKCγ activates the protein kinase system in neurons, thereby changing the phosphorylation

state of the substrate, and is considered to be a central molecular integrator of nociceptive signalling. It is worth noting that PKCγ is involved in central sensitization [26] as well as synaptic remodelling of neurons and long-term potentiation. Moreover, many studies have also indicated that some enhanced processes of reactivity, 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 participates in the pathogenesis of chronic migraine [27]. In addition, spinal protein kinase C is involved in the induction and maintenance of both the persistent spontaneous flinching reflex and contralateral heat hyperalgesia in rats [28]. Huang focused on the signalling pathway of PKCγ showing that the CCR5/PKCγ may result in the maintenance of BCP in rats [8]. However, findings in rACC regions related to PKCγ-mediated pain have not been well established. In our present study, PKCγ expression in the rACC was markedly upregulated in rats with mechanical allodynia and thermal hyperalgesia is markedly upregulated suggesting that the increased PKCγ expression participated in BCP formation. Subsequently, we further investigated whether PKCγ in the rACC fufill a critical role in the development of BCP in rats. Histological examination and western blot analysis showed that the number of PKCγ-immunoreactive neurons in the rACC was significantly decreased following the microinjection of LV-PKCγ/shRNA. In addition, the decrease in PKCγ expression after LV-PKCγ/shRNA administration alleviated hind paw mechanical allodynia and thermal hyperalgesia. Malmberg found that in PKCγ knockout mice, acute pain was not significantly affected, while chronic pain was weakened, consistent with our experiments [10]. The antiallodynic effects of PKCγ antagonists have also been reported in other animal models of chronic pain [8]. Collectively, these results demonstrate that overexpression of PKCγ subunits in the rACC neurons of tumour-bearing rats leads to the development of BCP.

Recombinant lentiviral vectors, a universally used gene delivery systems, can be used to infect both intermitotic cells and mitotic cells. After a virus binds to a cell, its genes can be incorporated into the genomes of cells as a stable cytogenetic component that can be passed on to daughter cells during cell division. In addition, the pathogenic lentiviral genes have been deleted; thus, recombinant lentiviral vectors are used to express short interfering RNA (siRNA) [29]. One approach for delivering siRNA in vivo is to clone siRNA sequences into plasmid vectors as short hairpin RNAs (shRNAs). Viral delivery of shRNA expression cassettes allows efficient transduction into brain neurons. In our previous study, we successfully transfected LV-GluN2B/shRNA into rACC neurons and relieved the debilitating pain of bone cancer by selectively decreasing GluN2B expression levels in the rACC [24]. To further explore whether the PKC subunit in rACC neurons is essential for BCP, we injected PKCγ/shRNA recombinant lentiviral vectors into the bilateral rACC to silence the PKCγ subunits in rACC neurons after establishing the tumour-bearing rat model. Following intra-rACC administration of LV-PKCγ/shRNA, western blot in concert with histological examination demonstrated a marked reduction in the protein expression levels of PKCγ in the rACC. Recombinant lentiviral vectors can safely and persistently reciprocally regulate the expression of target genes. Compared with protein inhibitors, lentiviral vectors have the advantages of robust targeting and high specificity and are not limited by the half-life of the drug. Lentiviral vectors can maintain a stable blood concentration by integrating the harboured gene into the host genome and thus provide an experimental basis for specific long-term downregulation of PKCγ expression in rACC. In the current study,

we found that the analgesic effect did not diminish until postoperative day 21, possibly because RNA interference inhibited the function of the PKCγ gene and persistently impacted the PKCγ protein expression. In this experiment, hyperalgesia was noted on postoperative day 7, and the level of pain was not appreciably reduced until postoperative day 14. This phenomenon occurred because one week was needed for the gene harboured by the recombinant lentiviral vectors 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. This finding proves that upregulation of PKCγ facilitates the development of BCP, whereas the degree of pain sensitivity in rats did not fully return to normal. This result may be related to both the interference effect of our target sequence, which only partially downregulated the expression of PKCγ, and other mechanisms of BCP. Therefore, a follow-up study should exclude additional contributing factors and obtain more accurate results. Besides, we need to screen out more representative genes that affect the development of BCP.

Conclusions

In summary, this study is the first to show that mechanical allodynia and thermal hyperalgesia develop following intra-tibial implantation of mammary carcinoma cells, accompanied by enhanced PKCγ expression in the rACC region, whereas the silencing of PKCγ protein expression in rACC neurons via bilateral rACC microinjection of shRNA/PKCγ recombinant lentivirus reversed mechanical allodynia and thermal hyperalgesia. These results are significant and suggest that PKCγ subunits in rACC neurons result in the development of BCP.

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, shRNA: Short hairpin RNA, IRs: Immunopositive reactions, IOD: Integrated optical density, siRNA: Short interfering 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 analysed 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 Supplementary Files section.

Figures

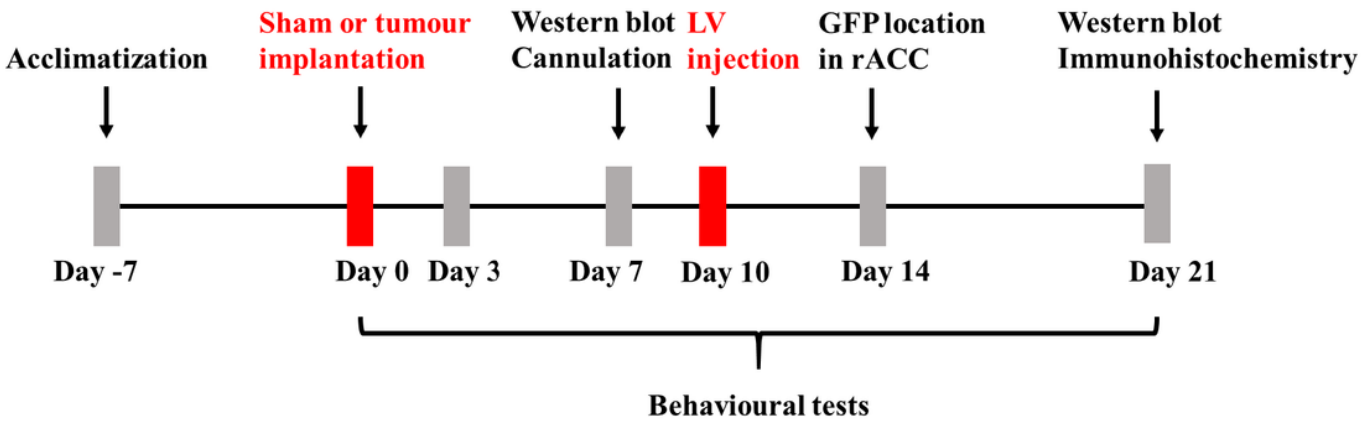

Figure 1

Flow chart of the establishment of tumour-bearing rat model, lentiviral intervention measures and examinations on different time points.

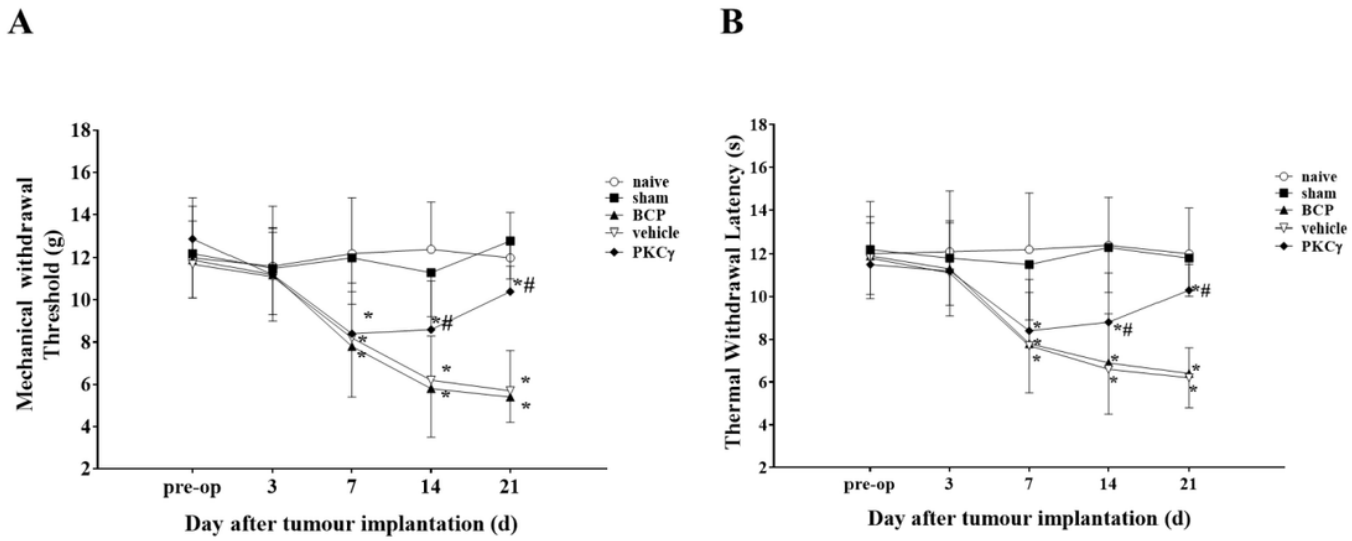

Figure 2

Behavioural tests after intra-tibial implantation and rACC microinjection of lentiviral vectors. MWT measurement on different time point in five groups (A). TWL measurement on different time point in five groups (B). *P < 0.05 vs. naive or sham; #P < 0.05 vs. BCP or vehicle. (mean ± SD; n=6 rats per group). “Pre-op” indicates the time point of the baseline of MWT and TWL measurement before intra-tibial administration of rats, and “3”, “7”, “14” and “21” indicate 3, 7, 14 and 21 days after intra-tibial implantation, respectively.

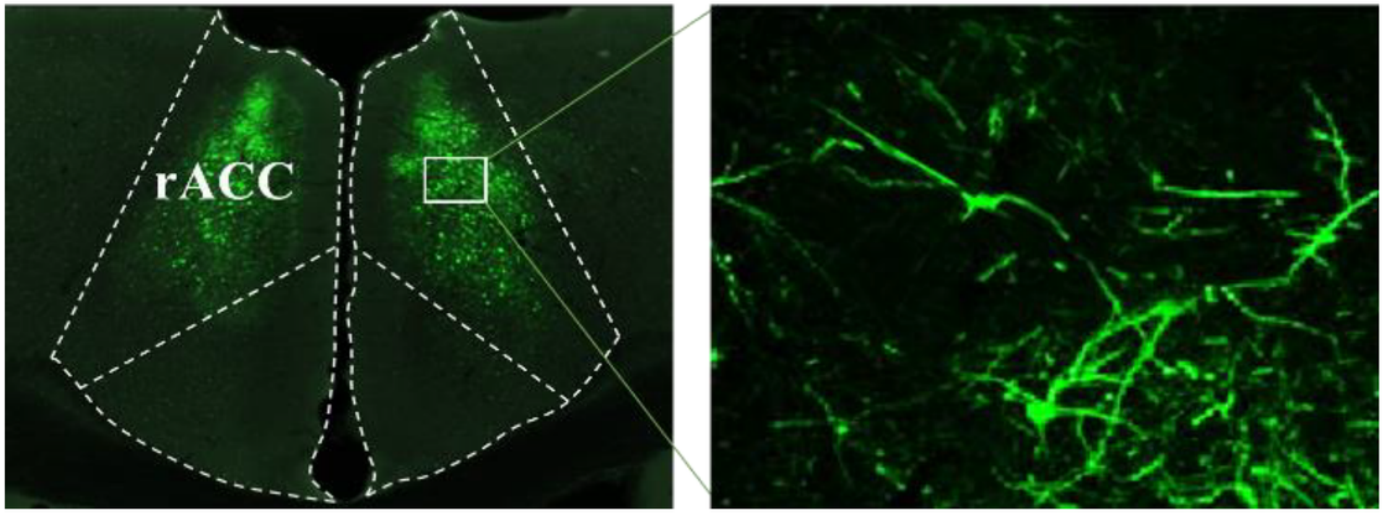

Figure 3

Representative coronal micrograph of PKCγ-GFP expression in the bilateral rACC, showing of the expression PKCγ in the rACC. Magnification:5× (left panel), 100× (right panel).

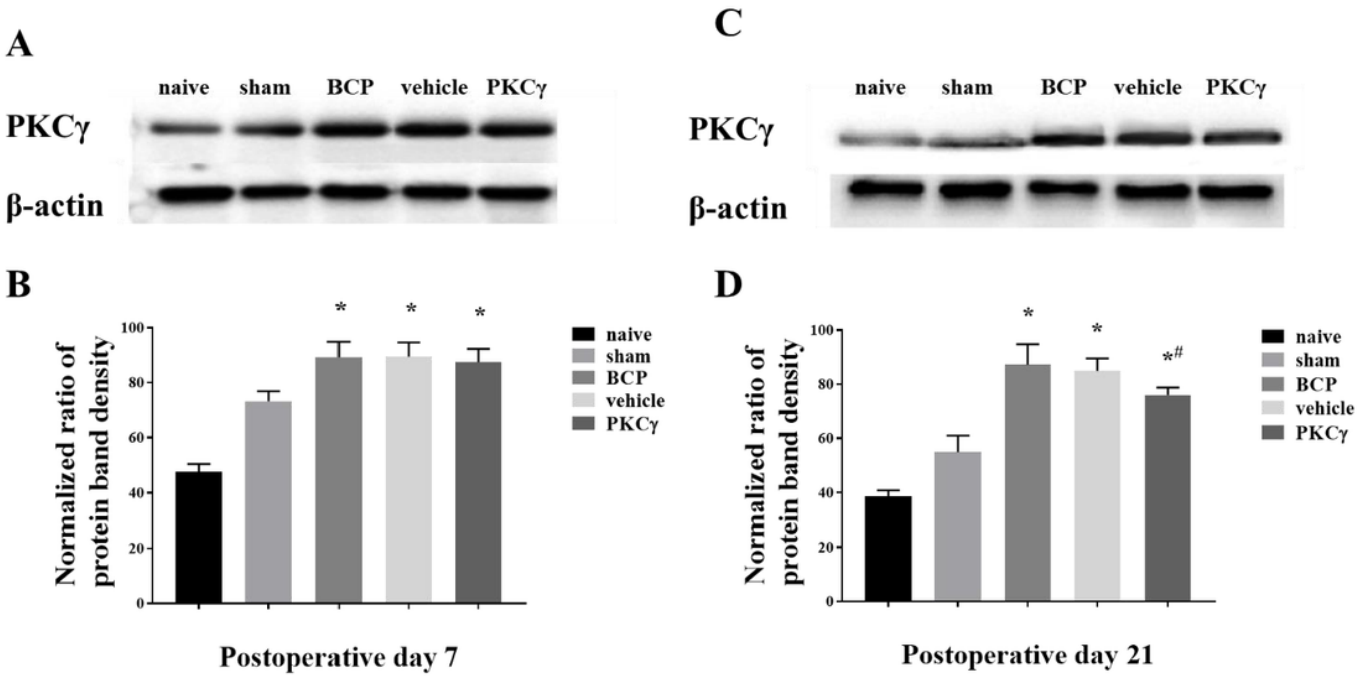

Figure 4

Expression of PKCγ protein in rACC neurons by western blot. Western blot analysis on 7 days after intratibial implantation (A). The relative band densities of A (B). Western blot analysis on 21 days after intratibial implantation (C). The relative band densities of C (D). *P < 0.05 vs. naive or sham; #P < 0.05 vs. BCP or vehicle; (mean ± SD; n=6 rats per group).

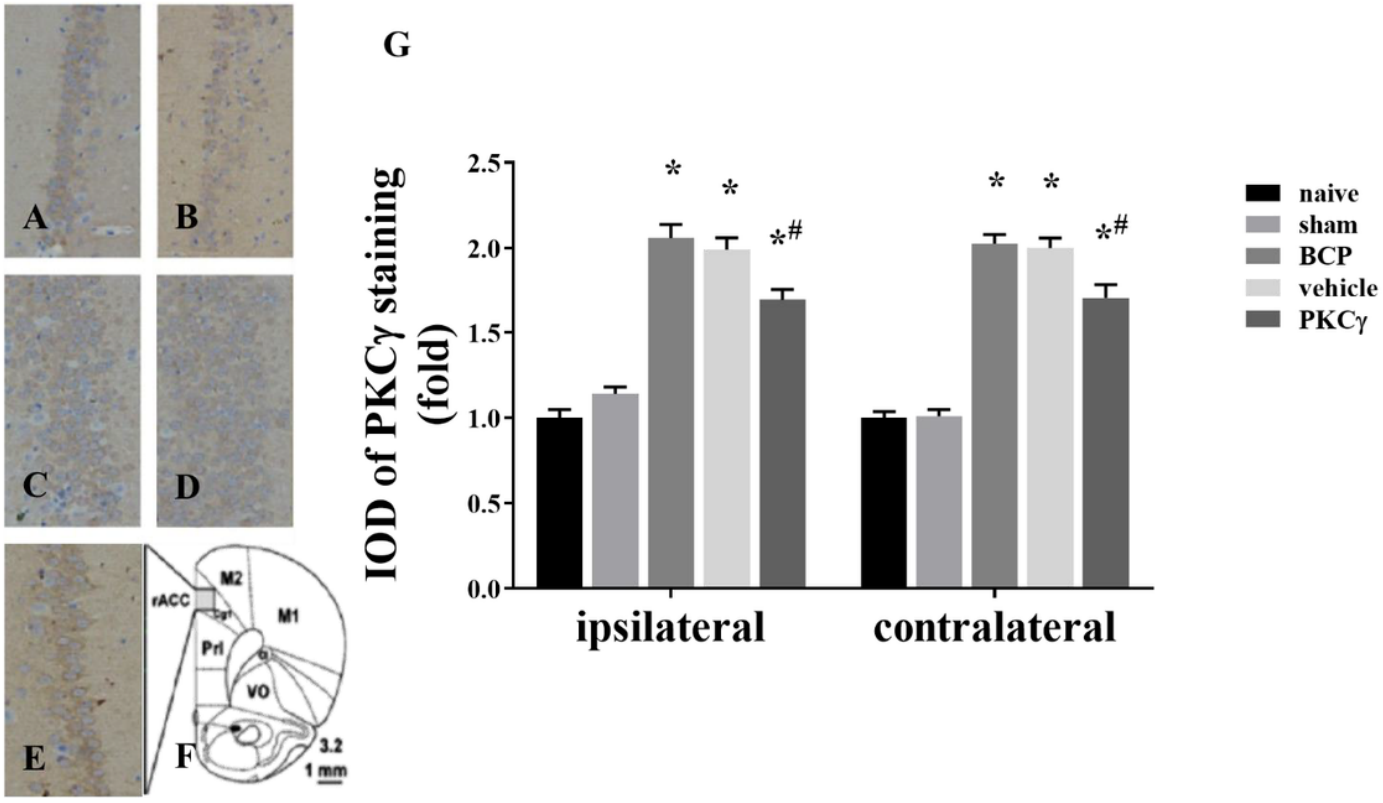

Figure 5

Immunohistochemical analysis of PKCγ in the rACC neurons after bilateral rACC microinjection. Photomicrographs of coronal sections depicting representative examples of IRs of PKCγ on day 21: naive group (A); sham group (B); BCP group (C); vehicle group (D); PKCg group (E). Schematic illustration of coronal sections illustrating the microinjection sites in rACC (F). The IOD fold of PKCγ staining (G) *P < 0.05 vs. naive or sham; #P < 0.05 vs. BCP or vehicle; (mean ± SD; n=3 rats per group).

Supplementary Files

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