The role of PKCγ subunit of rACC neurons in the development of bone cancer pain in rats

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Research article

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Abstract

Background To explore the role of PKCγ subunit of rACC neurons in the development of bone cancer pain in rats. Methods 40 adult female SD rats were divided into five groups: blank control group (Naive group), sham operation group (Sham group), bone cancer pain group (BCP group) empty lentiviral vector group Vehicle group and PKCγ/shRNA recombinant lentiviral vector group (PKCγ group). 10μl MADB-106 rat mammary cancer cells suspension was inoculated into the tibia bone marrow cavity of rats in BCP group and Vehicle group similarly. 10μl saline was inoculated into the proximal tibia bone marrow cavity of rats in Sham group. In the PKCγ group, the rats were taken the same treatment as the BCP group, and then 10μl shRNA/PKCγ recombinant lentivirus was injected into the bilateral rACC on the 7th day. The mechanical withdrawal threshold and thermal withdrawal latency were measured every 3 days to assess the rat pain behavior. Immunohistochemistry, Western blotting technology and immunofluorescence were used to detect the expression of PKCγ subunits in rat rACC neurons after operation. Results: From the 3rd day after operation, the mechanical withdrawal thresholds in BCP group, Vehicle group and PKCγ group were significantly decreased than those in Naive group and Sham group (P<0.05). Compared with the BCP group and Vehicle group the mechanical withdrawal threshold in the PKCγ group increased significantly (P<0.05). On the 3rd postoperative day, the thermal withdrawal latency in BCP, Vehicle and PKCγ groups was significantly longer than those in Naive and Sham groups P 0.05). From the 14th postoperative day, the TWL in PKCγ group was shorter than that in BCP and Vehicle groups (P<0.05). Western blot analysis showed that the expression of PKCγ in rACC neurons on the 14th day after operation in rats of BCP and Vehicle groups were significantly higher than that in Naive group and Sham group. (P<0.05) However, in the PKCγ group, the expression of PKCγ in rACC neurons was significantly lower than that in BCP group (P<0.05). Conclusion: Up-regulation of PKC subunit of rACC neurons in bone cancer pain rats contributes to the development of pain sensitivity in bone cancer.

Background

The pathogenesis of bone cancer pain remains largely unknown, and there is still no effective treatment[1, 2].The anterior cingulate cortex (ACC) is an important part of the cerebral cortex, especially the rostral ACC rACC which is associated with pain perception and regulation[3-5].

Under the persistent action of noxious stimulation, the function and structure of neurons or synapses in the ACC undergo long-term changes, which is known as plasticity. As an important signaling molecule in cells, PKC plays an important 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 plays an important role in the treatment of noxious stimulation in the dorsal horn of the spinal cord. Does rACC neuronal PKCγ play an important role in the formation of bone cancer pain? In this study, the role of PKCγ subunit of rACC neurons in the development of bone cancer pain was studied in a rat model of bone cancer pain. The mechanical withdrawal threshold and thermal withdrawal latency were measured to assess the rat pain

behavior. Immunohistochemistry, Western blotting technology and immunofluorescence were used to detect the expression of PKCγ subunits in rat rACC neurons after operation.

Methods

Construction of lentiviral vectors expressing PKCγ shRNA

The virus was packaged using the shRNA interference sequence TGAATGTGCACCGACGCTG plasmid pLVTHM (Shanghai Jikai Gene Co., Ltd，Shanghai，China) and the lentiviral packaging plasmid constructed with the highly efficient and specific rat PKCγ gene. The shRNAs were cloned into lentiviral vector pLVTHM-GFP (Shanghai Jikai Gene Co., Ltd，Shanghai，China). To generate the lentivirus, the recombinant vector and packaged plasmids were cotransduced into 293T cells using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Lentivirus with the human U6 promoter carrying shPKCγ were generated as previously described[8]. He final titer of recombinant virus was 1.25×10^9 TU/ml.

Preparation of carcinoma cell

the MADB-106 cells were maintained in Dulbecco's Modified Eagle's Medium(DMEM)，supplemented with 10% Fetal bovine serum，100 unit/ml penicillin，and 100 μg/ml streptomycin，and cultured at 37°C in the humidified atmosphere of 5% CO2，and then passaged weekly according to ATCC guidelines. For adminstration，cells were detached by scraping and then centrifuged at 900 rpm for 3 min. The pellet was suspended in Hank's balanced salt solution. Cells in logarithmic growth phase were selected for experiments and then used for intra-tibial injection.

Animals and grouping

40 female adult 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 accordance with the recommendation of the Principles of Laboratory[9] and the ethics committee of the International Association for the Study of Pain[10]. 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) prior to the onset of the experiments (Permit number: KYLL-2017(LW)017). The number of animals was used as little as possible and their suffering was minimized to the lowest degree according to IASP guidelines[10]. All rats were maintained in the following identical conditions: A controlled temperature of 22of˚C, a 12 h light/dark cycle and *ad libitum* access to food and water. 1 week later, the rats were randomly and equally divided into five groups: normal control group (Naive group), sham operation group (Sham group), Bone cancer pain model group (BCP group), empty lentiviral vector group （Vehicle group）and PKCγ/shRNA recombinant lentiviral vector group (PKCγ group). Normal control group (Naive group): The experimenters were blinded to the pharmacological treatment while processing data and making exclusion decisions. Naïve group healthy rats without any treatment. Sham operation group

(Sham group):unilateral intra-tibial injection of Normal saline. Bone cancer pain model group (BCP group): unilateral intra-tibial injection with 10 μl MADB-106 cells (cell density 4.8×10^9/ml) (from Cancer Institute of Concord Medical University of Chinese Academy of Medical Sciences). The initial treatment of Vehicle group and PKCγ group were the same as that of BCP group.

Recombinant lentivirus administration into rACC

Rats were implanted with stainless steel cannula for intra-rACC drug infusions. For microinjection studies, rats were anesthetized with intraperitoneal chloral hydrate (40 mg/kg), and were firmly fastened into a brain stereotactic apparatus with the lambda and bregma at 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,2.5 mm beneath the surface of the skull] or the prefrontal cortex(PFC) [2.6 mm anterior to bregma, 0.6 mm lateral from the mid-line, 3.7 mm beneath the surface of the skull] in-line with the atlas of Paxinos and Watson[11]. A 1μL 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. Animals were allowed to recover for one week before the next experimental procedure. Before and At the end of the experiment, brains were sectioned for cresyl violet staining to verify cannula position and injection site. A week after operation, rACC were injected per hemisphere both with PKCγ/shRNA recombinant lentivirus 6.2×10^6 particles(1μL) in PKCγ/shRNA group and empty vector recombinant lentivirus 6.2×10^6 particles(1μL) in Vehicle group over a 5 min period.

Establishment of rat BCP model

Rat Bone Cancer Pain Model was established as previously described[4]. The rats were anesthetized with an intraperitoneal injection of 10% chloral hydrate (300m/kg). Superficial incisions were made in the skin overlying the patella to expose the tibia head with minimal damage. A 23-gauge needle was inserted at the site of intercondylar eminence and pierced 5-10mm below the knee joint into the medullary cavity of tibia. In PKCγ/shRNA group and BCP group the Needle was then removed and 10 μL of mammary carcinoma cell suspension or 10 μL of heat-killed mammary carcinoma cell suspension into a 50 μL micropipette. The needle was slowly inserted into the tibia cavity and injected in the carcinoma cells. The injection site was closed with bone wax quickly 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.

Pain thresholds analysis

Before baseline testing, the rats were habituated to the testing environment for 5 days. Baseline data were tested both before and after using the von Frey hair stimulation. 3 rats that showed obviously different data between these two tests were discarded. For the remaining animals internalized in the subsequent studies, the average of these two baseline tests was recorded as a baseline data. The experimental rats were placed in a plastic cage (10x10x15cm) with a Plantar Von Frey TM Dynamic Plantar Stimulator at

the bottom, and the cage was placed on a wire mesh plate for experimental operation and observation. After 15 min accommodation, 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 hair with logarithmically incremental stiffness (1.0, 2.0, 4.0, 6.0, 8.0,15.0 and 20.0g) were applied perpendicularly to the mid-plantar surface (avoiding the less sensitive tori) of each hind paw. The stimulus lasted for 10 seconds, and the interval between each measurement was 10 minutes. The minimum stimulus that caused rat paw withdrawal was defined as the mechanical withdrawal threshold.

Rats were placed under a cage on a glass plate that was elevated to allow maneuvering 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 onset of radiant heat application to withdrawal of the rat's hind paw was defined as the thermal withdrawal latency. The glass plate was kept dry and clean during the measurement. Both hind paws were tested independently with a 5 min interval between trials so that pain can be restored to normal. A cut-off time of 20 s was imposed on the stimulus duration to prevent tissue damage. Each rat was tested on the paw three times and the average value was taken.

Western blot and immunofluorescence

A total of 14 days after operation, each group of rats were anaesthetized with pentobarbital prior to the perfusion of 100 ml NS through the ascending aorta and then rapidly sacrificed by decapitation. The rACC tissues were immediately removed five rats randomly selected at each time point for measurement) and snap frozen in liquid nitrogen until all the samples were collected for immunohistochemical DAB staining to detect the expression of PKCγ in rACC tissues. On the 7th day after operation, brain tissue neurons were taken from each of the five groups of rats, and the expression level of PKCγ subunit was measured by Western blot and immunofluorescence. On the 14th day after operation, the same operation as on the 7th day after surgery was performed again.

Fluorescent microscope test

After the perfusion, the rACC tissues were immediately removed, post-fixed in the same fresh fixative for 4 h at 4 °C, and placed in 30% sucrose in 0.1 M phosphate buffer for 24 h at 4°C. The rACC tissues were cut into 20-μm-thick frontal sections with a cryostat. The sections were washed with PBS, mounted onto clean glass slides, dried at 4 °C(protected from light), and cover-slipped with PBS containing 50% glycerin. The sections were observed with a fluorescent microscope (Leica, Wetzlar, Germany).

Statistical analysis

All results from the data analysis are shown as mean ± standard deviation (SD). An ANOVA test, followed by Student-Newman-Keuls (SNK) was used to compare the quantitative data between groups. A p value of less than 0.05 (two-tailed) was considered to indicate a statistically significant difference. Graph displays were performed using GraphPad Prism Software version 7.0.

Results

Inter-group comparison of mechanical withdrawal thresholds within the surgical side. There was no statistically significant difference between the Naive and Sham groups on the 3rd, 7th, 14th, and 21th days after surgery and preoperatively (P>0.05). However, the BCP, Vehicle and PKCγ groups began to decrease from the 7th day postoperatively and were significantly lower than that before surgery. The difference was statistically significant. (P<0.05). This trend continued until the 21th day after surgery. From the 7th day of operation, BCP、Vehicle and PKCγ groups were lower than that of Naive and Sham groups. (P<0.05) On the 14th and 21st days after operation, the PKCγ group was significantly higher than that of the BCP and Vehicle group (P<0.05)

(Table 1).

Inter-group comparison of thermal withdrawal duration within the surgical side. There was no statistically significant difference between the Naive and Sham groups on the 3rd, 7th, 14th, and 21th days after surgery and preoperatively (P>0.05). Compared with preoperative levels, the BCP , Vehicle and PKCγ groups showed significantly prolonged thermal withdrawal duration from 7 days after surgery (P<0.05 or P<0.01). This difference continued until the 21th day postoperatively. From the 7th day postoperatively, the thermal withdrawal duration of the BCP, Vehicle and PKCγ groups was higher than that of the Naive and Sham groups (P<0.05 or P<0.01). On the 14th and 21st days after operation, the PKCγ group was significantly lower than that of the Vehicle and BCP group (P<0.05 or P<0.01)

 (Table 2).

In order to assess PKCγ protein expression in rACC neurons following the development of mechanical pain and thermal sensitization in rats, rACC brain tissue was taken on the 7th day after intrapatellar injection of breast cancer cells when the rats showed mechanical pain and thermal pain. The expression of PKCγ in the brain of rats in each group was exmined by western blot. The expression levels of PKCγ protein in the BCP, Vehicle and PKCγ groups were higher than those in the Naive and Sham groups, (P<0.05) (Fig 1).

In order to examine the changes of PKCγ protein expression in rACC neurons, rACC brain neurons were taken from each group on the 14th day after rACC neurons were given with PKCγ/shRNA recombinant lentivirus vector. Compared with the Naive and Sham groups, the expression of PKCγ protein was up-regulated in the BCP⎕Vehicle and PKCγ groups (P<0.05) as assessed by Western blotting (P<0.05). Compared with the BCP and Vehicle group, the expression of PKCγ protein in the PKCγ group was decreased (P<0.05) (Fig 2).

Comparison of the number of bilateral PKCγ-positive neurons in rACC after rACC-administered recombinant lentiviral vector of PKCγ/shRNA by immunohistochemistry. PKCγ-positive neurons in rACC neurons were assessed after PKCγ/shRNA recombinant lentiviral vector administration in rACC of rats in each group on the 14th day after operation. We observed an increased expression of PKCγ protein in the BCP, Vehicle and PKCγ groups compared with the Naive and Sham groups (P<0.05). However, compared with the BCP and Vehicle group, the expression was decreased in the PKCγ group (P<0.05) (Fig 3).

the lentivirus was packaged into viral particles in packaging cell 293T cells, and under fluorescent microscope, the virus particles showed strong green fluorescence(Fig 4).

The detection of lentiviral neurocyte transduction. Rats received rACC GFP-expressing lentivirus and were sacrificed 7 days later. rACC tissues were fixed in a 4% formalin/sucrose gradient and cryosectioned for examination under a fluorescence microscope.(Fig 5)

Discussion

This study found that changes in pain-related behaviors such as mechanical withdrawal threshold and mechanical withdrawal duration in rats with bone cancer pain resulted in up-regulation of PKCγ subunit expression in rACC neurons. Recombinant lentiviral vector of PKCγ/shRNA was administered in rACC to silence the PKCγ subunit, resulting in reduced pain in rats. These results indicate that the PKC subunit of rACC neurons plays an important role in the development of pain sensitivity in bone cancer.

Pain is one of the symptoms in terminal cancer patients and has been described as a deep, burning-like pain with severe emotional reactions. So far, although there are opioid, diphosphonate, radiotherapy, chemotherapy and surgery for relieving cancer pain, it is still reported that many cancer patients have inadequate and undermanaged pain control[12, 13], One of the reasons may be related to the unclear

mechanism of bone cancer pain. Therefore, it is of important clinical significance to explore the mechanism of bone cancer pain for improving the treatment of patients. Recent studies have shown that the excitability of neurons on the upper spinal cord such as ACC and the enhancement of synaptic transmission play an important role in the development of chronic pain[14]. The ACC is an important structure of the limbic system and reflect affective and motivational aspects of pain.

Many study results showed that ACC especially rostral ACC participated in nocuity information transmission and regulation. Unpleasant sensations caused by noxious stimuli or pain can also activate ACC[15]. Resection of peripheral cortical tissue including ACC can reduce the patient's pain and emotional response, but not affect the intensity and location of the pain stimulus. The latest BOLD further directly proves the important role of ACC in emotional regulation in human subjects. When given a certain stimulus, the negative emotions increase and the activity of ACC also rapidly increases[16-18]. These studies suggest that enhanced nerve excitability of rACC plays an important role in the generation of bone cancer pain. PKCγ as an important signal transduction molecule in neurons activates the protein kinase system in neurons, thereby changes the phosphorylation state of the substrate. PKCγ is also involved in synaptic remodeling of neurons and long-term potentiation. Studies have shown PKCγ in peripheral nerve and spinal dorsal horn plays an important role in the processing of noxious stimulation which suggests that PKCγ in rACC neurons may be vital in the enhancement of nerve excitability and pain sensitivity of rACC neurons, and no relevant reports have been reported. The results of this study showed that up-regulation of PKCγ subunit expression in rACC neurons was accompanied by pain in rats, suggesting that this subunit may be involved in the development of pain sensitivity in bone cancer. To further explore whether the PKC subunit of rACC neurons play a major role in bone cancer pain, we injected PKCγ/shRNA recombinant lentiviral vectors into the bilateral rACC to silence the PKCγ subunits of rACC neurons. We observed an increase of the mechanical withdrawal threshold and the shortening of the thermal withdrawal duration, suggesting that rat's hyperalgesia was reduced. This confirms that the PKCγ subunit of rACC neurons plays an important role in the development of rat bone cancer pain. 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[19]. Follow-up study should exclude more influencing factors and get more accurate results.

recombinant lentiviral vectors is a common used gene-delivery system, which can infect the intermitotic cell 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 progeny during cell division. At the same time, the pathogenic genes of lentivirus have been deleted, so recombinant lentiviral vectors are used to express small interfering RNA (short interference RNA, siRNA). recombinant lentiviral vectors can play the role of down-regulating the expression of target gene safely and persistently. Compared with drug therapy, lentiviral vectors have the advantages of strong 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.

This experiment still showed pain or hyperalgesia on the third day of lentivirus administration in rACC, but the degree of pain was significantly reduced on the 7th day. This is mainly because it takes about 4 days for the target gene of the recombinant lentiviral vector to integrate into the target cell genome and express normally. From table 1 and table 2, we observed that the degree of pain was significantly reduced after administration of the lentiviral vector PKCγ/shRNA. This phenomenon shows that the use of genetic analgesic treatment can indeed achieve effective results, but at the same time we also observed that the degree of pain in rats did not fully return to normal. It may be related to both the interference of our target sequence, which can only partially down-regulate the expression of PKCγ, and other mechanisms of bone cancer pain. Therefore, on the one hand, we need to screen out more effective interference target sequences, or use multi-segment interference sequences synergistically to achieve more efficient silencing of PKCγ expression. On the other hand, we should also explore other analgesic methods for bone cancer pain.

Conclusions

In summary, this study confirmed that the expression of PKC in rACC was significantly decreased, and significant analgesic effect was observed, After lentivirus PKC /shRNA was injected into rACC in BCP rats, indicating that transgenic analgesic therapy could achieve effective analgesic effect.

List Of Abbreviations

ACC: Anterior cingulate cortex   rACC: Rostral ACC LTP: Long-term potentiation   PWL: Paw withdrawal latency

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

HF, GW, KL and MS submitted Ethics application, participated in provision of teaching sessions, collected data and prepared manuscript. ZF and GC carried out compilation of data and performed statistical analysis. RW and XL 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.

Tables

Due to technical limitations, the tables have been placed in the supplementary file section.

Figures
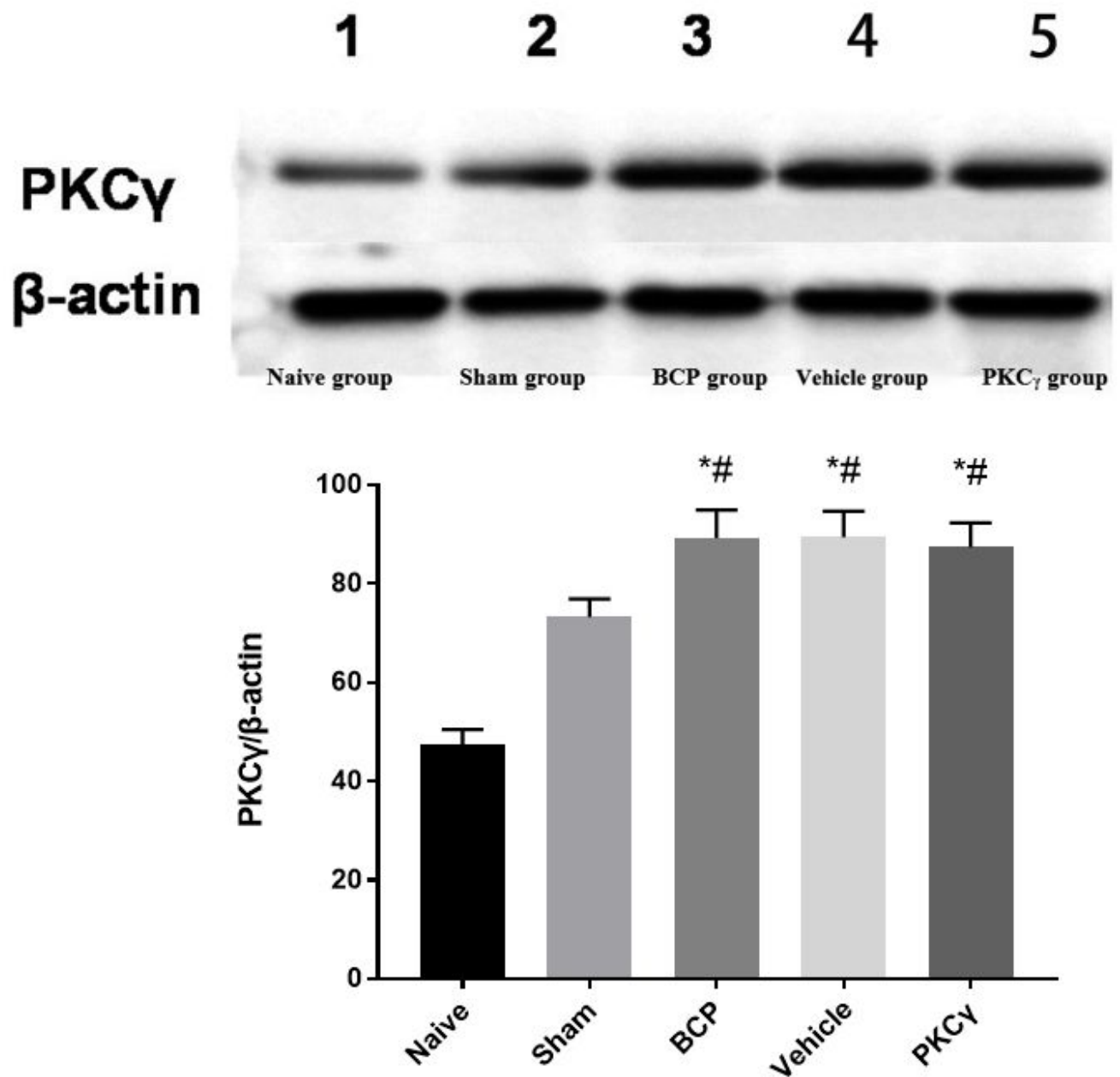
Figure 1

Western blotting in the expression of PKCγ subunits in neurons of ACC brain regions in rats 7 days after operation.*P < 0.05，compared with naive group; #P <0.05, compared with Sham group.
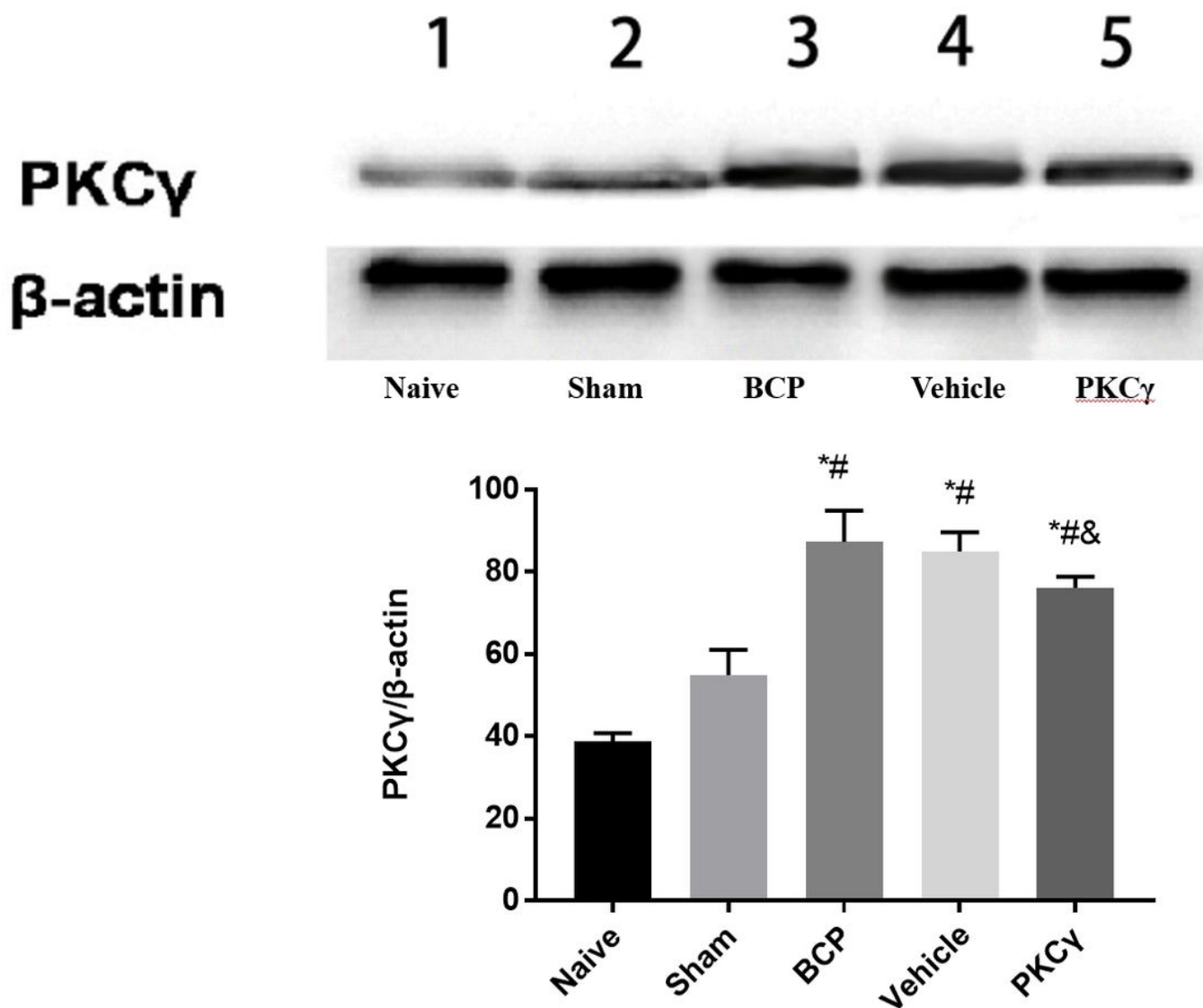
Figure 2

The expression of PKCγ subunits in rACC neurons after rACC-administered. *P < 0.05, compared with naive group; #P < 0.05, compared with Sham group；&P <0.05, compared with BCP and Vehicle group.
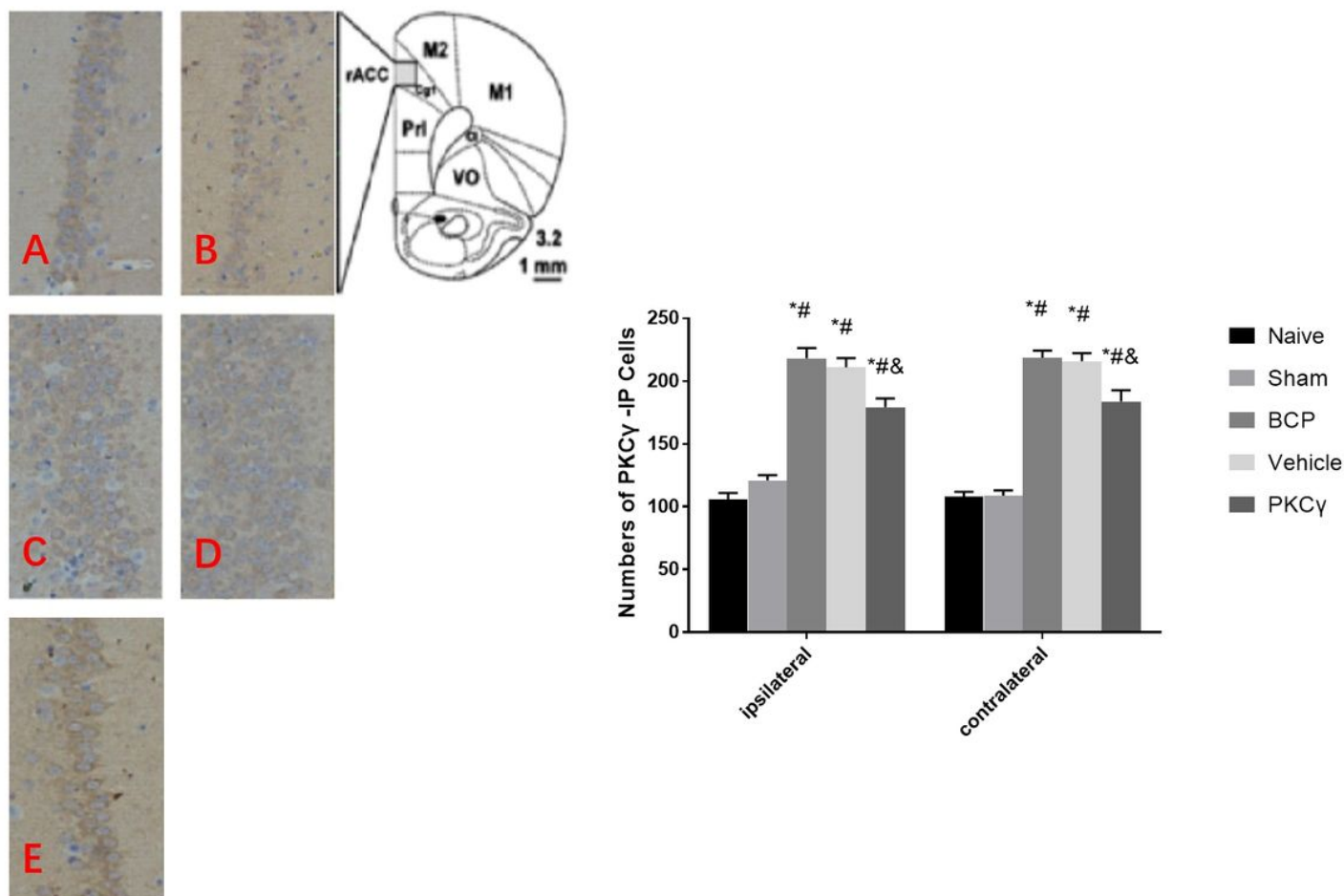
Figure 3

The expression of PKCγ subunits in rat bilateral rACC neurons after rACC administered recombinant lentiviral vector of PKCγ/shRNA by immunohistochemistry. *P < 0.05, compared with naive group; #P < 0.05, compared with Sham group；&P <0.05, compared with BCP and Vehicle group. Immunohistochemistry graph A. Naive group B. Sham group C. BCP group D. Vehicle group E. PKCγ group
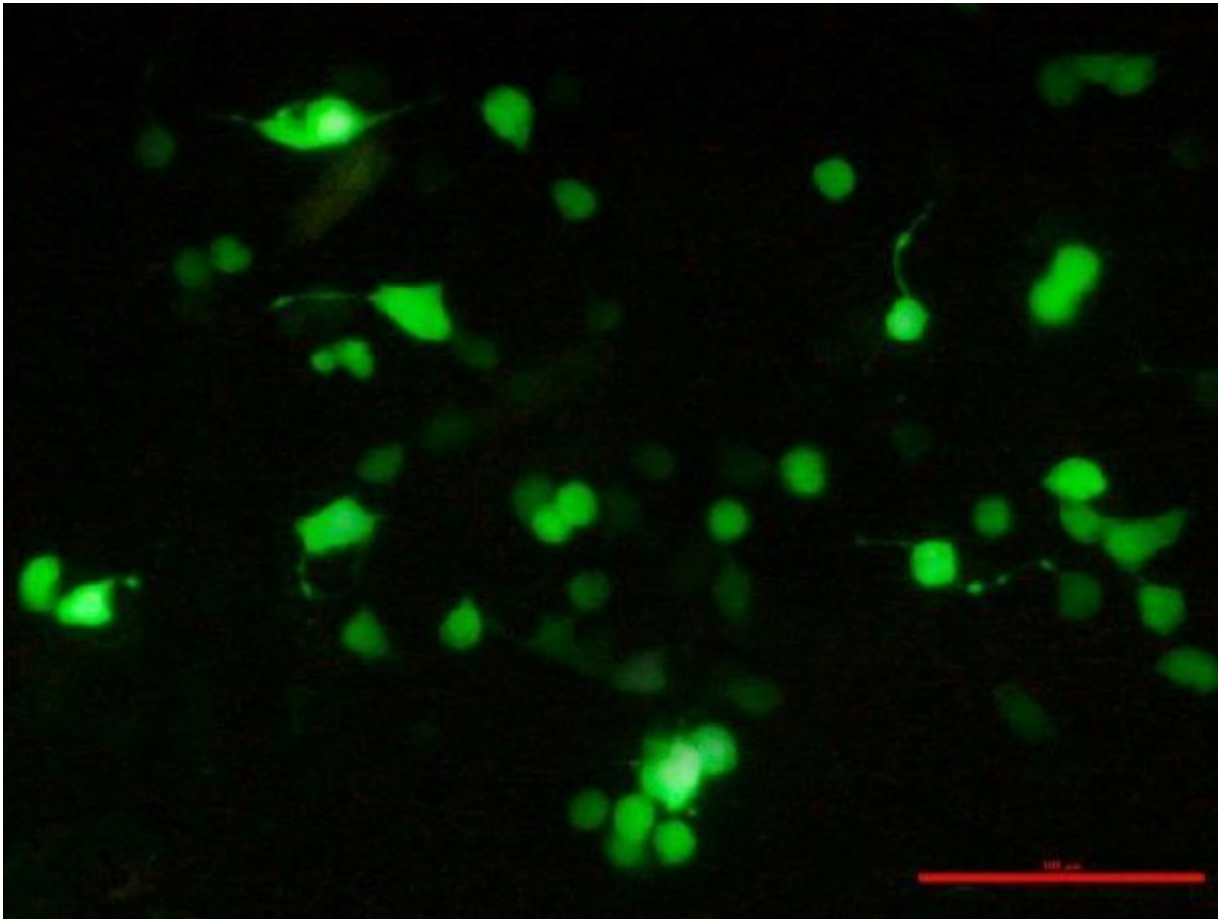
Figure 4

The lentivirus was packaged into viral particles in packaging cell 293T cells, and under fluorescent microscope, the virus particles showed strong green fluorescence
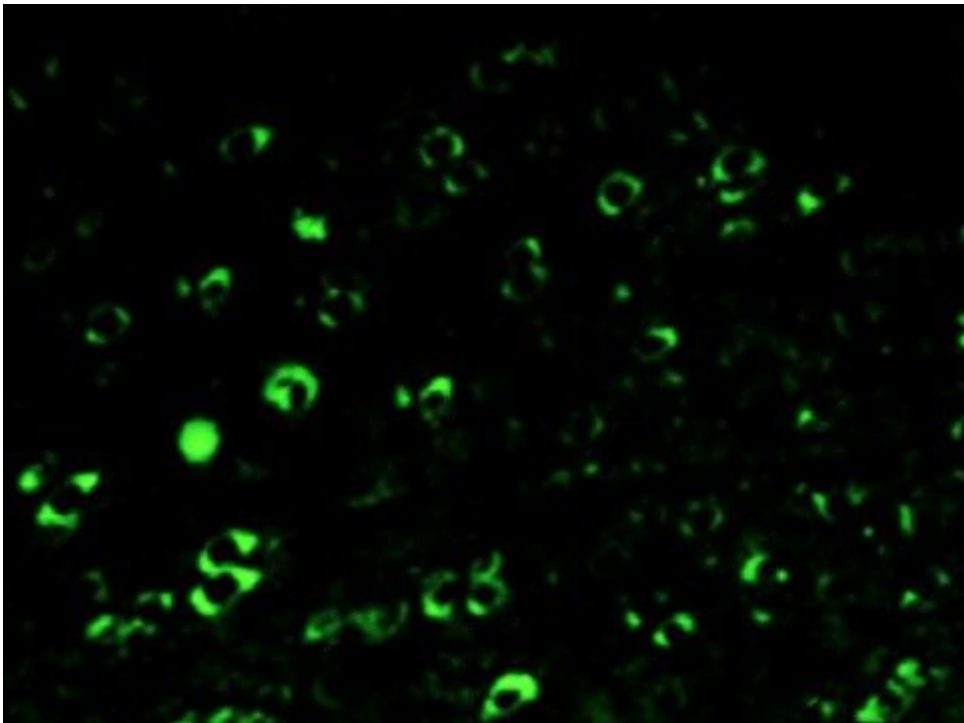
Figure 5

The detection of lentiviral neurocyte transduction. Rats received rACC GFP-expressing lentivirus and were sacrificed 7 days later. rACC tissues were fixed in a 4% formalin/sucrose gradient and cryosectioned for examination under a fluorescence microscope.