Review Article
Effect of migraine on expression of PACAP and CGRP in trigeminal nerve of rats

Feng Han

Department of Human Anatomy, Jilin Medical University, No. 5 Jilin Street, Jilin 132013, Jilin Province, China

Abstract: The changes of mood, PACA and CGRP in migraine model rats compared with normal rats were explored. A total of 40 adult healthy male SD rats were randomly assigned to control group (con group), sham operation group, model group, and inhibition group. The stimulation electrodes were located in trigeminal ganglion in model and inhibition groups. In the sham operation group, the electrodes were fixed at the ganglia, but no electrical stimulation was applied. In addition, no treatment was given to the con group, while PACA and CGRP inhibitors were given to the inhibition group. The results of pain behavior confirmed the success of the model and inhibition groups. The levels of PACAP, CGRP, dopamine, 5-hp, TNF-α, IL-6, and IL-1β in the model group were higher than those in the con group (P < 0.05). PACAP, CGRP, dopamine and 5-hp in the inhibition group were lower than those in the model group (P < 0.05), while TNF-α, IL-6 and IL-1β in the inhibition group were higher than those in the model group (P < 0.05). The degree of depression and anxiety in the model group was more severe than that in the con group, while that in the inhibition group was relieved (P < 0.05). Additionally, PACAP and CGRP were negatively correlated with TNF-α, IL-6, and IL-1β, respectively. Compared with normal rats, the levels of PACAP and CGRP in migraine model rats increased, and the depression and anxiety increased. PACAP and CGRP can be used as therapeutic targets.

Keywords: Migraine, trigeminal nerve, pituitary adenylate cyclase activating peptide, calcitonin gene-related peptide

Introduction

As a nervous system disease [1], migraine is a primary headache [2], and the global incidence is about 11.7% [3]. Migraine patients often show moderate to severe headache, nausea, vomiting, and they have pain sensitivity to light and sound [4]. Migraine is an important factor of disability in neurologic diseases. Trigeminal ganglion (TG) is an important site for headache attack [6]. TG activation and sensitization will stimulate the release of calcitonin gene-related peptide (CGRP), pituitary adenylate cycle activating peptide (PACAP) and other neurotransmitters [7], thus causing multiple pain in the brain.

PACAP is a kind of bioactive neuropeptide, which can effectively activate camp [8]. Many researchers have studied the effect of PACAP on the pathogenesis of headache. Tuka [9] studied the level of PACAP-38 in patients with primary headache, and found that the content of PACAP-38 in serum was greatly increased compared with that before the onset of the disease and believed that PACAP-38 could be used as a marker of primary headache. Zhang [3] established a migraine model by electrical stimulation and measured the levels of PACAP, PAC1, VPAC1, VPAC2, and CGRP in the trigeminal nerve of rats. The results showed that PACAP plays a crucial role in headache attack through PAC1 receptor. Schytz [10] believes that PACAP can activate camp in TG pain receptors through PAC1 receptor, thus giving rise to pain.

CGRP is a variable fragment of calcitonin gene, which contains 37 amino acids [11]. CGRP is an effective vasodilator [12], which can participate in the transmission of pain [13]. Warfvinge [14] discussed the location and function of CGRP and its receptor CLR, RAMP1 in rat brain, and thought that the expression of CGRP and its receptor combination in central nervous system was related to its role in migraine and oth-

er physiological processes. Cernuda morollón [15] found that the level of CGRP in the peripheral blood vessels of migraine patients increased, and considered that CGRP could be a biomarker of chronic migraine.

In order to provide experimental data for the development of headache mechanism and treatment, this study was designed to construct a migraine model by using parasagittal dural electrical stimulation in rats.

Materials and methods

Materials and reagents

Animal materials: A total of 40 adult healthy male SD rats, 12-15 months old, weighing 155-175 g, were purchased from Hunan Shrek Jingda Experimental Animal Co., Ltd. Approved by the ethics committee of Affiliated Hospital of Jilin Medical University, the operation process is strictly in line with the guidelines for the care and use of experimental animals (NIH publication, 1996 revision, no. 85-23). The rats were randomly assigned to control group (con group), sham operation group, model group, and inhibition group.

All animal experiments were carried out at Jilin Medical University.

Reagent: PACAP 6-38 (PACAP antagonist) was purchased from Selleck Company in the United States, Catalog No. s8416; CGRP 8-38 (CGRP antagonist) was purchased from Sigma in the United States, product No. scp0060.

PACAP ELISA kit was purchased from Shanghai Jingkang Bioengineering Co., Ltd., product No.: JK-(a)-0222; CGRP ELISA kit was purchased from Shanghai yuanmu Biotechnology Co., Ltd., product No.: ym-qp12294; 5-HT ELISA kit was purchased from Shanghai Jingkang Bioengineering Co., Ltd., product No.: JK-(a)-1537; dopamine ELISA test kit was purchased from Shanghai Qincheng Biotechnology Co., Ltd., product No. qc12415-b.

Rat tissue protein extract was purchased from Shanghai Beibo biological company, batch No. bb18011; BCA protein concentration test kit was provided by Biyun Biotechnology Institute, batch No. p0012; PACAP and CGRP anti-1, anti-2 Goat anti rabbit (HRP cross-linking) and β-actin were purchased from Shanghai Abcam Company.

Method

Construction of migraine model: In this study, the TG models of model group and inhibition group were constructed by electrical stimulation. The rats were anesthetized and fixed on the operating table. The dura was exposed by craniotomy. The electrodes were fixed under the skull cap for 9.2-9.8 M. The right trigeminal nerve was electrically stimulated (3.0 mA, 5 ms, 5 Hz). The process lasted for 5 min. PACA and CGRP inhibitors were adopted to inhibit the expression of PACA and CGRP in the inhibition group. In the sham operated group, the electrodes were fixed at the trigeminal ganglion without electrical stimulation. No treatment was carried out to the con group.

Evaluation of pain behavior: The rats in each group were placed in a suitable environment, and the times of scratching, rotating, and climbing cage were evaluated 2 hours later. Scoring standard: 1 point for 10 times of scratching, 0.1 point/time later; 1 point for 2 times of rotation, 1 point/time later; 1 point for 2 times of climbing cage, 1 point/time later. A more serious pain gets a higher score. If the score is no less than 6 in one hour, the model is constructed successfully.

Pain threshold measurement: The minimum pressure value of the withdrawal reaction of rats was measured by dynamic acupuncture pain detector. The pressure value was called the mechanical pain claw retraction threshold (MWT), which was tested once every 5 minutes for three times. The thermal tolerance of the foot bottom of the injured side limb was collected by the thermal pain tester. The time from the beginning of irradiation to the lifting of the hind paw was called thermal pain latency (TWL).

Detection of PACAP, CGRP, 5-HT and dopamine in plasma by ELISA: Five days after the establishment of the model, blood samples were sampled from tail vein of each group, followed by 3×10^3 r/min centrifugation for 20 min, and the supernatant was detected by ELISA. The whole process shall be carried out in strict accordance with the specification standard.

Western blot assay of PACAP and CGRP in trigeminal nerve: Five days after the establish-
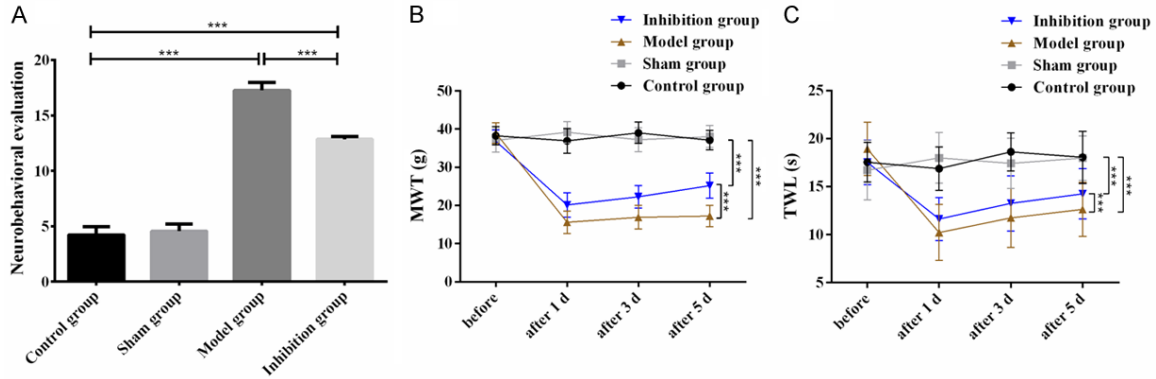
Figure 1. Pain behavior evaluation results of each group. A. Behavioral score 2 hours after each modeling. B. MWT change before and after modeling in each group, 5 days before and after modeling. C. TWL change before and after modeling in each group, 5 days after modeling.

ment of the model, after the pain threshold experiment, the rats in each group were decapitated, the brain was taken out, and the trigeminal nerve was separated and placed in liquid nitrogen for testing. The trigeminal nerve tissue was cut into pieces, and the protein extract (lysate:protease inhibitor:phosphatase inhibitor = 98:1:1) was put into the pre-cooled cell, centrifuged for 15 min, 1.2×10^4 r/min, and the supernatant was taken. SDS-PAGE electrophoresis was employed to separate the protein, and the protein was transferred to the NC membrane, which was kept for 1 h at room temperature (the closing solution was 5% skim milk PBS solution). After that, PACAP (1:50000) and CGRP (1:1000) were added into the reactor and the reactor was placed overnight at 4°C. Using PBS solution for cleaning, the operation repeated three times, followed by adding secondary antibody (HRP cross-linking, 1:50000), and continued to stand for 1 h at room temperature. Finally, PBS solution was used to wash the membrane, and the enhanced chemiluminescence method was used for visualization. The internal reference protein is β-actin (1:200), and the relative expression of the protein to be tested = the gray value of the strip to be tested/that of the β-actin strip. Beta-actin, PACAP, CGRP, and secondary antibody (HRP cross-linking) were all purchased from the Abcam company.

Standard depression and anxiety scale [16]: The development of the standard depression and anxiety disorder scale refers to the Zhang's study [16], and the depression and anxiety degree were evaluated on the 5th day after modeling. The higher the score is, the less depression or anxiety is.

Statistical analysis

The above index data were input into spss20.0 software package (Asia analytics formally SPSS China) for statistical analysis. The measurement data were expressed as the mean ± SD. The data comparison method between groups was carried out using the one-way ANOVA, and the data comparison method within groups was carried out using the paired t-test. The correlation analysis was analyzed by the Pearson analysis. All data are double tailed. P < 0.05 implies a significant difference.

Result

Pain behavior assessment for modeling judgment

As shown in **Figure 1A**, there was no significant difference between the con group and the sham operation group in behavioral score (P > 0.05), while the behavioral score of the model and inhibition groups was greatly higher than that of the con group (P < 0.05). The behavioral scores of model group and inhibition group were more than 6, indicating that the two groups were successfully modeled. As shown in **Figure 1B** and **1C**, there was no significant difference between the con group MWT and TWL in the experimental period (P > 0.05), and the sham operation group had the same results, while there was no significant difference between the two groups MWT and TWL in the same period (P > 0.05). During the experi-
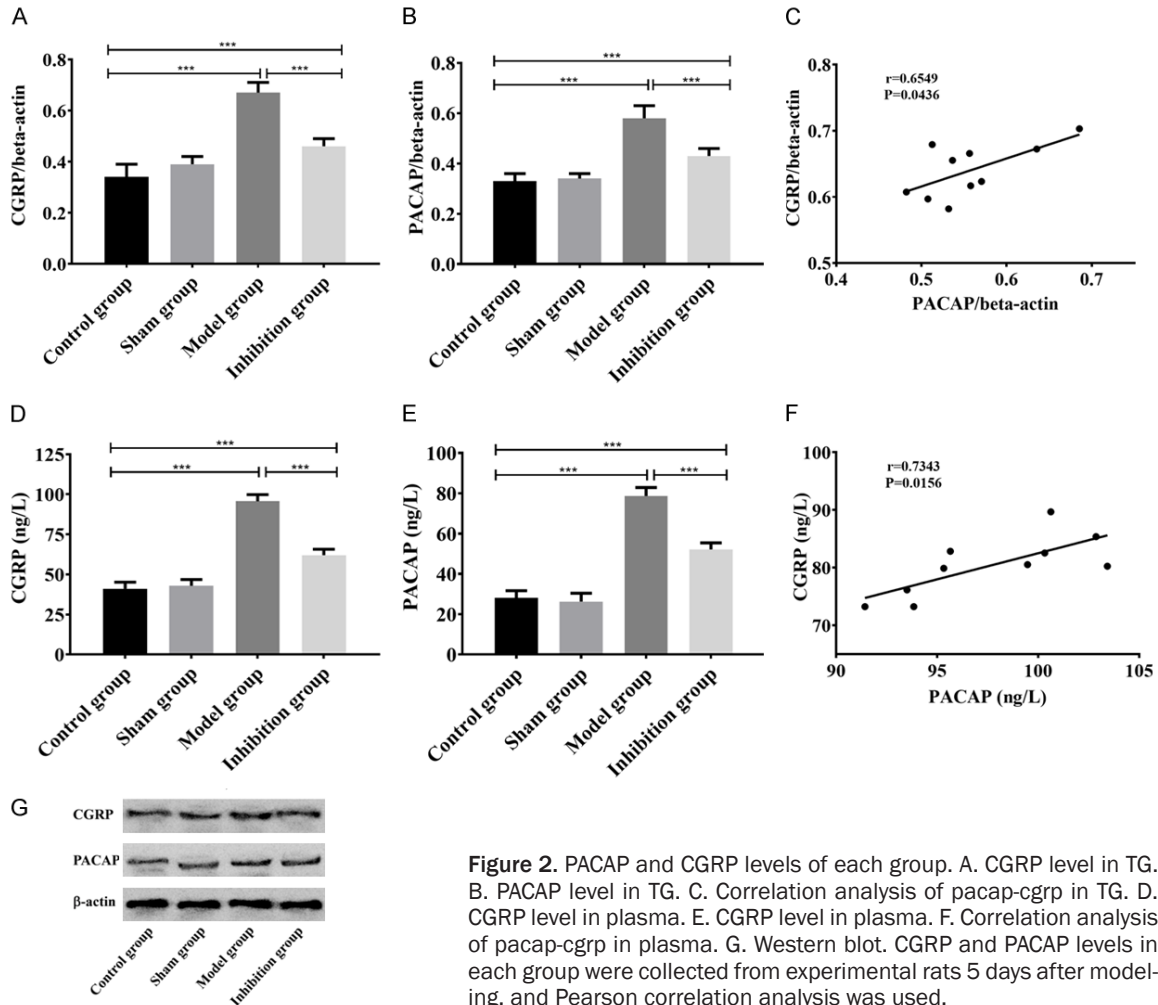
Figure 2. PACAP and CGRP levels of each group. A. CGRP level in TG. B. PACAP level in TG. C. Correlation analysis of pacap-cgrp in TG. D. CGRP level in plasma. E. CGRP level in plasma. F. Correlation analysis of pacap-cgrp in plasma. G. Western blot. CGRP and PACAP levels in each group were collected from experimental rats 5 days after modeling, and Pearson correlation analysis was used.

mental period, MWT and TWL in the model and con groups decreased 1 day after the establishment of the model (P < 0.05), and there was no significant change in MWT and TWL in the model group (P > 0.05), while MWT and TWL in the con group increased gradually (P > 0.05), and MWT and TWL in the model group were lower than those in the con group (P < 0.05).

Expression of PACAP and CGRP

As shown in **Figure 2**, there was no significant difference in PACAP and CGRP in trigeminal nerve and serum between the con group and the operation group (P > 0.05). In comparison with the con group, PACAP and CGRP in trigeminal nerve and plasma of the model group and inhibition group increased greatly (P < 0.05). In comparison with the model group, PACAP and CGRP in trigeminal nerve and plasma in the inhibition group were greatly lower (P < 0.05).

Pearson correlation analysis revealed a positive correlation between PACAP and CGRP (P < 0.05).

Emotional changes in rats

This study compared the depression and anxiety of experimental rats in each group. As shown in **Figure 3**, there was no significant difference in depression and anxiety between the con group and the sham operation group (P > 0.05). Compared with the con group, the depression and anxiety scores of the model group and the inhibition group decreased greatly. In comparison with the model group, the depression and anxiety scores of the inhibition group were greatly higher (P < 0.05). As shown in **Figure 4**, there was no significant difference in 5-HT and dopamine between the con group and the sham operation group (P > 0.05). 5-HT and dopamine in the model group and the
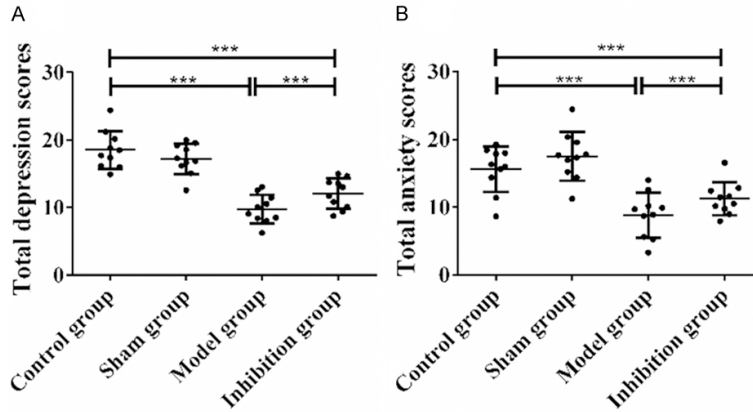
Figure 3. Comparison of depression and anxiety in each group. A. Comparison of total depression scores of each group. Comparison of total anxiety scores of each group. B. Collection of total depression and anxiety scores from experimental rats 5 days after modeling.
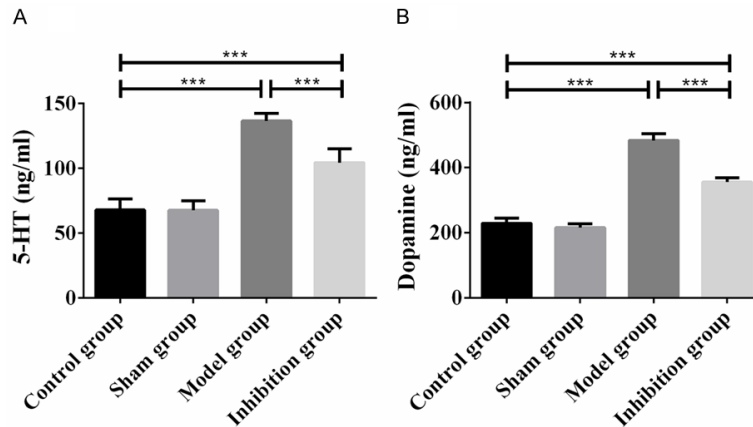
Figure 4. 5-HT and dopamine changes in TG model. A. Comparison of 5-HT levels in each group. B. Dopamine levels in each group. The above data were collected from blood samples of experimental rats 5 days after modeling.

inhibition group were statistically higher than those in the con group (P < 0.05), and 5-HT and dopamine in the inhibition group were significantly lower than those in the model group (P < 0.05). Compared with the con group, the model group showed significant depression and anxiety.

Correlation analysis of PACAP, CGRP and neurotransmitter

As shown in **Figure 5**, there was no correlation between PACAP and CGRP and 5-HT (PACAP vs 5-HT: r = 0.6157, P = 0.0581; CGRP vs 5-HT: r = 0.6194, P = 0.0562), while there was a negative correlation of PACAP with dopamine (r = -0.7763, P = 0.0083), and there was a positive

correlation of CGRP with dopamine (r = 0.6882, P = 0.0278).

Levels of proinflammatory cytokines TNF-α, IL-6 and IL-1β

As shown in **Figure 6**, there was no significant difference in the levels of pro-inflammatory cytokines between the con group and the sham operation group (P > 0.05). The levels of pro-inflammatory cytokines in the model group and the inhibition group were significantly higher than those in the con group (P < 0.05). The levels of pro-inflammatory cytokines in the inhibition group were significantly higher than those in the model group (P < 0.05).

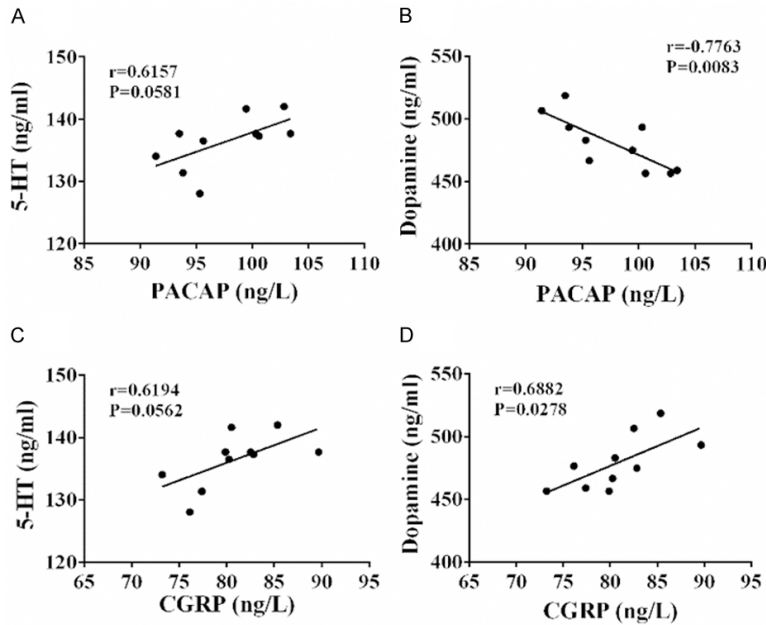*Correlation analysis of PACAP, CGRP and proinflammatory cytokines*

As shown in **Figure 7**, PACAP was negatively correlated with TNF-α, IL-6 and IL-1β (PACAP vs TNF-α: r = -0.6412, P = 0.0457; PACAP vs IL-6: r = -0.7329, P = 0.0159; PACAP vs IL-1b: r = -0.6394, P = 0.0465). CGRP was negatively correlated with TNF-α, IL-6 and IL-1b (CGRP vs TNF-α: r = -7559, P = 0.0114; CGRP vs IL-6: r = -8596, P = 0.0014; CGRP vs IL-1b: r = -8066, P = 0.0048).

Discussion

Migraine is not only the third most prevalent disease, but also the seventh most disabling cause [17]. The pathogenesis of migraine is related to vasoconstriction and relaxation [18]. TG, as the main regulatory center of cerebral blood vessels, plays a crucial role in the transmission of pain. Tardiolo et al. [19] believe that the activation of TG vascular system will lead to the generation of pain. When TG vessels are activated, they will secrete vasoactive neuropeptides such as PACAP and CGRP [20]. The expression of PACAP is the key link of TG's phys-

Figure 5. Correlation Analysis of PACAP, CGRP and neurotransmitter. A. Correlation analysis of PACAP vs 5-HT. B. Correlation analysis of PACAP vs dopamine. C. Correlation analysis of CGRP vs 5-HT. D. Correlation analysis of CGRP vs dopamine. All the above data were collected from blood samples of experimental rats 5 days after modeling, using person correlation analysis.

PACAP and CGRP [20], and PACAP and CGRP are involved in the development and transmission of pain. Schtyz et al. [10] suggested that PACAP activated PAC1 receptor, which resulted in the increase of camp in the dural pain receptor, and finally promoted the development of migraine. Vasodilation may be an essential factor in migraine development [20]. Brain et al. [12] believed that the extravascular release of CGRP can promote blood circulation and cause hyperemia. Therefore, the increase of CGRP level will stimulate the increase of blood flow and promote the spread and development of pain. In addition, these results also suggest that inhibition of PACAP and CGRP may be beneficial to migraine relief.

iological function [21]. Edvinsson et al. [22] discussed the relationship between PACAP and migraine, and believed that PACAP and its receptor could be used as therapeutic targets. CGRP is the trigger point of migraine mechanism; regulating its expression level in migraine patients may improve their symptoms. Silberstein et al. [23] found that the CGRP antibody fremanezumab can effectively alleviate the frequency of headache in migraine patients.

The results of pain behavior in this study showed that the model group had lower tolerance to pain, while inhibition of PACAP and CGRP was beneficial to the enhancement of tolerance. In this study, the levels of PACAP and CGRP in trigeminal nerve and plasma were measured. The results revealed that the levels of PACAP and CGRP in the model group were higher than those in the con group (P < 0.05), while the levels of PACAP and CGRP in the inhibition group were lower than those in the model group (P < 0.05). These results imply that the increase of PACAP and CGRP is related to migraine. The correlation between PACAP and CGRP suggests that they may be involved in the same pathway. The relationship between PACAP and CGRP and migraine is complex and mutual. TG can induce the release of

Zhang et al. [16] thought that most of the migraine rats showed depression and anxiety when studying the depression and anxiety behaviors of the chronic migraine rats. This study also studied the depression and anxiety of each group. The results of depression and anxiety scale and neurotransmitter showed that migraine rats showed significant depression and anxiety, and the levels of 5-HT and dopamine increased, which was in line with the results of Zhang et al. In addition, the correlation between PACAP, CGRP, and neurotransmitters was analyzed. The results revealed that there was no relationship between PACAP and CGRP and 5-HT (P > 0.05), but there was a negative relationship between PACAP and dopamine (P < 0.05) and a positive relationship between CGRP and dopamine (P < 0.05). It has been found in a previous study [24] that a-CGRP can cause the release of dopamine in rat brain. Therefore, the increase of CGRP level in migraine rats can cause the increase of dopamine and other neurotransmitters, so it has a positive correlation. Takei et al. [25] found that PACAP can increase the number of the immunoreactive neurons, increase the protein, and thus increase dopamine metabolism. In addition, the increase of dopamine level in
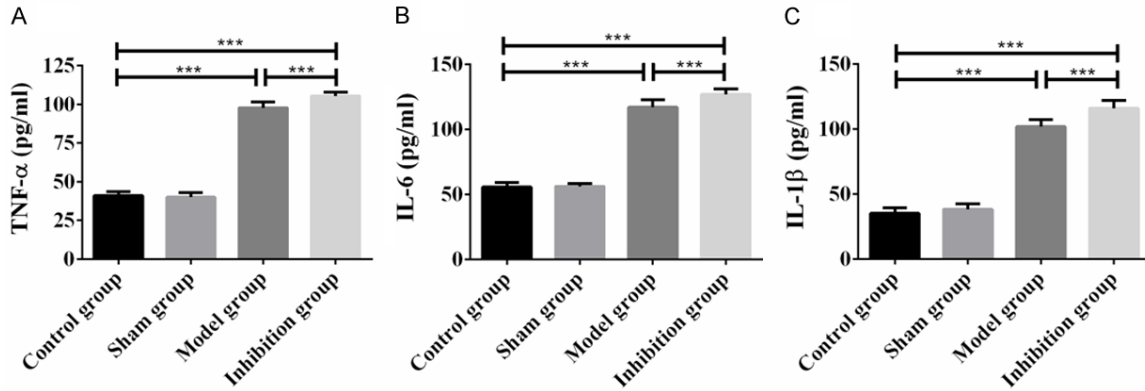
Figure 6. Comparison of TNF-α, IL-6 and IL-1β in each group. A. Comparison of TNF-α. B. Comparison of IL-6 level in each group. C. Comparison of IL-1β level in each group. All the above data were collected from blood samples of experimental rats 5 days after modeling.
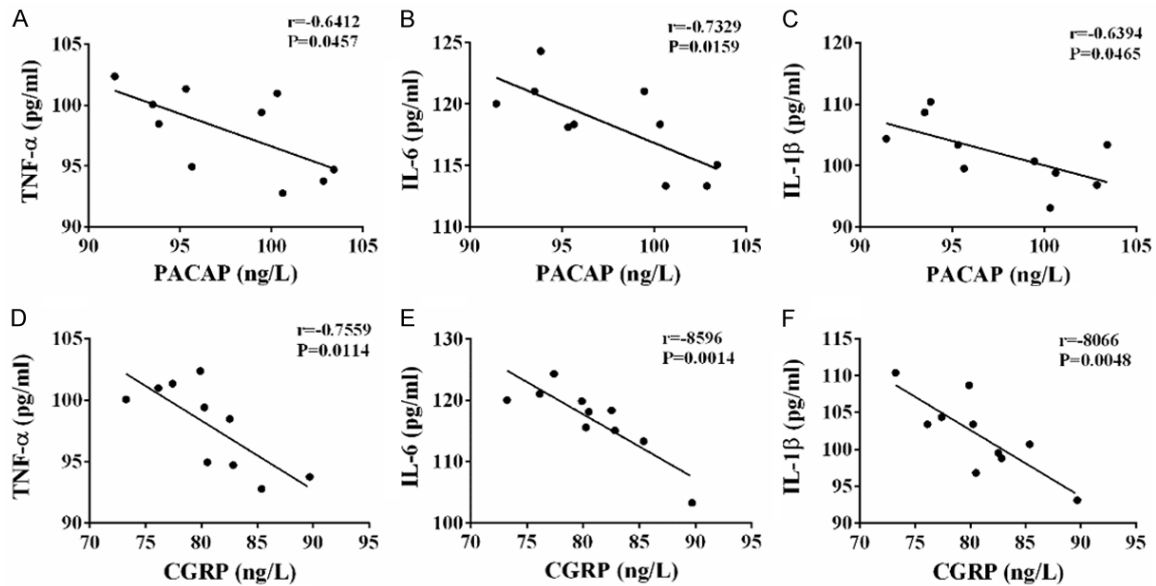
Figure 7. Correlation analysis of PACAP, CGRP and proinflammatory cytokines. A. PACAP-TNF-α correlation analysis. B. PACAP-IL-6 correlation analysis. C. Pacap-IL-1β correlation analysis. D. CGRP-TNF-α correlation analysis. E. Cgrp-IL-6 correlation analysis. F. CGRP-IL-1β correlation analysis. All the above data were collected from blood samples of experimental rats 5 days after modeling, using Pearson correlation analysis.

migraine rats may indicate that CGRP is more effective than PACAP.

This study analyzed the levels of proinflammatory cytokines in migraine rats, and found that the levels of proinflammatory cytokines in migraine rats increased, but the inflammation in migraine rats increased after inhibiting the expression of PACAP and CGRP. In addition, we also analyzed the correlation between PACAP, CGRP and proinflammatory cytokines, and found that PACAP and CGRP were negatively correlated with proinflammatory cytokines.

Some studies [26] have verified that CGRP has an anti-inflammatory effect and can inhibit the development of inflammation, which may be the reason for the negative correlation between CGRP and pro-inflammatory cytokines. Azuma et al. [27] found that the levels of proinflammatory cytokines including IL-1β and IL-6 in PACAP deficient mice were higher than those in normal wild-type mice, and believed that PACAP could inhibit the synthesis of proinflammatory cytokines, which may be the reason why PACAP was negatively correlated with proinflammatory cytokines. Although PACAP and CGRP can

inhibit the secretion of pro-inflammatory cytokines, the elevation in the levels of pro-inflammatory cytokines in migraine rats suggests that there are other substances involved in the regulation of pro-inflammatory cytokines.

In our study, the changes of PACAP and CGRP in migraine model rats were studied, and the correlation between PACAP and CGRP, neurotransmitters and proinflammatory cytokines was studied. This study found that PACAP and CGRP may be involved in the same pathway, so in the future experimental design, we will discuss which pathway they are involved in together with migraine mechanism and its role in the pathway, so as to provide more accurate information for migraine treatment sites.

To sum up, this study compared the expression of PACAP and CGRP in trigeminal nerve of the con group, sham operation group, model group and inhibition group. It was considered that PACAP and CGRP were secreted at a high level in trigeminal nerve of migraine rats, and the symptoms of migraine were relieved after the treatment with their inhibitors. Therefore, PACAP and CGRP could be used as treatment targets when formulating treatment strategies for migraine.

Disclosure of conflict of interest

None.

Address correspondence to: Feng Han, Department of Human Anatomy, Jilin Medical University, No. 5 Jilin Street, Jilin 132013, Jilin Province, China. E-mail: han9598610@126.com

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