Research Article

MOLECULAR PAIN

Molecular Pain
Volume 19: 1–13

DOI: 10.1177/17448069231152129
journals.sagepub.com/home/mpx
SAGE

Temporal alterations of pituitary adenylate cyclase activating polypeptide and its receptors in a rat model induced by recurrent chemical stimulations: Relevant to chronic migraine

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Abstract

Background: Migraine is a common type of primary headache with disabling brain dysfunction. It has been found that pituitary adenylate cyclase activating polypeptide (PACAP) is involved in the pathogenesis of migraine, however, the role of PACAP and its receptors in chronic migraine remains unclear. Therefore, the present study aimed to explore the changes of PACAP and its receptors in different duration after recurrent dural inflammation soup stimulations and to investigate the co-expression between PACAP and calcitonin gene-related peptide (CGRP). **Methods**: Adult male rats were implanted with cannula surrounding superior sagittal sinus, which was followed by dural infusion of inflammatory soup (IS) or normal saline (NS). The rats were randomly divided into 4 groups (n = 8 for each group): IS stimulation for seven days (IS-7 group), IS stimulation for 14 days (IS-14 group), IS stimulation for 21 days (IS-21 group), and NS control for 21 days (CON group). The facial mechanical withdrawal threshold was daily measured during the whole experiment. The behavioral changes (ipsilateral and bilateral face grooming behavior) in a plastic cage of rats were observed and recorded. The expression of PACAP, its receptors (PAC1, VPAC1, VPAC2), and CGRP in the trigeminal ganglia (TG) and the trigeminal nucleus caudalis (TNC) was examined by immunohistochemistry. Immunofluorescence was used to explore the co-expression of PACAP, PAC1 receptor, and CGRP after repeated IS administration in the TG. **Results**: The ipsilateral facial grooming time of IS-21 group displayed an apparent increase than CON group after repeated stimulation on day 2, while significant differences were observed on day 14. No differences were found between the IS-21 and CON group in bilateral facial grooming. Dural IS stimulation induced a significantly decrease in facial mechanical withdrawal thresholds. PACAP positive cells in the regions of TNC were gradually decreased with the IS days increasing. PACAP and PAC1 receptor expression in the TG had a trend of increasing first and then decreasing. There was no significant difference in expression of VPAC1 and VPAC2 in the TG and the TNC. Immunofluorescence showed that PACAP was mainly expressed in TG neurons. PACAP and PAC1 receptor co-expression decreased gradually after repetitive IS stimulation. While the co-expression between PACAP and CGRP reached the peak in IS-7 group after repetitive IS stimulation, and then decreased. **Conclusions**: This study demonstrated that repetitive chemical stimulations

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induced a gradual decrease of PACAP in the TNC, while the PACAP and PAC1 receptor expression in TG showed dynamical changes of increasing first and then decreasing after repeated IS administration. These results suggested exhaustion of PACAP could be involved in the duration of chronic migraine and implied PACAP may contribute to the pathology of migraine through the PAC1 receptor, which was associated with CGRP.

Keywords
Pituitary adenylate cyclase activating polypeptide, PAC1 receptor, calcitonin gene-related peptide, migraine, inflammatory soup

Date Received: 27 June 2022; Revised 5 November 2022; accepted: 4 January 2023

Introduction

Migraine is a neurological disorder that has been listed as the second most disabling disease worldwide by the World Health Organization.[1] The clinical features include unilateral pulsating headache associated with nausea, vomiting, photophobia and phonophobia. According to epidemiological reports, the one-year prevalence of migraine is 17.1% in women and 5.6% in men,[2] which significantly burdens on both sufferers and society.

Activation of dural meningeal nociception releases neuropeptides, such as calcitonin gene-related peptide (CGRP), pituitary adenylate cyclase activating polypeptide (PACAP), and vasoactive intestinal peptide (VIP) from nerve endings of perivascular nerve fibers,[3,4] resulting in mast cell activation, degranulation, and subsequently release of inflammatory mediators such as interleukin (IL)-6 and vascular endothelial growth factor (VEGF). Previous research suggested that sensitization of dura-sensitive peripheral nociceptors could lead to enhanced responses in central neurons.[5] Inflammatory soup (IS) has been successfully used to study the mechanisms of meningeal and trigeminovascular nociception. Using repeated dural IS infusions in rats, it can modeled the repeated episodic activation of the trigeminovascular system seen in patients with recurrent migraine headache.[6]

Pituitary adenylate cyclase activating polypeptide belongs to VIP-glucagon-growth hormone releasing factor-secretin superfamily.[7] There are two forms of PACAP: 38 amino acid peptide (PACAP38) and 27 amino acid form (PACAP27). PACAP38 is the predominant form in the CNS. In this manuscript, PACAP-38 was referred to simply as PACAP. PACAP plays a vital role in vasodilation, immune system, inflammation[8] and primary headache.[9] In migraine patients, intravenous infusion of PACAP can trigger migraine-like headaches.[10] Vasoactive intestinal peptide causes vasodilation of meningeal arteries for a short time (1–5 min), while PACAP can trigger delayed activation and sensitization of central trigeminal neurons after 90 min.[11] PACAP levels in the plasma were significantly lower in the interictal of migraineurs than in the healthy control. In contrast, increased peptide levels were measured in the ictal period.[12] Sumatriptan can reduce PACAP release in patients with migraine.[13] Furthermore, an MRI study revealed significant and sustained vasodilation of extracranial arteries following PACAP administration.[14] PACAP-induced migraine attacks are associated with altered connectivity of several large-scale functional networks.[15]

Pituitary adenylate cyclase activating polypeptide exerts actions through three G protein receptors: PACAP-selective PAC1 receptor (Adcyap1r1), vasoactive intestinal peptide receptor 1 (VPAC1) and vasoactive intestinal peptide receptor 2 (VPAC2). Among them, VPAC1 and VPAC2 are the receptors shared by PACAP and VIP, whereas PAC1 receptor has a high affinity for PACAP.[16] PACAP has a strong vasodilatory effect on the middle meningeal artery, which may be related to the high expression of PAC1 receptor in the middle meningeal artery.[17] Inhibition of the PAC1 receptor with a monoclonal antibody reduces nociceptive TNC activity.[11] Sandor et al. found that an overall excitatory role of PACAP in pain transmission originating from both exteroceptive and interoceptive areas, which is also involved in central sensitization. This can be explained by the signal transduction mechanisms of its identified receptors, both PAC1 and VPAC activation leads to neuronal excitation.[18]

The role of PACAP in migraine strikingly resembles clinical efforts with CGRP. CGRP is a neuropeptide selectively expressed by the calcitonin gene, which has been proven to be a validated target in migraine. In TG, Eftekhari et al. found co-expression of PACAP and CGRP was found in around 20% of the immunoreactive neurons.[19] In vivo data demonstrates that PACAP induces the release of CGRP from the TNC.[20] However, another study showed that PACAP administration does not induce the release of CGRP in a human model of migraine.[21] It suggested that PACAP-induced effects on trigeminal activation may be not directly affect on CGRP-mediated mechanism. At present, the interaction between PACAP and CGRP remains unclear.

Therefore, a rodent model of chronic migraine was established by stimulating the superior sagittal sinus with inflammatory soup of rats. This study investigated the temporal changes and the co-expression of PACAP, CGRP and PACAP

receptors to determine the pathophysiological mechanism of PACAP in migraine.

Methods

Animals

Male Sprague-Dawley rats (weight 180–200 g) were obtained from the Laboratory Animal Center of the Chinese PLA 302 Hospital. All animals in this experiment were approved by the Research and Education of the Laboratory Animals Center at Chinese PLA General Hospital and consistent with the ethical guidelines for investigations of experimental pain in conscious animals.[22] All animals were housed in individual cages with adequate food and water in a temperature (21°C ± 3°C) and humidity-controlled (50 ± 10%) room with 12 h dark-light cycle.

Surgery

As previous research,[23] the experimental apparatus and detailed stimulation procedures were described. Rats were anesthetized intraperitoneally (i. p.) with 1% pentobarbital sodium (45 mg/kg) and placed onto a stereotaxic apparatus with a heating blanket under the body. The scalp was incised along the midline to expose the skull. The left frontal bone (1 mm caudal to the bregma and 2 mm lateral to the midline) was slowly drilled to expose the dura mater. A plastic cap with a stainless steel inner cannula (RWD Life Science Co., Ltd., Shenzhen, China) was implanted in the drilled cranial window. Two small screws were implanted around the cannula and fixed it to the skull with dental cement. The cannula was sealed with an obturator cap to prevent obstruction by scar tissue.

Experimental procedures

Thirty two rats were randomly divided into 4 groups: inflammatory soup stimulation for 7 days (IS-7 group, $n = 8$), inflammatory soup stimulation for 14 days (IS-14 group, $n = 8$), inflammatory soup stimulation for 21 days (IS-21 group, $n = 8$), and normal saline for 21 days (CON group, $n = 8$). Since the animals would be in a pain state, the sample size of rats was restricted in minimum necessary to yield statistical significance. For immunohistochemistry and immunofluorescence, sample size was estimated based on our previous experience.

The rats were given three days to acclimatize prior to surgery. Researchers used Von Frey filaments to touch their outer canthus, inner canthus, and vibrissal pad to test apparatus and measure the baseline thresholds of rats. A cannula was implanted as described earlier.[23] Following surgery, all rats rested for three days to recover the facial mechanical withdrawal thresholds to baseline. According to the corresponding final concentration, the ratio of 1 mM histamine, 1 mM serotonin, 1 mM bradykinin, and 0.1 mM prostaglandin E2 was mixed into inflammatory soup (IS). The rats in the IS-7, IS-14, and IS-21 groups received a 10 μL IS once daily for seven days, 14 days, and 21 days, respectively. The rats in CON group received a 10 μL normal saline once daily for 21 days followed the same timeline of the IS-21 group. The infusion time was controlled for over 5 min. The connection was kept for 10 min to prevent the inflammatory soup from spilling and ensure the absorption of medicine.

Facial mechanical withdrawal threshold

The facial mechanical withdrawal threshold was measured 10 min before IS/NS stimulation and 30 min after administration. The experimenters were blinded to the grouping during the tests. Six patches of facial skin (bilateral vibrissal pads, bilateral outer and inner canthus) were tested using traditional Von Frey filaments (North Coast Medical Co., Ltd., USA) with the recommended force values (26, 15, 10, 8, 6, 4, 2, 1.4, 1, 0.6, and 0.4 g), up to 3 to 6 s until a positive response. The thresholds were determined using the "up-down" paradigm,[24] set at 26 g when the rats did not respond to the 26 g filament test. The average of three measurements was calculated to obtain the experimental withdrawal threshold for each rat. The positive responses were observed: withdrawal reaction, escape, attack response, or asymmetrical facial grooming.[25]

Behavioral test

The free behavior of rats in plastic cages was observed. The experimenters were blinded to the grouping during the tests. The behavior was videotaped for 10 min, starting 10 min before stimulation and 30 min after IS/NS administration in the cage. The high-definition camera was positioned to ensure that the field of view was directly above the rats' cage. All personnel were prohibited from staying in the observation room during the recording period to reduce the stimulation of external environment and human factors on behavior.[26] The video analysis focused exclusively on ipsilateral and bilateral face grooming behavior. Melo-Carrillo et al.[27] found that the animals that received the IS infusion displayed an unusual pattern in facial grooming which was called ipsilateral hindpaw facial grooming. The rats performed a more intense facial grooming behavior with only one hindpaw and always ipsilateral to the place of the cannula implantation.

Immunohistochemistry

The expression of PACAP, PAC1, VPAC1, VPAC2, and CGRP in the trigeminal ganglion (TG) and the TNC were measured using an immunohistochemistry assay. To avoid the measuring acute changes, the rats were sacrificed and sampled 24 h after the final inflammatory soup stimulation

or normal saline infusion. TNC and bilateral TG were embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA, USA). The samples were placed in liquid nitrogen and frozen for 10–15 sec and then were cut into 20 μm-thick serial sections on a freezing microtome (CM1850; Leica, Wetzlar, Germany). The sections were blocked with fresh goat serum, which was mixed with 100 μL goat serum (ZLI-9021, ZSGB-Bio, China), 50 μL 10% TritonX-100, and 850 μL PBS, and incubated in a 37°C incubator for 20 min. Then, the sections were incubated with respective diluted primary antibodies for anti-PACAP (1:80, sc-25439, Santa Cruz Biotechnology, USA), anti-PAC1 (1:400, ab54980, Abcam, USA), anti-VPAC1 (1:50, sc-30019, Santa Cruz Biotechnology, USA), anti-VPAC2 (1:50, sc-30020, Santa Cruz Biotechnology, USA), and anti-CGRP (1:50, ab81887, Abcam, USA) at 4°C overnight. After washing off the primary antibodies, the sections were stained with anti-mouse/rabbit secondary antibodies (1:1, KIT-5030, Maixin Biological Technology, China) at room temperature for 2 h.

Immunofluorescence

The frozen sections were blocked using goat serum and then incubated at 4°C overnight with respective diluted primary antibodies for anti-PACAP (1:50, ab174982, Abcam, USA), anti-PAC1 (1:400, ab54980, Abcam, USA), anti-CGRP (1:50, ab81887, Abcam, USA) and anti-NeuN (1:3000, MAB337, Merck Millipore, Germany). After washing off the primary antibodies, the sections were incubated with goat anti-rabbit (1:500, A-11034, Thermo Fisher, USA) and goat anti-mouse (1:1000, A-21424, Thermo Fisher, USA) at darkroom temperature for 3 h. Immunohistochemistry and immunofluorescence were observed through a 20x magnification of TG and 40x magnification of TNC on a microscope (DP73, Olympus, Tokyo, Japan). Six sections were selected from each rat TG at 20 magnification, and one image was selected from each section. Three sections were selected from each rat TNC at 40 magnification, and bilateral images were selected from each one. The average integrated density of six images was defined as the final result for each animal.

Statistical analysis

SPSS 19.0 software was used for the statistical analysis, GraphPad Prism 6.0 was used to generate the graphs. Levene's test analyzed data homogeneity and distributions. Abnormally distributed data were evaluated using Kruskal–Wallis test, and normally distributed data were investigated using analysis of variance (ANOVA) and Fisher's least significant difference test when variance was regular or Dunnett's T3 test with irregular variance. All data were reported as mean ± standard deviation, and a P-value <0.05 was considered statistically significant.

Results

Decreased facial mechanical withdrawal thresholds by dural inflammatory stimulation

This study tested six aspects of the face (the bilateral inner and outer canthus, bilateral vibrissal pads) before and after IS/NS administration. First, the facial mechanical withdrawal threshold was tested before IS/NS administration to measure sensitization of the trigeminal nerve Figure 1. The mechanical thresholds among six aspects in IS-21 group decreased gradually with increased stimulation days, while those in CON group remained mostly unchanged. The mechanical threshold of bilateral inner canthus in IS-21 group decreased from the second day (14.25 ± 7.94 vs. 26±0, $P < 0.01$) and decreased significantly for 21 days (1.122 ± 1.63 vs. 26 ± 0, $P < 0.001$) (Figure 1(a)). After seven days of IS administration, the mechanical thresholds of bilateral outer canthus were significantly lower than CON group (2.66 ± 5.01 vs. 26 ± 0, $P < 0.001$) (Figure 1(b)). Similarly, for the vibrissal pads, the IS-21 group exhibited an apparent decreased mechanical threshold between day 3 (11.81 ± 8.57 vs. 26 ± 0) until day 17 (2.89 ± 5.28 vs. 26±0) ($P < 0.001$) (Figure 1(c)). The curves of inner canthus and vibrissal pads groups were overlapping, while the curves of outer canthus were above the two groups, as depicted in Figure 1(d). This illustrated that the thresholds of outer canthus decrease more slowly than the inner canthus and vibrissal pads. However, no significant differences were observed among the bilateral inner and outer canthus, bilateral vibrissal pads in IS-21 group from day 1 to day 7 ($P > 0.05$).

Second, the daily changes of facial mechanical withdrawal threshold after IS/NS administration were measured. IS-21 group exhibited a greater decrease than CON group in all six aspects of the face as the day progressed (Figure 1(e)–(g)). Compared with the outer canthus, the differences in mechanical thresholds of the inner canthus and vibrissal pads in the IS-21 group on day 1 (24.63 ± 3.89 vs. 10.88 ± 3.83 vs. 13 ± 5.83) and 2 (20.75 ± 7.76 vs. 9.75 ± 5.65 vs. 11.13 ± 7.97) were statistically significant ($P < 0.01$) (Figure 1(h)).

Increased ipsilateral facial grooming time by dural inflammatory stimulation

This study tested two nociceptive behaviors (ipsilateral and bilateral facial grooming). The facial grooming was daily measured before and after IS/NS administration. There were no significant differences in ipsilateral facial grooming before IS/NS administration (Figure 2(a)). However, as illustrated in Figure 2(b), the ipsilateral facial grooming of IS-21 group (28.67 ± 18.03) displayed an apparent increase than CON group (13.00 ± 10.00) after repeated IS administration on day two, while significant differences of IS-21 group (28.00 ± 9.09) were observed on day 14 than CON group (8.40 ± 8.85) ($P < 0.05$). Additionally, no differences were found between

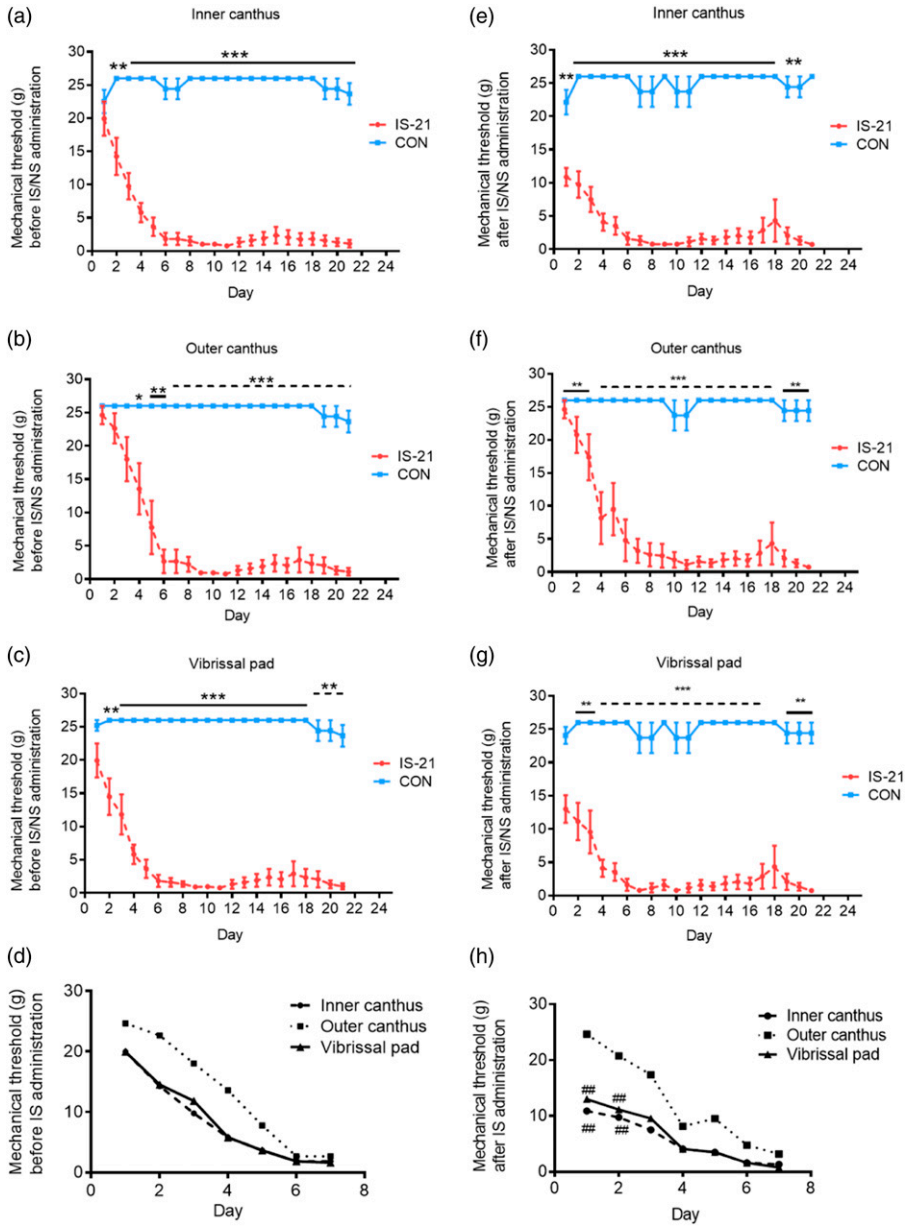

Figure 1. Facial mechanical withdrawal thresholds decreased by dural inflammatory stimulation. The mechanical thresholds of (a) Inner canthus, (b) outer canthus and (c) vibrissal pads before IS administration decreased gradually with increased stimulation days in IS-21 group on the four left graphs. (d) the thresholds of the outer canthus decrease more slowly than those of the inner canthus and vibrissal pads. The right four graphs showed the mechanical thresholds of (e) Inner canthus, (f) outer canthus, (g) vibrissal pads decreased gradually with increased stimulation days in IS-21 group after IS administration. (h) Compared with the outer canthus after IS administration, the differences in mechanical thresholds of the inner canthus and vibrissal pads in IS-21 group on day 1 and 2 were statistically significant ($^{\#\#}P < 0.01$). Data are presented as the mean ± SD, $n = 8$. Compared with the CON group: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Compared with outer canthus: $^{\#\#}P < 0.01$

IS-21 and CON groups in bilateral facial grooming before or after IS/NS administration (Figure 2(c) and (d)). The ipsilateral facial grooming behavior of rodents was observed in a variety of migraine models,[27,28] which was consistent with our results. From video recordings, rats' frequent and long-term ipsilateral facial grooming behavior was observed, proving that the behavior mimics the clinical feature of patients during migraine attacks.

Changes in the expressions of PACAP, CGRP, and PACAP-related receptors in TG and TNC after repeated IS administration

Immunohistochemistry analysis investigated changes in the expressions of PACAP, CGRP, and PACAP-related receptors in TG and TNC after repeated IS/NS administration. PACAP expression in TNC decreased gradually with increasing IS

administration days (Figure 3(b)). The number of IS-7 group (59.62 ± 11.68) was the highest among the other three groups. IS-21 group (31.00 ± 3.59) was lower than CON group (41.71 ± 8.24) ($P < 0.05$). PACAP expression in TG reported an increasing trend, which later decreased (Figure 3(c)). The number of PACAP expression in IS-14 group (58.86 ± 11.81) was the highest. The PACAP expression level of IS-21 group (41.13 ± 8.22) was lower than that of CON group (64.00 ± 9.21) ($P < 0.001$).

PAC1 receptor was decreased slowly as the time passed in TNC (Figure 3(e)): the expression level of IS-21 group (32.67 ± 6.77) was significantly lower than CON groups

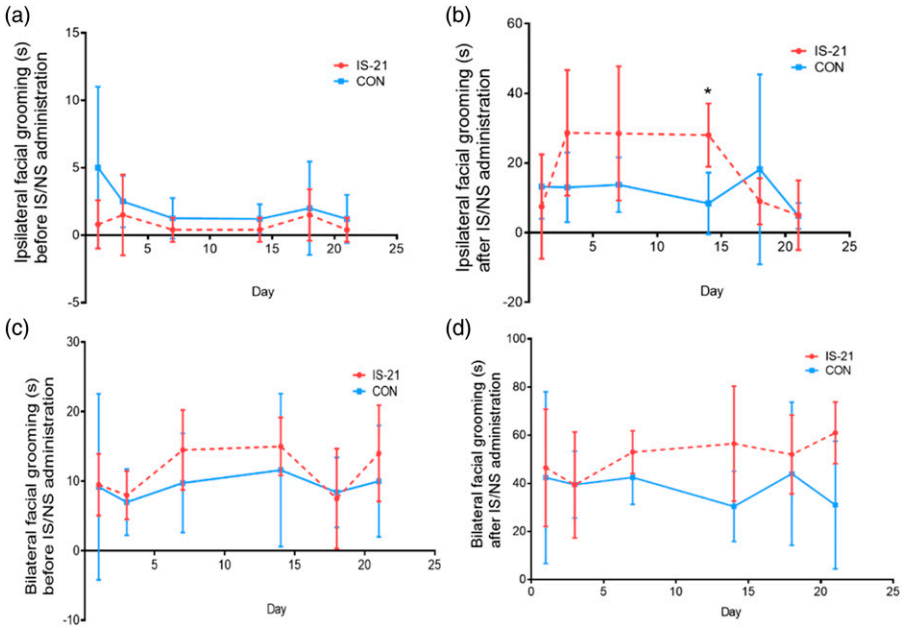

Figure 2. The ipsilateral facial grooming was induced by recurrent chemical stimulations. Ipsilateral facial grooming (a, b) and bilateral facial grooming (c, d) before and after repeated IS/NS administration in 21 days. Data are presented as the mean ± SD, $n = 8$. The ipsilateral facial grooming time of IS-21 group displayed an apparent increase than CON group after repeated stimulation on day 2, while significant differences were observed on day 14 ($^*P < 0.05$). As for bilateral facial grooming, no apparent differences were found before and after IS/NS administration.

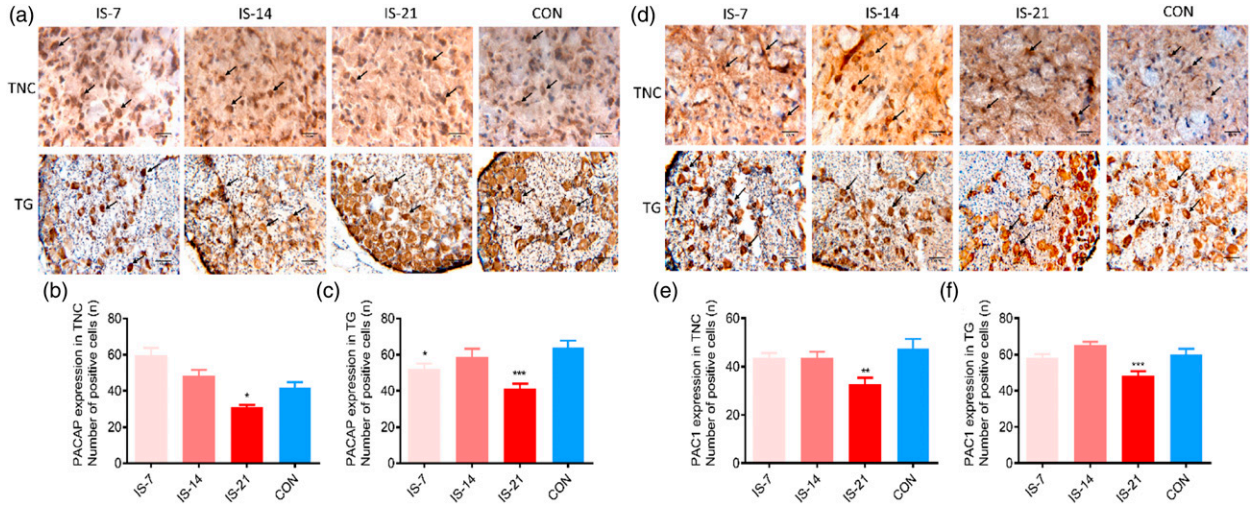

Figure 3. Expression of PACAP and PAC1 in TG and TNC after repeated IS/NS administration. Data are presented as the mean ± SD, $n = 8$. (a, d) Immunohistochemistry staining of PACAP and PAC1 in the TNC and TG. The average number of positive cells were showed at 20 magnification of the TG and at 50 magnification of the TNC. (b) PACAP expression in TNC decreased gradually with increasing IS administration days. (c) PACAP expression in TG reported an increasing trend, which later decreased. (e, f) PAC1 receptor showed similar changes as PACAP in the TNC and TG. Compared with the CON group: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

(47.43 ± 10.52) ($P < 0.01$). Similar to the expression trend of PACAP, the level of PAC1 in the TG increased initially until day 14 and then decreased gradually by the repeated IS administration (Figure 3(f)). PAC1 receptor expression in IS-14 group (65.13 ± 5.57) was slightly higher than IS-7 group (58.14 ± 5.46). Expression was decreased in the IS-21 group (48.42 ± 6.32) compared to the CON group (61.67 ± 7.53) ($P < 0.001$).

Different from the changes in PAC1 receptor, VPAC1 and VPAC2 receptor in TNC had a trend of decreasing first and then increasing (Figure 4(b) and (e)). No significant difference was observed among the four groups in the TNC. In TG, VPAC1 and VPAC2 receptor levels increased slightly from day 7 to 14 (Figure 4(c) and (f)). The numbers of VPAC1 and VPAC2 in IS-21 group were not statistically significant compared with CON group, respectively.

Calcitonin gene-related peptide expression in TG and TNC decreased gradually with the prolongation of repeated IS administration (Figure 5). Compared with CON group (TG: 27.17 ± 5.19; TNC: 41.57 ± 5.53), IS-21 (TG: 15.13 ± 2.59; TNC: 32.17 ± 5.27) group was significantly lower in TG ($P < 0.001$) and TNC ($P < 0.01$).

The co-expression of PACAP, CGRP, and PAC1 receptor decreased in the TG after repeated IS administration

Immunofluorescence explored the co-expression of PACAP, PAC1 receptor, and CGRP after repeated IS administration, and further elucidation its role in the pathogenesis of migraine chronification. This study suggested that PACAP was expressed in neurons, and the expression level was consistent with immunohistochemistry results (Figure 6). The number of positive cells co-expressing PACAP and neurons in TG increased initially and then decreased with increasing days of IS administration. Second, as presented in Figure 7, the co-expression between PACAP and CGRP in TG gradually decreased after repeated IS administration. The co-expression of PACAP and CGRP in the IS-7 group (13.00 ± 2.28) was the highest, and IS-7 group was significantly higher than CON group (8.75 ± 1.71) ($P < 0.01$). IS-21 group (7.20 ± 1.30) was slightly lower than CON group in co-expression. Third, similar to the trend of co-expression of PACAP and neurons, the co-expression of PACAP and PAC1 receptor decreased gradually with increased IS infusion days (Figure 8). There was no significant difference among the four groups.

Discussion

This study successfully established a chemical simulation model using IS administration to dural surrounding superior sagittal sinus, which reliably mimics chronic migraine presentation confirmed by these behavioral data. Similar to unilateral headache in migraineurs, it was found that ipsilateral facial grooming time significantly increased in IS-21 group. This spontaneous behavior is characterized by a more intense facial grooming performed with hindpaw, ipsilateral to the place of cannula implantation and IS infusion. Similarly, a study showed that the ophthalmic branch of the trigeminal nerve sensitization leads to ipsilateral hindpaw facial

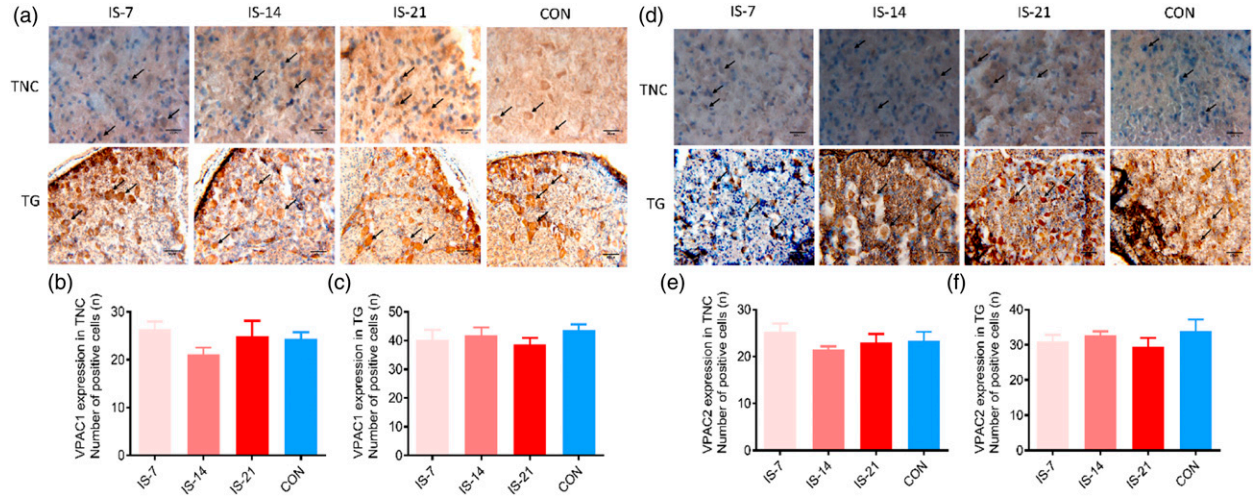

Figure 4. Expression of VPAC1 and VPAC2 in TG and TNC after repeated IS/NS administration. Data are presented as the mean ± SD, $n = 8$. (a, d) Immunohistochemistry staining of VPAC1 and VPAC2 in the TNC and TG. The average number of positive cells were showed at 20 magnification of the TG and at 50 magnification of the TNC. (b, e) Both VPAC1 and VPAC2 receptor in TNC showed a trend of decreasing first and then increasing. (c, f) In TG, VPAC1 and VPAC2 receptor levels increased slightly from day 7 to 14. No significant difference was observed among the 4 groups in the TNC and TG.

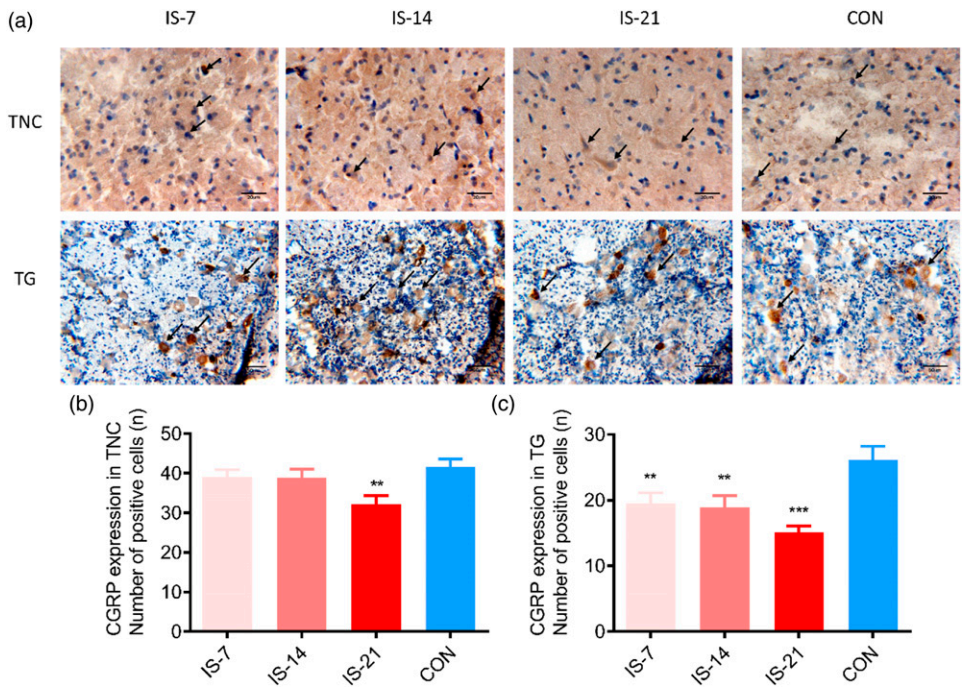

Figure 5. Expression of CGRP in TG and TNC after repeated IS/NS administration. Data are presented as the mean ± SD, $n = 8$. (a) Immunohistochemistry staining of CGRP in the TNC and TG. The average number of positive cells were showed at 20 magnification of the TG and at 50 magnification of the TNC. (b, c) CGRP expression in TG and TNC decreased gradually with the prolongation of repeated IS administration. Compared with CON group, IS-21 group was significantly lower in TG ($P < 0.001$) and TNC ($P < 0.01$). Compared with the CON group: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

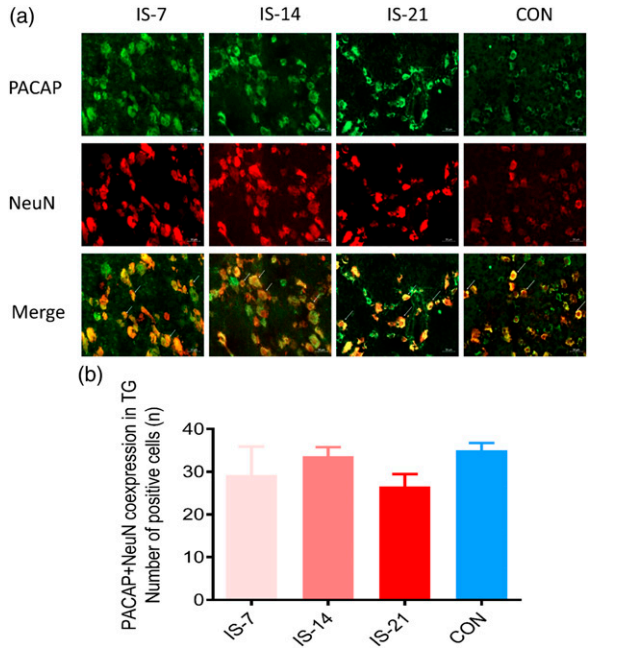

Figure 6. The co-expression of PACAP and neurons in the TG after repeated IS administration. (a) Immunofluorescence staining of PACAP and neurons in the TG. (b) The number of positive cells co-expressing PACAP and neurons in TG increased initially and then decreased with increasing days of IS administration. Data are presented as the mean ± SD, $n = 8$.

grooming.[28] In addition, previous study also verified that the time of spontaneous resting behavior increased in IS group while it decreased in the exploration behavior.[27] These observations could correlate to the clinical symptoms during the migraine attack. This model conforms to the ethical standards of experimental animals and was suitable for establishing a chronic migraine model more than one week.

Inflammatory soup has been successfully used to study the mechanisms of meningeal and trigeminovascular nociception. Using repeated dural IS infusions in rats, Michael et al. modeled the repeated episodic activation of the trigeminovascular system seen in patients with recurrent migraine headache.[6] The significant decrease in the thresholds is due to the successive stimulation with IS which is more than 8 times IS infusion. To avoid the measuring acute changes, the rats were sacrificed and sampled 24 h after the final IS stimulation. In this study, the mechanical withdrawal threshold curve of IS-21 group decreased gradually with the increasing time of IS administration. After one week of IS administration, the mechanical withdrawal thresholds of IS-21 group were significantly lower than CON group. In contrast, the mechanical withdrawal thresholds among outer canthus, inner canthus, and vibrissal pad remained unchanged in CON group as time passed. Moreover, the mechanical withdrawal threshold of lateral canthus decreased more slowly than the inner canthus and perinasal region as the experimental days progressed. It

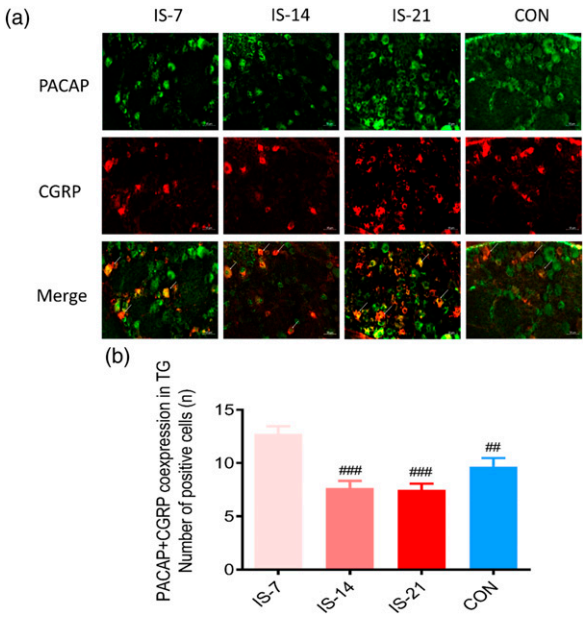

Figure 7. The co-expression of PACAP and CGRP in the TG after repeated IS administration. (a) Immunofluorescence staining of PACAP and CGRP in the TG. (b) The co-expression between PACAP and CGRP in TG gradually decreased after repeated IS administration. Data are presented as the mean ± SD, $n = 8$. Compared with the IS-7 group: $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$.

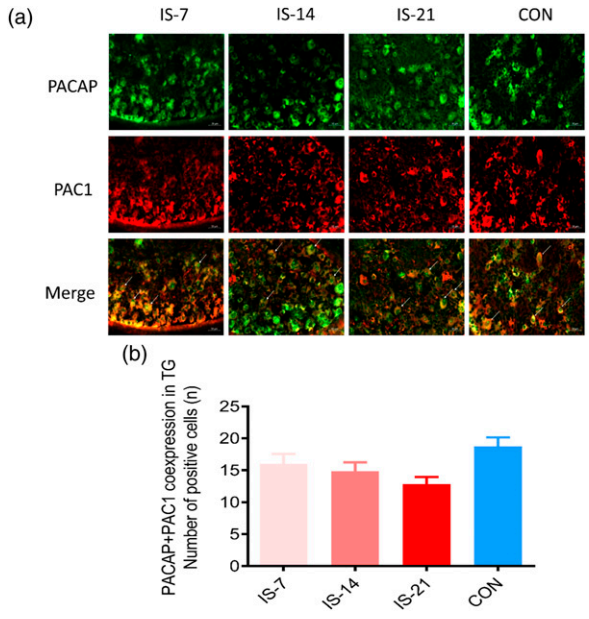

Figure 8. The co-expression of PACAP and PAC1 in the TG after repeated IS administration. (a) Immunofluorescence staining of PACAP and PAC1 in the TG. (b) The co-expression of PACAP and PAC1 receptor decreased gradually with increased of stimulus time. Data are presented as the mean ± SD, $n = 8$.

might be related to the difference in TNC activation caused by the transmission of nociceptive behavior from different branches of the trigeminal nerve. There may be more neurons in the inner canthus and perinasal region in the maxillary branch of the trigeminal nerve (V2) than in the ocular branch of the trigeminal nerve (V1) of the outer canthus peripheral sensitivities are faster.[29,30]

Activating the trigeminovascular system (TGVS) releases neuropeptides, resulting in mast cell degranulation and central sensitization.[31,32] Pituitary adenylate cyclase activating polypeptide plays an important role in the pathogenesis of migraine. Intravenous infusion of PACAP can trigger migraine-like headaches in migraineurs.[10] The central nociceptive effect of PACAP has also been observed in several mouse pain models, suggesting that PACAP function in pain transmission is excitatory.[18] Markovics et al. found that there was no change in the meningeal blood flow in response to nitroglycerin (NTG) injected in the first 2 hours in PACAP gene deletion group,[19] while there is no evidence of chronic changes. In this study, PACAP expression in TNC decreased with the increasing chemical stimulation days, while in TG first increased and then decreased. The current study results provided clear immunohistochemical evidence that PACAP release peaked and then depleted in TG neurons after chronic repeated chemical stimulation. This is consistent with the low level of PACAP in peripheral blood of patients with chronic migraine in clinical trials.[33]

The trigeminal ganglia represent a neuromodulator system, composed of various cell types such as pseudounipolar neurons and satellite glial cells (SGCs), thought to be implicated in the progression of cranial pain. Frederiksen et al. found that PACAP was expressed in neuronal somas and SGCs in both rat and human TG.[34] PAC1 was observed in neuronal somas and SGCs, while VPAC 1 and VPAC2 were observed in SGCs in rat TG. However, other study showed that PAC1 was mainly expressed in SGCs, which envelop the neurons in the trigeminal ganglion.[35] Many clinical studies proved that intravenous PACAP induced a delayed migraine attack but not VIP.[11,36] This difference may be related to the specific receptors PAC1, as being highly selectively for PACAP. The other two receptors (VPAC1 and VPAC2) have a similar affinity for VIP and PACAP.[37] It was found that the variation tendency of PAC1 receptor expression in the trigeminal nucleus and trigeminal ganglion was consistent with that of PACAP. Immunofluorescence results disclosed the co-expression of PACAP and its specific receptor PAC1 in TG. There was no significant correlation between the expression of PACAP and VPAC1 and VPAC2 in the trigeminal nucleus and trigeminal ganglion. Notably, all three receptors are expressed during the initial stage of IS stimulation, then specific receptor PAC1 expression was increased with the extension of time. The results suggested that migraine chronification could be mediated by the PACAP-PAC1 pathway.

In fact, preclinical data found AMG 301, a selective human monoclonal antibody inhibitor of the PAC1 receptor, to be as

effective as sumatriptan in inhibiting evoked nociceptive activity in the trigeminocervical complex in rats, supporting further investigation of AMG 301 in the treatment of migraine.[38] However, Ashina et al.[39] have recently published a clinical trial using AMG 301 on migraine attacks, and the result was negative. It is unclear whether targeting the PAC1 receptor will be effective in certain subpopulations of migraine only or whether the patients need to be stratified by the presence of cranial autonomic symptoms. Syed et al.[17] reported that PAC1 receptor antagonist could effectively block the dilation of meningeal vessels in migraine patients. Intraventricular application of PAC1 receptor antagonist inhibited dural nociceptive-evoked action potentials in central trigeminovascular neurons.[11] These studies supported the potential role of PACAP and receptor PAC1 in the developing central sensitization of pain in migraine.

Calcitonin gene-related peptide is found in neurons throughout the body,[40] and approximately 50% of trigeminal neurons express CGRP. SGCs and neurons have been proposed to participate in a positive feedback loop of CGRP synthesis and release maintaining a state of heightened inflammation and sensitization.[3] Several studies have found that CGRP is involved in photophobia, peripheral sensitization, neurogenic inflammation, and cortical spreading depression (CSD) in the pathogenesis of migraine.[29] Saliva CGRP of patients with chronic migraine levels were elevated during the headache phase of the attack and returned to near baseline values following successful treatment with a rizatriptan.[41] A case–control study demonstrated that interictal serum CGRP, VIP, and PACAP levels in chronic migraine were significantly higher than those in episodic migraine.[42]

However, this study found that CGRP was decreased in TG and TNC with repeated IS stimulation. It is possible that the release of CGRP reactivity decreased and consumption increased after long-term repeated IS stimulation, resulting in the gradual depletion of the reserve pool in the nervous system. Furthermore, it was observed PACAP and CGRP co-expression existed in TG and decreased gradually after stimuli of/for one week. In the chronic migraine model, the increased PACAP release resulted in the activation of neurons and central sensitization, promoting CGRP release, which aggravated the severity of headache. In another mouse model of chronic migraine, repeated NTG administration significantly increased the number of CGRP-R and PACAP-R neurons in TG, and PACAP may promote the release of endogenous CGRP through PAC1 receptor.[43] Another study showed that a possible synergistic effect of increased RAMP1 levels on both CGRP and PACAP receptor cAMP signaling.[44] Taken together, as two important neuropeptides, CGRP and PACAP might interact in migraine pathogenesis. Further research into their co-release relationship and receptor antagonist is necessitated to provide a foundation for developing emerging migraine drugs in the future.

Conclusion

Repetitive chemical stimulation of rat model can be used to mimics chronic migraine presentation. This study demonstrated that daily IS infusion induced a gradual decrease of PACAP in the TNC. While the PACAP and PAC1 receptor expression in TG showed dynamical changes of increasing first and then decreasing after repeated IS administration, implying excessive release and exhaustion of PACAP is involved in the duration of chronic migraine, which was associated with CGRP. These results also suggested PACAP may contribute to the pathology of migraine through the PAC1 receptor, and PACAP-PAC1 receptor pathway activation could promote central sensitization in migraine.

Appendix

Abbreviations

PACAP:	pituitary adenylate cyclase activating polypeptide.
CGRP:	calcitonin gene-related peptide.
VIP:	vasoactive intestinal peptide.
VEGF:	vascular endothelial growth factor.
TG:	trigeminal ganglia.
TNC:	trigeminal nucleus caudalis.
IS:	Inflammatory soup.
NS:	normal saline.
VPAC1:	vasoactive intestinal peptide receptor 1.
VPAC2:	vasoactive intestinal peptide receptor 2.
TGVS:	trigeminovascular system.
NTG:	nitroglycerin.
SGCs:	satellite glial cells.
CSD:	cortical spreading depression.

Acknowledgments

We thank all the participants in this study. The authors would like to thank the 302 Hospital of the PLA General Hospital and the 304 Hospital of the PLA General Hospital for their technical assistance.

Author contributions

All authors read and approved the final manuscript. Hangfei Wu performed experiments, analyzed data, prepared figures, and drafted the manuscript. Zhao Dong performed the pre-experiments, collected the data, and drafted the manuscript. Yinglu Liu and Qing Zhang performed the pre-experiments and analyzed the data. Mingjie Zhang and Guanqun Hu analyzed the data and generated the figures. Shengyuan Yu and Xun Han designed and monitored this study and edited the manuscript. Shengyuan Yu and Xun Han contributed equally to this paper.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by National Natural Science Foundation of China (81901134, 82101288, 82171208).

Ethical approval

Our study was approved by the Committee on Animal Use for Research and Education of the Laboratory Animals Centre at Chinese PLA General Hospital (Beijing, China). All animals were used in accordance with the ethical guidelines for experimental pain in conscious animals.

Data availability

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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