学　位　論　文

The role of calcitonin gene-related peptide in migraine prevention

by botulinum toxin type A

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Abstract

Objectives: Calcitonin gene-related peptide (CGRP) is currently considered to be a major contributing factor in migraine headache. Botulinum toxin type A (BTXA) was found to be effective in migraine prevention. However, the mechanism of action in patients was unknown. Using injection as in clinical setting, the study aimed to determine whether BTXA could decrease the sensitization of the trigeminovascular nociceptive system through the reduction of CGRP action.

Methods: Adult male Wistar rats were pretreated with normal saline solution or BTXA before KCl application to induce cortical spreading depression (CSD) or NaCl application as a control. Regional cerebral blood flow at parietal cortex was measured for 90 min after KCl or NaCl application. Tissues from trigeminal ganglion (TG) and trigeminal nucleus caudalis (TNC) were then collected for CGRP and c-Fos measurement respectively.

Results: BTXA pretreatment significantly decreased the cumulative blood flow and number of hyperemic peaks induced by KCl. Numbers of CGRP positive cells at TG and c-Fos positive cells at TNC were also reduced by BTXA.

Conclusion: BTXA pretreatment reduced CGRP production and release from the TG leading to lessen CSD production and persistent activation of TNC which played a major role in migraine headache.

INTRODUCTION

Migraine affects 17% of female and 8% of male population in Europe.[1] It was estimated that in Europe 18.5 billion Euros per year was lost due to migraine.[2] World Health Organization named migraine as 1 of 20 most disabling diseases.[3] Therefore, migraine is a public health problem with great impact on individual and society.

There are 2 types of migraine treatment: abortive and preventive treatment. Abortive treatment is used for alleviating acute headache attack; preventive treatment,

for reducing headache frequency and severity. Preventive treatment is often used in patients with attack frequency more than 15 episodes per month or patients with chronic migraine.[4]

Preventive treatment has gained interests in recent years after it was found that frequent use of medications for abortive treatment could cause a condition known as medication overuse when headache severity increased and did not response to conventional treatment.[5] Thus, development of preventive anti-migraine drugs that do not lead to medication overuse becomes important.

Botulinum toxin is produced by *Clostridium botulinum* and acts as a neurotoxin. Purified botulinum toxin type A (BTXA) was the first bacterial toxin to be approved by US Food and Drug Administration (FDA) under the trade name Botox® for use in muscular disorders and cosmetic treatments.[6] Unexpectedly, it was found that migraine patients who received Botox® injection for cosmetic reasons had reduced headache frequency and severity.[7] In 2010, Botox® injection was approved for headache prevention in patients with chronic migraine.[8] However, the sites and the mechanisms by which BTXA reduces and prevents migraine headache in vivo are unknown.

Several lines of evidence supported the role of CGRP in migraine pathophysiology. Blood level of CGRP was found to be increased during migraine attack in patients.[9] Intravenous injection of CGRP was found to often cause migraine-like headaches in patients with migraine.[10-12] Additionally, CGRP-induced migraine-like headaches could be reduced by a triptan.[12] CGRP receptor antagonists such as

olcegepant and telcagepant were also found to reduce vasodilation, neurogenic inflammation and migraine headache. Clinical trials of CGRP blocking antibodies and CGRP receptor blockers demonstrated promising results for effective migraine therapy.[13] Additionally, onabotulinumtoxinA treatment for 1 month in chronic migraine patients could reduce interictal plasma CGRP level in a responders group.[14]

In animal studies, cortical spreading depression (CSD) generated a calcium-dependent release of endogenous CGRP from rat neocortical slices.[15] The CSD could be inhibited by CGRP receptor antagonists suggesting the role of CGRP in initiating and maintaining CSD. Drugs with preventive anti-migraine activities were shown to reduce CSD while antiepileptic drugs without the efficacy on migraine such as oxcarbazepine had no effect on CSD.[15, 16]

In this study CSD was induced in rats by direct application of KCl crystal on the surface of cerebral cortex which produced typical changes in cerebral blood flow.[17] Thus, changes in cerebral blood flow could be used to signify corresponding changes in the CSD. Additionally, KCl application could also cause a release of CGRP from trigeminal ganglia in primary cell culture.[18]

Activation of the cranial pain pathway by CSD in migraine headache produces an increase in neuronal activity of the pain pathway including TNC which receives input from the trigeminal ganglion. To determine the level of TNC activation by CSD, the expression of c-Fos was measured.[19]

The objective of this study was to determine how BTXA affect the trigeminovascular nociceptive system (TVNS) using CSD model in rats. As an

effective migraine preventive medication, BTXA should be able to reduce CSD production and CGRP synthesis by the trigeminovascular nociceptive system.

MATERIALS AND METHODS

Male Wistar rats between the ages of 8–10 weeks and the body weights of 300–350 g were purchased from National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand. Animals were housed under standard conditions and allowed free access to food pellets and tap water. Rats were allowed to acclimatize to housing conditions for 1 week before the experiment. All experiments were conducted in accordance with the approved standard guidelines for animal experimentation of the Kagawa University and the Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes by National Research Council of Thailand 1999.

Animals were pretreated for 3 days with either NSS or BTXA at 3 different dosages before CSD induction. During the blood flow measurement, NSS-pretreated rats received either NaCl (n = 8) or KCl (n = 8). Similarly, BTXA-pretreated rats received either NaCl (n = 24) as a control or KCl (n = 24) crystal to induce cortical spreading depression (CSD) which is considered to be the basis for migraine generation.[20]

In the BTXA-pretreated group, rats were injected with 40 μl of BTXA (Botox®, Allergan Inc.) using microliter syringe (Hamilton Company, USA) at 3, 10

and 30 units/kg body weight at eyebrows of the rats on both sides (20 μl each side) 3

days prior to NaCl or KCl application. As a negative control, normal saline solution

(NSS) was administered instead of BTXA.

Induction of migraine-like phenomena

Migraine headache is associated with a generation of CSD.[21] Several methods

including application of KCl can be used to induce CSD in animal model.[16] Rats were

anesthetized with intraperitoneal injection of pentobarbital sodium at a dose of 50

mg/kg body weight and ventilated with a positive pressure ventilator (Rodent

ventilator model 683, Harvard Apparatus, South Natick, USA) through tracheotomy

openings. Blood pressure at femoral artery was monitored continuously with an intra-

arterial pressure transducer (Gould P23 Statham, USA). Blood pressure data was

digitized and recorded using a data acquisition system for further off-line analysis

(PowerLab, ADInstruments, CO, USA). After tracheotomy and cannulation, rats were

placed on a surgical frame and their heads fixed to the head holder. A craniotomy of 2

mm in diameter was performed on the parietal bone at 7 mm posterior and 1 mm

lateral to the bregma. Dura mater was cut to expose the cerebral cortical surface. To

prevent drying and hypothermia, the cortical surface was superfused with warm

artificial cerebrospinal fluid (CSF).[17] To induce CSD, the artificial CSF perfusion was

halted then a 3-mg solid KCl was placed directly on the surface of the parietal cortex.

The crystal was allowed to dissolve completely on the cortical surface without

reapplication of the artificial CSF. NaCl crystal was used instead of KCl crystal for

rats in control group.[22]

Measurement of focal cerebral hyperemia

To measure the cerebral blood flow, an anterior craniotomy of 2 mm in

diameter was performed on the parietal bone at 1 mm anterior and 1 mm lateral to the

bregma. To prevent drying and hypothermia, the cortical surface was superfused with

artificial CSF.[17] A fiber optic pencil probe of the Blood Flow Meter (Powerlab,

ADInstruments, CO, USA) was placed perpendicularly with a distance of 2 mm above

the cortical surface. The 780 nm wavelength laser beam was used to measure changes

in blood flow under the probe. Data was recorded for 90 min and the maximum

amplitude of each hyperemic peak was measured for all peaks observed during a 60-

min period after KCl application. Amplitudes for every hyperemic peak during the

entire 60 min period were summed to produce cumulative blood flow in 1 hour. After

cerebral blood flow measurements, rats were transcardially perfused with 250 ml of

ice-cold phosphate buffered saline (PBS) with a pH of 7.4. The cervical part of spinal

cord and trigeminal ganglions (TG) were removed and immersed in 4%

paraformaldehyde in 0.1 M PBS, pH 7.4 for subsequent measurement of c-Fos and

CGRP.

Measurement of c-Fos expression at trigeminal nucleus caudalis

The caudal medulla (3 mm caudal to the obex) to the first cervical cord were fixed overnight in 4% paraformaldehyde in 0.1 M PBS pH 7.4, then placed in a cryoprotective solution consisting of 30% sucrose in 0.1 M PBS, pH 7.4. The tissue was cut into transverse serial sections at 30 μm thickness using a cryostat at −20°C (Leica CM 1580, Germany). Serial sections were collected 1 in every 5 sections and then rinsed in 0.1 M PBS. All sections were prepared for c-Fos immunochemistry staining as described by Supornsilchai W.[22] The number of c-Fos positive cells in lamina I and II of trigeminal nucleus caudalis was determined using image analysis software (ImagePro® Plus; Media Cybernetics Inc., Bethesda, Maryland, USA).

Measurement of CGRP expression at trigeminal ganglion

TG tissue were fixed overnight in 4% paraformaldehyde in 0.1 M PBS pH 7.4 and then paraffin processed and transverse 3 μm thick sections were cut and deparaffinized before immunostaining. TG paraffin sections were stained for CGRP by immunoperoxidase staining as described by Chatchaisak D.[23] The immunostaining TG sections were viewed under a confocal microscope and CGRP-immunopositive neurons were manually counted. Every fifth section of TG from each rat was chosen for counting of both positive and negative immunoreactive (IR) TG neurons.

Statistical analysis

The results are expressed as mean±S.E.M. Data was evaluated using Student's

t-test or one-way ANOVA followed by the Student-Newman-Keuls test. P-values less

than 0.05 were considered statistically significant.

RESULTS

Changes in Cerebral Blood Flow by KCl

KCl application to the cerebral cortex produced repeated cycles of cerebral

hyperemia while NaCl application did not produce any hyperemic peaks (Figure 1). For

NSS-pretreated group, KCl induced an increased in cerebral blood flow with the

average cumulative blood flow amplitude of $16.72 \pm 2.77 \times 10^3$ Blood Perfusion Unit

(BPU) (Figure 2). The average number of peaks in 1 hour of the NSS-pretreated group

was 11.80 ± 1.11 peaks/h (ranging from 10 to 14 peaks) (Figure 3). The results confirmed

that only KCl but not NaCl could induce CSD resulting in episodic increases of

cerebral blood flow (Figure 1).

Effect of BTXA on Cerebral Blood Flow

After BTXA injection, no apparent facial muscle weakness or ptosis were

observed. Drooling and dyspnea were also absent even after the highest dose of

BTXA. The averaged cumulative blood flow amplitude of the BTXA-pretreated groups

at the dosage of 3, 10 and 30 units/kg body weight were 6.28 ± 1.70, 4.68 ± 0.87 and

$1.16 \pm 0.31 \times 10^3$ BPU, respectively (Figure 2). These blood flow amplitudes of BTXA

pretreated groups were significantly less than that of the NSS-pretreated group.

Compared to NSS-pretreated group, BTXA at the dosage of 3, 10 and 30 units/kg

body weight significantly reduced the averaged hyperemic peak numbers per hour to

9.20 ± 0.20, 6.40 ± 0.51 and 3.00 ± 0.32 peaks/h, respectively (Figure 3).

Therefore, BTXA decreased cerebral blood flow induced by CSD through a

reduction in cumulative flow amplitude and number of flow peaks.

Effect of KCl on the Number of c-Fos Positive Cells at Trigeminal Nucleus Caudalis

KCl increased the number of c-Fos positive cell at lamina I and II of TNC

(Figure 4 and 5). The average number of c-Fos positive cells in KCl group (16.21 ± 2.23

cells) was significantly higher than that of the NaCl group (1.50 ± 0.76 cells). Therefore,

KCl application on the cortical surface increased activity of the cranial pain pathway

as indicated by an increased number of c-Fos positive cells at TNC.

Effect of BTXA on the Number of c-Fos Positive Cells Induced by KCl at Trigeminal

Nucleus Caudalis

BTXA pretreatment significantly reduce the numbers of c-Fos positive cell at

TNC to 3.04 ± 0.59 cells when compared to the number of cells in NSS-pretreated group

(Figure 5). The result indicated that BTXA could reduce the activity of TNC neurons

leading to a reduction in pain transmission through the trigeminovascular nociceptive

pathway.

Effect of KCl on the Number of Cells Expressing CGRP mRNA at Trigeminal Ganglion

When compared with the NaCl group, KCl produced a significant increase in number of CGRP-positive cells per number of total cells ratio at the trigeminal ganglion (Figure 6). The averaged CGRP-positive cells per total cells of KCl control group and of NaCl group were $57.81 \pm 2.22\%$ and $39.11 \pm 0.49\%$ respectively (Figure 7).

Effect of BTXA on the CGRP-positive Cells Induced by KCl at Trigeminal Ganglion

BTXA at 30 units/kg dosage significantly reduced the percentage of CGRP-positive cells per number of total cells at the trigeminal ganglion to $35.71 \pm 3.82 \%$ when compared with that of NSS-pretreated group (Figure 7).

DISCUSSION

This is the first study to investigate the effects of BTXA, administered on the facial area in a similar manner as in standard clinical setting, on parameters related to the migraine pathophysiology using rat CSD model. For treatment of chronic migraine in human, the recommended total dosage by FDA and Allergan is 155 units given intramuscularly[24] which may be comparable to the dosage of 3 units/kg in this study. Additionally, our preliminary study demonstrated that the maximum effect of BTXA is reached 3 days following the initial administration which is in agreement with a previous study by Antonucci et al.[25] In summary, results from this study confirmed

that BTXA at dosages comparable to the clinically recommended dosage could reduce changes associated with CSD production and migraine headache.

In rat, application of KCl induced CSD that stimulated the trigeminovascular nociceptive system (TVNS) as indicated by increased c-Fos positive cells at the trigeminal nucleus caudalis (TNC).[17] It was proposed that CSD caused a release of many neuropeptides such as substance P, nitric oxide (NO) and calcitonin gene-related peptide (CGRP) from nerve terminals to adjacent meninges and nerves resulting in vasodilation, neurogenic inflammation and activation of the TVNS.[26]

In this study changes in cerebral blood flow signify corresponding changes in CSD. CSD, recorded as direct current (DC) shift, produced corresponding changes in regional cerebral blood flow (rCBF).[22] Hyperemic peak frequency (number of peaks/h) was found to be directly correlated with the CSD frequency. However, the amplitude of increased blood flow was not directly correlated with the amplitude of DC shift. Additionally, paracetamol was shown to reduce the hyperemic amplitude but not the DC-shift or CSD amplitude.[22] Therefore, individual hyperemic peak amplitude was not determined by CSD but by vasodilators such as CGRP released from perivascular nerves in response to CSD.

In the current study, BTXA reduced both the number of hyperemic peaks per hour and the cumulative amplitude of hyperemic peaks. Therefore, it can be concluded that BTXA could both suppress CSD production as determined by reduced number of hyperemic peaks per hour and decreased cerebral vasodilatation as determined by reduced cumulative blood flow. Because of the direct relationship between hyperemic

peak frequency and CSD wave frequency, it can be concluded that BTXA suppresses CSD production and eventually migraine headache.

BTXA mainly acts as a proteolytic enzyme to degrade the SNAP-25 protein, a type of SNARE protein. The SNAP-25 protein is required for vesicle fusion that releases neurotransmitters from the nerve endings. BTXA specifically cleaves these SNAREs, so prevents secretory vesicles from docking/fusing with the nerve synapse plasma membrane and releasing neurotransmitters such as acetylcholine at the neuromuscular junction resulting in muscle relaxation.[27]

Moreover, results from animal and in vitro experiments showed that BTXA reduced pain by decreasing the release of neuropeptide related to pain modulation including CGRP, glutamate, substance P and bradykinin.[28, 29] In a rat model, subcutaneous BTXA injection suppressed nitroglycerin-induced CGRP and SP-like immunoreactivity in the jugular plasma and in the medulla oblongata.[30] BTXA could also be retrogradely transported along the axon to affect SNAP-25 protein at distant sites in the central nervous system.[25]

Current study showed that BTXA reduced both the number and the amplitude of hyperemic peaks indicating a parallel reduction in CSD and hyperemia, respectively. It is most likely that injected BTXA is retrogradely transported along ophthalmic division (VI) of the trigeminal nerve to affect SNAP-25 protein at both the peripheral and central branches of the axon.[25] The reduction of CGRP release at the peripheral branches of TG neurons surrounding cerebral blood vessels by BTXA

reduced vasodilatation and cerebral hyperemia as indicated by decreased number of CGRP-positive cells at the trigeminal ganglia.

Centrally, BTXA most likely reduced neurotransmitter glutamate, NO and/or CGRP release from the presynaptic TG nerve terminal causing a decreased activation of the TNC as supported by the reduction of c-Fos expression and the number of cells positive for c-Fos at lamina I and II of TNC. As a result, transmission of pain signal along the TVNS was reduced leading to decreased central sensitization.[31]

Because the number of hyperemic peaks was reduced by BTXA, it was also possible that BTXA could directly inhibit the production of CSD thereby decreasing the stimulation of the TVNS and subsequent production of c-Fos, nNOS and CGRP induced by CSD. However, no direct evidence from this study or from previous studies that demonstrated direct inhibition of CSD production by BTXA. Future experiments are required to determine the direct effect of BTXA on CSD generation. Although the mechanism of CSD generation in migraine patients is not known, it has been shown that CSD could activate meningeal nociceptors.[32, 33] The most likely explanation is that BTXA reduces the stimulatory effects of CSD on the trigeminovascular nociceptive pathway. However, the possibility of BTXA inhibition of upstream events such as the accumulation of glutamate that could trigger the generation of CSD and the release of CGRP and other peptides could not be ruled out.

In conclusion, BTXA pretreatment decreased CGRP production and release by TG neurons at the perivascular nerves leading a reduction in CSD production as indicated by a decrease in the number of cerebral hyperemic peaks per hour. Centrally,

BTXA also reduced activation of the TNC as indicated by decreased the number of neurons expressing c-FOS. By acting both at the peripheral and central locations, BTXA prevent the sensitization of the trigeminovascular nociceptive pathway which is an underlying basis of migraine headache. Results from the current study demonstrated the important role of CGRP in migraine pathogenesis and in the future development of preventive migraine therapy.

ACKNOWLEDGEMENT

DISCLOSURE

Conflict of interest: None.
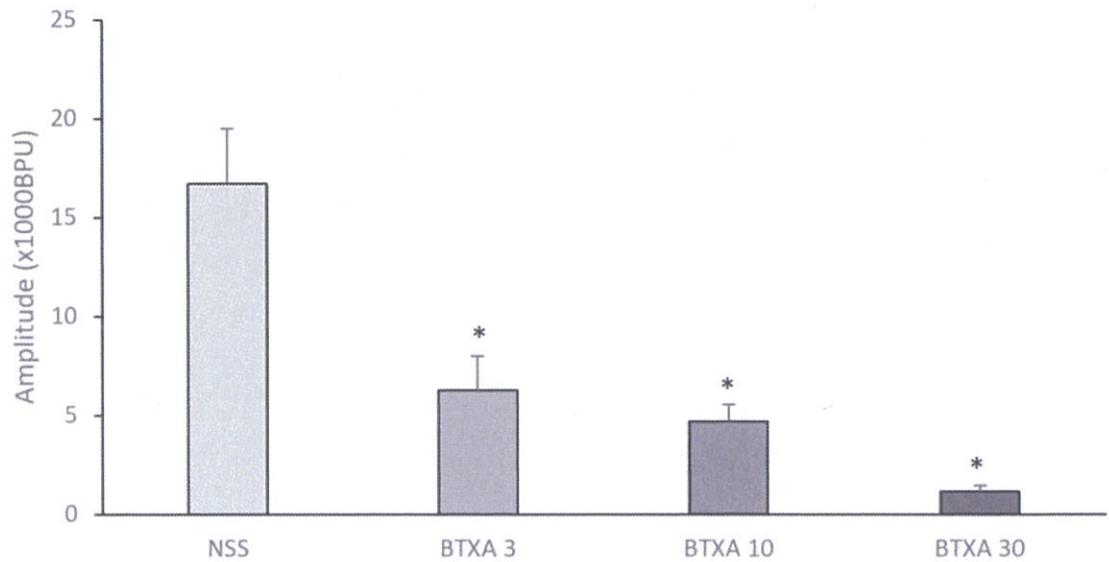
REFERENCES

1.   Stovner LJ and Hagen K. Prevalence, burden, and cost of headache disorders. *Curr Opin Neurol* 2006; 19: 281-5.
2.   Olesen J, Gustavsson A, Svensson M, Wittchen HU and Jonsson B. The economic cost of brain disorders in Europe. *Eur J Neurol* 2012; 19: 155-62.
3.   Matak I, Riederer P and Lackovic Z. Botulinum toxin's axonal transport from periphery to the spinal cord. *Neurochem Int* 2012; 61: 236-9.
4.   Pringsheim T, Davenport WJ and Becker WJ. Prophylaxis of migraine headache. *Cmaj* 2010; 182: 16.
5.   Eross EJ. Patient page. Chronic migraine and medication-overuse headache. *Neurology* 2006; 66: E43-4.
6.   Whitcup SM, Turkel CC, DeGryse RE and Brin MF. Development of onabotulinumtoxinA for chronic migraine. *Annals of the New York Academy of Sciences* 2014; 1329: 67-80.
7.   Silberstein S, Mathew N, Saper J and Jenkins S. Botulinum toxin type A as a migraine preventive treatment. For the BOTOX Migraine Clinical Research Group. *Headache* 2000; 40: 445-50.

8.   Jackson JL, Kuriyama A and Hayashino Y. Botulinum toxin A for prophylactic treatment of migraine and tension headaches in adults: a meta-analysis. *Jama* 2012; 307: 1736-45.

9.   Goadsby PJ, Edvinsson L and Ekman R. Vasoactive peptide release in the extracerebral circulation of humans during migraine headache. *Annals of neurology* 1990; 28: 183-7.

10.   Lassen LH, Haderslev PA, Jacobsen VB, Iversen HK, Sperling B and Olesen J. CGRP may play a causative role in migraine. *Cephalalgia* 2002; 22: 54-61.

11.   Hansen JM, Hauge AW, Olesen J and Ashina M. Calcitonin gene-related peptide triggers migraine-like attacks in patients with migraine with aura. *Cephalalgia* 2010; 30: 1179-86.

12.   Asghar MS, Hansen AE, Amin FM, et al. Evidence for a vascular factor in migraine. *Annals of neurology* 2011; 69: 635-45.

13.   Edvinsson L. CGRP receptor antagonists and antibodies against CGRP and its receptor in migraine treatment. *British journal of clinical pharmacology* 2015; 80: 193-9.

14.   Cernuda-Morollon E, Ramon C, Martinez-Camblor P, Serrano-Pertierra E, Larrosa D and Pascual J. OnabotulinumtoxinA decreases interictal CGRP plasma levels in patients with chronic migraine. *Pain* 2015; 156: 820-4.

15.   Tozzi A, de Iure A, Di Filippo M, et al. Critical role of calcitonin gene-related peptide receptors in cortical spreading depression. *Proceedings of the National Academy of Sciences of the United States of America* 2012; 109: 18985-90.

16.   Costa C TA, Rainero I, Cupini LM, Calabresi P, Ayata C, Sarchielli P. Cortical spreading depression as a target for anti-migraine agents. *The journal of headache and pain* 2013; 14: 62.

17.   Maneesri S, Patamanont J, Patumraj S and Srikiatkhachorn A. Cortical spreading depression, meningeal inflammation and trigeminal nociception. *Neuroreport* 2004; 15: 1623-7.

18.   Durham PL, Niemann C and Cady R. Repression of stimulated calcitonin gene-related peptide secretion by topiramate. *Headache* 2006; 46: 1291-5.

19.   Ingvardsen BK, Laursen H, Olsen UB and Hansen AJ. Possible mechanism of c-fos expression in trigeminal nucleus caudalis following cortical spreading depression. *Pain* 1997; 72: 407-15.

20.   Supronsinchai W, Hoffmann J, Akermann S and Goadsby PJ. KCl-induced repetitive cortical spreading depression inhibits trigeminal neuronal firing mediated by 5-HT1B/1D and opioid receptor. *The journal of headache and pain* 2013; 14: P69-P.

21.   Ayata C. Cortical spreading depression triggers migraine attack: pro. *Headache* 2010; 50: 725-30.

22.   Supornsilpchai W, le Grand SM and Srikiatkhachorn A. Cortical hyperexcitability and mechanism of medication-overuse headache. *Cephalalgia* 2010; 30: 1101-9.

23.   Chatchaisak D, Srikiatkhachorn A, Maneesri-le Grand S, Govitrapong P and Chetsawang B. The role of calcitonin gene-related peptide on the increase in transient receptor potential vanilloid-1 levels in trigeminal ganglion and trigeminal nucleus caudalis activation of rat. *J Chem Neuroanat* 2013; 47: 50-6.

24.   Ashkenazi A and Blumenfeld A. OnabotulinumtoxinA for the treatment of headache. *Headache* 2013; 2: 54-61.

25.   Antonucci F, Rossi C, Gianfranceschi L, Rossetto O and Caleo M. Long-distance retrograde effects of botulinum neurotoxin A. *J Neurosci* 2008; 28: 3689-96.

26.   Moskowitz MA. Genes, proteases, cortical spreading depression and migraine: impact on pathophysiology and treatment. *Functional neurology* 2007; 22: 133-6.

27.   Arezzo JC. Possible mechanisms for the effects of botulinum toxin on pain. *Clin J Pain* 2002; 18: S125-32.

28.   Peng Chen Z, Morris JG, Jr., Rodriguez RL, Shukla AW, Tapia-Nunez J and Okun MS. Emerging opportunities for serotypes of botulinum neurotoxins. *Toxins* 2012; 4: 1196-222.

29.   Caputi CA. Effectiveness of BoNT-A in the treatment of migraine and its ability to repress CGRP release. *Headache* 2004; 44: 837-8.

30.   Shao Y-F, Zhang Y, Zhao P, et al. Botulinum Toxin Type A Therapy in Migraine: Preclinical and Clinical Trials. *Iranian Red Crescent Medical Journal* 2013; 15: e7704.

31.   Pradhan AA, Smith ML, McGuire B, Tarash I, Evans CJ and Charles A. Characterization of a novel model of chronic migraine. *Pain* 2014; 155: 269-74.

32.   Zhang X LD, Noseda R, Kainz V, Jakubowski M, and Burstein R. Activation of meningeal nociceptors by cortical spreading depression: implications for migraine with aura. *J Neurosci* 2010; 30: 8807-14.

33.   Zhao J and Levy D. *Modulation of intracranial meningeal nociceptor activity by cortical spreading depression- A reassessment.* 2015.
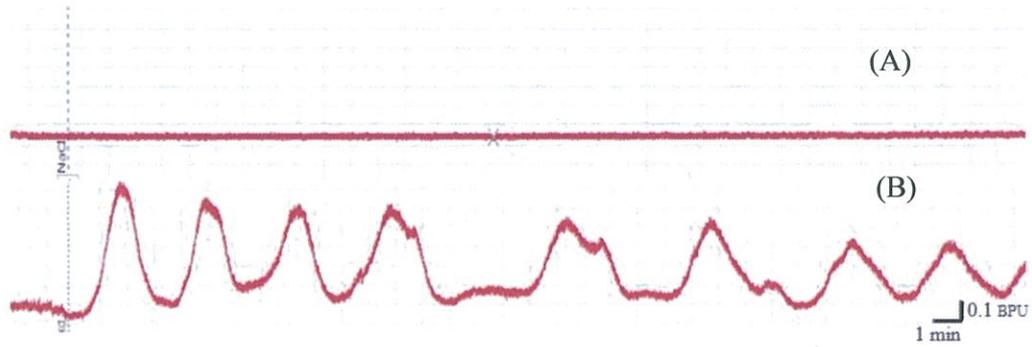
Figure 1. Changes in cerebral blood flow. Application of 3 mg KCl (B) but not 3 mg NaCl (A) produced cyclical increase in local cerebral blood flow. BPU, Blood Perfusion Unit.


Figure 2. Total (cumulative) cerebral blood flow during 60 min of recording. BTXA reduced the total increase in blood flow induced by KCl. Data are expressed as mean+S.E.M; $n = 8$ in each groups. All differences are significant at $P < 0.05$. Asterisk (*) indicates significant difference between control (NSS) group and other groups.
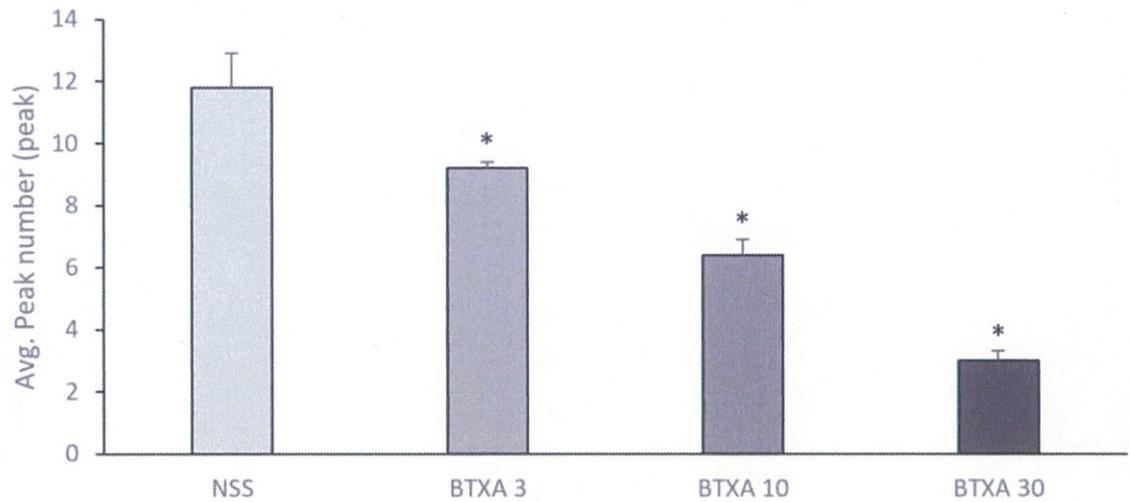
Figure 3. The average number of hyperemic peaks. BTXA reduced the number of hyperemic peaks during the recording period. Data are expressed as mean+S.E.M; $n = 8$ in each group. All differences are significant at P < 0.05. Asterisk (*) indicates significant difference between control group and other groups.
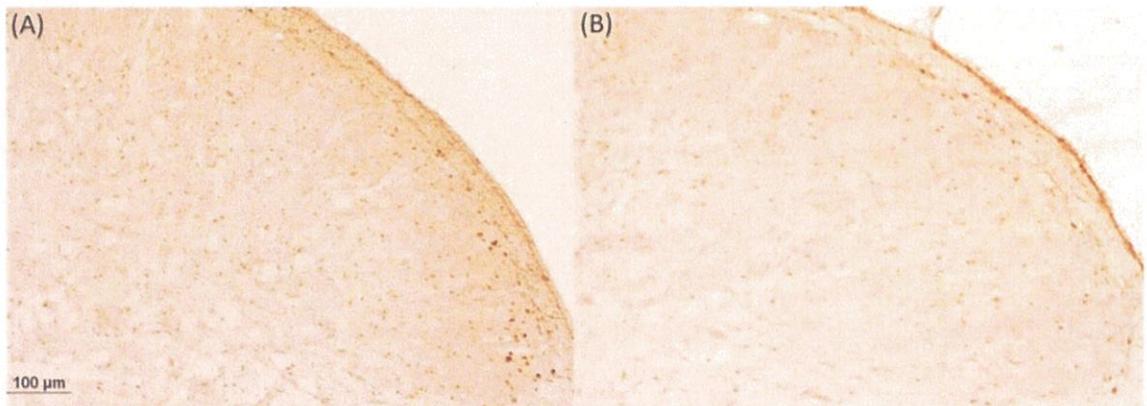

Figure 4. Expression of c-Fos at the TNC. (A) Increased c-Fos expression induced by KCl. (B) Reduction of c-Fos expression by BTXA pre-treatment. Marker scale bar indicates a length of 100 μm.
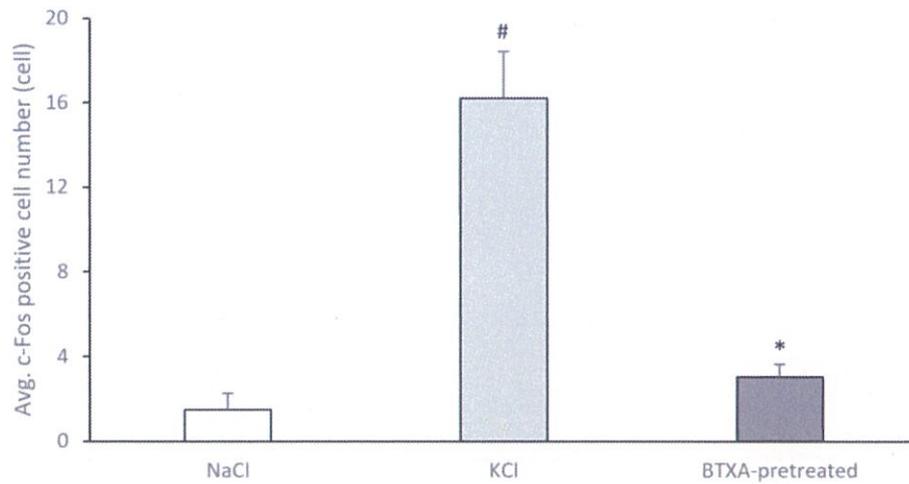
Figure 5. Number of c-Fos positive cells in lamina I and II of TNC. BTXA reduced the number of c-Fos positive cells activated by KCl. Data are expressed as mean+S.E.M; n = 8 in each group. All differences are significant at P< 0.05. Hash mark (#) indicates significant difference between NaCl group and KCl group; Asterisk (*), significant difference between KCl group and BTXA-pretreated groups.
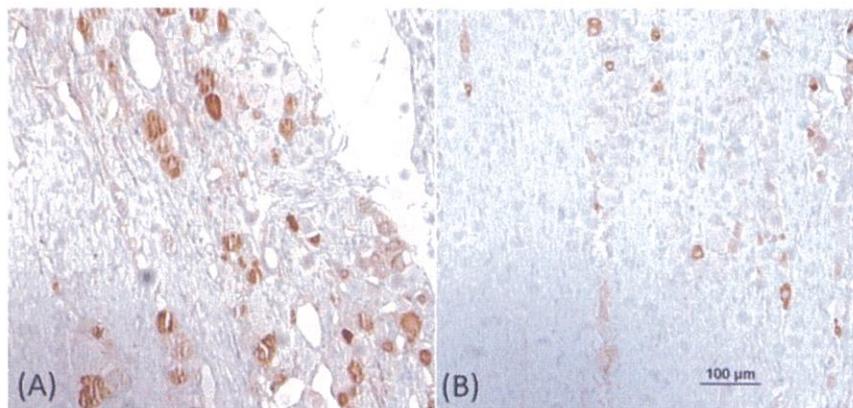
Figure 6. CGRP expression in TG. (A) Increased CGRP expression induced by KCl; (B) Reduction of CGRP expression by BTXA pretreatment. Marker scale bar indicates a length of 100 μm.
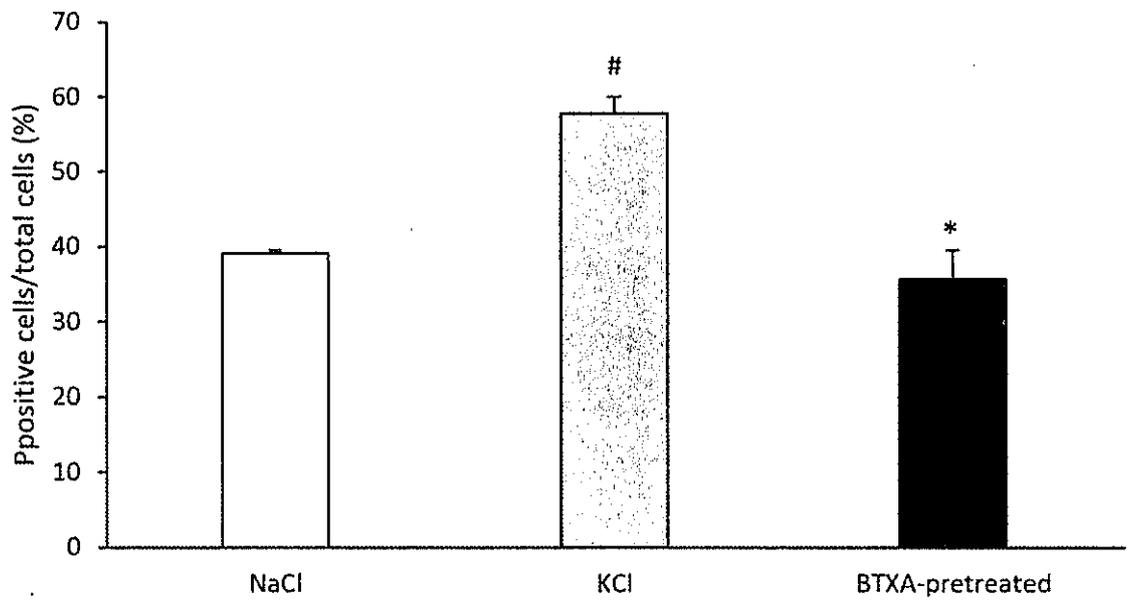
Figure 7. Percent of CGRP-positive cells at TG. BTXA decrease the percent of CGRP-positive cells induced by KCl. Data are expressed as mean+S.E.M; n = 8 in each group. All differences are significant at P < 0.05. Hash mark (#) indicated significant difference between NaCl group and KCl group; Asterisk (*), significant difference between KCl group and BTXA-pretreated groups.