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# 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

Duangthip Chatchaisak [a], Anan Srikiatkhachorn [b], Supang Maneesri-le Grand [c], Piyarat Govitrapong [a,d], Banthit Chetsawang [a,*]

[a] Research Center for Neuroscience, Institute of Molecular Biosciences, Mahidol University, Salaya, Nakhonpathom 73170, Thailand
[b] Department of Physiology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand
[c] Department of Pathology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand
[d] Center for Neuroscience and Department for Pharmacology, Faculty of Science, Mahidol University, Bangkok, Thailand

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ABSTRACT

Calcitonin gene-related peptide (CGRP) and transient receptor potential vanilloid-1 (TRPV1) play an important role in the development of pain and migraine pathogenesis. Increase in plasma CGRP levels is associated with delayed migraine-like attacks in migraine patients. Although several lines of evidence have indicated a key role of CGRP in migraine pain, its mechanisms remain unclear. In this study, we aimed to investigate the functional role of CGRP on trigeminal nociceptive pathway by determining the alteration in TRPV1 levels in trigeminal ganglion (TG) and the activation of trigeminal nucleus caudalis (TNC) of rat. Post intravenous injection of CGRP (600 ng/kg) at 60 min significantly increased the levels of TRPV1, CGRP, phosphorylated protein kinase C and phosphorylated cyclic AMP responsive element-binding protein in TG of rats. The number of small and medium TRPV1 and CGRP positive immunostaining neurons accompanying with co-localization of TRPV1 with CGRP neurons were significantly increased in TG of CGRP-injected rats. The sustained increase in c-Fos expression in TNC neurons was also observed in CGRP-injected rats. These results indicate that CGRP may participate in trigeminal nociceptive system sensitization by induced increase in TRPV1 and CGRP levels in TG neurons and activation of the central neurons in TNC.



## 1. Introduction

Migraine is believed to be a trigeminovascular disease (Goadsby et al., 2009). Migraine pain appears to be the consequence of multiple pathophysiological changes, which are related to activation of primary sensory neurons in trigeminal ganglion (TG) and secondary sensory neurons in trigeminal nucleus caudalis (TNC). TG and TNC are likely to be sites of action of calcitonin gene-related peptide (CGRP), a neuropeptide, which, it is hypothesized may be involved in migraine pain (Storer et al., 2004; Jenkins et al., 2009; Lennerz et al., 2008; Cady et al., 2011). A marked increase in CGRP plasma level is observed during migraine headache (Edvinsson and Uddman, 2005). In addition, plasma CGRP levels from migraine sufferers with and without aura are considerably higher than those of healthy non-sufferers (Fusayasu et al., 2007; Goadsby et al., 1990), and its level becomes even higher during migraine attacks (Fan et al., 2009). High doses of CGRP intravenous injection can induce dilation of the cerebral cortical pial arteries/arterioles. However, this was probably secondary to a decrease in blood pressures (Petersen et al., 2005a). CGRP infusion also induces delayed headache in migraine patients with aura (MA) (Hansen et al., 2010). However, it remains unclear why CGRP is able to cause a delayed headache in migraine sufferers.

CGRP is found in 35–50% of all trigeminal neurons and in over 90% of those with cell diameters less than 15 μm (Lazarov, 2002). CGRP-receptor is a member of the G protein-coupled receptor family, and is present in the peripheral and central nervous system

* Corresponding author. Tel.: +66 87 9241078; fax: +66 2 4411013.
*E-mail address:* banthit.che@mahidol.ac.th (B. Chetsawang).

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(Edvinsson and Goadsby, 1995), trigeminovascular system and TNC (Lennerz et al., 2008). CGRP-activated CGRP-receptors affect several systems, including neurogenic inflammation (recruiting immune cells, and activating sensory neurons) in peripheral tissue (Cady et al., 2011) and vasodilation of meningeal vessels (Xu et al., 2009; Zhang et al., 2007; Nicoletti et al., 2008). CGRP receptors activation induces several intracellular downstream effector molecules such as cAMP, PKA, PKC, mitogen-activated protein kinases (MAPKs) and $K^+$ channel (Brain and Grant, 2004; Vause and Durham, 2009; Sun et al., 2004).

Transient receptor potential vanilloid-1 (TRPV1) is originally named VR1 and is a member of transient receptor potential (TRP) calcium ion channels, which respond to many direct stimuli, exogenous and endogenous, both in physical and chemical forms. These ion channels can be phosphorylated by a variety of protein kinases, including calcium/calmodulin-dependent protein kinase II (CaMKII), PKA (Vetter et al., 2008) and PKC (Jeske et al., 2009; Xu et al., 2009). The PKC induces phosphorylation of TRPV1, leading to enhancement of the translocation of the channel from cytoplasm to cell membrane (Xu et al., 2009). TRPV1 is considered as a marker of nociceptive primary afferent neurons (Tominaga and Tominaga, 2005). TRPV1-immunopositive neurons are mostly small and medium size trigeminal neurons, and send their unmyelinated fibers to terminate at laminae I and II of trigeminal sensory nuclei caudalis (Bae et al., 2004), which play a pivotal role in pain sensitization.

In the trigeminovascular system, CGRP and TRPV1 are co-localized in trigeminal neurons innervated cranial vascular and neurons in TNC (Bae et al., 2004; Price and Flores, 2007). Activation of TRPV1 results in increase in the release of CGRP, glutamate and substance P (SP) in TG neurons. Increasing evidence has emphasized the increase in the activity of peripheral nociceptors inducing changes in the expression of various proteins in the trigeminal ganglionic neurons (Durham and Garrett, 2010). Taken together, high plasticity of this system has been proposed to explain the changes in the clinical features of headache. However, the underlying mechanism by which CGRP promotes sensitization in trigeminal nociceptive system, which relates to migraine pain, remains unclear.

Thus, in the present study we investigated the modulatory role of CGRP on activation of intracellular signaling cascades, phosphorylated PKC and phosphorylated cyclic AMP responsive element-binding protein (p-CREB), and their consequent role in inducing the increase in TRPV1 and CGRP levels in TG. We also investigated the functional role of CGRP on activation of neurons in TNC by observing the induction in c-Fos expression.

## 2. Methods

### 2.1. Animals

Male Wistar rats, 250–300 g, were obtained from National Laboratory Animal Center, Mahidol University, Salaya, Nakhonpathom, Thailand. All procedures were performed under anesthesia using intraperitoneal (i.p.) injection of 60 mg/kg sodium pentobarbiturate (Nembutal®). Control rats were injected intravenously (i.v.) with 15 μl/100 g 0.9% normal saline solution (NSS) into their femoral veins. CGRP-injected rats were i.v. injected with CGRP (MW 3789) (Sigma–Aldrich, St. Louis, MO) dissolving in NSS (15 μl/100 g) into their femoral veins. Our preliminary study on various doses and times of CGRP administration affecting TRPV1 levels in TG had been tried and we found that post i.v. injection with 300 ng/kg CGRP for 45 and 60 min and 600 ng/kg CGRP for 45 min did not alter the TRPV1 levels in rat TG when compared to the control-NSS-injected rats. However, post i.v. injection with 600 ng/kg CGRP for 60 min tended to increase TRPV1 levels in TG. Therefore 600 ng/kg CGRP was chosen throughout this study. The effect of CGRP injection on blood pressure of rat was checked by recording mean arterial pressure (MAP), systolic pressure (SP) and diastolic pressure (DP). The i.v. injection with 600 ng/kg CGRP slightly decreased MAP, SP and DP. The study was conducted in accordance with the NIH Guidelines on the Care and Use of Animals and the protocol was approved by the Institute of Molecular Biosciences Animal Care and Use Committee (MB-ACUC), Mahidol University, Thailand (COA. NO. MB-ACUC 2011/002).

### 2.2. Western immunoblotting

Animals were decapitated under anesthesia, and the trigeminal ganglion and trigeminal nucleus caudalis (1–2 mm caudal to the obex) were rapidly removed. Tissues were homogenized in ice-cold lysis buffer containing 150 mM NaCl, 50 mM Tris (pH 7.4), 1 mM phenylmethanesulfonylfluoride (PMSF), 1 mM EDTA, 10% TritonX-100, 0.5% Na deoxycholate, 0.1% SDS, phosphatase inhibitor (100 mM NaF, 100 mM $Na_3VO_4$ and 400 mM sodium tartate dehydrate) and protease inhibitor (Cell Signaling, Beverly, MA). Protein determination assay was performed according to Lowry method. Protein samples were denatured and separated by 10% SDS-polyacrylamide gel-electrophoresis and then transferred onto a PVDF membrane (Amersham Biosciences, Piscataway, NJ). Membranes were incubated with 3% non-fat milk in 0.1% Tween-Tris-buffered saline (TBST) for 1 h at room temperature and incubated overnight at 4 °C with primary antibodies, goat anti-VR1 (P-19): sc-12498 (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-phospho-PKC (pan) (βII Ser660): #9371 (1:1000 dilution; Cell Signaling), rabbit anti-phospho-CREB (Ser133): #9198 (dilution 1:1,000 dilution; Cell Signaling), and mouse anti-c-Fos: OP53 (1:1,000 dilution; Calbiochem, Darmstadt, Germany), followed by horseradish peroxidase (HRP)-conjugated goat-anti IgG (1:20,000 dilution; Cell Signaling), HRP-conjugated rabbit-anti IgG (1:5000 dilution; Cell Signaling) and HRP-conjugated mouse-anti IgG (1:10,000 dilution; Cell Signaling) for 1.5 h. Chemiluminescence ECL Plus-Western Blotting detection reagents were used to detect the immunoblots, which were quantified by densitometry analysis with Scion image program (National Institutes of Health, Bethesda, MD, USA). For normalizing protein loading of gels, immunoblots were re-probed with mouse anti-β-actin antibody: MAB1501 (1:10,000 dilution; Chemicon International, Temecula, CA).

### 2.3. Immunohistochemistry

Animals were deeply anesthetized and perfused transcardially with 250 ml of ice-cold phosphate buffered saline (PBS) pH 7.4. TG and TNC were removed and immersed in 4% paraformaldehyde in 0.1 M PBS pH 7.4. Using the free-floating method, tissues were placed in 30% sucrose overnight and transverse 30 μm thick sections were cut with a cryomicrotome at −20 °C. In paraffin section method, tissues were paraffin processed and transverse 3 μm thick sections were cut and deparaffinized before immunostaining.

TG paraffin sections were stained for VR-1 and CGRP by immunoperoxidase staining. TNC sections were stained for c-Fos by immunoperoxidase staining using free-floating sections. In brief, TG sections were warmed with antigen retrieval solution (citrate buffer; Dako, Glostrup, Denmark) in a microwave oven. Endogenous peroxidase activity was inhibited with Dako Peroxidase Blocking Reagent (Dako) for 5 min, and non-specific staining was blocked with antibody diluent (Dako) for 10 min. Sections were incubated with primary antibodies; goat anti-VR-1 (1:200 dilution; Santa Cruz) and rabbit anti-CGRP: C8198 (1:6000 dilution; Sigma–Aldrich) overnight at 4 °C. Endogenous peroxidase activity in TNC sections was inhibited with 1% $H_2O_2$ in 50% methyl alcohol for 30 min, and non-specific staining was blocked with antibody diluent (Dako). Sections were incubated with primary antibody, rabbit anti-c-Fos: sc-253 (1:2000 dilution; Santa Cruz Biotechnology) overnight at 4 °C. Sections then were incubated with HRP-conjugated anti-IgG. Antigen visualization was carried out using EnVision™ Detection System (Dako) and sites of peroxidase activity were detected under a light microscope following treatment with 3,3′-diaminobenzidene tetrahydrochloride (0.005% DAB, 30% $H_2O_2$ in 0.05 M Tris–HCl, pH 7.2). The immunostaining sections were viewed under a confocal microscope. TG neurons were classified as small, medium and large based on their cellular cross-sectional area being less than 400 $\mu m^2$, 400–800 $\mu m^2$ and more than 800 $\mu m^2$, respectively. TRPV1- or CGRP-immunopositive neurons were manually counted in TG sections. One staining section of TG per rat with regular TG neuron distribution was chosen for counting cells. All positive and negative immunoreactive (IR) TG neurons in each section were counted. The ratio of positive IR neurons/total neurons and the mean value of each experimental group were calculated. The number of c-Fos positive immunostaining neurons in three different levels of TNC lamina I and II from obex to cervical of spinal cord were manually counted. One c-Fos staining section per TNC level/rat was chosen for counting cells. Fos immunoreactive cells were counted and expressed as number of cells per section.

For immunofluorescence staining, TG paraffin sections were warmed in a microwave oven and non-specific staining blocked as described in the process of immunoperoxidase staining. Sections were incubated overnight with a combination of antibody against VR-1 (1:200 dilution) and antibody against CGRP (1:6000 dilution). After washing with PBS, sections were incubated with tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-rabbit IgG and fluorescein isothiocyanate (FITC)-conjugated anti-goat IgG (Dako) for 60 min at 37 °C. Sections were mounted using Fluorescence Mounting Medium containing 4′,6-diamidino-2-phenylindale (DAPI) and viewed under a confocal microscope.