Current Neurobiology 2010; 1 (1): 82-88

Expressional change in calcitonin gene-related peptide, receptor activity modifying protein 1 (RAMP1) and bradykinin 1 receptor after experimental inflammation in rat trigeminal ganglia.

Kim A Kristiansen and Lars Edvinsson

Glostrup Research Institute, Department of Clinical Research, Glostrup Hospital, Ndr Ringvej 69 DK-2600 Glostrup, Denmark

Abstract

Inflammation is believed to play a crucial role in the onset and maintenance of migraine. Other key players in migraine are calcitonin gene-related peptide (CGRP) and its receptor components receptor activity modifying protein 1 (RAMP1) and calcitonin like receptor (CLR). In this study we provoked an inflammatory response in rat trigeminal ganglia following tissue culture. We show the bradykinin 1 receptor is immunohistochemically localized to the neuron nuclei in fresh ganglia and after tissue culture the localization has shifted to solely in the cytoplasm of some of the neurons. We also found that CGRP aggregated into distinct vesicles/granules upon tissue culture.

Key words: Calcitonin gene-related peptide, Cytokines, Inflammation, Migraine, Trigeminal ganglia

Accepted January 11 2010

Introduction

Several lines of evidence indicate that glutamate and calcitonin gene-related peptide (CGRP) are involved at different levels of the pain pathways activated in migraine attacks. CGRP is elevated in cranial venous outflow during pain in primary headaches and CGRP receptor blockers are effective in migraine therapy [1;2]. In a manner similar to CGRP, studies have suggested elevated levels of glutamate in migraine sufferers [3-5]. Neuronal tracing studies have shown that the intracranial pain pathways with sensory trigeminal C- and Aδ-fibres project to lamina I/II and lamina III/IV, respectively, in the trigeminal nucleus caudalis (TNC) and corresponding regions at the C1-2 levels [6;7]. The fibres store mainly CGRP, but also substance P, pituitary adenylate cyclase activating peptide (PACAP) and nitric oxide [8-10]. The signal is then transmitted to the second order of neurons in the brain stem [11] and these neurons then use glutamate as signalling molecule.

In the trigeminal ganglion, glutamate is co-localized with CGRP [12]. In addition, neurons with glutamate may have 5-HT receptor subtypes [12;13] and also various types of calcium channels [14]. Together evidence suggest an intimate role for these signal molecules and related receptors. CGRP and its receptor may participate in inflammatory responses, hence, CGRP probably plays a crucial role of initiating and maintaining migraine [15]. A number of studies have shown involvement of neurogenic inflammation following activation of the trigeminal pathway [16]. Cytokines play a prominent role in inflammation and are involved in regulation of downstream proteins that facilitate the inflammatory response [17]. Different cytokines often possess similar functions in the regulatory pathways; since they can act as backup if one cytokine is blocked. There is accumulating evidence that interleukin 6 (IL6) has both pro- and anti-inflammatory functions depending on circumstances [18]. Cytokines are involved in elevated signalling between neurons and satellite glial cells (SGC) in the trigeminal ganglion during inflammation [19-22].

The bradykinin receptors, G-protein-coupled receptors, are divided into the constitutively expressed bradykinin 2 receptor (B2R), distributed throughout the central and peripheral tissues. The bradykinin 1 receptor (B1R) which is not expressed under normal physiological conditions but rapidly upregulated following inflammation [23]. B1R is up-regulated mainly by the pro-inflammatory cytokine IL1β but also others such as TNFα and IL2 can up-regulate the B1R expression [23-25]. In rats B1R was upregulated following enhanced expression of the pro-inflammatory cytokines IL 1β and IL6 [26;27] and these cytokines are found in predominantly in activated immune cells [28]. Thus, B1R is strongly linked to nociception and pain as the receptor has been found in pain transmitting tissues [23;29].
The aim of this study was to examine the relation of CGRP, the CGRP receptor, (using RAMP1 as a marker), and the B1R, combined with organ culture as a way to induce activation of the cytokine response in the trigeminal ganglion. The

response to organ culture was studied using immunohistochemistry and the cytokine regulated proteins downstream of pro-inflammatory cytokines were visualized.

At resting conditions, in fresh ganglia, the B1R is expressed in neuronal nuclei. Organ culture of TG slices elicited an activation and relocation of the B1R; this may be a new way to induce an inflammatory reaction in the TG, involving different cell types but allowing them to interact.

Materials and Methods

Material

Sprague Dawley rats (n = 12; male, b. w. 250 g) were used for tissue acquisition. The animals were kept at controlled temperature and humidity with free access to food and water. The experimental procedures were approved by the Danish veterinary authorities. The animals were terminated using CO_2 and decapitation. Immediately after decapitation the trigeminal ganglia were dissected out of the animals and transferred to 1xTris buffered saline (TBS) on ice for transport to the laboratory.

Treatment of trigeminal ganglia

Immediately after dissection the fresh TG were directly transferred to either Trizol solution (Invitrogen, Denmark) for RNA extraction or embedded in Tissue-Tek (Sakura Finetek, Denmark) and snap frozen on dry ice for immunohistochemistry.

TG used for tissue culture was immediately transferred to 1 ml of Dulbeccos modified eagle medium (DMEM containing 1g/L D-Glucose, L-Glutamine and Pyruvate) (Gibco, Invitrogen, Denmark) and incubated in a heat incubator at 37ºC in the presence of 5% CO_2 for 24 h. To avoid infections a mixture of penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (25 µg/ml) (Invitrogen, Denmark) was added to the media prior to incubation.

RNA extraction

Following the manufacturers protocols, total RNA preparations were obtained using a Trizol RNA isolation kit (Invitrogen, Denmark). Briefly, the TG was homogenized using lysing matrix D tubes in a FastPrep fp120 machine (both MP Biomedicals, France). The homogenates were suspended in 1 ml of Trizol and centrifuged at 4 ºC to remove fat and debris. The supernatant was transferred to a new tube for further processing. The samples were incubated at room temperature for 5 min and 0.2 ml of 1-Bromo-3-chloro-propane (Sigma, Denmark) was added. The samples gently mixed by hand and incubated at room temperature 3 min before centrifuging at 12.000 g for 15 min at 4 ºC. After phase separation 400 µl of the upper RNA containing phase was transferred to a new tube and an equal amount of 70% ethanol was added. The obtained RNA was washed through a series of on column binding and washing steps before resuspending in 30 µl DEPC water. RNA concentration and purity was assessed using a NanoDrop 2000c machine (Thermo Scientific). Both 260/280 ratio and 260/230 ratio was measured using 1 µl RNA dilution for analysis in the NanoDrop and typical values for both ratios were between 1.8 - 2.0 indicating a high degree of purity. Typical RNA concentrations were of 100 ng/µl RNA or more.

RT-PCR

Reverse transcription of total RNA to single strand cDNA was carried out using a RT^2 First strand kit (SABiosciences, through Tebu-bio, Denmark) in a GeneAmp PCR system 2400 (Perkin Elmer, Demark) using 800 ng of RNA in a 20 µl reaction volume using random hexamers as primers. Real-time PCR was performed on a 7500 fast Real-Time PCR detection system (Applied Biosciences, Denmark), using 25 µl reactions with 1.13 µl of cDNA as template in a premade assay plate from SABioscience (PARN-021) detecting 84 different "common" cytokines and 6 controls (Tebu-bio, Denmark). On each plate the detection was performed in triplicate and run for 40 cycles. The expressions were normalized to the internal on plate controls to account for differences in cDNA reaction efficiency.

The data was analysed using the online program RT^2 profiler PCR array data analysis, provided by SABiosciences http://www.sabiosciences.com/pcr/arrayanalysis.php). The triplicate group of fresh samples were chosen as control group and compared to the culture treated triplicate group. For direct comparison Ct values and fold regulation were extracted from the dataset and are depicted in table 1.

Immunohistochemistry

Fresh and *in vitro* cultured trigeminal ganglia were transferred to Tissue-Tek, snap frozen on dry ice and stored at - 80°C until further processing. Sectioning of the frozen TG was carried out in a CM3050s cryostat (Leica, Denmark) with a section thickness of 16 µm. The TG sections were placed on superfrosted tissue slides and left to dry out for no more than 2 hrs. Afterward the samples were fixed for 20 min using Stefanini´s fixture (2% paraformaldehyde and picric acid 2%), rinsed/permeabilized three times in 1x phosphate buffered saline (PBS) containing 0,25% triton X 100. Then

samples were blocked for 1 hr to prevent unspecific staining using 1x PBS with 0,25% triton X 100 and 1%BSA (dilution buffer) mixed with 5% donkey serum. Then slides were incubated overnight with primary antibodies (see table 2) diluted in a buffer containing 2% donkey serum. The following day slides were washed three times in 1x PBS and incubated with secondary antibodies (table 2) for 1 hr followed by triple washing in 1x PBS. The tissue samples were then counterstained for DNA using the DNA specific dye Hoechst (Invitrogen) 1:5000 diluted in 1xPBS with 5 min incubation followed by three washes with 1xPBS. Finally the slides were mounted with Clearmount mounting media (Invitrogen) and left at 60 ºC for 30 min to dry before proceeding to image capturing.

Table 1. For both cytokines the RT-PCR cycle threshold (Ct) is much higher for the fresh tissue compared to the cultured tissue. The differences present large upregulation (calculated as ΔΔCt) for both cytokines, the highest being IL6. Data are presented as mean +/- standard error of the mean (S.E.M.), n = number of experiments.

	Fresh Ct mean n= 3	Tissue culture Ct mean n= 3	Fold upregulation
IL6	39.98 +/- 0.45	23.85 +/- 0.75	507.92
Lif	33.56 +/- 1.72	25.16 +/- 0.88	307.72

Experiments were setup as triple staining where all samples were stained with Hoechst to detect nuclei, with Goat polyclonal anti Bradykinin B1R antibody to localize the B1R and also with either Rabbit polyclonal anti RAMP1 antibody or Rabbit polyclonal anti CGRP antibody to study CGRP/CGRP receptor localization with respect to B1R localization. All staining were conducted as doublets, negative control omitting primary antibodies were routinely made in all staining batches.

Microscopy

Staining intensity and staining patterns in the samples was recorded using a Nikon C1 confocal microscope (Nikon, Sweden). The microscopy was carried out using a 60x oil immersion lens with NA 1.4 in xy mode with an averaging of 6 scans in order to lower the contribution of unspecific random noise seen as background. The pictures were acquired using frame λ, meaning that the different laser channels were collected one by one before next z position was acquired. Fluorescence intensity measurements were performed as a region of interest for all samples. The laser channels used was 405nm excitation with filter 450/35 collecting Hoechst staining, 488 nm excitation with filter 515/30 collecting Dylight 488 and 543 nm excitation with filter 605/75 collecting Dylight 549. Image and data analyses were conducted using NIS basic research software (Nikon, Japan) and graph visualization was performed in Excel. Fluorescence intensities were normalized to the values for fresh cytoplasm/nuclei which were set to 1.

Table 2. All the antibodies used for immunohistochemical experiments are listed including working solutions.

Antibody type	Name	Concentration	Manufacturer
Primary	Goat polyclonal anti Bradykinin B1R	1:200	Santa Cruz Biotechnology, USA (sc-15048)
Primary	Rabbit polyclonal anti RAMP1	1:100	Santa Cruz Biotechnology, USA (sc-11379)
Primary	Rabbit polyclonal anti CGRP	1:800	Euro-Diagnostica, Sweden (2263B47-1)
Secondary	Dylight 488 Donkey-anti-Goat	1:200	Jackson Research, USA (705-485-003)
Secondary	Dylight 549 Donkey-anti-Rabbit	1:200	Jackson Research, USA (711-505-152)

Results and Discussion

Up regulation of pro-inflammatory cytokines following incubation

Our first aim was to examine if the tissue culture model of TG elicits an inflammatory type of response and if it is suitable for study of the effects of inflammation on neuron-satellite glia cell (SGC) signalling in the trigeminal ganglia. Hence, we investigated the influence of 24 hour incubation of TG sections and compared to fresh TG using a microarray

system. The main finding was a strong significant up-regulation of the pro-inflammatory cytokines IL6 and Leukemia inhibiting factor (lif), using a specifically designed qRT-PCR array for the study of "common cytokines" (Table 1).

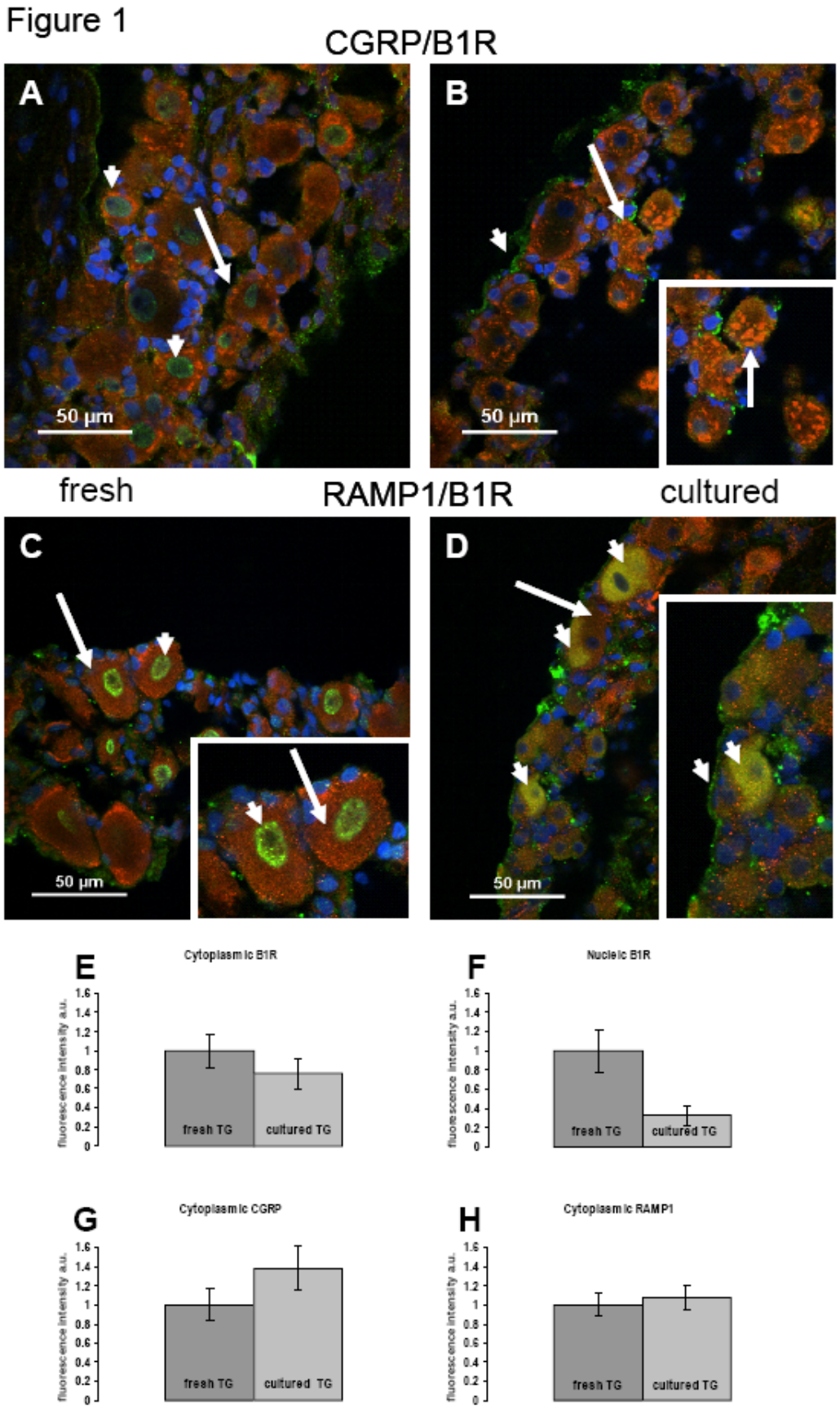

Figure 1.Immunohistochemical staining patterns A-D and fluorescence intensities E-H found in rat TG.

Blue colour is Hoechst/DNA staining, green colour is B1R staining and red colour is CGRP or RAMP1 staining A-D. A) Fresh TG stained for CGRP/B1R. B1R is seen to localize primarily to the larger nuclei of the neurons (green and arrowheads). CGRP (red and arrow) is localized to the cytoplasm of the neurons, occasionally in vesicles/granules. Nuclei of both neurons and SGC are stained blue. B) Cultured TG stained for CGRP/B1R. B1R is seen not to localize to the nuclei of the neurons anymore. The distribution has shifted and the B1R is now localized to the SGC band to the outside of the neurons (green and arrowheads). CGRP (red and arrow) is localized to the cytoplasm of the neurons, and the vesicular/granular distribution has intensified (arrows and insert). Nuclei of both neurons and SGC are stained blue. C) Fresh TG stained for RAMP1/B1R. B1R is seen to localize primarily to the larger nuclei of the neurons (green, arrowheads and insert). RAMP1 (red, arrows and insert) is localized to the cytoplasm of the neurons. Nuclei of both neurons and SGC are stained blue. D) Cultured TG stained for RAMP1/B1R. B1R is seen not to localize to the nuclei of the neurons anymore. The distribution has shifted and the B1R is now localized to cytoplasm in some of the neurons and to the SGC band to the outside of the neurons (green, arrowheads and insert). RAMP1 (red and arrow) is localized to the cytoplasm of the neurons. Nuclei of both neurons and SGC are stained blue. E) Fluorescence intensity of cytoplasmic B1R. No significant difference was found when comparing fresh and cultured TG. F) Fluorescence intensity of nucleic B1R. Significantly higher fluorescence was found for the fresh TG compared to the cultured TG. G) Fluorescence intensity of cytoplasmic CGRP. No significant difference was found when comparing fresh and cultured TG but there is a tendency towards higher fluorescence in the cultured ganglia. H) Fluorescence intensity of cytoplasmic RAMP1. No significant difference was found when comparing fresh and cultured TG but there is a slight tendency towards higher fluorescence in the cultured ganglia. Scale bar; 50 µm.

The distribution of CGRP, RAMP1 and B1R

The CGRP, RAMP1 and B1R distribution was examined in fresh and cultured TG using immunohistochemistry combined with confocal microscopy, using the fluorescence intensities in both nuclei and cytoplasm as a measure of abundance of the three studied molecules. In this study we found that B1R is expressed in the nuclei of the neurons larger and not in the nuclei of the smaller SGC (fig. 1A & C). There was no relation between this receptor and the expression of CGRP and RAMP1. As previously observed CGRP is mainly expressed in small and medium sized neurons of the TG [9]. The B1R was found at significantly higher levels in fresh nuclei compared to nuclei in the cultured TG (fig. 1A & C green colours and arrowheads compared to 1B & D and 1F). We show that at least some of the B1R is (re)located to the SGC zone surrounding and guarding the TG neurons after tissue culture (fig. 1B arrowhead and 1D insert left arrowhead, green colour). No net difference in B1R fluorescence intensity can be detected when comparing fresh and cultured TG (fig 1A-D, green colours & E), but B1R can be found locally in some neurons (fig. 1D, green colours) as reported previously [29]. To the best of our knowledge we are the first to report of the B1R to localize to the nuclei of fresh TG. It is obvious from both micrographs as well as fluorescence intensity measurements that there is significantly higher expression of B1R in nuclei from fresh samples compared to cultured samples. The localization of B1R in the cytoplasm of cultured TG, as seen in fig 1D and 1D inset (arrowheads, green colours), is in accordance with earlier observations in injured mouse dorsal root ganglion (DRG) neurons [29]. SGC have recently been shown to facilitate signalling to and from neurons and play an active part in inflammation [19;22].
We observed CGRP immunoreactivity in the cytoplasm of neurons of middle to small size (fig. 1A-B red colours) which is in accordance with previous observations [9]. The SGC were without CGRP immunoreactivity in fresh ganglia. Organ culture slightly altered the net expression of CGRP towards higher expression in the neurons and SGC (fig. 1A+B & G). There was a redistribution of CGRP in the cytoplasm when comparing fresh and cultured neurons. In fresh neurons a broad unspecific staining was found with sparse tendency towards granular/vesicular occurrence of the CGRP (fig 1A & B red colours and arrows). In the cultured neurons CGRP were mostly found in granules/vesicles, sometimes surrounding the nucleus and sometimes as a more undefined granular staining (fig 1B and insert red colours and arrows). We have seen similar distribution patterns of CGRP in human TG (unpublished data). A possible explanation for CGRP to become localized in granules/vesicles could be that the neurons are preparing to release the neurotransmitter out into the surrounding tissue of the ganglia, i.e. the CGRP is docked for trafficking.

The CGRP receptor consists of calcitonin like receptor (CLR) and RAMP1; upon activation these two elements join and activates intracellular signalling [30]. As seen for CGRP, no overall difference in fluorescence intensity between fresh and cultured TG was found for RAMP1 (fig. 1H). RAMP1 is located in the cytoplasm of both fresh and cultured neurons (fig 1C & D) where it seems to be equally distributed between smaller and larger neurons.

Large size neurons were sometimes seen to have RAMP1 immunoreactivity in their cytoplasm close to the cell membrane (fig 1C). Upon culture; some SGC were now seen to be positive for RAMP1 (fig 1D). These observation suggest that neurons that are CGRP positive can signal to other neurons and SGC that posses the CGRP receptor; we suggest this is morphological evidence for the possibility of intraganglionic transmission.

Conclusion

To summarise we can conclude it is possible to induce inflammation in rat trigeminal ganglia tissue by the use of *in vitro* culturing of the tissue. We show the B1R is localized to the nuclei in fresh TG. In the inflamed tissue the B1R has moved out of the nuclei and disappeared, perhaps being utilized or consumed (bound and internalized) in the ganglia. We found that the neuropeptide CGRP localized to distinct structures in the cultured neurons. These strongly stained structures maybe granules/vesicles prepared by the neurons for trafficking of the neuro-messenger out of the cell bodies to the peripheral ends of the axons.

Competing interests

The authors state no conflict of interest

Authors' contribution

KAK planned and conducted all the experimental work and wrote the manuscript. LE designed and planned the experiments and assisted in writing the manuscript.

Acknowledgements

This work was supported by the Lundbeck Foundation via the Lundbeck Foundation Center for Neurovascular Signalling (LUCENS).

References

1. Edvinsson L: Blockade of CGRP receptors in the intracranial vasculature: a new target in the treatment of headache. Cephalalgia 2004; 24:611-622.
2. Edvinsson L: CGRP-receptor antagonism in migraine treatment. Lancet 2008;372:2089-2090.
3. Martinez F, Castillo J, Rodriguez JR, Leira R, Noya M: Neuroexcitatory amino acid levels in plasma and cerebrospinal fluid during migraine attacks. Cephalalgia 1993;13:89-93.
4. Ferrari MD, Odink J, Bos KD, Malessy MJ, Bruyn GW: Neuroexcitatory plasma amino acids are elevated in migraine. Neurology 1990;40:1582-1586.
5. Cananzi AR, D'Andrea G, Perini F, Zamberlan F, Welch KM: Platelet and plasma levels of glutamate and glutamine in migraine with and without aura. Cephalalgia 1995;15:132-135.
6. Liu Y, Broman J, Edvinsson L: Central projections of sensory innervation of the rat superior sagittal sinus. Neuroscience 2004;129:431-437.
7. Liu Y, Broman J, Edvinsson L: Central projections of the sensory innervation of the rat middle meningeal artery. Brain Res 2008;1208:103-110.
8. Uddman R, Tajti J, Hou M, Sundler F, Edvinsson L: Neuropeptide expression in the human trigeminal nucleus caudalis and in the cervical spinal cord C1 and C2. Cephalalgia 2002;22:112-116.
9. Tajti J, Uddman R, Moller S, Sundler F, Edvinsson L: Messenger molecules and receptor mRNA in the human trigeminal ganglion. J Auton Nerv Syst 1999;76:176-183.
10. Christensen ML, Eades SK, Fuseau E, Kempsford RD, Phelps SJ, Hak LJ: Pharmacokinetics of naratriptan in adolescent subjects with a history of migraine. J Clin Pharmacol 2001;41:170-175.
11. Liu Y, Broman J, Zhang M, Edvinsson L: Brainstem and thalamic projections from a craniovascular sensory nervous centre in the rostral cervical spinal dorsal horn of rats. Cephalalgia 2009;29:935-948.
12. Ma QP: Co-localization of 5-HT(1B/1D/1F) receptors and glutamate in trigeminal ganglia in rats. Neuroreport 2001;12:1589-1591.
13. Hou M, Kanje M, Longmore J, Tajti J, Uddman R, Edvinsson L: 5-HT(1B) and 5-HT(1D) receptors in the human trigeminal ganglion: co-localization with calcitonin gene-related peptide, substance P and nitric oxide synthase. Brain Res 2001;909:112-120.
14. Xiao Y, Richter JA, Hurley JH: Release of glutamate and CGRP from trigeminal ganglion neurons: Role of calcium channels and 5-HT1 receptor signaling. Mol Pain 2008;4:12.
15. Goadsby PJ, Edvinsson L, Ekman R: Vasoactive peptide release in the extracerebral circulation of humans during migraine headache. Ann Neurol 1990;28:183-187.
16. Moskowitz MA: Neurogenic versus vascular mechanisms of sumatriptan and ergot alkaloids in migraine. Trends Pharmacol Sci 1992;13:307-311.
17. Rothwell NJ, Hopkins SJ: Cytokines and the nervous system II: Actions and mechanisms of action. Trends Neurosci 1995;18:130-136.
18. Kocer A, Memisogullari R, Domac FM, Ilhan A, Kocer E, Okuyucu S, Ozdemir B, Yuksel H: IL-6 levels in migraine patients receiving topiramate. Pain Pract 2009;9:375-379.
19. Capuano A, De CA, Lisi L, Tringali G, Navarra P, Dello RC: Proinflammatory-activated trigeminal satellite cells promote neuronal sensitization: relevance for migraine pathology. Mol Pain 2009;5:43.

20. Damodaram S, Thalakoti S, Freeman SE, Garrett FG, Durham PL: Tonabersat inhibits trigeminal ganglion neuronal-satellite glial cell signaling. Headache 2009;49:5-20.
21. Garrett FG, Durham PL: Differential expression of connexins in trigeminal ganglion neurons and satellite glial cells in response to chronic or acute joint inflammation. Neuron Glia Biol 2009;1-12.
22. Thalakoti S, Patil VV, Damodaram S, Vause CV, Langford LE, Freeman SE, Durham PL: Neuron-glia signaling in trigeminal ganglion: implications for migraine pathology. Headache 2007;47:1008-1023.
23. Calixto JB, Medeiros R, Fernandes ES, Ferreira J, Cabrini DA, Campos MM: Kinin B1 receptors: key G-protein-coupled receptors and their role in inflammatory and painful processes. Br J Pharmacol 2004;143:803-818.
24. Calixto JB, Cabrini DA, Ferreira J, Campos MM: Kinins in pain and inflammation. Pain 2000;87:1-5.
25. Talbot S, Theberge-Turmel P, Liazoghli D, Senecal J, Gaudreau P, Couture R: Cellular localization of kinin B1 receptor in the spinal cord of streptozotocin-diabetic rats with a fluorescent [Nalpha-Bodipy]-des-Arg9-bradykinin. J Neuroinflammation 2009;6:11.
26. Cunha FQ, Poole S, Lorenzetti BB, Ferreira SH: The pivotal role of tumour necrosis factor alpha in the development of inflammatory hyperalgesia. Br J Pharmacol 1992;107:660-664.
27. Davis AJ, Perkins MN: The involvement of bradykinin B1 and B2 receptor mechanisms in cytokine-induced mechanical hyperalgesia in the rat. Br J Pharmacol 1994;113:63-68.
28. Waeber C, Moskowitz MA: Migraine as an inflammatory disorder. Neurology 2005;64:S9-15.
29. Rashid MH, Inoue M, Matsumoto M, Ueda H: Switching of bradykinin-mediated nociception following partial sciatic nerve injury in mice. J Pharmacol Exp Ther 2004;308:1158-1164.
30. Lennerz JK, Ruhle V, Ceppa EP, Neuhuber WL, Bunnett NW, Grady EF, Messlinger K: Calcitonin receptor-like receptor (CLR), receptor activity-modifying protein 1 (RAMP1), and calcitonin gene-related peptide (CGRP) immunoreactivity in the rat trigeminovascular system: differences between peripheral and central CGRP receptor distribution. J Comp Neurol 2008; 507:1277-1299.