

Gender differences in the neurochemical response of trigeminal ganglion neurons to peripheral inflammation in mice

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It is well established that the majority of headache and other trigeminal nerve-associated disorders have higher prevalence in females than in males. However, the pathogenesis of many chronic trigeminal pain conditions, such as trigeminal neuralgia, migraine and temporo-mandibular disorders, is still not known. One of the proposed mechanisms involve calcitonin gene-related peptide (CGRP), which is considered the most important neuropeptide in the trigeminal system. In various animal models of trigeminal nerve-associated disorders concentration of CGRP has been shown to be increased in trigeminal ganglia (TG). Moreover, intraganglionic release of CGRP has been shown to modulate neuronal transmission of pain signals. In most of these models, pathological changes in the trigeminal system are accompanied by inflammation within peripheral endings of TG neurons. The aim of the present study was to investigate the relation between gender and neurochemical changes in trigeminal ganglia evoked by peripheral inflammation, induced by Complete Freund Adjuvant (CFA) administration. Our studies show significant increase in CGRP expression in female mice, comparing to male mice. Furthermore, we demonstrate, that activation of trigeminal nociceptors by peripheral inflammation causes significant increase in expression of IL-1 β , IL-6, TNF and BDNF in male mice, comparing to female mice. This phenomenon may be involved in clinically observed gender-dependent differences in the frequency of both migraine and other trigeminal nerve-related facial pain disorders.

Key words: CGRP, BDNF, TNF, IL-1 β , IL-6, trigeminal nerve

Clinical evidence indicates that female to male ratios of the migraine prevalence range from 2: 1 to 3:1 (Buse et al. 2013). Females report to use more prescription and non-prescription medications for headache and greater headache-related disability compared with males (Buse et al. 2013). However, mechanisms underlying differences in prevalence between sexes in migraine and other trigeminal nerve-related disorders are so far poorly understood. Many studies point out possibility of sex hormones (mainly 17- β estradiol and progesterone) influence (Gupta et al. 2007a,b). Similarly, such conditions as pregnancy, menarche and menopause have been shown to be associated with changes in migraine frequency and severity (Gupta et al. 2007a).

Although migraine constitutes a major health and economic problem, affecting up to 15–20% of general population, its pathophysiology is largely unknown (Buse et al. 2013). Migraine is best understood as a neurovascular disorder. It is strongly suggested, that trigeminal activation, resulting both from peripheral and central sensitization, as well as vasodilatation, are responsible for the headache phase of the migraine (Bigal et al. 2013). Functioning of trigeminal system is regulated by many factors, including neuropeptides. Calcitonin gene-related peptide (CGRP) is a neuropeptide widely distributed in the peripheral and central nervous system, which is believed to be a major contributor to a neurogenic inflammation underlying several disorders involving craniofacial structures. It is considered the most important neuropeptide in the trigeminal sensory pathways (Samsam et al. 2001). CGRP occur in two isoforms – CGRP α and CGRP β .

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(Bigal et al. 2013). Both isoforms are similar in their pharmacology (Watkins et al. 2013), but differ in tissue distribution. CGRP α is found predominantly in TRPV1-expressing sensory neurons while CGRP β is mainly present in enteric nervous system (Silberstein and Edvinsson 2013). Both forms have been demonstrated to play protective role in experimentally induced colitis (Thompson et al. 2008). Moreover, CGRP is also considered a factor responsible for gastric mucosa protection (Brzozowski et al. 2005, 2012). Immunohistochemical and radioimmunological studies have demonstrated that CGRP α is produced in the ventral and dorsal root neurons as well as in trigeminal system (Bigal et al. 2013). CGRP α presence has been proven in 35–50% of all the TG neurons (Lazarov 2002). Moreover, receptors for CGRP have been detected within trigeminal ganglia both on glial cells (satellite glia and Schwann cells) and neurons, which suggests, that CGRP can regulate the excitation of trigeminal cells (Capuano et al. 2009). CGRP is released in a paracrine/autocrine manner in the trigeminal ganglia, which suggest its role as a local modulator. Various studies have proven importance of CGRP in the regulation of trigeminal neurons function (Messlinger et al. 2012). On the other hand, application of TNF and IL-1 β to cultured rat TG ganglia has been shown to increase expression of CGRP (Bowen et al. 2006, Bałkowiec-Iskra et al. 2011). Furthermore, tooth pulp inflammation has been shown to increase CGRP concentration in rat TG ganglia (Tarsa et al. 2010). CGRP has been shown to facilitate neurogenic inflammation and nociception in peripheral tissues. CGRP is stored in perivascular nerve terminals surrounding most of the

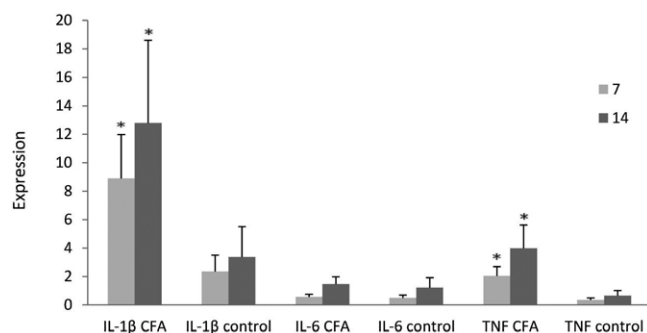


Fig. 1. Expression of IL-1 β and TNF was significantly increased in CFA-treated male mice both on 7th and 14th day after CFA administration compared to control. Data reported as mean \pm SEM, * $P < 0.05$.

blood vessels, thus modulating vascular tone, including cranial blood vessels (Gupta et al. 2007a). Moreover, increased concentration of CGRP has been detected in serum and saliva of patients suffering from acute migraine and cluster headaches (Goadsby and Edvinsson 1993, 1994). Increased CGRP concentration in jugular venous plasma has been reported during migraine in two studies (Goadsby et al. 1990, Goadsby and Edvinsson 1993). However Tvedskov and coauthors (2005) has found no change in CGRP concentration both during migraine attacks without aura and remission phase. CGRP has been shown to participate in the pathology of rhinosinusitis and temporomandibular joint disorder (TMD). CGRP antagonists have been shown to be effective in the treatment of acute migraine, confirming its role in the pathophysiology of migraine (Gupta et al. 2007a, Bigal et al. 2013). Thus, CGRP is considered to act as a mediator of trigeminal signaling during migraine.

Brain derived neurotrophic factor (BDNF) is a mediator of plasticity at central synapses (Cohen and Greenberg 2008) and modulator of nociceptive signaling (Merighi et al. 2008, Latremoliere and Woolf 2009). It is a neurotransmitter with a definite role in the regulation of the trigeminal system function. BDNF expression and production in trigeminal ganglia has been shown to be increased by peripheral inflammatory reaction within peripheral endings of trigeminal nociceptors in tooth pulp (Tarsa et al. 2010). Moreover, BDNF has been shown to be co-expressed with CGRP in cultured TG neurons (Bałkowiec-Iskra 2011). Thus, BDNF can be a candidate mediator of plasticity at first-order synapses in trigeminal nociceptive pathways (Buldyrev et al. 2006) with implications for pathophysiology of migraine and other primary headaches (Latremoliere and Woolf 2009).

Peripheral inflammatory reaction has been shown to influence the function of the nervous system. Proinflammatory cytokines, such as IL-1 β and TNF have established role in the modulation of neurons function (Bałkowiec-Iskra 2010). TNF, in addition to its neuroprotective properties (Figiel 2008) has been shown to cause sensitization of both TTX-resistant sodium channels and transient potential receptor vanilloid type 1 (TRPV1) (Jin and Gereau 2006). TRPV1 receptor function has been also shown to be modulated by IL-1 β . Both, IL-1 β and TNF have been shown to act as direct nociceptors activators (Binshtok et al. 2008) resulting in increased excitability and sensitiza-

tion of e.g. trigeminal neurons (Flake and Gold 2005, Vaughn and Gold 2010). Peripheral inflammatory reaction in the area of trigeminal nociceptors has been shown to increase expression of CGRP and BDNF in trigeminal ganglia (Tarsa et al. 2010). Moreover, TNF has been shown to stimulate expression and secretion of both BDNF and CGRP from cultured trigeminal ganglia neurons (Bałkowiec-Iskra et al. 2011).

In the present study we employed animal model of trigeminal pain, induced by Complete Freund Adjuvant (CFA) administration, to study the role of gender in the trigeminal ganglia neurochemical response to peripheral inflammation. Two-months old, weighting from 23 g to 27 g, C57BL mice (Warsaw Medical University of Warsaw Laboratories, Warsaw, Poland) were used for this study. All the procedures were approved by the Institutional Animal Care and Use Committee of the Medical University of Warsaw, and conformed to the Policies on the Use of Animals and Humans in Neuroscience Research approved by the Society for Neuroscience. A model of oro-facial inflammation was obtained by local injection of 30 μ l of CFA [Adjuvant Complete H37Ra (Bacto, Difco Laboratories, Detroit MI, USA)] to the mice left whisker pad (total 22 mice, 6 female mice and 16 male mice, respectively). Controls were injected with the same volume of saline and into the same area as CFA-treated mice (total 14 mice, 4 female mice and 10 male mice, respectively). Other mice, which were not injected, served as a pure control (total 9 mice, 4 female and 5 male mice, respectively). Seven and 14 days after CFA injection mice were scarified by spinal cord dislocation (27 mice on 7th day – 14 female mice and 13 male mice; 18 male mice on 14th day). Both TG ganglia were removed and frozen immediately in (-80°C) for RT-PCR analysis. Briefly, total RNA was extracted using TRI reagent (Sigma-Aldrich). Quantitative PCR was performed in duplicates using the DNA Engine Real Time Fluorescences Detection System Opticon 2 (MJ Search/Bio Rad) in a final volume of 10 μ l including Maxima SYBR Green qPCR MasterMix (Thermo Scientific – Fermentas), 1 μ l cDNA, and 0.3 μ M of each forward and reverse primers. The forward primers were: 5'-CAA CTT GAT GTA TGA AGG CTT TGG T-3', 5'- ACT TTT ATT GGT CTC AAG TCA GTG TAC AG – 3' (BDNF), 5'- ATA ACA GCC CCA GAA TGA AG -3', 5'-CAA TAC TAA GAA GGA TGC AAA T-3' (CGRP α), 5'-GTC ACT AGC AGC TCC AGG AAG A-3', 5'-TCT ATC AGG ATG

CAA GCA TAC AC-3' (CGRP β), 5'GAG GAT ACC ACT CCC AAC AGA CC3', 5'AAG TGC ATC ATC GTT GTT CAT ACA 3'(IL-6), 5'CAT CTT CTC AAA ATT CGA GTG ACA A3', 5' TGG GAG TAG ACA AGG TAC AAC CC 3' (TNF), 5' GGG AAC TGT GCA GAC TCA AAC TC 3', 5' AAA GAA GAA GAT GGA AAA GGC GGT T3' (IL-1 β). Melting curve analysis was used to check the specificity an amplified product. Relative quantification was performed using Pfaffl's method and GAPDH (5' TCT CCC TCA CAA TTT CCA TCC CAG 3', 5' GGG TGC AGC GAA CTT TAT TGA TGG 3') as reference gene. All the statistical analyses were done in statistical package SAS (v.12.1). For the analysis of the repeated multidimensional observations the Generalized Linear Mixed Models (GLMM) were used. To select the best model for the observed relations the Generalized Chi-Square test divided by degree of freedom was employed. Results are expressed as the means, standard errors and confidence intervals obtained from the model. The *P*-value of 0.05 was considered as significant.

To study the effect of peripheral inflammatory reaction evoked by CFA administration on TG function we measured the expression of BDNF, CGRP and three proinflammatory cytokines – IL-1 β , IL-6 and TNF, separately in the left and the right trigeminal ganglia, both in male and female mice, 7 and 14 days after induction of inflammation. However, on 14th day only male mice were included. In our previous studies, where we used tooth-injury induced model of trigeminal pain in rats, expression of these cytokines has been

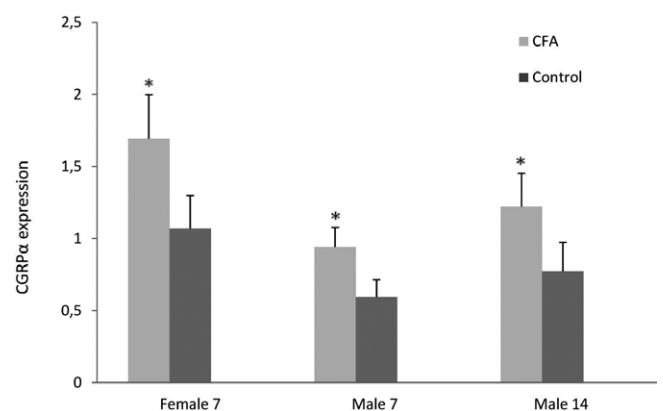


Fig. 2. Expression of CGRP α was significantly increased in both male and female mice 7th and 14th days after CFA administration. Data reported as mean \pm SEM, * *P* < 0.05.

shown to be increased in trigeminal ganglia (Tarsa et al. 2010).

In the present study the expression either of cytokines or CGRP and BDNF did not differ between the left and the right trigeminal ganglia. However, as CFA was injected into left whisker pad, we analyzed all the data separately and in the manuscript we present values obtained from the left TG.

CFA administration caused significant increase in the expression of both IL-1 β and TNF in both female and male CFA-treated mice (male mice – Fig. 1; female mice on 7th day: IL-1 β – 0.5307 ± 0.2290 in CFA group vs. 0.1394 ± 0.0716 in control group; $P < 0.02$; TNF – 0.7086 ± 0.2738 in CFA group vs. 0.1152 ± 0.053 in control group; $P = 0.0004$). We observed increase in expression of IL-1 β and TNF between days 7th and 14th, but it was not statistically significant (Fig. 1). Expression of IL-6 did not differ between groups, however was statistically significantly higher in CFA treated group on the day 14th, comparing to the day 7th (1.45 ± 0.541 vs. 0.56 ± 0.17 ; $P = 0.015$).

Either on day 7th or 14th expression of CGRP α differed significantly between CFA-treated and control groups (Fig. 2). However, we did not observed differences in both CGRP β and BDNF expression between experimental groups. As in behavioral observation all of the mice treated with CFA showed symptoms of facial allodynia and hypersensitivity (as measured by von Frey filaments, data not shown) and non-specific symptoms of pain, such as hyperactivity and anxiety only till day 5 after CFA administration, we presume

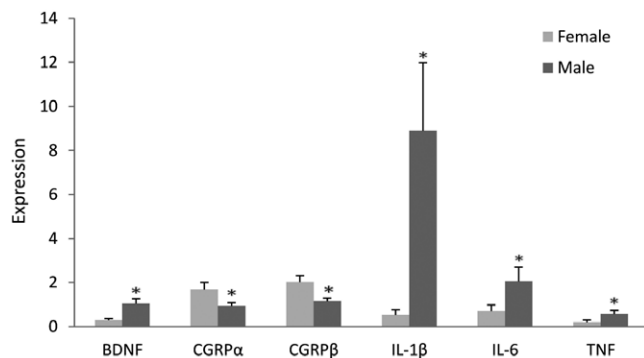


Fig. 3. Expression of BDNF, IL-1 β , IL-6, TNF was significantly increased in CFA-treated male mice comparing to female mice. Expression of both CGRP α and CGRP β was significantly increased in female mice. (7th day after CFA administration). Data reported as mean \pm SEM, * $P < 0.05$.

that on days 7th and 14th BDNF expression could already be normalized. Similarly, Krzyżanowska and colleagues (2011, Krzyżanowska and Avendano 2012) and described hypersensitivity lasting 4 days after injection of CFA. Stucky and others (2011) found that cutaneous allodynia is most pronounced 30 minutes after application of inflammatory soup to the dura.

The most important and intriguing findings of the present study are the differences in the expression of CGRP, BDNF and proinflammatory cytokines between male and female mice.

In the present study we show, that expression of BDNF is significantly increased in male trigeminal ganglia comparing to female TG (1.04 ± 0.22 vs. 0.28 ± 0.07) (Fig. 3). It indicates, that BDNF expression may be regulated not only by CGRP (as shown previously, Tarsa et al. 2010) but also by proinflammatory cytokines. Moreover we show, that expression of both CGRP α and CGRP β has been significantly increased in female trigeminal ganglia comparing to male trigeminal ganglia (CGRP α 1.7 ± 0.3 vs. 0.94 ± 0.14 ; $P < 0.002$; CGRP β 2.01 ± 0.3 vs. 1.15 ± 0.14 ; $P < 0.0004$) (Fig. 3). To the authors knowledge, gender-dependent differences in CGRP concentration was studied only by Stucky and coauthors (2011). In that study no sex differences in CGRP-encoding mRNA was shown, however, females had lower baseline of mRNA encoding 3 components of CGRP receptor (RAMP1, CLR and RCP). Nevertheless, the study employed model of migraine induced by dural application of inflammatory soup, which may explain differences between results.

Pain reaction and activity of neurons are regulated by inflammation. Proinflammatory cytokines are present in sensory ganglia and are suggested to influence the activity of neurons. In our study expression of IL-1 β , IL-6 and TNF was significantly increased in male trigeminal ganglia, comparing to female TG (Fig. 3). It could be related to the immunosuppressive action of CGRP, which has been shown to decrease production of pro-inflammatory cytokines from macrophages and dendritic cells (Holzmann 2013). Observed increased expression of proinflammatory cytokines in male trigeminal ganglia may also be associated with the function of satellite glial cells (SGC). Sensory ganglia (including TG) lack a blood-brain barrier and enclose a high number of satellite glial cells (SGC). SGC directly associate with neuronal soma, which provide physical support and protective barrier. Moreover,

SGC have been shown to respond to neuronal activity with the secretion of factors, such as prostaglandins and NO, that can in turn modulate neurons activity. According to Capuano and colleagues (2009), during neurogenic inflammation trigeminal satellite cells are activated and release pro-inflammatory mediators. These mediators can reduce the firing threshold of sensory neurons. It can be postulated, that in our model increased expression of proinflammatory cytokines in male TG decreased sensitivity of trigeminal neurons, thus preventing its hyperactivation. However, it's worth stressing that we have not yet assessed the synthesis and release of cytokines, which may differ from its mRNA expression. Further studies are necessary to explain direct role of proinflammatory cytokines, including IL-1 β , IL-6 and TNF in the function of trigeminal neurons.

Previous studies from our and other laboratories indicated that the neuropeptide CGRP is expressed by a large subset of TG neurons both *in vivo* (Buldyrev et al. 2006, Tarsa et al. 2010) and *in vitro* (Bowen et al. 2006, Bałkowiec-Iskra et al. 2011). Bowen and colleagues (2006) have shown that the proinflammatory cytokine TNF increases CGRP expression and secretion in cultured TG neurons. Although such conditions as migraine or trigeminal neuralgia are more frequently observed in females, association of gender and CGRP expression in model of CFA-evoked TG dysfunction has never been assessed before. In the present study we show, that expression of CGRP in female TG is statistically significantly higher comparing to male TG. Moreover, we show significantly increased expression of proinflammatory cytokines in male mice comparing to female mice, which suggests that influence of peripheral inflammatory reaction on nociceptors may be gender-dependent.

Our studies indicate that differences in prevalence of TG nerve – related disorders between male and female may be a result of distinct reaction of TG ganglia cells (neurons and satellite glia cells) to activation of trigeminal nociceptors. TG neurons in female mice showed increased expression of CGRP α , the neuropeptide which is thought to act as the main mediator of trigeminal signaling during migraine. Our results indicate that response of TG ganglia to peripheral inflammatory reaction is gender-dependent, what may explain differences in frequency and severity of trigeminal nerve – associated disorders observed between women and men. This phenomenon should be further studied.

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