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Research Submission

Diet-Induced Obesity Enhances TRPV1-Mediated Neurovascular Reactions in the Dura Mater

Balázs Marics, MSc; Barna Peitl, MD, PhD; Kitti Pázmándi, PhD; Attila Bácsi, PhD; József Németh, PhD; Orsolya Oszlács, MSc; Gábor Jancsó, MD, DSc; Mária Dux, MD, PhD

Objective.—Exploring the pathophysiological changes in transient receptor potential vanilloid 1 (TRPV1) receptor of the trigeminovascular system in high-fat, high-sucrose (HFHS) diet-induced obesity of experimental animals.

Background.—Clinical and experimental observations suggest a link between obesity and migraine. Accumulating evidence indicates that metabolic and immunological alterations associated with obesity may potentially modulate trigeminovascular functions. A possible target for obesity-induced pathophysiological changes is the TRPV1/capsaicin receptor which is implicated in the pathomechanism of headaches in a complex way.

Methods.—Male Sprague-Dawley rats were fed a regular (n = 25) or HFHS diet (n = 26) for 20 weeks. At the end of the dietary period, body weight of the animals was normally distributed in both groups and it was significantly higher in animals on HFHS diet. Therefore, experimental groups were regarded as control and HFHS diet-induced obese groups. Capsaicin-induced changes in meningeal blood flow and release of calcitonin gene-related peptide (CGRP) from dural trigeminal afferents were measured in control and obese rats. The distribution of TRPV1- and CGRP-immunoreactive meningeal sensory nerves was also compared in whole mount preparations of the dura mater. Metabolic parameters of the animals were assessed by examining glucose and insulin homeostasis as well as plasma cytokine concentrations.

Results.—HFHS diet was accompanied by reduced food consumption and greater fluid and energy intakes in addition to increased body weight of the animals. HFHS diet increased fasting blood glucose and insulin concentrations as well as levels of circulating proinflammatory cytokines interleukin-1β and interleukin-6. In obese animals, dural application of the archetypal TRPV1 agonist capsaicin resulted in significantly augmented vasodilatory and vasoconstrictor responses as compared to controls. Diet-induced obesity was also associated with enhanced basal and capsaicin-induced CGRP release from meningeal afferents ex vivo. Except for minor morphological changes, the distribution of dural TRPV1- and CGRP-immunoreactive afferents was similar in control and obese animals.

Conclusions.—Our results suggest that obesity induced by long-term HFHS diet results in sensitization of the trigeminovascular system. Changes in TRPV1-mediated vascular reactions and CGRP release are pathophysiological alterations that may be of relevance to the enhanced headache susceptibility of obese individuals.

Key words: headache, obesity, transient receptor potential vanilloid 1, calcitonin gene-related peptide, meningeal blood flow, trigeminal nociception

Abbreviations: CGRP calcitonin gene-related peptide, EIA enzyme-linked immunoassay, HFHS high-fat, high-sucrose, i.p intraperitoneally, IB4 Griffonia simplicifolia isolectin B4, IL-1β interleukin-1β, IL-6 interleukin-6, IRMA immunoradiometric assay, PU perfusion units, SIF synthetic interstitial fluid, sIL-6R IL-6 soluble receptor,

From the Department of Pharmacology and Pharmacotherapy, University of Debrecen, Debrecen, Hungary (B. Marics, B. Peitl, J. Németh); Department of Immunology, University of Debrecen, Debrecen, Hungary (K. Pázmándi and A. Bácsi); Department of Physiology, University of Szeged, Szeged, Hungary (O. Oszlács, G. Jancsó, and M. Dux).

Address all correspondence to B. Marics, Department of Pharmacology and Pharmacotherapy, University of Debrecen, Debrecen, Hungary, email: maricsbalazs@gmail.com

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TNFα tumor necrosis factor α, TRP transient receptor potential, TRPA1 transient receptor potential ankyrin 1, TRPV1 transient receptor potential vanilloid 1

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INTRODUCTION

Migraine is a common and disabling neurological disorder that seems to be potentiated by obesity. Obese persons have increased risk of developing migraine and may suffer from more frequent and severe headache attacks.[1] Clinical and experimental observations suggest multiple links between the pathophysiology of obesity and the primary headache migraine,[2] among which the role of calcitonin gene-related peptide (CGRP) released from trigeminal afferents might be of particular relevance.[3]

CGRP is regarded as one of the key mediators in both nociceptive transmission and meningeal arterial vasodilatation that are critical pathophysiological components of headaches.[4-6] Current migraine therapies are based on reducing CGRP effects by either inhibiting its release from meningeal nociceptors (triptans acting on 5-$HT_{1B/1D}$ receptors) or blocking the CGRP receptor (nonpeptide CGRP receptor antagonists "gepants").[7] Administration of humanized monoclonal antibodies targeting CGRP or its receptor appears also a promising new strategy in the therapy of migraine.[8,9] However, available anti-migraine drugs are not without limitations. Triptans are ineffective in a significant percentage of migraine sufferers, their frequent use may lead to medication overuse headache and they are contraindicated in patients with risk factors for cardiovascular disease that are often associated with obesity.[10,11] Furthermore, the development of gepants has been interrupted due to side effects. Thus, migraine remains a condition calling for additional therapeutic approaches.[12]

Conflict of Interest: None.

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Chemosensitive primary afferent neurons which express the transient receptor potential (TRP) vanilloid 1 (TRPV1) receptor and may also co-express other members of the TRP receptor family represent a unique population of trigeminal ganglion neurons.[13-17] They play a fundamental role in nociception and their peptidergic population releasing CGRP and/or substance P mediates neurogenic vascular reactions in the meninges.[13,18-20] Chemosensitive nociceptors represent a promising target for novel migraine therapeutics.[21,22] TRPV1 is a molecular integrator of various nociceptive stimuli. It can be activated by low pH, noxious heat and different endogenous and exogenous agents, such as endovanilloids (eg, anandamide) and capsaicin, or alcohol, a well-known headache trigger.[19,23,24] Factors related to tissue damage or inflammation may sensitize the receptor by increasing the probability of channel opening on stimulation.[25,26] Enhanced activity of the trigeminovascular nociceptive pathway may result from sensitization of TRPV1 receptors.[27] Prolonged activation of trigeminal afferents may also increase the excitability of second order neurons in the caudal trigeminal nucleus.[28] Processes of peripheral and central sensitization are considered as significant pathophysiological mechanisms of primary headaches. Earlier findings from our laboratory have also proved a capsaicin-induced vasoconstrictor response in dural blood vessels that was present even after functional degradation of trigeminal chemosensitive afferents.[18] Expression of TRPV1 receptors in a wide variety of non-neuronal tissues including smooth muscle cells in different vascular beds may explain the vasoconstrictor effect of capsaicin.[29]

Modification of neuronal and vascular TRPV1 receptor function resulting in altered activity of the trigeminovascular system may represent a link between obesity and migraine pathophysiology. Chronic expansion of adipose tissue producing bioactive molecules with immunoregulatory functions, oxidative and nitrosative stress and microvascular

dysfunction are pathophysiological correlates of obesity[30] that may modulate trigeminovascular functions as well.[31,32] These interrelated disturbances may influence TRPV1 channel function leading to enhanced sensitivity of the trigeminal nociceptive pathway. Recent experimental studies demonstrated an increased capsaicin-induced activity of the trigeminal pain pathway in obese mice.[33,34]

The present experiments were initiated to examine the effects of high-fat, high-sucrose (HFHS) diet-induced obesity on the function of trigeminovascular nociceptive afferents in the rat. Therefore, TRPV1-dependent meningeal nociceptor function was evaluated in control and obese rats by studying capsaicin-evoked meningeal vascular responses, the ex vivo release of CGRP from dural afferents and the immunohistochemical demonstration of TRPV1- and CGRP-immunoreactive nerves of the dura mater. The plasma levels of proinflammatory cytokines as well as glucose and insulin homeostasis were also determined in control and obese animals to gain further insight into possible mechanisms of obesity-related trigeminal neurovascular alterations.

METHODS

Animals and Diet.—Experiments were approved by the Ethical Committee for Animal Care of the University of Szeged and the University of Debrecen. Study procedures were carried out in accordance with the Directive 2010/63/EU of the European Parliament. All efforts were made to minimize the number of animals used and their suffering.

Male Sprague-Dawley rats (6 weeks old, weighing 150-170 g, Charles River Laboratories, Hungary) were housed in an environmentally controlled room (12-hours light/dark cycle, 22 ± 2°C, 50-70% relative humidity) and fed according to our recently published protocol.[35] Animals in the control group ($n = 25$) received a regular diet containing standard rodent chow (3.20 kcal/g, 59% carbohydrate, 32% protein, 9% fat; diet code: S8106-S011 SM R/M-Z + H, ssniff Spezialdiäten GmbH, Germany) and tap water. Another group of animals was placed on an HFHS diet ($n = 26$) consisting of high-fat chow (4.56 kcal/g, 35% carbohydrate, 20% protein, 45% fat; diet code: 824018, Special Diets Services, UK) and 5% sucrose solution made with tap water. Body weight, food and fluid intake of the animals were measured regularly and daily calorie intake was calculated. Diets were provided ad libitum for 20 weeks. All experiments were carried out after the completion of the dietary treatment period in 26-week-old animals.

Analysis of Fasting Blood Glucose, Plasma Insulin, Interleukin-1β (IL-1β), and Interleukin-6 (IL-6) Concentrations.—Following an overnight fast, rats were anesthetized with intraperitoneally (i.p.) administered thiopental sodium (100 mg/kg, Sandoz, Austria). The carotid artery was cannulated for blood sampling. After a short stabilization period, blood glucose was determined with a glucometer (Accu-Chek, Roche Diagnostics, Hungary, detection range: 0.6-33.3 mmol/L) and additional samples were taken to Eppendorf cups. Blood samples were centrifuged for 2 minutes at 10,000*g* and 4°C. The plasma was aliquoted, frozen and stored at −70°C for later analysis. Plasma concentration of insulin was measured by immunoradiometric assay (IRMA) using an insulin IRMA kit (Institute of Isotopes, Hungary, detection range: 8.6-861 μU/mL, sensitivity: 2.8 μU/mL). IL-1β (Thermo Scientific, USA, detection range: 25.6-2500 pg/mL, sensitivity: 12 pg/mL) and IL-6 (Life Technologies, USA, detection range: 23.5-1500 pg/mL, sensitivity: 5 pg/mL) levels were assessed by enzyme-linked immunoassay (EIA) specific for rat according to the manufacturer's instructions.

In Vivo Recordings of Meningeal Blood Flow.—Changes in meningeal blood flow were measured in an open cranial window preparation.[18,36] Control and obese rats were anesthetized with thiopental sodium (100 mg/kg, i.p.). The femoral artery was cannulated on one side for the measurement of systemic blood pressure. The body temperature of the animals was recorded with a thermoprobe inserted into the rectum and was kept at 37-37.5°C with a feedback-controlled heating pad. The trachea was cannulated to allow spontaneous breathing throughout the experiment. The head of the animal was fixed in a stereotaxic frame and the

skin overlying the skull was opened. A cranial window was drilled into the parietal bone to expose the dura mater. Blood flow was recorded with a needle-type probe of a laser Doppler flowmeter (Perimed, Sweden) positioned over a branch of the middle meningeal artery lying distant from visible cortical blood vessels.

The exposed dura mater in the cranial window was covered with 40 μL of synthetic interstitial fluid (SIF) containing (in mM): 135 NaCl, 5 KCl, 1 $MgCl_2$, 5 $CaCl_2$, 10 glucose and 10 Hepes, pH 7.4. Stimulation of the dura mater was performed by topical application of capsaicin (100 nM and 10 μM, Sigma-Aldrich, Germany), CGRP or histamine (both at 100 μM, Sigma-Aldrich) at a volume of 40 μL. Solutions were removed after 5 minutes and the dura mater was washed repeatedly with SIF to allow the blood flow to return to the basal level. Blood flow was measured in perfusion units (PU). Data on meningeal blood flow and systemic blood pressure were processed with the Perisoft program (Perimed, Sweden). Basal blood flow was determined as the mean flow during a 3-minute period prior to the stimulation of the dura mater. Percentage changes in meningeal blood flow in response to capsaicin, CGRP and histamine were determined as mean flow values within the 5-minutes application period calculated separately at one-minute intervals relative to the basal flow. A stock solution of capsaicin (32 mM) was prepared with the aid of 6% ethanol and 8% Tween 80 in saline and was further diluted with SIF. All the other drugs were dissolved in SIF immediately before use.

Measurement of CGRP Release in Ex Vivo Dura Mater Preparation.—An ex vivo rat dura mater preparation was used to measure basal and stimulated CGRP release from meningeal afferents.[37] Control and obese rats were decapitated following deep anesthesia with thiopental sodium (150 mg/kg i.p.). After removing the skin and the muscles, the skull was divided into halves along the midline. The cerebral hemispheres were removed, skull halves were washed at room temperature for 30 minutes in SIF, then placed in a humid chamber and the cranial fossae were filled with 300 μL SIF. Samples of the superfusate were collected with a micropipette at periods of 5 minutes for CGRP measurement. A control sample was taken to determine basal CGRP release. Then the dura mater was stimulated with capsaicin at concentrations of 10 and 100 nM. 100 μL of samples diluted with 25 μL EIA buffer were placed into Eppendorf cups and immediately frozen at -70°C for subsequent analysis. EIA method was used for CGRP determination (SPI-Bio, Bertin Pharma, France). The CGRP concentrations of the superfusates were expressed in pg/mL. Changes induced in CGRP release by capsaicin were expressed as percentage changes relative to the basal release.

Immunohistochemistry.—The distribution of TRPV1- and CGRP-immunoreactive nerve fibers was studied in dural whole mount preparations of rats not used in in vivo blood flow recordings or in ex vivo CGRP release experiments previously. Control and obese animals were anesthetized deeply with thiopental sodium (150 mg/kg, i.p.) and perfused transcardially with physiological saline followed by 4% paraformaldehyde in phosphate buffer (pH 7.4). The skin and muscles of the skull were removed and the skull was divided into halves along the sagittal suture. After removing the brain, samples of the dura mater containing branches of the middle meningeal artery were cut out, postfixed for 2 hours in the same fixative and processed for staining with the indirect immunofluorescence technique using a rabbit polyclonal antiserum raised against the TRPV1 receptor (1:500, Alomone Laboratories, Israel) in combination with a monoclonal mouse anti-CGRP antibody (1:500, Sigma-Aldrich, Germany). IgGs labeled with Cy3 and DL488 were used as secondary antibodies (both 1:500, Jackson Immunoresearch Laboratories, USA). Whole mount preparations of the dura mater were examined under a confocal fluorescence microscope (ZEISS LSM 700, Germany).

Statistics.—All values were expressed as means $\pm$ SEM. Statistical analysis of the data was performed using Statistica 12 (StatSoft, USA). In both dietary groups, normality in terminal body weight was proved by the Shapiro-Wilk test. For the statistical comparisons of CGRP and cytokine concentrations,

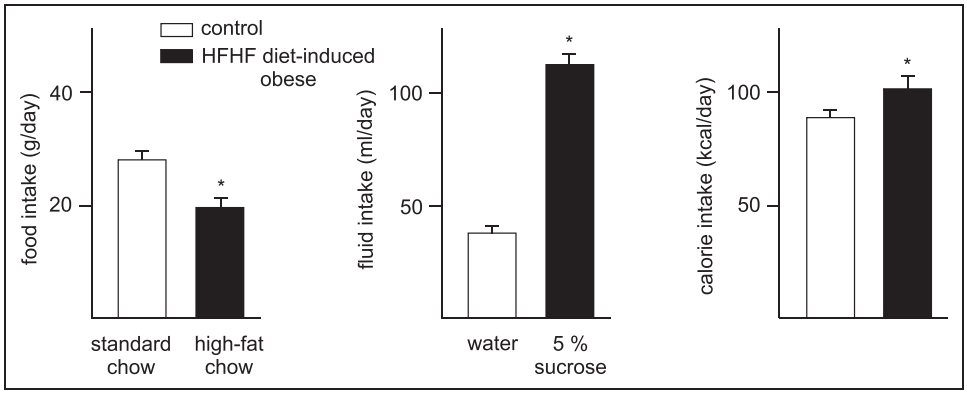

Fig. 1.—Average daily food, fluid, and calorie intakes of rats maintained on regular or HFHS diet (n = 17 in both groups). *Statistically different from the control.

meningeal blood flow changes, food and fluid intake of the animals the Student's t-test was used for group sizes of $n \geq 10$ and the Mann-Whitney U-test for independent measurements of group sizes $n < 10$. One-way ANOVA followed by the Bonferroni test was used to compare metabolic parameters of the animals. A probability level of $P < .05$ was regarded as statistically significant.

RESULTS

Characterization of the Diet-Induced Obesity Model.—On average, the HFHS group of animals consumed smaller quantities of food ($P < .001$) and greater amount of fluid ($P < .001$) than animals fed with a regular diet. The mean daily caloric intake of HFHS rats significantly exceeded the control group ($P < .001$), resulting in a significantly higher body weight at the end of the dietary period (616.5 ± 11 g in control and 740.5 ± 15 g in obese animals, $P < .001$, $n = 17$ for both groups, Fig. 1). Diet-induced obesity led to elevations in fasting blood glucose (5.75 ± 0.13 vs 6.54 ± 0.33 mmol/L, $P = .049$, $n = 8$ and 9) and plasma insulin concentrations (17.46 ± 3.31 vs 48.51 ± 8.67 μU/mL, $P = .006$, $n = 8$ and 9) in addition to circulating levels of the proinflammatory cytokines IL-1β (59.04 ± 2.99 vs 170.04 ± 23.78 pg/mL, $P < .001$, $n = 8$ and 9) and IL-6 (56.39 ± 2.15 vs 114.46 ± 11.32 pg/mL, $P < .001$, $n = 8$ and 9) as indicated recently[35] (Fig. 2). Concentration of all blood markers were above the lower limit of detection. Since body weights indicating the obesity status of the animals were different and normally distributed, we regarded the dietary groups accordingly as control and HFHS diet-induced obese groups.

Diet-Induced Obesity Potentiates Increases in Meningeal Blood Flow Elicited by TRPV1 Activation.—The basal blood flow values were in the same range in control and obese rats amounting to 245.2 ± 18.1 and 244.1 ± 19.6 PU ($P = .96$), respectively. In control rats, topical administration of capsaicin at 100 nM concentration induced a moderate increase in meningeal blood flow reaching significance in the last two minutes of the 5 minutes application period ($P = .048$ and .035, respectively, $n = 8$). Blood flow increasing effect of the same capsaicin concentration was more robust in obese animals throughout the whole application period, it reached $20 \pm 3.1\%$ increase in the last minute ($P \leq .023$, $n = 9$). This increase significantly exceeded the effect measured in control rats ($P \leq .049$, Fig. 3A,B). Administration of capsaicin at 10 μM reduced meningeal blood flow in both groups of animals. In control animals, decrease in meningeal blood flow varied in the range from $4.7 \pm 2.7\%$ to $2.7 \pm 2.5\%$ ($P \leq .33$, $n = 6$), while this range was 14.5 ± 2.9 to $8 \pm 2.8\%$ ($P \leq .036$, $n = 6$) in obese rats. Blood flow reducing effect of 10 μM capsaicin was significantly stronger in obese animals from the second to the fourth min compared to controls ($P \leq .037$, Fig. 3A,C). Histamine and CGRP acting directly on endothelial- or smooth muscle cells of

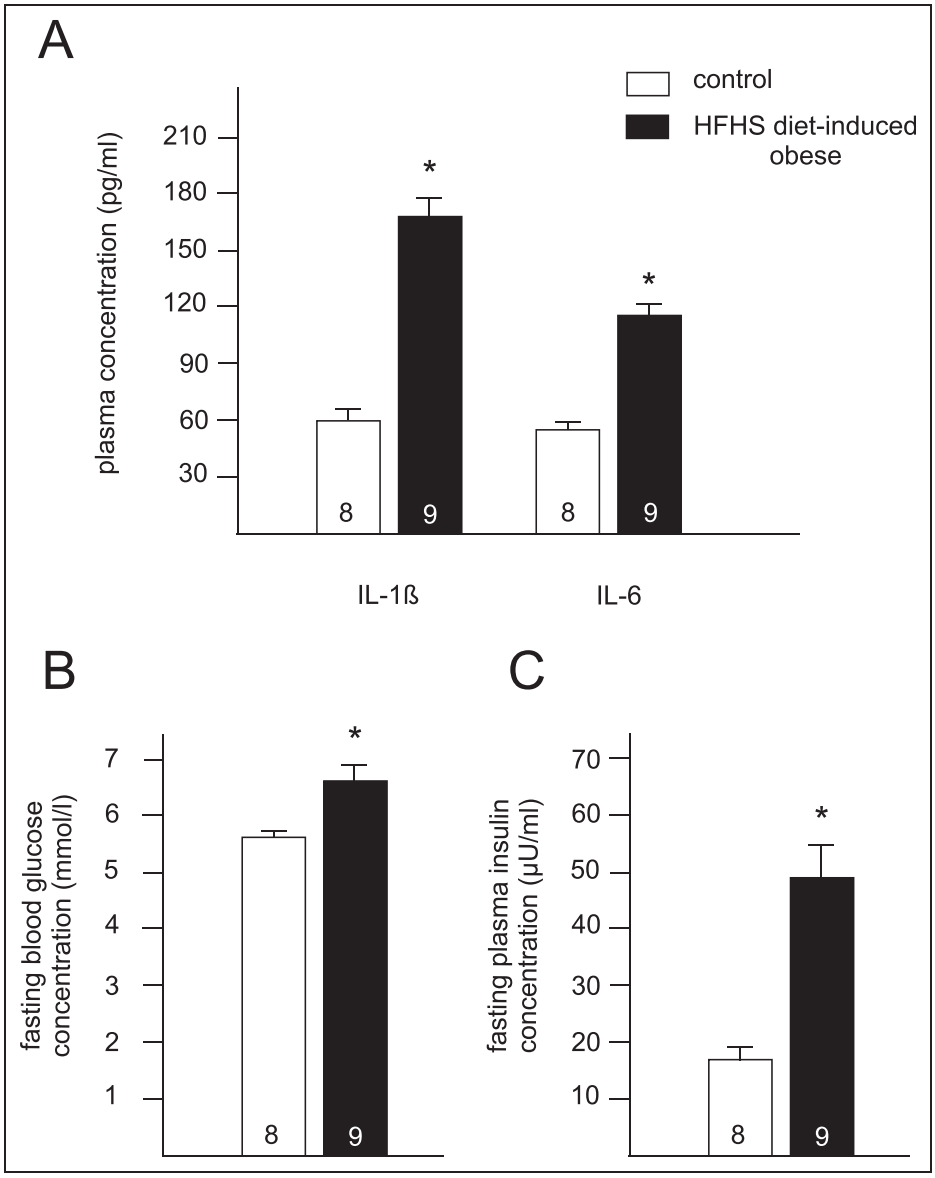

Fig. 2.—Effect of diet-induced obesity on plasma concentrations of (A) interleukin-1β (IL-1β), interleukin-6 (IL-6), (B) fasting blood glucose, and (C) fasting plasma insulin concentrations. The number of experiments is indicated in the bars. *Statistically different from the control.

blood vessels induced significant increases in meningeal blood flow in both control ($P \leq .015$, $n = 8$ both) and obese ($P \leq .035$, $n = 8$ and 10, respectively) rats. No difference regarding the vasodilatation induced by histamine ($P \leq .98$) and CGRP ($P \leq .92$) administrations could be observed between control and HFHS diet-induced obese animals (Fig. 3D,E).

Systemic blood pressure of animals was in the same range as we have published recently.[35] It was 138 ± 12 and 142 ± 18 mmHg in control and obese rats, respectively. Drugs administered topically to the dura mater failed to influence systemic blood pressure.

Diet-Induced Obesity Enhances Basal and TRPV1-Mediated CGRP Release.—In the ex vivo dura mater preparation of obese animals we measured a significantly increased basal CGRP release. Unstimulated CGRP concentration was 16 ± 1.5 in

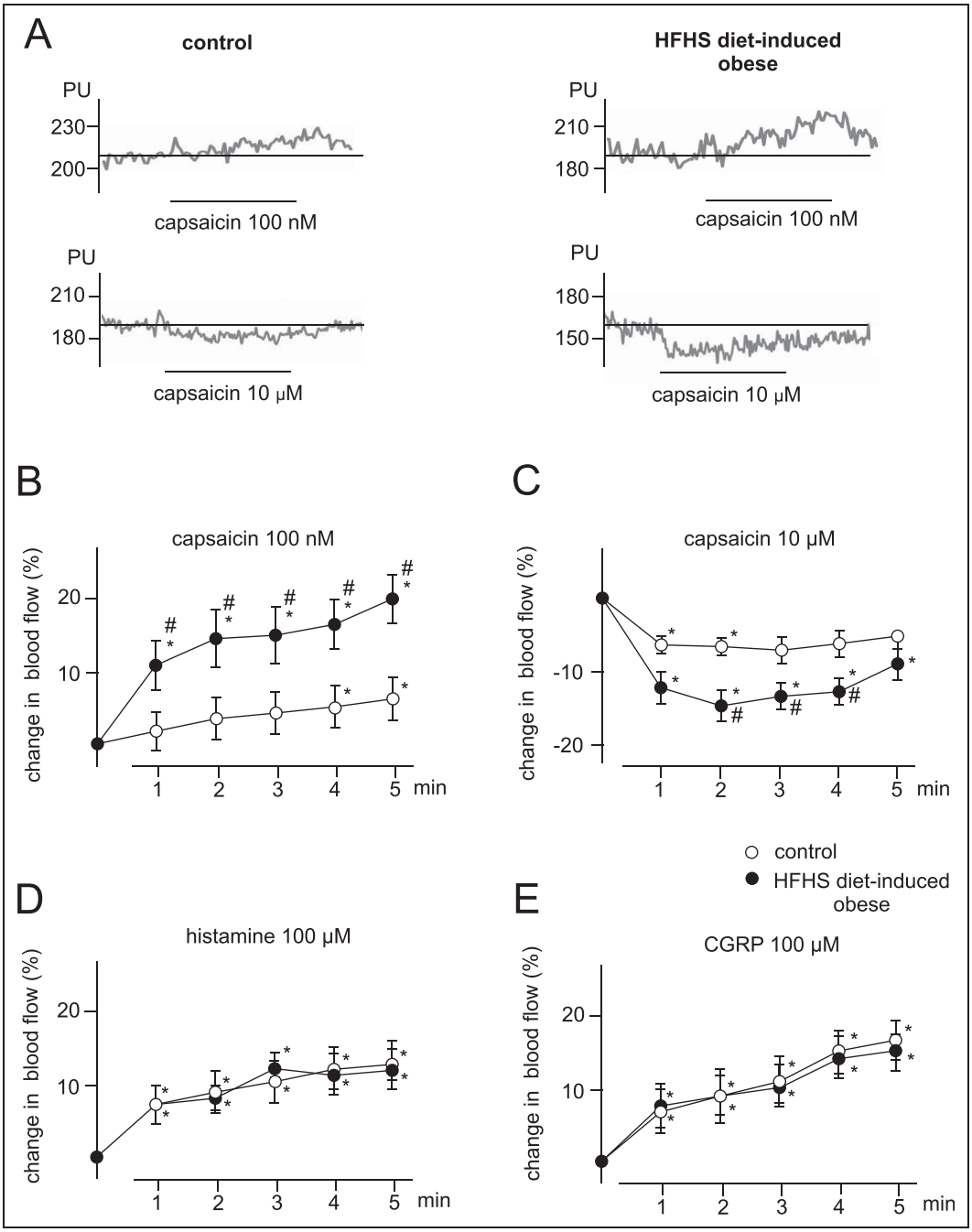

Fig. 3.—Effect of TRPV1 receptor activation on meningeal blood flow. (A) Original recordings and (B-E) statistical evaluation of blood flow-modifying effects of topical applications of capsaicin (100 nM and 10 µM), histamine and CGRP (100 µM both). *Statistically different from the basal flow; #Statistically different from the control.

control and 42.1 ± 6.6 pg/mL in obese rats ($P = .005$). Stimulation with capsaicin significantly enhanced the release of CGRP from meningeal afferents in skull preparations obtained from both control and obese animals. Capsaicin at a concentration of 10 nM elevated CGRP release to 34.7 ± 2.3 pg/mL (196.6 ± 21.2% of basal release, $P < .001$, n = 7) and at 100 nM concentration to 75.6 ± 7 pg/ml (612.7 ± 58.9% of basal release, $P < .001$, n = 6) in rats maintained on a regular diet. In obese animals, capsaicin at 10 nM concentration increased the release of CGRP to 120 ± 27.6 pg/mL

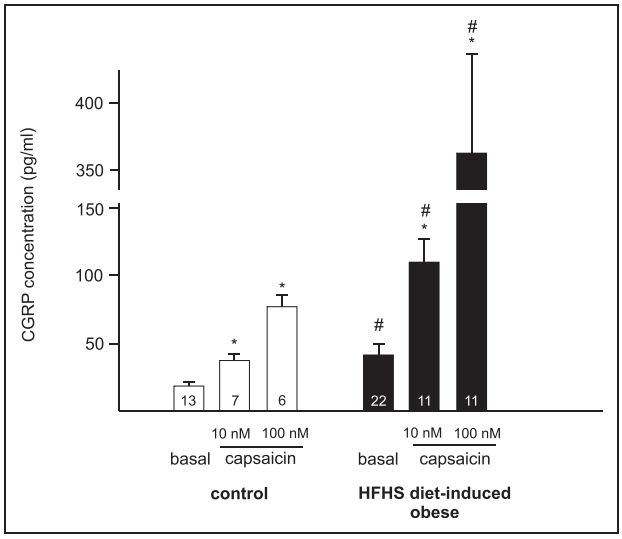

Fig. 4.—TRPV1 receptor activation-induced release of CGRP from meningeal afferents. CGRP concentrations (pg/mL) measured after topical application of SIF and capsaicin (10 and 100 nM). The number of experiments is indicated in the bars. *Statistically different from the basal release; #Statistically different from the control.

(300.1 ± 60.1% of basal release, $P = .004$, n = 11) and at 100 nM to 358.1 ± 95.5 pg/mL (853.1 ± 76% of basal release, $P = .004$, n = 11). HFHS diet-induced obesity resulted in a significantly greater CGRP release in response to both capsaicin concentrations as compared with the control ($P = .027$ and $P < .048$, respectively, Fig. 4).

Effect of HFHS Diet on TRPV1- and CGRP-Immunoreactive Nerve Fibers of the Dura Mater.—In the dura mater of control animals TRPV1- and CGRP-immunoreactive nerve fibres were distributed over the whole supratentorial dura mater. Many TRPV1-immunoreactive nerves were seen running in small nerve bundles in association with dural blood vessels. Single axons were also observed in avascular regions of the meningeal tissue, ie, in areas at a distance from larger blood vessels, where they formed loose nerve plexuses. CGRP was colocalized with TRPV1 in most of these nerve fibres. Although in whole mount dura preparations of obese rats, distribution of TRPV1- and CGRP-immunoreactive afferents was similar to that seen in control dura mater preparations, many single axons showed characteristic changes. While CGRP-immunoreactivity was distributed evenly in the afferents of control dura, in whole mounts of obese animals the immunoreactivity was observed in the form of a string of pearls (Fig. 5).

DISCUSSION

This study was undertaken to study the effect of a state of obesity on trigeminovascular TRPV1 receptor function, a pivotal component of headache mechanisms. Therefore, rats were fed on an HFHS Western-type diet to create a metabolic and immunological condition that may also characterize obese headache sufferers.

HFHS diet altered the eating behavior of animals indicating that dietary treatment influenced processes involved in the regulation of food intake and energy balance.[38] The lower food consumption and higher fluid and calorie intakes in HFHS-fed animals are in agreement with previous reports in which the energy density and palatability of the diet were manipulated in a similar way.[39-41] The accumulation of adipose tissue was accompanied by impairments in glucose and insulin homeostasis and a low-grade systemic inflammatory milieu. The present findings demonstrate that our animal model of diet-induced obesity possessed many of the metabolic alterations that have been previously suggested to be associated with obesity as well as with the pathophysiology of migraine.[42-44]

The function of trigeminal nociceptors can be assessed by studying vascular responses in relation to neuropeptide release from dural afferents. Previous studies have confirmed that the meningeal vasodilatory action of capsaicin is mediated by the activation of TRPV1 receptors in chemosensitive afferents and the consequent release of CGRP from their peripheral endings.[18] Changes in metabolic conditions may modify the functionality of trigeminal chemosensitive neurons. Meningeal TRPV1 expressing nerves in streptozotocin-induced diabetic rats were found impaired showing reduced neurogenic sensory vasodilatation, decreased capsaicin-evoked CGRP release and reduction in the number of TRPV1-immunoreactive nerve fibers of the dura mater.[45] This study provides the first direct evidence that diet-induced obesity in rats leads to augmented

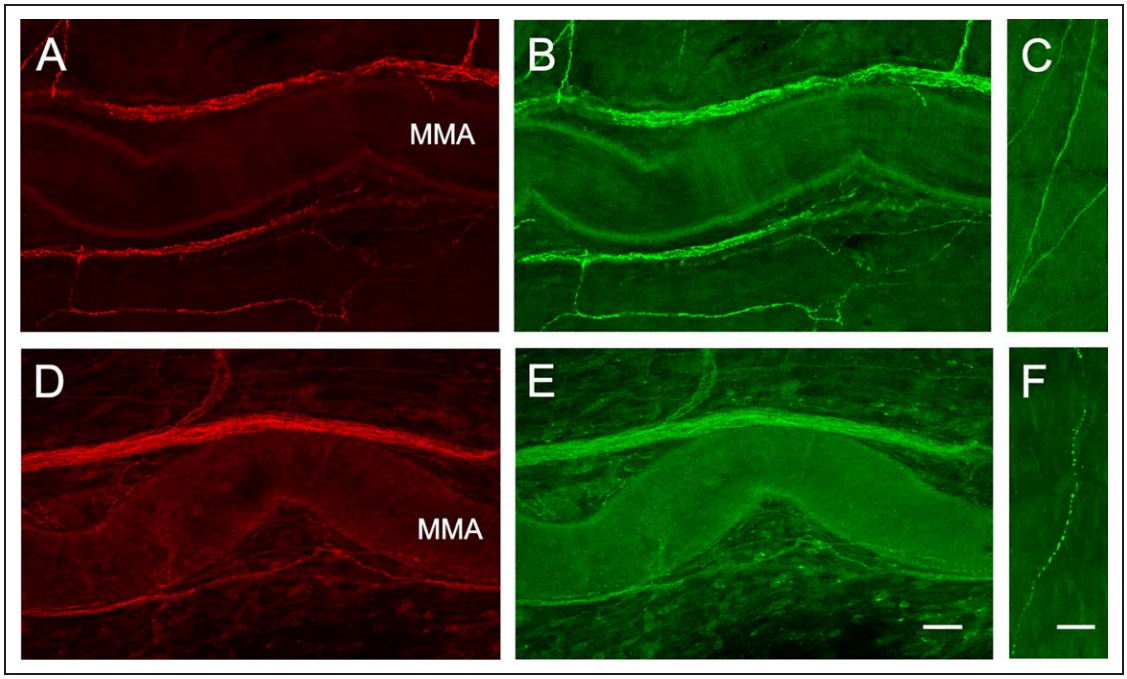

Fig. 5.—Immunohistochemical photomicrographs showing the distribution of TRPV1- (A, D) and CGRP- (B, C, E, F) immunoreactive nerve fibers in the dura mater of control (A-C) and HFHS diet-induced obese (D-F) rats. Distribution of TRPV1- and CGRP-immunoreactive afferents is similar in the dura mater preparations of control and obese animals. The pearl-like distribution of CGRP-immunoreactivity may be the morphological correlate of enhanced CGRP release in obese animals. Scale bar on E represents 50 μm and applies for A, B, D, and E; scale bar on F represents 25 μm and applies for C and F, MMA= branch of the middle meningeal artery. [Color figure can be viewed at wileyonlinelibrary.com]

vasodilatory responses in meningeal blood vessels following activation of trigeminal TRPV1 receptors. Enhanced vascular reactions were selective for capsaicin since neither CGRP- nor histamine-induced vascular reactions in diet-induced obese animals differed from the control. Measurement of capsaicin-induced CGRP release in ex vivo dura mater preparations of the obese rats indicates that an increased CGRP release from meningeal nociceptive afferent nerves may account for the enhanced capsaicin-induced vasodilatation. The current results are in accordance with recent studies in our laboratory that furnished evidence for an enhanced CGRP-mediated neurogenic sensory vasodilatation in the meninges of obese animals on the activation of transient receptor potential ankyrin 1 (TRPA1) channels by the environmental irritant acrolein.[35] However, TRPA1 receptors are expressed only in a subset of TRPV1-positive chemosensitive primary afferent nerves.[46]

Although most studies dealing with headache mechanisms focus on trigeminal peptidergic afferents, it should be emphasized that a large population of TRPV1 expressing neurons does not contain neuropeptides.[47] Non-peptidergic nociceptive primary sensory neurons can be identified through their binding of the plant lectin, Griffonia simplicifolia isolectin B4 (IB4). The possible role of non-peptidergic nociceptive afferents in the pathomechanism of headaches remains to be elucidated. It is conceivable that peptidergic and non-peptidergic TRPV1-expressing neurons provide parallel afferent pathways for the transmission of nociceptive signals.[21] Activation of peptidergic neurons induces increases in meningeal blood flow but release of CGRP from their central terminals in the trigeminal nucleus has presumably only a presynaptic modulatory effect controlling the neurotransmitter release in other populations of primary afferents.[48] Non-peptidergic TRPV1 expressing trigeminal neurons fail to contribute to local neurogenic vascular reactions in the dura mater, but they may provide a direct transmission of nociceptive signals to second

order neurons of the pain pathway.[21] Obesity-related factors that alter the function of TRPV1 receptors in CGRP-containing meningeal afferents may also have the potential to affect TRPV1 in non-peptidergic trigeminal sensory neurons.

Earlier observations in our laboratory demonstrated a capsaicin-induced vasoconstriction in meningeal blood vessels that appeared parallel with the CGRP-induced vasodilatation and was independent of neural processes.[18] The mechanism of capsaicin-induced vasoconstriction is not completely clarified. It is generally regarded as a direct vascular action of capsaicin,[49-51] but contribution of TRPV1 receptors expressed in vascular smooth muscle cells cannot be excluded.[29] Our present findings indicated that in dural arteries both the vasodilatory and the vasoconstrictor actions of capsaicin were enhanced in obese animals.

The mechanisms responsible for obesity-associated changes in capsaicin-evoked meningeal vascular responses and CGRP release are likely to involve complex dose- and time-dependent interactions among multiple causative factors, which may appear already at an early stage of the dietary period enabling them to act chronically on the trigeminovascular system. High-fat diet has been shown to increase calcium influx in both cultured trigeminal ganglion neurons and vascular smooth muscle cells of cerebral arteries,[34,52] therefore alterations in calcium homeostasis in trigeminal primary afferents and meningeal vasculature could potentially contribute to altered neurocrine and vasoconstrictor responses under basal conditions and to stimulation with capsaicin in obese animals.

The increased basal and capsaicin-induced CGRP release that was measured in the ex vivo dura mater preparation of HFHS diet-induced obese animals may result from a sensitization of the TRPV1 receptor. Sensitization may occur in several ways such as modifications in gating properties of the ion channel, increased trafficking of the receptor to cell membrane or increased production of the TRPV1 protein.[22] In the dura mater whole mount preparations of lean and obese animals, in accord with previous data,[45,53] the density and distribution of TRPV1- and CGRP-immunoreactive nerve fibers were similar. It is noteworthy that microscopic examination revealed characteristic structural changes in dural afferents of diet-induced obese animals. The pearl-like appearance of CGRP-immunoreactivity might be the morphological correlate of enhanced CGRP release.[54,55] Despite the known limitations of immunohistochemistry to detect subtle changes in receptor protein content, our findings indicate that obesity-associated potentiation of capsaicin-induced neurogenic sensory vasodilatation was brought about in the absence of obvious changes in TRPV1-immunoreactivity of dural afferents. Sensitization of the TRPV1 receptor is more likely the consequence of the release of proinflammatory agents produced in the adipose tissue,[56] which may also influence neuropeptide-mediated vascular reactions. Indeed, we measured increased plasma concentrations of the proinflammatory cytokines IL-1β and IL-6 in obese animals. IL-1β may directly modify the gating properties of TRPV1 to noxious stimuli in primary sensory neurons while leaving the recruitment of the channel to plasma membrane unaffected.[57] Additionally, IL-1β has been shown to enhance basal and capsaicin-induced CGRP release in the rat trigeminal ganglion neurons,[58,59] whereas the intraplantar injection of this cytokine augmented the capsaicin-induced neurogenic sensory vasodilatation without altering the vascular action of CGRP.[60] In the trigeminal ganglion, IL-1β has also been demonstrated to increase the production of prostaglandin E_2,[59] which may lower the heat activation threshold of TRPV1 and increase capsaicin-evoked responses in sensory neurons[61-63] as well as the release of CGRP in slices of the trigeminal nucleus caudalis.[64] IL-6 in conjunction with its soluble receptor (sIL-6R) may have abilities to reduce heat threshold and increase heat-activated inward currents in rat dorsal root ganglion cells with a possible involvement of TRPV1.[65] Both IL-1β and IL-6/sIL-6R complex have been found to potentiate heat-stimulated CGRP release from an in vitro rat skin preparation.[66] The obesity-related enhancement in systemic and cerebral oxidative stress along with the marked hyperinsulinemia might also contribute to changes in TRPV1-mediated trigeminovascular responses.[67,68] A major role of the proinflammatory

cytokine tumor necrosis factor α (TNFα) is not very likely since its serum level was not affected in our diet-induced obesity model.[35] This, however, does not exclude the involvement of local TNFα production in meningeal TRPV1 sensitization.[69]

The particular combination of high-fat chow and sucrose solution applied in this study seems to be a reliable method to correspond certain criteria of both experimental and human obesity. However, it remains unclear whether the present findings are specific to dietary factors or whether they can be attributed to the obese state itself regardless of the etiology. This uncertainty associated with animal models of diet-induced obesity should be taken into consideration when interpreting the data.

Sex-related differences affecting the distribution and metabolic function of adipose tissue may modify the effect of diet-induced obesity on nociceptor function in females.[70] Additionally, the female sex hormone estradiol may modify nociceptor function by acting directly on the TRPV1 ion channel or by increasing its expression in dorsal root ganglion neurons.[71] Since this study was undertaken exclusively in male rats, extrapolation of the findings to females requires some caution.

Obesity and TRPV1 receptor function may have a versatile connection. Recent experimental results attributed a potential therapeutic effect of TRPV1 receptor stimulation in obesity since capsaicin dose-dependently increased intracellular calcium concentration in preadipocytes that inhibited their differentiation.[72]

Considering the central role of chemosensitive trigeminal afferents in the pathomechanism of headaches, both TRPV1 receptor agonists (desensitizing the channel protein) and antagonists have potential in migraine therapy. A novel approach to modulate TRPV1 receptor function may be the development of drugs preventing the phosphorylation of the receptor, thereby counteracting the increased probability of channel opening. Therefore, kinase inhibitors preventing the sensitization of the receptor may provide a promising strategy for migraine therapy, especially in obese persons, where altered TRPV1 receptor function seems to be an important pathophysiological factor.[22,73]

In conclusion, our study demonstrates that obesity induced by long-term HFHS feeding increases TRPV1-mediated CGRP release and neurogenic sensory vasodilatation in the dura mater encephali of rats. Augmented trigeminovascular responses on TRPV1 activation occur without obvious changes in TRPV1- and CGRP-immunoreactivity of meningeal nociceptors. HFHS diet-induced obesity also results in enhanced capsaicin-induced vasoconstriction, which may also be of relevance to obesity-headache relationship. The enhanced vasoconstrictor activity of meningeal blood vessels may impair the effective removal of pain-producing tissue mediators involved in the induction or aggravation of headache attacks or may delay the restoration of tissue homeostasis.

STATEMENT OF AUTHORSHIP

Category 1

(a) Conception and Design

Balázs Marics, Gábor Jancsó, Mária Dux

(b) Acquisition of Data

Balázs Marics, Kitti Pázmándi, József Németh, Orsolya Oszlács, Mária Dux

(c) Analysis and Interpretation of Data

Balázs Marics, Barna Peitl, Attila Bácsi, Gábor Jancsó, Mária Dux

Category 2

(a) Drafting the Manuscript

Balázs Marics, Gábor Jancsó, Mária Dux

(b) Revising It for Intellectual Content

Barna Peitl, Kitti Pázmándi, Attila Bácsi, József Németh, Orsolya Oszlács

Category 3

(a) Final Approval of the Completed Manuscript

Balázs Marics, Barna Peitl, Kitti Pázmándi, Attila Bácsi, József Németh, Orsolya Oszlács, Gábor Jancsó, Mária Dux

REFERENCES

1. Chai NC, Scher AI, Moghekar A, Bond DS, Peterlin BL. Obesity and headache: Part I – a systematic review of the epidemiology of obesity and headache. *Headache.* 2014;54:219-234.

2. Chai NC, Bond DS, Moghekar A, Scher AI, Peterlin BL. Obesity and headache: Part II – potential mechanism and treatment considerations. *Headache.* 2014;54:459-471.
3. Recober A, Goadsby PJ. Calcitonin gene-related peptide: A molecular link between obesity and migraine? *Drug News Perspect.* 2010;23:112-117.
4. Buzzi MG, Moskowitz MA. The trigeminovascular system and migraine. *Pathol Biol (Paris).* 1992;40:313-317.
5. Edvinsson L, Goadsby PJ. Neuropeptides in the cerebral circulation: Relevance to headache. *Cephalalgia.* 1995;15:272-276.
6. Russo AF. Calcitonin gene-related peptide (CGRP): A new target for migraine. *Annu Rev Pharmacol Toxicol.* 2015;55:533-552.
7. Karsan N, Goadsby PJ. CGRP mechanism antagonists and migraine management. *Curr Neurol Neurosci Rep.* 2015;15:25.
8. Bigal ME, Walter S, Rapoport AM. Therapeutic antibodies against CGRP or its receptor. *Br J Clin Pharmacol.* 2015;79:886-895.
9. Dux M, Messlinger K. New options for migraine treatment: Focus on CGRP blocking antibodies. *Drugs Future.* 2015;40:589-599.
10. Kristoffersen ES, Lundqvist C. Medication-overuse headache: Epidemiology, diagnosis and treatment. *Ther Adv Drug Saf.* 2014;5:87-99.
11. Tajti J, Majláth Z, Szok D, Csáti A, Vécsei L. Drug safety in acute migraine treatment. *Expert Opin Drug Saf.* 2015;14:891-909.
12. Diener H-C, Charles A, Goadsby PJ, Holle D. New therapeutic approaches for the prevention and treatment of migraine. *Lancet Neurol.* 2015;14:1010-1022.
13. Huang D, Li S, Dhaka A, Story GM, Cao Y-Q. Expression of the transient receptor potential channels TRPV1, TRPA1 and TRPM8 in mouse trigeminal primary afferent neurons innervating the dura. *Mol Pain.* 2012;8:66.
14. Ichikawa H, Sugimoto T. VR1-immunoreactive primary sensory neurons in the rat trigeminal ganglion. *Brain Res.* 2001;890:184-188.
15. Jancsó G, Kiraly E, Jancsó-Gábor A. Pharmacologically induced selective degeneration of chemosensitive primary sensory neurones. *Nature.* 1977;270:741-743.
16. Jancsó N, Jancsó-Gábor A, Szolcsányi J. Direct evidence for neurogenic inflammation and its prevention by denervation and by pretreatment with capsaicin. *Br J Pharmacol Chemother.* 1967;31:138-151.
17. Viana F. Chemosensory properties of the trigeminal system. *ACS Chem Neurosci.* 2011;2:38-50.
18. Dux M, Sántha P, Jancsó G. Capsaicin-sensitive neurogenic sensory vasodilatation in the dura mater of the rat. *J Physiol.* 2003;552:859-867.
19. Dux M, Deák É, Tassi N, Sántha P, Jancsó G. Endovanilloids are potential activators of the trigeminovascular nocisensor complex. *J Headache Pain.* 2016;17:53.
20. Messlinger K, Hanesch U, Baumgärtel M, Trost B, Schmidt RF. Innervation of the dura mater encephali of cat and rat: Ultrastructure and calcitonin gene-related peptide-like and substance P-like immunoreactivity. *Anat Embryol (Berl).* 1993;188:219-237.
21. Dux M, Sántha P, Jancsó G. The role of chemosensitive afferent nerves and TRP ion channels in the pathomechanism of headaches. *Pflüg Arch Eur J Physiol.* 2012;464:239-248.
22. Meents JE, Neeb L, Reuter U. TRPV1 in migraine pathophysiology. *Trends Mol Med.* 2010;16:153-159.
23. Nicoletti P, Trevisani M, Manconi M, et al. Ethanol causes neurogenic vasodilation by TRPV1 activation and CGRP release in the trigeminovascular system of the guinea pig. *Cephalalgia.* 2008;28:9-17.
24. Numazaki M, Tominaga M. Nociception and TRP channels. *Curr Drug Targets CNS Neurol Disord.* 2004;3:479-485.
25. Holzer P. The pharmacological challenge to tame the transient receptor potential vanilloid-1 (TRPV1) nocisensor. *Br J Pharmacol.* 2008;155:1145-1162.
26. Julius D. TRP channels and pain. *Annu Rev Cell Dev Biol.* 2013;29:355-384.
27. Vaughn AH, Gold MS. Ionic mechanisms underlying inflammatory mediator-induced sensitization of dural afferents. *J Neurosci.* 2010;30:7878-7888.
28. Noseda R, Burstein R. Migraine pathophysiology: Anatomy of the trigeminovascular pathway and associated neurological symptoms, cortical spreading depression, sensitization, and modulation of pain. *Pain.* 2013;154(Suppl 1):S44-53.
29. Tóth A, Czikora A, Pásztor ET, et al. Vanilloid receptor-1 (TRPV1) expression and function in

the vasculature of the rat. *J Histochem Cytochem Off J Histochem Soc.* 2014;62:129-144.
30. Singer G, Granger DN. Inflammatory responses underlying the microvascular dysfunction associated with obesity and insulin resistance. *Microcirc.* 2007;14:375-387.
31. Borkum JM. Migraine triggers and oxidative stress: A narrative review and synthesis. *Headache.* 2016;56:12-35.
32. Peterlin BL, Sacco S, Bernecker C, Scher AI. Adipokines and migraine: A systematic review. *Headache.* 2016;56:622-644.
33. Rossi HL, Luu AKS, DeVilbiss JL, Recober A. Obesity increases nociceptive activation of the trigeminal system. *Eur J Pain Lond Engl.* 2013;17: 649-653.
34. Rossi HL, Broadhurst KA, Luu ASK, et al. Abnormal trigeminal sensory processing in obese mice. *Pain.* 2016;157:235-246.
35. Marics B, Peitl B, Varga A, et al. Diet-induced obesity alters dural CGRP release and potentiates TRPA1-mediated trigeminovascular responses. *Cephalalgia.* 2016: DOI: 10.1177/0333102416654883 [Epub ahead of print].
36. Kurosawa M, Messlinger K, Pawlak M, Schmidt RF. Increase of meningeal blood flow after electrical stimulation of rat dura mater encephali: Mediation by calcitonin gene-related peptide. *Br J Pharmacol.* 1995;114:1397-1402.
37. Ebersberger A, Averbeck B, Messlinger K, Reeh PW. Release of substance P, calcitonin gene-related peptide and prostaglandin E2 from rat dura mater encephali following electrical and chemical stimulation in vitro. *Neuroscience.* 1999;89:901-907.
38. Guyenet SJ, Schwartz MW. Clinical review: Regulation of food intake, energy balance, and body fat mass: Implications for the pathogenesis and treatment of obesity. *J Clin Endocrinol Metab.* 2012;97: 745-755.
39. Chen W-P, Ho B-Y, Lee C-L, Lee C-H, Pan T-M. Red mold rice prevents the development of obesity, dyslipidemia and hyperinsulinemia induced by high-fat diet. *Int J Obes.* 2008;32:1694-1704.
40. Frye M, McMurtry I, Orton EC, Fagan K. Use of fat-fed rats to study the metabolic and vascular sequelae of obesity and beta-adrenergic antagonism. *Comp Med.* 2009;59:242-248.
41. Lindqvist A, de la Cour CD, Stegmark A, Håkanson R, Erlanson-Albertsson C. Overeating of palatable food is associated with blunted leptin and ghrelin responses. *Regul Pept.* 2005;130:123-132.
42. Cavestro C, Rosatello A, Micca G, et al. Insulin metabolism is altered in migraineurs: A new pathogenic mechanism for migraine? *Headache.* 2007; 47:1436-1442.
43. Koçer A, Memişoğullari R, Domaç FM, et al. IL-6 levels in migraine patients receiving topiramate. *Pain Pract.* 2009;9:375-379.
44. Uzar E, Evliyaoglu O, Yucel Y, et al. Serum cytokine and pro-brain natriuretic peptide (BNP) levels in patients with migraine. *Eur Rev Med Pharmacol Sci.* 2011;15:1111-1116.
45. Dux M, Rosta J, Pintér S, Sántha P, Jancsó G. Loss of capsaicin-induced meningeal neurogenic sensory vasodilatation in diabetic rats. *Neuroscience.* 2007;150:194-201.
46. Kobayashi K, Fukuoka T, Obata K, et al. Distinct expression of TRPM8, TRPA1, and TRPV1 mRNAs in rat primary afferent neurons with adelta/c-fibers and colocalization with trk receptors. *J Comp Neurol.* 2005;493:596-606.
47. Price TJ, Flores CM. Critical evaluation of the colocalization between calcitonin gene-related peptide, substance P, transient receptor potential vanilloid subfamily type 1 immunoreactivities, and isolectin B4 binding in primary afferent neurons of the rat and mouse. *J Pain off J Am Pain Soc.* 2007;8:263-272.
48. Messlinger K, Lennerz JK, Eberhardt M, Fischer MJM. CGRP and NO in the trigeminal system: Mechanisms and role in headache generation. *Headache.* 2012;52:1411-1427.
49. Duckles SP. Effects of capsaicin on vascular smooth muscle. *Naunyn Schmiedebergs Arch Pharmacol.* 1986;333:59-64.
50. Pórszász R, Porkoláb A, Ferencz A, Pataki T, Szilvássy Z, Szolcsányi J. Capsaicin-induced non-neural vasoconstriction in canine mesenteric arteries. *Eur J Pharmacol.* 2002;441:173-175.
51. Toda N, Usui H, Nishino N, Fujiwara M. Cardiovascular effects of capsaicin in dogs and rabbits. *J Pharmacol Exp Ther.* 1972;181:512-521.
52. Wilde DW, Massey KD, Walker GK, Vollmer A, Grekin RJ. High-fat diet elevates blood pressure and cerebrovascular muscle Ca(2+) current. *Hypertens.* 2000;35:832-837.
53. Shimizu T, Toriumi H, Sato H, et al. Distribution and origin of TRPV1 receptor-containing nerve

fibers in the dura mater of rat. *Brain Res.* 2007; 1173:84-91.
54. Knyihár-Csillik E, Tajti J, Samsam M, Sáry G, Slezák S, Vécsei L. Effect of a serotonin agonist (sumatriptan) on the peptidergic innervation of the rat cerebral dura mater and on the expression of c-fos in the caudal trigeminal nucleus in an experimental migraine model. *J Neurosci Res.* 1997;48:449-464.
55. Messlinger K, Hanesch U, Kurosawa M, Pawlak M, Schmidt RF. Calcitonin gene related peptide released from dural nerve fibers mediates increase of meningeal blood flow in the rat. *Can J Physiol Pharmacol.* 1995;73:1020-1024.
56. Schaible H-G. Nociceptive neurons detect cytokines in arthritis. *Arthritis Res Ther.* 2014;16:470-
57. Camprubí-Robles M, Planells-Cases R, Ferrer-Montiel A. Differential contribution of SNARE-dependent exocytosis to inflammatory potentiation of TRPV1 in nociceptors. *FASEB J.* 2009;23:3722-3733.
58. Capuano A, De Corato A, Lisi L, Tringali G, Navarra P, Dello Russo C. Proinflammatory-activated trigeminal satellite cells promote neuronal sensitization: Relevance for migraine pathology. *Mol Pain.* 2009;5:43.
59. Neeb L, Hellen P, Boehnke C, et al. IL-1β stimulates COX-2 dependent PGE_2 synthesis and CGRP release in rat trigeminal ganglia cells. *PloS One.* 2011;6:e17360.
60. Herbert MK, Holzer P. Interleukin-1 beta enhances capsaicin-induced neurogenic vasodilatation in the rat skin. *Br J Pharmacol.* 1994;111:681-686.
61. Gu Q, Kwong K, Lee L-Y. Ca2+ transient evoked by chemical stimulation is enhanced by PGE2 in vagal sensory neurons: Role of cAMP/PKA signaling pathway. *J Neurophysiol.* 2003;89:1985-1993.
62. Lopshire JC, Nicol GD. The cAMP transduction cascade mediates the prostaglandin E2 enhancement of the capsaicin-elicited current in rat sensory neurons: Whole-cell and single-channel studies. *J Neurosci.* 1998;18:6081-6092.
63. Moriyama T, Higashi T, Togashi K, et al. Sensitization of TRPV1 by EP1 and IP reveals peripheral nociceptive mechanism of prostaglandins. *Mol Pain.* 2005;1:3.
64. Jenkins DW, Langmead CJ, Parsons AA, Strijbos PJ. Regulation of calcitonin gene-related peptide release from rat trigeminal nucleus caudalis slices in vitro. *Neurosci Lett.* 2004;366:241-244.
65. Obreja O, Biasio W, Andratsch M, et al. Fast modulation of heat-activated ionic current by proinflammatory interleukin 6 in rat sensory neurons. *Brain J Neurol.* 2005;128:1634-1641.
66. Oprée A, Kress M. Involvement of the proinflammatory cytokines tumor necrosis factor-alpha, IL-1 beta, and IL-6 but not IL-8 in the development of heat hyperalgesia: Effects on heat-evoked calcitonin gene-related peptide release from rat skin. *J Neurosci.* 2000;20:6289-6293.
67. Chuang H, Lin S. Oxidative challenges sensitize the capsaicin receptor by covalent cysteine modification. *Proc Natl Acad Sci U S A.* 2009;106:20097-20102.
68. Van Buren JJ, Bhat S, Rotello R, Pauza ME, Premkumar LS. Sensitization and translocation of TRPV1 by insulin and IGF-I. *Mol Pain.* 2005;1:17.
69. Khan AA, Diogenes A, Jeske NA, Henry MA, Akopian A, Hargreaves KM. Tumor necrosis factor alpha enhances the sensitivity of rat trigeminal neurons to capsaicin. *Neuroscience.* 2008;155:503-509.
70. Mauvais-Jarvis F. Sex differences in metabolic homeostasis, diabetes, and obesity. *Biol Sex Differ.* 2015;6:14.
71. Cho T, Chaban VV. Expression of P2X3 and TRPV1 receptors in primary sensory neurons from estrogen receptors-α and estrogen receptor-β knockout mice. *Neuroreport.* 2012;23:530-534.
72. Zhang LL, Yan Liu D, Ma LQ, et al. Activation of transient receptor potential vanilloid type-1 channel prevents adipogenesis and obesity. *Circ Res.* 2007;100:1063-1070.
73. Brederson J-D, Kym PR, Szallasi A. Targeting TRP channels for pain relief. *Eur J Pharmacol.* 2013;716:61-76.