

**Diet-induced obesity enhances TRPV1-mediated  
neurovascular reactions in the dura mater**

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**ABSTRACT**

**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-1 $\beta$  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-

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3 induced CGRP release from meningeal afferents *ex vivo*. Except for minor morphological changes,  
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5 the distribution of dural TRPV1- and CGRP-immunoreactive afferents was similar in control and  
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7 obese animals.  
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10 **Conclusions:** Our results suggest that obesity induced by long-term HFHS diet results in  
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12 sensitization of the trigeminovascular system. Changes in TRPV1-mediated vascular reactions and  
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14 CGRP release are pathophysiological alterations that may be of relevance to the enhanced  
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16 headache susceptibility of obese individuals.  
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For Peer Review

## 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<sup>1</sup>. Clinical and experimental observations suggest multiple links between the pathophysiology of obesity and the primary headache migraine<sup>2</sup>, among which the role of calcitonin gene-related peptide (CGRP) released from trigeminal afferents might be of particular relevance<sup>3</sup>.

CGRP is regarded as one of the key mediators in both nociceptive transmission and meningeal arterial vasodilatation that are critical pathophysiological components of headaches<sup>4-6</sup>. Current migraine therapies are based on reducing CGRP effects by either inhibiting its release from meningeal nociceptors (triptans acting on 5-HT<sub>1B/1D</sub> receptors) or blocking the CGRP receptor (non-peptide CGRP receptor antagonists “gepants”)<sup>7</sup>. Administration of humanized monoclonal antibodies targeting CGRP or its receptor appears also a promising new strategy in the therapy of migraine<sup>8,9</sup>. 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<sup>10,11</sup>. Furthermore, the development of gepants has been interrupted due to side effects. Thus, migraine remains a condition calling for additional therapeutic approaches<sup>12</sup>.

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**<sup>13-17</sup>. They play a fundamental role in nociception and their peptidergic population releasing CGRP and/or substance P mediates neurogenic vascular reactions in the meninges<sup>13,18-20</sup>. Chemosensitive nociceptors represent a

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3 promising target for novel migraine therapeutics<sup>21,22</sup>. TRPV1 is a molecular integrator of various  
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5 nociceptive stimuli. It can be activated by low pH, noxious heat and different endogenous and  
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7 exogenous agents, such as endovanilloids (e.g. anandamide) and capsaicin, or alcohol, a well-known  
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9 headache trigger<sup>19,23,24</sup>. Factors related to tissue damage or inflammation may sensitize the  
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11 receptor by increasing the probability of channel opening upon stimulation<sup>25,26</sup>. Enhanced activity  
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13 of the trigeminovascular nociceptive pathway may result from sensitization of TRPV1 receptors<sup>27</sup>.  
14  
15 Prolonged activation of trigeminal afferents may also increase the excitability of second order  
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17 neurons in the caudal trigeminal nucleus<sup>28</sup>. **Processes of peripheral and central sensitization** are  
18  
19 considered as significant pathophysiological mechanisms of primary headaches. Earlier findings  
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21 from our laboratory have also proved a capsaicin-induced vasoconstrictor response in dural blood  
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23 vessels that was present even after **functional degradation** of trigeminal chemosensitive afferents  
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25<sup>18</sup>. Expression of TRPV1 receptors in a wide variety of non-neuronal tissues including smooth muscle  
26  
27 cells in different vascular beds may explain the vasoconstrictor effect of capsaicin<sup>29</sup>.  
28  
29 Modification of neuronal and vascular TRPV1 receptor function resulting in altered activity of the  
30  
31 trigeminovascular system may represent a link between obesity and migraine pathophysiology.  
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33 Chronic expansion of adipose tissue producing bioactive molecules with immunoregulatory  
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35 functions, oxidative and nitrosative stress and microvascular dysfunction are pathophysiological  
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37 correlates of obesity<sup>30</sup> that may modulate trigeminovascular functions as well<sup>31,32</sup>. These  
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39 interrelated disturbances may influence TRPV1 channel function leading to enhanced sensitivity of  
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41 the trigeminal nociceptive pathway. Recent experimental studies demonstrated an increased  
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43 capsaicin-induced activity of the trigeminal pain pathway in obese mice<sup>33,34</sup>.  
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48 The present experiments were initiated to examine the effects of high-fat, high-sucrose (HFHS)  
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50 diet-induced obesity on the function of trigeminovascular nociceptive afferents in the rat.  
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55 Therefore, TRPV1-dependent meningeal nociceptor function was evaluated in control and obese  
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3 rats by studying capsaicin-evoked meningeal vascular responses, the *ex vivo* release of CGRP from  
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5 dural afferents and the immunohistochemical demonstration of TRPV1- and CGRP-immunoreactive  
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7 nerves of the dura mater. The plasma levels of proinflammatory cytokines as well as glucose and  
8  
9 insulin homeostasis were also determined in control and obese animals to gain further insight into  
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11 possible mechanisms of obesity-related trigeminal neurovascular alterations.  
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## 17 **METHODS**

### 18 **Animals and diet**

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20 Experiments were approved by the Ethical Committee for Animal Care of the University of Szeged  
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22 and the University of Debrecen. Study procedures were carried out in accordance with the Directive  
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24 2010/63/EU of the European Parliament. All efforts were made to minimize the number of animals  
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26 used and their suffering.  
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31 Male Sprague Dawley rats (6 weeks old, weighing 150-170 g, Charles River Laboratories, Hungary)  
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33 were housed in an environmentally controlled room (12-hour light/dark cycle,  $22 \pm 2$  °C, 50-70 %  
34  
35 relative humidity) and fed according to our recently published protocol <sup>35</sup>. Animals in the control  
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37 group (n = 25) received a regular diet containing standard rodent chow (3.20 kcal/g, 59 %  
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39 carbohydrate, 32 % protein, 9 % fat; diet code: S8106-S011 SM R/M-Z+H, ssniff Spezialdiäten  
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41 GmbH, Germany) and tap water. Another group of animals was placed on a HFHS diet (n = 26)  
42  
43 consisting of high-fat chow (4.56 kcal/g, 35 % carbohydrate, 20 % protein, 45 % fat; diet code:  
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45 824018, Special Diets Services, UK) and 5 % sucrose solution made with tap water. Body weight,  
46  
47 food and fluid intake of the animals were measured regularly and daily calorie intake was  
48  
49 calculated. Diets were provided *ad libitum* for 20 weeks. All experiments were carried out after the  
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51 completion of the dietary treatment period in 26 weeks old animals.  
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### Analysis of fasting blood glucose, plasma insulin, interleukin-1 $\beta$ (IL-1 $\beta$ ) 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  $\mu$ U/ml, sensitivity: 2.8  $\mu$ U/ml). IL-1 $\beta$  (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<sup>18,36</sup>. 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



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2  
3 laser Doppler flowmeter (Perimed, Sweden) positioned over a branch of the middle meningeal  
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5 artery lying distant from visible cortical blood vessels.  
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8 The exposed dura mater in the cranial window was covered with 40  $\mu$ l of synthetic interstitial fluid  
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10 (SIF) containing (in mM): 135 NaCl, 5 KCl, 1 MgCl<sub>2</sub>, 5 CaCl<sub>2</sub>, 10 glucose and 10 Hepes, pH 7.4.

11  
12 Stimulation of the dura mater was performed by topical application of capsaicin (100 nM and 10  
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14  $\mu$ M, Sigma-Aldrich, Germany), CGRP or histamine (both at 100  $\mu$ M, Sigma-Aldrich, Germany) at a  
15  
16 volume of 40  $\mu$ l. Solutions were removed after 5 min and the dura mater was washed repeatedly  
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18 with SIF to allow the blood flow to return to the basal level. Blood flow was measured in perfusion  
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20 units (PU). Data on meningeal blood flow and systemic blood pressure were processed with the  
21  
22 Perisoft program (Perimed, Sweden). Basal blood flow was determined as the mean flow during a  
23  
24 three minute period prior to the stimulation of the dura mater. Percentage changes in meningeal  
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26 blood flow in response to capsaicin, CGRP and histamine were determined as mean flow values  
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28 within the 5 min application period calculated separately at one-minute intervals relative to the  
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30 basal flow. A stock solution of capsaicin (32 mM) was prepared with the aid of 6% ethanol and 8%  
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32 Tween 80 in saline and was further diluted with SIF. All the other drugs were dissolved in SIF  
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34 immediately before use.  
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### 43 **Measurement of CGRP release in *ex vivo* dura mater preparation**

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45 An *ex vivo* rat dura mater preparation was used to measure basal and stimulated CGRP release  
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47 from meningeal afferents<sup>37</sup>. Control and obese rats were decapitated following deep anesthesia  
48  
49 with thiopental sodium (150 mg/kg i.p.). After removing the skin and the muscles, the skull was  
50  
51 divided into halves along the midline. The cerebral hemispheres were removed, skull halves were  
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53 washed at room temperature for 30 min in SIF, then placed in a humid chamber and the cranial  
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55 fossae were filled with 300  $\mu$ l SIF. Samples of the superfusate were collected with a micropipette at  
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3 periods of 5 min for CGRP measurement. A control sample was taken in order to determine basal  
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5 CGRP release. Then the dura mater was stimulated with capsaicin at concentrations of 10 and 100  
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7 nM. 100  $\mu$ l of samples diluted with 25  $\mu$ l EIA buffer were placed into Eppendorf cups and  
8  
9 immediately frozen at -70 °C for subsequent analysis. EIA method was used for CGRP determination  
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11 (SPI-Bio, Bertin Pharma, France). The CGRP concentrations of the superfusates were expressed in  
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13 pg/ml. Changes induced in CGRP release by capsaicin were expressed as percentage changes  
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15 relative to the basal release.  
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## 22 Immunohistochemistry

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24 The distribution of TRPV1- and CGRP-immunoreactive nerve fibers was studied in dural whole  
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26 mount preparations of rats not used in *in vivo* blood flow recordings or in *ex vivo* CGRP release  
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28 experiments previously. Control and obese animals were anesthetized deeply with thiopental  
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30 sodium (150 mg/kg, i.p.) and perfused transcardially with physiological saline followed by 4%  
31  
32 paraformaldehyde in phosphate buffer (pH 7.4). The skin and muscles of the skull were removed  
33  
34 and the skull was divided into halves along the sagittal suture. After removing the brain, samples of  
35  
36 the dura mater containing branches of the middle meningeal artery were cut out, postfixed for 2 h  
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38 in the same fixative and processed for staining with the indirect immunofluorescence technique  
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40 using a rabbit polyclonal antiserum raised against the TRPV1 receptor (1:500, Alomone  
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42 Laboratories, Israel) in combination with a monoclonal mouse anti-CGRP antibody (1:500, Sigma-  
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44 Aldrich, Germany). IgGs labeled with Cy3 and DL488 were used as secondary antibodies (both  
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46 1:500, Jackson Immunoresearch Laboratories, USA). Whole mount preparations of the dura mater  
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48 were examined under a confocal fluorescence microscope (ZEISS LSM 700, Germany).  
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## 57 Statistics

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3 All values were expressed as means  $\pm$  SEM. Statistical analysis of the data was performed using  
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5 Statistica 12 (StatSoft, USA). In both dietary groups, normality in terminal body weight was proved  
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7 by the Shapiro-Wilk test. For the statistical comparisons of CGRP and cytokine concentrations,  
8  
9 meningeal blood flow changes, food and fluid intake of the animals the Student's t-test was used  
10  
11 for group sizes of  $n \geq 10$  and the Mann-Whitney U-test for independent measurements of group  
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13 sizes  $n < 10$ . One-way ANOVA followed by the Bonferroni test was used to compare metabolic  
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15 parameters of the animals. A probability level of  $p < 0.05$  was regarded as statistically significant.  
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## 22 RESULTS

### 23 Characterization of the diet-induced obesity model

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25 On average, the HFHS group of animals consumed smaller quantities of food ( $p < 0.001$ ) and greater  
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27 amount of fluid ( $p < 0.001$ ) than animals fed with a regular diet. The mean daily caloric intake of  
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29 HFHS rats significantly exceeded the control group ( $p < 0.001$ ), resulting in a significantly higher  
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31 body weight at the end of the dietary period ( $616.5 \pm 11$  g in control and  $740.5 \pm 15$  g in obese  
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33 animals,  $p < 0.001$ ,  $n = 17$  for both groups, Fig. 1). Diet-induced obesity led to elevations in fasting  
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35 blood glucose ( $5.75 \pm 0.13$  vs.  $6.54 \pm 0.33$  mmol/l,  $p = 0.049$ ,  $n = 8$  and 9) and plasma insulin  
36  
37 concentrations ( $17.46 \pm 3.31$  vs.  $48.51 \pm 8.67$   $\mu$ U/ml,  $p = 0.006$ ,  $n = 8$  and 9) in addition to circulating  
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39 levels of the proinflammatory cytokines IL-1 $\beta$  ( $59.04 \pm 2.99$  vs.  $170.04 \pm 23.78$  pg/ml,  $p < 0.001$ ,  $n =$   
40  
41 8 and 9) and IL-6 ( $56.39 \pm 2.15$  vs.  $114.46 \pm 11.32$  pg/ml,  $p < 0.001$ ,  $n = 8$  and 9) as indicated recently  
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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

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3 The basal blood flow values were in the same range in control and obese rats amounting to  $245.2 \pm$   
4  $18.1$  and  $244.1 \pm 19.6$  PU ( $p = 0.96$ ), respectively. In control rats topical administration of capsaicin  
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7 at  $100$  nM concentration induced a moderate increase in meningeal blood flow reaching  
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10 significance in the last two minutes of the  $5$  min application period ( $p = 0.048$  and  $0.035$ ,  
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12 respectively,  $n = 8$ ). Blood flow increasing effect of the same capsaicin concentration was more  
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14 robust in obese animals throughout the whole application period, it reached  $20 \pm 3.1$  % increase in  
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16 the last minute ( $p \leq 0.023$ ,  $n = 9$ ). This increase significantly exceeded the effect measured in  
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18 control rats ( $p \leq 0.049$ , Fig. 3 A, B). Administration of capsaicin at  $10$   $\mu$ M reduced meningeal blood  
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20 flow in both groups of animals. In control animals, decrease in meningeal blood flow varied in the  
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22 range from  $4.7 \pm 2.7$  % to  $2.7 \pm 2.5$  % ( $p \leq 0.33$ ,  $n = 6$ ), while this range was  $14.5 \pm 2.9$  to  $8 \pm 2.8$  % ( $p$   
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24  $\leq 0.036$ ,  $n = 6$ ) in obese rats. Blood flow reducing effect of  $10$   $\mu$ M capsaicin was significantly  
25  
26 stronger in obese animals from the 2nd to the 4th min compared to controls ( $p \leq 0.037$ , Fig. 3 A, C).  
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28 Histamine and CGRP acting directly on endothelial- or smooth muscle cells of blood vessels induced  
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30 significant increases in meningeal blood flow in both control ( $p \leq 0.015$ ,  $n = 8$  both) and obese ( $p \leq$   
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32  $0.035$ ,  $n = 8$  and  $10$ , respectively) rats. No difference regarding the vasodilatation induced by  
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34 histamine ( $p \leq 0.98$ ) and CGRP ( $p \leq 0.92$ ) administrations could be observed between control and  
35  
36 HFHS diet-induced obese animals (Fig. 3 D, E).

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43 Systemic blood pressure of animals was in the same range as we have published recently<sup>35</sup>. It was  
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45  $138 \pm 12$  and  $142 \pm 18$  mmHg in control and obese rats, respectively. Drugs administered topically  
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47 to the dura mater failed to influence systemic blood pressure.

#### 52 Diet-induced obesity enhances basal and TRPV1-mediated CGRP release

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55 In the *ex vivo* dura mater preparation of obese animals we measured a significantly increased basal  
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57 CGRP release. Unstimulated CGRP concentration was  $16 \pm 1.5$  in control and  $42.1 \pm 6.6$  pg/ml in  
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3 obese rats ( $p = 0.005$ ). Stimulation with capsaicin significantly enhanced the release of CGRP from  
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5 meningeal afferents in skull preparations obtained from both control and obese animals. Capsaicin  
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7 at a concentration of 10 nM elevated CGRP release to  $34.7 \pm 2.3$  pg/ml ( $196.6 \pm 21.2$  % of basal  
8  
9 release,  $p < 0.001$ ,  $n = 7$ ) and at 100 nM concentration to  $75.6 \pm 7$  pg/ml ( $612.7 \pm 58.9$  % of basal  
10  
11 release,  $p < 0.001$ ,  $n = 6$ ) in rats maintained on a regular diet. In obese animals capsaicin at 10 nM  
12  
13 concentration increased the release of CGRP to  $120 \pm 27.6$  pg/ml ( $300.1 \pm 60.1$  % of basal release,  $p$   
14  
15  $= 0.004$ ,  $n = 11$ ) and at 100 nM to  $358.1 \pm 95.5$  pg/ml ( $853.1 \pm 76$  % of basal release,  $p = 0.004$ ,  $n =$   
16  
17 11). HFHS diet-induced obesity resulted in a significantly greater CGRP release in response to both  
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19 capsaicin concentrations as compared with the control ( $p = 0.027$  and  $p < 0.048$ , respectively, Fig.  
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21 4).

#### 22 23 24 25 26 27 28 29 **Effect of HFHS diet on TRPV1- and CGRP-immunoreactive nerve fibers of the dura mater**

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31 In the dura mater of control animals TRPV1- and CGRP-immunoreactive nerve fibres were  
32  
33 distributed over the whole supratentorial dura mater. Many TRPV1-immunoreactive nerves were  
34  
35 seen running in small nerve bundles in association with dural blood vessels. Single axons were also  
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37 observed in avascular regions of the meningeal tissue, i.e. in areas at a distance from larger blood  
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39 vessels, where they formed loose nerve plexuses. CGRP was colocalized with TRPV1 in most of  
40  
41 these nerve fibres. Although in whole mount dura preparations of obese rats, distribution of  
42  
43 TRPV1- and CGRP-immunoreactive afferents was similar to that seen in control dura mater  
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45 preparations, many single axons showed characteristic changes. While CGRP-immunoreactivity was  
46  
47 distributed evenly in the afferents of control dura, in whole mounts of obese animals the  
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49 immunoreactivity was observed in the form of a string of pearls (Fig. 5).

#### 50 51 52 53 54 55 56 57 **DISCUSSION**

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

10  
11 HFHS diet altered the eating behavior of animals indicating that dietary treatment influenced  
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13 processes involved in the regulation of food intake and energy balance<sup>38</sup>. The lower food  
14  
15 consumption and higher fluid and calorie intakes in HFHS-fed animals are in agreement with  
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17 previous reports in which the energy density and palatability of the diet were manipulated in a  
18  
19 similar way<sup>39-41</sup>. The accumulation of adipose tissue was accompanied by impairments in glucose  
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21 and insulin homeostasis and a low-grade systemic inflammatory milieu. The present findings  
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23 demonstrate that our animal model of diet-induced obesity possessed many of the metabolic  
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25 alterations that have been previously suggested to be associated with obesity as well as with the  
26  
27 pathophysiology of migraine<sup>42-44</sup>.

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29 The function of trigeminal nociceptors can be assessed by studying vascular responses in relation to  
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31 **neuropeptide** release from dural afferents. Previous studies have confirmed that the meningeal  
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33 vasodilatory action of capsaicin is mediated by the activation of TRPV1 receptors in chemosensitive  
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35 afferents and the consequent release of CGRP from their peripheral endings<sup>18</sup>. Changes in  
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37 metabolic conditions may modify the functionality of trigeminal chemosensitive neurons.

38  
39 **Meningeal TRPV1 expressing nerves in streptozotocin-induced diabetic rats were found impaired**  
40  
41 **showing reduced neurogenic sensory vasodilatation, decreased capsaicin-evoked CGRP release and**  
42  
43 **reduction in the number of TRPV1-immunoreactive nerve fibers of the dura mater**<sup>45</sup>. The present  
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45 study provides the first direct evidence that diet-induced obesity in rats leads to augmented  
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47 vasodilatory responses in meningeal blood vessels following activation of trigeminal TRPV1  
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49 receptors. Enhanced vascular reactions were selective for capsaicin since neither CGRP- nor  
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3 histamine-induced vascular reactions in diet-induced obese animals differed from the control.  
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5 Measurement of capsaicin-induced CGRP release in *ex vivo* dura mater preparations of the obese  
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7 rats indicates that an increased CGRP release from meningeal nociceptive afferent nerves may  
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9 account for the enhanced capsaicin-induced vasodilatation. The current results are in accordance  
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11 with recent studies in our laboratory that furnished evidence for an enhanced CGRP-mediated  
12  
13 neurogenic sensory vasodilatation in the meninges of obese animals upon the activation of  
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15 transient receptor potential ankyrin 1 (TRPA1) channels by the environmental irritant acrolein<sup>35</sup>.  
16  
17 However, TRPA1 receptors are expressed only in a subset of TRPV1-positive chemosensitive  
18  
19 primary afferent nerves<sup>46</sup>.  
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21 Although most studies dealing with headache mechanisms focus on trigeminal peptidergic  
22  
23 afferents, it should be emphasized that a large population of TRPV1 expressing neurons does not  
24  
25 contain neuropeptides<sup>47</sup>. Non-peptidergic nociceptive primary sensory neurons can be identified  
26  
27 through their binding of the plant lectin, Griffonia simplicifolia isolectin B4 (IB4). The possible role  
28  
29 of non-peptidergic nociceptive afferents in the pathomechanism of headaches remains to be  
30  
31 elucidated. It is conceivable that peptidergic and non-peptidergic TRPV1-expressing neurons  
32  
33 provide parallel afferent pathways for the transmission of nociceptive signals<sup>21</sup>. Activation of  
34  
35 peptidergic neurons induces increases in meningeal blood flow but release of CGRP from their  
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37 central terminals in the trigeminal nucleus has presumably only a presynaptic modulatory effect  
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39 controlling the neurotransmitter release in other populations of primary afferents<sup>48</sup>. Non-  
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41 peptidergic TRPV1 expressing trigeminal neurons fail to contribute to local neurogenic vascular  
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43 reactions in the dura mater, but they may provide a direct transmission of nociceptive signals to  
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45 second order neurons of the pain pathway<sup>21</sup>. Obesity-related factors that alter the function of  
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47 TRPV1 receptors in CGRP-containing meningeal afferents may also have the potential to affect  
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49 TRPV1 in non-peptidergic trigeminal sensory neurons.  
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3 Earlier observations in our laboratory demonstrated a capsaicin-induced vasoconstriction in  
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5 meningeal blood vessels that appeared parallel with the CGRP-induced vasodilatation and was  
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7 independent of neural processes<sup>18</sup>. The mechanism of capsaicin-induced vasoconstriction is not  
8  
9 completely clarified. It is generally regarded as a direct vascular action of capsaicin<sup>49-51</sup>, but  
10  
11 contribution of TRPV1 receptors expressed in vascular smooth muscle cells cannot be excluded<sup>29</sup>.  
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14 Our present findings indicated that in dural arteries both the vasodilatory and the vasoconstrictor  
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16 actions of capsaicin were enhanced in obese animals.  
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19 The mechanisms responsible for obesity-associated changes in capsaicin-evoked meningeal  
20  
21 vascular responses and CGRP release are likely to involve complex dose- and time-dependent  
22  
23 interactions among multiple causative factors, which may appear already at an early stage of the  
24  
25 dietary period enabling them to act chronically on the trigeminovascular system. High-fat diet has  
26  
27 been shown to increase calcium influx in both cultured trigeminal ganglion neurons and vascular  
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29 smooth muscle cells of cerebral arteries<sup>34,52</sup>, therefore alterations in calcium homeostasis in  
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31 trigeminal primary afferents and meningeal vasculature could potentially contribute to altered  
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33 neurocrine and vasoconstrictor responses under basal conditions and to stimulation with capsaicin  
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35 in obese animals.  
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40 The increased basal and capsaicin-induced CGRP release that was measured in the *ex vivo* dura  
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42 mater preparation of HFHS diet-induced obese animals may result from a sensitization of the TRPV1  
43  
44 receptor. Sensitization may occur in several ways such as modifications in gating properties of the  
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46 ion channel, increased trafficking of the receptor to cell membrane or increased production of the  
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48 TRPV1 protein<sup>22</sup>. In the dura mater whole mount preparations of lean and obese animals, in accord  
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50 with previous data<sup>45,53</sup>, the density and distribution of TRPV1- and CGRP-immunoreactive nerve  
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52 fibers were similar. It is noteworthy that microscopic examination revealed characteristic structural  
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54 changes in dural afferents of diet-induced obese animals. The pearl-like appearance of CGRP-  
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3 immunoreactivity might be the morphological correlate of enhanced CGRP release<sup>54,55</sup>. Despite the  
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5 known limitations of immunohistochemistry to detect subtle changes in receptor protein content,  
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7 our findings indicate that obesity-associated potentiation of capsaicin-induced neurogenic sensory  
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9 vasodilatation was brought about in the absence of obvious changes in TRPV1-immunoreactivity of  
10  
11 dural afferents. Sensitization of the TRPV1 receptor is more likely the consequence of the release of  
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13 proinflammatory agents produced in the adipose tissue<sup>56</sup>, which may also influence neuropeptide-  
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15 mediated vascular reactions. Indeed, we measured increased plasma concentrations of the  
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17 proinflammatory cytokines IL-1 $\beta$  and IL-6 in obese animals. IL-1 $\beta$  may directly modify the gating  
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19 properties of TRPV1 to noxious stimuli in primary sensory neurons while leaving the recruitment of  
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21 the channel to plasma membrane unaffected<sup>57</sup>. Additionally, IL-1 $\beta$  has been shown to enhance  
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23 basal and capsaicin-induced CGRP release in the rat trigeminal ganglion neurons<sup>58,59</sup>, whereas the  
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25 intraplantar injection of this cytokine augmented the capsaicin-induced neurogenic sensory  
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27 vasodilatation without altering the vascular action of CGRP<sup>60</sup>. In the trigeminal ganglion, IL-1 $\beta$  has  
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29 also been demonstrated to increase the production of prostaglandin E<sub>2</sub><sup>59</sup>, which may lower the  
30  
31 heat activation threshold of TRPV1 and increase capsaicin-evoked responses in sensory neurons<sup>61–</sup>  
32  
33<sup>63</sup> as well as the release of CGRP in slices of the trigeminal nucleus caudalis<sup>64</sup>. IL-6 in conjunction  
34  
35 with its soluble receptor (sIL-6R) may have abilities to reduce heat threshold and increase heat-  
36  
37 activated inward currents in rat dorsal root ganglion cells with a possible involvement of TRPV1<sup>65</sup>.  
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39 Both IL-1 $\beta$  and IL-6/sIL-6R complex have been found to potentiate heat-stimulated CGRP release  
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41 from an *in vitro* rat skin preparation<sup>66</sup>. The obesity-related enhancement in systemic and cerebral  
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43 oxidative stress along with the marked hyperinsulinemia might also contribute to changes in  
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45 TRPV1-mediated trigeminovascular responses<sup>67,68</sup>. A major role of the proinflammatory cytokine  
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47 tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) is not very likely since its serum level was not affected in our diet-  
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3 induced obesity model <sup>35</sup>. This, however, does not exclude the involvement of local TNF $\alpha$   
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5 production in meningeal TRPV1 sensitization <sup>69</sup>.  
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8 The particular combination of high-fat chow and sucrose solution applied in this study seems to be  
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10 a reliable method to correspond certain criteria of both experimental and human obesity. However,  
11  
12 it remains unclear whether the present findings are specific to dietary factors or whether they can  
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14 be attributed to the obese state itself regardless of the etiology. This uncertainty associated with  
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16 animal models of diet-induced obesity should be taken into consideration when interpreting the  
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18 data.  
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21 Sex-related differences affecting the distribution and metabolic function of adipose tissue may  
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23 modify the effect of diet-induced obesity on nociceptor function in females <sup>70</sup>. Additionally, the  
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25 female sex hormone estradiol may modify nociceptor function by acting directly on the TRPV1 ion  
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27 channel or by increasing its expression in dorsal root ganglion neurons <sup>71</sup>. Since, the present study  
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29 was undertaken exclusively in male rats, extrapolation of the findings to females requires some  
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31 caution.  
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34  
35 Obesity and TRPV1 receptor function may have a versatile connection. Recent experimental results  
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37 attributed a potential therapeutic effect of TRPV1 receptor stimulation in obesity since capsaicin  
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39 dose-dependently increased intracellular calcium concentration in preadipocytes that inhibited  
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41 their differentiation <sup>72</sup>.  
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45 Considering the central role of chemosensitive trigeminal afferents in the pathomechanism of  
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47 headaches, both TRPV1 receptor agonists (desensitizing the channel protein) and antagonists have  
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49 potential in migraine therapy. A novel approach to modulate TRPV1 receptor function may be the  
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51 development of drugs preventing the phosphorylation of the receptor, thereby counteracting the  
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53 increased probability of channel opening. Therefore, kinase inhibitors preventing the sensitization  
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3 of the receptor may provide a promising strategy for migraine therapy, especially in obese persons,  
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5 where altered TRPV1 receptor function seems to be an important pathophysiological factor <sup>22,73</sup>.  
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7 In conclusion, our study demonstrates that obesity induced by long-term HFHS feeding increases  
8  
9 TRPV1-mediated CGRP release and neurogenic sensory vasodilatation in the dura mater encephali  
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11 of rats. Augmented trigeminovascular responses upon TRPV1 activation occur without obvious  
12  
13 changes in TRPV1- and CGRP-immunoreactivity of meningeal nociceptors. **HFHS diet-induced**  
14  
15 obesity also results in enhanced capsaicin-induced vasoconstriction, which may also be of relevance  
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17 to obesity-headache relationship. The enhanced vasoconstrictor activity of meningeal blood vessels  
18  
19 may impair the effective removal of pain-producing tissue mediators involved in the induction or  
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21 aggravation of headache attacks or may delay the restoration of tissue homeostasis.  
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**FIGURE LEGENDS****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.

**Fig. 2**

Effect of diet-induced obesity on plasma concentrations of (A) interleukin-1 $\beta$  (IL-1 $\beta$ ), 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.

**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  $\mu$ M), histamine and CGRP (100  $\mu$ M both). \*: statistically different from the basal flow, #: statistically different from the control.

**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.

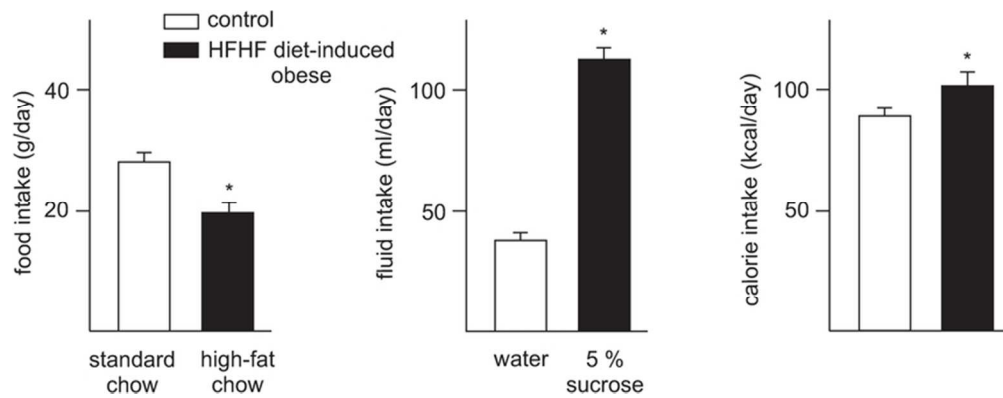
**Fig. 5**

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

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represents 50  $\mu\text{m}$  and applies for A, B, D and E, scale bare on F represents 25  $\mu\text{m}$  and applies for C and F, MMA: branch of the middle meningeal artery.

For Peer Review



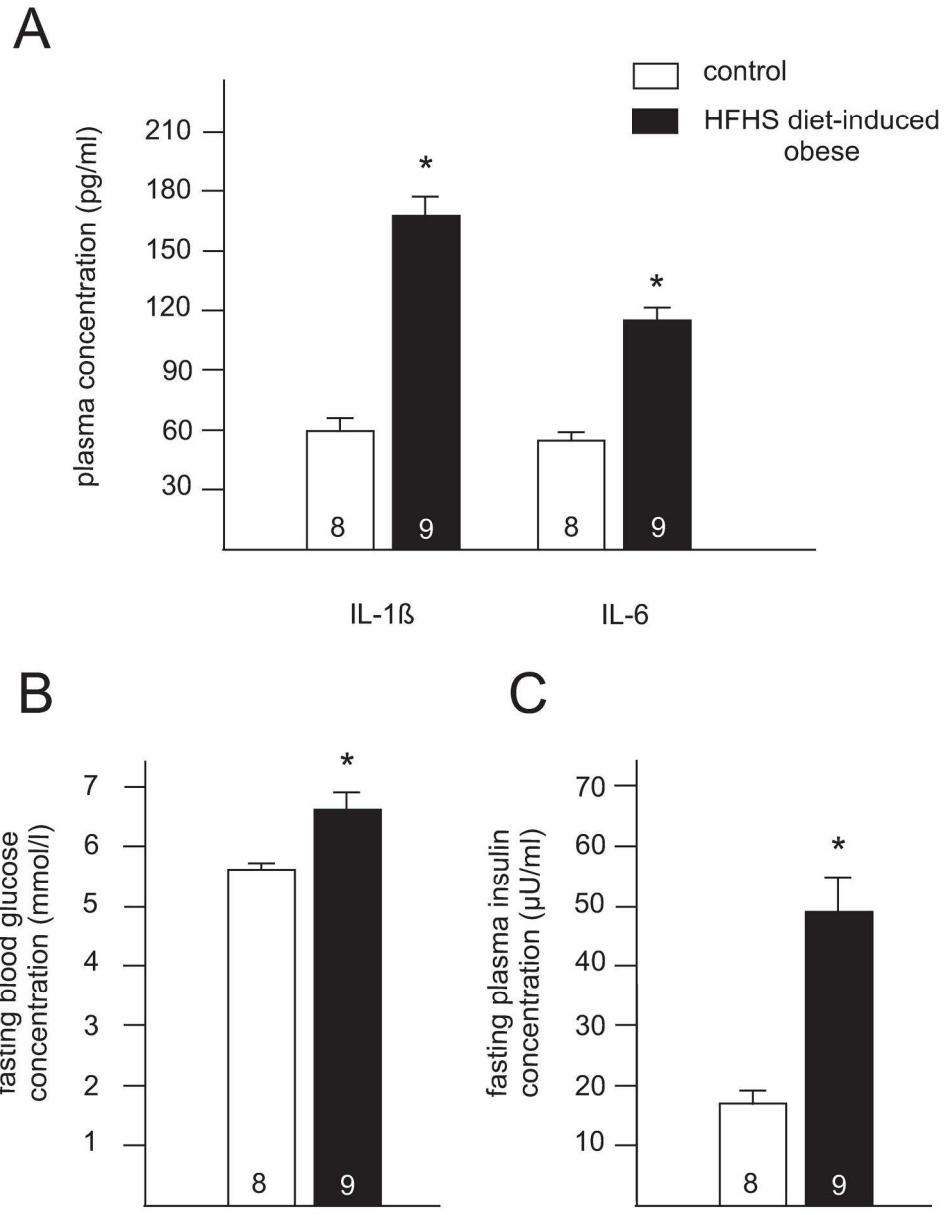
Average daily food-, fluid- and calorie intakes of rats maintained on regular or HFHS diet (n = 17 in both groups) \*: statistically different from the control.

72x27mm (300 x 300 DPI)

Peer Review

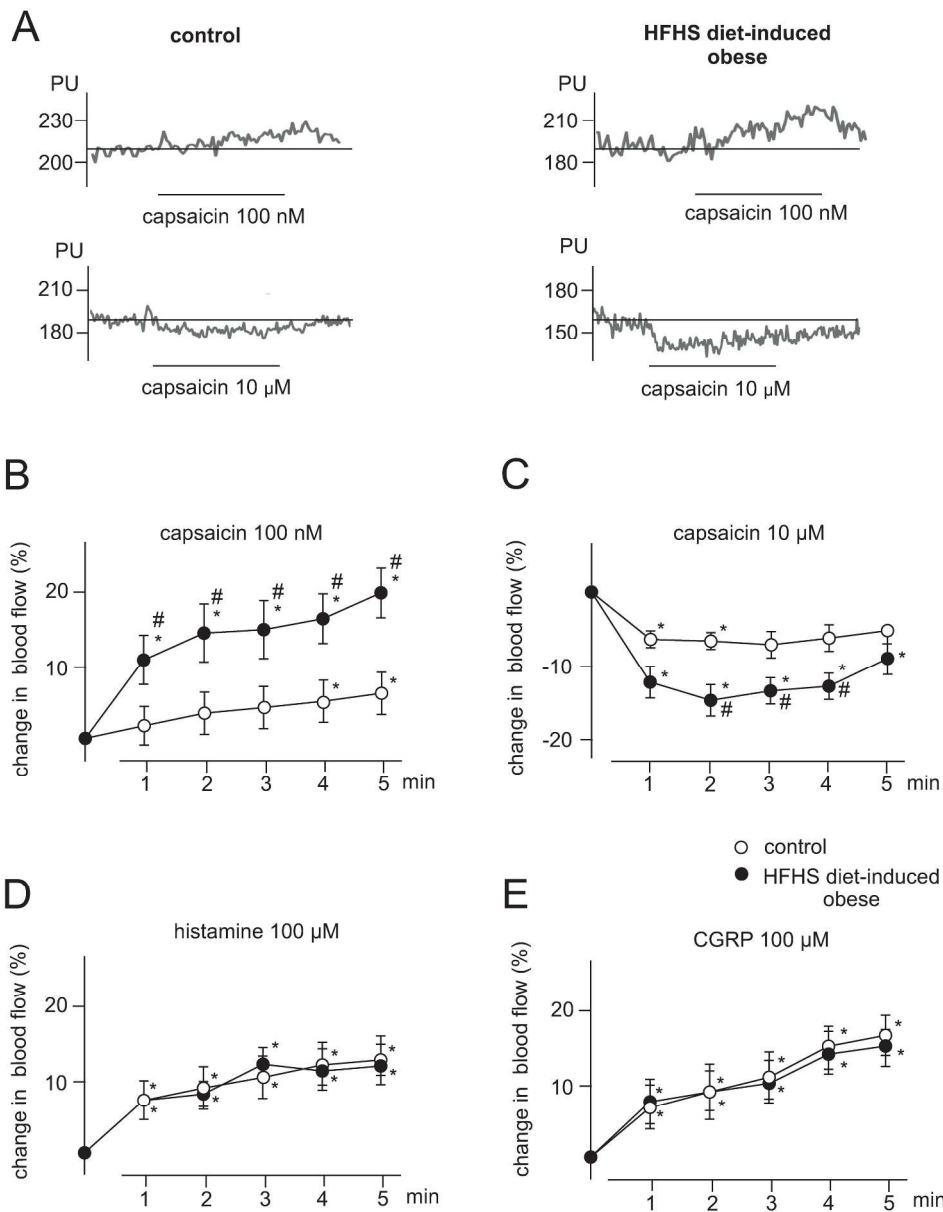
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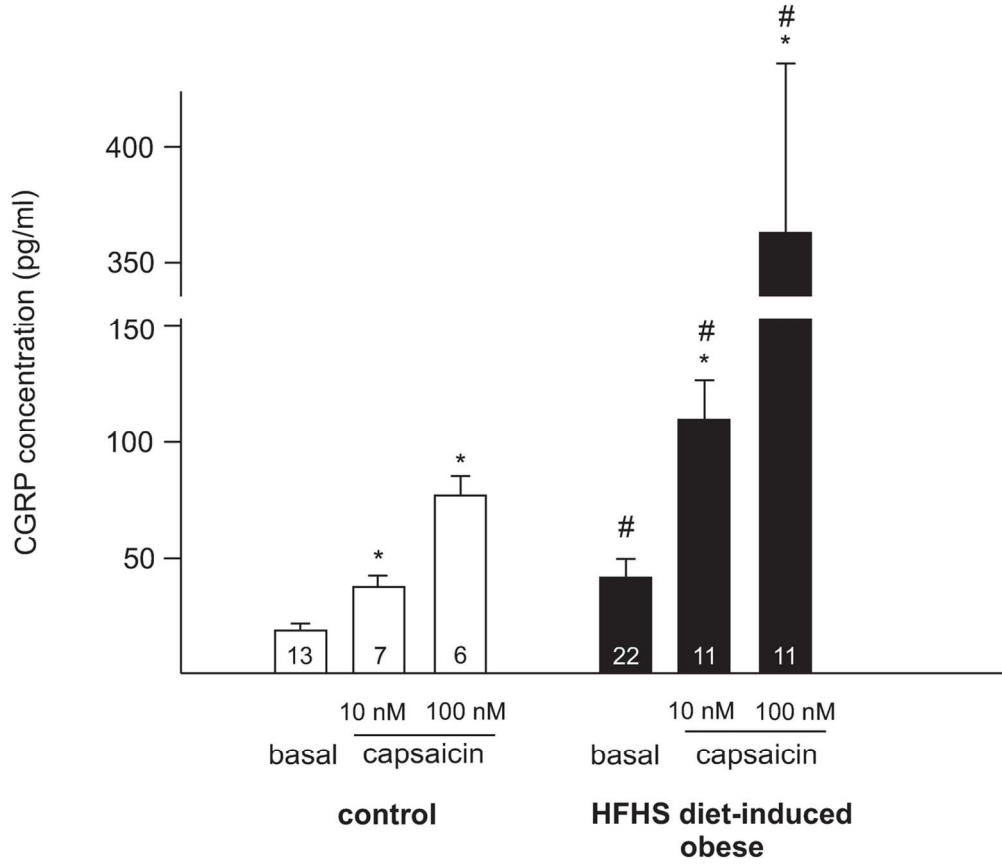
Effect of diet-induced obesity on plasma concentrations of (A) interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-6 (IL-6), (B) fasting blood glucose and (C) fasting plasma insulin concentrations. The number of experiments is indicated in the bars. \*: statistically different from the control.

181x230mm (300 x 300 DPI)



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  $\mu$ M), histamine and CGRP (100  $\mu$ M both). \*: statistically different from the basal flow, #: statistically different from the control.

242x307mm (300 x 300 DPI)

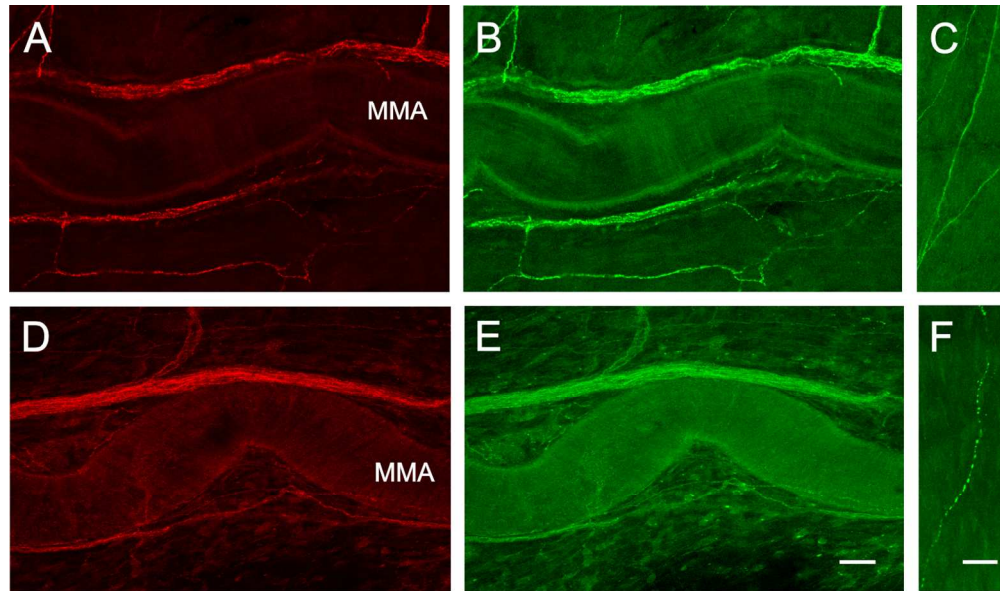


TRPV1 receptor activation-induced release of CGRP from meningeal afferents. CGRP concentrations (pg/ml) measured after topical application of SIF and capsaicin (10 and 100 nM). The number of experiments is indicated in the bars. \*: statistically different from the basal release, #: statistically different from the control.

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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  $\mu$ m and applies for A, B, D and E, scale bare on F represents 25  $\mu$ m and applies for C and F, MMA: branch of the middle meningeal artery.

136x80mm (300 x 300 DPI)

Review