FOS EXPRESSION IN HYPOCRETINERGIC NEURONS IN C57B1/6 MALE AND FEMALE MICE AFTER LONG-TERM CONSUMPTION OF HIGH FAT DIET

ZDENO PIRNIK, JANA BUNDZIKOVA, JENS D. MIKKELSEN[1], BLANKA ZELEZNA[1], LENKA MALETINSKA[2], ALEXANDER KISS

Laboratory of Functional Neuromorphology, Institute of Experimental Endocrinology, Slovak Academy of Sciences, Vlarska 3, 833 06 Bratislava, Slovak Republic, [1]Department of Translational Neurobiology, NeuroSearch A/S, Ballerup, Denmark, [2]Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic
e-mail: ueenkiss@savba.sk

Objective. It is generally known that hypocretin (Hcrt) neurons in lateral hypothalamus (LH) are involved in feeding behaviour. The aim of this study was to reveal the activity response of Hcrt neurons, as measured by Fos protein incidence, to prolonged high fat (HF) diet in the LH of both genders of C57B1/6 mice.

Methods. Standard (St) and high fat (HF) diets were available to mice for 16 weeks and thereafter the animals were perfused transcardially with fixative. Then the brains were removed, soaked with 15 % sucrose in 0.1 M phosphate buffer (PB), and cryo-sectioned throughout the hypothalamus into 35 μm thick coronal sections. Fos/Hcrt co-localizations were processed by employing avidin-biotin-peroxidase (ABC) complex and diaminobenzidine chromogen for Hcrt labeling. Fos immunoproduct was intensified by nickel chloride as a black color inducer. Evaluation of the incidence of Fos/Hcrt co-labeled perikarya was performed using a computerized Leica light microscopy.

Results. The effect of of mice gender, applied diet, and gender plus applied diet on the activation of Hcrt neurons was found. Turkey´s test revealed significant ($p<0.05$) rise in Fos labeled Hcrt neurons in male vs. female mice after consumption of both types of diets: St (44.64 ± 2.28 % vs. 1.47 ± 0.195 %, resp.) and HF (44.15 ± 3.77 % vs. 24.32 ± 0.7 %, resp.). This showed that HF diet significantly elevated the number of activated Hcrt neurons only in female mice (24.32 ± 0.7 % in HF fed vs. 1.47 ± 0.195 % in St fed, $p<0.05$). The body weight and accumulation of body fat in animals (body fat weight expressed as % of body weight) were influenced by gender and applied diet, although the body fat weight was influenced by HF diet (more noticeably in females).

Conclusion. The data indicated a positive correlation between body weight, fat gain, and Hcrt activities in females but not in males, thus accentuating the importance of the gender impact.

Key words: Fos - Hypocretin neurons - Lateral hypothalamus - High fat diet - Male and female C57B1/6 mice

Hypocretin (referred also as orexin) is a term for two neuropeptides, Hcrt-1 and Hcrt-2. Both of them are derived from the same precursor (DE LECEA et al. 1998; SAKURAI et al. 1998) and both of them serve as brain neurotransmitters. Hcrt axons and terminals have been detected in many regions of the rodent brain in differ-

Corresponding author: Alexander Kiss, PhD., D.Sci., Laboratory of Functional Neuromorphology, Institute of Experimental Endocrinology, Slovak Academy of Sciences, Vlarska 3, 833 06 Bratislava, Slovakia. Phone.: (42102)54772800, Fax.:(42102)54774247, e-mail: ueenkiss@savba.sk

ent amount (Trivedi et al. 1998; Cutler et al. 1999; Date et al. 1999). On the other hand, the occurrence of the Hcrt cell bodies has only been found within the lateral hypothalamus (Wagner et al. 2000; Novak and Albers 2002; Baldo et al. 2003), especially in the perifornical area and the adjacent zona incerta region (Peyron et al. 1998). Hypocretins are associated with several physiological functions such as the modulation of sleep-wake cycle (Wagner et al. 2000; Willie et al. 2001; de Lecea and Sutcliffe 2005) and behavioral state (D'Anna and Gammie 2006; Harris and Aston-Jones 2006), alteration of sympathetic and cardiovascular functions (Samson et al. 1999; Antunes et al. 2001; Ciriello et al. 2003), and control of appetite and energy metabolism (Griffond et al. 1999; Liu et al. 2001; Cai et al. 2001; Williams et al. 2001). Although several brain regions including the hypothalamic arcuate, paraventricular, ventromedial, and dorsomedial nuclei are involved in the regulation of energy homeostasis (Kalra et al. 1999), the lateral hypothalamic area (LHA) has been designated for long time as a centre of feeding and its activation has been shown to be associated with increased food intake and body adiposity (Williams et al. 2000, 2001).

Hcrt seems to stimulate the food intake in several animal species including sheep (Sartin et al. 2001), pigs (Dyer et al. 1999), and rats (Muroya et al. 2004). Hcrt gene expression increase has been observed following fasting (Sakurai et al. 1998) and insulin-induced hypoglycemia (Griffond et al. 1999). Central administration of Hcrt-1 is associated with increasing of daytime feeding and Hcrt neurons sense glucose availability to regulate their activity (Baye et al. 2000). Hcrt neurons also interact intimately with other appetite-regulating neuronal systems. In spite of the large number of data accumulated, evidences about the Hcrt implication in the obesity are still ambiguous. The present study was designated to reveal the activity of Hcrt neurons in the LHA after long-term consumption of high fat diet in both genders of C57Bl/6 mice with an attempt to better understand the sensitivity of Hcrt neurons to the obesity development. Dual Fos/Hcrt immunohistochemistry was employed to analyze the activity of Hcrt neurons in the LHA.

Materials and Methods

Animals. C57Bl/6 male and female mice obtained from Institute of Molecular Genetics (Prague, Czech Republic) were housed 4-5 per cage in a room with controlled light (12 h/day), humidity (40 %), and temperature (23 °C). Seven weeks old mice of both genders were subdivided into standard (St-1, obtained from Velaz, Kolec, Czech Rebublic) and high fat diet fed groups (n = 9 males/females per group). The standard (St) and high fat (HF) diets were available for 16 weeks, i.e. until the 23rd week of the mice age and throughout the whole study all animals had free access to food and water. HF diet consisted of 13 %, 60 %, and 27 % calories as protein, fat and carbohydrate, respectively, according to protocol by Kopecky et al. (1996). Principles of laboratory animal care and all experimental procedures followed the local Ethical Guidelines for Animal Experiments and the Law of the Czech Republic, No. 246/1992.

Tissue processing. For immunohistochemical study, from each group three randomly selected adult C57Bl/6 mice were used (three males and females each from St and HF diet group). To avoid the effect of diurnal variations on the Fos-expression, the experiment was performed between 9:00 and 11:00 a.m. The animals were anesthetized intraperitoneally with a mixture of xylazine/ketamine (rate: 16 mg/kg and 100 mg/kg; obtained from Spofa, Prague, Czech Rep.) and perfused transcardially with 50 ml of 0.1 M phosphate buffer (PB, pH 7.4) containing 4 % paraformaldehyde, 0.1 % glutaraldehyde, and 10 % picric acid (w/w). Then the brains were removed, post-fixed in the same fixative overnight at 4 °C, and infiltrated with 15 % sucrose in 0.025 M PB for 48 h at 4 °C. Before sectioning, the brains were rapidly (20 sec) frozen in cold isopentane (-30/-40 °C) followed by an additional cooling (30 sec) in dry ice and placed into a Reichert cryocut device to adjust to – 16 °C for 1 h. Coronal sections of 35 μm thickness were cut from the whole hypothalamus, i.e. from the optic chiasm (Bregma + 0.02 mm) to the end of the lateral hypothalamus (Bregma -2.8 mm), using the mouse brain atlas (Frankin and Paxinos 1997) as a guideline. The sections were collected as free floating in cold (4 °C) PB. The body weight of mice was measured once a week during the duration of experiment, the body fat weight (it means: subcutaneous, abdominal, perirenal and gonadal fat together) was measured after cleaning.

Immunohistochemistry. The sections were repeatedly washed in cold PB followed by a preincubation in 3 % H_2O_2 for 40 min at room temperature. They were incubated with a polyclonal Fos protein antiserum (1:2000, No. 94012), diluted in 0.1 M PB containing

4 % normal goat serum (Gibco, Grand Island, NY, USA), 0.5 % Triton X-100 (Koch-Light Lab. Ltd., Colnbrook, Berks, England), and 0.1 % sodium azide (Sigma Chemical Ltd., St. Louis, MO, USA) for 48 h at 4 °C. After several rinses in PB, the sections were incubated with biotinylated goat-antirabbit IgG (1:500, VectorStain Elite ABC, Vector Lab., Burlingame, CA, USA) for 90 min at room temperature. Next PB rinses were followed by incubation with the avidin–biotin peroxidase complex (1:250) for 90 min at room temperature. PB washing was followed by washing in 0.05 M sodium acetate buffer (SAB, pH 6.0). The Fos antigenic sites were visualized with 0.0266 % 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) dissolved in SAB containing 0.003 % H_2O_2 and 2.5 % nickel ammonium sulfate (Sigma), for 5 min. The metal-intensification of DAB produced black staining in the labeled nuclei. After several washes in PB, the Fos-positive sections were incubated with Hcrt-1 antibodies (1:2000) using the same procedure as described above. Second immunoreactions were visualized in 0.05 M Tris (pH 7.4) containing 0.0125 % DAB chromogen and 0.003 % H_2O_2. To reach the appropriate yellow color, the developing time was monitored under the light microscope. Finally, the sections were rinsed in 0.05 M Tris followed by 0.05 M SAB, mounted into 0.1 % of gelatine dissolved in 0.0125 M SAB, air-dried, coverslipped with Permount (Sigma), and examined under Leica DMLS light microscope using the mouse brain atlas (Frankin and Paxinos 1997). Immunostaining of negative control, which did not show any antiserum immunolabeling, included substitution of the primary antisera with normal rabbit serum, and sequential elimination of the primary or secondary antibody from the staining series.

Cell counting. Counting of Fos-Hcrt and Hcrt immunoreactive cells within the LHA (Bregma - 1.34 mm to - 1.46 mm) was performed separately in each side of 35 µm thick serial coronal sections employing a computerized system that included Leica DMLS light microscope equipped with a Canon digital camera (PowerShot S40). The quantitative assessment was performed from the captured images in a computer screen obtained from 3-4 brain sections per animal. The percentage of activated Hcrt neurons was calculated by formula: 100 x (amount of Hcrt neurons displaying Fos signal/ total Hcrt immunolabeled perikarya), and was expressed unilaterally per one section/animal. Representative sections were captured by computerized system that included microscope equipped with a Canon digital camera.

Statistical evaluation. All data represent the mean ± S.E.M. For statistical comparison two-way analysis of variance (ANOVA) for the factors of gender and applied diet were used with subsequent *post hoc* Tukey's tests, $p<0.05$ being considered statistically significant.

Antisera. The Fos (No 94012) and Hcrt-1 (No 99006) antisera were generated in the lab of Dr. J.D. Mikkelsen (NeuroSearch A/S Ballerup, Denmark). The specificity and sensitivity of Fos and Hcrt-1 antisera have already been tested previously (Kiss et al. 1988; Woldbye et al. 1996; Mikkelsen et al. 1998, 2001).

Results

At the end of experiment, after 16 weeks of HF and St diets, the statistical analysis revealed impact of gender ($F_{1,7} = 6.768$, $p = 0.0315$) and applied diet ($F_{1,7} = 25.363$, $p = 0.001$) on total body weight in C57B1/6 mice (Tab 1). Gender differences in body weight were already observable at the beginning of the experiment, male C57B1/6 mice had higher body

Table 1
Body weight, body fat weight and accumulation of body fat (%) in C57B1/6 male and female mice (n = 3/group) after 16 weeks of St and HF diets intake

	Female		Male	
	St diet	HF diet	St diet	HF diet
Body weight (g)	22.24 ± 2.03	35.2 ± 2.9*	30.84 ± 0.36*	35.9 ± 0.14
Body fat weight (g)	1.17 ± 0.4	7.56 ± 1.34*	0.95 ± 0.23	4.34 ± 0.61
Accumulation of body fat (%)	5.09 ± 1.31	21.19 ± 2.02*, x	3.12 ± 0.81	12.11 ± 1.74+

* $p < 0.05$ vs. female mice on St diet, + $p < 0.05$ vs. male mice on St diet, x $p < 0.05$ vs. male mice on HF diet

Legenda k obrázkom

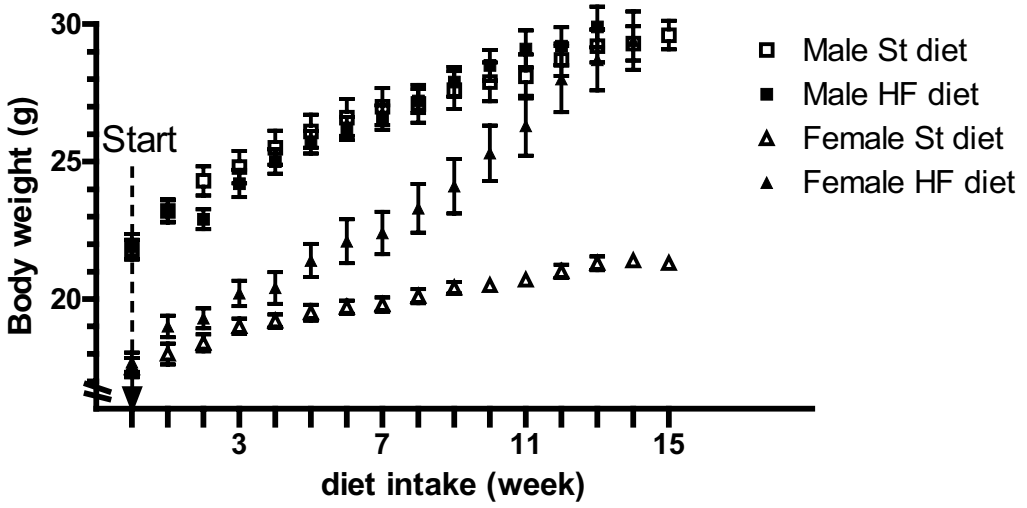

Fig. 1 Time-course in body weight progress during the first 15 weeks after St and HF diets consumption in C57B1/6 male and female mice (n = 9/group). Arrow indicates the beginning of the experiment.

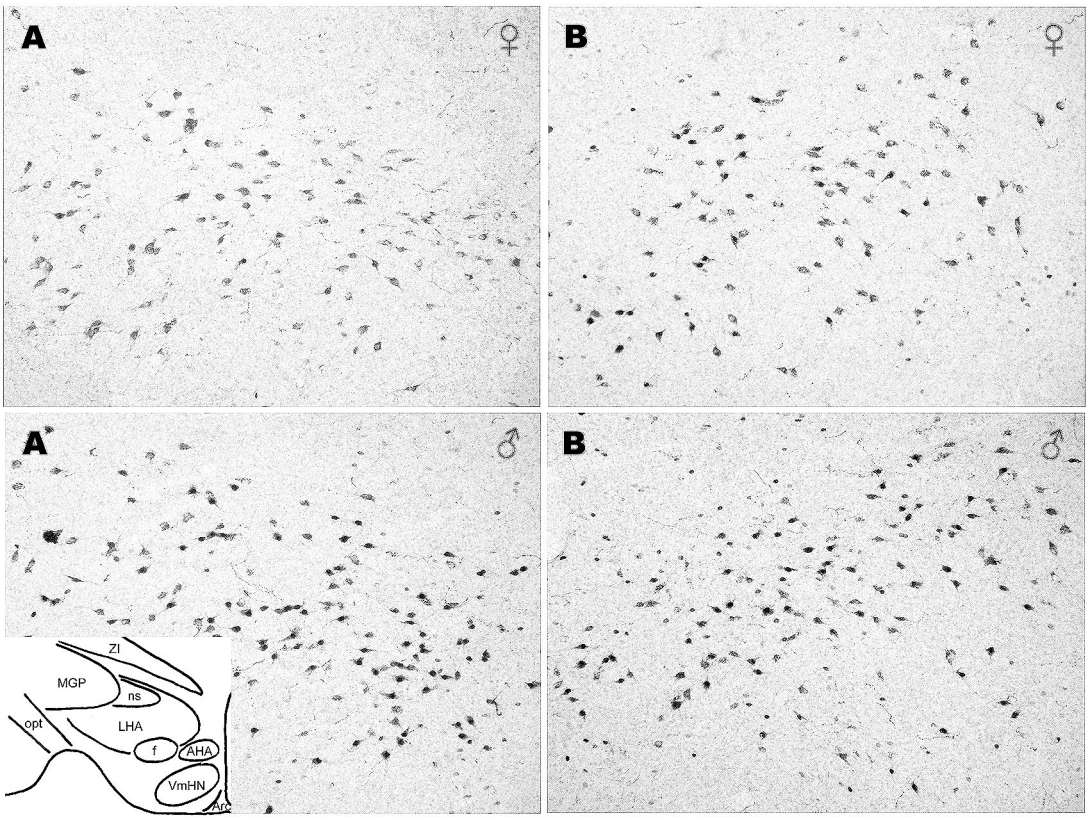

Fig. 2 Pattern of the analysed LHA in the mouse hypothalamus. Light microscopic pictures (magnification 71x) demonstrating co-localized Fos-Hcrt immunopositive neurons in female (♀) and male (♂) mice after long-term intake of St (A) and HF (B) diets. opt – optic tract, MGP – medial globus pallidus, ZI – zona incerta, ns – nigrostriatal bundle, LHA – lateral hypothalamic area, f - fornix, AHA – anterior hypothalamic area, VmHN – ventromedial hypothalamic nucleus, Arc – arcuate hypothalamic nucleus

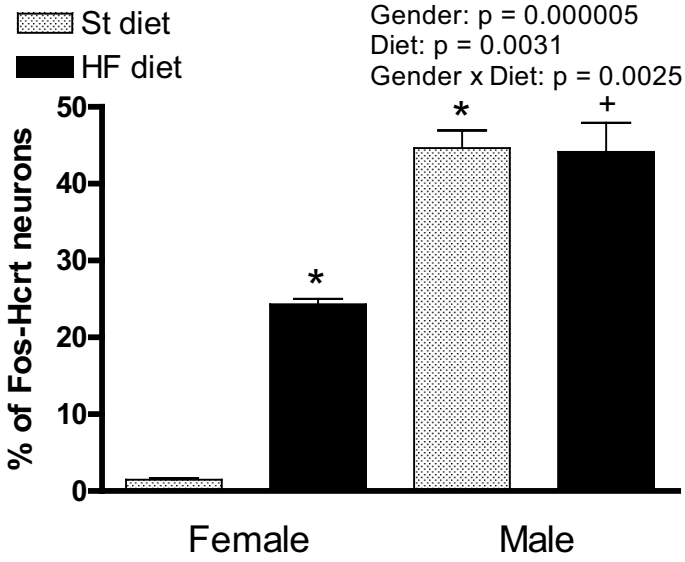

Fig. 3 Expression of the quantity pattern of activated Hcrt neurons in the LHA of C57B1/6 male and female mice after 16 weeks of St and HF diets intake (n = 3/group). * = p<0.05 vs. female mice on St diet, + = p<0.05 vs. female mice on HF diet

weight than the female mice of the same age (Fig 1). In control animals fed with St diet, as demonstrated by time course measurements (Fig. 1), the body weight parallelly increased in both genders during the duration of experiment. Positive effect of prolonged HF diet consumption on the weight gain was observed only in female group of animals, started at 3-5 weeks after beginning of the diet (Fig 1) and progressed till the end of experiment. After 16 weeks of the HF diet the female mice weighted as much as the male mice (Tab. 1).

At the end of experiment, body fat weight in all experimental animals was influenced only by consumed diet ($F_{1,7}$ = 39.59, p = 0.000235) although more noticeably in female mice (p < 0.05, Tab 1). Accumulation of body fat in animals (body fat weight expressed as percent of body weight) was influenced by applied diet ($F_{1,8}$ = 66.156, p = 0.000039) and gender ($F_{1,8}$ = 12.8673, p = 0.007114) and *post hoc* Tukey test (p < 0.05) revealed impact of HF diet in both genders (Table 1)

The distribution of cell bodies immunoreactive for hypocretin was easily identifiable, the Hcrt cells occurred laterally, above and below the fornix (Fig 2). Statistical analysis of activated Hcrt neurons in the LHA revealed effect of mice gender ($F_{1,7}$ = 154.88, p = 0.000005), applied diet ($F_{1,7}$ = 19.49, p = 0.0031), and gender x applied diet ($F_{1,7}$ = 21.25, p = 0.0025, Fig 3). *Post hoc* Turkey´s test revealed statistically significant (p<0.05) rise in Fos labeled Hcrt neurons in male vs. female mice after consumption of both types of diets: St (44.64 ± 2.28 vs 1.47 ± 0.195 %) and HF (44.15 ± 3.77 vs 24.32 ± 0.7 %, Fig 3). In control female C57B1/6 mice fed with St diet, there was only a minimal number of activated Hcrt neurons in the LHA. On the other hand, only in this gender the long term consumption of HF diet significantly elevated the number of activated Hcrt neurons (24.32 ± 0.7 vs 1.47 ± 0.195 %, p<0.05, Fig 3). The number of Hcrt neurons did not statistically differ among evaluated sections and their average number was 105.6 ± 1.6 expressed unilaterally per one section/animal.

Discussion

In this study we compared the effect of prolonged HF diet on the activity of Hcrt neurons in both genders of mice. In C57Bl/6 female mice after 16 weeks of HF diet, we found increased body weight as well as body fat weight accompanied by an elevated Fos-immunoreactivity in Hcrt neurons of the LHA. Under the same conditions, small differences in the body weight, prominent differences in body fat weight, and no differences in the number of activated Hcrt neu-

rons were observed in the LHA of the males. We also noticed distinct gender differences in the number of Fos immunostained Hcrt neurons in the LHA in control group of animals fed with St diet. These data indicate a positive correlation among the body weight, fat gain, and Hcrt activities only in female mice which clearly speaks out for the involvement of a gender impact.

There is evidence regarding the increased body weight, fat mass, and serum leptin levels and a parallel activation of Fos-immunoreactivity in the LHA of C57Bl/6J male mice after long-lasting consumption of HF diet (Lin and Huang 1999; Xin et al. 2000). The quantitative differences in Fos activated neurons presented in the above mentioned studies, however, can not be directly linked to Hcrt activated neurons observed in the present study since the lack of the phenotypic characterization of neurons. There is no doubt that hypocretin-neuropeptides, which are exclusively synthesized in neurons of the lateral, posterior, and perifornical hypothalamus, have widespread projections throughout the CNS (Peyron et al. 1998; Preti 2002; Taheri et al. 2002). The major function, emerging from the studies dealing with these neuropeptides, is the regulation of sleep and wakefulness (Estabrooke et al. 2001). However, since leptin receptors were found in these lateral hypothalamic cells (Meister 2000), they can be activated by metabolic challenges (Diano et al. 2003) and they innervate brain regions influencing food intake and body weigh including the hypothalamic arcuate, paraventricular, and dorsomedial nuclei, indicate that the Hcrt system may play an important role in the central control of appetite (Edwards et al. 1999; Martynska et al. 2005; Samson et al. 2005). Recent studies have also indicated that hypocretin neurons are activated in the positive correlation with the triglyceride levels (Chang et al. 2004) and that they can alter their intrinsic electrical activity according to ambient fluctuations at the levels of nutrients and appetite-regulating hormones. These intriguing electrical responses are the strongest candidates to date for the elusive neural correlates of after-meal sleepiness and hunger-induced wakefulness. Hypocretin/orexin neurons may thus directly translate rises and falls in body energy levels into different states of consciousness (Burdakov and Alexopoulos 2005).

The differences observed in the activation of Hcrt neurons in the LHA between female and male mice under control conditions may be observed under a number of other conditions such as 1) circadian variation in sleep-wake behavior, 2) stressful conditions and 3) impact of sexual differences. While to the first issue of the above mentioned points, it has been shown that the activation in Hcrt neurons is minimal during the first hours of the daylight phase and its maximum is reached during nocturnal phase of the day (Estabrooke et al. 2001). Although in our experiment we started first with female and then followed by male mice, both were performed in the morning hours in awake animals. Thus circadian effect will probably not account for the explanation of spontaneous Fos expression discrepancies in Hcrt neurons. Stress is another factor which has been shown to be able to affect the activity of the LHA Hcrt neurons. Evidence of a direct neuroanatomical and physiological inputs from stress responsive corticotropin-releasing factor (CRF) peptidergic system onto hypocretin neurons has been given and also by stress triggered hypocretin release has been demonstrated (Winsky-Sommerer et al. 2004). The level of corticosterone has been shown to be increased in a dose-dependent manner after intracerebroventricular injection of orexin, and it remained high for at least 60 min and orexin mRNA levels, but not the melanin-concentrating hormone mRNA ones, have been also demonstrated to be elevated after cold stress in the lateral hypothalamic area (Ida et al. 2000). In addition, gender differences in response to stress have also been well documented in the literature (Hinojosa-Laborde et al. 1999; Kudielka et al. 2004). For example, differences in hypothalamo-pituitary-adrenocortical (HPA) axis, body weight, and feeding responses to immobilization stress has been shown to be more robust among female than male rats (Faraday et al. 2005). Effect of stress can not be excluded from our study, however, its possible interference with our findings needs to be elicited by further studies.

In rodents, but also in other species, different feeding behavior associated with different spectrum of gonadal hormones has been recognized (Gentr and Wade 1976; Laviano et al. 1996). There is evidence about sex differences in hypothalamic regulation of body weight in male and female rats (Lenard et al. 1991). In the above mentioned work, although lesion of the LHA resulted in hypophagia, hypodipsia, and body weight loss in male rats, only female animals exhibited hyperphagia and weight increase after the ventromedial hypothalamic nucleus was destroyed. Moreover, in the lateral hypothalamus greater immunoreactivity of orexin A has been found in females

than male rats (Taheri et al. 1999) and similar sex differences in the amount of hypothalamic orexin 1 receptor mRNA have been also described (Johren et al. 2001). On the other hand, there was also shown that hypothalamic orexin A content was lower at late proestrus relative to all other stages of the estrous cycle in female rats (Russell et al. 2001). Although we did not monitor the individual stages of the estrous cycle in female mice, there was also shown that the high fat diet may induced alterations in serum steroid and free fatty acids concentrations that might affect several reproductive processes (Whyte et al. 2007). We noticed distinct gender differences with respect to the number of activated Hcrt immunopositive population of the LHA neurons, unambiguously altered in different manner in females, which can be explained by the impact of sex differences on the metabolic state and the LHA neurons. Although diet-induced satiated obese mice, compared with lean mice, had significantly increased the number of Fos neurons in the LHA (Lin and Huang 1999) this situation can not be associated with activation of Hcrt neurons observed in our study because prolonged HF diet may activate, besides Hcrt neurons, also another phenotype of LHA neurons. Most potential candidate for this phenotype of cells might be melanin-concentrating hormone (MCH) cells since MCH injection into the lateral ventricle of rats has been shown to increase the food consumption suggesting that MCH, likewise Hcrt, participates in the hypothalamic regulation of body weight (Qu et al. 1996; Shimada et al. 1998). On the other hand, using phosphorylated cAMP response element-binding protein (pCREB), as a marker of neural activity, gonadectomy in males enhanced and in females attenuated the response of MCH neurons to glucose, which has been restored by testosterone and estrogen replacement in males and females, respectively (Mogi et al. 2005). Thus distinct amount of Hcrt neurons observed in our study and different amount of activated neurons in the LHA represented by single Fos immunoreactivities in other studies (Lin and Huang 1999; Xin et al. 2000) may indicate for their different sensitivity or physiological role in male and female genders and may indicate for gender differences of the LHA role in food regulation during HF diet intake.

In summary, the data of the present study indicate that the activation of Hcrt neurons in the LHA during the development of HF diet obesity is gender dependent.

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