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Research Report

A positive change in energy balance modulates TrkB expression in the hypothalamus and nodose ganglia of rats

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ABSTRACT

Brain-derived neurotrophic factor (BDNF) and its TrkB receptor play critical roles in the synaptic activity and plasticity of mature neurons and enhance adult neurogenesis. Furthermore, treatment with BDNF has been found to attenuate weight gain or even cause weight loss and appetite suppression in rats. The aim of this study was to look at the effect of nutrient intake on BDNF concentrations and cellular proliferation in the brain. Adult male Wistar rats were given one of three diets for 6 weeks: high-carbohydrate, high-fat or high-fat pair-fed diets. Rats were sacrificed at the end of the feeding period and BDNF concentrations in the dorsal vagal complex (DVC), hypothalamus and plasma were measured by ELISA on protein extracts of these samples. Cellular proliferation in the DVC was quantified by Ki-67 immunohistochemistry. Neither BDNF levels nor proliferation were modified by the diet. Secondly, using rats that received the same diets, real-time PCR was performed in the DVC, hypothalamus and nodose ganglia in order to compare TrkB receptor levels. The results showed significantly lower TrkB levels in the hypothalamus and nodose ganglia of fasted rats receiving the high-fat diet when compared to the other groups. These two complementary methodological approaches suggest that there is a relationship between long-term dietary intake and BDNF. More precisely, TrkB expression is more responsive to energy states than to diet composition. An increment in energy stores thus triggers decreased BDNF anorexigenic signaling at the receptor level in the hypothalamus and nodose ganglia, but not in the DVC.

1. Introduction

The peripheral and central neural pathways controlling food intake are probably highly flexible and it is now acknowledged that dietary conditions can affect the neural networks controlling food intake. These properties of neural circuits may be responsible for changes to energy intake and, as a consequence, may result in the onset of overweight and obesity. Neuronal plasticity mechanisms, consisting particularly in desensitization of the gut brain axis to anorexic signals are likely to be at the origin of this phenomenon. Neuronal plasticity is the ability of the nervous system to adapt its

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structural organization to new situations that result from developmental or environmental changes as well as other factors (e.g. injuries) that may affect conditions in the nervous system and the whole (living) organism. For instance, if a subject (rat or mouse) is subjected to a high-fat diet, the neural pathways responsible for controlling meal size become much less efficient, causing an increase in meal size and elevated body weight (Savastano and Covasa, 2005; Paulino et al., 2008). Neurotrophins play an important role in the proliferation, differentiation and survival of neurons in the peripheral and central nervous systems during development (reviewed by Barde (1990)). In addition, some neurotrophins, and particularly BDNF, play critical roles in the synaptic activity and plasticity of mature neurons (Hennigan et al., 2007). Brain-derived neurotrophic factor (BDNF) is one of the trophic nerve growth factors. In the brain, BDNF exerts a trophic action on retinal, cholinergic and dopaminergic neurons, while in the peripheral nervous system it acts on both motor and sensory neurons (reviewed by Lebrun et al. (2006)).

There is evidence that BDNF and its high-affinity receptor TrkB contribute to the control of food intake and body weight. The central administration of BDNF has been found to attenuate weight gain or even cause weight loss and appetite suppression in rats (Lapchak and Hefti, 1992; Pelleymounter et al., 1995). Also, BDNF serum levels have been correlated to eating disorders. During a study in human subjects, serum BDNF levels were found to be significantly lower in female patients with anorexia or bulimia nervosa than in age-matched, normal female control subjects. In addition, a significant and positive correlation between serum BDNF levels and body mass index was detected in all of the subjects (Nakazato et al., 2003). Similarly, BDNF plasma levels were seen to be strongly correlated with the symptoms checklist 90 Revised (SLC-90R) questionnaire in a total of 78 anorexia nervosa patients (Mercader et al., 2008). In animal studies, BDNF has also been shown to act as an anorexigenic factor in the dorsal vagal complex (DVC). Indeed, in a study by Bariohay et al. (2005), BDNF infusion in the DVC of adult rats induced anorexia and weight loss. Furthermore, the BDNF protein content decreased significantly in the DVC after food deprivation for 48 h but increased after re-feeding. This same study also suggested that BDNF could be a common downstream effector of leptin and CCK and involved in their synergistic action. Indeed, acute and repeated peripheral leptin injections, as well as peripheral CCK treatment, induced a rise in BDNF protein levels in the DVC (Bariohay et al., 2005).

Although these processes are of importance to the onset of obesity and changes in eating behavior, our understanding of their molecular and cellular bases remains very poor. The aim of the present study was therefore to look at the effects of long-term nutrient intake on BDNF-related parameters. Our hypothesis was that the levels of BDNF and its receptor in specific brain areas could be dependent on the type of dietary regimen. Two studies were thus carried out to investigate this hypothesis. The first explored the influence of a high-fat (HF) diet on BDNF levels in the brain and on cellular proliferation using the Ki67 marker (expressed during active phases of the cellular cycle), while the second considered the effects of this HF diet on the level of TrkB receptor expression in specific brain areas.

2. Results

2.1. *Body weight and food intake*

Rats were fed one of three diets for 6 weeks: a standard high-carbohydrate diet (HC), a high-fat diet ad libitum (HF) or a high-fat diet pair-fed (HF-pf). As expected, the HF group displayed higher body weights than the high-carbohydrate (HC) and the high-fat pair-fed (HF-pf) groups over the last 10 days of the dietary treatment (F (6, 68) = 4.8, $P < 0.001$). On the last day of the experiment, the average body weights of the rats were 494.2 ± 13.5 g in the HF group and 406.8 ± 5.5 g and 397.22 ± 10.4 g in the HC and HF-pf groups, respectively. Both body weight values and the cumulative energy intake of the animals are presented in Fig. 1. The HF group displayed a significantly higher energy intake (387.5 ± 8.1 kJ) than the HC (348.4 ± 8.3 kJ) and the HF-pf (329.4 ± 8.2 kJ) groups, (F (2, 67) = 13.14, $P < 0.001$). The energy intake of HC and HF-pf animals did not differ significantly from each other.

2.2. *BDNF levels, cellular proliferation and TrkB receptor expression in the brain*

During the first set of experiments, BDNF levels were measured in the dorsal vagal complex (DVC), hypothalamus and serum of rats after the 6-week dietary treatment. These parameters were not affected by diet: F (2, 13) = 0.04, $p = 0.85$; F (2,13) = 0.79, $p = 0.48$ and F (2,13) = 0.79, $p = 0.48$, respectively. Also, cellular proliferation as measured in terms of Ki-67 protein levels in the DVC, did not significantly differ between the dietary groups: F (2, 13) = 0.32, $p = 0.77$. During the second study, levels of TrkB receptor expression were measured in the hypothalamus, DVC and nodose ganglia of the rats. TrkB mRNA levels in the hypothalamus were affected by diet: F (2, 13) = 4.92, $p = 0.026$ (Fig. 2). Indeed, rats fed the HF diet had lower TrkB expression levels (0.84 ± 0.18) than rats fed the HC (1.58 ± 0.23) or HF-pf diets (1.57 ± 0.18), $t(13) = 2.52$, $p = 0.026$ and $t(13) = 2.80$, $p = 0.047$, respectively (Fig. 3). Similarly, in the nodose ganglia, TrkB mRNA levels were affected by diet: F (2,13) = 4.92, $p = 0.026$. The results showed a decrease in TrkB mRNA expression in rats receiving the high-fat diet (0.69 ± 0.047) compared to the two other dietary groups (1.29 ± 0.25 and 1.16 ± 0.18) (Fig. 3). However, TrkB levels were not significantly affected by diet in the DVC of the rats (F (2, 14) = 0.36, $p = 0.71$) (Fig. 3).

3. Discussion

The objective of this study was to verify whether the levels of BDNF and its receptor in specific brain areas could be dependent on the type of dietary regimen. The results did not demonstrate any diet-related variations in BDNF levels or in cellular proliferation (as measured in terms of Ki-67 protein). By contrast, they showed significantly lower levels of TrkB expression in the hypothalamus and nodose ganglia of fasted rats that had received the high-fat diet *versus* the control dietary groups. These findings thus provide new evidence for a variation in BDNF receptor dynamics consequent on a positive change in the energy balance.

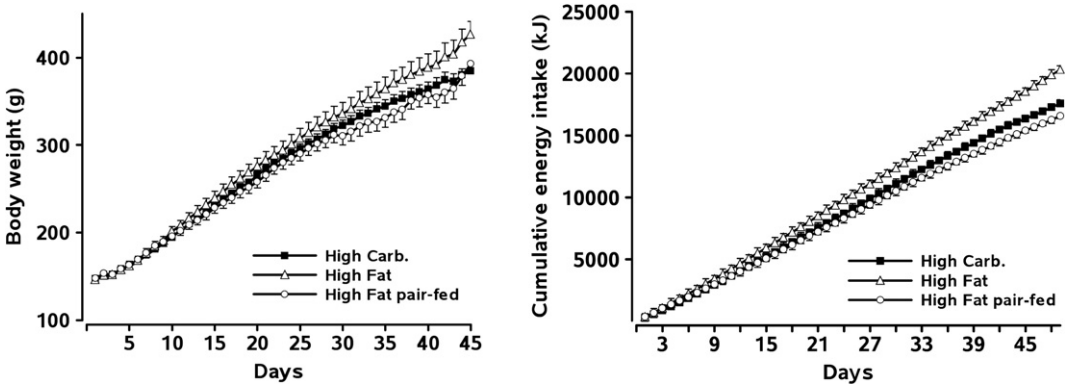

Fig. 1 – Body weights and cumulated daily energy intake of rats fed either a standard high-carbohydrate, high-fat or high-fat pair-fed diet for 6 weeks ($n=18$). Data are expressed as the mean ± SEM. $^{*}P<0.05$.

Although BDNF levels had previously been found to be affected by feeding status, the present study indicated that long-term feeding with a high-fat diet did not produce significant differences in BDNF levels and cellular proliferation. Indeed, other studies in rats have demonstrated higher levels of BDNF in the DVC after a 48-hour period of food deprivation as well as after peripheral short-term treatments with leptin or cholecystokinin (CCK) (Bariohay et al., 2005). Because our HF animals displayed an increase in body weight, we might have expected higher leptin levels and therefore higher BDNF levels. Also, a previous study involving a 3-month alternate-day feeding schedule had been found to lead to increased levels of BDNF in the hippocampus, cerebral cortex and striatum of mice (Duan et al., 2001). The results of the present study could be explained by the fact that BDNF plays an important role as a neurotransmitter in addition to being a neurotrophin. Indeed, even at low concentrations, BDNF has been found to excite neurons in the hippocampus, cortex and cerebellum, and also to depolarize neurons just as rapidly as the neurotransmitter glutamate, even at a concentration more than a thousand-fold lower (Kafitz et al., 1999). Neurotrophin-induced depolarization results from the activation of sodium ion conductance which is reversibly blocked by K-252a, a protein kinase blocker that prefers tyrosine kinase Trk receptors (Kafitz et al., 1999; Delmas and Coste, 2003). This suggests that the BDNF levels determined during the present study may have been representative not only of its activity as a neurotrophin but also as a neurotransmitter. Furthermore, Ki-67 levels did not vary significantly as a function of diet. This marker is frequently used to detect cellular proliferation in healthy tissues because it is expressed by proliferating cells throughout active phases of the cell cycle, but is absent from resting cells. In addition, because the half-life of Ki-67 is 90 min, its detection in situ provides an instant localization of newly-generated cells (Scholzen and Gerdes, 2000; Charrier et al., 2006a). When used in brain structures that have been shown to harbor neural stem/progenitor cells, this marker can therefore constitute an index of neurogenesis modulation. This was confirmed in the DVC during out study, because the immunohistochemical Ki-67 labeling pattern matched the distribution of neurogenic proliferation previously characterized in adult rats using classical neurogenesis assays *in vivo* and *in vitro* in this structure (Bauer et al., 2005; Charrier et al. 2006a; Chigr et al., 2009). However, it should be borne in mind that neurogenic systems in an adult brain do not only yield neuronal progenies.

Interestingly, our results showed a decrease in TrkB expression levels in both the hypothalamus and nodose ganglia of rats fed the high-fat diet ad libitum when compared to the other dietary groups. It might appear counter-intuitive that BDNF itself was not affected, even though expression of its receptor was significantly reduced in some brain areas, but such discrepancies have been observed elsewhere in the literature. Indeed, there is a growing number of reports on physiological signaling adaptations that involve changes to the expression of BDNF and/or TrkB which are either parallel (Kim et al., 2004; Rage et al., 2002; Bracken and Turrigiano, 2009; Fumagalli et al., 2009) or different/opposite (Huber et al., 2000; Givalois et al., 2001; Marmigère et al., 2001; Marmigere et al., 2003; Silhol et al., 2005, 2007; Rage et al., 2002; Huang and Stahler, 2009). It should also be noted that when TrkB receptors are affected, the physiological modulations can differ between isoforms (Silhol et al. 2005, Rage et al., 2002) or even specifically affect one isoform rather than others (Skup et al. 2002; Rage et al., 2002; Bracken and Turrigiano 2009).

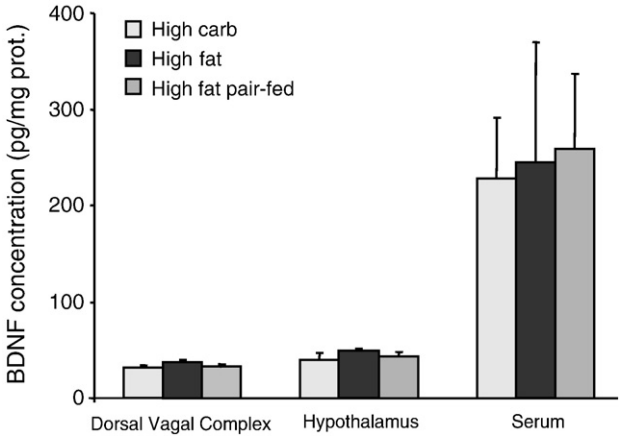

Fig. 2 – BDNF concentrations (pg/mg prot.) in the DVC, hypothalamus and serum of rats fed either a standard high-carbohydrate, high-fat or high-fat pair-fed diet for 6 weeks ($n=6$). Data are expressed as the mean ± SEM.

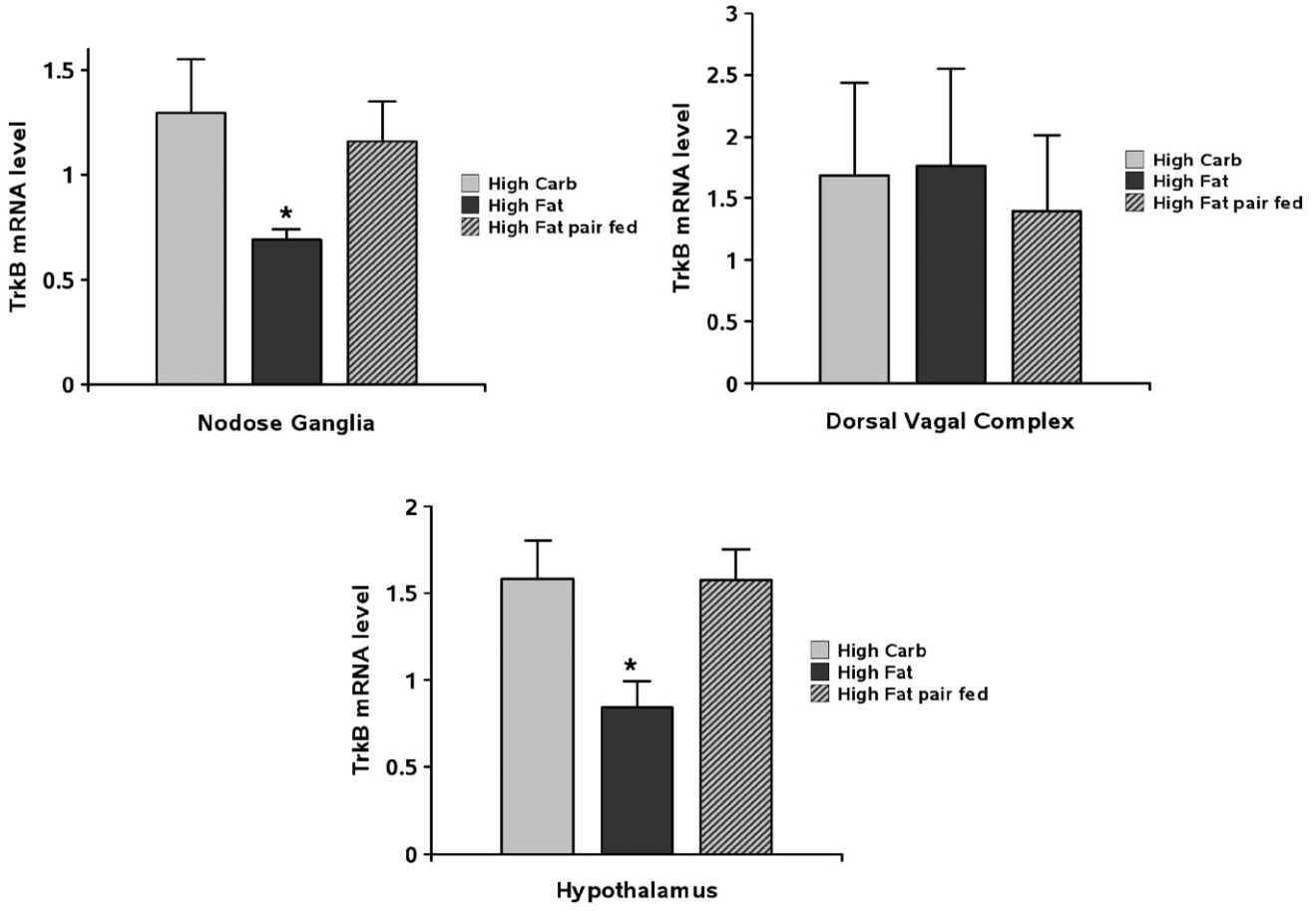

Fig. 3 – Expression of the tyrosine kinase receptor (TrkB) in the hypothalamus, dorsal vagal complex and nodose ganglia of rats fed either a standard high-carbohydrate, high-fat or high-fat pair-fed diet for 6 weeks ($n=6$). Data are expressed as the mean ± SEM. *$P<0.05$.

Moreover, the "classical" enzymatically-active FL isoform of TrkB receptor was recently shown to undergo down-regulation by one of the truncated isoforms, T1 (Carim-Todd et al., 2009). Overall, therefore, TrkB expression is regulated independently of BDNF availability in numerous instances, as explicitly quoted by Huang and Stahler (2009).

Our results were thus in line with others regarding the dietary modifications that affect TrkB expression. For example, in a study conducted on rats, anorexia induced by immobilization stress (IS) was shown to affect BDNF expression: significant variations in BDNF expression, as well as an increase in TrkB mRNA levels 24 h post-IS, were found in the DVC of rats that had undergone immobilization stress *versus* control animals (Charrier et al., 2006b). BDNF has been shown to be inversely related in older adults to appetite (Stanek et al., 2008) and to increased energy expenditure (Wang et al., 2007a), and to exert anorexigenic effects in rats when injected in the ventromedial and paraventricular nuclei of the hypothalamus (Wang et al., 2007b,c). TrkB agonists have also been found to reduce food intake and ameliorate obesity in mice (Tsao et al., 2007). Furthermore, the fact that the pair-fed animals displayed similar levels of TrkB expression as the high-carbohydrate group lead us to think that the effect obtained might actually be a result of increased body adiposity and body weight. Our results therefore suggest that TrkB expression is responsive to energy states rather than diet composition. An increment in energy stores thus triggers weaker BDNF anorexigenic signaling at the target level in the hypothalamus and nodose ganglia but not in the DVC. Furthermore, a diet

Table 1 – Composition of diets.

Ingredient	High-carbohydrate diet (HC)	High-fat diet (HF)
	(g kg^{-1})	
Total milk protein	140	170
Cornstarch	623	436
Fat (soybean oil)	40	225
Sucrose	100	71
Cellulose	50	50
Choline chloride	2	2
Vitamin mix	10	10
Salt mix	35	35
Energy content (kJ g^{-1})	15.2	19.6
Energy (protein)	14%	14%
Energy (fat)	10%	43%
Energy (carbohydrate)	76%	43%

Table 2 – Primers used for real-time PCR.

Gene	Forward primer (5′–3′)	Concentration (nM)	Reverse primer (5′–3′)
Ribosomal 18S	ACGGAAGGGCACCACCAGGAG	500	GACCCACCACCCACGGAAACG
TrkB (exon 2)	AGCCTTCTCCAGGCATCGT	500	CGGGTCAACGCTGTTAGGTT

containing high levels of saturated fat and refined sugar had been found to lower BDNF levels in the hippocampus to an extent that compromised cognitive function in rats (Molteni et al., 2002). The diet used during the present study was made up of polyunsaturated fats (soybean oil) and small quantities of refined sugars. This supports the hypothesis that the effect of diet on BDNF function is due to the energy density of the diet itself, and indicates that widely-consumed, high-energy diets can influence crucial aspects of neuronal and behavioral plasticity associated with the function of BDNF. Our conclusion is that the HF diet did indeed modulate TrkB expression in the hypothalamus and nodose ganglia of rats, but it perhaps not as a result of a direct relationship.

These findings may explain why obese subjects find it more difficult to lose weight or feel satiated. Furthermore, they also suggest that TrkB agonists could constitute a therapy for metabolic disorders (Lin et al., 2008). Finally, the fact that receptor levels varied as a function of diet in both the hypothalamus and nodose ganglia suggests a dual location for BDNF-related modifications to the control of food intake.

In conclusion, the data generated using these two complementary methodological approaches indicated that energy balance states can alter the neural networks that control food intake and are suggestive of a relationship between energy balance and BDNF.

4. Experimental procedures

4.1. *Dietary treatments*

Fifty-four male Wistar rats (Harlan Laboratories, France), initially weighing 120±10 g, were housed individually in a temperature and humidity controlled room under a 12:12 light:dark cycle (lights on at 11:00). All experimental procedures complied with the guidelines of the French National Animal Care Committee. After one week of adaptation to the environmental conditions, during which the rats were fed a standard diet, they were randomly allotted to three dietary groups ($n=18$), each receiving one of three diets: a standard high-carbohydrate diet (HC), a high-fat diet (HF) or a high-fat pair-fed diet (HF-pf) (INRA, Jouy-en-Josas, France) (Table 1). HF-pf rats received the high-fat diet in quantities corresponding to the caloric density eaten by the HC group the previous day. Food was therefore provided ad libitum to HC and HF animals but not to those in the HF-pf group. It was supplied in a single helping at the beginning of the activity period for all groups, in a liquid form (powder/water ratio of 1/1) in order to minimize spillage and enable more precise food intake measurements. Water was available ad libitum. After six weeks of the dietary treatment, the rats from each group were divided again into three different groups for BDNF-dosage by ELISA ($n=6$), Ki-67 protein immunolabeling ($n=6$) and the assessment of TrkB receptor expression ($n=6$). All animals were sacrificed at the beginning of their activity period after fasting overnight.

4.2. *Fresh and fixed tissue sampling and immunolabeling quantifications*

For fresh tissue sampling, rats were euthanized to collect both blood and the intestine, hypothalamus, nodose ganglia and dorso-vagal complex (DVC). All samples from one rat were collected on ice within a maximum time of 13 min and rinsed with phosphate buffer saline (PBS). Blood was mixed with a solution of EDTA (ethylenediaminetetraacetic acid, 70 μL/ml) and centrifuged for 15 min (6000 rpm, 4 °C) in order to collect the plasma. All samples were frozen instantly with liquid nitrogen and then stored at −80 °C until protein extraction and assay using a conventional two-site ELISA (Enzyme-linked ImmunoSorbent Assay). The BDNF Emax immunoassay (Promega, Charbonniere, France) was performed according to the manufacturer's protocol. For each sample, 30 μg of total proteins were used to determine the BDNF content. The sensitivity of the assay was 15 pg/ml, and cross-reactivity with other related neurotrophic factors was less than 3%. The within-assay variability was less than 3%, and all relevant comparisons were made during the same assay. BDNF levels were determined as picograms per milligram of total protein, and relative changes to BDNF levels between experimental groups were expressed as a percentage of control values.

For fixed tissue sampling, the rats were euthanized with a lethal injection of pentobarbital sodium (90 mg/kg, i.p.). The thoracic cage was opened and the animals perfused intracardially with 500 mL saline buffer at 37 °C followed by 1000 mL of 4% paraformaldehyde in PBS. The forebrain and spinal cord were exposed from the cervical spinal segment C2 to the olfactory bulb; the entire forebrain, the cerebellum and the first two cervical spinal segments were removed. This portion of the CNS was cryoprotected using 15% sucrose in PBS for 2 h and then stored in 30% sucrose in PBS with sodium amide to prevent bacterial contamination. The brain region of interest was localized using a stereotaxic atlas (nucleus of the solitary tract at the mid-area postrema level; bregma −13.8 to −13.6 mm). Transverse 40 μm-thick sections corresponding to the regions of interest were cut with a cryostat at −22 °C and collected in PBS. The slices were collected for processing as a free-floating section and were immunostained for Ki67 protein according to the avidin–biotin peroxidase method. Sections were incubated in PBS containing 2% normal goat serum and 0.3% Triton X-100 for 1 h at room temperature, then incubated in the primary antiserum containing the polyclonal pAb5 antibody raised against Ki67 protein diluted 1:50 for 48 h at 4 °C. The sections were then washed in PBS, incubated in biotinylated goat anti-rabbit antiserum for 24 h, washed, and incubated for 1 h in the avidin–peroxidase complex. Staining

was developed in 0.05% diaminobenzidine (DAB) with 0.03% hydrogen peroxide. The DAB reaction was halted by washing several times in PBS, and the sections were then mounted on gelatin-coated slides. After drying overnight, the sections were cleared in an ascending series of ethanol baths (70, 95, and 100%, 10 min in each bath) followed by a 5-min bath in xylene. They were then mounted on gelatin-coated slides and cover-slipped. To control for staining variability between days, each immunohistochemistry run contained matched sections from all the experimental groups. For immunolabeling quantifications, the sections were examined under a light microscope; for each rat and each brain region, five corresponding sections were chosen for further cell counts, and the sections were carefully selected to determine a constant location in the brain. The sections thus analyzed were magnified under a Zeiss Z-1 axio-imager (40×) and image processing was performed using Axiovision 4.0 imaging software. New cells appeared in purple whereas the background remained slightly yellowish. Immunolabeling quantifications were processed manually through a blind test. Only cells pertaining to the solitary tract at the sub-postremal level were counted.

4.3. *Tissue collection procedure and TrkB receptor expression in the brain determined by real-time PCR amplification*

Prior to the experimental days, rats from each group (HC, HF, HF-pf) were fasted overnight (with free access to water). On the experimental days, the rats were sacrificed and their hypothalamus, dorsal vagal complex and nodose ganglia were rapidly dissected out on ice (maximum of 13 min per rat), stored into trizol reagent, snap-frozen in liquid nitrogen and stored at −80 °C. Total RNA was extracted from a pool of four nodose ganglia (from two rats) or from one hypothalamus or one dorsal vagal complex with 200 μL of Trizol reagent (Invitrogen, Carlsbad, CA) and homogenized using a Polytron homogenizer. Total RNA concentrations were determined by measuring absorbance at 260 nm with a Nanodrop spectrophotometer (Labtek, Paris, France). A synthesis of strand complementary DNA (cDNA) was performed on 400 ng of total RNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Primers were designed using Primer Express software, and an 18 S protein was used as a housekeeping gene to normalize the mRNA abundance of each gene. BLASTN searches were conducted against GenBank to check the total gene specificity of the nucleotide sequences chosen for the primers (Table 2). PCR reactions were performed using a 7300 real-time PCR system (Applied Biosystems, Foster City, CA). Each cDNA was amplified in a 20 μL volume containing 15 μL of 2× SYBR Green master mix (Applied Biosystems, Foster City, CA) and 500 nM concentrations of the gene-specific primer. Thermal cycling conditions comprised an initial denaturation step at 95 °C for 10 min and 40 amplification cycles at 95 °C for 15 s and 60 °C for 1 min. The cycle threshold (C_T) for each sample was determined at a constant fluorescence threshold line. For each run, a melt curve was subsequently performed to analyze the products generated for contaminations resulting from residual genomic DNA amplification (using a control without reverse transcriptase) and/or from controls with no DNA template and no reverse transcriptase. Quantitative values for cDNA amplification were obtained from the threshold cycle number (C_t) at which the increase in signal was associated with exponential growth of the PCR products that were starting to be detected. The overall efficiency (E) of PCR was calculated from the slopes of the standard curves according to the equation: $E=[10^{-1/\text{slope}}]-1$. 18S protein was used as a housekeeping gene to normalize the mRNA abundance of our target gene. All cDNA were amplified with an efficiency higher than 98%, indicating that 18S and the target gene were amplified with the same efficiency. Gene expression was determined using the $2^{-\Delta Ct}$ formula where ΔCt = (Ct target gene − Ct 18S).

4.4. *Statistical analysis*

BDNF levels and cellular proliferation, total 24-hour food intake (g), and body weights (g) were analyzed separately using a one-way repeated measures ANOVA with time being the repeated measure and diet (HC *versus* HF *versus* HF-pf) as the factor. Plasticity parameters, including cellular regeneration and BDNF levels, were analyzed using a one-way measure ANOVA with diet (HC *versus* HF *versus* HF-pf) as the factor. Real-time PCR values of TrkB receptor expression in the brain were analyzed using one-way measures ANOVA with diet (HC *versus* HF *versus* HF-pf) as the factor. Descriptive data are presented as means ± SEM. For all analyses, the procedure used under SAS was PROC MIXED, which is specific for models with fixed and random variables. For both studies, significant differences in the main effects were tested using Tukey–Kramer's Post-Hoc test for multiple comparisons. All data were analyzed using the SAS statistical package (Version 8.02) and a probability of less than 5% was considered significant.

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