FEBS Letters xxx (2008) xxx–xxx

A putative physiological role of hypothalamic CNTF in the control of energy homeostasis

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Received 24 June 2008; revised 26 August 2008; accepted 10 October 2008

Available online

Edited by Laszlo Nagy

Abstract **Administration of CNTF durably reduces food intake and body weight in obese humans and rodent models. However, the involvement of endogenous CNTF in the central regulation of energy homeostasis needs to be elucidated. Here, we demonstrate that CNTF and its receptor are expressed in the arcuate nucleus, a key hypothalamic region controlling food intake, and that CNTF levels are inversely correlated to body weight in rats fed a high-sucrose diet. Thus endogenous CNTF may act, in some individuals, as a protective factor against weight gain during hypercaloric diet and could account for individual differences in the susceptibility to obesity.**

Keywords: Food intake; Arcuate nucleus; CNTF; Unbalanced diet; Obesity

1. Introduction

The hypothalamic arcuate nucleus (ARC) integrates changes in circulating levels of nutrients and hormones such as leptin and insulin to respond to the energy body requirements [1]. The alteration of hypothalamic hormonal and/or nutrients sensing contributes to the onset of obesity, which is usually associated to hypothalamic leptin resistance [2–5].

Ciliary neurotrophic factor (CNTF) has been first demonstrated to exert a trophic action on motor neurons of the ciliary ganglion [6]. This cytokine reduces food intake and body weight by activating leptin-like intracellular pathways in the ARC [7]. Interestingly, CNTF causes long-lasting weight loss in most obese patients and animal models bypassing leptin resistance [7–10]. Hence, CNTF has been considered as a promising therapeutic tool for the treatment of obesity and has prompted intense research aimed at identifying the cellular and molecular mechanisms underlying its potent anorexigenic properties. CNTF first binds to its specific receptor subunit (CNTFRα), a membrane glycosyl-phosphatidyl-inositol (GPI)-anchored. Binding of CNTF to CNTFRα induces heterodimerization of the β components of the receptor complex, leukaemia inhibitory factor receptor (LIFR) and gp130 [11,12], leading to the activation of MAP kinase (MAPK) and JAK-2/STAT-3 signaling pathways [13]. CNTF is also able to regulate hypothalamic and muscle AMP kinase (AMPK) [14,15].

The expression of CNTF and its receptor has been evidenced in the rodent brain. Nevertheless, to date, no exhaustive investigation has regarded the expression of CNTF and its receptor in the ARC. Besides, the role of CNTF as an endogenous modulator of energy homeostasis has not been yet determined. Indeed, correlation studies between CNTF gene disruption and body weight in mice or humans provided controversial data [16–20]. However, compensatory pathways cannot be excluded in such genetic approaches [21]. Thus, in this study, we have reconsidered the hypothesis of a physiological role of CNTF in the central control of energy homeostasis by using complementary morphological and biochemical approaches aimed at identifying the cellular sources and targets of CNTF and examining the impact of a high-sucrose or a high-fat diet on CNTF level in the rat ARC.

2. Materials and methods

2.1. Animal experimental procedure

Adult (9 week-old) Wistar rats (males and females) were fed for 6 weeks with either a chow, a high-sucrose (HS) or a high-fat diet adapted from previous studies [22]. Body weight was registered every week and food intake monitored for the two last weeks before the sacrifice. CNTF (0.3 mg/kg) or saline solution were injected (IP) 45 min before euthanasia. Recombinant CNTF, which crosses the blood–brain barrier [23], was produced as previously described [24]. Experiments were performed according to European legal requirements (Decree 86/609/EEC).

2.2. Plasma parameters determination

Plasma glucose, cholesterol, insulin, leptin and triglycerides were measured as previously described [25]. The homeostatic model assessment (HOMA) for insulin resistance was calculated using the HOMA version 2.2 calculator software (Diabetes Trials Unit, Oxford, UK).

2.3. CNTF in situ hybridization

A previously described CNTF oligonucleotide probe [26] (5′ CCA GAT AGA ACG GCT ACA GAG GTC CCG GCG GTG AAG GGT CAG AGG 3′) was labelled using terminal transferase and digoxygenin (DIG)-ddUTP according to the manufacturer's recommendations (Roche Applied Science, Meylan, France). Free-floating sections prepared from rat hypothalamus were incubated overnight at 42 °C

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0014-5793/$34.00
doi:10.1016/j.febslet.2008.10.018

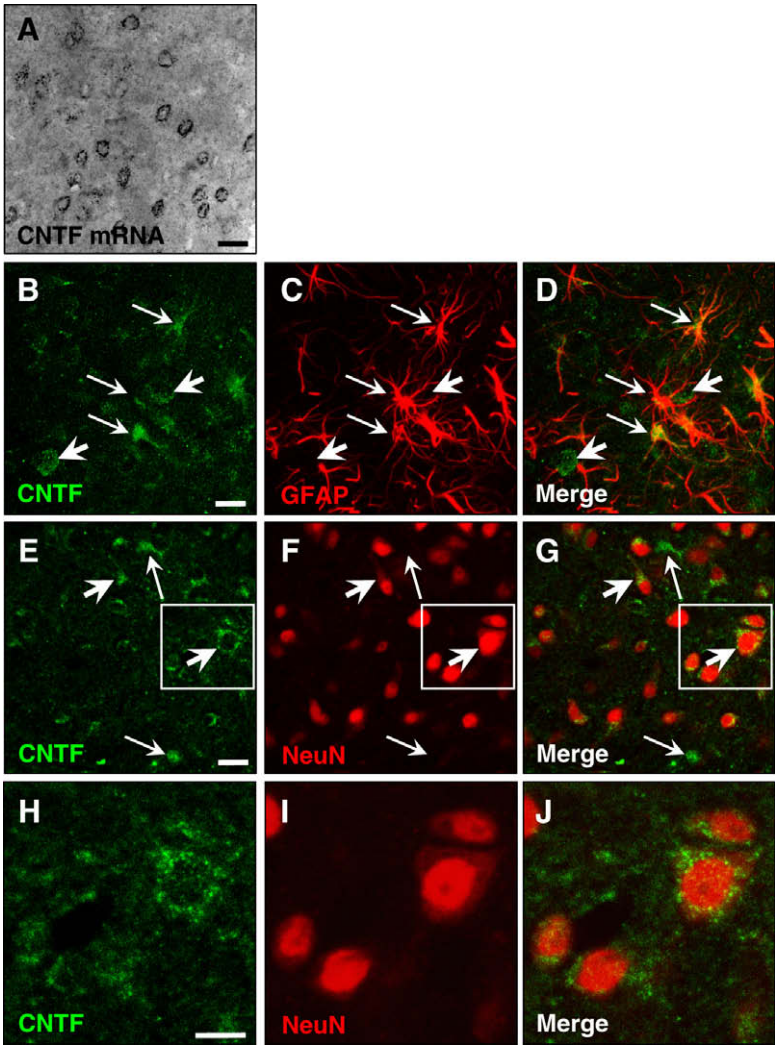

Fig. 1. Detection of CNTF mRNA by *in situ* hybridization and CNTF protein by multiple fluorescent immunohistochemistry. CNTF mRNA is present in rat ARC (A). CNTF-IF is found in astrocytes (arrows) where it overlaps with GFAP-IF (B–D) and in neurons (arrowhead) as shown on NeuN immunolaballed sections (E–J). Overlapping of 3 0.4 μm-thick focal planes. Scale bars = 20 μm for A–D; 10 μm for E–G.

with labelled CNTF probe (0.2 nM). Following several washes as previously described [27], labelled cells were detected by a DIG-DNA detection kit (Roche). Controls were assessed in the presence of the sense probe.

2.4. Fluorescent immunohistochemistry

The immunohistochemical analyses were performed as previously described with slight adaptations [28]. Hypothalamic sections (50 μm) were incubated with goat anti-CNTF (1:200, R&D Systems, Minneapolis, MN, USA), goat anti-CNTFRα (1:50; Santa Cruz Biotechnology, CA, USA) or rabbit anti-p42/44 MAPK (1:200; Cell Signaling Technology, Beverly, MA, USA). The chosen antibodies cross-react with the rat forms of CNTF and CNTFRα [29,30]. Multiple stainings were performed using a combination of monoclonal mouse anti-GFAP (Glial Fibrillary Acid Protein; 1:500; Sigma) or anti-NeuN (Neuronal nuclei; 1:100; Chemicon, Temecula, CA, USA) immunoglobulins. Primary antibodies were visualized with FluoProbes-488 (FP-488; Interchim, Montluçon, France) or cyanine-5 (Cy5; Jackson Immunoresearch Laboratories; Suffolk, UK) conjugated antibodies (1:400). Immunofluorescence (IF) was examined by confocal microscopy (Zeiss LSM 510 system, Germany). Optical sections were taken through the *Z* axis at 0.4 μm intervals and averaged three times. Quantification was performed with ImageJ 1.36 b software (NIH, USA). Briefly, CNTF levels and GFAP coverage were assessed by measuring the integrated fluorescence densities or the area fractions, respectively, within the reproductive contours of a whole ARC and after background subtraction.

2.5. Western blots

Whole hypothalamus samples were homogenized as previously described [31]. Proteins (50 μg) were subjected to SDS/PAGE and Western blot analysis using the anti-CNTF antibody. All Western blots were normalized to β-tubulin (1:1000; Cell Signaling Technology). Blot quantification was performed by using Bio1D software (Vilbert Lourmat, Marne-la-Vallée, France).

2.6. Quantitative real-time PCR

A Fast SYBR Green Master mix (Applied Biosystems, Courtaboeuf, France) was used to analyze CNTF mRNA expression level in the ARC. The accumulation of PCR products was measured directly by monitoring fluorescence intensity with a StepOne™ Real-Time PCR System (Applied Biosystems). Expression levels of mRNA were calculated after normalization with the housekeeping gene β-actin. Nucleotide sequences of the specific primers used were as follows: 5′-GGACCTCTG TAGCCGTTCTATCTG-3′ (sense) and 5′-GGTACACCATCCACT

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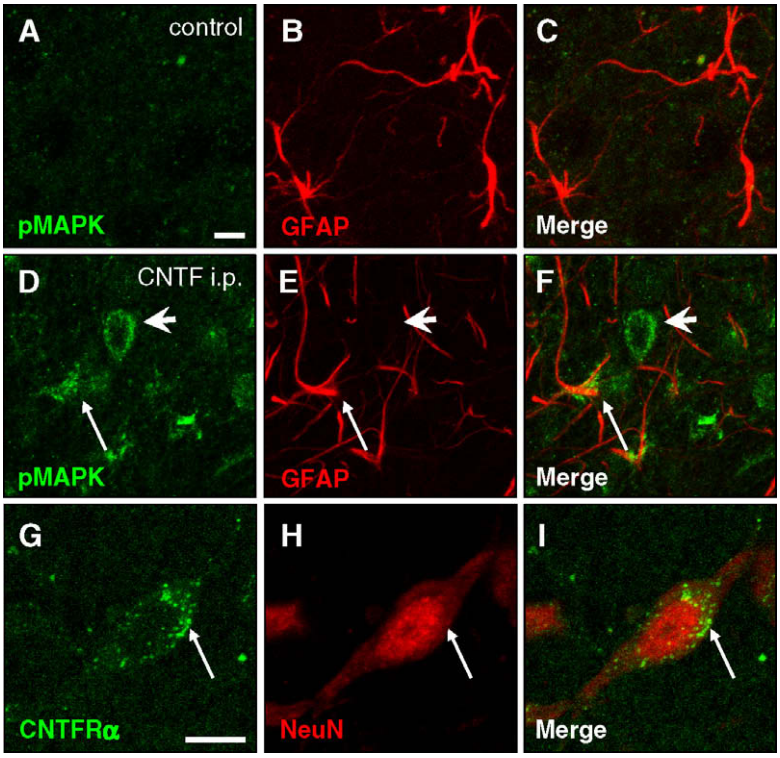

Fig. 2. Multiple fluorescent immunohistochemistry. Co-detection of pMAPK and GFAP in saline (A–C) and CNTF-treated (D–F) rats. A single i.p. injection of CNTF induces MAPK phosphorylation in GFAP-positive (arrow) and GFAP-negative (arrowhead) cells. Co-detection of CNTFRα and NeuN (G–I). CNTFRα-IF is evidenced in the cytoplasm of a NeuN-positive cell. 0.4 μm-thick focal planes. Scale bars = 10 μm for A–F; 15 μm for G–I.

GAGTCAAGG-3′ (antisense) for CNTF; 5′-CTATCGGCAAT GAGCGGTTCC-3′ (sense) and 5′-TGTGTTGGCATAGAGGTCTT-TACG-3′ (antisense) for β-actin.

2.7. Statistics

Data are represented as the mean percentage of control ± S.E.M. Unpaired Student's *t*-test was carried out with Statview (SAS Institute, NC, USA). The correlation coefficient was used to examine the relationship between hypothalamic CNTF levels and body weight in control, HS and HF fed animals. In all comparisons, results were considered significant if $P < 0.05$.

3. Results

3.1. Identification of a local source of CNTF in the rat ARC

3.1.1. CNTF gene expression. Digoxygenin-labelled antisense CNTF probes evidenced a perinuclear staining in some ARC cells (Fig. 1A). No staining was observed with the sense probe (not shown). Based upon double immunostaining experiments combining anti-CNTF and anti-GFAP or anti-NeuN antibodies, CNTF-IF appeared to emerge from approximately 90% of astrocytes (Fig. 1B–D) and 75% of neurons (Fig. 1E–J).

Table 1
Final body weights and physiological parameters measured in control, high-sucrose and high-fat diet fed animals.

	C	HS	HF
Final body weight, g	265.28 ± 2.7	281.9 ± 4.3**	276.7 ± 5.8
Energy intake, kcal/day	53.8 ± 1.5	58.2 ± 0.9*	57.2 ± 1.3
Relative adipose tissue, % BW	3.2 ± 0.2	4.5 ± 0.2***	5.4 ± 0.5***
Relative liver weight, % BW	2.6 ± 0.06	2.53 ± 0.05	2.52 ± 0.06
Plasma			
Glucose, g/l	1 ± 0.02	1 ± 0.03	1.09 ± 0.05
Triglycerides, g/l	0.71 ± 0.16	1.1 ± 0.25	0.97 ± 0.23
Cholesterol, g/l	1 ± 0.07	0.95 ± 0.11	0.83 ± 0.03
Insulin, ng/ml	0.74 ± 0.07	1.11 ± 0.08*	1.47 ± 0.94**
Leptin, ng/ml	1.73 ± 0.15	3.03 ± 0.19**	3.53 ± 0.44*
HOMA index	0.79 ± 0.1	1.19 ± 0.1*	1.78 ± 0.2***,£

*$P < 0.05$ versus control.
**$P < 0.005$ versus control.
***$P < 0.001$ versus control.
£$P < 0.05$ versus HS.

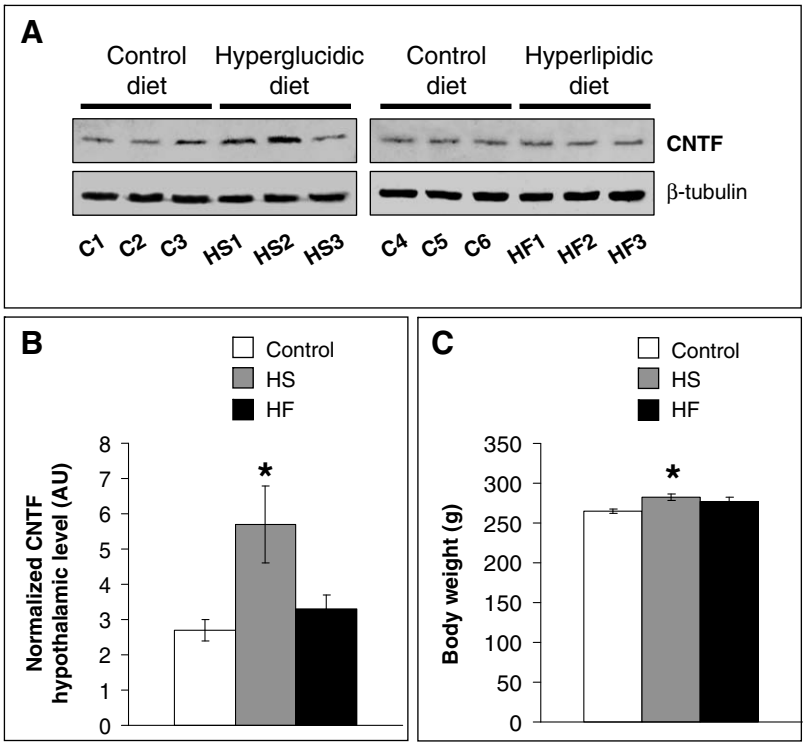

Fig. 3. Western blot analysis aimed at evaluating the impact of a high-sucrose (HS) or a high-fat (HF) diet on CNTF levels in the rat hypothalamus. Compared to control and HF groups, the HS diet induces a significant 2-fold increase in the mean CNTF hypothalamic levels (AB) and a significant 6.4% increase in body weight (C). Unpaired Student's *t*-test $^{*}P < 0.005$.

It is to note that other hypothalamic nuclei, such as the paraventricular nucleus and the lateral hypothalamic area, expressed CNTF. However, the ARC exhibited the highest amount of CNTF (not shown).

3.2. CNTF induces MAPK phosphorylation in the rat ARC

To identify the cellular types exhibiting functional CNTF receptors in the rat ARC, the effect of CNTF on MAPK phosphorylation was examined. While pMAPK levels were low in control animals (Fig. 2A), treated animals exhibited a dramatic increase in MAPK phosphorylation both in astrocytes and neurons, as revealed by detecting GFAP [Fig. 2D–F; arrow pointing at an astrocyte (GFAP-positive cell); arrowhead pointing at a neuron (GFAP-negative cell)]. The neuronal expression of CNTFRα was corroborated since CNTFRα-IF appeared in the shape of large puncta, presumably corresponding to receptor clusters, in NeuN-positive cells (Fig. 2G–I).

3.3. Impact of a different hypercaloric diets on hypothalamic CNTF level

To test the hypothesis of a relationship between the hypothalamic expression of CNTF and the control of energy homeostasis, two unbalanced diets were compared, high-sucrose or high-fat, on CNTF levels in the hypothalamus and the ARC. The endocrine and metabolic parameters are summarized in Table 1. HS but not HF diet induced a significant increase in body weight associated with increased energy intake. Both hypercaloric diets induced a significant increase in relative subcutaneous adipose tissue, insulinemia, leptinemia and HOMA index (Table 1). Plasma glucose, triglycerides and cholesterol levels were not affected. The HS diet induced a significant (2-fold) increase in CNTF hypothalamic levels compared to control and HF groups ($P < 0.005$; Fig. 3A and B). While no association was evidenced between CNTF hypothalamic levels and body weight in control and HF animals ($r = 0.2774$ in control group; $r = 0.1732$ in HF group; $P > 0.1$; Fig. 4A and C), a significant inverse correlation appeared in HS animals ($r = 0.8592$; $P < 0.001$; Fig. 4B). Indeed, in these conditions, animals with lower body weight exhibited higher amounts of CNTF in the hypothalamus. This result was corroborated in the ARC by evaluating both CNTF-mRNA contents (0.99 in overweight-resistant versus 0.40 in the heaviest rats; qRT-PCR values normalized to β-actin–mRNA) and CNTF-IF (Fig. 5A, D, G). It is noteworthy that this increase in CNTF expression was specific to the ARC. In overweight-resistant animals fed a HS diet, the mean CNTF-IF increase in the ARC could reach +160% ± 3 ($P < 0.001$; Fig. 5J) and was associated to a significant decrease (−65% ± 10; $P < 0.01$) in the astrocytic coverage (Fig. 5B, E, H, K). Besides, a positive correlation was found between body weight and body weight gain in HS diet fed rats ($r = 0.9801$; $P < 0.001$).

4. Discussion

Several studies attempted to correlate CNTF genotype and body mass. Except in a report showing that the homozygous null mutation of the CNTF gene is associated to increased body mass in humans [19], no link was found between CNTF gene disruption and body weight [16,20]. However, compensa-

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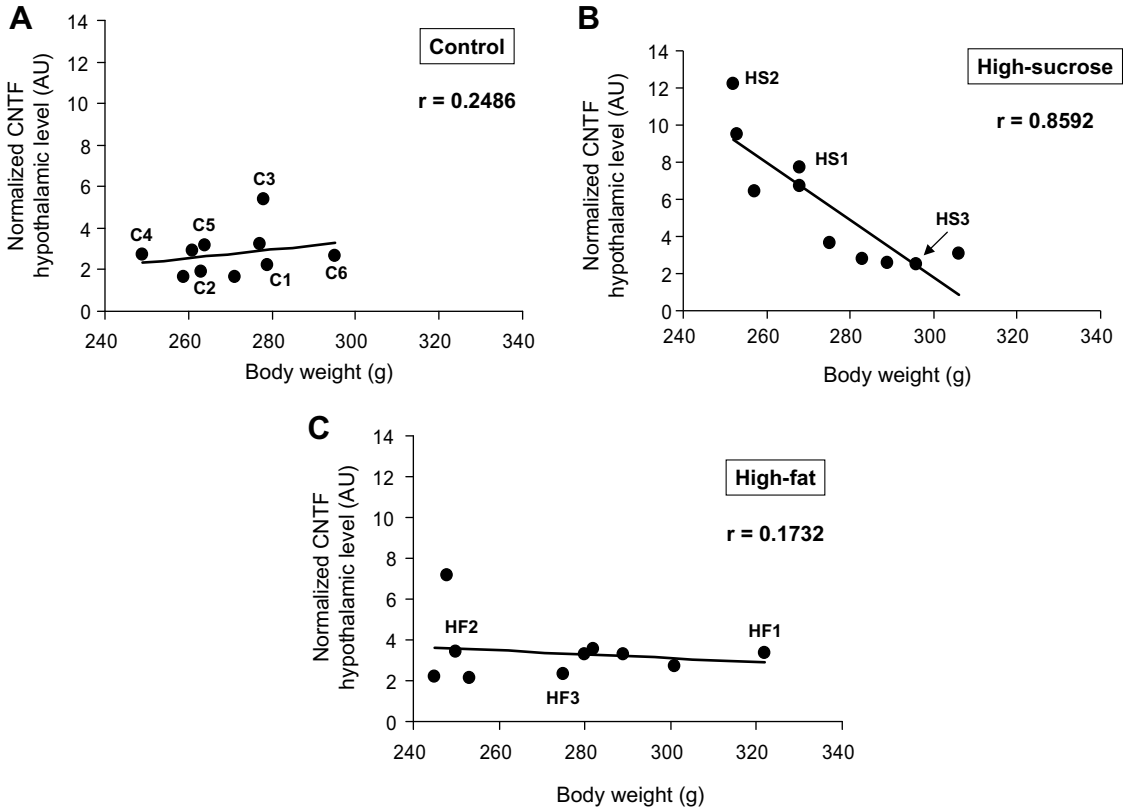

Fig. 4. CNTF hypothalamic levels determined by Western blot negatively correlates with body weight in HS diet fed (B) but neither in control (A) nor in HF diet fed rats.

tory mechanisms cannot be excluded. Indeed the permanent disruption of the orexigenic NPY gene does not affect body weight or feeding behaviour [32,33] although ablation of NPY-expressing neurons in adults causes rapid starvation [21].

To reconsider the possibility for CNTF to represent an endogenous anorexigenic factor, we have developed an alternative strategy aimed at evaluating the impact of two unbalanced hypercaloric diets (high-sucrose or high-fat) on the local production of CNTF in the rat ARC.

Here we first have demonstrated that neurons and astrocytes express both CNTF and functional CNTFRα, as evidenced by MAPK phosphorylation in response to exogenous CNTF. Furthermore, the hypothalamic CNTF expression was increased and inversely correlated with body weight in HS rats. This suggests that endogenous CNTF could protect a fraction of individuals against diet-induced weight gain and account, at least partially, for the individual variations toward susceptibility to develop high-carbohydrate-induced obesity. In the light of previous reports (i.e. [7,8]), we can assume that the curbing effect of CNTF on weight gain could be linked to its anorexigenic properties. The anorexigenic action of CNTF can be attributed to its expression not only in the ARC but also in the paraventricular nucleus and the lateral hypothalamic area, as observed in our laboratory. Nevertheless, the highest hypothalamic amounts of CNTF were found in the ARC and the increased expression of CNTF in obesity resistant rats was specific to the ARC, suggesting a crucial role of this nucleus in the endogenous effect of CNTF on the regulation of energy homeostasis.

Direct evidence for CNTF release has not been yet demonstrated in vivo. Nevertheless, evidence was presented for release of CNTF from cultured astrocytes [34]. Besides, a direct intracellular action may also constitute a plausible mechanism of CNTF action, as demonstrated in vitro [35]. This possibility deserves further investigation.

Interestingly, the increase in CNTF levels observed in a proportion of HS diet fed rats paralleled a retraction of astrocytic processes. Astrocytes are known to play a pivotal role in the regulation of food intake by monitoring circulating hormones/nutrients and communicating with neurons [36–38]. Moreover, they modulate synaptic number and activity in response to peripheral signals [39–42]. However, whether these modifications are beneficial or harmful in situations of unbalanced energy status needs further investigation. An intrinsic inhibition of CNTF on glial coverage is unlikely because previous studies have demonstrated that CNTF does not reduce but instead stimulate GFAP production and astrocytic network extent in the brain [43,44].

It is noteworthy that HS and HF diets similarly affect insulin, leptin and glucose circulating levels but only HS modifies CNTF expression and astrocytic coverage in the ARC. This clearly indicates that diet composition plays a key role in neuronal organisation of hypothalamic nuclei and may account for the individual variations of predisposition to develop obesity.

In conclusion, our data show that CNTF may be considered as an endogenous modulator of energy homeostasis in the

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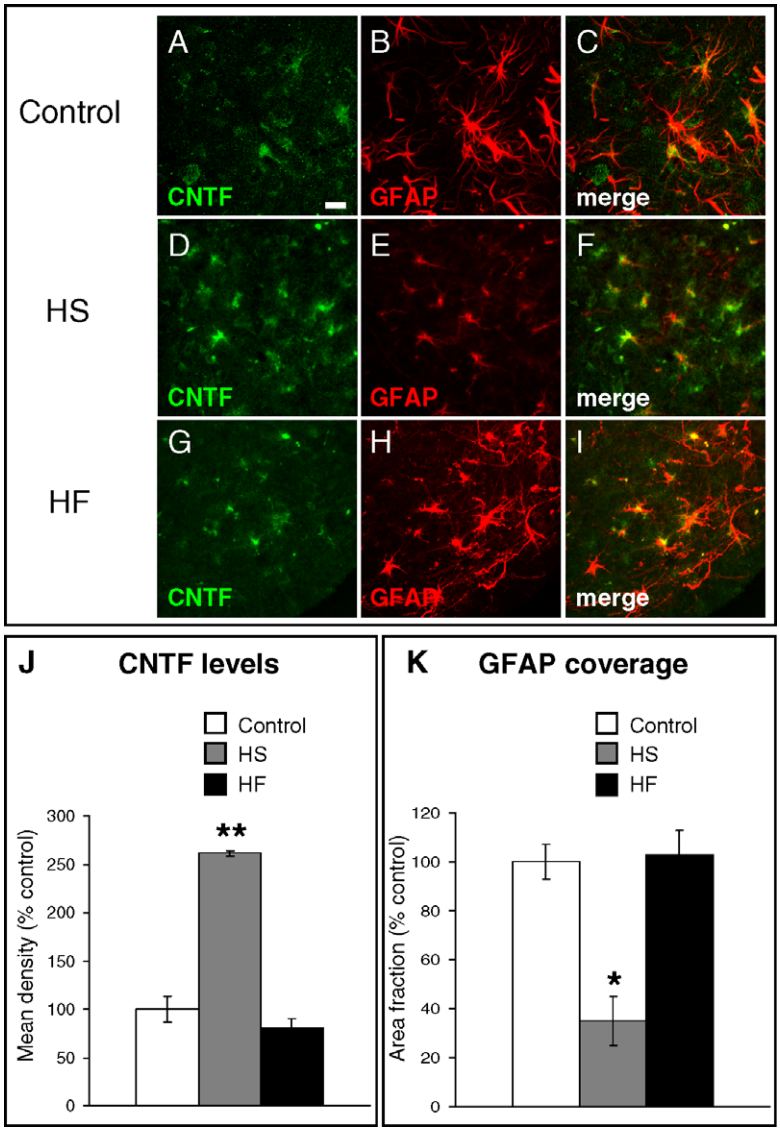

Fig. 5. Co-detection of CNTF and GFAP by immunofluorescence reveals that CNTF level increases while GFAP coverage decreases in a fraction of HS diet fed rats compared to control and HF groups (A–I). These alterations are estimated at +160% for CNTF (J) and −65% for GFAP (K). Scale bar = 10 μm.

ARC that possibly protects some individuals against the onset of diet-induced obesity.

Acknowledgements: We thank Dr. Monique Lavialle for her helpful comments. This work was supported by the "Agence Nationale pour la Recherche" (ANR-06-JCJC-0107 to CMV).

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