The anorexigenic cytokine ciliary neurotrophic factor stimulates POMC gene expression via receptors localized in the nucleus of arcuate neurons

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Ciliary neurotrophic factor (CNTF) is a 22-kDa cytokine expressed mainly in the nervous system by neuronal and glial cells. Originally, it was shown to promote the survival of ciliary ganglion neurons (6, 26). Today, we know that its spectrum of activities is much broader since it includes the differentiation and/or survival of a variety of nervous cells such as motor neurons, oligodendrocytes, and astrocytes (29, 38, 50). In an initial clinical trial designed to test the efficacy of a CNTF analog to prevent motor neuron degeneration, some patients suffered substantial weight loss (39). Ever since then the mechanisms by which CNTF induces weight loss have been deciphered using animal models, and it has been shown that CNTF mimics the ability of leptin, an adipocyte-secreted cytokine, to decrease food intake and produce fat loss. Moreover, and interestingly, CNTF is able to bypass leptin resistance in a diet-induced obesity model, a more representative model of human obesity (19).

Similar to leptin, CNTF acts through neurons located in hypothalamic centers that control energy homeostasis, including the arcuate nucleus (ARC) (36). The ARC contains two main neuronal populations that exert opposite effects: neuropeptide Y (NPY)-producing neurons and proopiomelanocortin (POMC)-synthesizing neurons, which stimulate and inhibit appetite, respectively. The anorexigenic action of exogenous CNTF has been attributed to a decrease in NPY gene expression (63) and an increase in POMC transcription (1) in rats.

Functional CNTF receptor encompasses three subunits. CNTF first binds to its specific CNTF receptor (CNTFRa), which induces heterodimerization of the “β”-components of the receptor complex, glycoprotein 130 kDa (gp130) and leukemia inhibitory factor receptor (LIFR)-β (14, 15, 30). In rodents, CNTF shares signaling cascades with leptin in the ARC. Indeed, the β-component dimerization initiates the association with Janus-activated kinase-2 (JAK2), and this subsequently phosphorylates specific tyrosine residues on the intracellular domains, creating docking sites for Src homology-containing signaling components. Briefly, this leads to the inhibition of AMP-activated protein kinase (55) and the activation of the phosphoinositide 3-kinase/Akt, mitogen-activated protein kinase/extracellular signal-regulated kinase, and signal transducer and activator of transcription 3 (STAT3) pathways (14, 15, 44, 53). Phosphorylated STAT3 forms a dimer that translocates to the nucleus, where it activates the transcription of target genes such as that encoding POMC (54). The activation of this signaling pathway by CNTF is negatively modulated by members of the suppressor of cytokine signaling family of proteins (11). Interestingly, we have shown that leptin but not CNTF is able to induce protein-tyrosine phosphatase-1B expression. In addition, CNTF signaling was not affected by protein-tyrosine phosphatase-1B overexpression, which may at least partially explain its efficacy in the leptin-resistant state (9).

Recently, the contribution of endogenous CNTF to the physiological control of energy homeostasis has been proposed with the finding of a body weight-dependent production of CNTF in the rat ARC (57). Nevertheless, the comprehension of the physiological significance of neural CNTF action was still incomplete because CNTF lacks a signal peptide (49) and thus may not be secreted by the classical exocytosis pathways. Several studies have indicated that CNTF may be released in

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pathological states such as brain lesions or inflammation (23, 51, 62). However, the synthesis of CNTF is not limited to these conditions. Thus, we propose here that besides its extracellular action in pathological situations, endogenous CNTF may also act intracellularly to take part in the control of energy homeostasis.

We have shown previously that CNTF distribution shares similarities with that of its receptor subunits in the rat ARC. Indeed, a majority of neurons and astrocytes express both CNTF and CNTF-Rα, and both β-components of the receptor are ubiquitous in the rat ARC (57), suggesting the possibility of an intracellular effect of the cytokine, as previously envisaged in cell culture (40). The aim of the current work was to validate this hypothesis in vivo and to evaluate the impact of such a mechanism on the hypothalamic control of energy homeostasis. Here, we demonstrate that CNTF and its receptors interact in the nuclei of ARC cells, including anorexigenic POMC neurons. Moreover, CNTF stimulation of hypothalamic isolated nuclei increased Akt phosphorylation and POMC gene transcription. Taken together, these data indicate that intracellular CNTF may exert an endogenous anorexigenic action via the activation of receptors localized in the cell nucleus that leads to the stimulation of POMC gene expression. These data provide a novel plausible mechanism of CNTF action in the hypothalamic regulation of energy homeostasis.

MATERIALS AND METHODS

Animals and housing. All experiments were performed in agreement with European legal requirements (Decree 86/609/EEC) and approved by the local committee on Animal Welfare. Thirty 3-mo-old Wistar male rats (Rattus norvegicus) were used. They were housed under 12:12 h-light-dark cycles (lights on 0700, lights off 1900), with access to food and water ad libitum. They were euthanized at the same time of the day (between 1400 and 1600).

SH-SY5Y cells. The human neuroblastoma cell line (SH-SY5Y) was obtained from Dr. B. Dufy (UMR 5543; Centre National de la Recherche Scientifique, Bordeaux-II University) and cultured according to the method of Benomar et al. (10).

Immunofluorescence. The immunohistochemical analyses were performed as described previously (57) in 10 rats. Hypothalamic floating sections (50 μm thick) were incubated with a combination of goat anti-CNTF (1:200; R & D Systems, Minneapolis, MN), goat anti-CNTF-Rα (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-LIFR (COOH terminus) (1:1,000; Santa Cruz Biotechnology), mouse anti-glial fibrillary protein (GFAP, 1:500; Sigma, St-Quentin-Fallavier, France), rabbit anti-gp130 (NH2 terminus) (1:200; Santa Cruz Biotechnology), rabbit anti-JAK2 (1:100; Santa Cruz Biotechnology), rabbit anti-Akt (1:100; Cell Signaling Technology), mouse anti-neuronal nuclei (NeuN; 1:1,000; Millipore, Molsheim, France), and rabbit anti-POMC (1:5,000; Phoenix Pharmaceuticals, Burlingame, CA) antibodies. Primary antibodies were visualized by using FluoroProbes-488 (FP-488; Interchim, Montluçon, France) or cyanine-5 (Cy5; Jackson Immunoresearch Laboratories, West Grove, PA)-conjugated donkey anti-goat, rabbit, mouse, or sheep antibodies (1:400). The choice of antibodies was based on their cross-reactivity for the rat forms of our molecules of interest and on their previous characterization on nervous system sections (8, 17, 24, 35, 61).

Moreover, their specificity was tested on Western blots and with several controls: incubation with blocking peptides (1 h at room temperature prior to addition to sections; Santa Cruz Biotechnology) and omission of the antibodies. In all cases, immunofluorescence was reduced to background levels. Moreover, the specificity of CNTF antibody was tested on brain sections coming from CNTF-knockout mice (generously given by Prof. R. Sendtner (37). Some sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI) or ethidium homodimer-2 (EtH-2; 2 μg/ml, 1 h; Invitrogen-Molecular Probes), a membrane-permeable fluorescent dye that binds to nucleic acids, allowing the visualization of the different cellular compartments.

Microscopy and image acquisition. Immunofluorescence was examined by confocal microscopy (Zeiss MRC 1024ES; Zeiss Microscopy, Jena, Germany). Series of optical sections were collected through the z-axis at 0.4- or 0.2-μm intervals and averaged three times. Four lasers at 450, 488, 543, and 633 nm were used for the excitation of DAPI, FP-488, EtH-2, and Cy5, respectively. Images were taken through a ×40 or a ×63 oil immersion objective. The images used for three-dimensional cell reconstructions were scanned with a zoom of 3.5.

Image processing and analysis. CNTF and its receptor subunit immunofluorescence exhibited a punctiform-like pattern. To investigate the distribution of these punctae through the ARC cells, 0.2-μm-thick focal planes of EtH-2-stained cells were acquired and processed for 3D reconstructions. Confocal image stacks were automatically converted to a format enabling their processing by programs developed using the free-FD software libraries (2). The segmentation was then performed as follows. The cell and nucleus contours were delineated manually on each EtH-2 colored focal plane, and a surface model of the plasmic membrane (Fig. 3, gray) and the nuclear envelope (Fig. 3, red) was generated. A third model was created to point immunofluorescent spots (Fig. 3, green). The distribution of green spots was quantified in the cytoplasm and the nucleus on three different cells from three different sections and three different rats using Imaged 1.36b software (National Institutes of Health).

Immunogold electron microscopy. Rats were perfused with a fixative solution (2% paraformaldehyde, 0.5% glutaraldehyde; Electron Microscopy Sciences, Washington, PA). Briefly, bilateral ARC were microdissected from hypothalamic slices, dehydrated, and embedded in LR White resin (Sigma). Ultrathin sections (70 nm) were cut and transferred to nickel grids with a formvar/carbon film for postembedding immunogold labeling. These sections were saturated and incubated with a combination of primary antibodies raised against CNTF (1:50), CNTF-Rα (1:100), LIFR (1:200), or gp130 (1:50) overnight at 4°C. The sections were then incubated with donkey anti-goat- and/or anti-rabbit immunoglobulin coupled to 10- or 6-nm colloidal gold particles, respectively (1:30; Aurion Costerwegs, Wageningen, The Netherlands). Sections were finally contrast-stained in a 5% uranyl acetate solution and observed with a transmission electron microscope at 80 kV (EM 2081; Philips, Eindhoven, The Netherlands), and pictures were taken using a charge-coupled device wide-angle camera (AMT). Control experiments were performed by omitting primary antibodies. For each group of particles, deposits were performed to evaluate particle size segregation. Diameter means ± SE were calculated, and particle diameters were plotted in a frequency distribution. Immunogold particles localized in the cytoplasmic, nuclear, and extracellular compartments were counted manually.

Preparation of nuclear and cytosolic extracts. Hypothalamic samples from 10 rats were homogenized, and then nuclear, cytoplasmic, and membrane fractions were prepared as described previously (21).

Briefly, whole hypothalami were homogenized individually in a nuclear homogenization buffer containing 20 mM Tris, pH 7.5, 10 mM NaCl, and 1 mM MgCl2. Nonidet P-40 at the final concentration of 0.15%, 10 mM NaF, 20 mM β-glycerophosphate, 1 mM PMSF, 1 mM Na3VO4, 2 μg/ml leupeptin, and 2 μg/ml aprotinin was then added. Homogenates were centrifuged for 5 min at 1,500 rpm. Supernatants were kept as cytosolic extracts, and nuclear pellets were resuspended in 0.5 volume of nuclear homogenization buffer and centrifuged at 1,500 rpm for 5 min twice again. Integrity of the nuclei was examined on a small part of the pellets using a toluidin blue staining.

Stimulation of isolated nuclei with CNTF or leptin. The nuclei were resuspended in a stimulation buffer containing 50 mM Tris·HCl, pH
5.5, 1 mM MgCl2, 2 mM EGTA, 10 mM NaF, 20 mM β-glycerol phosphate, 1 mM PMSF, 1 mM Na3VO4, 2 μg/ml leupeptin, and 2 μg/ml aprotinin before being incubated for 10 min at 37°C with CNTF (1 nM) or leptin (10 nM) in the presence or not of ATP (5 μM). Proteins (50–80 μg) were resolved by SDS-PAGE and electrotransferred to nitrocellulose. Membranes were then incubated with the appropriate primary and secondary antibodies, and targeted proteins were visualized by enhanced chemiluminescence reagents (ECL detection kit).

**Immunoprecipitation.** Proteins (500 μg) were incubated overnight at 4°C with 10 μl of CNTFRα, LIFR, or gp130 antibodies and lysis buffer containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, and 0.5% Nonidet P-40 for the binding step. Then protein A (40 μl) was added to the preparation and incubated at 4°C for 2 h to capture the immune complex. After a spin at 13,000 rpm for 3 min, the pellets were resuspended in 500 μl of lysis buffer and centrifuged for 3 min at 13,000 rpm again. We performed this step three times before subjecting the proteins to SDS-PAGE and Western blot experiments using CNTFRα and gp130 antibodies. Positive control experiments were performed by immunoprecipitating our samples and blotting the membranes with the same antibodies. In parallel, negative control experiments were carried out by immunoprecipitating our samples with irrelevant antibodies raised against Lammmer protein kinase and protein-tyrosine phosphatase 1B.

**Western blots.** Proteins (50 μg) were subjected to SDS-PAGE and Western blot analysis using the same antibodies as mentioned above in addition to mouse anti-phospho-tyrosine 100 (Millipore, Temecula, CA), rabbit anti-p44/p42 ERK (Thr202/Tyr204), rabbit anti-44/42 ERK, rabbit anti-p-JAK2 (Tyr1007/1008), rabbit anti-JAK2, rabbit anti-p-Akt (Ser473), rabbit anti-Akt, rabbit anti-p-STAT3 (Tyr705), and rabbit anti-STAT3 (Cell Signaling Technology). All Western blots were normalized to the corresponding total proteins. Blot quantification was performed by using BioID software (Vilber Lourmat, Marne-la-Vallée, France).

**Nuclear run-on reaction.** Nuclei isolation from the hypothalamus of five rats was adapted from the previously published method (46). Briefly, hypothalami were isolated from five anesthetized Wistar rats. Hypothalamic samples (~70 mg) were rapidly homogenized in ice-cold buffer A [15 mM HEPES, pH 7.5, 60 mM KCl, 3 mg/ml BSA, 300 mM sucrose, 5 mM each of EDTA and EGTA, 1 mM dithiothreitol (DTT), 0.5 mM spermidine, 0.5 mM PMSF, and 2 μg/ml each of leupeptin and aprotinin]. Following centrifugation at 700 g at 4°C for 10 min, the resulting pellet containing crude nuclei was suspended in ice-cold buffer B (similar to buffer A, except for 0.1 mM each of EDTA and EGTA and the addition of 5 mM MgOAc) and centrifuged in the same condition as described above. The final nuclei pellets were suspended in storage buffer (40% glycerol, 75 mM HEPES, pH 7.5, 60 mM KCl, 15 mM NaCl, 5 mM MgAc, 0.1 mM each of EDTA and EGTA, 1 mM DTT, 0.5 mM spermidine, and 2 μg/ml each of leupeptin and aprotinin), frozen immediately in liquid nitrogen, and stored at −80°C. To determine transcription of POMC gene in response to CNTF, a qRT-PCR based on the nuclear run-on technique was used as described previously (28). Briefly, frozen nuclei were thawed on ice, and equal volumes of nuclei and reaction buffer (20% glycerol, 100 mM KCl, 10 mM MgCl2, 4.5 mM DTT, 1.2 mM ATP, 0.6 mM each of CTP, TTP, and UTP, 80 U/ml RNase inhibitor, and finally, 1 nM of CNTF) were mixed and incubated at 22°C for 45 min. RNA was then extracted using Trizol (Invitrogen) according to the manufacturer’s recommendations.

**Quantitative RT-PCR.** Nascent RNA from the nuclear run-on reactions was reverse transcribed using the Super-Script II RNase H-system (Invitrogen), as described previously (9). The resulting cDNAs were submitted to quantitative PCR. The PCR primer sequences used were as follows: POMC forward 5'-AGGTTAAAGGACGATGAC-3', reverse 5'-CGTCTATGAGGAGTCTGAC-3'; NPY forward 5'-ATGCTAGTAACAAAAAG-3', reverse 5'-ATGATGTGTCGAGAAGG-3'; and 18S forward 5'-TCCCGGAGAAGTTGACACAT-3', reverse 5'-CTTC-CCATCCTACGGTCCTTC-3'. Real-time PCR was carried out using the Step One apparatus (Applied Biosystems) and the Fast SYBR Green Master Mix (Applied Biosystems). A ratio of specific Nascent POMC-RNA/18S amplification was calculated to correct for any difference in efficiency at RT.

**Recombinant CNTF and leptin.** Human CNTF and ovine leptin were produced by Protein Laboratories Behovot as described previously and validated (16, 18, 57).

**Statistical methods.** A two-tailed Student t-test was used to compare the frequency distribution patterns, and a Friedman test was carried out to detect significant differences between different treated samples. These tests have been performed with StatView (StatView 4.0; Abacus Concepts, Berkeley, CA) and with AnaStats.fr (Rilly-sur-Vienne, France). Data are presented as means ± SE. Results were considered significant if P < 0.05.

**RESULTS**

**CNTF and its receptor subunits are expressed in anorexigenic neurons of the ARC.** We have demonstrated previously that CNTF was expressed in 90% of astrocytes and 75% of neurons in the rat ARC (57). Because CNTF administration decreases food intake, we addressed the possibility for the cytokine to be produced in anorexigenic neurons. Thus we have detected POMC by immunohistochemistry in rats. Both approaches evidenced the presence of a punctiform CNTF-immunofluorescent staining in 100% of anorexigenic POMC neurons of the ARC in rat (Fig. 1, A–H). The specificity of CNTF staining was validated on hypothalamic sections obtained from CNTF-knockout mice (Fig. 1, I and J). Moreover, all POMC neurons of the rat ARC exhibited an immunoreactivity for CNTF receptor subunits, namely CNTFRα (Fig. 2, A–C), LIFR (Fig. 2, D–F), and gp130 (Fig. 2, G–I). It is of note that the immunofluorescence for CNTF and its receptors was particularly intense in the cell nucleus (Fig. 2, arrows), as evidenced with the DAPI counterstaining on 0.4-μm-thick focal planes (Fig. 1, A–D) or on 1.2-μm-thick stacks of high-magnification scans (Fig. 1, E–H) in POMC neurons as well as in POMC-immunonegative cells. This nuclear staining appeared in clustered dots, which colocalized with the heterochromatin protein 1 (not shown), a positive regulator of active transcription in euchromatin (33), suggesting a potential role of nuclear CNTF in the regulation of gene transcription. Finally, a specific cytoplasmic and extracellular staining was also evidenced for CNTF, CNTFRα, LIFR, and gp130, which are known to exist as soluble forms (14, 41, 43, 52).

**CNTF and its receptor components are localized in the nucleus of rat ARC cells.** To confirm and quantify the presence of CNTF and its receptors in the ARC cell nuclei, we have counterstained hypothalamic sections with EtH-2, a fluorescent molecule that stains nucleic acids with a high affinity and allows the delimitation of the different cell compartments. We then estimated the relative nuclear and cytoplasmic contents of CNTF and its receptors from three-dimensional reconstructions of high-magnification confocal stacks. We found that CNTF and its different receptor partners were located mainly in the nuclear compartment. Indeed, 65 ± 21% of CNTF (Fig. 3, A and G), 77 ± 11% of CNTFRα (Fig. 3, B and G), 87 ± 5% of LIFR (Fig. 3, C and G), and 67 ± 10% of gp130 (Fig. 3, D and G) staining were located in the nucleus of rat ARC cells. Besides, a staining of CNTF receptor subunits was observed close to the cell membrane (Fig. 3, B–D). It is also noteworthy that the predominant
nuclear distribution of CNTF and its receptor subunits was observed throughout the hypothalamus. In addition, JAK2, a signaling molecule associated with CNTF receptor subunits, was also present in the cell nucleus (Fig. 3, E and G). Finally, Akt, a downstream signaling element, exhibited a nuclear localization that reached 63 ± 22% in the ARC cells (Fig. 3, F and G).

CNTF and its receptor subunits may interact in the cell nucleus of rat ARC cells. Previous in vitro studies have demonstrated that the activation of CNTF receptor required the heteromerization of its subunits. To check the possibility that these molecules form a CNTF tripartite complex in the ARC cells, we performed multiple immunogold stainings followed by a transmission electron microscopy analysis. Four pairs of proteins were tested, according to the host species of precharacterized antibodies (see MATERIALS AND METHODS): CNTF/ LIFR, CNTF/gp130, CNTFRα/LIFR, and CNTFRα/gp130. For each group of particles, deposits were performed to eval-

Fig. 1. Ciliary neurotrophic factor (CNTF) is present in proopiomelanocortin (POMC) neurons of the rat arcuate nucleus. A–H: POMC and CNTF were detected by multiple immunofluorescence followed by a confocal laser scanning. Sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI). I and J: detection of CNTF immunofluorescence in wild-type (WT) and CNTF-knockout mice (CNTF−/−). A–D: 0.4-μm-thick focal planes. E–H: 1.2-μm-thick stacks of high-magnification scan (×63). I and J: epifluorescence observation. Scale bars = 50 (A–D), 10 (E–H), and 50 μm (I and J).

Fig. 2. CNTF receptor subunits are present in POMC neurons of the rat arcuate nucleus. POMC and CNTF receptor-α (CNTFRα; A–C), leukemia inhibitory factor receptor (LIFR; D–F), and glycoprotein 130 kDa (gp130; G–I) were detected by multiple immunofluorescence followed by a confocal laser scanning. Sections were counterstained with DAPI; 0.4-μm-thick focal planes. Scale bar = 15 μm.
uate particle size segregation. Diameter means ± SE were calculated, and particle diameters were plotted in a frequency distribution (Fig. 4A). The difference was highly significant [5.579 ± 0.17 (n = 72) vs. 9.522 ± 0.1 (n = 84), P < 0.0001; 2-tailed Student’s t-test]. Despite the use of a postembedding method chosen to facilitate the accessibility of the antibodies to the deep cellular compartments such as the cell nucleus, the tissue integrity was preserved, and the main organelles, including rough endoplasmic reticulum, mitochondria, nuclear envelope, heterochromatin, and euchromatin, were easily identified. The same four pairs of proteins, as mentioned above, were tested. Omission of the primary antibody validated the specificity of the immunolabeling. CNTF and its receptor subunits were observed in both the cytoplasm and the nucleus of the ARC cells. The nuclear localization represented 57 ± 11, 65 ± 16, 72 ± 12, and 52 ± 7% of the total CNTF, CNTFRα, LIFR, and gp130 immunostaining, respectively (Fig. 4B). The concomitant detection of CNTF and its receptor subunits confirmed the proximity of CNTF and LIFR (Fig. 4C), CNTF and gp130 (Fig. 4D), CNTFRα and LIFR (Fig. 4E), and CNTFRα and gp130 (Fig. 4F) in the cell nucleus. The cytoplasmic compartment also contained CNTF and its receptor subunits. This staining was particularly intense near the rough endoplasmic reticulum. Moreover, single or double staining of CNTF and its receptor subunits was also evidenced in nuclear pores (not shown). Finally, it is of note that an extracellular staining was observed for CNTF, CNTFRα, and gp130. This soluble fraction was estimated at 17, 12, and 5% for CNTF, CNTFRα, and gp130, respectively (Fig. 4B).

We next performed a Western blot analysis from fractionated hypothalamic samples. These biochemical data corroborated the common nuclear location of CNTF and its receptor subunits in ARC cells. These experiments showed a mixed distribution of CNTF, CNTFRα, and gp130 between the cytoplasmic (Fig. 5A, lane C) and the nuclear (Fig. 5A, lane N) compartments and revealed that LIFR and gp130 were also
The quantitative variability of cellular distributions between the three-dimensional reconstructions and the Western blot may be due to the fact that the analyses were performed on ARC cells and on whole hypothalamic extracts (which contain heterogeneous cell populations), respectively. The purity of the different fractions was verified by detecting different cytoplasmic or nuclear proteins, such as $\beta$-tubulin and GFAP (cytoplasmic) and NeuN (nuclear). Moreover, the examination of cresyl violet stained fractions under a light microscope confirmed the purity and the integrity of the isolated nuclei. The possibility for CNTF receptor subunits to interact in the cell nucleus was assessed by coimmunoprecipitations from hypothalamic nuclear fractions. As shown in Fig. 5B, gp130 was found to coimmunoprecipitate with CNTFR$\alpha$ and LIFR, and CNTFR$\alpha$ coimmunoprecipitated with LIFR and gp130. The immunoprecipitation with irrelevant antibodies raised against Lammer protein kinase and protein-tyrosine phosphatase 1B confirmed that the immunoprecipitated complexes were specific (not shown).

**CNTF stimulates POMC transcription in isolated hypothalamic nuclei.** Because nuclear CNTF was found in ARC POMC neurons, we assessed its capability to modulate POMC transcription in isolated nuclei from hypothalamus. A 45-min incubation with CNTF (1 nM) increased the level of nascent POMC-RNA significantly ($P < 0.05$, $n = 5$; Fig. 6). By contrast, CNTF did not alter the transcription of NPY gene, and leptin treatment had no significant effect on the transcription level of our genes of interest (Fig. 6).
CNTF stimulates signaling pathways in isolated hypothalamic nuclei. We next investigated the effects of nuclear CNTF on the activation of signaling pathways after the incubation of hypothalamic nuclear extracts from 10 different rats with CNTF. Western blot analysis using anti-p-JAK2 and anti-p-Akt antibodies showed that a 10-min incubation with CNTF (1 nM) increased their phosphorylation levels (Fig. 7A). The band intensity analysis of the blots (after normalization with total JAK2 or total Akt) evidenced a significant increase of the JAK2 ($P < 0.0005$) and Akt ($P < 0.005$) phosphorylation levels compared with control conditions (Fig. 7B). It is of note that phosphorylation of these proteins was not affected by leptin (10 nM) and was prevented in the absence of ATP. Moreover, CNTF did not induce the phosphorylation of ERK42/44 in nuclear extracts, but it induced that of STAT3 in two out of 10 animals (data not shown). We performed the same experiment on nuclear fractions extracted from a neuronal cell line (SH-SY5Y), which expressed POMC. These cells served as a negative control since, unlike gp130, CNTFRα or LIFR were found mainly in the cytoplasm. The incubation of SH-SY5Y nuclear extracts with CNTF (1 nM) was not associated with increased phosphorylation of JAK2 and Akt (not shown).

**DISCUSSION**

Anorexigenic properties of CNTF have conferred to this cytokine a promising therapeutic potential in the treatment of obesity. Besides, several studies have tended to show that endogenous CNTF can also represent a new modulator of energy homeostasis. Indeed, a null mutation in CNTF gene has been associated with a significant increase in body mass in humans (25, 42), and variants in CNTF or CNTFRα gene in humans have been associated with a lower age at the onset of eating disorders (22). Furthermore, hypothalamic neurons controlling food intake represent a significant source of CNTF that varies in inverse ratio with body weight in rats fed a hyperca-
Nuclear CNTF regulates POMC gene expression in the rat ARC

A

ATP  +  +  +  +  -
CNTF  +  +  +  -  -
Leptin  +  +  +  -  -
pJAK-2  -  -  -  -  -
tot-JAK-2  -  -  -  -  -
pAkt  -  -  -  -  -
tot-Akt  -  -  -  -  -

B

Normalized levels (% of control lane)

ATP  CNTF  Leptin  pJAK-2  pAkt

**  *  

Fig. 7. CNTF stimulates Akt signaling in hypothalamic isolated nuclei. Isolated nuclei were obtained from individual fresh rat hypothalami and incubated in the presence or not of ATP (5 μM), CNTF (1 nM), and leptin (10 nM) for 10 min at 37°C. They were then subjected to SDS-PAGE and Western blot analysis. A: montage of nitrocellulose membranes blotted with an anti-phospho (p)-JAK2 or an anti-p-Akt before being stripped and blotted a 2nd time with an anti-total (tot) JAK2 or anti-tot Akt. B: quantification of the blots. The data are presented as the %control condition ± SE. *P < 0.05 and **P < 0.01 compared with control condition with paired Student t-test; n = 10 rats.

loric diet, suggesting a protective action of the cytokine in a fraction of individuals against diet-induced weight gain (57). However, how hypothalamic CNTF contributes to the control of energy balance is still an open question since it lacks a signal peptide and thus may not be released by the classical exocytotic pathway (49). In the current study, we show for the first time that CNTF can exert a direct intracrine action by activating its receptor in the cell nucleus of ARC cells. This leads to the stimulation of POMC expression and may account for the anorexigenic effect of the cytokine.

A variety of hormones (i.e., leptin and insulin) and growth factors (i.e., leukemia inhibitory factor and brain-derived neurotrophic factor) have been shown to operate in an intracrine manner, and most of them have been reported to translocate and bind to the cell nucleus (47). However, although CNTF has been described previously in the nuclear compartment of neurons and glial cells throughout the brain (3–5, 27, 35), the evidence of CNTF in the nucleus of ARC neurons was unexpected. This result was validated not only by the diversity of the approaches used in our study (immunohistochemistry, 3D reconstructions, and Western blot from fractionated cells) but also by the implementation of several control experiments (omission of the primary antibody, competition with blocking peptide, and use of CNTF-knockout mice). Moreover, we have tested several antibodies obtained from different species and raised against different domains of the protein, and this comparative study confirmed CNTF to be present not only in the cytoplasm but also in the nucleus of ARC cells. The mechanisms underlying the nuclear translocation of CNTF have not been elucidated yet. CNTF gene can be cotranscribed in mouse and human with that encoding zinc finger protein 91, a nuclear proliferative and antiapoptotic factor (Entrez Gene: ZFP91-CNTF transcription unit) (56), but no nuclear targeting signal (NTS) was found for CNTF. Nevertheless, alternative NTS-independent pathways exist for nuclear transport (reviewed in Ref. 59). In vitro studies have indicated that CNTF could reach the nucleus by a facilitated transport mechanism in oocytes and astrocytes (4, 5). Moreover, the fact that we evidenced both CNTF and LIFR passing through the nuclear envelope by the same pore could suggest a receptor-mediated translocation, as demonstrated previously for IL-1, another signal peptide lacking cytokine (13).

Our data indicate that CNTF receptor subunits are present not only at the membrane and in the cytoplasm but in the nucleus of ARC cells as well. This observation is in accord with several studies showing that intracrine factors can usually act both at the membrane and in the nucleus (47). Furthermore, CNTF receptor subunits may associate in the cell nucleus, as demonstrated using transmission electron microscopy and coimmunoprecipitation from nuclear extracts. To our knowledge, among the different CNTF receptor subunits, LIFR is the only one that has been described previously in cell nucleus, notably in neurons (17, 20).

Among the different cell types constituting the ARC, POMC-expressing neurons appeared as a privileged place for nuclear CNTF to activate its receptor complex. Indeed, all the partners are present in the nucleus of these neurons, and the stimulation of nuclear extract with CNTF induced a specific increase in POMC gene expression. The important transcriptional activity of the activated CNTF receptor complex is illustrated by the high degree of colocalization between these proteins and HP1, a positive regulator of active transcription in euchromatin (33). By comparison, NPY-nascent RNA levels were not affected by CNTF. In the hypothalamus, POMC neurons play a crucial role in the regulation of energy homeostasis by controlling food intake, energy expenditure, and glucose metabolism (45). They integrate nervous and circulatory signals to respond to the energy body requirements and in turn release the anorexigenic α-MSH toward second-order neurons located in different hypothalamic regions, such as the paraventricular nucleus and the lateral hypothalamic area. Furthermore, POMC neurons have been shown to mediate the anorexigenic effect of exogenous CNTF. Indeed, CNTF injection induces an increase in POMC expression (1), and the ablation of gp130 in POMC neurons prevents CNTF from activating STAT3 phosphorylation in these neurons and from inhibiting food intake (32). Thus, here we demonstrate that endogenous CNTF could take part in the control of energy homeostasis by regulating POMC gene expression via the
activation of its receptor subunits in the cell nucleus of these neurons. The signaling cascades involved in such a regulation need to be elucidated. According to our results, we can assume that an Akt-dependent pathway mediates the effect of nuclear CNTF on POMC transcription. Indeed, we observed JAK2 and Akt in the nucleus of ARC cells, and the stimulation of nuclear extracts with CNTF induced the phosphorylation of both proteins. This hypothesis is reinforced by the fact that Akt can be activated directly in the cell nucleus in different cellular models. Moreover, POMC gene transcription is known to be regulated by signaling pathways, including JAK2 and Akt (7, 31). The role of STAT3 cannot be totally excluded since CNTF induced its phosphorylation in two out of 10 animals. By contrast, leptin was unable to induce Akt and JAK2 phosphorylation in isolated nuclei, indicating a specific effect of CNTF. Moreover, the presence of CNTFRα and/or LIFR in the nucleus seems to be necessary to activate these signaling pathways since in the SH-SY5Y neuroblastoma cell line, which exhibits none of these subunits in the nucleus, CNTF failed to activate the JAK2/Akt signaling pathways in isolated nuclei.

The impact of our data may not be restricted to the comprehension of the central control of energy homeostasis but extended to therapeutic applications. Indeed, the use of a cell penetrating modified CNTF (48, 58), which crosses the cell membrane and translocates to the nucleus, can retain all or part of the CNTF activities without inducing the side effects usually caused by the cytokine (i.e., fever, cough, asthenia) (12). In conclusion, our results indicate that endogenous CNTF could activate its receptor complex in the nucleus of ARC cells, including POMC neurons. This mechanism, which is not shared by leptin, may contribute to the regulation of POMC gene expression and thus to the control of energy homeostasis. The involvement of such a process in the protective action of endogenous CNTF against diet-induced weight gain deserves further investigation. Nevertheless, these data could influence future drug discovery efforts for the development of new therapeutic targets against obesity.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

O.C., A.A., D.C., and J.D. performed the experiments; O.C. analyzed the data; O.C. interpreted the results of the experiments; O.C. and C.-M.V. prepared the figures; A.G., M.T., and C.-M.V. did the conception and design of the research; C.-M.V. drafted the manuscript.

REFERENCES


