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Ciliary neurotrophic factor infused intracerebroventricularly shows reduced catabolic effects when linked to the TAT protein transduction domain

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Abstract

Ciliary neurotrophic factor (CNTF) regulates the differentiation and survival of a wide spectrum of developing and adult neurons, including motor neuron loss after injury. We recently described a cell-penetrant recombinant human CNTF (rhCNTF) molecule, formed by fusion with the human immunodeficiency virus-1 transactivator of transcription (TAT) protein transduction domain (TAT-CNTF) that, upon subcutaneous administration, retains full neurotrophic activity without cytokine-like side-effects. Although the CNTF receptor is present in hypothalamic nuclei, which are involved in the control of energy, rhCNTF but not TAT-CNTF stimulates signal transducers and activators of transcription 3 phosphorylation in the rat hypothalamus after subcutaneous administration. This could be due limited TAT-CNTF distribution in the hypothalamus and/or altered intracellular signaling by the fusion protein. To explore these possibilities, we examined the effect of intracerebroventricular administration of TAT-CNTF in male adult rats. TAT-CNTF-induced weight loss, although the effect was smaller than that seen with either rhCNTF or leptin (which exerts CNTF-like effects via its receptor). In contrast to rhCNTF and leptin, TAT-CNTF neither induced morphological changes in adipose tissues nor increased uncoupling protein 1 expression in brown adipose tissue, a characteristic feature of rhCNTF and leptin. Acute intracerebroventricular administration of TAT-CNTF induced a less robust phosphorylation of signal transducers and activators of transcription 3 in the hypothalamus, compared with rhCNTF. The data show that fusion of a protein transduction domain may change rhCNTF CNS distribution, while further strengthening the utility of cell-penetrating peptide technology to neurotrophic factor biology beyond the neuroscience field.

Keywords: adipose tissue, ciliary neurotrophic factor, fusion protein, hypothalamus, leptin, protein transduction domain, signal transducers and activators of transcription 3.

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Ciliary neurotrophic factor (CNTF) is a 23 kDa cytoplasmic protein expressed in both the PNS and CNS beginning in the late embryonic period (Stöckli *et al.* 1991). CNTF shares structural and functional properties with the interleukin-6 family of cytokines (Bazan 1991; Mcdonald and Hendrickson 1993) and is thought to play a major role in the adult nervous system's early response to lesions (Kirsch *et al.* 2003). CNTF acts through a trimeric membrane receptor complex composed of the extracellular, membrane anchored CNTF binding subunit alpha, and two signaling transducing, transmembrane subunits, leukemia inhibitory factor receptor-β, and gp130 (Davis *et al.* 1993). Engagement of the CNTF receptor complex leads to activation of the janus kinase

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This study is dedicated to our colleague and friend Francesco Langone, who passed away this May.

Abbreviations used: BAT, brown adipose tissue; CNTF, ciliary neurotrophic factor; EP, epididymal; i.c.v., intracerebroventricular; JAK, janus kinase; PBS, phosphate-buffered saline; pSTAT3, phospho-STAT3; rhCNTF, recombinant human CNTF; RP, retroperitoneal; SDS, sodium dodecyl sulfate; STAT, signal transducers and activators of transcription; TAT, transactivator of transcription; TAT-CNTF, CNTF-protein transduction domain fusion protein; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; UCP1, uncoupling protein 1.

(JAK)/signal transducers and activators of transcription (STAT) signaling pathway, and the phosphorylation of STAT3 (Davis *et al.* 1993; Boulton *et al.* 1995).

Ciliary neurotrophic factor is well known for its ability to support the survival of motoneurons *in vivo* and *in vitro* (Arakawa *et al.* 1990; Sendtner *et al.* 1990). Exogenously administered CNTF prevents lesion-mediated motoneuron degeneration in the early postnatal period and greatly reduces functional and morphological changes in *pmn*/*pmn* mice, an autosomal mutant characterized by progressive motoneuron degeneration (Sendtner *et al.* 1990, 1992; Kuzis and Eckenstein 1996). These pre-clinical results encouraged clinical trials of CNTF for amyotrophic lateral sclerosis (Sendtner *et al.* 1994; Miller *et al.* 1996a,b). However, daily subcutaneous dosing of CNTF in humans led to severe side-effects, notably weight loss, anorexia, and an increase in mortality (Miller *et al.* 1996a,b).

The effects of CNTF on body weight in animal models share similarities with leptin-mediated body weight loss (Halaas *et al.* 1995; Henderson *et al.* 1996). The CNTF receptor is found in the hypothalamus, a target of leptin responsible for many of its effects on energy metabolism (Davis *et al.* 1991; Tartaglia *et al.* 1995). CNTF-induced weight loss appears to be mediated mainly by its direct action on proopiomelanocortin cells, a population of neurons in the arcuate nucleus of the hypothalamus critically involved in energy homeostasis (Janoschek *et al.* 2006). However, CNTF's action on energy metabolism is likely independent of the leptin system, as CNTF is able to correct the obesity and diabetes associated with leptin deficiency and/or resistance (Gloaguen *et al.* 1997).

Ciliary neurotrophic factor lacks a classical signal peptide sequence that serves to direct cellular egress of newly synthesized polypeptides. It is likely that CNTF resides within the cell, only to be released after injury (Stöckli *et al.* 1989; Kirsch *et al.* 2003). Strategies which facilitate intracellular entry of CNTF following administration would thus be of therapeutic interest. To mimic this property of CNTF, we linked human CNTF to a protein transduction domain (TAT-CNTF) (Schwarze *et al.* 1999) derived from the human immunodeficiency virus TAT protein (named TAT-CNTF) to promote cellular uptake following injection (Rezende *et al.* 2009). Cell-penetrating peptide-mediated delivery of CNTF has been reported previously to rescue neurological impairments induced by amyloid β-peptide in mice (Qu *et al.* 2008). The TAT-CNTF-protein described by Rezende *et al.* (2009) supported the survival of cultured chicken embryonic sensory dorsal root ganglion neurons as did recombinant human CNTF (rhCNTF), and subcutaneous administration of TAT-CNTF reduced motoneuron loss following sciatic nerve transection in neonatal rats. However, unlike rhCNTF, TAT-CNTF given to neonatal rats did not affect animal weight or fat tissue mobilization.

The reasons for TAT-CNTF inability to induce changes in body weight remain to be fully explained. The structural and functional modifications induced by the fusion of TAT to CNTF are not known and, conceivably, TAT-CNTF could have followed routes alternative to those normally used by CNTF to reach the hypothalamic areas involved in the control of energy metabolism (Pan *et al.* 1999). Also, TAT-CNTF distribution after subcutaneous administration may limit its ability to achieve effective concentrations in the hypothalamus. In addition, fusion of TAT to CNTF may have modified the CNTF capacity to induce intracellular signaling in hypothalamic neurons. To further explore these questions, we have now examined the effects of intracerebroventricular (i.c.v.) TAT-CNTF administration in male adult rats.

Materials and methods

Animals

Ten-week-old male Wistar rats weighting about 300 g were obtained from the University of Campinas Multidisciplinary Center for Biological Investigation in Laboratory Animal Science (CEMIB). Rats were housed in plastic cages in a ventilated rack (Alesco model 9902.001; Alesco, Campinas, SP, Brazil) with a 12 h light–dark cycle at 22 ± 1°C. Animals had *ad libitum* access to standard rodent chow and water throughout. All experiments were conducted in accordance with the principles and procedures described by the NIH Guidelines for the Care and Use of Experimental Animals and were approved by the State University of Campinas Committee of Animal Care (Protocols CEEA-UNICAMP 509-1 and 878-2).

Recombinant ciliary neurotrophic factor proteins

Recombinant human CNTF and TAT-CNTF were produced as previously described (Rezende *et al.* 2009). Protein concentrations were determined using a double beam Perkin-Elmer lambda 25 UV/Vis spectrophotometer (Perkin-Elmer, Fremont, CA, USA), and the absorption coefficient at 280 nm calculated according to Gill and von Hippel (1989) (taken as 1.27/mg cm^2 for rhCNTF and 1.16/mg cm^2 for TAT-CNTF).

Intracerebroventricular cannula placement and infusion

For i.c.v. cannula placement rats were anaesthetized with xylazine (27 mg/kg, i.p.) and ketamine (14 mg/kg, i.p.). A guide cannula (23 G-15 mm in length) was implanted in the lateral ventricle, using a stereotaxic frame (Insight, Ribeirão Preto, SP, Brazil) (coordinates relative to bregma for the tip of the guide cannula: 0.2 mm anteroposterior, 1.5 mm lateral, 3.8 mm ventral) (Paxinos and Watson 1986). The cannula was kept in place with the aid of a metal screw attached to the skull and acrylic cement. The guide cannula was blocked with a nylon thread of 15 mm until the start of i.c.v. administration. Rats were allowed to recover for 6 days. Correct guide cannula positioning was confirmed in the sixth day post-surgery by infusing 2 μL of angiotensin II (10 μg/mL in phosphate-buffered saline; PBS). Rats that presented a dipsogenic response to angiotensin II infusion were used.

Evaluation of TAT-CNTF metabolic effects following i.c.v. administration

On the seventh day post-cannula placement, 23 rats were randomly assigned to four treatment groups. The following i.c.v. infusions were carried out every 12 h for 4 days: TAT-CNTF (2.5 μg/8 μL; $n = 8$), rhCNTF (2.3 μg/8 μL; $n = 5$), leptin (5 μg/8 μL; $n = 5$), and PBS ($n = 5$). Rats were weighed after each infusion. Twenty-four hours after the last infusion all rats were killed by CO_2 asphyxiation. Interscapular brown adipose tissue (BAT), retroperitoneal (RP), and epididymal (EP) white fat pads were collected and weighted. Samples of BAT (50 mg), RP and EP (200 mg of each) were taken from each rat and stored at −80°C for DNA fragmentation analysis. A second sample of 50 mg of BAT was collected and stored at −80°C for uncoupling protein 1 (UCP1) expression analysis. A 2 mm^3 fragment of each tissue was fixed in buffered formaldehyde (0.1 M phosphate buffer and 4% *p*-formaldehyde, pH 7.4) for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) analysis.

DNA fragmentation analysis

DNA was extracted from stored samples of BAT, RP and EP by mincing and homogenizing (30 s at 4°C) in 750 μL of 50 mM Tris–Cl, 10 mM EDTA 1% Triton X-100, pH 8.1, using a Polytron PTA generator (Brinkmann Instruments, Westbury, NY, USA) set at minimum speed. Homogenized samples were incubated for 20 min at 4°C and then centrifuged at 14 000 *g* for 15 min at 4°C. The supernatant (containing fragmented DNA) was collected and kept at 4°C, and the pellet resuspended in 500 μL of 50 mM Tris–Cl, 10 mM EDTA, 1% sodium dodecyl sulfate (SDS), pH 8.1, and incubated at 37°C for 30 min (genomic DNA fraction). Both DNA fractions were phenol/chloroform extracted. RNA was digested by a 30 min incubation at 37°C with 1 μg of Rnase A (Sigma, St Louis, MO, USA). The DNA concentration of both fractions was determined using the PicoGreen Kit (Invitrogen, Carlsbad, CA, USA). DNA fragmentation percentage of each sample was defined as: fragmented DNA/(fragmented DNA + genomic DNA) × 100 (Gullicksen *et al.* 2004).

In situ apoptosis detection by TUNEL

The previously fixed adipose tissue samples were paraffin embedded and cut into a series of six sections of 6 μm of thickness, at intervals of 60 μm. TUNEL reaction (Gavrieli *et al.* 1992) was performed with the TdT-FragELTM DNA Fragmentation Detection Kit (QIA33; Merck, San Diego, CA, USA), according to the manufacturer's instructions. All steps were performed at ambient temperature, except the TdT enzyme reaction. In brief, sections were deparaffinized and permeabilized with proteinase K/10 mM Tris, pH 8.0 (1 : 100; 5 min). Endogenous peroxidase was quenched with 3% H_2O_2/methanol (5 min). The sections were incubated first with equilibration buffer (30 min), followed by TdT enzyme solution (90 min, at 37°C) and then stop buffer (5 min). Labeled DNA was detected by applying blocking buffer (10 min) followed by peroxidase-conjugated streptavidin solution (30 min) and 3,3′-diaminobenzidine reaction. Methyl green was used as counterstain.

Detection of UCP1 expression in brown adipose tissue

Brown adipose tissue samples were minced and homogenized for 30 s in 750 μL of 50 mM Tris–Cl, pH 8.1, 10 mM EDTA, 1% protease inhibitor cocktail (Sigma), 1% Triton X-100 at 4°C, using a 20 s Polytron PTA set at maximum speed. Proteins were coagulated with an equal volume of phenol/chloroform. After centrifugation (16 000 *g*), the aqueous phase was removed and 750 μL of methanol were added to precipitate the proteins. The supernatant was removed by centrifugation (12 000 *g*), and the pellet washed three times with methanol and then dried under a stream of air. The pellet was resuspended in 50 mM phosphate buffer, pH 7.4, 5% β-mercaptoethanol, 10 mM EDTA, 1% protease inhibitor cocktail (Sigma), 1% SDS, and protein concentration determined by the Bradford method (Bradford 1976). Total protein from each animal (3 μg) was eletrophoretically separated by SDS–polyacrylamide gel electrophoresis following standard procedures (Laemmli 1970). The Kaleidoscope (BioRad, Hercules, CA, USA) pre-stained molecular weight markers were employed. Gels were electroblotted to a nitrocellulose membrane (120 V, 90 min) (Towbin *et al.* 1979). Equal protein loading was assessed with Ponceau S staining of membranes and optical density analysis of various protein bands. Membranes were blocked with PBS–Tween (50 mM phosphate buffer, pH 7.4, 150 mM NaCl, and 0.05% Tween 20) containing 5% non-fat dry milk. Membranes were then incubated with anti-UCP1 antibody (sc-6529; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted (1 : 1000) in PBS–Tween containing 3% bovine serum albumin (12 h, 4°C), followed by washing with PBS–Tween and incubation with horseradish peroxidase-labeled secondary antibody (1 : 10 000; Zymed, South San Francisco, CA, USA). Reactive bands were detected with the SuperSignal West Pico chemiluminescent kit (Pierce, Rockford, IL, USA).

Evaluation of JAK/STAT signaling by western blot

On the seventh day post-cannula placement, 28 rats were randomly assigned to four treatment groups: TAT-CNTF (2.5 μg/8 μL; $n = 8$), rhCNTF (2.3 μg/8 μL; $n = 8$), leptin (5 μg/8 μL; $n = 8$), and PBS ($n = 4$). Rats were anesthetized with pentobarbital (50 mg/kg, i.p.) and then received one i.c.v. infusion. Five or twenty minutes after the infusion of TAT-CNTF, rhCNTF or leptin, and 20 min after PBS infusion, rats were decapitated, the hypothalamus was collected, and immediately frozen in liquid nitrogen. For total protein, samples were homogenized in 1% Triton X-100, 50 mM phosphate buffer, pH 7.4, 1 mM sodium pyrophosphate, 1 mM sodium fluoride, 5 mM EDTA, 1 mM sodium vanadate, 1% protease inhibitor cocktail (P8340; Sigma), 7 M urea, and 2 M thiourea (10% w/v). Sample homogenization was carried out at 4°C using a Polytron 20 s generator set at maximum speed for 30 s. Insoluble materials were removed by centrifugation (12 000 *g*, 4°C, 15 min). Protein concentration was determined using the Bradford method. One hundred micrograms of total protein extract from each animal was eletrophoretically separated by SDS–polyacrylamide gel electrophoresis and electroblotted to a nitrocellulose membrane (Laemmli 1970; Towbin *et al.* 1979). Membranes were blocked with PBS–Tween containing 5% non-fat dry milk and then incubated with an anti-phospho-STAT3 (pSTAT3) antibody (Tyr705; Cell Signaling, Beverly, MA, USA) diluted (1 : 1000) in PBS–Tween containing 3% bovine serum albumin (12 h at 4°C). Membranes were washed with PBS–Tween and incubated with horseradish peroxidase-labeled secondary antibody (1 : 10 000; Zymed). Reactive bands were detected with the SuperSignal West Pico chemiluminescent kit. To detect total STAT3 expression membranes were stripped with a glycine–HCl solution, pH 2.7, and immunoblotting performed using an anti-STAT3 antibody

(Santa Cruz). Results are expressed as the ratio of pSTAT3 : STAT3 relative optical densities, with the PBS group taken as 1.0.

Immunohistochemistry

On the seventh day post-cannula placement, 12 rats were randomly assigned to four treatment groups: TAT-CNTF (2.5 μg/8 μL; $n = 3$), rhCNTF (2.3 μg/8 μL; $n = 3$), leptin (5 μg/8 μL; $n = 3$), and PBS ($n = 3$). Rats were anesthetized with pentobarbital (50 mg/kg, i.p.) and then received one i.c.v. infusion. Twenty minutes after infusion of TAT-CNTF, rhCNTF, leptin or PBS, rats were perfused transcardially with saline followed by 200 mL of freshly prepared fixative (0.1 M phosphate buffer and 4% *p*-formaldehyde, pH 7.4). The brain was carefully removed, post-fixed for 24 h in the same fixative, and cryoprotected in 30% sucrose. Brains were frozen in dry ice and cut into 30 μm coronal sections on a cryostat. Sections were stored at 4°C in 0.02% sodium azide containing PBS until used. For pSTAT3 immunostaining, sections containing the arcuate nucleus of the hypothalamus were pre-treated 20 min with an aqueous solution of 1% NaOH/1% H_2O_2, 10 min with 0.3% glycine, and finally 10 min with 0.03% SDS (Munzberg *et al.* 2003). The sections were then blocked for 1 h with 3% bovine serum albumin in PBS/0.5% Triton X-100. Following an overnight incubation at 4°C with pSTAT3 antibody (rabbit anti-pSTAT3, 1 : 3000 in blocking solution), the sections were washed with PBS, incubated with a biotinylated secondary donkey anti-rabbit antibody (1 : 100 in blocking solution), and then treated with avidin-biotin complex solution for 1 h. The signal was developed by reaction with 3,3′-diaminobenzidine/nickel to give a black precipitate. Morphology was analyzed by Nissl staining of adjacent coronal sections. Staining was visualized using a brightfield microscope and pictures captured with a digital camera.

Statistical analysis

A two-way ANOVA with repeated measures, followed by a Bonferroni *post hoc* test was used to analyze the effect of treatments on rat body weight. An ANOVA followed by a Student–Newman–Keul's *post hoc* test was used to analyze the effect of treatments on adipose tissue weight and DNA fragmentation. A paired *t*-test was used to analyze the effect of treatments on STAT3 phosphorylation and UCP1 expression. Results are expressed as mean ± SEM and the level of significance was considered $p < 0.05$.

Results

Metabolic effects of TAT-CNTF CNS administration

A 4-day i.c.v. infusion of TAT-CNTF produced a 3.1% reduction in body weight relative to control animals, while rhCNTF and leptin infusions diminished body weight by 11.2% and 6.5%, respectively (Fig. 1). These treatments had a significant effect on body weight ($F_{3,144} = 13.91$; $p < 0.0001$), although TAT-CNTF caused a more modest reduction of body weight compared with rhCNTF ($p < 0.001$). Moreover, the effect of TAT-CNTF on body weight was similar to that of leptin during the first 2 days ($p > 0.05$), while by treatment end the body weights of TAT-CNTF infused and control (PBS) rats did not differ significantly ($p > 0.05$).

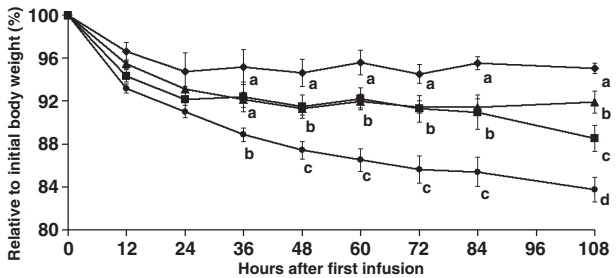

Fig. 1 TAT-CNTF given i.c.v. causes a significantly smaller weight loss compared with rhCNTF and leptin. Rats received an i.c.v. infusion every 12 h over 4 days (total of eight dosings) of: (▲) TAT-CNTF (2.5 μg/8 μL; $n = 8$); (●) rhCNTF (2.3 μg/8 μL; $n = 5$); (■) leptin (5 μg/8 μL; $n = 5$) (◆) PBS ($n = 5$). Data (mean ± SEM) are the percentage of body weights relative to onset of treatment. Absence of a common letter indicates statistical significant difference ($p < 0.05$) between those data points.

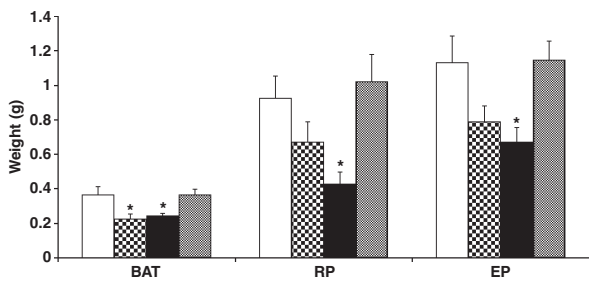

Fig. 2 TAT-CNTF given i.c.v. does not reduce adipose tissue weights. Rats received an i.c.v. infusion every 12 h over 4 days of: (▨) TAT-CNTF, (■) rhCNTF, (⊠) leptin, or (□) PBS. All animals were killed 24 h after the last infusion and the weights of interescapular brown adipose tissue (BAT), retroperitoneal (RP), and epididymal (EP) white adipose tissue samples were measured. Data are mean ± SEM ($n = 4$); *$p < 0.05$ versus PBS.

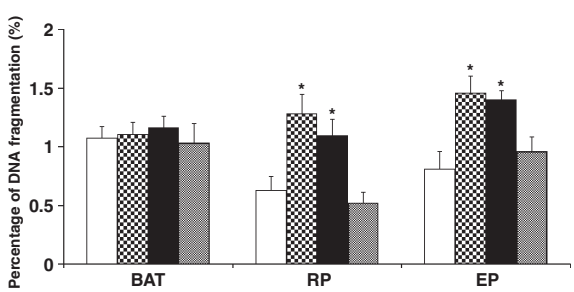

Fig. 3 TAT-CNTF does not increase DNA fragmentation in adipose tissues. Rats received an i.c.v. infusion every 12 h for 4 days of: (■) rhCNTF, (▨) TAT-CNTF, (⊠) leptin, or (□) PBS. The percentage of DNA fragmentation was measured in interescapular brown adipose tissue (BAT), retroperitoneal (RP) or epididymal (EP) white adipose tissue samples were measured. Data are mean ± SEM ($n = 4$); *$p < 0.05$ versus PBS.

The weights of BAT, RP, and EP fat pad were not altered by TAT-CNTF infusion, whereas rhCNTF significantly reduced the weights of these tissues ($p < 0.05$; Fig. 2). Leptin infusion led to a significant reduction of BAT weight, as well ($p < 0.05$).

The reduction of RP and EP weight after rhCNTF i.c.v. infusions was associated with an increase in the percentage of fragmented DNA ($p < 0.05$; Fig. 3). TUNEL$^+$ cells displaying adipocyte morphology were also detected in RP and EP of rhCNTF-treated animals (Fig. 4). Leptin i.c.v. infusions increased the percentage of fragmented DNA in RP and EP, and the frequency of TUNEL$^+$ cells. In keeping with the absence of weight reduction in the fat pads of TAT-CNTF infused animals, apoptosis was not detected in any tissue sampled from this group.

An increase in UCP1 expression in BAT is a characteristic effect of leptin and rhCNTF administration. To assess whether TAT-CNTF would also cause this effect, western blot analysis of UCP1 expression in BAT was performed. Rats receiving TAT-CNTF i.c.v. for 4 days had no increase in UCP1 expression, unlike the 50% increase observed in rhCNTF- or leptin-treated animals ($p < 0.05$; Fig. 5).

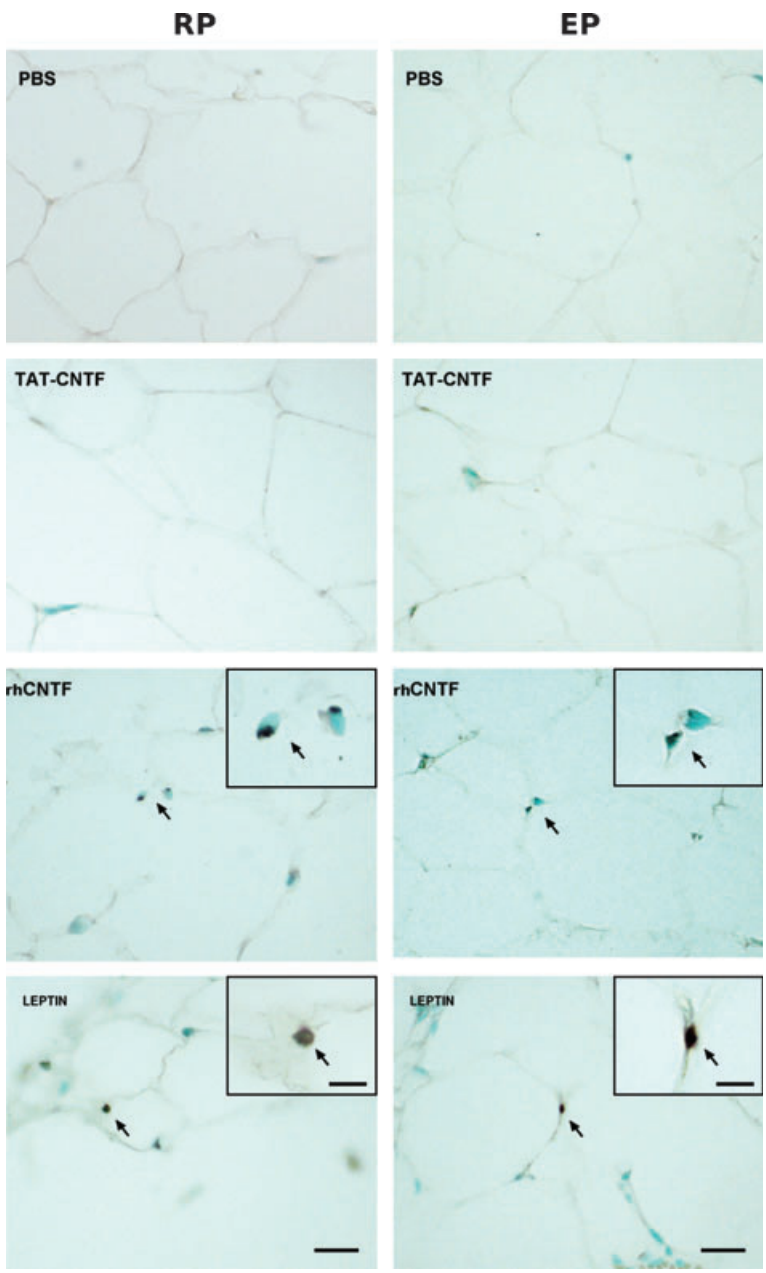

Fig. 4 TAT-CNTF treatment does not cause *in situ* apoptosis (TUNEL staining). TUNEL labeling was performed in histological sections from retroperitoneal (RP) and epididymal (EP) white adipose tissue taken from rats that received TAT-CNTF, rhCNTF, leptin, or PBS i.c.v. infusion every 12 h for 4 days. Counterstaining was with methyl green. Arrows indicate TUNEL$^+$ cells in the leptin- and rhCNTF-treated groups. Inset: arrow indicates a TUNEL$^+$ nucleus. Bar, 20 μm (inset: 7 μm).

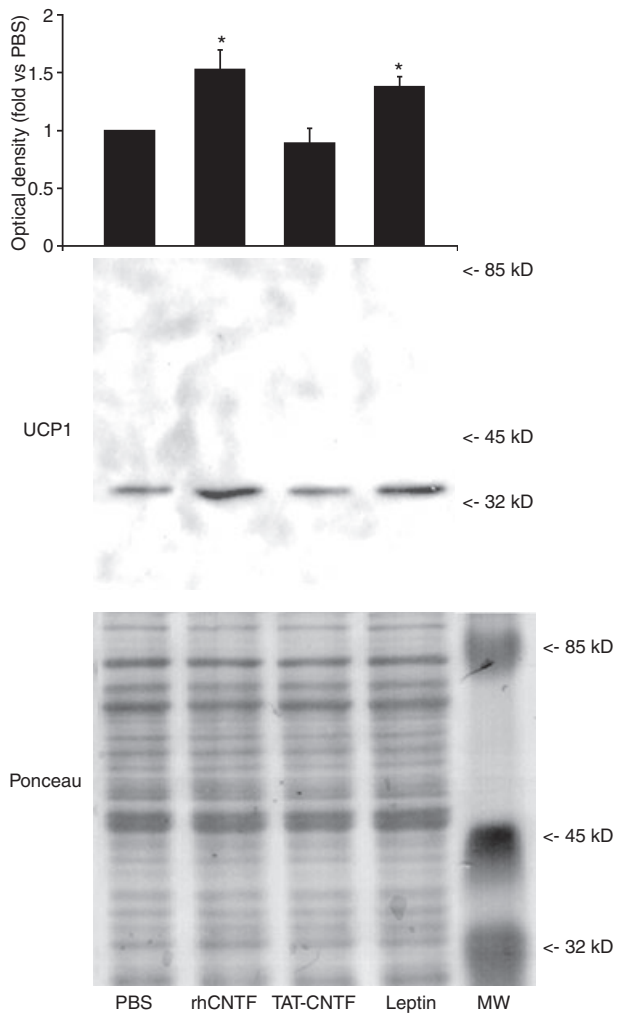

Fig. 5 TAT-CNTF given i.c.v. does not increase UCP1 expression in interescapular brown adipose tissue (BAT). The expression of UCP1 in BAT of rats that received TAT-CNTF, rhCNTF, leptin, or PBS i.c.v. infusion every 12 h for 4 days was measured by western blot. Data are mean ± SEM ($n = 4$); *$p < 0.05$ versus PBS. A representative immunoblot together with a Ponceau S stained membrane is shown below the graph.

JAK/STAT signaling

Signal transducers and activators of transcription 3 phosphorylation in the hypothalamus was induced following a single i.c.v. dosing of rhCNTF, leptin or TAT-CNTF (Fig. 6). The increase in STAT3 phosphorylation reached significance in rhCNTF-infused animals already after 5 min, further increasing by 20 min. In the TAT-CNTF- and leptin-infused groups, phosphorylated STAT3 levels were significantly elevated only after 20 min ($p < 0.05$).

Phospho-STAT3 immunostaining in brain coronal sections was observed 20 min after rhCNTF, TAT-CNTF or leptin i.c.v. administration (Fig. 7). Control rats (PBS) showed virtually no pSTAT3 labeled cells (Fig. 7a). Rats infused with rhCNTF displayed a dense population of cells with nuclear staining distributed in the arcuate nucleus of the hypothalamus and around the third ventricle (Fig. 7c). In agreement with pSTAT3 western blots, TAT-CNTF infused rats had a smaller number of pSTAT3 labeled cells in the hypothalamus when compared with rhCNTF-treated rats. Phospho-STAT3 immunolabelled cells in the TAT-CNTF group were closer to the ventricle wall (most of them being ependymal cells), with virtually no pSTAT3 positive cells in the arcuate nucleus (Fig. 7d). Leptin-infused animals showed some pSTAT3 labeled cells in the arcuate nucleus of the hypothalamus and along the ventricle wall (Fig. 7b).

Discussion

Ciliary neurotrophic factor is a neurokine with neuroprotective effects and has been considered a candidate for the treatment of neurodegenerative diseases such as amyotrophic lateral sclerosis (Sendtner *et al.* 1992, 1994). In spite of positive pre-clinical findings, trials of CNTF for human motor neuron disease resulted in marked side-effects, including cachexia and a pronounced immune response (Miller *et al.* 1996a,b; Ettinger *et al.* 2003). We recently demonstrated that fusing rhCNTF to a protein transduction domain derived from the HIV-1 TAT protein (TAT-CNTF) produced a chimeric molecule which retained the capacity to reduce motoneuron loss following sciatic nerve transection but unlike rhCNTF, did not adversely affect body weight or fat tissue mobilization (Rezende *et al.* 2009). Although STAT phosphorylation is required for CNTF-induced weight loss (Janoschek *et al.* 2006), in the hypothalamus only rhCNTF-induced STAT3 phosphorylation (Rezende *et al.* 2009). We thus hypothesized possible differences in the ability of TAT-CNTF to act on brain regions involved in energy metabolism and body weight control.

The apparent inactivity of TAT-CNTF at the hypothalamic level was examined by dosing the fusion protein i.c.v. in adult male rats. Daily i.c.v. infusion of TAT-CNTF-induced weight loss, although the effect was smaller than that elicited by rhCNTF or leptin. As TAT-CNTF and rhCNTF were diluted in a Tris–HCl-buffered saline while PBS injection was used as control, we cannot rule out the possibility that buffer differences contributed to the small weight loss mediated by TAT-CNTF when compared with PBS. However, as TAT-CNTF and rhCNTF were diluted in the same buffer, differences in effects observed between the two molecules can most likely be attributed to the presence of a protein transduction domain linked to CNTF. Moreover, 4 days of TAT-CNTF i.c.v. infusion did not reduce the weights of either RP and EP white adipose tissue, or interescapular BAT. Not surprisingly, TAT-CNTF neither induced DNA fragmentation in adipose tissues nor increased UCP1 expression in BAT. In addition, acute i.c.v. administration of TAT-CNTF produced a less intense STAT3

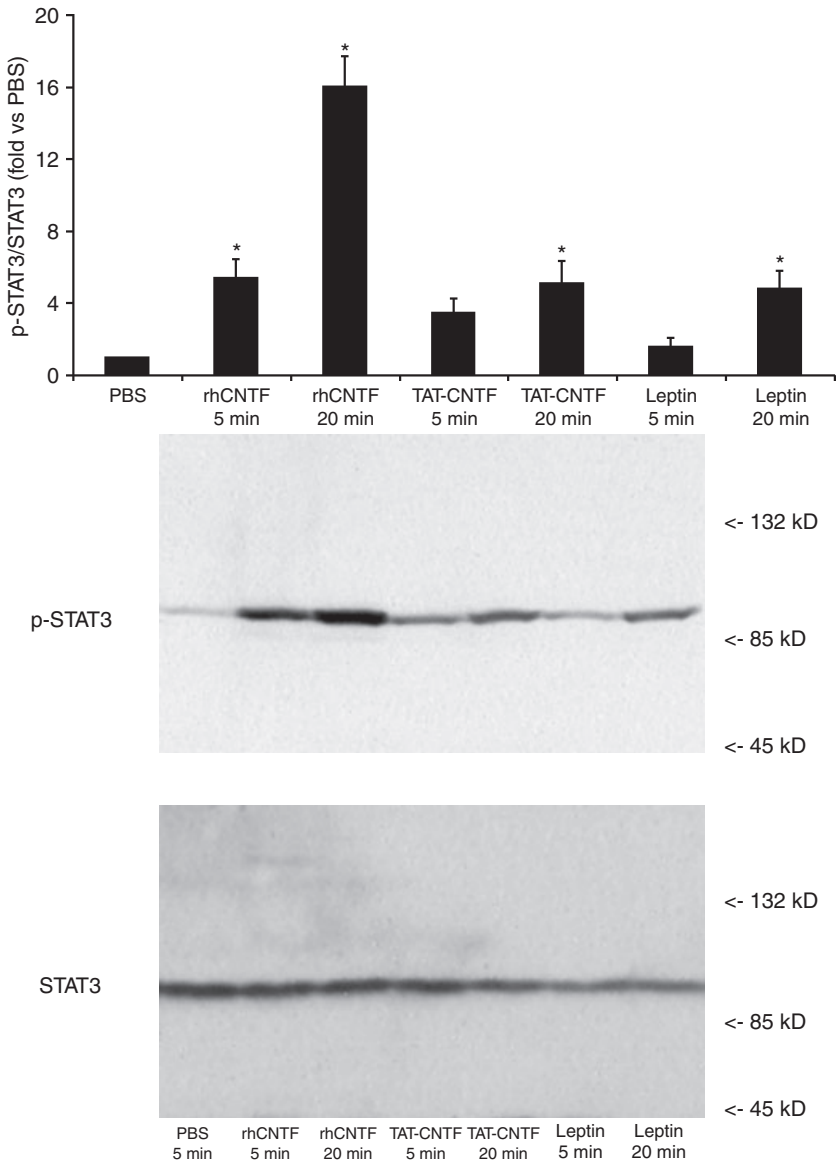

Fig. 6 Western blot analysis of STAT3 phosphorylation in hypothalamus of rats acutely treated with TAT-CNTF or rhCNTF. Rats received an i.c.v. infusion of TAT-CNTF (2.5 μg/8 μL, $n = 8$), rhCNTF (2.3 μg/8 μL, $n = 8$), leptin (5 μg/8 μL, $n = 8$), or PBS ($n = 4$). STAT3 phosphorylation in the hypothalamus was measured 5 or 20 min after the infusions. Data are mean ± SEM ($n = 4$ for each time point), and are expressed as the ratio of pSTAT3 : STAT3 band density (PBS=1.0); $^*p < 0.05$ versus PBS. A representative immunoblot is shown below the graph.

phosphorylation in the hypothalamus compared with rhCNTF. These observations suggest that TAT fusion to CNTF modified the latter's ability to act on hypothalamic nuclei involved in metabolic control.

Ciliary neurotrophic factor acts through a trimeric membrane receptor complex leading to the activation of the JAK/STAT signaling pathway and the phosphorylation of STAT3 (Davis *et al.* 1993; Boulton *et al.* 1995). Activation of STAT3 is a key step for CNTF-mediated neuroprotection in an adult mouse facial nerve lesion model (Schweizer *et al.* 2002; Kirsch *et al.* 2003). STAT phosphorylation is also essential for CNTF-induced body weight loss, as demonstrated in proopiomelanocortin cell-specific gp130 knockout mice (Janoschek *et al.* 2006). In support of the motoneuron survival data, rhCNTF and TAT-CNTF-induced STAT3 phosphorylation in the lumbar spinal cord, although rhCNTF gave a more robust response (Rezende *et al.* 2009). Interestingly, in the hypothalamus, only rhCNTF-induced STAT3 phosphorylation (Rezende *et al.* 2009). Possible structural and functional modifications induced by fusion of TAT to CNTF have not been explored; conceivably, TAT-CNTF could have reached the CNS by a route(s) distinct from that taken by CNTF (Pan *et al.* 1999). Subcutaneous administration of TAT-CNTF may limit its ability to achieve concentrations similar to rhCNTF in the spinal cord and hypothalamus. The amount of TAT-CNTF reaching the spinal cord, however, appears sufficient to elicit a neuroprotective action on motoneurons. Qu *et al.* (2008) showed that CNTF fused to the protein transduction domain TAT (p11-CNTF) has increased blood–brain barrier permeability, greater brain parenchyma penetration, and induces neurological improvement in amyloid β-peptide-treated mice. However, these

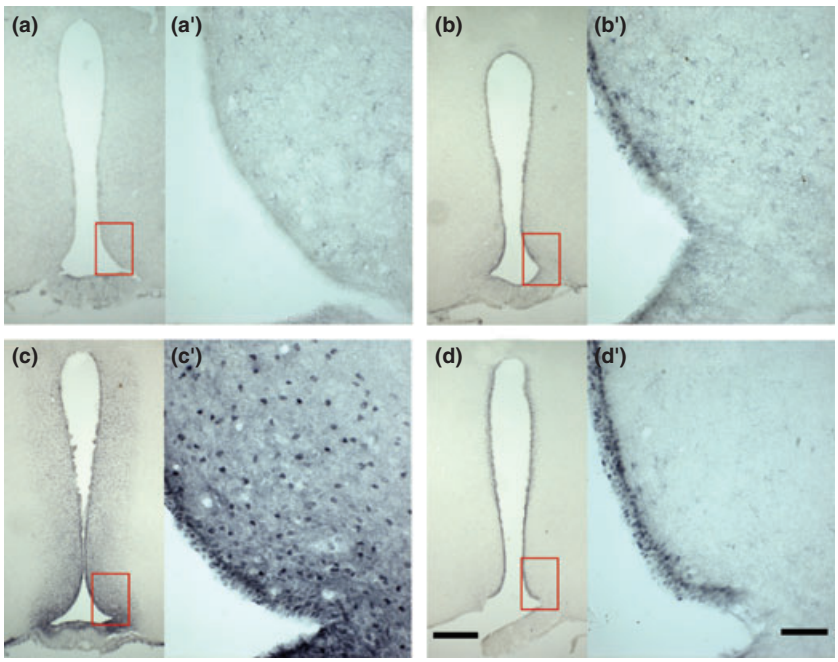

Fig. 7 Immunohistochemical analysis of STAT3 phosphorylation in hypothalamus of rats treated acutely with TAT-CNTF or rhCNTF. Rats received an i.c.v. infusion of TAT-CNTF (d) (2.5 μg/8 μL, $n = 3$), rhCNTF (c) (2.3 μg/8 μL, $n = 3$), leptin (b) (5 μg/8 μL, $n = 3$), or PBS (a) ($n = 3$). Labeling of STAT3 phosphorylation was performed 20 min after the infusions. (a′), (b′), (c′) and (d′) are high-magnification images of (a), (b), (c) and (d) (inset squares). Scale bars: (a), (b), (c) and (d), 300 μm; (a′), (b′), (c′) and (d′), 50 μm.

authors did not report any body weight effects associated with p11-CNTF treatment, nor was the signaling ability of p11-CNTF evaluated. It is possible that p11-CNTF reaches the hypothalamus, but is unable to induce signaling events in hypothalamic neurons.

Weight loss induced by rhCNTF and leptin is mediated via the arcuate nucleus of hypothalamus, mainly on proopiomelanocortin-expressing neurons (Elmquist *et al.* 1999; Schwartz *et al.* 2000; Elmquist 2001; Lambert *et al.* 2001; Anderson *et al.* 2003; Janoschek *et al.* 2006). The weight loss seen after daily i.c.v. dosing of rhCNTF or leptin confirms earlier reports (Qian *et al.* 1998; Pu *et al.* 2000; Duff *et al.* 2004). The lack of pSTAT3-immunolabeled cells in the arcuate nucleus after TAT-CNTF i.c.v. injection, in contrast to rhCNTF, proposes that the smaller weight loss seen with TAT-CNTF could result from impaired action of this molecule on proopiomelanocortin neurons.

Hypothalamic STAT3 phosphorylation induced by acute rhCNTF or leptin i.c.v. administration was similar to previous reports, as was the temporal pattern of STAT3 activation *in vitro* (Kaur *et al.* 2002; Carvalheira *et al.* 2005; Steinberg *et al.* 2006). STAT3 phosphorylation was less robust in TAT-CNTF-treated rats when compared with rhCNTF-treated animals, in keeping with the observation that pSTAT3 immunopositive cells in the former group are closer to the ventricle walls with virtually no pSTAT3 positive cells in the arcuate nucleus. This suggests that a large proportion of TAT-CNTF remains within the ependymal cell layer. One cannot rule out that TAT-CNTF is rapidly taken up and sequestered by ependymal cells within the ventricle wall. STAT3 phosphorylation is an indirect measure of TAT-CNTF presence, and further experiments are needed to demonstrate that TAT-CNTF is present following i.c.v. administration. Another possibility is that TAT-CNTF penetrates the brain parenchyma but is unable to induce cell signaling events. However, we have previously shown that in cultured cells TAT-CNTF and rhCNTF are equally competent in inducing STAT3 phosphorylation (Rezende *et al.* 2009). A saturable transport system for CNTF across the blood–brain barrier has been described (Pan *et al.* 1999), and its activation could change the hypothalamic microenvironment, rendering neurons more responsive to CNTF. Evaluation of this last possibility will require additional investigation.

Daily treatment with TAT-CNTF did not affect fat pad weight, indicating a lack of fat mobilization in adipose tissue. Furthermore, rhCNTF and leptin (but nor TAT-CNTF) appeared to stimulate energy expenditure, as judged by their ability to induce UCP1 expression in BAT. CNTF reportedly increases UCP1 expression in BAT and is linked with thermogenesis (Ott *et al.* 2002; Bluher *et al.* 2004). Indeed, Bluher *et al.* (2004) demonstrated that CNTF induces body weight reduction mainly by increasing UCP1 expression concomitant with reduced food intake. In mice with diet-induced obesity, CNTF given systemically caused a greater body weight loss than in pair-fed controls (Bluher *et al.* 2004). Moreover, in UCP1 deficiency-induced obese mice (UCP1-DTA mice), weight loss caused by CNTF treatment was similar to pair-fed controls. The present data for UCP1 point to a lack of increase in energy expenditure in TAT-CNTF-treated rats, and is in keeping with the smaller weight reduction in the TAT-CNTF group compared with rhCNTF.

Our data are the first to show that centrally administered rhCNTF increases UCP1 expression in BAT. Although a direct action of CNTF on BAT cannot be excluded, our results suggest that rhCNTF acts on hypothalamic neurons controlling sympathetic innervation of BAT (Scarpace and Matheny 1998). In the case of systemic leptin administration, the increase in BAT UCP1 expression depends on the integrity of the sympathetic innervation of this adipose tissue (Scarpace and Matheny 1998). CNTF and leptin activate similar hypothalamic regions, which are connected with central sympathetic pathways, proposing that the UCP1 increase in BAT is mediated by a neuronal mechanism. In fact, proopiomelanocortin cells from the arcuate nucleus project directly to sympathetic neurons in the spinal cord (Elias *et al.* 1998). Because i.c.v. administration of TAT-CNTF failed to increase UCP1 expression in BAT, CNTF fusion to a protein transduction domain may have modified CNTFs ability to act on proopiomelanocortin neurons. Indeed, acute TAT-CNTF i.c.v. dosing did not produce pSTAT3 immunostaining of arcuate nucleus cells in the hypothalamus.

In food-deprived animals, exhaustion of stored fat leads to a reduced adipocyte volume, while central inhibition of food intake results in consumption of fat reserves and apoptosis of adipocytes (Qian *et al.* 1998; Gullicksen *et al.* 2003; Duff *et al.* 2004). The mechanism underlying this apoptosis is unknown, but occurs after CNTF or leptin administration (Qian *et al.* 1998; Gullicksen *et al.* 2003; Duff *et al.* 2004). rhCNTF- and leptin-treated animals exhibited increased DNA fragmentation and $TUNEL^{+}$ adipocytes in RP and EP fat pads, but neither marker was seen in TAT-CNTF-treated rats. These observations strengthen the view that TAT-CNTF has reduced metabolic effects on adipose tissue.

In conclusion, our data show that rhCNTF fused to the TAT protein transduction domain retains its ability to induce intracellular signaling in hypothalamus, but displays a different CNS distribution when compared with rhCNTF. Protein transduction domain technology may thus have applications beyond its classical property of allowing the movement of large proteins across the plasma membrane (Kilic *et al.* 2003, 2004; Qu *et al.* 2008; Rezende *et al.* 2009). The ability of TAT-CNTF to protect neurons from death without significant induction of metabolic effects makes it a candidate molecule for the treatment neurodegenerative diseases.

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