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Ciliary neurotrophic factor fused to a protein transduction domain retains full neuroprotective activity in the absence of cytokine-like side effects

Alexandre C. Rezende,* Daniele Peroni,# Andrè S. Vieira,* Fabio Rogerio,* Rafael L. Talaisys,‡ Fabio T. M. Costa,‡ Francesco Langone,* Stephen D. Skaper† and Alessandro Negro#

*Department of Physiology and Biophysics, State University of Campinas, Campinas, Brazil

†Departments of Pharmacology and Anesthesiology and #Veterinary Science, University of Padova (CRIBI), Padova, Italy

‡Department of Microbiology and Immunology, State University of Campinas, Campinas, Brazil

Abstract

Ciliary neurotrophic factor (CNTF) is a multifunctional cytokine that can regulate the survival and differentiation of many types of developing and adult neurons. CNTF prevents the degeneration of motor neurons after axotomy and in mouse mutant progressive motor neuronopathy, which has encouraged trials of CNTF for human motor neuron disease. Given systemically, however, CNTF causes severe side effects, including cachexia and a marked immune response, which has limited its clinical application. The present work describes a novel approach for administering recombinant human CNTF (rhCNTF) while conserving neurotrophic activity and avoiding deleterious side effects. rhCNTF was fused to a protein transduction domain derived from the human immunodeficiency virus-1 TAT (transactivator) protein. The resulting fusion protein (TAT-CNTF) crosses the plasma membrane within minutes and displays a nuclear localization. TAT-CNTF was equipotent to rhCNTF in supporting the survival of cultured chicken embryo dorsal root ganglion neurons. Local or subcutaneous administration of TAT-CNTF, like rhCNTF rescued motor neurons from death in neonatal rats subjected to sciatic nerve transection. In contrast to subcutaneous rhCNTF, which caused a 20–30% decrease in body weight in neonatal rats between postnatal days 2 and 7 together with a considerable fat mobilization in brown adipose tissue, TAT-CNTF lacked such side effects. Together, these results indicate that rhCNTF fused with the protein transduction domain/TAT retains neurotrophic activity in the absence of CNTFs cytokine-like side effects and may be a promising candidate for the treatment of motor neuron and other neurodegenerative diseases.

Keywords: ciliary neurotrophic factor, fusion protein, motor neuron, neuroprotection, protein transduction domain, weight loss.

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Ciliary neurotrophic factor (CNTF) is a 23 kDa cytoplasmic protein which is expressed in both the PNS and CNS beginning in the late embryonic period (Barbin *et al.* 1984; Stöckli *et al.* 1991). CNTF exhibits a variety of activities, affecting both the differentiation and survival of neuronal and glial cells (Ernsberger *et al.* 1989; Oppenheim *et al.* 1991; Louis *et al.* 1993), and also shares structural and functional properties with the cytokines interleukin-6, leukemia inhibitory factor, interleukin-11, and oncostatin M (Bazan 1991). For this reason, CNTF is often referred to as a 'neurokine' (Taga and Kishimoto 1992). CNTF promotes the survival of motor neurons in culture (Arakawa *et al.* 1990; Oppenheim *et al.* 1991), and prevents the degeneration of motor neurons after axotomy (Sendtner *et al.* 1990) and in mouse mutant progressive motor neuronopathy (Sendtner *et al.* 1992). These pre-clinical observations encouraged the potential for CNTF as a therapy for human motor neuron disease.

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Address correspondence and reprint requests to Alessandro Negro, CRIBI, University of Padova, Via G. Colombo, 3, 35121 Padova, Italy. E-mail: alessandro.negro@unipd.it

Abbreviations used: CD, circular dichroism; CNTF, ciliary neurotrophic factor; DRG, dorsal root ganglion; HIV, human immunodeficiency virus; JAK, just another kinase; mNGF, mouse nerve growth factor; P2, postnatal day 2; PBS, phosphate-buffered saline; PTD, protein transduction domain; rhCNTF, recombinant human CNTF; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; STAT, signal transducers and activators of transcription; TAT, transactivator.

However, these trials revealed adverse events, such as anorexia and weight loss, and increased deaths at the highest dose level (Miller *et al.* 1996a,b).

In addition to preventing neuronal cell degeneration, systemically administered CNTF causes fever, acute-phase response, weight loss, and cachexia in experimental animals (Shapiro *et al.* 1993; Dittrich *et al.* 1994; Fantuzzi *et al.* 1995; Espat *et al.* 1996; Henderson *et al.* 1996). The effects of CNTF on body weight change and food intake in mice are transient (Fantuzzi *et al.* 1995; Espat *et al.* 1996), and their mechanism is unknown. Receptor subunits for CNTF share sequence similarity with the receptor for leptin (Chen *et al.* 1995), an adipocyte-derived cytokine involved in body weight homeostasis (Henderson *et al.* 1994; Campfield *et al.* 1995; Halaas *et al.* 1995; Pelleymounter *et al.* 1995). CNTF corrects obesity and diabetes associated with leptin deficiency and resistance, indicating an action independent of the leptin system (Gloaguen *et al.* 1997). A recent study suggests that recombinant human CNTF (rhCNTF) reduces weight partly by regulating nuclear respiratory factor 1 and mitochondrial transcription factor A (Liu *et al.* 2007). As such, CNTF is considered as an anti-injury factor and anti-obesity agent (Zvonic *et al.* 2005; Watt *et al.* 2006a).

Ciliary neurotrophic factor lacks a classical signal peptide sequence that serves to direct polypeptides out of the cell following synthesis. It is likely that CNTF resides within the cell, only to be released after injury. Strategies which facilitate intracellular entry of CNTF following administration would thus be of therapeutic interest. To this end, we designed a fusion protein between a protein transduction domain (PTD) (Schwarze *et al.* 1999) derived from the TAT (transactivator) transduction domain of human immunodeficiency virus (HIV) and human CNTF, to promote cellular uptake following injection. The TAT fusion protein strategy is well suited for intracellular translocation of proteins (Gump & Dowdy 2007). The TAT-CNTF protein thus obtained retained *in vitro* neurotrophic activity comparable to rhCNTF, and rescued axotomized motor neurons following sciatic nerve transaction. Unlike rhCNTF, the fusion protein did not affect animal weight or fat tissue mobilization. These data validate the application of designing fusion proteins with a PTD for potential therapeutic benefit (Gump & Dowdy 2007).

Materials and methods

Construction of pTAT-ciliary neurotrophic factor bacterial expression vector

The pTAT-CNTF plasmid was constructed to express the basic domain (amino acids 47–57) of HIV-1 TAT fused with human CNTF. The coding sequence for human CNTF was amplified by PCR using the pT7.7 CNTF vector (Negro *et al.* 1991) as template and the following primers: 5′-GCTCTAGAATGGCTTTCACAGAGCATTCACCG-3′ and 5′-GCGAATTCACATTTTCTTGTTGTTAGCAATA-3′. The PCR product was digested with *Xba*I and *Eco*RI restriction sites and cloned into *Nhe*I and *Eco*RI sites of pTAT-Ngb plasmid (Peroni *et al.* 2007), which contains an N-terminal six-histidine sequence, followed by the transduction domain of the TAT protein.

Expression and purification of TAT-ciliary neurotrophic factor fusion protein

BL21(DE3)LysS *Escherichia coli* transformed with pTAT-CNTF plasmid were plated on Luria broth agar-coated Petri dishes and grown at 37°C overnight. The cells were then suspended in 2 L of Luria broth and cultured at 37°C until reaching an absorbance of OD_{600} = 0.6. Protein expression was induced by adding 0.4 mM isopropyl-β-D-thiogalactopyranoside. After 3 h, the cells were harvested by centrifugation, washed with phosphate-buffered saline (PBS), and lysed by sonication with *buffer A* (200 mM NaCl and 20 mM Tris/HCl, pH 8). The lysate was cleared by centrifugation (30 min at 11 000 *g*) and then incubated with 3 mL of wet Ni(II) nitriloacetic acid agarose in *buffer A* for 1 h; 1 × 10 cm column was filled with the protein-adsorbed agarose resin and exhaustively washed with *buffer A*. The TAT-CNTF fusion protein was eluted with *buffer B* (200 mM NaCl, 20 mM Tris/HCl, and 250 mM imidazole, pH 8) and dialyzed against *buffer A*. The purity of TAT-CNTF was evaluated on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and stained with Coomassie brilliant blue. The purified protein was analyzed also by reverse phase HPLC using a C4 Jupiter column (Phenomenex, Torrance, CA, USA). Molecular mass were determined using an ESI-MS Mariner System 5220 (Applied Biosystems, Foster City, CA, USA) equipped for time-of-flight analysis. Spectra were deconvoluted with Data Explorer 4.0.0.1 software (Applied Biosystems). The obtained molecular weight of 22 780.1 was in good agreement with a theoretical molecular mass of 22 799 for the fusion protein; rhCNTF was produced as described previously (Negro *et al.* 1991), with minor modifications. The protein concentration was calculated using a double-beam Perkin-Elmer (Waltham, MA, USA) lambda 25 UV/Vis spectrophotometer, 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). Far-UV circular dichroism (CD) spectra (200–250 nm) were recorded at 22–24°C using a Jasco (Tokyo, Japan) model 715 spectropolarimeter and a protein concentration of 100–200 μM; results were expressed as mean residue ellipticity. The final spectrum was obtained by averaging five scans, and the baseline corrected by subtracting the spectrum contributed by the buffer.

In vitro transduction of TAT-ciliary neurotrophic factor

Chinese Hamster Ovary cells and SH-SY5Y neuroblastoma cells were routinely cultured at 37°C/5% CO_2 in Roswell Park Memorial Institute medium supplemented with 10% fetal calf serum, penicillin (100 U/mL), and streptomycin (100 μg/mL). For transduction, cell monolayers were washed with PBS and incubated in OptiMEM (Invitrogen, Gaithersburg, MD, USA) then incubated with TAT-CNTF (100 nM) or rhCNTF (100 nM) for 5–10 min, washed with PBS and fixed with 2% *p*-formaldehyde. Protein transduction was evaluated by immunocytochemistry. Cells were permeabilized with 0.5% Triton X-100/PBS at 4°C for 10 min, saturated with 1% bovine serum albumin in PBS, and

incubated at 37°C for 2 h with anti-CNTF monoclonal antibody 8E2 (Cazzola *et al.* 1993). The cells were next washed with PBS and incubated at 37°C for 30 min with alkaline phosphatase-conjugated secondary antibody. After washing with PBS, alkaline phosphatase activity was detected using an alkaline phosphatase detection kit (Chemicon, Temecula, CA, USA), following the manufacturer's instructions. Cells were observed using a Zeiss Axiovert 100 microscope (Carl Zeiss, Oberkochen, Germany) equipped with a 16-bit digital charged-coupled device video camera (MicroMAX; Princeton Instruments/Roper Scientific Trenton, NJ, USA). Alternatively SH-SY5Y cells were lysed in SDS sample buffer and boiled for 5 min, and proteins were resolved on 12% SDS–PAGE followed by western blotting on immobilon-P membranes (Millipore, Bedford, MA, USA) Non-specific binding sites were blocked with 3% bovine serum albumin in PBS before incubation with anti-CNTF monoclonal antibodies for 1 h at 22°C. The membrane was rinsed twice and then incubated for 1 h with alkaline phosphatase-conjugated secondary antibody (Sigma, St Louis, MO, USA) and developed with nitro blue tetrazolium chloride and 5-bromo-3-indolyl-phosphate (Roche, Indianapolis, IN, USA).

Assay for neurotrophic activity

Survival of purified chicken embryonic day 10 dorsal root ganglion (DRG) neurons was assayed essentially as described previously (Skaper *et al.* 1990). Briefly, neurons were seeded at a density of ≈2000 cells/well in laminin- and polyornithine-coated 96-well tissue culture plates (Falcon BD Biosciences, San José, CA, USA) containing 100 μL culture medium (Dulbecco's modified Eagle's medium, 2 mM L-glutamine, 100 U/mL penicillin, and 10% fetal calf serum. Following a 48 h treatment with TAT-CNTF or rhCNTF, or mouse nerve growth factor (mNGF) (Chemicon), cultures were fixed with 2% glutaraldehyde and viable neurons were counted under phase contrast microscopy.

STAT3 phosphorylation *in vitro* and *in vivo*

The human neuroblastoma cell line SH-SY5Y was obtained from Dr June Bidler (Sloan Kettering Institute for Cancer Research). The cells were cultured at 37°C in a humidified incubator under 5% CO_2 using a 1 : 1 mixture of Ham's F12 and Eagle's minimal essential medium supplemented with 10% (v/v) fetal calf serum, 50 U/mL penicillin, and 50 μg/mL streptomycin. At 80% confluence, cells were placed in serum-free medium for 1–5 h before initiation treatment (1 nM TAT-CNTF or rhCNTF). After several times (0–60 min), cell monolayers were harvested in SDS–PAGE sample buffer and proteins (50–100 μg) were resolved by 8% SDS–PAGE following by western blotting as described below. Two-day-old (postnatal day; P2) Wistar rat pups (n = 12) were anesthetized by placing on crushed ice for 3 or 4 min, until painful stimulation ceased to elicit movements. Experimental procedures were approved by the Committee on Animal Care of the State University of Campinas (protocol 509-1). The left sciatic nerve was transected at the sciatic foramen level and ~3 mm of the distal stump was removed. The musculature was repositioned and the skin was closed with two stitches of 8-0 silk suture. After surgery, the rats were allowed to recover from hypothermia under an incandescent lamp. Rats received subcutaneous administration of rhCNTF (1.2 μg/g, n = 4) or TAT-CNTF (1.2 μg/g, n = 4) dissolved in PBS. Dosing was carried out 30 min prior to sciatic nerve transection, immediately following axotomy, and 2 h post-axotomy. The control group was treated at the same times with PBS (n = 4). One hour after the last dosing, rats were killed by decapitation and the spinal cord and hypothalamus were 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 (Metrohm, Riverview, FL, USA) 20s 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 sample was eletrophoretically separated by 8% SDS–PAGE and electroblotted to a nitrocellulose membrane. Membranes were blocked with PBS–Tween containing 5% non-fat dry milk and then incubated with an anti-p-signal transducers and activators of transcription 3 (STAT3) 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 HRP-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). To detect total STAT3 expression, membranes were stripped with a glycine–HCl solution, pH 2.7, and immunoblotting was performed using an anti-STAT3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Animal treatments

Wister rat pups at P2 (n = 58) were anesthetized by hypothermia as described above. The left sciatic nerve was transected at the sciatic foramen level and ~3 mm of the distal stump was removed. For local treatment, immediately after the lesion, a piece of gel-foam soaked with 20 μL of rhCNTF (0.3 μg/μL in PBS; n = 7) or TAT-CNTF (0.3 μg/μL and 0.15 μg/μL in PBS; n = 7 and n = 6, respectively) was placed in direct contact with the proximal nerve stump. The control group received a piece of gel-foam soaked with the same volume of PBS (n = 5). The musculature was repositioned and the skin was closed with two stitches of 8-0 silk suture. After surgery, the rats were allowed to recover from hypothermia under an incandescent lamp and were returned to their mothers. For systemic treatment, rhCNTF and TAT-CNTF dissolved in PBS were administered subcutaneously. The animals received rhCNTF (1.2 μg/g, n = 7) or TAT-CNTF (0.3 μg/g, n = 6; 0.6 μg/g, n = 8; and 1.2 μg/g, n = 7). Dosing was carried out 30 min prior to sciatic nerve transection, immediately following axotomy, 2 h post-axotomy, and then daily (between 12:00 and 13:00 h) for 5 days. The control group was treated at the same times with PBS (n = 5). A further group of 12 non-operated animals was treated with daily subcutaneous doses of 1.2 μg/g rhCNTF (n = 4), 1.2 μg/g TAT-CNTF (n = 4), or PBS (n = 4) between P2 and P7, after which time they were killed. The selection of CNTF dosing was based on those used in previous *in vivo* studies by our group (Oliviera *et al.* 2002; Rezende *et al.* 2008) and others (Sendtner *et al.* 1990; Kwon and Gurney 1994).

Evaluation of body weight

All animals were weighed daily and prior to treatment, using an Acculab V-200 balance (Sartorius, Goetting, Germany) with a precision of ± 0.01 g. Data were analyzed through the repeated measures ANOVA, followed by Duncan's multiple range test (SAS System, SAS Institute, Cary, NC, USA).

Tissue preparation

On the fifth day post-axotomy, rats were anesthetized with sodium pentobarbital 4% (0.1 mL/20 g; i.p.) and perfused through the heart with saline followed by 40 mL of freshly prepared 4% *p*-formaldehyde in PBS (0.1 M; pH 7.4). The lumbar spinal cord was carefully dissected and post-fixed in the same fixative for 24 h at 4°C. The specimens were rinsed with water, dehydrated with increasing concentrations of ethanol (70–100%), and embedded with paraffin. Transverse serial sections (8 μm) from the L4 and L5 segments of the lumbar enlargement were cut and transferred to chrome alum gelatin-coated slides and stained with cresyl violet. Interscapular brown adipose tissue was collected from operated animals treated with daily subcutaneous doses of 1.2 μg/g rhCNTF or TAT-CNTF, or PBS and immediately immersion fixed in 4% *p*-formaldehyde/0.1 M PBS, pH 7.4. The tissue specimens were maintained in the same fixative for 24 h at 4°C and then washed in PBS, dehydrated through a graded series of ethanols to 96% and embedded in hydroxyethyl methacrylate (Historesin – Leica 7022-18-500; Leica Microsystems GmbH, Wetzlar, Germany). Embedded material was sectioned (thickness: 2 μm), mounted on slides and stained with toludine blue. Photomicrographs (spinal cord and adipose brown tissue) were taken using a photomicroscope (Leica DMLB) equipped with a digital camera (Leica DC 300F).

Motor neuron counting

For each animal, motor neuron counting was performed in 20 serial sections (the first out of every five) of levels L4 and L5 stained with cresyl violet. Cell counting was undertaken by an observer blind to the treatment. Only large multipolar motor neurons of the ventrolateral group (lamina IX) containing a clearly visible nucleolus in the plane of section were counted (Rogerio *et al.* 2002). The unoperated side of the spinal cord was used as control. Neuronal survival ratio, defined as the ratio between the motoneurons counted in the operated and control sides, was subjected to ANOVA followed by the Student–Newman–Keul's test (GRAPHPAD INSTAT Software, San Diego, CA, USA).

Results

Synthesis, characterization, and neurotrophic activity of TAT-CNTF

Ciliary neurotrophic factor lacks a signal peptide that normally directs polypeptides out of the cell following synthesis and, as such remains within the cell only to be released after injury. We designed a fusion protein between the TAT transduction domain of HIV and human CNTF, to facilitate cellular uptake following injection. The TAT fusion protein strategy is well suited for intracellular translocation of proteins (Gump and Dowdy 2007). Purification of TAT-CNTF to homogeneity was achieved under non-denaturing conditions by affinity chromatography using Nickel-nitrilotriacetic acid resin. The purified protein exhibited an apparent molecular weight of 28 kDa when subjected to SDS–PAGE under reducing conditions (Fig. 1a). Reverse phase HPLC analysis showed a unique sharp peak at around 68% acetonitrile (Fig. 1b). Mass spectrometry indicated a molecular weight of 26 525.61 Da, in good agreement with a calculated molecular weight of 26 525.33 Da. Antibodies against rhCNTF recognized TAT-CNTF (Cazzola *et al.* 1993), demonstrating the correctness of the construct (data not shown).

Ciliary neurotrophic factor is a member of a large cytokine family of proteins that display a four α-helix bundle topology (McDonald *et al.* 1995). The far-UV CD spectrum of TAT-CNTF showed a maximum at 194 nm and two minima centered at 208 and 221 nm, confirming the presence of a high percentage of α-helical secondary structure, as described for rhCNTF (Negro *et al.* 1991) (Fig. 1c), indicating that the TAT domain and histidine tag fused to CNTF did not alter its secondary structure (compare the secondary structures of rhCNTF and TAT-CNTF).

Ciliary neurotrophic factor and related cytokines induce the activation of receptor-associated just another kinase (JAK)/Tyk tyrosine kinases and subsequent recruitment and phosphorylation of STAT proteins, in particular STAT3 (Lelièvre *et al.* 2001). Phosphorylated STAT proteins dimerize and translocate to the nucleus where they activate target gene transcription (Heinrich *et al.* 1998; Levy and Darnell 2002). Incubation of human neuroblastoma SH-SH5Y cells, which express an intact CNTF receptor complex (Kaur *et al.* 2002), to rhCNTF or TAT-CNTF caused a rapid and sustained increase in STAT3 phosphorylation (Fig. 2).

Ciliary neurotrophic factor is capable of supporting the survival of cultured chicken embryonic day 10 DRG neurons (Barbin *et al.* 1984), and this culture system was used to evaluate the biological activity of TAT-CNTF. Robust survival of DRG neurons was observed at the morphological level in cultures supplemented with 1 nM rhCNTF, 1 nM TAT-CNTF, or 1 nM mNGF, but not TAT-green fluorescent protein. These results are presented quantitatively in Fig. 3, with EC_{50} of 0.037 nM, 0.042 nM, and 0.076 nM for rhCNTF, TAT-CNTF, and mNGF, respectively. The neurotrophic activity of TAT-CNTF is thus likely attributable to the CNTF domain, with no interference from either the TAT domain or histidine tag. This is not unexpected, as the 14 amino acid N-terminus of CNTF is not required for neurotrophic activity (Negro *et al.* 1997).

TAT fusion proteins readily translocate into cells (Gump and Dowdy 2007). Incubation of Chinese Hamster Ovary cells with TAT-CNTF resulted in internalization of the fusion protein within minutes, while rhCNTF showed no such effect (Fig. 4a and b). TAT-CNTF was present mainly in the

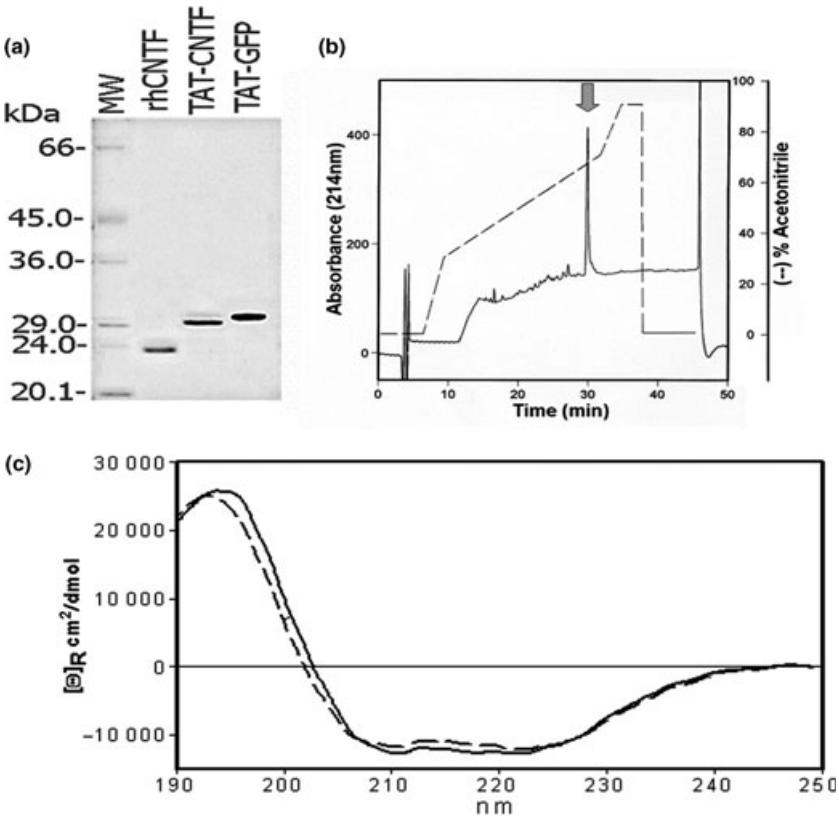

Fig. 1 Characterization of recombinant CNTF proteins. (a) Purified TAT-green fluorescent protein (GFP), TAT-CNTF, and rhCNTF (2 μg each) were subjected to SDS–PAGE (12% polyacrylamide) and the gel stained with Coomassie brilliant blue. (b) Reverse phase HPLC profile of purified TAT-CNTF. The fusion protein (arrow) elutes from a C4 reverse phase column at a high concentration of acetonitrile (65%), as reported for rhCNTF (Negro *et al.* 1991). (c) CD analysis of secondary structures of TAT-CNTF (- - -) and rhCNTF (—). CD spectra were recorded at 25°C using a Jasco 810 spectropolarimeter and 2 μM of each protein. The presence of minima at 220 and 208 nm and a maximum at 195 nm is indicative of a high α-helical content.

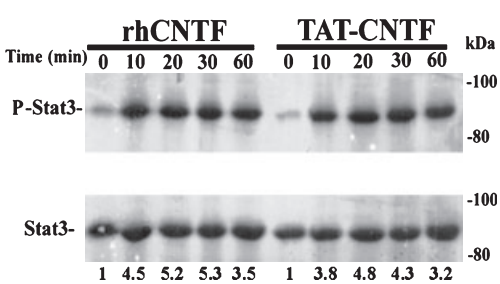

Fig. 2 TAT-CNTF and rhCNTF induce STAT3 phosphorylation in human SH-5Y5Y neuroblastoma cells. Cells were incubated with 1 μM each of rhCNTF or TAT-CNTF. Cell lysates were subjected to western blotting and probed with phospho-STAT3 (P-Stat3) antibody, stripped, and reprobed for total STAT3. A representative blot is shown, with values for fold-increase given below each lane (normalized to total STAT3 amounts). Similar results were obtained in three other experiments.

nucleus, as observed for natural CNTF (Bajetto *et al.* 1999). This is further illustrated by immunoblot of SH-SY5Y neuroblastoma cells incubated with TAT-CNTF or rhCNTF (Fig. 4, bottom panel), whereby TAT-CNTF (but not rhCNTF) is found intracellularly and decreases with time. The low molecular weight of CNTF, together the presence of several basic residues in the TAT domain may facilitate CNTF nuclear localization.

TAT-CNTF rescues axotomized motor neurons after sciatic nerve transection

The neuroprotective action of TAT-CNTF was evaluated using peripheral nerve lesion in neonatal rats. Rodent motor neurons are highly susceptible to injury when the peripheral nerve is transected during the first week of life (Schmalbruch 1987; Pollin *et al.* 1991), perhaps because CNTF expression in peripheral nerve is very low during the period CNTF-responsive neurons undergo developmentally programmed cell death (Stöckli *et al.* 1991). CNTF prevents the degeneration of these motor neurons after axotomy, and the effect is most evident between P2 and P7 (Sendtner *et al.* 1990). Subcutaneous or local injection of either rhCNTF or TAT-CNTF each reduced the morphological degenerative changes in spinal cord motor neurons following sciatic nerve axotomy (Fig. 5), in keeping with earlier reports for CNTF (Sendtner *et al.* 1990). These results are presented quantitatively in Fig. 6, where both rhCNTF and TAT-CNTF are seen to significantly reduce this motor neuron loss by comparable extents, demonstrating the biological efficacy of TAT-CNTF.

Body weight

While performing the nerve lesion experiments, we observed a decrease in body weight of rat pups treated subcutaneously with rhCNTF, in comparison to pups treated with TAT-CNTF or PBS. Rats receiving rhCNTF by this route displayed a 25% reduction in body weight versus rats treated with TAT-

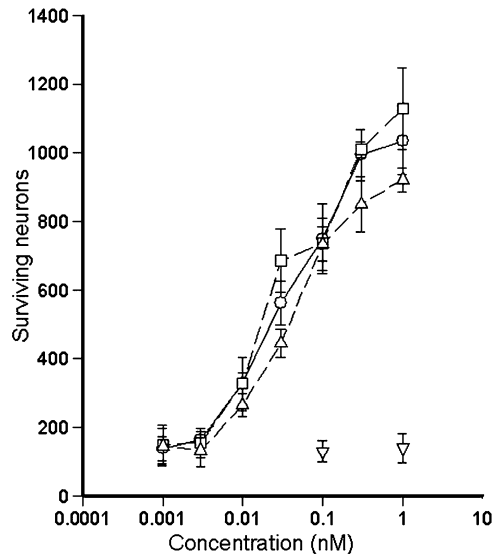

Fig. 3 Quantitative assessment of TAT-CNTF neurotrophic activity. Chicken embryonic day 10 DRG neurons were cultured for 48 h with the indicated concentrations of rhCNTF (□), TAT-CNTF (○), TAT-green fluorescent protein (▽), mNGF (△), or medium only. Cultures were then fixed and viable neurons counted under phase contrast microscopy. Values are numbers of surviving neurons (mean ± SD) of quadruplicate cultures from each of two experiments.

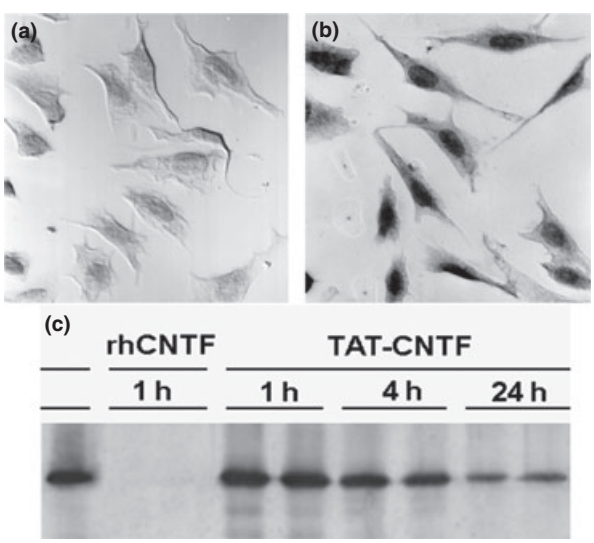

Fig. 4 *In vitro* transduction of TAT-CNTF. CHO cells were incubated 30 min with 10 μM rhCNTF (a) or 10 μM TAT-CNTF (b). After washing with PBS, cells were immunostained using antibodies against human CNTF. Note the internalization of TAT-CNTF, but not rhCNTF. (c) SH-SY5Y neuroblastoma cells were incubated for 1, 4, and 24 h with 100 μM rhCNTF or 100 μM TAT-CNTF. Following cell lysis, proteins were separated on 12% SDS–PAGE, transferred to Immobilon-P membranes, and immunostained with anti-rhCNTF antibodies. Note the internalization of TAT-CNTF at 1 h (but not rhCNTF), which decreases by 24 h.

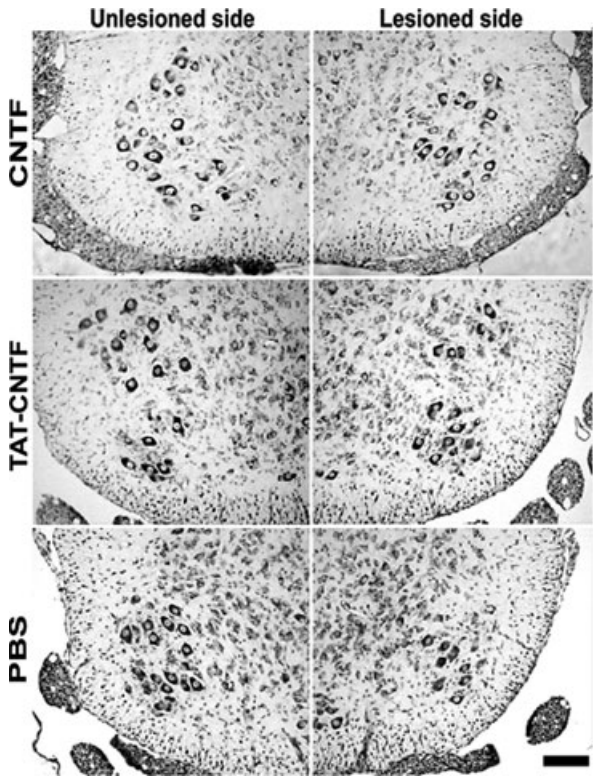

Fig. 5 Representative spinal cord lumbar sections of 7-day-old rats treated subcutaneously with rhCNTF or TAT-CNTF. P2 pups were subjected to sciatic nerve transection, and received rhCNTF (1.2 μg/g), TAT-CNTF (1.2 μg/g), or PBS daily for 5 days. The population of motor neurons in the ventrolateral group of the lesioned side (PBS only) are reduced in number and appear shrunken, while cells in animals treated with rhCNTF or TAT-CNTF are similar to unlesioned control. Scale bar, 100 μm.

CNTF or PBS (Fig. 7). CNTF-induced-cachexia has been known for some time (Henderson *et al.* 1996), and presents a serious impediment in the therapeutic application of this protein to human neurological diseases, such as motor neuron disease (Miller *et al.* 1996a).

Loss of body weight in animals and humans is associated with alterations in adipose tissue. The effect of rhCNTF and TAT-CNTF administration on interscapular brown adipose tissue of neonatal rats treated between P2 and P7 was examined, and rhCNTF was found to significantly decrease tissue weight versus either the TAT-CNTF or PBS groups (Fig. 8, upper panel). Brown adipose tissue from the rhCNTF group showed a dramatic decrease in lipid vesicles, in contrast to the abundance of lipid vesicles in both the TAT-CNTF and PBS groups (Fig. 8, lower panel).

TAT-CNTF does not induce STAT3 phosphorylation in hypothalamus

Ciliary neurotrophic factor-induced weight loss is associated with activation of hypothalamic neurons (Watt *et al.* 2006b).

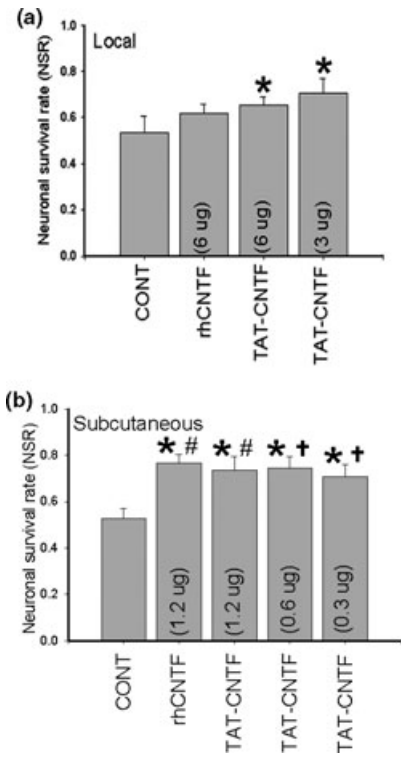

Fig. 6 TAT-CNTF rescues axotomized motor neurons from death. Following sciatic nerve transaction at P2, one cohort of rats (panel a) received locally rhCNTF (6 μg, $n = 7$) or TAT-CNTF (6 or 3 μg, $n = 6$ and 7, respectively). A second cohort of lesioned rats (panel b) were dosed subcutaneously once daily with either rhCNTF (1.2 μg/g, $n = 7$) or TAT-CNTF (1.2 μg/g, $n = 7$; 0.6 μg/g, $n = 8$; 0.3 μg/g, $n = 6$, respectively). The ratio between the number of ventrolateral motor neurons counted in the lesioned and contralateral sides (neuronal survival ratio; NSR) of lumbar enlargement at 5 days after sciatic nerve transection was determined. NSR values are given as mean ± SD). NSR values for rats in all TAT-CNTF treatment groups differed significantly ($^*p < 0.05$) from their respective PBS (control) groups, but were not different ($p > 0.05$) from the corresponding rhCNTF group Moreover, daily subcutaneous dosing of rhCNTF or TAT-CNTF (1.2 μg/g) was more efficacious than the locally applied protein (6 μg/g) ($^\#p < 0.05$), while rats treated subcutaneously with 0.3–0.6 μg/g TAT-CNTF had NSR values significantly greater ($^\dagger p < 0.05$) than rats which received 6 μg rhCNTF locally.

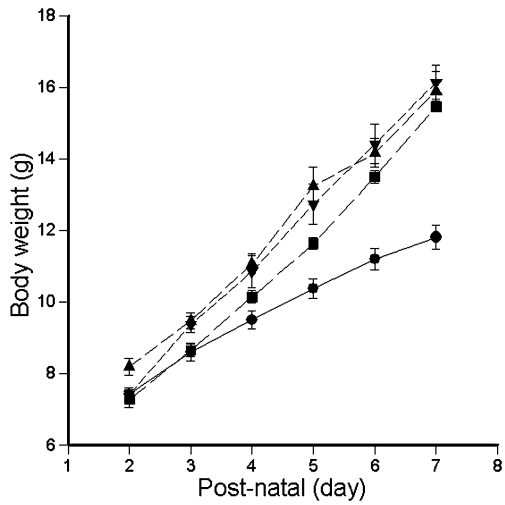

Fig. 7 TAT-CNTF, unlike rhCNTF does not affect body weight of rat pups. Neonatal rats at P2 were dosed subcutaneously daily for 5 days with (●) 1.2 μg/g rhCNTF ($n = 7$), (▼, ■) TAT-CNTF (0.3 or 1.2 μg/g; $n = 6$ and 7, respectively) or (▲) PBS ($n = 5$). Weight gain in rats treated with TAT-CNTF did not differ significantly from the control group between P2 and P7. After three days of treatment with rhCNTF, rat pups showed a reduction in body weight gain when compared with the other groups ($p < 0.05$). At P7 rats which received rhCNTF had ~25% lower body weight than pups which received TAT-CNTF. Data are mean ± SEM.

Subcutaneous administration of rhCNTF to P2 rat pups induced robust STAT3 phosphorylation in the spinal cord, while TAT-CNTF treatment resulted in a significantly less intense signal (Fig. 9, left panels). In the hypothalamus, in contrast, rhCNTF but not TAT-CNTF, caused a significant increase in STAT3 phosphorylation (Fig. 9, right panels).

Discussion

The power of neurotrophic factors to regulate neuronal cell survival in the developing nervous system and to promote also survival after injury or protect neurons in toxin-mediated disease models in animals has encouraged the idea that such proteins could be applied in the treatment of neurodegenerative disease. One such molecule, CNTF, is capable of rescuing motor neurons after axotomy (Sendtner *et al.* 1990) and after nerve lesion in mutant progressive motor neuronopathy mice (Sendtner *et al.* 1992; Sagot *et al.* 1995). In spite of positive pre-clinical findings, trials of CNTF for human motor neuron disease resulted in severe side effects, including cachexia and a marked immune response (Miller *et al.* 1996a,b; Turner *et al.* 2001; Ettinger *et al.* 2003). To circumvent these deleterious effects, we engineered a chimeric molecule in which rhCNTF was fused to a PTD derived from the HIV-1 TAT protein. The TAT-CNTF fusion protein crossed the plasma membrane within minutes and displayed a nuclear localization, and was equipotent to rhCNTF in supporting the survival of cultured sensory ganglion neurons. Local or subcutaneous administration of both TAT-CNTF and rhCNTF rescued motor neurons from injury following sciatic nerve transection in neonatal rats. While rhCNTF given subcutaneously significantly decreased body weight and caused fat mobilization in brown adipose tissue, TAT-CNTF lacked such side effects.

Protein transduction strategies are well suited for intracellular translocation of proteins (Gump and Dowdy 2007). The TAT sequence and PTD are capable, in principle, of crossing

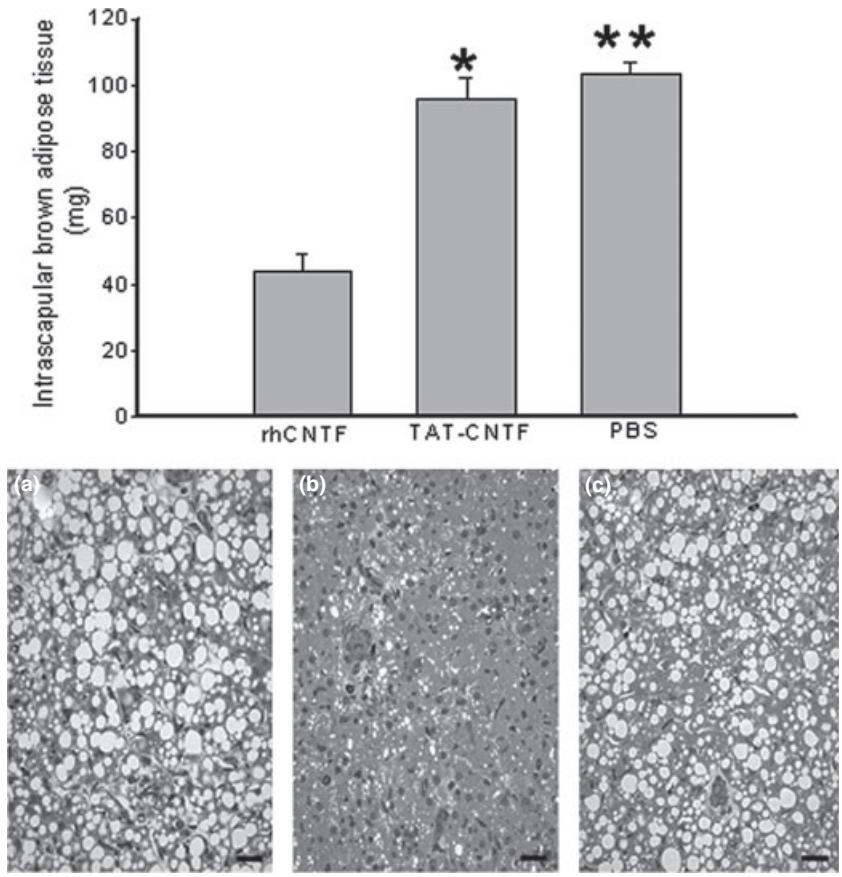

Fig. 8 Recombinant human CNTF (rhCNTF) decreases brown adipose tissue weight in rat pups. Upper panel: rat pups (P2) were dosed subcutaneously daily for 5 days with 1.2 μg/g of rhCNTF, 1.2 μg/g of TAT-CNTF or PBS, after which time intrascapular brown adipose tissue was collected. Values are mean ± SEM; $*p < 0.05$ and $**p < 0.001$ versus rhCNTF. Lower panel: rhCNTF, but not TAT-CNTF decreases lipid vesicle content of brown adipose tissue. Sections of intrascapular brown adipose tissue were taken from P7 rat pups dosed subcutaneously daily for 5 days with PBS (a), 1.2 μg/g of rhCNTF (b), or 1.2 μg/g of TAT-CNTF (c) beginning on P2. Tissue from pups that received rhCNTF displayed adipocytes with a few, small fat droplets (b), in contrast to tissue from pups treated with TAT-CNTF (c) or PBS (a). Note the similarity in tissue morphology between (a and c), with cells closely packed and showing a nucleus in an eccentric position and fat droplets of varying size (bar, 50 μm).

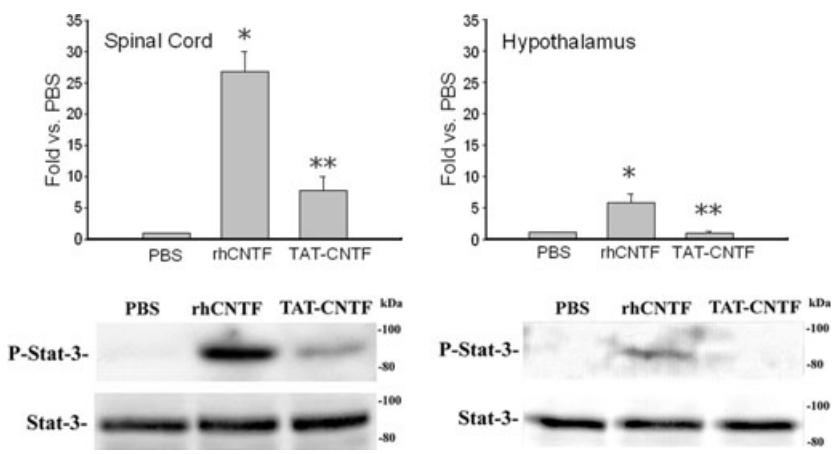

Fig. 9 Differential effects of rhCNTF and TAT-CNTF on STAT3 phosphorylation in neonatal rat spinal cord and hypothalamus. Rat pups at P2 received subcutaneously either rhCNTF or TAT-CNTF (1.2 μg/g body weight). An equal volume of PBS was given to control rats. Phospho-specific immunoreactivity was determined as described under Materials and methods, and quantified by densitometric analysis and expressed relative to total STAT3 protein (mean ± SD, three independent experiments, expressed as fold-increase over PBS-treated group). A representative immunoblot is shown for spinal cord (left panels) and hypothalamus (right panels); $*p < 0.04$ versus PBS and $**p < 0.05$ versus rhCNTF.

the plasma membrane in any cell type. For example, a TAT-β-galactosidase fusion protein injected in the rat tail vein was found widely distributed in brain (Schwarze *et al.* 1999). In this study, subcutaneous treatment with rhCNTF (1.2 μg/g/day) or TAT-CNTF (0.3–1.2 μg/g/day) was more effective than local administration of rhCNTF alone. Moreover, the

neuronal survival ratio in rats receiving subcutaneous TAT-CNTF (0.3 μg/g/day) was similar to the obtained with 1.2 μg/g/day of rhCNTF, suggesting that the PTD of TAT-HIV did not compromise the neurotrophic action of CNTF. Importantly, TAT-CNTF given subcutaneously did not alter neonatal development between P2 and P7 or nor induce a failure to thrive, the latter action being responsible for weight loss in adult rats treated with CNTF (Zhang *et al.* 1995; Henderson *et al.* 1996).

Ciliary neurotrophic factor and related cytokines induce the activation of receptor-associated JAK/Tyk tyrosine kinases and subsequent recruitment and phosphorylation of STAT proteins, in particular STAT3 (Lelièvre *et al.* 2001) Phosphorylated STAT proteins dimerize and translocate to the nucleus where they activate target gene transcription (Heinrich *et al.* 1998; Levy and Darnell 2002). Endogenous CNTF acts directly on lesioned motor neurons as a retrograde signal and is responsible for the induction of STAT3 signaling in these cells but not the maintenance of STAT3 activation beyond the first day after axotomy (Kirsch *et al.* 2003). CNTF has been shown to activate the mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt signaling pathways by recruitment and phosphorylation of the tyrosine phosphatase-2, and interaction of these pathways with STAT3 signaling has been reported (Hirano *et al.* 1997; Alonzi *et al.* 2001; Lelièvre *et al.* 2001). In cultured avian motor neurons, CNTF-stimulated survival was dependent on activation of the phosphatidylinositol 3-kinase/Akt pathway, which was induced by a JAK/STAT-dependent mechanism (Dolcet *et al.* 2001). Whether or not these intracellular signaling pathways play a role in the response of motor neurons to axotomy in wild-type animals remains to be established. These observations suggest that STAT phosphorylation at the site of injury occurs at the somal rather than the axonal level. Retrograde transport of CNTF is increased after peripheral nerve injury (Curtis *et al.* 1993) along with synthesis in the sciatic nerve after lesion (Sendtner *et al.* 1992), proposing a model in which CNTF released from Schwann cells at the lesion site activates the CNTF receptor complex located at the proximal axon stump. The complex is then internalized and retrogradely transported to the cell body where STAT3 is activated by receptor-associated JAK kinases (Haas *et al.* 1999) and then translocated to the nucleus. The rescue effect of TAT-CNTF on axotomized lumbar motor neurons at a dose of 0.3 μg/g/day is not inconsistent with a receptor-mediated action, and insertion of TAT-CNTF in the lipid bilayer through its PTD region may favor CNTF interaction with cell surface receptors. Indeed, both TAT-CNTF and rhCNTF induced STAT3 phosphorylation in the spinal cord on neonatal rats, although only rhCNTF was active in the hypothalamus. The reason for this difference is not known, but is consistent with the effects of rhCNTF (but not TAT-CNTF) on body weight.

Ciliary neurotrophic factor is thought to induce body weight loss by activation of the JAK/STAT pathway, similar to leptin (Lambert *et al.* 2001; Zvonic *et al.* 2003a; Ott *et al.* 2004). Activation of CNTF receptors on adipocytes can regulate energy homeostasis in these cells (Halaas *et al.* 1995; Lambert *et al.* 2001; Zvonic *et al.* 2003b; Ott *et al.* 2004) CNTF could induce weight loss also via a central action, by reducing neuropeptide Y expression in neurons of the hypothalamic arcuate nucleus (Xu *et al.* 1998). This nucleus mediates appetite suppression and weight loss in response to CNTF and leptin (Anderson *et al.* 2003). Lastly, intracerebroventricular injection of CNTF in rats reduced adipose tissue mass in epididimal, inguinal, and tissue retroperitoneal adipose tissue and in intrascapular brown adipose tissue (Duff *et al.* 2004). The weight-reducing effects of CNTF are seen in both leptin-deficient (*ob/ob*) and leptin-resistant (*db/db*) mice, suggesting that CNTF effects are not mediated by the release of leptin or activation of leptin receptors. Thus, the effects of CNTF and leptin on body weight, adipose tissue, and energy expenditure likely invoke distinct but converging hypothalamic neuronal pathways.

The absence of weight loss in rats treated with TAT-CNTF could, conceivably, be because of the protein's inability to activate signaling pathways in both adipocytes and hypothalamic neurons (assuming that TAT-CNTF is capable of crossing the blood–brain barrier). In this regard, and unlike TAT-CNTF, rhCNTF is unable to induce STAT3 phosphorylation in hypothalamic neurons *in vivo*, although both proteins stimulated STAT3 phosphorylation in cultured SH-SY5Y neuroblastoma cells. This distinction *in vivo* may underlie the inability of TAT-CNTF to provoke weight loss and fat mobilization. Interestingly, TAT-CNTF-induced STAT3 phosphorylation in spinal cord did not reach the level achieved with rhCNTF, suggesting that TAT-CNTF is able to translocate into cells in the proximity of the administration site, but unable to reach more distant CNS sites (brain parenchyma). Subcutaneously administered CNTF crosses the blood–brain barrier rapidly, with a rate of entry (K_i) of 4.60 (± 0.78) × 10^{-4} mL/g min, considerably faster than that of the 99mTc-albumin control (Pan *et al.* 1999). The K_i was reduced more than threefold by addition of excess unlabeled CNTF, indicating that CNTF transport from blood to brain achieves saturation (Pan *et al.* 1999). That TAT-CNTF follows the same route cannot be discounted.

In conclusion, we describe a TAT-CNTF protein which retains full neurotrophic activity and rescues axotomized motor neurons. The fusion protein did not affect animal weight or fat tissue mobilization. These data support the application of designing fusion proteins with a PTD for potential therapeutic benefit, including neurodegenerative diseases without undesirable collateral effects.

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References

Alonzi T., Middleton G., Wyatt S., Buchman V., Betz U. A. K., Müller W., Musiani P., Poli V. and Davies A. M. (2001) Role of STAT3 and PI 3-kinase/Akt in mediating the survival actions of cytokines on sensory neurons. *Mol. Cell. Neurosci.* **18**, 270–282.

Anderson K. D., Lambert P. D., Corcoran T. L., Murray J. D., Thabet K. E., Yancopoulos G. D. and Wiegand S. J. (2003) Activation of the hypothalamic arcuate nucleus predicts the anorectic actions of ciliary neurotrophic factor and leptin in intact and gold thioglucose-lesioned mice. *J. Neuroendocrinol.* **15**, 649–660.

Arakawa Y., Sendtner M. and Thoenen H. (1990) Survival effect of ciliary neurotrophic factor (CNTF) on chick embryonic motoneurons in culture: comparison with other neurotrophic factors and cytokines. *J. Neurosci.* **10**, 3507–3515.

Bajetto A., Schettini G. and Chimini G. (1999) Nuclear localization of ciliary neurotrophic factor in glial cells. *Brain Res.* **818**, 565–569.

Barbin G., Manthorpe M. and Varon S. (1984) Purification of the chick eye ciliary neuronotrophic factor. *J. Neurochem.* **43**, 1468–1478.

Bazan J. F. (1991) Neuropoietic cytokines in the hematopoietic fold. *Neuron* **7**, 197–208.

Campfield L. A., Smith F. J., Guisez Y., Devos R. and Burn P. (1995) Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. *Science* **269**, 546–549.

Cazzola F., Battiston S., Fabris M., Negro A., Corona G. and Callegaro L. (1993) Production and characterization of monoclonal antibodies to human ciliary neurotrophic factor with defined epitope recognition. *Hybridoma* **12**, 259–270.

Chen H., Charlat O., Tartaglia L. A. *et al.* (1995) Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in db/db mice. *Science* **84**, 491–495.

Curtis R., Adryan K. M., Zhu Y., Harkness P. J., Lindsay R. M. and DiStefano P. S. (1993) Retrograde transport of ciliary neurotrophic factor is increased by peripheral nerve injury. *Nature* **365**, 253–255.

Dittrich F., Thoenen H. and Sendtner M. (1994) Ciliary neurotrophic factor: pharmacokinetics and acute-phase response in rat. *Ann. Neurol.* **35**, 151–163.

Dolcet X., Soler R. M., Gould T. W., Egea J., Oppenheim R. W. and Comella J. X. (2001) Cytokines promote motoneuron survival through the Janus kinase-dependent activation of the phosphatidylinositol 3-kinase pathway. *Mol. Cell. Neurosci.* **18**, 619–631.

Duff E., Li C. L., Hartzell D. L., Choi Y.-H., Della-Fera M. A. and Baile C. A. (2004) Ciliary neurotrophic factor injected icv induces adipose tissue apoptosis in rats. *Apoptosis* **9**, 629–634.

Ernsberger U., Sendtner M. and Rohrer H. (1989) Proliferation and differentiation of embryonic chick sympathetic neurons: effects of ciliary neurotrophic factor. *Neuron* **2**, 1275–1284.

Espat N. J., Auffenberg T., Rosenberg J. J., Rogy M., Martin D., Fang C. H., Hasselgren P. O., Copeland E. M. and Moldawer L. L. (1996) Ciliary neurotrophic factor is catabolic and shares with IL-6 the capacity to induce an acute phase response. *Am. J. Physiol.* **271**, R185–R190.

Ettinger M. P., Littlejohn T. W., Schwartz S. L. *et al.* (2003) Recombinant variant of ciliary neurotrophic factor for weight loss in obese adults. A randomized, dose-ranging study. *J. Am. Med. Assoc.* **289**, 1826–1832.

Fantuzzi G., Benigni F., Sironi M., Conni M., Carelli M., Cantoni L., Shapiro L., Dinarello C. A., Sipe J. D. and Grezzi P. (1995) Ciliary neurotrophic factor (CNTF) induces serum amyloid A, hypoglycaemia and anorexia, and potentiates IL-1 induced corticosterone and IL-6 production in mice. *Cytokine* **7**, 150–156.

Gill S. C. and von Hippel P. H. (1989) Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.* **182**, 319–326.

Gloaguen I., Costa P., Demartis A. *et al.* (1997) Ciliary neurotrophic factor corrects obesity and diabetes associated with leptin deficiency and resistance. *Proc. Natl Acad. Sci. USA* **94**, 6456–6461.

Gump J. M. and Dowdy S. F. (2007) TAT transduction: the molecular mechanism and therapeutic prospects. *Trends Mol. Med.* **13**, 443–448.

Haas C. A., Hofmann H. D. and Kirsch M. (1999) Expression of CNTF/LIF receptor components and activation of STAT3 signaling in axotomized motoneurons: evidence for a sequential postlesional function of the cytokines. *J. Neurobiol.* **41**, 559–571.

Halaas J. L., Gajiwala K. S., Maffei M., Cohen S. L., Chait B. T., Rabinowitz D., Lallone R. L., Burley S. K. and Friedman J. M. (1995) Weight-reducing effects of the plasma protein encoded by the obese gene. *Science* **269**, 543–546.

Heinrich P. C., Behrmann I., Müller-Newen G., Schaper F. and Graeve L. (1998) Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *Biochem. J.* **334**, 297–314.

Henderson J. T., Seniuk N. A., Richardson P. M., Gauldie J. and Roder J. C. (1994) Systemic administration of ciliary neurotrophic factor induces cachexia in rodents. *J. Clin. Invest.* **93**, 2632–2638.

Henderson J. T., Mullen B. J. M. and Roder J. C. (1996) Physiological effects of CNTF-induced wasting. *Cytokine* **8**, 784–793.

Hirano T., Nakajima K. and Hibi M. (1997) Signaling mechanisms through gp130: a model of the cytokine system. *Cytokine Growth Factor Rev.* **8**, 241–252.

Kaur N., Wohlhueter A. L. and Halvorsens W. (2002) Activation and inactivation of signal transactivator and activators of transcription by ciliary neutrotrophic factor in neuroblastoma cells. *Cell Signal* **14**, 419–429.

Kirsch M., Terheggen U. and Hofmann H.-D. (2003) Ciliary neurotrophic factor is an early lesion-induced retrograde signal for axotomized facial motoneurons. *Mol. Cell. Neurosci.* **24**, 130–138.

Kwon Y. W. and Gurney M. E. (1994) Systemic injections of ciliary neurotrophic factor induce sprouting by adult motor neurons. *NeuroReport* **5**, 789–792.

Lambert P. D., Anderson K. D., Sleeman M. W. *et al.* (2001) Ciliary neurotrophic factor activates leptin-like pathways and reduces body fat, without cachexia or rebound weight gain, even in leptin-resistant obesity. *Proc. Natl Acad. Sci. USA* **98**, 4652–4657.

Lelièvre E., Plun-Favreau H., Chevalier S., Froger J., Guillet C., Elson G. C. A., Gauchat J.-F. and Gascan H. (2001) Signaling pathways recruited by the cardiotrophin-like cytokine/cytokine-like factor-1 composite cytokine. *J. Biol. Chem.* **276**, 22476–22484.

Levy D. E. and Darnell J. E. Jr (2002) STATs: transcriptional control and biological impact. *Nat. Rev. Mol. Cell Biol.* **3**, 651–662.

Liu Q.-S., Wang Q.-J., Du G.-H., Zhu S.-Y., Gao M., Zhang L., Zhu J.-M. and Cao J.-F. (2007) Recombinant human ciliary neurotrophic factor reduces weight partly by regulating nuclear respiratory factor 1 and mitochondrial transcription factor A. *Eur. J. Pharmacol.* **563**, 77–82.

Louis J.-C., Magal E., Takayama S. and Varon S. (1993) CNTF protection of oligodendrocytes again natural and tumor necrosis factor-induced death. *Science* **259**, 689–692.

McDonald N. Q., Panayotatos N. and Hendrickson W. A. (1995) Crystal structure of dimeric human ciliary neurotrophic factor determined by MAD phasing. *EMBO J.* **14**, 2689–2699.

Miller R. G., Petajan J. H., Btyan W. W., Armon C., Barohn R. J., Goodpasture J. C., Hoagland R. J., Parry G. J., Ross M. A. and Stromatt S. C. (1996a) A placebo-controlled trial of recombinant human ciliary neurotrophic (rhCNTF) factor in amyotrophic lateral sclerosis. rhCNTF ALS Study Group. *Ann. Neurol.* **39**, 256–260.

Miller R. G., Bryan W. W., Dietz M. A., Munsat T. L., Petajan J. H., Smith S. A. and Goodpasture J. C. (1996b) Toxicity and tolerability of recombinant human ciliary neurotrophic factor in patients with amyotrophic lateral sclerosis. *Neurology* **47**, 1329–1331.

Negro A., Corona G., Bigon E., Martini I., Grandi C., Skaper S. D. and Callegaro L. (1991) Synthesis, purification and characterization of human ciliary neurotrophic factor from *E. coli*. *J. Neurosci. Res.* **29**, 251–260.

Negro A., Grassato L., Polverino De Laureto P. and Skaper S. D. (1997) Genetic construction, properties and application of a green fluorescent protein tagged ciliary neurotrophic factor. *Protein Eng.* **10**, 1077–1083.

Oliviera A. L., Risling M., Negro A., Langone F. and Cullheim S. (2002) Apoptosis of spinal interneurons induced by sciatic nerve axotomy in the neonatal rat is counteracted by nerve growth factor and ciliary neurotrophic factor. *J. Comp. Neurol.* **447**, 381–393.

Oppenheim R. W., Prevette D., Yin Q.-W., Collins F. and MacDonald J. (1991) Control of embryonic motoneuron survival *in vivo* by ciliary neurotrophic factor. *Science* **251**, 1616–1618.

Ott V., Fasshauber M., Meier B., Dalski A., Kraus D., Gettys T. W., Perwitz N. and Klein J. (2004) Cilary neurotrophic factor influences endocrine adipocyte function: inhibition of leptin via PI 3-kinase. *Mol. Cell. Endocrinol.* **224**, 21–27.

Pan W., Kastin A. J., Maness L. M. and Brennan J. M. (1999) Saturable entry of ciliary neurotrophic factor into brain. *Neurosci. Lett.* **263**, 69–71.

Pelleymounter M. A., Cullen M. J., Baker M. B., Hecht R., Winters D., Boone T. and Collins F. (1995) Effects of the obese gene product on body weight regulation in ob/ob mice. *Science* **269**, 540–543.

Peroni D., Negro A., Bähr M. and Dietz G. P. H. (2007) Intracellular delivery of Neuroglobin using HIV-1 TAT protein transduction domain fails to protect against oxygen and glucose deprivation. *Neurosci. Lett.* **421**, 110–114.

Pollin M. M., McHanwell S. and Slater C. R. (1991) The effect of age on motor neuron death following axotomy in the mouse. *Development* **112**, 83–89.

Rezende A. C., Vieira A. S., Rogério F., Rezende L. F., Boschero A. C., Negro A. and Langone F. (2008) Effects of systemic administration of ciliary neurotrophic factor on Bax and Bcl-2 proteins in the lumbar spinal cord of neonatal rats after sciatic nerve transection. *Braz. J. Med. Biol. Res.* **41**, 1024–1028.

Rogerio F., de Souza Queiroz L., Teixeira S. A., Oliveira A. L., de Nucci G. and Langone F. (2002) Neuroprotective action of melatonin on neonatal rat motoneurons after sciatic nerve transection. *Brain Res.* **926**, 33–41.

Sagot Y., Tan S. A., Baetge E., Schmalbruch H., Kato A. C. and Aebischer P. (1995) Polymer encapsulated cell lines genetically engineered to release ciliary neurotrophic factor can slow down progressive motor neuronopathy in the mouse. *Eur. J. Neurosci.* **7**, 1313–1322.

Schmalbruch H. (1987) Loss of sensory neurons after sciatic nerve section in the rat. *Anat. Rec.* **219**, 323–329.

Schwarze S. R., Ho A., Vocero-Akbani A. and Dowdy S. F. (1999) In vivo protein transduction: delivery of a biologically active protein into the mouse. *Science* **285**, 1569–1572.

Sendtner M., Kreutzberg G. W. and Thoenen H. (1990) Ciliary neurotrophic factor prevents the degeneration of motor neurons after axotomy. *Nature* **345**, 440–441.

Sendtner M., Schmalbruch H., Stöckli K. A., Carroll P., Kreutzberg G. W. and Thoenen H. (1992) Ciliary neurotrophic factor prevents degeneration of motor neurons in mouse mutant progressive motor neuronopathy. *Nature* **358**, 502–504.

Shapiro L., Zhang X.-X., Rupp R. G., Wolff S. M. and Dinarello C. A. (1993) Ciliary neurotrophic factor is an endogenous pyrogen. *Proc. Natl Acad. Sci. USA* **90**, 8614–8618.

Skaper S. D., Facci L., Milani D., Leon A. and Toffano G. (1990) Culture and use of primary and clonal neural cells, in *Methods in Neurosciences* (Conn P. M., ed), Vol. 2, pp. 17–33. Academic Press, San Diego, CA.

Stöckli K. A., Lillien L. E., Näher-Noé M., Breitfeld G., Hughes R. A., Raff M. C., Thoenen H. and Sendtner M. (1991) Regional distribution, developmental changes, and cellular localization of CNTF-mRNA and protein in the rat brain. *J. Cell Biol.* **115**, 447–459.

Taga T. and Kishimoto T. (1992) Cytokine receptors and signal transduction. *FASEB J.* **6**, 3387–3396.

Turner M. R., Parton M. J. and Leigh P. N. (2001) Clinical trials in ALS: an overview. *Sem. Neurol.* **21**, 167–175.

Watt M. J., DzamKo N., Thomas W. G., Rose-john S., Ernst M., Carling D., Kemp B. E., Febbraio M. A. and Steinberg G. R. (2006a) CNTF reverses obesity-induced insulin resistance by activating skeletal muscle AMPK. *Nat. Med.* **12**, 541–548.

Watt J. A., Bone S., Pressler M., Cranston H. J. and Paden C. M. (2006b) Ciliary neurotrophic factor is expressed in the magnocellular neurosecretory system of the rat in vivo: evidence for injury- and activity-induced upregulation. *Exp. Neurol.* **197**, 206–214.

Xu B., Dube M. G., Kalra P. S., Farmerie W. G., Kaibara A., Moldawer L. L., Martin D. and Kalra S. P. (1998) Anorectic effects of the cytokine, ciliary neurotropic factor, are mediated by hypothalamic neuropeptide Y: comparison with leptin. *Endocrinology* **139**, 466–473.

Zhang F., Richardson P. M., Holland D. P., Guo Q. and Tatton W. G. (1995) CNTF or (−)-deprenyl in immature rats: survival of axotomized facial motoneurons and weight loss. *J. Neurosci. Res.* **40**, 564–570.

Zvonic S., Cornelius P., Stewart W. C., Mynatt R. L. and Stephens J. M. (2003a) The regulation and activation of ciliary neurotrophic factor signaling proteins in adipocytes. *J. Biol. Chem.* **278**, 2228–2235.

Zvonic S., Story D. J., Stephens J. M. and Mynatt R. L. (2003b) Growth hormone, but not insulin, activates STAT5 proteins in adipocytes in vitro and in vivo. *Biochem. Biophys. Res. Comm.* **302**, 359–362.

Zvonic S., Baugh J. E. Jr, Arbour-Reily P., Mynatt R. I. and Stephens J. M. (2005) Cross-talk among gp130 cytokines in adipocytes. *J. Biol. Chem.* **280**, 33856–33863.

