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Ciliary Neurotrophic Factor Overexpression in Neural Progenitor Cells (ST14A) Increases Proliferation, Metabolic Activity, and Resistance to Stress During Differentiation

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Neurotrophic factors exert considerable neuroprotective and neurorestorative effects in neurodegenerative diseases. Because neuronal progenitor cells have, at least in part, the potency to restore degenerated neuronal networks, transgenic high-dosage expression of neurotrophins by these cells in neurotransplantation may be advantageous. In the present study, a retroviral vector containing the gene of rat ciliary neurotrophic factor (rCNTF) was permanently transfected into a striatal neuronal progenitor cell line. Qualitative and quantitative analyses demonstrated a sustained expression of the transgene; i.e., rCNTF was present at the mRNA level and protein level. Moreover, cocultivation in separate chambers of transgenic CNTF-ST14A cells and CNTF-dependent TF1 cells exerted typical biological effects, such as increased proliferation and differentiation of the TF1 cells, indicating the functional integrity of the secreted recombinant neurotrophin. The CNTF-ST14A cells displayed improved stress response compared with native ST14A cells under differentiation conditions, i.e., at the nonpermissive temperature of 39°C and after staurosporine exposure, respectively. This effect coincided with a relatively reduced apoptosis rate and a raised metabolic activity of CNTF-ST14A cells at 39°C. Neurotransplantation of CNTF-ST14A cells in the rat quinolinic acid model of Huntington's disease showed a significant and sustained decline in pathological apomorphine-induced rotations compared with parental ST14A cells. We conclude that sustained functional transgene CNTF production improves stress response as well as metabolic activity, making CNTF-ST14A cells a promising tool for neurotransplantation in the quinolinic acid model of Huntington's disease.

Key words: neuronal progenitor cells; neurodegenerative diseases; neurotransplantation; neurotrophic factors; transgene cell lines; metabolic activity; DNA synthesis; stress response; apoptosis; CNTF

The development and maintenance of functional integrity of the nervous system is controlled by a multitude of neurotrophic factors, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), GDNF, and ciliary neurotrophic factor (CNTF; Mufson et al., 1999). It has become increasingly clear that these factors are not monofunctional but rather exert regulatory and protective effects on various types of neurons in the central and peripheral nervous systems (Sendtner et al., 1990, 1992; Ip et al., 1991, 1993; Friedman et al., 1992; Hagg and Varon, 1993; Larkfors et al., 1994; Segal and Greenberg, 1996). An important example in this respect involves CNTF. Originally, this neurotrophin was found to prevent degeneration of traumatically and genetically disturbed neurons in mice (Sendtner et al., 1990, 1992). Its efficacy in neurodegenerative animal models could be demonstrated in neurotoxin-induced models (Emerich et al., 1996; Mittoux et al., 2000) as well as genetically, huntingtin-induced models of Huntington's disease (HD; Saudou et al., 1998). For these reasons, CNTF is a promising drug for therapeutic approaches in neurodegenerative diseases such as HD.

However, a major drawback of therapeutic trials in HD models with CNTF has been the need for continuous delivery to target sites, because CNTF is inactivated very quickly in vivo (Emerich et al., 1996, 1997). Moreover, it has to be delivered distinctly in the striatum, because CNTF action in the hypothalamus leads to remarkable weight loss (Henderson et al., 1994). Therefore, alterna-

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tive approaches have been developed, e.g., transplantation of neuronal progenitor cells, based on speculation on their ability to restore neuronal circuitry and neurotrophic balance right at the target site.

A well-established cell line suitable for this purpose in animal models is the rat striatal progenitor cell line ST14A, which is immortalized by the temperature-sensitive SV40 promoter, which allows unlimited proliferation at the permissive temperature of 33°C and a proliferation stop and differentiation at the nonpermissive temperature of 39°C, the physiological temperature of the rat brain (Cattaneo and Conti, 1998). ST14A cells have been investigated broadly in vitro and in neurotransplantation, and their phenotype is well characterized (Lundberg et al., 1997; Cattaneo and Conti, 1998). Because CNTF exerts the above-mentioned multiple neuroprotective effects and because native ST14A cells produce it at a very low level, we hypothesized that the overexpression of CNTF could provide trophic and commitment signals for ST14A cells. This would allow designing of CNTF-overexpressing ST14A cells as a promising tool for transplantation and could bring to the host cells surrounding the transplanted cells a positive restorative environment. Therefore, we engineered these ST14A cells with permanent expression and secretion of the neurotrophin CNTF. The transgenic overexpression of CNTF improves proliferation rates, metabolic activity, and stress response of ST14A cells in vitro under differentiation conditions. Moreover, ipsilateral apomorphine-induced rotations in rats could be significantly reduced by CNTF-ST14A cells within 2 weeks after striatal neurotransplantation in the quinolinic acid (QA) model of HD.

MATERIALS AND METHODS

Cell Cultures

The generation and characterization of the ST14A cell line, a conditionally immortalized embryonal striatal progenitor cell line with temperature-dependent restriction of the immortalizing oncogene expression, have been previously described (Cattaneo and Conti, 1998). ST14A cells produce neurotrophins at low levels, especially CNTF, and can be genetically manipulated for sustained expression of transgenes in vitro (Cattaneo and Conti, 1998; Torchiana et al., 1998; Hurlbert et al., 1999). After transplantation to the embryonic and adult rat brain, they do not induce tumor formation (Cattaneo et al., 1994; Lundberg et al., 1996, 1997).

Cells were cultured as monolayers in Dulbecco's MEM-Glutamax I (DMEM) supplemented with 50 IU/ml penicillin, 60 μg/ml streptomycin, and 10% inactivated fetal bovine serum (FBS; all solutions from Gibco BRL Life Technologies, Heidelberg, Germany) in a humidified atmosphere containing 5% CO_2 at either the permissive temperature of 33°C or the nonpermissive temperature of 39°C. Culture volumes varied between 100 μl in 96-well plates (Falcon Becton Dickinson, Heidelberg, Germany) and 20 ml in 185 cm^2 culture flasks (Nunc, Wiesbaden, Germany) depending on the number of cells needed for further analysis. Cells were generally allowed to grow close to confluence or to complete confluence but never to superconfluence. Adherent cells were harvested by the trypsin/EDTA method (10% trypsin/EDTA solution).

Cloning of the Rat CNTF Construct

Total RNA was extracted from rat brain according to standard protocols. After reverse transcription (RT) using Superscript (Gibco BRL), an aliquot of the resulting cDNA was polymerase chain reaction (PCR) amplified with a primer pair specific for rat CNTF (Genbank accession No. X17457), resulting in a 660 bp fragment covering the entire open reading frame. To allow for site-directed cloning, the PCR primers carried restriction enzyme recognition sequences for BamHI or NotI (primer 1: 5′-NotI-ACC AGC TCA CTT GTG TCC TG-3′; primer 2: 5′-BamHI-ACA GAG GTA TGA GCG AAT GG-3′). Purified PCR amplicons were directly cloned into pGEM-Teasy (Promega, Heidelberg, Germany), and the integrity of the inserts was verified by complete sequencing. After restriction digestion with BamHI/NotI, inserts were subcloned into the mammalian retroviral expression vector pRetro-on (Clontech, Heidelberg, Germany).

Retroviral Infection of Native ST14A Cells

Packaging cells Ecopack 293 (Clontech) were plated 12–18 hr before transfection, reaching about 80% confluence at the day of transfection. Cells were grown in DMEM/10% FCS without any antibiotic supplements. Ecopack 293 cells were transfected with the retroviral CNTF construct facilitated by the Lipofectamine Plus Reagent (Gibco BRL) as described elsewhere (Torchiana et al., 1998). Briefly, 24 hr after transfection, the packaging cells were rinsed with phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} (Gibco BRL) and resuspended in fresh complete medium. Native ST14A cells at a density of 8×10^5 cells per 60 mm culture dish were supplemented with conditioned medium from packaging cells containing the retroviral particles with the CNTF expression construct mixed with 4 μg/ml polybrene (Sigma, München, Germany). This procedure was repeated twice, but without adding polybrene. Thereafter, the virus-containing medium was replaced by fresh DMEM/10% FBS and antibiotics. At day 4, cells were trypsinized, and fresh DMEM containing 3 μg/ml puromycin (Sigma) was supplemented to select for transgenic cells. After 2 weeks of puromycin selection, resistant colonies were picked with sterile cotton wool sticks and expanded in 96-well plates. The clones were subsequently grown at 33°C in complete medium and subjected to characterization of transgene expression as described below.

Identification of CNTF-ST14A Clones by Flow Cytometry

Cells were fixed, permeabilized, and stained for detection of intracellular CNTF by standard procedures (Jung et al., 1993). Polyclonal goat anti-rat CNTF antibody (R&D Systems, Wiesbaden, Germany) served as the primary antibody and fluorescein isothiocyanate (FITC)-conjugated donkey anti-goat IgG (Dianova, Hamburg, Germany) as the secondary antibody. Flow cytometric analysis of 5,000 cells was performed with an EPICS XL cytometer (Coulter, Hialeah, FL). For exclusion of debris from analysis, an electronic gate in the light scattergram was applied. Mean green fluorescence intensity channels (MFI) were

TABLE I. Sequences of Primers and Detection Probes Used for Semiquantitative RT-PCR in the LightCycler Instrument

Name	Sequence[a]	cDNA coordinates[b]
rCNTF FP	5′ acc agc tca ctt gtg tcc tg 3′	38/−19
rCNTF RP	5′ gga gac aga ggt atg agc gaa t 3′	606/627
rCNTF donor	5′ agg tcc cgg cgg tga agg gtc a FITC 3′	26/47
rCNTF acceptor	5′ LC Red640 tgt ttg ctc tgc gaa agc cat ccc c p 3′	4/21
rβ-actin FP	5′ acc cac act gtg ccc atc ta 3′	478/497
rβ-actin RP	5′ gcc aca gga ttc cat acc ca 3′	800/819
rβ-actin donor	5′ gcc acg ctc ggt cag gat ctt cat FITC 3′	568/591
rβ-actin acceptor	5′ LCRed640 agg tag tct gtc agg tcc cgg cca p 3′	543/566

[a]"p" Denotes dephosphorylated. LC Red 640 is the specific fluorescence dye that allows FRET in the LightCycler.
[b]Starting point of counting from the adenosine residue of the initiation codon.

calculated and served as a measure of relative CNTF expression. A retrovirus-transfected ST14A clone without detectable CNTF expression served as a mock control in cocultivation and transplantation studies (mock-ST14A; see below).

Quantification of mRNA Levels in ST14A Clones

CNTF mRNA expression was analyzed by semiquantitative RT-PCR using the LightCycler instrument (Roche) after DNase I digestion of contaminating genomic DNA. Oligonucleotide sequences of primers and fluorescence-labeled probes are given in Table I. To standardize CNTF transcript levels, the housekeeping gene β-actin was quantitated in parallel samples as an external control. Incremental synthesis of specific amplicons was measured during the annealing step in each cycle online. LightCycler quantification software was used to calculate crossing points of amplification slopes and thereby to deduce the cDNA content in a semiquantitative manner.

Western Blotting

CNTF detection by immunoblotting was performed according to standard procedures (Gallagher et al., 1997). Primary antibodies were goat anti-rat CNTF (R&D Systems) and mouse anti-rat β-actin (Sigma), and secondary antibodies were donkey anti-goat IgG peroxidase conjugated (Jackson Immunoresearch Laboratories, West Baltimore, United Kingdom) and goat anti-mouse IgG peroxidase conjugated (Sigma), respectively. ECL detection reagents (Amersham, Arlington Heights, IL) were applied according to the manufacturer's instructions. As molecular weight markers, prestained broad range (Bio-Rad, Hercules, CA) and BOA protein marker (Mobitec, Göttingen, Germany) were used.

Cocultivation of ST14A Clones With TF-1 Cells

ST14A clones were cocultivated with TF-1 cells, a lymphoblastoid cell line that proliferates and differentiates in a growth factor-dependent manner (Kitamura et al., 1989). Briefly, 300,000 ST14A cells were seeded into the inserts of Costar six-well plates (Costar, Cambridge, MA) and cocultivated with 20,000 TF-1 cells in the outer wells. After 60 hr, ^{3}H-methylthymidine (Amersham) was added to achieve a final activity of 5 μCi/ml for the TF-1 cells. Twelve hours later, cells were harvested onto glass fiber filters (Titertek; Skatron, Lierbyen, Norway), and specific ^{3}H-thymidine incorporation in TF-1 cells was measured using a liquid β-scintillation counter. The results were expressed as counts per minute (cpm). In parallel, the extent of the TF-1 cell differentiation was semiquantitated microscopically and photodocumented.

Determination of DNA Synthesis Rates by ^{3}H-Thymidine Incorporation

Cells were cultured for 24 hr in triplicate in 96-well flat-bottomed plates (Falcon) at a concentration of 100,000 cells per milliliter in the presence or absence of serum and 10^{-7} M staurosporine. For the last 10 hr of incubation, cells were pulsed with ^{3}H-methylthymidine and subsequently harvested and measured as described for TF-1 cells.

Determination of Cellular Metabolic Activity by WST-1 Test

The WST-1 test (Roche) is based on measurement of enzyme activity of mitochondrial dehydrogenases cleaving tetrazolium substrate, resulting in colored formazan. Enzyme activity correlates with the metabolic activity of viable cells (Hipper and Isenberg, 2000). Cells were cultured for 24 hr under the same conditions as used for determination of DNA synthesis rates. For the last 4 hr of incubation, 10 μl WST-1 reagent per well were added. Optical density (OD) was determined at 450 nm with a spectrophotometer (Spectra II; Tecan, Crailsheim, Germany). Results were evaluated by using the software "easyWIN screening" from the manufacturer and presented as OD.

Apoptosis Assay

Cells were cultured for 24 hr under the same conditions as used for determination of DNA synthesis rates. After 24 hr of incubation, nonadherent cells were collected by transferring the supernatant cells to 1.5 ml conical reaction tubes (Eppendorf, Hamburg, Germany). Adherent cells were obtained by trypsin-EDTA treatment and transferred to the same reaction tubes. For quantitation of apoptotic cells, the TUNEL technique was applied as described previously (Gold et al., 1993; Mix et al., 1999). Flow cytometric analysis allowed for determination of apoptotic cells as percentage of green fluorescent cells, which exceeded the fluorescence of negative control cells that were prepared according to the manufacturer's instructions.

Quinolinic Acid Lesion and Neurotransplantation

All experimental procedures were conducted in accordance with the regulations and licensing of the local authorities.

Adult male Wistar rats aged 3–5 months were used and housed at 22°C ± 2°C under a 12 hr light/dark cycle, with free access to tap water and a standard diet. Stereotaxic surgery was conducted with animals under anesthesia with fentanyl (0.25 mg/kg) and droperidol (5 mg/kg). Lesions were made in the left caudate putamen by injection of 2 × 0.5 μl of QA (Sigma) dissolved in 0.1 M phosphate-buffered saline (titrated with 1 M NaOH to pH 7.4) delivered over 4 min each via a 26 gauge 5 μl Hamilton syringe. The coordinates with reference to bregma were 1) A = + 1.2, L = 2.8, V = –5.5 and 2) A = + 0.0, L = 3.6, V = –5.5 (Paxinos and Watson, 1992). QA lesions were made in four groups of rats. In the first group QA lesions were made without pretreatment (QA only, n = 6); in the remaining groups, QA lesions were made 10 days after injection of 1 μl DMEM (DMEM/QA, n = 17) or 100,000 viable CNTF-ST14A cells dissolved in 1 μl DMEM (CNTF-ST14A/QA, n = 5) or 100,000 viable mock-ST14A cells (mock-ST14A/QA, n = 6) into the left caudate putamen (coordinates with reference to bregma: A = + 0.5, L = 3.5, V = –5.5).

Apomorphine-induced rotation provides a sensitive and rapid behavioral correlate of striatal damage and has been used to evaluate the potential efficacy of tissue and/or cell grafts into excitotoxic lesions of the striatum (Norman et al., 1988; Björklund et al., 1994; Emerich et al., 1996). Two weeks after QA lesion, animals were tested for apomorphine-induced rotation (1.0 mg/kg in normal saline, injected subcutaneously) using a self-constructed automated rotometry device. Rotations measured over 30 min were defined as complete 360° ipsolateral turns and are reported as net differences between the two directions per minute.

Statistical Analysis

Data are expressed as mean ± SEM. Differences were tested with Student's *t*-test (two-tailed, unpaired) or were analyzed by ANOVA with the SPSS software package.

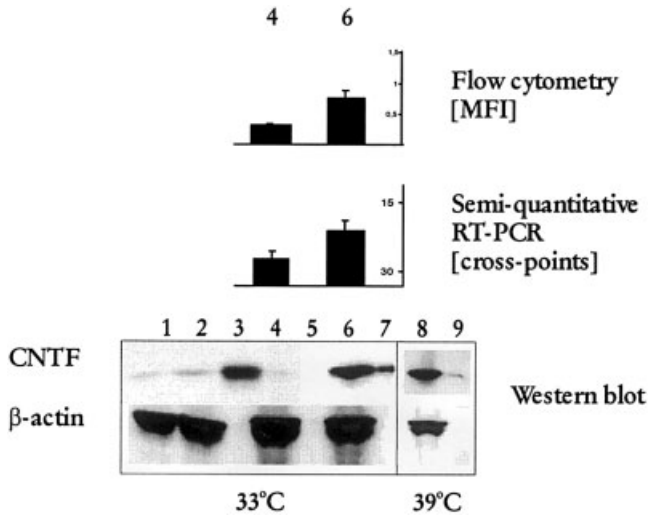

Fig. 1. Transcription, translation, and secretion of transgenic rCNTF in ST14A cells. Lower part: Western blotting for rCNTF and β-actin was performed for protein preparations from rat brain (lane 1), rat substantia nigra (lane 2), recombinant rCNTF (20 ng; R&D Systems, Wiesbaden, Germany; lane 3), native ST14A cells cultivated at the permissive temperature of 33°C (lane 4), supernatant from native ST14A cells cultivated at 33°C (lane 5), CNTF-ST14A cells cultivated at 33°C (lane 6), supernatant from CNTF-ST14A cells cultivated at 33°C (lane 7), CNTF-ST14A cells cultivated at nonpermissive 39°C (lane 8), and supernatant from the latter cells at 39°C (lane 9). Specific immunoreactive signals were detected at 24 kD (rCNTF) and 42 kD (β-actin). Upper part: For ST14A cells and CNTF-ST14A cells, rCNTF protein content and CNTF mRNA content were measured semiquantitatively by means of flow cytometry and RT-PCR, respectively. Results are expressed as mean fluorescence channels (MF) of the flow cytometric analysis and as cross-points (equal to PCR cycle numbers) of the LightCycler analysis as described in Materials and Methods.

RESULTS

Transgene ST14A Cells Secrete Functional CNTF In Vitro and In Vivo

After stable transfection of the retroviral CNTF expression vector in ST14A cells, we could measure an abundant clonal CNTF mRNA expression in several cell lines by semiquantitative RT-PCR, which was more than five times higher than CNTF expression in native ST14A cells (Fig. 1). The most robust clone, which underwent further characterization, displayed increased cytoplasmic CNTF protein content as shown by fluorescence-activated cell sorting (FACS) analysis (Fig. 1). Moreover, transgenic CNTF was demonstrable in Western blots performed in protein preparations under permissive conditions (33°C) and under nonpermissive conditions (39°C), indicating that CNTF expression is not silenced when the cells are forced to start differentiation. Under both conditions, CNTF was nearly absent in native ST14A cells.

Finally, we investigated whether the transgenic CNTF could exhibit typical biological responses in vitro and in vivo. Therefore, we cocultivated either native or CNTF-expressing ST14A cells with growth factor-dependent TF-1 cells and measured the proliferation rate of the TF-1 cells. As a main result, transgenic CNTF expression improved survival of TF-1 cells and raised their proliferation rate more than twofold compared with non-transfected and sham-transfected cells (mock-ST14A), respectively (Fig. 2). Proproliferative and differentiating effects are mimicked by addition of granulocyte-macrophage colony-stimulating factor (GM-CSF) to TF-1 cells, whereas growth factor-free culturing results in massive cell death and low ^{3}H-thymidine incorporation. Native and mock-transfected ST14A cells had an intermediate stimulatory effect on TF-1 cells compared with the discrete effects of GM-CSF supplementation and the large effect of CNTF-transfected cells. We could also demonstrate the characteristic polarized differentiation of TF-1 cells (Kitamura et al., 1989), which was most pronounced in cell cocultures with CNTF-transfected cells (Fig. 2B). These experiments have been performed more than 6 months after generation of CNTF-ST14A clones and permanent culturing. Thus, sustained production and secretion of functional transgenic CNTF are evident.

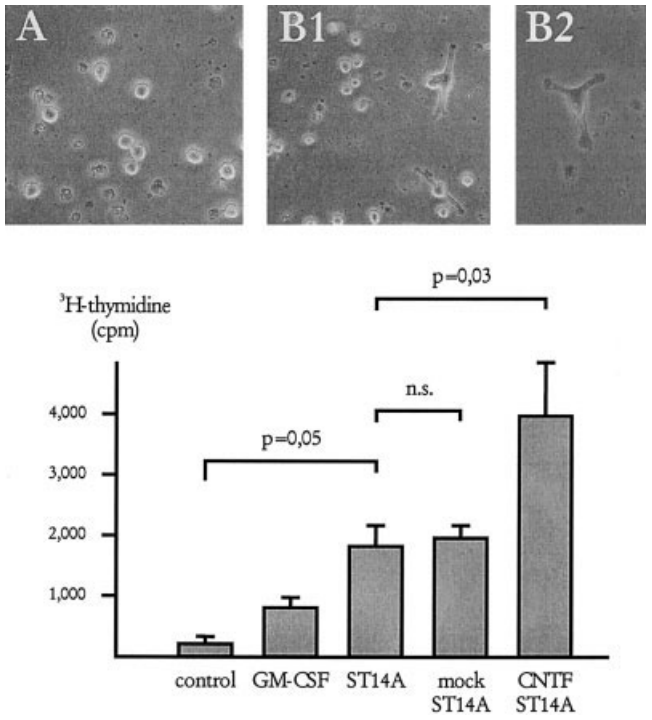

Fig. 2. Biological activity of transgenetically produced rCNTF as tested by coculturing of CNTF-transfected, sham-transfected ("mock"), and nontransfected native ST14A cells with TF-1 target cells. Proliferation assays measuring DNA synthesis via ^{3}H-thymidine incorporation were performed as described in Materials and Methods. Columns represent three experiments, with six replicates each. Significant differences were computed with Student's *t*-test. The microphotographs show the morphology of TF-1 cells after 60 hr. **A** demonstrates poor proliferation and many pycnotic, dead TF-1 cells in the control experiment without exogenous addition of neurotrophins or cocultivation. **B1** shows more viable cells and two adherent, differentiated TF-1 cells after cocultivation with ST14A cells. **B2** is a close-up of one tripolar TF-1 cell, which is typical of highly differentiated cells.

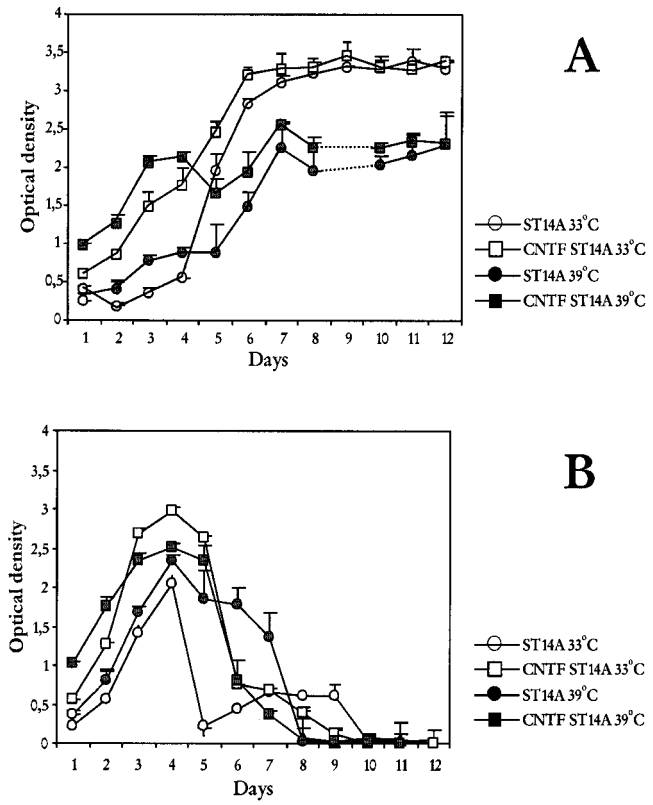

Fig. 3. Time course of metabolic activity of ST14A cells and CNTF-ST14A cells as measured by the WST-1 cleavage under permissive (33°C) and nonpermissive (39°C) conditions. Cell cultures started with 3,000 cells per well (100 μl). The cell number was chosen after pilot test experiments with cell numbers between 1,000 and 10,000 per well and daily medium exchange. At the concentration of 3,000 cells per well, cell growth was relatively uniform and close to confluence at the end of the observation time of 12 days by avoiding superconfluence. Culture medium was exchanged either every day (**A**) or never (**B**). WST-1 was added for the last 4 hr of each incubation. Symbols represent mean values ± SD of triplicate determinations.

As a proof of principle, we transplanted CNTF-ST14A cells in a rat model of HD. Transplantation of 100,000 CNTF-ST14A cells 10 days prior to ipsilateral striatal QA lesion chronically could ameliorate functional deficits. Apomorphine-induced ipsilateral rotations were counted as a marker for functional impairment. Not surprisingly, sham-operated animals (only lesion) and vehicle-transplanted animals were apomorphine inducible, accounting for 4,68 ± 1.92 (n = 6) and 4.4 ± 1.1 (n = 17) rotations per minute, respectively. CNTF-ST14A transplantation could reduce rotations to 1.4 ± 1.64 (n = 5). A retrovirally transfected ST14A clone without functional CNTF expression (mock-ST14A) did not show any functional benefit (5.1 ± 1.52 rotations per minute; n = 6).

Metabolic Activity and DNA Synthesis in CNTF-ST14A Cells vs. ST14A Cells Under Proliferating and Differentiating Conditions

WST-1 cleavage was assessed under permissive conditions at 33°C as well as nonpermissive, "differentiating" conditions at 39°C to address the question of whether transgenic CNTF production alters the viability and metabolic activity of ST14A cells during their differentiation process. The first tests were designed to be long term and, therefore, started with relatively few cells (3,000 cells per well of 96-well plates). When media were replaced daily, CNTF-ST14A cells exhibited higher rates of WST-1 cleavage than native ST14A cells during the first 4–5 days of culture (Fig. 3A). This was true for both permissive (33°C) and nonpermissive (39°C) conditions. Because proliferation rates as measured by DNA synthesis were not significantly different between CNTF-ST14A cells and ST14A cells at the nonpermissive temperature of 39°C (data not shown), this effect could be due either to less decline of the proliferation capability at 39°C in CNTF-ST14A cells or to increased metabolic activity in the transgenic cells per se. CNTF-ST14A cells reached a plateau value of metabolic activity earlier than ST14A cells at

TABLE II. Influence of Recombinant rCNTF on the Metabolic Activity of ST14A Cells*

Temperature (°C)	ST14A alone	ST14A + rCNTF	Increase (%)
33	0.29 ± 0.02	0.40 ± 0.01	29
39 (1 day)	0.57 ± 0.08	1.03 ± 0.06	45
39 (3 days)	0.29 ± 0.02	0.79 ± 0.01	62

*OD values of WST-1 test are given as mean values ± SD of three parallel estimations.

39°C, i.e., when the cells silenced their oncogene and lost their proliferating properties. When media were not replaced, WST-1 cleavage levels were comparable to those in cultures with media exchange during the first 4 days (Fig. 3B). Thereafter, initial proliferation—with the described difference between CNTF-ST14A cells and ST14A cells—was followed by "starvation," as media were exhausted and cell death occurred, with rapid decline of enzyme activity reflected by WST-1 cleavage. Not surprisingly, this process started at 39°C earlier in CNTF-ST14A cells than in ST14A cells, probably because starvation occurred earlier in the transgenic cells.

To determine whether the increased metabolic activity of CNTF-ST14A cells compared with native ST14A cells is dependent on the transfection of the CNTF gene or whether it can also be induced by exogenous CNTF, the native ST14A cells were incubated with 0.1 ng/ml recombinant rCNTF. As shown in Table II there was an increase of the metabolic activity of up to 62%, seen in proliferating as well as in differentiating ST14A cells in response to exogenous rCNTF at the concentration of 0.1 ng/ml. Higher concentrations had a similar effect, without a significant further increase (data not shown).

Metabolic Activity and DNA Synthesis in CNTF-ST14A Cells vs. ST14A Cells Under Exogenous Stress Conditions

As a next step, we applied experimental stress to the cell lines by either depleting serum or exposing the cells to the protein kinase C inhibitor staurosporine at the apoptosis-inducing concentration of 10^{-7} M (Ackermann et al., 1999; Fig. 4). CNTF-ST14A cells showed less inhibition of metabolic activity in response to both serum depletion (Fig. 4A) and staurosporine exposure (Fig. 4B). Along with augmented differentiation time, i.e., with increased incubation time at 39°C, the inhibitory response to serum depletion for 24 hr increased continuously, until it reached 100% on day 8 in both cell lines (Fig. 4A, left). In contrast to the metabolic activity, DNA synthesis was not increasingly inhibited in differentiating cells by serum depletion, either in CNTF-ST14A cells or in ST14A cells (Fig. 4A, right). After more than 3 days of cultivation at 39°C, the DNA synthesis had almost stopped in both cell lines. Therefore, stress effects on DNA synthesis were not registered at later points. For both parameters, metabolic activity and DNA synthesis, CNTF-ST14A cells showed a tendency of lower inhibition by serum depletion than

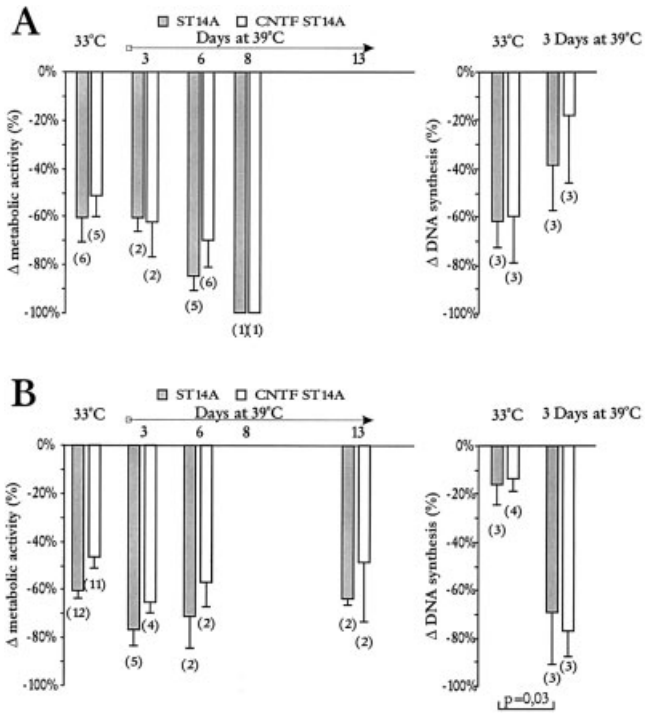

Fig. 4. Influence of serum depletion and staurosporine exposure on metabolic activity and DNA synthesis of CNTF-ST14A cells and ST14A cells under permissive (33°C) and nonpermissive (39°C) conditions. Cells were cultivated in 100 mm cell culture dishes, except for the last 24 hr, when cells were transferred to 96-well plates to allow for standardized measurement of WST-1 cleavage and ^{3}H-thymidine incorporation as described in Materials and Methods. The cell concentration in the 96-well plates was 10,000 cells per well (100 μl). This was chosen after pilot test experiments with cell concentrations between 1,000 and 20,000 per well, because at this concentration the cells grew relatively uniformly to confluence but never to superconfluence. Parallel cultures were exposed to the stress factors serum depletion (**A**) and staurosporine (**B**), respectively, during the final 24 hr of incubation in 96-well plates as described in Materials and Methods. The total incubation times at 39°C are indicated. Columns and bars represent mean values ± SEM of the numbers of experiments indicated in parentheses.

ST14A cells. However, this difference was not statistically significant. In contrast to the case in the starvation experiments, staurosporine-induced inhibition of metabolic activity increased only until day 3 at 39°C, reaching a maximum of only 70–80%; thereafter, it decreased again (Fig. 4B, left). In CNTF-ST14A cells, the staurosporine-induced inhibition of metabolic activity was lower than in ST14A cells at all time points. The effect of staurosporine on DNA synthesis (Fig. 4B, right) was low in proliferating cells (at 33°C) but high in differentiating cells (at 39°C), with no differences between CNTF-ST14A cells and ST14A cells, quite in contrast to the effect of serum depletion.

Apoptosis of CNTF-ST14A Cells vs. ST14A Cells

Finally, we investigated whether differences in proliferation rates are in accordance with altered apoptosis

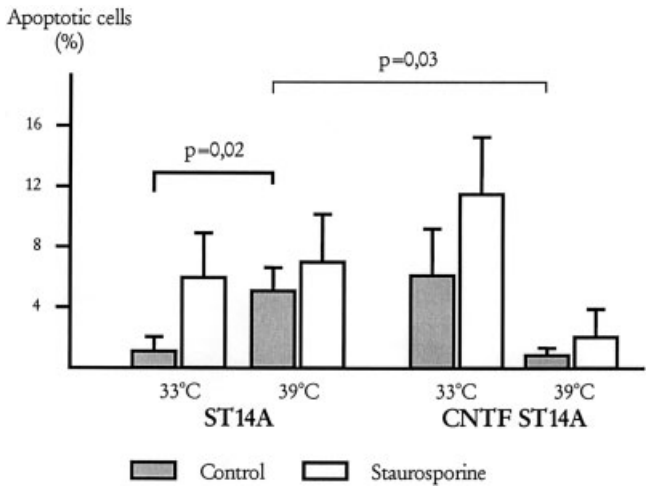

Fig. 5. Apoptosis of CNTF-ST14A cells and ST14A cells under permissive (33°C) conditions with and without staurosporine. Cells were cultivated as described for Figure 4. In addition, parallel cultures were exposed to staurosporine at a concentration of 10^{-7} M during the final 24 hr of incubation in 96-well plates as described in Materials and Methods. The total incubation time at 39°C was 3 days. Apoptotic cells were detected by TUNEL assay, and results are expressed as percentages of apoptotic cells from among total cells in culture. Columns and bars represent mean values ± SEM of three (staurosporine) and four (control) experiments. Significant differences were determined according to Student's *t*-test.

rates as measured by TUNEL assay of cells cultivated under nondifferentiating and differentiating conditions with and without staurosporine exposure (Fig. 5). Consistently with the higher metabolic activity of CNTF-ST14A cells compared with ST14A cells, after short-term incubation (3 days) at nonpermissive temperature (39°C), the apoptosis rate was significantly lower in CNTF-ST14A cells than in ST14A cells. The increase of apoptosis in response to staurosporine was not significant.

DISCUSSION

We demonstrate that transgene overexpression of CNTF in the striatal neuronal progenitor cell line ST14A improves proliferation rates, metabolic activity, and stress response in vitro under differentiation conditions. It has been stated that native CNTF accumulates cytosolically and is not released in the extracellular space because the protein lacks a signal peptide (Peterson et al., 2000). Our data show clearly that CNTF-ST14A cells overexpress recombinant CNTF mRNA, translate this transcript into functional proteins, and release, at least partially, the protein in the supernatant of the cell culture. Moreover, the secreted protein is able to exert trophin-like responses when TF-1 cells are cocultivated. Finally, CNTF-ST14A cells prevent behavioral deficits in QA-lesioned rats, which parental ST14A cells do not. Insofar as this beneficial effect has been hallmarked by CNTF-releasing, encapsulated fibroblasts in the same animal model (Emerich et al., 1996), a specific CNTF effect can be assumed for our in vitro and in vivo experiments.

Long-term administration of neurotrophic drugs to target sites involved in specific pathology is crucial for therapeutic success, and different approaches have been evaluated recently. To summarize two mainstream ideas, the neurotrophic drug is either transduced into host tissue by replication-deficient viral expression vectors or brought to target sites via transplantation of genetically engineered cells. The latter either serve as a permanent secretion reservoir or, if neuronal stem cells are used, provide a favorable neurotrophic milieu and may be able to differentiate into neurons (Emerich et al., 1994, 1996; Kordower et al., 1999, 2000a; de Almeida et al., 2001; Kim et al., 2002). All strategies could demonstrate, at least partially, functional recovery. Lentiviral expression vectors seem to have high transduction and expression rates, whereas cellular approaches might be more safe, insofar as transgene expression can be studied in vitro and limited to distinct cell populations. Retroviral vectors have repeatedly been used for transduction of ST14A cells (Benedetti et al., 1998; Torchiana et al., 1998). Although we introduced a tetracycline-responsive transactivator element (TetOn), all positive transgenic cell lines that displayed robust rCNTF expression could not be augmented by supplementing tetracycline in the culture media (data not shown). This spoiled the option of activating transgenic expression during transplantation studies by feeding animals with tetracycline. Nevertheless, because we could demonstrate sustained transgenic expression in vitro over several months and under permissive and nonpermissive temperature conditions, we decided to continue the in vitro characterization of CNTF-ST14A cells.

A major result of this study highlights a prometabolic effect of CNTF in these neuronal progenitor cells. Consistently, we have found WST-1 cleavage at higher levels in CNTF-ST14A cells compared with controls. This effect was demonstrated in basal proliferation at permissive and nonpermissive temperatures and during serum removal or staurosporine-induced stress, i.e., under variable conditions that might be important in neurotransplantation. There is some evidence that this reflects a direct CNTF effect; Johnson et al. (1994) have described a significant increase in cellular metabolism after binding of CNTF to its receptor.

Quantitative RT-PCR with an intron-spanning primer set for CNTFRα resulted in a specific amplification product in ST14A and CNTF-ST14 mRNA preparations (data not shown), raising the probability of an autocrine CNTF action via the complete high-affinity CNTF receptor (consisting of CNTFRα, LIFRβ and gp130). In addition, in vivo effects of CNTF-producing ST14A cells may not depend on receptor ligation; grafts of NGF- and CNTF-producing fibroblasts protected degenerating striatal neurons that do not express the appropriate tyrosine receptor kinase (Trk)- resp. CNTF receptor (Emerich et al., 1994; Shannon and Kordower, 1996; Kordower et al., 2000). The prometabolic CNTF effect in ST14A cells shownj in our study is of particular interest, in that the viability of the cells should be improved, and

especially HD pathology has been associated with chronic neuronal energy impairment (Beal et al., 1993; Brouillet et al., 1995).

In addition, when stress stimuli have been applied, CNTF-ST14A cells displayed a differential behavior compared with native ST14A cells. Serum depletion and starvation led to a high cell death rate in CNTF-ST14A cells, which exceeded rates in native cells. One might speculate about a direct relation between the improved metabolic activity and this failure to withstand an energy-depleted environment. A different result was observed when stress was induced by inhibition of this protein kinase C-dependent signal pathway through staurosporine, which induces apoptosis in several cell types (Ackermann et al., 1999), including glioma cell lines (Couldwell et al., 1994). CNTF-ST14A cells had increased resistance to proapoptotic stimulation and were able to recover from continuous staurosporine-exposure more pronouncedly; i.e., there was a significantly reduced apoptosis rate during staurosporine exposure under nonpermissive conditions at 39°C. This strongly points to an antiapoptotic effect of CNTF during cell differentiation. Additional evidence for the mode of this CNTF action remains to be found, e.g., by modulating CNTF-dependent signaling pathways (Segal and Greenberg, 1996; Mufson et al., 1999). CNTF could protect neurons from oxidative damage or changes in ionic balance, including calcium stores, Na/K ATPase, and chloride fluxes. Thus, trophic factors may act to increase endogenous levels of antioxidant enzymes or perhaps decrease the production of oxidative agents after excitotoxicity in addition to exerting classical trophic effects (Choi, 1988; Mattson et al., 1989, 1993).

The present study focused on basal effects of transgenetically augmented CNTF in neuronal progenitor cells in vitro. A considerable shift in the metabolic phenotype of ST14A cells arose from permanent transgenic expression. Neuronal transplantation data support the efficacy of these transgene cells in a model of HD. Advantages of the CNTF-ST14A overengineered and encapsulated fibroblasts may be a better integration, longer survival, and improved stress resistance at the target site. It will be necessary to address the immunohistochemical phenotype of CNTF-ST14A cells in the in vitro and in vivo situation in future studies and to concretize their therapeutic potency in animal models of acute and chronic neurodegeneration.

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