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Gene Expression Profiling of Ciliary Neurotrophic Factor-Overexpressing Rat Striatal Progenitor Cells (ST14A) Indicates Improved Stress Response During the Early Stage of Differentiation

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Neuronal progenitor cells delivering neurotrophic factors are a promising therapeutic tool for treatment of neurodegenerative diseases. Although several promising results have come from studies in different animal models, detailed knowledge of the action of neurotrophic factors in the CNS is still lacking. A clonally derived, immortalized rat striatal cell line (ST14A) expressing ciliary neurotrophic factor (CNTF) offers a stable and controlled background with which to analyze CNTF actions on the transcriptional level in CNS progenitor cells. To identify early transcriptional changes induced by CNTF expression, we transfected the CNTF gene into ST14A cells, which differentiate at the nonpermissive temperature of 39°C via suppression of the immortalizing SV40 large T antigen. This shows a CNTF-dependent hypoxic/ischemic stress response during the earliest stage of differentiation, with expression of specific transcripts and evidence of translational repression leading to decreased protein synthesis in the transfected cells. This process is mediated by the Ras/MAP kinase pathway and is accompanied by impaired proliferation and metabolism as well as signs of neuronal differentiation. The stress-like response in the early stage of differentiation improves the ability of the transfected cells to respond to and cope with a stressful environment in vivo. The present data indicate higher viability, longer life, and greater differentiation capacity of CNTF-ST14A cells if they are used for transplantation. We conclude that the stress-like response during the early stage of differentiation improves the ability of the CNTF-ST14A cells to respond and adapt to a stressful environment, which renders them useful candidate cells for in vivo trials of treatment for neurodegenerative diseases in animal models, e.g., of Huntington's disease. © 2003 Wiley-Liss, Inc.

Key words: neuronal progenitor cells; neurodegenerative diseases; CNTF; neurotrophic factors; transgene cell lines; gene expression profiling; stress response

New experimental therapeutic strategies for the treatment of degenerative neurological disorders imply grafting of embryonic tissue and delivery of neurotrophic factors to the central nervous system (CNS). Although several promising results have come from studies in different animal models, detailed knowledge of the interaction of neurotrophic factors in the complex regulatory network of the CNS is still lacking. Furthermore, the appropriate trophic factors, vehicles for delivery, and sites of implantation are still a matter of debate.

Several approaches have made use of the advantages of neuronal progenitor cell lines for neurotransplantation (Cattaneo and McKay, 1991; Lundberg et al., 1996, 2002; Englund et al., 2002). The main advantages are 1) high proliferative potency; 2) inducibility of differentiation by several means, such as serum deprivation, stimulatory substances, and temperature increase; 3) production of almost unlimited amounts of cells under standardized conditions; and 4) introduction of therapeutic genes by viral and nonviral transfection. Widely used for these purposes is the

This paper is dedicated to Prof. Dr. H.J. Meyer-Rienecker.

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ST14A cell line, which was originally generated by Cattaneo et al. (1994) from embryonic day 14 (E14) rat striatum primordia by retroviral transduction of the temperature-sensitive mutant of the SV40 large T antigen. ST14A cells have properties of CNS progenitor cells, including expression of nestin and the ability to differentiate in vitro into mature MAP2-positive cells. They are the first and so far the only immortalized cell line expressing antigens specific to striatal neurons. Furthermore, they express the same intracellular signaling molecules found in their in vivo counterparts (Conti et al., 1997; Ehrlich et al., 2001). The presence of a conditional oncogene allows regulation of the growth of the cells in vitro and in vivo. ST14A cells are particularly suitable for cell transplantation studies, in that the temperature for inactivation of the conditional oncogene corresponds to the body temperature of rodents. The cells have been successfully transplanted into embryonic and adult brain (Cattaneo et al., 1994; Lundberg et al., 1997) and were shown to differentiate into neurons and glial cells.

Furthermore, ST14A cells can be easily genetically manipulated. We were able to show that ST14A cells that had been permanently transfected with a retroviral vector containing the gene for rat ciliary neurotrophic factor (CNTF) produce and release biologically active CNTF and that these cells have improved stress response and metabolic activity (Weinelt et al., 2003).

CNTF is a member of a family of distantly related cytokines that includes leukemia inhibitory factor (LIF), oncostatin M, interleukin-6 (IL-6), interleukin-11 (IL-11), and cardiotrophin-1 (Segal and Greenberg, 1996). Its pleiotrophic effects include maintenance of pluripotentiality of embryonic stem cells (Conover et al., 1993), maintenance of adult forebrain neural stem cells (Shimazaki et al., 2001), stimulation of spinal cord and ciliary ganglia neuron survival (Magal et al., 1991; Unsicker et al., 1992), induction of cholinergic differentiation of sympathetic neurons (Saadat et al., 1989), and stimulation of terminal differentiation of neuronal as well as hematopoietic progenitor cells (Ip et al., 1994; Ip, 1998). Thus CNTF exerts protective functions in the peripheral nervous system (Sendtner et al., 1992; Apfel et al., 1993) as well as in the CNS (Hagg et al., 1992; Hagg and Varon, 1993; Clatterbuck et al., 1993; Emerich et al., 1996; Mufson et al., 1999; Linker et al., 2002). The protection of oligodendrocytes from apoptotic cell death (Louis et al., 1993) and of mice from demyelinating CNS disease (Linker et al., 2002) qualifies CNTF as a repair factor in the CNS. CNTF can also partly prevent the loss of striatal neurons and protect against behavioral deficits in a rodent model of Huntington's disease (HD; Emerich et al., 1996; Anderson et al., 1996). In a primate model of HD, human CNTF delivered by polymer-encapsulated, genetically modified hamster fibroblasts had a trophic influence on degenerating striatal neurons as well as on critical nonstriatal regions, such as the cerebral cortex, thereby preventing the degeneration of vulnerable striatal populations and cortical-striatal basal ganglia circuitries (Emerich et al., 1997).

The transplantation of genetically modified striatal progenitor cells releasing neurotrophic factors to protect themselves and surrounding neurons that are in danger of dying provides a rational, powerful, and promising therapeutic tool for the treatment of neurodegenerative disorders. ST14A cells overexpressing CNTF are promising candidates for this approach. For better understanding of the biological properties and regulatory pathways expressed in these cells during the early stage of differentiation, we analyzed the expression profiles of native and CNTF-transfected ST14A (CNTF-ST14A) cells under permissive and nonpermissive temperatures.

MATERIALS AND METHODS

Generation and Culture of CNTF-ST14A Cells

ST14A cells were originally derived from the embryonic day 14 striatum primordia of the Sprague-Dawley rat. Cells were immortalized by transduction of the temperature-sensitive mutant of the SV40 large T antigen together with a neomycin-resistance gene (Cattaneo and Conti, 1998). At the permissive temperature of 33°C, cell proliferation and nestin expression persist, whereas, at the nonpermissive temperature of 39°C, cell division and nestin expression cease. Furthermore, the stopping of proliferation is accompanied by differentiation of the ST14A cells into glial and neuronal cells. The latter develop preferentially to medium-sized spiny neurons upon exposure to reagents stimulating the mitogen-activated protein kinase (MAPK) and adenylyl cyclase pathways (Ehrlich et al., 2001).

For transfection with the CNTF gene, a retroviral vector was constructed and cloned as described in detail elsewhere (Weinelt et al., 2003). Briefly, total RNA was extracted from rat brain according to a standard protocol (Chomczynski and Sacchi, 1987). After reverse transcription (RT) using Superscript (Gibco BRL Life Technologies, Karlsruhe, Germany), 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 and NotI, respectively (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). Packaging cells Ecopack 293 (Clontech) were plated 12–18 hr before transfection, reaching approximately 80% confluency at the day of transfection. Plating density was 3×10^5 cells/cm^2. Cells were grown in Dulbecco's minimal essential medium-Glutamax 1 (DMEM; Gibco BRL) supplemented with 10% fetal bovine serum (FBS; Gibco BRL), but without any antibiotic supplementation. Packaging cells were transfected with the retroviral CNTF construct facilitated by the Lipofectamine Plus Reagent (Gibco BRL) as described else-

where (Torchiana et al., 1998). Briefly, 24 hr after transfection, the cells were rinsed with phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} (Gibco BRL) and resuspended in fresh complete medium.

For infection with the retroviral construct, native ST14A cells were seeded at a density of 4×10^5 cells per 60-mm culture dish; 24 hr later (day 2), medium was removed, and conditioned medium from packaging cells containing the retroviral particles with the CNTF expression construct mixed with 4 μg/ml polybrene (Sigma, München, Germany) was added for a further 6 hr. This procedure was repeated twice without adding polybrene. Thereafter, the virus-containing medium was replaced with fresh DMEM supplemented with 10% FBS and antibiotics. At day 4, cells were trypsinized, and fresh DMEM containing 3 μg/ml puromycin (Sigma) was added to select for transgene cells. This medium was renewed every fourth day. After 2 weeks of puromycin selection, resistant colonies were picked and expanded, and resulting clones were subsequently grown at 33°C in complete medium. The transgene expression was characterized by flow cytometry and Western blotting (Weinelt et al., 2003).

All cell lines were tested for the absence of mycoplasma by a photometric enzyme immunoassay (Roche, Mannheim, Germany), and one high-CNTF-expressing clone (CNTF-ST14A-31) was selected for further analysis. Cells were cultured as monolayers in DMEM supplemented with 50 IU/ml penicillin, 60 μg/ml streptomycin (both from Gibco BRL), and 10% FBS in a humidified 5% CO_2 atmosphere in 75-cm^2 culture flasks (Nunc, Wiesbaden, Germany) at either the permissive temperature (33°C, time zero) or the nonpermissive temperature (39°C for 3 hr or 24 hr). Cells were allowed to grow close to confluency, and adherent cells were harvested with the trypsin/EDTA method (10% trypsin-EDTA solution).

Cultivation of ST14A Cells With Exogenous CNTF

To test the effect of exogenous CNTF on gene expression of native ST14A cells, rat recombinant CNTF (R&D Systems, Wiesbaden, Germany) was added to subconfluent 75-cm^2 cultures of native ST14A cells at a final concentration of 10 ng/ml. Four cultures were performed: 1) 33°C without CNTF, 2) 33°C with CNTF for 24 hr, 3) 39°C without CNTF for 24 hr, and 4) 39°C with CNTF for 24 hr. At the end of cell culture, the cells were grown to close to confluency, and adherent cells were harvested with the trypsin/EDTA method.

RNA Preparation and High-Density Oligonucleotide Array Hybridization

Total RNA was extracted from 2–3 $\times$ 10^6 cells per test using an RNA extraction kit and following the manufacturer's instructions (Qiagen, Hilden, Germany). RNA concentrations were determined spectrophotometrically at 260 nm. RNA probes were labelled according to the supplier's instructions (Affymetrix, Santa Clara, CA) and hybridized as described by Teague et al. (1999). Essentially, first-strand synthesis was carried out with a T7-(dT)24 primer and SuperScript II reverse transcriptase (Gibco BRL) using a 10-μg total RNA sample. Second-strand synthesis was carried out according to the SuperScript Choice System (Gibco BRL) by *Escherichia coli* DNA-polymerase I, *E. coli* ligase, and RNaseH. Fragment end polishing was performed using T4-polymerase. An in vitro transcription reaction was used to incorporate biotin-11-CTP and biotin-16-UTP into the cRNA probe (BioArray HighYield RNA Transcript Labeling Kit; Enzo Germany). The fragmented cRNA was hybridized overnight (45°C) to the DNA microarrays RG-U34A (Affymetrix) with a capacity of approximately 7,900 genes. Washes were performed in the GeneChip Fluidics Station (Affymetrix) according to the producer's protocol. Staining was performed with R-phycoerythrin streptavidin (Molecular Probes, Göttingen, Germany), followed by an antibody amplification procedure using a biotinylated antistreptavidin antibody (Vector Laboratories, Burlingame, CA) and goat IgG (Sigma). Fluorescence intensities corresponding to the number of cRNA molecules hybridized to complementary oligonucleotides bound to the microarrays were estimated by laser scanning (Hewlett-Packard Gene Scanner). The scanning was carried out at 3 μm resolution and 488 nm excitation and 570 nm emission wavelength. Data were scaled based on total intensity with the Affymetrix GeneChip software. To demonstrate the reproducibility of the array data, we cultured the CNTF-ST14 cells in two completely independent experiments and analyzed the correlation of the gene expression levels of those genes that are called *present* in the Affymetrix software (see Fig. 1).

Data Analysis of Gene Expression Measurement by the Microarray Technique

The gene expression levels were calculated with commercially available software provided by Affymetrix. Data are given as -fold increase of gene expression in cells kept at the nonpermissive temperature of 39°C for 3 hr or 24 hr compared with the gene expression in cells kept at the permissive temperature of 33°C (time 0). A change in level of expression for any gene was considered significant if it was twofold or more. The "average difference" values for every transcript of each experiment were first scaled to an arbitrary fluorescence value according to the default settings in the Affymetrix software and then imported into Genespring 3.2.12 (Silicon Genetics, Redwood City, CA) for further analysis (filtering, clustering, statistics) and graphic visualization of the gene expression profile. The procedure has been described in detail by Iyer et al. (1999) and Silva (2000).

RT-PCR for Analysis of CNTFR-α Gene Expression

For gene expression analysis of the CNTFR-α subunit, total RNA from 2–3 $\times$ 10^6 cells of native and CNTF-ST14A cells was isolated by phenol-chloroform with Trizol (Sigma). Two micrograms of each extract were reversely transcribed with 120 ng random hexanucleotides and 200 U Superscript II (Gibco BRL). The PCR master mix consisted of 2.0 μl of 10× PCR buffer (Pharmacia, Freiburg, Germany), 2.5 mmol/liter $MgCl_2$, 1.0 mmol/liter each dNTP (Roche), 0.5 g/liter bovine serum albumin (BSA; New England Biolabs, Beverly, MA), 50 mg/liter dimethyl sulfoxide (Sigma), 2.0 U Taq DNA polymerase (Pharmacia), 0.5 μmol/liter amplification primers, 5 μl cDNA solution, and PCR-grade water at 20 μl. The cycle conditions were as follows: initial denaturation at 94°C for 4 min, followed by 40 cycles with denaturation at 94°C (30 sec), annealing at 62° (30 sec), elongation at 72°C (60 sec), and final extension at 72°C (10 min). Primers for a CNTFR-α gene

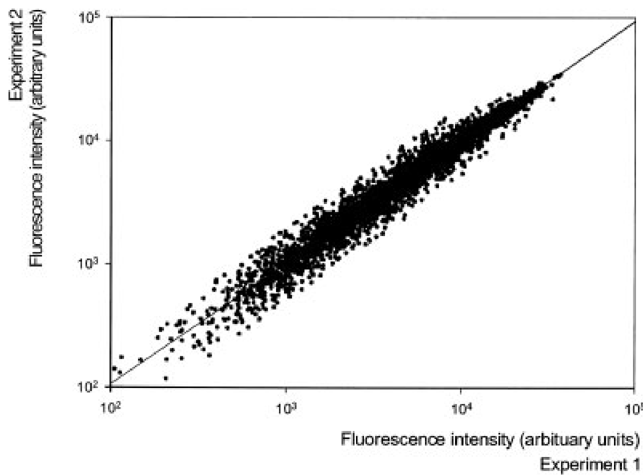

Fig. 1. Correlation of gene expression levels of 3,472 genes of CNTF-transfected ST14A cells in two parallel (independent cell culture) experiments demonstrating the reproducibility of the microarray experiments (correlation coefficient, r = 0.982). Cells were cultured at 33°C, and gene expression analysis was performed with the DNA microarray technique as described in Materials and Methods. All genes were called "present" by the Affymetrix software in both experiments.

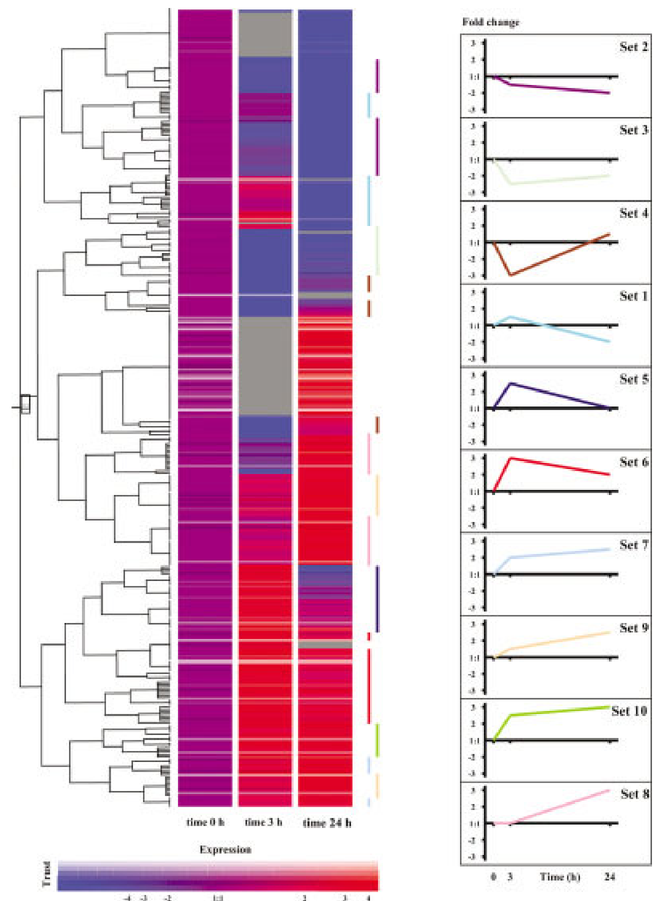

Fig. 2. Cluster graph analysis (GeneSpring) of the gene expression of CNTF-transfected ST14A cells (left). Changes in the expression levels from time 0 (0 hr, corresponding to cells cultures at 33°C) to time points of 3 hr and 24 hr (corresponding to cells cultured at 39°C for the indicated periods) are illustrated by colors according to the scale given at lower left. Ten sets of genes are constructed (right) according to similarities of their expression profiles during 3 hr and 24 hr of cell culturing at the nonpermissive temperature of 39°C. The average expression profiles (-fold change) of the 10 gene sets are shown.

fragment of 499 bp had the following sequences: 1) sense: tggtggtaacgag/atggc (junction primer spanning a 43-bp intron), 2) antisense: gttcttgagggctgggtc. For positive amplification control, the housekeeping β-actin gene was used, because this gene reveals no pseudogene organization, a problem that normally hampers the exact interpretation of PCR results on the cDNA level. Primers for β-actin cDNA fragments (341 bp) had the following sequences: 1) sense: catggatgacgatatcgctg, 2) antisense: acgtaggagtccttctgacc. M VI molecular weight marker from Roche was used for agarose electrophoresis.

RESULTS

Gene Expression Changes in CNTF-ST14A Cells at the Nonpermissive Temperature

Of the 8,740 oligonucleotids representing approximately 7,900 different genes and expression sequence tags (EST) of the rat genome, 353 transcripts changed substantially (twofold or more at at least one of the measured time points) in response to the nonpermissive temperature (39°C). The reproducibility of the data was high (r = 0,982); i.e., repeated experiments showed very similar gene expression profiles. This is illustrated in Figure 1, showing, from two independent experiments, expression levels of identical paired transcripts in CNTF-ST14A cells at 33°C.

For each gene, the ratio of mRNA levels at the nonpermissive temperature (39°C, 3 hr and 24 hr) to its level at the permissive temperature (33°C, time 0) was calculated and represented by color in Figure 2 (left). Two hundred eighty-one transcripts with likely expression at all three time points (all called "present") were clustered by k-means-clustering (Pearson correlation) into groups on the basis of similarity of their expression profiles. The procedure resulted in 10 different sets of genes with the average expression profiles shown in Figure 2 (right). Among the 281 transcripts described, 187 were genes with known full-length DNA sequence; 94 were unnamed ESTs. For 71 ESTs, highly probable genes could be identified by sequence similarity. Twenty-nine transcripts of the subset were represented twice or more on the array, either as multiple known full-length genes or as ESTs with substantial sequence similarity. The majority of them (n = 28) had identical or very similar expression profiles, pointing to the validity of the clustering method grouping genes with similar patterns of expression. Within the 10 gene sets, there are subgroups revealing similar expression pro-

TABLE I. Functional Gene Clustering Profile of Twofold or Greater Gene Expression Changes of CNTF-ST14A Cells After Transition From 33°C to the Nonpermissive Temperature of 39°C and Influence (Twofold or Greater Gene Expression Changes) of Exogenous CNTF on Native ST14A Cells*

Gene function	Gene name	Genebank accession No.	Effect of exogenous CNTF on native ST14A cells	
			33°C	39°C
Regulation of proliferation, apoptosis, growth, and cell cycle arrest				
Set 2	Amidophosphoribosyltransferase	D10853		
	Methionine adenosyltransferase	AB000717		
	APP-binding protein I	U90829		
	Topoisomerase related function protein (EST)	AA891891		
Set 3	Uridine kinase (EST)	AA859827		
Set 4	Cdk-activating kinase	X83579		
	1d2	A1137583	Up-regulated	Up-regulated
	Omithine decarboxylase	J04792		
	SMN	AF044910		
Set 1	Nkcc 1	AF086758		
Set 5	Lot 1	AA900750		
Set 6	PRG 1	X96437		
	Caspase 3	U49930		
	CDC2L (EST)	AA875127		
	DYRK	A1104012		
	SIRP	AF055056		
Set 7	β-cell translocation gene I	126268	Up-regulated	Up-regulated
Set 9	Interferon-inducible protein 9–27	X61381	Up-regulated	Up-regulated
	CADD4S	132591	Up-regulated	
	CRP 2	D17512		
	Synaptojanin	U90312		
Set 10	Integrin-associated protein CD47	AF017437		
	Connexin Cx26	X51615	Up-regulated	
	PKC delta	M18330		
Set 8	Cyclin G1	X70871	Up-regulated	Up-regulated
	Cyclin D1	D14014		
	LRF-1	M64250	Up-regulated	
	Protein S	U06230		
	Bax	S76511	Up-regulated	
	Mud-4	U70270		
Regulation of protein synthesis and processing				
Set 2	Peroxysomal targeting signal 1 regulator (EST)	AA892300		
Set 3	26S proteasome isoform p112-L	AJ006340		
	Initiation factor eIF-2Be	U19516		
	tRNA synthetase-like protein (EST)	AA875602		
Set 4	MPP	M57728		
Set 9	PHAS-1	U05014		
(Neuronal) differentiation and determination				
Set I	Pol II	X59608		
Set 5	Neuron glucose transporter	D13962		
	GABA-A receptor alpha-1	L08490		
	Fibroblast growth factor-5	D64085		
	Heparin-binding EGF-like growth factor	L05489		
	Amphiregulin	X5583		
Set 6	c-myc	Y00396		
	TGN 38 precursor	X53565		
	Tenascin C	U15550		
Set 7	Lipocortin V	AF051895		
	AML-1	L35271		
Set 9	Glucocorticoid receptor	M14058	Up-regulated	Up-regulated
	Galectin	AI172064		
	Latexin	X76985		
	Septapterm reductase	M30410	Up-regulated	Up-regulated
Set 10	sgk	L01624		
	β-Galactoside-alpha-2,6-sialyltransferase	M83143		

TABLE I continues to the next page

TABLE I. Functional Gene Clustering Profile of Twofold or Greater Gene Expression Changes of CNTF-ST14A Cells After Transition From 33°C to the Nonpermissive Temperature of 39°C and Influence (Twofold or Greater Gene Expression Changes) of Exogenous CNTF on Native ST14A Cells* (continued)

Gene function	Gene name	Genebank accession No.	Effect of exogenous CNTF on native ST14A cells	
			33°C	39°C
Set 8	Jagged 1	AA900503		
	P9K	X06916	Up-regulated	Up-regulated
	Mdm 2 (EST)	AI639488		
	Membrane attractin (EST)	AA869645	Up-regulated	Up-regulated
	LIMK-2a	D31874		
Stress response				
Set 2	Glutathione synthetase	AA852004	Down-regulated	Down-regulated
	CARP	U50736		
Set 1	Glutathione synthetase	L38615		
	TBF II	X74565		
	Gro	D11445		
	Transferrin receptor	M58040		
	Prodynorphin precursor	M32783		
Set 5	NAC-1	AF015911		
	JE	X17053		
	Glutathione-S-transferase Yb2	J02592		
	Cationic amino acid transporter 1	AA957917		
	TAO-1			
Set 6	XRCC I (EST)	AI010580		
	IRP 94	AF077354		
	UDP-glucuronosyl transferase	D38062		
	Diaphorase	J02679		
	Heme oxygenase	J02722		
	NADPH-cytochrome P450 reductase	E01524		
Set 7	Fra-1 (EST)	AA875032		
Set 9	Hexokinase II	S56464	Up-regulated	Up-regulated
	NF-E2 related factor 2 (EST)	AI177161	Up-regulated	Up-regulated
Set 10	JunB	X54686		
	c-Jun (EST)	AI175959		
	Hsp 27	M86389		
Set 8	Diacylglycerollcinase	S49760	Up-regulated	Up-regulated
	Amyloidogenic glycoprotein	X07648	Up-regulated	Up-regulated
	NADPH-dependent isocitrate dehydrogenase (EST)	AA892314		
	MAMA	AF065438	Up-regulated	Up-regulated
	Mtp 1	X57525	Up-regulated	Up-regulated
	Multidrug resistance protein	X96394		

*The sets of genes correspond to the expression profiles as illustrated in Figure 2.

files but different expression levels. For example, genes of sets 2–4 are all down-regulated, whereas genes of sets 7 and 10 show early and sustained up-regulation. Genes related to four major cell functions are listed with respect to their expression profiles in Table I. Interestingly, genes involved in the control of distinct cell functions belong to different gene sets, but the net result is mainly an influence in the same direction. For example, genes inducing mitosis and protein synthesis are down-regulated, whereas genes inhibiting growth and inducing apoptosis are up-regulated. Of utmost interest is the observation that almost all of the expressed genes, which are related to (neuronal) differentiation and to response to (oxidative) stress, are up-regulated in CNTF-ST14A cells after the start of temperature-induced differentiation.

Comparison of Gene Expression Changes in CNTF-ST14A Cells vs. Native ST14A Cells

To determine whether temperature increase alone results in the observed gene expression changes independently of the CNTF transfection, we compared the data on CNTF-ST14A gene clustering with the time-dependent gene expression profiles of native ST14A cells exposed to the nonpermissive temperature. The two experiments were merged in the Genespring software to disclose the behavior of the transcripts in native ST14A cells of genes clustered for CNTF-ST14A cells (Fig. 3). Seven of the ten comparative clusters showed similar average expression profiles in the two experiments. However, sets 6, 7, and 9 were different, with a striking

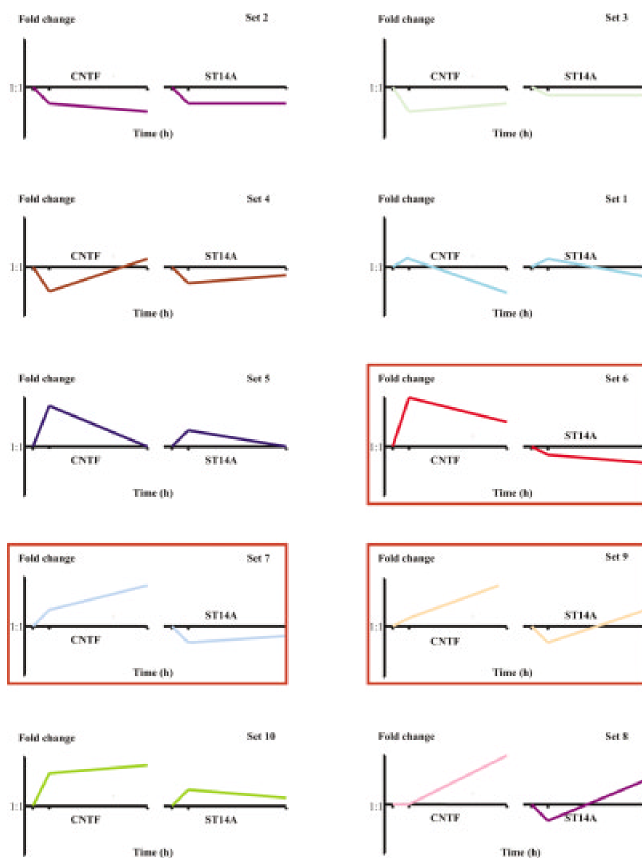

Fig. 3. Clusters of average gene expression profiles of CNTF-transfected ST14A cells in comparison with those of the same genes in native ST14A cells. The time points are 0 hr (33°C), 3 hr, and 24 hr, indicating the periods for which the cells were cultured at the nonpermissive temperature of 39°C. Seven of the ten comparative clusters showed similar average expression profiles in the two experiments. However, sets 6, 7, and 9 were different (boxed), with a striking common feature: Those genes, which were up-regulated at the early time point (3 hr) in the CNTF-ST14A cells, showed a reduced expression at the same time in the native ST14A cells.

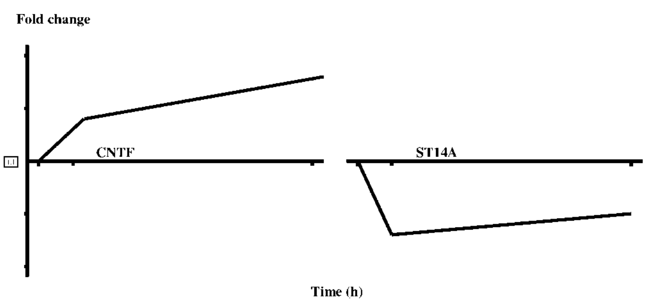

Fig. 4. Average expression profiles of those genes that differed significantly between CNTF-transfected and native ST14A cells (n = 23). The time points are 0 hr (33°C), 3 hr, and 24 hr, indicating the periods for which the cells were cultured at the nonpermissive temperature of 39°C.

common feature: Those genes, which were up-regulated at the early time point (3 hr) in the CNTF-ST14A cells, showed a reduced expression at the same time in the native ST14A cells.

The same result could be obtained with other filter methods. By building venn diagrams from both data sets and searching for the intersection, 144 genes were identified to be present in both data sets. The average expression profiles of theses 144 genes confirmed the above-mentioned differences at the early time point. This became even more evident when those genes with a similar (n = 68, confidence interval 99%) expression were excluded, leaving a subgroup of 78 genes with different expression profiles. We further filtered for those genes that matched the average expression profile of this subgroup (Fig. 4) as well as possible, i.e., up-regulation (increased by twofold or more) at 3 hr in the CNTF-ST14A cells and down-regulation (decreased by twofold or more) at the same time in the native ST14A cells. This resulted in 20 different transcripts (Fig. 5). Three of them are seen twice because of the redundancy of the microarray design, i.e., oligonucleotide probes of different regions of the same genes are present; 2 of the 20 transcripts were unidentified ESTs.

Effect of Exogenous CNTF on Gene Expression in Native ST14A Cells

To determine whether the changes in gene expression of CNTF-ST14A cells are dependent on the transfection of the CNTF gene alone or whether they can also be induced by exogenous CNTF, native ST14A cells were exposed to recombinant rat CNTF for 24 hr either under permissive conditions (33°C) or under nonpermissive conditions (39°C), and the transcription activity was analyzed again by microarray technology. As a main result, the expression of a total of 19 genes was regulated (changed by twofold or more) upon exposure to CNTF at 33°C and of 14 genes also at 39°C (see Table I). The direction of changes was congruent with that seen in CNTF-transfected cells upon induction of differentiation by temperature increase in all genes but one (the exception is Id2). In all but one of these genes, up-regulation was registered; i.e., only glutamine synthetase was down-regulated.

CNTFR-α Gene Expression

A RT-PCR analysis applying a semijunction primer system provided clear evidence of the expression of the CNTFR-α gene in both native and CNTF-transfected ST14A cells (Fig. 7).

DISCUSSION

ST14A striatal progenitor cells stop the proliferation-only state and start to differentiate when the temperature is raised from permissive 33°C to nonpermissive 39°C. This process is characterized by huge and complex changes in more or less all aspects of cellular regulation, which is reflected by differential gene expressions. We were inter-

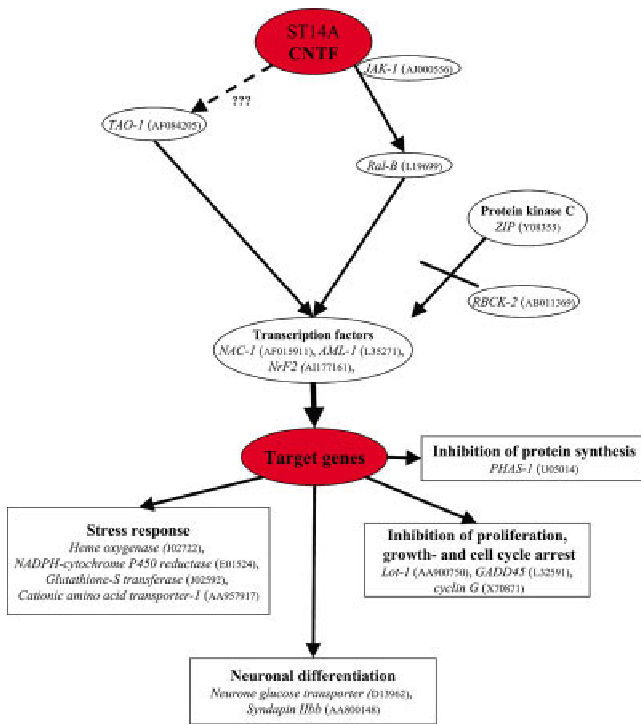

Fig. 5. CNTF-dependent signal transductions and regulatory responses in CNTF-transfected ST14A cells indicated by microarray expression analysis. After exclusion of genes with a similar expression profile in CNTF-transfected ST14A cells and native ST14A cells, a subgroup of 78 genes remained with different expression profiles. Those genes were further filtered, matching the average expression profile of this subgroup as well as possible, i.e., up-regulation (increased by twofold or more) at 3 hr in the CNTF-ST14A cells and down-regulation (decreased by twofold or more) at the same time in the native ST14A cells. This resulted in the 20 different transcripts given. Three of them are seen twice because of the redundancy of the microarray design; i.e., oligonucleotide probes of different regions of the same genes are present.

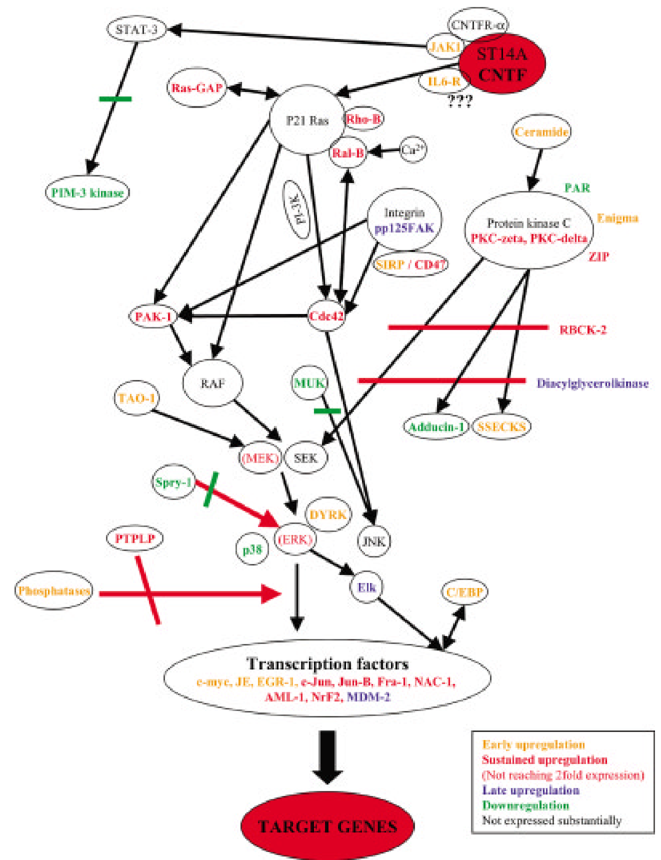

Fig. 6. Differentially expressed transcripts in CNTF-transfected ST14A cells at nonpermissive temperature (39°C) vs. permissive temperature (33°C) were matched with known signal transduction pathways obtained from the literature. The time course of regulation for each gene is given by colors as described in the **inset** (for details, see Discussion).

ested in comparing the gene expression changes during the early (initiation) stage of differentiation in CNTF-transfected and nontransfected striatal progenitor (ST14A) cells. Gene expression was detected by the Affymetrix microarray system, and evaluation of gene expression profiles was performed using the Genespring software. For the determination of substantial gene expression, we referred to the Affymetrix software matrix. Thereby, only those genes that had a "present" call at time 0 (0 hr) were included when the data were imported into the Genespring software. Most of the expressed genes could be classified into 10 sets according to their dominant expression profiles (Fig. 2) and into four groups according to their functional properties (Table I).

Genes indicating CNTF-dependent signaling were observed in set 6, showing an early up-regulation. The transcript of the CNTFR-α subunit could be detected in native as well as in CNTF-ST14A cells by RT-PCR analysis (see Fig. 7). However, CNTFR-α protein expression was not detectable by Western blotting (preliminary and unpublished data from M. Kirsch, Freiburg). Therefore, it remains an open question whether CNTF released by the CNTF-transfected cells (Weinelt et al., 2003) exerts receptor-mediated effects in an autocrine manner or whether other extra- or intracellular mechanisms may account for specific effects of CNTF transfection. Kordower et al. (1999) could show that transgenetic CNTF-secreting cells protected degenerating striatal neurons, which did not express the CNTF receptor. However, indirect effects may be involved in these in vivo findings. Alternatively, CNTF could bind to the IL-6/LIF receptor subunits and thereby induce signal transduction (Segal and Greenberg, 1996). Competitive binding of CNTF to the IL-6 receptor has been demonstrated, e.g., in CNTF-dependent regulation of fibrinogen gene expression in rat hepatocytes (Wang and Fuller, 1995). CNTF-mediated receptor activation in the CNTF-transfected ST14A cells is also suggested by up-regulation of JAK1, the receptor-associated kinase. Furthermore, we could detect C/EBP gamma and the C/EBP-related transcription factor. Members of the C/EBP family of transcription activators are involved in CNTF/LIF-mediated transcriptional activa-

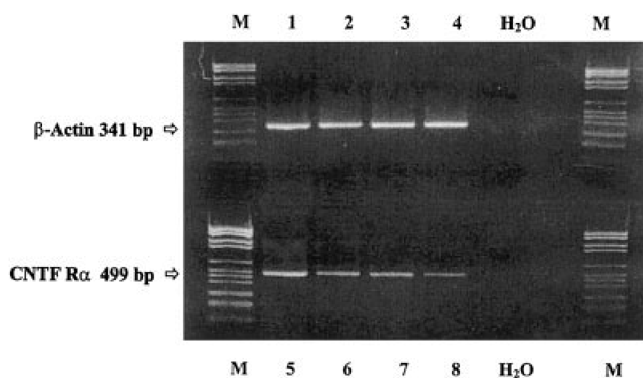

Fig. 7. Agarose gel electrophoresis of cDNA fragments of β-actin and CNTFR-α in native and CNTF-transfected ST14A cells. Preparations of rat brain served as positive controls. M VI from Roche (Mannheim, Germany) was used as molecular weight marker. **Lanes 1, 5:** cDNA rat brain; **lanes 2, 6:** cDNA ST14A (nativ); **lanes 3, 7:** cDNA CNTF-ST14A (clone 13); **lanes 4, 8:** cDNA CNTF-ST14A (clone 31).

tion (Symes et al., 1995; Davis, 1995). It is known that CNTF can activate multiple signaling pathways in parallel, including janus kinase (JAK)/STAT, MAP kinases, and phosphatidylinositol-3 kinase (PI-3 kinase; Yokogami et al., 2000). JunB, an immediate-early gene of the AP-1 complex, indicated as stress responsive in Table I, is also induced by CNTF and binds to C/EBP-like sites. JunB expression is essential for neuronal differentiation and negatively regulates cell growth (Schlingensiepen et al., 1994).

The regulation of signal transduction pathways was an early event in our experiments and was mediated predominantly via activation of MAP kinase-dependent signaling (Fig. 6), although JAK/STAT-dependent signal transduction is generally known to be the major pathway in CNTF-dependent cascades (Peterson et al., 2000). This might be due to the differentiating state of the cells, in that, in ST14A cells, the activation of the JAK/STAT pathway was related to proliferation (Cattaneo et al., 1996). PKC-dependent signaling seemed to be mostly suppressed. P21-Ras activation was followed by activation of the classical Raf/MEK pathway or by activation of other small GTPases, such as Cdc42. The involvement of the Raf/MEK/MAPK pathway with predominant ERK- and JNK-dependent activity is suggested mainly by indirect hints. Reasons for the fact that we did not find an up-regulation of all of the elements of the pathway could be low expression levels, short half-lives, or the restrictions we defined for expression level. In searching for single members of the pathway, such as MEK and ERK, there was an early up-regulation of these elements (as characteristic for gene sets 5 and 6), but it did not reach twofold expression level changes in comparison with time 0 (data not shown).

Many of the gene expression changes observed can be related to the switch of the CNTF-ST14A cells from proliferation to differentiation in response to temperature increase. On the other hand, there is clear evidence of a strong response to stress, mainly hypoxic/ischemic or oxidative stress; i.e., 1) components of the stress-sensitive MAP kinase pathway are up-regulated, whereas the p38 MAP kinase is down-regulated, and 2) protein biosynthesis is strongly inhibited, e.g., via down-regulation of translation-associated transcripts (Brostrom and Brostrom, 1998; Dever, 1999; Sheikh and Fornace, 1999; Holcik et al., 2000). Furthermore, 1) many of the proliferation- and growth arrest-related genes are also stress inducible, 2) various regulated transcripts in these sets are known to be induced by growth factor activity, and 3) there is clear evidence of CNTF-dependent signal transduction and cellular response.

EXPRESSION PROFILING OF CNTF-ST14A CELLS IN COMPARISON WITH NATIVE ST14A CELLS

The influence of CNTF transfection on the cellular response to nonpermissive temperature was assessed by comparing the transcription profile of these celles with the expression profile data of native, nontransfected ST14A cells in response to nonpermissive temperature. We could define a subgroup of genes (n = 20) whose regulation was clearly CNTF dependent. These transcripts were up-regulated early (time point 3 hr) in the CNTF-ST14A cells but remained unchanged or were even slightly decreased in the native ST14A cells. Functionally, the same features are evident as those described in Table I (Fig. 5).

Exogenous CNTF has induced the up-regulation of six and the down-regulation of one stress response-related genes in native ST14A cells at permissive as well as at nonpermissive temperatures congruently with the changes of gene expression in CNTF-transfected cells after transition to the nonpermissive differentiation-inducing temperature (see Table I). The same was true for 11 additional genes of other functional groups. Two of these genes belong to families of molecules that are already known to be up-regulated by CNTF, i.e., steroid receptors (Young et al., 1998) and connexins (Ozog et al., 2002). This finding suggests that exogenic CNTF partially mimics those effects that become apparent in CNTF-transfected cells after the start of differentiation.

Another important question is whether the differences between CNTF-transfected and native ST14A cells are due to nonspecific effects of the transfection process or are originally CNTF related. Our unpublished data on ST14A cells transfected with the glial cell line-derived growth factor (GDNF) have shown that these cells exert considerably different biological properties, e.g., prolonged lower metabolic and proliferative activity after differentiation compared with native ST14A cells, and only partially congruent gene expression changes with CNTF-ST14A cells. Seven of the twenty-three genes that showed twofold or more up-regulation 3 hr and 24 hr after temperature increase in GDNF-ST14A showed the same expression pattern in CNTF-ST14A cells. These seven genes are involved in basic mechanisms of metabolism and growth. Therefore, the result seems to be facilitated up-regulation of two groups of genes by CNTF transfection: 1) a minor group related to the basic regula-

tion of cell metabolism and cell growth, which is influenced, e.g., by different growth factors, and 2) a larger group of genes selectively influenced by CNTF. Members of the second group are, in particular, those involved in the stress response. For example, heme oxygenase is known to be increased when cells are exposed to hypoxia and shows a neuroprotective effect on adult rat ganglion cells after pressure-induced ischemia (Giaccia et al., 1992; Hegazy et al., 2000). It acts in concert with NADPH-cytochrome P450 reductase and down-regulates cellular heme and hemoprotein levels, thereby inactivating the most effective catalyst for formation of free radicals (Ewing and Maines, 1997). Heme oxygenase gene expression can be induced via activation of NF-E2-related factor 2 (Alam et al., 2000), which has been considered an essential component of the antioxidant response element (ARE)-binding transcriptional complex (Huang et al., 2000).

Up-regulation of PHAS 1 points to an inhibited protein biosynthesis, a hallmark of the cellular stress response (Holcik et al., 2000). Furthermore, transcripts of all ribosomal proteins and translation factors present on the microarray showed the same expression profiles, with an up-regulation at 3 hr in the native ST14A cells and no such regulation in the CNTF-transfected cells (data not shown). An early (3 hr) reduced metabolic activity in the CNTF-transfected cells has also been shown by the WST assay of metabolic activity. In later stages of differentiation, the in vitro experiments finally revealed a higher metabolic activity of the transfected cells compared with the native cells for more than 1 week at both 33°C and 39°C (Weinelt et al., 2003).

A strictly antiproliferative function is ascribed to Lot-1, GADD45, and cyclin G, which are selectively up-regulated in differentiating CNTF-ST14A cells. All three genes contribute to cell cycle arrest and apoptosis (Hughes et al., 1996; Shimizu et al., 1998; Okamoto and Prives, 1999; Azam et al., 2001). JAK-1 is clearly involved in CNTF signal transduction and may activate not only STAT but also the Ras/MAP kinase pathway (Rane and Reddy, 2000). Up-regulation of Ral-B, a Ras-related small GTPase, and TAO-1, a protein kinase that is highly expressed in the brain and activates MEKs in a stress-sensitive kinase cascade (Hutchison et al., 1998; Chen et al., 1999), provides clear evidence for an important role of MAP kinase-dependent signal transduction in our experiment.

AML-1, NAC-1, and neuron glucose transporter point to ongoing cell differentiation/determination. The neuron glucose transporter is specifically expressed in neurons, predominantly in the hippocampus, cerebral cortex, striatum, and granular layer of the cerebellum (Nagamatsu et al., 1993). A function in the stress response can be assumed, insofar as an increased uptake of extracellular glucose may provide the basis for glycolytic metabolism that is crucial for cell survival during hypoxia (Niitsu et al., 1999).

In vitro, CNS stem cells differentiate predominantly into astrocytes in the presence of CNTF (Johe et al., 1996). Our data indicate neuronal rather than astrocytic differentiation. We found, in addition to the described CNTF-specific regulated transcripts, further hints in this direction in the up-regulation of MAP2 in the CNTF-transfected cells at 24 hr, which had been excluded from our data set because of the Affymetrix decision matrix at 0 hr and 3 hr. Glial fibrillary acidic protein (GFAP), a marker of astrocytic differentiation, was not expressed in the native or in transfected cells at any time. The reason for the suggested neuronal development of the transfected cells could be that these progenitors are already committed to a certain fate. There are fundamental differences between lineage restriction in stem cells and differentiation of progenitor cells (McKay, 1997). The obvious Ras/MAP kinase-dependent signal transduction pathways used by CNTF in our experiment further argue against astrocytic differentiation (McKay, 1997).

Comparison with the expression profiles of non-transfected ST14A cells clearly shows a CNTF-dependent hypoxic/ischemic stress response during the earliest stage of differentiation (3 hr), with expression of specific transcripts and evidence of translational repression leading to decreased protein synthesis in the transfected cells. This is mediated by the Ras/MAP kinase pathway and is accompanied by impaired proliferation and metabolism as well as signs of neuronal differentiation. In vivo, CNTF is up-regulated in response to a variety of stress factors and protects mature neurons in the CNS and peripheral nervous system from degeneration arising from multiple etiologies (Peterson et al., 2000). One of these conditions is hypoxic/ischemic stress (Wen et al., 1995; Kumon et al., 1996; Lin et al., 1998; Mitchell et al., 1998; Ju et al., 1999; Park et al., 2000). The transgenically provided excess CNTF in our transfected cells seems to serve as an artificial signal mimicking a stress situation. The cells react in a "stress-like" manner because of the CNTF signaling.

CONCLUSIONS

The stress-like response in the early stage of differentiation improves the ability of the CNTF-ST14A cells to respond to and cope with a stressful environment in vivo. Less apoptosis and higher metabolic activity of the CNTF-transfected cells in later stages of differentiation (Weinelt et al., 2003) are the biological proof of an increased stress resistance. This renders CNTF-ST14A cells a favorable tool for transplantation experiments in animal models of degenerative CNS disorders involving the striatum. The present data indicate higher viability, longer lives, and greater differentiation capacity of these cells if used for transplantation. The demonstrated biological activity of CNTF released from the transfected cells should result in a sustained neurotrophic effect not only for the transplanted cells but also for surrounding neurons involved in the degenerative process. Our data support the utility of transplantation of genetically modified striatal progenitor cells and the delivery of neurotrophic factors by them for treatment of neurodegenerative disorders such as HD. Moreover, disclosure of several selectively and differentiation dependently up-regulated signal molecules in

CNTF-transfected cells may lead to identification of low-molecular-weight substances that could mimic the action of the neurotrophic factor with the advantage of being easier and safer (Borasio et al., 1996).

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