Neuroprotective Effect of a CNTF-Expressing Lentiviral Vector in the Quinolinic Acid Rat Model of Huntington's Disease

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Neurodegenerative diseases represent promising targets for gene therapy approaches provided effective transfer vectors. In the present study, we evaluated the effectiveness of LacZ-expressing lentiviral vectors with two different internal promoters, the mouse phosphoglycerate kinase 1 (PGK) and cytomegalovirus (CMV), to infect striatal cells. The intrastriatal injection of lenti-β-Gal vectors lead to 207,400 ± 11,500 and 303,100 ± 4,300 infected cells in adult rats, respectively. Importantly, the β-galactosidase activity was higher in striatal extracts from PGK-LacZ-injected animals as compared to CMV-LacZ animals. The efficacy of the system was further examined with a potential therapeutic gene for the treatment of Huntington's disease, the human ciliary neurotrophic factor (CNTF). PGK-LacZ- or PGK-CNTF-expressing viruses were stereotaxically injected into the striatum of rats, 3 weeks later the animals were unilaterally lesioned with 180 nmol of quinolinic acid (QA). Control animals displayed 148 ± 43 apomorphine-induced rotations ipsilateral to the lesion 5 days postlesion as compared to 26 ± 22 turns/45 min in the CNTF-treated group. The extent of the striatal damage was significantly diminished in the CNTF-treated rats as indicated by the 52 ± 9.7% decrease of the lesion volume and the sparing of DARPP-32, ChAT and NADPH-d neuronal populations. These results further establish that lentiviruses may represent an efficient gene delivery system for the screening of therapeutic molecules in Huntington's disease.   © 2001 Academic Press

Key Words: Huntington's disease; lentiviral vector; gene therapy; ciliary neurotrophic factor; quinolinic acid lesion model.

INTRODUCTION

Huntington's disease (HD) is an autosomal dominant inherited disorder involving the selective neuronal loss of striatal GABAergic output neurons and astrogliosis (Vonsattel and DiFiglia, 1998). The disease is characterized by motor and cognitive disturbances as well as psychiatric manifestations which usually appear in adult life and progress over one to two decades. Huntingtin, the gene causing Huntington's disease has been cloned in 1993 and the mutation identified as an unstable (CAG)n repeat in the 5′ coding region of the protein (The Huntington's disease

collaborative research group, 1993). Despite these important progresses, opening the possibility to generate genetic models of the disease (Mangiarini *et al.,* 1996; Reddy *et al.,* 1998; Hodgson *et al.,* 1999; Schilling *et al.,* 1999; Yamamoto *et al.,* 2000), the mechanisms accounting for the selective striatal degeneration are still largely unknown and there is currently no treatment available.

Several lines of evidence suggest that energetic defects and oxidative damage leading to secondary excitotoxicity and cell death may be implicated in HD process (Beal, 1992; Gu *et al.,* 1996). Consistent with this hypothesis, the intrastriatal injection of the tryp-

tophan metabolite quinolinate (QA), an *N*-methyl-d-aspartate (NMDA) receptor agonist, produces many pathological and neurochemical alterations observed in HD (Beal *et al.,* 1986). The chronic administration of the mitochondrial toxin 3-nitropropionic acid (3-NP) to rats and primates also results in selective striatal lesions (Palfi *et al.,* 1996). These models have been extensively used to develop strategies for the treatment of HD, such as cell replacement and neuroprotective approaches. A significant rescue of degenerating GABAergic neurons was observed following the grafting of fetal striatal cells or the administration of neurotrophic factors in QA-lesioned rats (Nakao *et al.,* 1999; Araujo and Hilt, 1997; Volpe *et al.,* 1998; Bemelmans *et al.,* 1999; Martínez-Serrano and Björklund, 1996; Kordower *et al.,* 1999a). In particular, CNTF initially identified for its ability to sustain the survival of parasympathetic neurons from embryonic chick ciliary ganglia was found to prevent behavioral deficits and striatal degeneration in QA-lesioned rats (Anderson *et al.,* 1996; Emerich *et al.,* 1996, 1997a). This protective effect was further confirmed in the QA and 3-NP primate models of Huntington's disease (Emerich *et al.,* 1997b; Mittoux *et al.,* 2000). In the latter study, the intracerebral and continuous delivery of CNTF at the time of appearance of striatal dysfunction and motor/cognitive deficits in six monkeys chronically treated with 3-NP not only protected neurons from degeneration but also alleviated motor and cognitive symptoms. The therapeutical value of both fetal grafts and CNTF delivery is presently investigated in several clinical trials (Kopyov *et al.,* 1998; St Hillaire *et al.,* 1998; Bachoud-Lévi *et al.,* 1998).

Neuroprotective strategies rely on the development of effective delivery systems leading to robust and long-term expression of the transgene and the presence of the protein in large area of the striatum. The transplantation of genetically engineered cells, the implantation of encapsulated cells releasing neurotrophic factors and more recently *in vivo* gene therapy approaches with an adenoviral vector have been assessed (Schumacher *et al.,* 1991; Emerich *et al.,* 1996; Bemelmans *et al.,* 1999). In the present study, we have used HIV-1-derived lentiviral vectors that have emerged as a promising gene delivery system in the CNS (Naldini *et al.,* 1996a; Klimatcheva *et al.,* 1999). We have previously demonstrated high and consistent transduction of neuronal cells with a self-inactivating vector (SIN) expressing the LacZ reporter gene in mice, rats, and primates (Bensadoun *et al.,* 2000; Déglon *et al.,* 2000; Kordower *et al.,* 1999b). In addition, the presence of the posttranscriptional element from the woodchuck hepatitis virus (Zufferey *et al.,* 1999) was shown to result in a three- to fourfold increase of the transgene expression level (Déglon *et al.,* 2000), similarly to what was observed in adeno-associated viruses (Loeb *et al.,* 1999; Paterna *et al.,* 2000).

To further optimize and validate this gene transfer system, we have now analyzed the transgene expression levels obtained with two internal promoters: the cytomegalovirus (CMV) and mouse phosphoglycerate kinase 1 (PGK) promoters and assessed the neuroprotective effect of CNTF in the rat QA-lesion model of Huntington's disease.

MATERIALS AND METHODS

Lentiviral Vector Production

The cDNA coding for a nuclear-localized β-galactosidase (LacZ) and the human CNTF cDNA (Aebischer *et al.,* 1996) were cloned in the SIN-W transfer vector (Déglon *et al.,* 2000). Two internal promoters were used: the mouse phosphoglycerate kinase-1 (PGK) and the cytomegalovirus (CMV) promoter. The packaging construct and the vesicular stomatitis virus G protein (VSV-G) envelope used in this study were the pCMVDR-8.92, pRSV-Rev, and the pMD.G plasmids described previously (Naldini *et al.,* 1996a; Dull *et al.,* 1998; Hottinger *et al.,* 2000). The viral particles were produced by transient transfection of 293T cells (Naldini *et al.,* 1996b). Forty-eight hours later the supernatant was collected, filtered and the particle content was determined by an ELISA assay (NEN Life Technologies, Boston, MA). High-titer stocks were obtained by ultracentrifugation. The batches of virus were tested for the absence of replication-competent retroviruses (RCR) (Naldini *et al.,* 1996a). The titers of the LacZ stocks were determined on 293T cells. The cells were plated at a density of 2×10^5 cells per well on six-well tissue culture dishes (Costar, Cambridge, MA). Serial dilutions of the viral stocks were added and the number of LacZ-infected cells was analyzed 48 h later. Titers were calculated by counting the number of blue foci per well and dividing it by the dilution factor. LacZ and CNTF expressing viruses resuspended in phosphate buffered saline (PBS)/1% bovine serum albumin (BSA) and matched for particle content (200,000 ng p24 antigen/ml as measurement by ELISA assay) were used for the *in vivo* experiments.

hCNTF ELISA Assay and Bioactivity Measurement

Quantitative analysis of *in vitro* and *in vivo* hCNTF production was performed using an ELISA assay (R&D Systems, Minneapolis, MN) according to the supplier's manual. 293T cells (1×10^5) were transiently transfected by calcium phosphate precipitation with the CNTF-expressing transfer vector or infected with the SIN-W-PGK-CNTF recombinant virus (p24 = 150 ng/ml). Two days later, conditioned media were obtained by incubating the cells in 1 ml of fresh media for 2 h.

The *in vivo* synthesis of CNTF was determined from punches taken around the injection sites and from the non-injected hemispheres ($n = 3$). The samples were sonicated in 500 μl PBS, containing a cocktail of protease inhibitors (pronase, thermolysin, chymotrypsin, trypsin, papain; Roche Pharma, Reinach, Switzerland). The supernatants were used for the ELISA assay.

Choline acetyltransferase activity was determined by measuring the synthesis of [^{3}H]acetylcholine from [^{3}H]acetylcoenzyme A on E14 rat spinal cord cultures as previously described (Zurn *et al.,* 1994; Zurn and Werren, 1994). Fifty microliters of conditioned media from 293T cells infected with the SIN-W-PGK-CNTF virus, transfected with the corresponding plasmid or from control cells, were added to the E14 rat spinal cord cultures.

Animals

Adult female Wistar rats (Iffa-Credo, France), weighing 180–200 g were used in these studies. The animals were housed in a controlled temperature room that was maintained on a 12-h light/dark cycle. Food and water were available *ad libitum.* The experiments were carried out in accordance with the European Community Council directive (86/609/EEC) for care and use of laboratory animals.

Injection of the Lentiviruses

The concentrated viral stocks were defrost and resuspended by repeated pipetting. Two microliters of CNTF or LacZ-expressing lentiviral vectors were injected into the *striatum* of pentobarbital anesthetized (45 mg/kg, i.p.) animals using a syringe (Hamilton, Reno, NV), with a 28-gauge blunt tip needle. The stereotaxic coordinates for the injection were: 1.2 rostral to bregma; 2.6 lateral to midline for the LacZ experiment or 2.2 for the QA-CNTF experiment; 4.5 ventral from the dural surface; with the mouthbar set at 3.3. The suspension was injected at 0.2 μl/min by means of an automatic injector (Stoelting Co., Wood Dale, U.S.A.) and the needle was left in place for 5 min. The skin was closed using a 6-0 Vicryl suture (Ethicon, Johnson and Johnson, Brussels). The animals were allowed to recover for 3 weeks before the injection of quinolinic acid.

β-Galactosidase Activity

One week after the injection of CMV- or PGK-LacZ-expressing viruses, the animals were sacrificed, perfused with ice-cold PBS containing 0.02% ascorbic acid and 5000 units of heparin (Liquemin, Roche, Reinach, Switzerland), and fixed in 4% paraformaldehyde for 24 h.

β-Galactosidase activity was detected by incubating sections in PBS containing 4 mM FeCN(II)/FeCN(III), 2 mM $MgCl_2$, 0.02% NP-40, and 0.4 mg/ml X-Gal. β-Galactosidase activities were also measured on a punch made over a 2-mm-thick transversal slices cut with a tissue chopper and covering most of the striatum. The samples were rapidly frozen on dry ice and kept at $-80°C$ until processing. The samples were sonicated (Microson Ultrasonic Cell Disruptor; Heat Systems, Farmingdale, NY; 10s/low power) in 500 μl of extraction solution provided with the β-galactosidase assay kit (Promega, Madison, WI). After centrifugation at 14,000 rpm for 10 min the supernatants were assayed according to the manufacturer's protocol.

Quinolinic Acid Lesion

Quinolinic acid (QA) (Sigma Chemical, St. Louis, MO) was dissolved in 2 M NaOH, the pH was adjusted to 7.4 and the volume completed with PBS, pH 7.4. Under pentobarbital anesthesia (45 mg/kg), the animals received an intrastriatal injection of 1 μl of quinolinic acid (180 nmol) using the following coordinates: 1.2 rostral to bregma; 3.2 lateral to midline; 4.5 ventral from the dural surface; with the mouthbar set at 3.3. The toxin was injected over 1 min, and the needle was left in place for an additional 3 min.

Behavioral Analysis

Apomorphine-induced rotational asymmetry was measured twice before the injection of the virus. Animals were injected subcutaneously with 1.0 mg/kg apomorphine (Amino AG, Neunhof, Switzerland) and placed into a test chamber (Rotoscan, Rotometer v5.06, Omnitech Instruments, Columbus, OH) for a 3-min

habituation period before a 45-min test session. Rotations were defined as complete 360° ipsilateral turns and were reported as the net difference between the two directions. Animals that did not display spontaneous turning behavior (less than 20 tours per 45 min) were selected. The animals were tested 1 and 2 weeks after the injection of the virus and 5, 9, and 13 days after QA administration. Results are expressed as the net difference between the total number of turns after and before the administration of QA. Ipsilateral turns were counted as positive turns, whereas contralateral turns were counted as negative turns.

Histological Processing

Two weeks after QA lesion, the animals were sacrificed and the brains processed for immunohistochemistry. The animals were anesthetized with a sodium pentobarbital overdose, transcardially perfused with heparinized PBS, and postfixed in 4% paraformaldehyde for approximately 24 h and finally cryoprotected in 25% sucrose/0.1 M phosphate buffer for 48 h. The brains were frozen in dry ice and coronal sections were cut on a sliding cryostat microtome (Cryocut 1800, Leica Microsystems, Nußloch, Germany) at a temperature of $-20°C$ and a thickness of 25 μm. The slices throughout the striatum were collected and stored in 48-well trays (Costar, Cambridge, MA) as free-floating sections in PBS containing 0.12 μM sodium azide. The trays were stored at 4°C until immunohistochemical processing. A rabbit polyclonal anti-β-galactosidase antibody was used for β-Gal staining (1:500, 5 Prime 3 Prime Inc., U.S.A.). To identify the type of infected cells, double-staining were performed with the neuronal marker NeuN (1:200: Chemicon, Temecula, CA) and the glial cell marker glial fibrillary acidic protein (1:50: GFAP) (Roche Pharma, Reinach, Switzerland). The mounted sections were analyzed by confocal scanning laser microscopy (Leica TCSNT, argon-Krypton laser). Fluorescent signals were collected, digitally color enhanced, and superimposed.

The evaluation of the lesion volume and the quantification of the cell loss was performed on six sections separated by 200 μm. These samples were processed by immunohistochemistry for glutamic acid decarboxylase 67 (GAD_{67}) (Chemicon, Temecula, CA), choline acetyltransferase (ChAT) (Roche Pharma, Reinach, Switzerland), dopamine and cAMP-regulated phosphoprotein of a molecular mass of 32 kDa (DARPP-32) (a generous gift from Dr. Greengard, Rockefeller Univ., NY), and neuronal nitric oxide synthase

(nNOS) (Chemicon International Inc.). Enzymatic staining for NADPH-diaphorase (NADPH-d) was performed as previously described (Ellison *et al.,* 1987). For immunohistochemical stainings, endogenous peroxidase activity was quenched with 0.1% diphenylhydrazine/PBS (37°C/30′) and washed three times in PBS. Free floating sections were incubated overnight in 5% Normal Goat Serum (NGS, Dako Diagnostics, Switzerland)/0.1 M phosphate buffered saline at 4°C, followed by an overnight reaction (except for GAD, where the sections were incubated for 3 days) with the respective antibodies: β-Gal (1:500); GAD (1:2000); ChAT (1:50), DARPP-32 (1:20,000), nNOS (1:1000) diluted in PBS/1% NGS solution. After three washings the sections were incubated with the corresponding biotinylated secondary antibody (Vector, Burlingame, CA; 1:200) for 2 h at room temperature, and bound antibody was visualized by using the ABC system (Vectastain ABC kit, Vector Laboratories, West Grove, U.S.A.), with 3,3′ diaminobenzidin (DAB Metal Concentrate, Pierce, Rockford, IL) as chromogen. The sections were mounted, dehydrated by passing twice through ethanol and toluol and coverslipped with Merckoglas. Secondary antibodies coupled to fluorescent chromophores (Jackson Immunoresearch Laboratories, West Grove) were used for LacZ (1:200: FITC-Goat Anti-rabbit), NeuN, and GFAP (1:500: CY3-Goat Anti-mouse). The sections were agitated at a speed of 100 rpm for 2 h at ambient room temperature and finally mounted on glass slides with FluorSave Reagent (Calbiochem, La Jolla, CA).

Cell Counting and Evaluation of the Lesion Size

The number of β-galactosidase-positive nuclei was determined by counting the number of DAB-stained nuclei on five sections covering the infection site over a 2.4-mm zone using a 20× objective. The total number of infected neurons was calculated by multiplying the number of sections by the average number of neurons between two sections (Tseng *et al.,* 1998). Counts of DARPP-32-, ChAT-, and NADPH-d-stained neurons were performed using a 20× objective on six sections adjacent to those analyzed for the lesion size and the values expressed as percentage of the neuron numbers on the nonlesioned side. The total striatal surface areas were considered for ChAT and NADPH-diaphorase countings, while, for DARPP-32, a sampling of the striatum was made by counting neurons on a quadrant placed at the bottom of the ventricle.
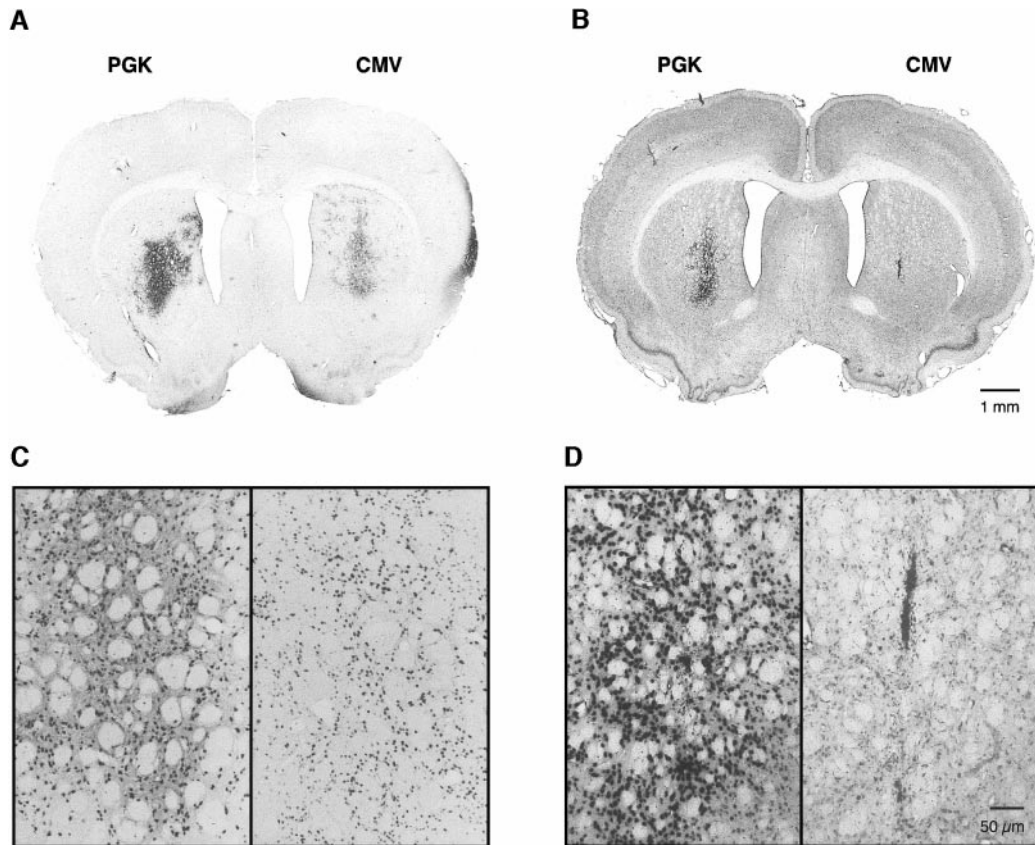
FIG. 1. Rat striatal sections showing LacZ immunoreactivity (A, C) or enzymatic activity (B, D) 1 week after the injection of the SIN-W-PGK and SIN-W-CMV-lentiviral vectors in the left and right hemispheres, respectively. (C, D) High power photomicrographs around the injection site showing the density of neurons infected and the higher LacZ expression level obtained with the SIN-W-PGK lentivirus as compared with the SIN-W-CMV vector.

The extent of the lesion produced by QA was determined by digitizing six sections per animal with a slide scanner and quantifying it with an image analysis public domain program (NIH-Image, Version 1.6.1, National Institutes of Health). The lesion size was determined from equidistant (200 μm) GAD-stained sections covering the center of the lesion. The border of the lesion was traced on GAD stained sections throughout the six sections and the corresponding volume of the lesion was determined and expressed as percentage of the total hemisphere volume.

Data Analysis

Data are expressed as mean $\pm$ SEM and evaluated for analysis of variance (ANOVA) followed by a Scheffe's PLSD post-hoc test (StatView 4.0, Version 3.2.6, Aladdin Systems, U.S.A.). The significance level was set at $P < 0.05$.

RESULTS

Transduction Efficiency and β-Galactosidase Expression Levels with SIN-W-PGK and SIN-W-CMV Vectors

To evaluate the transduction efficacy and the level of expression of β-galactosidase *in vivo,* 2 μl of SIN-W-PGK- and SIN-W-CMV-LacZ viruses were injected in the left and right hemisphere of three adult Wistar rats. The titers of the concentrated β-Gal-expressing viruses were 2.6×10^8 and 5.5×10^8 TU/ml, respectively. The histological analysis of striatal sections collected 1 week later showed that infected cells are present over 2.2 mm with $207,400 \pm 11,500$ and $303,100 \pm 4,300$ β-Gal-expressing cells in PGK- and CMV-injected animals (Figs. 1, 2A and 2B). A robust immunostaining of the nucleus and the fibers was observed in PGK-LacZ-infected cells, whereas the la-
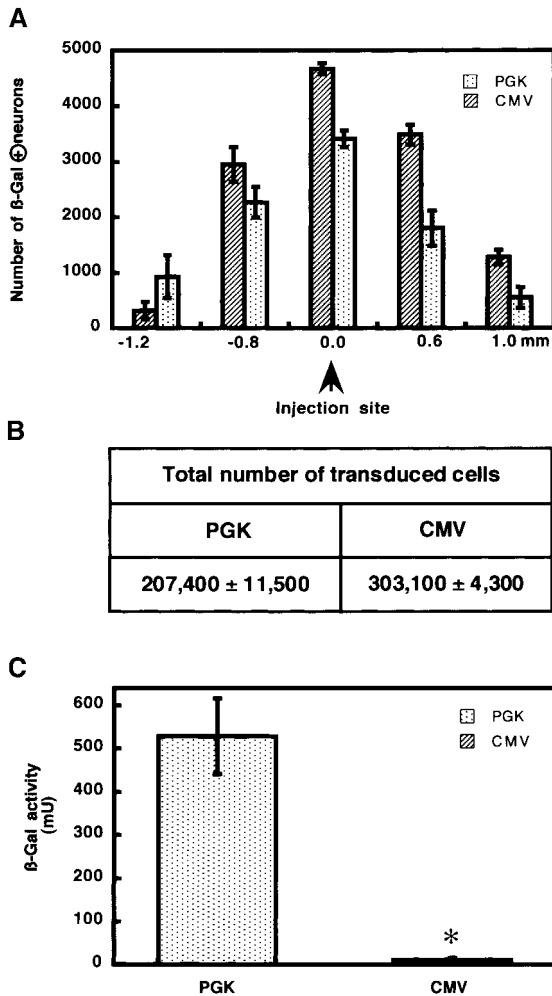
FIG. 2. (A) Evaluation of the diffusion of the lentiviral vector from the injection site and determination of the number of the LacZ-expressing cells on five immunostained sections. (B) Estimation of the total number of infected cells in the PGK- and CMV-LacZ-injected animals. (C) Measurement of β-galactosidase activity on striatal punches from PGK- and CMV-LacZ injected animals ($n = 5$). *$P = 0.0004$.

beling was limited to the nucleus with the CMV-LacZ vector (Figs. 1A and 1C). Similarly, the X-Gal enzymatic staining was more intense in the PGK-LacZ animals as compared with the CMV-LacZ group (Figs. 1B and 1D). To quantify the difference in transgene expression levels, striatal punches covering most of the infected area were used to measure β-galactosidase enzymatic activity in five additional animals. The activity was higher with the PGK-LacZ virus as compared with the CMV-LacZ virus (Fig. 2C). Control animals or animals injected with PBS did not display β-galactosidase activity (data not shown). In addition,

robust expression of the LacZ reporter gene was observed 12 months after the injection of SIN-W-PGK virus in the striatum of two rats (Fig. 3A). Long-term expression of β-galactosidase is not associated with neurotoxic effects as indicated by the unaltered expression of the neuronal markers, DARPP-32, ChAT, and nitric oxide synthase (nNOS) (Fig. 3B). These results establish that SIN-W-PGK lentiviral constructs allow the transduction of a large number of cells in the rat striatum and that high and sustained expression of the transgene is obtained with the housekeeping PGK promoter.

Neurotropism of VSV-G Pseudotyped Lentiviruses

The strong neurotropism observed after the injection of lentiviral vectors in the CNS has been attributed to the VSV-G envelope and/or the preferential activity of the CMV promoter in neurons (Naldini *et al.,* 1996). We therefore analyzed whether the use of the housekeeping promoter PGK alters the phenotype of transduced cells. Striatal sections from the SIN-W-CMV- and SIN-W-PGK-injected animals were immunostained with β-Gal and the NeuN neuronal or GFAP glial markers (Fig. 4). Confocal microscopic images showed that in agreement with previous studies with the CMV-LacZ construct (Naldini *et al.,* 1996b), essentially all β-Gal-positive cells were NeuN-positive (Fig. 4) with $1.80 \pm 0.75\%$ of GFAP/LacZ double-labeled cells. Similar results were obtained with the PGK-driven construct, suggesting that the neurotropism of the lentiviral vector is due to the VSV-G envelope.

Characterization of a CNTF-Encoding Lentivirus

Based on the above-mentioned data, the cDNA coding for the human CNTF was cloned into the SIN-W-PGK backbone. To evaluate the production of CNTF, 293T cells were infected with the CNTF-lentivirus (p24 = 150 ng/ml). Seventy-two hours later, the levels of CNTF present in the medium were determined by an enzyme-linked immunosorbent assay (ELISA). CNTF levels of 84.6 ± 16.8 ng CNTF/day were detected in the infected cells, whereas it was undetectable in control cells. The vector was shown to express bioactive CNTF as determined by an increased choline acetyltransferase (ChAT) activity on E14 rat mesencephalic primary cell cultures exposed to conditioned media from transfected or infected 293T cells (Table 1). The *in vivo* production of CNTF was measured on 3 Wistar rats injected with 2 μl of SIN-W-PGK-CNTF virus (200,000 ng p24/ml). One
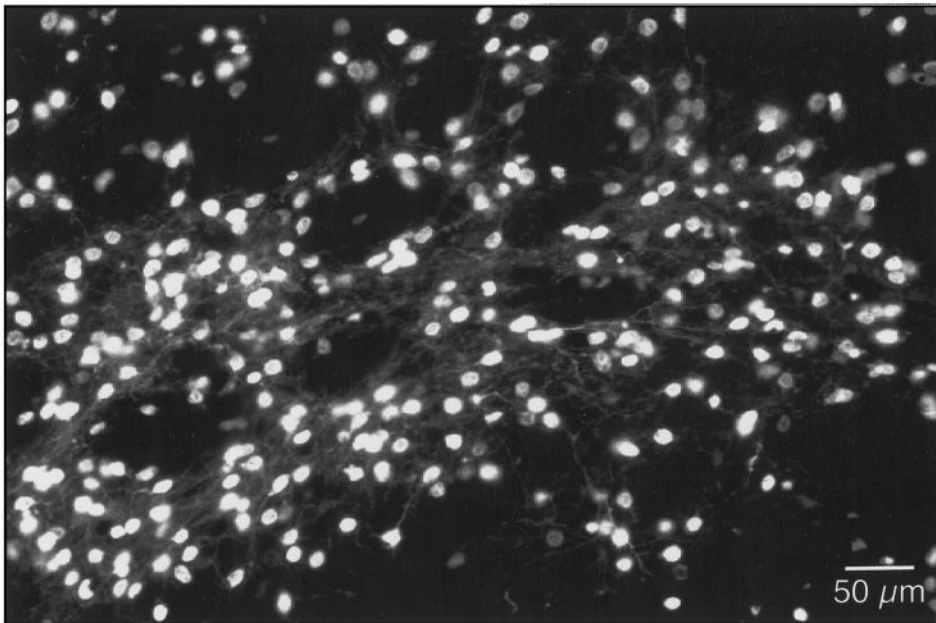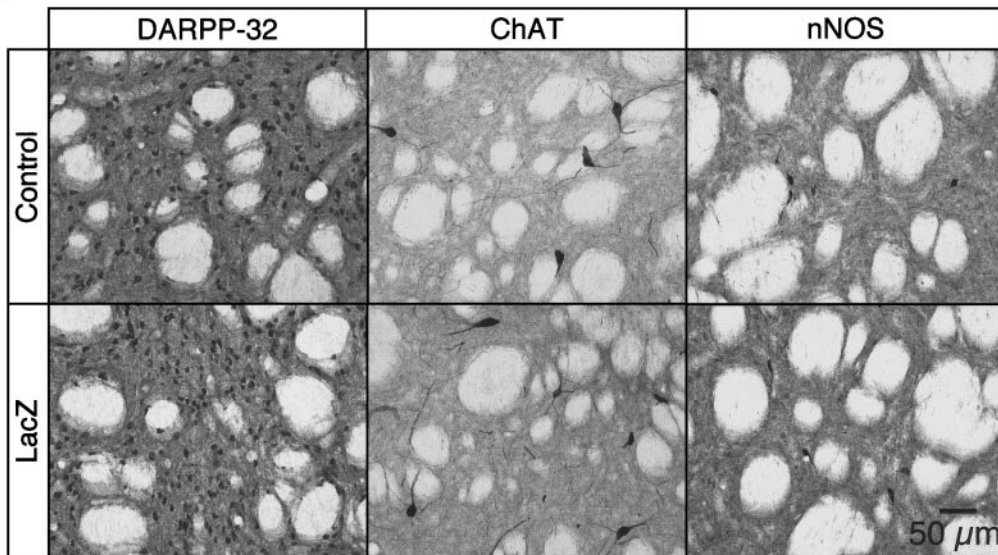
A

B

FIG. 3. (A) LacZ-immunostained striatal section showing the robust expression of the reporter gene 12 months postinjection of a SIN-W-PGK lentiviral vector. (B) The expression of the neuronal markers DARPP-32, ChAT, and nNOS were not altered by the long-term and sustained expression of β-galactosidase.

week later, the animals were sacrificed and a punch covering most of the striatum was excised. The tissue was homogenized and analyzed by ELISA assay for CNTF content. The concentration of CNTF was 0.94 ± 0.33 ng/mg protein in the injected site, whereas it was undetectable in the noninjected hemispheres.

Neuroprotective Effect of a CNTF-Expressing Lentivirus

Wistar rats were injected with lentiviruses encoding for CNTF ($n = 6$) or LacZ ($n = 6$). Three weeks later, the animals were lesioned with the intrastriatal injec-
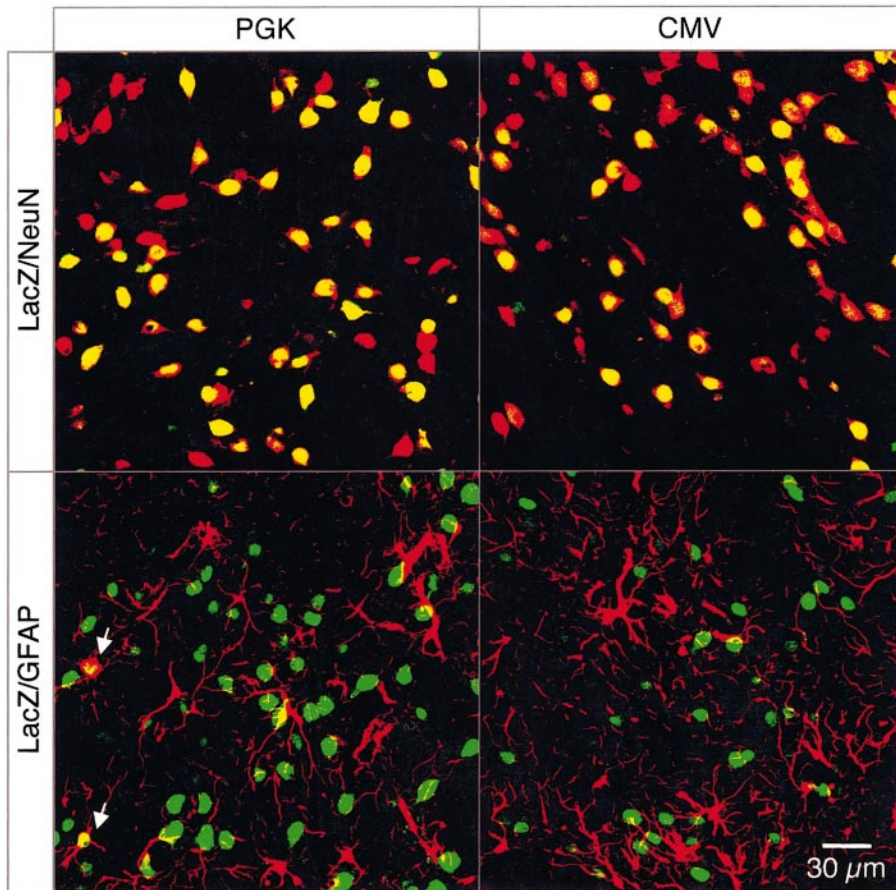
FIG. 4. Confocal microscope images of striatal sections from PGK-LacZ or CMV-LacZ injected rats stained for β-galactosidase (green) and the neuronal marker NeuN (red) or GFAP (red). Numerous NeuN⁺ neurons were infected with the LacZ-expressing lentivirus as indicated by the yellow nuclei resulting from the colocalization of β-Gal (Green) and NeuN (Red), whereas only scarce β-Gal-positive cells were double-stained for GFAP (arrows).

tion of 180 nmol QA. The striatal degeneration produced by the unilateral administration of QA resulted in an apomorphine-rotational asymmetry (Borlongan *et al.,* 1995; Nakao and Brundin, 1997). As expected, control animals injected with the LacZ-virus displayed ipsilateral turning in response to the dopamine agonist, 5, 9, and 13 days after QA administration (Fig. 5). In contrast, a significant reduction of the motor deficit was observed in the CNTF-treated animals (Fig. 5). Interestingly, a slower weight gain was observed in the CNTF animals (11.1 ± 3.2%) 12 days after QA lesion relative to the control group (19.5 ± 1.2%; $P = 0.0342$).

The glutamic acid decarboxylase (GAD) marker of GABAergic neurons, including the projecting neurons that make up for more than 90% of all striatal neurons was used to measure the damages caused by the in-

TABLE 1

Choline Acetyltransferase Activity Measured on E14 Rat Embryonic Spinal Cord Cultures Exposed to Conditioned Media from 293 T Cells Transfected or Infected with SIN-W-CNTF or from Mock-Infected Cells

Media	ChAT activity (%)
Control	100
Mock-infected 293T cells	116 ± 15
10 ng/ml rhCNTF	286 ± 4
293T Transfected: hCNTF transfer vector	362 ± 37
293T Infected: hCNTF lentivirus	317 ± 81

Note. The values are expressed as percentages of control cultures grown in the absence of neurotrophic factor. The addition of 10 ng/ml of recombinant hCNTF was used as a positive control. Each value represents the mean of two culture wells from two separate experiments. Data are expressed as the mean ± SEM.
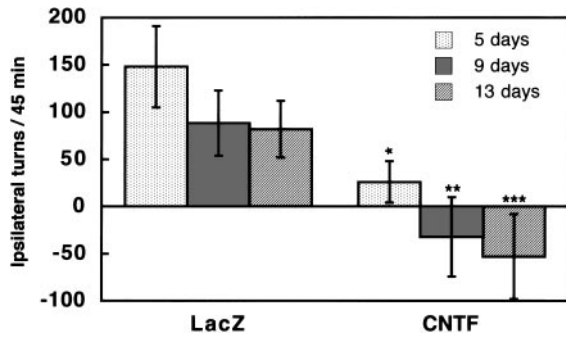
FIG. 5. Histogram showing the average changes in rotational asymmetry induced by apomorphine stimulation (1 mg/kg, s.q.) 5, 9, and 13 days after QA lesion. Positive values correspond to turns ipsilateral to the lesion. A significant reduction of the drug-induced rotational behavior was observed in the CNTF-treated animals ($n = 6$) as compared with the LacZ-injected animals ($n = 6$). Values are expressed as mean $\pm$ SEM. *$P = 0.031$, **$P = 0.051$, ***$P = 0.031$.

DISCUSSION

Over the past few years, the major obstacles toward the use of viral vectors for gene therapy applications has been the poor transduction efficiency often associated with transient and low transgene expression levels (Verma and Somia, 1997; Anderson, 1998). Several strategies are currently being investigated to overcome these limitations. Among them, the development of second/third generation of viral vectors (Romano *et al.,* 1999), the use of viruses with altered tropism (Russell and Cosset, 1999), the assessment of transcriptional and posttranscriptional regulatory elements (Walther and Stein, 1996; Srinivasakumar *et al.,* 1997) and the evaluation of methods to improve the transduction and diffusion of viruses in tissues (Ko-
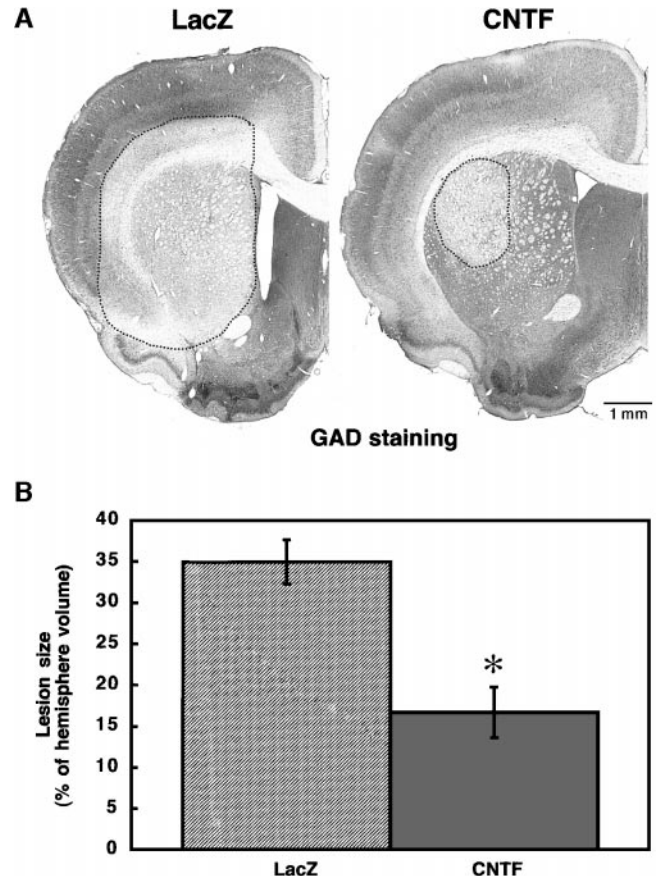
jection of QA (Fig. 6). In the LacZ-injected animals, a drastic reduction of the GAD-ir covering most of the striatum and in some cases adjacent regions was observed on the lesioned side. In contrast, the volume of the lesion was significantly reduced in the CNTF treated animals (Fig. 6) ($P = 0.0012$).

The cell degeneration was analyzed in different subpopulations of striatal neurons. The neuronal phosphoprotein DARPP-32 was used as a marker of the projecting GABAergic neurons affected in Huntington's disease. The administration of QA in LacZ-injected animals induced a near complete loss of DARPP-32 staining in the core of the lesion with only $26.2 \pm 6.2\%$ positive neurons throughout the affected area (Fig. 7). This loss of DARPP-32 immunoreactivity was however attenuated in the CNTF-treated animals compared to the control group ($73.7 \pm 6.9\%$; $P = 0.0004$) (Fig. 7).

To further assess the effect of CNTF administration, similar analyses were performed on two populations of interneurons. The cholinergic interneurons visualized with choline acetyltransferase (ChAT) immunostaining and the GABAergic interneurons identified with nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d). In control animals, the number of ChAT-ir and NADPH-d-ir cells was significantly reduced on the lesioned side (Fig. 7). In CNTF-treated animals, ChAT- and NADPH-d-positive neurons with long neuritic processes were seen even in sections close to the needle tract (Fig. 7). Cell counts further demonstrated that CNTF treatment rescues these two subpopulations of neurons (Fig. 7).

FIG. 6. (A) GAD immunohistochemical staining in the striatum of two rats showing the reduced lesion area in the CNTF-treated animal as compared with the LacZ-injected rat (40 and 7.6%, respectively). (B) lesion volume in control and CNTF-treated animals expressed as percentage of the hemisphere volume. Values are expressed as mean $\pm$ SEM. *$P = 0.0012$.
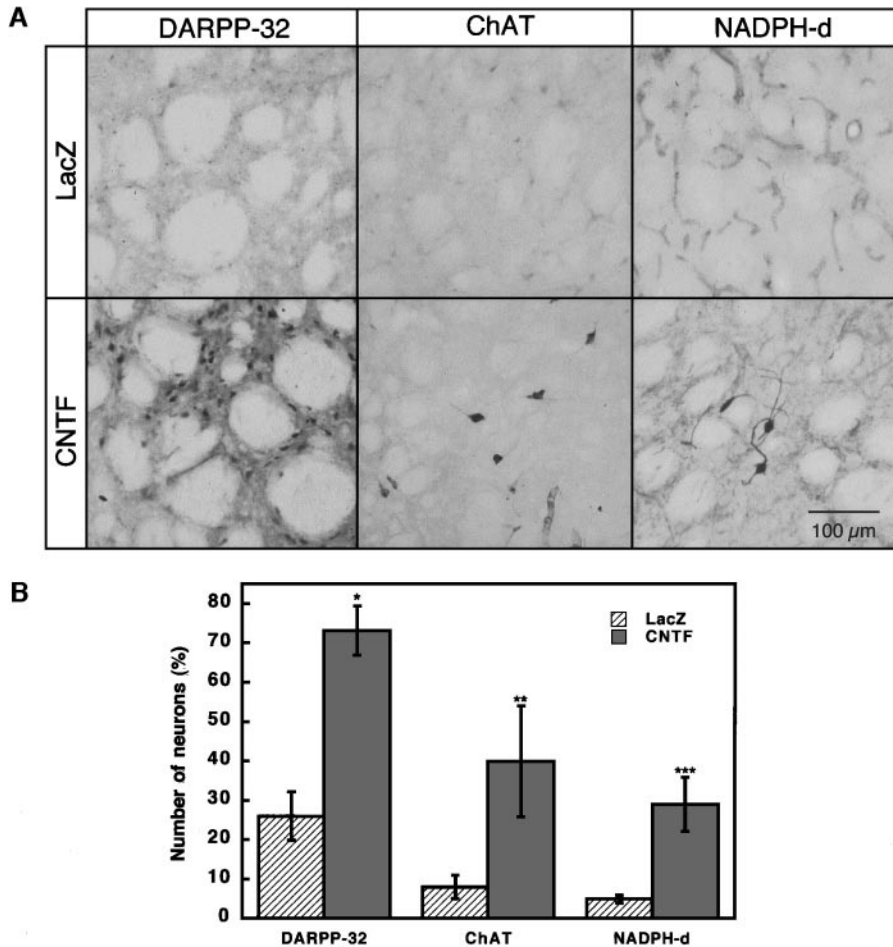
FIG. 7. (A) Micrographs showing DARPP-32, ChAT, and NADPH-d histochemical stainings in the striatum 2 weeks after QA lesion in animals injected with lentiviruses expressing β-Gal or CNTF. (B) Percentage of DARPP-32, ChAT, or NADPH-d-positive neurons on the right (lesioned) side versus left (nonlesioned) side. Values are expressed as mean ± SEM. *$P = 0.0004$, **$P = 0.0613$, ***$P = 0.0064$.

hutnicka *et al.,* 1999; Tenenbaum *et al.,* 1999; Zennou *et al.,* 2000).

In this context, lentiviral vectors from human, simian, feline, or equine origin appear as promising gene delivery vehicles for quiescent cells such as neurons and hematopoietic stem cells (Poeschla *et al.,* 1998; White *et al.,* 1999; Johnston and Power, 1999; Olsen, 1998). This property might however not apply to all postmitotic cells as suggested by the cell-cycle dependent transduction of hepatocytes (Park *et al.,* 2000) and the low transduction efficiency of muscle (Trono, 2000). In the CNS, high transduction efficiency and long-term expression of reporter genes have been reported (Naldini *et al.,* 1996b; Blömer *et al.,* 1997; Déglon *et al.,* 2000). In the present study, $2-3 \times 10^5$ infected cells spread over 2.2 mm were counted after the injection of 2 μl of concentrated stock of LacZ-

expressing viruses. The increased number of infected cells in the CMV-W-LacZ-injected hemispheres ($303,100 \pm 4,300'$) as compared to the PGK-W-LacZ ($207,400 \pm 11,500$) correlates with the higher titer of the latter batch (5.5×10^8 and 2.6×10^8 TU/ml, respectively). Compared to earlier studies, this represents a 10- to 30-fold increase in the number of infected striatal cells ($1-2 \times 10^4$ transduced cells) (Zufferey *et al.,* 1997; Mitrophanous *et al.,* 1999). The higher proportion of transduced cells might be due to differences in vector design, in target cells, and protocols used to determine the titers, in the procedures utilized to concentrate the viral stocks and/or in the methods employed to detect infected cells. Storage conditions aimed at minimizing the development of aggregates and noninfectious particles, as well as injection procedures insuring an optimal diffusion of the

viral particles may also explain the increased transduction efficiency. The importance of these parameters remains to be established.

In agreement with previous reports, the great majority of the transduced cells express the neuronal marker NeuN (Naldini *et al.,* 1996; Blömer *et al.,* 1997). If one considers that the striatum of rats contains approximately 2.8 million neurons (Oorschot, 1996), a single injection of a lentiviral vector is therefore sufficient to infect approximately 10% of the striatal neurons. Increasing the amount of viral particles, the number of sites and/or improving the vector properties (Zennou *et al.,* 2000) may further augment this proportion. This will be particularly important for the scale-up of the technology in primates (Kordower *et al.,* 1999b) and for the expression of intracellular proteins.

To maximize the transgene expression and avoid the transcriptional shut off over time, the appropriate promoter/enhancer combination has to be determined for each cell type. Lentiviral vectors expressing the LacZ reporter under the control of the CMV or the mouse PGK promoters were therefore compared. Similarly to what has been observed on purified motoneurons infected with LacZ-expressing lentiviral vectors *in vitro* (Cisterni *et al.,* 2000), higher β-gal activity was measured on striatal punches from PGK-LacZ-injected animals. A result which can't be explained by differences in viral titers or variability in the number of infected cells between the two groups.

As a first step toward the use of lentiviral vector as gene delivery system in HD, we have produced a lentivirus encoding the human CNTF. The administration of this molecule in a large brain structure, such as the striatum and the short half-life of the protein represent a major challenge for its clinical application. The diffusion of lentiviral vectors several millimeters from the injection site may overcome these limitations and provide multiple point sources of CNTF throughout the striatum, ensuring an optimal diffusion of the protein and maximizing its biological activity.

Previous studies have shown that the intracerebral administration of recombinant CNTF with minipumps or the implantation of encapsulated CNTF-releasing cells, 3 to 12 days prior to lesioning with QA, protect rat striatal neurons and prevent motor and cognitive deficits (Anderson *et al.,* 1996; Emerich *et al.,* 1997a). Although differences in the QA lesioning protocol and in the evaluation methods preclude any direct comparison with previous reports, our results demonstrated that the injection of a CNTF-expressing lentiviral vector and the continuous production of nanogram of CNTF partially reversed behavioral deficits. A number of factors can influence apomorphine-induced rotational behavior in QA-lesioned rats. Among them, the extent and localization of the lesion (Nakao and Brundin, 1997; Fricker *et al.,* 1996), the interval between the administration of QA and the behavioral analysis, and the interval between successive drug administration. Differences in these parameters may explain the observed decrease in rotational asymmetry over time contrary to what has been described by Nakao *et al.* (1996). These behavioral improvements were associated with a rescue of GAD, DARPP-32, ChAT, and NADPH-d-ir neurons in CNTF-treated animals. The mechanism of action of CNTF is however unclear. CNTF may directly interfere with the toxic effect of QA by altering the expression or function of glutamate receptors. This possibility seems unlikely as CNTF does not block the neurotoxic effect of glutamate *in vitro* (Mattson *et al.,* 1995) and prevents striatal degeneration of 3-NP-lesioned animals (Mittoux *et al.,* 2000). Striatal neurons are responsive to the cytokine (Buzas *et al.,* 1999) and brain injury leads to an up-regulation of CNTF and CNTFRα mRNAs in the wound area that correlates with the appearance of reactive astrocytes (Ip *et al.* 1993; Asada *et al.* 1995). CNTF activates the JAK-STAT signal transduction pathway (Hirano *et al.,* 1994; Bonni *et al.,* 1997) and induces the expression of immediate early genes (Bonni *et al.,* 1993; Gloaguen *et al.,* 1997). The development of microarray technology (Serafini, 1999) should offer new opportunities to dissect the intracellular signaling pathway and neuroprotective effect of CNTF and obtain a global view of the temporal program of gene expression in CNTF-treated animals.

Interestingly, rats treated with CNTF have a reduced weight gain as compared to LacZ injected animals. No weight loss was, however, reported with encapsulated cells genetically engineered to produce human CNTF (4–11 ng/day) implanted in the lateral ventricle (Emerich *et al.,* 1996, 1997a) or with the intracerebral administration of recombinant rat CNTF (9.4 μg/day) using an osmotic minipump (Anderson *et al.,* 1996). In contrast, the systemic delivery of CNTF (approximately 8 μg/day) in rat pups was associated with muscle wasting (Henderson *et al.,* 1994). This might be due to differences in the sites of administration of CNTF (CNS vs periphery) or in the diffusion of CNTF from the ventricles or in the parenchyma (Kordower *et al.,* 1999a). It has been proposed that CNTF-induced weight loss is the consequence of anorexia caused by a reduction of the hypothalamic appetite stimulating neuropeptide Y (NPY) (Xu *et al.,* 1998; Pu *et al.,* 2000). The intrastriatal injection of CNTF-expressing lentiviral vector may therefore affect NPY-

producing neurons located in the brain stem and arcuate nucleus of the basal hypothalamus. Inducible lentiviral vectors (Kafri *et al.,* 2000; Gingrich and Roder, 1998) need to be developed to further investigate the dose-dependent neuroprotective effects of CNTF, to perform toxicity studies, and finally to turn-off gene expression in cases of side effects.

Recently, it has been shown that cell death of primary rat striatal cultures transfected with a mutated huntingtin fragment is hampered by the addition of recombinant CNTF (Saudou *et al.,* 1998). This *in vitro* study suggests, for the first time, that CNTF may not only alter the degeneration of GABAergic neurons in neurotoxin-induced models of HD but also in a genetic model. The lentiviral-mediated administration of CNTF in the striatum of HD transgenic mice (Mangiarini *et al.,* 1996; Reddy *et al.,* 1998; Hodgson *et al.,* 1999; Schilling *et al.,* 1999; Yamamoto *et al.,* 2000) should further establish the potential therapeutic value of this molecule.

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