

Regulated Delivery of Glial Cell Line-Derived Neurotrophic Factor into Rat Striatum, Using a Tetracycline-Dependent Lentiviral Vector

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ABSTRACT

In this study, a tetracycline-regulated lentiviral vector system, based on the tetracycline-dependent transactivator rtTA2^S-M2, was developed for controlled expression of glial cell line-derived neurotrophic factor (GDNF) in the rat brain. Expression of the marker gene green fluorescent protein (GFP) and GDNF was tightly regulated in a dose-dependent manner in neural cell lines *in vitro*. Injection of high-titer lentiviral vectors into the rat striatum resulted in a 7-fold induction of GDNF tissue levels (1060 pg/mg tissue), when doxycycline (a tetracycline analog) was added to the drinking water. However, low levels of GDNF (150 pg/mg tissue) were also detected in animals that did not receive doxycycline, indicating a significant background leakage from the vector system *in vivo*. The level of basal expression was markedly reduced when a 10-fold lower dose of the tetracycline-regulated GDNF vector was injected into the striatum (3–11 pg/mg tissue), and doxycycline-induced GDNF tissue levels obtained in these animals were about 190 pg/mg tissue. Doxycycline-induced expression of GDNF resulted in a significant downregulation of the tyrosine hydroxylase (TH) protein in the intact striatum. Removal of doxycycline from the drinking water rapidly (within 3 days) turned off transgenic GDNF mRNA expression and GDNF protein levels in the tissue were completely reduced by 2 weeks, demonstrating the dynamics of the system *in vivo*. Accordingly, TH protein expression returned to normal by 2–8 weeks after removal of doxycycline, indicating that GDNF-induced downregulation of TH is a reversible event.

OVERVIEW SUMMARY

A neuroprotective treatment strategy for Parkinson's disease (PD) is of great interest, as it is aimed at preventing progression of the disease. GDNF has been of particular interest because of its potent actions on dopaminergic (DA) neurons, and symptomatic benefits have been reported in a clinical trial after delivery of recombinant GDNF protein into the putamen of PD patients. Considering the slow and progressive degeneration of nigral DA neurons, it is likely that GDNF should be delivered continuously into the brain parenchyma over several years for long-term effects. This can be accomplished by GDNF gene therapy, using recombinant viral vector systems for delivery of the GDNF gene into brain cells. However, findings regarding potential side effects have prompted the need for regulated GDNF ex-

pression. Development of a regulated GDNF vector system will lead to improved understanding of the mechanisms of neuroprotection after GDNF gene delivery in animal models of PD, as well as move viral vectors closer to clinical use.

INTRODUCTION

PARKINSON'S DISEASE (PD) is characterized by the loss of dopaminergic (DA) neurons in the substantia nigra (SN). The cause of this degeneration remains unclear and current therapy strategies are focused on symptomatic relief. However, increasing evidence indicates that glial cell line-derived neurotrophic factor (GDNF) exerts neuroprotective effects on degenerating DA neurons and several promising preclinical studies in animal models of PD indicate that a neuroprotective

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treatment strategy may be possible (Björklund *et al.*, 2000; Kirik *et al.*, 2004). This was highlighted in a phase I clinical study, in which improvements were found in five PD patients who received continuous infusion of recombinant GDNF protein (14.4 μg of GDNF per day) into the putamen (Gill *et al.*, 2003). These studies support the notion that continuous delivery of GDNF is crucial and that it needs to be delivered locally at the appropriate target site. In fact, the failure of a previous clinical trial has been attributed largely to an inappropriate form of delivery and dosage, in which GDNF was delivered to the ventricle at much higher doses (25 to 4000 μg of GDNF) as monthly bolus injections, resulting in no therapeutic benefits for the patients and reports of substantial side effects (Nutt *et al.*, 2003).

Gene transfer using recombinant lentiviral (rLV) vectors has been found to be effective for local delivery of GDNF at therapeutic doses. Previous studies have shown that rLV-mediated delivery of GDNF leads to rescue of DA neurons in both rodent (Bensadoun *et al.*, 2000; Déglon *et al.*, 2000; Rosenblad *et al.*, 2000; Georgievska *et al.*, 2002a,b) and primate (Kordower *et al.*, 2000) models of PD. However, findings in our laboratory suggest that continuous overexpression of GDNF at high doses (~ 2 ng/mg tissue) not only protects the nigrostriatal DA system but may also lead to adverse effects, such as aberrant sprouting in areas outside the striatum and downregulation of tyrosine hydroxylase (TH) in the preserved DA terminals (Georgievska *et al.*, 2002a). Similarly, long-term (13 months) overexpression of GDNF at equivalent doses can induce downregulation of TH in the intact striatum (Rosenblad *et al.*, 2003). These findings suggest that a regulated vector system may be needed. After delivery of the transgene it would be of value to be able to adjust the levels of GDNF and to withdraw the treatment, in the case of adverse effects.

In this study, we have therefore developed regulated rLV vectors, using the Tet-on system. Regulated expression of GDNF, as well as the reporter gene encoding green fluorescent protein (GFP), was developed on the basis of a system that incorporates a novel version of the reverse transactivator: rTA^S-M2. This version has been reported to be more sensitive to doxycycline and displays less basal activity (Urlinger *et al.*, 2000). Our results show that the inducible rLV vector system allows for efficient and rapid regulation of GDNF expression *in vivo* (when doxycycline is provided in the drinking water), and that turning off GDNF expression leads to restoration of TH expression in the intact striatum, demonstrating that this effect is reversible.

MATERIALS AND METHODS

Recombinant lentiviral vectors

Self-inactivating rLV vectors encoding either GFP or human GDNF under the human cytomegalovirus (hCMV) promoter, and containing a woodchuck hepatitis virus posttranscriptional regulatory element (rLV.CMV.GFP and rLV.CMV.GDNF [Zufferey *et al.*, 1998; Georgievska *et al.*, 2002b]), were used as control vectors for constitutive transgene expression. From the control vectors, an inducible two-vector system was constructed by standard recombinant DNA technology. The in-

ducible vector system incorporated one vector encoding the transactivator under the hCMV promoter (rLV.SM2), and another vector encoding either GFP (rLV.TRE.GFP) or GDNF (rLV.TRE.GDNF) under the Tet-responsive element (TRE). An outline of the vector system is shown in Fig. 1 and the details of all vector constructions are available on request. The rLV.rTA vector construct encoding the original transactivator was developed as previously described (Johansen *et al.*, 2002).

The rLV vectors were produced as previously described (Zufferey *et al.*, 1997; Georgievska *et al.*, 2002b). To determine the titer of the various vector batches, an RNA slot-blot technique in combination with serial dilution transduction of 293T cells was used, as previously described (Jakobsson *et al.*, 2003). The viral vectors described in this study were used in the range of $1\text{--}2 \times 10^8$ transducing units (TU)/ml.

Cell culturing and *in vitro* transduction

To study regulated transgene expression *in vitro*, we have used primary rat astrocytes, immortalized human embryonic kidney cells (293T), and rat-derived immortalized neural progenitor cell lines (HiB5 and RN33B cells). These were cultured as previously described (Englund *et al.*, 2000; Ericson *et al.*, 2002). For 293T, HiB5, and RN33B, 5×10^5 cells were transduced at a multiplicity of infection (MOI) of 1 for rLV.TRE.GFP or rLV.TRE.GDNF and at an MOI of 2 for rLV.SM2. Primary rat astrocytes (5×10^5) were transduced with rLV.TRE.GFP and rLV.SM2 at an MOI of 1 or 5 in various combinations. At 4 days posttransduction the culture medium was replaced with medium containing the desired concentration of doxycycline (ranging from 0.0001 to 10 $\mu\text{g}/\text{ml}$). Cell cultures that were transduced with the rLV.TRE.GFP and rLV.SM2 vectors were analyzed by fluorescence-activated cell sorting (FACS) after 72 hr of doxycycline treatment. The GFP-positive population was selected by excluding cells overlapping the profile of nontransduced cells. Cell cultures cotransduced with rLV.TRE.GDNF and rLV.SM2 were treated with doxycycline for 72 hr and the amount of GDNF released into the medium over a period of 10 hr was determined by enzyme-linked immunosorbent assay (ELISA), according to the sup-

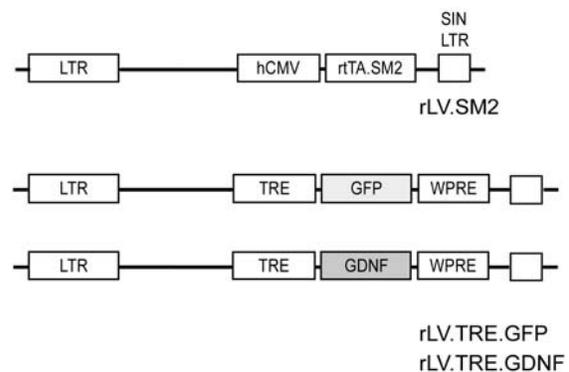


FIG. 1. Schematic outline of the vector system used in this study. SIN LTR, self-inactivating LTR; hCMV, human cytomegalovirus promoter; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element; SM2, rTA^S-M2; TRE, tetracycline-responsive element.

plier's recommendations (Promega, Madison, WI). The *in vitro* experiments were performed in duplicate or triplicate and repeated at least twice with different vector batches.

Experimental design for *in vivo* experiments

All experiments on animals were approved by and performed according to the guidelines of the Ethics Committee for Use of Laboratory Animals at Lund University (Lund, Sweden), and the animals were housed at two or three per cage under a 12-hr light:dark cycle with free access to food and water. Adult female Sprague-Dawley rats (225 g; total, 123) were used in two different experiments. In the first experiment, the inducible rLV vector system was evaluated for regulated expression of GFP and GDNF *in vivo* by injecting the vectors into the striatum. The experimental groups were as follows: (1) rLV.CMV.GDNF (control vector, 6.7×10^4 TU injected, $n = 10$), (2) rLV.TRE.GDNF plus rLV.SM2 at a 1:2 ratio (6.7×10^4 : 13.3×10^4 TU injected, $n = 20$), (3) rLV.TRE.GDNF plus rLV.SM2 at a 0.1:2 ratio (6.7×10^3 : 13.3×10^4 TU injected, $n = 10$), (4) rLV.TRE.GDNF plus rLV.SM2 at a 0.01:2 ratio (6.7×10^2 : 13.3×10^4 TU injected, $n = 10$), (5) rLV.TRE.GDNF only (6.7×10^4 TU injected, $n = 5$), (6) rLV.TRE.GDNF only, 10 times diluted (6.7×10^3 TU injected, $n = 5$), and (7) rLV.TRE.GFP plus rLV.SM2 at a 1:2 ratio (6.7×10^4 : 13.3×10^4 TU injected, $n = 9$). Half of the animals in experimental groups 2, 3, and 4, as well as five animals in group 7, were assigned to the "on" groups and received doxycycline at 1 mg/ml in the drinking water, in order to induce transgene expression. The doxycycline treatment was initiated 1 day after surgery and was maintained for 4 weeks before killing the animals.

In the second experiment, the regulated rLV vector system was used to study the reversibility of GDNF-induced down-regulation of TH in the intact striatum. For this purpose, 42 animals received injections of rLV.TRE.GDNF (1.7×10^4 TU injected) and rLV.SM2 (1.5×10^5 TU injected) at a 0.1:1 ratio into the striatum. The animals were then divided into seven subgroups ($n = 6$ per group), of which six groups received doxycycline in the drinking water (1 mg/ml) in order to induce GDNF expression (on-groups), and one group remained non-treated ("off"-group). Five of the on-groups were treated with doxycycline for 6 weeks, after which the treatment was terminated and subgroups of animals were killed at this time point (day 0) and at 3 days, 1 week, 2 weeks, or 8 weeks after removal of doxycycline from the drinking water. The remaining on-group was kept on doxycycline treatment throughout the course of the experiment (i.e., 14 weeks). In addition, two control groups ($n = 6$ per group) received injections of either rLV.CMV.GFP (2×10^4 TU injected) or rLV.CMV.GDNF (2×10^4 TU injected) into the striatum and were killed 6 weeks postinjection.

Surgical procedure

Animals were anesthetized with isoflurane before being placed into a stereotaxic frame. Injections were made with a 5- μ l Hamilton syringe fitted with a glass capillary, with an outer diameter of 60–80 μ m, in order to reduce nonspecific damage associated with the injection procedure. In the first experiment, a total volume of 2 μ l of the rLV vectors was injected into one site (1 μ l per deposit) in the right striatum at the following co-

ordinates: anteroposterior (AP), +0.5; mediolateral (ML), -2.8; and dorsoventral (DV), -5.0, -4.0, with the tooth bar set at -3.3. In the second experiment, a total volume of 3 μ l of the rLV vectors was injected into three sites (0.5 μ l/deposit) in the right striatum at the following coordinates: (1) AP, +1.4; ML, -2.6; DV, -5.0, -4.0; (2) AP, +0.4; ML, -3.4; DV, -5.0, -4.0; (3) AP, -0.8; ML, -4.4; DV, -5.0, -4.0, with the tooth bar set at -3.3. The injection rate was 0.5 μ l/min and the needle was left in place for an additional 3 min before withdrawal.

Histology

Four weeks after vector injection, animals that received rLV.TRE.GFP plus rLV.SM2 (experiment I) were deeply anesthetized (1 ml of pentobarbital per 225 g, administered intraperitoneally) and transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4. The brains were postfixed in PFA for 2–4 hr at 4°C and then kept in 30% sucrose for 2 days. The brains were cut on a freezing microtome at a thickness of 40 μ m and coronal sections throughout the striatum were collected in eight series. Expression of GFP was analyzed either by direct investigation of GFP autofluorescence, or by using a polyclonal antibody against GFP. Inflammatory responses were monitored with antibodies raised against OX-19 (T cells) and OX-42 (microglia and macrophages), and immunohistochemistry was performed according to standard methods, as previously described (Jakobsson *et al.*, 2003).

Primary antibodies used in this study, and working dilutions, were as follows: chicken anti-GFP (AB16901, diluted 1:5000; Chemicon International, Temecula, CA), mouse anti-NeuN (neuronal nuclei) (MAB377, diluted 1:1000; Chemicon International), mouse anti-OX-19 (MCA52G, diluted 1:100; Serotec, Oxford, UK), and mouse anti-OX-42 (MCA275G, diluted 1:200; Serotec). The secondary antibodies were all diluted 1:200 and included the following: biotinylated horse anti-mouse (BA-2001; Vector Laboratories, Burlingame, CA), biotinylated rabbit anti-chicken (G2891; Promega), Cy3-conjugated donkey anti-mouse (115-116-075; Jackson ImmunoResearch Laboratories, West Grove, PA), and fluorescein isothiocyanate (FITC)-conjugated donkey anti-chicken (703-096-155; Jackson ImmunoResearch Laboratories).

To estimate the number of GFP-expressing cells in the striatum, all cells in one series of sections were counted and the actual number was then estimated according to Abercrombie's formula (Abercrombie, 1946).

Biochemical analysis

All animals receiving rLV.TRE.GDNF, either alone or in combination with rLV.SM2, as well as animals receiving the control vectors (rLV.CMV.GFP and rLV.CMV.GDNF), were deeply anesthetized with sodium pentobarbital and decapitated. The brain was rapidly removed and the striatum and SN were dissected out, as previously described (Georgievska *et al.*, 2002b). For experiment II, the tissue was chopped in pieces and divided into two parts, with one part being used for the GDNF ELISA and the other part for the *in vitro* TH enzyme activity assay. After homogenization of the tissue and subsequent centrifugation (see below), total mRNA and genomic DNA were

extracted from the remaining pellets, thus allowing us to analyze protein levels, as well as mRNA and DNA levels, from the same samples. Transgenic GDNF mRNA levels and proviral DNA levels were analyzed by TaqMan polymerase chain reaction (PCR).

GDNF ELISA. Frozen tissue samples from the striatum and SN were sonicated in homogenization buffer (50 mM TRIS [pH 7.4], 150 mM NaCl, 1% Triton X-100, phenylmethylsulfonyl fluoride [1.7 $\mu\text{g}/\text{ml}$], leupeptin [1.0 $\mu\text{g}/\text{ml}$], aprotinin [10 $\mu\text{g}/\text{ml}$], and pepstatin [1.0 $\mu\text{g}/\text{ml}$]) at a tissue concentration of 30 mg/ml and centrifuged at $20,000 \times g$ for 10 min at 4°C. Tissue levels of GDNF in homogenates were determined by ELISA, according to the supplier's recommendations (Promega).

In vitro TH enzyme activity assay. The TH enzyme activity assay was performed according to Reinhard *et al.* (1986) and is based on the release of tritiated H₂O from L-[3,5-³H]tyrosine during the hydroxylation reaction. This assay measures the activity of nonphosphorylated TH enzyme (because phosphate groups are easily lost during homogenization of tissue) and is correlated mainly with the amount of TH protein present in tissue homogenates. Thus, this assay can be used to determine TH protein levels in striatal tissue samples. In brief, striatal tissue was sonicated in homogenization buffer [20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES, pH 6.1), 0.2% Triton] at 10 $\mu\text{l}/\text{mg}$ and centrifuged at $20,000 \times g$ for 10 min at 4°C. Four microcuries of L-[3,5-³H]tyrosine (Amersham Biosciences, Piscataway, NJ) was dissolved in 100 μl of L-tyrosine (500 μM ; Sigma, St. Louis, MO), 500 μl of MES (200 mM, pH 6.1), 12 μl of catalase (12,690 units; Sigma), and 688 μl of distilled H₂O. Sixty-five microliters of this cocktail was added to 10 μl of supernatant, together with 25 μl of the cofactor biopterin (0.6 mg/ml, dissolved in dithiothreitol [3 mg/ml]; Sigma-RBI, Natick, MA). The samples were incubated for 20 min at 37°C, the reaction was terminated by adding charcoal-HCl, and the samples were then centrifuged at $20,000 \times g$ for 15 min. One hundred microliters of the supernatant was transferred to a scintillation vial and 5 ml of scintillation liquid (Insta-Gel Plus; PerkinElmer Life and Analytical Sciences, Boston, MA) was added. The number of decays per minute was measured in a liquid scintillation counter (1214 RackBeta; LKB Wallac/PerkinElmer Life and Analytical Sciences).

Quantitative PCR. Total RNA and genomic DNA were extracted with TRIzol (Invitrogen, Carlsbad, CA) according to the supplier's recommendations. To remove remaining DNA contaminants from the RNA samples, these were treated twice with RNase-free DNase (DNA-free; Ambion, Austin, TX). The cDNA libraries were constructed according to the manufacturer's protocol (SuperScript II reverse transcriptase; Invitrogen), and SuperScript-negative controls were performed and found to be DNA free in all cases.

TaqMan PCR was conducted with an ABI PRISM 7700 real-time PCR system (Applied Biosystems, Foster City, CA). For quantification of transgenic GDNF mRNA levels, primers directed toward the WPRE element were used (WPREprim) and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) pri-

mers were used as reference mRNA primers. For quantification of proviral DNA, primers directed to a human immunodeficiency virus type 1 (HIV-1)-derived part of the lentiviral vector was used (LVprim), whereas primers against rat interleukin 2 (IL-2) were used as reference. In each reaction, 2 μl of template, corresponding to 50 ng of genomic DNA, was used. Primer sequences were as follows: WPREprim up, 5'-CCG TTG TCA GGC AAC GTG-3'; WPREprim down, 5'-AGC TGA CAG GTG GTG GCA AT-3'; WPREprim probe, 5'-FAM-TGC TGA CGC AAC CCC CAC TGGT-TAMRA-3'; GAPDH up, 5'-TGC ACC ACC AAC TGC TTAG-3'; GAPDH down, 5'-GGA TGC AGG GAT GAT GTTC-3'; GAPDH probe, 5'-FAM-TCC AGT ACA AGC ACG GTG AAC CAA TTCC-TAMRA-3'; LVprim up, 5'-ACT TGA AAG CGA AAG GGA AAC-3'; LVprim down, 5'-CAC CCA TCT CTC TCC TTC TAG CC-3'; LVprim probe, 5'-FAM-AGC TCT CTC GAC GCA GGA CTC GGC-TAMRA-3'; IL2 up, 5'-GCC TTG TGT GTT ATA AGT AGG AGG-3'; IL2 down, 5'-AGT GCC AAT TCG ATG ATG AGC-3'; IL2 probe, 5'-FAM-TCT CCT CAG AAA TTC CAC CAC AGT TGC TG-TAMRA-3'.

TaqMan Universal PCR master mix (Applied Biosystems) was used in a total reaction volume of 25 μl and the reaction conditions were as follows: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 sec at 95°C and 1 min at 60°C. The threshold was adjusted to fit with the linear phase. Relative mRNA and DNA levels were quantified by the comparative C_t method according to User Bulletin #2, supplied by Applied Biosystems (www.appliedbiosystems.com; <http://docs.appliedbiosystems.com/pebiiodocs/04303859.pdf> [accessed August 2004]). The efficiencies of the various PCRs were found to be comparable. The WPRE mRNA levels were calculated as the ratio of a pool consisting of a mixture of the six cDNA libraries from the nontreated off-group in experiment II, and the values from the TaqMan PCR are presented as arbitrary units (U). No significant levels of transgenic mRNA or proviral DNA were found in the contralateral hemisphere.

Statistical analysis

For statistical analysis a two-factor analysis of variance (ANOVA), followed by a post hoc test when appropriate, was performed with StatView software (SAS Institute, Cary, NC). Data are presented as means \pm SEM.

RESULTS

Regulated transgene expression in vitro

As an initial experiment, we confirmed that the synthetic M2 version of rTA (SM2) is more sensitive to doxycycline than the old version of rTA, in the lentiviral vector system. A dose-response analysis of 293T cells, transduced with rLV.TRE.GFP and either rLV.SM2 or rLV.rTA, demonstrated that a 10-fold lower dose of doxycycline was needed to achieve the same level of induction with SM2 version as with the old rTA (Fig. 2A). 293T cells transduced with rLV.TRE.GFP alone expressed low levels of GFP, suggesting that the minimal CMV promoter, to some extent, maintained constitutive expression in this cell line (9% GFP-positive cells). This basal expression was not in-

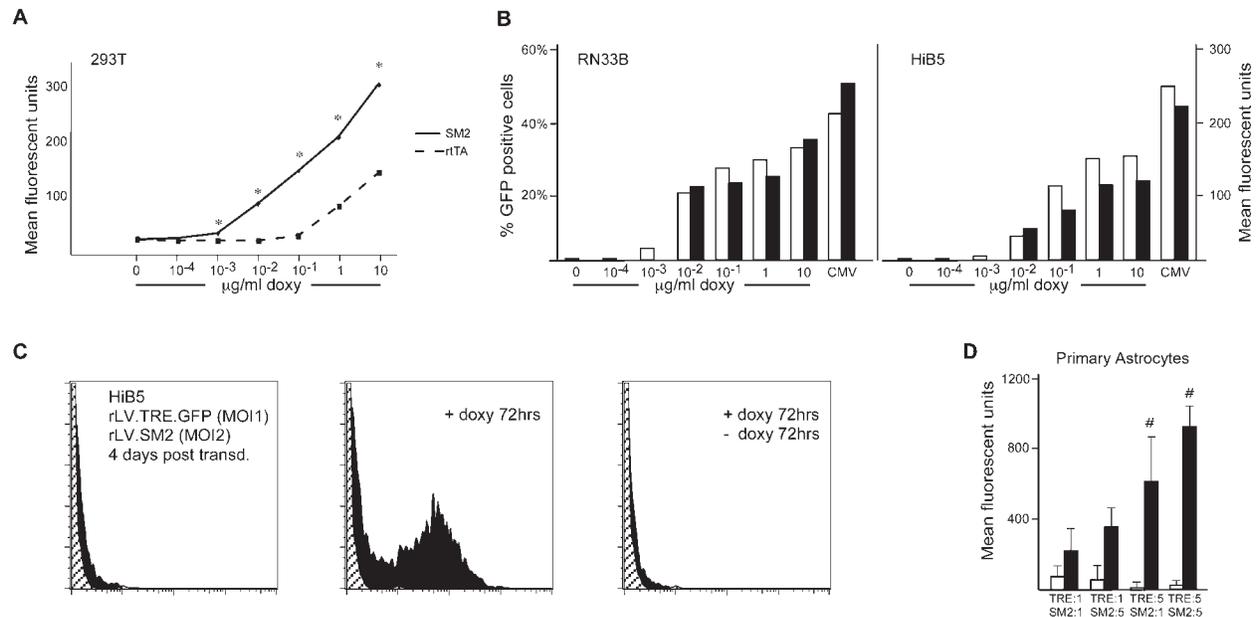


FIG. 2. Regulated GFP expression *in vitro*. **(A)** Dose-dependent expression in 293T cells, using either the synthetic M2 (solid line) or the original rTA transactivator (dashed line). *Significantly different from original rTA at the same doxycycline concentration, $p < 0.01$, Fisher's PLSD. **(B)** Dose-dependent GFP expression in neural progenitor cell lines RN33B and HiB5 transduced with rLV.SM2 (MOI of 2) and rLV.TRE.GFP (MOI of 1) at 7 days posttransduction and after 72 hr of doxycycline treatment. Open columns represent the ratio of GFP-expressing cells and solid columns represent the fluorescence intensity of GFP-expressing cells. **(C)** FACS profiles demonstrating the reversibility of doxycycline-induced GFP expression within 72 hr of removal of doxycycline. **(D)** GFP expression in primary astrocytes transduced with rLV.SM2 and rLV.TRE.GFP at various MOIs. Shaded columns, no doxycycline; solid columns, doxycycline at 1 $\mu\text{g/ml}$. #Significantly different from all other groups, $p < 0.01$, Fisher's PLSD.

creased by cotransduction of either the old or the SM2 version of rTA (7 and 8% GFP-positive cells).

Neural progenitor cell lines HiB5 and RN33B were transduced with rLV.TRE.GFP (MOI of 1) and rLV.SM2 (MOI of 2) and a dose-dependent response to doxycycline was observed, both in terms of number of GFP-expressing cells and the intensity of GFP fluorescence (Fig. 2B). GFP expression was detected in less than 0.1% of the double-transduced cells that were not treated with doxycycline (Fig. 2B), or in cells that received only the rLV.TRE.GFP vector (data not shown). Detectable induction of GFP expression was found when cells were cultured in doxycycline at $\geq 0.01 \mu\text{g/ml}$. Furthermore, after 72 hr of induction, removal of doxycycline from the medium for an additional 72 hr completely turned off GFP expression (Fig. 2C).

Using this vector system, GFP expression could also be induced in primary rat astrocytes (Fig. 2D). In this experiment, various combinations of MOIs were analyzed and the highest level of induction was observed when using an MOI of 5 for both vector constructs (Fig. 2D).

Transduction of HiB5 cells with rLV.TRE.GDNF (MOI of 1) and rLV.SM2 (MOI of 2) demonstrated a dose-dependent regulation of GDNF expression *in vitro*, similar to the regulation of GFP. At the highest doxycycline concentration (10 $\mu\text{g/ml}$), a 32-fold induction of GDNF release was observed, as compared with the noninduced group (1269 ± 170 versus $39.5 \pm 44 \text{ pg/100,000 cells per hour}$; Fig. 3). Detectable levels

of GDNF were not observed in cultures without doxycycline treatment (Fig. 3) or in cultures transduced only with the rLV.TRE.GDNF vector (data not shown).

Regulated transgene expression *in vivo*

To investigate the regulation of transgene expression *in vivo*, using this vector system, rLV.TRE.GFP and rLV.SM2 vectors were coinjected into the striatum of rats at a 1:2 ratio. In the on-group (doxycycline at 1 mg/ml in the drinking water for 4 weeks) we found GFP-expressing cells in all animals, whereas no GFP-positive cells could be detected in the off-group (Fig. 4A), either by analyzing GFP autofluorescence or by immunohistochemistry for GFP. Although four of five doxycycline-treated animals in the on-group showed a similar number of GFP-expressing cells in the striatum (374 ± 77 cells, $n = 4$; Fig. 4A), one animal had a substantially higher number of GFP-positive cells (2337 cells). As this value was 3 standard deviations from the group mean value (see above), this animal was considered an outlier and thus excluded from the analysis. The vast majority of GFP-expressing cells found in the on-group had a neuronal morphology and GFP expression colocalized with the neuronal marker NeuN (Fig. 4B).

Signs of an inflammatory response against the rLV vector constructs were monitored with antibodies raised against CD5 (OX-19) and CD11b (OX-42), detecting T cells and activated

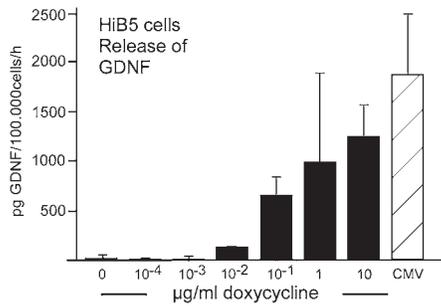


FIG. 3. Regulated GDNF expression *in vitro*. Shown is GDNF release from HiB5 cells transduced with rLV.SM2 (MOI of 2) and rLV.TRE.GDNF (MOI of 1) 7 days posttransduction and after 72 hr of doxycycline treatment. Hatched column represents GDNF release from a transduced control culture (rLV.CMV.GDNF, MOI of 1).

microglia, respectively. Except for some local damage at the injection site (caused by the penetration of the injection needle), no additional signs of adverse inflammation were observed in animals injected with only the rLV.SM2 vector or with both vector constructs, as compared with animals injected with an rLV.CMV.GFP control vector (data not shown).

To investigate the regulation of GDNF *in vivo*, rLV.TRE.GDNF and rLV.SM2 were injected into the rat striatum at various ratios (1:2, 0.1:2, and 0.01:2). Three different concentrations for the rLV.TRE.GDNF vector were thus used—concentrated, 1:10 dilution, and 1:100 dilution—whereas concentrated rLV.SM2 vector was used in all experiments. Detectable GDNF expression was found in all doxycycline-treated animals (doxycycline at 1 mg/ml in the drinking water for 4 weeks). In the group receiving concentrated rLV.TRE.GDNF (1:2 ratio), a 7-fold increase in GDNF tissue levels was observed in the on-group (1060 ± 600 pg/mg tissue), compared with the off-group (150 ± 10 pg/mg tissue [$p < 0.05$, Student–Newman–Keuls]; Fig. 4C). Whereas GDNF tissue levels in the on-group were comparable to levels obtained by injecting a similar dose of the nonregulated rLV.CMV.GDNF vector (620 ± 30 pg/mg tissue; Fig. 4C), the background GDNF levels in the off-group were well above the endogenous gene expression level on the contralateral, nontransduced side ($2\text{--}5$ pg/mg tissue). By diluting the rLV.TRE.GDNF vector further (0.1:2 and 0.01:2 ratios), background expression was substantially reduced to 11 ± 7 pg/mg tissue in the 1:10 dilution group and to 3 ± 3 pg/mg tissue in the 1:100 dilution group. This did, however, lead to lower levels of GDNF in the on-groups: 190 ± 90 pg/mg tissue in the 1:10 dilution group and 20 ± 10 pg/mg tissue in the 1:100 dilution group. Nevertheless, GDNF tissue levels obtained in the 1:10 dilution on-group (190 pg/mg tissue) would be considered functionally relevant on the basis of previous studies (see Björklund *et al.*, 2000). There was also evidence of elevated GDNF levels in the SN of the on-groups (180 ± 60 [concentrated], 34 ± 20 [1:10 dilution], and 8 ± 5 [1:100 dilution] pg/mg tissue) compared with the off-groups (29 ± 20 [concentrated], 4 ± 3 [1:10 dilution], and 2 ± 2 [1:100 dilution] pg/mg tissue).

The background GDNF expression observed in the off-groups was further analyzed in a separate set of animals. Injection of only rLV.TRE.GDNF into the striatum resulted in a

60% reduction of basal GDNF expression, compared with when rLV.SM2 was coinjected (88 ± 40 versus 230 ± 40 pg/mg tissue [$p < 0.05$, Student–Newman–Keuls]; Fig. 4D). Basal GDNF levels were further reduced by diluting the rLV.TRE.GDNF vector 10 times (35 ± 9 versus 88 ± 40 pg/mg tissue [$p < 0.05$, Student–Newman–Keuls]; Fig. 4D). This suggests that both the promoter (TRE) and the transactivator (SM2) contributed to the GDNF expression observed in the off-state.

Reversibility of GDNF-induced downregulation of TH

We then used the regulated rLV vector system to study the reversibility of GDNF-induced downregulation of TH in the intact striatum. For this purpose, rLV.TRE.GDNF and rLV.SM2 vectors were injected into the striatum at a 0.1:1 ratio, because these were the injection parameters that resulted in a significant induction of GDNF at functionally relevant levels, but in the absence of a detectable background expression in the nontreated group (see above).

GDNF tissue levels in the striatum after 6 weeks of doxycycline treatment were 93 ± 22 pg/mg tissue, compared with 10 ± 2 pg/mg tissue in the nontreated group (Fig. 5B). At this time point, TH protein levels, as determined by the *in vitro* TH enzyme activity assay (Fig. 5C), were significantly reduced in the doxycycline-treated animals ($57.4 \pm 5.1\%$ of contralateral side), compared with the nontreated group ($81.4 \pm 4.7\%$ of contralateral side). Similarly, expression of GDNF from the control rLV.CMV.GDNF vector (780 ± 140 pg/mg tissue) induced a significant downregulation of TH in the striatum ($37.3 \pm 4.2\%$ of contralateral side).

Using quantitative PCR it was possible to detect low, but significant, levels of transgenic GDNF mRNA in the off-group that did not receive doxycycline (0.96 ± 0.22 U). After 6 weeks of doxycycline treatment, 6-fold higher levels of transgenic GDNF mRNA were detected in the on-group (6.07 ± 1.22 U) (Fig. 5A), similar to the GDNF protein levels (see above). After removal of doxycycline from the drinking water, GDNF mRNA expression returned by 3 days (1.03 ± 0.22 U) to the same level as detected in the off-group, and remained at this low level throughout the experiment (Fig. 5A). GDNF protein levels in the striatum, as determined by ELISA, were significantly reduced at 3 days (39 ± 13 pg/mg tissue) and reached background levels by 2 weeks (5 ± 2 pg/mg tissue; Fig. 5B). Accordingly, 2 weeks after the removal of doxycycline, TH protein expression was significantly increased ($77.7 \pm 12.3\%$ of contralateral side) and by 8 weeks TH expression reached the same level ($83.5 \pm 8.0\%$ of contralateral side) as in the nontreated off-group (see above), and was not significantly different from an rLV.CMV.GFP-injected control group ($90.8 \pm 6.1\%$ of contralateral side) killed 6 weeks after vector injection (Fig. 5C).

Levels of proviral DNA in the samples were measured throughout the experiment, using primers detecting both vector constructs (rLV.TRE.GDNF and rLV.SM2). There was no difference in proviral DNA levels in groups killed 6 weeks (1.00 ± 0.11 U, $n = 12$) or 14 weeks (1.08 ± 0.17 U, $n = 12$) after vector injection, suggesting that transduced cells expressing the rtTA^S-M2 transactivator were not removed via an immune response. In accordance, one group of animals that received doxycycline throughout the 14 weeks of the experiment ex-

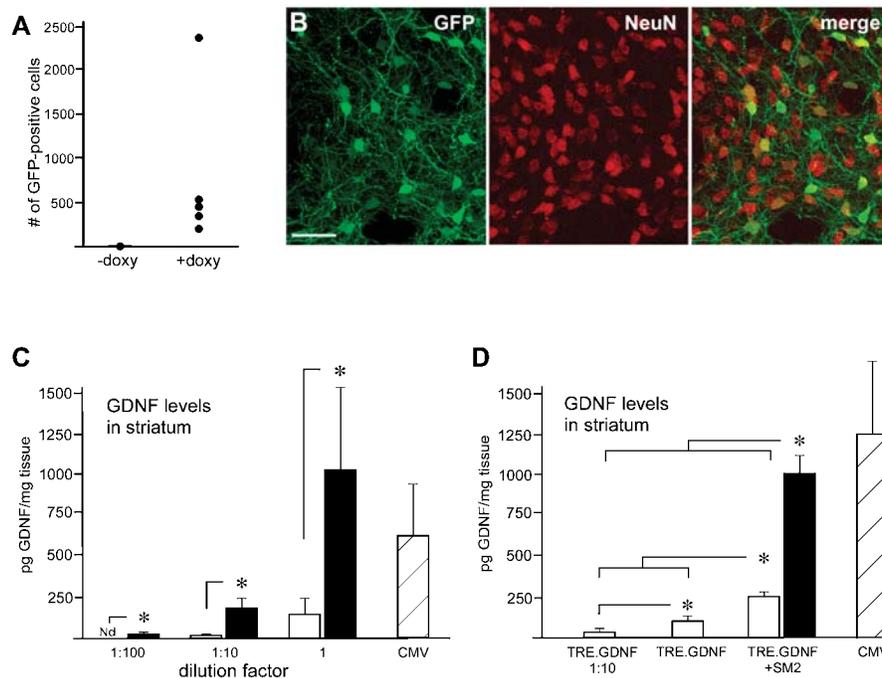


FIG. 4. Regulated transgene expression *in vivo*. **(A)** Quantification of the number of GFP-expressing cells in the striatum of animals treated with doxycycline (+doxy) and the lack of GFP-expressing cells in the nontreated animals (–doxy). **(B)** Stack of confocal images from the striatum of an animal treated with doxycycline for 4 weeks. Immunohistochemical staining of GFP (green) and the neuronal marker NeuN (red) displayed almost complete overlap. Scale bar: 40 μm . **(C)** GDNF tissue levels (picograms per milligram of tissue) in the striatum 4 weeks after vector injection. Dilution factor denotes the dilution of the rLV.TRE.GDNF vector. Open columns, no doxycycline; solid columns, doxycycline at 1 mg/ml in the drinking water; hatched column, rLV.CMV.GDNF control vector. **(D)** Analysis of the basal GDNF expression observed in the off-state. 1:10 indicates the dilution factor of the rLV.TRE.GDNF. Open columns, no doxycycline; solid columns, doxycycline at 1 mg/ml in the drinking water; hatched column, rLV.CMV.GDNF control vector. *Significantly different, $p < 0.05$, Student–Newman–Keuls.

pressed GDNF at similar levels (125 ± 21 pg/mg tissue) as in doxycycline-treated animals killed after 6 weeks (93 ± 22 pg/mg tissue).

DISCUSSION

The present results demonstrate the possibility to regulate GDNF gene expression *in vivo* after delivery of a tetracycline-inducible rLV vector system to the rat brain. Intra-striatal injections of the tetracycline-regulated GDNF construct (rLV.TRE.GDNF) and the transactivator-encoding construct (rLV.SM2) at a 1:2 ratio resulted in significant GDNF expression in the doxycycline-treated on-group (1060 pg of GDNF per milligram of tissue); however, substantial background expression was observed in the nontreated off-group (150 pg/mg tissue). The basal expression of GDNF in the off-group was efficiently reduced (11 pg/mg tissue) when a lower dose (10-fold dilution) of the rLV.TRE.GDNF vector was used. As a result, induced GDNF levels in the on-group receiving the diluted rLV.TRE.GDNF were also reduced (190 pg/mg tissue); however, this level of GDNF expression is within the therapeutic window and would be functionally relevant in animal models of PD (Björklund *et al.*, 2000). Furthermore, results from this study demonstrated that GDNF transgene expression was rapidly (by 3 days) turned off *in vivo* after removal of doxycy-

cline, and that GDNF-induced downregulation of TH was reversed 2–8 weeks after termination of doxycycline treatment.

Regulated transgene expression *in vitro* and *in vivo*

Tetracycline-regulated transgene expression, in the context of viral vectors, has been extensively studied with various types of vector systems and with different designs of the vectors (for review see, e.g., Toniatti *et al.*, 2004). In this study we have used a two-vector design, in which the transactivator (rtTA2^S-M2) is expressed from one vector construct and the transgene from another vector. Use of the rtTA2^S-M2 transactivator resulted in a 10-fold increase in the sensitivity of the transactivator to doxycycline, similar to what has been reported previously (Urlinger *et al.*, 2000). Induction of transgene expression from the regulated vectors (rLV.TRE.GFP and rLV.TRE.GDNF) was dependent on the dose of doxycycline and significant gene expression was detected *in vitro* even at a 0.01- $\mu\text{g}/\text{ml}$ concentration of doxycycline. Transgene expression could also be completely turned off within 72 hr of withdrawal of doxycycline from the medium, demonstrating the dynamics of the regulated system.

In the *in vitro* experiments, using neural progenitor cell lines, we did not observe any basal expression from the vector system (GDNF or GFP) in the absence of doxycycline. Similarly, injection of the rLV.TRE.GFP and rLV.SM2 vectors at a 1:2

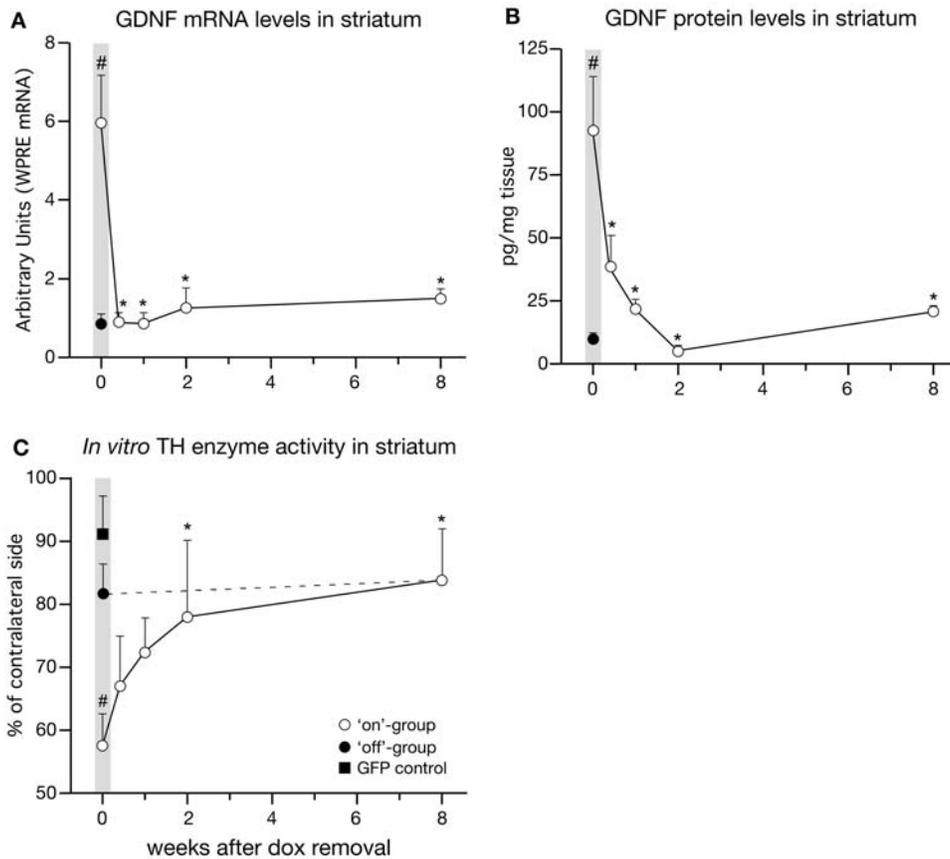


FIG. 5. Reversible effects of GDNF on TH expression in the intact striatum. (**A** and **B**) GDNF mRNA levels were measured by TaqMan PCR, using primers against the WPRE region (in order to detect only transgenic GDNF mRNA transcripts) and GDNF tissue levels were determined by ELISA. Significant induction of GDNF expression, as analyzed both at the mRNA (**A**) and protein (**B**) levels, is observed in animals treated with doxycycline (1 mg/ml in the drinking water) for 6 weeks (on-group, time point 0), compared with nontreated animals (off-group, time point 0). After removal of doxycycline, GDNF mRNA expression is already turned off by 3 days and reaches background levels that are maintained over 8 weeks (**A**). GDNF tissue levels are significantly reduced by 3 days and reach background levels by 1–2 weeks after doxycycline removal (**B**). (**C**) Induced GDNF expression in animals treated with doxycycline for 6 weeks (on-group, time point 0) was sufficient to significantly down-regulate TH protein (measured by *in vitro* TH enzyme activity assay), as compared with nontreated animals (off-group, time point 0). Two weeks after removal of doxycycline, TH expression was significantly increased and fully reversed by 8 weeks, at least to the same level as in the nontreated group, and did not differ significantly from an rLV.CMV.GFP-injected control group (GFP control, time point 0). #Significantly different from nontreated animals ($p < 0.05$); *significantly different from the expression level in the on-group at time point 0 ($p < 0.05$).

ratio into the striatum resulted in a significant induction of GFP expression in animals receiving doxycycline in the drinking water, whereas no GFP-positive cells could be found in the nontreated off-group. Koponen and collaborators (2003) have developed a similar two-vector lentiviral system, incorporating the rtTA2^S-M2 transactivator, for regulated expression of the marker protein β -galactosidase. The *in vitro* data presented in their study were comparable to the data reported here. In addition, the vector system in the Koponen *et al.* (2003) study was also injected into the rat striatum at a 1:2 ratio and the β -galactosidase expression was analyzed 10 days after vector injection and after 4 days of doxycycline treatment (daily intraperitoneal injections of 1 mg). Significant induction was seen in five of seven doxycycline-treated animals, whereas no background levels were observed in the nontreated group. This is in keeping with our data obtained for the regulated expression of the intracellular marker protein GFP.

Tight *in vivo* regulation of GFP expression has also been documented in studies using lentiviral vectors based on the Tet-off system (Régulier *et al.*, 2002). One of the major differences between the Tet-on and Tet-off systems is the dose of doxycycline required in the drinking water for successful regulation of gene expression in the brain. In our study, a relatively high dose of doxycycline (1 mg/ml in the drinking water) was needed to achieve efficient induction of GDNF expression, whereas a lower dose of doxycycline (0.2 mg/ml) did not induce any detectable gene expression (B. Georgievska and J. Jakobsson, unpublished observations). By contrast, a 0.2-mg/ml concentration of doxycycline in the drinking water is sufficient to repress gene expression, using the Tet-off system (Kafri *et al.*, 2000; Régulier *et al.*, 2002).

It has previously been shown that the transactivators used for the various tetracycline-responsive systems, including the one used in this study, can induce immunological reactions *in*

vivo (Favre *et al.*, 2002; Latta-Mahieu *et al.*, 2002). It is not clear to what extent this would be a problem in the brain after viral delivery. In the present study, we did not observe increased microglial activation (OX-42 staining) or infiltration of T lymphocytes (OX-19 staining) into the striatum. Furthermore, proviral vector DNA was stable up to 14 weeks after injection, suggesting that expression of the transactivator is well tolerated in the brain. Although our data indicate that the rTA2^S-M2 transactivator did not induce a significant immune response in the brain, the present study was not designed primarily to evaluate potential immunological responses to the transactivator.

Regulated expression of GDNF

In contrast to regulation of the marker gene GFP, results from the *in vivo* GDNF experiment showed that there was significant basal transgene expression in animals that did not receive doxycycline. The discrepancy between the different transgenes (GDNF versus GFP) can be explained by different properties of the proteins (secreted versus intracellular), different sensitivities between the detection methods used (immunohistochemistry versus ELISA), as well as the diverse regulation of the two transgenes. It is, however, clear from our data that results obtained *in vitro* or with reporter genes (such as GFP *in vivo*) may not be translatable to the regulation of functional proteins (such as GDNF). The basal expression observed in the GDNF experiment seemed to be the result of both low-level regulation-independent transcription from the TRE promoter, as injection of rLV.TRE.GDNF alone resulted in significant GDNF tissue levels (88 pg/mg tissue), and from residual activation of the transactivator in the absence of the inducer. Similar basal transgene expression of ciliary neurotrophic factor (CNTF) has been observed in the rat striatum after injection of a two-vector lentiviral system based on the Tet-off system (Régulier *et al.*, 2002). Repression of transgene expression by doxycycline resulted in a background level of 0.88 ng of CNTF per milligram of protein in the striatum, whereas a 15-fold induction was observed in the nontreated animals. Although the basal CNTF levels in the doxycycline-treated group were not sufficient to protect striatal neurons against a quinolinic acid lesion, the basal GDNF tissue levels detected in the noninduced animals in this study (150 pg/mg tissue) are well within the therapeutic range and would be functionally relevant in animal models of PD (Björklund *et al.*, 2000).

Reversible effects of GDNF on TH expression

We have previously observed that long-term and continuous overexpression of GDNF in the intact nigrostriatal DA system can induce a selective downregulation of TH (Rosenblad *et al.*, 2003). This downregulation is both time and dose dependent, such that it develops only after 6 weeks of continuous GDNF overexpression and is most pronounced (about 70% reduction) in animals in which the level of GDNF exceeds 700 pg/mg tissue (B. Georgievska, D. Kirik, and A. Björklund, unpublished data). In the present study, we therefore used the regulated rLV vector system to study the reversibility of GDNF-induced downregulation of TH in the intact rat striatum. Induced GDNF expression in doxycycline-treated animals caused a significant

downregulation of TH (57% of contralateral side) compared with the nontreated off-group (81% of contralateral side). Removal of doxycycline from the drinking water resulted in a rapid reduction in GDNF mRNA expression at 3 days, whereas the GDNF tissue levels were completely reduced by 2 weeks after termination of the doxycycline treatment. This indicates that transgene expression is rapidly turned off after removal of the inducer molecule, but that the already synthesized GDNF protein can be detected for a longer period, before it is completely cleared from the tissue. Accordingly, TH protein expression was significantly increased at 2 weeks (when GDNF protein was no longer detected in the striatum) and was completely reversed by 8 weeks (83% of contralateral side), reaching the same level as in the off-group. These results suggest that the downregulation of TH in the intact nigrostriatal DA system is dependent on the presence of GDNF in the striatum and that turning off GDNF expression can efficiently reverse this effect. However, the background expression of GDNF in the off-group was able to induce a small reduction in the TH protein levels (about 20%), supporting the need to develop more tightly regulated vector constructs for application in animal models of PD.

Local delivery of regulated lentiviral vectors into the brain, leading to high vector doses around the injection site, may compromise the tightness of gene regulation, as observed in previous dose-response experiments (Aurisicchio *et al.*, 2001; Lamartina *et al.*, 2002). Improving the vector design may counteract this effect and increase the tightness of regulation. Increased stringency has been achieved with vectors that incorporate a doxycycline-dependent trans-silencer (tTS) (Salucci *et al.*, 2002; Lamartina *et al.*, 2003; Mizuguchi *et al.*, 2003) and coexpression of tTS with the rTA2^S-M2 transactivator in muscle, using an adenoviral vector, significantly reduced basal expression (Salucci *et al.*, 2002; Lamartina *et al.*, 2003). Furthermore, it would be of value to develop an efficient one-vector system. This has proven to be problematic because interference of multiple genes and *cis*-acting elements may lead to poor regulating properties. However, a few reports have described the development of lentiviral vectors encompassing all necessary regulatory elements into a single vector (Vigna *et al.*, 2002; Vogel *et al.*, 2004). Regulated transgene expression from these vector systems has been demonstrated *in vivo*; however, it is not clear how these data will relate to the regulated expression of GDNF in the brain and this remains to be tested.

In conclusion, GDNF therapy for PD is of great interest because it offers not only symptomatic relief, as was reported from the latest phase I trial (Gill *et al.*, 2003), but more importantly it is a disease-modifying therapy aiming at protecting the further loss of DA neurons and thus preventing progression of the disease. *In vivo* gene transfer using lentiviral vectors is promising in that it would allow long-term expression of GDNF in the brain at functional levels. However, it is important to determine the optimal dosing and possible adverse effects associated with sustained high-level expression of GDNF and to develop the means to regulate gene expression safely and efficiently. The tetracycline-inducible lentiviral vector system described here could be useful for initial studies; however, it is clear that the vector system needs further improvements, including elimination of basal transgene expression and development of a single-vector system.

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