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Evidence for Disease-Regulated Transgene Expression in the Brain With Use of Lentiviral Vectors

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In this study we have developed and validated a novel approach of transgene regulation in the brain. By using lentiviral vectors that incorporate promoters of genes that are up-regulated during different pathological states, we were able to regulate transgene expression in accordance with the disease process. When using a glial fibrillary acidic protein promoter, efficient disease regulation in glial cells was achieved after an excitotoxic lesion or a 6-hydroxydopamine (6-OHDA) lesion. Transgene expression was physiologically regulated and displayed a dose-dependent increase depending on the severity of lesion. Efficient regulation was also achieved in neurons when using a preproenkephlin promoter in 6-OHDA-lesioned rats, allowing combined regulation and targeting. This disease-regulated approach allows control of transgene expression in the brain without the use of inducer molecules and without overexpression of transactivator proteins.

Key words: disease-regulated transgene expression; brain; lentiviral vectors

The use of lentiviral vectors allows high-level and long-term expression of a transgene in the brain (Azzouz et al., 2004; Deglon and Hantraye, 2005). By using such vectors or vectors based on other viruses, the potential of gene therapy has been demonstrated in a number of preclinical studies in various animal models of different brain disorders, including Parkinson's disease (PD), Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis, multiple sclerosis, stroke, and various lysosomal storage diseases (Davidson and Breakefield, 2003; Azzouz et al., 2004; Deglon and Hantraye, 2005). Despite these promising results, it is clear that the success of many gene therapy strategies will depend on further development of the gene transfer systems. These improvements include the development of regulated vectors, allowing appropriate dosage and reversal of treatment if necessary, and the development of targeted systems, allowing transduction of specific populations of cells in the CNS.

For the purpose of regulating transgene expression following gene delivery, several transgene control systems have been designed. These systems, such as the tetracycline and the rapamycine systems, are responsive to small molecules, including antibiotics, immunosuppressive agents, or hormone analogs, and allow regulated transgene expression in mammalian cells in vitro and in vivo (Weber and Fussenegger, 2004). However, for certain gene transfer strategies, these systems possess practical limitations. These limitations include 1) the requirement of overexpression of a protein of nonmammalian origin that serves as a transactivator, which may lead to immunological problems; 2) the requirement of specialized promoter sequences limiting targeting strategies; and 3) the requirement of exogenous administration of the inducing drug. In the brain, this is further complicated because of poor penetration of the blood–brain barrier by some of the inducer molecules. An alternative approach that is appealing, insofar as it avoids all of these problems, is to use an endogenous promoter from a gene that is specifically up-regulated during a pathological state. By using such a promoter to drive transgene expression, regulated gene delivery independent of a transactivator and its ligand would be possible. It would also be possible to combine regulated and targeted transgene expression by using promoters that are up-regulated in specific cell types.

We and others have shown that endogenous promoters placed within a lentiviral vector direct transgene expression to the predicted cell type, including neurons and astrocytes (De Palma et al., 2003; Jakobsson et al., 2003; Follenzi et al., 2004). However, it is has not been established that such vectors are specifically regulated with

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regard to different disease states in the brain. In this study, we have examined the dynamics of transgene expression from endogenous promoters of genes that are up-regulated following various experimental lesions to the brain. We used promoters from genes that have been extensively characterized in disease models in the brain and compared the expression levels from the lentiviral vectors with their endogenous counterparts. Our results suggest that disease-regulated transgene expression may be an attractive strategy in a number of gene therapy settings.

MATERIALS AND METHODS

Lentiviral Vectors

The lentiviral vectors expressing either β-galactosidase under the control of the 2.1-kb human glial fibrillary acidic protein (GFAP) promoter (LV-hGFAP; see Fig. 3A) or green fluorescent proein (GFP) under the control of a rat enkephalin promoter (2.7 kb of upstream sequence and 0.7 kb of exon I) was constructed by using standard cloning procedures (LV-rENK; see Fig. 6A). We used HIV-I-based lentiviral vectors that contained a self-inactivating long terminal repeat (LTR; Zufferey et al., 1997, 1998), a central polypurine tract (Follenzi et al., 2000), and the woodchuck hepatitis posttranscriptional regulatory element (Zufferey et al., 1999) in order to improve the transgene expression properties of the vectors (see Figs. 3A, 6A). Recombinant VSV-G pseudotyped lentiviruses were produced by transient transfection of 293T cells according to standard protocols (Zufferey et al., 1997). In brief, 293T cells grown in a 10-cm Petri dish were cotransfected with 20 μg of transfer vector, 15 μg of pBR-8.91, and 5 μg of pMDG by calcium phosphate precipitation. Medium was replaced after 16 hr, and viral vectors were collected after 24 hr and 48 hr. Lentiviral vectors were concentrated by using ultracentrifugation. A biological titer of the vectors was estimated by infection of 293T cells followed by TaqmanPCR on genomic DNA extracted from these cells. A functional titer (tranducing units/ml) was then calculated from a reference lentiviral vector, where a ubiquitous hCMV promoter drove transgene expression. The titer of the LV-hGFAP batch used in this experiment was estimated to be 5×10^8 TU/ml and the LV-rENK to be 1×10^9 TU/ml.

Surgical Procedures

All animal procedures were approved by and performed according to the guidelines of the Ethical Committee for Use of Laboratory Animals at Lund University. Lentiviral vectors were diluted ×2 with HBSS, and 1 μl was injected bilaterally into rat striatum as previously described. A thin glass capillary was attached to a Hamilton syringe to induce minimal damage from the injection. In total, 46 rats were injected with LV-hGFAP. Unilateral intrastriatal 6-hydroxydopamine (6-OHDA) lesion (n = 22) and ibotenic acid (IBO) lesion (n = 22) were performed as previously described (Jakobsson et al., 2004). Two animals were used as nonlesion control. Efficiency of 6-OHDA and IBO lesions were confirmed by using either tyrosine hydroxylase (TH) or NeuN immunohistochemistry (Fig. 1A,B). In total, 31 rats were injected with LV-rENK. The MFB-6-OHDA lesions (n = 29) were performed as previously described (Jakobsson et al., 2004). Two animals were used as nonlesion control. Efficiency of 6-OHDA lesions was confirmed by using TH immunohistochemistry (Fig. 1C). One animal was excluded from the study because of an incomplete 6-OHDA lesion. A single cell suspension of embryonic ventral mesencephalic tissue (14 days gestational age) was prepared according to established protocols (Bjorklund et al., 1983). In total, 50,000 cells were transplanted unilaterally into three sites of the striatum (n = 10) according to Cenci et al. (1993).

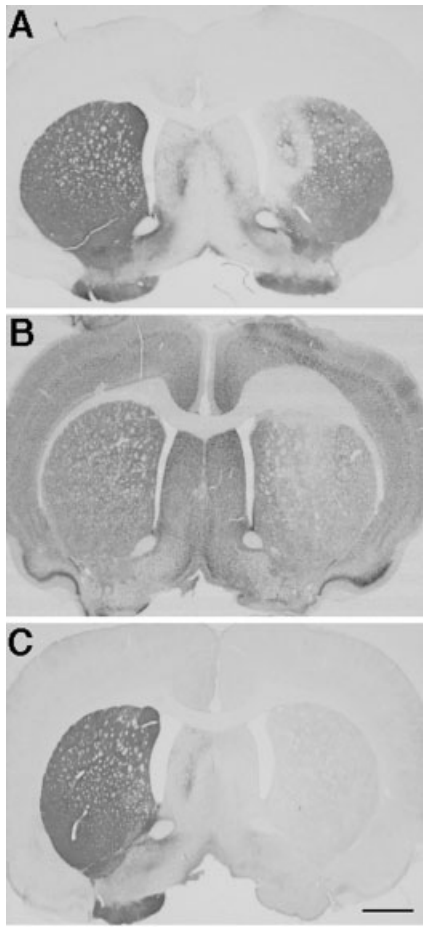

Fig. 1. Photomicrographs showing the effects of the lesions used in the study. **A:** The intrastriatal 6-OHDA lesion resulted in a partial dopamine denervation of the striatum as shown by the lack of TH staining in the striatum of the right hemisphere. **B** illustrates the loss of neurons in the striatum resulting from the IBO lesion of the striatum visualized by immunohistochemistry against NeuN. **C:** The animals that received injections of LV-rENK were lesioned by injections of 6-OHDA into the MFB. This resulted in a near-complete loss of TH fibers in the right striatum. Scale bar = 1.3 mm.

Histology

Animals selected for histology were perfused by using 250 ml of 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were then sectioned on a freezing-stage microtome at 40 μm in eight series. Lightfield and fluorescent immuno-

histochemical staining were performed as previously described (Jakobsson et al., 2003). The following antibodies and dilutions were used: anti-GFP (Chemicon, Temecula, CA; AB16901, lot No. 25060075, 1:1,000), anti-TH (Chemicon; MAB318, lot No. 0509010596, 1:2,000), anti-DARPP32 (Chemcion; AB1656, lot No. 24030679, 1:1,000), antiparvalbumin (Sigma, St. Louis, MO; P3088, lot No. 90K4850, 1:2,000), anti-Met-enkephalin (Chemicon; AB5026, 1:100), anti-β-gal (Promega, Madison, WI; Z3781, lot No. 11744609, 1:500), anti-NeuN (Chemicon; MAB377, lot No. 25040168, 1:100), and anti-GFAP (Dako, Carpinteria, CA; Z0334, lot No. 01096501, 1:1,000).

β-Gal Activity

Striatal tissue was dissected and then homogenized by using sonication in a Triton X-100-Tris buffer as previously described (Georgievska et al., 2004). The β-gal activity in the brains was estimated by using the FluoReporter lacZ/Galactosidase quantitation kit (Molecular Probes, Eugene, OR) according to the supplier's recommendations.

Quantification of GFP Expression

Quantification of intensity of GFP autofluorescence was done by computerized image analysis in ImageJ software (NIH). In brief, digital images of all sections from a series, containing GFP fluorescence, were obtained with the ×10 objective in a Leitz DMBRE microscope (Leica) linked to a digital camera connected to a Macintosh computer. Each image was then subdivided into 96 images from which the GFP signal was estimated and background subtracted. Data are presented as relative fluorescence. To quantify the number of GFP cells, sections were analyzed throughout the whole transduced area. Stereological analysis of the sections was performed by using the CAST-Grid system (Olympus Denmark), composed of an Olympus BH2 microscope, an X-Y step motor stage run by an IBM-compatible PC computer, and a microcator (ND 281; Heidnhain, Traunreut, Germany). Briefly, the CAST-Grid software (version 1.10) was used to delineate the area with the transduced cells by using the ×4 objective. In the ×100 objective, a counting frame was first placed randomly, to generate the first counting area, and then was systematically moved (between 100- and 300-μm steps in the X-Y directions) until the entire delineated region was sampled. The total number of transduced cells was calculated according to the optical fractionator formula (West et al., 1991).

Real-Time PCR

Total RNA and genomic DNA were extracted with Trizol (Invitrogen, La Jolla, CA) according to the supplier's recommendations. To remove the 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 endogenous enkephalin and GFAP expression, primers against the respective messenger RNAs were designed in Primer Express software (Applied Biosystems). Rat GAPDH primers were used as reference mRNA primers. For quantification of proviral DNA, primers directed to an HIV-I-derived part of the lentiviral vector was used (LVprim), whereas primers against the rat interleukin-2 (IL2) gene were used as reference. Primer sequences were as follows; GFAPprim up: 5′-AGG CCT TGA CCT GCG ACC-3′, GFAPprim down: 5′-GCG GAT TTG CCT CTC CAA-3′, GFAPprim probe: 5′-FAM-CCT TGC GCG GCA CGA ACG AG-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′; ENKprim up: 5′-CCA GCT GGA AGA CGA AGC AA-3′, ENKprim down: 5′-GCC CGA CCC TTC TCA TGA-3′, ENKprim probe: 5′-CCA TAG CGC TTC TGC AGC TCCT-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 AGGC-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, 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 using the comparative Ct-method according to the User Bulletin 2, supplied by Applied Biosystems (www.appliedbiosystems.com). The efficiencies of the various PCRs were found to be comparable.

Statistical Analysis

Statistical significance was analyzed via ANOVA, followed by a Fisher's post hoc test. $P < 0.05$ was considered significant. Data are presented as mean ± SEM.

RESULTS

Transgene Expression With the hGFAP-Lentiviral Vector

The setup for the experiment with the hGFAP promoter is outlined in Figure 2B,C. The hGFAP-lentiviral vector was injected bilaterally into the striatum of female SD rats (n = 46). Three weeks after vector injection, the rats received a unilateral lesion of the striatum, leaving the contralateral side intact, serving as an internal control. Two types of lesions were used: an intrastriatal 6-OHDA lesion or an intrastriatal IBO lesion. The 6-OHDA lesion leads to retrograde degeneration of dopaminergic fibers and a mild gliotic reaction within the striatum. The IBO lesion induces excitotoxic death of

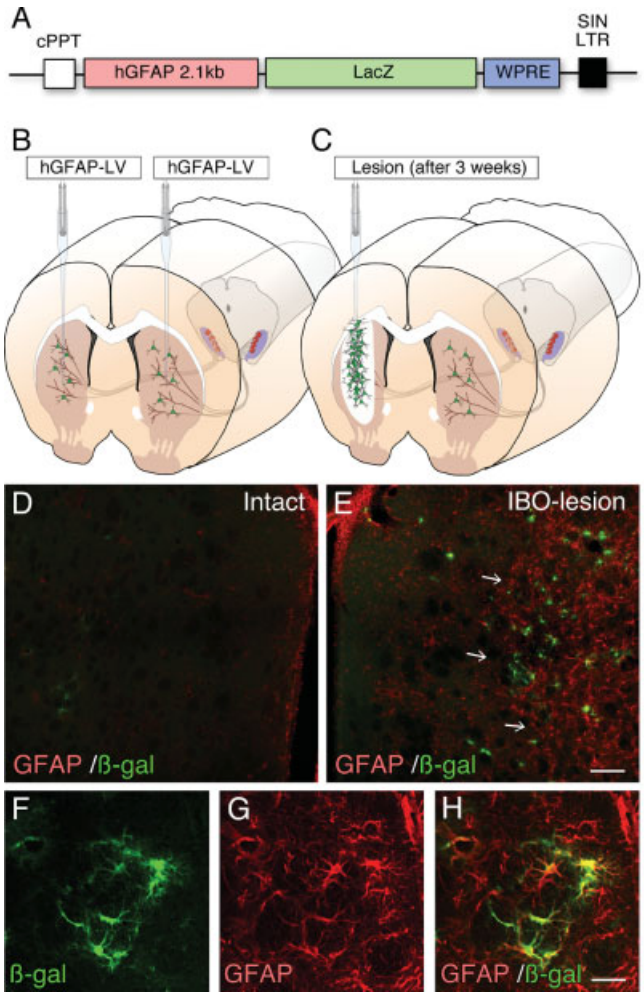

Fig. 2. **A:** Design of the lentiviral vector expressing β-gal under the control of a 2.1-kb human GFAP promoter. **B,C:** Experimental setup. The hGFAP-lentiviral vector was injected bilaterally into the striatum of rats, and 3 weeks later the rats received a unilateral intrastriatal lesion. Animals were sacrificed after 1, 4, or 9 weeks. One group of animals received a second lesion of the other kind after 8 weeks and sacrificed 1 week later. **D,E:** Photomicrographs of the striatum doubly stained by immunofluorescence for GFAP and β-gal. Pictures are from the same section and represent typical intact and IBO-lesioned striatum 1 week after the lesion. Note that expression of β-gal can be detected in areas of up-regulated GFAP expression (arrows). **F–H:** Confocal micrograph of IBO-lesioned striatum confirming that the great majority of β-gal cells are colabeled with GFAP. Scale bars = 200 μm in D (applies to D,E); 40 μm in H (applies to F–H).

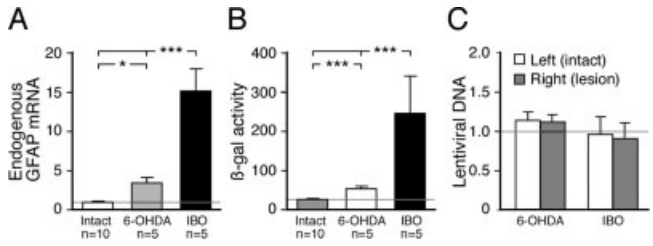

Fig. 3. **A:** Endogenous GFAP mRNA levels, estimated with Taqman PCR (presented as relative values of nonlesioned animals), 1 week after lesion. The 6-OHDA and IBO lesion leads to an up-regulation of GFAP mRNA that is dose-dependent on the severity of the gliotic reaction. Data are presented as relative mRNA levels compared with nonlesioned control. **B:** β-Gal activity of brain homogenates 1 week after lesion, revealing transgene expression levels that are dependent on the lesion severity. The β-gal activity of nonlesioned brains was not statistically different from that of noninjected animals. Data are presented as picograms β-gal per milligram wet tissue. **C:** Levels of proviral DNA, estimated with Taqman PCR and presented as relative values of nonlesioned animals injected with same amount of vector. No difference in proviral load was found in the experimental group, suggesting that the increased transgene expression was due to enhanced promoter activity. $*P < 0.05$, $***P < 0.001$, ANOVA followed by Fishers PLSD.

striatal neurons and leads to an intense gliotic reaction within the striatum.

The first group of animals was sacrificed 1 week after receiving the lesion. Brain sections analyzed using immunohistochemical staining revealed a loss of TH-expressing fibers in the striatum of animals receiving a 6-OHDA lesion, whereas animals receiving an IBO lesion displayed a loss of NeuN immunoreactivty in the area where the toxin was injected (data not shown). In terms of GFAP immunorectivity, there was intense staining in the area of the lesion in the IBO animals (Fig. 2D,E). 6-OHDA animals displayed a subtler up-regulation of GFAP expression. There was a marked increase of transgene-expressing cells on the lesioned side in animals receiving IBO (Fig. 2D,E). The vast majority of these cells colabeled with β-gal and GFAP (Fig. 2F,H). These data confirmed that a β-galactoisdase transgene was up-regulated in response to lesion, in agreement with what we have previously found when using a GFP transgene (Jakobsson et al., 2004).

To perform a more quantitative analysis, the striatum was dissected from lesioned rats. Protein, RNA, and DNA were then extracted from the tissue. The RNA was used for quantitative real-time RT-PCR toward endogenous GFAP messenger. This analysis revealed up-regulation of GFAP expression in animals receiving 6-OHDA lesion or IBO lesion. The up-regulation was 3.4-fold (6-OHDA) and 15-fold (IBO), reflecting the different intensities of the gliotic reaction of the two lesions (Fig. 3A). There was no apparent up-regulation of GFAP expression in animals receiving only virus compared with completely intact animals, confirming that the up-regulation of GFAP was due to the 6-OHDA and IBO lesions (data not shown). Quantification of enzymatic β-gal activity in protein extracts from these brains revealed a similar pattern. β-Gal levels in nonlesioned animals were not significantly different from those in noninjected animals. In lesioned animals, there was a dose-dependent up-regulation of β-gal activity, closely reflecting the endogenous GFAP expression. Thus, the β-gal levels detected in IBO-lesioned animals

were 230 ± 96 pg/mg (wet tissue) and in 6-OHDA lesion 52.6 ± 7 pg/mg compared with the intact side, 26.5 ± 2 pg/mg (Fig. 3B). Quantitative measurements of the level of proviral DNA in genomic DNA isolated from the brains revealed a similar ratio of transgenic DNA vs. a genomic control gene in all experimental groups (Fig. 3C). This suggests that the proviral DNA is to a great extent not amplified following lesion because of cellular proliferation. However, it should be noted that the DNA yield from IBO-lesioned striata was only 10% compared with nonlesioned or 6-OHDA-lesioned tissue (data not shown), complicating the interpretation of these results. Nevertheless, these results suggest that the internal hGFAP promoter within the lentiviral vector is regulated in a dynamic manner resembling the endogenous GFAP gene following lesion to the brain.

Two groups of animals were sacrificed 4 and 9 weeks after the lesion (Fig. 4A,D). Quantification of GFAP mRNA revealed a gradual decline in GFAP expression, demonstrating that the gliotic reaction diminishes with time (Fig. 4B,E). In 6-OHDA animals, the GFAP expression had returned to baseline levels already after 4 weeks, whereas, in IBO animals, there was still a 5-fold up regulation 9 weeks after lesion. The β-gal levels in these brains mimicked this gradual decline. In 6-OHDA animals, the β-gal activity returned to values comparable with those in nonlesion control at 4 weeks postlesion (Fig. 4C). In IBO animals, there was still elevated β-gal expression 9 weeks after the lesion, but there was a decline with time mimicking the endogenous GFAP expression (Fig. 4F).

Ten of the animals were lesioned a second time 8 weeks after the initial lesion (Fig. 4A,D). Animals that were initially lesioned with 6-OHDA received an IBO lesion (n = 5) and vice versa (n = 5). In the animals receiving IBO as the second lesion, there was, as expected, a robust up-regulation in endogenous GFAP expression (IBO 10.4-fold, compared with 1.3-fold in the 9 w group; Fig. 4B). Interestingly, there was also up-regulated β-gal activity in these brains (IBO 77.3 pg/mg compared with 24.5 pg/mg in the 9 w group), demonstrating that a lentiviral transgene can be inactivated and then once more activated in response to injury (Fig. 4C). At this time point, the activation of transgene expression was lower in relation to the activation of endogenous GFAP. The reason for this is unclear, but it may demonstrate an exhaustion of the reactive astrocyte response, which is unveiled by the sequential lesions. However, in the animals that were lesioned with IBO first, the GFAP response to the 6-OHDA lesions was similar in magnitude to the response in naïve animals, though not significant from the levels in animals 9 weeks after the IBO lesion.

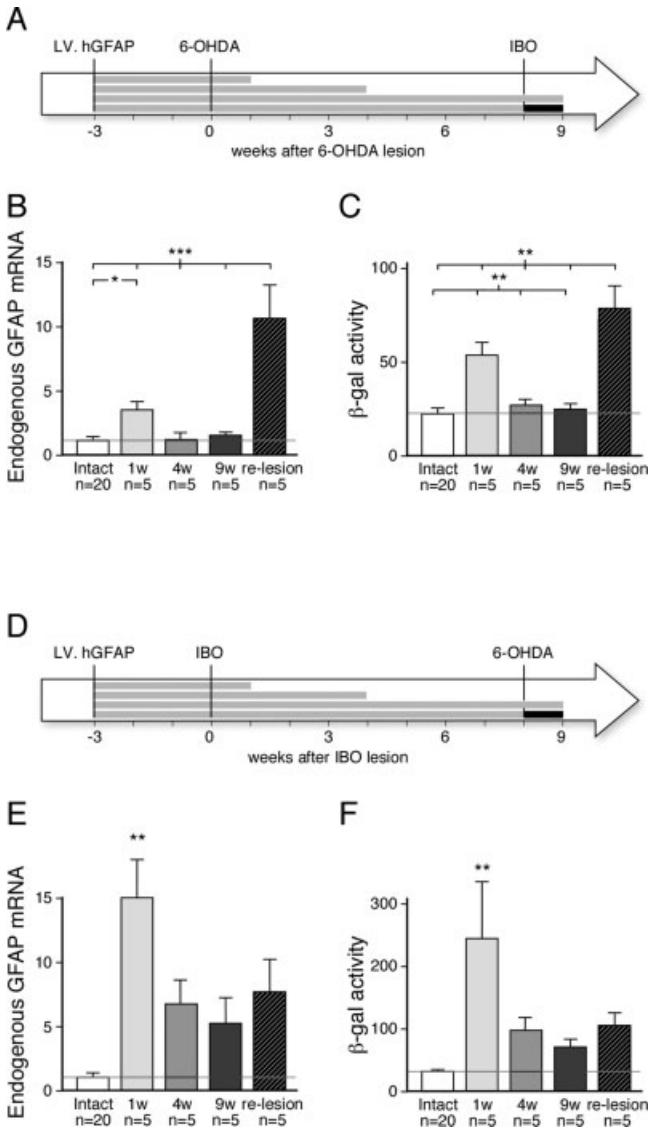

Fig. 4. Expression of β-gal over time and after a second lesion. **A,D:** Experimental setup. Three weeks after vector injection, animals were lesioned with either 6-OHDA or IBO and then sacrificed 1, 4, and 9 weeks after the lesion. A subset of animals received a second lesion 8 weeks after initial lesion. IBO-lesioned animals were then lesioned with 6-OHDA and vice versa. **B,E:** Endogenous GFAP mRNA gradually declined with time after both types of lesion. A second lesion led once again to up-regulation of GFAP mRNA. **C,F:** β-Gal activity in these brains mirrored endogenous GFAP expression. Note that a second lesion can reinduce β-gal expression, demonstrating that transgene expression can be dynamically up- and down-regulated after lesion. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, ANOVA followed by Fishers PLSD.

Transgene Expression With the rENK-Lentiviral Vector

The design for the experiment using the rENK promoter is outlined in Figure 5B. The majority of the animals (n = 29) received a unilateral injection of 6-OHDA into the midbrain-forebrain bundle 3 weeks after vector injection. This lesion leads to a unilateral loss of the dopamine neurons within the substantia nigra and an almost complete loss of dopaminergic innervation to the striatum. A subset of the animals (n = 10) was sacri-

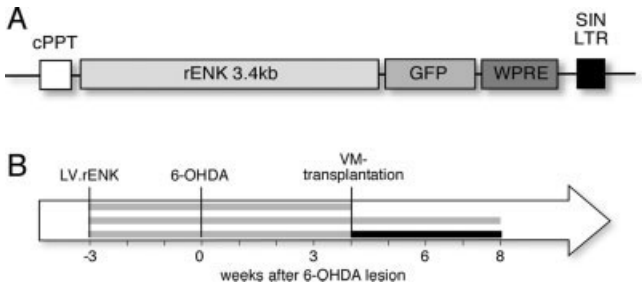

Fig. 5. Lentiviral vector with enkephalin promoter. **A:** Design of the lentiviral construct. The promoter is rat derived and consists of 2,700 bp of upstream sequence and 703 bp of exon I of the enkephalin gene. **B:** Experimental design.

ficed 4 weeks after the lesion. Expression of endogenous preproenkephlin (PPE) mRNA was quantified by using real-time PCR with a primer set directed toward exon III of the enkephalin gene. The up-regulation of PPE mRNA at the lesioned side was, in agreement with previous reports, estimated to be 2-fold at this time point (Fig. 6A). Quantification of GFP autofluorescence revealed an almost identical up-regulation of GFP expression compared with PPE mRNA (Fig. 6B,D–F). Twice as many GFP-expressing cells were detected on the lesioned side when combining immunohistochemistry with unbiased stereological quantification (Fig. 6C).

The remaining animals were divided into two groups (n = 19). One group was left for another 4 weeks and then sacrificed. PPE mRNA, GFP fluorescence, and number of GFP-expressing cells were similar in the 8-week group compared with the 4-week group, revealing a robust 2-fold up-regulation on the lesion side (Fig. 6A–C). The second group of animals received striatal transplants of ventral mescencephalic (VM) embryonic tissue at the 4-week time point. VM transplants into the striatum of Parkinsonian rats reinervate the lesioned area, leading to partially restored dopamine levels, and has been reported to reverse the up-regulation of PPE expression. In the transplanted group, PPE mRNA was restored to baseline levels on the lesioned side. In agreement with this was a reduction in GFP fluorescence as well as in number of GFP cells, suggesting that the restored dopamine levels from the effect of the transplant down-regulated transgene expression (Fig. 6D,E).

We performed a morphological analysis by using immunohistochemistry on animals from all groups, with identical results. The morphology of the GFP-expressing cells was exclusively neuronal. GFP cells colabeled with DARPP-32 (Fig. 7A–C), a marker for striatal interneurons, but not with parvalbumin (Fig. 7E–G), a marker for striatal interneurons, and not with the astrocyte marker GFAP (data not shown). A dense network of GFP-positive processes was found in the globus pallidus, output nuclei for enkephalinergic neurons (Fig. 7D). Double labeling with GFP and an antibody raised against Met-enkephalin revealed colocalization at the level of the cell bodies, and the GFP-positive processes were located in areas within the globus pallidus with intense Met-enkephalin immunoreactivity (Fig. 7H–K).

DISCUSSION

Development of cell type-specific gene transfer is important, in that it will allow genetic modification of only a subset of cells within the brain. This has consequences for both gene therapy and experimental gene transfer. More efficient gene therapy with reduced immune responses and more appropriate levels of transgene expression will be possible. Cell-specific gene transfer may also prevent or facilitate anterograde transport of the transgene product, something that, depending of the nature on the transgene, may be crucial for efficient gene therapy. In terms of experimental gene transfer, it will allow in vivo studies of gene function in isolated populations of cells, an effort that today requires the time-consuming and expensive establishment of transgenic mice.

In this study, we have continued our investigations of endogenous promoters in lentiviral vectors. We demonstrate that when using lentiviral vectors it is possible to locate the transgene product to specific cell populations including both neurons and glial cells. We also demonstrate that promoters within the lentiviral construct are regulated in response to physiological stimuli in a manner that greatly resembles how the endogenous counterpart is regulated. These results provide a proof-of-principle of disease regulation of a transgene in vivo and suggest that autoregulated disease-targeted transgene expression is possible. In contrast to classical regulatory systems, a strategy using endogenous promoters does not require expression of any transactivator protein and is not dependent on specialized promoter elements. Thus, this approach allows for a combination of targeted and regulated delivery that avoids potential immunological problems associated with overexpression of a transactivator protein. The hGFAP and rENK promoter used in our studies might not be the optimal choice of promoters for this strategy, because the choice of these promoters was based largely on the existence of previous reports in the literature. With the increasing knowledge of gene regulation during different pathological states, which is emerging from the use of techniques such as microarrays and proteomics, it should be possible to identify optimal promoters for autoregulated gene therapy in disorders such as stroke and multiple sclerosis.

Expression From the hGFAP Promoter

The 2.1-kb human GFAP promoter sequence used in this study has been extensively studied in different transgenic settings (Brenner et al., 1994; Messing and Brenner, 2003). GFAP is a intermediate filament protein expressed in mature astrocytes. GFAP expression is up-regulated in to the gliotic reaction that appears after lesions. In transgenic mice, the 2.1-kb human promoter has in most settings been demonstrated to confer trans-

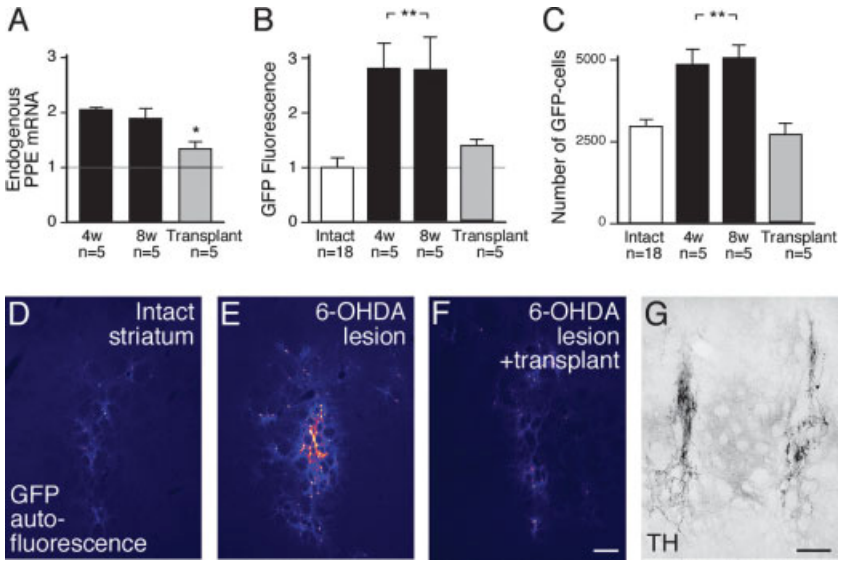

Fig. 6. Dopamine depletion and dopamine restoration regulate transgene expression. **A:** Endogenous PPE mRNA was up-regulated 4 and 8 weeks post 6-OHDA lesion. Transplantation of embryonic VM cells 4 weeks after the initial lesion normalizes PPE expression. Data are presented as lesion/intact. **B:** GFP expression estimated by measuring GFP fluorescence from PFA-fixed microscope slides revealed a similar up-regulation of GFP expression after 6-OHDA lesions and also a down-regulation after a VM transplant. Data are presented as relative fluorescence compared with nonlesion control. **C:** Unbiased stereological quantification of labelled cells immunostained for GFP confirmed up-regulation of transgene expression. **D–F:** Pseudocolored images of GFP autofluorescence revealed that the area of transduction was similar but the intensity of GFP expression was higher in lesioned striatum. **G:** Reinnervating tyrosine hydroxylase (TH)-expressing cells found in the striatum after VM transplantation. $^{**}P < 0.01$, ANOVA followed by Fishers PLSD. Scale bars = 200 μm in F (applies to (D–F); 200 μm in G.

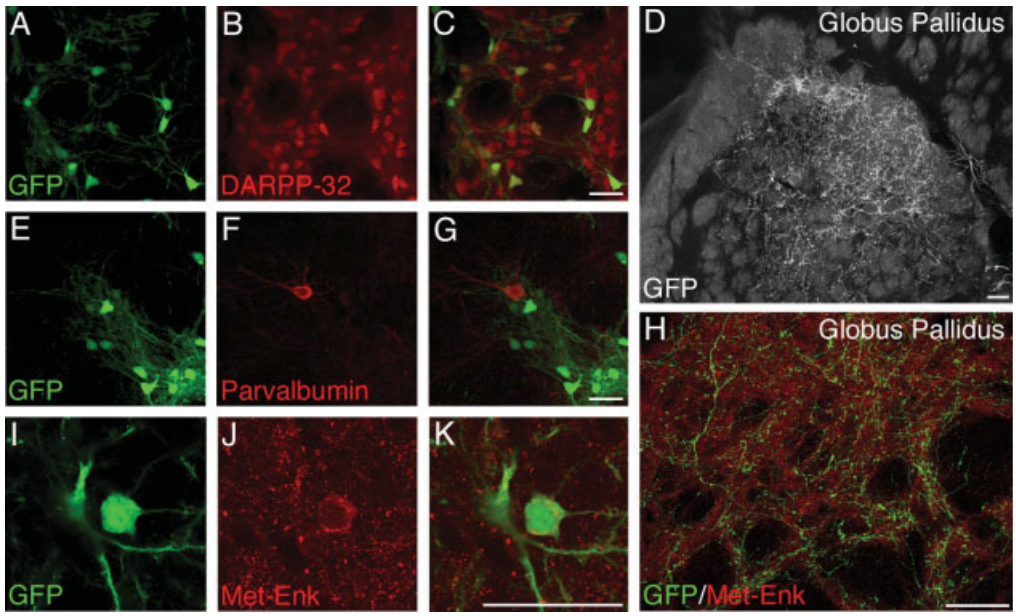

Fig. 7. GFP is expressed specifically in enkephalinergic neurons. **A–C, E–G:** Confocal images of tissue sections doubly stained for GFP-DARPP32 and GFP-parvalbumin, respectively. Note that all GFP-expressing cells colocalize with DAR PP-32. **D:** Darkfield image of GFP staining revealing a dense network of labeled fibers within the globus pallidus. **H–K:** Confocal images of tissue sections doubly labeled for Met-enkephalin and GFP revealing colocalization at the level of both the cell bodies and the synaptic terminals in the globus pallidus. Scale bars = 40 μm in C (applies to A–C); 200 μm in D; 40 μm in G (applies to E–G); 200 μm in H; 40 μm in K (applies to I–K).

gene expression to the appropriate cell type as well as being induced upon lesion (Brenner et al., 1994; Su et al., 2004). In a previous study, we found that the transcription from a lentiviral construct, having the hGFAP promoter, is up-regulated upon an intrastriatal IBO lesion. We now show, by using direct quantitative analysis of β-gal activity, that the magnitude of up-regulation is dependent on the intensity of the gliotic reaction. We used two different lesion models (6-OHDA and IBO) with distinct differences both in mode of action and regarding the gliotic reaction. The 6-OHDA lesion does not induce striatal cell death but instead cell death of the dopaminergic neurons situated in the substantia nigra, projecting to the striatum. The gliotic

response in the striatum to this degeneration is mild but still reproducible. The IBO lesion leads to selective excitotoxic cell death of the neurons within the striatum. The glial response to this lesion is very strong and includes mobilization of resident astrocytes and microglia as well as recruitment of infiltrating immune cells from the blood system. After IBO lesion, astrocytes within the striatum strongly up-regulate GFAP expression. Interesting and most encouraging is that astrocytes transduced by the hGFAP-lentiviral vector up-regulated β-gal expression in a dose-dependent manner, reflecting the severity of the lesion and the glial response. Furthermore, the transgene expression could be reinduced by a second lesion, confirming the dynamic and specific regulation of an endogenous promoter within a lentiviral construct.

Expression From the rENK Promoter

Expression of the enkephalin gene (*ENK*; also named *preproenkephalin A* and *proenkephalin A*) in the brain is restricted to a subset of neurons in the CNS, including the projection neurons in the striatum as well as neuronal populations in the olfactory bulb and the hypothalamic nuclei (Loughlin et al., 1995). *ENK* encodes various enkephalin peptides that act as neurotransmitters in the CNS. *ENK* consists of three exons separated by two introns, the precursor protein proenkephalin is encoded by exon III (Comb et al., 1992). From this precursor protein, proteolytic cleavage generates four copies of met-enkephalin and one copy of leu-enkephalin as well as several enkephalin-like molecules. The level of enkephalin peptides within the cell is regulated in a complex manner, including transcriptional, posttranscriptional, and posttranslational regulation (Agoston and Dobi, 2000; Le et al., 2003).

Enkephalin gene transcription is modulated by a number of ubiquitous transcription factors. The 200-bp located just upstream of transcription start is extremely well conserved among different species and contains binding sites for AP-1 (activator protein 1), NF-1 (nuclear factor 1), (ENK)CRE1, (ENK)CRE2, AP-2 and NF-κB (Capone et al., 1998; Joshi and Sabol, 1991; v. Agoston et al., 1998). Transgenic mice with this stretch of the ENK promoter fail to govern proper expression patterns of the transgene within the CNS, despite evidence for proper up- and down-regulation of transgene expression in response to physiological stimuli (Donovan et al., 1992; Hyman et al., 1994). The elements responsible for cell-specific expression of the ENK gene are not well characterized but are thought to be located upstream of these initial 200 bp. A transgenic mouse with a 3-kb stretch of the human ENK promoter failed to reproduce proper expression patterns within the striatum and the olfactory bulb (Borsook et al., 1992; Le et al., 2003), whereas a 2.7-kb rat promoter inserted into a nonintegrating herpes viral vector directed, at least to some extent, cell-specific expression after injection into rat brain (Kaplitt et al., 1994). These results suggest that the surrounding chromatin greatly influences the activity of the *ENK* promoter, making it particularly sensitive to positional effects. Hence, to achieve a proper of understanding of the function of various elements within the *ENK* promoter, either a large number of mouse strains must be established to avoid potential bias resulting from positional effects or alternative methods must be used. In fact, in a recent study by Sauer and coworkers, a cre/lox-based strategy was used to knock an ENK-driven transgene into the HPRT locus (Le et al., 2003). The HPRT locus is believed to reside within open chromatin during development and may therefore confer fewer positional effects on a transgene. Indeed, the mice produced by using this strategy conferred organ-specific transgene expression. However, the issues of cell-specific expression and response to various physiological stimuli were not reported.

Our data clearly demonstrate that elements located in close proximity to the transcription start (2.7 kb) of the *ENK* gene are necessary to drive cell-specific expression of a transgene reporter within the striatum. The fact that transgenic mice utilizing similar *ENK* promoter sequences fail to replicate appropriate expression patterns in the CNS, mostly resulting in low-level expression within the striatum, suggests that distant DNA elements are important in controlling expression of the *ENK* gene and may serve to imprint the enkephalin promoter during development. Our strategy avoids positional effects and developmental imprinting. Because the lentiviral vector integrates into a large number of active loci (Mitchell et al., 2004), the resulting effect of the surrounding chromatin will be an average of the numerous integrations of the viral vector. Furthermore, because the vector is injected in adulthood, potential effects of a global rearrangement of the chromatin and specific imprinting effects during development are avoided.

In PD, enkephalin expression is up-regulated in the indirect pathway as a response to dopamine depletion. This up-regulation occurs when more than 90% of striatal dopamine levels are lost (Steiner and Gerfen, 1998). Reintroducing dopamine by using a VM transplant reverses this up-regulation (Cenci et al., 1993). Experimental data suggest that abnormalities of the neurons within the indirect pathway are, at least to some extent, responsible for the inappropriate movements linked to today's treatment paradigms for PD (such as L-DOPA-induced dyskinesia; Steiner and Gerfen, 1998). However, not much is known about the molecular and electrophysiological properties of these neurons in various disease states. The use of lentiviral vectors with GFP as a marker gene allows for identification and visualization of neurons within the CNS, enabling electrophysiological analysis (Dittgen et al., 2004). In this report, we demonstrate identification and visualization of a specific neuronal population affected in PD. By using this vector, characterization of the electrophysiological properties of these neurons in various disease models is possible. Furthermore, the use of the ENK promoter allows for genetic modification of this specific neuronal population.

This opens up the way for experimental and therapeutical gene transfer approaches targeted to modulation of the indirect pathway.

Implications for Gene Therapy and Experimental Gene Transfer

The use of the VSV-G envelope to pseudotype lentiviral vectors broadens their tropism. However, VSV-G-pseudotyped lentiviral vectors have been considered mainly neurotropic (Blomer et al., 1997). The data from this study do not support such a hypothesis. When we measured proviral load in intact striatum or 6-OHDA-lesioned striatum, we found similar copy numbers. The 6-OHDA lesion leads to minimal changes in the cell pool within the striatum, including very limited proliferation of glial cells (Fallon et al., 2000). Thus, the increased transgene expression is due to increased promoter activity. In the IBO-lesioned striatum, the situation becomes more complex. The neuronal population is lost, and there is proliferation of resident glial cells and infiltration of cells from the blood system. This makes the real-time PCR analysis complicated, in that it is based on an endogenous reference gene. Thus, the number of cells used as reference will have changed, leading to an altered reference. The yield of DNA from the IBO-lesioned striatum was markedly lower (about 10-fold), reflecting the change in tissue composition and cell number. Still, the ratio of proviral DNA vs. endogenous DNA was similar to that in nonlesioned striatum. This supports the notion that the lentivector is taken up by glial cells at least to the same extent as neurons. Hence, the proposed neuronal tropism for VSV-G-pseudotyped lentiviral vector probably is due to higher transcriptional activity from ubiquitous promoters in neurons (e.g., hCMV and mPGK). This raises a few concerns. First, the number of proviral integrations after injection of lentiviral vectors into the brain is likely to be much higher than the number of cells actually expressing the transgene. Second, proviral integration is not preferentially located to a postmitotic cell population (neurons) but rather is evenly distributed to all cell types in the brain, including cells with the capacity to proliferate (e.g., astrocytes). This has to be taken into account when evaluating the potential risk for insertional mutagenesis after lentiviral gene delivery to the brain.

CONCLUSIONS

Our results suggest that an autoregulated disease-targeted transgene delivery is possible. The hGFAP vector described in this study could be a useful tool in relapsing neurological disorders in which up- and down-regulation of GFAP has been found to correlate with the pathological state. Thus, the vector could be injected into affected areas, and, when the patient has a disease attack, the transgene is expressed, achieves therapeutical benefits, and then automatically turns off its expression, thereby avoiding unwanted side effects when the patient is not in the pathological state. In contrast to classical regulatory systems, a strategy employing endogenous promoters does not require expression of any transcativator protein and is not dependent on specialized promoter elements. Thus, this approach allows for a combination of targeted and regulated delivery that avoids potential immunological problems associated with overexpression of a transactivator protein.

Furthermore, this report on the rENK vector describes a targeted genetic modification of a subpopulation of neurons that are affected in PD. By using lentiviral vectors, which are simple to produce, a large number of transgenic animals of different species can in a cost-effective manner be produced in a short time. This will allow for characterization of neuronal populations affected in various neurological diseases.

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