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SHORT COMMUNICATION
Lesion-dependent regulation of transgene expression in the rat brain using a human glial fibrillary acidic protein-lentiviral vector

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Abstract

The ability to regulate transgene expression will be crucial for development of gene therapy to the brain. The most commonly used systems are based on a transactivator in combination with a drug, e.g. the tetracycline-regulated system. Here we describe a different method of transgene regulation by the use of the human glial fibrillary acidic protein (GFAP) promoter. We constructed a lentiviral vector that directs transgene expression to astrocytes. Using toxin-induced lesions we investigated to what extent transgene expression could be regulated in accordance with the activation of the endogenous GFAP gene. In animals receiving excitotoxic lesions of the striatum we detected an eightfold increase of green fluorescent protein (GFP)-expressing cells. The vast majority of these cells did not divide, suggesting that the transgene was indeed regulated in a similar fashion as the endogenous GFAP gene. This finding will lead to the development of lentiviral vectors with autoregulatory capacities that may be very useful for gene therapy to the brain.

Introduction

The use of viral vectors to deliver foreign genes to the brain is highly effective and is now a widely used method. There are a number of different vectors that transduce cells in the brain in a slightly different manner (for a review see, e.g. Davidson & Breakefield, 2003). In our laboratory, we have investigated the characteristics of lentiviral vectors. These vectors have a number of appealing features including the abilities to efficiently transduce cells in the central nervous system, maintain long-term expression and direct expression to specific cell populations (Blömer *et al.*, 1997; Kordower *et al.*, 1999; Jakobsson *et al.*, 2003). The therapeutic effects of different genes delivered by lentiviral vectors have been documented in a number of animal models, both in rodents and primates, and examples include delivery of glial cell line-derived neurotrophic factor (GDNF) in models of Parkinson's disease (Georgievska *et al.*, 2002b; Kordower *et al.*, 2000) and ciliary neurotrophic factor (CNTF) in models of Huntington's disease (de Almeida *et al.*, 2001).

An aspect of gene delivery to the brain that is currently being developed is the ability to regulate transgene expression. Efforts have been made to develop systems based on different drugs or hormones including tetracycline, rapamycine and progestrone (Clackson, 2000). However, the proteins needed for transcriptional control have been reported to be immunogenic and there is often a substantial leakage in the systems (Clackson, 2000). We, therefore, decided to try another approach to regulate transgenic expression.

Up-regulation of glial fibrillary acidic protein (GFAP) is well documented in several animal models as well as in different pathological states in the human brain (Eng *et al.*, 2000). We, therefore, hypothesized that regulating the transgene with a GFAP promoter in a viral vector would give rise to a high transgenic expression in the lesioned brain and a substantially less pronounced expression in the normal, intact brain. This would provide an option of regulating expression by using the machinery of the host cell instead of depending on recombinant regulatory proteins.

In this study we used a lentiviral vector that drives transgenic expression under a human GFAP (hGFAP) promoter. The 2.1-kb element of the hGFAP promoter used here has been extensively studied and found to be up-regulated after several different stimuli both *in vitro* and *in vivo* in transgenic mice (Brenner *et al.*, 1994; Eng *et al.*, 2000). We have recently reported that this vector directs transgene expression to glial cells in a highly specific manner when injected into the rat striatum (Jakobsson *et al.*, 2003). Based on these previous findings we argued that this vector would be able to respond to the same transcription factors that regulate the endogenous GFAP expression in a lesioned brain. We, therefore, designed a study to compare the expression of green fluorescent protein (GFP) and GDNF under the control of the hGFAP promoter in the intact brain and after different lesions to the brain.

Materials and methods

Viral vectors

The construction of lentiviral vectors expressing GFP under either a 2.1-kb hGFAP promoter or a 1.5-kb rat neuron-specific enolase (rNSE) promoter (control vector) has been described elsewhere (Jakobsson *et al.*, 2003). In order to produce a lentiviral vector expressing GDNF under the control of the hGFAP promoter, the cDNA for human GDNF was released from a pHR.CMV.GDNF plasmid (Georgievska *et al.*, 2002b) using *BamH*I–*Sac*II digestion. This fragment was then ligated

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into the corresponding sites of the hGFAP.GFP vector. All three constructs used in this study contained the central polypurine tract and the woodchuck hepatitis post-transcriptional regulatory element that have been shown to enhance transduction in the brain (Zufferey *et al.*, 1999; Zennou *et al.*, 2001). Viral vectors were produced using transient cotransfection as previously described (Zufferey *et al.*, 1997).

The titre of the different batches was estimated using an RNA slot blot technique (von Schwedler *et al.*, 1993). A batch of vector (CMV.GFP) with a known titre was used as a standard in order to estimate a functional titre. The titres of the three batches used in this study were estimated to be 1.0×10^9 TU/mL (hGFAP.GDNF), 1.0×10^9 TU/mL (hGFAP.GFP) and 1.1×10^9 TU/mL (rNSE.GFP), where TU are transducing units.

Surgical procedures

All experiments performed on animals were approved by and performed according to the guidelines of the Ethical Committee for Use of Laboratory Animals at Lund University. A total of 28 young female Sprague-Dawley rats (225 g; B & K Universal, Solna, Sweden) were housed two to three per cage with free access to food and water under a 12 h light : dark cycle. The animals were anaesthetized with isoflurane (2% in a 40% oxygen/60% nitrogen mixture) throughout the surgery session. The animals were placed in a stereotaxic frame and the vector suspension was injected using the coordinates listed below.

In the experiment using GFP as the transgene, 1 μL of a three times diluted vector was injected bilaterally into the rat striatum at (coordinates from bregma and dural surface) anterior–posterior (AP), +1.2, medio-lateral (ML), ±2.5 and dorso-ventral (DV), −4.5 mm. A thin glass capillary was attached to a Hamilton syringe in order to produce minimal mechanical damage to the host brain. One group of animals ($n = 11$) was injected with hGFAP.GFP vector while the other group ($n = 9$) received injections of rNSE.GFP vector. The animals were divided into three groups 3 weeks after the vector injection. The first group (hGFAP, $n = 5$; rNSE, $n = 4$) received a unilateral striatal lesion using the excitotoxin ibotenic acid (IBO; Sigma, Stockholm, Sweden), a total of 1.5 μL of 10 μg/mL in 0.1 M phosphate buffer, pH 7.4, at three sites, coordinates (in mm) as follows: (i) AP, +0.2, ML, −3.0, DV, −5.5; (ii) AP, +0.2, ML, −3.0, DV, −4.0 and (iii) AP, +1.5, ML, +3, DV, −5.6 with the tooth bar set at −2.3 mm (Paxinos & Watson, 1986). The second group (hGFAP, $n = 5$; rNSE, $n = 4$) received a unilateral lesion of the medial forebrain bundle using 6-hydroxy dopamine (6-OHDA, a total of 4.5 μL of 3 mg/mL in 0.02% ascorbic acid) at the following (in mm) coordinates: (i) AP, −4.0, ML, – 0.8, DV, −8.0 and (ii) AP, −4.4, ML, −1.2, DV, −7.8 mm. The third group (one animal of each vector) was left intact.

In the experiment using GDNF as the transgene, concentrated viral vector was injected bilaterally into the striatum ($n = 4$) at three sites (1 μL per site) at the following coordinates: (i) AP, +1.4, ML, ±2.6, DV, −5.0, −4.0; (ii) AP, +0.4, ML, ±3.4, DV, −5.0, −4.0 and (iii) AP, – 0.8, ML, ±4.4, DV, −5.0, −4.0 and the tooth bar set at 0.0 mm. Four weeks after the vector injections, the animals received injections of a total of 21 μg 6-OHDA (calculated as free base; Sigma) divided into three 7-μg deposits into the right striatum according to Kirik *et al.* (1998). The injections were made at the following coordinates: (i) AP, +1.0, ML, −3.0, DV, −5.0; (ii) AP, – 0.1, ML, −3.7, DV, −5.0 and (iii) AP, −1.2, ML, −4.5, DV, −5.0 and the tooth bar set at 0.0 mm.

Histology

At 1 week postlesion the animals in the GFP experiment were deeply anaesthetized (1 mL/225 g pentobarbital i.p.; Apoteksbolaget, Sweden) and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were postfixed in paraformaldehyde for 2–4 h at 8 °C and then kept in 30% sucrose for 2 days. The brains were cut into 40-μm coronal sections on a freezing microtome throughout the striatum and collected in eight series.

In order to estimate the extent of the lesions, sections were stained against the neuronal marker NeuN (IBO lesion) or tyrosine hydroxylase (6-OHDA lesion). Inflammatory and glial responses were monitored using antibodies raised against OX-19 (T-cells), OX-42 (microglia) and GFAP. Cell division was investigated using an antibody raised against proliferating cell nuclear antigen (PCNA). Expression of GFP was analysed either by direct investigation of GFP autofluorescence or by using a polyclonal GFP antibody. Double labelling was performed for GFP + GFAP and GFP + PCNA. Immunohistochemistry was performed using standard methods as described previously (Jakobsson *et al.*, 2003). For PCNA staining the tissue was denatured in 1 M HCl for 30 min at 65 °C onto chrome alum-coated slides.

The primary antibodies used in this study and working dilutions are as follows: rabbit-α-GFAP (1 : 500, Z0334; DAKO, Glostrup, Denmark), chicken-α-GFP (1 : 5000, AB16901; Chemicon, Temecula, CA, USA), mouse-α-NeuN (1 : 1000, MAB377; Chemicon), mouse-α-OX-19 (1 : 100, MCA52G; Serotec, Oxford, UK), mouse-α-OX-42 (1 : 200, MCA275G; Serotec), mouse-α-PCNA (1 : 100, M0879; DAKO) and mouse-α-tyrosine hydroxylase (1: 2000, MAB318; Chemicon). The secondary antibodies were biotinylated goat-α-rabbit (BA-1000; Vector, Burlingame, CA, USA), biotinylated horse-α-mouse (BA-2001; Vector), biotinylated rabbit-α-chicken (G2891; Promega, Madison, WI, USA), Cy3-donkey-α-mouse (115-116-075; Jackson, West Grove, PA, USA), Cy3-donkey-α-rabbit (711-165-152; Jackson) and fluoroscein isothiocyanate-donkey-α-chicken (703-096-155; Jackson). All secondary antibodies were diluted 1 : 200.

Glial cell line-derived neurotrophic factor determination by ELISA

At 1 week after the intrastriatal 6-OHDA lesions, the animals that had received GDNF vectors were deeply anaesthetized with sodium pentobarbital and decapitated. The striatum and substantia nigra (SN) were dissected out as previously reported (Georgievska *et al.*, 2002b) and tissue levels of GDNF were then determined on brain homogenates using ELISA according to the supplier's recommendations (G3240; Promega).

Statistical analysis

A two-factor ANOVA was used for statistical analysis followed by a Fisher's PLSD posthoc test using Statview software (SAS Institute, Cary, NC, USA).

Results

GFP expression after excitotoxic lesion

GFP-positive cells could be detected in all striata injected with lentiviral vectors transferring GFP. In animals with IBO lesions, an eightfold increase in the number of GFP-positive cells was found in the lesioned striatum of animals injected with the hGFAP vector compared with the intact, contralateral side (3413 ± 1440, $n = 5$ vs. 455 ± 312 cells, $n = 5$, $P < 0.0001$; Fig. 1A and B). In animals injected with the control rNSE vector we found substantially fewer GFP-positive cells on the lesioned side (484 ± 80, $n = 4$ vs. 2191 ± 1101 cells, $n = 4$ in the contralateral striatum, $P < 0.001$; Fig. 1A). As expected, NeuN immunohistochemistry confirmed that a large proportion of the neurons in the lesioned striatum were lost (data not shown) explaining the loss of GFP-expressing cells in the animals injected with the control vector where a neuron-specific promoter drives transgene expression. The up-regulation of the hGFAP vector colocalized largely with the

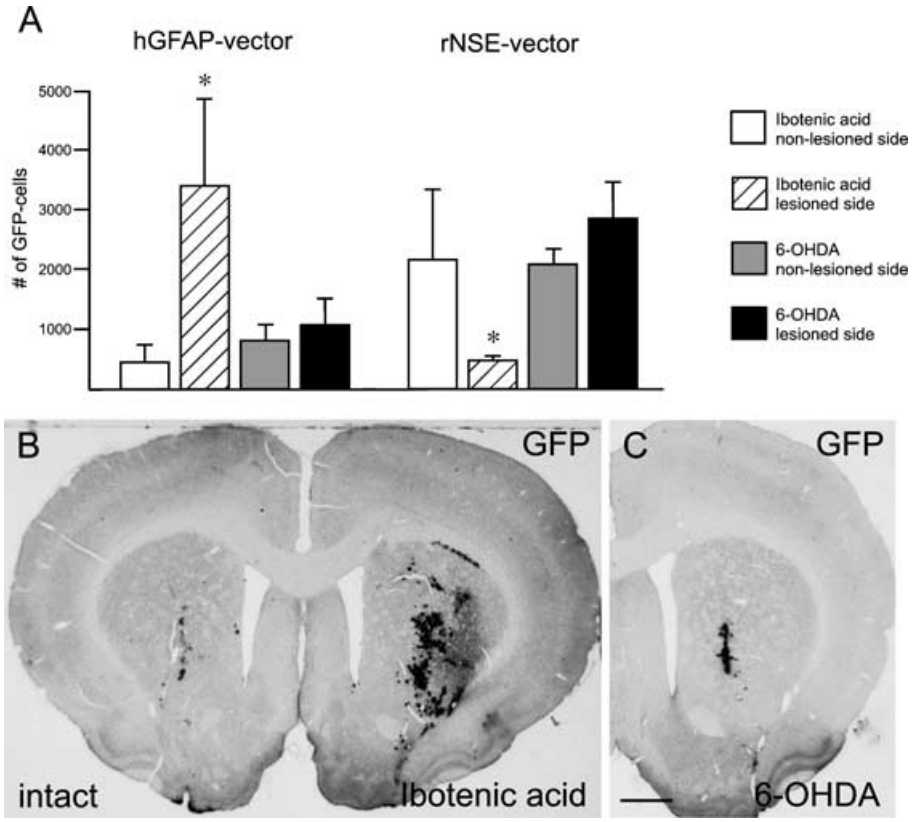

FIG. 1. (A and B) In animals lesioned with ibotenic acid a distinct up-regulation of GFP expression could be found when using the human glial fibrillary acidic protein (hGFAP) vector but not when using the rNSE vector. (A and C) In animals lesioned with 6-hydroxy dopamine (6-OHDA), GFP expression was found in an area close to the injection site and was not up-regulated on the lesioned side. Scale bar, 1 mm. $^{*}P < 0.001$, error bars, SD.

native up-regulation of GFAP (Fig. 2A–C), which was massive in an area surrounding the lesion while the necrotic centre of the lesion displayed almost no immunoreactivity against GFAP. On the contralateral side of the lesion, GFP expression could only be found in a very limited area surrounding the injection site and also here GFP expression colocalized with an up-regulated GFAP expression as a result of the mechanical injury by the injection needle (Fig. 2G–I). The GFP-positive cells found in animals injected with the hGFAP vector had glia-like morphologies and confocal analysis confirmed that a large proportion of these cells colocalized with native GFAP expression. When examining double labelling of GFP and PCNA, a few double-labelled cells ($< 5\%$) were found, indicating that the increase in GFP-positive cells could, to some extent, be explained by cell division (Fig. 2J–L). However, the majority of GFP-positive cells were negative for PCNA. The PCNA expression pattern was very similar to OX-19 staining (and, to some extent, OX-42 immunoreactivity) suggesting that the majority of newly formed cells were microglial cells or cells penetrating from the blood system (data not shown).

GFP expression after 6-hydroxy dopamine lesion to the medial forebrain bundle

In the animals that were lesioned in the medial forebrain bundle using 6-OHDA no increase in the number of GFP-expressing cells was found on the lesioned side in animals injected with the hGFAP vector (842 ± 281, $n = 5$ compared with 1092 ± 459 cells, $n = 5$, $P = 0.62$; Fig. 1A and C). In contrast to the animals lesioned with IBO, only a small increase of endogenous GFAP expression was found (Fig. 2D–F). Tyrosine hydroxylase immunohistochemistry showed that there was an almost complete loss of the nigrostriatal fibre innervation. The GFP-positive cells that were found were mainly situated around the injection site and colocalized with endogenous GFAP expression as seen in the specimens from the IBO group (Fig. 2D–F).

Glial cell line-derived neurotrophic factor expression after intrastriatal 6-hydroxy dopamine lesion

At 1 week after the intrastriatal 6-OHDA lesions, we found a threefold up-regulation of GDNF levels in the striatum as compared with the intact side (Table 1, $P < 0.01$). Interestingly, we also detected low levels of GDNF in the SN pars reticulata (SNpr; $\sim$0.1 ng/mg tissue; Table 1). This indicates that there was an anterograde transport of GDNF protein to the SNpr, which may be a consequence of a small subset of neurons expressing unspecifically from the hGFAP promoter in the striatum. The GDNF levels were, however, not up-regulated in the SNpr ipsilateral to the lesion (see Table 1) indicating that the increased levels of GDNF in the lesioned striatum were due to an increased GFAP expression in astrocytes in the damaged striatum.

Discussion

In this study we have taken advantage of the up-regulated GFAP expression that is found after a lesion to the brain. By driving transgenic expression from a lentiviral vector using a 2.1-kb DNA element taken from upstream of the hGFAP gene we found that transgene expression was regulated in a similar manner as the endogenous GFAP expression. This is a novel way of regulating transgene expression from a viral vector in the brain and has the advantage that

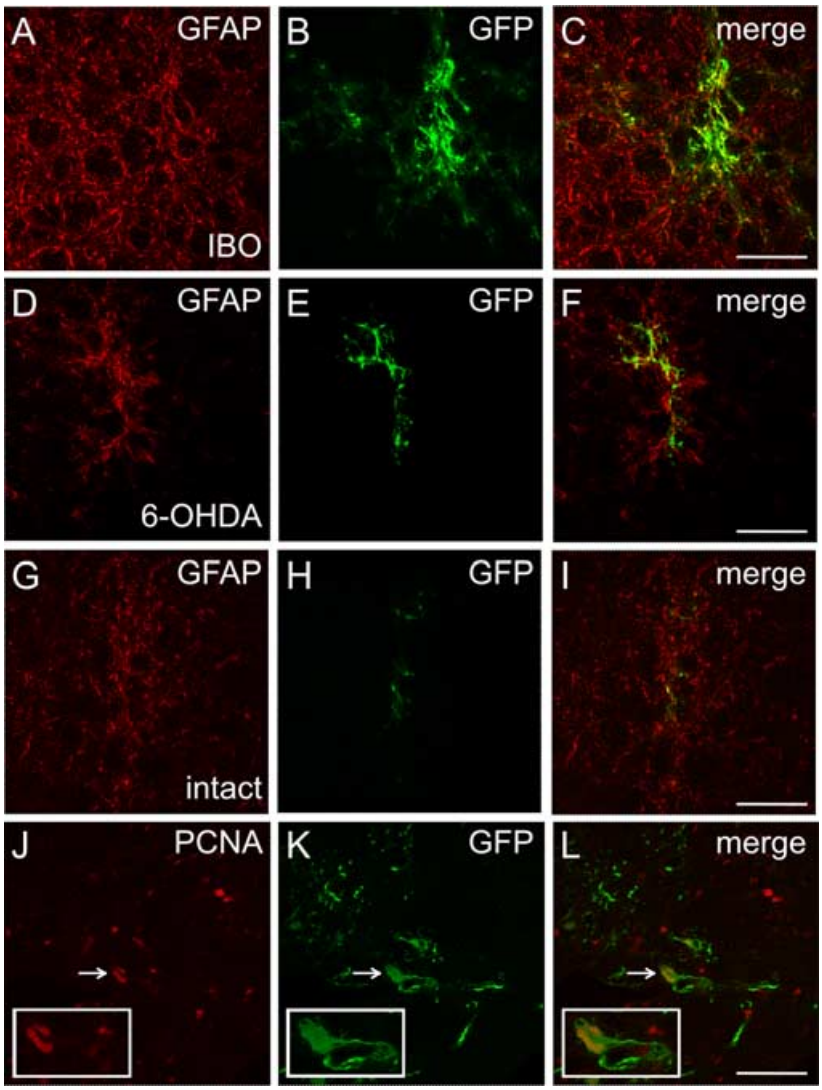

FIG. 2. (A–I) Confocal analysis of glial fibrillary acidic protein (GFAP) (red) and GFP (green) expression in the different lesion models used in this study. Note in particular the massive up-regulation of GFAP expression found after an ibotenic acid (IBO) lesion (A) and the colocalization of up-regulated GFP expression when GFP is driven by a human GFAP promoter (B and C). (J–L) Confocal analysis of proliferating cell nuclear antigen (PCNA)-GFP expression in a specimen lesioned with IBO. A small number of double-labelled cells were found although the majority of GFP-positive cells were negative for PCNA. Arrow indicates double-labelled profile. 6-ODHA, 6-hydroxy dopamine. Scale bars, (A–I) 200 μm, (J–L) 100 μm, inlay 50 μm.

no inducing molecule, such as doxycycline, has to be added and that no recombinant regulatory protein has to be coexpressed.

The fact that we were able to regulate two independent transgenes using two different lesion models demonstrates a solid proof of principle. However, when using high doses of viral vectors as in the GDNF experiment there is also substantial expression in the nonlesioned striatum. Further studies will confirm if it is possible to remove this background expression and to what levels the induced GFAP promoter will be able to express a transgene. It will also be of importance to analyse whether or not expression is codown-regulated with the endogenous GFAP expression at later time points.

TABLE 1. Expression of GDNF from the hGFAP.GDNF vector in the intact nigrostriatal system and 1 week after an intrastriatal 6-OHDA lesion

	Striatum	Substantia nigra
Intact	1.74 ± 0.29	0.13 ± 0.03
6-OHDA	$5.22 \pm 0.84^{*}$	0.10 ± 0.02

Values are presented as ng/mg tissue ± SEM ($n = 4$). *Significantly different from intact striatum ($P < 0.01$).

The up-regulation of transgene expression can be explained in two ways. On one hand it is known that there is cell division in the glial cell population in a lesioned brain area (Bignami & Dahl, 1995). In our case we studied this by performing immunohistochemistry for the cell cycle marker PCNA. Proliferating cell nuclear antigen is expressed during S-phase in dividing cells and its expression correlates well with other markers of mitosis such as Ki-67 and bromodeoxyuridine labelling (Abadie *et al.*, 1999; Kato *et al.*, 2003). On the other hand, our findings indicate that only a small minority of GFP-labelled cells (< 5%) were double labelled for PCNA, suggesting that it is unlikely that the increase in GFP-positive cells (eightfold) after excitotoxic lesion is due only to self-renewal of GFP-positive cells. In brain trauma models astrocytes have been shown to increase 1.8-fold 1 week after injury (Kato *et al.*, 2003), which is much less than the eightfold increase of GFP-expressing cells detected here. Therefore,

we believe that it is more likely that silent or near silent proviral copies integrated into the genome of host cells have been reactivated by the same cellular signals that activate the endogenous GFAP gene after a lesion.

In a previous study we found that the hGFAP.GFP vector directed transgene expression to astrocytes in a highly specific manner (Jakobsson *et al.*, 2003). In the present study when GDNF was used as the transgene and protein levels were measured using ELISA we were able to detect levels of the transgene in the SN. This correlates with previous studies in our laboratory where anterograde transport to the SNpr was found using a CMV promoter-based lentiviral vector (Georgievska *et al.*, 2002a,b). Explanations for this include a small subset of neurons expressing the GDNF transgene nonspecifically from the hGFAP promoter, possibly due to the random integration of the provirus leading to influence from surrounding enhancers and/or promoters (Lai *et al.*, 2002), or GDNF being taken up by striatal projection neurons and transported to the SN. In this study we could not detect any cells with neuronal profiles expressing the GFP transgene, which is in keeping with our previous results (Jakobsson *et al.*, 2003). The discrepancy between the two transgenes may also be due to the use of more virus vector (approx. 10 times more) in the GDNF experiments. However, the levels of GDNF were not up-regulated in the SNpr ipsilateral to the striatal lesion indicating that the up-regulation of transgene expression was specific to astrocytes.

The fact that transgenic expression was not up-regulated after the medial forebrain bundle lesion may be explained by the lack of a significant up-regulation of endogenous GFAP exceeding the pure mechanical trauma that takes place when the injection needle is inserted and the viral vector is injected. In the case of the up-regulated transgenic GDNF expression found after an intrastriatal 6-OHDA lesion, it should be kept in mind that this lesion is, in fact, a combined mechanical (injection needle) and toxin-specific effect.

It will be critical to develop efficient means of regulating transgene expression if gene therapy is to be used clinically in certain brain disorders. We report here a novel strategy that uses the host cell's own machinery to regulate expression in a lesion-dependent manner. Although further evaluation is necessary this may provide an alternative to systems that include inducer molecules and transgenic transactivators.

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Abbreviations

AP, anterior–posterior; DV, dorso-ventral; GDNF, glial cell line-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; hGFAP, human GFAP; IBO, ibotenic acid; ML, medio-lateral; NSE, neuron specific enolase; 6-OHDA, 6-hydroxy dopamine; PCNA, proliferating cell nuclear antigen; rNSE, rat NSE; SN, substantia nigra; SNpr, SN pars reticulata.

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