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Targeted Transgene Expression in Rat Brain Using Lentiviral Vectors

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Direct gene transfer to the adult brain is dependent on vectors that transduce non-dividing cells, such as lentiviral vectors. Another aspect of the development of gene therapy to the brain is the need for cell-specific transgene expression. Expression from vesicular stomatitis virus G-protein (VSV-G) pseudotyped lentiviral vectors has been reported to be mainly neuron specific in the brain. We constructed cell-specific lentiviral vectors using the neuron-specific enolase (rNSE) or the glial fibrillary acidic protein (hGFAP) promoters and compared them to the ubiquitous human cytomegalovirus promoter (hCMV), a hybrid CMV/β-actin promoter (CAG) and the promoter for human elongation factor 1α (EF1α). Our results showed that the hGFAP promoter was expressed only in glial cells, whereas rNSE was purely neuron specific, showing that VSV-G is pantropic in the rat striatum. We conclude that the VSV-G allows transduction of both glial and neuronal cells and the promoter dictates in what cell type the transgene will be expressed. The expression of transgenes exclusively in astrocytes would allow for local delivery of secreted transgene products, such as glial cell line-derived neurotrophic factor (GDNF), circumventing the anterograde transport that may induce unwanted side effects.

Key words: lentiviral vectors; cell-specific; glia; neuron; pseudotype

In recent years, lentiviral vectors have been shown to be powerful tools for gene transfer to the brain (Blomer et al., 1997; Kordower et al., 2000). These vectors can infect and integrate into non-dividing cells, and they show high transduction efficiency and long-term expression in the central nervous system (CNS) (Naldini et al., 2000). Moreover, these vectors encode no viral proteins that may evoke an immune response and therapeutic benefit has been demonstrated in animal models of severe CNS disorders, including Parkinson's and Huntington's diseases (Kordower et al., 2000; Trono, 2000; Regulier et al., 2002). Lentiviral vectors also hold great promise as experimental vectors in several settings in the brain, including stem cell research and development of transgenic animal models (de Almeida et al., 2002; Englund et al., 2002; Lo Bianco et al., 2002).

Most studies in the CNS using lentiviral vectors have thus far relied on strong promoters, such as the human cytomegalovirus promoter (hCMV) or the mouse phosphoglycerate kinase 1 promoter (mPGK) (Blomer et al., 1997; Kordower et al., 1999, 2000; Deglon et al., 2000). These promoters are considered ubiquitous and the expression pattern found after vector injection into the rat brain has therefore been considered a result of either vector properties or properties of the recombinant envelope used.

In several studies, transduction in the brain using lentiviral vectors has been found to be mainly neurotropic (Blomer et al., 1997; Kordower et al., 1999). This has been attributed to the glycoprotein of the vesicular stomatitis virus (VSV-G) (Sanders, 2002), which is used regularly to pseudotype these vectors (pseudotype refers to the use of an envelope protein derived from a virus different from vector virus) to achieve a broad transduction spectrum and allow for concentration using ultracentrifugation (Zufferey et al., 1997). No careful examination, however, has been carried out to determine the cause of neurotropism in VSV-G pseudotyped lentiviral vectors.

We designed a study that examines several different promoters, all inserted into HIV-I derived lentiviral vectors and used to drive expression of green fluorescent protein (GFP). All vectors were VSV-G pseudotyped and after production and concentration, injected into the striatum of rat brains. The promoters we used included both pan-cellular promoters and cell-specific promoters (Table I). We found that when using VSV-G-pseudotyped lentiviral vectors, it was indeed possible to direct transgene expression to specific cell populations, including both glial cells and neurons, and that it is possible to achieve high level of transgene expression in both cell subsets. We conclude that expression in rat brain when using VSV-G lentiviral vectors is highly dependent on the promoter that was used. This may prove useful for improvement of gene

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TABLE I. Promoters Used in This Study

Promoter	Abbreviation	Size	Reference
Human cytomegalovirus	hCMV	0.8 kb	(Blomer et al., 1997)
Hybrid CMV/β-actin	CAG	1.8 kb	(Niwa et al., 1991)
Human elongation factor 1α	EF1α	1.2 kb	(Mizushima and Nigata, 1990)
Rat neuron-specific enolase	rNSE	1.5 kb	(Forss-Petter et al., 1990)
Mouse glial fibrillary acidic protein	mGFAP	0.3 kb	(Miura et al., 1990)
Human glial fibrillary acidic protein	hGFAP	2.1 kb	(Brenner et al., 1994)

therapy strategies in the brain by enabling cell-specific transgene delivery.

MATERIAL AND METHODS

Transfer Plasmids

The sequences for the neuron-specific enolase promoter (rNSE) (Forss-Petter et al., 1990) and the glial fibrillary acidic protein promoter (mGFAP) (Miura et al., 1990) were amplified by PCR. The following PCR reaction was used: 90 sec at 95°C, followed by 30 cycles of 20 sec at 95°C, 45 sec at 60°C and 30 sec at 72°C. Primers (containing additional restriction sites: *Bam*HI or *Cla*I) were as follows: rNSE (up), GCT AAT CGA TGG GAC AGT AAA GGT GAT GGC; rNSE (down), ATC CGG ATC CGG ACT GCA GAC TCA GCC G; mGFAP (up), GTA TAT CGA TTTG ATC AAT GCG AAG CCA GGC; and mGFAP (down), TGA CGG ATC CAG GAG CGG CGC GCA GAG. Genomic rat DNA was used as a template for the rNSE promoter and plasmid rAAV.GFAP.GFP (Lundberg, unpublished data) for the mGFAP promoter. After subcloning into a TA-vector (Invitrogen) and sequencing, fragments were released with *Bam*HI-*Cla*I digestion and inserted into the corresponding sites of a pHR.CMV.GFP.W plasmid (kind gift of D. Trono, Geneva, Switzerland), replacing the hCMV promoter. The hGFAP promoter was released from plasmid gfa2 (kind gift of M. Brenner, Birmingham, AL; Brenner et al., 1994) with *Bam*HI-*Bgl*II digestion and inserted into the corresponding sites in a pCRII-vector (Invitrogen). The vector was then digested with *Acc*I-*Bam*HI and inserted into the *Bam*HI-*Cla*I sites of pHR.CMV.GFP.W. The new constructs were named pHR.rNSE.GFP.W, pHR.mGFAP.GFP.W, and pHR.hGFAP.GFP.W. The pHR.EF1α.GFP.W and pHR.CAG.GFP.W plasmids were kindly provided by N.B. Woods (Lund, Sweden).

Promoter choice in this study in the case of CAG, CMV, and EF1α was based on work carried out by several research groups, which have found these promoters particularly interesting for gene transfer when using lentiviral vectors (for example, see Ramezani et al., 2000). The cell-specific promoters (rNSE, hGFAP, and mGFAP) were chosen based on transgenic mouse strains expressing transgenes under the control of these promoters (see Table I for references).

In the case of the pHR.rNSE.GFP.W and pHR.hGFAP.GFP.W, a cPPT sequence was inserted as reported previously (Follenzi et al., 2000). All plasmids contained the Woodchuck hepatitis virus post-transcriptional regulatory element (W) that has been shown to increase transgenic expression (Zufferey et al., 1999).

Lentiviral vectors were produced as described previously (Zufferey et al., 1997). Briefly, the transfer plasmids pHR.CMV/rNSE/EF1α/CAG/mGFAP/hGFAP.WPRE were co-transfected with pMD.G and pCMVΔR8.92 into 293T cells, the supernatants were collected on Days 2 and 3 after transfection and concentrated by ultracentrifugation.

Determination of Viral Titre and Vector Functionality

The viral particle titre for all constructs was determined by RNA slot blot techniques (von Schwedler et al., 1993). In brief, RNA from the various vector preparations were purified, precipitated, bound to a nylon membrane and then hybridized to a 0.6-kb P^{32}-labeled DNA probe containing the sequence for WPRE. The amount of RNA was estimated using a PhosphorImager (BAS-5000, Fuji) and then compared to a known standard. To determinate the functional titre (TU, transducing units/ml) a serial dilution of the viral stock was applied to 10^5 293 T cells. The dilutions that resulted in 15% or less GFP-positive cells after 48 hr, were used for calculations. TU was only calculated for the construct containing the hCMV promoter. The RNA slot blot values were then used for comparison and estimation of TU for constructs containing the other promoters.

Functionality of all vectors was tested in vitro on 293T cells. All vectors, with the exception of the mGFAP vector, efficiently transduced this cell type. To validate functionality of the mGFAP vector, we did a subsequent transduction on rat primary astrocytes (Ericson et al., 2002). FACS analysis revealed that 45% of transduced astrocytes were GFP positive, confirming that the mGFAP vector was indeed functional (data not shown).

Surgical Procedure

A total of 68 young female Sprague-Dawley rats (B&K Universal, Stockholm, Sweden) were housed 2–3 per cage with free access to food and water under 12-hr light:dark cycle. All surgical procedures were approved by and carried out according to the guidelines of the Ethical Committee for Use of Laboratory Animals at Lund University. The animals were anesthetized with halothane (2% in air) and placed into a stereotaxic frame (Kopf Instruments, Tujunga, CA). The skull was exposed, holes drilled, and a total volume of 1–2 μl of the viral stocks was injected bilaterally into the striatum using a 10-μl Hamilton syringe. The amount of injected TU:s was approximately 10^5 per injection (with the exception for when long term expression was studied when using the hGFAP promoter where a 20-fold

lower dose were used). The following stereotactic coordinates were used: anterior-posterior (AP) +1.2, medial-lateral (ML) ± 2.5 and dorsoventral (DL) −4.5 (Paxinos and Watson, 1986), with the tooth bar set at 0 mm. When carrying out the double injections a thin glass tip was attached to the syringe to achieve a more distinct delivery (Nikkhah et al., 1995). The coordinates for the double injections were: AP +1.2, +0.2, ML ± 2.5, ± 3.5, and DL −4.5, −5.0.

Immunohistochemistry

At 3 or 6 weeks after viral injection the rats were anesthetized deeply with pentobarbital and perfused through the ascending aorta with isotonic saline followed by 250–300 ml of ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. The brains were removed and postfixed for a few hours in the same solution and then transferred to 25% sucrose in 0.1 M PB until calibrated and then sectioned on a freezing-stage microtome at 40 μm throughout the striatum. The sections were first rinsed in potassium-phosphate buffered saline (KPBS), and then treated with 3% H_2O_2 and 10% methanol in KPBS to quench endogenous peroxidase activity. After additional rinses in KPBS, sections were preincubated in KPBS containing 5% normal rabbit serum (NRS; Biotech Line AS, Denmark) and 0.25% Triton X-100 for 1 hr at room temperature and incubated overnight at room-temperature in a 1:5,000 dilution of chicken anti-GFP antibody (Chemicon, Sweden), 5% NRS, and 0.25% Triton X-100 in KPBS. The next day, sections were rinsed in 2% NRS in KPBS and incubated with biotinylated rabbit anti-chicken (1:200; G289A, Promega) in KPBS with 2% NRS for 2 hr at room temperature. After rinses in KPBS and incubation with an avidin-biotin-peroxidase complex (Vectastain Elite ABC Kit PK-6100; Vector Labs, Burlingame, CA) the reaction was visualized using 3,3-diaminobenzidine as a chromagen (0.5 mg/ml; Sigma, Sweden). The sections were mounted on chrome-alum coated slides, dehydrated in ascending alcohol concentrations, cleared in xylene, and coverslipped in DPX (BDH, UK).

Some sections from each construct were also double stained for GFP/GFAP, GFP/NeuN and GFP/Ng2. The sections were rinsed three times in KPBS before preincubation in 5% normal donkey serum (NDS) for 1 hr at room temperature and then incubated over night in 5% NDS, 0.25% Triton X-100 and chicken anti-GFP (1:5,000; Chemicon AB 16901)/rabbit anti-GFAP (1:500; DAKO, Z0334) or 5% NDS, 0.25% Triton X-100, and chicken anti-GFP (1:5,000)/mouse anti-NeuN (1:1,000; Chemicon MAB377) or 5% NDS, 0.25% Triton X-100, and chicken anti-GFP (1:5,000)/rabbit anti-Ng2 (1:500; kind gift from Dr. W.B. Stallcup, La Jolla, CA), respectively. After three rinses in KPBS and 5% NDS on the second day, sections were incubated in the dark for 2 hr in 2% NDS, 0.25% Triton X-100, and fluorochrome-conjugated secondary antibodies: donkey anti-chicken (1:400, FITC; Jackson, West Grove, PA)/donkey anti-rabbit (1:400, Cy3; Jackson) or donkey anti-chicken (1:400, FITC; Jackson)/donkey anti-mouse (1:400; Cy3; Jackson). After rinses in KPBS and mounting on chrome-alum-coated slides, the sections were coverslipped with PVA-DABCO.

Morphological Analysis

To quantify the number of transduced cells sections were analyzed throughout the whole transduced area. Stereological analysis of the sections was carried out 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 (Heidnhain, ND 281, Traunreut, Germany). Briefly, the *CAST-Grid v. 1.10* software was used to delineate the area with transduced cells using the 4× objective. With the 100× objective, a counting frame was first placed randomly to generate the first counting area, and then systematically moved (between 100–300 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).

Cell density in the corpus callosum was estimated by placing a fixed area (9,878 μm^2) by the injection site at the 4× objective and the cells found in the frame at 40× objective were counted. The same procedure was repeated on the two sections adjacent to the needle tract. The depth of the sections was estimated using the microcator (Heidnhain) and the number of cells/100 μm^3 were calculated.

To estimate the fraction of double-labeled cells, sections were analyzed by using a 40× objective, the appropriate UV-filter, and a photo frame on a microscope (Leitz DMBRE, Leica). Starting from the centre of the injection (dorsoventral and mediolateral) all GFP-expressing cells were counted and the fraction of cells also expressing GFAP or NeuN were noted. This was carried out on all sections were GFP-expressing cells were found throughout the striatum. About 100 positive double-labeled cells were confirmed further using confocal microscopy (Leitz DMRE, Leica).

Statistical Analysis

For statistical analysis, two-factor ANOVA was used followed by a Fisher's PLSD test when appropriate, using StatView software (Abacus Concepts, Berkeley, CA).

RESULTS

Transduction Using Ubiquitous Promoters

In all animals injected with vectors carrying the three ubiquitous promoters analyzed in this study (hCMV, EF1α, and CAG), a vast number of GFP-expressing cells were found. The pattern of distribution of the transduced cells throughout the striatum was similar using all constructs, with a higher cell density at the injection site, which declined further away from the centre of injection. In the striatum, most transduced cells displayed a neuronal morphology. The hCMV vector and the CAG vector were also able to transduce a few cells that had an astrocytic morphology. Expression level, judged by the intensity of GFP autofluorescence and GFP immunostaining was similar in the hCMV and EF1α specimens, whereas the CAG vector injections resulted in noticeably paler cells and less distinct fibre staining (Fig. 1A–F). The presence of anterograde transport, indicated by fibre staining in the

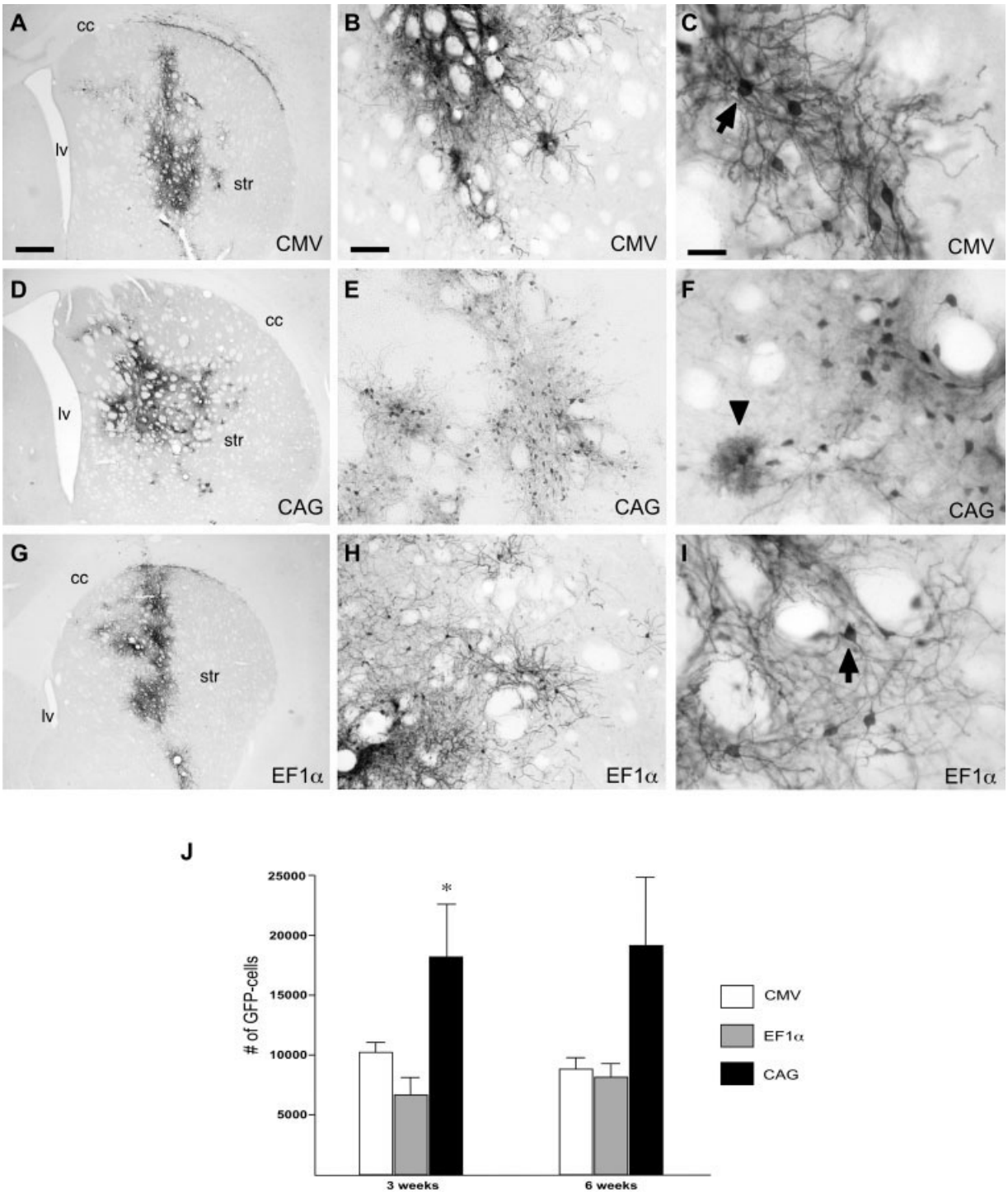

Fig. 1. Transgene expression in the striatum. **A–I:** Morphology and distribution of the GFP-expressing cells in the striatum when using various ubiquitous promoters. All vectors transduced mainly cells with neuronal morphology. Arrowhead indicates cell with astrocytic morphology, arrows indicate cells with neuronal morphology. Scale bar = 500 μm (A,D,G); 100 μm (B,E,H) and 35 μm (C,F,I). **J:** The number of GFP-positive cells found in the rat brain after different survival times (3 or 6 weeks). Cc, corpus callosum; lv, lateral ventricle; str, striatum. $^{\star}P < 0.01$; error bars represent SEM.

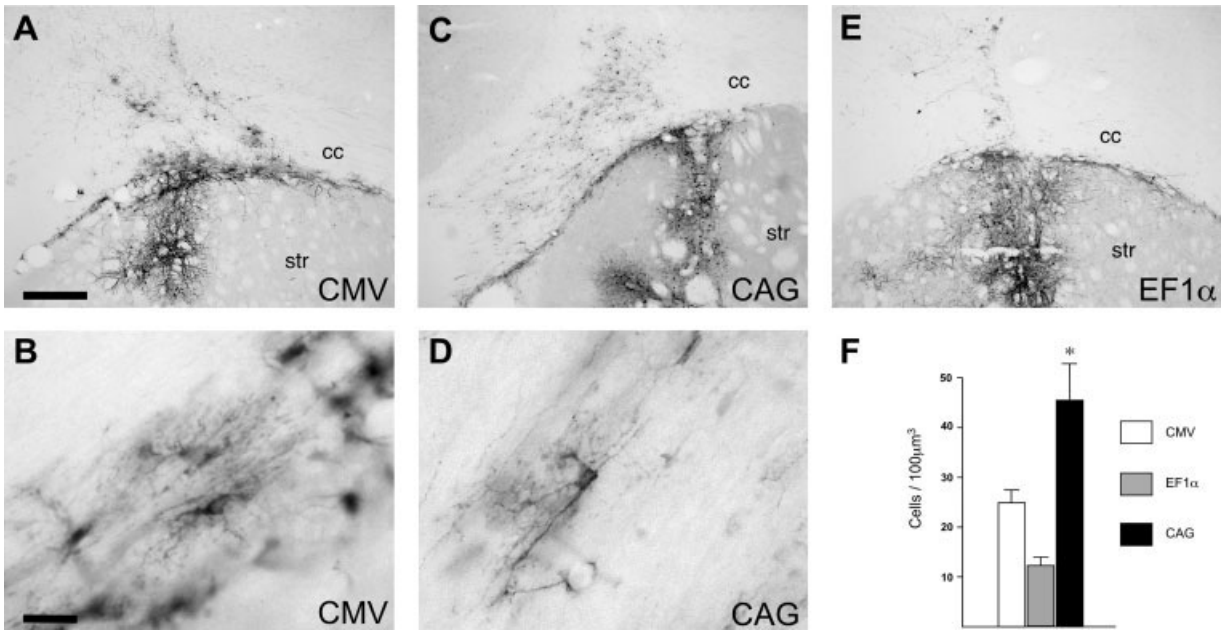

Fig. 2. Transgene expression in white matter. Differences in the expression in white matter were analyzed in the corpus callosum (cc) by the injection site. Vectors with the CMV enhancer (CMV and particularly CAG) showed a widespread transduction of the cc (**A,C**). In contrast, the EF1α promoter expressed GFP in significantly fewer cells (**E**). A large proportion of GFP-positive cells in the cc displayed an oligodendrocytic morphology (**B,D**). **F:** Analysis of the cell density in the cc (GFP-positive cells/100 μm^3) revealed significant differences between the different vectors. $\star P < 0.01$; error bars represent SEM. Cc, corpus callosum; str, striatum. Scale bar = 200 μm (A,C,E); 20 μm (B,E).

globus pallidus (an area innervated by the striatum) was found when using all three promoters (data not shown).

At 3 weeks, total number of GFP-positive cells arising from the hCMV, CAG, and EF1α vector injections were: hCMV, 10,411 ± 2,137 cells ($n = 6$); CAG, 18,484 ± 14,339 ($n = 12$); and EF1α, 6,798 ± 3,760 ($n = 12$). At 6 weeks the total number were: hCMV, 9,042 ± 3,136 ($n = 4$); CAG, 18,230 ± 14,638 ($n = 8$); and EF1α, 8,436 ± 2,819 ($n = 6$) (Fig. 1G). The number of GFP-positive cells was stable up to 6 weeks, the longest survival time studied here, using all vectors. The number of cells transduced relative to the number of transducing units that were injected was in the range of 7–18%; for hCMV, 9 ± 2% ($n = 10$); CAG, 18 ± 14% ($n = 20$); and EF1α, 7 ± 4% ($n = 18$).

To further study transduction efficiency, we injected a diluted viral stock (half the number of transducing units) of hCMV and CAG vector into either one or two injection sites in each hemisphere. This experiment revealed no increase in the number of transduced cells when using double injections of the hCMV vector: 1 site-hCMV, 8,495 ± 2,091 ($n = 5$); and 2 sites-hCMV, 8,681 ± 2,723 ($n = 6$). Injections of diluted viral stock did, however, result in a higher efficiency when compared to injection of a higher concentration: hCMV, 17 ± 5% ($n = 11$); and CAG, 30 ± 11% ($n = 8$). The data confirmed that using the CAG promoter resulted in a greater number of GFP-positive cells when compared to the hCMV and EF1α vectors (see above).

To investigate tropism of the vectors in white matter, we measured the number of GFP-expressing cells/100 μm^3 in the corpus callosum (cc). This white matter tract was transduced as a result of the backflow that occurs when the injection needle is withdrawn. By measuring the density of GFP cells, we found statistical differences between the three ubiquitous vectors: hCMV, 25 ± 12 cells/100 μm^3; CAG, 45 ± 34 cells/100 μm^3; EF1α, 12 ± 9 cells/100 μm^3 ($P < 0.01$, CAG different from CMV and EF1α; Fig. 2). Most GFP-expressing cells found in the cc presented an oligodendrocyte-like morphology (Fig. 2B,D), whereas a small proportion of the cells were likely white matter astrocytes because they expressed GFAP (Fig. 3D–F).

Examination of double labeling with GFP and NeuN revealed that most transduced cells were double labeled with this neuronal marker, although slight differences in the ratio could be observed between the constructs. The rations were as follows: hCMV, 82 ± 9% double-labeled cells; CAG, 86 ± 12% double-labeled cells; and EF1α 98 ± 2% double-labeled cells. Double staining with GFP/GFAP (astrocytic marker) and GFP/Ng2 (oligodendrocytic marker) revealed a few scattered double-labeled cells when using the hCMV or CAG promoter (Figs. 3A–I and 4A–C).

Transduction Using Cell-Specific Promoters

Injections of the vectors carrying the cell-specific promoters resulted in widespread transduction in the case

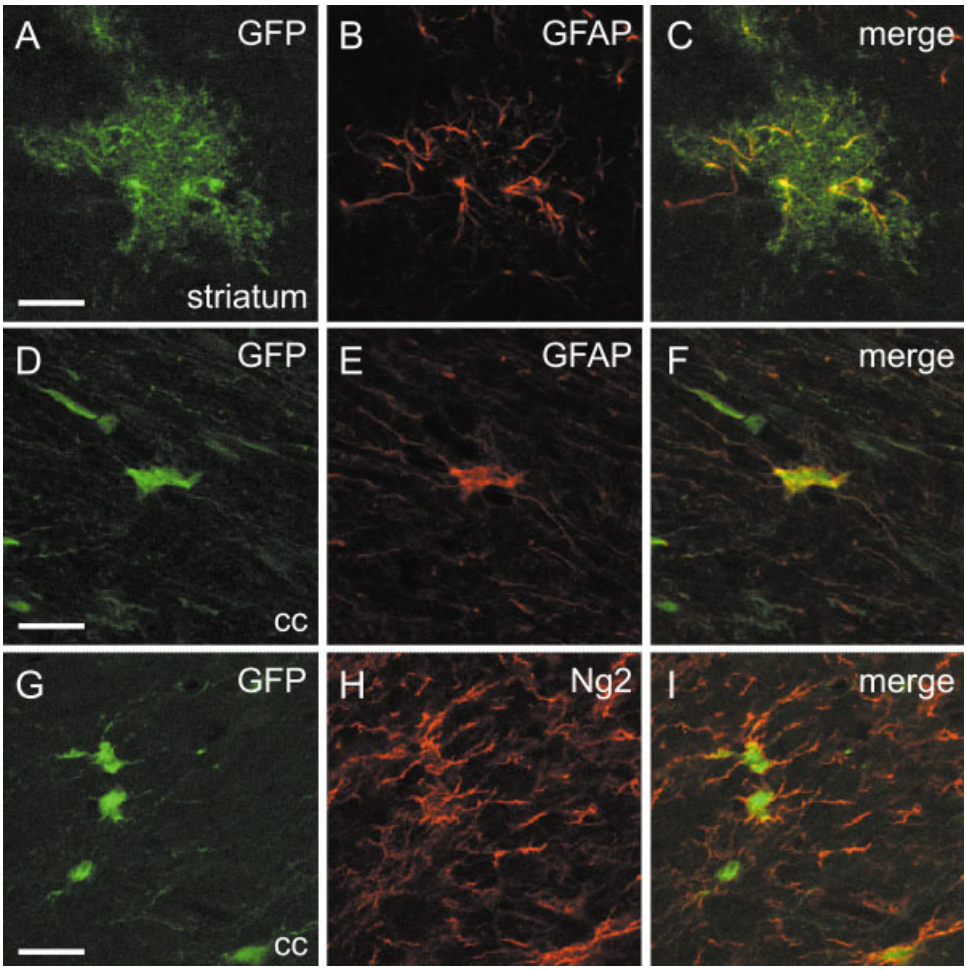

Fig. 3. Double staining with GFAP and Ng2. **A–F:** Confocal analysis of GFP (green) and GFAP (red) expression in brains injected with LV.CAG.GFP.W. Double-labeled cells could be found in both the striatum (A–C) and the corpus callosum (D–F). A few cells that were double labeled with the oligodendrocytic marker Ng2 (red) and GFP (green) could also be found when using the CAG promoter (**G–I**). Scale bar = 20 μm.

of rNSE and hGFAP, whereas the mGFAP vector only transduced a few cells with an astrocytic morphology. We therefore chose to use only the rNSE and the hGFAP for further analysis. The number of transduced cells was quantified using stereological methods and in animals that were injected with the LV.rNSE construct, 6,299 ± 3,760 (n = 10) cells were detected. Injections of LV.hGFAP resulted in 10,507 ± 4,084 GFP-positive cells (n = 6). The numbers of GFP-positive cells were in the same range as when using pan-cellular promoters (see above).

When using the rNSE-promoter, after 6 weeks we found numbers of GFP-expressing cells (8,518 ± 4,323 GFP-cells, n = 4) similar to the number after 3 weeks. To determine if expression was stable after 6 weeks when using the hGFAP promoter, we used a lower dose of vector (1/20). This was done to minimize the influence of upregulation of transcription factors that activate the endogenous GFAP promoter as a result of injecting a concentrated vector preparation. The number of GFP-expressing cells was found to be stable up to 6 weeks (3 weeks, 188 ± 74, n = 6; and 6 weeks, 133 ± 77, n = 6).

We quantified the ratio of cells that were double labeled with either NeuN/GFP or GFAP/GFP and used the ubiquitous vectors as controls. The targeting of the transgene expression was very specific. We found statistical differences ($P < 0.01$) in the way expression was directed to either the NeuN- or GFAP-positive cell populations, both in regard to the rNSE- and hGFAP-vectors, respectively, and in comparison to the pan-cellular promoters (Table II).

In specimens transduced with the LV.hCMV.GFP.W (Fig. 4A–C), the vast majority of the cells were GFAP negative, although single examples could be detected. The rNSE promoter did not express the transgene in any GFAP-positive cells (Fig. 4D–F); however, 98% of the transduced cells expressed the neuronal marker NeuN

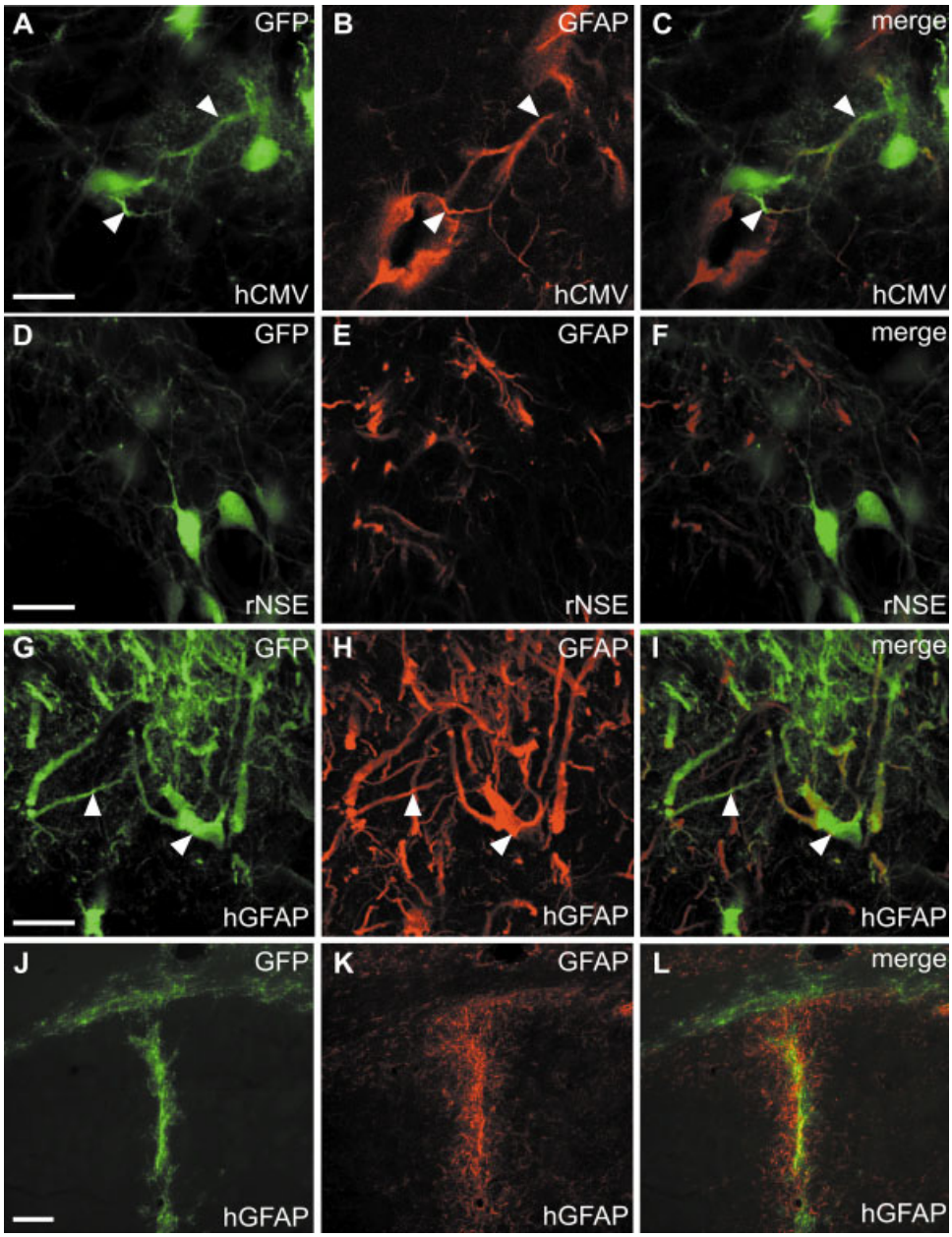

Fig. 4. Cell-specific transgene expression. **A–I:** Confocal analysis of GFP (green) and GFAP (red) expression. In brains injected with LV.hCMV.GFP.W (A–C) the vast majority of the cells were GFAP negative; however, single examples could be detected. The rNSE promoter did not express the transgene in any GFAP-positive cells (D–F). In animals receiving injections of LV.hGFAP.GFP.W, most GFP-expressing cells were GFAP positive (G–I). Arrowheads indicate double-labeled profiles. **J–L:** Photomicrographs at low magnification of striatum from a rat injected with LV.hGFAP.GFP.W. GFP expression was detected in the same area as the reactive astrocytes surrounding the needle tract, expressing GFAP. The total number of GFP expressing cells was similar to that detected using the other promoters, indicating that the VSV-G envelope is indeed equally efficient in transducing glial and neuronal cells. Scale bar = 20 μm (A–I); 200 μm (J–L).

(Table II). In the animals receiving injections of LV.hGFAP.GFP.W, most GFP-expressing cells (70%) were GFAP positive (Fig. 4G–I), showing that cell-specific targeted transgene expression can be achieved using VSV-G pseudotyped lentiviral vectors.

A morphological analysis underlined further the specificity of the two vectors. When using the rNSE-promoter, GFP expression was detected exclusively in cells with a typical neuronal morphology, and we were unable to find transduced cells in the cc (Fig. 5B). In the case of the hGFAP-promoter, the transduced cells displayed a morphology characteristic for astrocytes in the striatum and we found widespread transduction in white

TABLE II. Quantification of Double Labelling[†]

Promoter/Label (n = 6)	GFAP/GFP (%)	NSE/GFP (%)
rNSE	0	98.0 ± 2.6⋆
hGFAP	70.0 ± 8.4⋆⋆	0.6 ± 1.4⋆⋆
hCMV	8.6 ± 1.2	82 ± 6.3
CAG	11.8 ± 14	86.4 ± 14.2
EF1α	0	97.5 ± 1.7⋆

[†]Data is presented as mean ± SD.
⋆$P < 0.01$ (different form hCMV and CAG); ⋆⋆$P < 0.001$(different from all other groups); Fishers PLSD.

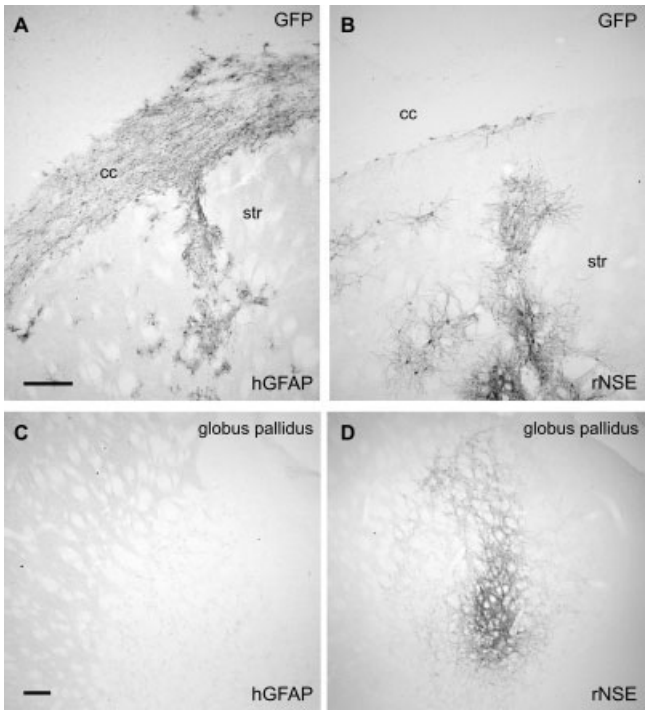

Fig. 5. GFP expression in animals injected with the rNSE- or hGFAP-vectors. **A,B:** Morphology and distribution of GFP-expressing cells in the striatum and the corpus callosum of animals injected with rNSE or hGFAP vectors. Note the absence of transduction in white matter when using the rNSE promoter. **C,D:** Anterograde transport of GFP was found in the globus pallidus of animals injected with rNSE vector but not in animals injected with hGFAP vector as indicated by GFP-labeled fibers. Cc, corpus callosum; str, striatum. Scale bar = 200 μm.

matter. The GFP expression co-localized to a large extent with native GFAP expression, which was upregulated around the needle tract (Figs. 4J–L and 5A). We found proof of anterograde GFP transport to the globus pallidus when using the rNSE promoter but not when using the hGFAP promoter (Fig. 5C,D).

DISCUSSION

We have used VSV-G pseudotyped lentiviral vectors carrying a variety of different promoters to drive transgenic expression in the rat brain. Several previous studies have reported a marked neurotropism when using this type of vector and this neuronal preference has been attributed largely to the VSV-G envelope (Blomer et al., 1997; Kordower et al., 1999). When carrying out careful examination of different promoters, however, we found that it is indeed possible to achieve a high level of transduction of glial cells and that the choice of promoter greatly influences the transduction pattern.

The three pan-cellular promoters that we used (hCMV, EF1α, and CAG) all transduced a high proportion of neurons. This is in line with previous reports where VSV-G pseudotyped lentiviral vectors were found to transduce mainly neurons when using ubiquitous promoters (hCMV and mPGK; Naldini et al., 1996; Blomer et al., 1997; Deglon et al., 2000). Based on these results, it has been assumed that the VSV-G envelope could be neurotropic (Mazarakis et al., 2001; Kang et al., 2002; Sanders, 2002). Pseudotyping of lentiviral vectors using other envelope proteins suggested that this was the case, because using the Ross River Virus glycoprotein changed the tropism toward a glial preference (Kang et al., 2002). Using the Mokola G protein resulted in a similar neurotropism as using VSV-G (Desmaris et al., 2001). The findings presented here, however, particularly the widespread transduction of glial cells when using the hGFAP promoter, suggest that promoter choice highly influences what cell type will be transduced. A possible explanation is that the ubiquitous promoters used in this study and elsewhere have a low activity in glial cells and therefore lead to preferential expression in neuronal cells.

Of the three cell-specific promoters we used, two of them (rNSE and hGFAP) led to many transduced cells and highly specific expression in regard to neurons and glial cells, respectively. The fact that only 70% of the GFP-positive cells were double labeled with GFAP when using the hGFAP promoter might be due to the low endogenous GFAP levels in the other 30% of the transduced cells, rather than expression in a non-GFAP-positive cell type (Stromberg et al., 1986). This was suggested further by morphological profiles presented by GFP-expressing cells, which indicated a glial phenotype. The mouse GFAP promoter used in this study consisted of a 256-base pair DNA sequence reported to be a minimal element for efficient and cell-specific expression (Miura et al., 1990). It may be that this small element lacked enhancer elements necessary to achieve detectable expression in the specific settings used here, a possible explanation for the very low efficacy of this vector.

In the brain, cell-specific transgene expression will be important because neighboring cells may have very different phenotypes. For example, a secreted transgene product, such as a trophic factor, could be delivered at many nuclei distant to the injection site if expressed by a neuron, due to anterograde protein transport within projecting axons of the cell. This would not be the case if the cell was glial, as demonstrated in this study where anterograde transport was found when using the hCMV, CAG, EF1α, and rNSE promoter but not when using the hGFAP promoter. This phenomenon has been shown recently using glial cell line-derived neurotrophic factor (GDNF) (Georgievska et al., 2002b) and has been suggested as one factor responsible for adverse effects detected after long-term, high-level GDNF delivery to the brain (Georgievska et al., 2002a). Glial-specific expression in that model of Parkinson's disease (6-hydroxydopamine) may circumvent some of these problems. Thus, using the cell-specific, highly effective vectors presented here may be part of developing a therapeutic gene transfer protocol for Parkinson's disease.

The fact that we used GFP as the transgene enabled a detailed morphological analysis; however, we carried out

no quantitative measurements of expression levels. It was clear that cells transduced with the CAG promoter were paler compared to the cells transduced using the other promoters. Furthermore, there were significantly higher numbers of transduced cells in the animals injected with LV.CAG. Taken together, this suggests that the CAG promoter was more effective. In vitro experiments in our lab (Jakobsson, unpublished observations) have shown that CNS-derived cell lines transduced using the CAG construct showed less variegation of transgenic expression over time compared to cells transduced using LV.hCMV or LV.EF1α. Resistance to variegation has been attributed to protection against downregulation, thus resulting in more effective transcription (Pikaart et al., 1998).

In vivo expression in rat brain using the hCMV promoter have been shown to be sustained for long periods, but the ratios of transduced cells and injected transducing units have been quite low (5–10%) (Blomer et al., 1997). This is in line with the transduction efficiency found in the present results. We were able to increase the efficiency, however, by diluting our vector preparation, although placing the injection at two sites did not have any effect on the number of transduced cells. One can envision that in concentrated viral suspension the virions were aggregated. After dilution of the saturated suspension, some virions were released from the aggregates resulting in increased transduction efficiency. The discrepancy between the number of injected units and number of transduced cells, however, might depend on multiple factors, including silent integrated proviruses or cells carrying multiple proviral copies, as well as a number of cellular and extracellular factors that may influence transduction (for example, Blomer et al., 1997). Interestingly, there was no difference in the number of GFP-positive cells between the LV.rNSE and the LV.hGFAP vectors. This finding suggests that there were non-transcriptional active proviral copies in glial cells or neurons in animals receiving the respective constructs.

In conclusion, the present study shows that VSV-G pseudotyped lentiviral vectors are excellent tools to achieve targeted transgene expression in neurons and glial cells in the brain. These vectors will most likely be highly valuable tools in regard to both gene therapy and experimental settings.

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