

Optimizing Polyethylenimine-Based Gene Transfer into Mammalian Brain for Analysis of Promoter Regulation and Protein Function

Barbara A. Demeneix, Mohamed Ghorbel, and Daniel Goula

1. Introduction

The efficient and safe introduction of genes into the central nervous system (CNS) is a difficult, yet much sought after objective. Two broad classes of aims can be distinguished. On the one hand, there is therapy in which the ultimate target will be the modification of an endogenous gene by homologous recombination or the remedial addition of a gene coding for a deficient protein. On the other hand, there are analytical approaches in which the aim may be either to determine the physiological relevance of a given protein by blocking or by bolstering its expression, or to dissect the regulatory mechanisms impinging on promoter function in an integrated setting. Furthermore, analysis of promoter regulation will be a prerequisite for creating constructs with optimized regulatory sequences for expressing therapeutic proteins in physiologically appropriate conditions. Microinjection of different permutations of a specific promoter into defined brain regions is a rapid and inexpensive method for assessing function and for mapping transcriptional regulatory elements. Indeed, using somatic gene transfer can provide results that otherwise could only be obtained by labor-intensive germinal transgenesis, an approach that also requires a great deal of organizational prowess and expense for maintenance of the numerous lines created.

Gene transfer into the CNS can be based on cell grafting or direct delivery. For direct delivery, a variety of viral or nonviral methods are available. In mammalian systems, reports have appeared that describe the use of adenoviruses, lentiviruses, herpesvirus, and adeno-associated virus derived vectors. In amphibians, vaccinia virus has also been applied. However, besides their

inherent safety problems in therapeutic settings, viral constructs are laborious to construct and verify. Moreover, their production in large quantities is often problematic. For these reasons, many groups have turned to synthetic, or nonviral, vectors to achieve gene transfer in the CNS. Two principal classes of synthetic vectors have been tested in the intact CNS: cationic lipids and cationic polymers. Here we describe the use of a cationic polymer, polyethylenimine (PEI). Indeed, of all the synthetic vectors so far tested in the mammalian CNS, low-molecular-weight PEIs (*see Note 1*) provide the most efficient gene delivery.

One of the most important features of PEI is its lack of toxicity *in vivo*. In the CNS the lesions inevitably created by microinjection into the brain tissue are no different following injection of carrier or injection of PEI/DNA complexes in carrier. This lack of toxicity with PEI is no doubt related to its high efficiency, which allows the use of very low amounts of DNA (in the nanogram range). The high efficiency of PEI is in turn related to its capacity for protonation (*1*). In PEI, one in three atoms is an amino nitrogen that can be protonated, which makes PEI the cationic polymer having the highest charge density potential available. Moreover, the overall protonation level of PEI increases from 20 to 45% between pH 7 and 5 (*2*).

PEI can be used for delivering plasmid DNA or oligonucleotides (*see Note 2*) to brains of adult and newborn mice (*3,4*) and rats (*5*). Moreover, it can be used in the CNS for intrathecal (*4,5*) or intraventricular delivery (*6*), the latter route being one that could be particularly useful for delivery of therapeutic proteins, such as nerve growth factors. Work ongoing in the laboratory is showing that the method can be used for up and down modulation of protein production in defined brains regions and for analysis of neuron-specific promoter regulation (*7,8; see Note 3*).

When starting a gene transfer protocol *in vivo*, it is always preferable, whether one's aim is promoter analysis or production of protein, to optimize delivery by examining the quantitative aspects of transgene expression in the region targeted with luciferase, and then the spatial aspects with a β -galactosidase (β -gal) construct. Indeed, the authors have found that the optimal ratio of PEI amine to DNA phosphate can vary according to species and brain region targeted (*see Note 4*). Such preliminary work will also enable one to test which promoter will perform best in a given cell population/developmental stage. For these reasons, the authors detail methods for extracting and assaying firefly luciferase (from *Photinus pyralis*) in the brain, this luciferase being the best reporter gene available for setting up gene transfer protocols. It is three orders of magnitude more sensitive than β -gal (*see Note 5*), and the fact that it can be quantified with precision is an overriding factor for choosing it for optimization of PEI:DNA ratios, amounts of DNA to be used, time-course evaluation, and promoter analysis. Other reporter genes, such as chloramphenicol acetyl

transferase (CAT) and β -gal can be quantified, but each has its drawbacks compared to luciferase. The chief problem with colorimetric quantification of β -gal expression in the CNS is excessive interference from endogenous enzymatic activity. Suppliers of kits for such methodology (such as Promega) recommend heat inactivation of endogenous enzymes. However, for the authors, such precautions have proven ineffective, and the extremely high background found throughout the mouse brain precludes precise quantification of expression of transgenes encoding β -gal. Similarly, when using histochemical procedures, it is vital to use appropriate fixation conditions to avoid interference from endogenous activity that can be high, particularly in the hippocampus. CAT is a good alternative for quantification, but, whichever method is chosen (usually enzyme-linked immunosorbent assay or the method of Seed and Sheen [9]), assay time is longer and the methodology laborious. Thus, the authors recommend starting off with luciferase, thereby determining, first, optimal PEI:DNA ratios, then kinetics and dose-response curves can be established (Fig. 1). Such experiments will also reveal the inherent variability of transfer efficiency in the target examined, and determine the need or not for normalization in experiments involving promoter regulation with luciferase.

However, β -gal remains one of the best markers for following spatial aspects of expression. Green fluorescent protein (GFP) is equally sensitive, but the fluorescent imaging, although esthetically pleasing, is not as satisfactory as standard light microscopy for anatomical detail. For this reason, the authors provide the methodology for β -gal revelation in whole brains. Indeed, histochemical analysis of β -gal expression on whole brains allows for rapid assessment of transfer efficiency and transgene distribution in small-sized samples (newborn mouse brains or regions of adult brains). The authors also provide a methodology for revealing β -gal expression on histological sections, a step that obviously permits more precise anatomical analysis, which is essential for determining brain regions and morphology of transfected neurons and glial cells. Furthermore, it is on such sections (prepared by vibratome or cryostat sectioning) that double labeling by immunocytochemistry can be performed to identify the cells expressing the transgenes. For instance, to identify neurons, one can use a neurofilament (NF) antibody or a Neuronal Nuclear Antigen (NeuN) antibody, and to identify astrocytes an antibody against glial acid fibrillary protein (GFAP) can be employed. Above all, one must remember that if one obtains just a few cells labeled with β -gal (a very insensitive method when dealing with transient expression *in vivo*), this level of efficiency will be more than sufficient to allow one either to proceed with promoter analysis using luciferase or to study the biological effects of a given gene of interest. Finally, the authors also suggest a very sensitive immunoradiographic method for measuring variation in expression of genes of interest at low levels (*see Note 5*).

Figure 1
Flow Chart for Optimizing PEI Transfection In Vivo

- Step 1** Preparation of endotoxin-free plasmid and verification of the plasmid quality.
- Step 2** Gel analysis of compaction with PEI.
- Step 3** Establishment of optimal PEI/DNA ratios by microinjection and/or stereotaxic delivery of a luciferase construct.
 Use volumes of 0.5–5 μL (according to site of injection, intrathecal or intraventricular).
 The authors suggest testing N:P ratios from 2 to 10. Express plasmid for 24 to 48 h in these initial tests.
- Step 4** Time course (1 d to 3 wk).
 Dose–response (DNA concentration) test concentrations from 100 to 500 ng/ μL .
- Step 5** Spatial distribution.
 Different possibilities exist for assessing spatial distribution:
 a. β -galactosidase histochemistry on whole brains or sections or immunocytochemistry.
 b. immunoradiography.
- Step 6** Expression gene of interest, if not used in **step 5**.
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Fig. 1. Flow chart for optimizing PEI-based gene delivery into the intact CNS. The authors recommend setting up this method with a luciferase reporter gene under a CMV promoter.

2. Materials

2.1. PEI and DNA Preparation

1. PEI: Branched 25 kDa PEI is available from Aldrich (St. Quentin Fallavier, France) in anhydrous form. Linear 22 kDa PEI is available from Euromedex (Souffleweysheim, France) at concentrations of 5 mM and 0.1 M. Both preparations should be stored at 4°C, having adjusted the pH to ≤ 4.0 .
2. Plasmids: For setting up in vivo gene transfer with PEI, one can use commercially available plasmids, e.g., pCMV-luciferase from Promega; pCMV(nls)-Lac-Z from Clontech (Montigny-le-Bretonneux, France). For CAT, the most efficient construct the authors have tested is pcis-CMV-CAT, provided by R. Debs (10).
3. Endotoxin-free plasmid DNA (see **Note 6**), prepared as in **Subheading 3.1., step 2**.
4. Appropriate restriction enzymes for verifying plasmids.
5. Agarose gels: 0.8% in 1X Tris Acetate EDTA (TAE) or Tris Borate EDTA (TBE) (11).
6. Spectrophotometer for measurements of DNA concentrations (OD_{260}) and purity of DNA ($\text{OD}_{260}/\text{OD}_{280} > 1.8$).

2.2. Condensation of DNA with PEI

1. Filtered (0.22 μm) 5% glucose solution.
2. Autoclaved 0.9% NaCl solution.
3. DNA resuspended in water at a final concentration of $<0.5 \mu\text{g}/\mu\text{L}$.
4. PEI solutions diluted extemporaneously to 0.1 M.
5. Sterile polypropylene tubes (1.5-mL).
6. Vortex.
7. Electrophoresis equipment for checking complexation (not required each time, but only for the first round of experiments).

2.4. Microinjections and Animal Care

1. Animals: adult and newborn OF-1 mice or Sprague-Dawley rats, both supplied by Iffa Credo (L' Arbresle, France).
2. Stereotaxic apparatus from David Kopf (Phymed, Paris, France). Stereotaxic coordinates for mice are determined according to Lehmann (*12*).
3. Micromanipulator (Narishige, supplied by OSI, Maurepas, France) and microcapillaries (ext diam 1 mm, OSI). Capillaries are pulled to ext diam of 10–15 μm .
4. Capillary puller (Narishige).
5. Hamilton syringe (10 μL) with a 21-gage needle (ext diam of 460 μm ; supplied by OSI).
6. Ice to anesthetize newborn mice (10 min on ice).
7. Sodium pentobarbital (Sanofi, France) diluted to 10% to anesthetize adult animals (70 mg/kg weight, ip).
8. Recovery chamber. Animals should be kept under an infra red lamp or a specially constructed heated cage until fully recovered from anesthesia and surgery.

2.5. Reporter Gene Revelation

2.5.1. Luciferase Activity in Brain Homogenates

1. Microdissection tools for dissecting out brain areas.
2. Ultra-Turrax (OSI) equipped with a small plunger to homogenize tissue samples directly in polyethylene tube.
3. Refrigerated benchtop centrifuge for Eppendorf tubes to pellet cell debris after homogenization, in order to recuperate supernatant for luciferase assay.
4. Luciferase assay kit from Promega. This system is based on the oxidation of luciferin by luciferase, in the presence of ATP and O_2 , with photon production.
5. Luminometer (model ILA-911 from Tropix, Bedford, MA) to quantify light emitted.

2.5.2. β -Galactosidase Revelation

2.5.2.1. DETECTION WITH X-GAL SUBSTRATE

1. X-gal (Eurogentec, Seraing, Belgium) sold in powder form. Both the powder and stock solution (40 mg/mL), prepared in dimethyl sulfoxide (DMSO) must be kept at -20°C .

2. Phosphate-buffered saline (PBS) 0.1 *M*.
3. EGTA.
4. Paraformaldehyde (PFA, Sigma, St. Quentin Fallavier, France). Prepare a stock solution of 20% in PBS, and store at -20°C .
5. MgCl_2 , 1 *M*.
6. Tween-20 (Sigma).
7. Heparin (10 U/L in 0.9% NaCl) for perfusion.
8. Peristaltic pump (Polylabo, Strasbourg, France) for perfusion of animals and fixation of tissues by perfusion.
9. Vibratome (Leica, Rueil-Malmaison, France) to section the tissues (20–40 μm thickness).
10. DMSO (Sigma) to make stock solutions of X-gal.
11. Small paintbrush to transfer tissue sections from one solution to another. Obtain from any art equipment supplier.
12. 25-mL sterile plastic vials (size no. 2) to collect sections
13. Potassium ferricyanide and potassium ferrocyanide (Sigma): Prepare 0.2 *M* stock solutions of each.
14. Alcohol series for dehydration (baths: 70, 95, 100% of ethanol).
15. Xylene, benzyl benzoate, and benzyl alcohol (all from Sigma) for delipidation of whole newborn brains or small blocks of tissue from adult brains.
16. Appropriate sized cover slips and glycerol (glycerol/PBS, 1V/3V) for mounting slides.
17. Plastic gloves. Benzyl benzoate and benzyl alcohol are irritants.
18. Glassware for delipidation solutions.

2.5.2.2. β -GALACTOSIDASE DETECTION USING F-DG (FLUORESC EIN-DI- β -GALACTOSIDE) AS SUBSTRATE

1. FDG (Molecular Probes, Leiden, The Netherlands): fluorescent substrate for β -gal.
2. PBS, 0.1 *M*.
3. DMSO (Sigma).
4. Small paint brush (*see Subheading 2.5.2.1.*).
5. Vibratome (Leica) for the same purpose as stated in **Subheading 2.5.2.1.**
6. Epifluorescence microscope equipped with activation/emission filters for fluorescein.

2.5.3. Double Immunocytochemical Revelation of β -Galactosidase and Neuron or glial Markers on Floating Sections

1. PBS, PBS–gelatin (0.2%).
2. Triton-X100 (Sigma).
3. Vibratome sections (40 μm).
4. Cromallun (Sigma).
5. Gelatinized slides prepared by dipping in a cromallun–gelatin solution (0.05/0.5% in distilled water) and dried overnight at 37°C .
6. Blocking solution: PBS–gelatin (0.2%).

7. Primary antibodies against β -gal and cell-specific markers (NF, NeuN, and GFAP) (Cappel, Organon Tetrika, Westchester, PA; Sigma; Dako, Glostrup, Denmark, respectively). To label neurons, NF can be replaced by NeuN antibody from Chemicon, Temecula, CA).
8. Coupling dye for labeling primary cell-marker antibodies (cy3.5; Fluorolink-AbTM). Labeling of the antibodies is carried out according to the manufacturer's instructions (Amersham, Les Ulis, France). The authors label the cell-specific antibodies (NF, NeuN, or GFAP), and use an antirabbit antibody coupled to fluorescein to reveal the β -gal immunoreactivity.
9. Mounting media: Vectashield (Biosys, Compiègne, France), or glycerol/PBS (1V/3V).
10. Fluorescent microscope (Olympus) equipped for visualizing fluorescein and Texas red emissions.
11. Small paintbrushes (*see Subheading 2.5.2.1.*)

2.5.4. CAT Assay

1. [¹⁴C]chloramphenicol (Amersham CFA, 57 mCi/mmol). Aliquots stored at -20°C .
2. Butyryl-CoA 10 mM (Sigma B1508, 100 mg). Aliquots stored at -80°C .
3. Tris-HCl buffer, 250 mM, pH 7.5.
4. 2,6,10,14-tetramethylpentadecane/xylene (TMPD, 2:1; Aldrich).
5. Miniature polybrene vials for scintillation counter (Packard, Meriden, CT).

2.6. Immunoradiography

1. Appropriate primary polyclonal antibody raised in rabbit against gene of interest.
2. Cryostat (Leica)
3. Cryostat sections (15–20- μm thick) cut at -20°C .
4. Cromallun–gelatin coated slides (*see Subheading 2.5.3.*).
5. Desiccator.
6. PFA (as in **Subheading 2.5.2.**).
7. PBS.
8. Bovine serum albumin (BSA; Sigma).
9. Normal goat serum (Sigma).
10. Donkey antirabbit [³⁵S]IgG (200–2000 Ci/mmol, 100 $\mu\text{Ci}/\text{mL}$). Amersham, Buckinghamshire, UK.
11. β -max films (Amersham).
12. Computerized image analysis system (Biocom, Les Ulis, France).

3. Methods

3.1. PEI and DNA Preparation

1. The stock solution (0.1 M) of PEI provided by Euromedex (22 kDa) is used as supplied. The 25 kDa obtained from Aldrich is prepared as follows: Weigh 4.5 mg of the solution, mix with 800 μL sterile water, thus obtaining a 0.1 M solution. Adjust the pH to ≤ 4.0 with 0.1 N HCl and the volume to 1 mL. Solutions are kept as aliquots at 4°C or at -20°C for long-term storage (>1 mo).

2. For plasmid DNA preparation and purification, the authors recommend use of Jetstar columns (Genomed, Research Triangle Park, NC, or Bad Oeynhausen, Germany). The system is based on anion exchange columns. According to the manufacturer's instructions and solutions supplied, bacteria resulting from maxiculture are lysed by alkali. Large membrane debris is eliminated using potassium acetate and centrifugation. The resulting supernatant is loaded on columns, and DNA is eluted with a solution containing $\sim 2 M$ NaCl, then centrifuged after isopropanol precipitation. The pellet is washed with 70% ethanol (-20°C), and is resuspended in water or (TE) at high DNA concentration ($\leq 5 \mu\text{g}/\mu\text{L}$ in TE). This is to ensure that when diluting DNA to its working concentration ($\leq 0.5 \mu\text{g}/\mu\text{L}$ in 5% glucose) the final TE concentration is not greater than 1 mM Tris/0.1 mM EDTA (Standard TE/10). These plasmid preparations are endotoxin free (*see Note 6*).
3. One microliter of each DNA preparation is diluted in 1 mL sterile water, and analyzed at 260 nm and 280 nm. According to Sambrook (**10**), 1 U DO_{260} corresponds to 50 $\mu\text{g}/\text{mL}$ double-strand DNA.
4. Agarose gel electrophoresis is used to verify that the plasmid DNA is not denatured, is free of RNA, and is mostly supercoiled. Restriction map analysis can be used to check constructions at this point. To this end, native and digested plasmids are analyzed using 0.8% agarose gel electrophoresis in TAE, with bromophenol blue and a DNA mol wt marker (**11**). The gel is observed on a UV transilluminator (312 nm), and photographed with Sony video equipment from OSI (Maurepas, France).

3.2. Condensation of DNA with PEI and Analysis

1. Plasmid DNA is diluted in sterile (0.22 μm filtered) 5% glucose to the chosen concentration (usually 0.5–2 $\mu\text{g}/\mu\text{L}$). After vortexing, the appropriate amount of a 0.1 M PEI solution is added, and the solution revortexed. The required amount of PEI, according to DNA concentration and number of equivalents needed, is calculated by taking into account that 1 μg DNA is 3 nmol phosphate and that 1 μL 0.1 M PEI is 100 nmol amine nitrogen. Therefore, to complex 10 μg DNA (30 nmol phosphate) with an amine/phosphate (N:P) (*see Note 4*) ratio of 5 eq PEI, one needs 150 nmol PEI (1.5 μL of a 0.1 M solution). To minimize pipeting errors, it is best to dilute PEI to 50 or 10 mM, but this should be done extemporaneously. Dilute DNA to 10 μg (final concentration 0.5 $\mu\text{g}/\mu\text{L}$) in 20 μL final 5% glucose. Add the necessary volume of PEI to form the desired N:P ratio, and, water qsp 20 μL .
2. When using a plasmid preparation for the first time, the authors recommend analysis of complexes by agarose gel electrophoresis (**Fig. 2**), and comparing their migration to that of naked DNA (plasmid alone, N:P ratio = 0) after adding 1–2 μL of bromophenol blue. This gives a gel retardation profile in which DNA–PEI complexes formed at N:P < 1 migrate similarly to naked DNA. At N:P > 6, complexes are so positively charged that they migrate to the negative pole (*see Note 4*).

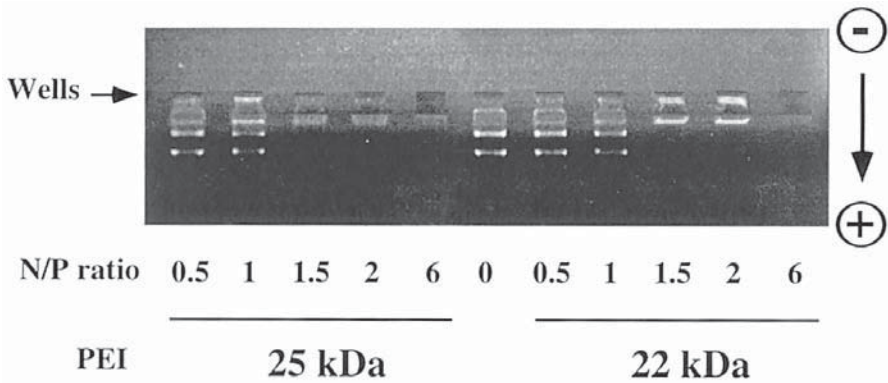


Fig. 2. Verification of DNA compaction by linear 22 kDa and branched 25 kDa PEI. A pCMV-luc construct (1 μ g) was mixed with PEI at various charge ratios, and DNA-PEI complexes were electrophoresed in 0.8% agarose gel stained with ethidium bromide. The position of wells and the direction of the electrophoretic migration are indicated on the right.

3.3. Injections

1. Obviously, all the procedures described herein that involve animals and their care must be conducted in conformity with appropriate institutional guidelines, that are in accordance with national and international laws and policies.
2. Adult (1–2-mo-old) mice are anesthetized using sodium pentobarbital (Sanofi, France) diluted to 10% in 0.9% NaCl. Animals are anesthetized by an ip injection (70 mg/kg). Adult mice or rats are placed in the stereotaxic apparatus. An incision is made to expose the cranial skull and a hole is made with a 21-gage needle at chosen stereotaxic coordinates. Between 0.5 and 5 μ L of complexes in 5% glucose are injected slowly (<5 min) with a Hamilton syringe adapted to a stereotaxic apparatus, small volumes are used for intrathecal injection and larger volumes for intraventricular injection. The needle is left in place for 2 min postinjection, to limit backflow from the injection site.
3. Newborn mice are anesthetized by hypothermia on ice, and 1 μ L of the complexes is injected with a microcapillary adapted to a micromanipulator. The head of the anesthetized pup is held manually for direct microinjection. Again, injections should be as slow as possible, and the capillary is left in place for at least a minute to limit backflow.
4. Animals are left in a recovery chamber until active. When optimizing PEI delivery, newborns are returned to the dam for 24 h before sacrifice; adults are kept for 72 h before sacrifice, because expression is usually maximal at these time points.

3.4. Sacrifice of Animals and Reporter Gene Detection

To quantify luciferase expression, animals are sacrificed by decapitation after anesthesia (Fig. 3). The dissected brains are separated into hemispheres, and

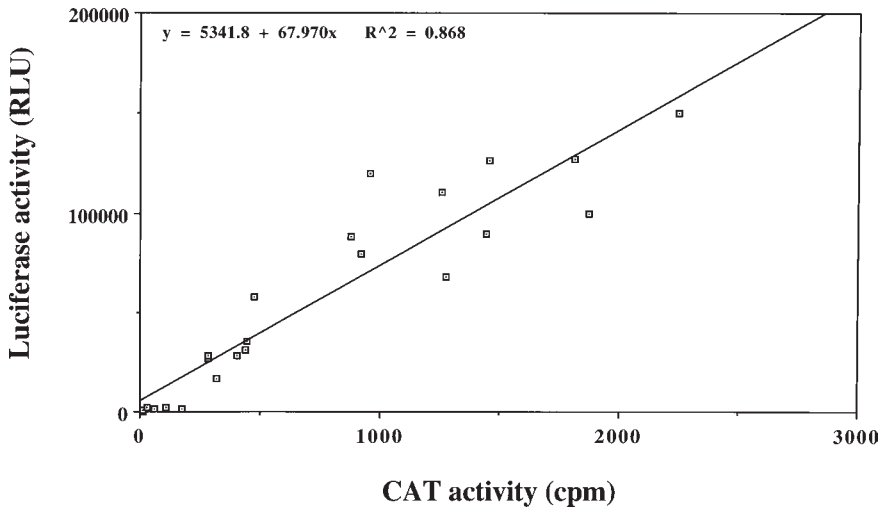


Fig. 3. Correlation between luciferase and CAT activities following PEI-based *in vivo* gene transfer in the newborn mouse brain. Plasmids (0.35 μg pCMV-luc and 0.15 μg pCMV-CAT in 2 μL 5% glucose) were complexed with PEI 22 kDa (4 Eq), and injected into the lateral ventricle. After 18 h, mice were anesthetized, decapitated, and brains removed for luciferase and CAT assays.

homogenized using an Ultra-Turrax, in 2-mL Eppendorf tubes containing 200 μL (for the newborn) or 500 μL (for the adults) of luciferase lysis buffer. Homogenates are centrifuged, and 20 μL of each supernatant mixed with 100 μL luciferase assay substrate and vortexed. The light emitted is quantified (in Relative Light Units [RLU]), using a luminometer.

β -galactosidase can be revealed by several means:

1. Histochemical X-gal revelation, whether on sections or carried out *in toto*, requires intracardial perfusion of the anesthetized animals, using a peristaltic pump. First, tissues are fixed by perfusing 2% PFA (in PBS), then fixation is continued by leaving tissue blocks overnight in the same solution. Tissues are then vibratome-sectioned, and sections incubated in a 0.8–1 mg/mL X-gal solution for 2–4 h (30°C). It is important to precede the fixation by perfusion with saline containing 10 U of heparine, to help remove blood and blood cells from the vessels. The fixative (2% PFA) can contain 1.25 mM EGTA and 2 mM MgCl_2 , which improves the X-gal reaction. To make the reaction mixture, the stock solution of X-gal (40 mg/mL in DMSO) is diluted to 0.8–1 mg/mL PBS containing: 0.1% Tween-20, 4 mM potassium ferricyanide, 4 mM potassium ferrocyanide, and 2 mM MgCl_2 .
2. Immunocytochemical revelation also requires fixed vibratome sections, mounted on cromallun–gelatin-coated slides. Polyclonal anti- β -gal monoclonal anti-GFAP

and monoclonal anti-NeuN antibodies are used in the authors' experiments. Monoclonal and polyclonal antibodies can be labeled using appropriate labeling kits from Amersham. The authors used cy3.5 (Fluorolinf-ab™), according to the manufacturer's instructions, to label monoclonal antibodies. Primary antibodies are diluted to the concentrations recommended by each manufacturer, in 0.1 M PBS (containing 0.2% gelatin, 0.3% Triton X-100, and 3% normal goat serum). Anti β -gal is revealed using fluorescein coupled antirabbit antibody. Sections are protected from light to avoid fading of the fluorescence, and are mounted with glycerol/PBS (1V/3V) or Vectashield (Biosys) and examined under a fluorescence microscope. Luciferase antibodies can also be used to follow transgene expression in a double-labeling protocol. However, there are currently some problems with obtaining good luciferase antibodies for in vivo work (see **Note 7**).

3. Fluorescein-DI- β -Galactoside (FDG) is another substrate for β -gal. Hydrolysis of FDG by β -gal results in the liberation of both a monogalactoside and fluorescein. This second product is easily detectable, and theoretically makes this method very sensitive. Its chief limitation for the authors is that it is not suitable for fixed tissues, so that it is difficult to obtain good morphology in brain preparations. Moreover, on unfixed tissue, as cells die, the fluorescein product diffuses out. Thus, the revelation procedure must be very fast. Also, it is not possible to perform double staining to identify cell types. This method, which has the theoretical advantage of higher sensitivity than X-gal, is in fact rather limited for in vivo studies.
4. *In toto* X-gal revelation requires intracardial perfusion of the anesthetized animals, using a peristaltic pump. Fixation and postfixation are performed as for vibratome sections, but organs are treated as whole mounts. Incubate tissues in a 0.8–1 mg/mL X-gal solution for 2–4 h (30°C), rinse in 0.1 M PBS (2 \times 5'), and transfer in series of ethyl alcohol under mild agitation: 70% (2 \times 2 h), 95% (overnight, and another bath of 1 h), 100% (2 \times 2 h). Dehydration times can be adapted, depending on the tissue size, to ensure thorough dehydration, one can leave the tissue in the second 100% alcohol bath overnight. Put dehydrated tissues into xylene (2 \times 2 h in glass), then transfer them into benzyl benzoate/benzyl alcohol (2V/1V) in a glass container until clarification.

N.B. Use gloves and glass containers at all steps involving benzyl benzoate and benzyl alcohol. These agents are irritants and also dissolve plastic.

3.5. Use of CAT Activity to Normalize Luciferase Expression

1. Before using a co-injected, ubiquitously expressed gene (*CMV-CAT*) to normalize for expression from a physiologically regulated transgene, it is appropriate to validate this approach by quantifying the correlation between the expression of two constitutively expressed genes co-injected into the same brain area. For this, two plasmids (0.35 μ g pCMV-luc and 0.15 μ g pCMV-CAT in 2 μ L 5% glucose) are complexed with PEI 22 kDa (4 eq), and co-injected into the brain area targeted. After 18 h, mice are anesthetized, decapitated, and brains removed for luciferase and CAT assays.

2. For CAT assay, transfer a 50- μ L supernatant aliquot to a 1.5-mL polypropylene tube, keep tube on ice before adding 40 μ L 0.25 M Tris-HCl buffer, pH 7.5. Start reaction by adding 10 μ L mixed solution of butyryl-CoA (0.53 mM; Sigma) and [14 C]chloramphenicol (0.01 mM, 1.85 kBq per tube; Amersham). After 3–5 s of vortexing, incubate 1 h at 37°C. Stop the reaction by adding 200 μ L TMPD:xylene solution (2:1). Vortex 20 s and place tube on ice. To separate products, centrifuge 5 min at 4°C (11,000g), remove 150 μ L supernatant, and quantify products in a scintillation counter (Wallac, Evry, France).
3. Assay luciferase on another sample of supernatant (*see Subheading 3.4.1.*).
4. Plot luciferase against CAT values from the same sample. Correlation should be significant.

3.6. Immunoradiography

This protocol is adapted from **ref. 13**.

1. The cryostat sections are desiccated at 4°C, and frozen at –80°C until used. After a 3-min fixation (4% PAF in PBS) at 4°C, sections are preincubated for 1 h in PBS supplemented with 3% BSA and 1% goat serum, then incubated overnight in an appropriate concentration of polyclonal primary antibody raised in rabbit.
2. After extensive washes in PBS, sections are incubated for 2 h at room temperature in donkey antirabbit [35 S] IgG.
3. After abundant washing, sections are air dried and apposed to β -max film for 1–2 d.
4. Optical density is measured by computerized image analysis.

4. Notes

1. A number of PEIs with different mean mol wt are available commercially. For example, preparations of branched PEI, synthesized to different degrees of polymerization, are available from Sigma or Aldrich (800, 50, or 25 kDa). Preparations of very low mol wt (0.7 and 2 kDa) are also available from Sigma, but do not complex DNA efficiently. The authors have found that the branched 25 kDa and linear 22 kDa (Euromedex) polymers work best in the CNS (**1,6**).
2. When using either the 22 and 25 kDa preparations to deliver oligonucleotides, one should only use phosphodiesteres, and not phosphorothioates. The authors have found (as has E. Saison Behmoaras, CNRS/MNHN, Paris) that complexes of PEI and phosphorothioates are of lower efficiency than PEI–phosphodiesteres complexes *in vitro* and *in vivo*.
3. A major area of discussion in the field at the moment is the physiological regulation and relevance of transient transfection which relies on following transcription from episomally located plasmids. However, the authors have found that, despite lack of integration into the genome, remarkably tight, physiologically appropriate regulation of cell-specific promoters can be obtained *in vivo*. One illustrative example is thyrotropin releasing hormone (TRH) promoter regulation in the hypothalamus. The authors have microinjected complexes containing 1 μ g of construct containing 900 bp of the rat TRH promoter upstream of the luciferase-

coding sequence, complexed with 22 kDa PEI into the hypothalamus of newborn mice in different thyroid states. In hypothyroid animals, transcription is twice that in normal, euthyroid animals, and, in hyperthyroid animals, transcription is reduced to half that of the normal group (7). This transcriptional regulation faithfully reflects the negative feedback effect of circulating thyroid hormone on hypothalamic TRH production. Another example of physiological regulation of transgenes introduced by somatic gene transfer includes Krox-24 gene in the newborn brain (8).

4. The authors have found that the optimal ratio of PEI amine:DNA phosphate (N:P ratio) can vary according to species (and perhaps also to site of injection). In mouse and *Xenopus* tadpole brains, the authors have consistently found that the N:P ratio of 6 provides the best transfection conditions. In contrast, when injecting PEI–DNA complexes into adult rat substantia nigra, the optimal ratio was 3 (5). It is important to note that increasing N:P ratio from 2 to 6 not only increases the overall charge of complexes, but also decreases complex size, complexes excluding ethidium bromide (BET) and becoming less visible in the gel. This greater condensation has been confirmed by ζ -sizing (6).
5. When assessing efficiency of gene transfer, one must take into account the sensitivity of the method used. Histochemical β -gal assay with X-gal is rather insensitive. The authors find that it gives roughly the same image of transgene distribution and number of cells labeled as an equivalent amount of GFP plasmid. It has been estimated that revealing a good GFP signal requires 10^5 – 10^6 molecules per cell to show up over background (14). For this reason, the authors have also tried immunocytochemistry with a polyclonal antibody against β -gal (Cappel), but this method is generally no more sensitive than histochemistry. However, it is important to note that one can obtain very good, and statistically significant, modulation of endogenous proteins with PEI-based gene transfer in systems in which transferring an equivalent amount of β -gal reveals no apparent transgene expression. This was the case for a recent series of experiments in which the authors transfected plasmids expressing either sense or antisense sequences of the dopamine transporter (DAT) into the rat substantia nigra (11). In conditions in which no β -gal activity could be revealed (0.5 μ g CMV- β -gal in 1 μ L 5% glucose), use of plasmid encoding DAT significantly increased DAT content. This was shown by immunoautoradiography and by biological measurements of dopamine uptake (11). For this reason, the authors recommend immunoautoradiography for following low levels of expression of genes of interest against which good antibodies have been raised.
6. Even though the authors own (unpublished) results show that the presence of small amounts (≤ 1 EU endotoxin/ μ g DNA) of endotoxins (also referred to as lipopolysaccharides [LPS]) have no deleterious effects on short-term (<4 d) expression in the CNS, there is no available data on the possible effects of their presence in the longer term. For this reason, the authors always use endotoxin-free plasmid preparations. Jetstar columns (Genomed) are recommended. The resulting DNA has ≤ 0.1 EU endotoxins/ μ g DNA. This value is not statistically

different from the values found in plasmids prepared with Qiagen Endotoxin-free columns.

If other methods of plasmid preparation are used, the authors recommend measurement of endotoxin content by Limulus Amebocyte Lysate Assay (LAL, or Coatest™ Endotoxin) manufactured by Charles River Endosafe, Charleston, SC, and distributed in Europe by Chromogenix Mölndal, Sweden. If values are ≥ 4 EU/ μ g DNA, then endotoxins should be removed by chromatography through Affi-prep™ Polymyxin Matrix (Bio-Rad, Hercules, CA), according to the manufacturer's instructions and endotoxin content reverified. After chromatography, values should be ≤ 0.05 EU/ μ g DNA.

7. To the authors' knowledge, the only reasonable luciferase antibody currently available is that from Cortex Biochem (San Leandro, CA, distributed by Europa Biochemistry in Europe). Variable results have been obtained with this polyclonal antibody, according to batch number. Promega commercialized a polyclonal antireflectively luciferase up until the end of 1997, but it has been withdrawn from circulation because of titer problems. However, the authors have obtained reasonable results with some batch numbers. Santa Cruz Biotechnology (Santa Cruz, CA) also produces a luciferase antibody, but the authors have not yet tested this antibody in vivo.

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