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Award Number: DAMD17-01-1-0773

TITLE: Non-invasive Gene Therapy of Experimental Parkinson's Disease

PRINCIPAL INVESTIGATOR: William M. Pardridge, M.D.

CONTRACTING ORGANIZATION: The University of California,
Los Angeles
Los Angeles, CA 90024-1406

REPORT DATE: September 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2003	Annual (Aug 27, 2002 - Aug 26, 2003)

4. TITLE AND SUBTITLE
Non-invasive Gene Therapy of Experimental Parkinson's Disease

5. FUNDING NUMBERS
DAMD17-01-1-0773

6. AUTHOR(S)
William M. Pardridge, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of California, Los Angeles
Los Angeles, CA 90024-1406

E-Mail: Wpardridgemednet.ucla.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The present research has developed a non-viral gene targeting technology, whereby the effects of a neurotoxin on the brain can be reversed shortly after the intravenous injection of a therapeutic gene medicine without the use of viral vectors. The brain gene targeting technology developed in this work creates an "artificial virus" which is comprised of non-immunogenic lipids and proteins, wherein the therapeutic gene is packaged in the interior of the gene delivery vehicle, which is called a pegylated immunoliposome (PIL). The PIL carrying the gene is a 85 nm "stealth" nanocontainer, which is relatively invisible to the body's reticuloendothelial system, which normally removes nanocontainers from the blood. The surface of the nanocontainer is studded with a receptor-specific monoclonal antibody (MAb). This MAb acts as a moleculr Trojan horse, and triggers the transport of the stealth nanocontainer across the 2 biologicial membrane barriers that separate the blood from the interior of brain cells. These barriers are the brain microvascular endothelial wall, which forms the blood-brain barrier in vivo, and the brain cell plasma membrane. Both barriers express the transferrin receptor, and the anti-receptor MAb enables the PIL to cross the membrane barriers via normal physiological transport processes usually used for endogeneous ligands such as transferrin. With this approach non-viral, non-invasive gene therapy of the brain is now possible.

14. SUBJECT TERMS
Neurotoxin, brain gene therapy, non-viral gene transfer

15. NUMBER OF PAGES
68

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

Neurotoxins can cause serious derangements in brain biochemistry that can compromise the cognitive and motor function of the individual. In the present studies an animal model of neurotoxin exposure is used, wherein the neurotoxin, 6-hyrdoxydopamine, is injected into a specific region of the rat brain called the medial forebrain bundle, followed approximately 4 weeks later by a biochemical picture resembling Parkinson's disease (PD). On the side of the brain where the neurotoxin is injected, there is a 90% reduction in the level of a key enzyme, tyrosine hydroxylase (TH), which is a rate-limiting enzyme involved in dopamine production. Dopamine is the neurotransmitter that is deficient in PD. One way that brain TH levels can be restored in conditions such as PD is through gene therapy, wherein the TH gene is given to the individual afflicted with PD. However, with the conventional approach to gene therapy of the brain, there are two serious problems. First, virtually all present-day approaches use viral vectors to carry the gene to brain cells. However, these viral vectors are either highly inflammatory (such as adenovirus or herpes simplex virus) or stably alter the host genome in a random way (retrovirus, adeno-associated virus), which can lead to insertional mutagenesis and cancer. These viruses do not enter the brain from blood, because they do not cross the blood-brain barrier (BBB). This creates the second problem with present-day approaches to gene therapy, which is the viral vector is administered to the brain by craniotomy and drilling a hole in the head. However, this only distributes the virus to a tiny region of the brain at the tip of the injection needle. What is needed is a non-invasive, non-viral form of brain gene therapy wherein the therapeutic gene can be administered intravenously without viral vectors followed by widespread expression of the exogenous gene throughout the brain. This is the goal of the present research.

The present research uses a completely new form of brain gene targeting technology which uses a novel gene delivery vehicle called pegylated immunoliposomes (PIL). PILs are comprised of non-immunogenic lipids and proteins, wherein the therapeutic gene is packaged within the interior of the gene delivery vehicle, which is called a pegylated immunoliposome (PIL). The PIL carrying the gene is an 85 nm "stealth" nano-container, which is relatively invisible to the body's reticuloendothelial system, that normally removes nano-containers from the blood. This stealth effect is created by conjugating approximately 2000 strands of 2000 Dalton polyethylene glycol (PEG) to the surface of the liposome carrying the gene inside. Approximately 1-2% of the tips of the PEG strands are studded with receptor-specific monoclonal antibodies (MAb). This MAb is a targeting ligand and acts as a molecular Trojan horse, which triggers the transport of the stealth nano-container across the two biological membrane barriers which separate the blood from the interior of brain cells: the brain microvascular endothelial wall, which forms the blood-brain barrier (BBB) in vivo, and the brain cell plasma membrane (BCM). Both the BBB and the BCM express a targeted receptor, in this case, the transferrin receptor (TfR), and the anti-TfR MAb enables the PIL to cross the membrane barriers via normal physiological transport processes which are usually used for endogenous ligands such as transferrin. With this approach, non-viral gene therapy, non-invasive gene therapy of the brain is now possible.

The TH expression plasmid is encapsulated in the interior of the 85 nm PIL which is targeted to rat brain with the OX26 murine MAb to the rat TfR. The TfRMAb-PIL carrying the plasmid DNA is injected intravenously in rats at a dose of 1-10 μg plasmid DNA per adult rat. These rats all have drug-confirmed experimental PD, owing to the intracerebral injection of the

6-hydroxydopamine neurotoxin into the brain four weeks earlier. The goal is to normalize the striatal TH activity based on both brain biochemistry assays, immunocytochemistry assays, and pharmacologic behavioral testing.

BODY

Original Statement of Work. The original statement of work outlined experiments in 5 areas:
(1) formulation of PILs (01 year)
(2) single dose efficacy study with SV40 promoter (01-02 years)
(3) toxicity study with focus on brain inflammatory response (02-03 years)
(4) single dose efficacy study with glial fibrillary acidic protein (GFAP) gene promoter (02-03 years)
(5) multi-dose efficacy study (03 year)

Progress toward these goals has been achieved on time. In the Progress Report a year ago, we submitted as an Appendix our manuscript, which was subsequently published in Human Gene Therapy (January, 2003), which is article 6 below in Publications. This work showed we completed all formulation goals and completed the single dose efficacy study with the SV40 promoter.

Progress in 02 Year. In the past 02 year, we completed the toxicity study following 6 weeks of multi-dosing (weekly dosing for 6 weeks), and this work is described in the attached Appendix 1, which is article 8 below in Publications. The following parameters were measured in our chronic dosing toxicity study:
Body weights
Organ histology
Serum chemistries (14 different assays)
Brain immunocytochemistry (ICC)

For the ICC study, we used antibodies to the following inflammatory antigenes: OX1 (leukocytes), OX6 (class II MHC), OX18 (class I MHC), OX35 (CD4 lymphocytes), OX42 (macrophages), and GFAP (astrocytes). As described in Appendix 1, there was no measureable toxicity with chronic intravenous administration of the PILs carrying the TH expression plasmid. We used Southern blotting to show that the TH gene was delivered to the brain (Appendix 1).

During the 02 year we also completed the construction of the TH expression plasmid under the influence of the brain specific promoter taken from the 5'-flanking sequence of the human GFAP gene. This work is described in Appendix 2, which is attached as a new manuscript that is presently submitted for publication. This manuscript shows the TH gene driven by the GFAP promoter completely normalizes striatal TH activity. The advantage of this form of the gene, as compared to the SV40 promoter gene, is that there is no ectopic TH gene expression in non-brain organs. Using the widely read SV40 promoter, we can normalize striatal TH with the PIL gene delivery technology, but we also increase TH gene expression in the liver 10-fold, as reported last year. Now, with the GFAP-TH gene, we see TH gene expression only in the target organ and not in peripheral tissues (Appendix 2).

KEY RESEARCH ACCOMPLISHMENTS

The following manuscripts were produced in the most recent year:

- Zhang, Y., Schlachetzki, F., and Pardridge, W.M. (2003): Global non-viral gene transfer to the primate brain following intravenous administration. Mol. Ther., 7: 11-18. **(Highlighted in 'Inside This Month' of *Molecular Therapy*; reviewed in March 22, 2003, *New Scientist;* reviewed in June, 2003 *Drug Discovery Today*, 8:473-474.)** This work is important because we now show that the PIL gene delivery technology that is so successful in rats is also working in primates, such as the Rhesus monkey. Moreover, the level of gene expression in the primate brain is 50-fold higher than gene expression in the rat brain.

- Zhang, Y., Boado, R.J., and Pardridge, W.M. (2003): Absence of toxicity of chronic weekly intravenous gene therapy with pegylated immunoliposomes. Pharm. Res., in press. **(INCLUDED AS APPENDIX 1)** This work is important because it shows that the PILs carrying a therapeutic gene can be chronically administered without any toxic effects.

- Zhang, Y., Schlachetzki, F., Li, J.Y., Boado, R.J., and Pardridge, W.M. (2003): Organ-specific gene expression in the Rhesus monkey eye following intravenous non-viral gene transfer. Submitted for publication. This work is important because it shows that tissue specific gene expression in the primate can be achieved with the use of tissue specific promoters coupled with the PIL gene targeting technology.

- Zhang, Y., Schlachetzki, F., Zhang, Y., Boado, R.J., and Pardridge, W.M. (2003): Normalization of striatal tyrosine hydroxylase and reversal of motor impairment in experimental Parkinsonism with intravenous non-viral gene therapy and a brain –specific promoter. Submitted for publication. **(INCLUDED AS APPENDIX 2)** This work is important because it shows that ectopic expression of the TH gene in non-brain organs is eliminated with the replacement of the widely read SV40 promoter with the brain-specific gene promoter taken from the 5'-flanking sequence of the human GFAP gene.

REPORTED OUTCOMES

(1) Manuscripts: listed below in Publications.

(2) Plasmids developed: tyrosine hydroxylase expression plasmids driven by either the SV40 or the GFAP promoter were produced as described in Appendix 2.

CONCLUSIONS

The US Army support of this work has led to the development of a transformational technology that revolutionizes our approach to gene therapy of the brain and to gene therapy of brain neurotoxin exposure. Unlike the conventional approach to brain gene therapy, we achieve the desired pharmacological effect without viruses and without craniotomy. The use of viral

vectors will probably never be widely used in gene therapy in humans, owing to their toxic effects. The use of craniotomy for delivering genes to the brain is problematic because the gene is only delivered to a tiny area of the brain. Moreover, craniotomy-based gene therapy of the brain in soldiers in the field is virtually impossible. We have created a form of brain gene therapy that could be administered to soldiers in the field.

This approach to gene therapy enables adult transgenics in 24 hours.

PUBLICATIONS FUNDED BY THIS WORK TO DATE:

1. Shi, N., Zhang, Y., Boado, R.J., Zhu, C., and Pardridge, W.M. (2001): Brain-specific expression of an exogenous gene following intravenous adminstration. Proc. Natl. Acad. Sci. U.S.A., 98: 12754-12759. **(Reviewed in *Nature Reviews-Neuroscience* 2: 852-853, December, 2001.)**

2. Pardridge, W.M. (2002): Drug and gene targeting to the brain with molecular Trojan horses. Nature Reviews-Drug Discovery, 1: 131-139.

3. Pardridge, W.M. (2002): Drug and gene delivery to the brain: the vascular route. Neuron, 36: 555-558. **(6th most frequently downloaded journal article in *Neuron*)**

4. Zhang, Y., Zhu, C., and Pardridge, W.M. (2002): Antisense gene therapy of brain cancer with an artificial virus gene delivery system. Mol. Ther., 6: 67-72.

5. Zhang, Y., Boado, R.J., and Pardridge, W.M. (2003): Marked enhancement in gene expression by targeting the human insulin receptor. J. Gene Med., 5: 157-163.

6. Zhang, Y., Calon, F., Zhu, C., Boado, R.J., and Pardridge, W.M. (2003): Intravenous non-viral gene therapy causes complete normalization of striatal tyrosine hydroxylase and reversal of motor impairment in experimental Parkinsonism. Hum. Gene Ther. 14: 1-12. **(Journal Cover Illustration)**

7. Zhang, Y., Schlachetzki, F., and Pardridge, W.M. (2003): Global non-viral gene transfer to the primate brain following intravenous administration. Mol. Ther., 7: 11-18. **(Highlighted in 'Inside This Month' of *Molecular Therapy*; reviewed in March 22, 2003, *New Scientist;* reviewed in June, 2003 *Drug Discovery Today*, 8:473-474.)**

8. Zhang, Y., Boado, R.J., and Pardridge, W.M. (2003): Absence of toxicity of chronic weekly intravenous gene therapy with pegylated immunoliposomes. Pharm. Res., in press. **(INCLUDED AS APPENDIX 1)**

9. Zhang, Y., Schlachetzki, F., Li, J.Y., Boado, R.J., and Pardridge, W.M. (2003): Organ-specific gene expression in the Rhesus monkey eye following intravenous non-viral gene transfer. Submitted for publication.

10. Zhang, Y., Schlachetzki, F., Zhang, Y., Boado, R.J., and Pardridge, W.M. (2003): Normalization of striatal tyrosine hydroxylase and reversal of motor impairment in experimental Parkinsonism with intravenous non-viral gene therapy and a brain –specific promoter. Submitted for publication. **(INCLUDED AS APPENDIX 2)**

APPENDICES:

Appendix 1

Zhang, Y., Boado, R.J., and Pardridge, W.M. (2003): Absence of toxicity of chronic weekly intravenous gene therapy with pegylated immunoliposomes. Pharm. Res., in press.

Appendix 2

Zhang, Y., Schlachetzki, F., Zhang, Y., Boado, R.J., and Pardridge, W.M. (2003): Normalization of striatal tyrosine hydroxylase and reversal of motor impairment in experimental Parkinsonism with intravenous non-viral gene therapy and a brain –specific promoter. Submitted for publication.

Appendix 1

Pharm. Res., in press (2003)

Absence of Toxicity of Chronic Weekly Intravenous Gene Therapy with Pegylated Immunoliposomes

Yufeng Zhang

Ruben J. Boado

William M. Pardridge[1]

Department of Medicine
UCLA School of Medicine
Los Angeles, CA

Running title: Gene therapy toxicity trial

[1]**To whom correspondence should be addressed at:**
Dr. William M. Pardridge
UCLA Warren Hall 13-164
900 Veteran Ave.
Los Angeles, CA 90024
(310) 825-8858
(310) 206-5163 (fax)
email: wpardridge@mednet.ucla.edu

Word count in text: 3769

ABSTRACT

Purpose. Plasmid DNA-based gene therapy involves episomal gene expression, and must be given on a chronic basis. Therefore, the purpose of the present study was to examine for toxic side effects of the chronic weekly intravenous administration of plasmid DNA delivered with non-viral gene transfer method using pegylated immunoliposomes (PIL).

Methods. A 7 kb expression plasmid encoding for rat tyrosine hydroxylase (TH) was encapsulated in PILs targeted with either the murine OX26 monoclonal antibody (MAb) to the rat transferrin receptor (TfR) or with the mouse IgG2a isotype control antibody. Rats were treated with weekly intravenous injections of 5 μg/rat/week of the TH expression plasmid DNA encapsulated in either the TfRMAb-targeted PIL or the mouse IgG2a-targeted PIL for a total period of 6 weeks. A third control group of rats was treated with saline.

Results. The animals treated with either saline, the TfRMAb-PIL or the mouse IgG2a-PIL had no measurable differences with respect to the following experimental parameters: body weights, 14 serum chemistries, organ histology for brain, liver, spleen, kidney, heart, or lung. Immunocytochemistry showed no evidence of inflammation in brain. The delivery to brain of the TH expression plasmid was confirmed with Southern blotting.

Conclusion. The PIL non-viral gene transfer method causes no toxic side effects following chronic weekly intravenous administration in rats.

Key words: gene therapy, brain, liposomes, non-viral gene transfer, inflammation

INTRODUCTION

An important issue with either viral or non-viral gene delivery systems is organ toxicity associated with the delivery vector (1). In the case of either adenovirus or Herpes simplex virus, the preexisting immunity to these viruses causes an inflammatory reaction (2,3). A single injection of either adenovirus or Herpes simplex virus into the brain causes inflammation leading to demyelination (4,5). More than 90% of the human population has a preexisting immunity to adeno-associated virus (6). Therefore, there is a need to establish non-viral gene transfer technology with minimal toxicity. The principal forms of non-viral gene transfer include the use of complexes of DNA/cationic polymers or the hydrodynamics injection method. Cationic polyplexes have a relatively narrow therapeutic index. A nitrogen/phosphate (N/P) ratio of 6-10 is necessary for gene expression in the lung following the intravenous injection of the cationic polymer/plasmid DNA complexes, whereas an N/P ratio >20 is lethal (7). The hydrodynamics method involves the rapid intravenous injection of a volume of saline greater than the existing blood volume of the animal. This results in transitory right heart failure and hepatic congestion causing a selective expression of plasmid DNA in the liver (8). This gene delivery method results in an increase in liver enzymes, and the mortality with this method can be as high as 40% depending on the salt solution injected (8).

An alternative form of non-viral gene transfer involves the use of pegylated immunoliposomes (PIL). In this formulation, the non-viral plasmid DNA is encapsulated in the *interior* of an 85 nm liposome that has a net anionic charge (9). The surface of the liposome is pegylated with several thousand strands of 2000 Da polyethyleneglycol (PEG). The pegylated liposome is then targeted to distant sites by conjugating a transporting ligand to the tips of 1-2% of the PEG strands. Peptidomimetic monoclonal antibodies (MAb) to either the transferrin

receptor (TfR) or the insulin receptor (IR) have been used to target PILs carrying expression plasmids to distant sites following intravenous injection (9,10). The PILs do not aggregate in saline, and have prolonged blood residence times (11). PILs have been administered intravenously to mice on a weekly basis for the treatment of brain cancer (12), and PILs have been given to rats for the treatment of experimental Parkinson's disease (13). PILs targeted with the TfRMAb have been used to deliver non-viral plasmid DNA to brain. Owing to the expression of the TfR on both the blood-brain barrier (BBB) and the neuronal plasma membrane, the TfRMAb-targeted PIL delivers the plasmid DNA to brain, as well as other organs rich in TfR, such as liver or kidney (9,14). However, to date, there has been no evaluation of the potential toxicity of repeat intravenous administration of PILs.

The purpose of the present study was to examine the potential toxicity of repeat weekly intravenous administration of PIL-encapsulated plasmid DNA that was targeted to tissues in the rat with either the murine OX26 MAb to the rat TfR, or PILs targeted with the corresponding mouse IgG2a isotype control antibody. The plasmid DNA used in the present studies is the clone 877 DNA which encodes for rat tyrosine hydroxylase (TH), as described previously (13). The delivery of the TH expression plasmid to brain with the TfRMAb-targeted PIL results in a normalization of striatal TH enzyme activity in brain of rats lesioned with a neurotoxin (13). For the present toxicity study, the TH expression plasmid DNA was encapsulated in either the TfRMAb-PIL or the mIgG2a-targeted PIL, and was injected weekly for 6 weeks at a dose of 5 μg/rat of PIL-encapsulated plasmid DNA. Body weights of the animals were determined during the treatment period, and at the end of the 6-week treatment, blood was obtained for measurement of 14 parameters of serum chemistry reflecting liver and renal function. Major organs were removed at the end of the treatment period for pathological analysis. In addition,

brain was examined in detail with immunocytochemistry using antibodies to multiple antigens that reflect underlying tissue inflammation. Immunocytochemistry of brain was performed with the mouse OX1 MAb to rat leukocytes, the mouse OX2 MAb to the rat class II multiple histocompatibility complex (MHC) antigen, the mouse OX18 MAb to the rat class I MHC antigen, the mouse OX35 MAb to the rat lymphocyte CD4 receptor, and the mouse OX42 MAb to the rat macrophage. Finally, the present studies used Southern blotting to confirm distribution of the TH expression plasmid in brain following targeting with the TfRMAb-PIL.

METHODS

Materials

POPC (1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine) and DDAB (didodecyldimethylammonium bromide) were purchased from Avanti-Polar Lipids Inc. (Alabaster, AL, USA). Distearoylphosphatidylethanolamine (DSPE)-PEG^{2000} was obtained from Shearwater Polymers (Huntsville, AL, USA), where PEG^{2000} is polyethylene glycol (PEG) of 2000 Daltons . DSPE-PEG^{2000}-maleimide was custom-synthesized by Shearwater Polymers. The LiposoFAST-Basic extruder and polycarbonate filters were from Avestin (Ottawa, Canada). [α-^{32}P]dCTP (3000 Ci/mmol) was from NEN Life Science Products Inc. (Boston, MA, USA). *N*-succinimidyl[2,3-^{3}H]propionate (^{3}H-NSP, 101 Ci/mmol), Sepharose CL-4B, and Protein G-Sepharose CL-4B were from Amersham Pharmacia Biotech (Arlington Heights, IL, USA). The nick translation system was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Exonuclease III was purchased from Promega (Madison, WI, USA); 2-iminothiolane (Traut's reagent) was obtained from Pierce Chemical Co. (Rockford, IL, USA). Mouse myeloma ascites containing IgG2a (κ) (mIgG2a), pancreatic DNase I with a specific activity of 2000 Kunitz units/mg, horse serum, mouse IgG1 isotype, mouse anti-glia fibrillary acidic protein (GFAP) monoclonal antibody (MAb), and glycerol-gelatin were from Sigma Chemical Co. (St. Louis, MO, USA). The anti-transferrin receptor monoclonal antibody (TfRMAb) used in these studies is the murine OX26 MAb to the rat TfR, which is a mouse IgG2a. TfRMAb and mIgG2a were individually purified by protein G affinity chromatography from hybridoma-generated ascites. The biotinylated horse anti-mouse IgG, Vectastain ABC kit, and 3-amino-9-ethylcarbazole (AEC) substrate kit were purchased from Vector Laboratories (Burlingame, CA, USA). Mouse anti-rat class I multiple histocompatibility complex (MHC) monoclonal antibody (OX18), mouse

anti-rat leukocyte CD45 (OX-1), mouse anti-rat lymphocyte CD4 (OX-35), mouse anti-rat class II MHC (OX-6), and mouse anti-rat macrophage CD11b (OX42) were purchased from Serotec (Raleigh, NC, USA). Optimal cutting temperature (O.C.T.) compound (Tissue-Tek) was purchased from Sakura FineTek (Torrance, CA, USA). Adult male Sprague-Dawley rats (weighing from 180-220g) were obtained from Harlan Breeders (Indianapolis, IN, USA).

Plasmid DNA preparation and radiolabeling

The tyrosine hydroxylase expression plasmid, driven by the SV40 promotor, and designated clone 877, was constructed as described previously (13). Clone 877 plasmid DNA was purified from *E. coli* with the Plasmid Maxi Kit and desalted per the manufacturer's instructions (Qiagen, Chatsworth, CA). The size of the DNA was confirmed by 0.8% agarose gel electrophoresis. DNA was labeled with ^{32}P-dCTP using nick translation. The specific activity of ^{32}P-DNA was 15-20 μCi/μg. The trichloroacetic acid precipitability was 99%.

PEGylated liposome synthesis and plasmid encapsulation

POPC (18.8 μmol), DDAB (0.6 μmol), DSPE-PEG^{2000} (0.6 μmol), and DSPE-PEG^{2000}-maleimide (0.2 μmol) were dissolved in chloroform, followed by evaporation, as described previously (14). The lipids were dispersed in 0.2 ml of 0.05 M Tris-HCl buffer (pH 7.0) and vortexed for 1 min followed by 2 min of bath sonication. Supercoiled DNA (200 μg) and 1 μCi of ^{32}P-DNA were added to the lipids. The dispersion was frozen in ethanol/dry ice for 5 min and thawed at room temperature for 25 min, and this freeze-thaw cycle was repeated five times to produce large vesicles with the DNA loosely entrapped inside. The large vesicles were converted into small (85-nm-diameter) liposomes by extrusion. The liposome dispersion was diluted to a lipid concentration of 40 mM, followed by extrusion five times each through two stacks each of 200- and 100-nm pore size polycarbonate membranes with a hand-held LiposoFAST-Basic

extruder as described previously (11). The mean vesicle diameters were determined by quasi-elastic light scattering using a Microtrac Ultrafine Particle Analyzer (Leeds-Northrup, St. Petersburg, FL, USA) as described previously (11).

The plasmid absorbed to the exterior of the liposomes was removed by nuclease digestion, and 6 units of pancreatic endonuclease I and 33 units of exonuclease III were added in 5 mM $MgCl_2$ to the liposome/DNA mixture after extrusion. After incubation at 37 °C for 1 h, the reaction was stopped by adding 20 mM EDTA. The nuclease digestion removed any exteriorized plasmid DNA, as demonstrated by agarose gel electrophoresis and ethidium bromide staining of aliquots taken before and after nuclease treatment, as described previously (11). The formulation prior to antibody conjugation is designated a pegylated liposome (PL) and the formulation after antibody conjugation is called a pegylated immunoliposome (PIL).

MAb conjugation to the PEGylated liposome encapsulated with DNA

TfRMAb or mIgG2a was thiolated and individually conjugated to the maleimide moiety of the PEGylated liposome to produce the PIL with the desired receptor specificity. PIL conjugated with the OX26 MAb is designated TfRMAb-PIL and PIL conjugated with the mIgG2a isotype control is designated mIgG2a-PIL. Either MAb or mIgG2a was radiolabeled with [^{3}H]-NSP as described previously (15). ^{3}H- MAb had a specific activity of >0.11 μCi/ug and a TCA precipitability of >97%. The MAb (3.0 mg, 20 nmol) was thiolated with 40:1 molar excess of 2-iminothiolane (Traut's reagent), as described previously (15). The thiolated MAb, which contained a trace amount of ^{3}H-labeled MAb, was conjugated overnight to the PEGylated liposome with encapsulated plasmid DNA containing a trace amount of ^{32}P-DNA. The unconjugated MAb and the oligonucleotides produced by nuclease treatment were separated from the PIL by Sepharose CL-4B column chromatography as described previously (11). The

number of MAb molecules conjugated per liposome was calculated from the total ^{3}H-labeled MAb radioactivity in the liposome pool and the specific activity of the labeled MAb, assuming 100,000 lipid molecules per liposome, as described previously (15). The average number of MAb molecules conjugated per liposome was 57 ± 12 (mean ± SD, n = 4 syntheses). The final percentage entrapment of 200 μg of plasmid DNA in the liposome preparation was computed from the ^{32}P radioactivity and was 30 ± 2 % (mean ± SD, n = 4 syntheses), or 60 μg of plasmid DNA.

Chronic intravenous administration of PIL encapsulated DNA

Adult male Sprague-Dawley rats weighing 200-220 g were anesthetized with ketamine (50 mg/kg) and xylazine (4 mg/kg) intraperitoneally. Animals were divided into 3 groups. PIL or saline was injected i.v. via femoral vein with a 30-g needle. The first group was injected with TfRMAb-PIL carrying clone 877 plasmid DNA at a dose of 5 μg per rat. The second group was injected mIgG2a-PIL carrying clone 877 plasmid DNA at a dose of 5 μg per rat. The third group was injected with saline. The average intravenous injection volume for all treatments was 300 μL. These intravenous treatments were given once a week for 6 consecutive weeks. Each week before injection, the body weight for each rat was measured. At 3 days following the 6th injection, the rats were anesthetized and blood was collected from the vena cava. Serum was stored at –20° C for serum chemistry measurements by auto analyzer in the UCLA Medical Center Clinical Laboratory. The rats were then sacrificed and organs were removed for immunocytochemistry.

Immunocytochemistry

Immunocytochemistry was performed by the avidin-biotin complex (ABC) immunoperoxidase method (Vector Laboratories). Brains were removed immediately after

sacrifice, and cut into three sagittal slabs. One slab was immersion fixed in cold 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS) for 24 h at 4°C. The second slab was fixed in cold 100% methanol for 24 h at -20°C. These slabs were cryo-protected in 20% sucrose in 0.1 M phosphate buffered water, pH=7.4 (PBW) for 24 h at 4°C, and 30% sucrose in PBW for 24 h at 4°C. Brains were embedded in O.C.T. medium, and frozen in dry ice powder. Frozen sections (20 μm) of rat brain were cut on a Mikron HM505E cryostat. Endogenous peroxidase was blocked with 0.3 % H_2O_2 in 0.3% horse serum-phosphate-buffered saline (PBS) for 30 min. Nonspecific binding of proteins was blocked with 10% horse serum in PBS for 30 min. Sections were then incubated in primary antibodies overnight at 4°C. Based on either results provided by the manufacturer or of pilot studies, the fixative (methanol or paraformaldehyde) was chosen so as to preserved the target antigenicity in the fixed tissue. For methanol-fixed brain sections, OX1 (5 μg/ml), OX18 (5 μg/ml), or OX35 (5 μg/ml) was used as the primary antibody; for paraformaldehyde-fixed brain sections, OX6 (5 μg/ml), OX42 (5 μg/ml), or mouse anti-GFAP MAb (1 μg/ml) was used as the primary antibody. Identical concentrations of isotype control antibody were also used as primary antibody. Mouse IgG1 was used as the isotype control antibody for OX18, OX1, and OX6 and mouse IgG2a was used as the isotype control antibody for OX35, OX42, and the mouse anti-GFAP MAb. After incubation and wash in PBS, sections were incubated in biotinylated horse anti-mouse IgG for 30 min. After development in AEC, sections were mounted with glycerol-gelatin and examined by light microscopy.

Hematoxylin and eosin staining of rat organs

The third sagittal slab of brain, as well as liver, spleen, kidney, heart, and lung of each rat were removed and immersion fixed in 10% formalin in 0.1 M phosphate buffer for 48 h at 4°C.

The fixed organs were embedded in paraffin and stained with hematoxylin and eosin and examined by light microscopy.

Southern blotting

Plasmid DNA was isolated with the Hirt procedure (16) from rat brain 3 days following the intravenous injection of saline, clone 877 encapsulated in TfRMAb-PIL, or clone 877 encapsulated in mIgG2a-PIL. Rat brain (100 mg) was homogenized in 2 ml lysis buffer (20 mM Tris pH=7.5, 10 mM EDTA, 1% SDS) containing 15 μg/ml DNase-free RNase A using a Polytron PT-MR 3000 homogenizer (Littau, Switzerland) at full speed for 10 sec. Samples were incubated for 30 min at 37°C. Proteinase K was added to a final concentration of 1 mg/ml and samples incubated for 2 hrs at 37°C. Nuclear DNA was precipitated overnight at 4°C in the presence of 1.1 M NaCl. Samples were centrifuged at 14000 rpm and 4°C for 30 min. Supernatants were extracted with phenol:chloroform and plasmid DNA precipitated with ethanol in the presence of 10 μg glycogen carrier. Aliquots of precipitated material were resolved by gel electrophoresis in 0.8 % agarose and blotted onto a GeneScreen Plus membrane (14). To prevent hybridization with the endogenous rat TH genomic DNA, membranes were hybridized with [^{32}P]-pGL2 clone 734 (17), which contains the clone 877 backbone but without the rat TH cDNA insert (13). Southern blot hybridization was performed as previously reported (14). Autoradiograms were developed with Kodak X-Omat Blue film and intensifying screens for 24 hrs at -70°C. Films were scanned with a Umax PowerLook III scanner, and images imported and cropped in Adobe Photoshop 5.5 on a G4 Power Macintosh.

Statistical analysis

Statistical differences at the $p<0.05$ level amongst different groups were evaluated by analysis of variance with Bonferroni correction.

RESULTS

The animals were divided into 3 groups depending on whether the rat was treated with weekly intravenous injections of (a) saline, (b) the TH expression plasmid encapsulated in mouse IgG2a targeted PILs, or (c) the TH expression plasmid encapsulated in the OX26 TfRMAb-targeted PILs. The body weights of the animals in the 3 treatment groups is shown in Figure 1, and there was no significant difference between the body weights of the animals in the 3 groups throughout the treatment period.

The results of the chemistry analysis of the serum taken 3 days after the 6^{th} weekly injection are shown in Table 1. There are no significant differences in any of the 14 different serum chemistries for any of the 3 treatment groups (Table 1). The organ histology in the rats sacrificed 3 days following the 6^{th} weekly treatment is shown in Figure 2 for brain cerebellum (Figure 2A), lung (Figure 2B), spleen (Figure 2C), liver (Figure 2D), heart (Figure 2E), and kidney (Figure 2F). The histology shown in Figure 2 is for organs removed from rats treated with the TfRMAb-PIL. The organ histology of these animals was normal (Figure 2), and was no different from the histology of organs taken from animals treated with either saline or the mIgG2a-PIL.

The results of the brain immunocytochemistry are given in Table 2. No OX1-immunoreactive leukocytes were found in brain in any of the 3 treatment groups, although there was immunopositive choroidal endothelium staining in all 3 groups (Table 2). There was an occasional OX6-immunoreactive class II antigen presenting cell in the meningial surface of all 3 treatment groups with no evidence of any parenchymal infiltration of class II immunopositive cells in any of the 3 treatment groups (Table 2). OX18 immunoreactivity indicative of the class I MHC antigen was found on capillary endothelium and in focal subependymal microglia, and the

same staining pattern was found in all 3 treatment groups (Table 2). OX35-immunoreactive CD4-lymphocytes were rare in brain with the same pattern in all 3 treatment groups (Table 2). OX42-immunoreactive microglia were found diffusely in the parenchyma throughout the cerebrum and cerebellum, with an identical pattern in all 3 treatment groups (Table 2). Immunoreactive GFAP astrocytes were found diffusely throughout the cerebrum and cerebellum, with the same pattern in all 3 treatment groups (Table 2). There was no immunoreactivity in brain with the non-specific mouse IgG1 (mIgG1), which is the isotype control antibody for the OX1, OX18, OX6, and the GFAP antibodies (Table 2). There was no immunocytochemical staining of brain using the non-specific mouse IgG2a (mIgG2a), which is the isotype control antibody for the OX35 and OX42 antibodies (Table 2).

The delivery of the TH expression plasmid to brain was verified with Southern blotting as shown in Figure 3 (Lane 3). No signal was detected in the saline treated animals (Lane 1, Figure 3) because these animals were not administered DNA. No hybridization signal was detected in the brain of animals treated with the expression plasmid encapsulated in the mIgG2a-PIL (Lane 2, Figure 3) because this isotype control antibody was unable to target the PIL across the blood-brain barrier and into brain cells.

DISCUSSION

These studies show that the repeat weekly intravenous administration of the PIL-based gene therapy in rats for 6 weeks causes no measureable toxicity in brain or peripheral tissues. In addition, these studies show that the chronic weekly intravenous administration of a TH expression plasmid encapsulated in TfRMAb-PILs causes no inflammation within the target organ, the central nervous system (CNS).

There is no general systemic toxicity following weekly PIL administration based on the observation that the body weights of the animals increase over the 6 week treatment period at the same rate for all 3 treatment groups (Figure 1). The PIL targets the plasmid DNA to TfR-rich organs such as the brain, liver, or spleen (9,14). The serum chemistries show normal hepatic function tests and an absence of an increase in serum bilirubin or liver enzymes (Table 1). In contrast, the intravenous injection of adenovirus in primates results in increased liver enzymes secondary to hepatic inflammation caused by reaction to the immunogenic viral vector (18). There is no change in serum electrolytes or other renal function tests (Table 1). The normal serum chemistry is paralleled by the normal organ histology for liver, speen, kidney, heart, lung, and brain (Figure 2). The serum chemistry and organ histology was examined at 3 days following the 6^{th} weekly injection, because prior work has shown the TH gene expression following PIL injection is maximal at this time (13).

The intracerebral injection of viral vectors, such as adenovirus or Herpes simplex virus, leads to inflammation of the brain, as evidenced by perivascular cuffing with lymphocytes and increased immunoreactivity for class I and class I MHC antigens in brain (2-5). Therefore, the present studies performed a detailed immunocytochemical analysis of brain to examine for any evidence of inflammation in the brain following the chronic delivery to brain of a TH expression

plasmid encapsulated in a TfRMAb-targeted PIL. The brain immunocytochemistry of the animals treated weekly with the TfRMAb-targeted PIL was compared to control groups of rats treated weekly with either saline or with mIgG2a-targeted PILs. There is an identical pattern of immunoreactivity in rat brain using OX1, OX6, OX18, OX35, OX42, and GFAP antibodies in immunocytochemical analysis of brain for all 3 treatment groups (Table 2). In these studies, the brain was fixed with either methanol or paraformaldehyde, depending on which was the optimal fixative for each antigen (Methods), and so as to preserve antigen recognition in the fixed brain. Chronic delivery of TfRMAb-targeted PILs to brain caused (a) no elevations in parenchymal class I (OX18) or II MHC (OX6), (b) no elevations in parenchymal infiltration by lymphocytes (OX35), leukocytes (OX1), or macrophages (OX42), and (c) no elevations in parenchymal gliosis (GFAP).

In summary, these studies demonstrate that non-viral expression plasmids can be delivered to organs with the PIL gene transfer method without toxic side effects when administered at a dose PIL-encapsulated plasmid DNA of 25 μg/kg. The chronic weekly intravenous administration of this dose of plasmid DNA encoding for rat TH and encapsulated in TfRMAb-targeted PILs causes no evidence of toxicity in either the target organ, brain, or in peripheral tissues, such as liver, spleen, kidney, heart, or lung. It is possible that toxic effects may be observed at higher doses, but the dose used in this study in rats was chosen because this dose is therapeutic in rats (13). Moreover, a much higher dose, 200 μg/kg, of PIL encapsulated plasmid DNA has been administered weekly to mice without evidence of toxicity (12). The need for high dosing of plasmid DNA with the PIL gene targeting method is unlikely because a dose of 12 μg/kg of PIL encapsulated plasmid DNA in adult primates results in levels of gene expression that are 50-fold higher than in rodents (10). The finding of a lack of toxicity

following chronic PIL administration is important because the PIL gene transfer method delivers to the target organ a non-viral plasmid that directs gene expression for only a finite duration (12,13). The expression plasmid is transcribed episomally, and is not permanently or randomly integrated into the host genome. Therefore, in order to sustain a pharmacologic effect with plasmid DNA-based gene therapy, it is necessary to administer the gene medicine on a chronic basis. The frequency of the administration is a function of the persistence plasmid expression in the target organ. Long-term gene expression is possible with viral vectors that permanently integrate into the host genome, but this approach is associated with the risk of insertional mutagenesis (1). An alternative approach to gene therapy is the chronic treatment with episomal-based plasmid DNA that is formulated in such a way that the DNA is able to target distant sites following intravenous administration. Prior work has shown that the PIL gene targeting method enables widespread expression of the exogenous gene in distant sites such as brain in mice, rats, and rhesus monkeys (9,10,11). The present studies show that the PIL-based gene therapy can be given chronically without the development of tissue toxicity in either the target organ, brain, or in peripheral tissues.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Neurotoxin Exposure Treatment Research Program of the US Department of Defense. Dr. Felix Schlachetzki assisted with the immunocytochemistry.

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Table 1. Summary of Serum Chemistry

ASSAY	UNITS	SALINE	mIgG2a-PIL	OX26-PIL
SODIUM	mM	143 ± 1	142 ± 1	140 ± 1
POTASSIUM	mM	4.4 ± 0.1	4.6 ± 0.1	4.6 ± 0.2
CHLORIDE	mM	100 ± 1	100 ± 1	100 ± 1
CO2	mM	29 ± 1	29 ± 1	27 ± 1
GLUCOSE	mg/dL	168 ± 8	160 ± 6	163 ± 4
CREATININE	mg/dL	0.45 ± 0.03	0.40 ± 0.01	0.45 ± 0.02
UREA NITROGEN	mg/dL	19 ± 1	21 ± 2	18 ± 1
TOTAL PROTEIN	g/dL	5.2 ± 0.1	5.3 ± 0.1	5.3 ± 0.1
ALBUMIN	g/dL	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.1
BILIRUBIN, TOTAL	mg/dL	0.35 ± 0.03	0.25 ± 0.05	0.33 ± 0.02
ALK PHOS	U/L	231 ± 27	212 ± 25	281 ± 11
AST (SGOT)	U/L	65 ± 5	59 ± 2	74 ± 6
ALT (SGPT)	U/L	54 ± 2	52 ± 3	59 ± 1
CALCIUM	mg/dL	9.4 ± 0.1	9.5 ± 0.2	9.2 ± 0.1

Data are mean ± S.E. (n= 6 rats in each of the 3 treatment groups).

Table 2. Summary of Immunocytochemistry

Antibody	Parameter	Fixative	Findings
OX1	leukocytes	methanol	positive choroidal endothelium same pattern in all 3 treatment groups
OX6	class II MHC	para.	occasional positive cell in meninges same pattern in all 3 treatment groups
OX18	class I MHC	methanol	weak staining of capillary endothelium focal sub-ependymal microglia same pattern in all 3 treatment groups
OX35	CD4-lymphocytes	methanol	minimal staining of brain and equal to mouse IgG2a control same pattern in all 3 treatment groups
OX42	macrophages	para.	diffuse immunoreactive microglia throughout cerebrum and cerebellum same pattern in all 3 treatment groups
GFAP	astrocytes	para.	diffuse immunoreactive astrocytes throughout cerebrum and cerebellum same pattern in all 3 treatment groups
mouse IgG1	control	methanol para.	no reaction (control for OX1, OX18) no reaction (control for OX6, GFAP)
mouse IgG2a	control	methanol para.	no reaction (control for OX35) no reaction (control for OX42)

para.=paraformaldehyde

LEGENDS TO FIGURES

Figure 1: The body weight of each rat in the 3 treatment groups was measured weekly during the course of treatment and the mean ± S.E. (n=6 rats per group) is shown. The OX26-PIL is the TfRMAb-targeted PIL, and the mIgG2a-PIL is the PIL targeted with the non-specific mouse IgG2a, which is the isotype control antibody for the OX26 MAb.

Figure 2: Hematoxylin and eosin staining of formalin fixed cerebellum (A), lung (B), spleen (C), liver (D), heart (E), and kidney (F), removed 3 days after the 6^{th} weekly intravenous injection of the TH expression plasmid encapsulated in the OX26 TfRMAb-targeted PIL. The magnification is the same in all panels and the magnification bar in panel A is 37 μm.

Figure 3: Southern blot analysis of rat brain with [^{32}P]-pGL2 clone 734. Lane 1, brain isolated from a saline-treated rat; lane 2, brain isolated from a rat injected with the TH expression plasmid encapsulated in the mIgG2a-targeted PIL; and lane 3, brain isolated from a rat injected with the TH expression plasmid encapsulated in the TfRMAb-targeted PIL. The migration of the DNA standards is indicated in the Figure. The expected ~7 kb plasmid DNA corresponding to the size of the TH expression plasmid is seen only in the brain of the rats treated with the TfRMab-targeted PIL (lane 3).

Figure 1

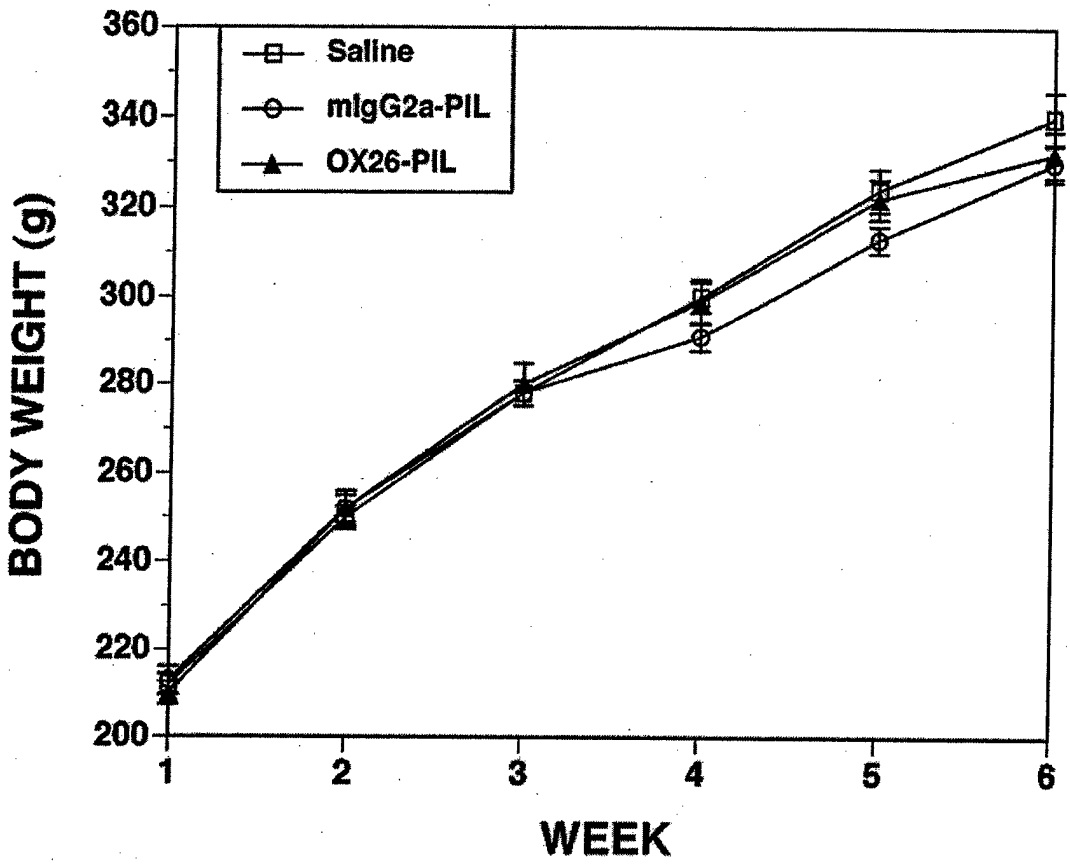

Saline
mIgG2a-PIL
OX26-PIL
BODY WEIGHT (g)
360
340
320
300
280
260
240
220
200
1
2
3
4
5
6
WEEK

Figure 2

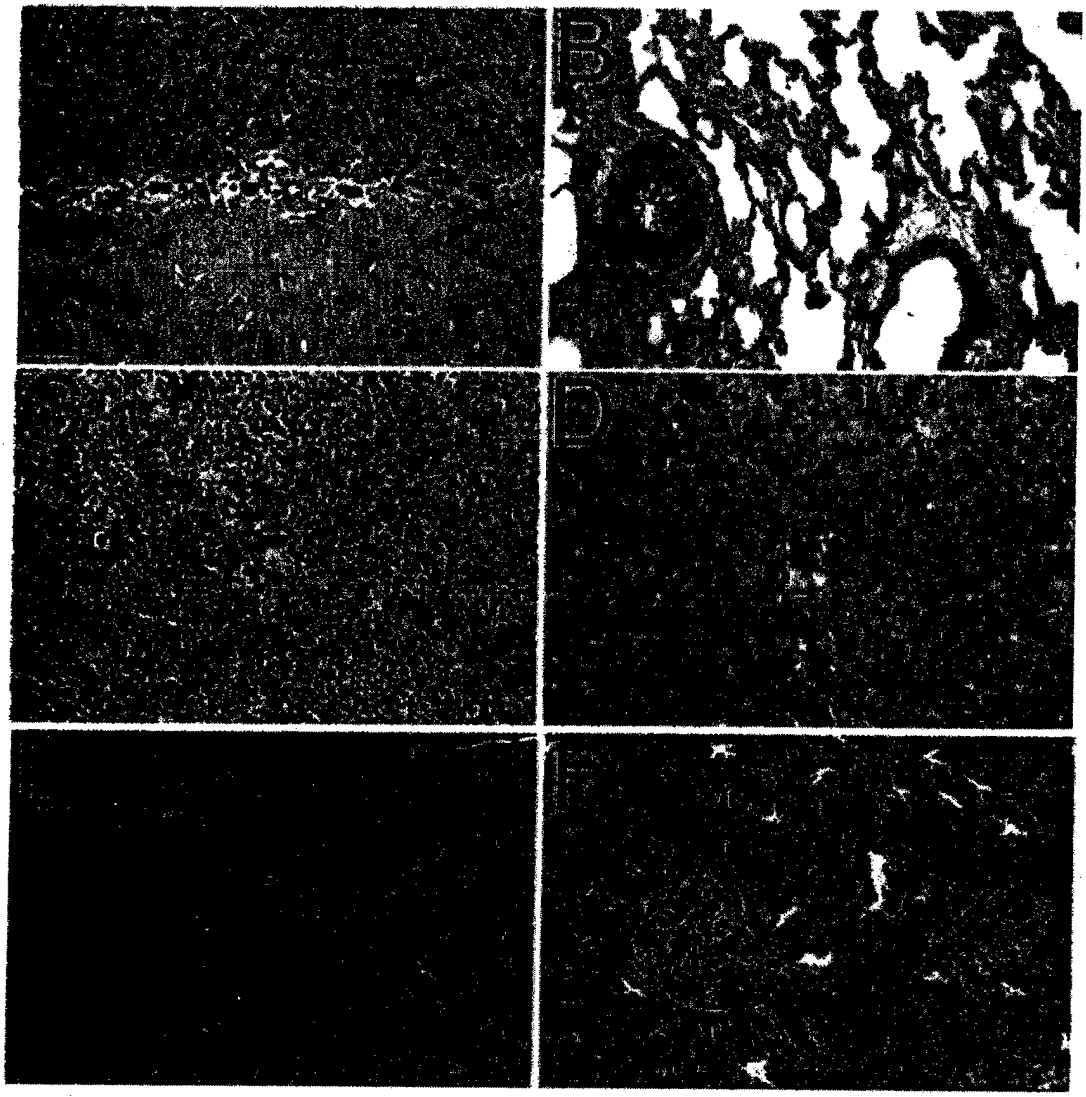
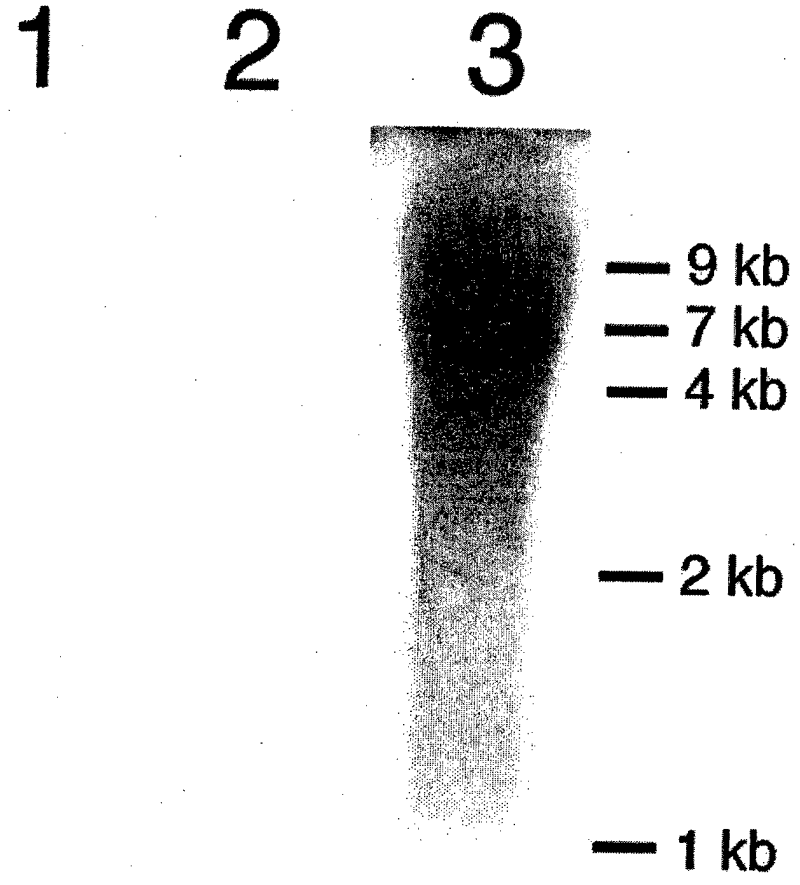


Figure 3



Appendix 2

Repair Neuroscience
Sr. Editor: Pat Levitt

Normalization of Striatal Tyrosine Hydroxylase and Reversal of Motor Impairment in Experimental Parkinsonism with Intravenous Non-Viral Gene Therapy and a Brain-Specific Promoter

Yun Zhang
Felix Schlachetzki*
Yu Feng Zhang
Ruben J. Boado
William M. Pardridge

Department of Medicine
UCLA
Los Angeles, CA 90024

Pages: 29
Words: Abstract (248); Introduction (574); Discussion (1230)
Figures: 5 **Tables**: 2

Running Title: Non-invasive, non-viral gene therapy of the brain

Acknowledgments: This work was supported by a grant from the Neurotoxin Exposure Treatment Research Program of the U.S. Department of Defense. F.S. was supported by the Ernst Schering Research Foundation.

***Current address:** Dept. of Neurology, University of Regensburg, Regensburg, Germany.

Correspondence: W. M. Pardridge, UCLA Warren Hall 13-164, 900 Veteran Avenue, Los Angeles, CA 90024. Phone: (310) 825-8858; Fax: (310) 206-5163; Email: wpardridge@mednet.ucla.edu

Keywords: brain gene therapy, blood-brain, barrier, transferrin receptor, tyrosine hydroxylase, Parkinson's disease

ABSTRACT

The goal of this work was to normalize striatal tyrosine hydroxylase (TH) activity with intravenous non-viral TH gene therapy, and at the same time eliminate ectopic TH gene expression in peripheral organs such as liver in the rat. TH expression plasmids, containing either the SV40 promoter or the glial fibrillary acidic protein (GFAP) gene promoter, were globally delivered to brain across the blood-brain barrier (BBB) following intravenous administration of pegylated immunoliposomes (PILs). The GFAP-TH or SV40-TH expression plasmids were encapsulated in the interior of 85 nm PILs, which were targeted across both the BBB and the neuronal cell membrane with a monoclonal antibody (MAb) to the transferrin receptor (TfR). Striatal TH activity was 98% depleted with the unilateral intra-cerebral injection of 6-hydroxydopamine. TH in the striatum ipsilateral to the lesion was normalized 3 days after the intravenous injection of 10 μg/rat of either the SV40-TH or the GFAP-TH plasmid DNA. Whereas the SV40-TH gene caused a 10-fold increase in hepatic TH activity, there was no increase in liver TH with the GFAP-TH gene. The GFAP-TH gene therapy caused an 82% reduction in apomorphine-induced rotation in the lesioned rats. Confocal microscopy using antibodies to TH, GFAP, and NeuN showed the GFAP-TH gene was selectively expressed in nigra-striatal neurons, with no expression in either cortical neurons, or astrocytes. These studies demonstrate that global delivery of exogenous genes to brain is possible with intravenous non-viral gene transfer, and that ectopic gene expression is eliminated with the use of brain-specific gene promoters.

Parkinson's disease (PD) is associated with a loss of dopaminergic neurons originating in the substantia nigra and terminating in the striatum (Mouradian and Chase, 1997; Mandel et al, 1999). The rate-limiting enzyme in dopamine synthesis is tyrosine hydroxylase (TH), and one approach to the treatment of PD is TH replacement gene therapy. The goals of TH gene therapy in PD are (a) the delivery of the TH gene to the majority of the nigral-striatal neurons resulting in the restoration of dopaminergic neurotransmitter release in the striatum, and (b) the selective expression of the TH gene in this region of brain without 'ectopic' TH gene expression in either the cortex or in non-brain organs. Ectopic TH gene expression could lead to unwanted increased dopaminergic activity in peripheral organs. The transduction of the majority of the nigral-striatal neurons with TH gene therapy is possible with a trans-vascular route to the brain. In this approach, the gene is administered intravenously followed by entry into brain across the blood-brain barrier (BBB). Since every neuron is perfused by its own blood vessel, the gene is targeted to the 'doorstep' of every neuron in the brain following a transvascular delivery route (Pardridge, 2002). Prior work with a non-viral expression plasmid driven by the widely expressed SV40 promoter demonstrated normalization of striatal TH activity in the 6-hydroxydopamine-lesioned rat brain using the pegylated immunoliposome (PIL) gene targeting technology (Zhang et al, 2003a). In this approach, the TH expression plasmid is encapsulated in an 85 nm pegylated liposome, which is targeted across both the BBB and the neuronal cell membrane with a peptidomimetic monoclonal antibody (MAb) to the transferrin receptor (TfR) following intravenous administration. The transduction of rat brain with the TH PIL gene therapy was confined to the nigral-striatal tract, and TH was not increased in the cortex of rat brain (Zhang et al, 2003a). The lack of TH gene expression in the cortex is due to the obligatory requirement of the TH enzyme for the biopterin co-factor (Hwang et al, 1998). The rate-limiting enzyme in the

biosynthetic pathway of biopterin, GTP cyclohydrolase I (GTPCH), is not expressed in cortex (Shimoji et al, 1999). However, GTPCH is expressed in peripheral tissues such as liver (Nagatsu et al, 1997). Consequently, intravenous TH gene therapy with the PIL technology and a widely expressed SV40 promoter led to ectopic gene expression in rat liver (Zhang et al, 2003a).

Ectopic gene expression can be eliminated with the combined use of the PIL gene targeting technology and brain-specific promoters. The peripheral expression of a β-galactosidase expression plasmid was eliminated when the transgene was driven by the 2 kb of the 5'-flanking sequence (FS) of the human glial fibrillary acidic protein (GFAP) gene (Shi et al, 2001a). Genes under the influence of the GFAP promoter are selectively expressed in brain, although the GFAP promoter enables gene expression in both neurons and astrocytes in brain (Kaneko and Sueoka, 1993; Galou et al, 1994). Since astrocytes do not express GTPCH or the biopterin cofactor (Nagatsu et al, 1997; Hwang et al, 1998), it is possible the use of the GFAP promoter, in lieu of the SV40 promoter, may enable neuronal expression of the TH gene and eliminate ectopic TH gene expression. Therefore, the purpose of the present studies was to produce a TH expression plasmid under the influence of the GFAP promoter, and to treat 6-hydroxydopamine lesioned rats with intravenous administration of the GFAP-TH plasmid DNA encapsulated in TfRMAb-targeted PILs.

MATERIALS AND METHODS

Materials

POPC (1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine) and DDAB (didodecyldimethylammonium bromide) were purchased from Avanti-Polar Lipids Inc. (Alabaster, AL). Distearoylphosphatidylethanolamine (DSPE)-PEG^{2000} was obtained from Shearwater Polymers (Huntsville, AL), where PEG^{2000} is 2000 Dalton polyethyleneglycol. DSPE-PEG^{2000}-maleimide (MAL) was custom synthesized by Shearwater Polymers. [α-^{32}P]dCTP (3000 Ci/mmol) and [3,5-^{3}H]-L-tyrosine (51.5 Ci/mmol) were from NEN Life Science Product Inc. (Boston, MA). N-succinimidyl[2,3-^{3}H]propionate (^{3}H-NSP, 101 Ci/mmol) and protein G Sepharose CL-4B were purchased from Amersham-Pharmacia Biotech (Arlington Heights, IL). The nick translation system was from Life Technologies Inc. (Rockville, MA). The 6-hydroxydopomine (6-OHDA), apomorphine, pargyline, catalase, (6R)-5,6,7,8-tetrahydrobiopterin (BH4), β-NADPH, L-tyrosine and charcoal were purchased from Sigma (St. Louis, MO); 2-iminothiolane (Traut's reagent) and bicinchoninic acid (BCA) protein assay reagents were obtained from Pierce Chemical Co. (Rockford, IL). Mouse myeloma ascites containing mouse IgG2a (mIgG2a) isotype control was from Cappel Division of ICN Pharmaceuticals (Aurora, OH). The anti-transferrin receptor monoclonal antibody (TfRMAb) used in this study is the murine OX26 MAb to the rat TfR, which is a mouse IgG2a. The anti-insulin receptor MAb used for gene targeting to human U87 glioma cells is the murine 83-14 MAb to the human insulin receptor (HIR). The TfRMAb, the HIRMAb, or the mIgG2a were individually purified with protein G affinity chromatography from hybridoma generated ascites. The pGfap-cLac plasmid (Brenner et al, 1994; Segovia et al, 1998) was provided by Dr. Jose Segovia, Centro de Investigacion y de Estudios Avanzados (San Pedro Zacatenco, Mexico). A

mouse monoclonal antibody against GFAP (clone G-A-5), a mouse monoclonal anti-tyrosine hydroxylase antibody, mouse IgG1 isotype control, and control rabbit IgG were purchased from Sigma (St.Louis, Ms). A mouse monoclonal antibody (MAB377) against neuronal nuclei (NeuN), a mouse monoclonal antibody (MAB5262) anti-neurofilament 200kD antibody, and an affinity purified rabbit polyclonal antibody (AB152) against tyrosine hydroxylase were obtained from Chemicon (Temecula, CA). Secondary antibodies used were Alexa fluor 488 donkey anti-mouse IgG and Alexa fluor 594 donkey anti-rabbit IgG (Molecular Probes, Eugene, OR).

Construction of tyrosine hydroxylase expression plasmid with GFAP promoter

The ~2 kb human GFAP promoter was obtained by PCR amplification using the pGfap-cLac plasmid. Custom oliogdeoxynucleotide primers were designed to amplify nucleotides 1-2210 of the human GFAP promoter region (GenBank accession # M67446). The forward, ATGGCTAGCGAGCTCCCACCTCCCTCTCTG, and reverse, ATGAAGCTTGCGAGCAGCGGAGGTGATGCG, primers contain NheI and HindIII sites, respectively, for directional cloning (Figure 1A). In addition, these primers have 3 unrelated nucleotides on the 5'-end to facilitate restriction endonuclease digestion. Custom primers were obtained from Biosource International (Camarillo, CA). PCR amplification of the GFAP promoter was performed using 50 ng plasmid DNA in a total volume of 50 µl Pfu DNA polymerase buffer (Stratagene). The reaction also contained 0.4 µM forward and reverse primers, 0.2 mM dNTPs, and 1 µl = 2.5 U Pfu-Turbo DNA polymerase (Stratagene). The sample was denatured 30 sec at 95°C, and amplified in 17 cycles of 30 sec at 95°C, 60 sec at 55°C (annealing) and 6 min 68°C (extension). PCR products were resolved by agarose gel electrophoresis and a major band of ~2kb corresponding to the human GFAP promoter was seen.

PCR products were purified using the Qiagen PCR purification kit (Valencia, CA), and double digested with NheI and HindIII for subcloning in the TH expression vector as described below.

The expression plasmid containing the complete open reading frame of the rat TH driven by the SV40 promoter is derived from the pGL2 plasmid and is designated clone 877 as described previously (Zhang et al, 2003a). The SV40 promoter was deleted from clone 877 by double digestion with NheI and HindIII, which cleaved at sites located upstream and downstream of the promoter, respectively (Figure 1A). The ~6.0 kb rTH-vector backbone fragment was purified by gel electrophoresis followed by centrifugation with a Spin X filter unit. The ~2 kb GFAP promoter was prepared as described above and subcloned at the same restriction endonuclease sites to form a GFAP-TH expression plasmid named clone 951 (Figure 1A). Positive clones were identified by restriction endonuclease mapping (i.e. NheI and HindIII), and its identity confirmed by DNA sequencing using the pGL2 sequencing primer 1 (Promega).

Synthesis of pegylated immunoliposomes

The TH-GFAP (clone 951) plasmid DNA or the SV40-TH (clone 877) plasmid DNA was encapsulated in PILs as described previously (Shi et al, 2001a; Zhang et al, 2002a; Zhang et al, 2003a). The liposome is 85-100 nm in diameter and the surface of the liposome is conjugated with several thousand strands of 2000 Da polyethyleneglycol (PEG). The tips of about 1-2% of the PEG strands are conjugated with either the mouse OX26 MAb to the rat TfR or the mouse 83-14 MAb to the human insulin receptor (HIR) or mIgG2a (Figure 1B). The mIgG2a is the isotype control for either the murine OX26 or 83-14 MAb. Any plasmid DNA not encapsulated in the interior of the liposome is quantitatively removed by exhaustive nuclease treatment (Shi et al, 2000). In a typical synthesis, 36-40 % of the initial plasmid DNA (250 μg) was encapsulated

within 20 μmol of lipid, and each liposome had a range of 69-73 MAb molecules conjugated to the PEG strands. The PIL conjugated with the OX26 TfRMAb is designated TfRMAb-PIL; the PIL conjugated with the murine 83-14 HIRMAb is designated the HIRMAb-PIL; and the PIL conjugated with the mouse IgG2a isotype control antibody is designated mIgG2a-PIL. The targeting MAb's are species specific and the HIRMAb-PIL is used for gene delivery to human cells and the TfRMAb-PIL is used for gene targeting to rat cells (Zhang et al, 2003b).

TH gene expression in cultured U87 human glioma cells

Human U87 glioma cells were plated on 60-mm collagen-treated dishes with MEM containing 10 % fetal bovine serum (FBS). When the cells reached 60 % confluence, the medium was removed by aspiration, and 6 ml of fresh medium containing 10% FBS was added to the cells, followed by the addition of 167 ul of the HIRMAb-PIL carrying the 951 DNA (4 ug plasmid DNA/dish). The cells were incubated for 2, 4 or 6 days, with 3 dishes at each time point, for measurement of TH enzyme activity. The cells were washed three times with cold wash buffer (5 mM potassium phosphate buffer), and then 400 ul of sonication buffer (wash buffer with 0.2 % triton X-100) was added to each dish. The cells were collected, and after a short vortex, the cells were sonicated for 30 seconds with a Branson Sonifier Cell Disruptor Model 185. The cell homogenate was centrifuged at 10,000 *g* for 10 min at 4 C. TH activity was measured with 200 ul of the supernatant as described previously (Zhang et al, 2003a).

6-Hydroxydopamine model

Adult male Sprague-Dawley rats (supplied by Harlan Breeders, Indianapolis, IN) weighing 200-230 g were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) intraperitoneally (ip). Animals received unilateral 6-hydroxydopamine (6-OHDA) injections into

the right medial forebrain bundle as described previously (Zhang et al, 2003a). Each animal received pargyline 30-60 min prior to surgery (50 mg/kg in normal saline, ip). Following pargyline administration, 4 μl of 2 ug/μl of 6-OHDA (prepared freshly in 0.2 μg/μl ascorbic acid) was injected over a 4-min period using a 10-ul Hamilton syringe with the following stereotaxic coordinates: 4.8 mm posterior to bregma, 1.1 mm lateral to bregma, and 8.0 mm below the dura. The syringe needle was left in place for 2 min after the injection to allow for diffusion of the toxin. Three weeks following the lesion, rats were tested for apomorphine-induced contralateral turning using 0.5 mg/kg of apomorphine injected intraperitoneally. Full (360^{o}), contralateral rotations only were counted over 20 min starting 5 min after apomorphine administration. Those rats turning >160 times in 20 min, or >8 rotations per min (RPM), were designated as having been successfully lesioned, and were treated 1 week later with TH gene therapy. In one series of experiments, the lesioned, apormorphine-responsive rats were divided into two groups: (1) control group: rats received 10 μg/rat of clone 951 DNA encapsulated in mIgG2a-PIL; (2) treatment group: rats received 10 μg/rat of clone 951 DNA encapsulated in TfRMAb-PIL. The PIL was administered via the femoral vein; 3 days later the rats were tested for apomorphine-induced rotation behavior and then sacrificed. In each group, half of the rats were used for TH immunocytochemistry, and the other half were used for confocol microscopy. In a different series of experiments, the two groups of lesioned, apomorphine-responsive rats were treated with either (1) 10 μg/rat of clone 877 DNA encapsulated in TfRMAb-PIL; or (2) 10 μg/rat of clone 951 DNA encapsulated in TfRMAb-PIL. These animals were sacrificed 3 days later for measurement of organ TH activity by a radio-enzymatic assay described previously (Zhang et al, 2003a).

Tyrosine hydroxylase assay

The TH activity assay was performed as described previously (Zhang et al, 2003a), and measures the conversion of L-[3,5-^{3}H]tyrosine to both [^{3}H]-H_2O and [^{3}H]-L-DOPA in a 1:1 stoichiometric relationship; the two metabolites are separated by charcoal, which selectively binds the amino acid. For the TH assay in rat organs, the liver, heart, lung, kidney, frontal cortex, and the dorsal striatum in both lesioned (ipsilateral) and nonlesioned (contralateral) sides of brain were frozen in dry ice. The counts per minute were converted to picomoles of L-dopa on the basis of the [^{3}H]tyrosine specific activity, and the results were expressed as picomoles of L-DOPA per hour per milligram protein.

Immunocytochemistry and confocal microscopy

The brains were removed and placed into coronal or sagittal rat brain matrices for immunocytochemistry and confocal microscopy, respectively. For immuncytochemistry, frozen sections were prepared as described previously (Zhang et al, 2003a). For confocal microscopy, brain tissue was then immersion fixed in 4% paraformaldehyde in 0.01 M phosphate buffered water (PBW), pH 7.4 and stored overnight at 4°C. After brief washing in 0.01 M PBW the brain slabs were cryoprotected in 30% sucrose in 0.1 M PBW, pH 7.4 for additional 12 hrs at 4°C. Followed by brief washing in 0.01 M PBW, the brains were placed in cryomolds filled with OCT embedding compound and rapidly frozen in powdered dry ice.

Tyrosine hydroxylase immunocytochemistry was performed on coronal sections by the avidin-biotin complex (ABC) immunoperoxidase method (Vector Laboratories). Frozen sections (20 μm) were incubated in either mouse anti-TH MAb (1 μg/ml) or mouse IgG1 isotype control (1 μg/ml) overnight at 4°C. The sections were incubated in biotinylated horse anti-mouse IgG

(35 μg/ml) for 30 min. After development in AEC, sections were scanned with a UMAX PowerLook III flatbed scanner with transparency adpater, and then cover-slipped.

Coronal sections (20μm) through the level of the substantia nigra, pars reticulata were cut from both hemispheres for confocal microscopy. After 30 min drying at room temperature (20°C) slides were washed and permeabilized using 0.01M PBS, pH 7.4 with 0.1% Triton-X 100 (PBST). Blocking for 1-2 hrs was performed with 10% preimmune donkey serum in 0.01M PBST at 20°C. Primary antibodies and all control studies with isotype IgG were used as follows: 15 μg/ml mouse monoclonal anti-NeuN antibody (Liu et al, 1998), 10 μg/ml mouse monoclonal anti-GFAP antibody (Debus et al, 1983), 2.5 μg/ml mouse monoclonal anti-TH antibody, 1 μg/ml mouse monoclonal anti-neurofilament 200kD antibody (Anderton et al, 1982), and 0.4 μg/ml affinity purified rabbit polyclonal anti-TH antibody (Horger et al, 1998). Sections were incubated overnight at 4°C with primary antibodies diluted in 3% bovine albumin in 0.01 M PBST. The secondary antibody, 488 donkey anti-mouse IgG (fluorescein labeled) and 594 donkey anti-mouse IgG (rhodamine labeled) were used at a concentration of 5 μg/ml diluted in 0.01 M PBST. After extensive washing in 0.01 M PBS all specimens were cover-slipped and slides stored at 4°C light protected.

Confocal imaging was performed employing a Zeiss LSM 5 PASCAL confocal microscope with dual argon and helium/neon lasers equipped with Zeiss LSM software for image reconstruction (LSM 5 PASCAL, version 3.2, Jena, Germany). All sections were scanned in multitrack mode to avoid overlap of the fluorescein (excitation at 488nm) and rhodamine (excitation at 568nm) channels. For acquisition of three-dimensional images, up to 20 serial images with a slice thickness between 1.6 – 3.7 μm were used. Pinhole size for each channel was kept as small as possible as to insure sufficient signal to noise ratio and highest spatial resolution

(126-145nm). Line density ranged from 0.19-0.45 μm using these settings. Detector gain and amplifier offset were optimized to reduce artificial background for each image. No amplifier gain was used. 3D image slices were scanned with a 1024x1024 resolution. All scanning parameters were kept constant. Image analysis was performed for each single image slice also in the 3D data stacks. Three dimensional images were reconstructed by projecting 6 consecutive planar views. To ensure a more objective measure of overlap, a co-localization feature was applied, that color-codes only regions in which both channels overlap at a threshold intensity level. Intensity of the fluorescent signal was measured in an arbitrary scale ranging from 0 (no signal) to 250 (highest signal) with respect to the individual image intensity profile. The intensity level was defined individually for each dataset using the integrated co-localization software and an overlap coefficient of 1 (Manders et al., 1993).

Statistical analyses

Statistically significant differences in different treatment groups were determined by analysis of variance (ANOVA) with Bonferroni correction using program 7D of the BMDP Statistical Software package developed by the UCLA Biomedical Computing Series. A p value <0.05 was considered significant.

RESULTS

GFAP-TH expression plasmid activity in U87 human glioma cells targeted with the HIRMAb-PIL

Human U87 cells express GFAP (Mandil et al, 2001) and also support TH gene expression in cell culture (Zhang et al, 2003a). Therefore, the biological activity of the GFAP-TH expression plasmid (clone 951) was measured in cultured U87 cells at 2, 4, and 6 days after the single application at day 0 of the plasmid DNA encapsulated in HIRMAb-targeted PILs. Clone 951 is well expressed, and the level of TH enzyme activity in the cells is comparable whether the TH gene is under the influence of either the SV40 promoter or the GFAP promoter (Table 1).

TH enzyme activity in brain and peripheral organs in lesioned rats treated with either the SV40-TH or the GFAP-TH expression plasmid targeted with the TfRMAb-PIL

The intra-cerebral injection of 6OHDA caused a 98% reduction in TH enzyme activity in the ipsilateral striatum compared to the contralateral or non-lesioned striatum (Table 2, saline treated rats). The TH enzyme activity in the cortex ipsilateral or contralateral to the lesion was no different, and was 98% reduced compared to the TH activity in the contralateral striatum (Table 2). The 6OHDA-lesioned, apomorphine-responsive rats were treated with SV40-TH gene therapy using clone 877 plasmid DNA encapsulated in the TfRMAb-targeted PIL. At 3 days following the single intravenous administration of 10 μg/rat of the clone 877 plasmid DNA, the TH activity in the ipsilateral or lesioned striatum was normalized and was not significantly different from the TH enzyme activity in the contralateral striatum (Table 2). SV40-TH gene

therapy caused no increase in TH in the cortex in brain, but did cause a 10-fold increase in TH enzyme activity in the liver (Table 2). In contrast, there was no increase in hepatic TH enzyme activity after intravenous administration of the GFAP-TH (clone 951) encapsulated in the TfRMAb-targeted PIL (Table 2). However, treatment with the GFAP-TH gene, similar to the SV40-TH gene, caused a complete normalization of the TH enzyme activity in the ipsilateral striatum of the 6OHDA lesioned, apomorphine-responsive rats (Table 2).

Reversal of motor impairment following GFAP-TH gene therapy

The lesioned rats that responded to apomorphine were separated into 2 groups and treated with clone 951 plasmid DNA (10 μg/rat) encapsulated in either mIgG2a-targeted PILs or TfRMAb-targeted PILs, and apomorphine-induced rotation behavior was measured in individual rats before and 3 days after intravenous gene therapy (Figure 2A,B). The apomorphine-induced rotation (mean RPM of 4 ± 3) in the rats treated with clone 951 encapsulated in the TfRMAb-PIL was reduced 82% compared to the apomorphine-induced roation (mean RPM of 22 ± 3) in the rats treated with clone 951 encapsulated in the mIgG2a-PIL (Figure 2C).

Neuronal expression of immunoreactive TH following GFAP-TH gene therapy

TH immunocytochemistry of coronal sections of rat brain is shown in Figure 3. There was complete normalization of the immunoreactive TH in the striatum of 6OHDA lesioned, apomorphine-responsive rats at 3 days after a single intravenous injection of clone 951 plasmid DNA (10 μg/rat) encapsulated in the TfRMAb-targeted PIL (Figure 3A, B, C). However, there was minimal immunoreactive TH detected in the striatum of 6OHDA lesioned, apomorphine-responsive rats at 3 days after a single intravenous injection of clone 951 plasmid DNA (10

μg/rat) encapsulated in the mIgG2a-targeted PIL (Figure 3D, E, F). The marked reduction in immunoreactive TH in the striatum of these rats correlates with the 98% reduction in striatal TH enzyme activity in the lesioned animals (Table 2).

The cellular origin of TH gene expression in brain of lesioned rats treated with the clone 951 DNA encapsulated in TfRMAb-PILs or mIgG-PILs was examined by confocal microscopy (Figures 4-5). The TH gene was selectively expressed in nerve terminals in the striatum on the side contralateral to the lesion with no overlap with NeuN immunoreactive neuronal cell bodies (Figure 4A). Virtually all of the nerve terminals were negative for TH in the ipsilateral striatum of lesioned rats treated with clone 951 encapsulated in the mIgG2a-PIL (Figure 4B). Conversely, the density of TH reactive nerve terminals in the ipsilateral striatum of lesioned rats treated with the clone 951 DNA encapsulated in the TfRMAb-targeted PIL was no different than in the contralateral, non-lesioned striatum (Figure 4A, C). The nerve terminals in the contralateral striatum that were immuno-positive for TH were generally immuno-negative for the 200 kDa neurofilament protein (Figure 4D), and there was preservation of the 200 kDa neurofilament fibers in the ipsilateral striatum of lesioned rats treated with the clone 951 encapsulated in the mIgG2a-PIL (Figure 4E). Conversely, the pattern of nerve terminals in the ipsilateral striatum of lesioned rats treated with clone 951 encapsulated in the TfRMAb-PIL was comparable to that in the contralateral striatum (Figure 4D, F).

The parallel expression of immunoreactive TH, GFAP, and NeuN in the striatum and substantia nigra is shown in Figure 5 for regions of brain ipsilateral to the lesion and in rats treated with the clone 951 plasmid DNA and encapuslated in the TfRMAb-PIL. For the striatum, separate views are shown for GFAP and TH immuno-staining in Figure 5A and B. The overlap image in Figure 5C indicates there is no co-expression of the TH in the GFAP-positive

astrocytes. Conversely, parallel immuno-staining for TH and NeuN in the substantia nigra shows there is expression of immunoreactive TH in NeuN immuno-reactive neuronal cell bodies (Figure 5 D, E, and F).

DISCUSSION

The results of these studies are consistent with the following conclusions. First, the ectopic expression of the TH gene in peripheral tissues such as liver following intravenous gene therapy is eliminated when the TH gene is under the influence of a brain specific promoter, such as the GFAP promoter (Table 2). Second, intravenous GFAP-TH gene therapy with clone 951 (Figure 1A) and the PIL gene targeting technology (Figure 1B) causes a normalization of striatal TH in the 6OHDA lesioned rat, but this is associated with no change in cortical TH (Table 2, Figure 3). Third, the normalization of striatal TH with intravenous GFAP-TH gene therapy is associated with an 82% reduction in apomorphine-induced rotation behavior (Figure 2). Fourth, the GFAP-TH gene is selectively expressed in neurons, not astrocytes, of the nigral-striatal tract (Figures 4 and 5).

The stability of the TH enzyme is linked to the availability of the biopterin cofactor, and the expression of the TH enzyme is found only in those regions of brain that express GTPCH, the rate-limiting enzyme in biopterin biosynthesis (Nagatsu et al, 1995; Hwang et al, 1998; Shimoji et al, 1999). However, GTPCH is also expressed in some peripheral organs such as liver (Nagatsu et al, 1997), which supports TH gene expression following intravenous gene therapy with an exogenous TH gene under the influence of a widely expressed promoter, such as the SV40 promoter. There is a 10-fold increase in hepatic TH activity in rats treated with clone 877, the SV40-TH, that is encapsulated in the TfRMAb-targeted PIL (Table 2). However, the ectopic expression of the TH gene in liver is eliminated with the use of a brain-specific promoter, such as the GFAP promoter (Table 2). These results parallel prior observations on β-galactosidase reporter gene expression. If the β-galactosidase gene was under the influence of the SV40 promoter, and was administered intravenously encapsulated in a TfRMAb-targeted PIL, then the

gene was expressed in both brain and TfRMAb-positive peripheral organs such as liver (Shi et al, 2001a,b). However, β-galactosidase gene expression in peripheral organs was eliminated if the gene was under the influence of the GFAP promoter (Shi et al, 2001a).

The PIL is able to deliver the TH gene to hepatocytes because the hepatic microcirculation is a sinusoidal network of highly porous capillaries. Conversely, peripheral organs such as heart or kidney are perfused by continuous endothelial barriers that block the egress into the organ of the circulating PIL. Unlike capillaries in the brain, which express high levels of the TfR (Jefferies et al, 1984; Pardridge et al, 1987), the endothelium of most peripheral organs do not express sufficient amounts of TfR to enable trans-capillary transport of the PIL. Consequently, there is no gene expression in these organs for either TH (Table 2) or for reporter genes such as β-galactosidase or luciferase (Shi et al, 2000; 2001a,b).

The GFAP-TH gene is expressed in neurons in the nigral-striatal tract following the intravenous administration of the clone 951 DNA encapsulated in the TfRMAb-targeted PIL, as demonstrated by confocal microscopy (Figure 4 and 5). Conversely, neuronal TH expression is not observed in the cortex based on either measurements of TH enzyme activity (Table 2) or by immuncytochemistry (Figure 3). In addition, the confocal microscopy shows that neuronal tracts immuno-positive for the 200 kDa neurofilament protein do not express TH in the striatum of the treated rat (Figure 4F). Neuronal expression of the TH transgene in the nigro-striatal tract is also indicated by the 82% reduction in apormorphine-induced rotation behavior following intravenous administration of the GFAP-TH in the TfRMAb-PIL (Figure 2). The ability of intravenous TH gene therapy to cause such a remarkable normalization of nigral-striatal TH expression in the 6OHDA lesioned model is due to the immediate regeneration in this pathway following neurotoxin administration. Providing at lease 25% of the nigral neurons survive the

chemical lesion, which is the case for the moderate dose (8 μg) of 6OHDA used in this study (Methods), there is intense sprouting of surviving neurons from the substantia nigra to the striatum following the lesion (Finkelstein et al, 2000; Parish et al, 2002). This sprouting continues until the density of the dopaminergic neurons, as measured by immunoreactive dopamine transporter, in the striatum is normal. Therefore, when the TH expression plasmid is delivered to substantia nigral neurons at 4 weeks after the lesion, the enzyme may be expressed in these cell bodies (Figure 5F) and transported to the regenerated terminals in the striatum (Figure 4C, 4F).

The expression of the GFAP-TH gene in brain is confined to neurons with no expression in astrocytes (Figures 4 and 5). The 5'-FS of the GFAP gene confers brain specificity of gene expression, but does not restrict gene expression to astrocytes (Kaneko and Sueoka, 1993; Galou et al, 1994). Astrocyte-specific expression requires the coordinate interactions of regulatory elements in both the 5'-FS and more distal parts of the gene, including the 3'-FS (Kaneko and Sueoko, 1993). Recent work in transgenic models demonstrate that the 5'-FS of the GFAP gene enables widespread neuronal expression of transgenes in brain (Zhuo et al, 2001). These findings parallel other observations that neurons secrete trans-acting factors that interact with the 5'-FS of the GFAP gene (Carvalho et al, 1999). The 5'-FS of the GFAP gene is completely methylated in peripheral tissues such as spleen but is hypo-methylated in neurons and astrocytes (Condorelli et al, 1997). In the presence of the entire GFAP gene, the neuron-suppressing elements in the 3'-FS prevent GFAP gene expression in neurons in brain in vivo (Kaneko and Sueoka, 1993). However, in the absence of the 3'-FS, the GFAP promoter can be used to direct brain specific expression of exogenous genes in neurons (Zhuo et al, 2001). The GFAP promoter also enables gene expression in astrocytes (Brenner et al, 1994). However, no TH gene

expression in astrocytes was detected in this study (Figure 5A-C). Similarly, the TH gene expression in cortical neurons was minimal (Figure 3). Neither astrocytes or cortical neurons express the GTPCH gene, and do not produce the biopterin cofactor necessary for TH enzyme activity (Nagatsu et al, 1995; Hwang et al 1998).

In summary, the present study demonstrates transduction of the entire striatum with TH gene therapy in the 6OHDA lesioned rat brain. The global expression of the TH gene in the entire striatum is possible because the exogenous gene encapsulated in PILs is delivered to brain via the trans-vascular route across the BBB. With transvascular approach to brain gene therapy, virtually every neuron in the brain is accessible to the exogenous gene following intravenous administration in either rodents (Shi et al, 2001a,b) or primates (Zhang et al 2003c). Ectopic expression of the TH gene in peripheral organs such as liver is eliminated with the combined use of a brain specific promoter and the PIL gene targeting technology. Brain TH gene expression is reversible and declines 50% at 6 days after a single intravenous administration (Zhang et al, 2003a). Therefore, chronic TH gene therapy requires repeat administration at periods determined by the persistence in brain of plasmid gene expression. Weekly intravenous brain antisense gene therapy resulted in a 100% increase in survival time in mice with intra-cranial brain cancer (Zhang et al, 2002b). Chronic TH gene with TfRMAb-targeted PILs has no toxic side effects and causes no change in serum chemistry, organ histology, or body weights, and induces no inflammatory reactions in brain (Zhang et al 2003).

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Table 1. Tyrosine hydroxylase (TH) activity in cultured U87 glioma cells following delivery of either SV40 promoter (clone 877) or GFAP promoter (clone 951) TH expression plasmid encapsulated in HIRMAb-targeted PIL

Days of incubation	TH activity (pmol L-Dopa/hour/mg_p)	
	Clone 877	clone 951
2	214 ± 14	231 ± 10
4	1458 ± 99	1576 ± 33
6	177 ± 10	311 ± 22

Mean ± S.E. (n= 3 dishes per time point). Clone 877 data from Zhang et al (2003a).

Table 2. Tyrosine hydroxylase in brain and peripheral organs in the rat 3 days after intravenous injection of gene therapy

Organs	Saline (pmol/hr/mg_p)	TfRMAb-PIL/877 (pmol/hr/mg_p)	TfRMAb-PIL/951 (pmol/hr/mg_p)
Ipsilateral striatum	128 ± 27	5177 ± 446**	5536 ± 395**
Contralateral striatum	6445 ± 523	5832 ± 391	5713 ± 577
Ipsilateral cortex	176 ± 30	132 ± 16	184 ± 38
Contralateral cortex	150 ± 36	150 ± 24	135 ± 25
Heart	29 ± 3	45 ± 8	31 ± 3
Liver	13 ± 2	130 ± 28**	18 ± 6
Lung	42 ± 13	74 ± 22	30 ± 6
Kidney	24 ± 2	35 ± 5	31 ± 8

**$p < 0.01$ difference from saline group (ANOVA with Bonferroni correction; n = 4 rats per group). Rats were lesioned with intra-cerebral injections of 6-hydroxydopamine; 3 weeks after toxin injection the rats were tested for apomorphine-induced rotation behavior; those rats testing positively to apormorphine were selected for gene therapy, which was administered intravenously 4 weeks after toxin administration; all animals were euthanized 3 days after gene administration

Figure 1. (A) Production of clone 951 from clone 877 and pGfa2-cLac. Clone 877 is an SV40 promoter (pro) driven rat tyrosine hydroxylase (TH) expression plasmid derived from pGL2 as described previously (Zhang et al 2003a). PGfa2-cLac is a human glial fibrillary acidic protein (GFAP) promoter driven β-galactosidase expression plasmid (Brenner et al, 1994; Segovia et al, 1998). The rat TH cDNA was released from clone 877 with NheI and HindIII, in parallel with the PCR-amplification of the GFAP promoter with an NheI 5'-primer and an HindIII-3' primer. The SV40 promoter of clone 877 was replaced with the GFAP promoter to produce clone 951. Both clones 877 and 951 contain a 200 nucleotide sequence within the 3'untranslated region (UTR), which is taken from the 3'-UTR of the bovine GLUT1 glucose transporter mRNA, and which optimizes gene expression via mRNA stabilization (Boado and Pardridge, 1998; Zhang et al, 2003a). (B) Diagram of a super-coiled TH expression plasmid encapsulated in an 85 nm pegylated immunoliposome (PIL) targeted to the rat transferrin receptor (TfR) with the OX26 murine monoclonal antibody (MAb).

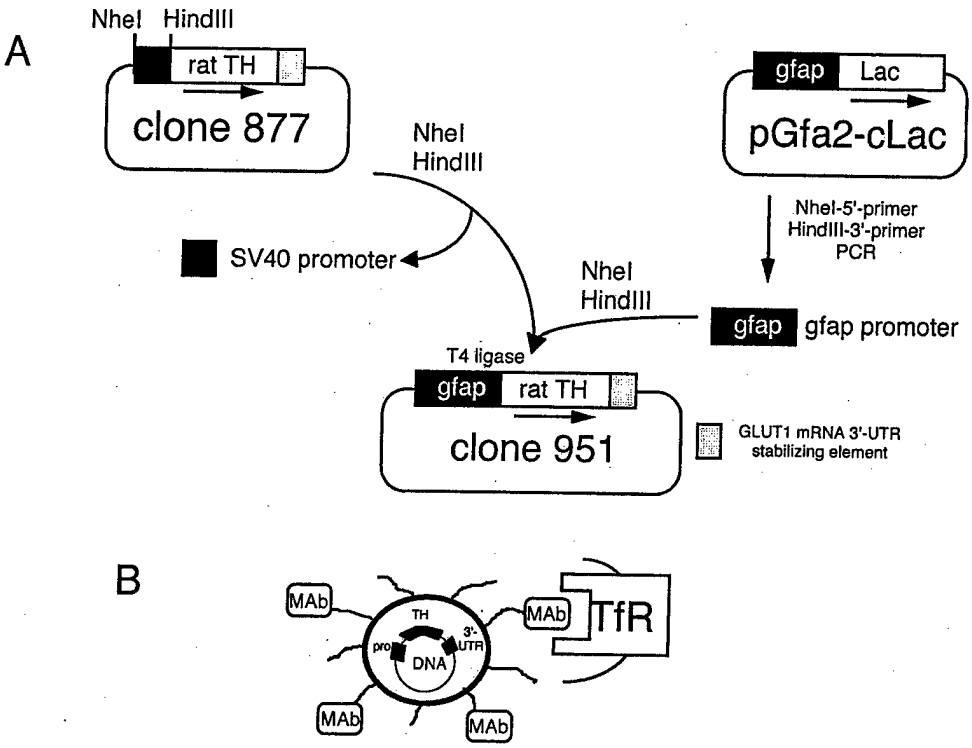
Figure 2. (A) Apomorphine-induced rotations per minute (RPM) over a 20 minute period measured in individual rats at 1 week before treatment and at 3 days after a single intravenous injection of 10 μg per rat of clone 951 plasmid DNA encapsulated in a PIL targeted with the mouse IgG2a isotype control antibody. (B) Apomorphine-induced RPM over a 20 minute period measured in individual rats at 1 week before treatment and at 3 days after a single intravenous injection of 10 μg per rat of clone 951 plasmid DNA encapsulated in a PIL targeted with the TfRMAb. (C) Comparison of the total rotations in the 2 groups at 3 days after treatment. The average RPM is 22 ± 3 and 4 ± 3 (mean ± SD) in animals treated with the mIgG2a-PIL and the

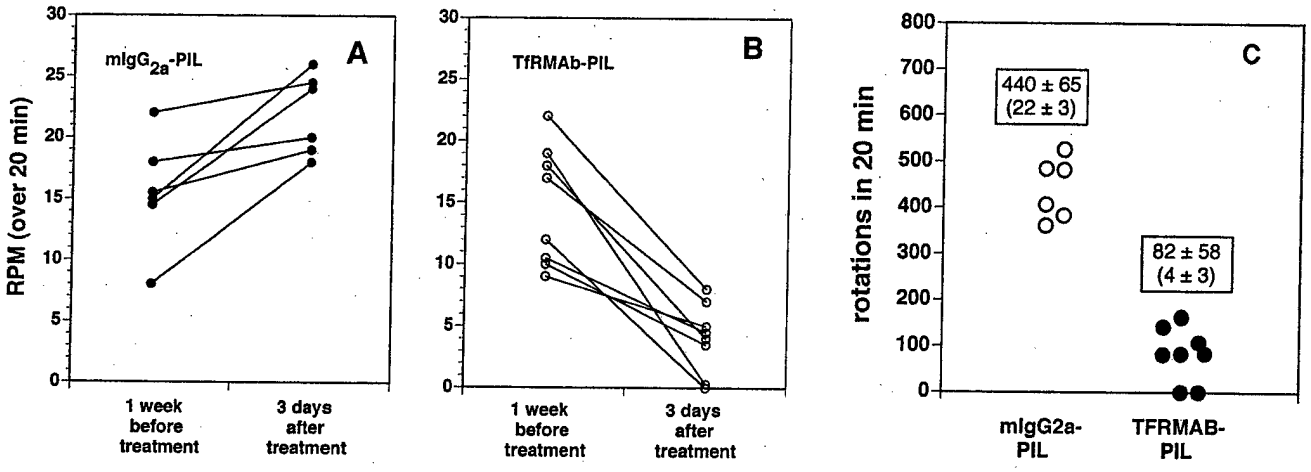
TfRMAb-PIL, respectively. The difference in rotation between the 2 groups is significant at the $p < 0.005$ level.

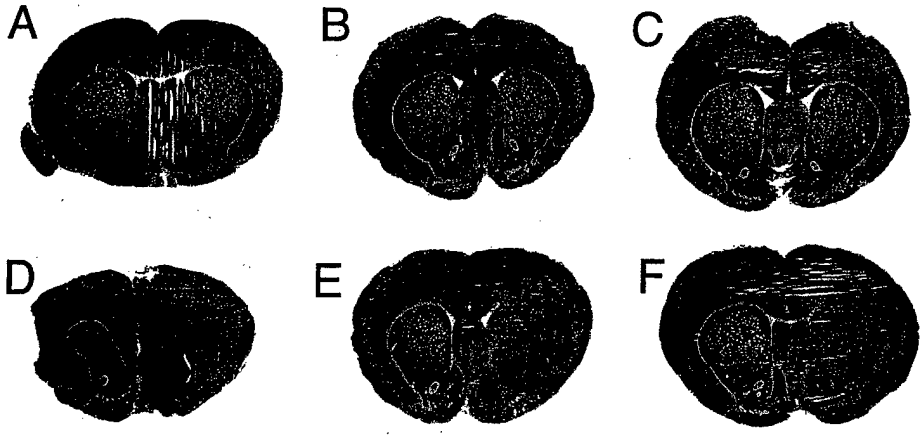
Figure 3. Tyrosine hydroxylase immunocytochemistry of rat brain removed 72 hours after a single intravenous injection of 10 ug per rat of clone 951 plasmid DNA encapsulated in PIL targeted with either the TfRMAb (Panels A, B, C) or with the mouse IgG2a isotype control (Panels D, E, F). Coronal sections are shown for 3 different rats from each of the two treatment groups. The 6-hydroxydopamine was injected in the medial forebrain bundle of the right hemisphere, which corresponds to right side of the figure. Sections are not counterstained.

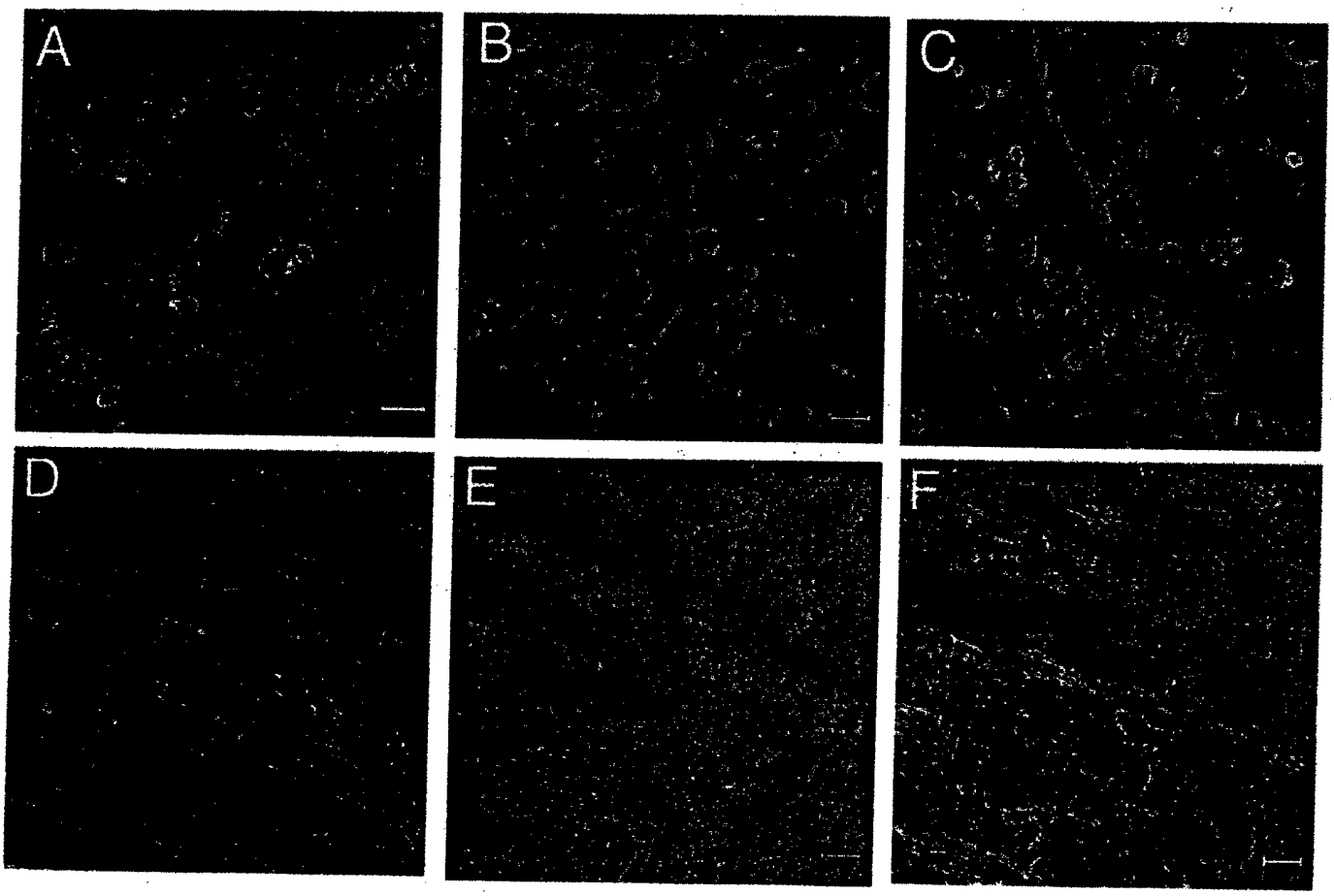
Figure 4. Confocal microscopy of striatum in 6OHDA-lesioned rats sacrificed at 3 days after intravenous injection of clone 951 plasmid DNA encapsulated in PILs targeted either with mouse IgG2a (panels B and E) or with the TfRMAb (panels A, C, D, and F). Panels A and D are from the striatum contralateral to toxin injection, and panels B, C, E, and F are from the striatum ipsilateral to toxin injection. Panels A-C show striatum co-labeled with a mouse monoclonal antibody to NeuN (green) and a rabbit polyclonal antibody to TH (red). Panels D-F show striatum co-labeled with a mouse monoclonal antibody to the 200 kDa neurofilament protein (green) and a rabbit polyclonal antibody to TH (red). All images were taken with a 40X objective, and the magnification bar in panel A is 20 μm. All images are 3-dimensional projection views of multiple planar images. The yellow color is an artifact from the 3-dimensional projection as there was no overlap observed in the single planar views.

Figure 5. Confocal microscopy of striatum (A-C) and substantia nigra (D-F) ipsilateral to the 6OHDA lesion in rats sacrificed at 3 days after intravenous injection of clone 951 plasmid DNA encapsulated in PILs targeted with the TfRMAb. Panels A and D show immune staining (green channel) with monoclonal antibodies to GFAP and NeuN, respectively. Panels B and E show immune staining (red channel) with a rabbit polyclonal antibody to TH. The overlap image of TH and GFAP in striatum is shown in panel C; the overlap image of TH and NeuN in substantia nigra is shown in panel F. Panels A-F were photographed with a 40X objective, whereas the size of panels D-F was increased with a 2X zoom. The inset of panel F is a 100X oil immersion view of co-labeling of TH (red), NeuN (green), and the overlap (yellow) in a neuron in the substantia nigra. The magnification bars in panels A and D are 20 and 10 μm, respectively. All images are 3-dimensional projection views of multiple planar images. The yellow color is an artifact from the 3-dimensional projection as there was no overlap observed in the single planar views.


A
NheI HindIII
rat TH
clone 877
NheI
HindIII
SV40 promoter
gfap Lac
pGfa2-cLac
NheI-5'-primer
HindIII-3'-primer
PCR
NheI
HindIII
gfap gfap promoter
T4 ligase
gfap rat TH
clone 951
GLUT1 mRNA 3'-UTR
stabilizing element
B
MAb
MAb
TfR
TH
pro
DNA
3'-UTR
MAb
MAb


mIgG2a-PIL
A
RPM (over 20 min)
30
25
20
15
10
5
0
1 week before treatment
3 days after treatment
TfRMAb-PIL
B
C
rotations in 20 min
800
700
600
500
400
300
200
100
0
440 ± 65
(22 ± 3)
82 ± 58
(4 ± 3)
mIgG2a-PIL
TFRMAB-PIL


A
B
C
D
E
F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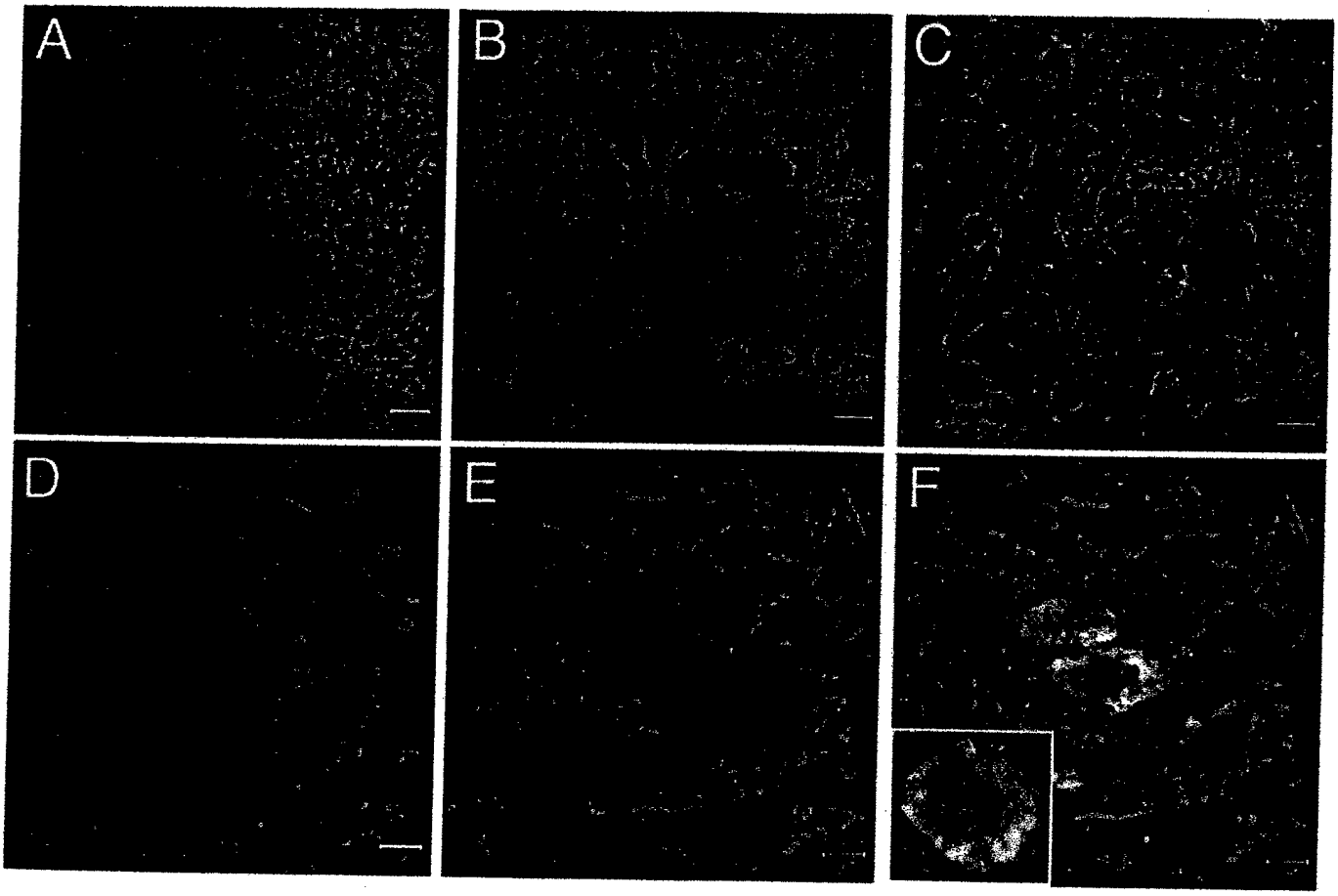
A
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