The Journal of Neuroscience, July 15, 1996, *16*(14):4449–4456

Double Transduction with GTP Cyclohydrolase I and Tyrosine Hydroxylase Is Necessary for Spontaneous Synthesis of L-DOPA by Primary Fibroblasts

Craig Bencsics,[1] Stephen R. Wachtel,[1] Sheldon Milstien,[2] Kazuyuki Hatakeyama,[3] Jill B. Becker,[4] and Un Jung Kang[1]

[1]*Departments of Neurology, Pharmacological and Physiological Sciences, and Committee on Neurobiology, The University of Chicago, Chicago, Illinois 60637,* [2]*Laboratory of Cell Biology, National Institute of Mental Health, Bethesda, Maryland 20892-4096,* [3]*Department of Surgery, University of Pittsburgh, Pittsburgh, Pennsylvania 15261, and* [4]*Department of Biopsychology, University of Michigan, Ann Arbor, Michigan 48104*

Gene transfer of tyrosine hydroxylase (TH) in animal models of Parkinson's disease (PD), using either genetically modified cells or recombinant virus vectors, has produced partial restoration of behavioral and biochemical deficits. The limited success of this approach may be related to the availability of the cofactor, tetrahydrobiopterin (BH_4), because neither the dopamine-depleted striatum nor the cells used for gene transfer possess a sufficient amount of BH_4 to support TH activity. To determine the role of BH_4 in gene therapy, fibroblast cells transduced with the gene for TH were additionally modified with the gene for GTP cyclohydrolase I, an enzyme critical for BH_4 synthesis. In contrast to cells transduced with only TH, doubly transduced fibroblasts spontaneously produced both BH_4 and 3,4-dihydroxy-L-phenylalanine. To examine further the importance of GTP cyclohydrolase I in gene therapy for PD, *in vivo* microdialysis was used to assess the biochemical changes in the dopamine-denervated striatum containing grafts of genetically modified fibroblasts. Only denervated striata grafted with fibroblasts possessing both TH and GTP cyclohydrolase I genes displayed biochemical restoration. However, no significant differences from controls were observed in apomorphine-induced rotation. This is partly attributable to a limited duration of gene expression *in vivo*. These differences between fibroblasts transduced with TH alone and those additionally modified with the GTP cyclohydrolase I gene indicate that BH_4 is critical for biochemical restoration in a rat model of PD and that GTP cyclohydrolase I is sufficient for production of BH_4.

Key words: tetrahydrobiopterin; Parkinson's disease; gene therapy; retrovirus vector; transplantation; catecholamine

Gene transfer technology has created new directions for investigation of CNS function and the potential for breakthroughs in therapy for many neurological disorders. By injection of viral vectors or transplantation of genetically engineered cells containing viral vectors into specific sites of the brain, the limitations of conventional experimental and therapeutic approaches can be overcome. Parkinson's disease (PD) is an attractive target for these new therapeutic approaches because the pathophysiology, degeneration of dopaminergic neurons of the substantia nigra, is well characterized. Furthermore, gene therapy with tyrosine hydroxylase (TH), the rate-limiting step in catecholamine biosynthesis, may avoid some complications associated with intermittent 3,4-dihydroxy-L-phenylalanine (L-DOPA) therapy by achieving continuous delivery of L-DOPA. Using this approach, several investigators have demonstrated partial restoration of abnormalities in rat models of PD (Wolff et al., 1989; Horellou et al., 1990; Fisher et al., 1991; During et al., 1994; Kaplitt et al., 1994).

For hydroxylation of tyrosine, TH requires Fe^{2+}, oxygen, and (6*R*)-(L-erythro-1′,2′-dihydroxypropyl)-2-amino-4-hydroxy-5,6,7,8-tetrahydropteridine (tetrahydrobiopterin, BH_4) as cofactors. Most cells previously used for gene transfer, including fibroblasts, do not synthesize BH_4. Furthermore, endogenous BH_4 levels in denervated striatum are insufficient to support TH activity (Levine et al., 1981; Nagatsu, 1983). Indicative of this problem, as reported by Uchida et al. (1992), exogenous BH_4 infusion is necessary for *in vivo* biochemical and functional effects of an immortalized fibroblast cell line transduced with TH. The critical role of BH_4 in PD can also be noted by the recent discovery of mutations in the GTP cyclohydrolase I gene, the rate-limiting step of BH_4 synthesis, in patients with hereditary progressive dystonia who manifest parkinsonian features (Ichinose et al., 1994). Yet, most recent investigations have focused primarily on modes of delivery, such as various types of cells and vectors, neglecting the issue of BH_4.

Clinically, long-term administration of exogenous BH_4 to PD patients is not practical because of poor penetration into the nervous system (Kapatos and Kaufman, 1981; Levine et al., 1987). Therefore, gene transfer of enzymes necessary to synthesize BH_4 could potentially provide a continuous and local source of BH_4. *De novo* biosynthesis of BH_4 requires three enzymes: GTP cyclohydrolase I (EC 3.5.4.16), 6-pyruvoyl-tetrahydropterin synthase, and sepiapterin reductase. Werner et al. (1990) found that

Received Dec. 13, 1995; revised March 26, 1996; accepted April 17, 1996.

This research was supported by R29 NS32080, Parkinson's Disease Foundation Junior Faculty Award, United Parkinson Foundation/the H.G. and Catharine Lieneman Memorial Fund, National Parkinson Foundation, Dystonia Medical Research Foundation, and Brain Research Foundation (U.J.K.). C.B. was supported by T32 NS07113, S.R.W. by T32 DA07255, and J.B.B. by NS22157. The full-length cDNA for human TH was kindly provided by Dr. K. O'Malley at Washington University, pLNCX by Dr. A. D. Miller at University of Washington, and pΔgHC by Immunex Corp. We thank Drs. G. Craviso and G. Kapatos for their advice on biochemical assays, and Dan Young, Anne Cahill, and Georgette Vosmer for their technical assistance.

Correspondence should be addressed to Dr. Un Jung Kang, Department of Neurology, MC 2030, University of Chicago, 5841 South Maryland Avenue, Chicago, IL 60637.

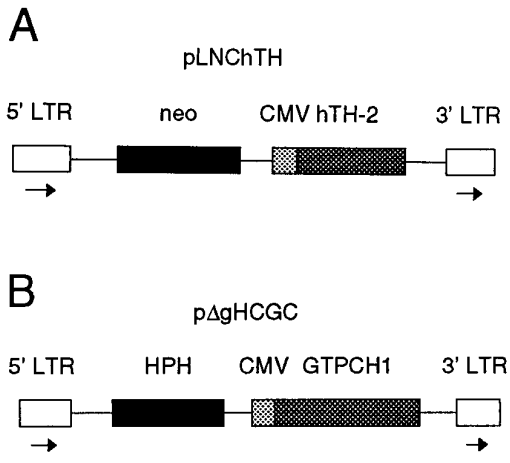

Figure 1. Schematic of the retroviral vector plasmids. *A*, pLNChTH contains the cDNA for human tyrosine hydroxylase type 2 (*hTH-2*) under the control of a cytomegalovirus promoter (*CMV*) and the selectable marker aminoglycoside phosphotransferase (*neo*) under the control of the retroviral long terminal repeat (*LTR*). *B*, pΔgHCGC contains the rat GTP cyclohydrolase I (*GTPCH1*) cDNA and the selectable marker hygromycin-B-phosphotransferase (*HPH*) within the same backbone as pLNChTH.

6-pyruvoyl-tetrahydropterin synthase and sepiapterin reductase are constitutively expressed in fibroblasts, whereas the rate-limiting enzyme for BH_4 synthesis, GTP cyclohydrolase I, was not. Thus, in the present study, we focused on determining genes necessary for synthesis of L-DOPA. To accomplish this, primary rat fibroblasts were genetically engineered with retroviral vectors to produce TH and BH_4 by double transduction with the cDNAs for human TH (Grima et al., 1987) and rat GTP cyclohydrolase I (Hatakeyama et al., 1991). We demonstrate that these fibroblasts spontaneously synthesize L-DOPA and BH_4 *in vitro* and that transplantation of these cells leads to enhanced TH function *in vivo*.

MATERIALS AND METHODS

Retroviral vector construction. The 1.7 kb *Bst*XI–*Hin*dIII-digested fragment of the human tyrosine hydroxylase type 2 (hTH2) cDNA (Ginns et al., 1988) was inserted into the *Hin*dIII–*Cla*I sites of a Moloney murine leukemia virus-derived plasmid, pLNCX (Miller and Rosman, 1989), downstream from the cytomegalovirus promoter (CMV) (Fig. 1*A*). The 0.9 kb *Bam*HI–*Hin*cII blunt-ended fragment of rat GTP cyclohydrolase I cDNA (Hatakeyama et al., 1991) was inserted into the *Bgl*II–*Cla*I blunt-ended sites of pΔgHC under the control of the CMV promoter (Fig. 1*B*). Whereas pΔgHC contains the gene for hygromycin-B-phosphotransferase (HPH) as a selection marker, pLNCX contains the gene for aminoglycoside phosphotransferase (*neo*) (Fig. 1). Production of viral producers was described previously (Kang et al., 1993). All selected producer clones had a titer of $>1 \times 10^5$ pfu/ml.

Transduction of primary fibroblasts with TH and GTP cyclohydrolase I. Isolation and culture of fibroblasts from adult female Fischer 344 rats were described previously (Kang et al., 1993). Primary fibroblasts (PF) were first infected with retroviruses expressing TH (LNChTH; Fig. 1*A*) and selected in the presence of 400 μg/ml G418. The cells surviving selection were pooled, and PFTH was established. To produce a cell line transduced with both TH and GTP cyclohydrolase I, PFTH was infected with retroviruses expressing GTP cyclohydrolase I (ΔgHCGC; Fig. 1*B*). These cells were doubly-selected in media containing G418 and hygromycin (150 μg/ml). The surviving cells were pooled and expanded into PFTHGC.

Immunohistochemical staining. Cells were plated in 8-well chamber slides. After attachment, the cells were fixed with 4% paraformaldehyde in 0.1 M PBS and immunostained with a polyclonal antibody against rat TH (Pel-freeze, Rogers, AR) at a dilution of 1:500 and a biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) as a secondary antibody. The signal was amplified by avidin and biotinylated horseradish peroxidase and the cells stained by adding 3,3′-diaminobenzidine tetrachloride dehydrate and hydrogen peroxide.

Biochemical assays. TH activity was measured according to the method of Nagatsu et al. (1964) as modified by Reinhard et al. (1986). Briefly, cells (5×10^6) were lysed by sonication in 10 mM phosphate buffer, pH 6.0, and 0.2% Triton X-100 and centrifuged at 12,300 × *g* for 10 min. The amount of protein in the supernatant was quantified with a Bio-Rad (Hercules, CA) protein determination kit. The assay contained 0.5 μCi of [^{3}H]tyrosine (51 Ci/mmol; Amersham, Arlington Heights, IL) and unlabeled tyrosine for a total of 167 μM, 100 μM DL-6-methyl-5,6,7,8-tetrahydropterin, 50 mM 2-(*N*-morpholino) ethanesulfonic acid (MES), pH 6.0, 5 mM dithiothreitol, and 3000 U/ml catalase in a 100 μl total volume and was incubated for 20 min at 37°C. The reaction was stopped by adding 1 ml of 7.5% charcoal in 1N HCl. The charcoal was pelleted, and the supernatant was collected for scintillation counting. The K_m of TH for BH_4 was determined by measuring TH activity in reactions containing varying amounts of BH_4 (0–200 μM). TH activity was measured as described above except that the reaction was incubated for 7 min.

GTP cyclohydrolase I activity was measured by modification of a previously described method (Duch et al., 1984). In brief, 7,8-dihydroneopterin triphosphate produced from GTP was oxidized with iodine to neopterin triphosphate and dephosphorylated with alkaline phosphatase to neopterin, which was then quantified by reverse-phase HPLC with fluorescence detection (Sakai et al., 1993).

For intracellular tetrahydrobiopterin measurements, adherent cells were harvested in 500 μl of 0.4 M perchloric acid (PCA)/0.1 mM EDTA and frozen at −80°C for HPLC analysis. The concentration of fully reduced tetrahydrobiopterin was determined by differential iodine oxidation in acid and base according to the methods of Fukushima and Nixon (1980). For L-DOPA measurements, confluent cells were incubated in Earl's balanced salt solution (EBSS) with 25 mM HEPES and 100 mM EDTA for 30 min at 37°C. The media and the cells were harvested in PCA solutions and frozen until analysis. The levels of L-DOPA in cells and media were analyzed by reverse-phase HPLC using a C18 column and an ESA Coulochem II electrochemical detector.

Surgical procedures: dopamine denervation and grafting. For all stereotaxic surgical procedures, female Fischer 344 rats (150–200 gm) were anesthetized with a mixture of ketamine (75 mg/kg), xylazine (3.8 mg/kg), and acepromazine (0.75 mg/kg). As described previously (Lucidi-Phillipi et al., 1995), 8 μg (freebase weight) of 6-hydroxydopamine (6-OHDA) was infused in 2 μl at the following coordinates: AP −4.4 mm, ML 1.2 mm relative to bregma, and DV −7.5 mm from dura (Paxinos and Watson, 1986). To prevent destruction of noradrenergic neurons, desipramine (12.5 mg/kg, i.p.) was administered 30 min before the infusion of 6-OHDA. Animals were allowed to recover 2–3 weeks before the cells were implanted. Only those rats with ipsilateral rotations of >400/hr induced by amphetamine (5 mg/kg, i.p.) were used for grafting experiments.

For grafting, PF, PFTH, or PFTHGC cells were washed, trypsinized, and suspended in Dulbecco's PBS. L-DOPA synthesis by PFTHGC cells was checked each time cells were grafted. Grafting was done only with cells below passage 15, because primary cells may senesce and survive poorly at higher passages.

Behavioral experiments. In this experiment, fibroblast cells were grafted at eight sites. One microliter of cell suspension (50,000 cells/μl) was infused at each site in dorso-lateral striatum (AP 1.0, ML 2.0 and 3.5, DV 5.0 and 4.0; AP 0.0, ML 2.5 and 4.0, DV 5.0 and 4.0) for a total of 400,000 cells per animal. To assess their response to changes induced by the transplantation of the genetically modified fibroblasts, animals were tested with apomorphine (0.05 mg/kg, s.c.) before and at 1, 2, and 3 weeks after grafting. An automated rotometer (San Diego Instruments, San Diego, CA) was used to record the number of rotations per hour. Data were analyzed by two-way ANOVA with repeated measures.

Microdialysis experiments. In this study, fibroblasts were grafted at four sites. One microliter of the fibroblast suspension (75,000 cells/μl) was infused (0.5 μl/min) at each site in the striatum [AP 1.5 and −0.5 mm, ML ±2.8 mm relative to bregma, and DV −4.0 and −3.5 mm below dura (Paxinos and Watson, 1986)] for a total of 300,000 cells per animal. Microdialysis probes, of vertical concentric design, with a 2 mm active area, were calibrated *in vitro* for relative recovery to assure consistency, but the data were not corrected for recovery (Wachtel and Abercrombie, 1994). A microdialysis probe was implanted into the 6-OHDA-denervated striatum equidistant from the grafts at the following coordinates: AP 0.5 mm, ML ±2.5 mm relative to bregma, and DV −5.5 mm

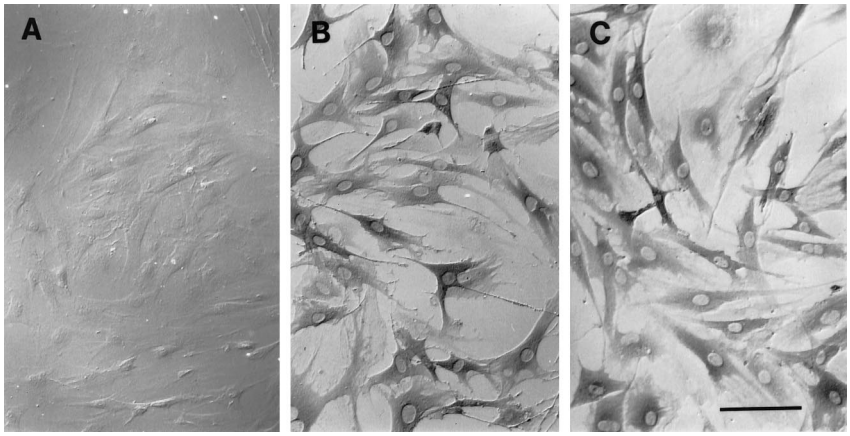

Figure 2. Immunostaining with polyclonal antibody against TH. *A*, Unmodified primary fibroblast cells (PF). *B*, Fibroblasts transduced with LNChTH (PFTH). *C*, Fibroblasts doubly transduced with LNChTH and ΔgH-CGC (PFTHGC). Scale bar, 100 μm.

below dura (Paxinos and Watson, 1986). Artificial CSF (147 mM NaCl, 2.5 mM KCl, 1.3 mM $CaCl_2$, and 0.9 mM $MgCl_2$, pH ~ 7.4) was perfused continuously through the probe at a rate of 1.5 μl/min. The microdialysis probe was left in place for 16–20 hr before dialysates were collected for HPLC analysis. NSD 1015 was administered intraperitoneally [100 mg/kg, dissolved in 0.9% (w/v) NaCl]. L-DOPA in dialysates (20 μl) was quantified at 15 min intervals by HPLC, as described above. Data were analyzed using ANOVA with repeated measures. Post hoc comparisons were conducted with Dunn's multiple comparisons test.

Time course experiments. Fibroblasts were grafted at eight sites. Two microliters of the cell suspension (75,000 cells/μl) were infused at each of four sites in the striatum (AP 1.2 and −0.3, ML 2.3 and 3.0, DV −4.0) and 1 μl at another four sites along the same needle tract (the same coordinates for AP and ML, DV −3.5) for a total of 900,000 cells per animal. As in the above microdialysis experiments, microdialysis probes were implanted equidistant between the graft sites (AP 0.45, ML 2.65, DV −5.5).

Immediately after all *in vivo* experiments, rats were anesthetized and transcardially perfused with 50 ml of saline followed by 250 ml of ice-cold 4% paraformaldehyde. Brains were removed, postfixed for 1 hr, and transferred to 30% sucrose until equilibrated. Thirty micrometer sections were cut and either stained with cresyl violet or immunoreacted for TH as described above.

RESULTS

In vitro characteristics of primary fibroblasts with TH and GTP cyclohydrolase I

Double transduction of primary fibroblast cells had no apparent effect on cell growth and morphology. All cell types (PF, PFTH, and PFTHGC) were tested for immunoreactivity with a TH-specific polyclonal antibody. Both PFTH and PFTHGC cells were immunoreactive for TH, whereas control PF cells were not (Fig. 2). The intensity of the staining for PFTH and PFTHGC cells in culture was stable throughout all passages (data not shown). Similarly, only PFTH and PFTHGC cells had significant TH activity (Table 1). The TH activities of these cells at difference passages ranged from 66.4 to 287.7 pmol/mg/min for PFTH cells and 198.5 to 430.3 pmol/mg/min for PFTHGC cells. Although we noticed some drift, the activity neither increased nor decreased consistently with increasing passage up to 27, the highest passage we have examined *in vitro*. A separate transduction could result in fibroblast cells with different transgene expression. Therefore, we used cells from the same infections whenever possible and monitored their transgene activities at regular intervals. The TH activity of PFTH and PFTHGC was comparable to bovine chromaffin cells (274.2 ± 72.8 pmol/mg/min), which have high endogenous TH expression. The K_m of TH was similar between PFTH and PFTHGC, suggesting that double transduction of cells or production of cofactor does not alter the biochemical properties of TH. Only PFTHGC cells had significant GTP cyclohydrolase I activity. Furthermore, expression of GTP cyclohydrolase I resulted in synthesis of biopterin, the majority of which was in the fully reduced form, BH_4 (Table 1). PFTHGC cells also produced significant amounts of L-DOPA; however, no detectable amount of L-DOPA was produced by PF or PFTH cells without addition of BH_4 (Table 1).

Cofactor requirement of fibroblasts with TH *in vitro*

To investigate the requirement for BH_4 in PFTH cells, L-DOPA was measured in the media of PFTH cells incubated with BH_4 (0–200 μM for 4 hr). In addition, intracellular BH_4 concentrations were measured in the cell pellets to estimate the amount of BH_4 taken up by the cells from the media. The BH_4 concentration in PFTH cells increased linearly as a function of the concentration of BH_4 in the media. In contrast, the level of L-DOPA in the media reached a plateau at a concentration of 50 μM BH_4 (Fig. 3*A*), indicating that TH was saturated at this concentration of cofactor. Similarly, L-DOPA synthesis by PFTHGC cells did not increase further with the addition of BH_4 (Fig. 3*B*), suggesting that the concentration of BH_4 within PFTHGC cells was enough to support maximal TH activity.

To confirm that synthesis of L-DOPA is specifically dependent on the activity of GTP cyclohydrolase I in PFTHGC cells, 2,4-diamino-6-hydroxypyrimidine (DAHP), a specific inhibitor of GTP cyclohydrolase I (Gal et al., 1978), was added to the culture

Table 1. Biochemical characterization of PF, PFTH, and PFTHGC cells

	Cell types		
	PF	PFTH	PFTHGC
TH activity (pmol/mg/min)	0.053 ± 0.57	148.2 ± 18.9 (n = 18)	271.6 ± 16.7 (n = 18)
K_m of TH for BH_4 (μM)	NA	110.98 ± 4.25	109.45 ± 5.25
GTPCH1 activity (pmol/mg/min)	0	0	12.6 ± 2.6
Total biopterin (pmol/10^6 cells)	0	0	139.2 ± 14
BH_4/total biopterin (%)	0	0	86.4 ± 1.8
L-DOPA in media (nmol/10^6 cells/hr)	0	0	2.16 ± 0.19

Biopterin production is expressed both as total biopterin and as the percentage of total biopterin fully reduced to BH_4. Values are the mean ± SEM from a representative set of multiple assays (n = 3 unless indicated otherwise). NA, Not applicable.

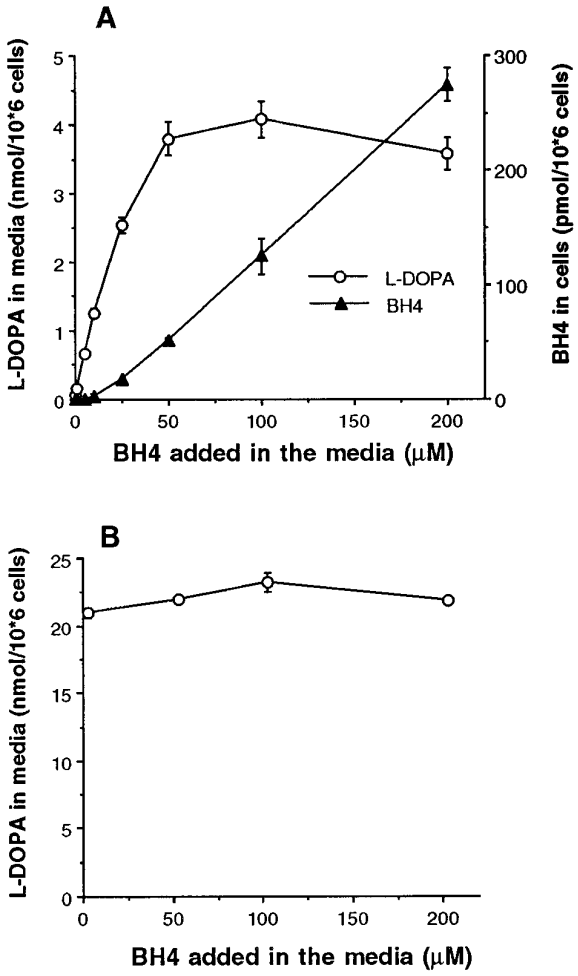

Figure 3. The effect of exogenous BH_4 in genetically modified fibroblast cells. *A*, Uptake of BH_4 and production of L-DOPA by primary fibroblasts transduced with hTH2 cDNA only (PFTH) incubated with exogenous BH_4. *B*, Production of L-DOPA by primary fibroblasts doubly transduced with hTH2 and rat GTP cyclohydrolase I cDNAs (PFTHGC). Confluent cells were incubated for 4 hr at 37°C in DMEM with 10% fetal calf serum with various concentration of cofactor (0–200 μM). Data represent mean ± SEM ($n = 3$).

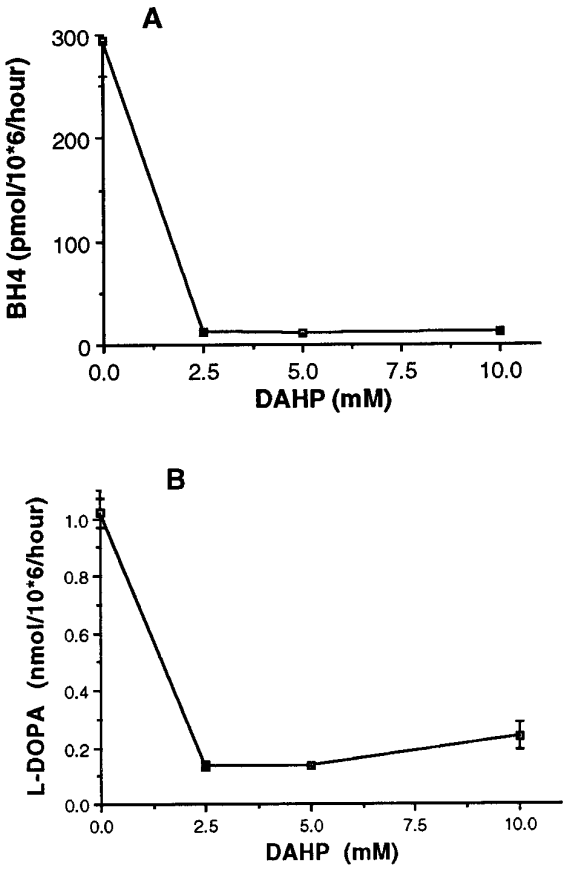

Figure 4. The effect of 2,4-diamino-6-hydroxy-pyrimidine (*DAHP*) on BH_4 production (*A*) and on L-DOPA production (*B*) in fibroblast cells cotransduced with TH and GTP cyclohydrolase I (PFTHGC). The cells were incubated for 1 hr at 37°C for the measurement. Data represent mean ± SEM ($n = 3$).

medium at concentrations of 2.5–10 mM for 19 hr at 37°C. GTP cyclohydrolase I inhibition by 2.5 mM DAHP reduced the level of BH_4 by 96% and L-DOPA by 86%, further indicating that GTP cyclohydrolase I activity is essential for BH_4 and L-DOPA production. Higher concentrations of DAHP did not further affect either L-DOPA or BH_4 production (Fig. 4).

Rotational behaviors of animals with fibroblasts grafted into 6-OHDA-denervated striatum

The three types of cells were grafted into the denervated striatum of rats with unilateral dopamine depletion. As noted previously (Kawaja and Gage, 1992; Lucidi-Phillipi et al., 1995), the genetically modified primary fibroblasts in the grafts survived well. There were no significant differences among all three types of genetically modified fibroblasts with respect to cell survival or graft size.

Rotation in response to apomorphine administration was monitored in animals grafted with the three types of fibroblast cells. Although there was no significant effect of the graft type ($F_{(2,18)} = 1.3239$) or interaction between graft type and time ($F_{(6,54)} = 0.2578$), all three groups showed moderate reduction of the rotations after grafting that persisted throughout the 3 week postgrafting period ($F_{(3,54)} = 8.2398$, $p < 0.001$) (Fig. 5).

In vivo biochemical characteristics of fibroblasts grafted into 6-OHDA-denervated striatum

The morphology of fibroblasts within a graft and a depiction of the relative location of the grafts and the microdialysis probe are shown in Figure 6. The *in vitro* differences in L-DOPA synthesis between the PFTH and PFTHGC cells were also apparent *in vivo*. The baseline dialysate level of L-DOPA was elevated only in the PFTHGC-grafted group. To assess further the *in vivo* function of these grafts, accumulation of L-DOPA was measured in dialysate after systemic administration of an aromatic L-amino acid decarboxylase (AADC) inhibitor, NSD 1015, 5 d after grafting. This method has the advantage of isolating *in vivo* TH activity from further metabolic steps such as AADC, monoamine oxidase (MAO), etc. (Westerink et al., 1990). In all groups, the increase in L-DOPA elicited by NSD 1015 peaked within 1 hr and decayed slowly for the duration of the experiment (4 hr) (Fig. 7). However, there was differential increase in L-DOPA levels induced by NSD 1015 among the three groups ($F_{(2,15)} = 59.43$, $p < 0.0001$). The group transplanted with PFTHGC cells displayed a significantly greater increase of L-DOPA than either the PFTH or the PF groups ($p < 0.01$). In contrast, in the group transplanted with PFTH, NSD 1015-induced L-DOPA accumulation was not signif-

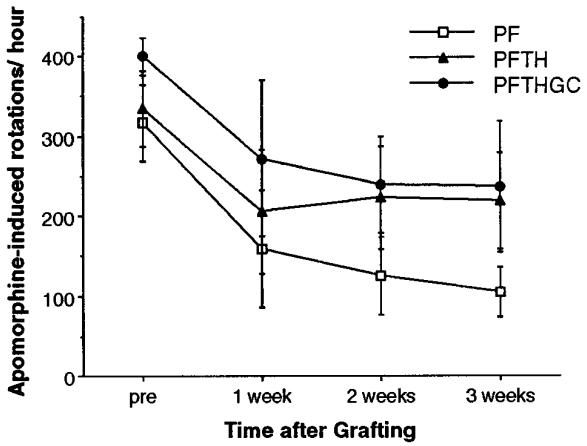

Figure 5. Apomorphine-induced contralateral rotation in unilateral 6-OHDA-lesioned rats with genetically modified grafts. The PF group had grafts of unmodified fibroblasts, PFTH had grafts of cells with the TH gene only, and PFTHGC had graft of cells with both TH and GTP cyclohydrolase I genes. Data represent mean ± SEM (n = 7).

icantly different from the control PF-implanted condition. The peak concentration of L-DOPA after NSD 1015 in the transplanted striatum was 47.4 ± 8.7 nM in the PFTHGC group, 2.6 ± 0.3 nM in the PF group, and 3.0 ± 0.4 nM in the PFTH group. In comparison, the peak concentration of L-DOPA after NSD 1015 in the intact striatum was 180.4 ± 11.8 nM.

In vivo characteristics of genetically modified grafts over time

To examine catecholamine production by PFTH and PFTHGC cells over time, L-DOPA, dopamine, dihydroxyphenyl acetic acid (DOPAC), and homovanillic acid (HVA) levels were measured at two time points, 4 and 14 d after grafting. L-DOPA, dopamine, DOPAC, and HVA were elevated only in dialysates from PFTHGC-grafted striata at 4 d after grafting. The PFTH-grafted striata showed no significant levels of L-DOPA, dopamine, DOPAC, or HVA at 4 d. Furthermore, by 14 d after grafting, the L-DOPA, dopamine, DOPAC, and HVA levels in PFTHGC-grafted striata decreased dramatically (Table 2). Immunostaining of both PFTH and PFTHGC grafts for TH showed immunopositive cells, but TH expression was more robust in PFTHGC grafts at 4 d after grafting (Fig. 8). However, by 14 d after grafting, immunostaining for TH was not consistently detectable (data not shown).

DISCUSSION

Expression of both TH and GTP cyclohydrolase I was achieved in primary fibroblasts by double transduction and selection with two different markers (G418 and hygromycin). By the nature of the bulk population of primary fibroblasts, there were some variations in the transgene expression, but they remained within a narrow range (Table 1). Transduction of PFTH cells with GTP cyclohydrolase I resulted in production of BH_4, further supporting the finding that the other enzymes in the biosynthetic pathway of BH_4 are constitutively expressed in fibroblasts (Werner et al., 1990). The amount of BH_4 in PFTHGC cells (Table 1) was comparable to the intracellular concentration in PFTH cells incubated with ~100 μM BH_4 and well above the concentration required for maximal production of L-DOPA (Fig. 3). Furthermore, whereas PFTH cells required addition of exogenous BH_4 to synthesize L-DOPA, fibroblasts transduced with both the TH and the GTP cyclohydrolase I genes synthesized L-DOPA spontaneously. The amount of L-DOPA produced by PFTHGC cells was similar to that synthesized by catecholamine cells and other genetically engineered cells incubated with exogenous BH_4 (Uchida et al., 1989; Wolff et al., 1989; Horellou et al., 1990; Ishii et al., 1990; Fisher et al., 1991; Owens et al., 1991; Anton et al., 1994).

Neither BH_4 nor L-DOPA production was detected from PFTH cells *in vitro.* This is consistent with previous data regarding a variety of cells genetically modified with only the cDNA for TH. These cell types include primary cells such as fibroblasts (Fisher et al., 1991; Kang et al., 1993), Schwann cells (Owens et al., 1991), astrocytes (Ridoux et al., 1994), and conditionally immortalized fetal ventral mesencephalic neuronal cells (Anton et al., 1994). Not all genetically engineered cells are devoid of BH_4. It has been shown that an endocrine tumor cell line can produce L-DOPA spontaneously (Horellou et al., 1990); however, immortalized cells or tumor cells are not suitable donor cells for gene therapy (Uchida et al., 1989; Wolff et al., 1989; Uchida et al., 1992). In contrast, primary fibroblasts provide an excellent vehicle for gene transfer, survive well in syngeneic rats, and do not lead to tumor formation (Fisher et al., 1991; Kang et al., 1993). Given the constitutive expression of the other enzymes in the pathway, one would predict that GTP cyclohydrolase I should be sufficient to achieve BH_4 production in other cell types as well including neuronal precursor cells.

The predominant indicator of the graft efficacy in unilaterally 6-OHDA-lesioned rats has been a reduction of apomorphine-induced rotation (Ungerstedt, 1971) because it is a relatively simple test to administer and there is a lack of better alternatives. Amphetamine-induced rotational response has been reported to be a better indicator of graft efficacy (Curran et al., 1993), but amphetamine does not release dopamine from most genetically modified cells. In the present experiments, all three groups including the control group showed significant reductions of rotational responses that persisted throughout the 3 week period, but there were no significant differences in reduction of apomorphine-induced rotations in either PFTH or PFTHGC group compared to the PF control. The reduction of the apomorphine-induced rotation by all the grafts is consistent with reports that damage induced by the grafts or the volume they occupy influences rotational responses (Barker and Dunnett, 1994; Isacson, 1995). In addition, apomorphine-induced rotation occurs as a result of postsynaptic changes that are not linear with respect to the degree of dopamine depletion (Hudson et al., 1993). Thus, changes in apomorphine-induced rotations after grafting may not accurately reflect biochemical restoration (Curran et al., 1993). Finally, our transgene expression was relatively short-lived, but a reversal of the denervation supersensitivity takes several days; therefore, a reduction of apomorphine-induced rotation would not be expected. To examine the behavioral consequences of transplants, especially genetically modified cells such as ours, development of paradigms incorporating spontaneous behaviors that more closely reflect the abnormalities of PD are sorely needed (Borlongan and Sanberg, 1995; Olsson et al., 1995). Therefore, in this study, we focused our attention on biochemical measures as direct evidence for the efficacy of gene therapy in animal models of PD.

The fact that L-DOPA was detected in microdialysates from 6-OHDA-denervated striata containing grafts of cells doubly transduced with TH and GTP cyclohydrolase I, but not in those with grafts of cells modified with only TH, further supports the idea that the denervated striatum does not have a sufficient amount of BH_4 to support TH activity (Uchida et al., 1992). The BH_4 concentration in dopaminergic terminals has been estimated to be in the range of 1–50 μm (Levine et al., 1981; Kapatos et al.,

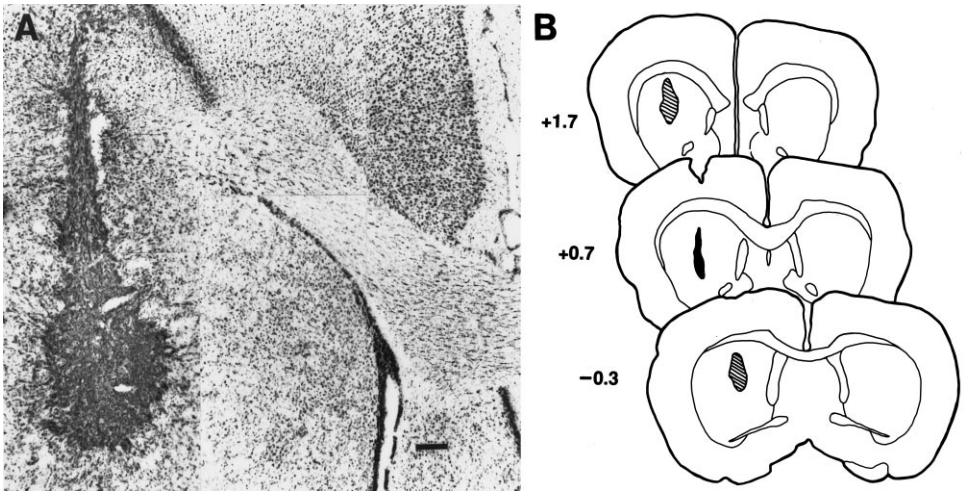

Figure 6. Histology of genetically modified fibroblast grafts and schematic of graft and microdialysis probe locations. *A*, Nissl-stained section showing the location of a representative graft expressing both TH and GTP cyclohydrolase I (PFTHGC) within the striatum. Scale bar, 200 μm. *B*, Schematic sections showing the relative positions of the grafts (*hatched*) and microdialysis probe (*solid*) between the two grafts.

1992). However, the BH_4 level in severely denervated striatum has been reported to be <25% of normal (Levine et al., 1981) and, therefore, is probably below the K_m for the TH. Partial reversals of biochemical and behavioral abnormalities have been noted after direct *in vivo* gene transfers of TH using herpes, adeno and adeno-associated viral vectors (During et al., 1994; Horellou et al., 1994; Kaplitt et al., 1994); however, given the low level of BH_4 in the denervated striatum, the source of BH_4 for viral transduced TH is unclear. In fact, addition of exogenous BH_4 was required to produce a significant amount of L-DOPA in striatal neuronal cultures transduced with TH by a herpes virus vector (Geller et al., 1995).

Accumulation of L-DOPA after inhibition of AADC was used to isolate the *in vivo* TH function from other variables such as DOPA decarboxylation, dopamine uptake, and metabolism of dopamine by subsequent enzymatic steps (Carlsson et al., 1972; Westerink et al., 1990; Robert et al., 1993). Our data demonstrate that in the absence of its metabolism, L-DOPA accumulates in the striatum of rats with PFTHGC grafts, supporting the idea that TH function is enhanced by this genetic modification. The clear-cut difference between the PFTH grafts and PFTHGC grafts illustrates the importance of GTP cyclohydrolase I in the TH gene transfer paradigm. Cells modified with both GTP cyclohydrolase I and TH displayed a higher level of TH activity *in vivo*, 18-fold higher, than either grafts of cells transduced with TH alone or grafts of control fibroblasts. Nevertheless, there was a small NSD 1015-induced increase of L-DOPA in TH- and control-grafted striata. This may have been attributable to some remaining endogenous TH activity in residual dopaminergic terminals or diffusion of L-DOPA from outside the striatum given the delayed peak effect compared to the PFTHGC group (Fig. 7).

To examine the longevity of the transgene expression, microdialysis experiments and histological examination were also conducted at two time points after grafting PFTHGC cells. The transgene expression was self-limited in that the levels of L-DOPA and dopamine were close to control levels (Table 2) and immunostaining was not detectable by 2 weeks after grafting. Such a limited long-term expression is similar, but somewhat shorter than other experiments with retroviral transduction of fibroblasts expressing AADC (Kang, 1995) or neurotrophic factors (Levivier et al., 1995; Lucidi-Phillipi et al., 1995). This may be because TH protein is relatively unstable when expressed in non-neuronal cells (Wu and Cepko, 1994). Al-

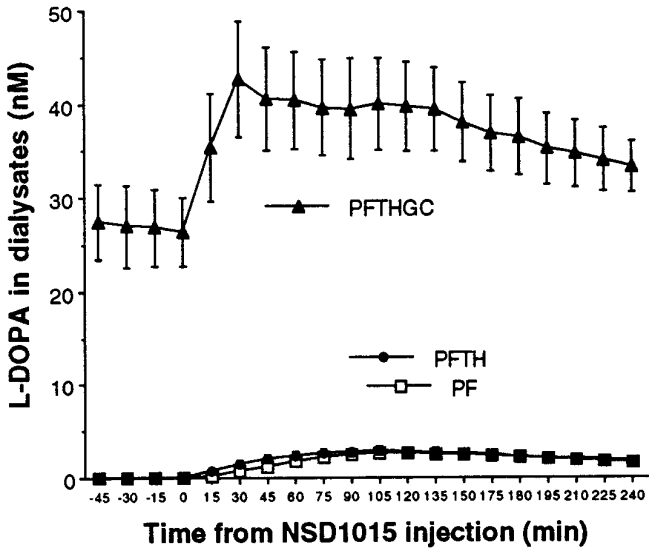

Figure 7. Microdialysis in striatum of freely moving rats with genetically modified fibroblast grafts. L-DOPA was measured by HPLC in the dialysates at 15 min intervals. Dialysates were monitored for 1 hr before and 4 hr after NSD 1015 (100 mg/kg, i.p.). Data represent mean ± SEM ($n = 6$) concentration of L-DOPA in 20 μl dialysate samples.

Table 2. Baseline catecholamine concentrations in the denervated striatum grafted with genetically modified cells doubly transduced with TH and GTP cyclohydrolase I

Cell type	L-DOPA (nM)	Dopamine (nM)	DOPAC (nM)	HVA (nM)
PFTHGC ($n = 6$)	170.03 ± 50.77	2.13 ± 0.27	67.03 ± 13.57	74.80 ± 2.14 ($n = 3$)
PFTH ($n = 4$)	0.45 ± 0.26	0.00 ± 0.00	0.88 ± 0.25	2.35 ± 0.27
PF ($n = 6$)	0.04 ± 0.04	0.00 ± 0.00	1.05 ± 0.46	ND
PFTHGC 14 d ($n = 4$)	3.37 ± 3.12	0.00 ± 0.00	2.19 ± 0.14	7.41 ± 1.02

Unless indicated otherwise, the microdialysis was performed 4 d after grafting in the denervated striata containing fibroblasts genetically modified with TH (PFTH), with both TH and GTP cyclohydrolase I (PFTHGC), or those without modification (PF). Because no significant levels of catecholamines were detected in PF and PFTH groups, only PFTHGC groups were followed for 14 d to assess their long-term expression. ND, Not done.

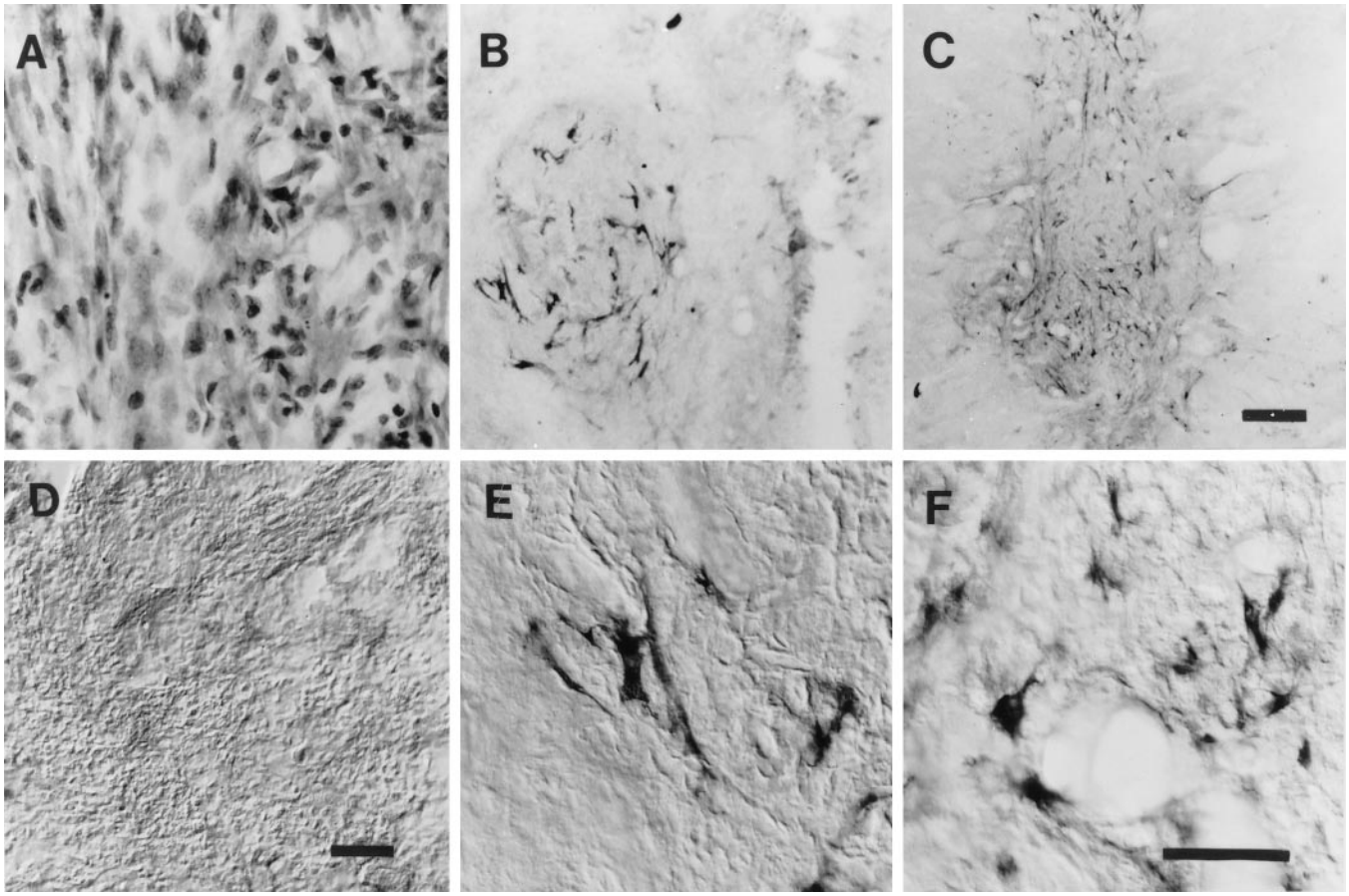

Figure 8. Histology and TH immunohistochemistry (*B–F*) of primary fibroblast grafts at 4 d. *A*, High-power (400×) view of Nissl staining showing the fibroblast morphology of PFTHGC cells shown in Figure 5. *B*, Fibroblast grafts modified with TH alone (PFTH). *C*, Fibroblasts doubly transduced with TH and GTP cyclohydrolase I (PFTHGC). *B* and *C* were taken at 100× magnification; scale bar, 100 μm. *D*, Unmodified fibroblast grafts at 200× magnification. Nomarski optics were used to demonstrate the morphology because the TH immunostaining was negative in this control PF graft. Scale bar, 50 μm. *E*, *F*, High-power (400×) views of the grafts in *B* and *C*. Scale bar, 50 μm.

though the duration may vary somewhat depending on the transgenes, cell types, and vectors used, consistent long-term expression of any transgenes has not been demonstrated in the literature. The reason for this failure of long-term expression *in vivo* is unknown, but is likely to be multifactorial. Improvements in long-term expression require systematic studies of promoters and their interactions with vectors, grafted cells, and host environments in addition to the stability of proteins. Nonetheless, the current combination of retroviral vectors and fibroblast cells serves as an extremely useful tool to investigate the effects of particular gene(s) *in vivo* in an animal model of PD.

In conclusion, we have shown that gene therapy for PD using primary fibroblasts may be much more effective by double transduction of fibroblast cells with GTP cyclohydrolase I in addition to TH. The rational design of gene therapy necessitates a systematic investigation of the genes required by the particular gene transfer vehicle to restore normal biochemical function. The present data clearly demonstrate that BH_4 is essential for spontaneous production of L-DOPA by primary fibroblast cells transduced with TH both *in vitro* and *in vivo*. Further, we have demonstrated that, in primary fibroblasts, transduction of GTP cyclohydrolase I is the only step required for production of BH_4. This important role of GTP cyclohydrolase I is applicable to most types of cells currently being explored for *ex vivo* gene therapy as well as for direct *in vivo* gene transfer of TH.

REFERENCES

Anton R, Kordower JH, Maidment NT, Manaster JS, Kane DJ, Rabizadeh S, Schueller SB, Yang J, Edwards RH, Markham CH, Bredesen DE (1994) Neural-targeted gene therapy for rodent and primate hemiparkinsonism. Exp Neurol 127:207–218.

Barker R, Dunnett SB (1994) Ibotenic acid lesions of the striatum reduce drug-induced rotation in the 6-hydroxydopamine-lesioned rat. Exp Brain Res 101:365–374.

Borlongan CV, Sanberg PR (1995) Elevated body swing test: a new behavioral parameter for rats with 6-hydroxydopamine-induced hemiparkinsonism. J Neurosci 15:5372–5378.

Carlsson A, Davis JN, Kehr W, Lindqvist M, Atack CV (1972) Simultaneous measurement of tyrosine and tryptophan hydroxylase activities in brain *in vivo* using an inhibitor of the aromatic amino acid decarboxylase. Naunyn Schmiedebergs Arch Pharmacol 275:153–168.

Curran EJ, Albin RL, Becker JB (1993) Adrenal medulla grafts in the hemiparkinsonian rat: Profile of behavioral recovery predicts restoration of the symmetry between the two striata in measures of pre- and postsynaptic dopamine function. J Neurosci 13:3864–3877.

Duch DS, Bowers SW, Woolf JH, Nickol CA (1984) Biopterin cofactor biosynthesis: GTP cyclohydrolase, neopterin and biopterin in tissues and body fluids of mammalian species. Life Sci 35:1895–1901.

During MJ, Naegele JR, O'Malley KL, Geller AI (1994) Long-term behavioral recovery in parkinsonian rats by an HSV vector expressing tyrosine hydroxylase. Science 266:1399–1403.

Fisher LJ, Jinnah HA, Kale LC, Higgins GA, Gage FH (1991) Survival and function of intrastriatally grafted primary fibroblasts genetically modified to produce L-DOPA. Neuron 6:371–380.

Fukushima T, Nixon JC (1980) Analysis of reduced forms of biopterin in biological tissues and fluids. Anal Biochem 102:176–188.

Gal EM, Nelson JM, Sherman AD (1978) Purification and characterization of enzymes involved in cerebral synthesis of 7,8-dihydrobiopterin. Neurochem Res 3:69–98.

Geller AI, During MJ, Oh YJ, Freese A, O'Malley K (1995) An HSV-1 vector expressing tyrosine hydroxylase causes production and release of L-DOPA from cultured rat striatal cells. J Neurochem 64:487–496.

Ginns EI, Rehavi M, Martin BM, Weller M, O'Malley KL, LaMarca ME, McAllister CG, Paul SM (1988) Expression of human tyrosine hydroxylase cDNA in invertebrate cells using a baculovirus vector. J Biol Chem 263:7406–7410.

Grima B, Lamouroux A, Boni C, Julien J-F, Javoy-Agid F, Mallet J (1987) A single human gene encoding multiple tyrosine hydroxylase with different predicted functional characteristics. Nature 326:707–711.

Hatakeyama K, Inoue Y, Harada T, Kagamiyama H (1991) Cloning and sequencing of cDNA encoding rat GTP cyclohydrolase I: the first enzyme of the tetrahydrobiopterin biosynthetic pathway. J Biol Chem 266:765–769.

Horellou P, Brundin P, Kalen P, Mallet J, Björklund A (1990) In vivo release of DOPA and dopamine from genetically engineered cells grafted to the denervated rat striatum. Neuron 5:393–402.

Horellou P, Vigne E, Castel M-N, Barnéoud P, Colin P, Perricaudet M, Delaère P, Mallet J (1994) Direct intracerebral gene transfer of an adenoviral vector expressing tyrosine hydroxylase in a rat model of Parkinson's disease. NeuroReport 6:49–53.

Hudson JL, Van Horne CG, Strömberg I, Brock S, Clayton J, Masserano J, Hoffer BJ, Gerhardt GA (1993) Correlation of apomorphine- and amphetamine-induced turning with nigrostriatal dopamine content in unilateral 6-hydroxydopamine lesioned rats. Brain Res 626:167–174.

Ichinose H, Ohye T, Takahashi E, Seki N, Hori T, Segawa M, Nomura Y, Endo K, Tanaka H, Tsuji S, Fujita K, Nagatsu T (1994) Hereditary progressive dystonia with marked diurnal fluctuation caused by mutations in the GTP cyclohydrolase 1 gene. Nature Genet 8:236–209.

Isacson O (1995) Behavioral effects and gene delivery in a rat model of Parkinson's disease. Science 269:856.

Ishii A, Hagihara M, Matsuura S, Uchida K, Kiuchi K, Kaneda N, Toya S, Kohsaka S, Nagatsu T (1990) Effect of (6*R*)- and (6*S*)-tetrahydrobiopterin on L-3,4-dihydroxyphenylalanine (DOPA) formation in NRK fibroblasts transfected with human tyrosine hydroxylase type 2 cDNA. Neurochem Int 17:625–632.

Kang UJ (1995) Genetic modification of cells with retrovirus vectors for grafting into the central nervous system. In: Viral vectors: gene therapy and neuroscience applications (Kaplitt MG, Loewy AD, eds), pp 211–237. San Diego: Academic.

Kang UJ, Fisher LJ, Joh TH, O'Malley KL, Gage FH (1993) Regulation of dopamine production by genetically modified primary fibroblasts. J Neurosci 13:5203–5211.

Kapatos G, Kaufman S (1981) Peripherally administered reduced pterins do enter the brain. Science 212:955–956.

Kapatos G, Hirayama K, Hasegawa H (1992) Tetrahydrobiopterin turnover in cultured rat sympathetic neurons: developmental profile, pharmacologic sensitivity, and relationship to norepinephrine synthesis. J Neurochem 59:2048–2055.

Kaplitt MG, Leone P, Samulski RJ, Xiao X, Pfaff DW, O'Malley KL, During MJ (1994) Long-term gene expression and phenotypic correction using adeno-associated virus vectors in the mammalian brain. Nature Genet 8:148–154.

Kawaja MD, Gage FH (1992) Morphological and neurochemical features of cultured primary skin fibroblasts of Fischer-344 rats following striatal implantation. J Comp Neurol 317:102–116.

Levine RA, Miller LP, Lovenberg W (1981) Tetrahydrobiopterin in striatum: localization in dopamine nerve terminals and role in catecholamine synthesis. Science 213:349–350.

Levine RA, Zoephel GP, Niederwieser A, Curtius H-C (1987) Entrance of tetrahydropterin derivatives in brain after peripheral administration: effect on biogenic amine metabolism. J Pharmacol Exp Ther 242:514–522.

Levivier M, Przedborski S, Bencsics C, Kang UJ (1995) Intrastriatal implantation of fibroblasts genetically engineered to produce brain-derived neurotrophic factor prevents degeneration of dopaminergic neurons in a rat model of Parkinson's disease. J Neurosci 15:7810–7820.

Lucidi-Phillipi CA, Gage FH, Shults CW, Jones KR, Reichardt LF, Kang UJ (1995) BDNF-transduced fibroblasts: production of BDNF and effects of grafting to the adult rat brain. J Comp Neurol 354:361–376.

Miller AD, Rosman GJ (1989) Improved retroviral vectors for gene transfer and expression. Biotechniques 7:980–989.

Nagatsu T (1983) Biopterin cofactor and monoamine-synthesizing monooxygenases. Neurochem Int 5:27–38.

Nagatsu T, Levitt M, Udenfriend S (1964) A rapid and simple radioassay for tyrosine hydroxylase activity. Anal Biochem 9:122–126.

Olsson M, Nikkhah G, Bentlage C, Björklund A (1995) Forelimb akinesia in the rat Parkinson model: differential effects of dopamine agonists and nigral transplants as assessed by a new stepping test. J Neurosci 15:3863–3875.

Owens GC, Johnson R, Bunge RP, O'Malley KL (1991) L-3,4-Dihydroxyphenylalanine synthesis by genetically modified Schwann cells. J Neurochem 56:1030–1036.

Paxinos G, Watson C (1986) The rat brain in stereotaxic coordinates. San Diego: Academic.

Reinhard JF, Smith GK, Nichol CA (1986) A rapid and sensitive assay for tyrosine-3-monooxygenase based upon the release of ^3H_2O and adsorption of [^{3}H]-tyrosine by charcoal. Life Sci 39:2185–2189.

Ridoux V, Robert JJ, Zhang X, Perricaudet M, Mallet J, Le Gal La Salle G (1994) The use of adenovirus vectors for intracerebral grafting of transfected nervous cells. NeuroReport 5:801–804.

Robert F, Lambas-Senas L, Ortemann C, Pujol J-F, Renaud B (1993) Microdialysis monitoring of 3,4-dihydroxypheylalanine accumulation after decarboxylase inhibition: a means to estimate in vivo changes in tyrosine hydroxylase activity of the rat locus ceruleus. J Neurochem 60:721–729.

Sakai N, Kaufman S, Milstien S (1993) Tetrahydrobiopterin is required for cytokine-induced nitric oxide production in a murine macrophage cell line (RAW 264). Mol Pharmacol 43:6–10.

Uchida K, Takamatsu K, Kaneda N, Toya S, Tsukada Y, Kurosawa Y, Fujita K, Nagatsu T, Kohsaka S (1989) Synthesis of L-3,4-dihydroxyphenylalanine by tyrosine hydroxylase cDNA-transfected C6 cells: application for intracerebral grafting. J Neurochem 53:728–732.

Uchida K, Tsuzaki N, Nagatsu T, Kohsaka S (1992) Tetrahydrobiopterin-dependent functional recovery in 6-hydroxydopamine-treated rats by intracerebral grafting of fibroblasts transfected with tyrosine hydroxylase cDNA. Dev Neurosci 14:173–180.

Ungerstedt U (1971) Postsynaptic supersensitivity after 6-hydroxydopamine induced degeneration of the nigro-striatal dopamine system. Acta Physiol Scand 82[Suppl 2367]:69–93.

Wachtel SR, Abercrombie ED (1994) L-3,4-Dihydroxyphenylalanine-induced dopamine release in the striatum of intact and 6-hydroxydopamine-treated rats: differential effects of monoamine oxidase A and B inhibitors. J Neurochem 63:108–117.

Werner ER, Werner-Felmayer G, Fuchs D, Hausen A, Reibnegger G, Yim JJ, Pfleiderer W, Wachter H (1990) Tetrahydrobiopterin biosynthetic activities in human macrophages, fibroblasts, THP-1, and T 24 cells. GTP-cyclohydrolase I is stimulated by interferon-γ, and 6-pyruvoyl tetrahydropterin synthase and sepiapterin reductase are constitutively present. J Biol Chem 265:3189–3192.

Westerink BHC, de Vries JB, Duran R (1990) Use of microdialysis for monitoring tyrosine hydroxylase activity in the brain of conscious rats. J Neurochem 54:381–387.

Wolff JA, Fisher LJ, Jinnah HA, Langlais PJ, Iuvone PM, O'Malley KL, Rosenberg MB, Shimohama S, Friedmann T, Gage FH (1989) Grafting fibroblasts genetically modified to produce L-dopa in a rat model of Parkinson disease. Proc Natl Acad Sci USA 86:9011–9014.

Wu DK, Cepko CL (1994) The stability of endogenous tyrosine hydroxylase protein in PC-12 cells differs from that expressed in mouse fibroblasts by gene transfer. J Neurochem 62:863–872.