Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 9011–9014, November 1989
Neurobiology

Grafting fibroblasts genetically modified to produce L-dopa in a rat model of Parkinson disease

(tyrosine hydroxylase/retroviral vector/6-hydroxydopamine transplantation)

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Communicated by J. Edwin Seegmiller, August 10, 1989

ABSTRACT **Rat fibroblasts were infected with a retroviral vector containing the cDNA for rat tyrosine hydroxylase [TH; tyrosine 3-monooxygenase; L-tyrosine, tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2]. A TH-positive clone was identified by biochemical assay and immunohistochemical staining. When supplemented *in vitro* with pterin cofactors required for TH activity, these cells produced L-dopa and released it into the cell culture medium. Uninfected control cells and fibroblasts infected with the TH vector were grafted separately to the caudate of rats with unilateral 6-hydroxydopamine lesions of the nigrostriatal pathway. Only grafts containing TH-expressing fibroblasts were found to reduce rotational asymmetry. These results have general implications for the application of gene therapy to human neurological disease and specific implications for Parkinson disease.**

A strategy for gene therapy, in which appropriate target cells are removed from the body, genetically modified in culture, and then implanted back into the body, has attracted considerable attention (1, 2). Recently, this strategy has been applied to the central nervous system by implanting genetically modified fibroblasts into the brain (3–5). The present study was undertaken to determine whether this approach could be used to reduce behavioral abnormalities in an animal model of human neurological disease.

Parkinson disease may be an ideal candidate for treatment with this approach since the neurological abnormalities are known to result from degeneration of a specific class of cells, the dopaminergic neurons of the nigrostriatal pathway. The clinical signs of the disease can be ameliorated by replacement of L-dopa, the precursor of dopamine. Fibroblasts genetically modified to produce L-dopa might be capable of altering the phenotype of this disease after implantation into the brain. To investigate this possibility, a rat fibroblast cell line was infected *in vitro* with a retroviral vector containing the rat cDNA for the enzyme tyrosine hydroxylase [TH; tyrosine 3-monooxygenase; L-tyrosine, tetrahydrobiopteridine:oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2]. This enzyme, in the presence of the cofactor tetrahydropterin, catalyzes the conversion of tyrosine to L-dopa. Since tyrosine and tetrahydropterin are readily available to cells of the brain (6), TH-expressing fibroblasts should be capable of producing L-dopa when implanted intracerebrally. In this report, we demonstrate that genetically modified fibroblasts can produce and release L-dopa *in vitro*, and they can reduce behavioral abnormalities in an animal model of Parkinson disease.

METHODS

A retroviral vector containing the full-length rat TH cDNA (LThRNL) was derived from the Moloney murine leukemia virus (Fig. 1). This vector expresses TH from the 5′ long terminal repeat (LTR) and the selectable bacterial neomycin-resistance gene (*neo*) from an internal Rous sarcoma virus (RSV) promoter (7). A 1688 base-pair (bp) *Bam*HI/*Sph* I fragment from the plasmid pTH-54 (8–9) containing the rat TH cDNA, a 1321-bp *Hin*dIII/*Sma* I fragment of *neo* from the plasmid pSV2neo (10), a 300-bp *Bam*HI/*Hin*dIII fragment from the plasmid pUCRH containing a modified RSV promoter (J.-K. Yee, personal communication), and LTR sequences derived from the LPL2 vector (11) were ligated and cloned by standard methods (12). The polyadenylylation signal within the RSV promoter was changed from AATAAA to AGCAAA by site-directed mutagenesis to allow full-length transcripts from the 5′ LTR promoter to the polyadenylylation site within the 3′ LTR.

Plasmid DNA containing the LThRNL provirus was transfected by the calcium phosphate method (13) into the amphotropic helper line PA317 (14), and cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (DMEM/FCS). Two days posttransfection, medium from these cells was filtered and used to infect the ecotropic helper line ψ-2 (15). Producer cells were selected for expression of *neo* by growth in medium containing 400 μg of the neomycin analog G418 per ml. The G418-resistant ψ-2 clone that contained the highest level of TH activity and produced the highest titer of virus (5×10^5 per ml) was used for subsequent production of virus.

Immortalized rat fibroblasts of the 208F cell line (16) were grown in DMEM/FCS and infected with LThRNL virus at a multiplicity of infection $<10^{-4}$ in the presence of 4 μg of Polybrene per ml. Twelve G418-resistant colonies were established and tested for expression of TH activity by a decarboxylase-coupled assay (17). Confluent 10-cm plates of cells were washed two times with Dulbecco's phosphate-buffered saline (PBS) without calcium or magnesium, scraped off the plates, and homogenized in 150 μl of ice-cold 50 mM Tris·HCl (pH 8.4) containing 50 mM sodium pyrophosphate and 0.2% Triton X-100. The sample was then

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Abbreviations: TH, tyrosine hydroxylase; LTR, long terminal repeat; RSV, Rous sarcoma virus.

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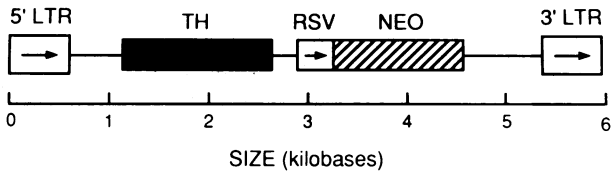

FIG. 1. Structure of LThRNL retroviral vector. This vector expresses TH from the 5' LTR and the selectable bacterial *neo* gene from an internal RSV promoter.

centrifuged at 32,000 × *g* for 15 min at 4°C to remove debris. The TH assay contained 20 μM [^{14}C]tyrosine, 1 mM 6-methyl-5,6,7,8-tetrahydrobiopterin, and 0.23 M potassium phosphate buffer (pH 6.0). Protein was measured by the method of Lowry *et al.* (18) using bovine serum albumin as a standard. The clone expressing the highest level of TH activity (208F/TH8) was selected for further study.

For immunohistochemical labeling of these cells *in vitro*, uninfected fibroblasts or 208F/TH8 fibroblasts were grown on microscope slides, fixed with 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100. Immunochemical labeling was performed with a monoclonal antibody to TH (Boehringer Mannheim) and the stain was developed by the avidin–biotin method (Vector Laboratories, Elite Kit) with nickel-intensified 3,3'-diethylaminobenzidine as the chromogen.

The fibroblasts were also examined for the production and release of L-dopa *in vitro*. Cultured cells were grown 24 hr in DMEM/FCS supplemented with 0.5 mM biopterin, a pterin precursor that fibroblasts can reduce to the required TH cofactor tetrahydropterin (19). Conditioned media and cells were collected separately, adjusted to 0.1 M perchloric acid (PCA) and 0.05 M EDTA, and centrifuged at 10,000 × *g* for 15 min at 4°C to remove precipitated material. The samples were analyzed for the presence of catecholamines and catecholamine metabolites by injection of PCA extracts onto a coulometric electrode array, gradient liquid chromatography system (CEAS model 55-0650, ESA, Bedford, MA), equipped with 16 electrochemical sensors and a refrigerated autosampler (20–22). The within-run precision for retention time (RT), concentration (C), ratio of lower potential sensor to the dominant sensor (LDR), and ratio of higher potential sensor to the dominant sensor (HDR) for L-dopa were 93.6%, 82.3%, 93.8%, and 90.2%, respectively. The standard gradient method was used as described (23) with two modifications: buffer A, 0.14% SDS in 0.1 M phosphate buffer (pH 3.2); buffer B, 40% methanol in 0.1% phosphate buffer (pH 3.35). Using the CEAS system, response parameters observed for L-dopa in chromatograms of standard solutions (RT = 4.02 ± 0.04, LDR = 0.50 ± 0.02, HDR = 0.18 ± 0.03; mean ± SEM; n = 3) and samples (RT = 3.98 ± 0.04, LDR = 0.46 ± 0.05, HDR = 0.19 ± 0.07; n = 18) were similar. Furthermore, addition of partially purified dopa decarboxylase to standards or samples (37°C for 2 hr prior to CEAS analysis) resulted in the elimination of the L-dopa peak and emergence of a peak indicative of dopamine. Finally, chromatograms of experimental samples supplemented with authentic L-dopa displayed a single L-dopa peak. The peak match for parameters (RT, LDR, HDR) in the sample to L-dopa standards, the elimination of the L-dopa peak of dopa decarboxylase, and precise coelution of L-dopa-supplemented samples confers a high degree of confidence in the identification of L-dopa produced by the cultured fibroblasts.

For implantation studies, adult female Sprague–Dawley rats received an injection of the catecholamine neurotoxin 6-hydroxydopamine (12 μg in 2 μl of saline containing 0.1% ascorbate) into the medial forebrain bundle [coordinates: anterior–posterior (AP) = −4.4, medial–lateral (ML) = 1.1, dorsal–ventral (DV) = −7.5; ref. 24]. Completeness of the lesion was assessed 10–30 days postinjection by either apomorphine- (0.1 mg/kg; s.c.) or amphetamine- (5.0 mg/kg; s.c.) induced rotational behavior. A total of 31 animals turning at a rate of >7 rotations per min (25) were included in the study (19 apomorphine tested, 12 amphetamine tested).

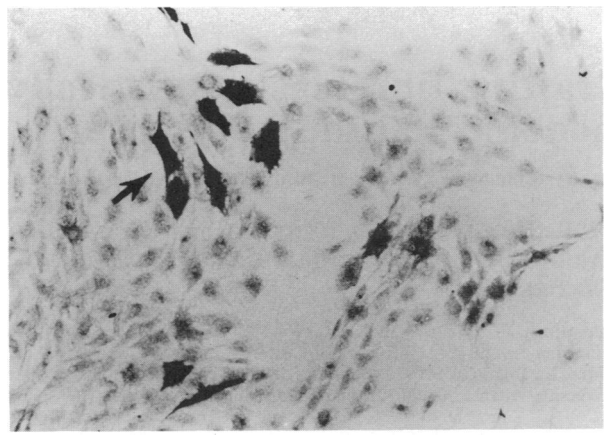

FIG. 2. Immunohistochemical staining for TH in 208F/TH8 cells. Cells with different immunoreactivities are shown. Arrow indicates fibroblasts with the most intense immunoreactivity. (×60.)

Cultured cells were suspended at a density of 80,000 cells per μl as described (4, 5) and were injected stereotaxically into several locations within rostral (AP = 1.4, ML = 2.0, DV = −3.5–4.5 to AP = 2.5, ML = 1.5, DV = −3.5–4.5) and caudal (AP = 0.4, ML = 3.0, DV = −3.5–4.5) areas of the denervated caudate. Fifteen of the 6-hydroxydopamine-lesioned rats received injections of noninfected fibroblasts to serve as controls. A total of 4 μl was delivered in two equal deposits over a 1 to 2–mm area at each site. Grafted rats were tested for rotational asymmetry 2 weeks after fibroblast implantation. Immediately after the behavioral test, brains were taken for histological examination. Rats were anesthetized and perfused transcardially with saline followed by 10% formalin. Brains were removed, postfixed overnight, transferred to a solution containing 30% (wt/vol) sucrose, and sectioned at a thickness of 40 μm on a freezing microtome. Some sections were stained with cresyl violet. Other sections were stained for TH or the fibroblast marker fibronectin (Organon Technica) as described above. Mounted sections were dehydrated and covered with a coverslip, and the size and placement of grafts was determined.

RESULTS

Control cells and cells infected with the TH vector were tested for production of functional TH activity by a decarboxylase-coupled assay. The TH activity of the 208F/TH8 fibroblasts was 2.9 pmol of L-dopa per min per mg of protein, while noninfected fibroblasts did not have detectable levels of TH. Immunohistochemical staining of 208F/TH8 cells for TH revealed a small percentage of darkly stained cells, with most cells staining only lightly (Fig. 2). Noninfected 208F fibroblasts did not stain specifically for TH.

Cell extracts and media from 208F/TH8 fibroblasts and control 208F fibroblasts were assayed for the production and release of catecholamines (Table 1). When supplemented

Table 1. L-Dopa concentration of cell extracts and medium

	L-Dopa, ng per 10^6 cells	
Cell type*	Cell extract	Conditioned medium
208F/control	ND	ND
208F/TH8	0.74 ± 0.01	782 ± 57

ND, not detectable.
*Cells were supplemented with biopterin.

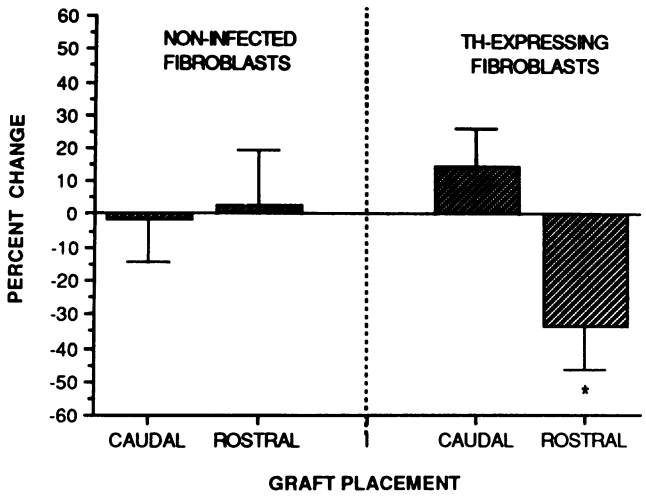

FIG. 3. Percent change in drug-induced rotations of fibroblast-implanted rats 2 weeks after transplantation. Since there was no apparent difference in the percent change between apomorphine- or amphetamine-induced rotations, data from both groups were pooled. Rats with TH-expressing cells grafted to rostral caudate areas showed a significant drop in rotations ($n = 7$; means ± SEM = 33.6% ± 12.7%; $P < 0.05$). A nonsignificant increase in rotations was observed for rats with TH-expressing fibroblasts implanted in caudal regions of the caudate ($n = 9$). Rotations of rats with noninfected cells were not different from pretransplant levels regardless of placement ($n = 11$).

with biopterin, a precursor of the required TH cofactor, 208F/TH8 cells were found to contain 0.74 ng L-dopa per 10^6 cells after 24 hr. Assay of the cell culture medium indicated that 782 ng of L-dopa was released from these cells during this period. Control 208F fibroblasts did not contain or release detectable levels of L-dopa. Neither 208F/TH8 nor control fibroblasts contained or released detectable levels of L-dopa metabolites including dopamine, 3,4 dihydroxyphenylacetic acid, homovanillic acid, norepinephrine, or 3-methoxytyramine.

These fibroblasts were tested for their ability to reduce rotational asymmetry in rats with unilateral lesions of the nigrostriatal pathway. Behavioral recovery was assessed for each animal 2 weeks after grafting by computing the percent change in posttransplant rotations from pretransplant scores. Rats with implants of TH-expressing fibroblasts injected into rostral caudate regions ($n = 7$) showed a significant reduction in drug-induced rotations [t (paired t test) = 2.52; $P < 0.05$; see Fig. 3]. In contrast, there was no significant change in the rotational behavior of rats with TH-expressing cells implanted in caudal areas of the caudate ($n = 9$). Rotational scores of animals with noninfected fibroblasts injected into either rostral ($n = 6$) or caudal ($n = 5$) areas of the caudate were unchanged from pretransplant levels.

Immunohistochemical staining for fibronectin indicated that 27 of 31 fibroblast grafts survived intraparenchymal transplantation into the denervated caudate (Fig. 4). Immunohistochemical staining for TH revealed near total absence of labeled fibers on the lesioned side. However, a few TH-positive fibers were observed in the vicinity of both control and infected fibroblast grafts. The 208F/TH8 fibroblasts in the graft could not be clearly identified with the TH antibody. The variable staining of these cells for TH expected from *in vitro* studies, in combination with higher background staining in the fibroblast grafts *in vivo*, prevented reliable identification of stained from unstained cells.

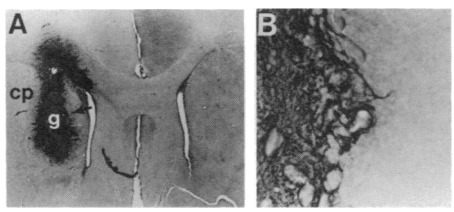

FIG. 4. Photomicrographs of fibroblast grafts to the caudate showing fibronectin immunoreactivity. G, Graft; cp, caudate putamen. Arrow in *A* indicates location of high-power photomicrograph in *B*. (*A*, ×10; *B*, ×120.)

DISCUSSION

This study demonstrates that fibroblasts infected with a retroviral vector containing the cDNA for rat TH can express functional TH enzymatic activity and produce and release L-dopa *in vitro*. While both infected and noninfected fibroblasts exhibit robust survival when implanted into rats with a unilateral lesion of the dopaminergic nigrostriatal pathway, there is a significant reduction in rotational asymmetry only in animals with grafts of L-dopa-producing fibroblasts. This functional effect on rotational behavior was observed 2 weeks after grafting.

Further studies are necessary to evaluate the potential long-term effects of grafted fibroblasts. Our previous studies have demonstrated that genetically modified fibroblasts implanted into the brain can survive and continue to express a foreign gene for at least 2 months (4). Although the fibroblasts used in the present study could not be clearly labeled for TH after implantation into the brain, *in vivo* labeling for TH by *in situ* hybridization techniques has been observed in recent preliminary work using a different cell line expressing higher levels of TH (26).

The location of TH-expressing fibroblast grafts within the denervated caudate was found to be an important factor in reducing rotational asymmetry. Grafts placed in rostral areas significantly decreased rotations, while grafts in caudal regions had no behavioral effect. This is in agreement with previous work showing that the effect of adrenal medulla grafts on rotational behavior is most pronounced in rostral caudate (27). These results support the idea of functional specificity within subregions of the caudate (28).

The mechanism through which L-dopa-producing fibroblasts reduce rotational asymmetry in the unilateral lesion model is unclear. However, previous studies using this model have demonstrated an increase in contralateral rotation after systemic administration of L-dopa (29). Presumably, the effect of L-dopa is mediated through its conversion to dopamine by residual dopa decarboxylase (30). It has been shown that 20% of decarboxylase activity remains within the caudate even after a 97% reduction in dopamine content resulting from a 6-hydroxydopamine lesion (31). Even after the unilateral destruction of 95% of nigrostriatal terminals within the caudate, dopamine is increased in both the lesioned and nonlesioned striata after systemic L-dopa administration (29). It seems likely that the L-dopa produced by the fibroblasts is also decarboxylated to dopamine by residual decarboxylase. The administration of L-dopa is currently one of the most effective treatments for Parkinson disease. However, global fluctuations in blood levels associated with dosing schedules may contribute to the occurrence of untoward side effects such as dyskinesia and the "on–off" phenomenon. The continuous local application of small amounts of L-dopa, attainable with direct minipump infusions or grafted cells, might avoid this problem.

In the same animal model as used in this study, adrenal medullary cell grafts are able to reduce rotational asymmetry to an extent equivalent to that reported here (32). Fetal substantia nigra cell grafts, however, have a much greater ability to reverse rotational abnormalities (28). The more complete restorative effect of fetal cell grafts in reducing

rotational asymmetry could be due, in part, to the reestablishment of synaptic connections between the grafted cells and host cells. Thus, the inability of fibroblast grafts to restore synaptic contacts may be a limiting factor in the efficacy of the fibroblast implants in this model.

The transplantation of adrenal medulla cells and fetal neurons is being actively investigated as a treatment for Parkinson disease (33–37). The ability, demonstrated in this study, to modify cells genetically to produce L-dopa broadens the search for the ideal type of cell for transplantation therapy of Parkinson disease. The 208F rat fibroblasts used in this study were chosen more for technical reasons than for actual clinical applicability. Rat 208F fibroblasts are easily infected *in vitro* with retroviral vectors and are known to survive for several months after implantation into the rat brain (4). Although we have not observed immunological responses or formations of tumors with these fibroblast grafts, the potential for developing such problems exists. An alternative approach would be to use autografts of primary fibroblasts or glia. Primary cells offer the theoretical advantage of decreased propensity for tumor formation and fewer problems of histoincompatibility. Further work is necessary to determine whether primary cells can be genetically manipulated for use in central nervous system grafting; the long-term survival and functional capabilities of these and other cells could also be examined.

These studies demonstrate that the ability to combine transplantation techniques with gene transfer is potentially a powerful approach to the treatment of human central nervous system dysfunction.

This research was supported by National Institutes of Health Grants HD00669, EY04864, HD20034, and AG06088; National Research Service Awards MH18398 and GM11013; Medical Scientist Training Program; Veterans Administration Merit Review Program; Sasakawa Fellowship; and grants from the Weingart and Gould Family Foundations, Athena Neurosciences, Pew Foundation, McKnight Foundation, The Margaret and Herbert Hoover Foundation, and the Office of Naval Research.

1. Anderson, W. F. (1984) *Science* **226,** 401–409.
2. Friedmann, T. (1989) *Science* **244,** 1275–1281.
3. Gage, F. H., Rosenberg, M. B., Wolff, J. A., Yee, J.-K., Xu, L., Schultz, C. & Friedmann, T. (1987) *Neuroscience* **23,** 795–807.
4. Rosenberg, M. B., Friedmann, T., Robertson, R. C., Tuszynski, M., Wolff, J. A., Breakefeld, X. O. & Gage, F. H. (1988) *Science* **242,** 1575–1578.
5. Shimohama, S., Rosenberg, M. B., Fagan, A.M., Wolff, J. A., Short, M. P., Breakefeld, X. O., Friedmann, T. & Gage, F. H., *Mol. Brain Res.*, in press.
6. Lovenberg, W. (1979) *Science* **204,** 624–626.
7. Xu, L., Yee, J.-K., Wolff, J. A. & Friedmann, T. (1989) *Virology* **171,** 331–341.
8. O'Malley, K. L. (1986) *J. Neurosci. Res.* **60,** 3–10.
9. Brown, E. R., Coker, G. T. & O'Malley, K. L. (1987) *Biochemistry* **26,** 5208–5212.
10. Southern, P. J. & Berg, P. (1982) *J. Mol. Appl. Genet.* **1,** 327–341.
11. Miller, A. D., Jolly, D. J., Friedmann, T. & Verma, I. (1985) *Proc. Natl. Acad. Sci. USA* **80,** 4709–4713.
12. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
13. Wigler, M., Silverstein, S., Lee, L.-S., Pellicer, A., Cheng, Y.-C. & Axel, R. (1977) *Cell* **11,** 223–232.
14. Miller, A. D. & Buttimore, C. (1986) *Mol. Cell. Biol.* **6,** 2895–2902.
15. Mann, R., Mulligan, R. C. & Baltimore, D. (1983) *Cell* **33,** 153–159.
16. Quade, K. (1979) *Virology* **98,** 461–465.
17. Iuvone, P. M. (1984) *J. Neurochem.* **43,** 1359–1368.
18. Lowry, O. H., Rosebrough, N. J., Farr, L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
19. Smith, I., Clayton, B. E. & Wolff, O. H. (1975) *Lancet* **i,** 328–329.
20. Matson, W. R., Langlais, P. J., Valicier, L., Gamache, P. H., Bird, E. & Mark, K. A. (1984) *Clin. Chem.* **30,** 1477–1488.
21. Langlais, P. J., Matson, W. R. & Bird, E. D. (1986) in *Monitoring Neurotransmitter Release During Behavior*, eds. Joseph, M. H., Fillenz, M., Macdonald, I. A. & Marsden, C. A. (Horwood, Chichester, U.K.), pp. 224–232.
22. Matson, W. R., Gamache, P. H., Beal, M. F. & Bird, E. D. (1987) *Life Sci.* **41,** 905–908.
23. Svendsen, C. N., Hrbek, C. C. & Bird, E. D. (1987) *Soc. Neurosci. Abstr.* **13,** 1474.
24. Paxinos, G. & Watson, C. (1982) *The Rat Brain in Stereotaxic Coordinates* (Academic, New York).
25. Schmidt, R. H., Ingvar, M., Lindvall, O., Stenevi, U. & Bjorklund, A. (1982) *J. Neurochem.* **38,** 737–748.
26. Fisher, L. J., Jinnah, H. A., Rosenberg, M. B., Langlais, P. J., Friedmann, T. & Gage, F. H. (1989) *Soc. Neurosci. Abstr.*, in press.
27. Herrera-Marschitz, M., Stromberg, I., Olsson, D., Ungerstedt, U. & Olson, L. (1984) *Brain Res.* **198,** 53–61.
28. Dunnett, S. B., Bjorkland, A., Schmidt, R. H., Stenevi, U. & Iversen, S. D. (1983) *Acta Physiol. Scand. Suppl.* **522,** 29–37.
29. Hefti, F., Melamed, E. & Wurtman, R. J. (1981) *J. Pharmacol. Exp. Ther.* **217,** 189–197.
30. Hornykiewicz, O. (1974) *Life Sci.* **15,** 1249–1259.
31. Melamed, E. & Hefti, F. (1984) *Adv. Neurol.* **40,** 149–157.
32. Becker, J. B. & Freed, W. J. (1988) *Brain Res.* **462,** 401–406.
33. Brundin, P., Strecker, R. E., Gage, F. H., Lindvall, O. & Bjorklund, A. (1988) *Ann. N.Y. Acad. Sci.* **537,** 148–160.
34. Lewin, R. (1987) *Science* **237,** 245–247.
35. Sladek, J. & Shoulson, I. (1988) *Science* **240,** 1386–1388.
36. Sladek, J. & Gash, D. M. (1988) *J. Neurosurg.* **68,** 337–351.
37. Perlow, M. J. (1987) *Neurosurgery* **20,** 335–342.