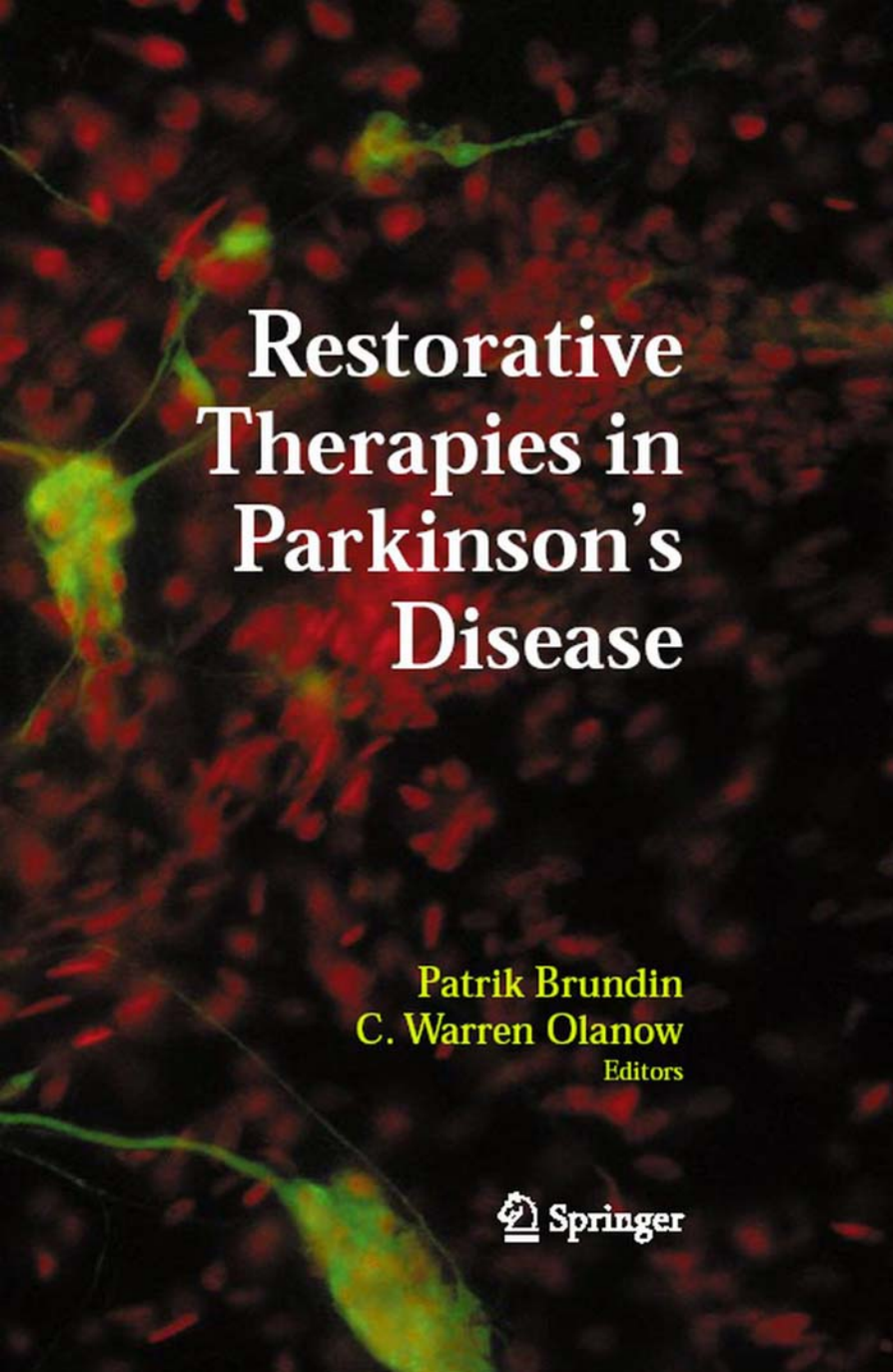
Restorative Therapies in Parkinson's Disease
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Patrik Brundin
Department of Experimental Medical Science
Wallenberg Neuroscience Center
Lund University
221 84 Lund
Sweden
patrik.brundin@med.lu.se

C. Warren Olanow
Department of Neurology
Mount Sinai School of Medicine
One Gustave L. Levy Place
Box 1137
New York, NY 10029
USA
warren.olanow@mssm.edu

*Cover Illustration:* Photomicrograph demonstrating dopaminergic neurons (stained green using an antibody against tyrosine hydroxylase) derived from human embryonic stem cells. The red label shows that the cells are human (stained using an antibody against human nuclear antigen). This type of stem cell-derived neuron is used for transplantation. Kindly provided by Ana Sofia Correia and Sergey V. Anisimov, Lund University.

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*To our families.*

# Preface

Existing pharmacological treatments for Parkinson's disease (PD) are largely focused on a dopamine replacement strategy. However, despite dramatic motor benefits with levodopa and other anti-parkinsonian agents, particularly in early disease, patients continue to suffer disability with chronic treatment and advancing disease. The majority of patients treated with levodopa for more than 5 years experience motor complications in the form of wearing off and dyskinesia. Further, disease progression is associated with the emergence of features that are not adequately controlled with dopaminergic therapies. These include freezing phenomena, gait dysfunction with postural instability, sleep disorders, autonomic dysfunction, psychiatric problems, and dementia. Further, current therapy does not prevent disease progression – indeed, there is concern that reactive oxygen species associated with the oxidative metabolism of levodopa may accelerate neuronal degeneration. Clearly a therapeutic approach for PD that slows, stops or reverses disease progression is an urgent unmet medical need.

Only three decades ago, a volume on restorative therapies in Parkinson's disease would have been better suited for the science fiction section of book stores. However, over recent years this field of investigation has matured into an area with an impressive array of scientific investigations, and several different approaches to restorative therapy have already been tested in clinical trials.

*Restorative Therapies in Parkinson's Disease* critically reviews these scientific and clinical studies, and considers whether replacing only the dopamine system is sufficient. It presents two main approaches to improving brain function in the patients: cell replacement and growth factor administration. The concept of neural transplantation entails replacing degenerated dopaminergic neurons with new dopamine cells that can integrate into the host brain circuitry and restore normal dopaminergic anatomy and physiology. Initial laboratory studies demonstrated that transplanted dopamine neurons derived from fetal mesencephalon had the capacity to survive, manufacture

dopamine, and restore motor function in models of PD. Open label clinical studies testing fetal nigral grafts implanted into the striatum reported modest to important therapeutic benefits with excellent restoration of striatal dopaminergic function based on PET and pathology studies. However, the results of these open label trials were not confirmed in double-blind, placebo-controlled trials. This volume discusses in detail these trials, and how transplant protocols might be improved in the future so as to enhance therapeutic benefits and avoid undesirable side effects. It also raises issues concerning research ethics, clinical trial design, and how the grafts best can be assessed using modern brain imaging methods.

The source of transplanted dopaminergic neurons has been an area of intense ongoing research. Of particular interest are stem cells which offer the potential to provide an unlimited supply of dopaminergic neurons for transplantation. These cells can be expanded in cell culture, and induced to differentiate into optimized dopamine neurons that can be tailored to avoid immune rejection. In this volume, experts describe the different forms of stem cells that have the capacity to differentiate into dopamine neurons, and discuss how these multipotent cells might contribute to future cell therapies of PD. Other approaches that have been or may be examined in the future are considered such as porcine xenografts.

A second approach to restoration of brain function described in this volume is the administration of growth factors in an attempt to restore function to host neurons. Similar to the case of neural transplantation, the initial enthusiasm spawned by small open label trials using infusions of glial cell line-derived neurotrophic factor (GDNF) into the brains of PD patients has not been matched by results obtained in a controlled study. Also safety issues with this procedure have been raised. Here too, however, there is still considerable hope for the future. Current studies utilized catheter delivery of trophic factors which may have limited diffusion of the protein and its potential to influence behavioral responses. Novel ways of delivering the growth factor, using genetically modified cells or viral vectors, may resolve both the current efficacy and safety issues.

These approaches to restore brain function in PD represent a pioneering form of translational research. *Restorative Therapies in Parkinson's disease* integrates the viewpoint of physicians and scientists with expertise in neuroscience, neurology, neurosurgery, brain imaging, viral vector biology, neurotrophic factors, developmental and stem cell biology. This research has provided insight into the organization of the normal and PD brain, offered great promise for those who suffer from this disabling disorder, and opened up the playing field for those interested in brain repair in acute and slowly progressing neurological disease. We are very grateful to all the contributors to this volume who have shared their expertise and thereby helped the research community more rapidly bring effective cures to the many patients in need.

P. Brundin, Lund, Sweden.
C.W. Olanow, New York, NY.
December 11, 2005

# Contents

# Contributors

**Barker, Roger A.**
Cambridge Ctr Brain Repair
Forvie site, Robinson Way
Cambridge CB2 2PY
Great Britain
e-mail: rab46@cam.ac.uk

**Behrstock, Shoshana**
Department of Anatomy and
Neurology
Waisman Center
University of Wisconsin-Madison
1500 Highland Avenue
Madison WI 53705-2280
USA

**Björklund, Anders**
Department of Experimental Medical Science
Division of Neurobiology
Wallenberg Neuroscience Center
BMC A11
SE-221 84 Lund
Sweden
e-mail: Anders.Bjorklund@med.lu.se

**Boer, Gerard J.**
Netherlands Institute for Neurosciences
Meibergdreef 47
1105 BA Amsterdam ZO
The Netherlands
e-mail: g.boer@nin.knaw.nl

**Brooks, David J.**
Division of Neuroscience
Imperial College
Hammersmith Hospital
London W12 ONN
Great Britain
e-mail: david.brooks@csc.mrc.ac.uk

**Brundin, Patrik**
Department of Experimental Medical Science
Wallenberg Neuroscience Center, BMC A10
SE-221 84 Lund
Sweden
e-mail: Patrik.Brundin@med.lu.se

**Cenci-Nilsson, M. Angela**
Department of Experimental Medical Science
BMC F11
Lund University
SE-221 84 Lund
Sweden
e-mail: angela.cenci_nilsson@med.lu.se

**Cesaro, Pierre**
Neurology Department
Inserm U. 421
CHU Henri Mondor
94010 Creteil
France
e-mail: pierre.cesaro@hmn.aphp.fr

**Cooper, Oliver**
Harvard/McLean Udall Parkinson's Disease Research Center
Neuroregeneration Laboratories
McLean Hospital
115 Mill St.
Belmont, MA 02478
USA
e-mail: ocooper@mclean.harvard.edu

**Fahn, Stanley**
Department of Neurology
College of Physicians and Surgeons, Columbia University
New York, New York 10032
USA
e-mail: Fahn@neuro.columbia.edu

**Freeman, Thomas B.**
Department of Neurological Surgery
Center of Excellence for Aging and Brain Repair
University of South Florida
4 Columbia Dr., Suite 730, Tampa, FL 33606
USA
e-mail: tfreeman@hsc.usf.edu

**Gage, Fred H.**
Laboratory of Genetics
The Salk Institute
La Jolla, CA 92037
USA
e-mail: gage@salk.edu

**Georgievska, Biljana**
Department of Experimental Medical Science
Wallenberg Neuroscience Center BMC A11
Lund University
SE-221 84 Lund
Sweden
e-mail: Biljana.Georgievska@med.lu.se

**Hagell, Peter**
Department of Health Sciences
P.O. Box 157
Lund University
SE-221 00 Lund
Sweden
e-mail: Peter.Hagell@med.lu.se

**Isacson, Ole**
Harvard/McLean Udall Parkinson's Disease Research Center
Neuroregeneration Laboratories
McLean Hospital
115 Mill St.
Belmont, MA 02478
USA
e-mail: isacson@hms.harvard.edu

**A. Jon Stoessl**
Pacific Parkinson's Research Centre,
University of British Columbia
Vancouver, Canada
e-mail: jstoessl@interchange.ubc.ca

**Kieburtz, Karl**
University of Rochester Medical Center
1351 Mt. Hope Ave., Ste.223
Rochester, NY 14620
USA
e-mail: karl.kieburtz@ctcc.rochester.edu

**Kim, Hyun-Jung**
Molecular and Cellular Pharmacology/ Waisman Center
University of Wisconsin-Madison
Madison, WI 53705
USA
email: hjkim@waisman.wisc.edu

**Kirik, Deniz**
Department of Experimental Medical Science
Wallenberg Neuroscience Center BMC A11
Lund University
SE-221 84 Lund
Sweden
e-mail: Deniz.Kirik@med.lu.se

**Kordower, Jeffrey H.**
Department of Neurological Sciences
Research Center for Brain Repair
Rush University Medical Center
1735 W. Harrison Street, Suite 300
Chicago, IL 60612
USA
e-mail: jkordowe@rush.edu

**Lang, Anthony E**
Movement Disorders Clinic
Toronto Western Hospital
University of Toronto
399 Bathurst St., 7McL
Canada M5T 2S8
e-mail:lang@uhnres.utoronto.ca

**Lange, Nicholas**
Statistical Neuroimaging Laboratory
McLean Hospital
115 Mill St.
Belmont, MA 02478
USA
e-mail: nlange@hms.harvard.edu

**Dr. D. Chichung Lie**
Institute of Developmental Genetics
GSF Research Center
Ingolstaedter Landstrasse 1
85764 Munich-Neuherberg
Germany
e-mail: chichung.lie@gsf.de

**Obeso, José A.**
Department of Neurology-Neuroscience
Clínica Universitaria and Medical School
University of Navarra, Pamplona
Spain
e-mail:jobeso@unav.es

**Olanow, Warren C.**
Department of Neurology
Mount Sinai School of Medicine
1 Gustave Levy Place
Annenberg 14-94
New York, New York, 10029.
USA
e-mail: warren.olanow@mssm.edu

**Paul, Gesine**
Department of Experimental Medical Science
Section for Neuroscience
Wallenberg Neuroscience Center, BMC A10
SE-22184 Lund
Sweden
e-mail: Gesine.Paul@med.lu.se

**Sanchez-Pernaute, Rosario**
Harvard/McLean Udall Parkinson's Disease Research Center
Neuroregeneration Laboratories
McLean Hospital
115 Mill St.
Belmont, MA 02478
617-855-3283
USA
e-mail: rosario_pernaute@hms.harvard.edu

**Sayles, Mark**
Cambridge Ctr Brain Repair
Forvie site, Robinson Way
Cambridge CB2 2PY
Great Britain
e-mail: rab46@cus.cam.ac.uk

**Sortwell, Caryl E.**
Department of Neurology
University of Cincinnati
P.O. Box 670525
Cincinnati, OH 45267-0525
USA
e-mail: caryl.sortwell@uc.edu

**Sossi, Vesna**
Department of Physics and Astronomy
University of British Columbia
Vancouver, Canada
email: vesna@physics.ubc.ca

**Studer, Lorenz**
Laboratory of Stem Cell & Tumor Biology,
Developmental Biology & Division of Neurosurgery
Memorial Sloan Kettering Cancer Center
New York, NY 10021
USA
e-mail: studerl@mskcc.org

**Svendsen, Clive Niels**
Department of Anatomy and
Neurology, Waisman Center
University of Wisconsin-Madison
1500 Highland Avenue
Madison WI 53705-2280
USA
e-mail: svendsen@waisman.wisc.edu

**Tabar, Viviane**
Laboratory of Stem Cell & Tumor Biology,
Division of Neurosurgery
Memorial Sloan Kettering Cancer Center
1275 York Ave., Box 71
New York, NY 10021
USA
e-mail: tabarv@mskcc.org

**Tai, Yu-Tzu**
Cambridge Centre for Brain Repair
University of Cambridge
Forvie Site
Cambridge
Great Britain

**Widner, Håkan**
Dept of Clinical Sciences
Lund University Hospital
S-221 85 Lund
Sweden
e-mail: Hakan.Widner@med.lu.se

**Zhao, Xinyu**
Department of Neurosciences
University of New Mexico
Albuquerque, NM 87131
USA
e-mail: xzhao@salud.unm.edu

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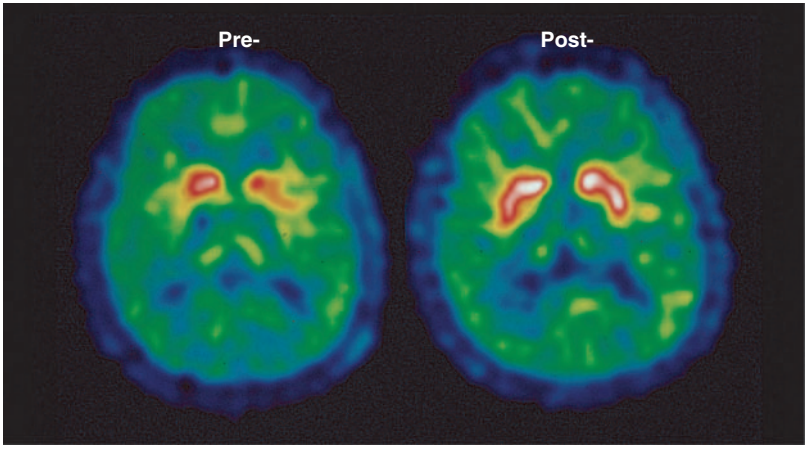


FIGURE 6.1. Flurodopa PET studies in a representative PD patient performed at baseline (**left panel**) and 6 months following bilateral fetal nigral transplantation into the posterior putamen. Note the classical picture of PD at baseline, with reduced striatal uptake particularly in the posterior putamen and the dramatic increase on each side following transplantation.

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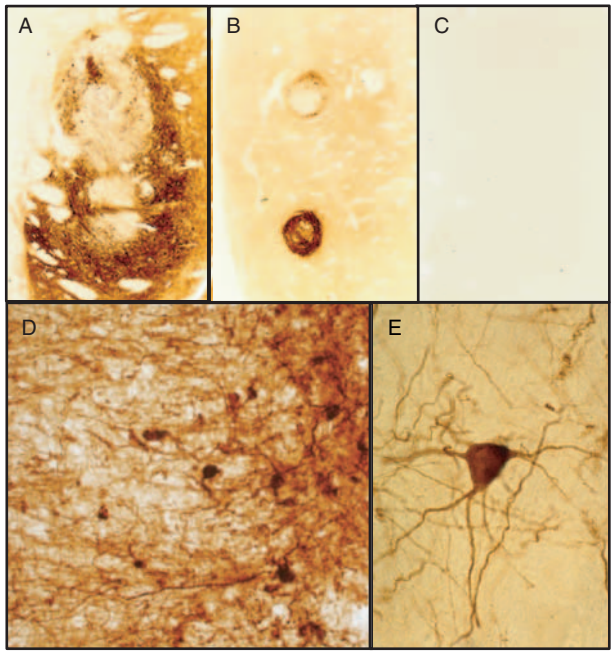
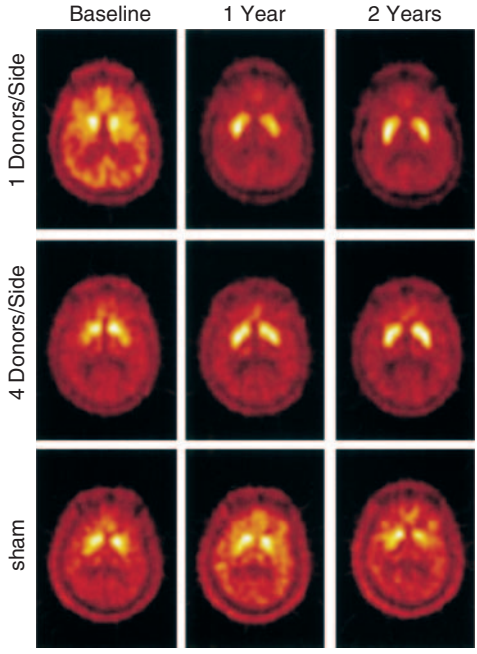



FIGURE 6.2. Tyrosine-hydroxylase immunoreactive staining of the striatum in PD patient receiving transplantation with 4 donors per side (**A**), 1 donor per side (**B**), or a sham procedure (**C**). Note that in the four-donor group, graft deposits have a cylindrical appearance with survival of more than 100,000 dopamine neurons per side. Grafts in the one-donor group have a more dense and circular appearance, with survival of approximately 30,000 TH-positive cells per side, although there still appeared to be continuous TH staining of striatum between graft deposits. High-power images show fibers extending from the graft deposit to provide innervation of striatum (**D**). Higher-power magnification shows normal-appearing implanted dopamine neuron (**E**).

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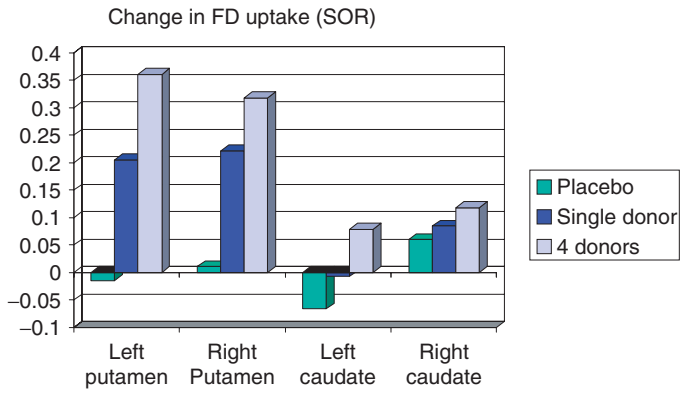


B

FIGURE 7.1. **A**. Axial PET images of 6-[$^{18}$F]-fluoro-L-dopa uptake in representative subjects following sham (bottom panel), one (top panel), or four (middle panel) donors per striatum. In the one- and four-donor subjects, there is an increase in tracer uptake seen at one year (middle column), with a further increase at two years (right column). In the sham-operated subject, fluorodopa uptake continues to decline (from Olanow et al., Ann. Neurol. 2003[40]). **B**. Mean change in striatal:occipital ratio of fluorodopa from baseline to two years following transplant. Note that changes are seen in both left and right putamen, but not in the caudate nuclei, which were not implanted (from Olanow et al., Ann. Neurol. 2003[40]).

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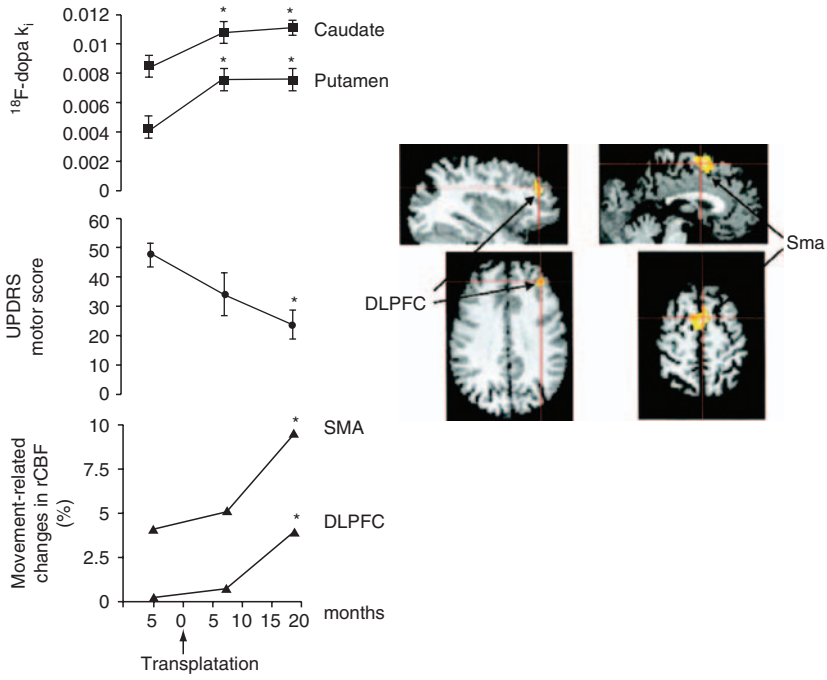


FIGURE 7.2. Increases in fluorodopa uptake following transplantation precede improvement in activation-induced increases in cerebral blood flow in supplementary motor area (SMA) and dorsolateral prefrontal cortex (DLPFC). Increases in the latter are associated with further clinical improvement (from Piccini et al., Ann. Neurol. 2000[1]).

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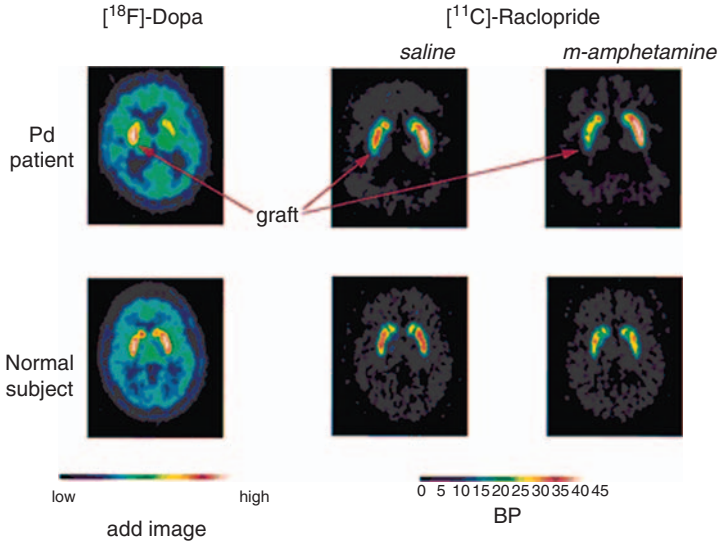


FIGURE 7.3. Fluorodopa uptake (**left panel**) and [$^{11}$C]raclopride binding (**middle** and **right panels**) in a Parkinson patient with a unilateral transplant in the right striatum (shown on the left side of the image). In the grafted striatum, fluorodopa uptake improves to near normal levels. Following saline injection (middle panel), raclopride binding is increased compared to control levels in the *non-grafted* striatum, with no response to intravenous methamphetamine (right panel). In contrast, raclopride binding is normal in the grafted striatum, with a further reduction following methamphetamine (as seen in controls). This reduction represents occupancy of dopamine receptors by dopamine released in response to the pharmacological stimulus (from Piccini et al., Nat. Neurosci. 1999[31]).

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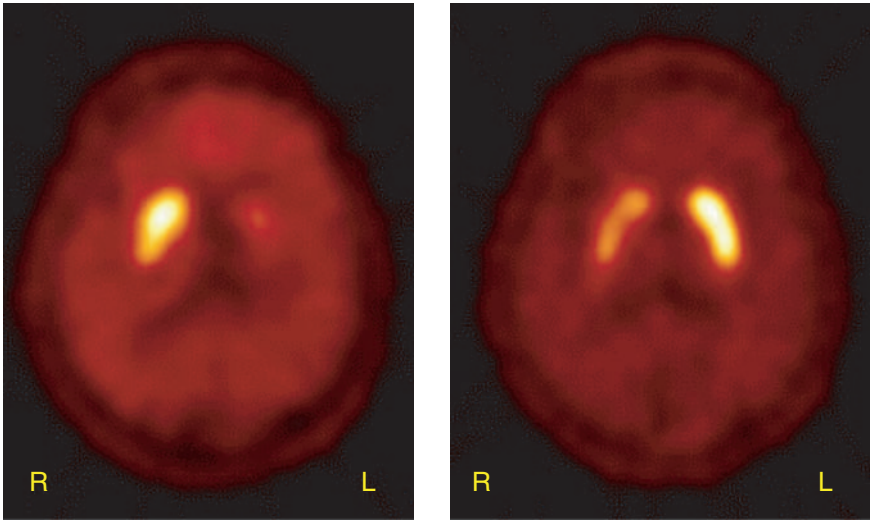


[$^{18}$F]Fluorodopa [$^{11}$C]Raclopride

FIGURE 7.4. Fluorodopa and raclopride PET scans in a patient with left hemibody dyskinesias 8 years following transplant. Note that there is a marked increase in fluorodopa uptake in the right caudate nucleus, while raclopride binding is reduced below control levels throughout the right striatum. The findings suggest aberrantly increased dopamine synthesis and release in the striatum contralateral to dyskinesias (UBC-TRIUMF PET team).

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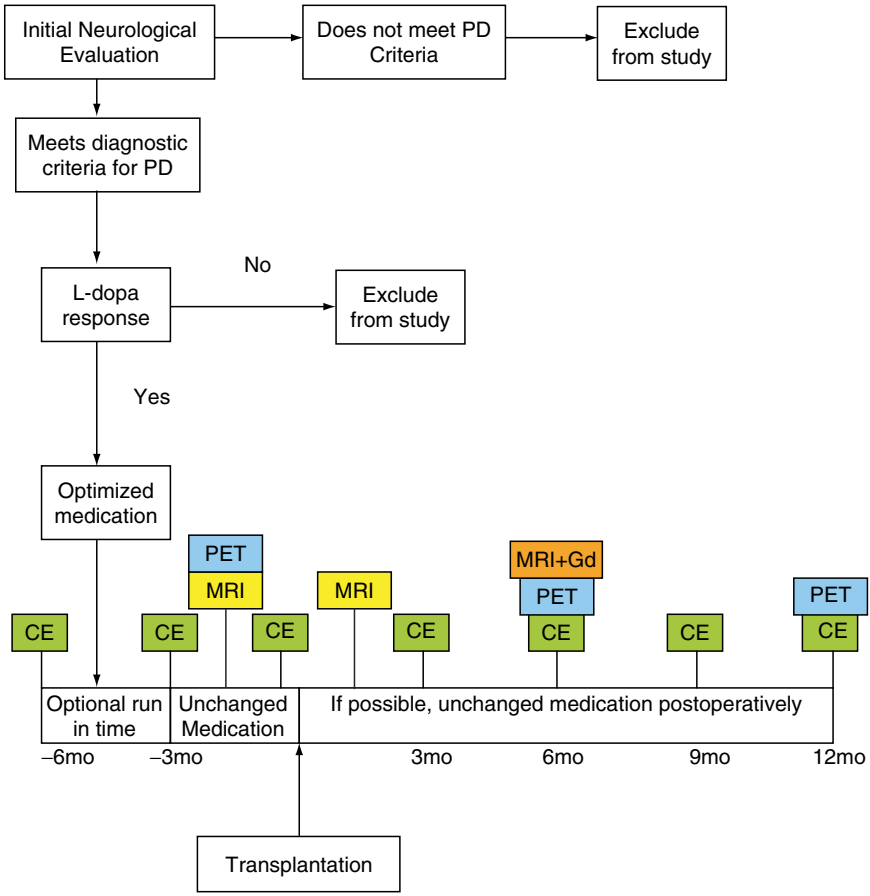


FIGURE 9.1. Schematic of the CAPIT protocol (modified from Langston et al., 1992).

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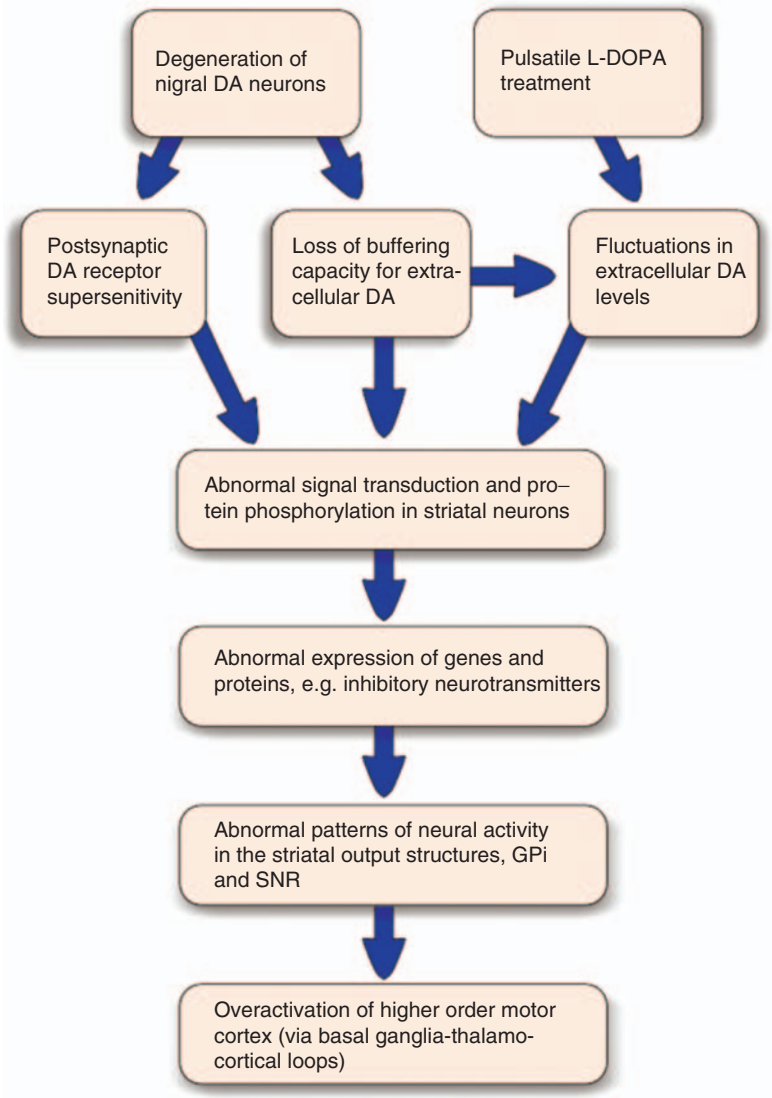


FIGURE 10.1. Flow chart illustrating the sequence of events at the basis of L-DOPA-induced dyskinesia according to commonly accepted notions. GPi, globus pallidus pars interna; SNR, substantia nigra pars reticulata.

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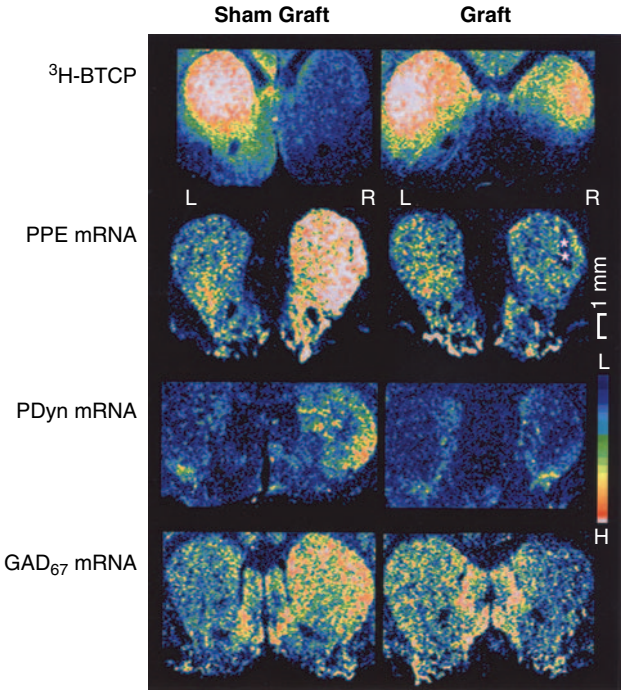


FIGURE 10.3. Pre- and post-synaptic effects of intrastriatal VM grafts in rats with unilateral 6-OHDA lesions that were treated with L-DOPA. In this study VM grafts were found to improve L-DOPA-induced "on" dyskinesia (see Fig. 2). Autoradiographic pictures of striatal sections from VM-grafted rats ("graft") are represented in the right-hand column, whereas pictures from sham-grafted controls ("sham graft") are shown in the left column. In all pictures, the DA-denervated and grafted side of the striatum is shown to the right (**R**), and the contralateral intact side is shown to the left (**L**). [$^3$H]BTCP binding autoradiography (which labels DA uptake sites in the striatum) showed a nearly complete restoration of striatal DA fiber density in the grafted animals. In situ hybridization histochemistry was used to measure the expression of mRNAs encoding for preproenkephalin (PPE), prodynorphin (PDyn), and glutamic acid decarboxylase (GAD$_{67}$). These transcripts are expressed in striatal medium-sized spiny neurons, and are known to exhibit a marked up-regulation following DA-denervating lesions and/or pulsatile L-DOPA treatment (compare lesioned side and contralateral intact side in the sham-grafted animals). The levels of all these transcripts were restored to normal values by the VM grafts (from Lee et al., 2000).

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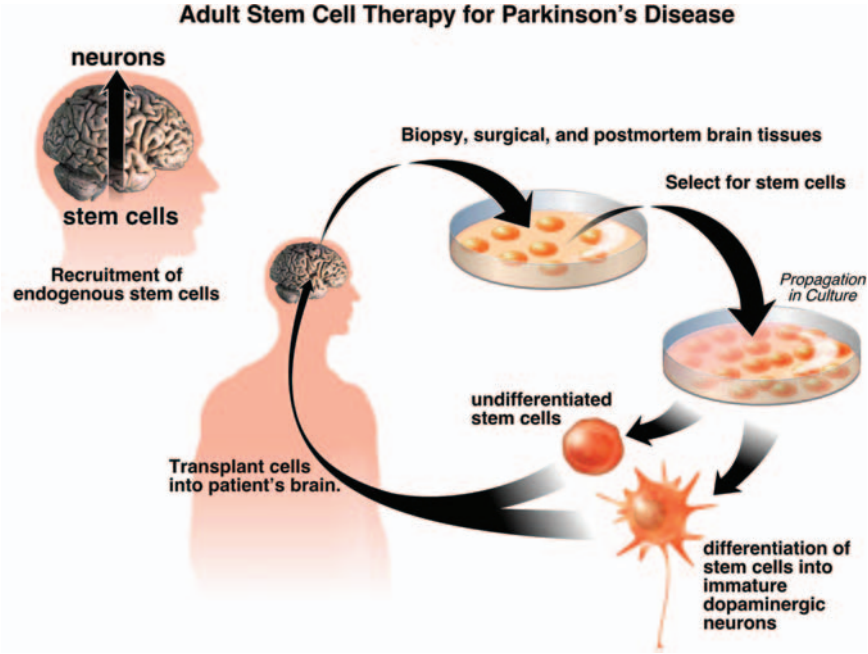


FIGURE 12.1. Schematic diagram showing strategies for using adult stem cells for treating PD. For transplantation therapy, stem cells can be isolated from surgical and post-mortem adult brain tissues, propagated in vitro, manipulated in vitro (e.g., genetically modified, differentiated, etc.) and transplanted into the brain of a patient with PD.

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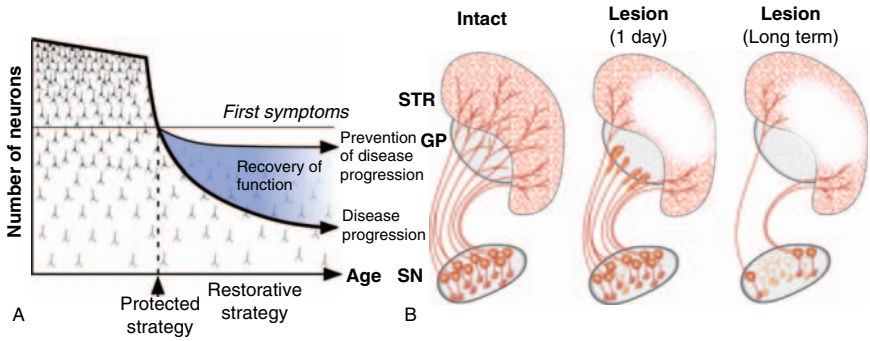


FIGURE 15.1. Protective treatment strategy for Parkinson's disease. **A**: The slow and protracted loss of nigral DA neurons in PD presents opportunities to intervene in the degenerative process and prevent the further progression of the disease. A protective "disease-modifying" treatment strategy would be relevant in the early phases of the disease, when a substantial portion of the nigral DA neurons remain and may involve treatment with neurotrophic factors, such as GDNF. **B**: The intrastriatal 6-OHDA lesion provides us with a progressive nigral cell degeneration model. Injection of 6-OHDA into the striatum induces an acute lesion of the DA axon terminals, followed by a slow retrograde degeneration of the nigral DA neurons.

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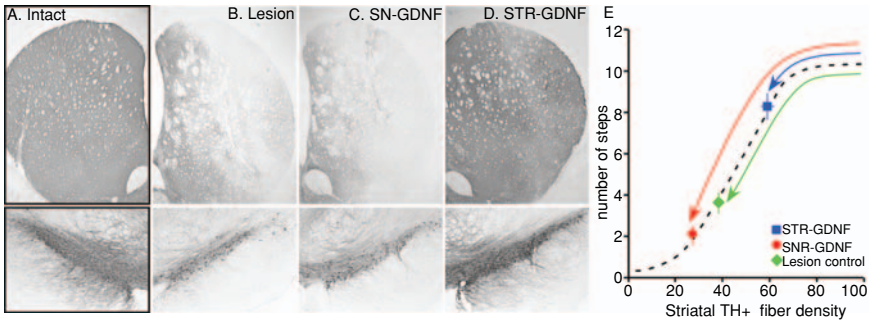


FIGURE 15.2. Protection of the nigrostriatal DA pathway after recombinant GDNF protein treatment. The photomicrographs show cross-sections from the central striatum and corresponding SN immunostained for TH representing four different conditions: (**A**) intact nigrostriatal system, (**B**) vehicle-treated lesion controls, (**C**) nigral GDNF injection group, (**D**) striatal GDNF injection group. Delivery of recombinant GDNF protein into the SN or striatum prior to an intrastriatal 6-OHDA lesion provides a significant protection of the nigral cell bodies as compared with the vehicle-treated controls. Protection of the DA terminals, on the other hand, can only be obtained following delivery of GDNF into the striatum. Note that the performance of the animals in the stepping test can best be explained by preservation of the striatal TH-positive fibers (**E**). The intrastriatal 6-OHDA lesion induces depletion of DA terminals accompanied with a reduction in the number of steps the animals can perform (green arrow in E). While administration of GDNF into the striatum preserves the TH-positive fibers and thus the normal motor function (blue arrow), the nigral GDNF group fails to be beneficial (red arrow). (Figure modified from data published in Kirik et al., 2000a).

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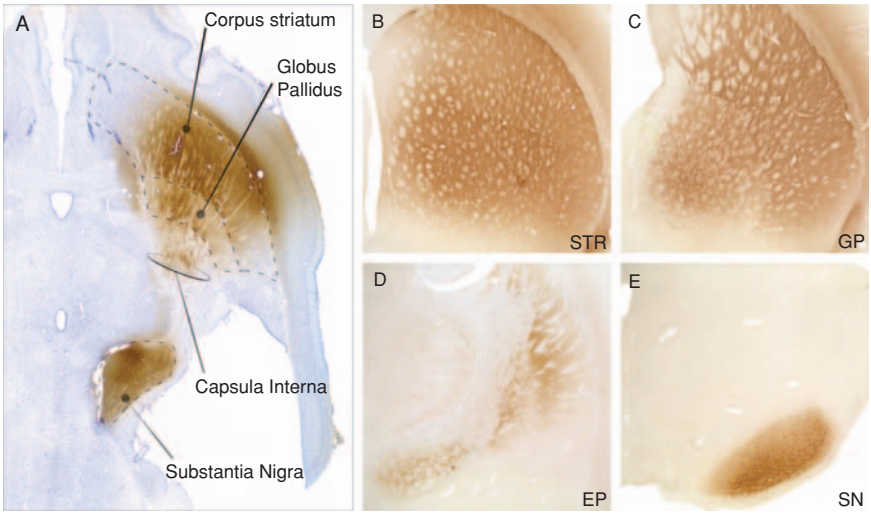


FIGURE 15.3. Expression and distribution of GDNF following rLV-GDNF injection into the striatum. **A**: A horizontal section immunostained for GDNF demonstrates the extensive diffusion of GDNF protein in the striatum, as well as transport and release of GDNF along the striatopallidal and striatonigral projections. In this particular animal, rLV-GDNF was injected into the striatum after a complete 6-OHDA lesion, demonstrating that the transport of GDNF to the GP and SN pars reticulata was in the anterograde direction. Coronal sections from the striatum (**B**) and the different striatal output nuclei (**C–E**) further illustrate the distribution of GDNF. (Figure modified from data published in Georgievska et al., 2002a).

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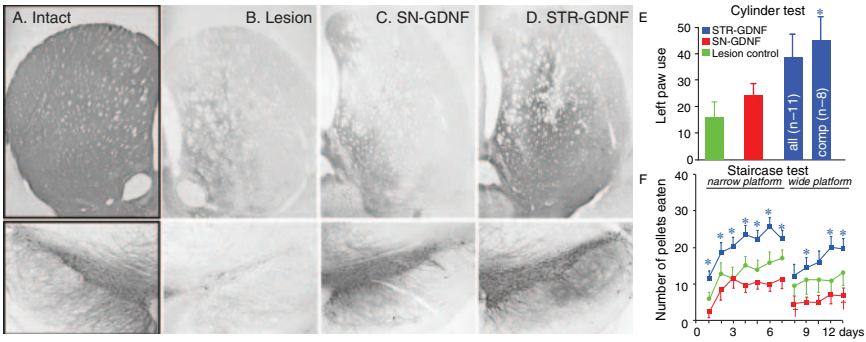


FIGURE 15.4. Protection of the nigrostriatal DA pathway after rAAV-mediated GDNF delivery. The photomicrographs show cross-sections from the central striatum and corresponding SN immunostained for TH representing four different conditions: (**A**) intact nigrostriatal system, (**B**) GFP vector-treated lesion controls, (**C**) nigral rAAV-GDNF injection group, (**D**) striatal rAAV-GDNF injection group. Expression of GDNF protein in the SN or striatum prior to an intrastriatal 6-OHDA lesion provides a significant protection of the nigral cell bodies, as compared with the lesion controls. Protection of the DA terminals, on the other hand, can only be obtained following delivery of rAAV-GDNF into the striatum. The results from the cylinder test (**E**) and the staircase test (**F**) indicate that functional improvements were seen only after delivery of rAAV-GDNF into the striatum (coded by blue colors in E and F), while nigral rAAV-GDNF delivery appeared to be ineffective (compare red to green color). (Figure modified from data published in Kirik et al., 2000b).

## COLOR PLATES

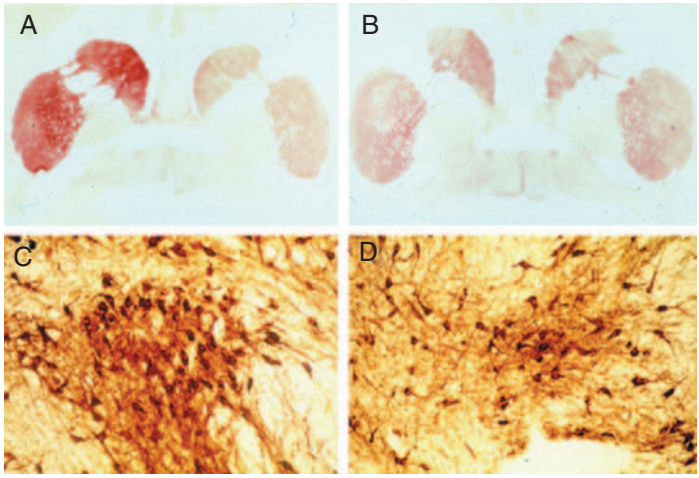


FIGURE 16.1. **A:** Section stained for TH immunoreactivity through the anterior commissure illustrating the increase in TH immunoreactivity within the right caudate nucleus and putamen after LVGDNF delivery to aged monkeys. **B:** Symmetrical and less intense staining for TH immunoreactivity in a monkey injected with LV βGal. **C:** There were greater numbers and larger TH-immunoreactive neurons within the substantia nigra of a LV GDNF-treated animal relative to (**D**) a LV βGal-treated monkey.

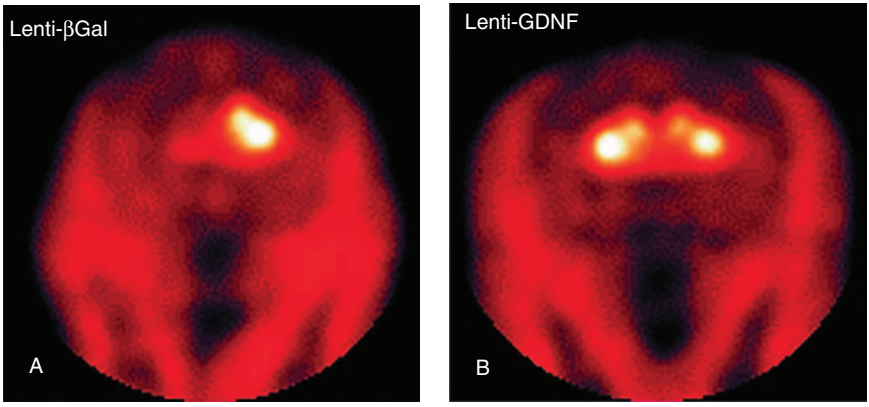


FIGURE 16.3. Positron emission tomography (PET) scan data evaluating the influence of LV GDNF on fluorodopa (FD) uptake in young adult MPTP-treated monkeys. **A:** After MPTP lesions, a comprehensive loss of FD uptake was seen within the right striatum of LV βGal-treated young adult monkeys. **B:** In contrast, FD uptake was enhanced in LV GDNF-treated monkeys. *K*i values (per minute) for the striatum are as follows: LV βGal left, 0.0091 ± 0.0004; LV βGal right, 0.0017 ± 0.0005; LV GDNF left, 0.0084 ± 0.0004; LV GDNF right, 0.0056 ± 0.0018.

# COLOR PLATES

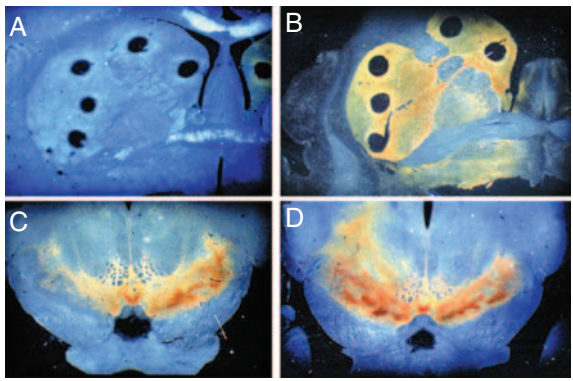


FIGURE 16.4. Low-power dark-field photomicrographs through the right striatum of TH-immunostained sections of MPTP-treated monkeys treated with (**A**) LV βGal or (**B**) LV GDNF. A: There was a comprehensive loss of TH immunoreactivity in the caudate and putamen of LV βGal-treated animal. In contrast, near normal level of TH immunoreactivity is seen in LV GDNF-treated animals. Low-power (**C** and **D**) photomicrographs of TH-immunostained section through the substantia nigra of animals treated with LV βGal (C) and LV GDNF (D). Note the loss of TH-immunoreactive neurons in the LV βGal-treated animals on the side of the MPTP-injection. TH-immunoreactive sprouting fibers, as well as a supranormal number of TH-immunoreactive nigral perikarya are seen in LV GDNF-treated animals on the side of the MPTP injection.

# 1

# Restorative Therapies for Parkinson's Disease: Current Status and Future Perspectives

ANDERS BJÖRKLUND

## 1. Introduction

This volume is about translational research: attempts to translate cell- and gene-based restorative approaches developed in experimental animal models and to assess their potential therapeutic efficacy in small, well-designed clinical trials in patients with Parkinson's disease (PD). This research has progressed along two parallel, but complementary, lines of investigation: *cell replacement*, where transplants of immature dopamine (DA) neurons are used to reinnervate and restore DA neurotransmission in the DA-dennervated striatum in PD patients; and *neurotrophic factor delivery*, where glial-cell-line-derived neurotrophic factor (GDNF) is infused intracerebrally in order to block degeneration of the nigral DA neurons and promote regeneration and recovery of function in remaining DA neurons.

The experimental basis for both these approaches is quite substantial and covers more than two decades of preclinical research in rodents and primates. The DA neuron transplantation technique was developed in the early 1980s. When the first trials were initiated in PD patients in 1987 there were quite extensive experimental data to indicate that intrastriatal transplants of immature DA neurons or neuroblasts, derived from the developing ventral mesencephalon, could reverse at least some aspects of the PD-like motor impairments in 6-hydroxydopamine (6-OHDA) lesioned rats. Similar, albeit more limited, data had also been generated in MPTP-lesioned primates (see Herman and Abrous, 1994; Björklund et al., 1994; Winkler et al., 2000, for comprehensive reviews). The first clinical trial using intraventricular infusions

Department of Experimental Medical Science, Wallenberg Neuroscience Center, Lund University, SE-221 84 Lund, Sweden
Anders.Bjorklund@med.lu.se

of recombinant GDNF protein was initiated in 1996. At this time data on the ability of intracerebrally infused GDNF to protect nigral DA neurons and stimulate DA turnover and function had been obtained in both rodents and primates (see Grondin et al., 2003; Kirik et al., 2004, for review). The MPTP and 6-OHDA lesion models are useful for basic studies of efficacy, mechanisms of action, modes of cell or protein delivery, etc.; but these toxin models do not replicate the underlying progressive pathology seen in human PD. The results obtained in preclinical studies in 6-OHDA-lesioned rats and MPTP-lesioned monkeys, therefore, are very valuable as a basis for the design of a clinical trial, but they are of limited value for prediction of the clinical outcome. For this reason, progress in the restorative therapy field has been characterized by a mixture of progress and failures where the results obtained in clinical trials have highlighted unresolved issues and problems to be resolved in further preclinical studies. Over time, a close interplay has developed between basic scientists and clinical investigators that has been critical to maintaining momentum and progress in neurorestorative research.

## 2. The Ups and Downs of Restorative Therapy

Clinical trials of fetal cell transplantation in PD developed in two phases. In the first phase, starting in 1987, a series of small open-label trials were undertaken with the aim of obtaining proof-of-principle for the DA cell replacement approach. From the results obtained in these trials we know for certain that human fetal mesencephalic DA neurons can survive long term in the striatum of PD patients, without any sign of being affected by the ongoing disease process, and that they can reinnervate parts of the DA-depleted striatum and provide a significant long-term restoration of striatal DA synthesis and storage, as revealed by $^{18}$F-DOPA PET (see Kordower et al., 1998; Hagell and Brundin; 2001, Lindvall and Björklund, 2004; Winkler et al., 2005, for recent reviews). A major issue raised by the open-label trials, however, is that the clinical outcome has been highly variable, not only between centers, but more importantly within groups of patients transplanted at the same centers using the same type of cell preparation and surgical technique. The best cases have demonstrated an excellent response with reductions in UPDRS motor scores of 40–60% in the *off*-state, while others have shown no or only minor benefits (Lindvall and Björklund, 2004; Winkler et al., 2005). Although graft survival and DA fiber outgrowth, as monitored by $^{18}$F-DOPA PET, show a similar marked variability from patient to patient, it is unlikely that graft survival alone can explain the variable clinical outcome. As discussed by Cesaro and Widner in Chapter 5, it is likely that other factors, such as composition, preparation and storage of the fetal mesencephalic tissue, immune and inflammatory mechanisms, immunosuppressive treatment, graft placement, and patient selection play an important role.

In the mid 1990s cell transplantation trials entered a second phase when the National Institutes of Health (NIH) decided to sponsor two sham surgery

controlled double-blind studies, the Denver-Columbia study (Freed et al., 2001) and the Tampa-Mount Sinai study (Olanow et al., 2003). The results of these trials have raised two major concerns: the modest efficacy, at best, of the procedure in the transplanted patients, and the development over time of *off*-state dyskinesias in a significant number of transplanted patients. The disappointing outcome has made it clear that we still do not fully know how best to apply DA cell transplantation to obtain consistent optimal results, and it highlights a number of important issues that need to be addressed and solved in further preclinical experiments. The NIH-sponsored trials, however, have been reassuring in that none of the control groups (which had undergone sham surgery and were evaluated in a blind manner) showed any significant, long-term benefit from the sham procedure. Placebo effects are known to be prominent in the treatment of PD patients (de la Fuente-Fernandez et al., 2001). These results suggest, however, that placebo effects and/or investigator bias may play a less important role in one-time surgical procedures where the assessment is protracted over many months or years after the intervention. The best argument against a pure placebo effect in the open-label trials may come from observations in patients with asymmetric disease who receive transplants only on one side—contralateral to the worst performing side—where the asymmetry gradually is reversed so that the worst side becomes the patient's best performing side. Intrastiatal DA neuron transplants can work, and sometimes work very well, in PD patients with advanced disease.

The clinical trials using intracerebral delivery of GDNF have seen similar setbacks. The initial trial, which was carried out by Amgen between 1996 and 1999, had to be terminated prematurely due to unwanted side effects, and the postoperative evaluation did not show any clinical improvement at any of the doses tested (Nutt et al., 2003). The design of this trial was based on previous experiments by Gash and co-workers in MPTP-treated monkeys, where recombinant GDNF was injected intracerebroventricularly (ICV) in bolus doses at monthly intervals (Gash et al., 1996; Zhang et al., 1997). The functional improvements observed in these primate experiments were not replicated in the clinical trial, not even at the very high doses of GDNF tested (up to 4,000 μg per injection in some of the patients). Further studies in rodents (Lapchak et al., 1997; Kobayashi et al., 1998; Kirik et al., 2000) as well as in one of the GDNF-infused patients from the Amgen trial (Kordower et al., 1999) indicate that the diffusion of GDNF from the cerebrospinal fluid into the brain parechyma is quite limited. GDNF administered ICV may thus not have reached the intended targets—the striatum and/or substantia nigra—at sufficient amounts in the Amgen trial. Indeed, the most prominent effects of GDNF on DA neuron survival, axonal sprouting, and functional recovery in 6-OHDA-lesioned rats or MPTP-lesioned monkeys have been observed after localized, sustained delivery of GDNF directly into the striatum or substantia nigra (Grondin et al., 2003, 2005; Kirik et al., 2004).

Based on these animal experimental data two open-label studies were initiated, in Bristol, UK, by Gill and co-workers (2003), and in Lexington, Kentucky, by Slevin and co-workers (2005). In these trials the patients received

continuous infusion of GDNF into the putamen, unilaterally or bilaterally, at escalating doses of up to 30–40 μg/day. The results reported from these two trials are quite promising: in both groups of patients there was a significant improvement of the UPDRS motor scores that persisted during the 1-month wash-out period (Slevin et al., 2005). In a follow-up report, Love et al., (2005) reported autopsy data from one of the five patients in the Bristol study that had received GDNF infusion unilaterally for over 3.5 years, which had been stopped 3 months before death (from a myocardial infarct). The microscopic analysis of this case showed a distinct increase in tyrosine hydroxylase (TH) staining in the putamen unilaterally, around the infusion site, matching an increase in $^{18}$F-DOPA uptake as seen by PET. This suggests that GDNF may have induced a sprouting response, or possibly an upregulation of the TH enzyme, at the site of infusion.

The data reported from the Bristol trial stimulated Amgen to reactivate their GDNF program and initiate a second multi-center trial, this time using continuous intraputaminal infusion of GDNF, essentially according to the Bristol protocol. The positive results reported from the Bristol and Lexington trials were not replicated, however, and the trial was terminated ahead of schedule due to the appearance of neutralizing GDNF antibodies in 4 of the 34 patients, and reports of cerebellar lesions in a primate toxicology study that was carried out in parallel with the clinical trial (see News@Nature online 05 October, 2004).

## 3. Entering a New Phase

Cell therapy and GDNF delivery are now entering a new phase. The negative results from the NIH and Amgen trials represent, of course, serious setbacks, but they are at the same time valuable in that they have raised a series of important questions and helped us to focus the preclinical research on a set of critical issues that need to be resolved in further experimental studies. The development of efficient and safe restorative therapies in PD will depend on a close interplay between systematic preclinical research, carried out in the best available animal models, and experience gained from carefully conducted trials in patients. The failure of the NIH and Amgen trials to replicate findings in rodent and primate models of PD has raised concerns about the validity of the toxin-based animal models and their value for predicting the outcome when the same procedures are applied in patients. This has led some investigators to play down the value of animal experimental work and dismiss the need for solid demonstration of efficacy and safety in animal models as a basis for the design of clinical trials. The 6-OHDA and MPTP lesion models have a weakness in that they do not accurately replicate the underlying disease process and the full spectrum of pathological changes seen in PD patients. These models are intrinsically non-progressive and they are designed to reproduce the nigral DA neuron cell loss, and the associated

DA-dependent functional impairments, seen in PD. Nevertheless, the toxin lesion models are highly useful for a range of more basic mechanistic studies, and they are essential as test beds for the development and perfection of the cell replacement approach, as well as the techniques for GDNF protein and gene delivery.

A second concern is whether DA neuron replacement, or neuroprotection aimed at the degenerating nigral DA neurons, will be sufficient. As Obeso and Lang emphasize in their chapter in this book (Chapter 3) restoration of dopaminergic function alone, and elective correction of the dopaminergic deficit, is unlikely to affect the natural history of PD, and may, therefore, be insufficient in the long run. As the disease progresses, PD patients will develop a variety of motor and non-motor symptoms that are insensitive to dopaminergic medication. These "non-dopaminergic" clinical manifestations are likely to be due to the involvement of neurons in other cortical, subcortical, and brainstem regions, possibly affected by the same disease process that affects the nigrostriatal DA system. This may, of course, limit the efficacy of dopaminergic restorative approaches, such as striatal DA neuron transplants and GDNF delivery. To the extent that these non-dopaminergic features are early manifestations in some PD patients, this may also restrict the potential use of DA-restoration therapies to defined subgroups of patients where DA neuron cell loss is the leading cause of disability. Nevertheless, there is every reason to believe that DA cell replacement and DA neuron protection can provide significant therapeutic benefit, improve the quality of life and extend the "good years" at least in a major subgroup of PD patients.

## 4. What Next?

The way forward in the restorative therapy field is through systematic preclinical studies aimed at a set of critical issues that need to be resolved in order to make further progress. These issues need to be defined, and the way to address them needs to be carefully discussed. The chapters contained in this volume do just that. They provide, for the first time, a comprehensive overview of the state of the art in the restorative therapy field, and a discussion of the challenges that face basic and clinical scientists in their efforts to translate preclinical research into successful clinical therapies. The main issues, as I see it, can be listed under the following seven headings:

1. *Perfection of the cell transplantation technique*. As discussed in detail in the chapter by Freeman and Brundin (Chapter 8), there has so far been no attempt to standardize the way fetal cell transplantation has been carried out at different centers. Almost all aspects of tissue procurement and handling has varied from one center to another: the dissection of the tissue, the age of the donor fetuses, the length and type of storage after dissection, the way the tissue is dissociated prior to grafting (into pieces or crude

cell suspensions), the amount of tissue grafted, and the composition of the medium used for storage and/or injection. As a result, the composition and the viability of the cell material used for transplantation is likely to vary significantly. Although systematic studies have not been carried out, there are good reasons to believe that prolonged cold storage or culture of the tissue may be detrimental to DA neuron survival and also change the composition of the cell material in an uncontrolled way. The use of solid tissue grafts (as done in the Tampa–Mount Sinai trial), makes it possible to use also somewhat older aborted fetuses (up to 9–9.5 weeks of gestation compared to 6–8 weeks for dissociated tissue). Solid tissue grafts, however, are likely to be more immunogenic and thus at a greater risk to be affected by the host's immune response.

2. *Patient selection*. Clearly, the optimal candidates for DA neuron replacement or GDNF delivery have not yet been identified. In most clinical trials patients were included at a relatively young age, usually in their 50s and 60s, which can in part be attributed to an overall higher agility in younger patients and to a smaller risk of concomitant diseases. The issue of patient selection for dopaminergic restorative therapy is discussed by several authors (see the chapters 3, 6, 8). As discussed above, it is essential that nigral DA cell loss be the leading cause of disabling symptoms, and that other, non-dopaminergic pathologies not be prominent. For this reason, older patients or patients with more advanced disease might be less suitable candidates for transplantation. Indeed, in the Denver-Columbia trial the younger patients, below the age of 60, showed significant improvement in motor performance, whereas patients above that age did not show any clinical improvement. And in the Tampa-Mount Sinai trial the less severely affected patients (UPDRS motor score <49) showed significantly better improvement as compared to patients with more severe disease (UPDRS motor score >49). It seems likely, although not yet proven, that the ability of the patient to respond to L-DOPA medication prior to the surgical intervention may be one of the important factors determining functional efficacy of dopaminergic restorative therapy.
3. *Graft efficacy and graft placement*. From available data it seems clear that good graft survival and reinnervation of the denervated striatum are necessary prerequisites for a good clinical response. It is equally clear, however, that the variable clinical outcome in transplanted patients cannot be explained by variability in DA neuron survival and axonal outgrowth alone. In the clinical trials performed so far, the DA neuron grafts have – with few exceptions – been placed in the putamen, and in some cases only in the posterior putamen. The reasons for selecting the putamen as the primary transplantation target are that this region exhibits the most marked reductions in DA content, and that it is the part of the striatal complex that is most closely linked to motor control. However, DA neuron degeneration is more widespread, and in most patients DA projections to limbic and cortical areas are also involved. Patients with more widespread DA neuron

cell loss, where the disease has progressed to involve also non-striatal areas, may be less responsive to DA neuron transplants confined to the putamen only. Ideally, graft placement should be tailored to each patient. One way to approach this would be to perform high-resolution $^{18}$F-DOPA PET scans prior to surgery in order to identify the areas, or subregions, where $^{18}$F-DOPA uptake is most severely reduced, as a basis for the selection of the optimal transplantation sites for each patient. This has so far not been attempted in any clinical trial.

4. *Immune and inflammatory mechanisms*. Animal experiments have shown that allografts of fetal VM tissue can survive for prolonged periods in the brain in the absence of any immunosuppression. However, in cases where the donor and host differ immunologically on both major and minor histocompatibility antigens the graft is likely to induce a long-lasting inflammatory response, accompanied by an upregulation of class-I and class-II antigens on the grafted cells, sustained expression of immunological markers, and macrophage and microglial activation at the graft site. The intensity of the immune reaction may increase over time and thus provide an ongoing immune/inflammatory process (see Barker and Widner, 2004, for a recent review). Shinoda et al. (1995,1996) have shown that this delayed inflammatory response may be detrimental to both survival and function of intrastriatal DA neuron transplants, and that the DA neurons may survive in a compromised state for a long time, with patches of activated microglia-macrophages and increased expression of class-II antigens. Interestingly, this is a picture similar to that observed in the two cases that have come to autopsy in the Tampa-Mount Sinai trial (Olanow et al., 2003). These patients showed a gradual clinical improvement over the first 6 months post-transplantation (i.e., during the time of immunosuppressive treatment), but the apparent benefits of the graft gradually disappeared after immunosuppression was terminated. The need for long-term immunosuppressive treatment needs to be explored more systematically. Such treatment may indeed be essential to allow the transplanted DA neurons to develop their full functional potential.
5. *Graft-induced dyskinesia*. The problem of graft-induced dyskinesias, as seen in the two NIH-sponsored clinical trials, is important to penetrate in detail. The mechanisms by which they are induced and the reason why they occurred in such high frequency in the Denver-Columbia and Tampa-Mount Sinai trials must be understood. Cenci and Hagell (Chapter 10) present a comprehensive overview of this problem and provide a list of eight different possible mechanisms, all of which can be explored in animal experiments. With few exceptions all previous transplantation studies in rodent and primate PD models have been performed in non-dyskinetic and non-L-DOPA-treated animals. The clinical observations made in the NIH trials, however, have stimulated renewed interest in the functional impact of intrastriatal DA neuron grafts in animals exposed to chronic L-DOPA treatment, and in animals exhibiting L-DOPA-induced dyskinesias. Experiments along these lines are now under way in several laboratories.

6. *Generation of DA neurons from stem cells*. The problems associated with the use of human fetal tissue makes it necessary to find alternative sources of cells for intracerebral grafting. Ideally, these cells should have the potential for expansion in large numbers in vitro and allow differentiation into fully functional dopaminergic neurons upon transplantation and/or after induction in vitro. Stem and progenitor cell technology has the potential to provide virtually unlimited numbers of defined and standardized cells for transplantation in PD patients. Three of the chapters in this volume discuss how this may be achieved using embryonic stem (ES) cells (Tabar and Studer, Chapter 13), adult-derived neural stem cells (Zhao et al., Chapter 12), or genetically engineered cells (Kim et al., Chapter 15). Another alternative, involving the use of embryonic porcine tissue, is discussed in the chapter by Barker at al. (Chapter 11). These are all techniques in early stages of development, and it is still too early to predict which strategy will turn out to be most successful. The xenotransplantation approach faces special hurdles, including the possible risk of so-called zoonotic infections by endogenous retroviruses, and the problems related to xenograft rejection.

   The most promising results so far have been obtained using mouse ES cells. The DA neurons generated from ES cells are clearly DA producing and express, at least in part, a dopaminergic mesencephalic neuronal phenotype, but it is unclear to what extent they differentiate into fully functional DA neurons of the correct type; i.e., DA neurons of the type normally present in the substantia nigra. There are good reasons to believe that only neurons expressing this *nigral* phenotype will be fully functional after transplantation to the striatum (see Isacson et al., Chapter 9; Thompson et al., 2005). This is important to keep in mind when evaluating the outcome of DA cell transplants in either animals or patients; the total number of surviving dopaminergic neurons (i.e., TH-positive cells) in the graft does not tell us how many of these are fully functional nigral neurons with extensive functional projections into the host striatum.

7. *Mode and site of delivery of GDNF*. The failure of GDNF to produce any significant clinical benefit in the latest Amgen trial raises the question of how, and where, GDNF should be delivered to produce optimal effects in PD patients. From animal experiments we know that GDNF can induce a functional upregulation in intact or lesioned nigral DA neurons by either the intraventricular, intranigral, or intrastriatal routes, although the intraventricular route requires considerably higher doses of GDNF, at levels that are prone to induce unwanted side-effects. In 6-OHDA-lesioned rats and MPTP-lesioned monkeys GDNF delivered directly into the striatum is capable of protecting degenerating or damaged nigrostriatal axons and terminals. This site of delivery may also be a factor in inducing axonal sprouting from spared DA neurons (Grondin et al., 2003; Kirik et al., 2004). This clearly speaks in favor of the intrastriatal delivery route, as used in the Gill et al. and Slevin et al. studies. From the animal experimental data,

however, it seems clear that intrastriatal GDNF is effective only in cases where a significant portion of the nigrostriatal projection remains intact, and thus that the efficacy is diminished in animals with advanced parkinsonism. In such cases, only intranigral delivery, acting via increased DA transmission in downstream striatal targets (substantia nigra and/or globus pallidus) may be more effective in providing symptomatic relief (Grondin et al., 2005). In PD patients, the optimal site of GDNF delivery may also depend on the site of the primary insult, whether acting on the axon terminals in the striatum or at the level of, or intrinsic to, the nigral cell bodies. In the latter case one may predict that combined GDNF delivery to both substantia nigra and striatum may be the optimal choice. The disappointing results reported from the second Amgen trial suggest that direct infusion of recombinant GDNF protein may not be ideal, and that other, gene-based modes of delivery should be tested. As discussed by Kirik et al. (Chapter 14) there are considerable experimental data in rodents and primates that in vivo or ex vivo gene delivery of GDNF can be highly effective. Trials are now underway to test this approach in PD patients, ether by encapsulated cells producing GDNF (see http://www.michaeljfox.org/news/article.php?id=111&sec=1) or by injection of recombinant adeno-associated viral (rAAV) vectors carrying the gene encoding for neurturin (which is a neurotrophic factor closely related to GDNF) (conducted by Ceregene, Inc. in San Diego). These approaches, if successful, may open a new phase in the development of a restorative therapy for PD based on long-term, intracerebral delivery of the GDNF family of neurotrophic factors.

## 5. Perspective

We are now entering a new phase in the development of restorative therapies for PD, a development that to a large extent will be driven by the application of novel stem cell and gene delivery technologies. Stem cell technology holds promise to turn cell transplantation from a highly experimental procedure into a clinically useful routine therapy, and ex vivo or in vivo gene delivery of neurotrophic factors may help to solve the problem of how to get these factors to the critical receptive sites, at physiological levels, over a large enough tissue volume in the patient's brain. The complexity of the biological and clinical problems, however, should not be underestimated. In cell engineering, the mechanisms involved in the regulation of DA neuron differentiation, connectivity, and function, and the role of the host tissue response, immune/inflammatory reactions, and the mechanism(s) of graft-induced dyskinesias need to be explored and controlled. In case of neurotrophic factor delivery, the issue of optimal dosing and the possible negative side effects associated with sustained high-level GDNF expression, as well as the safety and efficacy of GDNF expression from viral vectors, need to be carefully assessed. Progress

in this field should be made carefully and stepwise, with the full understanding that the techniques and procedures need time to be perfected. For this reason, further carefully planned open-label trials are required to answer major outstanding issues, before we can embark on double-blind trials on larger groups of patients. Clearly, controlled, blinded trials are needed to determine the true value and efficacy of cell- and gene-based therapies for PD. If such trials are conducted too early, however, before the techniques and procedures are fully developed, they may do more harm than good.

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# 2

# Restorative Therapies for Parkinson's Disease: Ethical Issues

Gerard J. Boer

## 1. Introduction

Improved health care and the many therapies and surgery methods for all kinds of previously life-threatening diseases have helped to increase the average lifespan of the world population. As a consequence, the incidence of disease connected with old age, in particular degenerative diseases of the brain, has increased: the human body outlives the normally aging brain. One of these diseases is Parkinson's disease (PD), in which the gradual loss of the neurons of the substantia nigra serving the dopaminergic input of the brain caudate/putamen complex seems the key cause of the prominent movement dysfunctions typically related to the disease. There is a relatively satisfactory pharmacological treatment for the early disease symptoms, which is based on promoting dopamine production to therapeutic levels in those nigra cells that are still present. The treatment is, however, not ideal. It fails in later stages of the disease when nigra cell loss has progressed, because it does not stop the neurodegeneration of the nigro-striatal system. For several decades now, PD has been subject of intense fundamental research aimed at bringing about repair of the brain in this large group of patients. In particular, the prospect of brain restorative therapies, such as cell or gene therapy, has raised enormous interest and is seen as a new method to tackle the disease more efficaciously. The ethical issues involved, both from the methodological angle as well as from the patient's point of view, involved in the development of such treatments are subject of the present chapter.

Department of Neuroregeneration, Netherlands Institute for Neurosciences, Amsterdam, The Netherlands
g.boer@nin.knaw.nl

## 2. Variety of New Treatment Approaches

Classic therapies for PD are all designed to restore striatal dopamine signaling, using oral preparations of the precursor L-DOPA, dopamine agonists, or drugs synthesized to enhance the efficacy of endogenous dopamine by inhibition of dopamine breakdown. In the early disease stages these therapies are effective, so brain restorative interventions are of more importance in the later stages of the disease when drug treatment starts to falter and major complications develop (Calne, 1993; Ahlskog and Muenter, 2001). Therapies like deep brain stimulation and lesioning (Benabid, 2003) are used to treat PD patients in cases extreme motor handicaps, but repair of the brain by cell or gene therapeutic surgery without lifelong or long-lasting post-operative care remains the ultimate goal. If successful, these therapies might even be considered in the early stages of PD in order to prevent the side effects of the current drug treatments.

Cellular therapies are meant either to replace lost nigra neurons or to supply cells that can compensate for the loss of dopamine production (cellular vs. molecular replacement) (Björklund et al., 2003). Molecular replacement may also be achieved by encapsulated dopamine-producing cell preparations, or by in vivo gene transfer for the enzyme system necessary for the dopamine production. Cell and gene therapy recently also became of importance for regenerative neurosurgery in different ways: namely, the introduction of neurotrophic factors, that may stop degeneration or direct regeneration, or of enzymes for GABA production that can counteract the imbalance of the striatal output pathways as achieved by electric hyperstimulation or GABA agonist infusion in the subthalamic nucleus (STN) in PD patients. Thus, restorative surgery in PD is developing in many directions.

Autologous neuroimplantation of adrenal medulla tissue, potentially producing dopamine, was the first neurorestorative clinical trial in PD (Backlund et al., 1985). Autologous peripheral dopaminergic neuron implants are yet another means of substituting for the lost dopamine in the striatum (Itakura et al., 1997). However, allotransplantation of fetal mesencephalic primary dopaminergic neurons became the new approach that was rather widely studied in patients. It can significantly suppress the disease symptoms in the later stages of PD, despite serious side effects (Dunnett et al., 2001). Further momentum in cell therapeutic approaches in PD was supplied by the potential of embryonic (ES) and somatic stem (SS) cells as source cells for neurotransplantation (Armstrong and Svendsen, 2000; Brazelton et al., 2000; Mezey et al., 2000; Donovan and Gearhart, 2001; Toma et al., 2001). Using human embryos and fetuses as sources of cell or tissue implants in the brain of patients with neurological or psychiatric disorders has not been and is still not acceptable for parts of society. Ethical considerations regarding the retrieval and use of this donor material have led to the drawing up of guidelines. The recent isolation of human ES (hES) cells, in order to grow and differentiate these cells for organ repair, has given new impetus

to societal and political debates on this issue (McLaren, 2001; Weissman, 2002). Consensus about the use of residual in vitro fertilization (IVF) preimplantation embryos is emerging, but the creation of these human blastocysts for therapeutic purposes will probably remain a never-to-be-solved ethical or moral problem, especially in view of the new finding of pluripotency of SS cells from embryonic, fetal, and adult origin.

Whereas new drugs or drug regimes are nowadays studied in humans following the gold standard of clinical pharmacological experiments (CPMP Working Party on Efficacy of Medicinal Products, 1990), the new restorative therapies imply surgical interventions for which formal guidance has not been developed. The new cellular and molecular interventions are usually irreversible types of surgery. None of the approaches has as yet developed into a standard therapy, i.e., they are still experimental approaches. Moreover, since the brain is the site of our mind, which, in interaction with the environment, determines personal identity and personality, the consequences of the putative restorative interventions in the brain must be considered carefully. Each and every new type of restorative brain surgery in PD (and in other neurological, neurodegenerative and psychiatric diseases) has separate safety and ethical issues.

## 3. How to Approach the Ethical Issues?

There are no guidelines on experimental brain surgery other than those of the general codes for protection of a patient, as set forth, for instance, in the *World Medical Association Declaration of Helsinki* (update 2000), and the "Convention for the Protection of Human Rights and Dignity of the Human Being with regard to the Application of Biology and Medicine" (Council of Europe, 1997). These and other guidelines deal with aspects of free and informed consent of human beings involved in experimentation (including patients unable to give consent, or those having a mental disorder), the professional standards of the experimental intervention, the risk/benefit ratio of experimentation for the patient, and the obligation for approval by a competent body performing an independent examination on the scientific merits of the study and a review of its ethical acceptability. Guidance on the level and quality of basic knowledge that should validate an experimental surgical intervention, or on the design of the experimental surgery to obtain meaningful and interpretable data, is simply not available. In this respect, the field of experimental (brain) surgery differs from the field of clinical pharmacology, where strict guidance is given, for instance, in the ECC guide *Good Clinical Practice* for trials on medicinal products (1990). Although cell transplantation sometimes has aspects of a pharmacological treatment (if we think of cells as biological mini-pumps of molecules), an irreversible surgical treatment is generally not comparable with reversible drug intake.

Clear guidance is thus not available in the field. Thus, on the one hand, discussion about the ethical issues of new invasive clinical handling in PD

may best be guided by the four general moral principles (Beauchamp and Childress, 1989): 1) human beings and their autonomy should be respected, 2) what is good should be done ("beneficence"), 3) what is bad should be avoided ("non-maleficence"), and 4) what is just should be based on the fair distribution of the available means, on respect for human rights, and on morally acceptable legislation. On the other hand, one may adhere to the rule that any experimental treatment "....must conform to generally accepted scientific principles and should be based on adequately performed laboratory and animal experimentation" (World Medical Association, 2000). This tenant is, however, not very precise. Medico-ethics committees or institutional review boards—nowadays obligatory consultation bodies in all published guidelines for human clinical research—cannot always adequately judge the scientific quality of the animal studies. It is important that scientists themselves discuss the outcome of the fundamental studies as a step toward a clinical trial. The route to human fetal dopaminergic neurografts in PD patients has already undergone such a process (Freed, 1991; Boer, 1999; Peschanski et al., 1999), but other types of experimental, irreversible cell and gene therapeutic approaches in PD are still under debate in the arena of fundamental research.

## 4. Volume of Preclinical Studies Required

The 6-hydroxydopamine (6-OHDA) nigrostriatal-lesioned rat and mouse models of PD served as the most important and basic test models for putative cell and gene therapies. A more sophisticated model is the MPTP-treated non-human primate, which better mimics the motor disturbances observed in the idiopathic PD patient. Various studies in these models employed dopamine autografts of carotid bodies or adrenal medulla, neural allo- or xenografts of fetal mesencephalic cell preparations, as well as stem cell-derived implants, all placed in the striatum, to ameliorate many of the behavioral deficits caused by the lesion. In these models gene therapeutic strategies to induce dopamine expression or expression of GDNF (glial cell-derived neurotrophic factor) to counteract nigrostriatal degeneration also showed positive results on the primary motor outcomes.

It thus looks like the fundamental ethical requirements in clinical research (a plausible biological mechanism of repair can be presented and a sufficient number of successful animal studies has been reported) have been fulfilled to warrant safe trials on humans. However, this will always remain a matter of dispute as it is difficult to determine how much should be known. What level of pre-clinical research should be required before a clinical trial can be considered? And what measures must be taken to evaluate the benefit/risk outcome of the experimental treatment considering that irreversible surgery is taking place in the seat of the human mind, the brain?

## *4.1. Neuronal Supplementation Strategies*

Critical issues for the efficacy of *cell supplementation* strategies are the survival of the implanted cells, their stable dopaminergic phenotype at the moment of and after implantation, and, ideally, their capacity to reconstruct the damaged neuronal circuits. The latter means the occurrence of axonal growth and establishment of appropriate synaptic connections, as well as the reformation of organized input of the new neurons from the recipient brain. Animal PD model studies have not reached complete circuit reconstruction, as dopaminergic neurons had to be placed heterotopically in the (output) striatal area and not homotopically in the substantia nigra for functional recovery (Dunnett and Björklund, 1999). Axonal growth also carries the risk of aberrant connectivity, and this may include the possibility of adverse effects. Recent observations of an enhanced risk of dyskinesia in fetal mesencephalic graft-receiving PD patients indicates that adverse affects can occur (Hagell et al., 2002). The need to revert to animal research to find the exact mechanism that produces dyskinesia and how to prevent this from occurring shows that PD animal models are still valuable for the development of new restorative therapies in human.

The grafting of primary fetal nigra neurons, the implantation of autologous dopamine-producing peripheral cells or neurons, or of dopaminergic cell lines, as well as the placement of pre- or undifferentiated (embryonic or somatic) stem cells, all need to be tested for efficacy in functional repair in PD, and also with regard to safety. These cell implants differ with respect to purity, survival, differentiation, neurite outgrowth and maturation, migration and tumorigenesis aspects, and side effects and may thus differ as much as they do with respect to their efficacy in repairing or replacing the degenerated nigro-striatal system. One would like to see that the critical and safety issues of each of these approaches are subject to evaluation by publication of the rationale and justification of the new approach in the international literature.

The use of human fetal nigra neurons as allografts for the treatment of PD has been reviewed extensively, (e.g., Peschanski et al., 1999; Dunnett et al., 2001), and nobody has challenged the scientific validity of this approach in humans. The same can be said about the use of *porcine mesencephalic xenografts* for the supplementation capacity of these fetal neurons (Isacson and Breakefield, 1997). However, the immune rejection issues as well as the apparent risk of cross-species transfer of viral animal diseases and of zoonosis require higher safety standards than for allografts. These have presently not satisfied well enough to initiate clinical trials (Chapter 11: Barker et al., 2000; Harrower and Barker, 2004). Although some positive results with embryonic and neural (somatic) stem cells have been achieved in the 6-OHDA mouse model for PD, steps toward any clinical trials await a series of fundamental studies on the stability of these cells in vivo, their true neuronal action following differentiation either in vitro or in vivo, and their purification and growth as cell lines for mass production of transplants.

## *4.2. Molecular Substitution and Regeneration Strategies*

If each type of cell source for supplementation therapy in PD has its own individual safety aspects, the same must be true for various types of molecular therapy. Whether it concerns the implantation 1) of non-nigra or non-neuronal cells for dopamine release in the striatum or 2) of genetically modified cells for this purpose, or the use of 3) direct gene therapy that is either aimed at replacing the dopaminergic function, 4) at modulating the imbalanced output pathways of the striatum at the level of the STN, or 5) at generating factors like GDNF that may halt the degeneration and/or stimulate regeneration (Dunnett and Björklund, 1999; Kordower et al., 2000; Borlongan and Sanberg, 2002; Le and Frim, 2002), in each case separate background reviews on the rationale and justification for a clinical trial are necessary. Intracranial placement of implants with encapsulated cells that release dopamine or factors to maintain dopaminergic function is yet another approach (Zurn et al., 1996), which in principle may be reversible by removing the device from the brain ventricle or parenchyma. But the chronic exposure to dopamine by introduction of the dopamine-synthetic enzymes or to degeneration-inhibiting/regenerative factors may have side effects as well, since the brain is a very plastic organ in its neuronal circuitry and connectivity.

The critical issues for the implantation of cells for *molecular therapy* are comparable with those for supplementation of dopaminergic (nigra-like) neurons. In addition, however, their post-mitotic character, or, at least, their proliferation characteristics following in vivo placement, should be established. Since the cells have often been genetically modified and insertion of transgenes is relatively random in the cellular genome, the absence of tumorgeneity or the expression of abnormal toxic proteins must be established as well.

*Gene therapy*, administered either directly on the host brain tissue or indirectly, through implantation of ex vivo transduced cells, raises even more safety concerns as at present it is primarily based on the use of recombinant viruses for gene transfer. Gene transfer to post-mitotic neurons (and other neural cells) has been reached by several classes of viral vectors (Hermens and Verhaagen, 1998). Herpes simplex viral and adenoviral vectors for gene transfer into brain cells must be banned for clinical trials (except perhaps for the killing of a brain tumor), because of the toxicity for several neuronal subtypes. However, the use of adeno-associated viral (AAV) and lentiviral (LV) vectors may be feasible in patients, as absence of toxicity and long-term expression of the transgene are hallmarks for these vectors (Chapter 16: Kordower et al., 1999; Tenenbaum et al., 2003). However, due to the death of patients in clinical trials outside the field of nervous system deficiencies, gene therapy has come under a cloud (Gansbacher, 2003). So, even more than in cell therapy research, safety aspects should be a focus in gene therapy research for PD. Aberrant insertion of the transgene in the genome of the host, the occurrence of integration of virus sequences at uncharacterized integration sites (insertional mutagenesis), as well as the insertion of the transcriptional

active sequences that could result in activation of otherwise silent genes, may have devastating effects on the cells or may lead to disorganization or tumor formation in the nervous system (Connelly, 2002). And this cannot be corrected afterwards. Even the application of co-transduction with killer genes could have traumatic effects due to the inflammatory and immunogenic responses that will occur when activation of this safety system is indicated.

Notwithstanding the above, the results obtained so far in studies of in vivo and ex vivo AAV and LV vector-mediated gene therapy in PD animal models have made it clear that recovery of motor symptoms can be achieved without apparent side effects, and thus offer promise for clinical trials in patients (Freese et al., 1999; Le and Frim, 2002; Kordower et al., 2000; Raymon et al., 1997; Shen et al., 2000). Biosafety of AAV vectors is well accepted, and the use of (Parvovirus family) AAV subtype 2 vector has been cleared for phase I studies in human. The immune system is not challenged by the vector, no toxicity is observed, and inflammatory responses are limited to those related to the surgery. Moreover, the fear that the viral vector, once inside the patient, may recover its ability to cause disease is nil, as AAV2 is not known to be an etiological agent of any disease in humans (Tenenbaum et al., 2003).

During et al. (2001) were the first to present a rationale and justification for the use of AAV vector in the brain. AAV-GAD, a vector that introduces overexpression of glutamate decarboxylase (GAD) for GABA release and when infused in the STN, was able to mimic the therapeutic results of deep brain stimulation effects in the PD animal model. This publication preceded the clinical experimental treatment, which was recently presented as the first and only attempt to put a gene directly into an adult patient's brain in an attempt to treat a neurological disease. As in the case of fetal mesencephalic grafting, it is another example of how researchers take responsibility in the scientific community for the translation of animal-to-human experimentation.

## *4.3. Brain as Seat of Human Mind*

Restorative surgery in PD should arneliorate the various handicaps that accompany the disease but without causing severe side effects and without affecting the psyche of the patient in any negative way. The surgery takes place in the brain, the organ of which the activity also reflects the human mind, such as thinking, feeling, perception, will, learning and memory, as well as personal identity. Preclinical studies in animals are not always suitable for evaluating *changes in personality and personal identity* or, in case of neurotransplantation of embryonic or fetal tissue, *personality transfer*, that may occur as possible and unwanted adverse effects (Walters, 1988; HMSO, 1989; Linke, 1993; Birnbacher, 1995).

It is almost impossible to find exact descriptions of personality and identity of a human being in psychology or philosophy that can easily be used in "psychological safety" studies. However, personality and identity are distinct aspects of the individual (Northoff, 1996). Personality is said to be subject to

gradual changes and is scalable, whereas one's identity is unique and immutable. A pragmatic definition for the present purpose would be the following: personality is the complex of mental capabilities and physical performance of an individual, visible to or experienced by others, and absolutely or relatively quantifiable in human behavioral and physical tests. Identity, on the other hand, can be described as the interactively communicated and consistent self-consciousness of a person about his/her past, present, and future (Birnbacher, 1995, Romijn, 1997). In view of current neurobiological knowledge, it seems unlikely that personality or identity is determined by small isolated units of the brain. Characteristics of an individual are more based on the complex interaction of integrated neural networks that are formed by, and based on, numerous nerve cells, often grouped or layered in various sites throughout the central nervous system ("the brain is a collection of individually stupid neurons"). Cellular or molecular restorative neurosurgery of PD patients concerns a local treatment in the brain, and any notion of changes of personal identity, i.e., describing oneself as another person in time and space, are mere science fiction. If personality transfer by fetal neuron transplantation were possible at all, it would require the transplantation of large pieces of intact fetal brain, which, moreover, must be able to survive, to further mature, and to integrate as a network in an existing, fully developed (adult) brain. This is not feasible, as optimal survival conditions are different for each type of cell involved (Björklund, 1992). Physiological characteristics of an individual, however, may be transferable by transplantation, and personality changes (not transfer) cannot be excluded (Arendash and Gorski, 1982; Gage et al., 1984; Ralph et al., 1990; Boer, 1999)

In many cases, PD patients suffer from changes in personality. These changes are either directly or indirectly related to the disease. The physical, psychological, and social situation of patients is indirectly altered by the burden of their symptoms; their limited potentials; fear, depression, and stress; uncertainties about the future; and a possible loss of self-respect and self-confidence. If the burden of the disease symptoms were to be eliminated or alleviated by restorative surgery, many of these personality aspects should improve. The question, however, remains whether, e.g., neurotransplantation of immature neurons or neuroprogenitors will affect personality in an unwanted and irreversible way due to the locally altered morphology and functional cellular interaction in the brain. The aberrant neurite sprouting mentioned above may irreversibly steer neuronal circuitry toward improper functioning. If so, are these behavioral and physiological consequences morally acceptable; or, in other words, do the advantages of the treatment outweigh the subtle personality changes? Answers to such questions can only come from experimental treatments in human. The focus in clinical trials usually is on motor recovery. So far, when investigated in (neuro)graft-receiving patients, no changes in personality parameters have been noticed (McRae et al., 2003) or seen as contraindication (Ostrosky-Solis et al., 1988). However, psychological testing remains necessary for each and every new

(and irreversible) phase 1 study of restorative surgery in PD (and in other brain diseases eligible for this type of therapy).

## *4.4. Clinical Assessment Protocol*

Strategies to control efficacy of various types of restorative clinical trials in PD, is a second translational problem. Human experiments that give non-interpretable results are unethical (Felten, 1994). Shortly after the first clinical trials with intrastriatal implantation of fetal brain dopaminergic cell preparations in PD patients, it became obvious that there was a critical need for a degree of commonality between the methods for patient diagnosis and evaluation by the teams undertaking such treatments. Daily fluctuations make scientific evaluation difficult, whereas the data of different centers had to be compared to achieve sufficient numbers to provide definitive results. An international committee, created in 1990, formulated a series of recommendations for a common and minimum set of diagnostic and methodological core evaluations, called Core Assessment Program for Intracerebral Transplantations in PD (CAPIT-PD) (Langston et al., 1992). It comprises criteria for inclusion of patients, for working definitions and aspects of motor disturbance states, for motor and dyskinesia rating scales (like the UPDRS and Hoehn and Yahr), and time testing of motor behavior, for a pharmacological challenge test, for brain imaging, and, no less important, for a fixed time frame of evaluations to obtain a reliable baseline estimate of the pre-graft clinical status and the post-graft effects for a period long enough for the grafted neurons to mature and become functional. CAPIT-PD has never been completely embraced by the PD grafting field, partly because the program was considered too laborious (and costly) to be carried out in large-scale trials and partly because the grafting was applied as a treatment worth trying rather than as experimental therapy. Had all centers that performed neurotransplantation in PD patients used the CAPIT-PD, a wealth of comparative information could have been obtained instead of the present set of incomparable and seemingly conflicting results. The challenge for the clinical scientist, given the obligation to the test persons undergoing any restorative surgery, is to formulate a CAP that guarantees the collection of objective data on PD symptoms (Boer and Widner, 2002). Objective data are needed to compare pre- and post-intervention data as well as for comparison with a parallel CAP-evaluated randomly assigned reference patient group. Markers of surviving tissue, obtained with imaging techniques, are pivotal for the interpretation of clinical effects and essential for linking clinical effects with a causal mechanism. Within the Network of European CNS Transplantation And Restoration (NECTAR), the CAPIT has been updated and improved to allow comparable evaluation of all kinds of new experimental treatments in PD patients (Core Assessment Program for Surgical Interventional Therapies, CAPSIT-PD) (Defer et al., 1999; Widner and Defer, 1999).

Patient placebo effects and investigator bias will be largely eliminated, or at least minimized, by a series of well-defined quantitative measures of evaluation. Whereas from a strict methodological point of view, randomized double-blind placebo-controlled studies may be the way to go (Freeman et al., 1999), an answer to the question of whether cellular implants in the brain of PD patients are efficacious can then also be obtained with a intrapatient study design that does not involve sham-operated patients (see subhead 7 and Chapter 4).

## 5. Retrieval and Use of Donor Cells

Cell-restorative surgery in PD began with open trials of striatal placement of the patients' own adrenal medulla tissue (Backlund et al., 1985; Madrazo et al., 1987). This tissue was used experimentally as an alternative source of dopamine neurons in order to circumvent the ethical problems following the use of human fetal brain tissue (Boer, 1994). The cells of the adrenal medulla produce large quantities of catecholamines, for which dopamine is a precursor molecule. Animal models had shown recovery of the experimentally induced motor disorders. However, the clinical results of these non-neural autologous implants were poor, variable, and inconsistent in patients, which was later confirmed in monkey model studies. Though researchers are always eagern to develop an effective therapy (Boer, 1996, 1999), this history of adrenal medulla auto-implants in the brain of PD patients illustrates the difficulty of determining what level of prior animal experimentation is required to establish the efficacy and safety of a new clinical treatment (see subheads 2 and 4.1.) Significantly better functional effects in the parkinsonian rat and monkey models with implants of immature dopaminergic nigra neurons made a move toward the use of human embryonic or fetal tissue inescapable (Boer, 1999). Ethical guidlines had to be developed, as the cells were obtained from the remains of human abortions. Nowadays cell-based therapies to replace (or rescue) dopaminergic function in PD use cells obtained from less controversial sources or from sources "cells from the shelf" (Figure 2.1). However, each cell source seems to have its own particular set of ethical problems.

### *5.1. Adult Human*

When it had become obvious that adrenal implants in PD had failed, the idea of autologous transplants did not vanish, because of the advantage of avoiding immunological problems and not requiring a donor. Combined adrenal chromaffin/peripheral nerve tissue (Date et al., 1997) or chromaffin/Sertoli cell implants (Sanberg et al., 1997), or treatment with nerve growth factor (Date et al., 1997) to promote long-term survival of chromaffin cells for dopamine production have been studied. Other paraneural cells with dopamine excretion, such as stellate ganglion or globoid bodies, have shown

modest anti-parkinsonian effects (Itakura et al., 1997; Nakao et al., 2001; Arjona et al., 2003). These clinical studies were a corollary of a successful series of rat and monkey PD model studies. The ethical concerns arise from the possible loss of function due to the retrieval of (dopamine-producing) cells from elsewhere in the body (see subhead 6.1).

Yet another process of autologous transplantation is the collection of the patient's own SS cells in order to grow, differentiate, and implant them as dopaminergic neurons. There are no ethical objections to the retrieval as long as the isolation of these cells is a minimal burden for the PD patient which can subsequently be treated or ameliorated for its symptoms.

## *5.2. Human Embryo and Fetus—Primary Cells*

Although neural grafting in PD became a real test bed for the possibilities of neurotransplantation therapy in neurological diseases, it also opened the arena for discussion of the ethical acceptability of the use of human abortion remains in the clinic and laboratory. For decades, research on pre-viable and non-viable fetuses and their tissues has been carried out by embryologists and physiologists. These data can be found in handbooks on embryology and intrauterine development (Falkner and Tanner, 1978; O'Rahilly and Müller, 1987) and have contributed to measures that have helped to safeguard early prenatal human life. Thus, in itself, the use of prenatal tissues has not been seen as ethically objectionable (Gareth Jones, 1991). However, the concern arose that the use of embryonic and fetal cells in large groups of patients (not only PD, but also Huntington's disease patients, and patients with haematological, liver, thymic, and pancreatic disorders; McCullagh, 1987) might created a great demand, and thus encourage induced abortions that would otherwise not have occurred (donation as a noble and selfless act for the

FIGURE 2.1. (*caption continued*)
the human. These somatic stem (SS) cells appeared multipotent as well as may also be a source for growing dopaminergic transplants. Embryonic stem (ES) cells present in the inner cell mass of the pre-implantation embryo are easier to grow and expand than SS cells. Their potential as source cells for the preparation of differentiated cells of various organs, including the brain, is great. Hence, dopaminergic grafts may be obtained from "cell lines from the shelf". The pre-implantation embryos could come from the donation of spare embryos from a parents' in vitro fertilization (IVF) program or be created for the purpose of obtaining these ES cells from donated sperm and egg cells from adults (egg cells could perhaps also come from the remains of aborted female fetuses). Finally, an in vitro pre-implantation embryo as a source of stem cells could also be obtained through somatic cell nucleus transfer (SCNT, also called "therapeutic cloning"). The DNA of the patient is then placed in an enucleated donated egg cell, which would provide a method to solve the problems related to immunological rejection of transplants.

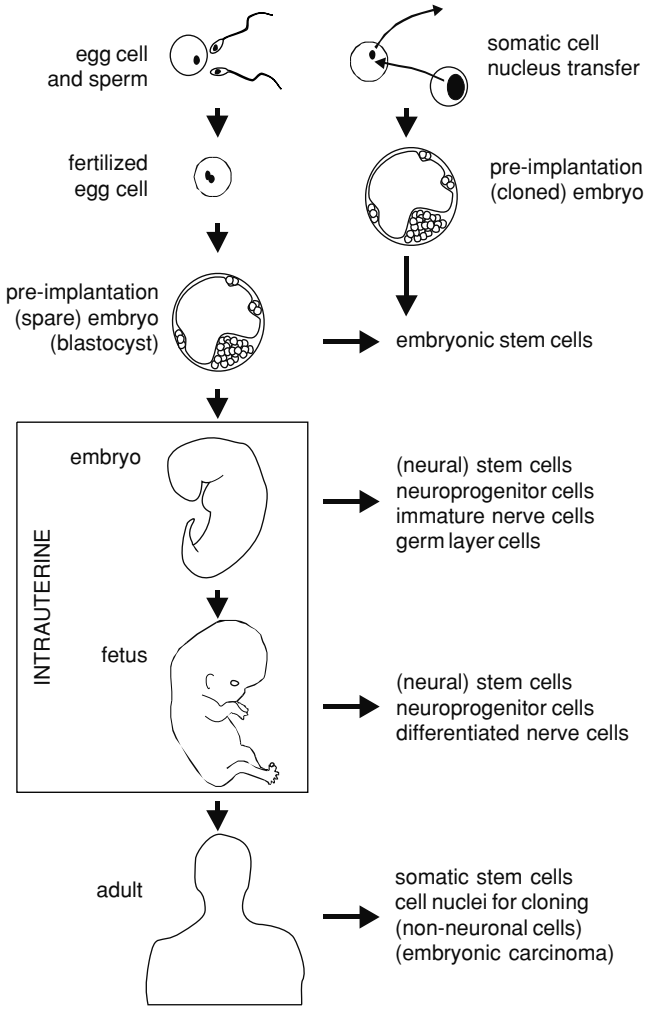


FIGURE 2.1. Present and possible future technical possibilities of obtaining dopaminergic transplants for human neural grafting in Parkinson's disease. The initial steps in experimental neurotransplantation were based on the use of mesencephalic tissue retrieved from human embryos and fetuses, and dissection or preparation of neurografts containing the immature but differentiated dopaminergic nerve cells of the substantia nigra. Several new developments have meanwhile taken place or are foreseeable. First, the embryonic or fetal remains of human abortion could also be the source of neural stem cells and neuroprogenitor cells (stem cells that passed the first stages of differentiation), which can be grown in culture and can be proliferated and differentiated for neurotransplantation. Neural stem cells also appear to be present in the adult brain, but it is unclear whether this can be a source of cells to grow for dopaminergic neurografts. Stem cells are also present in many other organs in the embryonic, fetal, and adult stages of

benefit of humanity). This issue can therefore not be completely separated from the ethical aspects of the decision on elective abortion (Boer, 1994, 1999).

The moral basis of the current legal practice of elective abortion in many countries is that the interest of the woman's physical and social health must be balanced against the interests and viability of (and the respect for) the embryo or fetus in utero, a human being-to-be. Many national and international organizations, national institutes of ethics, as well as for instance the Parliamentary Assembly of the Council of Europe and a Working Group of the European Commission, in addition to scientists in the field themselves (see Table 2.1) have provided ethical guidelines for the subsequent use of body remains for experimental and clinical research (Peel rapport, 1972; CNESVS, 1984, 1990; BMA, 1988; Boer, 1994; De Wert et al., 2002). Despite marked differences, they all aim to solve the above-mentioned ethical problem by trying to achieve complete separation between the decision about abortion and the possible donation of the remains (the so-called *separation principle*) (Boer, 1999; De Wert, 2002). The situation would then be similar to the generally accepted use of organs or tissue from deceased babies, children, or adults. Leaving aside that some give the embryo and fetus absolute worthiness of protection as life, which makes the use of the resultant material

Table 2.1. Guidelines for the Use of Human Embryonic or Fetal Tissue for Experimental and Clinical Neurotransplantation and Research (as Formulated by the Network of European CNS Transplantation And Restoration, NECTAR)*

1. Tissue for transplantation or research may be obtained from dead embryos or fetuses whose death resulted from legally induced or spontaneous abortion. Death of an intact embryo is defined as absence of respiration and heartbeats.
2. It is not allowed to keep intact embryos or fetuses alive artificially for the purpose of removing usable material.
3. The decision to terminate pregnancy must under no circumstances be influenced by the possible or desired subsequent use of the embryo or fetus and must therefore precede any introduction of the possible donation. There should be no link between the donor and the recipient, nor designation of the recipient by the donor.
4. Neither the procedure nor the timing of abortion must be influenced by the requirements of the transplantation activity when this would be in conflict with the woman's interest or would increase embryonic or fetal distress.
5. No material can be used without informed consent of the woman involved. This informed consent should, whenever possible, be obtained prior to abortion.
6. Screening of the woman for transmissible diseases requires informed consent.
7. Nervous tissue may be used for transplantation as suspended cell preparations or tissue fragments
8. All members of the hospital or research staff directly involved in any of the procedures must be fully informed.
9. The procurement of embryos, fetuses, or their tissue must not involve profit or remuneration.
10. Every transplantation or research project involving the use of embryonic or fetal tissue must be approved by the local ethical committee.

*Published on behalf of NECTAR by Boer (1994).

a crime of "complicity after the fact" (Bopp and Burtchaell, 1988), according to these guidelines, even if induced abortion is regarded as unethical, it cannot be concluded that it is inherently wrong to save lives with donated abortion remains (Robertson, 1988; Boer, 1994).

Of course, informed consent should be obtained for the donation. The main additional requirements should be that the timing and method should not compromise efficient medical handling in the interest of the woman and the conceptus, and that no remuneration be involved. The consent procedure and how much one can deviate from abortion procedures in order to obtain useful tissue without violating efficient handling remain open to differences in opinion (Boer, 1999; De Wert et al., 2002). In none of the informed consent procedures of any of the guidelines, has a role been set aside for the sire. In view of the equal legal positions of men and women (in Western societies), a father may have legal rights in this decision, similar to the case of donation of organs from a deceased newborn or child. On the other hand, some women do not want (for personal reasons) to involve the begetter in the process of terminating the pregnancy, or the begetter may be unknown at the time of abortion. These are practical arguments for not routinely seeking consent of the begetter (Boer, 1994) and sticking to consent of the woman. In case of a mutual good parental relationship, the begetter's consent can of course be sought.

Modifications of the method of abortion for the purpose of getting suitable material for transplantation could involve an additional burden for the woman, and thus conflict with her health interests. This objection vanishes for modifications that impose little or no additional risk on the woman. However, postponement of the abortion may be emotionally burdensome as well as being not in agreement with the above-mentioned separation principle (Boer, 1999; De Wert et al., 2002). Adhering to this separation principle has a strategic function, as it ensures that abortion and the use of the remains are separate practices, thereby circumventing any of the accusations of complicity or moral taint as mentioned above. A change in the method does not jeopardize the procedure of separation of decisions.

Although the treatment of PD patients with primary dopaminergic neurons from the donated remains of human elective abortions seems morally and ethically defendable, the therapeutic outcome has been variable. Moreover, successful treatment requires the use and donation of brain tissue from up to 10 abortions, which logistically is an impossible mission. A proof of principle was thus established, but the efficacy of the neuronal supplementation was still poor, if it is to be more successful in the future, it should be based on more readily available cell sources for equal and fair availability for large groups of PD patients. Alternatives are cultured *cell lines*, either grown from SS cells or cells from carcinomas (see subhead 5.1), or proliferated and differentiated from early human embryonic (pluripotent) stem cells, germ line cells, or fetal neuroprogenitor cells (Figure 2.1). The second alternative is implantation of neural grafts grown from animal sources *(xenografting)*.

## *5.3. Human Embryo or Fetus—Stem Cells*

The finding that the ES cells from the inner cell mass (ICM) of the human pre-implantation embryo at the blastocyst stage can be isolated, propagated, and differentiated into many different types of cells in vitro, including dopaminergic neurons, raised the possibility of growing transplants for engraftment in PD patients (Chapter 13). Theoretically, as these pluripotent ES cells can multiply indefinitely, the ICM of a single human pre-implantation embryo would be sufficient to treat large cohorts of patients (Palacios et al., 1995; Rohwedel et al., 1998; Thomson et al., 1998). However, the precise conditions to achieve and harnass cell lines for dopaminergic neuron supplementation are far from established (Deacon et al., 1998; Taylor and Minger, 2005) and research using human pre-implantation embryos remains necessary (and not only in view of the search for cell therapy for PD, but also for diseases like Huntington's disease, leukaemia, stroke, heart failure, diabetes mellitus, burns, etc.). Human cells with the pluripotency of ES cells can be found also as embryonic carcinoma (EC) cells (see subhead 5.1), and as embryonic germ (EG) cells from the germinal primordium of the post-implantation embryo (Shamblott et al., 2001). Furthermore, neuroprogenitor cells from the embryonic or fetal brain can be used as source cells to grow dopaminergic cell grafts. Finally, the SS cells and/or progenitor cells from the embryonic or fetal organs or from umbilical cord blood appeared to have the multipotency to form neural cells. Ethically speaking, the source or donor situation is quite different and requires separate discussion.

### 5.3.1. Post-Implantation Embryo

When stem cells or progenitor cells are retrieved from the post-implantation human embryo, the situation is similar to when primary neurons are retrieved and used for immediate grafting. The guidelines for donation following elective abortion thus also hold for the use of the remains for isolation and proliferation of neuroprogenitor, SS, or EG cells. Two main aspects are different. Informed consent must, of course, be obtained for a different research goal: the establishment of a cell line for research or possible cell therapy. Secondly, screening of transmissible diseases in the individual donating the cells can be omitted as safety tests can be performed on the cultured cells.

### 5.3.2. Surplus Pre-Implantation Embryo

So far, the potency of mammalian ES cells for expansion and differentiation appears to be superior to that of SS and other types of pluripotent or multipotent cells. There is thus a strong scientific wish as well as a need to explore ES cells from human blastocysts as source cells in order to grow transplants (see also subhead 5.5). The creation of human blastocysts is a current practice in IVF programs that help to fulfill the desire for parenthood of couples with particular reproduction problems. In the research preceding this type of

treatment of infertility, human blastocysts had been created as a means to an the end of developing the methods. The in vitro creation and sacrifice of human pre-implantation embryos is still mainly limited to research in the reproductive field (infertility treatment, causes of congenital diseases and miscarriage, and improvement of techniques and quality of IVF). It is licensed under strict control of regulatory bodies in some countries and completely forbidden in others. This is indicative of the differences in moral views of human embryo in vitro pre-implantation in different societies.

Due to the burden of the necessary hormonal treatment, current practice in IVF protocols is that the collection of egg cells is performed once. After IVF and some days of growth and quality control in the dish, blastocysts are then implanted in the uterus for pregnancy or deep-frozen for a second trial or subsequent pregnancies. At some point the latter are no longer necessary and regarded as "surplus" embryos (also called rest, residual, spare, or supernumerary embryos). Although extreme pro-life activists claim that these human-beings-to-be must not be destroyed and can only be thawed for implantation in a womb, pragmatism leads to destruction. If the use of the remains of an aborted post-implantation embryo can be ethically justified, the use of "surplus" embryos cannot be rejected. The protection of the in vitro human blastocyst, a liquid-filled tissue sphere with undifferentiated cells (Scothorne, 1968; Singer, 1990) should not be held in higher esteem than that of an intrauterine human embryo in which organogenesis into a visible human-being-to-be has taken shape. It reflects the principle of relative worthiness of protection of an embryo, which gives increasing protection as intrauterine development progresses (HER, 1994).

Many, but not all, of the guidelines developed for the retrieval and donation of the embryonic or fetal remains after elective abortion (Table 2.1) are of value also for the donation of "surplus" embryos. The decision not to implant opens the door for donation. The removal and cultivation of cells from such otherwise destroyed pre-implantation embryos could be regarded as analogous to tissue donation after elective abortion. In these cases either being conception in vivo or in vitro, the use of embryo cells is not intended a priori, and consent for donation is sought for the use of the remains, albeit at different embryonic ages. The IVF couple (not just the woman) should provide informed consent, the separation principle should be maintained (no IVF embryo created nor designated by the couple for a particular patient), and no elongated in vitro growth of the intact blastocyst toward post-implantation developmental stages should be sustained (Table 2.2).

### 5.3.3. Pre-Implantation Embryo—Creation and Therapeutic Cloning

Whereas the use of "surplus" embryos largely corresponds with the use of remains following elective termination of pregnancy, the use of blastocysts created solely for experimental or therapeutic use introduces a new moral problem. Here human life is created for the purpose of sacrificing it as a cell

Table 2.2. Proposed Points of Guidance for the Use of "surplus" IVF Embryos for Experimental and Clinical Research Toward Stem Cell-Based Cellular Therapies

1. It is not allowed to keep embryos alive artificially for the purpose of reaching the developmental stage of an in vivo post-implantation embryo (upto 10 days post-fertilization).
2. The decision to destroy so-called "surplus" embryos must not be influenced by the desired use and must precede the question of possible donation. There should be no link between the donor couple and the recipient, nor designation of the recipient by the donor except for possible treatment of the IVF offspring of the couple.
3. No material can be used without informed consent of the gamete providers involved.
4. The procurement of "surplus" embryos must not involve profit or remuneration.
5. All members of the hospital or research staff directly involved in any of the procedures must be fully informed.
6. Every research project involving the use of rest IVF embryos must be approved by the local ethical committee.

bank to grow therapeutic cell lines. Moreover, the use of the created embryo does not improve the quality of future in vitro procreation and, hence, the quality of life for persons conceived this way, i.e., does not future IVF children (McLaren, 1996). For restorative cell surgery, the creation of the human blastocyst must be regarded as a pure instrumental use of a human-being-to-be, and so a re-evaluation of its ethical acceptance is needed.

Pre-implantation embryos can be created not only with donated gametes, but also through somatic cell nuclear transfer (SCNT) in an enucleated donated egg cell (Wilmut et al., 1997; Cibelli et al., 2001). So far in non-human mammalian species only, since the report on successful hSCNT (Hwang et al., 2004) has recently been withdrawn (Normile et al., 2006). SCNT has been called therapeutic cloning, in order to distinguish it from reproductive cloning (which is forbidden almost everywhere). Therapeutic cloning has the advantage of creating a human blastocyst that contains ES cells of the genotype of the patient eligible for cellular therapy. It circumvents the problems of tissue rejection by the recipient and saves the patient from the unsatisfactory and troublesome life-long treatment with immunosuppressive drugs (Wolf et al., 1998).

In the many publications on the above-mentioned issue as well as in the public and political domains, the creation and use of human pre-implantation embryos for therapeutic purposes is a controversial issue that is unlikely ever to reach consensus. Societal and religious views on the human value of and respect for human life at the early stages of morula (4–5 days) and blastocyst (5–10 days after fertilization) simply differ too much (De Wert et al., 2002; Matthiesen, 2002; Oduncu, 2003). For many it is difficult to maintain moral principles that forbid the creation of the primitive stages of an embryo, if its cells may be an endless source of great therapeutic potential for a variety of severe or life-threatening diseases of adults. This includes PD patients for whom there is otherwise no satisfactory cure (see also subhead 5.5).

Hansen (2002) even argued that the moral status of a blastocyst created through SCNT is "found to be more clearly not equivalent to that of a human being" than a blastocyst created by gametes. Fewer ethical problems for the collection of stem cells in the former case therefore arise. For the time being, some form of consensus seems to have been reached to first make use of surplus human pre-implantation embryos to develop methods and prove efficacy in the patient (McLaren, 2001; Outka, 2002). When it has been shown that the creation of pre-implantation embryos is really needed, perhaps the temporary ban could be lifted.

Some fundamental rules, however, remain of importance: the providers of the gametes to perform IVF or SCNT must donate under the regime of informed consent for the purpose of the creation of the pre-implantation embryo, and donation must under no circumstances be obtained under pressure or for remuneration. Moreover, permission from a local medico-ethics committee should be obligatory (McLaren, 2001; Nuffield Council on Bioethics, 2000, Dutch Health Council, 2002).

## *5.4. Animal*

In view of the limited availability of human embryonic or fetal tissue, xenogenic tissue is seen as an alternative tissue for grafting (Chapter 11). Animal nerve cells make use of a similar molecular repertoire to serve very similar functions of cellular communications and activity responses in the brain as human nerve cells (Isacson and Breakefield, 1997). Moreover, human neuroprogenitor cells transplanted in the germinal ventricular zones of the postnatal developing rat brain take part in rat brain development as if they were rat cells (neurons are formed that migrate and settle in a network of genuine rat cells; Flax et al., 1998). Xenografting makes use of this chimeric plasticity of undifferentiated or immature mammalian nerve cells. Indeed, pig fetal mesencephalic grafts placed in rat models for neurodegenerative diseases like PD exhibit allograft-like morphology and a remarkable axonal target specificity as well as a functional restoration of impaired motor behaviour (Huffaker et al., 1989; Isacson et al., 1995; Galpern et al., 1996). For scientific, practical, and ethical reasons, in particular, embryonic tissue from porcine origin is being investigated as a neural graft (Dunning et al, 1994; Advisory Group on the Ethics of Transplantation, 1996; Nuffield Council on Bioethics, 1996; Daar, 1998). Pigs have been selected because of their brain size, and because there is extensive experience in large-scale breeding of these animals for food. Also there are ongoing attempts to apply porcine organs for transplantation in humans.

The ethical discussion in xenografting covers the welfare and choice of animals as source of transplants, as well as the dangers of infections and long-term immunosuppressive treatment of the patient, and the psychological acceptance by the recipient. Many of these points have also been discussed in

view of the shortage of organs for patients suffering from end-stage organ failure (Nuffield Council on Bioethics, 1996). Animal protectionists challenge and oppose the use of specially bred animals as a source of transplants, since the special breeding conditions—necessary to control the pathogen status of the source animals—would introduce yet another violation of animal integrity and autonomy, and would compromise animal welfare in a new type of factory farming. Ethically speaking, there should be no difference between breeding animals for food or breeding them to harvest cells or organs for transplantation (Daar, 1998), providing that suffering can be kept to a minimum. With all due respect for the life of animals, the integrity and autonomy of a pig should not be viewed on a human level. One might even say that breeding for transplants serves a higher goal. Any validation of welfare should, however, be weighed against the potential benefit to patients, and animals used for animal-to-human transplants are to be protected by animal acts.

The greatest concerns connected with xenotransplantation is that of the possibility of pathogens', specifically viruses and prions, jumping the species gap (Butler, 1998) and the necessary combination with life-long immunosuppression treatment (see subhead 6.2). These problems are absent when genetically modified animal cell lines are used, encapsulated in semi-permeable polymers for the local release of neuroregenerative or neuroprotective proteins in the nervous system. Cells from lower animals or even invertebrates can even be applied with no ethical objections against the source from society.

## *5.5. Proportionality and Subsidiarity*

Possible objections on the use of human embryos for research and therapy are also connected to the ethical principles of proportionality and subsidiarity. The *principle of proportionality* is translated as follows: the use must serve an important goal in the interest of human health. It is difficult to claim that isolating cells from human abortion remains or from "surplus" or created pre-implantation embryos is disproportional. In many societies elective abortion is legally accepted, pre-implantation embryos have been and still are used for research into the causes or treatment of infertility. It would be inconsistent to reject research on cell replacement therapies that may lead to treatment of severely handicapping and yet untreatable diseases like PD.

The *principle of subsidiarity* in restorative surgery with human cell implants implies that the goals of research or application cannot be reached with alternative sources for suitable cells other than the human blastocyst, embryo, and fetus, or cannot be reached by other methods than cell therapy. First of all, one has to realize that research on cellular replacement therapy is presently focusing on the establishment of cell lines for transplantation. An endlessly proliferative cell line with the capacity to form dopaminergic neurons would theoretically eliminate the need to obtain new material for separate PD patients ("dopaminergic transplants from the shelf"). This is not only true for the hES

cells as a source cell from pre-implantation embryos, but also for the pluripotent hEG cells and the multipotent neuroprogenitor cells from human embryos and fetuses. Calling a halt to this research would obstruct important new medical developments in embryo-use-saving methods that could moreover be applied in larger cohorts of patients. However, the finding that pluri- and multipotent stem cells are everywhere in the human body, also in adulthood, raised new criticism from people who do not accept the use of human embryonic and fetal sources (Oduncu, 2003). SS cells (also called "adult" stem cells) have now been isolated from bone marrow, liver, skin, fat tissue, umbilical cord blood, and the CNS. Their potencies seem remarkable and they are described as "blood into brain, brain into blood cells" (Bjornson et al., 1999; Brazelton et al., 2000; Mezey et al., 2000; Toma et al., 2001). The message that the use of cells from human blastocysts, embryos, and fetuses should be banned hampers new developments as well. However, recently the capacity of SS cells to transdifferentiate has been questioned, and the moment, the potencies of proliferation and differentiation of ES cells are superior to those of SS cells. Limiting the field to the use of SS cells would delay research in clinically operational and efficacious cellular treatments in PD (and many other organ-failure diseases). So, although the principle of subsidiarity is meant to express concern for the moral value of the embryo, it is a sign of ethical one-dimensionality to present every alternative which does not use early human embryos a priori as being morally superior (De Wert, 2002). Xenografting as an alternative for the use of allografts is at present no alternative either. From the perspective of animal ethics, one may question whether it is reasonable to breed and kill animals in order to obtain transplants when, e.g., residual human IVF embryos can be used that would otherwise be discarded.

Cell supplementation therapy is just one route to restorative surgery in PD. Logically, the homotopic placement of new dopaminergic nigra neurons that develop the proper output and input connections following implantation would be ideal (Dunnett et al., 2001). However, the principle of subsidiarity should also be considered with respect to other methods that may have a comparable therapeutic effect, rather than restoration of lost parts of the nigro-striatal system. Deep brain stimulation, thalamic lesions (Benabid, 2003) or chronic delivery of nigra-protecting GDNF (either via infusion, delivered from encapsulated cells, or from genetically modified autologous cells) (Kordower et al., 2000; Gill et al., 2003; Slevin et al., 2005) are therapies that are in various stages of clinical development. New approaches may be envisaged as well, such as in vivo stimulation of neurogenesis in the substantia nigra (Zhao et al., 2003). However, none of these methods can be seen as a standard treatment for PD, and outcomes never fully ameliorate all aspects of the disease symptoms. So, all these strategies need to be developed in parallel. The variability in the disease phenomena of PD patients may even require a repertoire of restorative and symptom-correcting methods for truly efficacious treatments.

## 6. The Recipient Patient

Animal "models" for PD are only of relative value because they do not mimic the disease in terms of long-term chronic neurodegeneration nor do they completely match the symptomatology and prognoses in the human situation (Bankiewicz et al., 1993). Final efficacy of any neurotransplantation approach will thus need clinical trials, with the risk of negative effects in human beings. In general, one may say that a new experimental approach should be presented in written form to a scientific committee of experts in the field, who should evaluate the proper step towards human experimentation as well as the study design. The ethical principles of value and scientific validity for initiating such trials are generally regarded to be fulfilled if immature nigra neurons are to be used (see subhead 3). For other types of cellular and molecular restorative surgeries in PD a decision-to-go has been taken or is still under discussion. Fetal nigra-containing grafting in PD patients has in fact been a first test bed for all of the new types of restorative neurosurgery, and has initiated ethical discussions on the conditions, source, and donation of the cells to be implanted (see subhead 5). However, an ethical evaluation concerning favorable risk-benefit ratio, informed consent, and respect for the patient involved in the study should be considered as well.

### *6.1. Informed Consent*

It goes without saying that participation in any restorative surgery project must be voluntary and that there is a right to withdraw consent at any time (United Nations, 1948; Boer, 1994; BMA, 1988). PD patients, though vulnerable, are still able to understand their situation. They are therefore regarded as competent to give informed consent to be a subject in experimental therapies. In advanced stages of the disease their mental state is often—but not always—impaired, but not to such an extent that it affects their communication, understanding, and expression of free will (Koller, 1987). The assessment of competence/decision-making capacity is the primary responsibility of the investigator. As a general rule, a candidate subject can be considered competent when the nature of the information, the consequences and risks of being a research participant, and the possiblility to refuse are understood. Considering the fact that invasive irreversible experimental surgery is involved, a second opinion on the assessment of competence/decision-making could be sought from a physician who is not involved in the research project. Some patients may have difficulty in expressing choice, and others may be unduly susceptible to the harm and stress of being a research subject. Researchers could minimize this vulnerability by including family members or patient representatives in the decision-making process. Oral and written information should be provided. Moreover, informed consent alone can never be an argument to initiate clinical experimentation. Putative efficacy, biosafety, and experimental design are equally important.

## 6.2. *Biosafety*

Biosafety has multiple facets, as there are various types of experimental restorative neurosurgery in PD. Human fetal primary brain cells as striatal implants will raise other safety concerns than the application of viral vector-mediated gene therapy in the nigro-striatal pathway, or xenografting combined with immune suppression treatment.

### 6.2.1. Transmissible Diseases From Human Transplants

Testing for transmissible human diseases of the donor tissue is a necessity. In case of a short time interval between the retrieval of the transplant from human abortion remains and the actual surgery, the tests can only be performed on the blood of the woman. These tests should thus be performed prior to the elective abortion, which requires her separate consent (Boer, 1994). This is routine procedure in all existing protocols of neurotransplantation involving embryonic mesencephalic tissues. The recently adopted EC Directive of the European Parliament and Council on setting standards of quality and safety for the donation, procurement, testing, processing, storage, and distribution of human tissues and cells (2003), requires serology tests for HIV1 and 2, hepatitis B and C, *Treponema pallidum*, and HTLV-I and II. Storage and hibernation of the abortion remains make it possible to carry out these tests without blood sampling from the woman. However, so far, for primary cells, this is done at the expense of neuronal cell survival following grafting (Chapter 8, Frodl et al., 1994). Contaminations contraindicate the direct use for implantation.

The above tests are of course also indicated when allogeneic ES, EG, fetal neuroprogenitor, or SS cells are to be used to grow dopaminergic transplants in vitro, or when cell lines from human embryonic teratocarcinoma are to be applied. However, the cell cultures can be tested for pathogens during the proliferation, differentiation, and storage phase before final use.

### 6.2.2. Zoonosis Following Xenotransplantation

The greatest concern connected with xenotransplantation is that of the possibility of pathogens, specifically viruses and prions, jumping the species gap (Butler, 1998). Animal viruses could be transmitted to humans (zoonosis), as evidenced by the large disease outbreaks in humans of the Ebola and Marburg monkey viruses, of the simian-derived HIV AIDS virus, and more recently of bird flu (Webster et al., 2005). Barker et al. (2000) formulated a series of specifications that should be fulfilled. They comprise microbiological specification of the pig strain, biosecurity of animal production, sterile tissue collection, creation of a tissue archive and safety database, and an investigation of porcine endogenous retroviruses (PERVs). Even assuming that the use of domestic pigs—which have been in contact with humans for ages—as source animals for cells will be less dangerous, zoonosis cannot be

completely avoided by pathogen-free breeding. PERVs are integrated into the genome, as are retroviral DNA sequences in the human genome and other mammalian species (Weiss, 1998). Zoonosis could therefore also be a result of DNA recombination and adaptation, leading to the expression of known or newly formed retroviruses. Though not directly pathogenic for humans, pathogenicity of porcine viruses can change unpredictably when they cross species. The chance of cross-species infection (Patience et al., 1997; Martin et al., 1998) increases with the closeness of contact of grafted and host cells following neurotransplantation, and the reduced competence of the immune system of the immunosuppressed graft-receiving patient. In a worst-case scenario, xenotransplants could introduce a very infectious, or possibly lethal pathogenic virus that would not only affect the graft recipient but could also (through human-to-human contacts) lay humanity open to a new plague (the Trojan xenotransplant) (Butler, 1998; Bach et al., 1998). To the xenograft recipient, the benefit of a successful transplant will certainly outweigh the risk of any subsequent unwanted effect of infection by a pig virus. To society, however, the possibility of setting off a new human epidemic requires fundamental virology studies before any ethical judgements are passed.

So far, patients who have received pig organ or tissue transplants (Nasto, 1997; Stoye et al., 1998; Heneine et al., 1998) or who were dialyzed with pig kidneys (Patience et al., 1997) have shown no signs of porcine virus-induced pathogenesis. Clinical studies with porcine embryonic mesencephalic dopaminergic grafts in the brain should still include long-term post-operative screening on the expression of porcine endogenous retroviruses in serum samples (Isacson and Breakefield, 1997). Possible consequences for the patient when hazardous viruses do show up have hardly been considered in the neurotransplantation field. If it affects just the patient, it is to be regarded as side effect. If it becomes a highly transmissible, life-threatening disease, at the extreme it could mean isolation of the xenotransplant-receiving person.

## *6.3. Risk/Benefit Aspects*

Experimental restorative neurosurgery is not without risk for the PD subject involved (Boer, 1999). In all cases mentioned in head 2 this type of surgery involves intracranial manipulations after drilling a hole in the skull, either under local or complete anaesthesia. The risks of the surgery, up to opening of the skull, are relatively mild. The risks involved in going into or through the brain parenchyma are more severe, since, despite extensive pre-clinical studies, the dangers of placement of cells are sometimes not fully predictable. However, the ethical principles of beneficence and non-maleficence must prevail at all times (see subheads 3 and 4), and a net therapeutic outcome should always the expectation.

Clinical neurotransplantation in PD subjects has so far been carried out either with autologous chromaffin cell-containing tissue, or with mesencephalic allo- or xenografts obtained from, respectively, electively aborted

human fetuses or specifically bred pregnant pigs. Sterile handling and tests for transmissible diseases prior to implantation (see subhead 6.2) are the first measures for safety, and can be seen as routine in any transplantation surgery. Adrenal medullary tissues as an autologous graft source for the chromaffin cells were abandoned as cell survival is poor and—in some centers this approach was regarded as a therapy rather than experimental treatment—morbidity and mortality were the side effects. Using human fetal mesencephalic grafts in PD, researchers have only recently identified the side effect of uncontrollable hyperkinesias in "off" phases (periods of increased motor disability) (Freed et al., 2001; Hagell et al., 2002). Here one has to go back to the animal research to investigate the cause before new, adapted—if feasible at all—clinical trials can be initiated. The benefit of fetal mesencephalic grafting is said to be limited by poor dopaminergic neuron survival and insufficient integration and reinnervation within the deficient striatum of the PD patient (Brundin et al., 2000). This is certainly the case for porcine xenografts, the clinical trials with which were apparently performed too early, as in rodents; e.g., cyclosporin A-mediated immune suppression protects porcine grafts only during the first weeks and failes in the long-term (Larsson et al., 2001).

Transient psychotic symptoms have been reported in some cases of mesencephalic engraftment in PD subjects (Freeman et al., 1999). Safety concerns regarding personality transfer or changes (even emphasized for porcine grafts; Linke, 1993; Coffman et al., 1998), and of negative alterations in cognitive or mental functioning have not been reported. In fact, they were not to be expected as the approach was a cautious one, with only minute volumes of cell suspensions or small fragments used for grafting (see subhead 4.3). The risk/benefit ratio is thus neither uniformly low nor very high, but at the present stage fetal mesencephalic grafting should not be recommended as a therapy. The findings should, however, not block further trials, as significant recovery is seen in many patients, and harmful hyperkinesias in the "off" state are not frequently seen and may depend on the particular transplantation protocol applied. In fact, the results so far must be seen as encouraging for other types of restorative neurosurgery because of the relatively mild, or at least infrequent, signs of harm in comparison with the severe incapacitating symptoms in PD (non-maleficience). Critical evaluation of the preclinical results in animal PD models, published as a review, that deal with efficacy on motor recovery in relation to the morphology and function nigro-striatal pathway, and with such safety aspects, must be seen as a prerequisite (see subhead 4).

The safety aspects of molecular restorative treatments using AAV viral vectors for the overexpression of GAD for enhanced GABA release in the STN have been dealt with (see subhead 4.2), and here one has to see whether the expectation of a low risk/benefit ratio will hold in the first current clinical trial (During et al., 2001). For any other approach, whether it is the application of cografted cells supporting the mesencephalic dopaminergic transplants, implants of immortilized cell lines with dopaminergic phenotype, paraneural

cell implants that secrete neurotrophic or growth factors to halt nigro-striatal degeneration, or direct gene therapy for trophic factors to rejuvenate the system (Kirik et al., 2004), the risk/benefit aspect still has to be discussed, as do the definitive answers on efficacy, preferably in PD non-human primate models. This certainly is the case for ES-cell-derived transplants in PD subjects, for whom the way from hype to hope for an "dopaminergic neuronal transplant from the shelf" is very long (Chapter 13).

## 7. Experimental Design

Clinical neurotransplantation in PD began with open trials of striatal placement of the patient's own adrenal medulla tissue (Backlund et al., 1985). The studies immediately raised the question of whether enough basic studies had been performed to justify such an experimental clinical treatment. The team that performed the first stereotaxic neurotransplantation in PD argued that the frustrating lack of treatment for advanced PD patients had weighed heavily in their considerations, and that the respective national and university-based ethical committees agreed to start this enterprise (discussions at the Eric K. Fernström Foundation Symposium on "Neural grafting in the human CNS," Lund, Sweden, 18–22 June 1984). The outcome of this first study was negative with respect to efficacy. Later, Madrazo et al. (1987) reported significant benefits, but the (afterward overenthusiastic) interpretation of trial data could not be replicated by other groups, and the method was therefore primarily a failure (see subhead 4). This history of adrenal medulla auto-implants in the brain of PD patients illustrates the difficulty of determining what level of prior animal experimentation is required to establish the efficacy and safety of a new clinical treatment. It also illustrates that a move toward the first clinical trial is often prompted by the absence of any effective treatment (Boer, 1996, 1999). Finally, it stimulated discussion on the need for an experimental design for such open-label studies that would give interpretable results. The "birth" of CAPIT-PD was the result (see subhead 4.4). CAPIT-PD improved patient selection, with reproducible pre-treatment measures of the motor scores as baseline, a 1–2-year follow-up period with fixed protocols for read-out of motor scores, and graft survival as key elements.

The value of CAPIT-PD was recently further established by a systematic review and meta-analysis of its measures from study groups that embraced this protocol in their mesencephalic grafting studies (Polgar et al., 2003). Consistent trends demonstrating recovery on many outcome measures could be identified, and recommendations for best practice reported. Ethically speaking it should be highly recommended that researchers put their standardized acquisition data in a CAPIT-PD database, as this would allow a more powerful statistical analysis for evaluating the overall benefits and harm of any type of reconstructive neurosurgery in PD. It saves many subjects from undergoing experimental surgery.

## *7.1. Double-Blind Sham-Controlled Studies*

The variable outcome among PD subjects following neurografting has led to the notion that results may be influenced by placebo effects and investigator's bias. To investigate further the efficacy of this new medical technique, double-blind, sham-surgery-controlled studies were proposed and initiated. Sham surgery is a completely new approach in restorative surgery, which had never received a critical evaluation in scientific literature until Freeman et al. (1999) published a plea in favor of this approach in experimental cell supplementation therapy in PD. Organ transplantation cannot be performed with sham surgery ("take the heart out and put it back") to control for bias or placebo effects, but adding cells by injection could in principle be performed under the rules of the gold standard of pharmacological studies. Placebo effects are not uncommon in the clinic, nor in PD studies (Shetty et al., 1999; Goetz et al., 2002), and investigator bias with overenthusiastic interpretations leading to surgeries or drug treatments that were later abandoned for lack of efficacy are not uncommon in clinical science (Albin, 2002). Moreover, De la Fuente-Fernandez and Stoessl (2002) found an effect of placebo administration on striatal dopamine release in PD subjects, and suggested the release was related to signal expectation of reward. Freeman et al. (1999) pleaded for sham (imitation) surgery under the following three conditions: 1) it should address an important research question that cannot be answered by a study with an alternative design that poses a lower risk to the subjects, 2) there must be preliminary but not conclusive evidence that the intervention is effective, and 3) the treatment should be sufficiently developed so that it is unlikely that it will become obsolete before the study has been completed. The latter two criteria pose no problems, as it is generally accepted that neurotransplantation surgery can be effective; there is need for improvement and perfection, but adverse effects are not too prominent (see subhead 6.3) (Brundin et al., 2000; Dunnett et al., 2001). The criterion for discussion is, therefore, the first one.

Sham controls were introduced with full-blown pre- and postoperative patient assessment and surgery, except that the dura was not penetrated and no needle insertion was performed (Freeman et al., 1999). The procedure is thus no control for the implantation surgery, but only controls for the placebo effects of the event of the surgery and of the pre- and postsurgical evaluations. At the outset, with the initiation of such studies meanwhile finalized and reported on (Freed et al., 2001; Olanow et al., 2003), critics claimed that it was too early to include large-scale sham-controlled studies with 30–40 patients when it was obvious that dopaminergic neuron survival was insufficient and transplant methods far from optimal (Widner, 1994). Moreover, surgical implantation of cells in the brain cannot simply be compared with a pharmacological treatment for which extensive guidelines are published, and a double-blind, placebo-controlled design is the gold and often legally required standard (CPMP Working Party on Efficacy of Medicinal Products,

1990). In pharmaceutical research, placebo is not known to produce any adverse effects, and subjects on the placebo do not risk positive harm (harm of commission), but only the harm of omission or exclusion (London and Kadane, 2002). Sham surgery carries risks, and it is thus undeniable that performing surgery with no potential benefit fails to minimize the risk of positive harm. The highest standards of research design were said to be in conflict with the ethical standards of beneficence and non-maleficence (Macklin, 1999). However, this ignores the fact that research ethics take into account not only the interests of the research subjects but also the interests of biomedical research, of the category of patient (aspirational benefit in contrast to direct or collateral benefit for the study subject), and of society at large (Dekkers and Boer, 2001; Albin, 2002). Freeman et al. (1999) stated that these risks are "reasonable" in relation to the possible benefits (sham patients undergo one imitation surgery and will then be elected for an effective treatment later on). Thus, if the chosen research question cannot be answered by a trial that poses fewer risks to subjects, then the use of a sham-surgery control may be a necessary component of a sound clinical trial. It is precisely this which remains the controversial issue: the alleged imbalance between the risks to subjects of the placebo group versus potential benefits to them and to society as a whole. Advocates argue that placebo surgery reduces the bias and increases the objectivity in result analysis, which would protect the public at large from a potentially dangerous procedure being available as a result of incorrect interpretation of test results. The latter points are therefore the crucial ones to consider in determining whether sham neurosurgery is acceptable and whether solid answers to a restorative neurosurgical research question can be obtained without sham control.

The plea against sham-surgery controls by Macklin (1999), London and Kadane (2002), and Clark (2002) is mainly based on the notion that researchers use individuals as a means-to-an-end for the study. Sham surgery is said to violate principles of respect for the autonomy, beneficence, and justice, whereas the validity of free and informed consent from the patients is undermined by therapeutic misconception. However, the lack of consensus on this point in itself may not be an argument against sham surgery. Lack of consensus has not prevented the first clinical neural grafting trials in a balanced, fairly discussed, and ethically acceptable fashion. If there is no other study design that will provide interpretable data, sham-controlled studies may be acceptable (Freeman et al., 1999; Dekkers and Boer, 2001; Albin, 2002). A significant number of PD patients worldwide have received an implant without evidence of a long-lasting therapeutic effect, whereas there is compelling evidence that the clinical course of the engrafted PD patients parallels the development of the graft measured with the surrogate marker F-dopa uptake in PET scans (as animal studies predicted) (Lindvall, 1999; Piccini et al., 1999; Dunnett et al., 2001). Following this, imitation surgery may not be needed, an aspect underestimated in the line of thinking in favor of sham surgery by Freeman et al., (1999). The imitation-surgery control

patients of the double blind studies indeed revealed no improvement of motor ratings (Freed et al., 2001; Olanow et al., 2003).

The necessity of sham-surgery control was additionally challenged on the basis of the so-called principle of equipoise (London and Kadane, 2002). In pharmacological studies, two drugs may be compared in a gold standard procedure to see whether a new compound is equally or more effective than an existing compound of proven effectiveness. The equipoise states here that there is no need for any patient group to be knowingly sacrificed in terms of welfare or body integrity. For PD patients there are pharmacological treatments available, albeit not sufficiently able to cure the symptoms in the late stages of the disease. Comparison of old and newly treated patient groups is thus possible. But this requires a solid standardized core assessment protocol, CAP-PD, with measures that are as objective as possible.

## *7.2. Plea for the CAP*

Human experiments that gave non-interpretable results are unethical (Felten, 1994), but so are unnecessary control treatments in human beings. Sham surgery should not be the default control method (Albin, 2002). However, this requires the use of a solid standardized CAP-PD, based on quantitative measures, and on a rigid schedule running from the pre-operative period through to post-convalescence (Boer and Widner, 2002). The latter is important, as it has been shown that it takes years before the full effects are reached following, e.g., human fetal nigra grafting. The application of a maximally objective CAP guarantees that data are collected that can also be compared with a parallel CAP-evaluated randomly assigned reference group or to groups that are subjected to other surgical interventions. Objective markers of surviving tissue, using imaging techniques, should also be recognized as pivotal for the interpretation of the clinical effects and should be used to link the clinical effects with a causal mechanism (Brooks et al., 2003). Negative scans combined with a modest clinical improvement indicate non-cellular therapy or non-molecular therapy-derived effects.

Other experimental irreversible surgical interventions in PD, such as placement of electrodes for deep brain stimulation (DBS) (Benabid et al., 1991; Benabid, 2003) and strategic lesions in the outflow pathways of the basal nuclei (Tasker et al, 1983), or gene therapeutic interventions to enhance the insufficient dopamine release (During et al., 1998) to modulate the output pathways in the STN (During et al., 2001) or to stop the degeneration of the dopaminergic nigra cells (Zurn et al., 1996; Kordower et al., 2000; Gill et al., 2003), would also require a proper unbiased control method to determine long-term efficacy and the absence of severe side effects. A validated PD-dedicated CAP would then be most valuable as an evaluation instrument, even though specific aspects may have to be skipped or added (e.g., repeated PET scans to determine dopaminergic function may be less effective when lesion treatment is applied). A maximally objective CAP-PD remains of value for open trials, too,

as pre- and post-treatment measures can be compared satisfactory (Boer and Widner, 2002).

## 8. Summarizing Remarks

The field of restorative neurosurgery in PD is booming. A variety of restorative cellular and molecular neurotherapies are under investigation ever since a proof of principle for (partial) recovery of the PD motor disturbances was reported following human fetal mesencephalic grafting in the caudate/putamen complex of PD subjects. These outcomes validated the rationale behind the approach: dopaminergic neuronal input in the striatum can be restored, and the decreased motor anomalies of the patient relates to neuronal cell survival and dopamine release. These results ethically justify further clinical experimentation in neurotransplantation surgery using embryonic or fetal tissues containing the immature dopaminergic neurons. These tissues, available after legislated or legal elective abortion, may be retrieved under two main conditions: 1) donation with informed consent after the definitive decision on abortion and 2) no remuneration or designation of the recipient patient (the separation principle). The variability and limited efficacy of cellular supplementation with immature mesencephalic dopaminergic neurons, as well as the logistical problems to compile (or store) sufficient disease-free and homogeneous cellular material for grafting have led to the search for alternatives that repair the degenerated nigro-striatal system.

Porcine xenografts, autologous transplants of adrenal medulla chromaffin cells with support of co-transplanted cells or molecular pre-treatment, implants of immortalized cells with dopaminergic phenotype, and finally dopaminergic cells proliferated and differentiated from ES, EG, or SS cells are the alternatives for cell supplementation therapy in PD. None of these approaches has as yet given a better answer to the PD problem. Some have reached phase I clinical trials; others are still in the animal laboratory research phase. However, all these approaches have raised new ethical questions, so that one cannot say that these alternatives have solved the ethical problems for groups in society that categorically condemn the use of any human embryonic or fetal cells for research or therapy. Each of these approaches has to deal with the aspects of tissue collection as well as with new safety aspects for the recipient patient of the clinical study.

Negative moral judgment about the use of human donor cells appears to be inversely related to the age of the human source. Ethical discussions on the use of in vitro human pre-implantation embryos as the source for ES cells provoked societal and political debate more strongly than at the time when the use of abortion remains was introduced as a possible therapeutic treatment for neurodegenerative diseases. The use of residual, otherwise destroyed IVF embryos must, however, be compared with the use of the tissue remains after elective abortion. The moral value of human life at the pre-implantation

stage of the blastocyst should not be placed above that of a post-implantation embryo in which organogenesis has started to create the appearance of a human body. In ethical evaluations of the human value of prenatal life the principle of growing relative protection with age is the generally accepted and main view. In addition, the use of ES cells is meant to develop cell lines for transplantation, so that, theoretically, large cohorts of patients may be treated with cells from a single abortion or a single surplus IVF embryo. The creation of human blastocysts, either from donated egg cells and sperm (e.g., IVF protocol) or from donated egg cells used for SCNT with the somatic nucleus of the patient (therapeutic cloning to obtain genetically identical ES cells to grow autologous transplants) must be regarded as using human life as a means-to-an-end for transplantation. Here one has to weigh the notion that in vitro human early life is not yet a "person," and the potential good for a person with a noncurable disease such as PD (or other life-threatening and severely handicapping diseases). At the present time, also in view of the recent findings that pluri- and multipotent SS cells might be isolated from adult organs as well as from umbilical cord blood, and may be possible to differentiate into virtually every cell type, a proof of principle for efficacious and safe transplantation with ES-derived neurons in PD should be presented before the need for creation of embryos has been decided upon. For cellular therapy in the nervous system it may not even be necessary to initiate therapeutic cloning to circumvent immune rejection, as the brain is an immunoprivileged site.

Besides methods to replace the lost dopaminergic input of the denervated striatum by cell placement, molecular interventions are also being developed, and clinical tests have started. Here, the safety aspects for, e.g., gene therapy in the CNS are to be considered. The brain is the organ of our mind, the complex network of our neurons make up our human behavior. Careful animal studies should always precede any clinical trial, taking into account possible side effects in functional capacity of the brain. Though neural grafting of embryonic mesencephalic tissue was found not to alter psychological and physiological measures to a great extent in PD subjects, any other putative but irreversible cellular or molecular treatment should reconsider these aspects. The field of restorative neurosurgery would be well served if the scientists involved in a clinical trial of any new approach were to publish the scientific and ethical justification of their program. This would allow discussion in the scientific and public domain, and help to take away the often publicly expressed distrust in "scientists playing God" with gene and human embryonic cell therapeutic means.

The publication of Freeman et al. (1999), which justified the double-blind sham-controlled study on the genuine efficacy of embryonic mesencephalic grafts in PD subjects is an excellent example of how discussion can be provoked and how it can lead to an open exchange of arguments on a sensitive topic: the human as research subject in irreversible experimental treatments. Freeman et al. (1999) stated that placebo effects and observer bias should be

eliminated in a meaningful study that includes patients. However, the gold standard of the double-blind placebo-controlled investigation model from the pharmacological field cannot be simply translated to restorative neurosurgery. Indeed, human experiments that gave non-interpretable results are unethical, but unnecessary control treatments in human beings are also. The more so because the risks of imitation surgery and further medical treatment and testing cannot be ignored, and informed consent is easily biased by therapeutic misconception for PD patients who have high hopes for any new therapy (Dekkers and Boer, 2001). These aspects may thus violate the basic ethical principles of beneficence, non-maleficence, and autonomy. The strict application of a CAP, including quantitative measures for the motor capacities, would very much help to avoid bias in the evaluation of the outcome of any type of restorative neurosurgery in PD. It can also render superfluous the need for the gold standard of double-blind placebo-controlled pharmaceutical studies in the experimental treatment of PD. Objective comparison between pre- and postsurgery measures is then possible as well as comparison between the new experimental therapies (as is done in clinical pharmacology when an old and a new drug are compared for their efficacy).

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# 3

# Evolution of Parkinson's Disease and Treatment Requirements: What New Treatments are Needed and the Role of Striatal Grafting

José A Obeso[1] and Anthony E Lang[2]

## 1. Introduction

The introduction of levodopa in the late 1960s revolutionized the treatment of Parkinson's disease (PD) and served as a reference for clinical neuropharmacology in general. This major therapeutic advance was based on the prior discovery of striatal dopamine (DA) deficiency secondary to cell loss in the substantia nigra pars compacta (SNc) in the brains of patients with PD (Hornykiewicz and Kish, 1987). Such precise definition and the profound improvement caused by levodopa, even in patients with longstanding severe disability, led to the equation of PD with striatal DA deficit (Hornykiewicz and Fish, 1987; Cotzias et al., 1967). However, it was soon realized that a large proportion of patients rapidly developed erratic motor responses and a variety of dyskinetic movements that had never been encountered before the initiation of levodopa (Yarh et al., 1969; Marsden and Parkers, 1976). Naturally, the largest effort during several decades of subsequent research concentrated on understanding the origin of such "levodopa-related" motor complications and on developing newer therapeutic strategies to better control the typical motor features of PD. Considerable success has been achieved in that regard (Lang and Lozano, 1998). Indeed, as a result of the various treatment options, the period of clinical stability has been prolonged to about 10 years after diagnosis and the life span of patients with PD has been significantly increased (Poewe and Wenning, 1996). The net effect is that the clinical picture of PD has changed substantially with respect to what was common in the pre-levodopa era. As a result, the management of PD has to be understood

[1]Department of Neurology-Neuroscience, Clínica Universitaria and Medical School, University of Navarra, Pamplona, Spain
jobeso@unav.es
[2]Movement Disorders Clinic, Toronto Western Hospital, University of Toronto, Toronto, Ontario, Canada
lang@uhnres.utoronto.ca

nowadays from a dual perspective: first, the beneficial effects and complications associated with chronic levodopa treatment (Table 3.1A) and, second, the consequences of long-term evolution and disease progression (Table 3.1B). Accordingly, current treatment options have to deal with a combination of motor and non-motor manifestations that were not commonly seen 15–20 years ago. In this chapter, we shall contend that major therapeutic developments for PD should not be uniquely focused on restoring DA striatal deficiency but also, and perhaps preferentially, on controlling the progressive course of the underlying neurodegenerative process (Lang and Obeso, 2004).

## 2. Clinical Problems

Current management of PD has to deal with a variety of different problems that arise mainly as a consequence of disease progression and the iatrogenic effects of levodopa treatment (Lang and Lozano, 1998a; Obeso et al., 2000a). PD may now be classified according to the main clinical manifestations throughout its evolution and the response to levodopa as described below (Table 3.2).

### 2.1.1. Early Stage

In the initial years after diagnosis (i.e., the first 5 years approximately), the typical motor manifestations predominate. The motor signs are relatively mild and predominate in one body segment or hemibody. Treatment with levodopa or a dopamine agonist provides a dramatic benefit, so that patients can easily cope with daily living activities, maintain their jobs, and even continue to enjoy challenging pastimes that they engaged in before the development of PD.

### 2.1.2. Intermediate Stage

The motor features become generalized after some 5–10 years of evolution (Poewe and Wenning, 1996). The response to levodopa is still associated with a marked benefit but the duration of this effect generally lasts for less than 4–5 hours and dyskinesias are often present. Thus, in the "on" medication state patients are perfectly capable of carrying out any motor activity although these may be limited, in some 30% of cases, by severe dyskinetic movements. Typically, motor fluctuations take the form of a "wearing-off" response or "end-of-dose" deterioration, where patients cycle following each levodopa dose between the "on" (good mobility) and "off" (return of parkinsonism) motor states (Marsden and Parkers, 1976; Obeso et al; 2000).

### 2.1.3. Advanced Stage

After some 10–15 years of evolution, motor complications are present in the great majority of patients (Marsden et al., 1981; Schrag et al., 2000). In the "off" state, patients are very disabled, incapable of looking after themselves or engaging in routine daily life activities. Dyskinesias are invariably found during

the "on" state. In addition, diphasic dyskinesias or "beginning- and end-of-dose" dyskinesias as well as "off" dystonic spasms and postures are also present in at least 50% of patients (Obeso et al., 2000; Marsden et al., 1981). The latter two are frequently very disabling as they are commonly accompanied by pain, dysphoria, anxiety, etc. Gait and equilibrium problems may also be present and not resolved completely in the "on" medication state. Psychiatric complications (i.e., hallucinations, confusional delirium, etc) are very common and require specific treatment with atypical neuroleptics. Quality of life is severely impaired.

#### 2.1.4. Multisystem Stage

A fair proportion of patients nowadays reach old age (i.e., >70) despite suffering from PD for as long as 15–20 years or more. At this stage, all of the above problems are still present, but the clinical picture is dominated by a plethora of motor and non-motor features that do not respond to dopaminergic medications (some may even be accentuated by this treatment, e.g., freezing) nor to surgical treatment with deep brain stimulation. Thus, cognitive impairment, falls and gait difficulties, dysautonomia, speech problems, excessive diurnal sleepiness, etc., become the major sources of disability (Lang and Obeso, 2004). These manifestations probably represent the extension of the pathological process to involve extra-nigral brain areas (Agid et al 1987).

The evolution outlined above describes the average patient; some may evolve more slowly and others more rapidly, particularly with respect to problematic motor complications and dopa-resistant clinical features.

## 3. Current Treatment Options (Table 3.2)

The treatment of PD has expanded considerably over the last decade to include several drugs acting on the dopaminergic system, drugs with novel (non-dopaminergic) mechanisms of action and functional neurosurgery (Lang and Obeso, 2004; Lang and Lozano, 1998b; Rascol et al., 2002). We shall briefly summarize the main results from the perspective of the unmet treatment needs and what cell therapy can add to the current therapeutic armamentarium of PD.

### *3.1. Early treatment: Role of DA agonists*

Three controlled clinical trials (involving a total of 961 patients) have demonstrated that initial treatment using a DA agonist (ropinirole, pramipexole, or cabergoline) rather than standard levodopa (i.e., Sinemet or Madopar, three times per day) is associated with a significant reduction in the probability of developing motor complications (dyskinesias or motor fluctuations) but a

similar degree of clinical improvement (Rascol et al., 2000; Rinne et al., 1999; Parkinson Study Group, 2000). Such in effect is thought to be mediated by providing more continuous dopaminergic stimulation in contrast to the pulsatile pattern provoked by standard levodopa treatment (Obeso et al., 2000a; Olanow and Obeso, 2000). It is well known, however, that the great majority of patients (some 70–80%) initiated on a DA agonist require supplemental treatment with levodopa after 5–7 years of treatment, which could potentially defeat the initial preventive effect against developing motor complications. It is noteworthy that experimental evidence in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) monkey model suggests that the addition of levodopa to animals previously treated with a DA agonist is also associated with less dyskinesias than those treated with levodopa alone (Jenner, 2004; Belanger et al., 2003). In addition, recent experimental findings in both the MPTP monkey model and the 6-hydroxydopamine (6-OH-DA) rat rotatory model indicate that the combined use of levodopa with a decarboxylase inhibitor and a catechol-O-methyltransferase (COMT) inhibitor, entacapone, is also associated with a reduced incidence of dyskinesias as well as fluctuations (Jenner, 2004; Olanow and Stocchi, 2004). There is an ongoing trial in "de novo" patients to clinically validate these experimental findings.

## *3.2. Treatment of Motor Complications*

Several pharmacological options are available to treat the "wearing off" phenomenon and "peak of dose" dyskinesias when they are relatively mild in the *intermediate* stage of evolution. These include longer-acting DA agonists, controlled-release levodopa preparations, COMT, and MAO-B inhibitors, etc. However, after some 10 years of evolution, the majority of patients have progressed to the point of experiencing more incapacitating and bothersome motor complications that are very difficult to control with standard medications. In these circumstances striking therapeutic benefit may be achieved with continuous parenteral infusions of a DA agonist (apomorphine, lisuride) or intraduodenal levodopa or with functional neurosurgery using deep brain stimulation (DBS) of the subthalamic nucleus (STN) or the globus pallidus pars interna (GPi).

### 3.2.1. Infusions

Several years ago it was shown that intravenous delivery of levodopa (Fabbrini et al., 1988) or subcutaneous administration of lisuride or apomorphine (Obeso et al., 1987; Vaamonde et al., 1991) could markedly ameliorate complex motor fluctuations and reduce dyskinesias, thus widening the so-called "therapeutic window." It has been consistently shown that such beneficial effect is more robust in patients with "wearing off" than in those with complex fluctuations and dyskinesias (Chase et al., 1989; Obeso et al., 1994; Stocchi et al., 2002). The practicalities of the portable systems for the

infusions and the initial psychiatric complications associated with 24-hour drug delivery dampened enthusiasm and limited widespread utilization of infusions for the treatment of PD. More recently, however, several groups have reported marked therapeutic success using infusion pumps to deliver apomorphine or lisuride subcutaneously or enteral levodopa during the day only (Manson et al., 2002; Stocchi et al., 2002; Nyholm et al., 2003). In general, the results are favorable and in keeping with the original findings in the 1980s. Time spent in the "off" state is significantly reduced (by approximately 60–70%) and dyskinesias are diminished, but this takes weeks to months to accomplish. It must be stressed, however, that in many patients the applicability of such infusions is limited. The major shortcomings are—

1. Complex fluctuations and dyskinesias are often ameliorated but rarely, if ever, resolved. This leads, in many patients, to complicated interactions between the effects of the ongoing infusion and those of supplementary acute prn dopaminergic drug doses (oral or parenteral bolus).
2. Night-time mobility and early morning "off" period dystonia are not significantly improved.
3. The pumps and associated technology still require considerable maintenance and attention.
4. Psychiatric and behavioral complications may be dose-limiting.

### 3.2.2. Functional Neurosurgery

Surgery for PD began in earnest in the mid-1940s, well before effective drug treatments were available, and was mainly directed at alleviating tremor. The therapeutic benefit was often low, probably because the stereotactic surgical techniques in use were not very accurate and the pathophysiology of PD was poorly understood. In recent years, there has been a resurgence in the use of neurosurgical treatments for PD. This has been largely driven by the development of a pathophysiological model of the basal ganglia mainly based on findings in the MPTP-treated monkey (The Deep Brain Stimulation for Advanced Parkinson Disease Study Group 2001). In these parkinsonian animals it was found that DA deficiency leads to a series of functional changes in basal ganglia circuits among which increased and abnormal neuronal firing of the STN is of paramount importance. The STN uses glutamate as neurotransmitter and exerts a powerful excitatory drive onto the major output nuclei of the basal ganglia, the globus pallidus pars interna (GPi), and substantia nigra pars reticulata (SNr), which utilize the inhibitory neurotransmitter gamma amino butyric acid (GABA). As a result, cortical and brainstem targets of basal ganglia efferent activity become overinhibited. Furthermore, it was shown that reduction of the abnormal neuronal activity of the STN (via a lesion or reversible chemical blockade) led to marked motor improvement and restoration of the electrophysiological and biochemical abnormalities in the output nuclei. These findings set the stage for a revitalization of pallidotomy first and the subsequent introduction of DBS

of the STN or GPi. Pallidotomy can only be performed unilaterally with sufficient safety and is now infrequently applied for surgical treatment of PD.

DBS of the STN or GPi is the fundamental surgical approach currently used in patients with severe motor complications. There is insufficient valid data to allow a proper comparison of the results of these targets. Therapeutic benefit is quite similar with the exception that levodopa daily dose may be significantly reduced (by an average of 40%) in patients treated with STN-DBS but not reduced at all in those treated with GPi-DBS. In addition, targeting the motor region of the STN appears easier than the homonymous area of the GPi and, probably due to the different sizes of the targets (STN more compact), GPi DBS requires more energy, causing the pulse-generator battery life to be shorter. As a result, most centers in the world mainly utilize DBS of the STN for the treatment of PD. Surgery of the thalamus has been virtually abandoned, as it is almost exclusively effective for tremor, which is also well controlled by STN-DBS (as well as GPi DBS). In recent years numerous studies (The Deep Brain Stimulation for Advanced Parkinson Disease Study Group, 2001; Kumar et al., 2000; Kleiner-Fisman et al., 2003; Krack et al., 2003; Rodriguez-Oroz et al., 2004) have demonstrated the potential for GPi or STN DBS to convey striking improvements in all classical motor features of parkinsonism, as well as levodopa-related motor complications. Recently, follow-up series have shown that benefit to levodopa-responsive cardinal symptoms of PD is maintained for more than 4–5 years (Kleiner-Fisman et al., 2003; Krack et al., 2003; Rodriguez-Oroz et al., 2004). However, the greatest benefit obtained with DBS-STN does not substantially surpass the patient's current best antiparkinsonian response to levodopa. This leaves a number of disabling medication-resistant symptoms, such as speech impairments, postural instability and falls, unaffected and a proportion of patients followed for 5 or more years have been shown to develop progressive dementia (Krack et al., 2003) presumably as they would have without this intervention. The major limitations of DBS for PD are—

1. A 1–2 % risk of severe intracranial hemorrhage during the surgical procedure.
2. Stimulation-induced behavioral abnormalities and persistent cognitive dysfunction.
3. High cost of the equipment.
4. Relatively high labor demand in order to adjust both the stimulation parameters and oral medication.

## *3.3. Psychiatric Complications*

Motor complications and other problems appearing in the long-term evolution of PD such as depression, insomnia, constipation, sialorrhea, urinary urgency, etc., lead to the use of considerable polypharmacy in patients with advanced

PD. As a result, a variety of drug-induced psychiatric complications are common in these patients. In addition, cognitive dysfunction as part of PD is an important predisposing factor to these psychiatric complications. Nocturnal myoclonus, vivid dreams, and nightmares may give way to nocturnal confusion. Daytime hallucinations (typically visual) may or may not be preceded by these nocturnal symptoms. These may evolve into a frank delirium or a paranoid psychosis. In a proportion of patients, psychiatric complications are more specific and restricted to behavioral abnormalities such as increased appetite (both food and sexual), gambling, compulsive behavior, concern about spousal fidelity, etc. The introduction of atypical neuroleptics (i.e., clozapine, quetiapine) have improved the management of psychiatric complications and now these symptoms can usually be controlled without aggravating parkinsonism in the majority of instances. In patients without overt cognitive deterioration, psychiatric complications may be the initial manifestation of this problem, which then drastically complicates management and quality of life.

## *3.4. Treatment of Non-Dopaminergic Features*

The progressive evolution of PD leads to the eventual development of a variety of motor and non-motor manifestations (Table 3.1) insensitive to treatment with dopaminergic drugs and consequently, to functional neurosurgery, as well. Currently, most of these problems do not have specific treatment (Table 3.2),

TABLE 3.1. Main Clinical Complications Associated With Long-Term Evolution of Parkinson's Disease

| | |
|---|---|
| **A. Levodopa related** | |
| Motor fluctuations | "Wearing-off"<br>Complicated "end of dose" or "on"–"off" |
| Dyskinesias | "On" or "peak dose"<br>Diphasic<br>"Off" dystonia |
| Non-motor fluctuations | Akathisia, "off" pain, other sensory |
| Autonomic, psychiatric | Mood changes[a]<br>Akathisia[a] |
| Psychiatric complications | Nocturnal parasomnia<br>Behavioral disorders<br>Hallucinations, delirium |
| **B. Related to spreading of the neurodegenerative process** | |
| Cognitive impairment; dementia[b] | |
| Depression | |
| Falls and equilibrium problems | |
| Autonomic disturbances | |
| Severe hypophonia and swallowing difficulties | |
| Neck and trunk fixed flexor posture; severe scoliosis and other skeletal deformities. | |

[a]Not related to dose timing
[b]Psychiatric complications are more likely in patients with underlying cognitive impairment.

Table 3.2. Evolution of Parkinson's Disease and Therapeutic Challenges

| Stages | Disease years | Main clinical manifestations | Established therapeutic approaches | Unmet treatment needs |
|---|---|---|---|---|
| Initial | 1–5 | Focal cardinal motor features | Dopaminergic drugs | Slow/halt progression |
| Intermediate | 6–10 | Bilateral motor features: "wearing-off" and dyskinesias | Attempts at more continuous dopaminergic stimulation<br>Surgery | Reduce/eliminate side effects |
| Advanced | 10–20 | Motor complications;<br>Psychiatric complications, gait and equilibrium problems | Surgery<br>Atypical neuroleptics | Treatment of non-dopaminergic motor problems |
| Multisystemic | >20 | Falls<br>Dementia<br>Autonomic disturbances<br>Sleepiness | None available | Treatment of multisystem manifestations |

with the possible exception of mild cognitive dysfunction, which may respond to cholinesterase-inhibiting drugs (e.g., donepezil, rivastigmine), successfully used for early Alzheimer's disease.

## 4. Therapeutic Needs

The previous sections indicate that, in the absence of complicating factors, the cardinal features of PD and levodopa-related motor complications can be adequately controlled in the majority of patients with available therapeutic measures. If the clinical manifestations of PD remained restricted to the cardinal motor features, a favorable long-term evolution could be guaranteed with today's available resources. However, the quality of life of patients deteriorates particularly as a consequence of gait and equilibrium difficulties, autonomic dysfunction, and cognitive impairment. These so-called "non-dopaminergic" clinical manifestations are very likely related to involvement of neurons in numerous cortical, subcortical, brainstem, and peripheral autonomic sites by the same neurodegenerative process that affects the nigrostriatal system (Lang and Obeso, 2004). Thus, it may be said that the major unmet therapeutic challenge in PD is to find neuroprotective strategies capable of halting disease progression both in and particularly beyond the nigrostriatal dopamine system. If in addition, the clinical consequences of the neurodegenerative process both in the SNc and beyond the basal ganglia could be restored and controlled, a major therapeutic breakthrough would

have been achieved. Can we envisage such a development from the perspective of dopamine replacement cellular strategies? The answer, we believe, on the one hand will depend upon the mechanisms involved in the origin and progression of PD and on the other on the extent and adequacy of grafting to restore the deficit.

## *4.1. Beginning and Progression of Parkinson's Disease*

The clinical onset of motor symptoms of PD coincides with a loss of about 60–80% of striatal dopaminergic terminals and around 40–50% of cell bodies in the SNc (Kish et al., 1988; Javoy-Agid et al., 1982). Pre- and postsynaptic compensatory mechanisms within the nigrostriatal DA system are classically believed to allow the basal ganglia to adapt to progressive DA loss. Increased DA turnover due to increased activity of surviving SNc cells has been considered as the most important (Agid et al., 1973). However, recent experimental findings in the MPTP monkey model have shown that compensatory mechanisms involving DA synthesis and turnover as well as postsynaptic DA receptors occur late in the evolution of SNc damage, once the parkinsonian manifestations are clinically evident (Bezard et al., 2001). Such studies have also demonstrated that increased neuronal activity in the STN precedes the onset of motor manifestations (Bezard et al., 1999) and is not associated with hypoactivity of the external segment of the globus pallidus as predicted by the classic model. This has led to the suggestion that perhaps the initial disruption of basal ganglia function is not due to striatal DA depletion but to derangement of other pathways controlling STN (and other nuclei) activity (Obeso et al., 2000b). In this regard the recent pathological study of Braak and colleagues (Braak et al., 2003), which analyzed a large number of brains with Lewy body disease, is especially relevant. According to these authors the pathological process in PD begins with involvement of the dorsal IX-X motor nucleus and/or intermediate reticular zone as well as the anterior olfactory nucleus (stage I) and spreads up the brainstem eventually reaching sensory association areas of neocortex and premotor areas (stage VI). Accordingly, SNc degeneration is a relatively late event (their stage III) in the evolution of PD. Clearly, these findings require confirmation and the concept may need some refinement, but undoubtedly they fit well with the evolution of PD toward a multisystem disorder and also the frequent clinical observation that symptoms such as depression, anxiety, focal pain, detrusor hyperreflexia, rapid eye movement (REM) behavioral disorder, etc., may precede the clinical diagnosis of PD sometimes by several years.

Regardless of how and where PD begins, there is no doubt that the neurodegenerative process involves several brain regions aside from the SNc and many neurotransmitter systems beyond the DA system. Such multisystem involvement is the basis for the typical clinical manifestations (i.e., hallucinations, dementia, falls, and equilibrium problems, autonomic dysfunction, excessive diurnal sleepiness, etc.) that characterize the advanced stage of

evolution (Table 3.2). Present evidence, therefore, indicates that neurodegeneration of the SNc and the consequent striatal DA depletion is only one component, albeit notoriously relevant in clinical practice, of a much more widespread multi-systems process. When patients with PD are experiencing a "poor response" to their medications, it is largely because the manifestations of the disease beyond the nigrostriatal dopamine system are predominating or are preventing more effective use of dopaminergic therapies.

In view of the above, how could restoration of the striatal DA deficit influence the evolution of PD? A convenient scenario would be that the functional, downstream abnormalities associated with SNc degeneration and dopamine deficiency are responsible for the extension of the pathological process. According to this "one-hit" hypothesis, the spread or progression of neuronal degeneration beyond the SNc would be a secondary phenomenon (a "domino-like" effect) (Rodriguez et al., 1998). In such circumstances, it is logical to hope that perfect compensation for the striatal DA deficiency might not only improve the early levodopa-sensitive symptoms but also halt disease progression (Holden 2002). In this regard it is relevant to note that the parkinsonian state is associated with hyperactivity of several sub-cortical glutamatergic nuclei (i.e., STN, centro-median/parafascicular nucleus of the thalamus and pedunculopontine nucleus [PPN] of the brainstem). In animal models of PD (6-OH-DA and MPTP) it has been shown that reducing glutamatergic efferent activity from the STN or PPN reduces DA cell loss in the SNc (Takada et al., 2000; Nakao et al., 1999). However, the widespread neuronal involvement of advanced PD is not limited to areas receiving glutamatergic projections or dopaminergic innervation. Thus, it is unlikely that the pathological extension of the neurodegenerative process in PD could be set off by striatal DA deficiency alone. It seems more realistic to assume that SNc degeneration is just one step in a chain of events occurring with different temporal clinical expression. Once nigral dopaminergic neurons become involved it is possible that the disease in this location is accelerated by pathogenetic factors more directly related to these neurons than others, for example oxidative stress, increased iron levels, the presence of neuromelanin etc. However, the weight of evidence suggests that this in not the primary source of the widespread pathology that accounts for the increasing disability as the illness progresses.

## *4.2. What is the role of DA-restoring strategies?*

The DA deficit in patients with PD may be compensated for mainly by implanting dopaminergic cells in the striatum or by inducing the sprouting of dopaminergic fibers to reinnervate the striatum. Considerable disappointment has been caused in the neurological community by the failure of two recent double-blind, placebo-controlled trials to show significant motor improvement in patients implanted with fetal mesencephalic cells (Freed et al., 2001; Olanow et al., 2003). This was further reinforced by the development of

"off" medication dyskinesias that did not resolve after withdrawing all antiparkinsonian drugs and, in some more disabled patients, required GPi DBS. However, some patients did show considerable improvement, more in keeping with the experience of earlier open-label studies (Lindvall 1997; Björklund et al., 2003). For instance, in the Freed et al. study (Freed et al., 2001) less advanced patients (i.e., Unified Parkinson' Disease Rating Score of about 30 in the "off" medication state pre-operatively) did show significant benefit, although they were also the ones more prone to develop severe "off" dyskinesias. These patients typically had a younger age of onset and exhibited the best motor responses to levodopa. A similar conclusion comes from the Olanow et al. (Olanow et al., 2003) study where the group of transplanted patients with an "off" UPDRS score of <49 obtained significantly greater benefit than the sham-treated group.

Technical problems may have jeopardized the clinical outcome of the two double-blind studies conducted in USA accounting for the overall poor results. These include how the cells were prepared and stored before transplantation or the way immunosuppression was given (Chapter 8). It is likely that a better understanding of the neurobiology of the implanted cells and the anatomy and physiology of the striatum will eventually lead to refinements in grafting approaches and better clinical outcomes (Björklund et al., 2003). Efforts in this direction have already started. It is possible then, that the striatal DA deficit of PD could be perfectly compensated sometime in the future. Will that substantially improve upon the current state of the art? Possibly—however, we believe that the overall impact will not be great.

If one selects the best responders to bilateral striatal grafting from the the initial open studies (Hagell et al., 1999) as well as those from the double-blind trials, the degree of clinical improvement obtained does not surpass in any aspect (and generally does not even match) the benefit currently achieved with infusions of dopaminergic drugs or bilateral DBS of the STN or GPi. In the very best scenario, that is, achieving full striatal reinnervation and, therefore, restoring pre-synaptic modulatory mechanisms (Kink et al., 2004), one would expect to improve some specific features of bradykinesia such as reaction time, movement time for sequential movements, etc., that are more resistant to complete control with levodopa and DBS. Presumably, "off" medication dyskinesias would not be a long-term complication and indeed, "on" dyskinesias would probably improve. One can also envisage improvement in the performance of more complex behavioral tasks (i.e., the Wisconsin sorting card test, the tower test, etc.) or in specific domains sensitive to DA deficiency, such as time estimation and reward-oriented behavior. However, the clinical significance and impact on patients' daily lives of such changes, over and above the improvements currently obtainable, is uncertain and quite questionable. In addition, resting tremor does not appear to respond well to striatal grafting, even when considering patients with good clinical results.

## 5. Conclusions

We are forced to conclude that achieving a perfect restoration of the dopaminergic deficit, even reconstruction of the nigro-striatal pathway, will not lead to a major change in the natural history of PD. The role of striatal grafting in the future management of PD is uncertain. Perhaps it could eventually contribute to less costly, more effective, and less complicated dopa-responsive symptom control, although before this goal can be achieved considerable scientific and technological advances over the current state of the art will be required. However, more importantly, what the field needs is to develop strategies to stop or even prevent the more widespread neurodegenerative process. This is more likely to come through advances in our understanding of the factors that initiate the disease process in the first place and those that drive its relentless progression in the second. Until such aims are achieved, the cardinal motor features may be effectively controlled by drugs and DBS, which provide a reasonably good quality of life for some 10–15 years after diagnosis and this will be further improved as new pharmacological therapies successfully address the motor complications. In the later stages, a time that many proponents of cellular therapies have argued is the prospect for such treatment due to the "failure" of current medications, the clinical scene is dominated by other aspects of the biology of the disease process with an increasing contribution of non-dopaminergic features to the level of persistent disability.

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# 4

# Clinical Trial Design Issues

Karl Kieburtz

Clinical trials designed to assess restorative therapies in Parkinson's disease present several methodological challenges. By their nature restorative therapies are likely to be novel and therefore have poorly characterized efficacy as well as safety. Furthermore, many of these therapies are likely to be administered by surgical manipulation of the brain. Because of the invasive means of administration, there has been, and will be, a debate regarding appropriate controls and study design. In addition, advanced Parkinson's disease, the likely target for such interventions, is associated with a highly variable disease state and prior studies have demonstrated a moderate to marked placebo effect in intervention studies. The longitudinal clinical course of the disease state likely to be studied is also quite variable and prone to respond to many types of modulation. Given the relatively labor-intensive nature of the surgical interventions, sample size for the trials will likely be small. This will require efficient, perhaps novel, trial designs to detect efficacy as well as safety. Lastly, the resource-intensive nature of the interventions and the complexity of the disease state suggest that functional or quality-of-life measures be used as primary outcomes. Further discussion of the ethical issues, research questions, primary outcome measures, issues regarding randomization, blinding and control groups, statistical analysis, and specific trial designs will be discussed below.

## 1. Ethical Considerations

All research involving human subjects needs to follow the basic principles of respect for person, justice, and benevolence. Respect for persons is primarily

University of Rochester Medical Center, Rochester, New York, Department of Neurology, Box 673, 601 Elmwood Avenue, Rochester, NY 14642, USA
karl.kieburtz@ctcc.rochester.edu

demonstrated by informed and voluntary consent to participate, the right to withdraw from a trial without prejudice, and the respect for subject privacy and confidentiality. The principle of justice is demonstrated by free and equal access of appropriate patients to the research, but without coercion and with sensitivity to the needs of vulnerable populations. The principal of benevolence is manifested by an attempt to minimize risks in relation to potential benefits. With regard to clinical trials of restorative therapies in Parkinson's disease, there has been a great deal of discussion of ethical considerations, particularly regarding the use of sham operative controls in the two published trials of embryonic cell grafting (Chapter 6; Freed et al., 2001; Olanow et al., 2003).

In addition to the above general ethical considerations there are special concerns for randomized clinical trials. Randomized clinical trials are a rigorous research methodology designed to evaluate both the effectiveness and safety of interventions. Randomization allows (when effective) for equal distribution of known and unknown prognostic factors among treatment groups. Although randomization provides for important methodological advantages and helps to minimize selection bias, there must be a situation of genuine uncertainty among experts in the field regarding the relative merits of the treatments being studied in a randomized trial. This state of genuine uncertainty has been named clinical equipoise (Freedman, 1987). This state of clinical equipoise suggests that experts in the field do not know which treatment is superior and have legitimate differences of opinion regarding possible superiority. Even though a single individual may have a strongly held opinion regarding the superiority of a specific treatment, the situation of clinical equipoise still exists if uncertainty prevails among the broader community of informed investigators. It is then ethically justified to assign research subjects to the treatment arms by chance and the methodologic benefits of the randomized study are achieved. With the current status of restorative therapies for Parkinson's disease there is little debate that randomized trials are ethically justified especially in advanced Parkinson's disease. Furthermore, there is little debate that restorative therapies should be compared to best available or standard therapy as a comparator arm. The major questions arise as to whether some type of "placebo" intervention should also be provided along with the standard medical therapy and what the nature of that placebo intervention should be.

In the two published studies of fetal cell transplantation in Parkinson's disease sham-operative procedures were employed as a placebo intervention (Olanow et al., 2003; Freed et al., 2001). The specifics of the sham-operative procedures differ between the two studies. In the Freed study burr holes were placed under local anesthesia, but no instrumentation was passed through the dura mater. In the Olanow study burr holes were placed under laryngeal mask general anesthesia, but the inner table of the skull was not penetrated. In addition, placebo-surgery subjects received antibiotics and cyclosporine. The debate has been intense regarding these research designs. Most of the critiques of the experimental designs (occasionally with mistaken notions about

these designs) have been negative (Clark, 2002; London and Kadane, 2002; Dekkers and Boer, 2001; Gillett, 2001; Weijer, 2002; Macklin, 1999). But the designs have been supported at least by the investigators (Freeman et al., 1999). These were not the first nor the only sham surgical procedures in clinical trials. A recent sham procedure in the evaluation of arthroscopic surgery for osteoarthritis of the knee (Mosley et al., 2002; Mosley et al., 1996) also provoked debate (Horng and Miller, 2002). The historical underpinnings of much of the debate go back to the sham-operative controlled trial of internal mammary ligation (Cobb et al., 1959) and subsequent discussion of design of trials evaluating surgical procedures (Beecher, 1961). The interested reader of these references will find the discussion often heated, with strongly held positions. The discussion will be confusing for those without any formal training in ethics, but several common themes emerge. The most important principle invoked is that of benevolence, the need to minimize risks in relation to potential benefits. Several commentators argue that sham surgery can never be justified because the risks the subjects are exposed to are not associated with any potential benefits. However, to my mind, the situation is not too dissimilar from phase I clinical trials, where healthy human subjects are exposed to experimental interventions with the intention of determining the dose that is associated with side effects. As these individuals are healthy, they have no possible chance of benefit from the exposure to the drugs and know that the drugs will be titrated to levels that induce side effects. Hence their only possible outcome is one of harm. Such phase I trials are done every day and little criticism is raised regarding the principle of benevolence. Perhaps this situation is not right, but this is the current environment in which clinical trials of surgical procedures will be conducted. There have been at least two specific proposals (Tenery et al., 2002; Horng and Miller, 2003) for an ethical framework for the evaluation and use of sham procedures in clinical trials. In this context the authors propose that sham procedures should have the risk minimized, with the procedures being limited to those necessary to provide blinding regarding the intervention. In this circumstance, the authors argue that conscious-sedation associated with skin incisions may have been sufficient to suggest to subjects that they underwent the surgical procedure of fetal cell engraftment. Other chapters in this book more fully discuss the ethical implications of sham surgical procedures and will offer alternative designs (Chapter 2). It is probably safe to say that the discussion regarding this issue will continue and that it is likely that randomized studies of surgical procedures with some degree of sham operative controls will continue in the future.

## 2. Identifying the Primary Research Question

An essential task in designing a clinical trial is identifying the primary research question. The trial is more likely to be successful if it is designed to definitively answer one specific clinical question rather than attempting to address questions regarding the mechanism of disease causation or a

fundamental biological principle. In the situation of fetal cell engraftment questions regarding whether fetal cells are able to be successfully engrafted and make appropriate neural connections in the host are not well addressed by clinical trials. On the other hand, the specific questions of whether fetal cell engraftment as a procedure can enhance the quality of life, or motor function, in patients with advanced Parkinson's disease are likely to be addressable. It is important that designers of clinical trials of restorative therapies focus the design of the trial on answering specific clinical questions that are amendable to analysis in a controlled clinical trial. The reasons for success or failure of the trial may hinge on underlying biological principles such as cell number and survivability, but these issues are poorly addressed or impossible to answer via standard clinical trial designs. These issues need to be more fully addressed in preclinical non-human primate studies. Investigators need to avoid the temptation to focus on more "biological" questions (such as graft survival), and focus on questions of "clinical" significance.

## 3. Choosing a Good Primary Outcome Measure

There is a relatively large number of outcome measures that have been used in clinical trials for Parkinson's disease. Most of these measures have focused on the motor manifestations of Parkinson's disease. Scales such as Unified Parkinson's Disease Rating Scale (UPDRS) assess features such as rigidity and bradykinesia. In addition, in advanced Parkinson's disease diaries or other rating scales of "on" (medication working) and "off" (medication not working) states have been used, as the more advanced state of Parkinson's disease is characterized by a fluctuating response to pharmacologic therapies. However, advanced Parkinson's disease is characterized by impairments in many spheres beyond the motor system, including cognition, autonomic function, and sleep. Standard scales of Parkinson's disease poorly capture these aspects. Furthermore, in advanced Parkinson's disease, scales like the UPDRS are prone to modulation by placebo pharmacologic treatments (Goetz, 2000). Given that standard PD scales capture a limited domain of PD disability and that they are prone to placebo effects, investigators should use more standard scales of general disability or quality-of-life as primary outcome measures. Such scales are used in other disabling disorders of the elderly and their use in Parkinson's disease may give some context and generalizability to the results of such novel interventions. In particular, if quality-of-life is assessed, then the costs of quality-adjusted life years gained can be calculated and compared to other resource intensive interventions. A disadvantage of using quality-of-life or general disability scales is that they are relatively subjective. It is precisely with these sorts of outcome measures that most commentators suggest some form of blinding regarding a surgical intervention.

## 4. Selecting Subjects and Sample Size

Studies of restorative therapies in Parkinson's disease, whether fetal cell or embryonic stem cell engraftment or the placement of viral vectors or virally transformed cells, will most likely be relatively small. In addition to these interventions being novel with unknown safety profiles, there is a great deal of effort expended in acquiring the material to be surgically administered to the subject. Hence these trials are likely to be relatively small and will only be designed to detect major therapeutic benefits. Perhaps this is appropriate given that potent pharmacologic therapies are available to treat many of the aspects of the illness, albeit modestly. Given the relative innovation and potential risk it may be appropriate for such restorative therapy trials to be attempts to provide major therapeutic benefits, e.g., slowing the rate of progression by 50% or 100%.

Relative care must also be taken in selecting research subjects. Advanced Parkinson's disease is relatively common disorder and is generally experienced in individuals over 60 years of age. In contrast, half the subjects in the Freed trial were less than 60 years old at the time of entry. Furthermore, the average age of onset of symptoms was in the 40s. This is also true of other surgical intervention trials, including deep brain stimulation studies. Whether and how representative this sample is of subjects with advanced Parkinson's disease in general is debatable. Investigators will be faced with a dilemma of recruiting relatively small sample sizes to restorative therapy trials yet attempting to have that group be as representative as possible.

Investigators also must be vigilant regarding cognitive impairment in advanced PD. Given the complexities and novelty of restorative therapy trials, an accurate comprehension of risks and possible benefits is important. Some means of assuring informed consent should be used. Investigators also need to be diligent in recruiting women and individuals from minority ethnic and racial backgrounds.

## 5. Randomization

Random allocation to treatment group helps to minimize selection bias: that is, the conscious or unconscious effort to put certain kinds of patients in one treatment group and other kinds of patients in the control group. Although matching patients based on certain characteristics (age, duration of Parkinson's disease) may be useful, this is not a sufficient replacement for randomization because of its ability to balance groups regarding both known and unknown confounding variables. Random allocation to treatment groups does not suggest haphazard assignment; rather, that the allocation is determined by a preset random mechanism. The likelihood of balance for potentially confounding variables between two treatment groups is greater as the size of the study is increased. It remains possible for groups to differ in important characteristics

due to chance alone when sample sizes are small, such as is anticipated in restorative therapy trials, and analytic methods may be needed to take into account such differences when comparing the treatment groups. Randomization is likely to be an important aspect of any well-designed study of restorative therapies in Parkinson's disease.

Randomization will usually be to restorative therapy or to no additional intervention. The current knowledge base about restorative therapies supports a state of clinical equipoise regarding these two treatment options, presuming that all subjects receive best available medical therapy. There seems to be little debate regarding the ethical justification for randomization, or for these two treatment options. Most of the debate surrounds how to "blind" this random treatment assignment.

## 6. Blinding or Masking

When either the investigator or the patient is unaware of the specific intervention (i.e., experimental or control) that is being administered, the study is called blinded or masked. When only the research subject does not know the treatment assignment, the study is called single-blinded. When neither the subject nor the investigator are aware of the treatment assignment the study is said to be double-blinded. When both the subject and the investigator are aware of the treatment assignment, the study is called unblinded or open label. Like randomization, blinding is used to prevent bias in the conduct and interpretation of the trial (observer bias). In unblinded trials both patients and investigators may overestimate treatment effects or underestimate adverse effects. In surgical intervention trials it is impossible for the surgeon not to be aware of the actual treatment assignment. This is not a methodologic shortcoming, however, as the outcome measures are assessed by someone other than the surgeon. The use of a "blinded rater" who is unaware of the nature and the timing of the intervention is a useful methodologic tool in such trials. This individual may evaluate subjects prior to the intervention and long enough after the intervention such that the external characteristics of the subject will not be sufficient to unblind them as to whether surgery has or has not taken place. To achieve a double-blind status the subject will also need to not know whether surgery has taken place. This presents the ethical difficulties listed above, for if the subject undergoes enough of the procedures to mimic the situation of surgery they may be exposed to risk beyond what is justified by the scientific advantages of double-blinding. There has been substantial argument, in the literature cited, regarding whether the scientific rigor of double-blinded, randomized controlled trials are of sufficiently greater degree than open trials to warrant the exposure of human subjects to the additional risks of the sham interventions. Since the scientific rigor of randomized, double-blinded trials is substantial in relation to other trial designs, attempting to find ways to minimize whatever additional risks are associated with sham procedures is imperative.

Investigators can only get a clear estimate of the impact of an intervention by comparing it with a control group that receives an alternative treatment or a placebo. Although some studies have relied on historical controls (a group of similar disease state followed off treatment in the past) such studies tend to over estimate treatment efficacy. It is important to ensure that the selection criteria for the study participants, the quality of the recorded data, quality of ancillary services, and the evaluation of response are all similar if historical controls will be used. As this is an unlikely scenario, a historical control group is usually considered inferior. As indicated above, the use of a placebo is ethically justified if there is a state of clinical equipoise. The majority of commentators have suggested that in trials of restorative therapies such as fetal cell engraftment that randomization to a surgical intervention and standard medical therapy is appropriate. The published studies used sham surgical procedures to create a "double-blind" study. Those who object to sham surgical interventions to promote masking of treatment assignment suggest that randomization to standard medical therapy or surgery be unblinded to the subjects. Blinded investigators could still be used to measure the primary outcome measure but this would not be useful if the primary outcome measure is a standard disability scale or quality-of-life scale which has important subjective elements. In that circumstance, the (unblinded) subject will be an important factor in completing the primary outcome measure. Thus, unblinded studies may be prone to observer bias. Other commentators (Chapter 2; Boer and Widner, 2002) have suggested that a prolonged use of standardized assessment tools prior to surgery will help to eliminate observer bias, but this kind of non-experimental design of following an individual's course before and after an intervention will not produce evidence of sufficient quality to form the basis for therapeutic recommendations. A formal experimental design is required to provide high-quality evidence.

## 7. Specific Trial Designs

The two basic trial designs usually considered are parallel-group and cross-over studies. In parallel-group trials subjects are allocated to a given treatment arm and prospectively followed for a set time period. The main potential drawback of a parallel-group design is that it requires more subjects than other designs and is likely to be the most costly and labor intensive. Subjects dropping out from parallel-group designs are usually assessed according to the treatment group to which they were originally assigned (intention to treat analysis). If not all data is available from all evaluations, there are techniques available to adjust for this missing data, although there is substantial controversy about the most appropriate techniques. In contrast, in the cross-over design each subject receives a sequence of treatments. In double-blinded cross-over trials neither the patient or the examiner knows the sequence of the experimental intervention and the control intervention.

In such trials, patients serve as their own controls which provides for a saving in sample size compared with parallel group studies because of the variability of within patient differences is usually less than the variability of between patient differences. In essence the proposal of Boer and Widner (2002) is of an open-label cross-over design in which subjects are followed prior to and after surgery using themselves as controls. Given that it is impossible to study subjects in the reverse order, i.e., in the transplantation situation and then in the control situation, there cannot be randomization of the order of the states. Thus this proposal is neither randomized nor double-blind, and is subject to observer and subject bias that randomization and blinding would help to reduce.

It is likely that restorative therapy trials in Parkinson's disease will require the use of parallel-group designs with an active intervention arm and a no-intervention control arm, with the possibility of the group initially assigned to the control group's receiving the active intervention later if it proves to be safe and effective. This mechanism was incorporated in the prior sham-controlled trials of fetal cell engraftment. The thorny issue will be when and how to blind the randomized treatment arms.

## 8. Reflections and Recommendations on Trial Design

Much of the ethical debate regarding the fetal cell engraftment trials occurred prior to the results of those trials. Most of the concern was regarding the risks inherent in the design for the sham-surgical group. Now that the results are available from both trials, it is clear that those who received engraftment had very modest if any benefit from the procedure, as neither study showed a statistical benefit (Chapter 6). However, between 15% and 50% of the engrafted groups developed dyskinesias in the unmedicated state. These dyskinesias were severe enough at times to require additional surgical intervention. Although there had been some minimal discussion of this complication in prior open-label case series of transplantation, it had not been identified as a serious safety issue prior to these studies (Hagell et al., 1999; Lindvall, 1998, 1999). This complication was observed exclusively in the subjects who received engraftment, and in the Freed study was associated with PET imaging suggesting better engraftment (Ma et al., 2002), although this was not the case in the Olanow study (Chapter 10). Taken together these data suggest that it was the engrafted group who in fact was exposed to greater risks. They had actual surgical manipulation of the brain, had a possible complication of dyskinesias off medications, and had no apparent therapeutic benefit. The ethical commentators have been surprisingly quiet on this ironic twist of their prior concerns. The results of these trials have been used by others as a justification for the trial designs, given that prior to these randomized studies possible side effects were minimized, the investigators who were proponents of engraftment had relatively unbridled enthusiasm, and

patients sought the operation. For example, in their recent proposal, Boer and Widner (2002) continue to be relatively enthusiastic about grafting stating that ". . . it is generally accepted that the neurotransplantation surgery is effective though still to be improved and perfected and that adverse experiences have not been prominent." These comments are surprising in the light of the objective data they discuss, which are to the contrary. Thus discussion on these issues at times remains entrenched to prior positions and is relatively resistant to modification by the new data as they emerge. Fortunately at this time few patients are seeking or receiving fetal cell grafts for PD. Still the approach holds promise. How are investigators to move forward at this time?

Although a decade has passed since the published transplantation trials were initiated, it is clear that further preclinical work is needed to refine transplantation techniques. Astounding progress has been made in demonstrating that such cells may be transplanted and that successful engraftment will occur. Further refinement of the source, number, and storage of cells prior to engraftment and of the mechanisms and locations of delivery in non-human primate brain need further elucidation. With these refinements it is possible that this technique could provide robust benefit to patients with Parkinson's disease. Once these refinements have been made and there is relatively widespread support among investigators in the field for the transition to human experimentation, what will be the next steps? The core assessment proposals (CAP) made by Boer and Widner (2002) are certainly a good initial start. As mentioned above, in a CAP subjects will serve as their own historical controls with rigorous assessment on a routine schedule prior to intervention and afterwards. Although this form of observation is likely to overestimate treatment effects, if no treatment effects are observed, there is little point in further experimentation. Presuming that such a method would find benefits, as such observations have been made in the past, the next appropriate step is likely a pilot efficacy study. A pilot efficacy study could be done in a randomized fashion with subjects randomized to the restorative therapy versus continued best medical management. Evaluators should be blind to the treatment status, although subjects will be aware of whether they have received the operation or not. Outcome measures should be relatively objective, given the unblinded status of the subjects. Measures could include timed motor tasks, UPDRS scores by blinded evaluators, and imaging measures of cell engraftment. Again this design presents a likelihood of false-positive results, but takes advantage of the rigor of randomization. Should such pilot efficacy studies again demonstrate benefit, larger studies confirming efficacy should be proposed. These should be randomized, double-blind controlled trials. The primary outcome measures should be either quality-of-life measures or general health-related disability measures (e.g., Medical Outcome Surveys short form 36 or 12). I think it is ethically responsible, in fact imperative, for subjects to receive some sham elements of the operative procedure to promote blinding to treatment assignment. These elements could be limited to local anesthesia infiltration of the skin with skin incisions, administration of

conscious sedation sufficient to induce amnesia regarding the event, and saline infusions of antibiotics or immunosuppressive agents if such are intended as part of the trial design. Subjects should be taken to the operative suite and to any recovery unit, similar to those subjects who have received an operative intervention. All other evaluations prior to randomization should be identical and subjects should be fully eligible and prepared to undergo either procedure. The post-treatment evaluation schedule should also be identical. These limited sham procedures should be sufficient to generate genuine uncertainty on the part of the subject regarding their actual treatment assignment. Assessing the subject's guess as to their treatment assignment at annual intervals would also be appropriate. This type of approach is likely in keeping with other proposals and the general ethical framework for such types of studies (Horng and Miller, 2002; Tenery et al., 2002; Albin, 2002; Emanuel and Miller, 2001).

These recommendations regarding this sequence of trial designs are based on my experience as a clinician and clinical trialist. Others with more formal training in biostatistics, ethics, and transplantation science are likely to disagree. Unfortunately, this debate is not well informed by one important constituency, namely, the patients who are potential subjects in this type of research. Very little research has been done in rigorous fashion assessing patients preferences and attitudes regarding research design. While some commentators have suggested that this may be a vulnerable population of patients who are not well positioned to make important decisions regarding the ethical constraints of clinical research, it certainly seems unreasonable to not have at least inquired of the subjects regarding their understanding and preferences regarding these important issues. As part of an NIH-funded effort in gene therapy for Parkinson's disease, we have just begun quantitative efforts to assess the attitudes of both researchers and patients regarding the risks and benefits of various research designs. In particular we have developed, through focus groups and patient testing, common language descriptors of open and blinded study designs and have assigned potential risks and benefits associated with novel restorative interventions. Subjects are then asked about their attitudes and preferences regarding a blinded and open design. A similar questionnaire has been prepared for surveying scientists involved in Parkinson's disease research. In addition to these efforts, we have developed an informed consent document for a "mock" gene therapy trial and are presenting this to subjects with Parkinson's disease, after obtaining informed consent for the process. The subjects then review the "mock" consent form as if there were a real trial, in which they might consider participation. We then review with them their decision regarding participating and go through trade-off scenarios regarding risks and benefits and how these affected their decision regarding participating. Our initial data suggest that subjects participate in this "pretend" consent process with good enthusiasm and they are able to articulate their legitimate concerns. The vast majority of

subjects involve family and caregivers in their decision-making process. Although these are just the initial steps in this type of elicitation of subject preferences, we believe the community of researchers needs to foster this kind of endeavor to generate the data necessary to incorporate the important opinions of affected patients and families regarding research design. I do not believe it is sufficient for us merely to consider our own opinions and preferences in these important matters.

## 9. Investigator Conflicts of Interest

Potential restorative therapies for PD are based on novel and brilliant preclinical research involving fetal cells, stem cells, and viral vectors for gene transfer. Our understanding of cellular biology and regulation has been increased enormously by these efforts, and they may lead to therapeutic advances. Many of the preclinical investigators have taken the opportunity to create for-profit business arrangements around their preclinical discoveries. This situation presents no major concern as long as such investigators exclude themselves from the clinical human experiments to evaluate the therapeutic potential of these discoveries. All too often investigators who have made a fundamental observation feel compelled to remain involved throughout the clinical investigation. If they have a financial interest in the intervention, there is a clear conflict-of-interest, real or perceived. Independent (no conflicts) investigators should design and conduct clinical trials of novel restorative therapies. Investigators who persist despite their financial conflicts-of-interest threaten the entire restorative therapy program, and even clinical research in general.

## 10. Summary

Clinical trials of restorative therapies in Parkinson's disease will present important methodological challenges. The ethical considerations for appropriate clinical trial design are diverse and complicated. The reported sham surgery controlled trials have provoked a great deal of discussion. There are prospects for therapeutic benefit, but also current evidence of harm from fetal cell grafting. Most investigators agree the techniques for administering restorative therapies need further refinement via pre-clinical research. Ultimately, such procedures will need to be tested in individuals with Parkinson's disease. A proposal for a sequence of clinical trial designs is presented that has increasing rigor, and exposes subjects in the sham-control situation to minimal additional risks. To continue the informed debate regarding appropriate clinical trial design, further appreciation and analysis of patients' perceptions and preferences are needed.

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# 5

# Lessons Learned From Early Clinical Neural Grafting in Parkinson's Disease

Pierre Cesaro[1] and Håkan Widner[2]

## 1. Introduction

Early clinical trials with embryonic neural tissue transplantation for repair strategies in Parkinson's disease (PD) have been successful in demonstrating proof-of-principle that the approach can be successful, and that results from animal experimentation can be translated into a clinical application.

There are several reviews summarizing the main findings from the early clinical trials (e.g. Olanow et al., 1996; Lindvall, 1997; Dunnett and Björklund, 1999; Björklund and Lindvall, 2000; Winkler et al., 2005). The aim of this chapter is not primarily to reiterate these findings, but to highlight some of the aspects in the translational research that have not been fully appreciated and recognised.

The review is limited to stereotaxic implantation of embryonic neural tissue, disregarding open surgical approaches and use of non-neuronal or non-embryonic tissue such as adrenal medullary tissue or carotid bodies.

### *1.1. Rationale for Early Clinical Trials*

The basic rationale for the first clinical trials was to try to translate the observations made in animal models of neurodegenerative disorders into a clinical setting with a human disease. Parkinson's disease was chosen as a model disorder, mainly because it represented a major clinical disorder with an unmet medical need in the advanced cases, and because there were possibilities to address additional scientific issues experimentally in different animal models. If proven successful in Parkinson's disease, experience and techniques gained from these endeavors could be employed for other disorders. This has been the case, for example, in Huntington's disease (Peschanski et al., 2004).

[1]Neurology Department, Inserm U. 421, Creteil, France
pierre.cesaro@hmn.aphp.fr
[2]Department of Clinical Neurosciences, Lund University Hospital, Lund, Sweden
Hakan.Widner@med.lu.se

Exploratory and investigational trials cannot be taken to address issues such as efficacy and cost effectiveness, or to address superiority over other techniques. However, pilot studies and open-label trials generate observations which can help predict the expected level of effects with regard to generated data for size-effects and group-sizes in order to design controlled trials. Important observations can be made provided careful evaluations programs are in place.

The transplantation programs have generated two sets of evaluation techniques, summarized in the CAPIT (Core Assessment Programme for Intracerebral Transplantations) (Langston et al., 1992) and the CAPSIT (Core Assessment Programme for Surgical Interventions and Transplantation) protocols in Parkinson's disease (Defer et al., 1999). The first protocol, CAPIT, although laborious and complex, provides a uniform set of recommendations for how to assess patents prior to and after implantation. The revised version, CAPSIT, provides means to compare effects between different neurosurgical approaches, such as deep brain stimulation and transplantations. Experience from these protocols has been important for patient selection in other functional neurosurgical procedures (Lang and Widner, 2002).

In the preparatory phases of a new technique, safety aspects are important. Transplantation of viable tissue into the brain involves several risks, directly linked to the surgery, the donor tissue, and the host treatment, along with biological effects of the graft. A sentence in the report on the first successful graft (Lindvall et al., 1990) stating that "there were no complications" represents six years of preparatory investigations regarding implantation approaches, donor tissue screening protocols and sterility, recipient treatment with judicial levels of immunosuppression and antibiotics, as well as handling of medication in the postoperative phase in relation to clinical effects. This kind of "homework" is necessary for any group to launch a transplantation program without experiencing any unexpected side effects. The implantation technique in the Swedish program has been subjected to revision twice, with a major difference between the first two patients, documented in Lindvall et al. (1989) and later surgeries (Lindvall et al., 1990). The policies regarding sterility and drug management has been summarized in (Widner et al., 2002). A large number of serological and bacterial cultures from the remains of induced abortions provided a panorama of bacterial and viral contaminations to be expected, as well as a baseline for assessment of the microbiological risks entailed. Prophylactic antibiotic treatments and screening measures against bacterial infections were designed accordingly.

## 2. Clinical Effects

Transplantation of embryonic neural tissue can be performed in humans, and human embryonic dopaminergic neurons can survive the transplantation process. There is no indication that a disease process negatively affects the

transplanted cells, but the endogenous remaining dopaminergic neurons appear to degenerate relentlessly. There is no data to suggest that implantation of embryonic neural tissue per se positively or negatively affects the rate of progression, but immunosuppressive treatment, when employed, may be beneficial (see section 3). The net clinical benefits of grafting in a patient with progressive disease is a balance between the rate of decline in the endogenous nigro-striatal system and the re-innervation achieved by the grafted cells. Grafts effects are dependent on graft survival and these being sufficient number of cells grafted (see Chapter 8). The donor tissue must be embryonic. There is a very narrow time limit for when the tissue can be grafted, between 5 and 7 weeks post-gestation for human tissue, and only 5–40% survive the implantation process. Multiple donors are needed to achieve sufficient graft effects. Better survival rates are achieved with pretreatment of the donor tissue with factors inhibiting, e.g., apoptotic cell death and free radicals. The grafts integrate into the host brain circuitries and function over a long period of time. There are consistent observations with functional assessments, clinical tests of, e.g., movement speed, that are paralleled by increased signal in quantitative imaging using PET (positron emission tomography). In a few cases these findings are in turn paralleled in a demonstration of graft survival determined histologically at autopsy (Kordover et al., 1995). The findings have all convincingly and clearly demonstrated that the clinical observed effects can be related to grafted cells, provided a sufficient number of cells survive, and that these cells can result in sufficient re-innervation levels. It is not known at what critical threshold re-innervation is sufficient to result in clinically meaningful benefits, but is estimated to be in the range of 100,000 surviving dopamine neurons per side of the brain, resulting in about 50% re-innervation density (Hagell and Brundin, 2001). Only limited clinical effects have been observed if the patients have received tissue from one donor only (Defer et al., 1996). When tissue has been cryopreseved prior to implantation only marginal, if any, clinical effects were observed (Spencer et al., 1992).

It has been possible to achieve a consistent increase in [$^{18}$F]-dopa positron emission tomography (FD-PET) signal. This indicates graft survival and the clinical functional improvement correlates with the increase in signal. There is also a dose-effect of the grafts (Cochen et al., 2003). Additionally, the PET-scan evidence of a re-afferentiation of the supplementary motor area indicates functional graft effects and that grafts release dopamine (Piccini et al., 1999, 2000).

The range of clinical beneficial effects has been wide. The cardinal signs of PD—rigidity and akinesia—are readily and consistently improved following grafting. It is possible to achieve beneficial effects on tremor, just as L-dopa can affect tremor, although in most programs patients with tremor being the dominating symptom have not been grafted. Less well characterized and quantified, but subjectively reported by several patients, is an improvement in gait ability and reduced incidences of gait freeze and freezing-related falls. Patients also report a marked improvement in stability and greater ease of finding their

equilibrium, which could be related to the reduced episodes of falls. In patients with bilateral grafts, improvement in speech ability has been found (Peschanski et al., 1994). There are examples of patients who have returned to an active professional life after years of illness-related inability to work. In the MPTP-patients there have been clear improvements in speech, swallowing, and reduction in drooling, as well as reduction in very troublesome eyelid dyspraxia. These effects were observed to appear gradually over time after surgery without any change in the regular anti-parkinsonian medication (Widner et al., 1992). Gait was clearly positively affected in one patient. The quality of life has been shown to improve significantly for most patients, obscured mainly by intercurrent disease (Hagell et al., 2000).

In the well-documented early clinical trials there have been no serious side effects of the surgery and limited side effects of the immunosuppressive treatment. None of the side effects observed in these early clinical trials have been a serious challenge to the approach and treatment policy

The technical aspects of grafting are highly complex, and if several crucial factors in the preparation of the donor tissue and implantation technique are disregarded, it is unlikely to result in any functional effects. The risks involved are not inconsequential, with clinically catastrophic hemorrhages and infections including abscesses following neural tissue transplants.

## *2.1. Comparison With Deep Brain Stimulation in the Subthalamic Nucleus*

There are no direct comparisons between the effects obtained by transplantation of embryonic neural tissue and other techniques. There are strong arguments for a randomised controlled trial that so far has not been implemented (Peschanski et al., 1999; Cesaro, 2004). There are several centers that have experience with both transplantation and other neurosurgical techniques, but no observations have been published.

In an attempt to compare the what it is possible to accomplish, a retrospective assessment of all available patients with a 2-year follow-up period, operated bilaterally with either embryonic neural tissue grafts, or implanted with electrodes in the subthalamic nucleus with high-frequency stimulation (deep brain stimulation, DBS-STN) was made in Lund. The patients were selected according to CAPIT criteria, and all had complication-phase-related difficulties with i.a. fluctuations, hyperkinesias, and "off" related dystonia. All 9 patients with bilateral transplants and idiopathic Parkinson's disease (the MPTP cases and one MSA case were omitted, and none of the unilaterally grafted patients were included), and 11 consecutive cases with DBS-STN were included. The evaluation was performed identically, at a defined "off" condition with 12 hours off medication. In the postoperative phase the DBS stimulators were turned off for at least 2 hours.

Table 5.1A–C gives the details on the patient material and observed immediate side effects and effects on drug reduction and time in "off/on" with

TABLE 5.1. Retrospective Comparison Between Patients in the Lund Series of Transplantation and Deep Brain Stimulation in the Nucleus Subthalamicus (DBS-STN)*

**A.** Group comparisons[a]

| | n | Age at surgery (yr) | Disease duration (yr) |
|---|---|---|---|
| STN | 11 | 68 (51–71) | 15 ± 7 |
| Transplantation | 9 | 51 (41–68) | 13 ± 2 |

**B.** Side effects up to 24 months[b]

| | Dyskinesias (peak of dose) | Confusion | Transient cognitive impariment | Speech distur-bance |
|---|---|---|---|---|
| STN | 0/11 | 1/11 | 0/11 | 5/11 |
| Transplantation | 2/9 | 2/9 | 3/9 | 0/11 |

**C.** Effects at 0 and 24 months[c]

| | L-dopa equivalents | | | % time in "off" | | % of with dys-kinesias | |
|---|---|---|---|---|---|---|---|
| | Pre-op | 24 mo | Reduction | Prep-op | 24 mo | Pre-op | 24 mo |
| STN | 720 ± 590 | 450 ± 315 | −40% | 35 | 13 | 27 ± 18 | 13 ± 14 |
| Transplan-tation | 760 ± 420 | 460 ± 346 | −37% | 33 ± 16 | 16 ± 13 | 16 | 5 |

*All patients with a diagnosis of Parkinson's disease with bilateral grafts, with a follow-up time of 24 months after the final grafting session (n = 9) has been included, and compared with patients that received bilateral DBS-STN with a 24 months follow-up (n = 11). No randomisation, nor matching have been made and selection criteria differs slightly. DBS-STN data is based in part on data included in the DBS-STN Study Group study, and is reported in N Engl J Med.
[a]Pre-operative group characteristics.
[b]Side effects encountered in the immediate peri-operative period and during the 24-month observation period. All side effects were mild and transient or tolerated by the patients.
[c]Table of effects observed pre-operatively (over a 3-month period, 3 observations) and at 24 months. The anti-parkinsonism medication is transferred into L-dopa equivalents and summed, and given as mean ± 1 SD, when available. The time spent in "off", and time in "on" with dyskinesias is based on patient diaries recorded over a minimum period of 5 days prior to assessment.

dyskinesias, based on diary information. Figure 5.1A–C demonstrates the effects based on defined "off"-condition in items in the UPDRS ratings (unified Parkinson's disease rating scale), motor part (III), and subscores for various symptoms. The overall effects obtained are very similar, and side effects observed were limited, transient, and well accepted by patients. The clinical effects have been of value for the patients operated on with either technique, but the adjustments with the DBS devices have tended to be numerous as have medical adjustments.

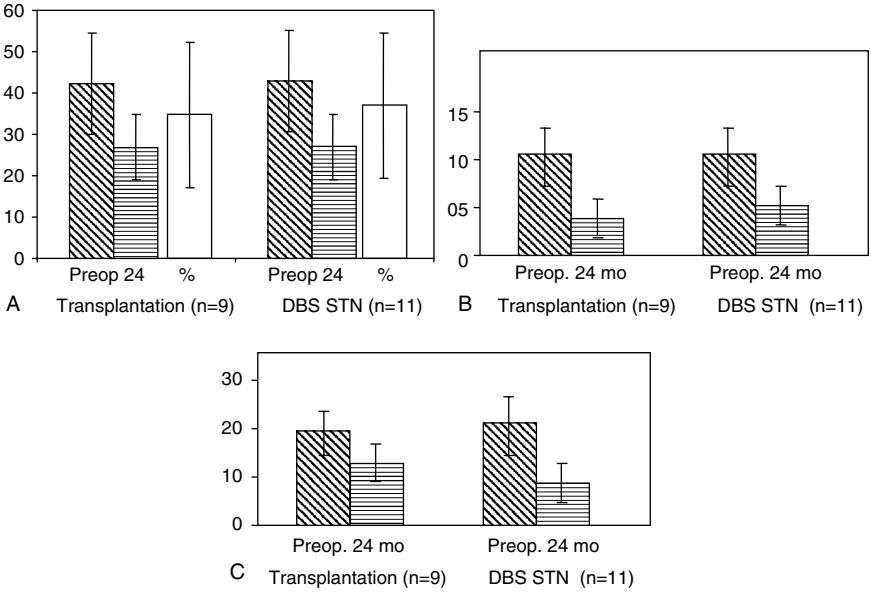



FIGURE 5.1. **A.** Retrospective comparison between patients in the Lund series of transplantation and deep brain stimulation in the nucleus subthatlamicus (DBS-STN). Graph shows the pre-operative (hatched bar) and 24-month data (lined bar) for UPDRS part III motor score in defined "off" (12 hours off medication and for DBS–STN at least 2 hours off stimulation and 12 hours off medication) as a mean ± 1 SD, and the percentage reduction between the pre-operative and 24-month period (open bar). The pre-operative period consists of a minimum of three independent tests over a period of a minimum of 3 months. Mean ± 1 SD, when available. **B.** Subsection from the UPDRS part III for the rigidity data, with a sum for ratings in the defined "off" condition for the neck, upper limbs (left and right), and lower limbs (left and right). Total score is 20. Graph shows the pre-operative (hatched bar) and the 24-month data (lined bar) for rigidity scores in the UPDRS part III motor score in defined "off" (12 hours off medication and for DBS–STN at least 2 hours off stimulation and 12 hours off medication) as a mean ± 1 SD. **C.** Subsection from the UPDRS part III for the items rating movement speed ability (pronations/supinations, hand flexions, finger tappings, and gait), with a sum for ratings in the defined "off" condition. Total score is 28. Graph shows the pre-operative (hatched bar) and the 24-month data (lined bar) for rigidity scores in the UPDRS part III motor score in defined off (12 hours off medication and for DBS–STN at least 2 hours off stimulation and 12 hours off medication) as a mean ± 1 SD.

One patient who was grafted unilaterally, showed an apparent unilateral improvement. This resulted in a very difficult situation to medicate, and due to difficulties in obtaining a sufficient amount of donor tissue, a DBS-STN was performed on the other side of the graft. The clinical effects balance each other well, and the regular medication has been drastically reduced. The patient now suffers from "off"-phase-related dystonias on the side of the

DBS device that cannot be properly adjusted to avoid them, but they are controlled by regular injection of botolinium toxin. The patient has apart from this intermittent diplopia and speech difficulties, but lives completely independently and is active in sports. The graft is stable in its effects.

It can be concluded based on these observations that no technique is far superior to the other and that there are advantages and disadvantages with either technique. The main difference is the better availability of DBS, whereas transplants may have long-term advantages. The observations presented here make it even more important to perform a randomized controlled trial comparing the two treatment options.

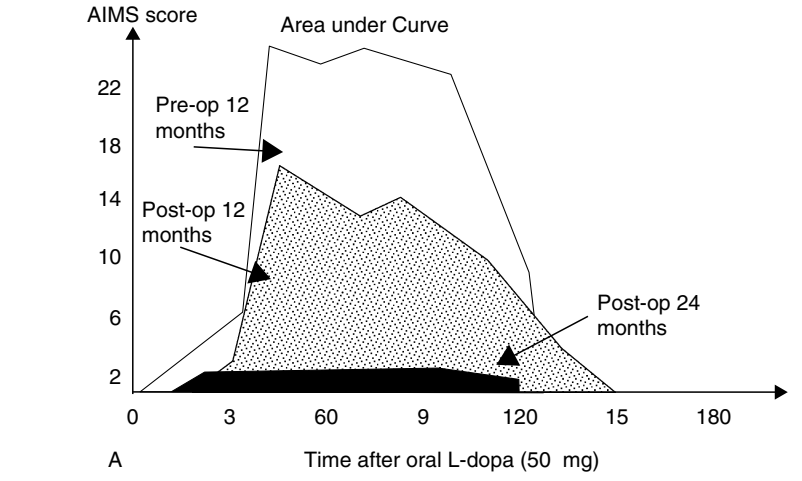
## *2.2. Dyskinesias*

The development of severe "off"-medication dyskinesias, sometimes called "run-away" dyskinesias, was the focus of debate and research after the observations in the first double-blind trial (see Chapter 10). Retrospective analysis of the effects in the open-label trials have not been able to find the same type of dyskinesias (Hagell et al., 2002) described in the Freed et al. study (2001).

Dyskinesias can appear following transplantation, but the patterns vary and depend in part on the presence of dyskinesias prior to surgery and the drug policy. The pattern of dyskinesias is also very dynamic. For example, one patient had typical peak-of-dose dyskinesias pre-operatively and after 3–6 months developed more pronounced dyskinesias, necessitating reduction of the regular anti-parkinsonian medication, and then developed transiently bi-phasic dyskinesias. After an additional 6 months, dyskinesias in this patients subsided, to the point that they were a minimal clinical nuisance to the patient.

Additional observations from patients with severe MPTP-induced parkinsonism can be of interest. One patient suffered from severe peak-of-dose dyskinesias on each dose of 50 mg L-dopa (which were dose limiting) along with visual hallucinations. The patient was grafted, and the amount of dyskinesias was quantified during single-dose L-dopa tests (Fig. 5.2 A). By 24 months, the area-under-the-curve (AuC) of dyskineisas was reduced by >90%. The second patient experienced severe bi-phasic dyskinesias pre-operatively. The dyskinesias tended to be very severe in the afternoon and evening,

FIGURE 5.2. **A.** Chart of Area under the Curve (AuC) for Abnormal Involuntary Movements Score (AIMS) for patient 1 of the MPTP patients grafted. Dyskinesias were rated every 20th minute during a single-dose L-dopa test using the AIMS rating scale (maximum number 28 for each time point) pre-operatively and at 12 and 24 months after bilateral grafting. The tests were performed at defined "off" conditions, 12 hours off medication, and at each time point a test dose of 50 mg L-dopa + carbidopa was given on fasting. The daily dose of anti-parkinsonian medication was kept stable over the whole period, at 250 mg / day. The pattern was typical peak-of-dose dyskinesias, which were reduced at 24 months by 97%. **B.** AuC chart for AIMS score for patient 2 of the grafted MPTP-patients. Over 22 months L-dopa medication over



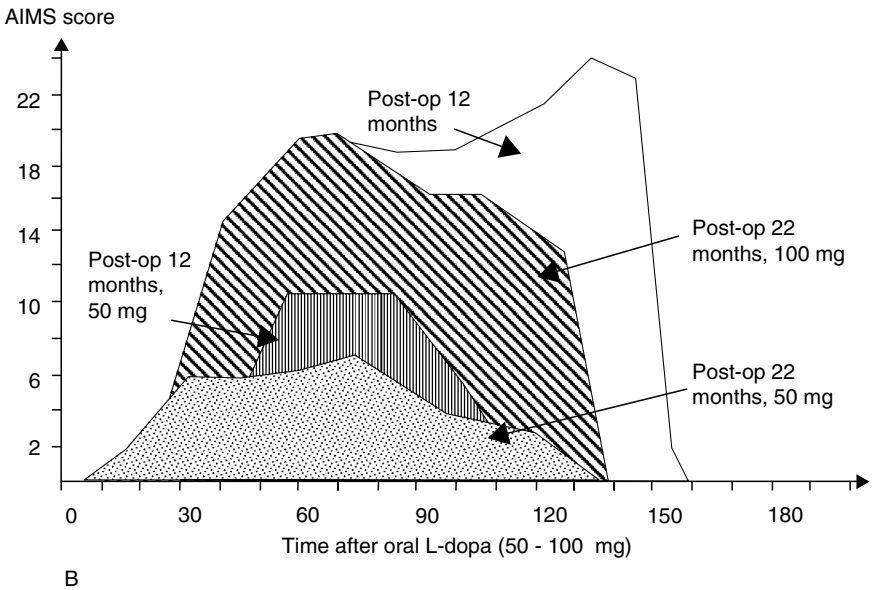




FIGURE 5.2. (*caption continued*)
the day had to be reduced due to accumulation over the day of effects, with a build-up of dyskinesias in the evening. All tests were performed in defined "off" conditions (12 hours without medication), and the patient received a test dose. At the pre-operative and 22-month time point a dose of 100 mg L-dopa + carbidopa was given. Due to a reduction of the total daily dose from 500 mg to 250 mg by 12 months, 50 mg was given as a test dose at 12 and 22 months postoperatively. There was a further reduction to 150 mg/day from 18 months onward. The pattern of dyskinesias was clearly bi-phasic during the pre-operative phase, and this pattern changed to typical peak-of-dose pattern from 12 months onward. There was a reduction in AuC AIMS from pre-operative (100 mg) to 22 months (50 mg) of >70%. The dyskinesias during the day were also reduced.

with the patient becoming exhausted. After the grafting, there was a prolongation of the L-dopa effects, leading up to an accumulation of the drug effects over the day and evening, aggravating the dyskinesias. When the L-dopa dose was reduced, the patient still had good anti-parkinsonian effects, and had reduced dyskinesias in the afternoon. When the patient was tested in the morning, with a formalized L-dopa challenge test, there was reduced AuC dyskinesia as well (Fig. 5.2 B). However, due to the changed L-dopa dose, the patient was tested with different challenge doses after 22 months, 100 mg and 50 mg. The AuC for the latter dose was >70% lower than the pre-operative values (Widner et al., 1992).

The latter pattern is indicative of a graft that can take up L-dopa and produce and store more dopamine, and this may lead to an accumulation. If tested in the "defined off condition" dyskinesias may be present as a more pronounced "early morning benefit." If drugs are withdrawn for longer periods of time, say, 24 or 36 hours, this type of "defined-off" dyskinesia subsides. It is likely that the dyskinesias observed in the second randomized trial (Olanow et al., 2003) are of this type and do not constitute a major new clinical problem.

In the Swedish series, 6 out of 14 patients did have some kind of dyskinesia prior to and after surgery (Hagell et al., 2002). Two patients reduced their dyskinesias after surgery, possibly as a consequence of drug reductions or successful re-innervation of the post-synaptic neurons, normalizing the receptors. One patient has experienced "on-phase" dyskinesias that were similar to what was observed prior to the surgery, in spite of reduction of anti-parkinsonism medication. Another patient has experienced marked "on-phase" dyskinesias in spite of marked reduction of anti-parkinsonism medication. The patient has received DBS-Gpi with good effects, has no major clinical difficulties with very low medication, and there are indications of a functional graft. Two patients have experienced "defined-off" dyskinesias but none that persist over a longer period of drug withdrawal. When taking regular mediation the dyskinesias do not cause clinical difficulties.

The severe "off-phase"-related dyskinesias observed in one study have not been observed in any of the open-label programs. The appearance of that kind of dyskinesia lies inherently in the technical aspects of that particular program.

Transplantation can induce dyskinesias, but also modify the type and severity of the abnormal movements, as well as reduce them. Rational understanding of the mechanism behind this warrants further investigations, but not a complete moratorium on grafting.

## 3. Immune Responses

### *3.1. Immunosuppressive Treatment*

Immune responses in the brain are complex but cannot be ignored when it comes to repair strategies involving cellular transplants. The immune responses are regulated, and depend on several factors. Rejecting neural

grafts produce and elicit cytokine responses that may contribute to altered functional effects observed in patients. The previously held view that the brain was an absolute "immunologically privileged site" allowing indefinite survival without rejection of grafts of cells has proven to be wrong. Thus the brain should be regarded as a site where immune responses can occur, albeit in a modified form, and under certain circumstances these are as vigorous as those seen in other peripheral sites (Widner and Brundin, 1988).

Patients in the Swedish program receive a slightly modified traditional protocol for kidney grafting, with cyclosporin A at 5–8 mg/kg starting 2 days prior to implantation aiming at a concentration of 200–250 ng/mL at time of surgery, and a maintenance level of about 100–150 ng/mL. In addition 2 mg azathioprine, and an IV dose of 500 mg methylprednisolone at time of implantation, with oral 100 mg prednisolone being reduced to a maintenance dose of 10 mg/day after 2–3 months are given. The treatment is maintained for 12 months after the last surgery, and is then slowly tapered. A majority of patients have been on immunosuppressive treatment for about 18 months, some for 5 years. In all cases, withdrawal has not resulted in any rejection episodes.

Tracers specific for inflammatory responses, particularly microglia, PK11195, can be used to detect massive infiltration around the grafts, and studies with this marker have been performed prior to and after withdrawal of immunosuppression in a limited number patients. There is no indication of any increase in microglia cells after stopped treatment in these tests, but activated microglia and other signs of rejection has been observed histologically (Kordower et al., 1997).

The side effects of the immunosuppression have been limited, with a skin cancer in one patient, reactivation of varicella virus, and increased osteoporosis and reduced creatinine clearance. Prophylactic calcium/vitamin D3 treatment should be recommended (Widner, 2002).

## *3.2. Effects of immunosuppression on disease progression*

There are only a few PD patients that have ever been treated with extensive immunosuppression. One patient has been sequentially scanned with FD-PET. The progression rate of the non-grafted side estimated from the decline in [$^{18}$F]-dopa uptake was found to be 6% while on triple-drug immunosuppression (cyclosporin A, azathioprine, and prednisolone) over a 3.5-year period (Fig. 5.3). The rate of [$^{18}$F]-dopa decline after cessation of the immunosuppressive treatment increased to 11.1% over a 5-year period (Piccini et al., 1999), but not in the grafted side. This observation needs of course to be confirmed by other studies, but may indicate that inflammatory responses, or possibly the use of compounds such as cyclosporin may affect disease processes. It is of interest to note that the non-immunosuppressive neuroimmunophilins have so far not been strongly protective with a negative first trial, and a second one with so far marginal effects. A trial in de novo patients is currently ongoing. Perhaps the immunosuppressive and anti-inflammatory

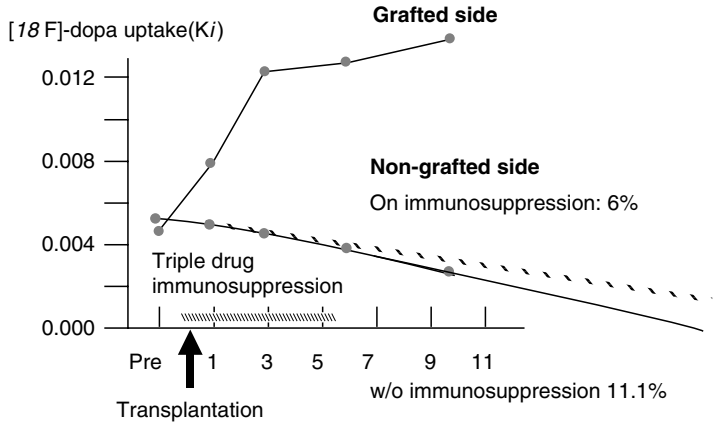



FIGURE 5.3. Rate of progression of the presynaptic marker [$^{18}$F]-fluorodopa, quantified by PET scanning, in an unilaterally transplanted patient. During a period of 60 months, the patient was treated with triple drug immunosuppression, consisting of cyclosporin A, azathioprine, and prednisolone, with a long period of 12 months of tapering doses. There were also reduced levels of anti-parkinsonian medication during this time period, and from months 33 to 48, the patient was without any anti-parkinsonism medication (no L-dopa, no dopamine agonists, or similar). From month 48 onward, the patient was treated with increasing doses of L-dopa, between 300 and 500 mg/day. Repeated [$^{18}$F]-fluorodopa PET scans during this period decreased with a rate of 6.6 % year. After 60 months all immunosuppression was withdrawn, and with an annual decline in the sequential [$^{18}$F]-fluorodopa PET scans of 11.1%. On the grafted side, the grafted dopaminergic cells survived and integrated into the host circuitry and completely normalized the [$^{18}$F]-fluorodopa PET scan signal by year 3.

effects of immunophilins are important as well, in addition to the direct effects on neuronal metabolism.

## 4. Cognitive Functions

The information on effects on cognitive functions after grafting is limited. The most plausible explanation is that patients have been carefully screened not to have any hints of cognitive impairment at the time of inclusion into a transplantation program. There are always risks of transient reactions in the peri-operative period when PD patients are operated, and these reactions along with transient hallucinations and confusion after surgery occur among transplanted patients as well.

The oldest patient grafted was 68 at the time of surgery. This patient developed transient depression several months after the surgery and after 5 years

cognitive decline ensued. The patient developed clear dementia and succumbed to this 7 years after grafting, with a disease history of more than 21 years of PD. Similarly, 3 patients of 16 grafted have developed cognitive defects with signs of sub-cortical dementia, which is in accordance with the expected rate of dementia.Neural tissue transplantation seems not to protect against, nor induce, dementia in PD (Leroy et al., 1996).

## 5. Future Clinical Trials

That neural tissue grafts in PD can be highly efficacious is now established. The principal findings in animal experiments have been confirmed in humans. It is possible to achieve long-term graft survival in patients with PD. It is also possible to normalize dopamine transmission in a patient with long-standing disease and progressive disease. The challenge is to demonstrate efficacy in a larger number of patients, and to demonstrate that neural tissue transplants offer an additional treatment advantage over other treatment modalities.

From a theoretical standpoint, young-onset PD patients may benefit the most from transplantation. When the complications start to appear, often within 5 years of disease onset, these patients are looking at another 25–30 years with the disease.

The ideal transplant candidate should have time to wait for the protracted graft effects to appear and patients should be aware of the long-term perspective.

If more critical and immediate help is needed, DBS should be tried.

A treatment modality that has the potential to reverse and repair the underlying disease process is highly attractive. However, for practical purposes, the technique is limited to low numbers of patients, unless alternatives to the dependence on aborted donor tissue material can be used. Xenografts may be one alternative, provided immune responses can be handled. Neuronal stem cells or various types of genetical approaches may also be alternatives, provided these approaches can achieve the same functional effects as neuronal grafts can. Until then, transplants should be considered to be a potential therapeutic alternative. Provided the transplantation technique is performed judiciously and under strict adherence to the basic principles defined in animal and human experimentation, more patients are likely to benefit form the procedure.

## 6. Lessons Learned

1. Transplantation of embryonic neural tissue has been a successful example of translational research.
2. There are clinically relevant effects, sufficient to meet a medical need obtained after grafting, within the ranges of other techniques.

3. Evaluations programs can be performed and detect effects and graft mechanisms in vivo.
4. The disease process is largely unaffected by grafts alone, and graft do not protect against development of dementia.
5. Chronic immunosuppression may not be negative, but rather may have additional beneficial effects on the disease course and progression.
6. Dyskinesias may develop after grafting, but do not constitute a major clinical problem. The grafts may also improve or eliminate dyskinesias.
7. A randomized controlled trial comparing the effects of DBS-STN with transplantation should be performed.

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# 6

# Fetal Nigral Transplantation as a Therapy for Parkinson's Disease

C. W. Olanow[1] and S. Fahn[2]

## 1. Introduction

Parkinson's disease (PD) is the second-commonest neurodegenerative disorder, affecting an estimated 1,000,000 persons in the United States alone. Resting tremor, bradykinesia, rigidity, and gait disturbance with postural instability are the cardinal clinical features of the disease (Olanow, 1996). The pathologic hallmark of PD is degeneration of neuromelanin-containing dopaminergic neurons of the substantia nigra pars compacta (SNc) coupled with intracytoplasmic proteinaceous inclusions or Lewy bodies, resulting in a reduction in striatal dopamine. Degeneration in PD can also be found in the dorsal motor nucleus of the vagus, the nucleus basalis of Meynert, the locus coeruleus, and peripheral autonomic neurons (Forno, 1996; Braak et al., 2003). Current treatment is based on a dopamine replacement strategy, primarily using the dopamine precursor levodopa (Olanow et al., 2001). In the early stages of the illness levodopa treatment is very effective, but long-term therapy is complicated by the development of motor complications (fluctuations in motor response and dyskinesias) in the majority of patients (Fahn 1992, 2000). In addition, disease progression is associated with the emergence of features that do not respond to dopaminergic therapy (e.g., postural instability, freezing of gait, autonomic dysfunction, and dementia) that are likely related to degeneration of non-dopaminergic neurons (Olanow et al., 2001). These problems limit the utility of levodopa and can represent a source of profound disability to PD patients despite the best of modern therapies. The introduction of surgical therapies, such as deep brain stimulation, represents a major advance, but these treatments are associated with their own potential adverse effects, including those related to the surgical procedure, the

[1]Department of Neurology, Mount Sinai School of Medicine, New York, New York 10029
warren.olanow@mssm.edu
[2]Department of Neurology, College of Physicians and Surgeons, Columbia University, New York, New York.
Fahn@neuro.columbia.edu

implantation system, and stimulation itself (The DeepBrain stimulation for PD Study Group, 2001). No therapy has yet been established to have a neuroprotective effect in PD or to restore function to disabled PD patients. There is thus a present and critical need for a therapy that slows disease progression and/or restores neurological function.

Transplantation of dopaminergic neurons is a rational consideration as a novel therapy for PD because: (i) degeneration of the nigrostriatal dopaminergic system is responsible for the major motor features of PD; (ii) dopamine replacement provides dramatic clinical benefits to virtually all PD patients; (iii) dopamine neurons fire tonically and provide relatively constant synaptic dopamine levels; (iv) non-physiologic pulsatile replacement of dopamine (with levodopa) is associated with the development of motor complications; and (v) transplantation of dopaminergic neurons offers the potential of restoring dopamine in a more physiologic manner and thereby providing anti-parkinson benefits without motor complications (Olanow et al., 1996).

In the laboratory, implanted fetal dopaminergic neurons have been shown to be able to survive, reinnervate the dennervated striatum, and ameliorate parkinsonian features in rodent and non-human primate models of PD (Björklund and Steveni, 1979; Perlow et al., 1979; Björklund et al., 1981; Brundin and Björklund, 1987; Sladek et al., 1986; Redmond et al., 1986; Bakay et al., 1987; Bankiewicz et al., 1990). Transplanted fetal nigral allografts exhibit normal electrical firing patterns (Wuerthele, 1981), demonstrate spontaneous synthesis and release of dopamine (Schmidt et al., 1982), and form normal appearing synaptic connections with host neurons (Mahalik et al., 1985). Benefits in animal models of PD depend on the site of implantation, the type of tissue employed, and the continued presence of implanted cells, (Brundin et al., 1988; Dunnett et al., 1988). Thus, neither intrastriatal grafts of non-dopaminergic tissue nor dopaminergic grafts implanted into non-dopaminergic regions such as the cerebellum provide motor benefits in these models, illustrating the neurochemical and structural specificity of transplantation.

While most transplant research aimed at treating PD has focused on human fetal nigral dopamine neurons, a number of alternate sources of dopamine producing cells have been studied. These include adrenal medullary cells, sympathetic ganglia, carotid body glomus cells, PC-12 cells, neuroblastoma cells, porcine fetal nigral cells, retinal pigmented epithelial cells, and more recently dopamine neurons derived from stem cells. This chapter will review the current status of transplantation in PD with emphasis on the recent NIH-funded prospective, double-blind clinical trials of fetal nigral transplantation and prospects for the future.

## *1.1. Clinical Trials of Transplantation in PD*

The first reports of transplantation in PD utilized autologous adrenal medullary cells. Initial human trials were without significant clinical benefit

(Backlund et al., 1985; Lindvall et al., 1987), but one report described "dramatic amelioration" of motor dysfunction in two PD patients following transplantation of adrenal medullary cells into the caudate nucleus using a direct intracranial approach (Madrazo et al., 1987). This magnitude of benefit could not be replicated in subsequent open-label trials, the procedure was associated with considerable morbidity related to the need for both intracranial and intra-abdominal operations, and only a few surviving cells were detected post-mortem (Goetz et al., 1985; Allen et al., 1989; Olanow et al., 1990; Jankovic, 1989; Peterson et al., 1989). Accordingly, the procedure has been abandoned. One lesson learned from these studies was the strong potential for overenthusiasm to bias interpretation of open-label surgical trials .

Clinical trials rapidly followed using stereotactic techniques to deliver dopamine neurons derived from human embryonic ventral mesencephalon. Open-label studies demonstrated the feasibility of performing this procedure, and reported clinical benefits in PD patients particularly with respect to motor scores during the practically defined "off" period (Olanow et al., 1996; Lindvall, 1994; Lindvall et al., 1989, 1990, 1992; Freed et al., 1990, 1992; Peschanski et al., 1994; Freeman et al., 1995; Hauser et al., 1999). Benefits in some transplanted patients were reported to be sustained; and in a few, motor features were able to be controlled without the need for levodopa. Striatal fluorodopa (FD) uptake on positron emission tomography (PET) was significantly increased in some of these studies consistent with graft survival (Freeman et al., 1995; Hauser et al., 1999; Sawle et al., 1992; Lindvall et al., 1994; Remy et al., 1995) (Fig. 6.1). Post-mortem examinations performed in

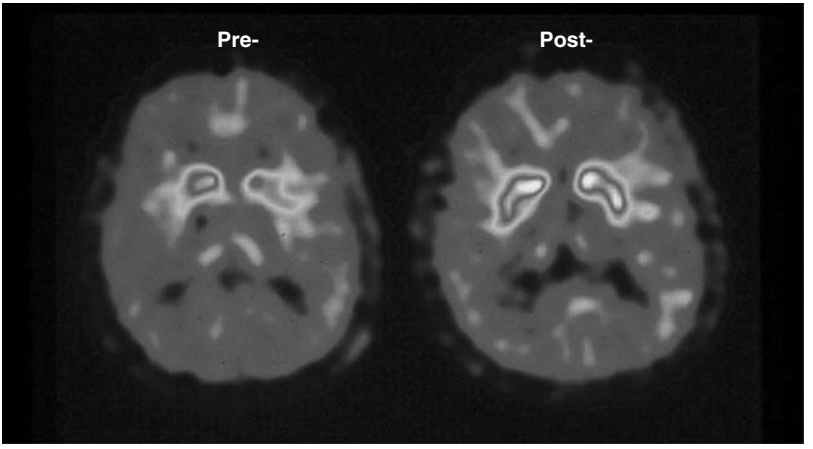


FIGURE 6.1. Flurodopa PET studies in a representative PD patient performed at baseline (**left panel**) and 6 months following bilateral fetal nigral transplantation into the posterior putamen. Note the classical picture of PD at baseline, with reduced striatal uptake particularly in the posterior putamen and the dramatic increase on each side following transplantation. (see color insert.)

small numbers of patients demonstrated survival of as many as 100,000 tyrosine hydroxylase (TH) immunoreactive neurons per side (Fig. 6.2), Transplanted dopamine neurons were normal appearing and provided extensive reinnervation of the striatum in an organotypic patch-matrix manner (Kordower et al., 1995). Transplanted areas demonstrated normal immunostaining for cytochrome oxidase, normal TH mRNA expression, and normal appearing host-graft and graft-host synapses on electron microscopy (Kordower et al., 1996). No evidence of host-derived sprouting was detected.

However, clinical results in these trials were inconsistent, and not all transplant studies reported positive results (Spencer et al., 1992). This may reflect the variability in patient selection, transplantation technique, and method of evaluation, although it is interesting that inconsistent results were also

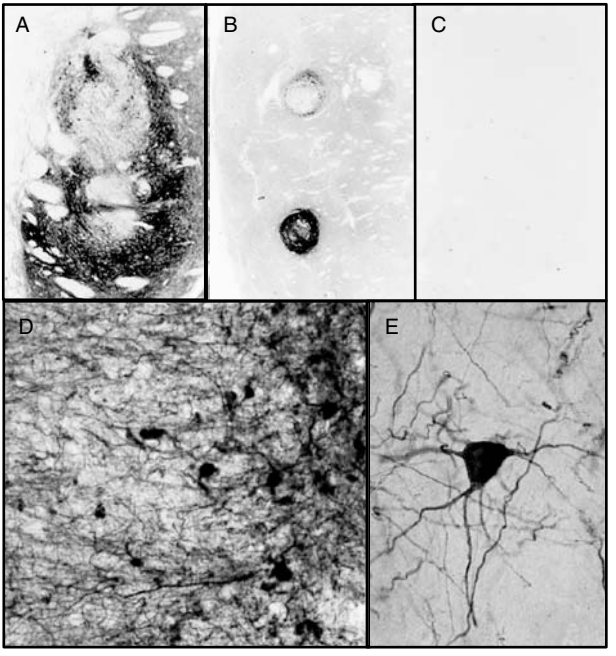


FIGURE 6.2. Tyrosine-hydroxylase immunoreactive staining of the striatum in PD patient receiving transplantation with 4 donors per side (**A**), 1 donor per side (**B**), or a sham procedure (**C**). Note that in the four-donor group, graft deposits have a cylindrical appearance with survival of more than 100,000 dopamine neurons per side. Grafts in the one-donor group have a more dense and circular appearance, with survival of approximately 30,000 TH-positive cells per side, although there still appeared to be continuous TH staining of striatum between graft deposits. High-power images show fibers extending from the graft deposit to provide innervation of striatum (**D**). Higher-power magnification shows normal-appearing implanted dopamine neuron (**E**). (See color insert.)

observed in patients who were treated at the same center with the same transplant protocol (Olanow et al., 1996; Lindvall, 1994). Some of the important transplant variables that are thought to be most likely to influence cell survival and/or clinical response include donor age (ideally 6.5–9 weeks post-conception (Freeman et al., 1991)), method of tissue storage (cultured vs fresh hibernation media), number of donors (1–4 per side), type of transplant (solid or suspension graft), target site (putamen, caudate nucleus or both), distribution of deposits (single large deposit or diffusely distributed throughout target), and use of immunosuppression (see Chapter 8, this volume, for a detailed review of transplant variables). The potential for placebo effect and physician bias in open-label trials also complicates interpretation of these studies (Freeman et al., 1999). To help clarify the risks and benefits associated with fetal nigral transplantation in PD, the National Institutes of Health in the United States sponsored two prospective, double-blind, placebo-controlled trials in patients with advanced PD.

## 2. Double-Blind Controlled Trials of Fetal Nigral Transplantation

### *2.1. The Colorado/Columbia Study*

The first of the NIH studies to be performed was a one-year, double-blind, placebo-controlled trial in which 40 advanced PD patients were randomly assigned to receive fetal nigral transplantation or a sham procedure (Freed et al., 2001). Tissue from two donors per side was stored in tissue culture for up to four weeks, and thereafter implanted bilaterally as two solid grafts (noodles) placed longitudinally into the superior and inferior putamen using a direct frontal approach. Patients in the placebo group received a twist drill burr hole, but a needle was not passed into the brain and no tissue was implanted. The neurosurgeon simulated all other aspects of the implantation technique as part of the study design. Immunosuppression was not employed. The primary endpoint of the study was the change between baseline and one year in a global quality of life measure of clinical benefit. UPDRS assessments of motor function and ADL scores were secondary outcome measures. Blinded FD-PET studies were performed at baseline and at the end of one year.

At the final visit there was no difference between transplanted and placebo patients with respect to the primary endpoint. Quality of life score changed by $0.0 \pm 2.1$ among the 19 transplanted patients and $-0.4 \pm 1.7$ in the sham surgery group ($P = 0.62$). But in the younger group (age <60), the mean global improvement was 0.5 in the transplanted subjects and –0.3 in the sham subjects. Total Unified Parkinson's Disease Rating Scale (UPDRS) scores during practically defined "off" were not significantly different between groups ($P = 0.11$), but in the pre-specified analysis for age effect

there was a significant improvement in the subset of patients younger than 60 years ($P = 0.01$), who improved by 28% compared to 15% for all transplanted subjects (figure 6.3). This benefit was even more pronounced when the analysis was restricted to the motor component of the UPDRS. The entire group of transplanted patients improved by 18% in comparison to baseline ($P = 0.04$), while the subset of patients less than 60 years of age improved by 34% ($P = 0.005$). Significant benefits were also noted on the Schwab–England disability scale, and again were primarily observed in younger patients (Fig. 6.3).

Fetal nigral transplantation was associated with a significant increase in striatal FD uptake on PET compared to sham control subjects ($P < 0.001$). At one year, transplanted patients had a mean 40% ± 42% increase in striatal FD uptake compared to baseline ($P < 0.001$), while patients in the sham-surgery group worsened by 2 ± 17 percent ($P = 0.40$). Post-mortem studies were performed in two patients who died of unrelated causes. All transplant tracts showed dopamine neurons with TH-positive cell counts of 6,840; 34,115; 36,796; and 38,392 in the four striata. Some inflammatory cells were

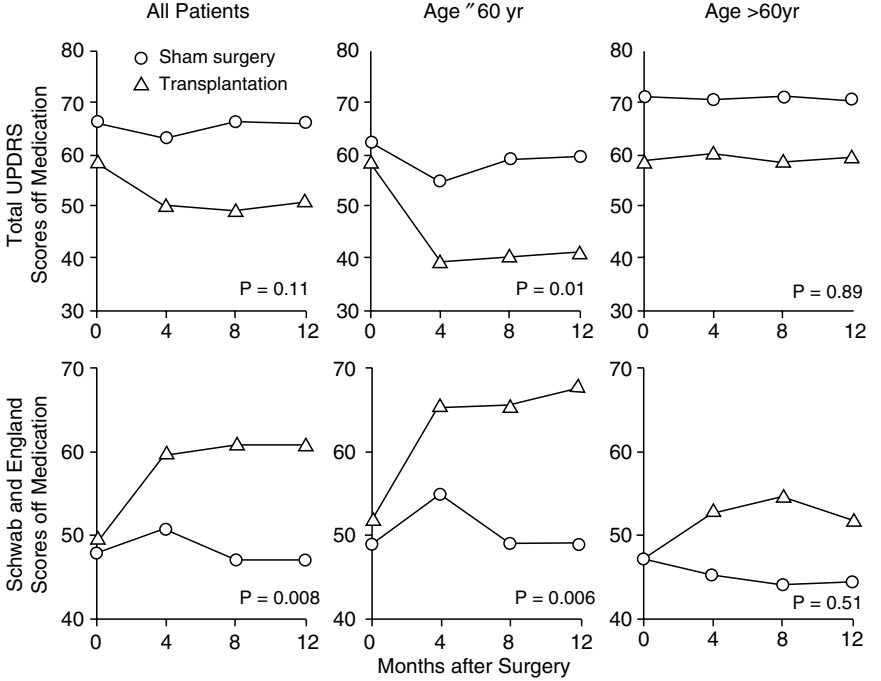


FIGURE 6.3. Total UPDRS (upper row) and Schwab and England disability scores (lower row) at 4 month visits for patients in the transplantation and sham-surgery groups while off medication. Note differences between transplanted and placebo patients and note that results were most striking in younger patients.

noted which stained positively for CD3 and HLA class II antigen. The authors did not feel that the degree of inflammation correlated with the number of surviving cells.

In general, the procedure was well tolerated, although transient adverse effects including confusion, hallucinations, or increase in psychosis were recorded more commonly in transplanted (N = 8) than in sham controls (N = 1). Following completion of the double-blind phase of the study, 5 of 33 patients who underwent transplantation (either during the study or immediately afterwards) developed dystonia/dyskinesia which persisted even after levodopa was reduced or discontinued (Freed et al., 2001; Greene et al., 1999). In some cases these abnormal movements were extremely severe and constituted a major source of disability for the patient.

## *2.2. The Mount Sinai/USF/Rush Study*

The second NIH sponsored study was a prospective double-blind, placebo-controlled, two year trial in which 34 advanced PD patients were randomized to one of three treatment groups: a) bilateral transplantation into the posterior putamen with tissue derived from 4 donors per side (12 patients); b) bilateral transplantation into the posterior putamen with tissue derived 1 donor per side (11 patients); or c) bilateral sham procedures (11 patients) (Olanow, 2003). Donors were aged 6–9.5 weeks postconception, and tissue was stored in cool hibernation media for no more than 48 hours prior to transplantation. Cells were implanted as solid grafts and deposits were placed no more than 5 mm apart in all three dimensions in order to enhance the likelihood of obtaining continuous striatal innervation between graft deposits. Placebo patients received a partial burr hole which did not penetrate the inner table of the skull. No needle tracts were made within the brain and no tissue was implanted. All other aspects of the operative procedure and postoperative care were identical. Patients in both treatment and control groups received immunosuppression with cyclosporine beginning two weeks before surgery and continuing for an additional six months following the surgical procedure. The primary endpoint was the change in UPDRS motor score performed during the practically defined "off" state (approximately 12 hours after the last evening dose of levodopa) between baseline and final visit at 2 years. Home diary measures of percent "on" time without dyskinesia and UPDRS scores in the "on" state were important secondary endpoints. FD-PET studies were performed at baseline, and at years 1 and 2 following transplantation.

An analysis of covariance (ANCOVA) showed that transplantation was associated with a significant increase in striatal FD uptake on PET ($P < 0.001$ on each side). Bilateral increased uptake was observed in patients in both the one- and four-donor groups. On the right side, striatal FD uptake (as determined by the striatal:occipital uptake ratio) was increased by $0.0145 \pm 0.0181$ in the placebo group (NS), by $0.2045 \pm 0.0492$ in the one donor group ($P < 0.007$ vs. placebo), and by $0.3600 \pm 0.0567$ in the four-donor group

($P < 0.001$ vs. placebo). Striatal FD uptake was not significantly different in the one- and four-donor groups, although there was a trend to a greater increase in the four-donor group. Similar findings were noted on the left side. The majority of increase in striatal FD uptake occurred within the first 12 months, but there was a further, albeit modest, increase in striatal FD uptake at month 24.

Post-mortem studies were performed on five patients who died during the course of the study or during subsequent follow up (see Fig. 6.2). Two patients were in the four-donor group, two in the one-donor group, and one in the placebo group. All died from causes unrelated to the transplant procedure. In the four-donor group, grafts were cylindrical in shape and contained large numbers of healthy-appearing dopamine neurons (approximately 70,000–120,000 cells per side) that extended processes into the striatum so as to provide TH innervation contiguously between graft deposits. In the one-donor group, graft deposits were smaller and more circular and contained fewer numbers of cells (approximately 30,000 per striatum). Nonetheless, they also provided extensive and continuous TH reinnervation of the striatum. There was virtually no TH staining in the striatum of patients in the sham group or in the non-transplanted regions of patients in the one- and four-donor groups. These findings were similar to previous reports in transplant patients who participated in open-label studies (42,43).

While the PET and post-mortem findings in this study are indicative of survival of implanted dopamine neurons, transplanted patients failed to show benefit in comparison to placebo with respect to the primary endpoint (an ANCOVA weighted for baseline UPDRS score did not show an overall benefit of transplantation versus a sham procedure; $P = 0.24$). Paired comparisons noted a trend in favor of transplantation but were also not significant, although a comparison of the four donor versus placebo groups just failed to meet statistical significance ($P = 0.096$). As shown in Figure 6.4A, transplanted patients experienced benefits through the first 6–9 months of treatment comparable to what had been reported in open-label studies, but deteriorated thereafter. It is noteworthy that deterioration in clinical benefit coincided with the timing of the cessation of cyclosporine, raising the possibility that immune rejection may have been responsible for the loss of efficacy. Indeed, pathology studies demonstrated increased immunostaining for CD45 (a marker for activated microglia and immune reactivity) in the immediate vicinity of graft deposits consistent with an immune response. A post hoc analysis did not reveal an age effect on the primary endpoint as reported by Freed et al. 2001, but stratification based on disease severity noted significant benefits of transplantation in the subgroup of patients with milder disease at baseline (Fig. 6.4b). No secondary endpoint was significantly improved in transplanted versus placebo patients. The procedure was well tolerated and there was no significant peri-operative morbidity or mortality. A blinded review of videotapes noted that all patients experienced peak dose dyskinesia at baseline (on-medication dyskinesia), which persisted but did not deteriorate

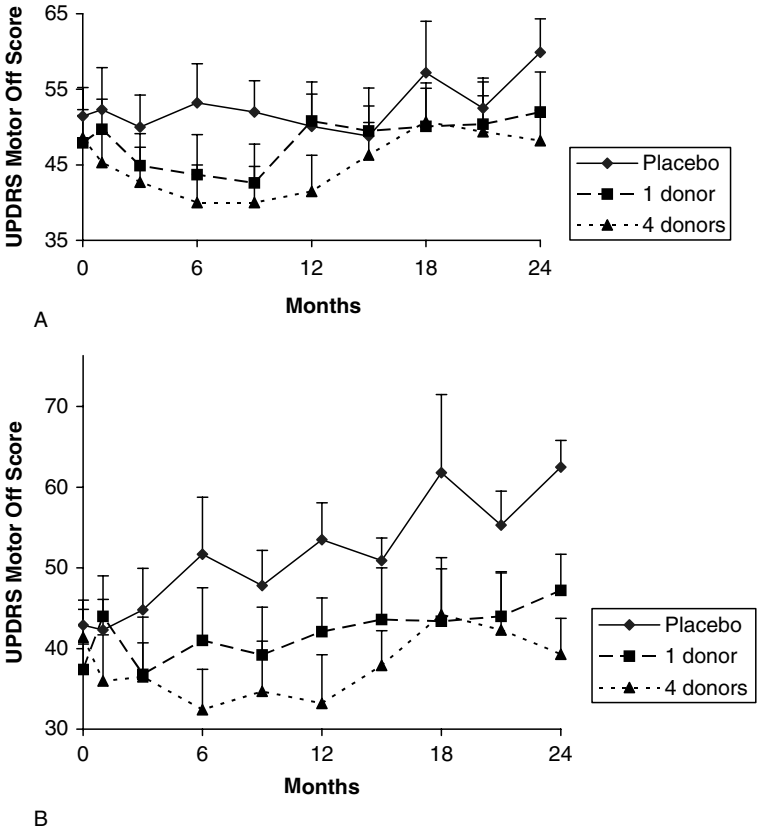


FIGURE 6.4. **A.** Mean (± SE) UPDRS motor score in the practically defined "off" state at each visit for patients in each of the three treatment groups. Note that patients in the one- and four-donor transplant groups were improved in comparison to placebo-treated patients after 6 and 9 months of treatment ($P < 0.05$), but deteriorated thereafter coincident with the cessation of cyclosporine. **B.** Mean (± SE) UPDRS motor scores in the practically defined "off" state at each visit for patients with less severe disease at baseline (UPDRS score ≤ 49). Note that in the subgroup of patients with less severe disease, transplantation was associated with an overall treatment effect ($P < 0.006$) despite the relatively small sample size. Patients in both the one- and four-donor group were significantly improved in comparison to placebo ($P < 0.005$).

following transplantation. In contrast, no patient had dyskinesia during the practically defined "off" state when patients had been off anti-parkinson medication for approximately 12 hours (off-medication dyskinesia). However, 13 of 23 transplanted patients developed this complication, while it was not seen in any of the placebo patients. Off-medication dyskinesias in these patients developed within 3–12 months of the transplant procedure, and were

characterized by asymmetric, stereotypic, rhythmic movements that predominantly affected the lower extremities and were associated with parkinsonism in other body regions. Higher levodopa doses were associated with a change in dyskinesia pattern to one that more closely resembled classical peak-dose dyskinesia, while levodopa withdrawal was associated with the disappearance of dyskinesias along with further worsening of parkinsonism. In three patients, dyskinesias were sufficiently severe as to warrant surgical intervention. Deep brain stimulation of the subthalamic nucleus (STN) provided dramatic reduction in dyskinesia in each case.

## 3. Commentary

A comparison of the two study protocols is provided in Table 6.1. Despite the promise of open-label studies, both of the double-blind trials of fetal nigral transplantation failed to meet their primary endpoint. Further, previously undescribed and potentially disabling off-medication dyskinesias were observed as a complication of transplantation in both studies. Accordingly, fetal nigral transplantation can not be recommended as a routine treatment for PD patients at the present time. While these results are disappointing, some encouragement can be taken from the observation that both studies provided FD-PET and pathology evidence for the survival of grafted dopamine neurons. There are also many lessons learned from these studies that might permit better results to be achieved with transplantation or other cell-based or gene therapies in the future. It still remains, however, to be determined if implanted cells can make proper connections with host striatal neurons, provide normal dopamine neurotransmission, and restore normal dopaminergic effects on basal ganglia circuitry. It also remains to be determined if superior results might be obtained with different transplant techniques, and more

TABLE 6.1. Comparison of Transplant Protocols in the two Double Blind Controlled Trials

| | Colorado/Columbia | Mount Sinai/USF/Rush |
|---|---|---|
| Donor age | 6-9 weeks post conception | 6-9 weeks post conception |
| Method of storage | Culture for up to 4 weeks | Hibernation medium up to 48 hrs |
| Number of donors | 2 per side | 1 or 4 per side |
| Type of transplant | Solid grafts (noodles) | Solid graft deposits |
| Target site | Superior and inferior putamen | Posterior putamen |
| Distribution of grafts | 2 noodles along the length of the putamen | 5 mm apart in all dimensions |
| Immunosuppression | None | Cyclosporine for 6 months |
| Primary endpoint | Global assessment of benefit | UPDRS motor in practically defined "off" state |
| Pre-specified secondary endpoints | 1. Age effect<br>2. UPDRS scores in practically defined "off" state | |

specifically with increases in the number and survival of transplanted cells. Some of the issues that might influence the results of the fetal nigral and other cell-based transplant procedures are considered below.

## *3.1. Patient Selection*

Correct diagnosis of PD is important, as there is no basis for considering that patients with atypical parkinsonism who suffer degeneration in the pallidum and striatum and don't improve with levodopa will benefit from transplantation. In the Freed et al. study, significant motor improvement was observed in younger transplanted patients (<60 years), while clinical outcomes were no different than controls in older patients (Freed et al., 2001). Olanow et al. did not find an age effect, but did report that patients with milder disease at baseline (UPDRS motor score < 49) were significantly improved in comparison to placebo while more severely affected patients were not (Olanow, 2003). The Lund group similarly found that patients with more severe disease based on FD-PET scan results had poorer results with transplantation. It has also been suggested that the best results of transplantation are found in patients who have a good response to levodopa (Freed et al., 2004) and in those who do not experience disability related to levodopa-unresponsive features (Björklund et al., 2003). Degeneration in non-dopaminergic neurons may result in features that cannot respond to dopamine cell transplant and could account for why the best results of transplant have been observed in younger and more mildly affected patients who show a good pre-operative response to levodopa. *These findings suggest that the best candidates for future trials of transplantation and other cell-based therapies should be PD patients who are young, have relatively mild disease, enjoy a good response to levodopa, and are relatively free of disability related to levodopa unresponsive features*.

## *3.2. Transplant Variables*

The optimal transplant protocol has not been defined (see Chapter 8). Both of the double-blind studies targeted the putamen, but it is possible that innervation of the caudate nucleus or specific subregions of the putamen might provide better clinical effects. Indeed, extrastriatal transplantation may be required for maximal benefit. It is not yet known how many donors to employ in order to reinnervate the putamen fully, or if complete reinnervation is required, as PD symptoms are not thought to emerge until there is a loss of approximately 50% of dopamine neurons. Transplant studies to date have employed between one and nine donors per side. One study found a trend favoring transplantation with implantation of four donors versus one donor per side, but this difference was not significant, and post-mortem studies indicate that more than 100,000 dopamine neurons per side can survive transplantation. This number is likely to be sufficient to reinnervate the putamen fully, but it is not known if all of these are nigrostriatal dopaminergic neurons as opposed to neurons that originate in

ventral tegmental area (VTA), or if they are all functionally intact. It is thus possible that larger numbers of functioning dopamine neurons are required in order to provide efficacy. Both of the double-blind trials used solid grafts, but it is possible that more homogeneous innervation and better results might be achieved with suspension grafts (Björklund, 2003).

## *3.3. Immunosuppression*

An important issue to consider is whether to employ immunosuppression. The brain has been considered to be an immunologically privileged site, but immune rejection can occur with allografts and even autografts (Fisher and Gage, 1993). Intracerebral grafts can survive in the absence of immunosuppression, but differences in expression of major and minor histocompatability antigens between graft and host could result in inflammatory reactions with microglial activation and up-regulation of immunological markers that could be detrimental to graft survival and function (Duan et al., 1993; Shinoda et al., 1995, 1996). It is possible that graft rejection contributed to the negative results obtained in the two double-blind trials, as neither employed long-standing immunosuppression and patients were exposed to multiple immunologically distinct donors. Indeed, histologic evidence of immune reactivity was observed following transplantation in both of these studies. Further, fewer dopaminergic cells survived transplantation in the Freed et al. study, where immunosuppression was not employed than in the Olanow et al. study, where cyclosporine was employed for 6 months (Freed et al., 2001; Olanow, 2003). Indeed, in the latter study it was suspected that an immune response may have still limited the clinical benefit of fetal nigral transplantation as initial benefits were lost following withdrawal of cyclosporine and there was increased CD45 immunostaining in grafted regions (Olanow, 2003). This is also in keeping with earlier findings from the same group, who reported increased HLA-DR immunostaining and numerous pan macrophages, T-cells, and B-cells in otherwise normal-appearing graft deposits (Kordower et al., 1997). In addition, each of the double-blind studies used solid tissue grafts, and it has been suggested that solid grafts are more likely to generate an immune response as they contain blood vessels that express major histocompatability type 1 antigens, which are intensely immunogenic (Baker-Cairns et al., 1996). In this regard it is noteworthy that the most striking and long-lasting clinical benefits observed to date have been reported by the Lund group, who used suspension grafts and employed long-term immunosuppression. *These observations suggest that need for long-term immunosuppression remains an open question, and that long-term immunosuppression may be required in order to obtain optimal results*.

## *3.4. Off-Medication Dyskinesia*

Off-medication dyskinesia is a type of dyskinesia that occurs when patients have been off levodopa for hours or even days (Freed et al., 2001; Greene

et al., 1999; Olanow et al., 2003, Hagell et al., 2002) and can be severe, disabling, and warrant an additional surgical intervention. Off-medication dyskinesias have not been observed in non-transplanted PD patients (Cubo et al., 2001), and were not detected in any of the sham-operated patients in the double-blind studies. They thus appear to be a direct complication of the transplant procedure, and an important hurdle that must be overcome before clinical trials of transplantation can be resumed. The precise basis for off-medication dyskinesias is not known (see Chapter 10). A detailed analysis comparing transplanted patients who developed off-medication dyskinesia with those who did not showed no difference in baseline characteristics, nor were there differences between groups in measures of striatal FD uptake, "on" dyskinesia scores, motor scores, or other components of the UPDRS at two years after transplantation or in the degree of change from baseline (Olanow et al., 2003).

In the Freed et al. study, subjects with off-medication dyskinesia were younger and had the greatest improvement in UPDRS scores. The authors proposed that off-medication dyskinesia is associated with continued fiber outgrowth from implanted dopamine neurons and excess dopamine release. In support of this concept, they observed that transplanted patients with off-medication dyskinesia had greater increases in striatal FD uptake on PET than did those who did not develop this complication (Ma et al., 2002). The scans also showed "hot spots" of increased FD uptake in the putamen. However, no difference in mean putaminal FD uptake was noted between patients with or without off-medication dyskinesia in the other double-blind study (Olanow, 2003), and striatal FD uptake levels did not return to normal, let alone supernormal levels, in either of the studies. Further a reduction, not an increase, in dyskinesia was reported in some patients in whom even larger numbers of surviving dopamine neurons were observed at post-mortem (Kordower et al., 1995).

Current evidence suggests that peak-dose levodopa-related dyskinesias are associated with pulsatile stimulation of striatal dopamine receptors (Obeso et al., 2000). Transplantation of dopamine neurons that release dopamine in a more physiologic manner might thus be expected to reduce the risk of dyskinesia. Indeed, Lee et al. observed a reduction in levodopa-induced dyskinesia in 6-OHDA-lesioned rodents following transplantation (Lee et al., 2000). It is possible that inhomogeneous transplant deposits result in variable dopamine release and pulsatile stimulation of receptors in specific regions of the striatum. Indeed, Ma et al. reported regional changes on PET following transplantation, supporting the notion that variable dopamine release might be a contributing factor to off-medication dyskinesia (Ma et al., 2002). However, hot spots were not detected in PET scans performed in the other double-blind trial (Olanow et al., 2003). It is nonetheless possible that transplantation is associated with variability in synaptic formation and/or altered dopaminergic connectivity, possibly as a result of immune-mediated damage. Studies in levodopa-treated monkeys testing the significance of "hot spots"

following transplantation are currently underway and will hopefully shed light on these concerns.

Hagell et al. suggested that preoperative on-dyskinesia score or long-term storage of dopamine neurons in tissue culture prior to transplantation are related to the development of off-medication dyskinesia (Hagell et al., 2002). However, Olanow et al. observed off-medication dyskinesias in more than half of transplanted patients despite using fresh cells that were not cultured and were stored for no more than 48 hours in a fresh hibernation media (Olanow et al., 2003). Further, they did not find a correlation between off-medication dyskinesia and "on-dyskinesia" scores at baseline or at any other time during the study.

Olanow et al. suggested that off-medication dyskinesias in at least some of their patients may be a form of diphasic dyskinesia based on their clinical pattern (Fahn, 2000; Ma et al., 2002), the presence of concomitant parkinsonism in other body regions, change of pattern in response to dopaminergic therapy (to a more typical peak dose dyskinesia with generalized chorea), elimination of dyskinesias following complete drug withdrawal, and the incomplete restoration of striatal FD uptake on PET studies (Olanow et al., 2003). The authors hypothesized that off-medication dyskinesia were related to partial, but incomplete, restoration of dopamine neurons and striatal reinnervation sufficient to induce a prolonged form of diphasic dyskinesias but not sufficient to provide an adequate anti-parkinsonian motor response. This concept suggests that transplantation of increased numbers of surviving and functional dopamine neurons with enhanced dopamine neurotransmission might both increase efficacy and reduce the risk of developing off-medication dyskinesias. In contrast to this view, Freed et al. point out that in their studies persistent dyskinesias developed long after transplantation in association with increased axonal growth and that the dyskinesias in their patients involved the entire body, including cranial regions where they strongly resembled the abnormal movements seen in the tardive dyskinesias syndromes, which are quite distinct from those seen in diphasic dyskinesias. *It is clear that a better understanding of the nature of off-medication dyskinesias, and the development of means to prevent them, is essential before additional trials of transplant therapy can be resumed. It also will be important to determine if this side effect will complicate other dopaminergic cell-based therapies such as stem cells, and studies to assess grafts in levodopa-treated dyskinetic animals should be performed before initiating clinical trials in PD.*

## *3.5. Ethical Issues*

In designing fetal nigral transplantation trials, ethical issues are an important consideration (Chapter 2). Fetal nigral transplantation studies utilize embryonic tissue acquired from women who undergo abortion. Accordingly, it is important to ensure that the study complies with prevailing regulatory, Institutional Review Board (IRB), and human rights issues. We also believe

it is crucial to perform double-blind, placebo-controlled studies in order to control for placebo effect and physician bias, even if this necessitates employing a sham-surgery group (Freeman et al., 1999; Olanow, 2005). Some surgeons have resisted placebo-controlled studies arguing that exposing patients to an incision without the prospect of benefit violates the principle of "do no harm" (Cohen, 1994). However, there are numerous examples of surgical procedures that were accepted into routine practice based on anecdotal observations only to be rejected following more formal investigations (e.g., gastric freezing, glomectomy for asthma, extracranial/intracranial bypass for carotid occlusion, internal mammary artery ligation, arthroscopy for knee pain, etc.) (Freeman et al., 1999; Olanow, 2005 and Chapter 4). The same is certainly true in PD, where reports of clinical benefits in open label surgical trials of fetal nigral transplantation, fetal nigral porcine transplantation, and GDNF were not confirmed in double-blind, placebo-controlled studies (Lindvall et al., 1989, 1990, 1992; Freed et al., 1990, 1992; Peschanskie et al., 1994; Freeman et al., 1995; Hauser et al., 1999; Fink et al., 2000; Gill et al., 2003; Watts et al., 2001). Transplantation therapies in PD are particularly good candidates for assessment in a double-blind trial as the treatment has much in common with drug therapies; namely, the intervention is relatively standardized and dose, distribution, and metabolism/degeneration, must all be addressed.

The feasibility of carrying out placebo controlled surgical trials in PD with minimal risk to placebo patients has now been well established (Freed et al., 2001; Olanow et al., 2003), and all participating patients are required to sign an IRB-approved informed consent which explains in detail the risks and benefits of participating in a placebo-controlled trial. Exposing a small number of patients to a placebo procedure in these trials can prevent thousands of individuals from undergoing the risks and costs of a surgical procedure that may not be effective and where the adverse event profile is incompletely defined. A recent survey of PD clinical investigators in North America found that 97% favored the employment of double-blind controlled trials for assessing cell-based and gene therapies in order to avoid the possibility of falsely declaring a study effective based only on open-label studies (Kim et al., 2005). Indeed, 47% felt the risk of passing a needle into the brain of placebo patients was justified in comparison to misstating the efficacy of a clinical trial. *Future trials of fetal nigral transplantation and other cell-based therapies will require positive results in a double-blind, placebo-controlled study in order to convince clinicians and regulators that the intervention is effective*.

## 4. Future Directions and Considerations

It is clear that fetal nigral transplantation for PD presently remains an experimental procedure and can not be recommended for general use at the present time. However, clinical trials performed to date suggest that improved

results might be obtained if surgery were restricted to younger patients with milder disease who primarily experience clinical features that are responsive to dopaminergic therapies. The need for long-term immunosuppression still needs to be resolved. The correct number of dopamine neurons to implant must also be determined. It is estimated that 5% of cells in the SNc are dopamine neurons and only about 5% of dopamine neurons survive transplantation. Approximately 20–30% of mesencephalic cells die during tissue preparation and 60–70% of cells die during the first week following transplantation (Fawcett et al., 1995; Barker et al., 1996; Sortwell et al., 2000). Increased numbers of surviving dopamine neurons could potentially yield enhanced clinical results and reduce the risk of off-medication dyskinesia. Research efforts have focused on ways of trying to improve this yield. Methods currently being investigated include pre- or post-transplant exposure of dopamine neurons to factors such as antioxidants, lazaroids, trophic factors, caspase inhibitors, and BCL-2 that promote their survival (Brundin et al., 2000a).

Anti-oxidants promote the survival of cultured dopamine neurons and improve dopamine cell survival, striatal TH-immunoreactivity, and behavioral effects when co-administered with ventral mesencephalic grafts (Brundin et al., 2000; Meyer et al., 1998; Agrawal et al., 2004). For example, Agrawal and colleagues showed enhanced survival of TH-positive dopamine neurons and increased behavioral effects when ventral mesencephalic cells were co-grafted with ascorbate and glutathione in a rat model of PD (Agrawal et al., 2004). Co-administration of lazaroids with fetal nigral transplantation has now been attempted in PD patients and reported to provide enhanced clinical benefits (Brundin et al., 2000b), but it is not possible to say with certainty how much the lazaroids contributed to the observed benefit in this single anecdotal report.

Neurotrophic factors can also promote the survival of cultured or implanted dopaminergic neurons (Stoder et al., 1995; Atlar et al., 1994) and might thereby enhance the functional effects of dopamine cell transplants. They can be administered by way of direct infusion into the striatum, co-transplantation of cells that secrete neurotrophic factors, or gene therapy. The large majority of SNc melanized neurons contain FGF-2 receptors, suggesting that this might be an important trophic factor for dopamine neurons (Tooyama et al., 1994). Indeed, Timmer et al. reported increased cell survival with enhanced striatal reinnervation and improved functional recovery when dopamine cell grafts were co-transplanted with Schwann cells engineered to overexpress FGF-2 (Timmer et al., 2004). Glial cell line-derived neurotrophic factor (GDNF) appears to be the trophic factor with the greatest capacity to protect dopaminergic neurons. Indeed, GDNF infusion enhances survival of nigral neurons, increases TH staining in the striatum, and improves behavioral features in both rodent and primate models of PD (Gash et al., 1995, 1996; Winler et al., 1996 and Chapter 14). Lentivirus delivery of GDNF similarly induces behavioral and anatomic benefits in both dopamine-lesioned

rodents and monkeys (Bensadovn et al., 2000; Kordower et al., 2000 and Chapter 16). The survival of grafted dopamine neurons and the extent of striatal reinnervation is also enhanced by GDNF in the 6-OHDA rat (Zawada et al., 1998; Granholm et al., 1997). Carotid body cells contain neurotrophic factors such as GDNF, Brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3). Co-transplantation of carotid body cells also enhances the survival of grafted fetal ventral mesencephalic dopaminergic cells, striatal TH immunoreactivity and functional recovery in 6-OHDA-lesioned rats (Shukla et al., 2004).

Anti-apoptotic agents have also been used to try and increase the survival of transplanted dopamine neurons. The Jun-N-terminal kinase (JNK) stress pathway is central to apoptotic neuronal death following exposure to toxins thought to be relevant to PD (Gearan et al., 2001; Chun et al., 2001). Mixed-lineage kinase (MLK) activation mediates activation of JNK (Xu et al., 2001), and MLK inhibitors protect against degeneration of cultured dopaminergic cells (Harris et al., 2002). MLK inhibitors also improve survival of fetal ventral mesencephalic cells, as well as graft size and fiber outgrowth in dopamine lesioned rodents (Boll et al., 2004). Caspases are terminal events in many forms of apoptosis, and caspase inhibitors block apoptosis in dopamine neurons and increase survival of dopaminergic grafts in hemiparkinsonian rats (Schierle et al., 2000).

Another approach that has been attempted involves transplantation of fetal ventral mesencephalic grafts into both the striatum and the SNc. This approach could theoretically provide dopamine reinnervation more extensively throughout the striatum as well as to regions of the basal ganglia and cerebral cortex that normally receive dopaminergic input from the SNc. Transplantation into both striatum and SNc of dopamine-lesioned rodents was reported to enhance the performance of complex tasks to a greater degree than that obtained with intrastriatal grafts alone (Baker et al., 2000). Mendez and colleagues transplanted fetal nigral dopamine cells into both the striatum and the SNc in a small numbers of PD patients in an open-label study. They reported clinical improvement and increased striatal FD uptake on PET (Mendez et al., 2002). The procedure was well tolerated and no patient developed off-medication dyskinesias. At autopsy, surviving TH-positive cells which stained positively for G-protein-coupled inward rectifying current postassium channel type 2 (Girk2) were noted to grow particularly well into the striatum, indicating the innervation specificity of the implanted cells (Mendez et al., 2005 and Chapter 8).

Transplantation of alternate dopaminergic cell types has also been explored as a means of increasing the number of cells available for transplantation and avoiding the societal and logistical problems associated with the use of human embryonic tissue. Transplantation of cells from the organ of Zuckerkandl, a paired organ located adjacent to the abdominal aorta, has been reported to provide functional improvement in parkinsonian rats (Espejo et al., 2001). Autologous sympathetic ganglia cells have been transplanted into a small

number of PD patients in open-label studies and reported to decrease "off" time (Nakao et al., 2001) and to amelriorate bradykinesia and gait dysfunction (Itakura et al., 1997). Transplantation of autologous carotid body cells improved motor function in Parkinsonian rodents and monkeys (Toledo-Aral et al., 2003; Luquin et al., 1999) and has been reported to provide long-lasting benefit in a small number of PD patients (Arjona et al., 2003). However, post-mortem studies demonstrated no evidence of graft integration into the brain, nor evidence that implanted cells secreted dopamine. Carotid body tissue undergoes atrophy with increasing age and the authors suggested that any improvement was due to the release of trophic factors and not restoration of the nigrostriatal system (Toledo-Aral et al., 2003). None of these procedures has been studied in double-blind placebo-controlled trials.

Implantation of fetal porcine nigral grafts in PD patients (see Chapter 11) has been reported to provide benefit in open-label studies (Fink et al., 2000), but benefits were modest and only small numbers of surviving cells were detected at post-mortem (Deacon et al., 1997). Further, a double-blind placebo-controlled trial failed to show benefits of transplantation with porcine nigral cells in comparison to placebo (Watts et al., 2001). Retinal pigmented epithelial cells can secrete levodopa, survive transplantation, and are relatively resistant to immune rejection when transplanted attached to gelatin microcarriers (Spheramine®). Spheramine transplantation has been reported to provide benefits to parkinsonian rats and monkeys as well as to PD patients in an open-label trial and off-medication dyskinesia has not been observed (Subramanian, 2002; Watts et al., 2003). Double-blind controlled trials of spheramine are currently underway.

Stem cells have attracted considerable interest as a means of generating large numbers of optimized dopamine neurons for transplantation and are discussed in detail in other chapters in this book (see Chapters 12 and 13). Embryonic stem (ES) cells are derived from the blastula layer of the embryo and can be expanded and induced to differentiate into dopamine neurons. Factors such as Nurr-1, sonic hedgehog, trophic factors (particularly FGF), ascorbate, and cytokines have been shown to promote the differentiation of ES cells to dopamine neurons (Lindvall et al., 2004; Snyder et al., 2005). Transplanted ES cells are capable of spontaneously differentiating into dopamine neuronal phenotypes and have been shown to provide motor and imaging benefits to 6-hydroxydopamine-lesioned rodents, although benefits are modest and there are only a small number of surviving TH-positive cells (Björklund et al., 2002). Further, many of the transplanted animals developed teratoma-like tumors. More recently, transplanted dopamine neurons derived from monkey ES cells were reported to be associated with behavioral improvement and increased striatal fluorodopa uptake on PET in MPTP-lesioned monkeys (Takagi et al., 2005). Here too, however, the number of surviving TH-positive cells was very small. Autologous stem cells derived from the cerebral cortex, bone marrow, or umbilical cord matrix have attracted much interest because immunosuppression is not required and they avoid

societal concerns related to the use of embryonic tissue. However, these types of stem cells preferentially differentiate into glia, and only small numbers can be induced to differentiate into dopamine neurons and survive transplantation (Lindvall et al., 2004).

In general the number of surviving, stem-cell-derived dopamine neurons that survive transplantation is small, and results in animal models of PD are modest and not yet comparable to what has been observed with fetal nigral cells (which failed in double-blind controlled clinical trials in PD patients). Many issues remain to be resolved before stem cells can be seriously considered for clinical trial in PD. The type of stem cell to use, the way to induce differentiation into dopamine neurons and the optimal transplant protocol remain to be defined. In addition, extensive preclinical testing is required to ensure that behavioral effects are at least comparable to what was obtained with fetal nigral transplant and to assess their side effect profile particularly with respect to the risk of tumor formation and off-medication dyskinesias.

It is also important to consider the potential limitations of strategies such as fetal nigral transplantation that primarily aim to restore dopaminergic function (Lang et al., 2004 and Chapter 3). PD pathology involves more than just the nigrostriatal system; indeed, degeneration and/or Lewy bodies are also found in cholinergic neurons of the nucleus basalis of Meynert, norepinephrine neurons of the locus ceoruleus, serotonin neurons of the raphe, as well as neurons of the pedunculopontine nucleus, dorsal motor nucleus of the vagus, peripheral autonomic neurons, and cerebral cortex (Forno, 1996; Braak et al., 2003). Degeneration of the SNc likely accounts for the cardinal motor features of PD, and it is reasonable to consider that dopaminergic transplants might restore function to this system in a more physiologic manner than can be accomplished with levodopa, and thereby provide levodopa-like benefits without the associated motor complications. However, it is also likely that pure dopamine transplants will not affect the motor and non-motor features of the disease (e.g., postural instability, autonomic dysfunction, and dementia) that likely derive from degeneration of non-dopaminergic neurons and can also represent a major source of disability. Transplantation into multiple targets or using transplanted cells to deliver trophic factors or other protective substances might have a more widespread effect than has been achieved with current protocols. It is also possible that restoring dopaminergic tone early in the course of PD might prevent degeneration in non-dopaminergic regions that occurs secondary to the loss of dopamine neurons. For example early dopamine restoration might prevent overactivity of the STN and excitotoxic damage in target neurons (Rodriguez et al., 1998).

Despite the enormous promise of transplantation as a treatment for PD, disappointing results in double-blind clinical trials, the development of unanticipated adverse effects, and the intrinsic limitations of a purely dopamine therapy offer a sobering reality test. It is still possible that better clinical results can be obtained with different transplant protocols, off-medication dyskinesia can be prevented, and dopamine cells can be utilized in such a way

as to have more widespread clinical benefits. It is nonetheless clear that much still needs to be accomplished before transplantation can be considered as a viable therapy for PD.

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# 7

# Imaging of the Parkinsonian Brain in Relation to Restorative Therapy

A. Jon Stoessl,[1] Vesna Sossi,[3] and David J. Brooks[2]

**Abstract**
Functional imaging has provided objective evidence that grafts of fetal dopaminergic tissue can survive in Parkinson's disease patients and that their dopamine storage capacity correlates with clinical improvement and normalization of brain metabolism. It has also demonstrated that such implants can release normal levels of dopamine after an amphetamine challenge. Despite this, clinical responses to grafts have been very variable and dyskinesias off medication have been a serious adverse event. This review discusses current imaging knowledge concerning graft function and possible mechanisms underlying lack of response and dyskinesias.

## 1. Introduction

Positron emission tomography can be extremely useful for the in vivo assessment of graft integrity and function following transplantation for Parkinson's disease. Broadly speaking, PET can be used to assess (i) neurochemical function and integrity and (ii) restoration of functional circuitry. As discussed below, it should be noted that while one might not expect the latter to take place in the absence of the former, the two processes are not entirely correlated. Thus, while fluorodopa PET might improve within a few months of the transplant procedure, with a relatively early plateau and little further improvement over time, there may be a delay in the restoration of function in the Cortico-Striatal-Thalamo-Cortical (CSTC) circuitry (Piccini et al., 2000).

[1]Pacific Parkinson's Research Centre, University of British Columbia, Vancouver, B.C., Canada.
jstoessl@interchange.ubc.ca
[2]Division of Neuroscience, Imperial College, Hammersmith Hospital, London, United Kingdom
david.brooks@csc.mrc.ac.uk
[3]Department of Physics and Astronomy, University of British Columbia, Vancouver, Canada.
vesna@physics.ubc.ca

# 2. Basic methodological approaches

## *2.1. Measurement of Presynaptic Dopaminergic Integrity*

### 2.1.1. Fluorodopa PET

There are three major approaches that have been applied to the measurement of presynaptic dopaminergic function. The most widely used, particularly in the transplant field, is PET with the levodopa analog 6-[$^{18}$F]-fluoro-L-dopa (FD). FD is taken up by monoaminergic neurons (and possibly other cells, including glia) and decarboxylated by L-aromatic amino acid decarboxylase (L-AAAD) to fluorodopamine (FDA). In the normal brain $^{18}$F activity is trapped in the striatum as FDA and its metabolites, at least for the first 90–120 minutes following tracer administration. Thus, $^{18}$F transport is essentially unidirectional and an influx or uptake constant, Ki, can be determined using multiple time graphical analysis (Patlak and Blasberg, 1985; Martin et al., 1989) and used to quantify the functional integrity of dopaminergic nerve terminals. FD uptake has been shown to correlate with nigral cell counts in humans and in monkeys with MPTP-induced parkinsonism (Snow et al., 1993; Pate et al., 1993). However, because AAAD is subject to compensatory changes and pharmacological regulation, FD PET studies may overestimate terminal density (Cho et al., 1997). FD uptake also correlates with motor function off medication, as determined by the Unified Parkinson's Disease Rating Scale (UPDRS), particularly scores of bradykinesia (Vingerhoets et al., 1997). Numerous studies have demonstrated increased FD uptake following fetal mesencephalic transplantation for Parkinson's (Freeman et al., 1995; Wenning et al., 1997).

### 2.1.2. Measurement of the Dopamine Transporter

A number of tropane (cocaine)-based tracers that bind to the membrane dopamine transporter (DAT) have been developed for use with PET or single photon emission computed tomography (SPECT). Other non-tropane options are [$^{11}$C]-*d-threo*-methylphenidate (MP) and $^{11}$C-nomifensine. DAT imaging has been widely used to assess dopaminergic integrity in patients with PD, and to assess disease progression and the effects of various pharmacological interventions on the rate of progression (Nurmi et al., 2000; Marek et al., 2001, 2002). The density of DAT binding reflects dopaminergic nerve terminal density and correlates loosely with motor function although, because the DAT is subject to compensatory changes and pharmacological regulation (Nurmi et al., 2000; Vander et al., 1995; Guttman et al., 2001; Wilson and Kish, 1996; Lee et al., 2000), PET/SPECT studies using DAT ligands should be interpreted with caution with respect to innervation density. Although dopaminergic grafts appear to express the DAT (Kordower et al., 1996), imaging studies following transplantation have been

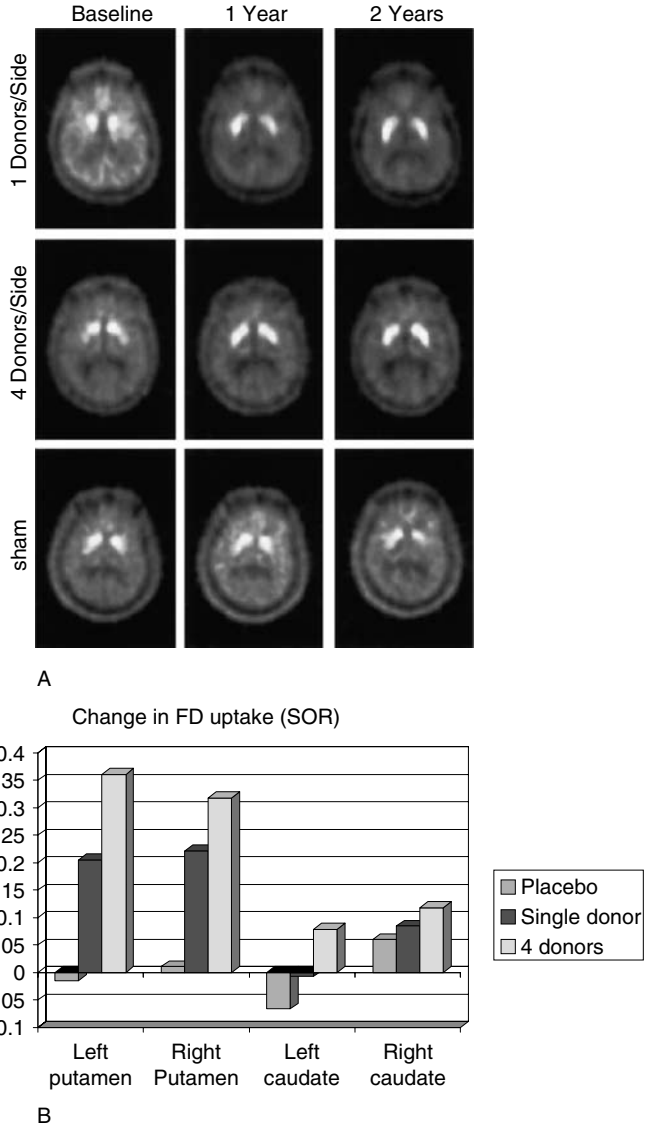


FIGURE 7.1. **A**. Axial PET images of 6-[$^{18}$F]-fluoro-L-dopa uptake in representative subjects following sham (bottom panel), one (top panel), or four (middle panel) donors per striatum. In the one- and four-donor subjects, there is an increase in tracer uptake seen at one year (middle column), with a further increase at two years (right column). In the sham-operated subject, fluorodopa uptake continues to decline (from Olanow et al., Ann. Neurol. 2003[40]). **B**. Mean change in striatal:occipital ratio of fluorodopa from baseline to two years following transplant. Note that changes are seen in both left and right putamen, but not in the caudate nuclei, which were not implanted (from Olanow et al., Ann. Neurol. 2003[40]). (See color insert.)

quite limited. A recent study suggests that DAT binding may not be restored to any significant degree following transplantation, even when FD uptake improves (Cochen, 2003).

### 2.1.3. The Vesicular Monoamine Transporter Type 2 (VMAT2)

The last direct approach to imaging the integrity of monoaminergic nerve terminals is the use of [$^{11}$C]dihydrotetrabenazine (DTBZ). This compound binds to the vesicular monoamine transporters that are responsible for packaging dopamine (and other monoamines) into synaptic vesicles. [$^{3}$H]DTBZ binding has been shown to correlate with other measures of dopaminergic nerve terminal density (Kilbourn, 1997) and, compared with the DAT, VMAT2 expression is thought to be relatively insensitive to regulatory changes (Vanderborght et al., 1995; Wilson and Kish, 1996). There are no reports of VMAT2 binding in grafts, although it is assumed that the VMAT2 is expressed. Unfortunately, DTBZ PET is currently available at only very few centers worldwide and there are no data on the expression and survival of VMAT2 in mesencephalic grafts.

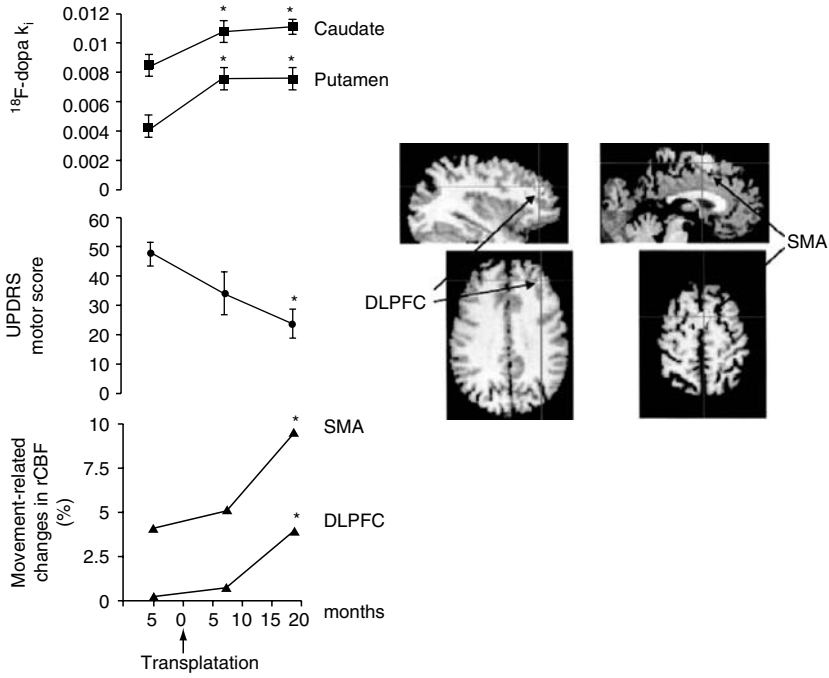


FIGURE 7.2. Increases in fluorodopa uptake following transplantation precede improvement in activation-induced increases in cerebral blood flow in supplementary motor area (SMA) and dorsolateral prefrontal cortex (DLPFC). Increases in the latter are associated with further clinical improvement (from Piccini et al., Ann. Neurol. 2000[1]). (See color insert.)

### 2.1.4. Indirect Measures of Graft Integrity by Assessment of Dopamine Release

[$^{11}$C]raclopride is the most widely used PET ligand for the D2 receptor. As its affinity for the D2 receptor is in the low nM range, it is subject to competitive displacement by endogenous dopamine (Seeman et al., 1989; Volkow et al, 1994). It is also unable to attach to D2 receptors which have been temporarily internalized into the cell soma after binding of endogenous dopamine. Thus, medications known to increase levels of synaptic DA, such as levodopa (Tedroff et al., 1996; de la Fuente-Fernandez et al., 2001), amphetamine (Laruelle et al., 1997; Breier et al., 1997), and methylphenidate (Volkow et al., 2001), all result in reduced [$^{11}$C]raclopride binding, as does transcranial magnetic stimulation (Strafella et al., 2001, 2003) and motor activity (Goerendt et al., 2003; Ouchi et al., 2002). Thus, improvement of dopaminergic transmission by a functioning graft should be associated with increased DA release both basally and in response to medications such as amphetamine (Fig. 7.3 Piccini et al., 1999).

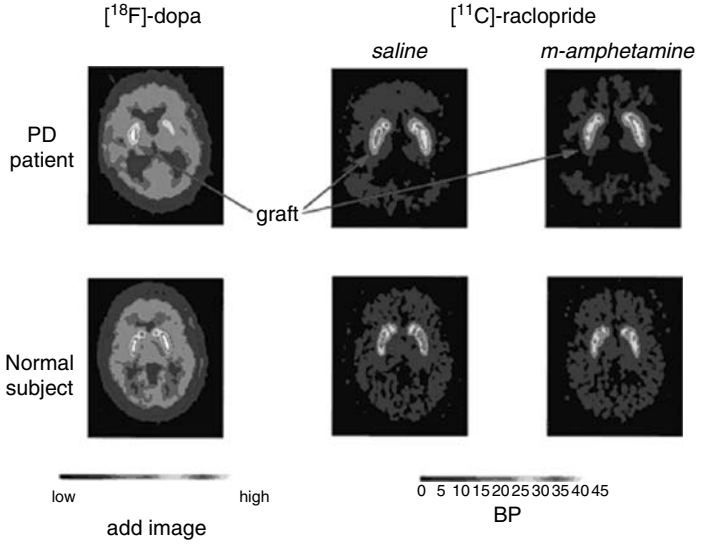


FIGURE 7.3. Fluorodopa uptake (**left panel**) and [$^{11}$C]raclopride binding (**middle** and **right panels**) in a Parkinson patient with a unilateral transplant in the right striatum (shown on the left side of the image). In the grafted striatum, fluorodopa uptake improves to near normal levels. Following saline injection (middle panel), raclopride binding is increased compared to control levels in the *non-grafted* striatum, with no response to intravenous methamphetamine (right panel). In contrast, raclopride binding is normal in the grafted striatum, with a further reduction following methamphetamine (as seen in controls). This reduction represents occupancy of dopamine receptors by dopamine released in response to the pharmacological stimulus (from Piccini et al., Nat. Neurosci. 1999[31]). (See color insert.)

### 2.1.5. Indirect Measures of Restored CSTC Function

Altered dopaminergic output to the striatum results in a number of changes in cerebral blood flow or glucose metabolism in regions downstream from the striatum (Playford et al., 1992; Eidelberg et al., 1990; Feigin et al., 2001). Resting glucose metabolism is relatively increased in the pallidum and decreased in frontal areas, while the blood flow response to activating motor tasks is attenuated in the supplementary motor and prefrontal cortex. These changes are reversed by dopaminergic therapies (Feigin et al., 2001; Jenkins et al., 1992) or by deep brain stimulation of the globus pallidus or subthalamic nucleus (Fukuda et al., 2001; Ceballos-Baumann et al., 1999). Changes in blood flow, glucose metabolism, and patterns of inter-connectivity of structures in the CSTC loop can similarly be used to assess functional integration of grafts.

## 3. Clinical Studies in Transplant

### *3.1. Evidence of Restoration of Dopaminergic Integrity*

Numerous open and two double-blinded studies implanting fetal mesencephalic cells into striatum for Parkinson's have demonstrated associated

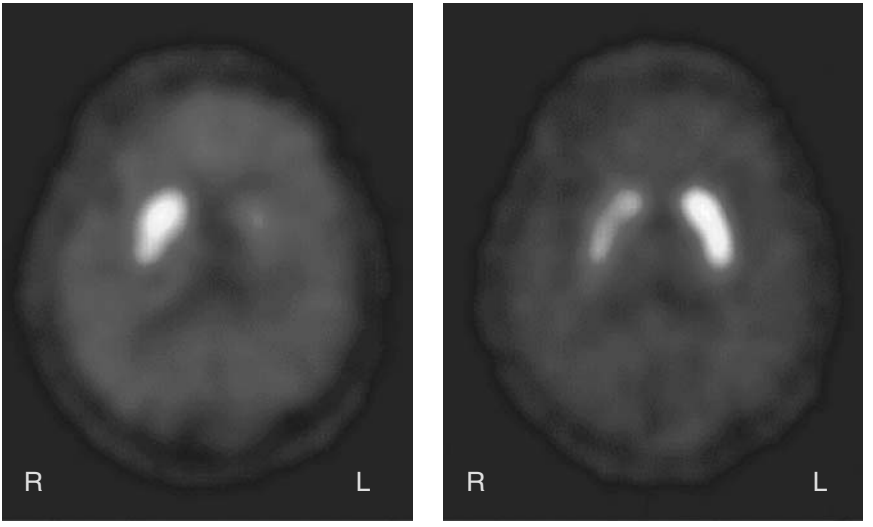


FIGURE 7.4. Fluorodopa and raclopride PET scans in a patient with left hemibody dyskinesias 8 years following transplant. Note that there is a marked increase in fluorodopa uptake in the right caudate nucleus, while raclopride binding is reduced below control levels throughout the right striatum. The findings suggest aberrantly increased dopamine synthesis and release in the striatum contralateral to dyskinesias (UBC-TRIUMF PET team). (See color insert.)

increases in FD uptake (Freeman et al., 1996; Wenning et al., 1997; Cochen, 2003; Hauser et al., 1999; Freed et al., 2001 and see Chapters 5 and 6). This striatal increase in FD uptake can be detected by 6 months following surgery, with further increases out to at least one year, but without substantial further increases. In one reported case putamen FD uptake had normalized by three years and this was sustained for over 10 years (Piccini et al., 1999).

The original unblinded studies suggested a loose but significant correlation between increased FD uptake and improvement in clinical function withdrawn from medication. However, while two recent double-blind studies of fetal transplantation have demonstrated significant increases in FD uptake (Fig. 7.1), the degree of clinical improvement was modest (Freed et al., 2001) or non-significant (Olanow et al., 2003 see Chapter 6). In the latter study, clinical improvement relative to placebo was evident during the first 6 months following the transplant procedure, while patients were receiving cyclosporin, but declined thereafter. Although graft rejection following withdrawal of immunosuppression is a possibility, the improvement in FD uptake was sustained, which would be against this. It is also of interest that, whereas increases in FD uptake were seen early after the transplant in most unblinded studies, in one, a corresponding recovery of activation induced supplementary motor cortex blood flow increase was not seen until two years later, and occurred in parallel with clinical improvement (Piccini et al., 2000, Fig. 7.2).

Although fetal transplants are known to express the dopamine transporter (Kordower et al., 1996), there has been only one study in which an attempt was made to image restoration of DAT using PET or SPECT following transplantation (Cochen, 2003). Interestingly, although FD uptake was significantly enhanced following the procedure, there was no improvement in DAT binding. The reasons for this are unclear, though it is possible that immature fetal dopamine cells show delayed expression of DAT relative to AAAD activity.

There are no published studies of VMAT binding studied using PET following transplantation, and, indeed, we are not aware of any post-mortem studies demonstrating restoration of VMAT expression.

## *3.2. Complications of Transplantation*

The two major complications of the transplant procedure are failure of clinical recovery and the emergence of severe dyskinesias that may persist despite drastic reduction or even discontinuation of levodopa therapy (Freed et al., 2001; Olanow et al., 2003 see Chapter 10). Perhaps the most perplexing finding is the observation that both double-blind studies of fetal transplantation failed to show meaningful improvements in clinical function, except in younger or milder subsets of cases, despite significant improvement in FD uptake, at times reaching near normal values. One obvious potential explanation for this is that, while the grafts may have intrinsic ability to decarboxylate FD and trap it as FDA, they may not release

dopamine in an appropriate physiological manner or functionally integrate into the host brain and form synaptic connections. This is somewhat difficult to reconcile with the observations of normalized supplementary motor cortex blood flow on activation and pallidal-thalamic resting glucose metabolism following the transplant, although the former was seen in an open label study with younger subjects (Piccini et al., 2000) and in which different surgical techniques were employed. Another possibility deserves consideration. FD uptake is simply a measure of radioactivity trapped within the striatum. Although it is assumed that it corresponds to decarboxylation of FD to FDA, and its subsequent storage within synaptic vesicles, it is conceivable that radioactivity could be trapped outside of vesicles without functional dopaminergic reinnervation. This possibility, which may admittedly seem unappealing at first look, deserves further exploration.

The pathogenesis of post-transplant dyskinesias off medication is similarly uncertain. One study demonstrated asymmetric increases in FD uptake particularly in the ventral striatum of patients with persistent dyskinesias off medication following transplantation (Ma et al., 2002). The open Swedish series (using a different surgical technique) noted mild "off" dyskinesias in a minority of cases. Here, the severity of dyskinesia among the patients was inversely correlated with FD uptake prior to surgery rather than post implant increases and also correlated with the amount of fetal tissue implanted (Hagell et al. 2003). The authors argued against off dyskinesias arising as a consequence of excessive dopaminergic function.

In the Mt. Sinai–Tampa–Chicago double-blind study, there was a 56% incidence of dyskinesias off medication following surgery (Olanow et al., 2003). There was again no obvious relationship between the emergence of increased dyskinesia and increases in FD uptake. However, in two patients who underwent surgery with identical techniques in a pilot study from this group, dyskinesia was associated with increased FD uptake and/or greater than expected DA release following levodopa (Huang et al., 2003). In one patient with severe "off-medication" hemibody dyskinesia, FD uptake increased in the caudate nucleus (Fig. 7.4). As this region was not grafted (and in most patients showed either no improvement, or continued decline in FD uptake following surgery), this suggests that the graft encroached upon the caudate nucleus and may have been releasing excessive amounts of dopamine. In both patients, [$^{11}$C]raclopride binding in the medication free state was markedly reduced below the levels seen in normals or patients with PD. This could reflect unrecognized damage to the striatum, but a more likely explanation is that remaining striatal D2 receptors were occupied by excessive release of DA. Furthermore, both patients showed a substantial increase in DA release following oral levodopa, as measured by further reductions in [$^{11}$C]raclopride binding. Whether or not such increases exceed those expected for the duration and severity of PD in these patients is, however, not clear.

## 4. Conclusions

Embryonic dopaminergic transplants have been shown to improve disability and normalize the resting patterns of brain metabolism and activation during volitional limb movements in selected PD cases. Grafts can also restore levels of striatal dopamine storage capacity and dopamine release after amphetamine challenges. However, in two major double-blind trials, a dissociation between graft function, as evidenced by striatal FD uptake, and clinical improvement was evident. This may reflect the fact that restoration of non-regulated dopamine storage and release during the early stages of maturation of the transplants is not sufficient to restore function of cortical-basal ganglia loops and, therefore, only results in partial symptomatic relief. Restoration of striato-cortical neurotransmission and movement-related cortical activation is likely to require the establishment of both afferent and efferent synaptic contacts with the host.

Interestingly, it appears to be younger, less affected patients who respond best following transplantation, possibly because functional integration of the grafts into the host brain is more effective.

A major concern is the development of severe "off" dyskinesias in up to half of transplanted subjects. It remains unclear whether "off" dyskinesias are a characteristic feature of dopamine cell replacement—the Swedish experience argues against this viewpoint, whereas two double-blind trials suggest aberrant ventral striatum or caudate innervation may be a factor. Clearly, the mechanism underlying this important adverse event will need to be elucidated if cell therapies for PD, such as stem cell approaches, are to be further developed.

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# 8

# Important Aspects of Surgical Methodology for Transplantation in Parkinson's Disease

Thomas B. Freeman[1] and Patrik Brundin[2]

## 1. Introduction

It is without doubt that the surgical technique is extremely important for successful outcome of neural transplantation in Parkinson's disease (PD). As is apparent from other chapters in this volume (see Chapters 5 and 6), the outcome between clinical grafting studies has varied dramatically, both in terms of the beneficial effects attained and the occurrence of unwanted side effects. In the discussion on whether neural transplants are effective in PD, insufficient attention has probably been paid to details in the surgical protocols. In reviews of the clinical transplantations trials, differences in surgical protocols between the studies are typically mentioned briefly, and there is rarely a critical discussion on which surgical aspects are crucial for clinical success. Most probably there are several surgical protocols that can result in effective transplants in PD patients. On the other hand, the methodology is unlikely to already have been developed to its fullest potential because many parameters have not yet been explored in detail in systematic experiments. It is already clear that neural grafting is not a simple technology and suboptimal strategies in one or more of several crucial steps can definitely lead to failure. Each of the several steps in transplantation surgery can be viewed as a link in a chain. For a successful outcome this chain has to be strong, but no transplantation protocol is better than the weakest link in the chain. Therefore, attention to small details can be very important when discussing neural transplantation methodology. In this chapter we describe the current state-of-the art concerning embryonic donor tissue, graft tissue storage and preparation, graft injection technique, and the role of different brain targets. We conclude

[1]Department of Neurological Surgery, Center of Excellence for Aging and Brain Repair, University of South Florida, Tampa, FL.
tfreeman@hsc.usf.edu
[2]Neuronal Survival Unit, Department of Experimental Medical Science, Wallenberg Neuroscience Center, Lund, Sweden.
Patrik.Brundin@med.lu.se

that while the accumulated knowledge on critical parameters in transplant technology is impressive, the significance of several methodological factors remains to be explored in more detail and an optimal protocol has yet to be developed.

## 2. Donor tissue procurement

Several studies in experimental animals have shown that only immature neural tissue will survive grafting. Until a viable and safe source of stem cells that can be used for grafting in PD is available (Roybon et al., 2004; Chapter 13), human tissue containing cells that develop into dopamine neurons has to be obtained from embryos or fetuses. It is worth pointing out that, according to the classical human developmental studies of O'Rahilly and Müller, the term *embryo* should be used until 8 weeks after conception (O'Rahilly and Müller, 1987). Only thereafter is the term *fetus* appropriate. In this chapter, we have attempted to follow these definitions as closely as possible. The fact that embryos or fetuses are required as donor tissue for neural grafting has raised several ethical and practical issues which we discuss briefly in the following sections.

### *2.1. Ethical Issues*

The use of aborted embryos or fetuses as donor tissue raises ethical concerns regarding, e.g., informed consent, the type of abortion technique, and the fundamental issue of whether the use of tissue from induced abortions is ever ethically justifiable. For a detailed discussion of the ethical issues of using embryonic/fetal tissue for transplantation, see Chapter 2 by G.J. Boer. Briefly, in the particular context of our chapter which discusses grafting technique, it is pertinent to mention that ethical guidelines typically state that the transplantation procedure must not change when the abortion is performed and the abortion should not be modified in a way that involves increased medical risk for the woman. Some would argue that using tissue from spontaneously aborted embryos is more ethically acceptable, and indeed such tissue has also been utilized in some clinical trials (Madrazo et al., 1991, 1998). However, from neurobiological and safety standpoints this type of donor tissue is less suitable (Freeman and Olanow, 1991). First, the viability of brain cells dissected from spontaneously aborted embryos/fetuses is likely to vary dramatically depending on how long the embryo/fetus has been dead in the uterus. Second, the reason that the embryo/fetus undergoes spontaneous abortion could be, e.g., genetic abnormality or intrauterine infection (Cramer and Wise, 2000), both of which would render the brain tissue unsuitable for transplantation. Third, the increased difficulty in predicting when tissue will be available makes elective transplantation surgery hard to plan, and the simultaneous use of more than one donor problematic.

## 2.2. Embryo Staging

Dopamine neurons will only survive grafting if harvested from sufficiently young donor embryos or fetuses. The maximal age of the donor tissue is dependent upon the type of grafting technique used. As discussed in more detail in the following section, it is clear that donor age is an extremely important surgical parameter in neural grafting in PD. Therefore assessing the age of the donor embryo/fetus correctly is vital for successful grafting.

The most accurate way to stage embryos is to measure crown-to-rump length (CRL). This correlates well to age (Table 8.1). In practical terms, it is often not possible to measure the CRL since the embryos are partially destroyed during the abortion procedure. In some cases, the CRL has been estimated prior to the abortion using ultrasound (Gustavii, 1989). With an experienced operator, the ultrasound-based estimates of CRL are very accurate. This procedure has the advantage that it also establishes whether the embryo is viable prior to the abortion and therefore if the brain tissue is suitable for grafting. The use of low-pressure aspiration abortion increases the chances of obtaining embryonic brain tissue that is sufficiently intact for transplantation and the procedure is also very safe for the woman undergoing abortion (Gustavii, 1989; Nauert and Freeman, 1994). The Carnegie collection of embryos provides an excellent catalogue that make it possible to correlate different external features of the embryo with its age (O'Rahilly and Müller, 1987). One word of caution, however: the Carnegie collection is based on formalin-treated embryos, which may have shrunk by up to 10% during the fixation procedure.

When there is uncertainty about the age of the embryo, e.g., in the absence of data from an ultrasound, other strategies to determine the developmental stage of the donor tissue are necessary. Since routine abortion procedures

Table 8.1. Development of the Human Embryo and Fetus and Its Relationship to the Development of the Mesotelencephalic Dopamine System

| Embryonic age (post-ovulatory days) | Carnegie stage | Approximate CRL (mm) | Development of tyrosine hydroxylase immunoreactivity |
|---|---|---|---|
| 44 | 18 | 13–17 | Stained cells first seen in ventricular zone |
| 47.5 | 19 | 17–20 | |
| 50.5 | 20 | 21–23 | |
| 52 | 21 | 22–24 | |
| 54 | 22 | 23–28 | Neuritic extensions and nigrostriatal pathway begin to develop |
| 63 | Early fetal—second trimester | 50–54 | Innervation of the striatum starts in the putamen |

rarely result in intact embryos, it is often not possible to measure the CRL after the abortion. A mathematical model using morphometric features of the embryo has been developed to facilitate staging embryos that have been partially destroyed (Evtouchenko et al., 1996). With increasing experience, it is also possible to estimate the age of the embryo based on anatomical features of the mesencephalon (see section 2.5 on Tissue Dissection for further discussion).

## *2.3. Optimal Donor Age*

As already mentioned, the age of the donor tissue is highly important when grafting in PD. Early studies in experimental animals established that there was a crucial developmental time window when immature dopamine neurons could be harvested and survive subsequent grafting. The animal studies also revealed that the transplantation technique influenced the donor age window. The upper donor age limit using rat mesencephalic tissue is about 17 days post conception when grafts are prepared as solid tissue pieces (Simonds and Freed, 1990; Stenevi et al., 1976). In contrast, if the tissue is dissociated into a cell suspension, the survival of rat dopamine neurons drops dramatically if the donor age exceeds 15–16 days (Brundin et al., 1985a, 1988). Conversely, if cells are harvested from embryos that are *too* immature, mesencephalic neuronal progenitors do not appear capable of adopting a dopaminergic phenotype after grafting. In an interesting study, Sinclair and coworkers examined the proliferation and survival of mesencephalic cells harvested from rat embryos and grafted with the cell suspension technique to the striatum (Sinclair et al., 1999). Using an elaborate experimental design where they labeled graft cells with the mitosis marker bromodeoxyuridine either before or after transplantation surgery, they found that virtually only post-mitotic dopaminergic neurons survived dissection and transplantation. Thus, when grafted cells underwent cell division after transplantation, they almost never developed into dopaminergic neurons. Alternatively, if newly divided cells differentiated into dopamine neurons after grafting they certainly did not survive long, since they could not be detected at the end of the experiment 5 weeks after surgery. One implication of this study is that grafting tissue from extremely young embryos may result in low numbers of dopaminergic neurons. The first evidence of the dopamine-synthesizing enzyme tyrosine hydroxylase (TH) during the ontogeny of the rat mesencephalon appears around 12 days of age (Specht et al., 1981). Thus, the earliest age when the rat dopamine neurons can be grafted using the cell suspension method is shortly after they begin to express TH. By 14.5 days of age, which approaches the upper donor age limit for mesencephalic cell suspension grafts (Brundin et al., 1985a, 1988), the TH-immunopositive neurons have extended the first axons to the striatum (Specht et al., 1981). Taking tissue from very young embryos may not only adversally influence dopamine neuron development in the graft, it also increases the risk of contaminating the neural tissue with

mesenchymal tissue that is attached to the developing central nervous system. Such tissue poses a safety risk because it can differentiate into undesirable non-neural tissue types. There are at least two autopsy reports in the literature suggesting that non-neural tissue was inadvertently included in transplant tissue in PD patients (Folkerth and Durso, 1996; Mamelak et al., 1998). Not only is this tissue ineffective in reversing the neurological symptoms, if it grows uncontrollably it puts the patient's life at risk.

What is the optimal donor age when using human mesencephalic tissue? For obvious reasons this has not been possible to study systematically in human patients. The information is largely based on comparisons between the developmental biology of the mesencephalon of humans and rodents, and on xenografting studies where human mesencephalic tissue has been implanted into the striatum of immunosuppressed rats. Unfortunately, the xenografting literature in this area was initially inconsistent due to differences between studies regarding how the age of the embryos was determined. Gynecologists have traditionally used the number of weeks from the last menstruation to denote the age of human embryos and fetuses. This can be inaccurate since pregnant women may not present a precise medical history and, moreover, there can be significant variation in the number of days between the last menstruation and the time point when fertilization takes place. Therefore, developmental biologists denote the age of the embryo in post-fertilization or post-ovulatory weeks. The latter term is recommended by the authoritative work on human development from O'Rahilly and Müller (1987), since post-fertilization has been used by some to denote when the embryo is implanted in the uterus. In the early transplant literature describing the survival of dopamine neurons taken from embryos of different ages, the distinction between post-menstrual weeks and post-fertilization weeks was not clear. For example, in the first of our own studies (Brundin et al., 1986) we used the term *post-conceptional* even though the embryonic ages presented were really post-menstruation.

Developmental studies on human embryos show that the first immunostaining for TH in the mesencephalon appears in cells located close to the ventricular space at around 44 post-ovulatory days (Freeman et al., 1991; Silani et al., 1994), suggesting that, approximately, this would be a good time to harvest the cells for transplantation using the cell suspension method (Table 8.1). These findings are both a confirmation and a more detailed extension of earlier observations (Olson et al., 1973; Pickel et al., 1980). After an additional 10 days of development, the mesencephalic dopamine neurons begin to extend their first axons rostrally (Table 8.1), and one additional week later the first terminals are formed in the putamen (Freeman et al., 1991). By analogy with findings in rat tissue, one would predict that somewhere between 54 and 63 post-ovulatory days would represent the upper donor age limit for human mesencephalic dopamine neurons if grafted in the form of a cell suspension.

While these predictions based on developmental studies are interesting, they cannot replace information obtained from human-to-rat xenografting

studies. In one study, the optimal donor age for solid grafts of human mesencephalic donor tissue implanted directly into the striatal parenchyma has been estimated at 44–65 days post-ovulation (Freeman et al., 1995b). There are other studies employing human mesencephalic tissue from up to 12-week-old fetuses that report good graft survival and function with intrastriatal solid implants. In contrast, for dissociated human mesencephalic tissue the optimal donor age has been estimated at 34–56 post—ovulatory days (Freeman et al., 1995b) and 44–54 days post-ovulation (equivalent to 13–28 mm CRL) (Brundin, 1986, 1992), and within these donor age ranges it has not been possible to detect meaningful differences in graft sizes. The difference regarding upper donor age limit between solid and cell suspension grafts may not seem very important at a first glance. However, it has practical implications since a significant number of routine abortions are conducted when the embryo is between 8 and 12 weeks old, and dissociated mesencephalic tissue from these embryos cannot be used for transplantation to PD patients. While data obtained in human-to-rat xenografting studies suggest that tissue from 34 post-ovulatory-day-old embryos can be grafted successfully, there is need for caution. At ages younger than 44 days, it is difficult reproducibly to separate the mesencephalic neural tissue from the overlying anlagen for meninges. The risk for contaminating the graft with mesenchymal tissue is clearly evident when the human embryo has a CRL smaller than 13 mm, and therefore the tissue is not suitable for implantation into humans.

## *2.4. Sterility*

For obvious safety reasons it is important to avoid infections in the transplant tissue. The three main possible sources for infections in embryonic/fetal donor tissue are the maternal blood, contamination from the vagina during the abortion, and contamination during the dissection, tissue preparation, and surgery (Holt et al., 1997). Many clinical programs have chosen to screen for common pathogenic viruses in the women undergoing abortion. Thus, screening for human deficiency virus (HIV), hepatitis A (HAV) and B viruses (HBV), cytomegalovirus (CMV), and herpes simplex (HSV I and II) is performed (Brundin, 1992). Embryos from women who are positive for HIV, HAV, or HBV are excluded. Likewise, material obtained from those exhibiting IgM or high IgG titers against CMV and HSV, suggestive of a primary infection or reactivation of an earlier one, is not used. Bacterial contamination of vaginal flora is common in the tissue that is obtained from the abortion. Once the embryonic brain tissue is identified, it can be rinsed repeatedly with a few milliliters of sterile saline or buffer, effectively removing most bacteria. It is important to use a sterile hood and that those involved be dressed in sterile gear because the identification and dissection of the brain tissue in the aborted material can be both difficult and time consuming.

## *2.5. Tissue Dissection*

It is relatively easy to identify and dissect the ventral mesencephalon in the few cases when the embryo is retrieved intact. In most cases, however, the embryo/fetus is partially destroyed during the abortion, making brain dissection a difficult task. According to the Lund University experience, it is possible to identify the ventral mesencephalon in 50–75% of the attempts (Brundin, 1992). Detailed knowledge of the embryonic brain anatomy is essential to maximize the chances of finding the desired donor tissue. Several anatomical landmarks can help identify the mesencephalon, so the vast majority of dopamine neurons, and as few non-dopaminergic cells as possible, are included in the graft. The objective of this chapter is not to provide a detailed dissection guide because photographs of the developing human mesencephalic dopamine system and dissection guides have already been published elsewhere (Brundin, 1992; Brundin and Sauer, 1991; Freeman and Kordower, 1991; Frodl et al., 1994). Nonetheless, a brief outline of how to dissect the human embryonic mesencephalon is still warranted. Using a dissection microscope, iridectomy scissors, and watchmaker's forceps (for details regarding instruments, see [Brundin, 1992]) the central nervous system is dissected free and separated from overlying skin. A rostral cut in the brainstem is made just caudal to the border of the mesencephalon and diencephalon (Fig. 8.1). The caudal limit of the substantia nigra anlagen is at the tuberculum interpedunculare of Hochstetter, which forms the borders of a fossa that has a doughnut-like shape (Freeman and Kordower, 1991; Freeman et al, 1991; Hochstetter, 1919). In a Carnegie stage 20 embryo (around 21–23 mm CRL) the rostrocaudal length of the dissected piece is around 2 mm. Finally, two cuts are made so that only the ventral one third to one half of the mesencephalon is retained (Fig. 8.1). The dissection of the human ventral mesencephalon encompasses dopaminergic neurons both in the substantia nigra and in the adjacent ventral tegmental area (VTA). It is important to clarify, however, that even following careful dissection as few as 3–10% of the dissected ventral mesencephalic cells are estimated to be dopaminergic (Sauer and Brundin, 1991; Silani et al., 1994). Both morphological and neurochemical evidence suggests that some of the cells in the dissected human mesencephalon differentiate into serotonergic neurons when grafted into rats (Stromberg et al., 1989, 1991), although serotonergic neurons were not observed when similar tissue was implanted into PD patients (Kordower et al., 1996). Serotonergic neurons have also been found in rat nigral tissue allografted to the striatum, where they have been reported to give rise to a serotonergic hyperinnervation (Ishida et al., 1998; Takeuchi et al., 1991; Wright et al., 1991). It has been suggested that non-dopaminergic neurons in the ventral mesencephalic graft tissue could contribute to the generation of involuntary movements that have been seen after transplantation in some patients. Specifically, it has been proposed that serotonergic neurons derived from the primoridum of the mesencephalic raphe nucleus could promote graft-induced dyskinesias (Lane et al., 2006; Chapter 10).

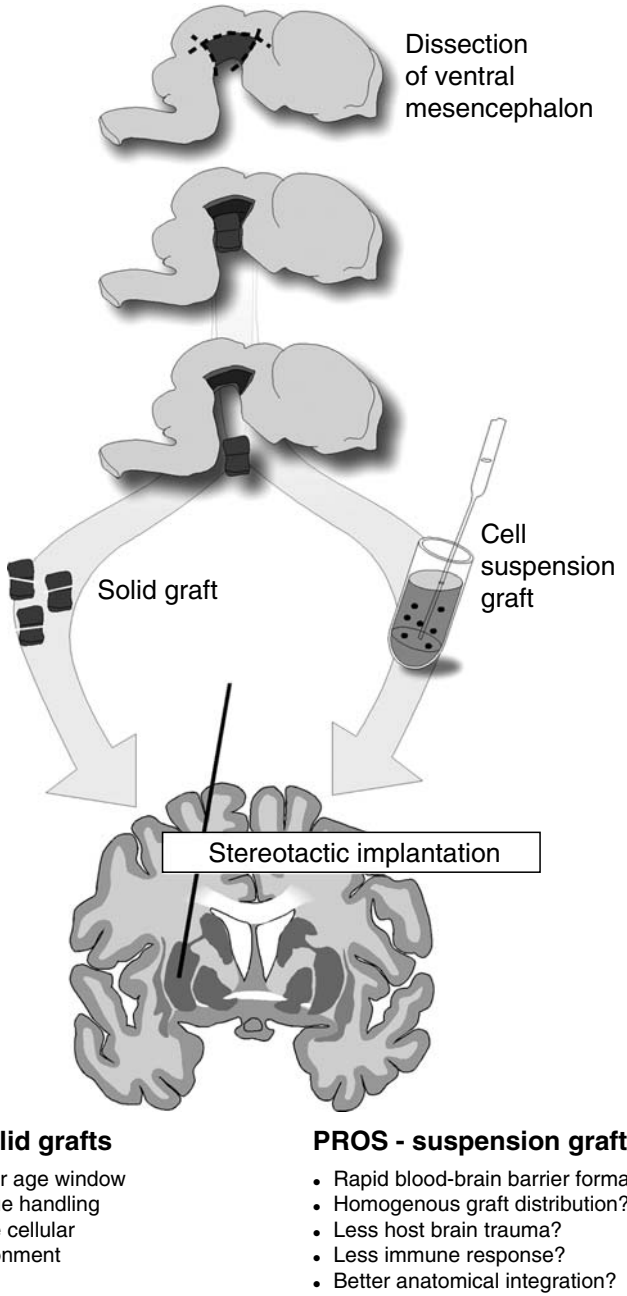


FIGURE 8.1. Schematic drawings illustrating the dissection of ventral mesencephalon, preparation of the donor tissue as a cell suspension or as solid tissue grafts, and the stereotactic injection of a transplant into the putamen. Boxes summarize established and suggested pros with each respective type of tissue graft.

The precise part of the mesencephalon that is grafted determines not only to what extent non-dopaminergic neurons are included, but also the type of dopamine neurons that are implanted. There are major differences in the projections and the physiological features of dopamine neurons from the substantia nigra pars compacta (that primarily innervate the caudate and putamen) and those located in the VTA (that innervate limbic and cortical regions). This may impact both on the degree of beneficial effects, as well as the risk for unwanted side effects. *First*, the dopamine neurons of the VTA do not appear to reinnervate the striatum as readily as those from the substantia nigra. Early studies in rats suggested that dopaminergic neurons from the VTA, which coexpress cholecystokinin, could survive grafting (Schultzberg et al., 1984). However, they did not innervate the striatum to a great extent and only reached areas immediately adjacent to the implant. Therefore, these cells were unlikely to contribute to the functional efficacy of the graft. In contrast, neurons derived from the more lateral parts of the grafted ventral mesencephalon (i.e., destined to become substantia nigra neurons) provided a new dopaminergic innervation extensively throughout the host striatum (Schultzberg et al., 1984). A recent study has elegantly extended these findings and shown that the dopaminergic neurons in mouse mesencephalic grafts that innervate the host striatum also express the potassium channel protein called Girk2, which normally is almost exclusively found in substantia nigra neurons (Thompson et al., 2005). These Girk2-positive cells were primarily angular in shape, and located around at the periphery of the grafts. The majority of the dopaminergic neurons located in the core if the implants were smaller, more round in shape, and presumably derived from VTA primordium because they did not express Girk2. Instead they were immunoreactive for the calcium-binding protein calbindin, which is normally found in most dopaminergic neurons of the VTA. In contrast to the Girk2-positive dopamine neurons, they did not extend axons into the surrounding host striatum, but instead innervated, e.g., the frontal cortex (Thompson et al., 2005). Similar observations were recently made post-mortem in two PD patients grafted with cell suspensions of embryonic nigral tissue (Mendez et al., 2005). Thus, around 70% of TH-immunopositive neurons in grafts located in the putamen also expressed Girk2 and were found at the periphery of the implants. Around 40% of the TH-neurons coexpressed calbindin, suggesting that they came from the VTA, and tended to lie in the center of the graft tissue (Mendez et al., 2005).

*Second*, it has been speculated that dopamine neurons from the VTA, which differ from those in the substantia nigra regarding several physiological properties (see Chapter 9), could underlie some of the unwanted side effects of mesencephalic grafts in PD (see Chapter 10, for further discussion). This theory assumes that the limited innervation they can provide of the host striatum gives rise to a non-physiological dopamine release. This hypothesis currently seems less plausible in view of the recent demonstrations that dopamine neurons derived from the VTA in embryonic mouse (Thompson et al., 2005)

and human (Mendez et al., 2005) ventral mesencephalon do not innervate the striatum to a great extent.

## 3. Tissue Preparation

There are two fundamentally different approaches to preparing the embryonic ventral mesencephalic tissue before it is implanted into the brain of a PD patient. The tissue can be grafted as solid tissue, either one single piece or after being cut into several smaller blocks, or the mesencephalic tissue is mechanically disrupted into single cells or small aggregates. There exist methodological variations of the two techniques, and both approaches have been reported to be successful in different clinical studies. In this section, we discuss the impact of tissue preparation on the size of the donor age window, graft survival, reinnervation of the host brain, and the host immune response to the transplant.

### *3.1. Solid Grafts*

Solid grafts of nigral tissue can survive well in the anterior chamber of the eye (for review see Olson et al., 1985) and this experimental model was studied extensively in the 1970's. In contrast, initial reports indicated that solid mesencephalic transplants survived poorly when grafted intraparenchymally (Björklund and Stenevi, 1984; Stenevi et al., 1976). It was postulated that the lack of vascular supply limited survival of the solid grafts when transplanted directly into the brain parenchyma. In comparison, solid nigral grafts were found to survive well in proximity to the vascularized ventricular surface or an aspiration cavity made one week earlier in the cortex (Stenevi et al., 1985; Björklund and Stenevi, 1984; Rosenstein and Brightman, 1978). Subsequently, solid pieces of rat (Stromberg et al., 1985) and human (Freeman and Kordower, 1991; Stromberg et al., 1989) embryonic mesencephalic tissue were demonstrated to survive transplantation intraparenchymally. Typically the size of the tissue pieces were around 0.5 mm in diameter. This stimulated the use of solid grafts in clinical trials. Thus, solid grafts have been used clinically, with preliminary evidence of efficacy in mid-stage PD patients (Freeman et al., 1995a; Hauser et al., 1999). Another type of solid tissue transplant, which has first been maintained in explant tissue culture, has been also used in several patients (Freed et al., 2001). In this case, the dissected embryonic tissue is extruded through a thin glass pipette so that it forms an elongated shaped tissue strand. These tissue pieces have been maintained in vitro for 1–4 weeks from the time of harvesting to the time of implantation (Freed et al., 2001).

### *3.2. Cell Suspension Grafts*

Cell suspension mesencephalic grafts were originally developed by Björklund and colleagues in 1980, as an adaptation of a dissociated cell culture protocol to the transplantation paradigm (Björklund et al., 1980). The technique

has been described in several earlier review articles (Björklund and Dunnett, 1992; Brundin et al., 1985b; Brundin and Strecker, 1991). Briefly, the technique involves a step where the tissue is incubated in a digestive enzyme, e.g., trypsin, in order to cleave intercellular bonds. Following removal of the enzyme through rinsing, the tissue is mechanically dissociated, typically by repeated passing through the tip of a fire-polished Pasteur pipette or the plastic tip of a micropipette. Consequently, the tissue is transferred into a mixture of single cells and small aggregates of cells. The vigor of the mechanical dissociation procedure and the size of the opening in the pipette govern how many single cells versus small aggregates are obtained. Some protocols include the presence of DNase in the dissociation medium. This enzyme digests DNA that has leaked out from dead and disrupted cells. Thereby the adhesiveness of the cells is reduced and the cell suspension is much easier to handle, e.g., when trying to obtain multiple graft aliquots of equal size. Stickiness of the cell suspension can particularly be a problem when using human donor tissue, which is why DNase is recommended in clinical protocols (Brundin, 1992; Brundin and Sauer, 1991).

## *3.3. Comparing Solid Versus Cell Suspension Transplants*

Unfortunately very few studies have directly compared solid and dissociated nigral tissue grafts. Moreover, different versions of the two techniques are available and make it difficult to generalize any differences in, e.g., survival and integration that may exist. Also, some of the differences may only be relevant in the animal models in which they were studied. We know very little about their relevance to the specific conditions that apply during clinical transplant surgery. Despite all these caveats, it is still interesting to review the literature and identify areas in which the two technical approaches to neural transplantation may generate divergent results (summarized in Fig. 8.1).

### 3.3.1. Solid Versus Suspension Transplants: Tissue Collection, Cell Survival, and Graft Integration

There are certain theoretical and practical advantages to the clinical use of solid mesencephalic transplants. As mentioned above, solid transplants provide a longer donor age "window" than suspension grafts, making it easier to obtain more starting material for each patient. Solid grafts also have the practical advantage that the surgeon can personally be responsible for tissue handling, eliminating the need for extra tissue handlers to be available during the surgery. Dopamine neurons transplanted in solid pieces of mesencephalic tissue are surrounded by appropriately organized mesencephalic glia, which may be important for the survival and maturation of the dopaminergic cells. Axotomy-induced cell death of adult nigral neurons is more extensive when the axotomy is closer to the cell soma (Reis et al., 1978), which would speak in favor solid grafts that entail less trauma to the donor cells than the cell suspension procedure. Nonetheless, in a study that examined cell death in

embryonic rat ventral mesencephalic tissue it was clear that many cells die in conjunction with the initial dissection, regardless of whether the tissue was left intact or dissociated afterwards (Emgard et al., 2002). After mechanical tissue dissociation, several additional cells died or exhibited a dramatically deranged morphology over the ensuing 4 hours. This study did not address if cells would fare better if they had been left as whole ventral mesencephalic tissue pieces for the same time (Emgard et al., 2002). Indeed, as mentioned earlier there are few direct comparisons of solid and cell suspension grafts in the same study. In a comparison of cell suspension grafts and strand grafts prepared from embryonic rat mesencephalon, it was claimed that the survival of dopamine neurons was better in the strand grafts (Clarkson et al., 1998). These data have to be interpreted with great caution, since the survival rate of dopaminergic neurons in the cell suspension group was at least 10-fold lower than expected from several earlier studies. In another study, which instead focused on human-to-rat mesencephalic xenografts, the frequency of transplants that survived was compared between solid and suspension transplants (Freeman et al., 1995b). The survival was similar with the two methods, but the variability appeared greater with the solid implants. Since no quantification of the number of surviving dopaminergic neurons was performed, it is difficult to draw definite conclusions. Finally, in terms of information on cell survival from clinical studies, information is limited to a small number of studies where the brains of patients have been examined morphologically after transplantation (Freed et al., 2001; Kordower et al., 1995, 1996, 1997a, 1998a,b; Mendez et al., 2005; Olanow et al., 2003; for review see Hagell and Brundin, 2001). It is difficult strictly to compare results obtained with cell suspension versus solid transplants in humans also, because the numbers of observations are low and multiple parameters vary between the different studies. For example, age and disease stage of patients, age of the donor tissue, amount of tissue grafted, type of implantation instrument used, numbers of implants per site, and, importantly, immunosuppressive regimens have differed. Disregarding these important factors for the moment, there are published clinical data on the number of surviving cells per donor when using three approaches: the tissue strand method, solid tissue grafts, and the cell suspension technique. With tissue strands, on average 15,000 dopamine neurons survived per donor (Freed et al., 2001); with solid tissue grafts the estimates vary from 25,000 to 50,000 (Kordower et al., 1996; Olanow et al., 2003); and with the cell suspension technique it has been calculated that 30,000–50,000 dopamine neurons survive per donor (Mendez et al., 2005). These latter observations are in good agreement with a survival rate of 35,000–40,000 dopamine neurons per donor that was reported for human mesencephalic cell suspensions xenografted to immunosuppressed rats (Frodl et al., 1994).

The cell suspension method also has some advantages over transplanting solid tissue blocks. First, it is easier to distribute graft tissue at multiple sites using a microsyringe. If a homogenous cell suspension is obtained, it is possible to graft cells of identical quality at each site. However, there is a mixture

of single cells and aggregates in the routine cell suspension method that has been employed in clinical trials. With this preparation, the variability in the number of surviving dopamine neurons can be as high as 100-fold between different implants made from the same cell suspension (Karlsson, 2001). Variability is reduced with the so called "microtransplantation" variant of the cell suspension method (Nikkhah et al., 1994). With this technique, the cells are dissociated into a single cell suspension, subjected to a centrifugation step, and then injected through a narrow glass capillary, which causes little trauma to the host brain. While the minimal trauma to the target structure may be particularly favorable for the transplanted cells, the drawback with this method is that the tissue preparation step is relatively harsh and many cells are lost already before the injection step (for discussion see Brundin et al., 2000a). Second, the fact that the internal tissue structure in the graft tissue is disrupted in cell suspension implants has been suggested to facilitate the integration of the new cells with the host and promote the formation of efferent and afferent connections to and from the surrounding brain. For example, such claims have been made when comparing grafts of solid versus dissociated striatal tissue (Watts et al., 2000). Both grafts of solid and dissociated nigral tissue have repeatedly been demonstrated to clearly innervate the host brain in rodents and humans. Whether the two types of transplants differ regarding their capacity to innervate the host brain is not clear. When it comes to connections from the host to the transplant, these have been demonstrated using morphological (Doucet et al., 1989) and electrophysiological (Fisher et al., 1991) techniques for cell suspension transplants. There are also host afferents innervating *solid* nigral grafts. This has been shown by electrophysiological recordings from transplants at the same time as stimulating different host brain regions (Arbuthnott et al., 1985). At the end of the day, there is no firm evidence showing that the connections with the host brain are more abundant in cell suspension than solid grafts.

### 3.3.2. Solid Versus Suspension Transplants: Impact on Host Immune Responses

The immune response in the brain in response to transplants is definitely milder than in most other body locations. Thus, there is the idea that the brain is an immunologically privileged site (Barker and Billingham, 1977). This does not mean, however, that immunological rejection of transplants cannot occur in the brain (Widner and Brundin, 1988). For example, intracerebral neural allografts (same species of donor and host, but genetically dissimilar individuals) can be rejected, especially if the host immune system is stimulated by a high level of donor antigens. Experimentally, this can be achieved, e.g., by inclusion of highly immunogenic cells in the transplant tissue (Duan et al., 1997a) or by peripheral immunization using skin allograft from the same donor strain that provided the intracerebral neural transplant (Duan et al., 1997b).

Three differences between solid tissue and cell suspension neural transplants could be particularly important for the ability to evoke immune rejection. First, with cell suspension grafts, a relatively thin injection cannula can be used, thereby minimizing the trauma to the host brain. This may reduce the risk of an intense local inflammatory response. A localized inflammation at the transplant site might promote the triggering of an immune rejection through up-regulation of antigen expression on the graft cells as well as increased activation and migration of host immunocompetent cells. Second, in cell suspension transplants the blood vessels are mostly derived through vascularization from the host brain, whereas in solid tissue transplants they are more likely to come from the donor tissue (Baker-Cairns et al., 1996; Broadwell et al., 1990; Broadwell et al., 1991) This has implications for graft rejection since endothelial cells express high levels of transplantation antigens and therefore are likely to trigger a vigorous rejection response if they are derived from the donor. Third, the blood–brain barrier forms more quickly in cell suspension grafts than in solid grafts (Rosenstein, 1987; Brundin et al., 1989).

Taken together, these studies in experimental animals tentatively suggest that solid grafts are more susceptible than cell suspension grafts to immune rejection. Some support for this idea comes from comparing the infiltrates of immune cells in the grafts between patients receiving either solid or cell suspension grafts. In PD patients that received solid tissue implants and a six month period of immunosuppression, there were numerous activated microglia, macrophages, T- and B-cells within the graft sites (Kordower et al., 1997b; Olanow et al., 2003). Similar evidence of an immune or inflammatory response has been obtained in post-mortem studies of patients implanted with nigral tissue strands and that did not receive any immunosuppression (Freed et al., 2001). By contrast, in a recent report on the post-mortem histology of grafts in two patients receiving cell suspension implants, followed by 6 months immunosuppression with cyclosporine A, there was only minimal microglial activation is described (Mendez et al., 2005).

So what are the implications of the host immune or inflammatory response to the solid nigral grafts? Obviously it does not necessarily lead to complete rejection of the implants, because the post-mortem examinations also reveal survival of numerous TH-immunoreactive cell bodies in the solid grafts (Freed et al., 2001; Kordower et al., 1996; Olanow et al., 2003) and from positron emission tomography (PET) studies it is clear that they have the capacity to take up fluorodopa (Freed et al., 2001; Hauser et al., 1999; Olanow et al., 2003). However, not only is survival of the graft tissue important for clinical efficacy, but also functional integration is required for the development of substantial clinical recovery in patients (Piccini et al., 2000). It is possible that solid grafts induce a mild immune response which is not strong enough to produce a complete graft rejection with removal of all the transplant tissue, but causes an intermittent dying back and regrowth of neuritic extensions. This theory would potentially explain the persistence of immature

morphology of synapses which has been described in solid grafts in PD patients (Kordower et al., 1996). Moreover, in human solid tissue transplants placed in PD patients, there is a conspicuous absence of beaded varicosities on the TH-immunopositive axons growing out from the transplants (Kordower et al., 1995, 1996). Beaded varicosities normally develop on dopaminergic axons by the 21st week of gestation (Pickel et al., 1980), and their absence in neurons from solid grafts could indicate constant remodeling of the neuritic tree, possible due to immune attack. By contrast, numerous beaded varicosities were seen on the dopaminergic axons derived from the transplants in post-mortem studies of patients who had received cell suspension grafts (I. Mendez, personal communication). This may indicate that they are not undergoing a rejection. There is also tentative evidence for loss of clinical efficacy seen after discontinuation of immunosuppression in patients grafted with solid tissue. In the study by Olanow et al. (2003), patients received either imitation operations as a placebo control, or solid grafts derived from either one or four donors per side into the postcommissural putamen. Significant clinical improvement in comparison control was demonstrated in both transplant groups at six months after surgery. This time point is equivalent to when cyclosporine treatment was discontinued. However, after discontinuation of cyclosporine, the improvements in symptoms gradually disappeared between months nine and twelve for both the low and high dose groups (in comparison to the subjects in the placebo arm of the trial). The temporal correlation of the discontinuation of cyclosporine followed by the loss of graft-induced efficacy suggests that an immune response may be involved in terminating the clinical benefit (Winkler et al., 2005). The late appearance of graft-induced dyskinesias at different time periods after discontinuation of cyclosporine treatment in these patients (Olanow et al., 2003) means that one cannot exclude that immune mechanisms also contribute to the triggering of involuntary movements (see Chapter 10; Winkler et al., 2005). However, there is no clear evidence that the loss of transplant efficacy was due to the number of remaining grafted dopamine neurons being low. In fact, between 80,000 and 120,000 dopamine neurons were still present on each side of the brain in a total of four patients who received four donors per side, and approximately 30,000 dopamine neurons survived on each side in the two patients who died and had received one donor per side (Olanow et al., 2003). A major challenge for the future will be to understand if immune rejection mechanisms can affect grafted neurons without actually causing their complete rejection and whether this inhibits their function.

## 4. Tissue Storage

Storage of donor tissue before transplantation presents several advantages. First, it is possible to pool brain tissue from abortions taking place on different days and thereby increase the amount of donor tissue available for each

surgical session. Second, the delay allows time for careful bacteriological and virological examination of the donor, so the risk for infection of the host can be eliminated. Third, the storage time could conceivably be used to expose the donor tissue to factors that improve graft performance, e.g., increase cell survival or axon outgrowth. Fourth, should immunological matching between donor and recipient turn out to be valuable in the future, it can be performed during the storage period. Fifth, informed consent regarding donation of tissue can be obtained from the woman undergoing abortion a significant time after the abortion has taken place. Then she is no longer dependent on the health care system for treatment and therefore can make her decision completely independently.

In principle three different approaches to storing embryonic mesencephalic tissue prior to grafting have been tested: explant or cell culture, freezing, and refridgeration. In the following section we briefly compare these methods and discuss their relevance to clinical transplantation trials in PD.

## *4.1. Comparison of Cryopreservation, Hibernation, and Cell Culture*

The longest possible duration that donor tissue can be stored prior to grafting is directly related to the exact storage method used. Freezing allows the donor tissue to be stored almost indefinitely, e.g., if it is maintained at temperatures below –90°C (for review of freezing methods see [Brundin and Sauer, 1991]). By contrast, the storage time when employing either tissue culture or refrigeration at temperatures above zero (called "hibernation") is limited (Brundin and Sauer, 1991). In the case of tissue culture, the dopaminergic neurons continue to develop *in vitro* and eventually reach a developmental stage when they do not survive additional handling and injection into the host brain. Using dissociated cell culture techniques, dopamine neurons can only be grown for a few days in vitro prior to transplantation (Brundin et al., 1988). After that, the survival rate of the neurons drops dramatically, as they have developed long processes and cannot survive the trauma of being redissociated from the culture dish. In explant cultures, the dissected mesencephalon is grown as a tissue piece. It can be maintained for at least 4 weeks and then survive subsequent grafting using the tissue strand method described earlier. During the time in culture, the tissue can be exposed to growth factors, such as insulin-like growth factor-I (IGF-I) and basic fibroblast growth factor (bFGF) which have been claimed to improve the survival of the grafted neurons (Clarkson et al., 2001). This method has been tested clinically, and tissue stored for between one and four weeks was implanted in the major double-blind placebo control clinical trial reported by Fahn, Freed and coworkers (Freed et al., 2001). The pitfall with this approach is that it is still not known how the culture period alters the composition of the grafted tissue. Possibly, certain glial and neuronal populations proliferate while

other cell populations die during the storage period. It has been speculated that changes in the tissue composition is one factor contributing to the runaway dyskinesias observed in the Fahn/Freed study (see Chapter 10; Björklund et al., 2003).

The method that allows for the longest storage period is freezing. Embryonic brain tissue from different brain regions has been successfully frozen at –90–196°C and thereafter grown in cell culture or grafted as solid pieces or cell suspensions. Generally, it is possible to obtain some survival of neurons but typically 30–70% more cells die as a consequence of the freezing step than if the same tissue had been grafted without storage. The outcome of these experiments are reviewed in detail other publications (Brundin and Sauer, 1991; Frodl et al., 1994). In the present context it is particularly interesting to review experiments done with frozen embryonic ventral mesencephalic tissue. Interestingly, in one clinical trial tissue that had undergone prior freezing was implanted unilaterally into the caudate nucleus of four presumed PD patients (Spencer et al., 1992). Clinical recovery and evidence for surviving grafts on PET was uncertain for these patients (Spencer et al., 1992). In one case which was found to suffer from striatonigral degeneration, the brain was examined post-mortem and only one purported TH-immunoreactive cell was detected in the graft region (Redmond et al., 1990). Experiments performed in animals have shown that dopaminergic neurons can survive transplantation after freezing when harvested from rat (Collier et al., 1988; Sauer et al., 1992), non-human primate (Collier et al., 1987, 1988) or human (Frodl et al., 1994; Redmond et al., 1988; Robbins et al., 1990) embryos. Experiments conducted with human tissue showed that the survival of grafted dopamine neurons that had been frozen was only around 40% of the survival obtained with fresh tissue (Frodl et al., 1994). In summary, freezing is an option for clinical trials, but since the additional loss of dopamine neurons with current protocols is dramatic it cannot be recommended when access to donor embryos is limited.

The final option for tissue storage is hibernation, i.e., refrigeration at around 4°C. This approach has been used extensively in clinical trials (Freeman et al., 1995a; Mendez et al., 2000a; Olanow et al., 2003). When an appropriate preservative medium is used, animal experiments show that rat and human dopamine neurons stored for 2–3 days survive grafting as a cell suspension equally well as when taken from fresh embryos (Nikkhah et al., 1995; Sauer and Brundin, 1991). When the tissue is stored for longer periods, e.g., 5–12 days, there is a gradual drop in survival of grafted dopamine neurons (Nikkhah et al., 1995; Sauer and Brundin, 1991). For rat tissue, this can be partially prevented by supplementing the preservation medium with lazaroids (Grasbon-Frodl et al., 1996) which inhibits lipid peroxidation. Glial cell line-derived neurotrophic factor (GDNF) has also been added to the preservation medium and it has been claimed that the survival of rat dopamine neurons is increased (Apostolides et al., 1998). These data should, however, be interpreted with caution since another study did not detect any

effect of GDNF when added to the hibernation medium (Petersen et al., 2000). From a theoretical standpoint it is conceivable that GDNF, which protects dopamine neurons from death in many other settings (see below), cannot elicit its protective effect on cells that have shut down their metabolic activity at 4˚C due to the low temperature (Brundin et al., 1999). Nonetheless, GDNF supplementation of hibernation medium has been employed in clinical trials, either alone (Mendez et al., 2000a) or in combination with lazaroids (unpublished data, Lund Clinical Neural Transplant Team) when the tissue has been stored up to 8 days.

## 5. Graft Size

Experiments in rodents have unequivocally shown that there is a close relationship between survival of grafted dopamine neurons and functional effects observed in the experimental animals. The consensus is that there is a threshold minimum number of neurons needed for amelioration of lesion-induced deficits to occur (for reviews see [Brundin and Hagell, 2001; Hagell and Brundin, 2001]). It is believed that more dopamine neurons lead to a faster onset, and to some extent greater degree, of functional recovery in rodents with experimental parkinsonism. At a certain graft size, addition of further numbers of dopamine neurons does not provide additional benefit, but a plateau effect is reached (Brundin and Hagell, 2001).

For PD patients the relationship between graft survival and clinical benefit is not well understood. Clearly, an absence of surviving dopamine neurons in transplants is never associated with symptomatic relief, but the minimum number of neurons required to observe a beneficial effect is not known. It has been suggested that around 100,000 dopamine neurons are needed in each putamen for major transplant-induced effects to occur (Hagell and Brundin, 2001). Likewise, it is not really know beyond what graft size in PD patients that there is no additional clinical benefit, or if grafts that are too large even can cause unwanted side effects. Using PET, it has been shown that neural grafts can normalize fluorodopa uptake in a restricted region such as the putamen (Piccini et al., 1999), and post-mortem studies suggest that the density of TH-immunoreactive fibers is close to normal in proximity to the cell suspension implants (Mendez et al., 2005). So far, no reports have indicated unwanted side effects as a consequence of transplants containing too many dopamine neurons. One study reported a correlation between the severity of graft-induced dyskinesias and a particularly high flurodopa uptake on PET scans in two small restricted parts of the putamen (Ma et al., 2002). This is more likely to have been a due to uneven distribution of graft tissue than too many dopamine neurons. Thus, patients exhibiting graft-induced dyskinesias have not exhibited greater generalized increases in flurodopa uptake in the whole grafted striatum than transplanted patients who did not experience the involuntary movements (Hagell et al., 2002; Ma et al., 2002). In summary,

there is reason to believe that large grafts with numerous surviving dopamine neurons are desirable in PD patients. In the following section we discuss how improved survival of transplanted dopamine neurons can be achieved.

## *5.1. Cytoprotective Additives/Procedures*

The survival rate of grafted rat and human dopamine neurons has been estimated at 1–20% in several studies in experimental animals (for review see [Brundin et al., 2000a]). The vast majority of cells die during the preparation of the tissue (Emgard et al., 2002) and during the first week after grafting (Barker et al., 1996; Emgard et al., 1999; Sortwell et al., 2000). As expected, information regarding survival of dopamine neurons in patients is limited and based on those cases (less than 25) who have come to post-mortem examination. Based on these, it is estimated that 1–15% of dopamine progenitors implanted survive in the grafts (Hagell and Brundin, 2001; Mendez et al., 2005; Olanow et al., 2003). Several neuroprotective strategies have been tested with varying degrees of success in attempts to increase the survival of grafted dopamine neurons in rodents. To review all of these in detail is beyond the scope of this chapter and the reader is referred other articles focused on this issue (Brundin et al., 2000a; Sortwell, 2003). In most cases they have been applied to the tissue prior to implantation, and in a few instances the host has also been treated with the protective agent. Briefly, they can be categorized into growth factors or drugs that primarily counteract oxidative stress, caspase activation, stress-activated protein kinases and anoikis (cell death due to insufficient cell-cell contact) (for reviews see Brundin et al., 2000a and Sortwell, 2003, and for more recent references see Boll et al., 2004; Macauley et al., 2004). Typically, the survival rate of grafted dopamine neurons increased by a factor of two to three. One of the highest reported survival rates for grafted dopamine neurons is around 50%. It was obtained when grafts were treated with a combination of a caspase inhibitor and a lipid peroxidation inhibitor, and implanted into rats that were rendered hypothermic during surgery in order to reduce the continued graft cell death in the host brain (Karlsson et al., 2005). Out of all the different neuroprotective protocols tested in rats, only two agents have so far been reported in clinical trials. The lipid peroxidation inhibitor lazaroid tirilazad mesylate was added to the ventral mesencephalic tissue grafted to 5 PD patients in Lund, Sweden (Brundin et al., 2000b), and GDNF has been added to grafts given to a number of patients in Halifax, Canada, (Mendez et al., 2000a) as well as two patients in Lund (unpublished data, Lund Clinical Neural Transplant Team).

Some interesting basic neurobiological issues can be raised concerning the application of neuroprotective protocols to the clinical setting. In the clinical lazaroid study, it was estimated that the same degree of reinnervation, as monitored using PET, and same level of symptomatic recovery were obtained by grafting 40–50% less tissue compared to previous trials in the same center

(Brundin et al., 2000b). Indeed, one primary gain of using neuroprotective protocols in clinical trials is that they open up the possibility of using less donor tissue per patient, thereby facilitating the logistics of each operation. A reduced amount of donor tissue has been dissociated in the same volume of vehicle as used in earlier trials. Thus, the cell density has been reduced with, e.g., around 50% when the lazaroid agent is added. A word of caution concerning this trial design is warranted. Most preclinical trials in experimental animals that have tested the effects of neuroprotective agents in grafts have examined whether their addition increases the number surviving dopamine neurons when compared to a cell suspension with an *equal* density of cells (see, e.g., Hansson et al., 2000; Nakao et al., 1994). Anoikis appears to play a role in the death of grafted dopamine neurons (Marchionini et al., 2003; Sortwell., 2003). It is unlikely that neuroprotective agents are equally effective at promoting survival of neural grafts at all cell densities. Indeed, one experience is that caspase inhibitors, which are effective under certain conditions (Hansson et al., 2000; Schierle et al., 1999) do not protect dopamine neurons in cell suspensions when the cell density is very low (Hurelbrink et al., 2001; Marchionini et al., 2004; Brundin, unpublished observations).

A second issue that is important to consider when translating basic science concerning neuroprotection findings to the clinical neural transplantation setting is the precise timing of the treatment. For example, there are several studies showing that GDNF treatment improves dopamine neuron graft survival in rats (see, e.g., Rosenblad et al., 1996; Sinclair et al., 1996 and reviews in Brundin et al. 2000a and Sortwell 2003). In the successful studies, the growth factor was applied to the graft by intracerebral infusion after transplantation surgery, not only during the tissue preparation. Nevertheless, in the clinical protocol used to treat nigral grafts with GDNF the tissue was exposed to the factor during a pre-transplantation tissue storage period at 4˚C (Mendez et al., 2000a). Although some experimental data indicates that GDNF may increase survival in this tissue storage paradigm (Apostolides et al., 1998), other data suggest that the growth factor is inactive when the cells are cooled down to 4˚C (Petersen et al., 2000). As mentioned earlier, at 4˚C the cells ought to be in a quiescent state and several metabolic processes, including growth factor signaling, could be arrested (Brundin et al., 1999). These two illustrations of pitfalls of translating of pre-clinical protocols into clinical practice illustrate that the neural transplantation technique is sensitive in every step. Small details in the protocols that initially appear insignificant may have profound influence on the outcome of the grafting procedure, and moving transplantation methods from the laboratory bench to the bedside is far from trivial.

## *5.2. Numbers of Donors*

The numbers of donors used for grafting in each PD patient has varied dramatically both between and within different clinical studies. The lowest number of donors per side of the brain has been one and the highest number is

seven (reviewed in Hagell and Brundin, 2001). As mentioned earlier, it is difficult to examine the role of one surgical factor, e.g., number of donor embryos, by comparing the outcomes between different studies simply because the grafting protocols differ in many respects. Only the study by Olanow and coworkers (Chapter 6 and Olanow et al., 2003) have compared the effects of different amounts of donor tissue in a systematic fashion, with all other experimental parameters maintained identical. Thus, the double-blinded study compared the effects of mesencephalic tissue from of one versus four donors implanted into the putamen on each side of the brain. As discussed above, the final outcome of the study was that neither grafted group exhibited a significant improvement compared to control at the predetermined endpoint 24 months after surgery (Olanow et al., 2003). Nevertheless, both transplanted groups showed equal signs of recovery compared to an imitation surgery control group at earlier time-points, before cyclosporine immunosuppression was discontinued. Two and four patients died due to causes unrelated to the clinical trial in the transplanted group receiving one and four donors per side, respectively. When their brains were examined post-mortem, 80,000–120,000 TH-immunoreactive dopamine neurons had survived per side in the four-donor patients and around 30,000 dopamine neurons on each side in the one-donor patients (Olanow et al., 2003). Based on this study, it is not possible to draw conclusions regarding what is the minimum or ideal number of donors needed for one patient. The study does show, however, that the amount of donor tissue, as expected, impacts on the number of dopamine neurons that survive. Earlier reviews of the literature have suggested that it is necessary to have at least around 100,000 surviving dopamine neurons in the putamen in order to achieve significant reductions in PD motor symptoms (Hagell and Brundin, 2001). The recent report from Mendez and coworkers showed that using the cell suspension method around 30,000–50,000 dopamine neurons survive grafting from one donor (Mendez et al., 2005). Based on these data, and the assumption that 100,000 surviving dopamine neurons in the grafts in one putamen are adequate, it would be necessary to graft 2–3 donors per putamen.

## 6. Transplant Injection Technique

Two fundamentally different approaches have been used for grafting. The open microsurgery technique which was advocated by Madrazo and coworkers has been described in detail elsewhere (Madrazo et al., 1990). It involves a small craniotomy and making a opening into the lateral ventricle. From this entrance point, the graft tissue is placed in the head of the caudate nucleus which borders onto the ventricle. The results with this technique have been variable and there is no PET evidence for robust graft survival (for review, see Rehncrona, 1997). The technique was also used with adrenal medulla grafting trials which have now terminated because the procedure was ineffective

and the surgery was associated with significant patient morbidity (e.g., psychiatric side effects) and mortality (Goetz et al., 1991). The open microsurgical approach for neural grafting has also now largely been abandoned.

The second approach is stereotactic surgery, which has been used both to implant cell suspensions and small solid tissue grafts into different targets within the basal ganglia. The advantages with this technique include that the surgery-related morbidity is relatively low and that it is possible to individually tailor the injections sites. Thus the reinnervation can reach the areas that are most denervated by the underlying disease process according to preoperative PET scans. Variations of the stereotactic transplantation method have been described in detail in published reports (Breeze et al., 1995; Freeman et al., 1998; Hitchcock et al., 1990; Mendez et al., 2000b; Peschanski et al., 1994; Rehncrona, 1997). In the following sections we do not provide methodological details, but instead briefly describe specific features that characterize the different stereotactic surgical approaches.

## *6.1. Instrumentation*

In most rat experiments, nigral grafts have been injected into deep brain sites through relatively thin cannulae, typically with outer diameters in the range 0.5–1.0 mm (reviewed in Rehncrona, 1997). Naturally, the clinical situation requires that the instrument be relatively long in order to reach the human basal ganglia, and this places special demands on the stability of the cannula. When the first two clinical neural grafting trials in Lund were performed, a highly rigid instrument with an outer diameter of 2.5 mm was used (Lindvall et al., 1989). The outcome in these first two PD patients was essentially negative, with a lack of graft survival on PET examinations and functional effects in the neurological assessment (Lindvall et al., 1989). One likely contributing reason for the poor outcome was that the outer diameter of the injection cannula was too large. In subsequent animal experiments we tested the instrument that had been used in the first two patients (Brundin et al., 1990). We compared the extent of host brain damage and the degree of survival between grafts injected into rat striatum using the instrument used clinically and two other instruments with cannulae with outer diameters 1.2 mm and 0.5 mm. The results clearly showed that the graft survival was greater with the two smaller instruments, and that signs of local brain damage (accumulation of blood cells, etc.) was greater in the rats implanted with the 2.5 mm instrument (Brundin et al., 1990). In the following 16 patients operated in Lund, an injection needle with an outer diameter of 1.0 mm has been used, and this has reached the target points through a rigid outer guide cannula that is 22 mm shorter and therefore never penetrates the site of the graft injection (Rehncrona, 1997). In these patients we have obtained PET evidence for graft survival (reviewed in Hagell and Brundin, 2001). Other centers using the stereotactic approach also use an injection instrument with an outer diameter of 1.0 mm or less (reviewed in Rehncrona, 1997). Later

experiments in rats have suggested that the use of a fine glass capillary (diameter around 50–70 μm) causes even less trauma at the injection site and therefore can improve transplant survival (Nikkhah et al., 1994). This technique, however, requires that the cells are dissociated into a single cell suspension—causing some additional cell death compared to less vigorously dissociated grafts—and it has not been tested in humans yet.

## *6.2. Numbers and Size of Graft Deposits*

The stereotactic technique allows that the tissue is distributed over multiple small sites in the striatum. With only one exception, the approach of multiple placements has been used in patients. In the exceptional case, Hitchcock and coworkers (Henderson et al., 1991; Hitchcock et al., 1990) made one single injection of 0.5–2.0 ml fluid containing tissue from one donor fetus into the head of the caudate nucleus. This is a large volume in relation to the structure it was injected into and presumably it must have caused significant local damage. There is no strong evidence that these grafts survived well or gave rise to functional benefits for the patients (Henderson et al., 1991). In other trials, the amount of cell suspension (a mixture of actual graft tissue and vehicle fluid) injected at each graft site has been in the range 20–30 μl (for review see Rehncrona, 1997). Typically each graft injection has been made slowly over several minutes and has been distributed over a distance of 7–14 mm in the target region. Whereas almost all centers have used a dorsal entry point into the striatum and made up to 14 injections per side of the brain (Freeman et al., 1995a, 1998; Rehncrona, 1997), Freed and collaborators have employed a frontal approach and injected 14–16 tissue strand nigral grafts along two trajectories in the longitudinal axis of each putamen (Breeze et al., 1995). Animal experiments suggest that human dopamine neurons xenografted to the rat can extend axons for up to 5 mm in the striatum (Brundin et al., 1986; Stromberg et al., 1986), and post-mortem findings in humans suggest that they grow up to 7 mm from the graft site (Kordower et al., 1995). With multiple injections into the human putamen it has been possible to space the grafts about 5 mm apart in the horizontal plane (Freeman et al., 1998; Rehncrona, 1997). The risk for graft-related hemorrhages is believed to be low (Rehncrona, 1997), and therefore the risks with making multiple placements have been considered manageable. Little is known, however, about what may be the optimal number of trajectories to make in each patient. It is conceivable that each graft injection increased the risks for other side effects than hemorrhaging. For example, when the severity of post-operative graft-related dyskinesias were examined in the Lund cohort of patients, a weak correlation was found to the number of graft injections made (Hagell et al., 2002). Obviously, the number of graft injections can covary with other crucial factors such as graft size, but there was no correlation with the postoperative increase in flurodopa uptake on PET scans (Hagell et al., 2002), indicating that the surgical trauma cannot be excluded

as a contributing factor to graft-induced dyskinesias. Interestingly, the highest frequency of off-medication, graft-induced dyskinesias (57%) has been reported with solid tissue grafts (Olanow et al., 2003). It could be speculated that they, by virtue of the size of the solid implants, cause more host brain trauma than tissue strand implants and cell suspension grafts which have been associated with a lower incidence of significant graft-induced dyskinesias (around 10–15%) (Freed et al., 2001; Hagell et al., 2002).

## *6.3. Graft Targets*

The striatum is highly organized in a somatotopic fashion (Delong et al., 1984; Romanelli et al., 2005). The role of the exact transplant location with relation to the underlying somatotopic organization has not been fully examined in humans, and modern imaging techniques are likely to facilitate this being more systematically explored. Three main issues have been discussed when it comes to graft targets. What can be achieved with unilateral versus bilateral grafts? What is the relative importance of placing transplants in the caudate nucleus versus putamen? Can transplantation to the homotypic location in the substantia nigra lead to additional clinical benefit? Since no clinical trials have addressed these questions in a systematic and controlled fashion, there are no definite answers available. However, some suggestions based on theoretical reasoning, animal experiments and scattered clinical observations in grafted patients can be made.

It is clear that unilateral grafts to the putamen can provide symptomatic relief on both sides of the body, but the effects appear to be greater contralateral to the implants, as expected (see, e.g., Piccini et al., 1999). In patients with a unilateral graft, the addition of a second transplant typically leads to additional benefit (Hagell et al., 1999). Taken together, there is a strong case for bilateral transplantation being better, although some investigators have questioned that it adds benefit beyond that obtained with unilateral implants (Clarkson and Freed, 1999).

Both the caudate nucleus and putamen undergo gradual loss of dopaminergic innervation in PD. Substantia nigra neurons innervating the putamen clearly die earlier in the disease process and therefore the reduction of dopamine is more pronounced in the putamen than in the caudate nucleus (Hirsch, 1994). The anatomical connections of the putamen, especially the postcommissural parts (Freeman et al., 1995a), strongly support that it is more involved in sensorimotor functions than the caudate nucleus. These observations suggest that the putamen should be the primary target for transplantation in PD, especially in cases that are not so advanced in the disease. Whether the addition of grafts in the caudate nucleus has additional effects is not known at this stage (Brundin et al., 2000b). It is conceivable, but unproven, that they can alleviate some of the non-motor, e.g., cognitive and emotional, symptoms seen in PD.

The dopamine neurons of the substantia nigra do not only release dopamine in the striatum, but also release transmitter from dendrites locally in the mesencephalon (Smith and Kieval, 2000). Transplants placed into the adult substantia nigra in rodent PD models do not extend many axons along the nigrostriatal pathway, but can provide a dopaminergic innervation locally (Winkler et al., 2000). It has been suggested that intranigral grafts can provide some additional benefits in rats grafted with tissue also in the striatum (Ramachandran et al., 2002; Winkler et al., 2000). Therefore a small number of patients operated in Halifax, Canada, has received implants into both the putamen and adjacent to the substantia nigra (Mendez et al., 2002). These initial trials show that the intranigral implants can survive and affect flurodopa uptake in the mesencephalon. In two patients who have been examined following death, the implants survived four—fold less well in the substantia nigra as compared to the putamen (Mendez et al., 2005). It was not possible to ascertain in this initial small group of patients whether the addition of the intranigral implants improved functional outcome beyond what can be achieved with grafts in the putamen. Further studies are required to examine whether transplantation of dopamine-producing cells into multiple sites, also outside the striatum, is more effective than grafting to the striatum alone.

## 7. Concluding Remarks—The Quest for a Perfect Protocol

As is apparent from this review, the surgical technique is extremely important when transplanting dopamine neurons to PD patients. While more than one neural transplantation protocol has produced good transplant survival and functional efficacy without major side effects, very small changes to a procedure can jeopardize graft survival and functional outcome. The research field is still at an early stage of developing the neural transplantation technique and we are far from having established a surgical protocol that can be considered perfect. Animal experimentation has played, and should continue to play, an important role in increasing our knowledge about surgical factors that influence graft outcome. However, a major problem with relying on animal experiments, in particular those performed in rodents, is that they only mimic clinical surgery and cannot address all the issues at stake. Experiments in non-human primates can partly fill this void and answer questions regarding, e.g., which tissue preparation results in the best fiber outgrowth and integration with the host. Notwithstanding the merits of animal experimentation, some aspects of neural transplantation methodology can only be developed in carefully designed surgical trials in patients. For such trials to be feasible on a larger scale, it is likely that we need alternative sources of donor cells. Probably, aborted embryos are associated with too many difficult practical and ethical issues for them to be considered suitable as donor tissue for large scale systematic neural transplantation trials. Therefore novel sources of

dopamine neurons, most likely derived from stem cells (Roybon et al., 2004; and Chapters 12 and 13), are needed before neural transplantation can be developed into a therapy for PD. Transplantation of stem-cell-derived neurons to the brain may introduce a new set of specific surgical variables that will require careful investigation, but existing knowledge obtained using primary embryonic tissue will no doubt constitute a valuable foundation onto which one can build such novel therapies.

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# 9

# Histopathological and Clinical Criteria for Analyzing Transplanted Human Dopamine Cells in Parkinson's Disease

Ole Isacson,[1] Nicholas Lange,[2] Oliver Cooper,[1] and Rosario Sanchez-Pernaute[1]

## 1. Introduction

There are new opportunities to address specifically the advancement of human cell transplantation for Parkinson's disease (PD) using in vivo and post-mortem biomarkers for substantia nigra (SN) dopamine (DA) neurons and validated clinical measures. Such neurohistological and functional tools facilitate post hoc comparative analyses of post-mortem tissue from patients who received cell transplantation procedures and findings may correlate with clinical data. Although positron emission tomography (PET) studies provide a valuable tool to assess graft survival and viability, interpretation is complicated because Fluoro-L-DOPA (F-DOPA) provides a mixed estimation of DA terminal density, storage capacity, and aromatic amine decarboxylase activity (see Chapter 7). Post hoc analyses are required to establish the effects (both therapeutic and adverse) of transplants on which optimized designs are discussed in this chapter. The varying clinical outcomes of cell transplantation in PD patients have been extensively documented, and may depend on methodological differences between clinical transplantation teams (Table 9.1.), as well as selection of recipient patients (see Chapters 5,6 and 8). A critical question that remains to be addressed is the correlation between clinical benefit and graft biology: DA cell survival, DA cell subtype, location and integration are factors likely to determine the functional outcome for each patient. However, the relative contribution of these factors is largely unknown, partly due to the

[1]Harvard/McLean Udall Parkinson's Disease Research Center, Neuroregeneration Laboratories, Belmont, MA
isacson@hms.harvard.edu; ocooper@mclean.harvard.edu
rosario_pernaute@hms.harvard.edu
[2]Statistical Neuroimaging Laboratory, McLean Hospital, Belmont, MA
nlange@hms.harvard.edu

TABLE 9.1. Transplantation Clinical Trials for Parkinson's disease*

| Group | Reference | Cases (N) | Type | Donor age (weeks) | Donors per side | Site | IS | Clinical benefit (% in off) | PET F-DOPA uptake | Dyskinesias | Autopsy TH cell number and reinervation |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Lund/ London* | (Lindvall et al., 1989) | 2 (open) | SUSP | 7–9 | 4 | C+P | + | – | – (6 mo) | 14 cases reviewed by Hagell (2002); off-phase dyskinesias minimal to mild in 8; moderate in 5; severe in 1; peak on-phase dyskinesias improved and time on without dyskinesias increased (although non significantly) | |
| | (Lindvall et al., 1990) | 2 (open) | SUSP | 6–7 | 4 | P | + | + | + | | |
| | (Lindvall et al., 1992) | 2 (open) | SUSP | 6–7 | 4 | P | + | +/+++ | + | | |
| | (Widner et al., 1992) | 2 (MPTP) | SUSP | 6–8 | 4 | P/C+P (Bi) | + | +++ | ++ (200%) | | |
| | (Wenning et al., 1997) | 6* | SUSP | 6–8 | 4 | P/C+P | + | + (~30%) 4/6 | ++ (60%) | | |
| | (Hagell et al., 1999) | 5* | SUSP | 6–8 | 4–8 | P/C+P (Bi) | + | + 3/5 | ++ (85%) see also Piccini (1999, 2000) | | |
| | (Brundin et al., 2000) | 5 (open) | SUSP (+laza-roids) | 5–7 | 3–5 | C+P (Bi) | + | + (~40%) | + (~60%) | | |
| *Creteil* | (Peschanski et al., 1994) | 2 (open) | SUSP | 6–8 | 2–3 | P | + | + | + | Worse in on | |
| | (Defer et al., 1996) | 5* (open) | SUSP | 6–8 | 2–3 | P/C+P | + | + | + (~60%) | Worse in on (3/5) | |
| *Brussels* | (Levivier et al., 1997) | 3 (open, safety study) | SUSP | 6–8 | 3–4 | P | + | + | +/++ | | |

*Continued*

TABLE 9.1. Transplantation Clinical Trials for Parkinson's disease*—cont'd

| Group | Reference | Cases (N) | Type | Donor age (weeks) | Donors per side | Site | IS | Clinical benefit (% in off) | PET F-DOPA uptake | Dyskinesias | Autopsy TH cell number and reinervation |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Halifax* | (Mendez et al., 2000) | 8 (open) | SUSP (+GDNF) | 6–9 | 3–4 | P (Bi) | + | + | + | Improved | 2 TH+ cells 98,000 – 200,000 SN(1) ~4300 (Mendez et al., 2005) |
| | (Mendez et al., 2002) | 3 (open) | SUSP (+GDNF) | 6–9 | 3–4 | P + SN (Bi) | + | + (~40%) | +(~40% in putamen) | | |
| *Denver* | (Freed et al., 1990) | 2(open) | SOLID (strands) | 5–6 | 1 | C+P | + | ± | NA | | |
| | (Freed et al., 1992) | 7* (open) | SOLID (strands) | 5–6 | 1 | P (Bi) | + (4) | + | + | | |
| | (Freed et al., 2001) | Double-blind sham 19 +14 open | SOLID (strands, up to 28 d in vitro) | 7–8 | 2 | P (Bi) | – | + (~30%) | + (~40%) see also Nakamura 2001 and Ma 2002 | Severe off-phase dyskinesias 15% | 2 TH+ cells (2000-22000 per track) |
| *Tampa* | (Freeman et al., 1995) | 4 (open) | SOLID (pieces) | 6.5–9 | 6–8 | P (Bi) | + | + (~30%) | +(~40%) | | 2 TH+ cells 80,000 and 135,000 (5000-50000/track) reinervation 2–7 mm (Kordower, 1995,1996) |
| | (Hauser et al., 1999) | 6* (open) | SOLID (pieces, hiber 2 days) | 6.5–9 | 3–4 | P (Bi) | + | + (30%) | + (55%) | Improved | |
| | (Olanow et al., 2003) | Double-blind | SOLID (pieces, | 6–9 | 1/4 | P (Bi) | + | ± (4 donors | + (~20% 1 donor, | Off-dyskinesias 56% (disabling in 13%) | 4 (2/2) TH+ cells 30,000 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | sham 23 (11/12) | hiber 2 days) | | | | | 4%) | 30% 4 donors) | (1 donor) and 100,000 (4 donors) |
| *L.A* | (Kopyov et al., 1996) | 22 (open) | SOLID | 6–10 | 2–4 | P (Bi) | + | + (18/22) | NA | |
| | (Kopyov et al., 1997) | 13 (blind volume) | SOLID | 6–9 | 2 vs. 3–4 | P (Bi) | + | low 10%-high 50% | NA | |
| | (Jacques et al., 1999) | 60* (retro-spective) | SOLID | 6–10 | 2–4 | P (Bi) | + | + | NA | |

* Not included in this summary are trials from New Haven,US, using cryopreserved tissue (no TH cells in autopsy)(Redmond et al., 1990; Spencer et al., 1992) and Birmingham, UK, and Cuba that were performed using tissue from embryos ≥10 weeks old, as well as open surgical procedures. Includes patients previously reported. Susp: cell suspension, solid: tissue pieces and strands ("noodles"). IS: immunosupression (Cyclosporin A). C:Caudate nucleus; P:Putamen; SN: Substantia nigra reticulata; Bi: bilateral; Clinical benefit was rated in most studies as mild or moderate (+) corresponding roughly to 30% improvement in the UPDRS motor scores in OFF, – however, different studies based outcome on scores or % time in ON or in OFF and rating was performed on different medication regimes. In general, all studies reported a decrease in the amount of L-DOPA and dopaminomimetic drugs although there is great variability in the amount of the reduction and the time after transplantation when it happened; only exceptionally patients have been off medication (3 in the Lund series although in one it was reintroduced 3.5 years after the transplant and the other 2 had MPTP-induced parkinsonism). F-DOPA PET uptake was increased (+) in most studies (average 40–60%) but reported PET studies were performed at variable time points.

scarcity of post-mortem data. PET data are usually taken as a surrogate marker, as F-DOPA uptake is a sensitive index of presynaptic DA function, but it has limited resolution and poor clinical and pathological correlation. For instance, one of the patients reported by Freed et al. (2001) who died had symmetric uptake in spite of a fivefold difference (6,000/30,000) in the number of TH+ grafted cells between sides. In addition, most studies tend to compare relative changes (%), which leads to considerable error, in particular when levels are low. In spite of these limitations F-DOPA uptake is a reliable indicator of graft survival and post hoc analyses of clinical outcome should exclude patients lacking positive changes in PET. Moderate clinical benefit has been reported in most transplantation studies (Table 9.1). Detailed case analysis on an individual basis will be required to identify the variables that are more relevant for successful interventions and can be used to set criteria for patient selection. These analyses should be complemented and correlated with post-mortem studies in order to determine the therapeutic and restorative potential of DA cell replacement and the impact of technical and methodological differences.

Methodological differences may lead to a varied cellular composition and long-term survival of the donor DA neuron populations and host integration of the graft. Fetal DA neurons can grow to restore lost neuronal pathways in the adult brain. In PD patients, transplanted human fetal ventral midbrain (VM) neurons have shown functional capacity (Freed et al., 2001; Lindvall and Hagell, 2000; Mendez et al., 2002; Piccini et al., 1999, 2000; Ramachandran et al., 2002). There are two major methods of preparing and transplanting DA neurons from fetal donor tissue (see Chapter 8). The first is based on dissecting VM tissue pieces without separating or counting cells. All reports of post-mortem analysis of transplanted PD patients were from patients receiving such *non*-dissociated (solid and strands) VM donor tissue pieces (Freed et al., 2001; Kordower et al., 1995, 1996). The second method involves cell suspension grafts, where cells are first dissociated using proteolytic enzymes, then counted and prepared for liquid infusion into the brain. Evidence of survival and partial reinnervation of the host brain has been reported for solid grafts (Freed et al., 2001; Kordower et al., 1995, 1996), but also serious side effects in the form of severe dyskinesias have been recently reported in two clinical trials using solid pieces of VM (Freed et al., 2001; Olanow et al., 2003). Interestingly, only mild (Hagell et al., 2002) or no (Cochen et al., 2003; Mendez et al., 2005) dyskinesias in OFF have been reported using cell suspension grafts. There are biological differences between solid and cell suspension transplants. The major differences include graft–host integration, vascularization, volume effects, and cell–cell interactions (Davis and Temple, 1994; Finsen et al., 1991; Isacson et al., 1998; Leigh, 1994; Mendez et al., 2005). The cellular composition of the graft may play a decisive role in treatment outcome. In transplants using fetal ventral midbrain, about 10% of the cells are DA, and a smaller percentage of these are A9-like neurons (Haque et al., 1997). Although the presence of other cell types in the

graft might be beneficial, for example by providing trophic support and increasing DA neuron survival, the other cells may also create a physical barrier between the grafted DA neurons and the host, preventing synaptic interaction with the host neurons (particularly in solid grafts) and favoring nonfunctional connections. The presence of other transmitter phenotypes, such as serotonergic and cholinergic, may even interfere with DA neurotransmission. The presence in grafts of DA neurons of the mesolimbic "A10" kind, which have lower levels of the dopamine transporter (DAT) and D2 autoreceptors (DAD2R) regulating synaptic levels of DA, compared to the nigrostriatal (A9) axons, may also produce sub-optimal neurotransmission.

## 2. Dopamine Neuronal Subpopulations in Human Midbrain

The human VM contains several functional subpopulations of DA neurons that can be identified by both specific biochemical markers (Table 9.2.) and connectivity to their target neurons. A9 DA neurons are localized in the substantia nigra pars compacta (SNc) region of the midbrain. The A9 neurons project to the putamen and control motor function. The A9 neurons are also

TABLE 9.2. Biochemical Markers in the Human VM

| Markers preferentially expressed in A9 | | | |
|---|---|---|---|
| Protein | Species | Method | References |
| DAT | Primate Human | ISH | (Haber, 1995) |
| $D_2$ receptor | Primate Human | ISH | (Haber, 1995) |
| Neuromelanin | Human | | (Sesack and Lewis, 1997) |
| GIRK-2 | Mouse (P7/Adult)<br>Rat<br>Human | IHC<br>ISH | (Chen et al., 1997; Inanobe et al., 1999; Karschin et al., 1996; Liss et al., 1999; Roffler-Tarlov et al., 1996; Schein et al., 1998; Mendez et al., 2005) |
| AHD-2 | Rat | IHC | (McCaffery and Drager, 1994) |
| EphB1 receptor<br>Ephrin B2 ligand | Mouse (P7) | ISH | (Yue et al., 1999) |
| mGluR1α | Primate | IHC | (Kaneda et al., 2003) |
| **Markers preferentially expressed in A10** | | | |
| Protein | Species | Method | References |
| Calbindin $D_{28}K$ | Rodent<br>Primate<br>Human | IHC<br>ISH | (Airaksinen et al., 1997; Choi et al., 2001; Hontanilla et al., 1998; Parent et al., 1996; Tan et al., 2000; Verney et al., 2001; Yamada et al., 1990) |
| Estrogen-β Receptor | Mouse (P7) | IHC | (Callier et al., 2002; Creutz and Kritzer, 2002; Kuppers et al., 2000; Wang et al., 2001) |
| BDNF/trkB | Rat Human | IHC | (Nishio et al., 1998; Seroogy et al., 1994) |

the most vulnerable DA neuron in PD. A10 neurons are localized in several nuclei of the ventral midbrain, including the ventral tegmental area (VTA). The A10 neurons project to the limbic region and are less vulnerable than A9 DA neurons in PD (Damier et al., 1999).

Several markers are differentially expressed in DA neurons projecting to motor areas: for example, G protein-coupled inward rectifying current potassium channel type 2 (GIRK2) (Mendez et al., 2005), and to limbic areas, calbindin-D28k. Calbindin is expressed in DA neurons projecting to the accumbens and is not expressed in cells projecting to the motor striatum (Gerfen et al., 1985; Haber, 1995; Hontanilla et al., 1997; Liang et al., 1996; Nemoto et al., 1999). Calbindin-positive DA neurons are relatively spared in PD (Damier et al., 1999; Fearnley and Lees, 1991; German et al., 1992; Gibb, 1992; Yamada et al., 1990) and in toxic (MPTP) and genetic (weaver mice) models (Gaspar et al., 1994; Graybiel et al., 1990). GIRK2 tetramers are almost exclusively expressed in the membrane of DA neurons and are functionally linked to D2 and GABA B receptors (Guatteo et al., 2000; Inanobe et al., 1999). A mutation in GIRK2 causes the weaver phenotype in mice, in which all DA neurons are present at birth but 50% degenerate in the first 3 weeks (Bayer et al., 1995) and all surviving DA neurons express CB (Gaspar et al., 1994). Since the VM DA neurons projecting to the motor putamen are selectively vulnerable in PD, the identification of cell specific (phenotypic) markers of this subpopulation is relevant for cell replacement and neuroprotective therapies (Bjorklund and Isacson, 2002).

## 3. Correlation Studies to Identify the Biological Determinants of Functional Outcome in Cell Therapy

A key scientific rationale for application of modern biostatistical methods to longitudinal multi-center PD data is their use in meta-analyses of CAPSIT (Widner and Defer, 1999) and CAPIT-PD (Langston et al., 1992) databases in multi-institutional controlled clinical trials (DeGruttola et al., 2001; Lange, 2003a) (Fig. 9.1). Repeated clinical and imaging outcomes are a key data feature of these meta-analyses. Such longitudinal measurements on the same patient as a major source of inter-subject variation can be controlled by "borrowing strength" from the group of subjects in order to make better predictions of outcomes for the individual subjects as lower variability for intercepts and slope differences due to treatment effects and other effects, such as age and stage (Efron and Morris, 1977; Zeger and Liang, 1986). The understanding of individual patient changes over time is possible only through the use of such repeated measurements. We have listed (Table 9.3.) quantitative measurements from our clinical tissue transplantation collaborations that follow the CAPIT protocol for data acquisition in trials depicted in Tab. 3. Data and candidate distribution types suggest modeling and biostatistical analysis strategies for each patient outcome. In formal analyses, the first steps are univariate; while multivariate combinations may be useful at later stages. For our

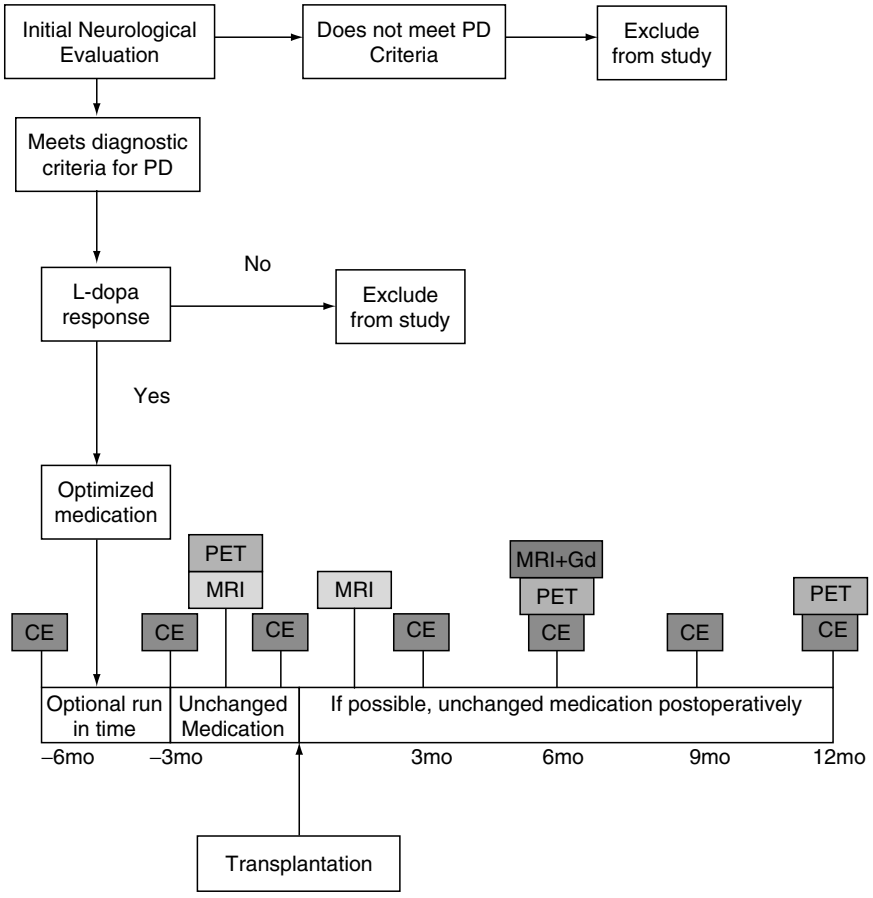


FIGURE 9.1. Schematic of the CAPIT protocol (modified from Langston et al., 1992). (see color insert.)

collaborations, all continuous and repeated measurements, such as UPDRS motor outcomes, are studied via linear mixed-effects models (Diggle et al., 2002; Laird et al., 1987; Laird and Ware, 1982; Lange and Ryan, 1989; Lange, 2003b). All count, categorical, and logistic repeated measurements, such as total TH+ cell counts, are studied via generalized linear mixed-effects models (Breslow and Clayton, 1993; Breslow and Lin, 1995; Wager et al., 2004). The pre/post paired, bilateral and unilateral F-DOPA PET spatial activity surfaces can be studied through use of random field and scale space methods (Siegmund and Worsley, 1995; Worsley, 1994; Worsley et al., 1992). Areas and peak heights of "hot spots" in subject-specific series of repeated PET images can be analyzed by linear mixed-effects models. Bayesian Markov chain Monte Carlo methods (Gelfand and Smith, 1990; Lange et al., 1992) are also useful, for understanding longitudinal cell count ratios.

TABLE 9.3. Categories To Be Used for the Individual and Comparative Analysis of PD Patients Treated by Cell Transplantation Using Different Biostatistical Techniques

| Category | Longitudinal measurements | Date type | Candidate probability distribution(s) |
|---|---|---|---|
| Clinical behavioral | UPDRS Motor (On/Off) | Continuous | Gaussian |
| | UPDRS Total | Continuous | Gaussian |
| | Dyskinesia scores | Continuous, discrete | Gaussian, multinomial, Poisson |
| | % time in Off | Continuous binary | Gaussian, logistic |
| Functional imaging | PET FDOPA $K_i$ Right and Left Putamen | Paired spatial activity surfaces | Gaussian, $F$, $\chi^2$, $\rho$ random fields |
| Morphology | Total graft Volume | Continuous | Gaussian |
| | Total TH+ cells | Cell counts | Poisson |
| | % TH/calbindin | Count ratios | Gaussian, MCMC |
| | % TH/Girk2 | | |

In order to determine clinical-pathological effects through analytic post hoc work, we have tested the use of linear mixed effects models on previously published data (Figs. 9.2 and 9.3). These post hoc analyses provide critical understanding about the effects of transplants and the progression of PD in transplant patients. We have quantified the correlation between PD severity and outcome and found that individual changes in both F-DOPA and UPDRS score depend significantly on pre-transplantation stage. This finding has potential impact on study design, patient inclusion criteria and the prediction of outcome.

## 4. Advanced Histopathological Methods: Confocal Microscopy, Stereology, and Automated Cell Counting

Confocal microscopy can be used to analyze tissue sections containing grafts prepared with immunofluorescence. Several spectrally similar dyes are separated by performing wavelength scanning of the specific single dyes in control sections of similarly prepared tissue and the data are translated to the regions of interest. This facilitates accurate interpretation of multiple labeling experiments and eliminates the bleed-through phenomenon. Three-dimensional reconstruction of co-localized signals can be investigated from Z-axis stacks of images captured by the confocal microscope. For example, bromodeoxyuridine in newly born cells and graft specific markers in transplantation experiments are confirmed by this gold-standard technique. Images acquired with this microscope can subsequently be incorporated into a stereology system to facilitate design-based quantification of the imaged structures. Stereology is

| Variable | Est. Coeff | SE | DF | *t*-value | *P*-valu |
|---|---|---|---|---|---|
| (Intercept) | 0.3470 | 0.0721 | 18 | 4.8104 | 0.0001 |
| Age Group | 0.0231 | 0.0228 | 17 | 1.0111 | 0.3261 |
| Post-Transplant | 0.1865 | 0.0456 | 18 | 4.0890 | 0.0007 |

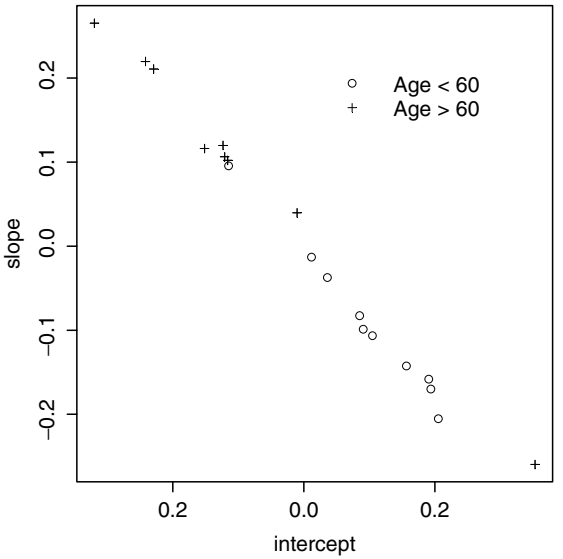


FIGURE 9.2. Re-analysis of Nakamura et al. (2001) using a linear mixed-effects model. To further understand group and individual post-transplantation changes in F-DOPA levels, a linear mixed-effects model was fit to published data of (Nakamura et al., 2001). The effects of both age group (less than or greater than 60 years) and pre- and post-transplantation time were included as possible covariates affecting F-DOPA levels. The effect of age group over time is not statistically significant. Hence there is a single overall group intercept (estimated group pre-transplantation baseline) and single overall group slope over time (post-transplant measurement). However, inspection of patient-specific pre- and post-changes in F-DOPA indicate that individual patients differ widely as they track along the group slope, and that pre-transplant F-DOPA level may in part determine post-transplant level. To investigate this possibility further, individual effects on intercept and on slope were included in the mixed-effects model. Individual intercepts and slopes are allowed as offsets to the overall intercept and slope. These estimates are shown in the graph, which reveals a strong negative correlation between individual intercepts and slopes. Patients with relatively low pre-transplant F-DOPA level tend to exhibit higher increases in F-DOPA than those with relatively high pre-transplant levels. In addition, older transplant patients tend to have lower pre-transplant F-DOPA levels than younger patients, as expected, yet their negative individual intercept-slope correlation does not appear to differ from that of the younger patients. Our more detailed, model-based analysis of the (Nakamura et al., 2001) data that accounts for individual patient differences has demonstrated a strong effect of transplantation on F-DOPA level, as is known, and has also provided more clues for expected individual post-transplant changes in F-DOPA levels depending on pre-transplantation levels.

| Variable | Est. Coeff | SE | DF | *t*-value | P-value |
|---|---|---|---|---|---|
| (Intercept) | 83.0934 | 6.0144 | 22 | 13.8158 | < 0.0001 |
| Time Post-Transplant | –2.1661 | 0.9737 | 22 | –2.2247 | 0.0367 |

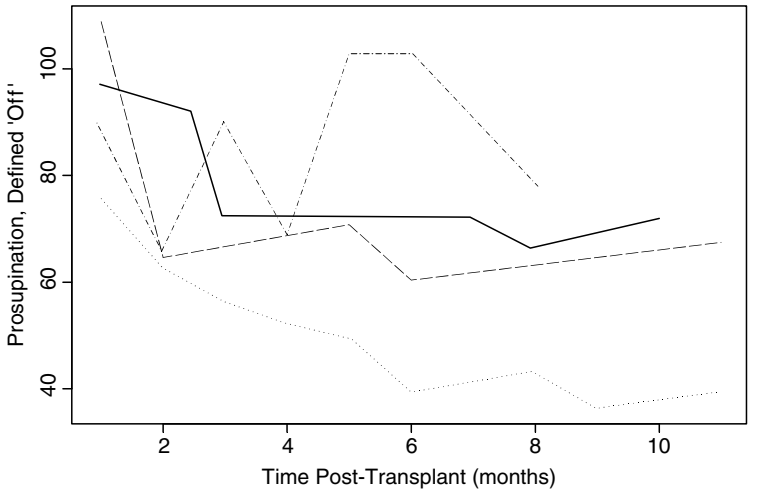


FIGURE 9.3. Re-analysis of Defer et al. (1996) using a linear mixed-effects model. Results on mixed-effects model fit to (Defer et al., 1996) data for prono/supination scores, 12-month post-transplantation, best "off." Individual intercepts and slopes are allowed offsets to the overall intercept and time slope. Our previous analysis (Fig. 9.2.) was limited by having only two measurements per patient over time. We extend our example by re-analyzing this dataset, which contains 5–13 repeated measurements of various UPDRS motor scores collected over time under the CAPIT protocol. The graph shows the changes over 12 months in prono-supination scores for 4 PD transplant patients in defined "Off" state (Hoehn and Yahr 4-5). As in the (Nakamura et al., 2001) data, shown in Fig. 9.2. again note the possible dependence of slope on intercept (after 0 months post-transplantation). We fit a linear mixed-effects model including time as the single covariate while allowing the individual intercepts and slopes. We find that there is a significant improvement in prono-supination scores over this 12-month period.

performed on stained sections representing a fraction of the structure or population to be quantified using a workstation with image capture equipment and software. The optimal fraction of the structure or population to be quantified is estimated by balancing the effective workload against the precision of the estimate (coefficient of error). Implementation of an effective stereological probe relies upon understanding the sampling required and the tissue preparation. The optical fractionator is an example of a global stereological probe that facilitates systematic random sampling of populations of objects, such as cells within relatively thick sections (40 μm) from the region of interest (West et al.,

1991). Structural volumes of grafted regions can be quantified using another example of a stereological probe, the Cavalieri estimator. The Cavalieri estimator is a global probe that relies upon the accurate counting of points in a projected, randomly orientated lattice that lies upon the image of a structure of interest. In a similar fashion to the implementation of the optical fractionator, a series of sections that samples the entire region of interest is randomly selected. Automatic 2-D quantification methods exist for identification of brightfield Fos expression (Wager et al., 2004; Young et al., 2001) that can be applied to images of human PD substantia nigra and transplantation. Automatic measurements of the sizes (area, perimeter), shapes (ellipsoidal axes) and average gray-levels of image objects are used against ground truth provided by manual touch counting to classify image objects as Fos-positive nuclei or not via an artificial neural network (ANN) classifier. Our ANN is a classical feed-forward net, a generalization of linked logistic regressions for more than two classes, with weight decay penalizing high-variance inputs to the hidden layer. Classification results derived from its smooth decision boundaries compare favorably, in terms of misclassification error rate, to classical linear and quadratic discrimination and nearest-neighbor methods, with no loss of overall accuracy in the contexts studied to date.

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# 10

# Dyskinesias and Neural Grafting in Parkinson's Disease

M. Angela Cenci[1] and Peter Hagell[2,3]

## 1. Introduction

In the past 20 years, intracerebral transplantation of embryonic ventral mesencephalic (VM) tissue has been looked upon as a particularly promising approach for the treatment of Parkinson's disease (PD). Among the many possible treatment options for the future, transplantation bore the promise of a truly curative approach: endogeneous, degenerating dopamine (DA) neurons would be substituted for by healthy DA-producing cells, restoring the damaged nigrostriatal circuit once and for all (Nikkhah and Brandis, 1995; Barker, 2000; Fricker-Gates et al., 2001). Hopes were fostered by the encouraging results produced by intrastriatal VM transplants both in animal models of PD (Björklund, 1992; Björklund and Stenevi, 1979; Herman and Abrous, 1994; Perlow et al., 1979) and in early open-label clinical trials (Lindvall, 1994; Lindvall and Hagell, 2000 and Chapter 5). The latter showed that embryonic VM tissue can engraft in the parkinsonian striatum and provide a local source of DA storage and release. In a majority of transplanted patients the grafts were found to ameliorate many of the symptoms of PD and to reduce the need for L-DOPA pharmacotherapy (Lindvall and Hagell, 2000). In addition to their immediate implications for PD, these results also suggested that neural cell replacement could develop into a radically new treatment approach for a wide range of neurological disorders (Gage et al., 1988; Lindvall and Björklund, 1992; Aichner et al., 2002; Turner and Shetty, 2003; Grisolia, 2002; Peschansky and Dunnett, 2002; Studer et al., 1998). This early enthusiasm was dampened by alarming reports from the first NIH-sponsored clinical

[1]Basal Ganglia Pathophysiology Unit, Department of Experimental Medical Science. Lund University, Lund, Sweden
angela.cenci_nilsson@med.lu.se
[2]Department of Health Sciences, Lund University, Lund, Sweden
[3]Section of Restorative Neurology, Lund University Hospital, Lund, Sweden
Peter.Hagell@med.lu.se

trial of neural transplantation, where a subgroup of patients had manifested a severe and persistent form of dyskinesia at late postoperative periods (Freed et al., 2001; Greene et al., 1999; Kolata, 2001 and Chapter 6). Other reports were soon to follow indicating that dyskinesias indeed can develop as a complication of intrastriatal VM grafting (Hagell et al., 2002; Ma et al., 2002; Olanow et al., 2003). These dyskinesias are a puzzling phenomenon that had not been foreseen by experimental studies of VM transplantation in animal models. This phenomenon does not presently lend itself to any simple explanation. In fact, current pathophysiological models are inadequate to explain the emergence of dyskinesia after interventions that can provide a source of continuous DA release in the striatum. Yet, understanding this issue appears essential in order to be able to plan further application of cell-replacement therapy in PD.

In this chapter, we shall first provide a general review of the clinical spectrum and pathophysiology of the dyskinesias that complicate the treatment of PD. We shall then discuss the effects of VM grafts on L-DOPA-induced dyskinesias that are present prior to transplantation surgery. Thereafter, we will specifically address the issue of graft-induced dyskinesia, viz., an apparently novel clinical entity that is caused by the intrastriatal grafts themselves. Finally, we shall provide a speculative review of possible mechanisms underlying the development of dyskinesia following intrastriatal VM transplantation.

## 2. Dyskinesias in Parkinson's Disease

### *2.1. Clinical Spectrum*

The main motor symptoms of PD are caused by the loss of striatal DA that results from nigral neuron degeneration. Pharmacological DA replacement by L-DOPA typically results in an excellent initial symptomatic response. However, within some years of treatment a significant proportion of patients develop motor complications, i.e., motor fluctuations and dyskinesias (Marsden et al., 1981; Quinn, 1998). Estimates based on published data indicate that both of these complications appear in about 40% of patients after 5–6 years of treatment (Ahlskog and Muenter, 2001). With time, especially in patients with young-onset PD, motor complications often increase in severity and to the point that drug therapy is no longer optimal (Marsden et al., 1981; Nutt, 1992; Quinn, 1998; Quinn et al., 1987).

Motor fluctuations appear as oscillations between good response to medication with no or minimal PD-related disability ("on" phases), and episodes of poor drug response with increased PD-related disability ("off" phases) (Quinn, 1998). Dyskinesias appear as hyperkinetic and dystonic abnormal involuntary movements and postures. Chorea (randomly "flowing," purposeless, dance-like movements) is the most common type of hyperkinesia, but other types (e.g., ballism, stereotypies) also occur (Marsden

et al., 1981; Nutt, 1992). Dystonia consists either of twisting movements that tend to be sustained at the peak of the movement (mobile dystonia), or of abnormal fixed postures that can be painful. Dystonic components are often intermingled with chorea (Luquin et al., 1992; Marconi et al., 1994; Marsden et al., 1981; Nutt, 1992; Quinn, 1998). While typically appearing as a consequence of L-DOPA therapy (Jankovic and Tintner, 2001), fixed dystonia is not necessarily a complication of dopaminergic drug therapy as it also appears in untreated patients and was described long before the emergence of effective therapy for PD (Stewart, 1898).

Dyskinesias appear in three temporal patterns in relation to the intake of antiparkinsonian medications. "On"-phase dyskinesias, which are the most common pattern, appear when brain and plasma levels of L-DOPA and DA are high ("peak-of-dose") but dyskinesias can also appear at low L-DOPA/DA levels ("off" phase dyskinesia), and when L-DOPA/DA levels are rising and falling, i.e., at the beginning and the end of the L-DOPA action cycle (biphasic dyskinesia) (Fahn, 1992; Luquin et al., 1992; Marsden et al., 1981; Nutt, 1992; Quinn, 1998). While there is individual variability in the phenomenology of dyskinesia at any point during the dopaminergic drug cycle, certain types are more commonly seen during specific phases (Nutt, 1992). "On"-phase dyskinesias are typically characterized by choreiform and choreo-dystonic movements that appear spontaneously but are provoked or exaggerated by, e.g., mental stress, speaking and physical activity (Durif et al., 1999; Luquin et al., 1992; Marsden et al., 1981; Nutt, 1992; Quinn, 1998). "On"-phase dyskinesias can affect virtually any muscle group, but predominate in the upper body (Luquin et al., 1992; Nutt, 1992). Biphasic dyskinesias typically manifest as stereotypic, repetitive, or ballistic movements, and/or dystonia, which most commonly affect the legs and feet (Luquin et al., 1992; Marsden et al., 1981; Nutt, 1992; Quinn, 1998). In the "off" phase, dystonic posturing, typically affecting the lower limbs, is the predominant type of dyskinesia, although rare cases of hyperkinesias during the "off" state also have been reported (Cubo et al., 2001; Luquin et al., 1992; Marsden et al., 1981; Nutt, 1992; Quinn, 1998). Phenomenologically, such hyperkinetic "off"-phase dyskinesias have resembled biphasic dyskinesias, thus appearing as repetitive, stereotypic and dystonic movements (Cubo et al., 2001; Hagell et al., 2002). "On"-phase, biphasic and "off"-phase dyskinesias may all coexist in the same patient and some patients may also experience dyskinesias in one part of the body while remaining parkinsonian ("off") in another (Fahn, 1992; Luquin et al., 1992; Marsden et al., 1981; Nutt, 1992).

## *2.2. Pathophysiological Aspects*

In principle, there are two prerequisites for the development of dyskinesias in PD: repeated pulsatile treatment with dopaminergic drugs, in particular oral L-DOPA, and degeneration of the nigrostriatal DA system (Bédard et al., 1999; Schneider, 1989), where the severity of the underlying disease process appears to be of greater importance than the duration of L-DOPA therapy

(Ahlskog and Muenter, 2001; Kostic et al., 2002). Disease severity is in turn correlated to the extent of putaminal DA depletion (Morrish et al., 1996). Thus, the loss of striatal DA innervation appears to play a determinant pathogenic role in the development of both dyskinesias and parkinsonian features in PD. Prolonged and massive loss of dopaminergic innervation causes increased expression and sensitivity of DA receptors in the striatum (Mishra et al., 1980; Rinne et al., 1990). Accordingly, DA receptor supersensitivity is regarded as an important factor at the basis of all types of dyskinesias (Klawans et al., 1977). The varying patterns of "on" versus "off" and biphasic dyskinesias have been proposed to derive from the heterogeneity of DA loss in PD, whereby denervation is greatest in the dorsal and posterior putamen, i.e., the region somatotopically related to the lower limbs (Kish et al., 1988). Low or intermediate brain levels of DA (such as those found when plasma levels of L-DOPA start to increase or decrease) would be sufficient to stimulate the highly sensitive DA receptors that are present in the most denervated putaminal locations. The gradually increasing DA concentrations would eventually stimulate DA receptors in more widespread striatal regions and dyskinesias would therefore become more generalized (Marconi et al., 1994). Although appealing in its straightforwardness, this explanation does not account for the fact that biphasic and "off"-phase dyskinesias improve when levels of striatal DA receptor stimulation increase, despite an ongoing DA receptor supersensitivity in the highly denervated, dorsoposterior putamen (Lhermitte et al., 1978). In an attempt to explain this paradox, Marconi et al. (1994) have postulated that biphasic and "on" phase dyskinesias are driven by different neural systems, possibly controlled by different DA receptor subtypes (i.e., D2 and D1 receptors, respectively). Some reciprocal, inhibitory connections between these systems would explain the transition from one type of dyskinesia to another when striatal DA levels rise or fall. This hypothesis is intriguing but speculative at the moment. There is a clear need for further investigation on the cellular effects of DA in the dyskinetic brain. At present, our understanding of the cellular and molecular mechanisms of dyskinesias is not only scattered, but also limited to the peak-of-dose pattern, which is the only category of dyskinesia to have been reliably modeled in animals so far.

In both rat and primate models of "on"-phase dyskinesia, the appearance of involuntary movements is accompanied by an abnormal upregulation of opioid precursor genes and ΔFosB-like transcription factors in the striatum (Doucet et al., 1996; Cenci et al., 1998; Andersson et al., 1999; Tel et al., 2002; Winkler et al., 2002). These observations have also been mirrored in humans. Thus, functional imaging studies using positron emission tomography (PET) have shown reduced striatal opioid receptor occupancy, indicating increases in endogenous opioid activity, in dyskinetic PD patients (Piccini et al., 1997). Histopathological analyses have also found increased putaminal ΔFosB immunostaining in post-mortem brain sections from dyskinetic PD patients as compared to non- dyskinetic PD cases and healthy controls (Cenci et al., 2002). In addition to opioids, striatal efferent neurons use the tachykinin, substance P as a peptide cotransmitter.

Interestingly, a recent PET study that utilized a substance P-receptor ligand as a tracer implicates excess substance P transmission in dyskinetic PD patients (Brooks, 2002). Taken together, these and other data have provided a basis for the current pathophysiological interpretation of dyskinesias as being dependent on a high, intermittent stimulation of striatal DA receptors causing downstream changes in gene and protein expression (Chase, 1998; Calon et al., 2000; Olanow and Obeso, 2000) (Fig. 10.1). In keeping with this concept, pharmacological treatments achieving sustained levels of DA receptor stimulation, such as long-acting DA agonists or continuous infusions of L-DOPA or DA agonists, have been shown to normalize the striatal expression of opioid precursor genes in animal models of PD (Engber et al., 1990; Gerfen et al., 1990; Morissette et al., 1999; Juncos et al., 1989). Moreover, such treatments can improve motor fluctuations and dyskinesias in PD patients (Colzi et al., 1998; Facca and Sanchez-Ramos, 1996; Stocchi et al., 2002).

## 3. The Influence of Neural Grafts on L-DOPA-Induced Dyskinesias: Observations in Experimental Animals

In the advanced stages of PD, there are few residual DA terminals in the striatum, causing reduced buffering capacity for exogenously derived DA. Thus, fluctuations in plasma L-DOPA concentration are more likely to cause fluctuations in striatal DA concentration and pulsatile stimulation of DA receptors (Olanow and Obeso, 2000). As reviewed above, pulsatile stimulation is thought to induce postsynaptic gene and protein changes that are causally linked to the emergence of dyskinetic movements. There are at least two ways by which intrastriatal VM grafts can counteract the fluctuating levels of DA-receptor stimulation consequent to pulsatile L-DOPA treatment. First, graft-derived DA axon terminals replenish a significant proportion of the DA reuptake sites that are lost with the degeneration of endogenous nigral neurons (Doucet et al., 1990). Microdialysis studies in 6-OHDA lesioned rats have shown that high-affinity uptake sites on striatal DA terminals play a crucial role in the clearance of extracellular DA formed from exogenous L-DOPA (Miller and Abercrombie, 1999). Accordingly, a single dose of L-DOPA produces a much larger elevation of extracellular DA levels in the DA-denervated striatum than it does in the intact striatum (Abercrombie et al., 1990). Second, VM grafts provide a stable source of DA release in a DA-denervated structure, normalizing both the expression and the sensitivity of striatal DA-receptors (see below). Indeed, in vivo microdialysis and voltammetric studies have shown normal, nonfluctuating concentrations of ambient DA in the striatal tissue surrounding VM grafts (Cenci et al., 1994, Cragg et al., 2000; Forni et al., 1989; Moukhles et al., 1994; Rioux et al., 1991; Strecker et al., 1987; Zetterström et al., 1986).

In line with the findings reviewed above, studies performed in animals models of L-DOPA-induced "on" phase dyskinesia have consistently shown an

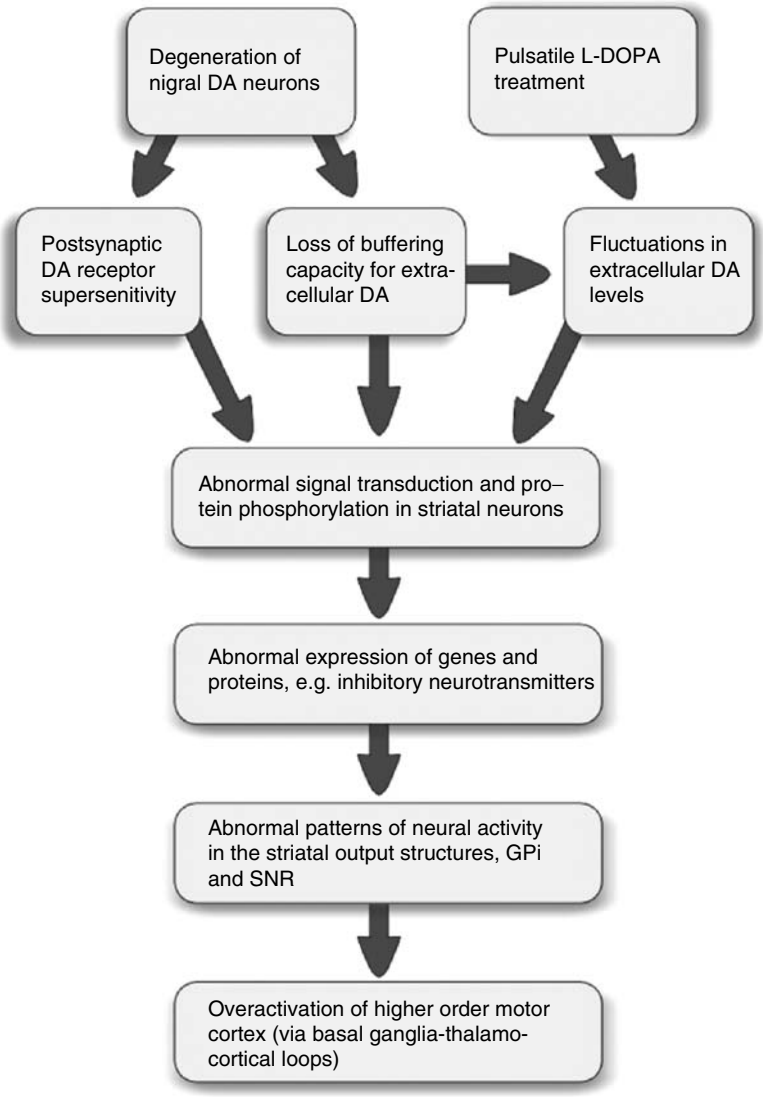


FIGURE 10.1. Flow chart illustrating the sequence of events at the basis of L-DOPA-induced dyskinesia according to commonly accepted notions. GPi, globus pallidus pars interna; SNR, substantia nigra pars reticulata. (see color insert.)

overall attenuation of dyskinesia severity following intrastriatal VM transplantation. A seminal study on this subject was provided by Lee et al. (2000). Lee and collaborators produced a rat model of "on" phase dyskinesias by giving 6-OHDA lesioned rats twice daily L-DOPA injections for 4 weeks. Thereafter, the animals received two standard implants of embryonic VM cell

suspension in the lateral caudate-putamen, and continued to be treated with L-DOPA treatment for an additional 3 months. During this postoperative period, VM-grafted animals, but not sham-grafted controls, showed a gradual reduction in their global "on" phase dyskinesia scores (Fig. 10.2). This effect

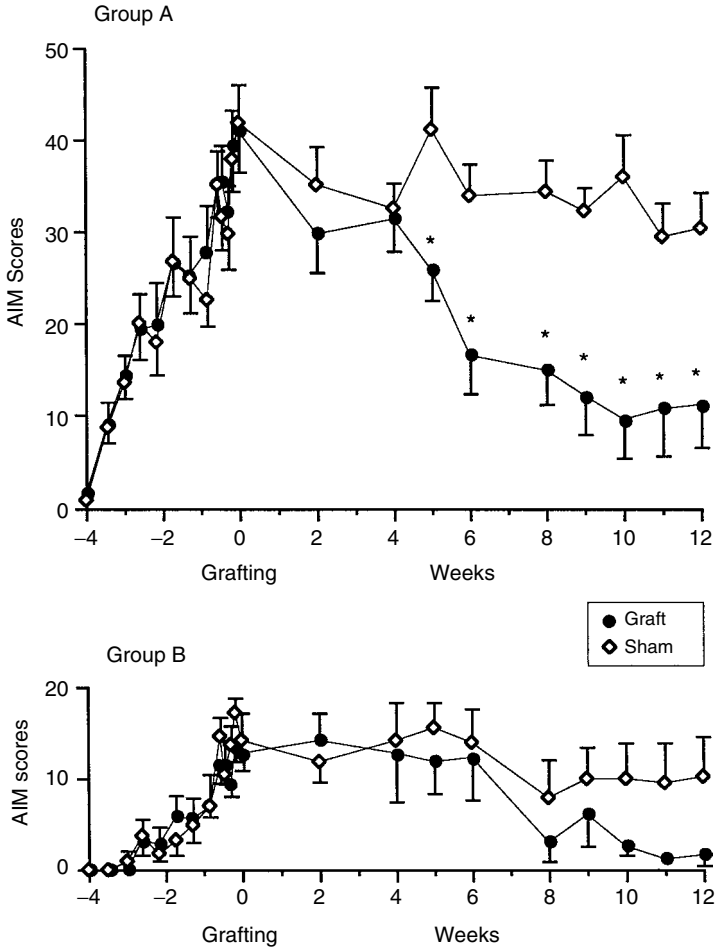


FIGURE 10.2. Intrastriatal VM grafts improve L-DOPA-induced "on"-phase dyskinesias in a rat model of Parkinson's disease. The diagrams show time course of rat abnormal involuntary movement (AIM) scores in animals with maximally severe dyskinesia prior to grafting (**Group A**), and animals with mild dyskinesia prior to grafting (**Group B**). In both groups, half of the animals received implants of VM tissue (**filled circles**), and the other half received sham grafts (**open circles**) in the sensorimotor part of the striatum at time 0. Rats were treated with L-DOPA for 12 weeks post-grafting, during which only the VM-grafted group showed a gradual decline in AIM scores. Asterisks indicate $P < 0.05$ vs. sham-grafted group (from Lee et al., 2000).

was accompanied by a normalization of dyskinesia-associated markers in the striatum such as preproenkephalin-, prodynorphin- and glutamic acid decarboxylase mRNA (Fig. 10.3). In the same study, Lee et al. (2000) used DA-transporter autoradiography in order to establish correlations between the extent of striatal DA innervation and the antidyskinetic effect of VM grafts. The severity of L-DOPA-induced dyskinesias in the grafted animals

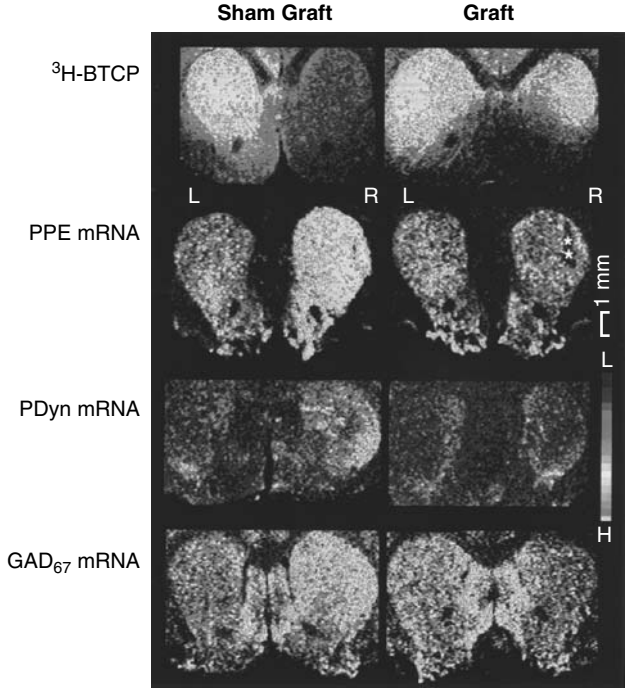


FIGURE 10.3. Pre- and post-synaptic effects of intrastriatal VM grafts in rats with unilateral 6-OHDA lesions that were treated with L-DOPA. In this study VM grafts were found to improve L-DOPA-induced "on" dyskinesia (see Fig. 10.2). Autoradiographic pictures of striatal sections from VM-grafted rats ("graft") are represented in the right-hand column, whereas pictures from sham-grafted controls ("sham graft") are shown in the left column. In all pictures, the DA-denervated and grafted side of the striatum is shown to the right (**R**), and the contralateral intact side is shown to the left (**L**). [$^3$H]BTCP binding autoradiography (which labels DA uptake sites in the striatum) showed a nearly complete restoration of striatal DA fiber density in the grafted animals. In situ hybridization histochemistry was used to measure the expression of mRNAs encoding for preproenkephalin (PPE), prodynorphin (PDyn), and glutamic acid decarboxylase (GAD$_{67}$). These transcripts are expressed in striatal medium-sized spiny neurons, and are known to exhibit a marked up-regulation following DA-denervating lesions and/or pulsatile L-DOPA treatment (compare lesioned side and contralateral intact side in the sham-grafted animals). The levels of all these transcripts were restored to normal values by the VM grafts (from Lee et al., 2000). (see color insert.)

was found to correlate inversely with the density of striatal DA fiber outgrowth. Interestingly, a nearly complete suppression of dyskinesias was seen in two rats with average striatal DA fiber density approximating 30% of normal values (Fig. 10.4). A reduction of L-DOPA-induced dyskinesia by intrastriatal VM grafts has now been reported also by two recent studies using the same rat model as in Lee et al. (Carlsson et al., 2005; Lane et al., 2005). Somewhat different results have however been reported by Steece-Collier et al. (2003). These authors found that intrastriatal VM grafts may worsen certain aspects of L-DOPA-induced dyskinesia in 6-OHDA lesioned rats. Steece-Collier and collaborators used an experimental paradigm similar to that in Lee et al. (2000), but with slightly different procedures for both transplantation and behavioral testing. In particular, VM graft recipients received one single implant in a rather central location within the striatum. Moreover, the rating scale used in this study differentiated between limb hyperkinesia and limb dystonia, whereas Lee et al., (2000) assigned one single score to all types of limb dyskinesias, whether hyperkinetic or dystonic in character. In

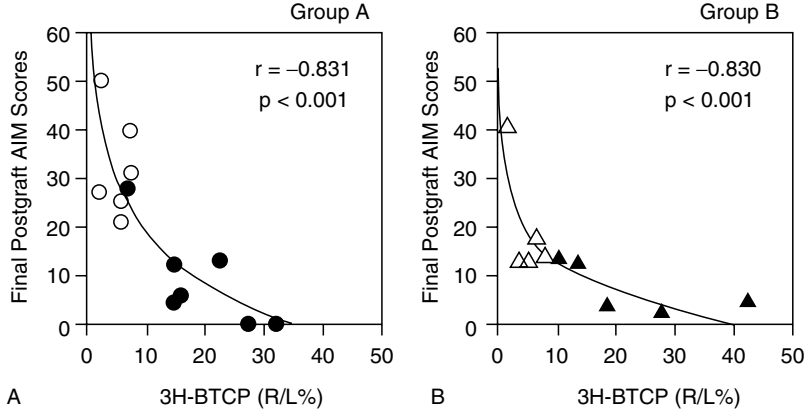


FIGURE 10.4. Scatter plots illustrating the relationship between the severity of L-DOPA-induced AIM scores (at 12 weeks post-grafting) and the density of graft-derived DA innervation in the same rats that are illustrated in Figs. 10.2 and 10.3. Final post-graft AIM scores were regressed on the logarithm of [$^3$H]BTCP binding (which provided a measure of striatal DA fiber density), showing a significant correlation between these two variables. In each animal, [$^3$H]BTCP binding levels on the 6-OHDA-lesioned and grafted side of the striatum were expressed as a percentage of the values found on the contralateral intact side (R/L%). **Group A** consists of rats that displayed maximally severe AIM scores pre-grafting, while **Group B** consists of animals with mild–moderate AIMs. Animals in each group had been randomly allocated to receive VM grafts (**filled symbols**) or sham grafts (**open symbols**). In both group A and group B the final post-graft AIM scores showed a sharp decrease when DA innervation densities in the graft-reinnervated striatum exceeded 15–20% of the values measured in the unlesioned control side (from Lee et al., 2000).

agreement with the data of Lee et al. (2000), VM grafts were found to reduce the expression of L-DOPA-induced rotation and dystonia, but they were also found to cause a significant aggravation of the hyperkinesias affecting the contralateral forelimb. Since Steece-Collier et al. (2003) did not examine the graft-induced reinnervation pattern, nor the post-synaptic effects of the grafts, it is not possible to propose an explanation to their interesting findings. However, very recent studies from this and other groups indicate that the pattern (extent and location) of graft-derived DA reinnervation may condition the profile of changes in L-DOPA-induced dyskinesia that is observed in the post-grafting period (Lane et al., 2005; Maries et al., 2005; Carlsson et al., 2005) (see also paragraph 4.5).

Very few studies have addressed the impact of embryonic VM grafts in non-human primate models of L-DOPA-induced dyskinesia. To the best of our knowledge, the only published study touching on this issue is one reported by Widner and Defer (1999), where MPTP-lesioned and chronically L-DOPA-treated squirrel monkeys received embryonic VM implants in the putamen. In agreement with the results of Lee et al. (2000), a significant alleviation of global "on" phase dyskinesia scores was found in the transplanted monkeys as compared to sham-grafted controls (Widner and Defer, 1999).

## 4. The Impact of Neural Grafts on Dyskinesias in Parkinsonian Patients

There is a discrepancy between the body of experimental findings reviewed above and the clinical experience with VM transplants in PD. To start with, different clinical trials of intracerebral transplantation in PD have provided contrasting results regarding the effects of the transplants on L-DOPA-induced "on" phase dyskinesias. Moreover, a substantial number of transplanted patients have recently been reported to display dyskinesias while off medications. Since "on"-phase dyskinesia and postoperative off-medication dyskinesia may represent two different entities (see below), the impact of neural grafts on dyskinesias will be reviewed separately for the two conditions.

### *4.1. Alterations in "On"-Phase Dyskinesias*

Open label clinical trials of intrastriatal VM grafts have yielded ambiguous and mixed results regarding the effects of the transplants on "on"-phase dyskinesias in PD. Thus, both increased and decreased dyskinesias have been reported following neural transplantation in parkinsonian patients (Brundin et al., 2000; Defer et al., 1996; Hagell et al., 1999; Hauser et al., 1999; Kopyov et al., 1996, 1997; Wenning et al., 1997; Widner et al., 1992). Possible explanations to this heterogeneous picture include the open-label design, differences between transplantation procedures and the fact that few

studies have included dyskinesias as a specific outcome or studied it in any detail. Most investigators have thus relied on clinical observations reported as narrative case summaries, and/or assessed dyskinesias merely in terms of their daily duration, as derived from patients' "on"/"off" diaries. Diary-derived data probably mainly reflect the daily amount of time spent with dyskinesias in the "on"-phase, since "on"/"off"-diaries do not specify dyskinesias as an isolated entity but only allow for recording of "on with dyskinesias." According to such diary-derived data a majority of grafted patients have been reported to experience clear reductions in daily motor fluctuations with about 40–50% reduced daily time spent in the "off" phase, coupled with improvements in "off" phase parkinsonism and increases in putaminal levels of $^{18}$F-labeled 6-L-fluorodopa (FD) uptake (see below) during the second postoperative year (Brundin et al., 2000; Hagell et al., 1999; Hauser et al., 1999; Mendez et al., 2000). Although the daily duration of the time spent "on with dyskinesias" in general has shown a beneficial postoperative development, both increases and decreases have been reported (Brundin et al., 2000; Hagell et al., 1999; Hauser et al., 1999; Wenning et al., 1997).

In contrast to studies relying on "on"/"off" diary data, Kopyov and collaborators (1996, 1997) assessed dyskinesia duration and intensity by means of the Obeso dyskinesia rating scale (Langston et al., 1992). They found significant improvements in both scores of about 30% between 6 and 18 months following transplantation, along with significant symptomatic relief, as assessed according to the Core Assessment Program for Intracerebral Transplantations (CAPIT) (Langston et al., 1992). However, upon closer consideration these data should be interpreted cautiously due to the ambiguity of the dyskinesia assessment tool (Goetz, 1999). The "intensity" score thus consists of a mixture between clinical observations and patients' perceptions and the "duration" score, similarly to that of the fourth section ("complications of therapy") of the Unified PD Rating Scale (UPDRS) (Fahn et al., 1987), relies completely on patient recall. These shortcomings are severely limiting, particularly considering the open label design, because it is known that untrained patients' accuracy in recognizing dyskinesias, at least if mild, is poor (Goetz et al., 1997). Moreover, reliance on recall in self-report of health experiences and disease episodes is, in general, of limited reliability (Hufford and Shiffman, 2002). Furthermore, the extent of graft survival in these patients is unknown since PET investigations of FD uptake were not performed (Kopyov et al., 1996, 1997). However, in another open label trial of bilateral intrastriatal grafts in two patients with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-(MPTP)-induced parkinsonism, Widner et al. (1992) evaluated dyskinesias prospectively using standardized observer-derived severity assessments. Patients were thus assessed by means of the Abnormal Involuntary Movements Scale (AIMS) (Guy, 1976) while performing standardized motor tests in the practically defined "off" phase (i.e., in the morning $\geq$ 12 hours after the last dose of antiparkinsonian medication

and ≥ 1 hour after arising) and at regular intervals throughout the effect of an individually standardized dose of L-DOPA (Langston et al., 1992). Clear and progressive reductions in the cumulative amount of dyskinesias during L-DOPA challenges were evident up to 2 years following transplantation. In parallel, parkinsonian symptoms improved and striatal levels of FD PET uptake showed a marked increase, indicating surviving grafts.

More recently, Hagell et al. (2002) carried out a blinded retrospective video analysis of the occurrence and severity of dyskinesias during practically defined "off" and the peak of the L-DOPA induced "on" response in 14 PD patients who had received uni- or bilateral intrastriatal human embryonic VM grafts up to 11 years earlier. This analysis did not reveal any significant effects regarding either the daily duration or severity of "on"-phase dyskinesias (Hagell et al., 2002), as assessed by the Clinical Dyskinesia Rating Scale (CDRS) (Defer et al., 1999; Hagell and Widner, 1999). However, individual cases showed clear long-term reductions in both the amount of time spent in "on" with dyskinesia and in dyskinesia severity (Brundin et al., 2000; Hagell et al., 1999, 2002). A lack of overall improvement in "on" phase dyskinesias post-grafting has recently been presented by Olanow et al. (2003). This study reported on the results from a two-year placebo-controlled double-blind randomized clinical trial (RCT) of bilateral transplantation of embryonic VM from one (n = 11) or four (n = 12) donors in each postcommissural putamen. The transplanted patients were compared to a group of patients who had been randomized to sham-surgery (n = 11). Neither "on"-phase dyskinesia severity, as rated by a blinded investigator using the AIMS, nor the time spent in "on" with dyskinesias, showed any significant changes or differences compared to sham-operated patients at 2 years after transplantation.

There have also been reports of increased dyskinesias following intrastriatal transplantation of DA-rich VM tissue (Brundin et al., 2000; Defer et al., 1996; Freed et al., 1992; Hagell et al., 1999; Jacques et al., 1999; Levivier et al., 1996; Peschanski et al., 1994; Wenning et al., 1997). Typically, these reports have indicated increased dyskinesias during the early phase (up to about 6–9 months) after grafting that have paralleled an increased FD PET uptake and a lengthening of the duration of the L-DOPA response. Interestingly, the increase in dyskinesias occurred prior to the development of a full graft-induced relief in parkinsonian motor symptoms. These abnormal involuntary movements were described as "on"-phase dyskinesias, which also is supported by the observation that they typically responded favorably to downward adjustments in medication (Freed et al., 1992; Hagell et al., 1999; Levivier et al., 1997; Peschanski et al., 1994; Wenning et al., 1997). Such increased dyskinesias during the early postoperative phase have been hypothesized to be due to immature grafts that have not yet formed major synaptic connections with host striatal neurons (Lindvall and Hagell, 2000). However, alternative explanations should be considered, in particular, delays in the normalization of striatal DA receptor sensitivity by the grafts (see below).

## *4.2. Off-Medication Dyskinesias Following Neural Transplantation in PD*

In 2001, Freed and collaborators reported the results from the first placebo-controlled double-blind RCT of neural transplantation for PD (Freed et al., 2001). Forty patients were enrolled, 20 of whom received bilateral grafts to the putamen, while 20 underwent sham-surgery. In this trial, human embryonic VM tissue was prepared into strands that were stored in explant culture for 1–4 weeks before being injected along two tracts in the axial plane, accessed by a transfrontal stereotactic approach (Table 10.1). No immunosuppression was given to the patients. One grafted patient died in a car accident before the end of the 12 months double-blind phase. At one year, the trial failed to detect any difference on its primary outcome measure, a global retrospective self-rating score, although motor function during the "off" phase and FD PET uptake were significantly improved in grafted patients but not in the placebo group. Following completion of the double-blind protocol at one year, 14 of the sham-operated patients received transplants and the full cohort of 33 surviving grafted patients were followed in an open label fashion for up to three years after surgery. Following improvement of parkinsonism during the first postoperative year, five of the grafted patients (15%) developed severe dyskinesias during the second and third year post-grafting. These dyskinesias persisted after substantial reduction in, or withdrawal of, dopaminergic drug therapy (Freed et al., 2001; Greene et al., 1999; Ma et al., 2002; Olanow et al., 2001). Amantadine reduced dyskinesias in two cases (Greene et al., 1999), and metyrosine (a DA synthesis inhibitor) was also reported to reduce dyskinesia severity (Olanow et al., 2001; see reply by Freed et al.). Three of the five patients subsequently required deep brain

TABLE 10.1. Overview of Grafting Protocols Used in Three Series of Patients Receiving Intrastriatal Grafts of Human Embryonic VM Tissue*

| | Tissue storage | Graft tissue | Surgical approach: graft placement | No. of trajectories (per putamen) | Immuno-suppression |
|---|---|---|---|---|---|
| Denver | 1–4 weeks | Strands | Axial; throughout the ventral and dorsal putamen | 2 | 0 |
| Lund | ≤ 12 hours (n = 12) | | | | |
| | 1–8 days (n = 2) | Cell suspension | Coronal; anterior, middle and posterior putamen | 3-7 | > 15 months (C+P+A) |
| Tampa | ≤ 48 hours | Solid pieces | Coronal; post-commissural putamen | 8 | 6 months (C) |

* VM, ventral mesencephalon; C, cyclosporine; P, prednisolone; A, azathioprine.

stimulation (DBS) of the internal segment of globus pallidum, which successfully ameliorated the dyskinesias (Olanow et al., 2001).

In a second placebo-controlled RCT, Olanow et al. (2003) implanted solid pieces of human embryonic VM bilaterally into the postcommissural putamen (see above; Table 10.1). Immunosuppressive treatment with cyclosporine was given for 6 months after surgery and patients were followed for 2 years. The trial failed to meet its primary outcome, i.e., group difference in UPDRS motor scores at 24 months, although the patients grafted with tissue from four donors showed progressive improvements up to 6-9 months after surgery (but deteriorated thereafter). Putamen FD PET uptake showed significant increase in grafted patients at 12 months, as compared to controls and non-grafted striatal areas, and remained largely stable at 2 years after transplantation. Thirteen out of 23 patients (56.5%) developed postoperative off-medication dyskinesias between 6 and 12 months after grafting. Dyskinesias consisted of stereotypic, rhythmic movements in the lower extremities. Dyskinesia severity appeared to be generally mild, as judged by mean scores of 3.2 and 2.7 out of a possible maximum score of 28 in the one- and four-donor group, respectively (no significant difference between groups), but were disabling and required surgery in three cases. Off-medication dyskinesia scores did not correlate with "on"-phase dyskinesia scores, UPDRS motor scores, amount of daily dopaminergic medication or striatal FD PET uptake.

The observations by Olanow and co-workers are similar to those from a retrospective video based re-evaluation of dyskinesias in 14 patients grafted in an open-label fashion up to 11 years earlier (Hagell et al., 2002). Immunosuppressive combination therapy (cyclosporine, prednisolone, and azathioprine) was given for more than 15 months following transplantation (Table 10.1). In this group of patients, off-medication dyskinesias increased or developed at late postoperative periods and typically manifested as activity-related choreiform movements intermingled with dystonic postures and repetitive, stereotypic, or ballistic, movements (Fig. 10.5). In eight patients, dyskinesias were mild and caused no distress or disability. In several of these cases, neither the patients nor their assessors had paid any previous attention to the dyskinesias, which were first revealed upon close re-examination of video recordings for the study. In the remaining six patients, dyskinesias were of moderate severity and in one patient they constituted a clinical therapeutic problem. In this patient, L-DOPA and DA agonists were withdrawn for up to nine weeks with no apparent effect on the severity of the dyskinesias (Hagell et al., 2002). Two of the patients had received tissue that had been stored for 1 to 8 days in the presence of glial cell line-derived neurotrophic factor (GDNF), whereas the other 12 patients had received fresh tissue (implanted up to about 12 hours following abortion). Conspicuously, the two patients grafted with stored tissue developed more pronounced postoperative off-medication dyskinesias (median global CDRS score: 15) than patients implanted with fresh tissue (median global CDRS score: 3.5). No correlation was found between the magnitudes of postoperative off-medication dyskinesias and antiparkinsonian

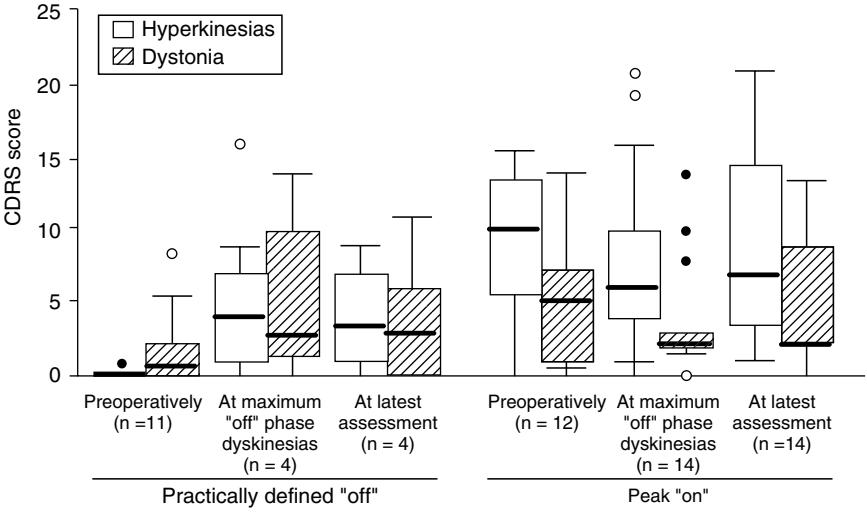


FIGURE 10.5. Hyperkinesias and dystonia, as assessed according to the CDRS (maximum score = 28), during practically defined "off" and peak "on" phases before transplantation, at the time of maximum postoperative dyskinesias (mean, 39.8 months), and at the latest assessment (mean, 44.6 months). **Solid horizontal lines** are median values, **boxes** are inter-quartile ranges, **error bars** are ranges. **Open** and **filled circles** are outliers and extremes (>1.5 and >3 box lengths from the 25th/75th percentiles, respectively). Hyperkinesias and dystonia during "off" increased significantly over time ($P < 0.001$ and $< 0.05$, respectively), whereas peak "on" dyskinesias did not (Friedman test). Data from Hagell et al., 2002. CDRS, Clinical Dyskinesia Rating Scale.

graft response ($r_s = -0.003$). Furthermore, these two phenomena also displayed different temporal developments following transplantation. Whereas dyskinesias reached their maximum at 24–48 months after transplantation, the antiparkinsonian response developed during the first postoperative year (Fig. 10.6).

Some interesting observations were made upon examination of the development of dyskinesias in individual patients. Thus, differential developments of off-medication and "on"-phase dyskinesias following grafting were observed in several patients (Fig. 10.7). Two patients with virtually no preoperative "off"-phase dyskinesias developed mild dyskinesias in "off" after grafting. Concomitantly, however, their preoperatively pronounced L-DOPA-induced peak "on" dyskinesias were reduced by more than 50% following the intake of an individually standardized L-DOPA dose (which was the same at all assessments). In another patient with no preoperative but pronounced postoperative "off" phase dyskinesias, virtually no change in peak "on" phase dyskinesia scores was observed. These observations suggest that grafts not only are able to induce or worsen "off"-medication dyskinesias but also, as discussed above, ameliorate L-DOPA-induced peak "on" dyskinesias, as would be predicted from animal models (see above).

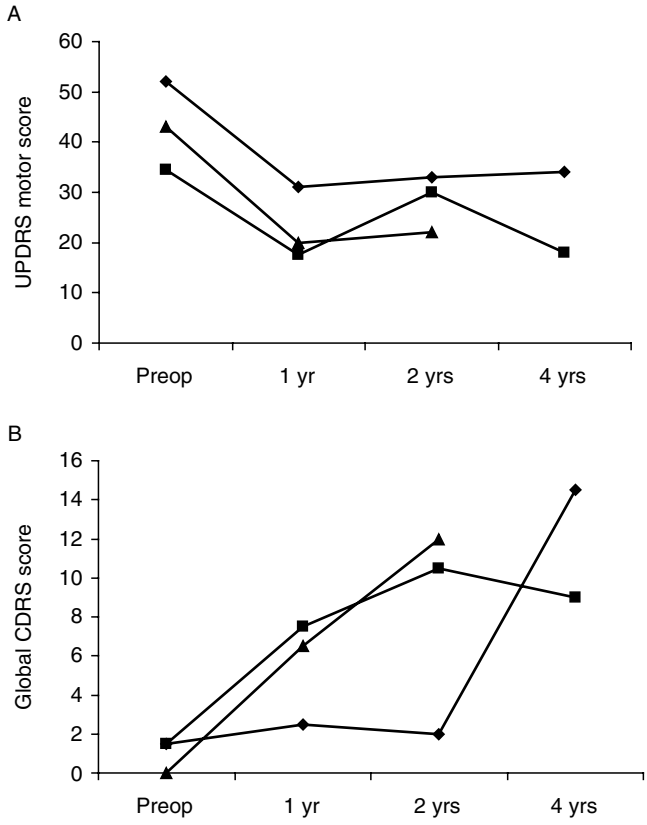


FIGURE 10.6. Evolution of clinical improvement and "off" dyskinesias after neural grafting in the three bilaterally grafted patients who showed the highest postoperative "off" phase dyskinesia scores in the Lund series of patients (Hagell et al., 2002). **A**: Maximum symptomatic relief (as assessed by the UPDRS motor score; maximum score = 108) occurred already by 12 months after transplantation, whereas **(B)** "off" phase dyskinesias (as assessed by the global CDRS score; maximum score = 28) were more protracted, not reaching their maximum until 24–48 months postoperatively. All scores are from the practically defined "off" phase (Langston et al., 1992). UPDRS, Unified Parkinson's Disease Rating Scale; CDRS, Clinical Dyskinesia Rating Scale.

### 4.2.1. Phenomenology of Off-Medication Dyskinesias Following Neural Transplantation in PD

In order to better understand post-operative off-medication dyskinesias, it may be of interest to consider their clinical characteristics and compare them with those seen in dyskinetic non-grafted PD patients. As reviewed above, all types of dyskinesias may occur at any point during the dopaminergic drug cycle (Nutt, 1992). However, dystonia predominates in the "off" phase and choreiform movements, with or without dystonic features, predominate during the "on" phase. Biphasic dyskinesias are typically

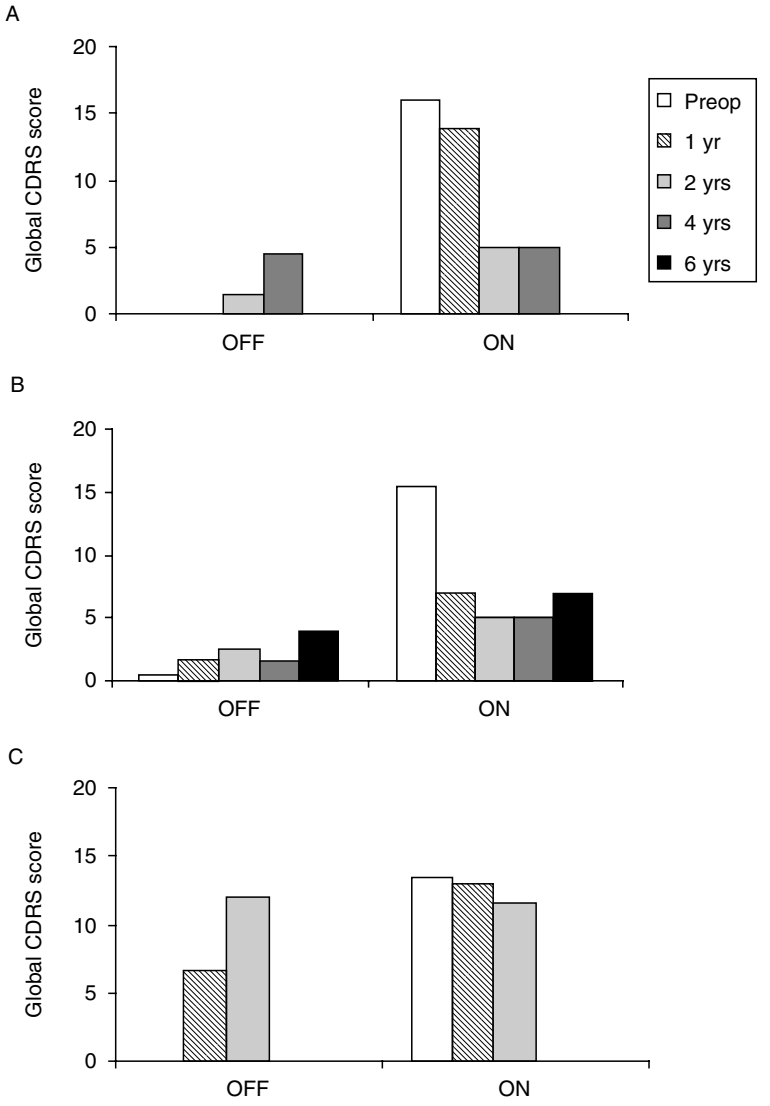


FIGURE 10.7. Differential developments of off-medication dyskinesias (as assessed during the practically defined "off" phase; **OFF**) and L-DOPA induced "on" phase dyskinesias (as assessed at the peak L-DOPA effect following intake of an individually standardized L-DOPA dose, which was the same at each assessment; **ON**) in three patients **(A–C)** from the Lund series grafted bilaterally with human embryonic VM tissue to the striatum (Hagell et al., 2002). Dyskinesia scores are global CDRS scores (maximum score = 28). CDRS, Clinical Dyskinesia Rating Scale.

characterized by repetitive movements and dystonia affecting the lower extremities. Reports of postoperative off-medication dyskinesias in transplanted PD patients have provided somewhat different accounts regarding the physical characteristics of the dyskinetic symptoms. Dystonia was reported to predominate in patients from the Denver/New York trial, although choreiform movements also occurred (Greene et al., 1999). Subsequently, it has been reported that "many of the features of the posttransplant dyskinesias resemble the biphasic dyskinesias that occur with L-dopa administration," but with an atypical topographic distribution to the head and upper extremities (Ma et al., 2002). Olanow et al. (2003) described the off-medication dyskinesias in their trial as being stereotypic, rhythmic movements of one or both the lower extremities, similar to biphasic dyskinesias. In the Lund series of patients, postoperative off-medication dyskinesias were described as choreiform movements intermingled with dystonic postures and repetitive movements (Hagell et al., 2002). When we re-examined these data for a more detailed description we found that dystonia was the predominant type of off-medication dyskinesias in 50% of the cases, repetitive movements in 14%, a combination of dystonic and repetitive movements in 14%, choreiform movements in 14%, and a combination of choreiform and dystonic movements in 7%. Postoperative off-medication dyskinesias were observed in all body parts. However, a closer examination of the anatomical score distribution at the time of maximal off-medication dyskinesias (i.e., between 11 and 132 months post-grafting) reveals a preferential distribution to the lower limbs. These observations are thus in line with the clinical description provided by Olanow et al. (2003) but not with that provided by Ma et al. (2002), where postoperative dyskinesias affected either the head and neck or the upper limbs. In summary, while there are some reported differences in the phenomenological features of the dyskinetic symptoms between these three centers, there appears to be a similar overall pattern of predominance, where postoperative off-medication dyskinesias in grafted PD patients primarily appear to resemble biphasic dyskinesias (Table 10.2).

TABLE 10.2. Dominating Types of Off-Medication Post-Transplantation Dyskinesias and "Off," Biphasic, and "On"-Phase Dyskinesias in Non-Grafted PD Patients[a]

| | Choreiform | Dystonic | Repetitive |
|---|---|---|---|
| Dyskinesias in non-grafted patients (Cubo et al., 2001; Luquin et al., 1992; Nutt, 1992) | | | |
| "Off"-phase dyskinesias | (+) | +++ | (+) |
| "On"-phase dyskinesias | +++ | ++ | (+) |
| Biphasic dyskinesias | (+) | ++ | +++ |
| Off-medication post-transplantation dyskinesias | | | |
| Denver (Greene et al., 1999) | + | +++ | n.r.[b] |
| Lund (Hagell et al., 2002) | + | +++ | ++ |
| Tampa (Olanow et al., 2003) | n.r. | n.r. | +++ |

[a] Key: +++, typical; ++, common, +, usually not predominant; (+), reported but less common; n.r., not reported.

[b] Ma et al.(2002) reported posttransplant dyskinesias in these patients to resemble biphasic dyskinesias.

## *4.3. Are Postoperative Off-Medication Dyskinesias a Novel Phenomenon?*

The emphasis on severe dyskinesias as an adverse effect of neural grafting in PD was prompted by the results from the first neural transplantation RCT by Freed and collaborators in 2001. However, clinical trials had been going on for over a decade in several countries, and it has been estimated that at least 300 PD patients had received human embryonic VM grafts prior to this report. Does this mean that severe off-medication dyskinesias had not occurred in grafted patients in earlier trials, or that open-label investigators had failed to report such events? The answer is most probably no in both instances. Indeed, troublesome post-grafting dyskinesias had been reported also before 2001. Defer and associates (1996) reported delayed (after 8–26 months) asymmetrical dyskinesias on the side contralateral to unilateral grafts, in three out of five cases. While described as "on" phase dyskinesias, attempts to counteract them by means of adjusting dopaminergic medication were not successful and doses of antiparkinsonian medication remained largely unchanged throughout the trial (Defer et al., 1996). In one patient, L-DOPA treatment actually had to be increased shortly after the late development of dyskinesias, by the end of the second postoperative year. This may indicate that the dyskinetic complications were of a similar nature to subsequently reported off-medication dyskinesias (Freed et al., 2001; Hagell et al., 2002; Olanow et al., 2003). From another transplantation center, Jacques et al. (1999) reported development of dyskinesias in a patient following bilateral grafts to the postcommissural putamen. While details are sparse, it was reported that these dyskinesias could not be alleviated by adjustment of the medical regimen and were severe enough to require pallidotomy, which effectively resolved them (Jacques et al., 1999).

The reason for the relative lack of reported post-grafting dyskinesias previous to the Freed trial may well be explained by two related factors. First, as illustrated in the study by Hagell et al. (2002), and probably also by Olanow et al. (2003), postoperative dyskinesias are often mild and do only rarely appear to reach a degree of severity at which they constitute a clinical problem. Second, as discussed above, lack of specific and sensitive dyskinesia assessment tools may have led investigators to unintentionally overlook milder degrees of dyskinesias. These factors may also be responsible for the relatively high incidences of off-medication dyskinesias reported in the two more recent studies that had been designed to assess this phenomenon (Hagell et al., 2002; Olanow et al., 2003). Although Freed et al. (2001) reported a lower (15%) incidence of postoperative dyskinesias than did Hagell et al. (2002) and Olanow et al. (2003), it is not clear whether there is an actual difference between the three studies. Indeed, if only severe dyskinesias that constitute clinical therapeutic problems are considered, the incidences reported by Hagell et al. (2002) and Olanow et al. (2003) can be estimated to 7-14% and 13%, respectively. While detailed and randomized video-based assessments of dyskinesias, using

specific and sensitive rating scales, were used in the two latter studies (Hagell et al. 2002; Olanow et al. 2003), no specific dyskinesia assessments appear to have been included in the protocol employed by Freed et al. (2001).

Taken together, available observations from clinical PD transplantation trials leave little doubt that human allogeneic intrastriatal grafts of embryonic DA-rich VM tissue are able to induce dyskinesias that can persist even in the absence of ongoing dopaminergic drug therapy. Such dyskinesias have hitherto been referred to as, e.g., "runaway", "off-phase," or "off-medication" dyskinesias. However, none of these terms appears sufficiently clear or specific for the phenomenon at hand. We therefore propose the term graft-induced dyskinesias (GID), which unequivocally refers to a causal relationship between the postoperative dyskinesias and the intracerebral graft in the striatum. Moreover, this term has the advantage of avoiding any reference to a temporal relationship with the intake of L-DOPA. Indeed, although these dyskinesias are usually examined when the patients are off medication, their modification during the L-DOPA dosing cycle remains to be clarified. Gaining insight into the pathophysiology of GID seems to be a very important goal to pursue. This will allow us to design DA cell replacement trials that can provide relief of parkinsonian disability without inducing abnormal, unwanted movements. In the following sections, we shall review data that can shed light on the possible mechanisms of GID. We shall first present some important clues that have been provided by neuroimaging studies in transplanted patients. We shall then review results and speculations that are based on preclinical studies in animal models.

## *4.4. Neuroimaging Studies of Graft-Induced Dyskinesias*

Most clinical trials of neural transplantation in PD have attempted to provide objective documentation on the extent of graft survival and graft integration in the host brain. Functional neuroimaging techniques have been particularly useful for this purpose and a majority of clinical transplantation trials have used FD PET to assess graft survival (see Chapter 7). FD PET provides a direct measure of DA storage capacity in the brain, and, indirectly, a measure of DA innervation density. Results from FD PET investigations performed in different trials have shown that VM grafts are able to normalize levels of FD uptake in grafted striatal areas (Piccini et al., 1999), although the uptake typically has increased from about 30% of normal values preoperatively to 50–60% during the second year after grafting (Hagell and Brundin, 2001). After the first reports of dyskinesia in transplanted PD patients, the question arose whether the development of GID was correlated with the levels of striatal DA innervation established by the grafts. In their retrospective study, Hagell and coworkers (2002) found a moderately strong inverse correlation between preoperative FD uptake in the putamen and the severity of GID ($r_s$ = −0.549), but not between GID and either the overall levels of postoperative putaminal FD uptake ($r_s$ = –0.132) or its increase as compared to preopera-

tive values ($r_s = -0.267$). Similarly, Olanow et al. (2003) reported a lack of correlation between putaminal FD uptake and GID in their RCT.

A PET study performed on 17 PD patients from the trial by Freed et al. (2001) examined not only the overall density of FD uptake, but also the spatial distribution of the changes in FD uptake produced by VM grafts in the putamen (Ma et al., 2002) Five of the patients exhibited GID ($DYS^+$), while 12 had not developed this complication ($DYS^-$). An interesting pattern of differences in FD uptake was found between these two groups. Compared to the nondyskinetic cases, the $DYS^+$ group showed significantly higher levels of FD uptake in the left, but not the right putamen (peculiarly, the left putamen was not always contralateral to the side of the body more affected by GID). Despite the observed increase in left putamen PET signal, none of the $DYS^+$ patients achieved supranormal levels of DA innervation, which is in agreement with the data reviewed above. When the spatial distribution of the PET signal was examined using a voxel-by-voxel approach, the $DYS^+$ patients exhibited spots of increased FD uptake in two discrete areas. The first was situated in the dorsal-posterior putamen. At 24 months post-transplantation, the PET signal in this region reached 60% of normal in the $DYS^-$ group and 80% of normal in the $DYS^+$ group. The second area was situated in a ventral putaminal region that showed relative sparing of intrinsic DA fibers. This region showed no significant post-operative change in FD uptake in the $DYS^-$ group, amounting to 57% of normal. By contrast, post-transplantation values reached approximately 73% of the normal mean values for this location in the $DYS^+$ group. When the dyskinetic patients were compared with the subgroup of nondyskinetic cases who had showed a similar postoperative improvement of parkinsonian symptoms, a statistically significant difference between the $DYS^+$ and $DYS^-$ groups persisted only in the ventral, but not in the dorsal location. Interestingly, 3 out of the 5 patients in the $DYS^+$ group had postoperative dyskinesias affecting primarily the head and the neck, i.e., regions that are somatotopically represented in the ventral putamen. The study thus suggested that GID could be associated with areas of relatively high DA innervation in restricted striatal regions. However, in the RCT by Olanow et al. (2003), no differences were reported either in regional or global levels of striatal FD uptake between patients who did and did not develop GID.

In addition to FD, the reversible DA D2 receptor ligand [11C] raclopride (RAC), has been used to study the function of VM grafts in patients affected by GID (Huang et al., 2003; Piccini et al., 2005). RAC PET takes advantage of a competition between endogenous DA and RAC for binding at D2 receptors. Thus, this technique can be used to estimate the levels of DA release in the striatum under normal circumstances and after pharmacological or physiological stimulation. An increase in striatal DA levels thus translates into a lower RAC binding potential compared to baseline values (de la Fuente-Fernández and Stoessl, 2002). The GID cases examined by Piccini et al. (2005) and Huang and colleagues (2003) showed lower baseline levels of RAC binding in the putamen compared to the values usually found in nontransplanted PD patients (Piccini et al. 2003). Decrease in baseline RAC binding potential compared to

nongrafted PD patients indicates the presence of VM grafts that are producing and releasing DA within the striatum, causing either a downregulation or increased occupancy of D2 receptors by DA. It should, however, be pointed out that a reduction of baseline RAC binding is not in itself a specific correlate of GID. Indeed, this finding was also reported in a RAC PET study performed on a transplanted PD patient who was not affected by any significant GID (Piccini et al., 1999). Piccini et al. (2005) also examined changes in RAC receptor binding induced by the administration of metamphetamine (which stimulates endogenous DA release) in eight patients from the Lund open label transplantation trials who displayed various degrees of GID. This study found no evidence of an association between GID and global or regional putaminal DA release either under basal conditions or after methamphetamine administration (Piccini et al., 2005). Instead of using metamphetamine, Huang et al. (2003) examined changes in RAC signal produced by the administration of L-DOPA in two GID-affected patients. Following this drug challenge, both patients showed a pronounced and prolonged reduction in RAC binding potential. It is difficult to determine the significance of the latter findings to the pathophysiology of GID, particularly because the study did not include any control data from either transplanted patients not affected by GID, from nongrafted PD patients, or from healthy subjects.

Taken together, PET studies in PD patients argue against the notion that GID results from supranormal levels of DA innervation in the graft-innervated striatum. However, data are either conflicting or insufficient regarding the regional distribution of the graft-derived dopaminergic reinnervation in the patients affected by GID. The possibility remains that imbalances in the distribution of DA storage and release within the striatum may contribute to the development of GID (Ma et al., 2002; Piccini, 2002). Moreover, studies using RAC PET in a larger number of transplanted patients are required to clarify the dynamics of striatal DA release in GID.

## *4.5. Graft-Induced Dyskinesias: Possible Cellular Mechanisms*

Several possible mechanisms have been proposed in the literature and/or discussed at scientific meetings to explain the development of GID. These mecha-

TABLE 10.3. Possible Causes and/or Risk Factors for Graft-Induced Dyskinesia

1. Excess levels of DA
2. Low-intermediate levels of DA
3. Patchy innervation and residual DA receptor supersensitivity in the host striatum
4. Abnormal graft-to-host synaptic contacts
5. Ectopic striatal innervation by A10 neurons
6. Nondopaminergic cells within the grafts
7. Nonspecific neuronal damage in the host striatum
8. Inflammatory and immune responses

nisms are listed in Table 10.3. Each of them is also listed and discussed below according to the same order. All of these mechanisms but the first in the list (i.e., "excess levels of DA") reflect some well documented features of VM graft action and/or composition. However, the relative role played by each factor in the development of GID is unknown. Because our knowledge of the mechanisms that trigger, maintain, or modulate dyskinesia in PD is largely incomplete, we are not yet in a position to reject any of the listed hypotheses. Thus, rather than providing simplified explanations, we will draw the reader's attention to the complex neurobiology of VM transplants, and we shall highlight the need for further experimental studies addressing the mechanisms of GID in animal models.

### 4.5.1. Excess Levels of DA

As discussed above, dyskinesias in PD are typically induced by L-DOPA, which is converted to DA in the brain and then exerts its action through stimulation of brain DA receptors. Virtually all forms of dyskinesia, even those that are primarily caused by nondopaminergic mechanisms, are attenuated by treatment with DA receptor antagonists (Gimenez-Roldan and Mateo, 1989; Rosin et al., 1978; Pfeiffer and Wagner, 1994). It is therefore understandable that a first tentative explanation of GID postulated excess DA due to continued fiber outgrowth from the VM transplants (Freed et al. 2001; Kolata, 2001). As reviewed above, data from FD and RAC PET investigations have failed to show supranormal levels of striatal DA innervation or release in patients affected by GID (Hagell et al., 2002; Ma et al., 2002; Olanow et al., 2003; Piccini et al., 2005). These data are in keeping with a body of knowledge gained from experimental studies. Results obtained in the rat 6-OHDA-lesion model of PD have consistently shown that VM grafts do not produce supranormal levels of DA fiber density (Doucet et al., 1990; Kirik et al., 2001) and/or DA release (Cenci et al., 1994; Cragg et al., 2000; Forni et al., 1989; Moukhles et al., 1994; Rioux et al., 1991; Strecker et al., 1987; Zetterström et al., 1986). In fact, the denervated striatal target appears to exert a strict homeostatic control on the extent of graft-derived DA innervation. One or more trophic factors are produced in the striatum upon DA denervation to support regrowth of DA axons (Carvey et al., 1996), and this stimulatory effect is reduced in rats, where part of the intrinsic DA system is left intact (Doucet et al., 1990; Kirik et al., 2001). When VM grafts are implanted in a partially DA-denervated striatum in the rat, the total striatal DA innervation density, i.e., the residual innervation from the host and the graft-derived innervation combined, never exceeds 70% of normal values (Kirik et al., 2001). Taken together, these results speak against the possibility that DA hyperinnervation may result from either large graft sizes or graft placement in striatal areas that are relatively spared from the neurodegenerative process in PD.

### 4.5.2. Low–Intermediate Levels of DA

The neuroimaging study of Ma et al. (2002) reported that GID is associated with focal areas of increased FD uptake within the grafted putamen, i.e., with

uneven and patchy graft-derived DA reinnervation. However, even within these areas, the restoration of DA activity is incomplete. If GID is caused by the dopaminergic component of the graft, it would then resemble biphasic dyskinesia rather than "on" phase dyskinesias in terms of pathophysiological mechanisms (Marconi et al., 1994). This concept is supported by phenomenological similarities between GID and biphasic dyskinesias (Table 10.2), although the topographical distribution of GID in Ma et al. (2002) did not follow the pattern typical of biphasic dyskinesias (see 4.2.1). As proposed by Ma and colleagues (2002), this discrepancy may be explained by the surgical approach used for transplantation in their study, whereby grafts where placed in the ventrocentral putamen, i.e., the region controlling head and neck movements (Ma et al., 2002). However, this explanation remains tentative, because other clinical transplantation trials may also have involved grafting of VM tissue to the ventrocentral putamen (Hagell et al., 1999; Rehncrona, 1997) without reporting any particular preference of GID for the neck and the head regions (Hagell et al., 2002). Nevertheless, the hypothesis that low-intermediate levels of striatal DA serve as a crucial trigger to GID remains highly plausible. Insufficient restoration of striatal DA levels could thus elicit dyskinesia without producing a significant amelioration of parkinsonian motor symptoms, similar to what is seen at the beginning and end of the L-DOPA action cycle (Marconi et al., 1994). It now remains to be explained why only a small proportion of the transplanted PD patients develops clinically significant GID, since all patients with surviving VM grafts would be expected to experience an incomplete restoration of striatal DA levels.

### 4.5.3. Patchy Innervation and Residual DA Receptor Supersensitivity in the Host Striatum

Although VM grafts do not provide supranormal DA reinnervation of the host striatum, a relative "hyperdopaminergic" state may be established by a combination of two factors: (i) uneven striatal reinnervation by the grafts (see above); and (ii) residual DA receptor supersensitivity in nonreinnervated striatal regions, which are reached by ambient DA that diffuses away from the grafted neurons. Both animal models and clinical studies are concordant in indicating that the distribution of graft-derived striatal DA innervation is uneven. Doucet et al. (1990) used [$^3$H] DA uptake autoradiography to assess the extent of striatal DA innervation after standard transplantation techniques in the rat. In the 6-OHDA-lesioned striatum the density of graft-derived DA innervation amounted to, on average, 55% of normal in the tissue surrounding the graft, but declined rapidly thereafter. At a distance of 1.8 mm from the graft–host border, the density of DA-rich fiber swellings (varicosities) was only approximately 13% of normal values. In the same study, an average 7% of the total neostriatal area was estimated to remain completely devoid of DA varicosities of graft origin. Following this study, different cellular and molecular markers have been used to address the question whether or not VM

transplants can reverse the supersensitivity of DA receptors also in nonreinnervated striatal regions. In rat models of PD, VM grafts were found to normalize the expression of D2 and D1 receptor mRNA and/or radioligand binding densities in the whole striatum (Chritin et al., 1992; Savasta et al., 1992; Dawson et al., 1991; Gagnon et al., 1991). Even when DA receptor supersensitivity was assessed at the signal-transduction levels (apomorphine-induced c-Fos and neuropeptide mRNA upregulation), reversal of the lesion-induced supersensitivity was seen also in striatal subregions that were not reached by graft-derived DA fibers (Cenci et al., 1992b; 1993). The effects of the transplant in non-reinnervated regions were thus attributed to a paracrine action ("volume transmission") of extracellular DA. Voltammetric recordings in the 6-OHDA-lesioned and VM-grafted rat striatum have shown that the low levels of evoked DA release are counterbalanced by an extended extracellular lifetime of the released DA, such that ambient levels of DA appear to be normal over a large extracellular sphere (Cragg et al., 2000). Yet the paracrine action of DA may not be sufficient to normalize all aspects of the denervation-induced striatal plasticity. Using in vivo electrophysiological recordings, Strömberg et al. (2000) have shown that striatal regions adjacent to VM grafts, but not directly innervated, maintain an increase in spontaneous firing rate and an enhanced responsiveness to apomorphine. Arguably, the amount of striatal tissue exhibiting residual supersensitivity after grafting would be comparatively extensive in humans, where the volume of the striatum is several-fold larger than it is in either rats or monkeys. It also remains unknown whether VM grafts are equally successful in normalizing striatal DA-receptor binding in the clinical setting as they are in animal experiments. In summary, many different lines of evidence, including a recent study in rats (Maries et al., 2005), lend credibility to the suggestion that patchy areas of DA reinnervation in an otherwise DA-depleted structure may predispose to GID. Yet, this hypothesis cannot be considered as proven. If GID were caused by diffusion of graft-derived DA to non-reinnervated areas it would be expected to exhibit maximal severity at early time points post-transplantation, followed by a gradual amelioration as the sensitivity of DA receptors is gradually restored to low, normal levels. In contrast, the occurrence of GID exhibits a delayed post-operative development in all the available clinical studies (Freed et al., 2001; Hagell et al., 2002; Olanow et al., 2003; Piccini et al., 2005).

### 4.5.4. Abnormal Graft-to-Host Synaptic Contacts

The vast majority of tyrosine hydroxylase (TH)-positive fiber endings present in the striatum are found in close apposition to the neck of dendritic spines, where they form symmetric synapses. Asymmetric synaptic contacts (presumably glutamatergic in nature) are found on the head of the spines that receive TH-positive boutons (Freund et al., 1984). This precise synaptic arrangement is thought to be essential to a fine DA-dependent gating of cortico- and thalamo-striatal inputs (Smith and Bolam, 1990; Lovinger and

Tyler, 1996). Grafted VM neurons are known to establish synaptic connections with the host striatal neurons (Jaeger, 1985; Bolam et al., 1987; Kordower et al., 1996; Mahalik et al., 1985). While the density of graft-derived DA synapses in the reinnervated portion of the striatum is close to normal, their subcellular distribution is not. An ultrastructural study of VM grafts in 6-OHDA-lesioned rats found that DA synapses were less frequent on dendrites and more abundant on neuronal perikarya in the reinnervated portion of the host striatum compared to the normal striatum. On dendrites, the proportion of contacts on spines was lower, and contacts on shaft higher than normal in the grafted animals (Freund et al., 1985). Similar findings were reported by Clarke et al. (1988) in a human-to-rat VM transplantation paradigm. It is possible that the failure of the grafts to restore a precise distribution of synaptic contacts on the host neurons results in an abnormal gating of corticostriatal inputs. This hypothesis finds support in several lines of evidence. Rats with intrastriatal VM grafts exhibit contralateral rotation and dyskinetic-like behaviours when administered with amphetamine (Carlsson et al., 2005; Lane et al., 2005). These abnormal behavioural responses (the contralateral rotation, at least) are strongly correlated with a hyperexpression of c-Fos in the graft-innervated portion of the host striatum, and they are abolished by a lesion of the host corticostriatal pathway (Cenci et al., 1994). These data would suggest that an inadequate gating of corticostriatal inputs by graft-derived DA synapses may cause dyskinesia via, e.g., an abnormal regulation of gene expression (Cenci et al., 1993) and synaptic plasticity in the striatum (Picconi et al., 2003) (Fig. 10.1). However, this interesting hypothesis requires experimental verification. This could be achieved by comparing the motor effects of VM grafts with those of non synapse-forming striatal implants (e.g., DA-secreting polymers, or DA-secreting non-neuronal cells).

### 4.5.5. Ectopic Striatal Innervation by A10 Neurons

The ventral midbrain contains functionally different groups of DA neurons, which have been denominated as A8, A9, and A10 since their first description (Dahlström and Fuxe, 1964). These nuclei form a fairly continuous cell system, which comprises, medially, the ventral tegmental area (A10), and laterally the pars compacta and pars lateralis of the substantia nigra (A9). Only the A9 group of midbrain DA neurons innervates motor areas within the striatal complex. The same group of DA neurons shows selective vulnerability to the neurodegenerative process at the basis of PD (for review, see Isacson et al., 2003). However, VM donor tissue used for transplantation contains the primordia of both A9 and A10 regions (Haque et al., 1997). Accordingly, light- and electron microscopic analyses of DA cells within VM grafts show a much larger heterogeneity of neuronal morphologies and sizes than seen in the A9 cell group in situ (Jaeger, 1985). A9 and A10 cells have different functional properties in terms of firing patterns and dynamics of DA release in response to different stimuli (Rice et al., 1997; Cenci et al., 1992; Cragg et al., 2002),

kinetics of DA reuptake (Blanchard et al., 1994), and capacity for axonal growth after transplantation (Schultzberg et al., 1984; Haque et al., 1997). Moreover, since DA receptor-mediated autoregulation of DA release is more efficient in A9 than A10 cells, the former would be less prone to cause abnormal fluctuations in DA levels in the graft innervated striatum (Isacson et al., 2003). In summary, several lines of evidence indicate that currently employed transplantation protocols cause part of the host striatum to receive an ectopic reinnervation by A10 donor cells. The relative amount of A10-derived innervation is likely to increase when VM donor tissue is stored before transplantation, causing in vitro-based selection of A10 neurons over A9 cells, due to higher sensitivity to oxidative stress in the latter (Isacson et al., 2003). A10 neurons can engraft in the host putamen, but are unable to restore normal DA-dependent functions and innervation patterns. Consequently, there is concern that this factor may contribute to an inadequate relief of parkinsonian motor symptoms and/or to the development of dyskinetic side effects after intrastriatal grafting (Isacson et al., 2003). Animal experiments that specifically address the role of A10-rich donor tissue as a risk factor for GID are therefore highly warranted.

#### 4.5.6. Nondopaminergic Cells Within the Graft

All the arguments developed so far build on the assumption that GID are exclusively mediated by the DA neurons within the transplants. It is therefore important to point out that only 5–11% of the cells within freshly dissociated VM tissue have a dopaminergic phenotype (Sauer and Brundin, 1991 and Chapter 8). The remaining 89% to 95% of the cells are either glia or nondopaminergic neurons, which contain a variety of neurotransmitters, such as GABA, serotonin, acetylcholine, and substance P (Doucet et al., 1989; Cenci et al., 1993, 1997; Jaeger, 1985). Storing or culturing VM tissue prior to transplantation is expected to further alter its cellular composition in favor of nondopaminergic cell types, which have a lower sensitivity to oxidative stress and/or a higher mitotic rate than do DA neurons (Fawcett et al., 1995; Zhou et al., 1994; Sauer and Brundin, 1991). At present, the contribution of transplanted nondopaminergic neurons to the development of GID is difficult to estimate. However, an increasing number of clinical and experimental studies are showing antidyskinetic effects by a wide range of substances that target nondopaminergic receptors in the brain (for review, see Rascol, 2000). Moreover, growing evidence from animal models of PD indicates that dyskinesias can be triggered by modulation of nondopaminergic receptors (Konitsiotis et al., 2000), signaling molecules (Andersson et al., 2001), and/or membrane-bound ionic pumps in the striatum (Andersson and Cenci, unpublished). Regarding the role of transplanted glial cells, it is worth pointing out that astrocytes are particularly abundant both within VM grafts and at the graft-host interface (Bolam et al., 1987; Barker et al., 1996; Reum et al., 2002). Many of these astrocytes show morphological features of activation, similar to those observed after injury of

brain tissue (Jaeger, 1985). Reactive astrocytes are known to produce cytokines and trophic factors that have profound effects on the activity and signal-transduction machinery of striatal neurons (see below). Culturing VM tissue for a few days prior to transplantation has been shown to cause an even larger representation of glial cells relative to neurons (Höglinger et al., 1998). In summary, the contribution of nondopaminergic graft tissue to the development of GID needs to be addressed under well-controlled conditions in animal models. Specific experiments need to be designed to clarify the effects of pregrafting storage on the composition and functional outcome of VM grafts. Unlike most transplantation experiments that have been performed in animals, several clinical transplantation trials have included a pregrafting step where embryonic VM pieces were stored or cultured for a few days or weeks. As reported above, among the Lund series of transplanted PD patients the most pronounced GID have occurred in the two cases implanted with VM tissue that had been stored for 1–8 days in the presence of GDNF (Hagell et al., 2002). The addition of trophic factors to the storage medium preserves a high number of DA neurons in the VM tissue but may alter the functional properties and growth potential of the grafts (Hoglinger et al., 2001).

### 4.5.7. Nonspecific Neuronal Damage in the Host Striatum

Dyskinesias with choreiform and/or dystonic features are encountered in some diseases characterized by striatal neuron degeneration, such as Huntington's disease (Lanska, 2000) and multiple system atrophy (Boesch et al., 2002), and following focal striatal lesions (Bhatia and Marsden, 1994). It is therefore worthwhile pointing out that intrastriatal tissue implantation inevitably produces a certain degree of nonspecific damage. In the study by Freed et al. (2001), grafts were placed along two 35–40-mm sagittal trajectories throughout each putamen, thus rendering a total "needle trauma" of 70–80 mm per putamen. Similarly, the majority of patients in the Lund series (Hagell et al., 2002) received grafts along five to seven 12–14 mm coronal trajectories (Hagell et al., 1999; Rehncrona, 1997), thus yielding a total "needle trauma" of 60–98 mm per putamen. This extent of striatal trauma has never been produced in any other surgical treatment for PD, and the impact of the surgery *per se* on parkinsonian motor symptoms and dyskinesias is therefore unknown. The possibility that some damage related to surgery may either cause or favor an uncontrolled motor response after transplantation is supported by studies performed in nonhuman primates, where small striatal lesions have been shown to cause dyskinetic motor responses following DA agonist treatment (Hantraye et al., 1990; Burns et al., 1995). Nonspecific tissue damage may upregulate the expression of cytokines and trophic factors, feeding forward to the mechanism listed as No. 8 in Table 10.3. The possible contribution of striatal damage to the development of GID is another issue that would lend itself to experimental verification in animal models of PD.

### 4.5.8. Inflammatory and Immune Responses

Some clinical observations suggest that chronic, partial graft rejection and/or the associated inflammatory response contributes to GID. In the first NIH-sponsored clinical transplantation trial, GID occurred during the second and third year after VM graft implantation (Freed et al., 2001), i.e., beyond the time point when a clinical benefit had been seen. Likewise, a delayed postoperative course was observed in the open-label trials performed in Lund (Fig. 10.3; Hagell et al. 2002; Piccini et al., 2005). In the second NIH-sponsored clinical transplantation trial, GID tended to develop at postoperative intervals no shorter than 6 months, which is the time point when the immunosuppressive treatment was discontinued (Olanow et al., 2003). Recently, Piccini etal. (2005) have provided some interesting observations from six patients transplanted in Lund and receiving immunosuppression for a mean of 29 months post-grafting. These patients showed continued increases in FD PET uptake and stable UPDRS motor scores up to 21-23 months after discontinuation of immunosuppression, indicating sustained survival and function of the grafts. However, GID scores were significantly elevated 23 months after withdrawl of immunosuppression as compared to 9 months before. This could indicate a link between low-grade inflammation around the graft and GID development, although GID had been observed also prior to the withdrawl of immunosuppression in three out of the six cases (Piccini et al., 2005). In some clinical trials, inflammation and microglial activation has been documented upon pathological examination of patients who happened to die for reasons unrelated to the surgery. In Freed et al. (2001), immunostaining with antibodies to the lymphocyte marker CD3 and HLA class II antigen revealed the occurrence of inflammatory cells in the transplant tracks and perivascular areas. Olanow et al. (2003) found that immunostaining for CD45, a marker of activated microglia and immune reaction, was increased in the transplanted striatum compared to controls, being particularly pronounced in and around graft deposits. Similar observations have been reported also in previous trials from autopsy findings performed at about 18 months postgrafting, and 12 months after discontinuation of cyclosporine treatment (Kordower et al., 1998). The ways by which immunological mechanisms may contribute to GID are at least twofold. First, a chronic and initially subclinic graft rejection may cause a reduction of graft size with time, feeding back to mechanism 2 in Table 10.3 ("low levels of DA receptor stimulation"). Second, inflammatory cells are known to release cytokines that can activate a number of intracellular and nuclear signaling pathways in the nearby neurons (Darnells et al., 1994; Hopkins and Rothwell, 1995; Rothwell and Hopkins, 1995). Of interest in this respect is the observation that cytokines can induce the expression of c-fos in the brain (Hisanaga et al., 1992; Niimi et al., 1996, 1997; Tchelingerian et al., 1997). In turn, high expression of Fos family proteins in striatal neurons correlates with the development of L-DOPA-induced dyskinesia in animal models (Doucet et al., 1996; Andersson et al., 1999; Winkler et al., 2002). There are thus a number of observations support-

ing the possibility that a late-onset low-grade inflammatory response may contribute to the development of GID. Again, this possibility lends itself to empirical examination in experimental animals.

## 5. Conclusions

While many fundamental questions are presently unresolved, some important conclusions can be drawn from the clinical data reviewed in this paper. First, intraputaminal embryonic VM grafts in human PD are indeed able to induce an unheralded form of dyskinesia that we have termed GID. Second, GID is unlikely to share the same pathophysiological basis as L-DOPA-induced "on" phase dyskinesias. Indeed, VM grafts have been found to produce improvement of L-DOPA-induced "on" phase dyskinesias in some patients who developed conspicuous GID. Third, GID does not seem to be a necessary side effect of efficacious DA-cell replacement in PD. Indeed, major improvement of parkinsonian disability postgrafting has been evident in patients who did not develop significant GID.

The mechanisms underlying the development of GID are presently unknown. Some clinical and experimental evidence suggests that GID is associated with patchy innervation of the host striatum and/or low-intermediate levels of DA receptor stimulation. However, these features are common to all intrastriatal VM grafts, and yet, severe GID have developed only in a small proportion of transplanted patients. It is therefore conceivable that the development of GID depends on several contributing mechanisms. While DA may be a necessary trigger to GID in all cases, the risk for GID development may be determined by factors related to the cellular composition of the grafts, which is expected to exhibit variations among patients and across clinical trials. The degrees of unspecific neuronal damage and inflammatory and immune responses in the grafted striatum are also likely to show individual variations. Preclinical research in animal models of VM transplantation and L-DOPA-induced dyskinesias has generated an impressive body of knowledge, which is now proving extremely useful for formulating hypotheses on the mechanisms of GID. We anticipate that pathophysiological questions of critical importance will be successfully addressed in animal experiments in the few years to come. Earlier animal studies on the effects of neural transplants on L-DOPA-induced dyskinesias were not designed to detect abnormal movements in the absence of L-DOPA administration. Hence, discrete dyskinesias may have occurred, but gone by unnoticed (Lane et al., 2005). Alternatively, the conditions predisposing to GID in the human transplantation trials, whether being relative graft size, graft location, graft composition, degree of host inflammatory response and/or storage of donor tissue prior to grafting, may not have been fulfilled in the experimental setting. The importance of such conditions is yet unknown but can easily be addressed in animal experiments that are specifically designed for this purpose. We also

anticipate that further neuroimaging studies in transplanted patients will provide important clues to the understanding of GID. Indeed, these studies will allow for a comparison between patients affected by severe GID and transplanted patients who are free from this complication. Neuroimaging techniques will provide an opportunity to examine some crucial issues, such as the dynamics of graft-derived DA release, and the degree of host tissue damage and/or inflammatory response.

The occurrence of GID has been discouraging for a field that was regarded as one of the most promising in the search for a curative approach to PD. However, as W. Maxwell Cowan and Eric R. Kandel have pointed out, "the history of science and medicine has taught us that the disappointments of today are often the prelude to tomorrow's success" (Cowan and Kandel, 2001, p. 595). We thus believe and hope that the many questions raised in the aftermath of GID will contribute to a considerable scientific advance not only in the development of cell-based therapies for PD, but also in the study of dyskinesias and DA-dependent motor control. Additionally, the occurrence of GID has taught us not to underestimate the risk of complex and unpredictable side effects when developing novel therapies for human neurological disorders. This is a lesson of outmost importance also for other emerging fields of translational neuroscience research, such as stem cells and gene therapy. Finally, while caution and patience are necessary, the occurrence of GID should not stop further systematic and goal-directed developments toward a cell-based restorative therapy for PD. Further clinical transplantation trials for PD are required. These should have well-defined scientific goals and should be accompanied by systematic efforts toward testing clinically relevant hypotheses in animal models.

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# 11

# Porcine Neural Xenotransplantation: Current Status

Roger A. Barker[1] and Mark Sayles[2]

## 1. Introduction

The allografting of embryonic neural tissue into the CNS of animals with experimental lesions and patients with neurodegenerative conditions has shown that this tissue survives, makes and receives synapses, and ameliorates behavioral deficits without inducing a significant rejection process. However, while complications from this approach have arisen recently in two double-blind placebo-controlled trials in patients with advanced Parkinson's disease, there have always been problems with the use of aborted human tissue which has lead to the search for alternative sources of cells, including embryonic porcine tissue. However, there are two major issues relating to the use of this tissue: (a) the risk of zoonotic infection especially with porcine endogenous retroviruses (PERVs) and (b) the loss of the tissue to an immunologically based rejection process. In this chapter we explore both these aspects of neural xenografts, and highlight that the major scientific problem at the current time relates more to rejection than infection. In this respect, the rejection of neural xenografts involves a number of pathways and thus a combination of strategies will be required to reduce the immune rejection response to an extent that it will be sensible to undertake proper clinical studies with porcine embryonic tissue.

The allografting of embryonic neural tissue into the CNS of animals has now reached the level of clinical trials with Parkinson's disease (PD) and more recently Huntington's disease (HD) (Bachoud-Levi et al., 2002; Barker, 2002). This is based on a background of experimental work which has shown that embryonic neural tissue allografted into the CNS survives, makes and receives synapses, and ameliorates behavioral deficits without inducing a significant

[1]Cambridge Centre for Brain Repair and Department of Neurology, Cambridge, United Kingdom
rab46@cam.ac.uk
[2]The Physiological Laboratory, Downing Street, Cambridge, CB2 3EG, United Kingdom
ms417@cam.ac.uk

rejection process (Barker and Dunnett, 1999a). However, there are major practical and ethical problems with the use of aborted human tissue in clinical programs of transplantation which prevents its widespread adoption and has lead to the search for alternative sources of cells, including other species such as the pig (Brevig et al., 2000).

Historically xenografts were the first neural transplants to be undertaken, but in the modern era, experimental work on neural xenotransplantation began as early as the 1980s, when neural cell replacement therapies were in their infancy (Björklund et al., 1982; Brundin et al., 1985). Embryonic pig neural tissue (at stage embryonic day 26–27) is held to be the most suitable material for transplantation into humans for a number of reasons (see Table 11.1). In particular, pig brains are of a similar size to ours and porcine tissue is preferred to that of non-human primates because they are have large litters and are amenable to genetic modification, making them attractive sources of whole organ grafts as well as neural cell grafts.

There has recently been a resurgence in interest surrounding porcine neural xenografts after it was shown that xenografted tissue appears to extend axonal and dendritic outgrowths over larger distances than equivalent allografted tissue (Armstrong et al., 2002; Deacon et al., 1994; Isacson et al., 1995; Wictorin et al., 1990). There is also evidence to suggest that xenografted cells migrate much more than allografted cells in the host CNS and may even migrate preferentially to regions of pathology (Hurelbrink et al., 2002). Despite these recent encouraging findings, there remain two key obstacles to the widespread clinical implementation of porcine neural xenografts: immune-mediated rejection and the potential risk of infection with porcine endogenous retroviruses (PERVs).

The first serious attempts at experimental neural xenografting were performed by Björklund and colleagues (Björklund et al., 1982) with the grafting of embryonic mouse ventral mesencephalon (VM) tissue into a cavity in

Table 11.1. Advantages and Disadvantages of Using Porcine Donor Tissue for Neural Transplantation

| Advantages | Disadvantages |
|---|---|
| The size and development of the porcine brain is similar to that seen in man | The risk of zoonotic infection, especially with PERVs |
| Easy to breed large litters | Immunological rejection |
| Can be transgenically modified | Different from cells originally lost in disease state |
| Long history of use for whole organ peripheral grafts | |
| Xenotransplanted, over allografted, tissue may have the primary advantage of a greater potential for sending fibers into the host brain—possibly through the avoidance of species specific barriers to axonal growth | |

the rat striatum. Grafts were seen to survive in 55% of cases and produced functional benefit in the absence of immunosuppression, although at 6 months post-grafting very few cells remained, with much of the graft mass being resorbed. The same group then demonstrated enhanced survival of embryonic mouse to rat neural xenografts treated with the immunosuppressive drug cyclosporin A (CsA) relative to non-immunosuppressed controls (Brundin et al., 1985). These early studies demonstrating unpredictable graft survival, which could be enhanced by immunosuppressive drug therapies, paved the way for investigations into the exact mechanisms of xenograft rejection in the immunocompetent host with a view to developing strategies to circumvent this immune reaction.

## 2. Xenograft Rejection

Immune-mediated graft rejection is a major hurdle to overcome before large-scale clinical trials using pig-derived xenogeneic tissue can be entertained. It is clear from studies in rats that xenografted tissue placed in the relatively immune-privileged CNS is rapidly rejected over a period of days to weeks through a combination of cellular and humoral immune processes. Specifically, porcine neural xenograft rejection has been shown to fundamentally involve T-lymphocytes, although there is clear evidence that antibodies and components of the complement cascade may also play some role e.g., (Barker et al., 2000; Larsson et al., 1999a). However, the relative contribution of T-cells, non-T cells (e.g., NK cells) and humoral mediators to actual tissue loss is at present unknown, and experiments designed to address this need to take into account differences in the pattern of rejection seen with concordant (between closely related species e.g. mouse to rat) and discordant (between two unrelated species, e.g., pig to human) grafts.

### *2.1. Mechanisms of Rejection: Cellular Responses*

The brain was once considered an "immunologically privileged site" for transplantation (Barker and Billingham, 1977; Raju and Grogan, 1977). However, it is now known that this privilege is not absolute, since histoincompatible tissue grafted into the CNS is rejected by the host immune system albeit more slowly than if the tissue were placed peripherally (Widner and Brundin, 1988). This rejection process appears directly related to the phylogenetic distance between donor and host (Mason et al., 1986; Pakzaban and Isacson, 1994) with the key cellular mediators of graft rejection being T lymphocytes (CD4+ and CD8+) and microglial cells (Brevig et al., 2000).

The mammalian brain is devoid of any true lymphatic vessels and is isolated from the circulation by the blood–brain barrier (BBB). However, perivascular spaces along larger blood vessels are thought to allow lymphatic drainage of the brain and so graft-derived antigen placed in the CNS could

gain access to the cervical lymph nodes, where it can activate naïve T-lymphocytes through indirect antigen presentation. Activated T-lymphocytes can cross the BBB to gain access to the brain parenchyma and interact with perivascular microglia which serve as antigen-presenting cells (APCs). In addition the necessary disruption of the BBB at the time of grafting would clearly aid the recruitment of peripheral components of the immune system, both cellular and humoral, and indeed once an immune response has been generated this will also serve to keep the BBB open.

Much evidence implicates T-lymphocytes as the main culprits of cell loss from xenografted tissue, and indeed with nude athymic rats neural xenografts survive indefinitely, e.g., (Hurelbrink et al., 2002). *In vitro* studies by Brevig and colleagues (Brevig et al., 1997) demonstrated that human T cell (CD4+ and CD8+) proliferation is induced by porcine embryonic tissue (T.P. Harrower, R.A. Barker, A. Dorling, unpublished observations). This study also showed that pretreatment of the porcine tissue with human serum reduced the proliferative response of human T cells, suggesting that such an approach might be useful clinically to improve graft survival, possibly by removing the more immunogenic cells. Antibodies against T-cells have also been employed to enhance the survival of intracerebral neural xenografts in rats (Okura et al., 1997; Wood et al., 1996). Okura and colleagues successfully used monoclonal antibodies (MAbs) generated against the αβ fragment of the rat T-cell receptor (anti-TCRαβ) and against the T-cell surface molecule CD2 (anti-CD2) to prolong the survival of mouse ventral mesencephalon tissue grafted into the rat lateral ventricle. In the absence of immunosuppressive treatment, there was complete graft rejection within 15 days. Administration of anti-CD2 alone gave no significant enhancement of graft survival, whereas in rats treated with the anti-TCRαβ MAb, graft survival was seen until day 35 post-transplantation, with T-cell infiltration being evident from day 15 onward. Both antibodies used together prolonged graft survival with functional efficacy until at least the longest time period examined in this study (20 weeks post-transplantation) with no evidence of T-cell infiltration at any time point in this group. Furthermore the authors showed that the induced tolerance is antigen specific; a second graft from the same donor strain was well tolerated, whereas a second graft from a different strain of donor mouse was rapidly rejected. Indeed recently Larsson and colleagues have explored the use of blockers to T-cell co-stimulatory molecules, as an alternative route to tolerance (Larsson et al., 2002; Larsson et al., 2003), so highlighting the value of targeting this arm of the immune response for xenograft survival.

However encouraging these results may be, it must be borne in mind that the studies either used concordant grafts or short survival times post-implantation. More recent work indicates that the situation is rather more complex for the long-term survival of discordant grafts such as from pigs to humans, in which NK cells (Larsson et al., 2001), host antibodies, and complement appear to contribute to rejection of the grafted cells (Brevig et al., 2000). For

example, Lena Larsson and colleagues (Larsson et al., 1999b) grafted embryonic mouse and rat VM tissue into the striatum of the phylogenetically distant guinea pig (D'Erchia et al., 1996) to examine the mechanisms involved in the rejection of discordant neural xenografts. Results demonstrated that mouse-to-guinea pig grafts were rapidly rejected, with no TH-positive cells remaining at 2 weeks post-transplantation. Rat-to-guinea pig grafts, however, survived well, with a high density of TH-positive neurons. Indeed there were no statistically significant differences in graft volume or TH immunoreactivity between the rat-to-rat allograft control group and the rat-to-guinea pig xenograft group. Therefore, there is some evidence to suggest that discordant graft rejection proceeds by a mechanism distinct from that involved in concordant graft rejection.

## *2.2. Mechanisms of Rejection: Humoral Responses*

A so-called "hyperacute" antibody-directed complement-mediated process results in the rejection of vascularised whole organ peripheral xenografts (e.g., pig-to-human) in a matter of minutes (Auchincloss and Sachs, 1998). Non-primate mammals and New World primates make large amounts of α-1,3-galactosyl terminated cell surface glycoproteins and glycolipids. In contrast, humans and other Old World primates produce antibodies against these sugars (Galili et al., 1984, 1993; Good et al. 1992), possibly due to their presence in bacteria colonizing the gut in the first few years of life. These anti-Gal antibodies are thought to bind their target epitopes on the surface of donor endothelial cells, recruit complement, and thereby initiate endothelial cell lysis in vascularised whole organ xenografts with platelet aggregation and formation of micro-thrombi leading to ischaemia and rejection of the grafted organ within minutes to hours (Brevig et al., 2000).

Since cellular neural xenografts are not dependent on intact donor-derived vasculature for survival it is traditionally thought that their rejection is entirely T-cell-mediated (Pakzaban and Isacson 1994). However, there is some evidence to suggest a role for humoral factors. Immunosuppression using CsA in experimental animal models of neural xenotransplantation (Brundin et al., 1989; Finsen et al., 1988) and in human PD patients receiving porcine VM grafts (Schumacher et al., 2000) does not consistently prevent rejection, although it does appear to prolong graft survival. MAbs against the T cell surface antigens CD4 and CD8 have been used to deplete recipient mice of either CD4+, CD8+ or both populations of T-lymphocytes in order to determine the relative involvement of these T-cell subtypes in the rejection of both concordant (rat-to-mouse) and discordant (human-to-mouse) transplants (Wood et al., 1996). Indefinite survival of concordant xenografts was achieved with administration of anti-CD4 MAbs, while depletion of CD8+ T-cells had no significant effect on graft survival. It was not possible to induce indefinite survival of discordant grafts using either anti-CD4 or anti-CD8, although CD4+ cell depletion prolonged graft survival for up to 60 days. Thus it would appear

that mechanisms other than the T-lymphocyte response are at least partially responsible for the rejection of discordant xenografts.

Although the BBB normally prevents immunoglobulins from the systemic circulation entering the CNS, at the time of surgery the necessary disruption to this barrier may allow preformed antibody to enter the graft. At a later stage antibodies may be generated in response to donor-derived antigen draining to the cervical lymph nodes, although quite how these would cross the BBB to enter the brain parenchyma is not clear.

The xenograft study by Larsson et al. (1999a) using immunoglobulin-deficient recipient mice also provides clear evidence for a role of antibody-directed complement-mediated rejection of xenografts; embryonic porcine VM cells survived better in immunoglobulin-depleted mice than in control mice. Work in our own laboratory further supported an antibody-directed complement-mediated process in the rejection of embryonic porcine VM tissue in 6-OHDA-lesioned rats (Barker et al., 2000). In this study xenografts were seen to be rejected over a period of 35 days, with the graft site becoming infiltrated with CD8+ cells and staining positive for IgM and complement component (C3) deposition. Transient depletion of complement components in recipient rats using cobra venom factor (CVF) prolonged graft survival, although it did not prevent rejection, by delaying the onset of the cellular immune response.

However, the view that the anti-pig antibodies target only the α-1,3-Gal epitope has been recently questioned. Sumitran et al. (1999) have demonstrated in vitro very low levels of the α-1,3-Gal epitope on embryonic porcine VM tissue, with much of this being restricted to endothelial cells and microglia. This same group went on to show that anti-Gal depleted and non-depleted human sera have an equal cytotoxic effect on porcine embryonic VM cells in the presence of complement. Furthermore this group identified three novel *non*-α-1,3-Gal epitopes on VM cells reactive with IgM present in the human serum and suggest that modification of discordant donor tissue may prove necessary if it is to survive in a host. This observation has now been validated and extended to apply to porcine neural stem cells (Harrower et al., 2002), and in addition other groups have also identified non-anti-Gal human anti-pig antibodies in normal healthy serum (Zhu and Hurst, 2002). Although humans possess several anti-pig antibodies in addition to anti-Gal, the Gal-reactive antibodies nevertheless remains by far the most important (Cooper, 2003), and so it would seem a logical step to develop transgenic pig lines deficient in this antigen. Two independent groups have now reported successful cloning of heterozygous α-galactosyltransferase knockout piglets (Dai et al., 2002; Lai et al., 2002) and recently, using a second-round of knockout and cloning strategy, four healthy female α-galactosyltransferase double knockout piglets have been produced (Phelps et al., 2003). Peripheral whole organ xenografts using these animals have now been undertaken with encouraging results (Kuwaki et al., 2005; Yamada et al., 2005).

In addition to xenografted cells expressing antigenic surface molecules such as α-1,3-galactosyl residues, they are made much more susceptible to anti-

body-directed complement-mediated attack through their lack of human specific complement inhibitor expression. A range of transgenic pig lines expressing human complement inhibitors such as DAF and CD59, and α-1,2-fucosyltransferase (H transferase, which modifies the cell surface carbohydrate phenotype, resulting in reduced α-1,3-Gal expression and decreased antibody binding) have been bred and characterized (Chen et al., 1998; Costa et al., 2002a,b; Cozzi et al., 1997; van Denderson et al., 1997), although in general the expression of these human complement regulatory proteins on embryonic neural tissue is low (Harrower et al., 2003). Nevertheless, using tissue from such embryonic transgenic pigs does not appear to affect their viability adversely, as evidenced by dopaminergic neuron xenograft survival in rats immunosuppressed with CsA (Deacon et al., 1998). Recently the same group lead by Ole Isacson has shown survival, for up to 12 weeks post-transplantation, of transgenic porcine VM tissue, expressing either the human complement inhibitor CD59 or human α-1,2-fucosyltransferase (H transferase), in primates rendered parkinsonian by MPTP administration (Cicchetti et al., 2003). The grafted tissue was given in combination with a novel immunosuppressive regime involving administration of murine monoclonal anti-C5 antibodies, designed to halt the complement cascade at C5 (thus preventing formation of the membrane attack complex (C5b-9)) and triple-drug therapy with cyclosporine A, methylprednisolone, and azathioprine. These results are clearly relevant to the pig-to-human xenograft situation, given the significant complement mediated lysis of porcine tissue caused by human serum (Sumitran et al., 1999).

Thus it is probable that combining transgenic porcine tissue designed to interfere with humoral mediators of xenograft rejection with inhibitors of the T-cell-mediated rejection process will be required for successful xenotransplantation, but how, exactly, that will be achieved remains to be seen.

## 3. Clinical Trials With Porcine Neural Xenografts

Despite experimental evidence suggesting that xenografts would be of little clinical benefit at the present time because of issues of rejection, small-scale clinical trials have been carried out in the USA in patients with PD and HD (Fink et al., 2000; Schumacher et al., 2000). Schumacher et al. (2000) reported the results of a one-year follow up assessment of 12 PD patients transplanted unilaterally with suspensions of E25–E28 porcine VM tissue into the striatum. These patients received either CsA monotherapy or porcine tissue treated with a monoclonal antibody against SLA-I. One patient involved in this study died from a pulmonary embolism at 7.5 months after transplantation and at post-mortem had only 638 surviving porcine-derived dopaminergic cells in the host striatum out of the 12 million transplanted. Despite the use of immunosuppression with cyclosporin A (CsA) (Deacon et al., 1997), lymphocyte infiltration was also seen in the graft region. This

extremely low survival rate correlates with the $^{18}$F-dopa PET data from the same study, which failed to demonstrate any significant increase in signal on the transplanted side. Taken together these two pieces of evidence cast doubt on the inference that the significant decrease in UPDRS scores reported by Schumacher and colleagues in some patients (average improvement 19%, $P = 0.01$) was due to dopamine release from transplanted neurons.

A second clinical trial of embryonic porcine VM xenotransplantation for PD, which remains unpublished, has been reported at meetings. In total 18 patients underwent surgery, 10 receiving embryonic porcine VM tissue and 8 sham surgery. At the 18-month follow-up assessment both groups showed similar modest (20–25% UPDRS) improvement; the effects of grafting thus being equal to the placebo effect. However, a full account of this study in a peer-reviewed journal is required before any further comment can be made.

These results serve to reinforce the need to better understand the process of rejection of discordant xenografts experimentally before any further clinical trials are planned.

## 4. Xenograft and Zoonoses

The use of pigs as a source of donor tissue, not only for neural and islet cell therapies but also whole organ xenografts (e.g., liver, heart), has raised concerns over the possibility of infectious diseases crossing the species barrier. Animal endogenous retroviruses (e.g., porcine endogenous retrovirus—PERV) are proviral DNA sequences integrated into the host genome, with pig chromosomes harboring at least 50 copies of PERV (Weiss, 1999). Unlike most bacterial and viral pathogens, endogenous retroviruses cannot be eliminated from potential donor animals by simple pathogen-free, closed breeding regimes and so retain the potential to cross species barriers at the time of grafting. Perhaps the most ominous argument against the introduction of large-scale clinical trials of porcine xenograft therapies rests on the now widely accepted conclusion that both HIV-1 and HIV-2 represent zoonotic infections (Gao et al., 1999). The vigour with which such viruses mutate adds further concern that PERVs could potentially infect human cells *in vivo* and mutate or combine with other human specific proviral sequences to create a lethal viral infection akin to HIV with the potential for human-to-human spread. Ironically, the development of immunosuppressive therapies to enable xenograft survival could potentially increase the host susceptibility to infection by graft-derived retroviruses, especially with respect to the complement cascade.

With a view to answering the questions surrounding xenografts and PERV infection, attempts have been made to establish the extent to which PERV shows human cell specific tropism in culture. However, it must be borne in mind that cultured cell lines and a whole animal are very different environments and while PERV might be able to infect human cells

in vitro, the situation may be very different in vivo. In vitro, Wilson et al. examined the ability of PERV to infect human cells in culture and showed that mitogenic activation of porcine peripheral blood monocytes could initiate production of infectious retrovirus, which in turn was capable of infecting co-cultured human cell lines (Wilson et al., 1998). In support of this infective potential of PERVs, an in vivo study by van der Laan and colleagues (van der Laan et al., 2000) provided the first direct evidence that porcine derived tissue transplanted into an immunocompromised mouse can result in cross-species infection. Non-obese diabetic, severe combined immunodeficiency (NOD/SCID) mice received grafts of pig pancreatic islet cells and were shown to have become infected with PERV in several tissue compartments, including the spleen, liver, kidney, pancreas, and intestine. Interestingly, infection was only seen in those tissue compartments in which there was tissue chimaerism, which may indicate that cell–cell contact is important in transmission of PERV from donor to host cells. In addition, the mice were severely immunodeficient, and even though they had become infected with PERV, none of them became ill or showed tissue pathology.

Crucially there has been no report of any human becoming infected with PERV following exposure to porcine tissue. However, most studies of this nature have been retrospective with limited porcine tissue survival and as such provide no evidence for a risk of infection, which is rather different from evidence for no risk of infection. A recent large-scale retrospective study of 160 patients treated with living pig tissue up to 12 years previously concluded there was no evidence for persistent PERV infection in any of the patients (Paradis et al., 1999). Patients were exposed to living pig tissue through either:—

1. extra-corporeal perfusion through pig liver, kidney, or spleen for various reasons;
2. pancreatic islet transplantation for diabetes mellitus;
3. pig skin grafts for burns; or
4. other porcine transplants.

Using PCR (for detection of PERV DNA), RT-PCR (for detection of PERV RNA, a marker for virions), and protein immunoblot antibody assays (for detection of exposure to PERV antigen), Paradis et al. (1999) demonstrated that in samples of peripheral blood lymphocytes and serum there was no evidence for persistent PERV infection in any patient examined. A group of 30 Russian patients treated with extracorporeal splenic perfusion (ECSP) tested positive for PERV DNA sequences, but this was shown to be due to the persistent presence of pig cells in the patients' circulation, presumably being flushed from the pig spleen during treatment. Thus in this particular group of patients, long-term (up to 8.5 years) survival of xenogeneic tissue was observed without immunosuppression. Although the conclusions of Paradis et al. are based on a large number of patients, it is worth considering that the only cells tested for infection were peripheral blood lymphocytes, which

cannot be productively infected with PERV in vitro (Wilson et al., 2000). Indeed it would appear from in vitro work that human epithelial cell lines are much more susceptible to PERV infection than are human B and T cells (Takeuchi et al., 1998), but that overall PERV infectivity is very low.

Therefore the evidence in support of concerns over PERV transmission remains equivocal. It is important to understand that the introduction of any new therapy must be subject to cost–benefit analysis and that the small, but significant, risk of infection with PERV may be acceptable if the predicted benefits of using porcine tissue are borne out. The UK Xenotransplantation Interim Regulatory Authority (UKXIRA) was set up in 1997 to advise the government on issues of safety and efficacy related to xenotransplantation and has been commissioned to report on the risk of disease transmission and the practicalities of transplanting pig organs. The most recent report, on the legality and ethics of xenotransplantation, has sparked controversy after it was reported in the UK press that the government had refused to publish the report because of concerns expressed therein over the possibility of the UK governments being sued for breaching international law if a HIV-like virus were to result and spread across the globe (Townsend, 2003).

Work to circumvent the potential problem of PERV transmission continues and may go some way toward allaying public concern. The generation of PERV knockout pigs may prove more difficult than originally thought since multiple copies of PERV genomes are present in normal pig genomes (Patience et al., 1997). Very recently antibodies against a PERV transmembrane envelope protein (p15E) were shown to neutralize PERV infectivity and might therefore be useful to create an antiretroviral vaccine (Fiebig et al., 2003). With the development of novel methods of preventing or at least reducing the risk of PERV infection, the potential benefits of xenografted over allografted tissue discussed below makes porcine neural xenografts increasingly attractive as a treatment for conditions such as PD.

## 5. Does Using Xenografted Tissue Offer a Primary Advantage to Allografted Tissue?

In both animal models of PD and in PD patients undergoing neural transplantation, grafted neurons (of ventral mesencephalic origin) are usually placed ectopically in the striatum of the recipient (Barker and Dunnett, 1999a or b; Barker, 2002). This is thought to impose an intrinsic limit to the potential benefit attainable from current transplantation regimes since the original neural circuitry involving projections to and from both the substantia nigra and striatum is not reconstructed. The ectopic graft placement and resulting incomplete repair of host neural circuitry is thought to account partially for the variable and imperfect alleviation of parkinsonian symptoms and possibly some of the serious side effects seen in PD patients undergoing allotransplantation with human embryonic VM tissue (Freed et al., 2001;

Freed 2002). It has become clear through experimental work that allografted tissue must be placed ectopically in the striatum since the inhibitory nature of the host white matter prevents long-distance circuit reconstruction by intranigral grafts, restricting axonal outgrowth to the graft mass itself, with no reinnervation of the striatal neuropil (Bentlage et al., 1999; Björklund et al., 1983; Dunnett et al., 1983). However, it has been argued that much more complete functional recovery with fewer side effects may result from placement of grafts in the substantia nigra and subsequent circuit reconstruction (Nikkhah et al., 1994). Xenografted tissue appears somewhat better at projecting long distances in host white matter, allowing xenografted neurons (e.g., pig to rat; human to rat) to extend axonal outgrowths over large distances to target sites specific for their region of origin (Armstrong et al., 2002; Deacon et al., 1994, 1999; Isacson et al., 1995, 1996; Widner and Brundin, 1988). Both pig-to-rat and human-to-rat intranigral primary ventral mesencephalic xenografts have shown remarkable ability to project axons along the nigrostriatal tract and reinnervate the host striatum, although the functional efficacy of these grafts is unknown (Isacson et al., 1995; Wictorin et al., 1992). There are two possible explanations for these differences in axonal outgrowth between allografted and xenografted neurons. It may be that pig and human neurons have a greater intrinsic growth capacity than rat neurons, arguably due to the longer gestational period of humans and pigs compared to that of rats, allowing genes needed for embryonic axonal growth to be expressed over a much longer time period in order to form long-distance synaptic connections in a much larger CNS (Fawcett, 1997). However, there is preliminary evidence in favour of an alternative explanation; species differences in CNS growth inhibitory molecules and/or their receptors may render xenografted axons less responsive to host specific inhibitory cues (van den Pol and Spencer, 2000; C. Hurelbrink and R.A. Barker, unpublished observations). There is much interest in this latter possibility since it has sparked speculation that the use of xenogeneic tissue as opposed to allogeneic tissue in a clinical setting may prove to have primary advantages in terms of circuit repair and therefore a more complete long-term alleviation of symptoms for PD patients.

In vitro work by van den Pol and Spencer (2000) demonstrated enhanced survival and axonal outgrowth from human (cortical) and rat (hippocampal and hypothalamic) neurons when co-cultured with heterospecific astrocytes (e.g., rat neurons with human astrocytes) rather than homospecific astrocytes (e.g., human neurons with human astrocytes). A recent in vivo study by Armstrong et al. (2002) provided evidence that xenografted porcine expanded neural precursor cells (ENPs) may be an even more flexible source of cells for use in neural transplant therapies where circuit reconstruction is required. Taking advantage of both the enhanced axonal outgrowth thought to be characteristic of xenografted neural tissue and the plastic nature of multipotential neural stem cells, this study aimed to examine the ability of porcine ENPs to differentiate into projection neurons and thereby reconstruct degenerate

neural circuitry in the CsA-immunosuppressed 6-OHDA-lesioned rat model of PD.

Embryonic (E26–27) porcine cortical neurons expanded in culture with FGF-2 and EGF were grafted into either the rat ventral mesencephalon or striatum. Although very few grafted ENPs differentiated into tyrosine-hydroxylase-positive neurons in both graft locations and no functional recovery was seen, there was extensive axonal outgrowth across the graft–host interface with projections along host fiber tracts to targets appropriate for the graft site. Intramesencephalic ENPs projected to the mediodorsal thalamus, caudate-putamen, ventral striatum, and the amygdaloid nuclei along the internal capsule and the medial forebrain bundle. Similarly those ENPs implanted in the striatum were shown to make local connections in the caudate-putamen and long-distance connections to the globus pallidus and substantia nigra. Crucially, staining for pig-specific synaptobrevin revealed synapse formation in the regions of gray matter in which ENP-derived axons were seen to ramify, suggesting functional integration of grafted neurons with existing host circuitry. Interestingly, the pattern of striatal reinnervation was significantly different between the two graft sites. Intramesencephalic grafts resulted in a more even distribution of re-innervation throughout the host striatum, whereas intrastriatal grafts were shown to reinnervate the dorsal striatum at a much higher density than the ventral striatum. Similar uneven re-innervation has been suggested in the past as a possible cause of the rather severe dyskinetic side effects seen in several PD transplant patients receiving human embryonic tissue directly into the striatum (Freed et al., 2001; Hagell et al., 2002; Ma et al., 2002). Although the results of Armstrong and colleagues show very few dopaminergic neurons developing from the grafted ENPs (of cortical origin) and no functional recovery on amphetamine-induced rotation behavioral testing, they do serve to demonstrate that a combination of increased axonal outgrowth, as a consequence of the xenograft paradigm, and possible future manipulations of neural stem cells to induce dopaminergic differentiation may provide the ideal cell source for repairing degenerating neural circuitry (Armstrong et al., 2002, 2003). Similar experiments using porcine embryonic VM-derived ENPs has shown recently that these cells too have the capacity for long-range circuit reconstruction (Armstrong et al., 2003).

In addition to enhanced axonal outgrowth, there is evidence to suggest that xenografted neurons have an increased propensity for migration within the adult host CNS compared to allografted cells (Hurelbrink et al., 2002). Indeed migration is a desirable property of transplanted neurones since the incomplete amelioration of symptoms seen with current neural replacement therapies for PD may be attributable to distant sites of pathology well outside of the primary graft region, for example in the cortex and other basal ganglia and brainstem nuclei (see Chapter 3). If grafted cells possessed the ability to migrate to and fully integrate with these distant sites of pathology symptomatic improvement would likely be considerably enhanced. Migration

is a well-documented characteristic of ENPs (Fricker et al., 1999; Rosser et al., 2000) but has not been as extensively studied with primary neural graft-derived cells. Most studies examining cell migration from primary neural grafts have been in neonatal hosts (e.g., Olsson et al., 1997). It is widely thought that the reduced capacity for neuronal migration seen when the same tissue is transplanted into an adult animal represents the absence of developmental guidance cues to which the grafted cells can respond. Some studies have reported much greater migratory capacity of graft-derived glial cells than neuronal cell types (e.g., Pundt et al., 1995).

Hurelbrink and colleagues grafted human fetal striatal tissue intrastriatally in quinolinic acid-lesioned athymic rats in order to follow the migration of grafted cells through staining for human-specific cellular markers (HuNu, Tau, GFAP, Ki67) (Hurelbrink et al., 2002). At 6 weeks very few grafted cells had migrated away from the graft core, and most differentiated cells were of a neuronal phenotype. At 6 months the graft core was much less dense, with many cells having migrated throughout the forebrain as far rostral as the olfactory bulb and caudal as the substantia nigra. Graft-derived cells differentiated into both neurons and glial cells, something that would be expected from a tissue such as human fetal striatum containing neural precursor cells. Hurelbrink et al. offer two alternative explanations for the extensive migratory capacity of the transplanted human cells. It remains possible that the longer gestational period and larger brain size of humans relative to rats may enable the human derived cells to respond to guidance cues for a more extended period. However, a rather more attractive proposition is that the xenotransplantation paradigm itself is responsible for the enhanced migratory capacity. An inability to respond to species-specific inhibitory cues may allow greater migration of xenografted cells than equivalent allografted cells and therefore enhance integration of grafted neurons with host circuitry and enable migration of cells to sites of pathology well outside the graft mass itself. It remains to be seen whether this extensive migratory potential is a feature of all xenografted neural tissues, and whether it can target preferentially sites of pathology. However, the possible advantages of such a characteristic are obvious in terms of brain repair for patients with conditions such as PD.

## 6. Conclusions

Despite the issues of immune-mediated rejection and the theoretical risk of infection with porcine endogenous retroviruses, xenogeneic neural tissues are an attractive source of material for use in brain repair surgery. Development of transgenic pigs in order to reduce antigenicity while limiting the risk of infection with PERVs may allow for the exploitation of the unique properties of xenografts to more completely reverse CNS circuit degeneration.

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# 12

# Adult-Derived Stem Cells for Transplantation in Parkinson's Disease

Xinyu Zhao,[1] D. Chichung Lie,[2] and Fred H. Gage[3]

## 1. Introduction

It has been known for many years that dopamine (DA)-releasing cells transplanted into the striatum can reverse motor symptoms in animal models for Parkinson's disease (PD) (reviewed by Björklund et al., 1986). This transplantation approach is based on the rationale that grafted DA-releasing cells will compensate for the loss of dopaminergic neurotransmission caused by the degeneration of neurons in the substantia nigra pars compacta. These early experimental findings have subsequently led to a number of clinical studies using mainly fetal mesencephalic CNS tissue as donor tissue and have produced promising results in some cases (reviewed by Björklund et al., 2003). However, the use of fetal tissue poses major problems, such as limited availability of fetal tissue; relatively low numbers of DA precursors and neurons in the grafted fetal tissue itself; and importantly, ethical concerns surrounding the use of fetal tissue, which is derived from aborted fetus. These problems greatly reduce the feasibility of transplantation strategies in PD, and it is therefore important to identify alternative cell sources for dopaminergic cell replacement.

Ideally, the alternate source for DA-releasing cells should have the following minimal characteristics:

- easily obtainable
- unlimited supply
- free of contaminating cell types
- ability to differentiate into fully mature DA neurons
- no tumorigenic potential

[1]Department of Neurosciences, University of New Mexico, Albuquerque, NM, 87131, USA
xzhao@salud.unm.edu
[2]Institute of Developmental Genetics, GSF Research Center, Ingolstaedter Landstrasse 1, 85764 Munich-Neuherberg, Germany
chichung.lie@gsf.de
[3]Laboratory of Genetics, The Salk Institute, La Jolla, CA.
gage@salk.edu

Recent studies have demonstrated that cells with the ability to proliferate and differentiate into neurons and glia reside in the adult CNS. In the following sections, we will discuss the potential of these so called adult-derived stem cells, focusing on adult-derived neural stem cells (NSCs) as a source for cell transplantation therapies in PD.

## 2. Adult-Derived Stem Cells In Vivo

Stem cells are defined as cells with the potential for extensive self-renewal and differentiation into one or more functionally different cell types. The most immature cells are embryonic stem (ES) cells, which can generate all types of organs and cells in a given species. In adult mammals, tissue-specific stem cells, e.g., in the skin, the hematopoetic system and the intestine, continue to generate new mature cells to compensate for cell loss from physiological wear and disease. For a long time it was believed that the adult CNS lacked such somatic stem cells, and the vulnerability of the adult CNS to disease and injury was thought to be at least in part due to the absence of these stem cells. However, it has now been demonstrated that neural stem cells (NSCs) with the potential to differentiate into neurons, astrocytes, and oligodendrocytes exist throughout the adult CNS of all mammals, including humans (Gage, 2002). What is the physiological function of these NSCs? And can they represent an endogenous reservoir of cells for transplantation in PD?

### *2.1. Neural Stem Cells Generate Functional Neurons in the Adult CNS*

In most areas of the adult CNS, dividing cells give rise to new astrocytes and oligodendrocytes (Horner et al., 2000a; Kuhn et al., 1997; Lie et al., 2002). However, in two restricted areas of the adult CNS, the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) and the subventricular zone (SVZ) of the lateral ventricle, new neurons arise from NSCs through the generation of rapidly dividing progenitor cells and differentiation of these progenitor cells into immature neurons (reviewed in Alvarez-Buylla and Garcia-Verdugo, 2002; Kempermann and Gage, 2000). Many of the immature neurons born in the SGZ die within the first 2 weeks after cell genesis. The surviving ones migrate into the molecular layer of the DG and differentiate into granule neurons (Kempermann et al., 2003). Within one month, they send axonal projections into the CA3 region and project dendrites into the outer molecular layer, similar to the pre-existing dentate granule neurons (Hastings and Gould, 1999; Markakis and Gage, 1999; Seri et al., 2001; van Praag et al., 2000b). Immature neurons born in the SVZ migrate long distances, through the rostral migratory pathway (RMP) toward the olfactory bulb. In the RMP, new neurons form chains and migrate through tubular structures formed by specialized astrocytes (Lois et al., 1996; Wichterle et al., 1997). Around 2

weeks after their birth, the first newborn neurons have reached the olfactory bulb and start to migrate radially to their final positions in the glomerular and periglomerular layers, where they differentiate into interneurons (Carleton et al., 2003; Winner et al., 2002). Recent studies have shown that, in both areas, new neurons develop the essential electrophysiological properties of mature neurons, such as the ability to generate action potentials, and they receive synaptic input, indicating that the new neurons are functional and integrate into the existing circuitry (van Praag, 2000b; Carleton 2003).

## *2.2. Adult Neural Stem Cells Generate New Neurons in Animal Models of Human Diseases*

Under physiological conditions, neurogenesis seems to balance naturally occurring neuronal cell death in the DG of the hippocampus and in the olfactory bulb. Recent studies suggest that endogenous NSCs can also contribute to repair in the adult CNS in disease and injury conditions. In injured brains, increased neurogenesis has been found in areas that have persistent neurogenesis in adult brain, DG of hippocampus, and SVZ of lateral ventricles (Kee et al., 2001; Liu et al., 1998; Yagita et al., 2001). Intriguingly, neurogenesis was also found in regions that are normally non-neurogenic in healthy brains (Nakatomi et al., 2002; Zhao et al., 2003).

Seizures induce neuronal cell death in various CNS regions. Increased precursor proliferation and neuronal differentiation have been observed following chemoconvulsant-induced seizure in the hippocampal DG and the SVZ/olfactory bulb system (reviewed in Parent and Lowenstein, 2002), indicating that increased neurogenesis might contribute to repair by compensation of seizure-induced neuronal cell death in these circuits. Global forebrain ischemia, a model for cardiac arrest or coronary artery occlusion, leads to selective death of defined vulnerable neuronal populations. In contrast to other hippocampal structures such as the CA1 region, the DG is relatively resistant to this insult. Interestingly, several groups have found that global forebrain ischemia promotes progenitor cell proliferation and neurogenesis in the adult DG (Kee et al., 2001; Liu et al., 1998; Yagita et al., 2001), suggesting that the resistance of the DG formation to global ischemia is related to its ability to generate new dentate granule neurons to replace degenerating ones.

These results indicate that a variety of insults can stimulate NSCs in neurogenic regions to replace dying neurons. In non-neurogenic regions, lesion-induced neuronal cell death can enhance the proliferation of local NSCs and can sometimes attract a large number of NSCs from adjacent neurogenic areas. In animal models of ischemic brain injury, there is significantly increased proliferation in endogenous neural progenitor cells, and these cells migrate to the hippocampus to regenerate damaged pyramidal neurons. Interesting, intraventricular infusion of growth factors greatly augments such a response, and treated animals also have significant functional recovery (Nakatomi et al., 2002). Several laboratories have observed the generation of new neurons in non-neurogenic areas in animal models for stroke (reviewed by Kokaia and

Lindvall, 2003). Most of the newborn neurons, however, display only markers of immature neurons and do not survive long. Moreover, the number of new neurons that survive is minimal in comparison to the number of neurons lost. In addition, some new neurons displaying abnormal migratory behavior were found in ectopic locations (Parent et al., 1997, 2002). Moreover, these new neurons contributed to aberrant network organization and in some cases showed altered physiological properties (Parent et al., 1997; Scharfman et al., 2000).

The behavior of endogenous NSCs has also been studied in animal models for Parkinson's disease (PD). Degeneration of dopaminergic neurons induced by MPTP or 6-OHDA increases the proliferation of NSCs in the substantia nigra (Kay and Blum, 2000; Lie et al., 2002; Zhao et al., 2003). Whether these NSCs give rise to new neurons is currently a controversial issue. One study reported increased dopaminergic neurogenesis following MPTP lesion and also suggested a basal turnover of dopaminergic neurons, with constant dopaminergic cell death and dopaminergic neurogenesis under physiological conditions (Zhao et al., 2003). However, other laboratories have not found any evidence for local neurogenesis following degeneration of dopaminergic neurons in the substantia nigra (Kay and Blum, 2000; Lie et al., 2002; Frielingsdorf et al., 2004).

Although some of these results suggest that there is a potential for self-repair from endogenous stem cells in the adult CNS, it becomes clear that this capacity for self-repair is extremely limited in most regions. Therefore, strategies have to be defined that can support this process, either by directly stimulating the endogenous stem cell population for cell replacement or by supplying additional cells through transplantation.

## 3. Adult-Derived Stem Cells for Transplantation

### *3.1. Source and Isolation of Neural Stem/Progenitor Cells From Adult Human*

Can adult NSCs be isolated and used for transplantation therapy? Recent advances in adult NSC research indicate that the answer is yes. The first important step toward using adult NSCs for clinical application is to isolate NSCs to relative high purity. There are at least two reasons why purity is important. First, a pure population of NSCs without contamination from other cell types, such as committed glial progenitors and proliferating hematopoetic progenitors, will make it much easier to determine the optimal conditions to differentiate NSCs into DA neurons both in vitro and in vivo. Second, high-purity NSC preparation may minimize immune responses triggered by other cell types upon transplantation. The biggest problem in the isolation of adult NSCs is that, currently, there is no specific NSC marker that can be used to identify and isolate these cells. Thus far, our understanding of the biology of adult NSCs has been primarily based on the analysis of cultured cells with stem-like characteristics and retrospective analysis in vivo.

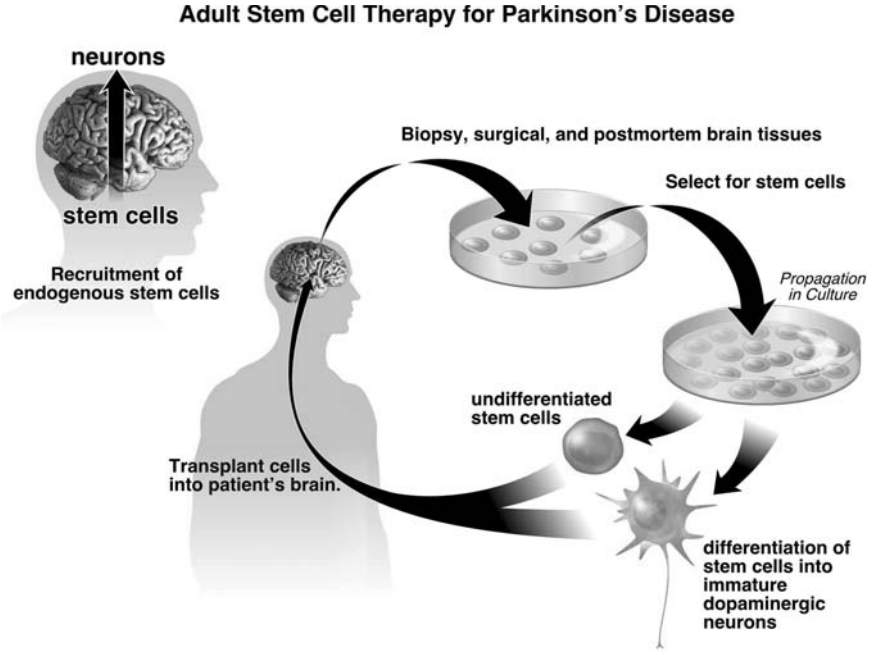


FIGURE 12.1. Schematic diagram showing strategies for using adult stem cells for treating PD. For transplantation therapy, stem cells can be isolated from surgical and post-mortem adult brain tissues, propagated in vitro, manipulated in vitro (e.g., genetically modified, differentiated, etc.) and transplanted into the brain of a patient with PD. (See color insert.)

Such limitations raise concerns about the in vivo relevance of the in vitro findings and hinder the use of cultured cells for transplantation therapy. Nevertheless, several methods have been reported to isolate adult NSCs from rodent brain. The resulting cells are likely a mixed population of neural stem and progenitor cells and are therefore called neural stem/progenitor cells (NSPCs). One method separates the fractions enriched with NSPCs based on cell size and density (Palmer et al., 1999). Promoter-based cell sorting has also been used, based on the expression of certain genes in NSPCs (Wang et al., 2000). NSPCs have been isolated from adult ependymal and SVZ of lateral ventricles using cell sorting based on the size of the cells and their binding ability to peanut agglutinin (PNA) (Rietze et al., 2001).

These methods have been adopted in isolating human NSPCs. One study that examined postmortem brain tissue from cancer patients who received BrdU injections for diagnostic purposes provided evidence that hippocampal neurogenesis is occurring in humans (Eriksson et al., 1998). Initially NSPCs were isolated from the human CNS regions that had previously been shown to be neurogenic in other mammalian species (SVZ and DG) (Kukekov et al., 1999; Palmer et al., 2001a; Pincus et al., 1998). Multipotent NSPCs have been

isolated from surgical specimens and postmortem tissue of donors of a broad age range, using the percoll gradient method. These cells can be propagated in vitro for about 30 divisions and they can differentiate into all three CNS lineages upon differentiation (Palmer et al., 2001a). Keyong et al. (2001) isolated multipotent NSPCs from fetal human brain using enhanced nestin and musahshi1 promoter-driven green fluorescence protein (GFP) and fluorescent-assisted cell sorting (FACS). The rationale for this approach is that nestin and musashi1 are proteins that are predominantly expressed in NSPCs. In vitro musashi1 and nestin promoters-defined cells were both self-renewing and multipotent, indicating that these methods allowed the isolation of NSPCs. Upon chimeric xenograft into fetal rat brain, these human NSPCs gave rise to both neurons and glia. Using this method, (Roy, Wang et al. 2000) isolated NSPCs from adult human hippocampus by transfecting cells with nestin promoter-EGFP followed by sorting GFP positive cells. The same group also used Ta1 promoter-driven hGFP to isolate committed neuronal precursor from adult human hippocampus.

More recently, Nunes and colleagues (Nunes et al., 2003) succeeded in isolating progenitor cells from non-neurogenic regions of the adult human brain. In this study cells were isolated from the human subcortical white matter using GFP FAC sorting based on the activity of a promoter for CNP2, which is expressed in early oligodendrocytes, and the expression of a surface marker for oligodendrocyte precursor (A2B5). Interestingly, cells purified by these methods not only differentiate into glial cells but also into functionally mature neurons in vitro, suggesting that these cells might be multipotent rather than glia-restricted progenitor cells. These isolated human NSPCs were also transplanted into the developing rat CNS. One month after transplantation, neurons and glia derived from human progenitor cells were found in the mature rat brain, indicating that the neurogenic potential of these cells was also present in vivo. Importantly, the ability to differentiate into neurons in vitro and in vivo was not dependent on prolonged culturing in the presence of growth factors, suggesting that white matter-derived progenitor cells possess an intrinsic neurogenic potential. Another source for adult NSCs is the olfactory bulb. Multipotent NSPCs have been isolated from the olfactory bulbs of mice and humans (Pagano et al., 2000). These cells can be isolated from a relatively small amount of surgically removed tissue, propagated in culture and grafted back into mouse brain. Some of the grafted cells express dopaminergic markers. (Parati et al., 2003). Taken together, these data demonstrate that cells with NSC properties can be isolated from different regions of the adult brain and provide a source for replacement therapy using adult-derived stem cells. Similar to other mammalian species, human brains contain NSCs that have the capacity to regenerate human brain throughout adulthood. Ethical concerns and lack of adequate techniques have restricted the study of cell genesis in the adult human brain, and there is currently no evidence that neurons or glia are regenerated in the diseased human CNS. However, the possibility of isolating cells with NSC-like characteristics from

different areas of the adult human brain fuels our hope that we will be able to use NSCs to repair the diseased human CNS.

## *3.2. Regulation of Neural Stem Cell Proliferation and Differentiation*

Understanding how NSC fate is regulated in adult brain is crucial for the success of cell replacement therapy using grafted NSCs. If NSCs are ubiquitously present in the adult CNS, why is adult neurogenesis restricted to the SGZ and SVZ? When transplanted into neurogenic regions in adult brain, NSCs derived from both neurogenic and non-neurogenic regions can give rise to neurons in vivo. In contrast, when transplanted into non-neurogenic regions in adult brain, adult NSCs, regardless of their origin in CNS, will differentiate into only glia (Lie et al., 2002; Shihabuddin et al., 2000; Suhonen et al., 1996). These findings indicate that adult NSCs from different regions are not fate restricted by their intrinsic programs. It is the extrinsic cues provided by the local environment that control their differentiation

### 3.2.1. Cellular Elements Regulating Neurogenesis

What are the cellular elements that control the proliferation and fate choice of NSCs? Functional studies in songbirds and anatomical studies in rodents have implied that the vasculature plays an important role in providing a neurogenic environment. NSPCs in the hippocampus, but not in non-neurogenic regions, proliferate in close proximity to blood vessels and dividing endothelial cells, suggesting that signals derived from the vasculature may regulate both neurogenesis and vasculogenesis (Palmer et al., 2000). The finding that factors promoting endothelial cell proliferation also increase neurogenesis also points to an important relationship between these two processes (Jin et al., 2002). In the adult songbird brain, a study showed that increased vasculogenesis stimulates neurogenesis by increasing the levels of endothelial cell-derived growth factors, such as vascular endothelial growth factor (VEGF) (Louissaint et al., 2002).

### 3.2.2. Molecular Regulation of Neurogenesis

The molecular mechanisms that control adult neurogenesis are under extensive investigation. Multiple growth factors, hormones, and neurotransmitters have been implicated in the regulation of neurogenesis, based on their ability to influence proliferation, differentiation and survival of NSCs in vitro and in vivo (reviewed by van Praag and Gage, 2002). The relationship between these different factors is currently not understood, and it is not clear whether all of them play a physiological role in the regulation of neurogenesis. However, given their potential to affect the behavior of NSCs in vivo, these factors and their receptors can be used (e.g., through gene therapy or growth factor delivery) to facilitate NSC transplantation therapy in PD.

### 3.2.3. Factors That Affect NSC Proliferation

Epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF-2) are commonly used as mitogens for the maintenance of NSCs in vitro (van Praag and Gage 2002a). These two growth factors may have effects on two distinct populations of mouse NSPCs. EGF-responsive NSPCs can be isolated from SVZ (Morshead et al., 1994). FGF-2 is a potent mitogen for NSPCs isolated from adult hippocampus and other brain regions (Palmer et al., 1999). EGF and FGF-2 have synergistic proliferation effects on NSPCs isolated from adult mouse brain (Ray, unpublished observation). It has been shown that both conditioned media and FGF-2 are required for NSPCs to survive when cultured at low density. A glycosylated form of Cystatin (CCg) has been shown to be an essential component of the conditioned media. CCg is expressed in the subgranular layer of the DG in adult hippocampus, and exogenous CCg can increase NSPC proliferation both in vitro and in vivo (Taupin and Gage, 2002). Conditioned media from CCg-expressing NSCs were essential for in vitro propagating of human NSCs from post-mortem brain (Palmer, 2001).

Other growth factors such as VEGF (Jin et al., 2002), brain-derived neurotrophic factor (BDNF) (Benraiss et al., 2001; Pencea et al., 2001), insulin-like growth factor (IGF-1) (Aberg et al., 2000; Arsenijevic et al., 2001; Hsieh et al., 2004), FGF-8 (Lie et al., 2002), and amphiregulin (Falk and Frisen, 2002) have also been shown to be sufficient to propagate NSCs in vitro or to enhance proliferation in vivo. Nerve growth factor (NGF) has been shown to affect NPC in vitro proliferation/differentiation (Cameron et al., 1998b). At this point, there is no direct evidence that any of these growth factors is an endogenous regulator of NSC proliferation. Moreover, the potential interaction of these growth factors with steroid hormones (estrogens, testosterone, glucocorticoids) (Cameron and Gould, 1994; Cameron et al., 1998c; Duman et al., 2001a) and neurotransmitters (glutamate, serotonine) (Banasr et al., 2001; Brezun and Daszuta, 1999; Cameron et al., 1995, 1998a; Duman et al., 2001b) that have been shown to influence proliferation of NSCs remains to be determined.

Based on their experiences with rodent NSPCs, several laboratories have developed the following conditions to culture NSPCs derived from adult human brains successfully. Isolated human NSPCs from white matter were propagated in a non-differentiated state in serum-free DMEM/F12/N1 supplement in the presence of FGF-2 (20 ng/ml), neurotrophin-3, (NT3) (2 ng/ml), and platelet-derived growth factor (PDGF)-AA (20 ng/ml)(Nunes et al., 2003). NSPC isolated from adult hippocampus were cultured in DMEM/F12/N2 with 2% PD-FBS and 10 ng/ml FGF-2 (Roy et al., 2000). NSPCs isolated from adult post-mortem tissues have been cultured in DMEM/F12/N2, supplemented with FGF-2 (20 ng/ml), EGF (20 ng/ml), PDGF-AB (20 ng/ml), and 25% CCg conditioned medium (Palmer et al., 2001b).

### 3.2.4. Factors That Affect NSC Differentiation

A crucial question is, can transplanted adult NSCs generate functional dopamine (DA) neurons? To use adult-derived NSCs for treating PD, it is critical to understand what factors influence NSC lineage determination. Many studies have been performed using in vitro cell culture systems because it is easier to control for individual factors. However, most differentiation treatments do not result in a large percentage of DA neurons.

There are currently no known physiological inducers of neuronal cell fate choice. In vitro, differentiation of adult NSCs into all three neural lineages, including neurons, is induced by withdrawal of mitogens. The neuronal differentiation can be enhanced by retinoic acid and increased cAMP levels (Nakagawa et al., 2002; Palmer et al., 1997; Takahashi et al., 1999). In addition, neurotrophins have limited influence on the neurotransmitter phenotype expressed in vitro (Takahashi et al., 1999). In vivo, increased generation of neurons has been observed following infusion/overexpression of BDNF (Benraiss et al., 2001; Pencea et al., 2001) and IGF-1 (Aberg et al., 2000). However, it is not clear whether this increase is caused by enhanced neuronal differentiation of adult NSCs or by enhanced survival of new neurons. IGF-1 can increase NSC proliferation in vitro and can also influence NSC fate in terms of what type of neurotransmitters the differentiated neurons secrete (Anderson et al., 2002). Activated Notch1 and Notch 3 have been found to restrict adult NSCs toward an astroglial lineage in vitro (Tanigaki et al., 2001). In addition, members of the bone morphogenetic protein (BMP) family instruct adult NSCs to adopt a glial cell fate (Lim et al., 2000). Interestingly BMPs are expressed by adult NSCs themselves. This autocrine gliogenic signal is inhibited by noggin, a secreted protein that blocks BMP signaling by binding to BMPs. In the neurogenic SVZ, noggin is secreted by ependymal cells in the lateral ventricle, thereby blocking the gliogenic effects of BMPs and permitting neuronal differentiation of adult NSCs. However, blocking of BMP by itself is not sufficient to induce the neuronal differentiation of adult NSCs but requires additional factors potentially derived from local astrocytes. Both fetal bovine serum alone or in combination with BMP and LIF have been used to differentiate embryonic NSCs into about 40% astrocytes (Nakashima et al., 1999). Such a combination also triggers adult NSCs to differentiate into mainly astrocytes (Nakashima and Gage, unpublished data).

Following long-term expansion, human and mouse embryonic neural progenitors can differentiate into tyrosine hydroxylase (TH) positive neurons in vitro following incubation with cytokines (LIF, glial cell line-derived neurotrophic factor (GDNF), IL-1b and IL-11). These TH positive neurons have functional DA neuron properties such as DA production and release in vitro (Storch, Paul et al., 2001). Neural progenitors derived from human fetus can be differentiated into 60.4% TH positive neurons in vitro after treatment with a combination of DA, forskolin and BDNF (Riaz et al., 2002). Can we make dopaminergic neurons from adult NSCs? NSCs derived from adult olfactory bulbs can differentiate into TH positive neurons after transplantation into

PD models of mice (Parati et al., 2003). In these cases, however, it is unclear if such TH positive neurons are functional DA neurons that are identical to the lost neurons in PD.

#### 3.2.5. Factors That Affect Survival, Migration, and Integration of New Neurons

Factors controlling later steps in neurogenesis, such as functional maturation, synapse formation, and integration into the neuronal circuit and survival, are currently unknown. Some mechanisms and molecules that control the remarkable long-distance migration of newly generated neurons through RMP have been described. Migrating neurons interact with their environment through expression of the polysialated glycoprotein neural cell adhesion molecule (PSA-NCAM), which is essential for proper migration, as a null mutation for NCAM or the deletion of the polysialic acid moiety results in migratory defects (Ono et al., 1994; Rousselot et al., 1995). Interactions via integrins between migrating neurons themselves (Jacques et al., 1998) as well as environment-derived short- and long-range chemorepulsive factors such as members of the ephrin-B family (Conover et al., 2000) and Slit (Wu et al., 1999) have also been demonstrated to direct the migration through the rostral migratory pathway.

## *3.3. Other Adult-Derived Stem Cells for Transplantation into CNS*

Stem cells have been found in adult tissues outside of the nervous system, including epidermal stem cells, liver stem cells, bone-marrow-derived stem cells (reviewed by (Pfendler and Kawase, 2003), and adipose tissue stem cells (Zuk et al., 2001). Nevertheless, because mesenchymal stem cells (MSCs) infrequently turn into brain neurons, the therapeutic value of MSCs for neurological diseases is still relatively low. Among these cells, bone–marrow-derived stem cells have triggered the most interest, because they are readily available from donors and patients themselves. Bone marrow contains both MSCs, which give rise to bone, cartilage, adipocytes, and stroma, and hematopoetic stem cells (HSCs), which give rise to all lineages of the blood. Grafted bone marrow and HSCs can be detected in many different types of tissues in recipients, including brain neurons (Pfendler and Kawase, 2003). Isolated MSCs containing bone marrow stromal cells have been shown to differentiate into neuron-specific enolase (NSE)-positive and NeuN (neuronal nuclei protein) positive neurons in vitro (Levy, Merims et al., 2003). It is still unclear if such double labeling of tissue-specific markers and grafted MSC markers is a result of transdifferentiation of MSCs or cell fusion between MSCs and other cells types. A type of multipotent adult progenitor cells (MAPCs) that are co-purified with MSCs have been isolated from both mouse and human bone marrow (Jiang et al., 2002; Reyes et al., 2001). MAPCs can be propagated in EGF, PDGF, and LIF (human MAPCS do not

require LIF) and can differentiate into most, if not all, somatic cells. MAPCs can be differentiated into midbrain TH positive neurons using similar protocols for ES cells. When MAPCs were treated with FGF-2 upon removal of other growth factors, MAPCS adopted a neural progenitor cell-like phenotype. Subsequent treatments with FGF8, SHH, and BDNF and co-culturing with astrocytes induced these cells to differentiate into dopa decarboxylase (DDC)-positive and TH-positive dopaminergic (neurons (Jiang et al., 2003; Keene et al., 2003). MSC-like cells have also been isolated from adipose tissue; these cells are called processed lipoaspirate (PLA) cells. PLA cells are very similar to MSC but have distinct gene expression profiles. PLA cells can also differentiate into neuron-like cells (Zuk et al., 2001). Further studies are needed to refine purification and control neuronal differentiation of these stem cells. With the development of better isolation and analysis techniques, we will gain further knowledge about adult-derived stem cells. Many of these cells can be potential sources for cell transplantation therapy to treat PD.

## 4. Adult-derived stem cells for cell replacement therapy in PD

### *4.1. Advantages of Using Adult-Derived Stem Cells for Replacement Therapy*

The ideal stem cells for treating PD should meet the following criteria:

- easily obtainable
- unlimited supply
- free of contaminating cell types
- ability to differentiate into fully mature DA neurons
- no tumorigenic potential

As discussed in other chapters, stem cells derived from embryonic and neonatal human sources have a great advantage for transplantation therapy, because these cells are easier to isolate and propagate in vitro. However, ethical issues limit the availability of such sources (see chapter 2).

One significant advantage of using adult-derived NSCs is that they can be isolated from post-mortem and surgical tissues, which poses minimal ethical concerns. These cells can be propagated and amplified in vitro using serum-free medium supplemented with growth factors to provide a sufficiently large quantity and relatively high purity sources for transplantation therapy. It is possible to build a bank of NSPCs from many different individual (post-mortem or surgical) donors. An immune matching process similar to that used for bone marrow/blood type matching can be performed to match the right stem cells with the right patients. In addition, stem cells can be isolated from surgical tissues (such as olfactory bulb) or bone marrow (MSCs) of the

patients themselves, and then be propagated and manipulated in vitro and grafted back into their brains. Such an approach avoids the immune responses from grafting cells isolated from other individuals.

A second advantage is that these adult stem cells can be genetically manipulated or marked using transfection or recombinant retrovirus-mediated infection before transplantation. Such manipulation is important because cell lineage-determination genes can be introduced into these cells in vitro to instruct them to differentiate into DA neurons in vivo. Trophic genes can also be introduced into these cells to make them more resistant to insult present in injured brain regions. These cells can also be engineered to self destruct after limited cell cycles in vivo to prevent tumor formation.

## *4.2. Problems Facing Stem Cell Transplantation Therapy*

The challenge of using adult stem cell therapy is multifaceted. Clinical trials using fetal tissues transplanted to PD patients have demonstrated the complexity and difficulty of cell replacement therapy and provided an insight into the problems facing transplantation using adult stem cells (Freed et al., 2001; Olanow et al., 2003; see chapters 5 and 6). Patients who received fetal tissue grafts have shown variable improvement and have in some cases displayed increased dyskinesia. Such a side effect may result from poorly functioning grafts and an immune response from the patients (see Chapter 10).

The biggest problem in stem cell grafting is that we are currently unable to control stem cell fate in vivo. Grafted cells are on their own to become neurons, glia, other cell types or tumors. In many cases, grafted NSCs become mostly glia. Even in the few cases in which grafted cells do become neurons, we only know that they express neuronal markers (TH, NeuN); we do not know if these new neurons can form the correct synapses and be functional (Yang et al., 2002).

Even though no tumor has been found in any NSPC or fetal tissue transplantation studies, the concern that stem cells with their proliferating potential will generate tumors after transplantation is still a serious one. Some recent findings have raised our alarm that such a concern is valid: certain studies have suggested that certain brain tumors may have stem cell origins (Noble and Dietrich, 2002). Genomic instability, including aneuploidy, is a hallmark of human cancer cells (Charames and Bapat, 2003). Neural progenitors from both embryonic and adult brain have much higher aneuploidy (an indication of genomic instability) than other somatic cell types, including lymphocytes (Rehen, McConnell et al., 2001; Zhao, Ueba et al., 2003). A transplantation study using mouse ES cells found that transplantation of mouse ES cells into mouse brain resulted in malignant tetratocarcinomas, whereas when xenotransplanted into rat brain these cells did not develop into tumors (Erdo et al., 2003). Before using adult-derived NSPC for cell replacement therapy, the oncogenic potential of these cells has to be addressed.

## *4.3. Improving Adult-Derived Stem Cells for Replacement Therapy*

How can we overcome these hurdles and augment the repair potential of stem cells? In vitro cultured NSCs have provided an unlimited source for cell transplantation. However, given our incomplete knowledge of how adult cell genesis is regulated under physiological conditions, and what factors are promoting or inhibiting neuronal differentiation of NSCs, it is currently difficult to devise logical strategies to enhance this neuronal differentiation capacity of NSCs. Nevertheless, recent studies have suggested that some growth factors might be able to either promote neurogenesis in otherwise gliogenic regions or enhance the repair from NSCs.

### 4.3.1. Stem Cell Transplantation in Combination With Growth Factor Delivery

Stem cell transplantation can be used in combination with growth factors to enhance the viability and differentiation of grafted cells. Some candidate factors are discussed here.

BDNF delivery to the adult forebrain by either continuous infusion or adenoviral overexpression in the ependymal layer results in enhanced proliferation in the SVZ and increased addition of neurons in the olfactory bulb, demonstrating that BDNF stimulates neurogenesis in the SVZ/olfactory bulb system in vivo (Benraiss et al., 2001; Pencea et al., 2001). Interestingly, in these studies, what may be newborn neurons in the striatum, septum, and hypothalamus were described in BDNF-treated animals but not in controls. Some of the newborn cells in the striatum also appeared to have differentiated into mature striatal spiny neurons, suggesting that BDNF might have not only the potential to recruit stem cells for neurogenesis in otherwise gliogenic regions but might also support their differentiation into region-specific phenotypes.

In rodents, transient global ischemia produces selective degeneration of hippocampal CA1 pyramidal neurons. FGF-2- and EGF-treated animals, however, showed approximately 40% recovery of the total number of CA1 pyramidal neurons lost by ischemia. BrdU labeling, retroviral lineage tracing, and inhibition of proliferation by administration of an antimitotic drug shortly after lesion provided evidence that a large number of the recovered pyramidal neurons were generated de novo in response to the combination of growth factor treatment and lesion (Nakatomi et al., 2002). Importantly, in this study, evidence was also found that functional synapses were formed on the regenerated neurons and that growth factor-treated animals performed significantly better in spatial learning tasks, suggesting that the recovered pyramidal neurons were participating in the hippocampal circuitry and contributed to behavioral recovery.

The combination of IGF-1 and physical exercise has been shown to significantly improve neurological conditions in an animal model of amyotrophic lateral sclerosis (ALS) (Kaspar et al., 2003). While the molecular conse-

quences of environmental enrichment are far from understood, these findings present us with the intriguing possibility that behavioral and environmental influences, potentially in conjunction with growth factor delivery, could be beneficial for stem cell therapy.

The effects of growth factors on regeneration have also been tested in animal models of PD. When fibroblasts expressing FGF-2 were co-grafted with fetal DA neurons into rat model of PD, FGF-2-expressing cells promoted the growth of the graft and improved functional recovery of animals (Takayama et al., 1995). In 6-OHDA-lesioned animals, transforming growth factor α (TGFα) infusion into the striatal parenchyma concomitant with or 2 weeks after lesion led to behavioral recovery (Fallon et al., 2000). Moreover, increased proliferation of precursor cells, migration from the SVZ toward the striatum, and differentiation into new dopaminergic neurons in the striatum was described in the TGFα–treated animals but not in the control animals. Based on these results, the authors suggested that TGFα in conjunction with signals arising from injury, recruited endogenous stem cells to generate new dopaminergic neurons in the striatum, which restored dopaminergic neurotransmission and caused behavioral recovery.

### 4.3.2. Stem Cell Transplantation in Combination With Gene Therapy

Despite extensive studies of PD, it is still not clear what changes occur in the affected brain area at molecular levels. What kind of trophic or toxic factors are expressed in lesioned regions? What signals stimulate or limit de novo neurogenesis in lesioned areas? Which factors will affect the viability and differentiation of endogenous and grafted NSCs? Knowing the answers to these questions will greatly enhance the success rate of cell transplantation therapy.

A number of signals regulating proliferation, neuronal migration, differentiation, survival, and connectivity during embryogenesis are reactivated in the injured adult environment. Interneurons surrounding dying neurons in the adult neocortex showed increased expression of neurotrophins (Wang et al., 1998), and glial cells in diseased areas acquired properties of radial glia (Leavitt et al., 1999), which act as a substrate for neuronal migration during development. Moreover, increased expression of mitogens for neural precursor cells was found in some lesions (Jin et al., 2002; Yoshimura et al., 2001). Transplantation of fetal immature neurons and neonatal and adult NSCs has been used to probe the lesioned environment of the adult CNS for its ability to recruit neural precursor cells for neuronal cell replacement. These experiments have shown that, in some specialized lesion models, the environment provides signals that are sufficient to stimulate the migration of transplanted fetal and neonatal derived precursor cells toward the lesioned area, their differentiation into neurons and the establishment of synaptic contacts (Fricker-Gates et al., 2002; Hernit-Grant and Macklis, 1996; Shin

et al., 2000; Snyder et al., 1997). In contrast, glial differentiation, but no neuronal differentiation of transplanted adult NSCs, has been observed in the lesion models tested so far (Vroemen et al., 2003) (Dziewczapolski, Lie et al., 2003), suggesting that environment-derived signals that can recruit adult NSCs are limited or that inhibitory factors for adult NSCs are predominant. In acute injuries such as stroke and spinal cord injury, factors that promote reactive gliosis and scar formation are up-regulated, and it is possible that they prevent neuronal differentiation of endogenous stem cells by promoting their glial fate choice (for review see Horner and Gage, 2000). The inhibitory effects of the glial scar (Pasterkamp et al., 1999; Stichel and Muller, 1998; Zuo et al., 1998), of extracellular matrix molecules (Haas et al., 1999; Jaworski et al., 1999; Jones and Tuszynski, 2002; Jones et al., 2002; Lemons et al., 1999) and of myelin derived-factors such as Nogo, MAG, and OMgp (Domeniconi et al., 2002; Liu et al., 2002; Wang et al., 2002a,b) on axonal outgrowth and regeneration in spinal cord injury are well documented. It is possible that the same factors interfere with the formation of functional synaptic connections of newborn neurons and their integration into existing circuits, thereby depriving them of target-derived trophic support and decreasing their survival rate. Given that axonal outgrowth and cell migration share similar pathways (Park et al., 2002), these inhibitory factors might also interfere with the migration of stem cells/immature neurons into the lesioned area.

Ideally, in conjunction with cell transplantation, we can deliver genes or gene-specific RNA interference (RNAi) to enhance the viability of grafted NSCs and direct grafted cells to differentiate into DA neurons, to help new DA neurons to form functional synapses, and to reduce glial scar and inflammation, all at the same time. These genes and RNAi can be either directly expressed in the NSCs before grafting or delivered by recombinant virus in parallell with cell transplantation.

### 4.3.3. Transplantation of Differentiated Cells

One alternative to stem cell transplantation is transplantation of partially differentiated progenitors that have been manipulated in vitro to become DA neurons. The advantages of such an approach are obvious. First, these cells have committed to a DA neuronal fate and are more likely to differentiate into mature DA neurons after transplantation. Second, one may be able to purify these cells based on their expression of DA neuronal marker (e.g., TH), thereby eliminating other contaminating cell types before transplantation. Third, these committed cells are less likely to be tumorigenic because they may have limited proliferative capacity. The challenge facing such an approach is that first one needs to efficiently differentiate a majority of the stem cells into DA progenitors in vivo. A combination of DA, forskolin, and BDNF has been shown to induce 60.4% of fetus-derived human neural progenitors into TH-positive neurons in vitro (Riaz et al., 2002). Such studies

need to be repeated and optimized for adult-derived NSPCs. Furthermore, we need to find the most effective differentiation stage of these cells for transplantation. Cells that have been differentiated too far down the road may have less plasticity to survive and differentiate in vivo after transplantation.

#### 4.3.4. Co-transplantation with Supporting Cells:

Another alternative is co-transplantation of NSPC with supporting cell types. Adult neurogenesis may be mechanistically different from embryonic neurogenesis because, in the embryonic stage, stem cells generate neurons before glia (Jacobson, 1991). During adult neurogenesis, multipotent NSCs are in intimate contact with the surrounding glia and the fate of NSCs can be affected by the microenvironment generated by glia and by other cellular, molecular and environmental factors in the adult brain. It has been shown that factors expressed by astrocytes can significantly affect the fate choice of adult-derived NSCs (Song et al., 2002) and ES cells (Nakayama et al., 2003) in vitro. Isolated astrocytes, endothelial cells or other cell types from postmortem substantia nigra of non-PD patients may be used to "prime" NSCs before transplantation or be co-transplanted with NSCs into the brain. These supporting cells can also be genetically manipulated to enhance DA neuronal differentiation.

#### 4.3.5. Recruitment of Endogenous Stem Cells for Cell Replacement Therapy

As discussed above, the lesioned adult brain may release signals to attract NSPCs and trigger them to differentiate and replace lost neurons (Kee et al., 2001; Liu et al., 1998; Nakatomi et al., 2002; Yagita et al., 2001; Zhao et al., 2003). As mentioned earlier, the possibility that endogenous stem cells may be used to replace dopaminergic neurons is an area of great controversy.

## 5. Challenges and Future Directions

At present our knowledge about adult NSC biology is very limited. It may be premature to evaluate the potential of NSCs to contribute to functional repair of the human CNS. There are several outstanding challenges that need to be overcome to help us to define the potential of adult stem cells for repair and to propose experimental strategies for the use of these cells in the diseased CNS:

### *5.1. To Identify and Isolate a Pure Population of Adult NSCs*

The prospective isolation of stem cells constitutes a key step in the study of hematopoiesis (reviewed in Weissman et al., 2001). Identification and separa-

tion of hematopoietic stem cells from other cell types based on their expression of surface markers allows the detailed characterization of this cell population. Thus far, our insight into the biology of adult NSCs has been primarily based on the analysis of cultured cells with stem-like characteristics and retrospective analysis in vivo. At this point, the identity and location of NSCs in vivo remains elusive, and no firm link between in vivo NSCs and cultured stem-like cells has been established. Such a limitation raises concerns about the in vivo relevance of the in vitro findings and hinders the use of cultured cells for transplantation therapy. The development of techniques to identify and isolate adult NSCs prospectively will provide a powerful tool to reevaluate our hypotheses and to characterize adult NSCs in detail.

## *5.2. To Be Able To Control Adult NSC Fate In Vitro and In Vivo.*

Cells with NSC-like characteristics have been isolated from several regions of the adult CNS. Do adult NSCs have different characteristics depending on their region of origin? Do adult NSCs have broad potential and differentiate into different neuronal subtypes or are they restricted by intrinsic determinants in the types of neurons they can generate? Until now it has only been demonstrated that adult NSCs can form functional interneurons in the olfactory bulb and local projection neurons in the hippocampal DG (Carleton et al., 2003; van Praag et al., 2002). However, one important requirement for therapeutic strategies using transplanted stem cells for PD is the ability of these cells specifically to regenerate dopaminergic neurons. Our current knowledge about the regulatory mechanisms of cell genesis in the adult CNS is incomplete and primarily descriptive. With the development of new technologies, such as functional genomics and proteomics, we will be able to identify molecular factors that govern the proliferation, fate choice, migration, integration and survival of new cells. The identification of regulators may provide potential triggers and targets for regulating the fate of transplanted NSCs.

## *5.3. To Characterize and Understand the Molecular and Cellular Changes in the Disease Environment*

How does PD alter the local environment into which NSCs will be transplanted? Which CNS cell types are affected in PD? It is important to recognize that not only defined subpopulations of neurons but also glial cells are killed by CNS injury. Until recently, neurodegenerative diseases such as PD have been thought of as diseases affecting specialized classes of neurons. However, it is becoming evident that neurodegeneration also elicits a glial reaction that potentially perpetuates neuronal cell death, and it is possible that the neurodegenerative process also affects glial cells. The behavior of NSCs is not only controlled by cell-autonomous signals but is also greatly influenced by environment-derived factors. In addition, it is evident that the function of mature nerve cells is highly dependent on an intact environment

that is created by many different cell types. It is therefore important to focus not only on the replacement of dying neurons but also on the creation of a cellular environment that supports the development and function of the new neurons.

### *5.4. To Understand the Function of Newly Generated Neurons From Grafted Stem Cells*

The observation that transplanted stem cells can be recruited to generate new neurons following lesion is highly exciting and reinforces our efforts to develop adult-derived stem cells for repair of the adult CNS. The observations that new neurons generated in response to epileptic injury in the hippocampus can be found in heterotopic locations (Parent et al., 1997, 2002), contribute to aberrant networks (Parent et al., 1997), and show altered physiological properties (Scharfman et al., 2000) reminds us that the uncontrolled generation of new neurons can also cause pathology (Parent and Lowenstein, 2002). Moreover, the contribution of new neurons to repair and functional improvement has not been unequivocally demonstrated, but has been assumed, primarily based on correlation of morphological and behavioral data. The study by Nakatomi and colleagues regarding the effects of FGF2 and EGF in global ischemia provides important evidence for a direct link between de novo neurogenesis and behavioral improvement (Nakatomi et al., 2002). However, the contribution of other factors, such as trophic support of injured neurons, axonal sprouting, and synaptic modulation by the growth factors, remains to be determined.

Our long-term goal is to introduce NSPCs into the substantia nigra or to mobilize local stem cells and instruct them to become DA neurons to replace lost neurons in PD. To achieve this goal, we first have to understand the molecular mechanism regulating adult NSCs and we also need to determine molecular changes occurring in the Parkinson's brain. These are complex and difficult questions, but with more and more effort being put into this research and better technology being developed, we are encouraged that the answers are on the horizon.

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# 13

# Embryonic Stem Cells for Grafting in Parkinson's Disease

Viviane Tabar[1] and Lorenz Studer[1,2]

## 1. Introduction

Embryonic stem (ES) cells are characterized by their extensive self-renewal capacity and the ability to differentiate into all cell types of the embryo proper, including germ cells. The isolation of human embryonic stem (hES) cells in 1998 (Thomson et al., 1998) caused great excitement about the potential of ES cells in regenerative medicine. Cell replacement therapy in neurodegenerative disorders has been suggested as a promising application of hES therapy given the devastating nature of these diseases and the lack of alternative approaches for cellular restoration in the CNS. Parkinson's disease has emerged as a key therapeutic target for cell replacement strategies in the CNS. This is due to several Factors, such as the rather selective loss of midbrain dopamine neurons in the substantia nigra, the extensive cell death at the time of clinical manifestation, and the experiences obtained from the transplantation of fetal midbrain dopamine neurons in experimental models in animals and in human patients. This chapter will discuss the suitability of ES cells as a source for cell transplants in Parkinson's disease. After a short introduction to the basic properties and the biology of ES cells, we will describe the various strategies that have been developed to control cell fate specification in vitro, and the application of ES-derived dopamine neurons in vivo in animal models of PD. The chapter will also discuss the current state of using human and non-human primate ES cells for dopamine neuron derivation and transplantation and the potential of therapeutic cloning in Parkinson's disease. A final section will be dedicated to the problems associated with translating ES cell work to the clinic and efforts to develop therapeutic cloning as a therapeutic option in preclinical models of PD.

[1,2] Laboratory of Stem Cell and Tumor Biology, Division of Neurosurgery and Developmental Biology, Memorial Sloan Kettering Cancer Center, New York, NY 10021
tabarv@mskcc.org
[2] Memorial Sloan Kettering Cancer Center, New York, NY 10021
studerl@mskcc.org

## *1.1. What Are ES cells?*

ES cells are derived from the inner cell mass (ICM) of a blastocyst, an early stage in embryonic development (3–4 days in mice, 5–6 days in humans). Cells in the ICM are known to give rise in vivo to the precursors of all three germ layers of the embryo, including germ cells. ES cells are obtained in vitro after culturing ICM cells on feeder layers, typically mouse embryonic fibroblasts (MEFs) that prevent cell differentiation. ES cells retain the full potential of the ICM and therefore provide a powerful source for deriving the large variety of cell types present in an organism. In addition to their wide differentiation potential, ES cells also possess the ability of nearly unlimited in vitro self-renewal by symmetric cell divisions. Mouse ES cells have revolutionized mouse genetics with the development of techniques to target individual genes by homologous recombination (Thomas and Capecchi, 1987; Doetschman et al., 1987). Other important genetic strategies that rely on the use of ES cells are ES-based mouse transgenesis (Goessler et al., 1986) and gene trapping (Goesskre et al., 1989; Soriano et al., 1986; Friedrich and Soriano, 1986), the generation of mouse chimeras (Martin, 1981; Evans and Kaufman, 1981) and purely ES derived mice by tetraploid embryo complementation (Nagy et al., 1990). Strategies for the controlled in vitro differentiation of mouse ES cells have been discussed for many years, though the scale of this effort remained modest up to the isolation of human ES cells. The possibility of translating in vitro strategies toward clinical use has clearly stimulated research in the field. Today, techniques for the differentiation and transplantation of mouse ES derived neural precursors have become routine and kits are available commercially to derive various neural cell types from mouse ES cells such as midbrain dopamine neurons and oligodendrocytes. Work on the directed differentiation of human ES cells has been largely driven by the need and potential for future clinical use. However, the long-term implications of human ES cell research might affect basic biology even more profoundly than direct therapeutic application given the unique opportunity to develop systematic genetic strategies to study human development and model human neural disease in vitro.

## *1.2. Advantages of ES Cells Compared to Other Stem Cell Sources*

Many cell sources have been explored to identify those most suited for the derivation of midbrain dopamine neurons. Those include neural stem cells, immortalized cell lines, non-neural somatic tissue stem cells, embryonic stem cells, and embryonic stem cell like cells generated by cloning or by parthenogenesis (for review, Perier and Studer, 2003). Neural stem cells may appear as the obvious source of dopamine neurons given their neural origin and their ability to proliferate extensively. However, despite intensive efforts, the derivation of true midbrain-type dopamine neurons from long-term expanded

naïve neural stem cells has not been achieved. Functional dopamine neurons have been derived in vitro from FGF2 expanded midbrain precursor cells (Studer et al., 1998). Transplantation of such in vitro generated precursor-derived dopamine (DA) neurons improves amphetamine-induced rotational asymmetry in 6-OHDA-lesioned rats (Yan et al., 2001). Alternative strategies demonstrated the induction of a dopamine neural-like phenotype from long-term expanded midbrain precursors (Carvey et al., 2001; Ling et al., 1998); however, the full neuronal and dopaminergic capacity of these neurons remains unclear. Developmentally based strategies for precursor cell conversion are an interesting area of research and new findings suggest a variety of strategies to improve DA neuron yield for neural precursors including low oxygen culture (Studer et al., 2000), ascorbic acid exposure (Yan et al., 2001), Nurr1 transfection (Wagner et al., 1999; Kim et al., 2003), or exposure to Wnts, particularly Wnt5A (Castelo-Branco et al., 2003). However, none of these strategies has overcome the difficulties associated with long-term proliferation and loss of the capacity to generate DA neurons (Yan et al., 2001). Immortalization of dopaminergic neurons or their precursors has been proposed as an alternative strategy to retain differentiation potential independent of in vitro proliferation (Rog et al., 2004). However, in addition to safety concerns associated with immortalization, no satisfactory human dopaminergic cell lines are currently available that yield robust in vivo function in an animal model of Parkinson's disease (e.g., (Liste et al., 2004)).

Non-neural stem cells such as hematopoietic, mesenchymal, skin, or muscle stem cells may appear as an attractive source for regenerative medicine due to the ease of availability and the possibility to perform autologous cell therapy. In the context of brain repair, the main issue is whether adult somatic non-CNS stem cells are capable of full neuronal and dopaminergic differentiation. Recent studies on the transdifferentiation of various stem cell types across traditional tissue boundaries and germ layers caused considerable interest and controversies (for review, (Wagers and Weissman, 2004)). Among the cell types proposed for use in neural replacement are bone marrow derived hematopoietic and stromal cells, including the multipotent adult precursor cells (MAPCs) (Jiang et al., 2002; Keener et al., 2002) and skin cells (Toma et al., 2001).

While the presence of tyrosine hydroxylase (TH) positive cells has been reported from adult MAPCs (Jiang et al., 2003, 2003), there is no evidence that such presumptive dopaminergic neurons derived from MAPCs or from any other somatic stem cell type outside the CNS are functional in vitro or in vivo.

The most critical advantages of ES cells compared with any other cell source are the extensive self-renewal, broad differentiation potential, and access to the earliest stages of neural development. Unlike most fetal and adult stem cell types, ES cells retain their full differentiation potential after long-term passage. However, the wide differentiation potential also poses a great challenge, requiring the development of techniques that yield selective

cell lineages. Random differentiation of ES cells in vivo typically results in teratomas, tumors composed of disorganized tissue derivatives of all three germ layers.

Access to the earliest stages of neural development in vitro is critical for the manipulation of regional specification of ES cell progeny. Neural development is guided by diffusible factors that pattern the neural plate and neural tube in both anterior-posterior and dorso-ventral orientation. Molecules that affect the patterning state of the developing neural tube in vivo are known to act at the very earliest stages of neural development, a period typically not accessible in neural stem cell biology. In contrast, ES cell differentiation strategies can be readily tuned to appropriate developmental windows resulting in highly successful strategies to direct regional identity (e.g., Lee et al., 2000; Wichterle, 2002; Barberi, 2003; Mizuseki et al., 2003). However, long-term expansion of any cell type in vitro carries the risk of accumulating genetic alterations. While ES cells are remarkably resistant against genetic alterations compared to other somatic cell types, chromosomal abnormalities such as recurrent gain of chromosomes 17q and 12 have been reported after long-term culture of hES cells (Draper et al., 2004), demonstrating the need for regular karyotyping of hES cells in vitro.

## 2. In Vitro Differentiation Strategies

### *2.1. Induction of Neural Differentiation*

The first key step toward generating midbrain-type DA neurons from ES cells is the ability to induce selective neural differentiation at the expense of non-neural derivatives and undifferentiated hES cells. There are three basic strategies to achieve neural fates: 1) embryoid body (EB)-based protocols; 2) stromal feeder-mediated neural induction; 3) default neural differentiation protocols. The first strategy (EB-based protocols) is based on the formation of embryoid bodies (EBs). EBs are formed upon aggregation of ES cells in suspension culture. The interaction of cells within EBs induces cell differentiation in a process mimicking gastrulation. Accordingly, derivatives of all three germ layers can be found in EBs (Doetschman et al., 1985; Weiss and Orkin, 1996). Various modifications of the basic protocol have been developed to enhance neural induction and to select and expand EB-derived neural precursors. One such strategy is based on the exposure to retinoic acid (RA) such as in the classic 4–/4+ protocol in which EBs are formed in the absence of RA for 4 days followed by 4 days of RA exposure (Bain et al., 1995). An alternative EB-based strategy is the exposure to conditioned medium derived from a hepatocarcinoma cell line (HepG2), which appears to induce neuroectodermal fates directly via an unknown activity present in the conditioned medium (Rathjen et al., 2002). A third EB-based strategy makes use of neural-selective growth conditions (Lee et al., 2000; Okabe et al., 1996; Brustle et al., 1999)

whereby EB progeny is kept under minimal growth conditions in serum-free medium leading to the selective survival of neural progenitor cells.

The second major approach (stromal feeder mediated neural induction) emerged from studies on the inducing properties of bone marrow-derived stromal cell lines that have been used for many years to support the growth of undifferentiated hematopoietic stem cells (Collins and Dorshkind, 1987; Croisille et al., 1994; Nakano et al., 1994; Sutherland, 1991). These stromal feeders, typically used at the preadipocyte stage, exhibit profound neural-inducing properties in co-culture with mouse and primate ES cells (Barberi et al., 2003; Kawasaki, et al., 2000, 2002a,b, Perrier et al., 2004). While the molecular nature of the stromal-derived inducing activity (often termed SDIA [Kawasaki et al., 2000]) remains elusive, the efficiency and robustness of neural induction under these conditions has made stromal feeder-mediated induction a very powerful approach widely used for both mouse and primate ES cell studies.

The third strategy (default neural differentiation) relates to conditions that achieve neural differentiation in the absence of EB formation or any co-culture requirements. The idea is based on developmental studies in xenopus, suggesting that primitive ectodermal cells undergo rapid neuronal differentiation in the absence of any extrinsic signals (Munoz-Sanjuan and Brivanlou, 2002). Several strategies have been developed in mouse ES cells to achieve neural differentiation under such conditions in non-adherent (Watanabe et al., 2005; Tropepe et al., 2001) or adherent (Ying et al., 2003) monocultures.

## *2.2. Induction of Dopaminergic Differentiation*

Most protocols for the dopaminergic differentiation of mouse ES cells are based on studies in explants that identified FGF8 and SHH as critical factors in midbrain DA neuron specification (Ye et al., 1998, 2001; Hynes et al., 1995a,b). The effect of SHH/FGF8 on ES-derived neural precursors was first described using an EB-based 5-step differentiation protocol (Lee et al., 2000). Under these conditions up to 34% of all neurons expressed TH, the rate-limiting enzyme in the synthesis of DA. A further increase in DA neuron yield (nearly 80% of all neurons expressing TH) was achieved using Nurr1 overexpressing ES cells (Kim et al., 2002). Midbrain dopaminergic differentiation was also obtained using co-culture of ES cells on the stromal feeder cell line (PA6) (Kawasaki et al., 2000) with 16% of all neurons expressing TH in the absence of SHH and FGF8. These results were initially interpreted as PA6 exhibiting a specific patterning action that promotes DA neuron fate (Hynes and Rosenthal, 2000). However, later studies demonstrated that neural precursors induced on stromal feeders can be shifted in anteroposterior and dorsoventral identity (Barber et al., 2003; Mizuseki et al., 2003). Stromal feeder-induced differentiation combined with SHH/FGF8 patterning yields up to 50% neurons expressing TH (Barberi et al., 2003) without requiring transgenic Nurr1 expression. Another transgenic modification

with an interesting effect on dopaminergic yield is the expression of $BclX_L$, which appears to achieve midbrain dopaminergic differentiation without requiring SHH/FGF8 exposure (Shim et al., 2004).

### *2.3. How To Define an Authentic Midbrain Dopamine Neuron In Vitro*

Numbers of TH neurons need to be interpreted carefully in all in vitro differentiation studies, as TH is an unreliable marker for identifying DA neurons. TH is expressed in other catecholaminergic neuron types such as noradrenergic and adrenergic cells and can be induced in many cell types under various unspecific external stimuli such as stress or hypoxia. It is therefore essential to use additional markers to confirm DA neuron identity and to perform functional studies in vitro and in vivo. The four key parameters to confirm DA neuron identity in vitro are 1) *biochemical markers* of dopaminergic fate, including expression of TH, DA transporter, aromatic acid decarboxylase, and the absence of other neurotransmitter markers such as glutamate decarboxylase or choline acetyl transferase; 2) *regional markers* demonstrating developmental progression of ES cell progeny from a Pax2-, Pax5-, Wnt1-positive population, to En1, Nurr1, Lmx1b, and Pitx3 expression; 3) *morphological markers*, including characteristic morphometric features by light and electron microscopy; 4) *functional markers*, including potassium-evoked DA release measures by HPLC analysis and extensive electrophysiological characterizations. For a detailed discussion of these in vitro parameters see (Perrier and Studer, 2003).

## 3. In Vivo Data

The first ES-cell based study that showed functional improvement in 6-OHDA-lesioned rats was based on the transplantation of low numbers of largely undifferentiated mouse ES cells isolated after short-term differentiation in EB cultures (Bjorklun et al., 2002). Spontaneous differentiation into large numbers of neurons with midbrain DA characteristics was observed. However, the clinical relevance of this approach is limited due to the high rate of tumor formation (> 50% of the animals with surviving grafts developed teratomas).

Remarkable functional improvement was obtained after transplantation of DA neurons derived from Nurr1-overexpressing mouse ES cells (Kim et al., 2002). This study reported behavioral restoration in 6-OHDA-lesioned rats, and demonstrated in vivo function via electrophysiological recordings from grafted DA neurons in slice preparations obtained from the grafted animals. However, transgenic expression of Nurr1 raises safety concerns that may complicate clinical translation. Another study showed behavioral recovery with DA neurons derived from naïve mouse ES cells (Barberi et al. 2003). Both regular mouse ES cells and ES cells derived after nuclear transfer (ntES cells)

from a cloned blastocyst were used in this study. $BclX_L$-expressing mouse ES cells also showed robust in vivo survival and function (Shim et al., 2004) as well as several additional studies using modified protocols (e.g., Inden et al., 2004; Yoshizaki et al., 2004; Baier et al., 2004; Nishimura et al., 2004).

## 4. Human Embryonic Stem Cells

Neural differentiation of hES cells has been achieved using a variety of strategies including EB (Carpenter et al., 2001; Zhang et al., 2001) default (Reubinoff et al., 2001), and stromal feeder protocols (Perrier et al., 2004). The differentiation of hES cells into midbrain DA neurons has been reported very recently (Perrier et al., 2004). This study was based on a stromal feeder-based neural differentiation approach. After 2–4 weeks of co-culture of hES cells on MS5 stroma primitive neuroepithelial structures emerge and proliferate. These structures were termed neural rosettes. Such neural rosettes express markers of early neural development, including Sox1, Pax6, Nestin, NCAM, and vimentin. Based on the characteristic tubular cytoarchitecture, it has been suggested that neural rosettes correspond to the neural tube stage in early development (Zhang et al., 2001; Li et al., 2005). However, neural rosettes do not express any of the characteristic markers that define specific domains within the developing neural tube with the exception of Pax6. Pax6, like Sox1, is expressed in the early neural plate and is not a definitive marker of the neural tube stage. Pax7, a marker of the dorsal spinal cord, hindbrain, and midbrain is expressed in cells that emerge from neural rosettes, but is typically absent within neural rosettes. This suggests that neural rosettes might correspond to the neural plate rather than the neural tube stage as at the time of neural tube closure dorso-ventral domains have been fully established. Upon trituration of hES-derived neural rosettes, cells are plated in the presence of FGF8 and SHH, leading to the sequential induction of transcription factors characteristic of midbrain development starting with the expression of Pax2, followed by co-expression of Pax2/En1, then En1/TH-expression. Upon differentiation, cultures with a high yield of DA neurons are obtained (up to 70% of all neurons express TH [Perrier et al., 2004]). Such hES-derived DA neurons exhibit potassium-evoked DA release as measured by HPLC analysis with electrochemical detection, depolarization-induced action potentials, and electron microscopic evidence of synaptic contacts using TH-immunogold labeling techniques. Several additional studies have been published recently showing the derivation of TH positive cells from hES cells using PA6 stroma (Zeng et al., 2004). PA 6 stroma in combination with GDNF exposure (Buy taert-Hoefen et al., 2004), or free-floating aggregate culture conditions (Schulz et al., 2004). However, none of these studies using human ES cells demonstrated robust survival of TH positive neurons in vivo after transplantation into $^6$OHDA lesioned rats. This suggests that hES-derived neurons that exhibit robust midbrain DA neuron characteristics

in vitro are either particularly vulnerable to cell death in vivo or loose dopaminergic identity upon transplantation. In contrast our own unpublished work, and recently published studies (Park et al., 2005), suggest that the non-DA neuron component within the graft shows robust in vivo survival.

More promising results in vivo emerged from studies with non-human primate cells grafted into rodent or primate hosts that yielded several hundreds (rodent host) to several thousand (primate host) TH positive cells in vivo (Sanchez-Pernaute et al., in press), and resulted in some functional improvement in monkeys (Takagi et al., 2005). While the first study was based on the protocols published previously for the differentiation of hES cells into dopaminergic neurons (Perrier et al., 2004), the second study developed an alternative approach whereby neurosphere like cultures are exposed to the combined action of FGF2 and FGF20, a growth factor expressed quite selectively within midbrain DA neurons in the adult brain.

Currently the key requirements for increased survival of hES-derived DA neurons remain unclear. However, the ability to generate unlimited numbers of cells highly enriched in midbrain DA neurons in vitro is a first step towards fulfilling the clinical promise of hES cells in Parkinson's disease and overcoming the challenge of controlling neuronal subtypes as discussed just a few years ago (Studer, 2001). The next milestone in the field will be robust in vivo survival and function that match or surpass the results currently obtainable using primary human fetal DA neurons (see Björklund et al., 2003).

## 5. Translational Aspects

Several issues remain to be addressed before making steps toward the first clinical trials with hES cell-derived dopamine in Parkinson's disease. Over 20 years of fetal tissue research have demonstrated that fetal midbrain DA neurons can survive and function long-term (>10 years [Piccini et al., 1999]) in the brain of Parkinson's patients. However, these studies have also shown limited efficacy in placebo-controlled clinical trials and demonstrated the potential for side effects (Freed et al., 2001; Olanow et al., 2003 and Chapter 6). The stem cell field will have to learn from the fetal tissue transplantation trials to better define and address the critical parameters that will take cell therapy in PD to the next level (Björklund et al., 2003). The derivation of highly purified populations of substantia nigra type DA neurons from hES cells is an important first step on this road. The main limiting factor to date concerns the poor in vivo survival and function of hES-derived midbrain DA neurons. Several strategies are underway to improve in vivo function, and it appears likely that this limitation will be overcome in the next few years. However, there will be several layers of safety concerns related to the use of hES progeny that also need to be addressed prior to any clinical efforts. While it is likely that the risk for teratoma formation can be largely eliminated using appropriate differentiation techniques, selective enrichment of inappropriate

neural fates in vivo has been observed, particularly the transient proliferation of forebrain type neuronal progenitors (Tabar V and Studer L, unpublished observations).

Furthermore, political hurdles currently prevent researchers in many countries from working with hES cell lines most appropriate for future clinical use. For example, all the cell lines currently listed on the US NIH registry of lines derived prior to August 9, 2001, have been in contact with mouse feeders. Such coculture conditions raise a host of safety concerns, including xenogenic viruses or mouse embryonic fibroblast-induced expression of an immunogenic molecules such as nonhuman sialic acid expression observed in hES cells (Martin et al., 2005). Such xenogenic markers may lead to the immunological elimination of hES cells and their progeny after transplantation into a human host. Completely xeno-free systems are required to resolve such concerns completely (Richards et al., 2002). Another important issue relates to the genetic stability of hES cells and their progeny (Droper et al., 2004) and to appropriate immunological matching to the host that may require the availability of a large number of genetically diverse "matched" hES cell lines.

In addition to hES-derived biological parameters there are also fundamental questions about the clinical role of cell transplantation in Parkinson's disease and the development of strategies that can predictably prevent potential graft-induced side-effects such as disabling "off"-dyskinesias reported in some of the patients undergoing fetal tissue grafting (Freed et al., 2001; Olanow et al., 2003 and see Chapter 10). While both biological and clinical hurdles need to be addressed toward clinical translation, hES cells provide a powerful resource to take on these challenges and to develop innovative strategies that go beyond generating unlimited numbers of midbrain DA neurons for transplantation and toward strategies of manipulating in vivo survival, host innervation outgrowth, and DA function.

## 6. Therapeutic Cloning

Therapeutic cloning or somatic cell nuclear transfer provides the possibility of generating genetically matched ES cells to an adult donor (Wakayams et al., 2001; Munise et al., 2000). While the role of the host immune response on the survival and function of fetal DA neuron allografts in Parkinson's disease is controversial, preclinical models of PD have been used as a proof of principle of therapeutic cloning in CNS disease (Barberi et al., 2003). Transplantation of genetically matched cells would provide an opportunity to eliminate immunological factors related to graft host mismatch and the effect of immunosuppressive therapy of graft survival and function. This question has become clinically more prominent after the results of the most recent placebo-controlled clinical trial with fetal tissue grafts (Olanow et al., 2003 and Chapter 6). This study showed clinical improvement in grafted patients 6 months after transplantation in post hoc analysis. However, at time

points beyond 6 months (when immunosuppressive drugs were withdrawn) graft function dramatically decreased. While the link between removing immunosuppressive drugs and loss of graft function is purely correlative, and graft survival has been reported in these patients beyond 6 months, it seems at least possible that immunological factors may affect dopamine graft function e.g., through factors secreted by activated microglia found in many of these grafts. In primates, cloning technology has faced multiple hurdles (Simerly et al., 2003) and up to recently the only genetically matched primate ES cell line had been established through parthenogenesis rather than cloning (Cibelli et al., 2002; Vrana, 2003). In fact, such parthenogenetic primate ES-like cells have provided a powerful system to study dopaminergic differentiation from primate ES cells in vitro and in vivo (Perrier et al., 2004; Sanchez-Pernaute et al.,2005). Recent progress in nonhuman primate nuclear transfer (Simerly et al., 2004) suggests that testing therapeutic cloning in Parkinsonian primates might become feasible. At this time the feasibility of human nuclear transfer remains to be determined. Progress in human nuclear transfer may provide additional resources including the generation of cloned hES cells lines from idiopathic PD patients. Such cells may be useful in studying the basic mechanisms of disease but could also provide a tool to address the fundamental question of whether DA neurons derived from a cloned PD hES cell line will be suitable for transplantation.

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# 14

# Genetically Modified Cells as a Source for Grafting in Parkinson's Disease

Hyun-Jung Kim,[1,2] Gesine Paul,[3] Yu-Tzu Tai,[4] and Clive N. Svendsen[2]

## 1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease and affects almost 1% of the population above the age of 50. PD was first described by James Parkinson in 1817. His essay on the "Shaking Palsy" reported the major symptoms of the disease, such as bradykinesia, resting tremor, and muscular rigidity (Parkinson, 2002). Most patients exhibit vegetative disturbances, with up to a third showing significant cognitive dysfunction (Lang and Lozano, 1998a,b), and almost 40% of PD patients are affected by depression (Oertel et al., 2001). The most conspicuous neuropathologic finding in PD is the progressive loss of dopamine (DA) neurons in the substantia nigra pars compacta (SNc).

In the mammalian ventral midbrain, DA neurons can be found in three different regions, the SNc, the ventral tegmental area (VTA), and the retrorubral field. DA neurons of the VTA project to the ventromedial striatum and cortical area and form the mesolimbic pathway, which is involved in emotional behavior and motivation. DA neurons in the SNc project to the dorsolateral striatum and release DA, an important neurotransmitter which controls movement. Thus, the loss of DA neurons of the SNc leads to a reduction of striatal DA levels (Agid, 1991), that is responsible for some of the cardinal symptoms of Parkinson's disease.

Currently, there is no treatment that can prevent or retard progression of the disease. Since the late 1960s, the main approach to treating PD has been the pharmacological alleviation of the symptoms caused by the striatal

[1]Molecular and Cellular Pharmacology, University of Wisconsin-Madison, Madison, WI 53706, USA
[2]Waisman Center, University of Wisconsin-Madison, Madison, WI 53705, USA
[3]Department of Experimental Medical Science, Neuronal Survival Unit, Wallenberg Neuroscience Center, Lund, Sweden.
Gesine.Paul@med.lu.se
[4]Cambridge Centre for Brain Repair, University of Cambridge, Forvie Site, Cambridge, UK
svendsen@waisman.wisc.edu

DA deficit. Although pharmacotherapy using L-dihydroxyphenylalanine (L-DOPA) or DA receptor agonists is effective in alleviating the symptoms of PD in early stages of the disease, chronic DA therapy is limited by disease progression and the development of therapy-related motor complications, such as dyskinesias (involuntary movements) or partly unpredictable motor fluctuations which significantly affect the patients quality of life (Brotchie et al., 2004; Miyawaki et al., 1997 and see Chapter 3).

One promising treatment option for PD is the restoration of the lost dopaminergic neurons by grafting immature embryonic ventral mesencephalic neurons (Olanow et al., 1996; Hauser et al., 1999; Madrazo et al., 1988; Lindvall et al., 1988, 1990, 1994; Björklund and Lindvall, 2000).

Grafting experiments have been performed in animal models of PD where the nigrostriatal dopaminergic system is destroyed, either by 6-hydroxydopamine (6-OHDA) in rats or by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in monkeys, and have shown that grafted dopaminergic neurons can survive, reinnervate the lesioned striatum, and improve motor function. Grafted dopaminergic neurons are active and can restore baseline DA synthesis (for review see Björklund, 1992, 2003).

DA neurons derived from human fetal tissue have been transplanted into 350 patients worldwide. The transplanted cells survive, function and partially reverse motor deficits in PD patients (Björklund et al., 2003 see Chapters 5 and 6), although there are concerns regarding possible side effects connected with transplants (Freed et al., 2001 see Chapters 6 and 10).

There are clearly major limitations related to the collection of fetal tissues. Differences in donor age may lead to heterogeneity of transplants, and the shortage of embryonic donor tissue prevents that this therapy is available for a larger number of patients (see Chapter 8). Therefore alternatives to using primary DA neurons from fetal tissue are sought. The ideal cell for transplantation in PD can be expanded indefinitely and produced on a large scale and is capable of differentiating into neurons that extend axons, form synapses, and release DA in a regulated fashion (see Chapter 12). Stem cells are such undifferentiated cells that have high proliferative potential, generate a wide variety of differentiated progeny, possess the capacity for self-renewal, and retain the multi-lineage potential over time (Gage, 2000). Also, stem cells can be genetically modified to generate DA neurons.

In this chapter we focus on genetically modifying cells to release DA and on the generation of DA neurons from stem cells.

## 2. Engineering Cells To Produce Dopamine

### *2.1. Ex vivo Gene Therapy Using Cells Genetically Modified To Release Dopamine*

The most direct way to get DA production within the brain may be to use gene therapy to deliver the rate-limiting enzyme of DA synthesis, tyrosine

hydroxylase (TH). The vector could simply be injected into the brain to infect host neurons, which would then produce DA. This approach has been tried in animal models with some success, but while direct gene therapy is an attractive idea and is being actively pursued (see Chapter 16), there remain serious practical and safety issues before translation to the clinic:

- inability to exactly control gene dosing following *in vivo* delivery
- accidental insertional mutagenesis as described in recent reports of a gene therapy trial in France (Pollack, 2003)
- forcing host neurons and glia to express the gene of interest may compromise their normal function

A parallel, alternative, and complementary approach to direct injection of live virus to the brain is using viruses to transduce cells in the culture dish. These cells can then be transplanted into the brain and release DA. This approach has a number of attractive features, including—

- Cells can be selected for gene dosing (DA release) prior to transplantation.
- The exact insertion sites can be documented from cloned cells and the disruption of normal oncogene regulation caused by the insertion can be checked.
- Healthy *ex vivo* cells will provide the DA delivery, not degenerating host cells.
- Because viral infection takes place *in vitro* and can be followed by extensive expansion of the cells in the absence of virus, there is far less danger of live replication competent virus transfer to the patient.

In order to achieve DA-producing cell lines, TH was introduced into several cell lines. Fibroblast 3T3, endocrine RIN cells, neuroblastoma NS20-Y, and neuroendocrine AtT-20 cells were engineered with a recombinant retrovirus encoding for human tyrosine hydroxylase (TH) (Horellou et al., 1990a,b). After transplantation into the striatum of 6-OHDA lesioned rats, cells survived to express TH and secreted high amounts of DOPA and/or DA into the surrounding host striatum. Slight behavioral improvement by cells engineered to produce DOPA and /or DA provided promise for ex vivo gene therapy. Additionally, human neural progenitors genetically modified with human TH were developed and the expression of TH could reversely be switched on or off by doxycycline, both in vitro and in vivo (Corti et al., 1999). In this study, a single adenovirus vector encoding human TH was under the control of a tetracycline responsive regulatory system. Human neural progenitor cells were infected and subsequently transplanted into the 6-OHDA-lesioned striatum of hemiparkinsonian rats. Even though these cells proved a useful vector for gene delivery into the CNS, behavioral improvement by transplanted cells was not reported. In a more recent study (Park et al., 2003), a human embryonic cell line was transfected with cDNA for TH and GTP cyclohydroxylase, and the cells subsequently transplanted into a rat Parkinson model. A partial behavioral effect was seen, however,

those studies need further evaluation and clarification of the mechanisms behind the partial behavioral recovery.

## *2.2. Generation of Dopamine Neurons Through Genetic Modification of Stem Cells*

Simply releasing DA from transplanted cells may not be sufficient to restore function. This repair may require synaptic contact from new DA-producing neurons. To restore lost DA neurons in Parkinson patients, we need to understand how DA neurons are generated or which molecules are required for the generation of DA neurons during development. Several essential molecules have been found to play important roles in the generation of DA phenotype, although the developmental mechanisms of DA neurons are not yet fully understood. Sonic hedge hog (Shh), a morphogenic signal factor released from the floor plate, induces a general ventral cell fate and is involved in the development of DA neurons (Hynes et al., 1995; Wang et al., 1995). Expressed in the dorsal neural tube, Shh was sufficient to induce DA neurons characterized by the expression of essential enzymes for the synthesis of DA. TH is not only the first but also the rate-limiting enzyme in catecholamine biosynthesis, and the expression of this enzyme correlates with the emergence and maturation of DA neurons. It is generally used as one of the markers for DA neurons. Briefly, DA is generated from one of the essential amino acids tyrosine. TH converts tyrosine into a catecholamine, DOPA, and DOPA can be further converted into dopamine by L-aromatic amino acid decarboxylare (AADC).

Fibroblast growth factor 8 (FGF8), produced by the isthmus organizer, is another essential soluble factor for the development of DA neurons, combined with Shh (Ye et al., 1998). Besides soluble factors, a subset of transcription factors is required to induce essential enzymes to produce DA and a DA phenotype (Burbach et al., 2003; Simon et al., 2003; Wallen and Perlmann, 2003). Nur(nuclear receptor)-related factor 1 (Nurr1) is a steroid/thyroid hormone receptor family expressed in DA neurons. The ligand of Nurr1 is unknown, so it is called an orphan receptor. Mice lacking Nurr1 were hypoactive, failed to generate midbrain dopaminergic neurons, and died within 2 days after birth (Castillo et al., 1998; Saucedo-Cardenas et al., 1998; Zetterstrom et al., 1997). The effect of Nurr1 in dopamine biosynthesis was specific to DA neurons of SNc and VTA (Castillo et al., 1998). Reduced levels of DA seen in heterozygous mice support a strong correlation between the expression levels of Nurr1 and dopamine synthesis (Zetterstrom et al., 1997). Nurr1 knockout mice showed loss of markers for a DA phenotype including aldehyde dehydrogenase (ADH 2), glial cell line-derived neurotrophic factor (GDNF) signal transducing receptor c-Ret, the DA D2 receptor, TH, AADC, and reduced expression of cyclin-dependent kinase inhibitor p57 kinase inhibitory factor ($p57^{kip2}$) (Joseph et al., 2003; Castillo et al., 1998; Saucedo-Cardenas et al., 1998; Zetterstrom et al., 1997).

Nurr1 activates TH by binding directly to a response element in the TH promoter site, *in vitro* (Iwawaki et al., 2000; Sakurada et al., 1999). Absence of Nurr1 did not affect neuroepithelial cells to adopt a normal ventral localization or neuronal phenotype as revealed by the expression of homeodomain transcription factor mesencephalic marker paired-like homeodomain transcription factor (Pitx3) and ventral marker hepatocyte nuclear factor (HNF3) at embryonic day 11.5, but failed to induce a full DA phenotype in late precursors (Saucedo-Cardenas et al., 1998). Dopaminergic precursor cells degenerate, lose the expression of Ptx-3 and show increased apoptotic cell death in the absence of Nurr1, showing that Nurr1 may be required for the survival of late dopaminergic precursor to committed into a fully differentiated DA phenotype (Saucedo-Cardenas et al., 1998). In heterozygous mice of Nurr1 (Nurr1+/–), which have a reduced expression of Nurr1, increased vulnerability of mesencephalic DA neurons to MPTP-induced injury (Le et al., 1999) has been observed. Although putative ligands of Nurr1 have not been identified, Nurr1 can hetero- dimerize with retinoic X receptor (RXR) and be activated by RXR ligands (Sacchetti et al., 2002; Aarnisalo et al., 2002; Forman et al., 1995). Recent evidence shows that $p57^{Kip2}$, the one of the cyclin-dependent kinase inhibitors of the cyclin-dependent kinase interaction protein (Cip)/Kip family, may play an important role in induction of a DA phenotype by direct protein–protein interaction with Nurr1 in the dopaminergic cell line MN9D cells (Joseph et al., 2003). However, Nurr1 can also activate transcription as a monomer indicating several pathways can be taken during development. X-ray crystallographic structure analysis of the ligand-binding domain (LBD) of Nurr1 at 2.2 Å resolution performed by Perlmann and colleagues revealed that the Nurr1 LBD has a canonical protein fold which resembles those of agonist-bound, transcriptionally active form (Wang et al., 2003). Several bulky hydrophobic residues result in the loss of a ligand binding cavity and Nurr1 lacks a "classical binding site" for coactivators, implicating that Nurr1 may act as a ligand-independent nuclear receptor (Wang et al., 2003).

There are a few genes known to be involved in the antero-posterior and dorso-ventral patterning of the developing brain. Engrailed 1 (En1), En2, Pitx3, Wnt1, paired box gene (Pax) 2 and Pax5 play critical roles in the midbrain pattering since targeted deletion of any of these genes resulted in a defect of midbrain development (Favor et al., 1996; Urbanek et al., 1994, 1997; McMahon and Bradley, 1990; Thomas and Capecchi, 1990; Wurst et al., 1994). Lmx1b is a member of the LIM homeodomain family and is expressed in the ventral midbrain DA region around E7.5 through the adulthood. Knockout embryos of Lmx1b lost the expression of Pitx3, however, TH expression was not affected, implicating that Lmx1b and Pitx3 may not have a direct effect on TH expression (Smidt et al., 2000). The expression of Pitx3, a bicoid-related homeobox gene, correlates with the appearance of mescencephalic DA neurons and the overexpression of Pitx3 induces AHD2 expression in the mouse embryonic stem (ES) cells, suggesting Pitx3

may be involved in the specification of DA neuronal lineage (Chung et al., 2005). En 1 and 2 are also expressed early in the midbrain and hindbrain. Double null mutants of En1 and En2 show that these genes are involved in survival and maintenance but not in induction of TH-positive neurons (Simon et al., 2001).

Although little is known about how cells differentiate into specific types of neurons such as DA neurons, there are a few genes known to be involved in neuronal commitment. Proneural basic helix-loop-helix (bHLH) proteins were initially identified from mutant flies which lacked subsets of external sense organs or bristles (Ghysen and Dambly-Chaudiere, 1989). These are transcription factors that typically function as either homo- or heterodimers and bind to a common DNA sequence (CANNTG) called the E box sequence (Murre et al., 1989b). The basic regions of bHLH proteins make contact with the DNA while the HLH domains are involved in dimerization (Murre et al., 1989a). Since the expression of TH is confined to mature neurons in vivo it seems plausible that neurogenesis has to occur no later than the induction of the DA phenotype. The detailed mechanism involved in neurogenesis and its specification to a DA phenotype need further investigation.

## *2.3. Transplantation of Nurr1 Expressing Cells Into Animal Model of PD*

Several studies have attempted dopaminergic neuron conversion of neural stem cells and precursor cells via overexpression of Nurr1. Wagner and colleagues induced a ventral mesencephalic dopaminergic phenotype in an immortalized multipotent neural stem cell line *in vitro* using the c17.2 (Wagner et al., 1999; Snyder et al., 1992). This cell line was originally derived from the developing mouse cerebellum and has been shown to differentiate into neurons, astrocytes and oligodendrocytes *in vitro* and *in vivo* (Snyder, 1992, 1995). When authors stably transfected the cells with Nurr-1, 30% of the cells expressed a neuronal phenotype, however, none of the cells expressed a DA phenotype upon transfection in vitro. A combination with a co-culture using astrocytes from the E16 rat ventral mesencephalon (VM) in combination with bFGF treatment resulted in 45% of the neurons expressing TH. However, the factors secreted by the astrocytes remain to be elucidated. Upon stimulation with KCl, cells released DA, acquired immunoreactivity for ADH-2, an enzyme selectively expressed in developing dopaminergic precursors within the VM (McCaffery and Drager, 1994) and expressed c-ret mRNA, the signaling receptor for GDNF. However, upon transplantation into the intact adult mouse striatum, many cells were lost. Few remaining surviving cells expressed TH, and the authors conclude that this indicates a stable dopaminergic phenotype. When Gage and colleagues transfected adult hippocampal precursor cells with Nurr1, they could demonstrate that Nurr1 binds directly to the TH promoter, resulting in

induction of TH (Sakurada et al., 1999). However, this induction was in the absence of neuronal differentiation and without the expression of other dopaminergic markers. In contrast to the above studies, Lee and co-workers demonstrated that Nurr1 was sufficient to induce dopaminergic differentiation in rat CNS precursors from various developmental stages (rat embryonic day 12–16) and regions of origin (cortex, VM, lateral ganglionic eminence)(Kim et al., 2003). Progenitor cells were proliferated and subsequently transfected with Nurr1 under the control of cellular elongation factorα (EFα) promoter. Nurr1 transfection induced TH expression and TH-positive neurons showed characteristics of dopaminergic phenotypes (AADC, dopamine transporter [DAT], vesicle-membrane-associated transporter [VMAT], Pitx3-expression) as well as spontaneous and evoked DA release. The yield of TH-positive neurons was further enhanced by adding ascorbic acid and B27. However, again upon grafting in a rat PD model, only a few TH-positive cells survived and cells expressed an immature neuronal morphology and no behavioral recovery was observed.

The difference in the effect of Nurr1–overexpression may well be explained by the different cell types used for transfection and different levels of overexpression. The latter study used short-term expanded precursors, whereas Wagner et al. used a cell line, and Gage and co-workers used long term expanded adult neural precursors. However, Nurr1-transfected cells show a lack of *in vivo* function (Wagner et al., 1999, Kim et al., 2003). Insufficient maturation and differentiation might be the reason, preventing necessary synaptic interaction with the host brain to allow long-term graft function.

Nurr1-overexpression seems to be more successful in ES cells. It has been shown that mouse ES cells can be differentiated into dopaminergic neurons via a multistep differentiation protocol or using a co-culture based method rewiring the inductive activities of stromal feeder layers (Kawasaki et al., 2000; Lee et al., 2000 and Chapter 13). Kim et al. used a cytomegalovirus plasmid (pCMV) driving expression of a rat Nurr1 cDNA to establish stable Nurr1 mouse ES cell lines. The yield of TH positive neurons was 10-fold higher compared to wildtype cells and was further enhanced with Shh and FGF8. These cells also expressed AADC, DAT, c-RET, Pitx3, and En-1 beside TH (Kim et al., 2003). Interestingly, Nurr1-transfected ES cells were able to survive, integrate, and reverse behavioral deficits in a rat Parkinson model. No ongoing mitosis or tumor formation was found in the grafts. However, long-term experiments need to be done to prove the safety of using ES cells *in vivo*. In another study, Chung et al. transfected mouse ES-cells with Nurr1 under EF1-α (Chung et al., 2002). This increased the differentiation of ES cells to dopaminergic cells by 4–5-fold in combination with factors such as Shh and FGF-4 and 8. Cells expressed TH and AADC, and some even expressed DAT, indicating a mature phenotype. The authors also report up-regulation of midbrain DA markers such as Pitx3, ADH2 and calbindin. Cells produced dopamine and released DA upon stimulation with KCl.

## 3. Future Directions

Clearly, ES cells could hold the future for producing DA neurons on demand (see Chapter 13). However, there have been problems converting human ES (hES) cells into DA neurons that survive transplantation. Most of the recent studies have only provided modest recovery and few surviving neurons with good fiber outgrowth (Perrier et al., 2004; Zeng et al., 2004). New methods for the growth, differentiation, and transplantation of DA neurons from hES cells will be developed over the next few years. Even if this approach is perfected, caution is still advised. It is possible that DA neurons from ES cells may produce dyskinesias as seen with DA neurons from primary fetal tissue (Freed et al., 2001). However, at least there is the opportunity with hES cells to modify them to control the DA release. This potentially could be through homologous recombination methods to insert inducible gene elements upstream of the TH promoter and generating DA neurons from these ES cell lines. In this way the release of DA might be regulated through doxycyline administration (Gossen and Bujard, 1992, 2002; Mansuy and Bujard, 2000).

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# 15

# Delivery of GDNF for Parkinson's Disease: Transition of a Neuroprotective Treatment Strategy From Basic Sciences to Clinical Application

Deniz Kirik,[1] Biljana Georgievska,[1] Soshana Behrstock,[2] and Clive Niels Svendsen[2]

## 1. Introduction

The cardinal symptoms of James Parkinson published his monograph on the symptoms of some of his patients, who displayed what he called the shaking palsy. The cardinal symptoms of Parkinson's disease (PD) are bradykinesia, rigidity, and resting tremor, and the disease is caused by a selective and progressive degeneration of dopaminergic neurons in the substantia nigra (SN) pars compacta in the midbrain. These neurons produce and release the neurotransmitter dopamine (DA) into the target structure striatum and modulate the activation of striatal projection neurons and thereby processing of motor information in the basal ganglia. DA neurons produce their transmitter through a two-step enzymatic reaction from dietary tyrosine. The first step is the conversion of tyrosine to L-3,4-dihydroxyphenylalanine (DOPA) by the rate-limiting enzyme tyrosine hydroxylase (TH), while the further conversion to DA is catalyzed by the aromatic acid decarboxylase (AADC) enzyme. DA is then stored into vesicular structures with the help of the vesicular monoamine transporter (VMAT).

There are a number of different hypotheses put forward that may explain why DA neurons are selectively vulnerable and degenerate in PD. Although it is highly likely that more than one factor contributes to the final outcome, one common denominator is the presence of DA, a readily oxidizable and potentially toxic substance. However, there are also clear familial cases of PD with mutations in specific genes, some of which do not link directly with DA synthesis and release

[1]Wallenberg Neuroscience Center, Department of Experimental Medical Science, Lund University, Lund, Sweden.
deniz.kirik@med.lu.se
Biljana.Georgievska@med.lu.se
[2]Department of Anatomy and Neurology, Waisman Center, University of Wisconsin-Madison, Madison, WI 53705
svendsen@waisman.wisc.edu

(Eriksen et al., 2003). But while the etiology of PD still remains largely unknown, pre-clinical data obtained from animal models promise significant advances in its treatment. This chapter will focus mainly on recent developments in the delivery of neurotrophic molecules locally at specific sites in the brain aiming at protecting the nigral DA neurons from degeneration.

## 2. Progressive Neurodegeneration in PD Allows for Early Intervention

Nigrostriatal neurodegeneration seen in PD has a slowly progressive nature, where several phases can be defined based on the clinical observations. These phases include a pre-clinical (asymptomatic) phase, early (mild) symptomatic phase, manifest PD, and end-stage (complication) phase (see Chapter 3). The pathological data from cross-sectional post-mortem nigral cell counts indicate that the mean preclinical disease duration in PD is about 4.6 years, and the nigral cell loss at the onset of the disease is about 50% (Fearnley and Lees, 1991). It is worth noting that with normal aging humans lose about 0.5% of the pigmented cell bodies in the nigra per year. However, the pattern of cell loss seen in aging differs from PD both by regional distribution and rate of cell loss. In normal aging the cell loss is evenly distributed in the pars compacta, while the major cell loss in the PD brain occurs in the ventrolateral and ventromedial parts of SN. During the first decade from the onset of neuronal loss in PD patients, there is about 45% decline in the age-adjusted cell counts. The loss of cells in the SN leads to loss of DA content primarily in the striatum, but also in other sites receiving DA input. Fiber degeneration follows an uneven pattern in the striatum: (1) Loss in the putamen is more substantial compared to the caudate nucleus, and (2) the caudal portions of the putamen are more severely depleted of DA (Nyberg et al., 1983; Kish et al., 1988).

The protracted degeneration of the nigral neurons in PD provides opportunities to intervene in the neurodegenerative disease process (Fig. 15.1A). It should be possible to protect the remaining DA projection neurons with the assumption that they will delay the continual progression of the disease. One class of agents that may have survival-promoting activities are the neurotrophic factors. A large number of trophic factors have been shown to protect dopaminergic neurons from various toxic insults in culture. Among these factors glial cell line-derived neurotrophic factor (GDNF) is particularly interesting due to its potent actions demonstrated in several models of nigral DA neuron degeneration in animals, including the MPTP model in mice and primates, 6-hydroxydopamine (6-OHDA) lesions or axotomy-induced degeneration in rats.

## 3. Intracerebral Injection of Recombinant GDNF Protein Proves Efficacy

One of the intriguing questions that is raised each time a trophic factor is found to be effective on dopaminergic cells is whether it will be able to

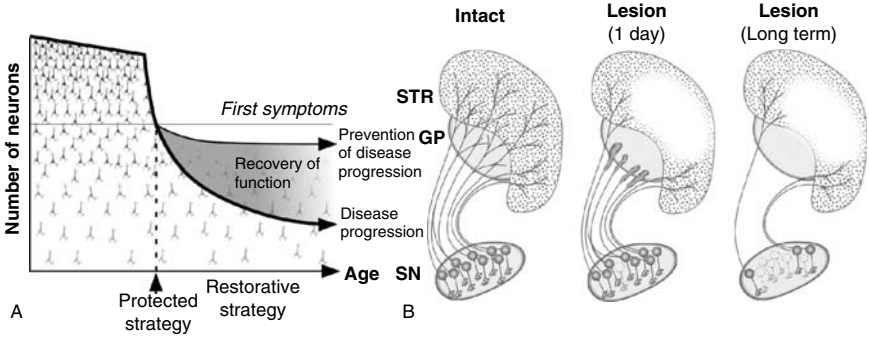


FIGURE 15.1. Protective treatment strategy for Parkinson's disease. **A**: The slow and protracted loss of nigral DA neurons in PD presents opportunities to intervene in the degenerative process and prevent the further progression of the disease. A protective "disease-modifying" treatment strategy would be relevant in the early phases of the disease, when a substantial portion of the nigral DA neurons remain and may involve treatment with neurotrophic factors, such as GDNF. **B**: The intrastriatal 6-OHDA lesion provides us with a progressive nigral cell degeneration model. Injection of 6-OHDA into the striatum induces an acute lesion of the DA axon terminals, followed by a slow retrograde degeneration of the nigral DA neurons. (See Color insert.)

promote regeneration from the damaged nigrostriatal neurons. This question has been addressed for GDNF in several animal models of PD. In mice, 10 μg GNDF given in the striatum 7 days after the MPTP injections had partial effects on restoring the DA levels in the striatum (Tomac et al., 1995). Although modest, these initial findings have stimulated other experiments in the hope of achieving better recovery. Single bolus injections of GDNF at 2–5 weeks after a complete 6-OHDA lesion in rats provided some effects in drug-induced rotation and protected a limited number of nigral DA cells, only after very high doses of GDNF (100-500 μg) were injected over the SN (Hoffer et al., 1994; Bowenkamp et al., 1995, 1996). When administration of the same dose of GDNF was further delayed to 9 weeks post-lesion, or given in the ventricular space, the effects were smaller and transient, even in animals where the injections were repeated three times (Lapchak et al., 1997).

It was only when a more progressive cell degeneration model was used in the rats, that the potency of GDNF to rescue DA cells was fully uncovered. In the partial lesion model, 6-OHDA is injected into the striatum, which results in a selective and progressive damage of the DA fiber terminals with a subsequent loss of nigral DA neurons (Fig. 15.1B). The initial effects of the 6-OHDA at the striatal DA terminals is relatively rapid and causes disintegration of fibers, leading to extensive loss of innervation within the first 24 hours, while the die-back of pre-terminal axons from the level of globus pallidus to SN is a slower process and is completed by 5–7 days. Fiber degeneration is then followed by cell death starting at about 1 week and progressing over several weeks thereafter.

In a series of experiments, where 50–85% of the nigral cells were injured by the intrastriatal 6-OHDA lesion, either repeated injections (every second or third day over a period of 2–4 weeks) or continuous infusion of GDNF (2.5 µg/day) using an osmotic minipump gave near complete protection of the cell bodies (Sauer et al., 1995; Winkler et al., 1996; Rosenblad et al., 1999, 2000a). This was assessed by quantification of remaining DA cell numbers within the nigra visualized by both immunohistochemical detection of TH and retrograde tracing of the nigrostriatal projection system by Fluorogold. Another important finding was that, once rescued, the nigral cells survived for several months after the withdrawal of the trophic support, suggesting that the rescued cells were not dependent on GDNF for further survival (Winkler et al., 1996). Although this cell survival effect could be demonstrated when GDNF was delivered to the lateral ventricles, over the substantia nigra or in the striatum, only the intrastriatal delivery route led to preservation of the axonal projections to the striatum and thus maintenance of normal motor performance (Kirik et al., 2000a) (Fig. 15.2). In cases where the initiation of striatal GDNF treatment was delayed for 2 weeks, thus allowing for the degeneration to proceed from striatum to nigra, the magni-

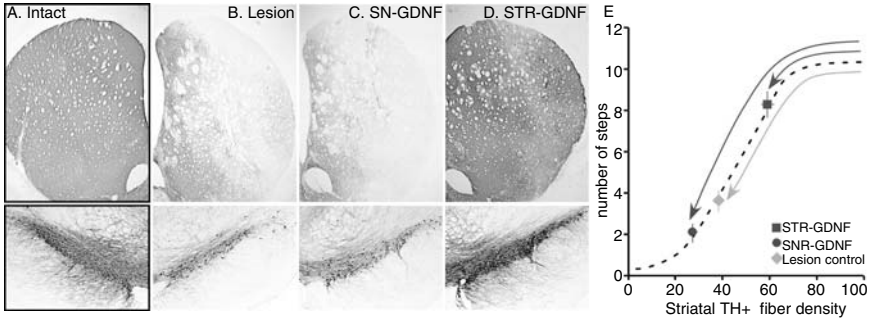


FIGURE 15.2. Protection of the nigrostriatal DA pathway after recombinant GDNF protein treatment. The photomicrographs show cross-sections from the central striatum and corresponding SN immunostained for TH representing four different conditions: (**A**) intact nigrostriatal system, (**B**) vehicle-treated lesion controls, (**C**) nigral GDNF injection group, (**D**) striatal GDNF injection group. Delivery of recombinant GDNF protein into the SN or striatum prior to an intrastriatal 6-OHDA lesion provides a significant protection of the nigral cell bodies as compared with the vehicle-treated controls. Protection of the DA terminals, on the other hand, can only be obtained following delivery of GDNF into the striatum. Note that the performance of the animals in the stepping test can best be explained by preservation of the striatal TH-positive fibers (**E**). The intrastriatal 6-OHDA lesion induces depletion of DA terminals accompanied with a reduction in the number of steps the animals can perform (green arrow in E). While administration of GDNF into the striatum preserves the TH-positive fibers and thus the normal motor function (blue arrow), the nigral GDNF group fails to be beneficial (red arrow). (Figure modified from data published in Kirik et al., 2000a). (See Color insert.)

tude of effects were greatly reduced, suggesting that the presence of fiber connections to the striatum was a prerequisite for GDNF actions to mediate functional protection and/or regeneration (Kirik et al., 2001). Intrastriatal GDNF delivery in this model also lead to prominent sprouting effects, especially in the globus pallidus. While the consequences of this sprouting remains unknown, in animals where striatal terminals are protected the presence of the excessive fiber innervation to the globus pallidus did not seem to hamper the motor function.

In summary, the findings of the rat studies suggests that there are at least three modes of action of GDNF on the lesioned nigrostriatal DA neurons: (1) *An acute protective effect at the site of toxin injection*, i.e., protection of terminals when given in the striatum or protection of cell bodies when given in the SN; (2) *rescue of injured DA neurons,* which can be achieved via administration of GDNF in the SN, the striatum or the ventricle; (3) *axonal sprouting from the spared DA neurons,* which occurs only at the site where GDNF is delivered.

The neuroprotective and restorative effects of GDNF have also been evaluated in MPTP-lesioned monkeys. In these studies, recombinant GDNF protein was delivered either as single or repeated bolus injections intraventricularly at doses up to 1,000 μg (Gash et al., 1996; Zhang et al., 1997; Gerhardt et al., 1999), or by continuous intracerebroventricular (ICV) or intraputaminal infusion at 5-15 μg/day (Grondin et al., 2002). The GDNF administration in these studies was initiated 1–3 months after the MPTP lesion, at a time when the neurodegeneration is nearly complete, with less than 20% nigral DA neurons remaining and >95% reductions in the striatal DA content (Gash et al., 1996; Gerhardt et al., 1999; Grondin et al., 2002). Administration of GDNF in the chronically lesioned monkeys provided an improvement in the motor performance, as measured on a primate PD rating scale (Gash et al., 1996; Zhang et al., 1997; Grondin et al., 2002). These functional changes were mainly associated with increased DA turnover and release in the GP and SN (Gash et al., 1996; Gerhardt et al., 1999; Grondin et al., 2002), and an increase in the number of nigral DA neurons expressing TH. However, only small increases in the density of TH-positive fibers were observed in the lesioned striatum (Grondin et al., 2002), suggesting that the GDNF-induced functional improvements in monkeys with advanced parkinsonism are most likely mediated by the upregulated DA function in the SN and GP, and that the remaining DA terminals in the striatum have a minor effect in these cases.

## 4. Clinical Trials Using Recombinant GDNF Infusions

Over the last decade the intrastriatal 6-OHDA lesion model in the rat and the MPTP lesion model in the primates has helped us to test the validity of neuroprotective treatment strategies. Clearly, most of the clinically relevant data

regarding the potential of GDNF as a neuroprotective factor is achieved using these models. On the other hand, one should also emphasize that this does not mean that the models replicate the human disease. One of the drawbacks is that the toxin-induced degeneration happens over a short time window and in a synchronized fashion where all the cells are affected from the same insult at the same time. Whereas PD patients probably suffer from multiple insults, including intrinsic risk factors that render the cells vulnerable, and extrinsic toxins that may substantiate the neuronal damage further, leading to a heterogeneous degree of injury in the patient. Thus it is likely that at a given time the nigrostriatal system of a PD patient contains some cells with an atrophic profile, and possibly damaged and retracted axons, while others may be relatively intact and continue to synthesize, store, and release DA in a normal physiological way.

While the animal experiments reported very promising data on neuroprotective properties of GDNF, a trial in humans concluded that GDNF delivered into the ventricles with bolus injections had no effect on the progression of PD (Nutt et al., 2003). Interestingly, results from a single patient from this trial who had received monthly bolus injections of recombinant GDNF protein intraventricularly (25–300 μg) over a period of 14 months was also published in 1999 (Kordower et al., 1999). According to this report, the patient continued to worsen following the treatment and showed side effects, including nausea, loss of appetite, hallucinations, depression, and inappropriate sexual conduct, some of which were temporarily related to the injections. The pathological findings indicated that there was negligible TH-immunoreactivity in the putamen and the extent of TH-positive innervation seen in the caudate nucleus was similar to what would be seen in a patient who had not received GDNF. Thus, GDNF had failed to improve clinical function or prevent nigrostriatal degeneration. Although this individual patient was in the later stages of PD and may not have had surviving DA neurons able to respond to GDNF, this trial most likely failed overall as GDNF cannot penetrate into deep brain structures from the ventricular system (Lapchak et al., 1998). It is clear that in PD the caudal part of the putamen is most affected, a region that does not lie near the lateral ventricle and therefore would not be expected to receive much GDNF.

Despite this negative data, GDNF may still be a promising factor to treat PD providing there is the appropriate site-specific delivery (Brundin, 2002). Local and continuous infusion of GDNF into the putamen would allow for direct protection and regeneration of the dopamine terminals affected in PD. In a recent clinical trial, GDNF was delivered directly into the putamen using mechanical pumps (Gill et al., 2003). Five patients showed significant clinical improvements and reductions in dyskinesias without side effects. Positron emission tomography (PET) scans showed a significant increase in DA storage within the putamen, suggesting a direct effect of GDNF on DA function. This improvement was also evident at later stages of infusion (Patel et al., 2005). Although a small and open trial, this study showed that large doses of GDNF are safe and have significant positive effects on DA function in

patients with PD. A second open-label study also showed pronounced effects of unilateral infusion of GDNF directly into the putamen in close agreement with the Gill study (Slevin et al., 2005). Furthermore, one patient has come to post-mortem and showed significant sprouting of TH fibers around the site of infusion (Love et al., 2005). Although important for "proof of concept" the potential drawbacks are that pumps are complicated to implant, need constant refilling and ultimate replacement, and importantly, diffusion of GDNF from the catheter tip is limited, so large areas of the putamen may not benefit from neuroprotection and neuroregeneration. Thus in the long term, direct *in vivo* gene transfer using viral vectors, or *ex vivo* gene transfer by implanation of genetically modified cells (as described in chapter 16) may provide a more long-lasting and practical approach to delivering large proteins such as GDNF to the brain of patients with PD.

## 5. In Vivo Gene Transfer of GDNF

The fact that the affected DA neurons may remain in dysfunctional states in PD, while their loss is likely to happen over long periods, suggests that neurotrophic factors can restore function in dysfunctional or atrophic neurons in the degenerating nigrostriatal system, as well as preventing further cell loss. However, given the chronic and progressive nature of PD, it is likely that the factor should be administered continuously, over months or years, for long-term survival and function. For this reason, local production of the neurotrophic factor may offer distinct advantages over repeated protein injections. During the last decade, new means of direct in vivo delivery of GDNF to the striatum and/or SN has been achieved using viral vectors. Efficient long-term expression of GDNF has been reported with three different recombinant viral vector systems in the rodent: (1) adenovirus (Ad), (2) adeno-associated virus (AAV), and (3) lentivirus vectors (LV). Each one of these vector systems holds promise for successful gene transfer of therapeutic proteins to non-dividing cells (Björklund et al., 2000).

### *5.1. Viral Vector Systems for GDNF Gene Delivery to the Brain*

Ad vectors have a large cloning capacity of about 8 kb, can be generated at very high titers, and can infect both dividing and non-dividing cells. The viral DNA remains as an episome in the nucleus and is, therefore, most adequate for transient expression of transgenes in non-dividing cells. The Ad vectors used thus far have the disadvantage that the transduced cells express adenoviral proteins that may cause inflammation and trigger host immune reactions toward the infected cells, which may reduce transgene expression over time and contribute to the variable long-term expression of the transduced protein seen in several in vivo studies using first generation Ad-vectors (Choi-Lundberg et al., 1997, 1998; Connor et al., 1999).

AAV is a single-stranded DNA virus that belongs to the parvovirus family. Although the AAV serotype 2 has been the most commonly used AAV vector to this point, there are at least ten different serotypes of AAV that have been isolated in human and primates (AAV 1-10). The sequence divergence in the capsid genes for the different serotypes affects the interactions between AAV and target cell receptors and thereby also the tissue tropism of different serotypes. The wild-type (wt) AAV virus depends on the co-infection of a helper virus (e.g. adenovirus) to complete its lytic cycle, thus termed dependovirus. The 4679 bp genome of wt AAV contains two open reading frames encoding four regulatory (rep) and three structural (cap) proteins and is framed by two inverted terminal repeats (ITRs). The two major rep proteins (Rep78 and Rep 68) bind to the ITRs and mediate site-specific integration of AAV into the human genome. The recombinant AAV (rAAV) vector has 96% of the wild-type genome removed, leaving only the two ITRs, which are sufficient for packaging and integration (not site-specific due to the lack of Rep78/68). The rAAV vectors can integrate and stably express their transgene product in non-diving cells, including neurons, and the absence of viral genes minimizes the expression of foreign proteins, and hence the risk of triggering host immune responses. Studies using reporter genes have shown that the rAAV vector is efficient in transducing non-dividing cells, mainly neurons, in the adult CNS. Although the rAAV vectors carrying the AAV serotype 2 capsid infects cells only at the site of injection in the CNS, the transgenic protein inserted by the viral vector is expressed both at the cell body level and also transported intra-axonally to the terminals. If the transgene product is a secreted protein (such as GDNF), then it will be released both at the cell body level and from the terminals at the target nuclei (Kirik et al., 2000b).

Recombinant LV (rLV) vectors are derived from the highly pathogenic HIV viruses, which belong to and share the useful properties of the onco-retroviral vectors. In addition, they can stably integrate themselves into the genome of non-dividing target cells. Furthermore, a large cloning capacity of about 9 kb make them highly favorable for long-term expression of large transgenes in the nervous system. In the current versions of the HIV-1-based rLV vector, about 60% of the viral genome has been eliminated and the particles are pseudotyped with the G envelope protein of the vesicular stomatitis virus (VSV). This gives the vector the capacity to infect a broad range of tissues, including the nervous system tissues. To increase the safety of the rLV vectors, self-inactivating vectors have been engineered where part of the viral promoter present in the long terminal repeat (LTR) has been deleted. This abolishes the transcriptional activity of the LTR and the production of full-length vector RNA in transduced cells, thereby minimizing the risk of emergence of replication-competent recombinant viral particles. Upon in vivo delivery to the brain, VSV-G pseudotyped rLV vectors lead to very high transduction efficiencies, particularly in the striatum and the SN, and in both sites the majority of the transduced cells are neurons, although a small number of astrocytes and oligodendrocytes will express the transgene (Naldini et al., 1996a, b; Blömer

et al., 1997; Georgievska et al., 2002b). Similar to the rAAV vectors, the transduction is limited to the site of injection. Thus, when injected in the striatum rAAV and rLV vectors lead to production and release of GDNF in the striatum, as well as in target sites of the striatal projection neurons (i.e., globus pallidus, entopeduncular nucleus, and SN pars reticulata) (Kirik et al., 2000b; Georgievska et al., 2002a, b), as illustrated in Figure 15.3.

## *5.2. GDNF Gene Delivery in Animal Models of PD*

Several studies have reported delivery of GDNF into the brain using viral vectors in rodent and primate lesion models of PD. In the intrastriatal 6-OHDA-lesion model in rats, efficient protection of the nigral DA neurons has been observed following GDNF gene delivery into the SN and/or striatum using either rAd (Bilang-Bleuel et al., 1997; Choi-Lundberg et al., 1997, 1998), rAAV (Mandel et al., 1997, 1999; Kirik et al., 2000b) or rLV (Rosenblad et al., 2000b; Georgievska et al., 2002a, b) vectors. The magnitude of protection of DA neurons in the SN in the different studies ranges between 60 and 75% using the rAd , 80–95% using the rAAV, and about 70–85% using the rLV

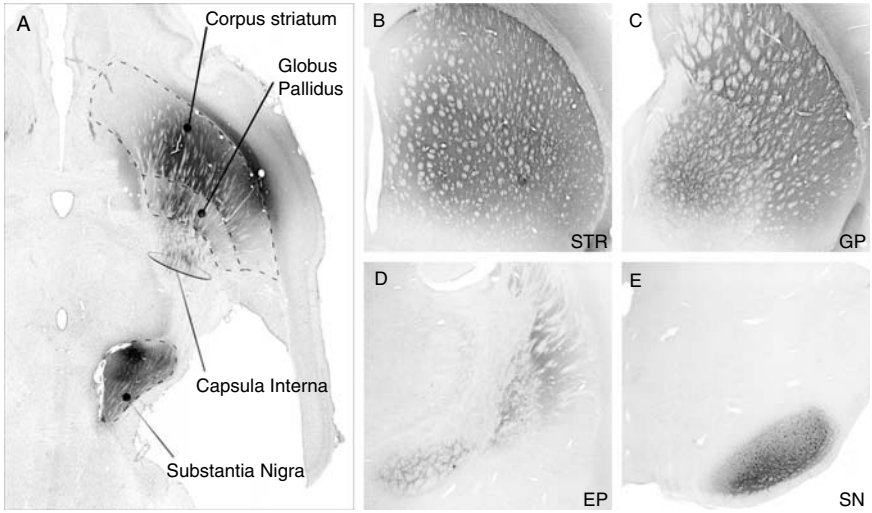


FIGURE 15.3. Expression and distribution of GDNF following rLV-GDNF injection into the striatum. **A**: A horizontal section immunostained for GDNF demonstrates the extensive diffusion of GDNF protein in the striatum, as well as transport and release of GDNF along the striatopallidal and striatonigral projections. In this particular animal, rLV-GDNF was injected into the striatum after a complete 6-OHDA lesion, demonstrating that the transport of GDNF to the GP and SN pars reticulata was in the anterograde direction. Coronal sections from the striatum (**B**) and the different striatal output nuclei (**C–E**) further illustrate the distribution of GDNF. (Figure modified from data published in Georgievska et al., 2002a). (See Color insert.)

vectors. One important aspect when comparing delivery by pumps vs. genes is that the amount of GDNF produced in vivo by the infected cells is in the order of 0.09–2.1 ng/mg wet tissue, as determined by ELISA measurements. Experiments performed using injections or infusions of GDNF protein using pumps delivered quantities that are about 3–4 orders of magnitude higher. Yet the protection of nigral neurons against 6-OHDA lesions achieved by viral vectors is clearly as efficient as the protein injections. This could be explained by the fact that the ED50 for GDNF is around 1 ng/ml for DA neurons in vitro, and GDNF that is released by transduced host cells is glycosylated differently from recombinant GDNF, which may confer extra stability. Whereas large bolus injections of recombinant GDNF may well not be stable following release from the citrate carrier buffer and has to diffuse long distances to get to their targets, GDNF released by host cells will only have very small distances to targets.

While the behavioral assessments of the animals reported in the first GDNF gene delivery studies are very limited, detailed behavioral characterization was carried out in a few studies using the rAAV vectors in the rat model and rLV vectors in both rat and primate models. When the rAAV vectors were used to express GDNF (or the marker gene GFP as a control) in the striatum and/or SN (SN, STR, SN+STR) prior to a four-site intrastriatal 6-OHDA lesion (Kirik et al., 2000b), the following was observed: In tests of motor performance assessed at 3–5 weeks after the lesion, all animals were severely impaired in the drug-induced and spontaneous behavioral tests, indicating that the initial impact of the 6-OHDA lesion was the same in all experimental groups. Over the following weeks, significant recovery was seen in the STR-GDNF group, leading to a progressive decline in the amphetamine rotation test between 7 and 16 weeks after the lesion. Neither the SN nor the SN+STR groups differed from the control groups at any time point. Between 16 and 23 weeks, the animals were tested in the spontaneous motor tests, termed the cylinder and the skilled paw use tests. In both tests, the affected paw use in the STR-GDNF group had recovered reaching to close-to-normal performance (Fig. 15.4). Histological evaluation in this material revealed that recovery in function could not be explained by the number of surviving TH-positive cells in the SN, as cellular protection was seen in all GDNF-treated animals (see Fig. 15.4). In the two groups that received vector injections in the nigra (SN and SN+STR groups), there was an extensive sprouting dorsal and rostral to the nigra, leading to disorganization of the TH-positive fibers. Sprouting fibers were seen extending to the subthalamic nucleus, entopeduncular nucleus, and the ventral parts of thalamus, but failed to reach the striatum. On the other hand in the STR-GDNF group the fibers in the MFB had a normal appearance and there was a very intense sprouting in the globus pallidus. Regenerating fibers were also found extending into the caudal and central parts of the striatum. The extent of TH-positive fibers in the striatum was correlated with the behavioral recovery seen in this group.

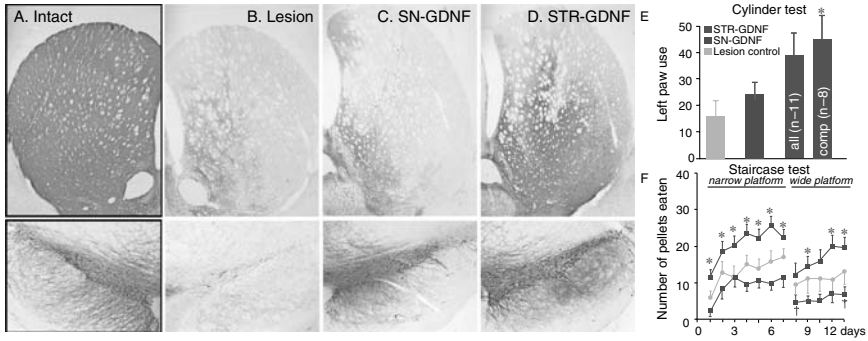


FIGURE 15.4. Protection of the nigrostriatal DA pathway after rAAV-mediated GDNF delivery. The photomicrographs show cross-sections from the central striatum and corresponding SN immunostained for TH representing four different conditions: (**A**) intact nigrostriatal system, (**B**) GFP vector-treated lesion controls, (**C**) nigral rAAV-GDNF injection group, (**D**) striatal rAAV-GDNF injection group. Expression of GDNF protein in the SN or striatum prior to an intrastriatal 6-OHDA lesion provides a significant protection of the nigral cell bodies, as compared with the lesion controls. Protection of the DA terminals, on the other hand, can only be obtained following delivery of rAAV-GDNF into the striatum. The results from the cylinder test (**E**) and the staircase test (**F**) indicate that functional improvements were seen only after delivery of rAAV-GDNF into the striatum (coded by blue colors in E and F), while nigral rAAV-GDNF delivery appeared to be ineffective (compare red to green color). (Figure modified from data published in Kirik et al., 2000b). (See Color insert.)

In another experiment, rLV vectors encoding the GDNF or GFP transgenes were delivered into the striatum 3 weeks prior to a three-site intrastriatal 6-OHDA lesion, and the animals were repeatedly tested on a number of drug-induced and spontaneous motor behaviors over 9 months (Georgievska et al., 2002a). The results of the post-lesion behavioral testing showed that the rLV-GDNF-treated animals initially displayed a lower turning bias in the amphetamine-induced rotation, which was further reduced over the following months until the asymmetry was completely abolished. In contrast, the rLV-GDNF treated animals remained impaired in the other motor tests, including the apomorphine rotation and spontaneous behavioral tests, such as the stepping test, skilled paw reaching in the staircase test, and the cylinder test. Histological analysis of the nigrostriatal DA system revealed that there was a clear protection of the nigral DA neurons in the rLV-GDNF group. Along the nigrostriatal DA pathway there was also a preservation and extensive sprouting of the TH-positive fibers in the globus pallidus, entopeduncular nucleus and SN, corresponding to areas where GDNF protein was released. However, the striatal TH-positive fiber innervation was reduced to the same extent as in the rLV-GFP control group. Using two different tracing methods of the nigrostriatal DA system, it was revealed that the striatal

DA terminals were structurally preserved in the striatum in the GDNF-treated animals, however they failed to express TH (Fig. 15.5).

## *5.3. Differential Effects of GDNF in Primates Versus Rodents*

The mechanism underlying the GDNF-induced downregulation of TH in the 6-OHDA-lesioned rats is unclear. Similar down-regulation of the TH protein has been observed in the intact rat striatum, after induction by long-term overexpression of GDNF, delivered using the same rLV vector (Rosenblad et al., 2003). The time- and dose-dependent effects of GDNF on the intact nigrostriatal DA system have been further studied (Georgievska et al., 2004) and showed that GDNF initially induced an increase in the striatal DA turnover (1–6 weeks), which was accompanied by an enhanced amphetamine-induced rotation towards the injected side, indicating an enhancement of the DA system on the transduced side. The TH down-regulation developed only after 6 weeks of continuous GDNF overexpression and the magnitude of down-regulation seemed to be related to the level of GDNF overexpression, such that the effect was most pronounced in animals where

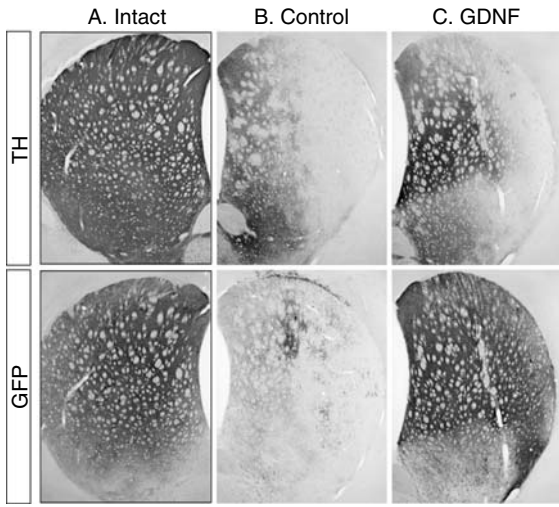


FIGURE 15.5. Down-regulation of TH in preserved DA terminals following rLV-mediated GDNF delivery into the striatum. The photomicrographs show cross-sections from the central striatum immunostained for TH or GFP representing three different conditions: (**A**) intact nigrostriatal system, (**B**) GFP vector-treated lesion controls, (**C**) rLV-GDNF injection group. Anterograde tracing of the nigrostriatal DA pathway using transgenic GFP demonstrated a near-complete protection of the DA terminals in the GDNF-treated group, compared to the lesion controls. However, there was a lack of TH expression in the preserved DA terminals in the dorsal and lateral parts of striatum. (Figure modified from data published in Georgievska et al., 2002a).

the GDNF level in the striatum exceeded 0.7 ng/mg tissue. Although GDNF induced an extensive down-regulation of the TH protein, the DA tissue levels were maintained at close to normal levels at all time points. These data suggest that the TH down-regulation may not be a sign of impaired function, but rather an adaptation of the DA neurons to a sustained activation. This would be consistent with a time-dependent compensatory mechanism, where continuous activation of the DA neurons by GDNF is followed by a compensatory down-regulation of the TH protein, in order to maintain DA transmission within the normal range. This is further supported by the observation that the DA turnover and amphetamine-induced rotation start to decline at the time when TH is down-regulated (i.e., at 6 weeks) and the motor asymmetry is completely lost by 19 weeks.

While this model is consistent with the observation made on intrastriatal GDNF delivery in rodents, it does not readily match the data obtained in primates. We have observed that rAAV-mediated overexpression of GDNF at even higher levels (9–18 ng/mg tissue) in the caudate-putamen in intact marmosets resulted in a two-fold increase, rather than decrease, in the TH protein levels at 3 months after vector injection (Eslamboli et al., 2005). Similar results have been seen in aged rhesus monkeys after long-term expression of GDNF (3 months) in the caudate-putamen and SN using rLV vector delivery (Kordower et al., 2000). The GDNF levels in this study were significantly lower than in the marmoset study (0.2–0.3 ng/mg tissue at 8 months after virus delivery). Nevertheless, GDNF augmented the DA function, as measured by increased striatal DA storage (using F-DOPA uptake in PET imaging), striatal TH-positive fiber density, and TH mRNA levels in the SN. Thus, it appears that there are differential effects of GDNF on DA function and TH gene expression in primates versus rodents. However, the neuroprotective effects of vector-mediated GDNF delivery in primate PD models are as efficient as in the rodent model. Delivery of rLV-GDNF one week after the MPTP administration resulted in a complete protection of the nigral DA neurons in all GDNF-treated animals and about 70–80% sparing of the TH-positive fibers in the striatum (Kordower et al., 2000). This was accompanied by a highly variable functional recovery, despite the fact that all treated animals exhibited a near-complete protection of the nigrostriatal DA system.

## 6. Ex Vivo Gene Transfer of GDNF

GDNF protein produced in the rLV or rAAV transduced neurons is not only expressed at the level of the cell bodies, but also transported anterogradely along the efferent projections. As a result, when striatal projection neurons are transduced, GDNF is released at three principal striatal targets, globus pallidus, entopeduncular nucleus, and SN pars reticulata, at biologically active levels (about 0.2 ng/mg tissue, as measured in the SN) sufficient to induce extensive sprouting of the nigrostriatal fibers in these structures. It is

possible that this aberrant sprouting-particularly in the entopeduncular nucleus and SN is functionally detrimental and acts to block recovery in spontaneous motor behavior that would otherwise have been obtained by GDNF-induced regeneration of the striatal DA afferents. Since the magnitude of aberrant sprouting seems to be related to the level of GDNF expression, it may be possible to avoid, or limit, these effects by adjusting the vector dose (and hence the GDNF expression). Alternatively, it should be possible to develop constructs that can limit GDNF delivery to the striatum, e.g., by using vectors targeted to glial cells or by ex vivo gene transfer using transplants of cells genetically engineered to secrete GDNF (see Chapter 15).

*Ex vivo* gene therapy is an alternative approach to direct injection of live virus to the brain. Indirect gene therapy uses viruses to transduce cells in vitro, which can then be transplanted into the brain to release GDNF. This method of gene therapy has a number of attractive features, including that (i) cells can be selected for gene dosing (protein release) prior to transplantation; (ii) the exact insertion sites from cloned cells can be documented and the disruption of normal oncogene regulation caused by the insertion can be checked; (iii) healthy ex vivo cells will provide the protein delivery, not degenerating host cells; and (iv) viral infection takes place in vitro thereby reducing the danger of live replication competent virus transfer to the patient. Furthermore, in some cases transplanted genetically engineered cells migrate to cover a large portion of the putamen, thereby providing GDNF and its trophic effects to a greater area than achievable with other delivery methods.

Different cell types can be genetically engineered in vitro to express GDNF and then transplanted to determine the in vivo effects. Transplanting engineered GDNF-producing astrocytes into the substantia nigra before a 6-OHDA lesion in the mouse prevented the loss of nigral cell bodies, provided partial protection of striatal dopaminergic fibers, and ameliorated rotational behavior (Cunningham and Su, 2002). While these results show promise for ex vivo gene therapy using GDNF-producing astrocytes, the level and pattern of GDNF expression post-transplantation into the nigra was not reported, and the function of cells transplanted into the striatum following a lesion still needs to be demonstrated. Park et al (2001) have shown that bone marrow cells can be genetically engineered in vitro to express GDNF and that after intravenous transplantation into MPTP-lesioned mice there is protection of nigral neurons and striatal fibers. While the duration of efficacy, specific targeting of GDNF to affected brain regions, and benefit to parkinsonian-like behaviors still need to be addressed, intravenous delivery of marrow-derived GDNF-producing cells to the brain provides another proof-of-concept for ex vivo GDNF gene therapy. An immortalized neural stem cell line genetically engineered in vitro to express GDNF and transplanted into a mouse model of PD engrafted well in the striatum, maintained high levels of GDNF for at least 4 months, prevented the degeneration of nigral dopamine neurons, and reduced behavioral impairment (Akerud et al., 2001). Additionally, using a lentiviral vector for ex vivo gene transfer, rodent neural stem cells have been

genetically engineered to express GDNF, and following transplantation these cells were shown to increase the survival of co-transplanted dopamine neurons (Ostenfeld et al., 2002). These cells do not have immortalizing genes and may therefore be safer candidates for clinical trials. Such studies confirm the potent effects of GDNF for PD and demonstrate the potential of neural stem cells for ex vivo gene therapy.

The manipulability in vitro and ability to deliver functional levels of GDNF in vivo make neural stem cells (NSCs) ideal for ex vivo gene therapy. NSCs can grow as either monolayers or free-floating aggregates termed "neurospheres" and maintain the potential to form neurons and glia (see Chapter 12). Rodent NSCs, described above, provide proof-of-concept for these cells as an ideal vehicle for ex vivo gene therapy. Ultimately, however, human cells are required for translation into the clinic. Human NSCs have been isolated from the germinal zones of post mortem fetal brain tissue, an advantage over human embryonic stem cells since fetal cells make only neural tissue and do not produce teratomas (see Chapter 13). Techniques for the growth, differentiation, and transplantation of human NSCs have now been refined (Ostenfeld and Svendsen, 2003). The advantages of these cells are that they can be cultured for extended periods of time, induced to differentiate into various neural phenotypes and genetically modified (Wu et al., 2002). Furthermore, these cells survive transplantation, have extensive fiber outgrowth and can migrate to cover a wide area of the striatum (Ostenfeld et al., 2000; Englund et al., 2002). We have now shown that human NSCs can be modified using lentivirus to release GDNF using a regulated promoter system, and survive transplantation with continued GDNF release for up to three months in both the rodent and primate brain (Behrstock et al, 2006).

## 7. Future Perspectives

For PD, treatments include pharmacologically replacing DA, grafting fetal DA cells, and muting hyper-excited regions by lesions or high frequency stimulation. However, pharmacological intervention has decreased efficacy and increased side effects with disease progression, fetal grafts may be ineffective, and altering nuclei activity can be invasive and irrevocable. The first proof-of-concept for a disease-modifying approach in PD has now been demonstrated using direct infusion of purified recombinant GDNF protein into the brain parenchyma of PD patients. This initial success should open new initiatives, including the development of GDNF gene transfer for clinical trials. In order to achieve this goal, several practical and safety issues remain to be addressed before direct gene therapy can be translated to the clinic. These include (i) the inability to exactly control gene dosing following in vivo delivery; (ii) the accidental insertional mutagenesis as described in recent reports of a gene therapy trial in France (Hacein-Bey-Abina et al., 2003); (iii) forcing host cells to express a gene of interest that may compromise

their normal function; (iv) possible immune issues following expression of viral proteins on host cells which may stimulate an immune response; and (v) viral transport to ectopic sites which can result in aberrant, detrimental sprouting. As the direct gene therapy field is moving forward, we expect these issues to be addressed in future experimental studies, which will then form the basis for the design of the clinical trials. Indeed, there is currently one clinical trial going ahead in the USA using a direct viral approach for another growth factor, neurturin. However, ex vivo gene therapy involving transplantation of human NSCs genetically modified to produce regulated GDNF does not involve injection of live virus to the brain and thus overcomes some of the above concerns. Clearly, regulated delivery is needed as well as more primate work before moving to clinical trials. But there is an interesting future ahead which will ultimately lead to novel ways to produce neuroprotection and neuroregeneration of the degenerating nigrostriatal circuitry in normal aging and PD.

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# 16

# In Vivo Gene Therapy as a Potential Treatment for Parkinson's Disease

Caryl E. Sortwell and Jeffrey H. Kordower

## 1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder that affects 1 percent of the population above the age of 60. While relatively rare genetic forms exist (Mouradian, 2002), its cause in the vast majority of sporadic cases remains to be established. The cardinal features of PD (bradykinesia, resting tremor, and rigidity) result from a loss of striatal dopamine secondary to degeneration of dopaminergic neurons in the substantia nigra pars compacta (SN). Motor dysfunction worsens and additional incapacitating features such as dementia and depression can appear as the disease progresses (see Chapter 3). After decades of research, levodopa remains the most potent antiparkinsonian treatment. However, virtually all patients will ultimately suffer from disabling dyskinesia and wearing-off effects, making the use of levodopa as well as dopaminergic agonists, problematic. It is essential that novel therapeutic strategies be designed to provide potent long-term benefit without disabling side effects for these patients.

Gene delivery systems are ideal for delivering therapeutic molecules to specific regions of the CNS. Via gene therapy, a piece or pieces of DNA placed into a carrying vector encoding for a substance of interest can be introduced into cells. Gene therapy can be applied ex vivo (genetically modifying cells in culture and then grafting those cells) or in vivo (genetically modifying host cells). In vivo gene therapy is when a vector carrying a gene of interest is introduced directly into the brain and not through a cell mediator. The vectors most commonly used are viral-derived such as adenovirus (AV), adenoassociated virus (AAV), lentivirus (LV), and herpes simplex virus (HSV). An ideal vector should have (1) high concentration, allowing many cells to be infected, (2) easy and reproducible production, (3) the ability to integrate in a site specific location in the host chromosome, or to be successfully maintained as a stable episome, and (4) lack of components that could elicit an immune response. Such vectors are currently available (Bensadoun et al.,

Department of Neurology, University of Cincinnati, Cincinnati, OH 4567-0525, USA
caryl.sortwell@uc.edu
jkordowe@rush.edu

2000; Deglon et al., 2000; Mandel et al., 1999). In some circumstances, it would be advantageous if the vector had (4) a regulatable transcriptional unit and (5) the ability to target a desired cell type (Jin et al., 1996; Sun et al., 2003). These additional modifications are the subject of ongoing experimentation. However, for current clinical use, it is not clear that all of these requirements must be presently met. Each vector has particular advantages and disadvantages and researchers worldwide are focusing on improving and developing each of these vector systems to optimize gene delivery and minimize toxicity. This ability of vectors to be delivered in a site-specific fashion might diminish or eliminate side effects that result from peripherally administered drugs acting at unintended targets. The present review will examine the use of the in vivo gene delivery systems in animal models of PD and discuss the relative strengths and weaknesses of each approach.

## 2. Gene Transfer of Dopamine Biosynthetic Enzymes

The earliest approach of utilizing gene therapy for PD involved the transfer of dopamine biosynthetic enzymes to the striatum of experimentally lesioned rats and non-human primates in order to facilitate symptomatic relief due to the loss of striatal dopaminergic transmission (Table 16.1). Dopamine biosynthesis in the brain occurs through a series of steps beginning with the uptake of the amino acid L-tyrosine from the bloodstream. Next, L-tyrosine is hydroxylated by the enzyme tyrosine hydroxylase (TH), which requires tetrahydrobiopterin ($BH_4$) as a cofactor, which in turn requires the GTP-cyclohydrolase-1 (GTPCH1) enzyme for its synthesis. The hydroxylation of L-tyrosine yields L-dihydrophenylalanine (L-DOPA) which is in turn decarboxylated by the enzyme L-aromatic acid decarboxylase (AADC, also known as dopa decarboxylase) to produce dopamine (DA). The rate-limiting step in DA biosynthesis is the conversion of L-tyrosine to L-DOPA by TH; L-DOPA in turn is converted so rapidly to DA that under normal conditions the L-DOPA levels in the brain are insignificant. In the parkinsonian striatum, both $BH_4$ and AADC are deficient (Gjedde et al., 1993; Lloyd et al., 1970; Nagatsu et al., 1987).

Initial experiments designed to replenish striatal dopaminergic transmission utilized gene transfer of TH via injections to the striatum of a helper-containing herpes simplex virus (HSV) vector to the 6-hydroxydopamine (6-OHDA)-lesioned striatum of rats (During et al., 1994). While this study demonstrated partial improvement in apomorphine-induced rotations along with significant increases in striatal TH enzyme and extracellular DA, TH transgene expression was relatively modest with maximal expression 4 days post transfection. A similar outcome was demonstrated utilizing the adenovirus vector construct to deliver the TH transgene (Horellou et al., 1994), with the delivered TH transgene primarily expressed by striatal astrocytes. Studies have also examined the effectiveness of AADC delivery using

TABLE 16.1. Gene Transfer of Dopamine Biosynthetic Enzymes*

| Enzyme | Vector | Species/ lesion type | Morphological outcome | Striatal biochemisry | Behavioral outcome | Duration of expression | Reference |
|---|---|---|---|---|---|---|---|
| **TH** | HSV | Rat/6-OHDA | TH ir TH mRNA | TH enzyme ↑ DA ↑ | Apomorphine 64% ↓ | 4 d – 1 yr | During et al., 1994 |
| **TH** | AV | Rat/6-OHDA | TH ir (astrocytes) | Not conducted | Apomorphine 22% ↓ | 2 wks | Horellou et al., 1994 |
| **AADC** | AAV | Rat/6-OHDA | AADC ir | L-DOPA, DA, DOPAC ↑, AADC activity ↑ | Not conducted | 6 m -1 yr | Leff et al., 1999 |
| **AADC** | AAV | Rhesus monkeys/ MPTP | AADC ir | FMT uptake ↑ HVA, L-DOPA to DA rates normalized | Not conducted , | 8 wks | Bankiewicz et al.2000 |
| **TH/AADC** | AAV | Rat/6-OHDA | TH/AADC ir | DOPAC ↑ | Apomorphine 70% ↓ | 6 wks | Fan et al., 1998 |
| **TH/AADC** | Plasmid DNA-liposome complex | Rat/6-OHDA | TH/AADC ir (astroctyes) | Not conducted | Apomorphine 50% ↓ | 1-6 wks | Imaoka et al., 1998 |
| **TH/AADC** | HSV | Rat/6-OHDA | TH/AADC ir | DA, DOPAC ↑ | Apomorphine 80% ↓ | 7 months | Sun et al., 2003 |
| **TH/GTPCH1** | AAV | Rat/6-OHDA | TH/GTPCH1 ir (neurons) | L-DOPA ↑ | Apomorphine 0% ↓ | 3 wks -1 yr | Mandel et al., 1998 |
| **TH/GTPCH1** | AAV | Rat/6-OHDA Complete and partial | TH ir (neurons) | L-DOPA, DA ↑ | Complete lesions: Apomorphine 25% ↓, Partial: 60% ↓ Improvements: cylinder test, amphetamine rots | 15 wks | Kirik et al., 2002 |
| **TH/AADC/ GTPCH1** | AAV | Rat/6-OHDA | TH ir (neurons) | DA, $BH_4$ ↑ | Apomorphine 60% ↓ | 7-12 months | Shen et al., 2000 |
| **TH/AADC/ GTPCH1** | AAV | Macaque monkeys/ MPTP | TH, AADC, GCH ir (neurons) | DA ↑ (n = 1) | Improvement: manual dexterity (n=1) | 9 wks | Muramatsu et al., 2002 |
| **TH/AADC/ GTPCH1** | LV | Rat/6-OHDA | TH, AADC, CH1 ir (neurons) | DA, DOPAC ↑ | Apomorphine 48% ↓ | 5 months | Azzouz et al., 2002 |

* ir = immunoreactive; ↑ = increase; ↓ = decrease; FMT = 6-[$^{18}$F]fluoro-L-*m*-tyrosine.

adeno-associated virus (AAV)-mediated gene transfer to the striatum of 6-OHDA lesioned rats (Leff et al., 1999) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned rhesus monkeys (Bankiewicz et al., 2000). In these studies animals were administered peripheral L-DOPA in order to observe a functional consequence of AADC gene delivery. Behavioral evaluations were not conducted in either of these studies; however, striatal biochemical evaluations indicated normalization of many dopaminergic markers in the transduced striatal areas at 6 months (rats) and 8 weeks (monkeys) post-vector injection.

Investigators report improvement on several behavioral and biochemical outcome measures in experiments that utilized cotransduction of the rat striatum with TH and AADC (Fan et al., 1998; Sun et al., 2003; Imaoka et al., 1998) in order to provide transduced striatal cells with a more complete biochemical machinery for DA synthesis. Greater reductions in apomorphine-induced rotations were observed when striatal cells were contransduced with both enzymes as compared to gene transfer of TH alone using either the AAV (Fan et al., 1998) or helper-free HSV (Sun et al., 2003) vector delivery system. Co-expression of both enzymes was evident in one study for up to 7 months (Sun et al., 2003). Similarly, AAV cotransduction of striatal cells in rats with both TH and GTPCH1 transgenes yielded improved L-DOPA and DA production in both a complete and partial 6-OHDA lesion model (Mandel et al., 1998; Kirik et al., 2002). Although significant improvements in motor performance were demonstrated in one study (Kirik et al., 2002), motor function was only normalized under partial lesion conditions. Lastly, and more recently, triple transduction with TH, AADC, and GTPCH1 has been reported utilizing either AAV or lentiviral constructs (Shen et al., 2000; Muramatsu et al., 2002; Azzouz et al., 2002). Co-expression of all three transgenes was confirmed in medium spiny neurons of the striatum in rats for as long as 1 year post injection (Shen et al., 2003) and for 9 weeks in nonhuman primates (Muramatsu et al., 2003).

Substantial progress has been made in terms of efficiency of gene transfer of DA biosynthetic enzymes in animal models fo PD. The development of lentiviral vectors with a large cloning capacity allows for the simultaneous encoding of three different transgenes (Azzouz et al., 2002). Furthermore, although initial studies have demonstrated down-regulation of transgene overtime *in vivo* (During et al., 1994; Leff et al., 1999; Mandel et al., 1998), recent studies using AAV and lentiviral vectors demonstrate consistent long-term gene expression (Shen et al., 2000; Azzouz et al., 2002). However, critical work with gene delivery of biosynthetic enzymes remains to be performed. For example, no study to date has demonstrated complete normalization of functionality after near complete lesions, suggesting that very advanced PD patients with severe nigrostriatal degeneration would be suboptimal candidates for this approach. It also needs to be acknowledged that the gene delivery of biosynthetic enzymes is a symptomatic approach and, as with levodopa therapy, will not impact the underlying disease process.

Therefore, its long-term utility, as the nigrostriatal system continues to degenerate may be suboptimal.

## 3. Antiapoptotic Gene Therapy Strategies

Advances in our understanding of how nigral DA neuron loss may occur in PD have provided novel gene therapy targets for investigation. Changes in the levels of apoptosis-related factors in the nigrostriatal region of post-mortem PD brains have been documented (Nagatsu, 2003), including an increase in the concentration of the anti-apoptotic protein bcl-2 (Mogi et al., 1996) and higher activities of the proapoptotic factors caspases 1 and 3 (Mogi et al., 2003). Although the exact role that these specific factors, and the apoptotic cell death process in general, plays in PD remains controversial (Tatton et al., 2003), this doesn't preclude the possibility that viral vector-mediated delivery of antiapoptotic factors could confer protection against nigral DA neuron degeneration.

Several studies have examined the ability of gene transfer of bcl-2 (Yamada et al., 1999; Natsume et al., 2001) and caspase inhibitor gene delivery (Eberhardt et al., 2000; Mochizuki et al., 2001; Crocker et al., 2001) to protect nigral DA neurons from experimental toxins (Table 16.2). In contrast to gene transfer of DA biosynthetic enzymes to the striatum *following* lesions of the nigro-striatal pathway, experiments delivering antiapoptotic genes inject the vector prior to the making of a neurotoxic lesion. This timing therefore makes the interpretation of these studies somewhat difficult since it is unclear whether the vector of interest is mediating protection from insult or merely interfering with the means by which the toxin works. For example, intrastriatal injection of 6-OHDA produces a gradual loss of nigral neurons that can continue for at least sixteen weeks (Sauer et al., 1994). Studies in which the efficacy of antiapoptotic genes have been evaluated using this model have delivered the vector of interest one week prior to intrastriatal 6-OHDA injection (Yamada et al., 1999; Natsume et al., 2001; Crocker et al., 2001). The neurotoxin MPTP produces decreased numbers of nigral neurons over the course of 4 days following intraperitoneal (IP) injection (Jackson-Lewis et al., 1995) or for at least 28 days following injection of MPP+ into the striatum of rats (Fallon et al., 1997). In experiments employing antiapoptotic gene therapy, vector delivery has occurred 1–2 weeks prior to MPTP administration (Eberhardt et al., 2000; Mochizuki et al., 2003). Therefore, these antiapoptotic gene transfer experiments should be strictly viewed as investigations of neuroprotective strategies as opposed to addressing the capacity to augment the functionality of previously injured DA neurons. Nontheless, neuroprotective strategies can serve to stabilize the DA neuron cell population thereby providing a set of cells whose activity can be augmented to ameliorate symptoms and prevent or slow further degeneration. Coupled with the ability to diagnose and intervene early in PD, neuroprotection provides for an extremely valuable approach.

TABLE 16.2. Gene Transfer of Anti-Apoptotic Factors for Neuroprotection*

| Factor | Vector/site/ Timing | Species/lesion type | Morphological outcome | Striatal biochemistry | Behavioral outcome | % Transduced/ duration of expression | Toxicity | Reference |
|---|---|---|---|---|---|---|---|---|
| **Bcl-2** | HSV/SN/ 1 week prior | Rats/Striatal 6-OHDA | 100% ↑ SN (THir) 3 weeks post HSV | Not conducted | Not conducted | Not reported/ 3 weeks | 40% fewer SN cells due to vector | Yamada et al., 1999 |
| **Bcl-2** | HSV/SN/ 1 week prior | Rats/Striatal 6-OHDA | 20% ↑ SN (THir) 3 weeks post HSV | Not conducted | Amphetamine 89% ↓ | Not reported/ Not conducted | No significant differences from PBS | Natsume et al., 2001 |
| **XIAP** | AV/Striatum 1 week prior | Rats/Striatal $MPP^+$ | 100% ↑ SN (THir) 18 days post AV | No protection of striatal DA, HVA, DOPAC | Not conducted | Data not shown/1 week | No significant differences from PBS | Eberhardt et al., 2000 |
| **Apaf-1 DN** | AAV/Striatum 2 weeks prior | Mice/MPTP (i.p.) | 133% ≠ SN (THir) 2 wks post AAV | Not conducted | Amphetamine asymmetry Bilateral lesion | Not reported/ 2 weeks (ICC) | No reporter control | Mochizuki et al., 2001 |
| **Caspase-1 DN** | AAV/Striatum 2 weeks prior | Mice/MPTP (i.p.) | 0% ↑ SN (THir) 2 wks post AAV | Not conducted | Not conducted | Not reported/ 2 weeks (ICC) | No reporter control | Mochizuki et al., 2001 |
| **NAIP** | AV type 5 Striatum 1 week prior | Rats/Striatal 6-OHDA | 33% ↑ SN (THir) 4 wks post AV | Not conducted | Amphetamine 85% ↓ | 59%/4 weeks | No PBS Control | Crocker et al., 2001 |

* ir = immunoreactive; ↑ = increase; ↓ = decrease; No PBS control = only expression plasmid and reporter plasmid groups examined, which does not allow for assessment of possible cytotoxicity due to viral infection.

Antiapoptotic gene transfer strategies also differ from DA biosynthetic enzyme gene transfer in that the target cells are the nigral DA neurons themselves rather than striatal neurons. Genes of interest under this approach must be expressed by a significant population of nigral DA neurons and translated to biologically relevant levels while simultaneously avoiding cytotoxicity that is occasionally associated with specific viral vectors. The first study using this strategy investigated the transfer of Bcl-2 using HSV vector injections to the SN of rats prior to striatal 6-OHDA lesions (Yamada et al., 1999). This report demonstrated a doubling of nigral DA neuron survival with Bcl-2 gene transfer; however, injections of this same vector construct to unlesioned rats induced toxicity in 40% of SN neurons. HSV-mediated gene transfer of Bcl-2 yielded more modest protection of nigral DA neurons (20% increase) and significant reductions in amphetamine-induced rotations in a subsequent report from the same laboratory (Natsume et al., 2001). This follow-up study appeared to have resolved the issue of toxicity to nigral DA neurons previously associated with the HSV Bcl-2 vector.

Caspase inhibition with adenoviral (AV) gene transfer of X-chromosome-linked inhibitor of apoptosis (XIAP) protects nigral neurons following intrastriatal MPP+ injections (Eberhardt et al., 2000). However, while cell bodies were spared, there was no preservation of striatal DA, HVA, or DOPAC. Injection of AAV encoding the dominant negative form of Apaf-1 (blocks the Apaf-1/caspase-9 pathway) to the striatum of MPTP-treated mice protected nigral DA neurons and partially reversed amphetamine-induced rotations (Mochizuki et al., 2003). In contrast, AAV-mediated gene transfer of the dominant negative form of caspase-1 failed to preserve nigral neurons and did not produce functional benefits (Mochizuki et al., 2003). Lastly, intranigral injections of an adenovirus encoding for the caspase-3 inhibitor, neuronal apoptosis inhibitor protein (NAIP)-protected 33% more nigral DA neurons than a control vector (Crocker et al., 2001). NAIP-treated rats also exhibited an 85% reduction in amphetamine-induced rotations. This study was unique in that the percentage of DA neurons transduced by the AV vector was specifically examined with 59% of the dopaminergic cells being transfected. Collectively, while antiapoptotic gene transfer strategies display promise in their ability to protect nigral DA neurons from neurotoxins, only one study has directly examined the impact of this approach on striatal DA and DA metabolite levels (Eberhardt et al., 2000). No significant protection of striatal DA biochemistry was observed. This would indicate that the possibility that DA neurons are spared from degeneration by antiapoptotic gene transfer but the state in which the cell remains does not truly recapitulate DA neuron functionality. For this reason, a contransduction strategy has been employed where antiapoptotic gene transfer was paired with gene delivery of glial cell line-derived neurotrophic factor (GDNF) in an attempt for antiapoptotic genes to protect nigral perikarya and the GDNF gene to preserve striatal innervation (Natsume et al., 2001; Eberhardt et al., 2000). Cotransduction with the caspase inhibitor XIAP and GDNF afforded

complete protection of striatal DA levels, while gene delivery of GDNF alone provided only partial protection (Eberhardt et al., 2000).

## 4. Neurotrophic Factor Gene Therapy

### *4.1. Neuroprotection*

A great deal of research has been conducted investigating neuroprotective strategies using gene delivery of neurotrophic factors in a variety of animal models of neurodegenerative diseases. Specifically for PD, there is an expansive literature devoted to the neurotrophic molecules for dopaminergic nigrostriatal neurons. Direct injections of acidic or basic fibroblast growth factor (FGF), epidermal growth factor (EGF), persephin (PSP), neurturin (NTN), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), interleukin-1ß (IL-1ß), ciliary neuronotrophic factor (CNTF), and glial cell line-derived neurotrophic factor (GDNF) to either the SN or the striatum in animal models of PD have all elicited positive effects of varying degrees on DA neuron survival or striatal innervation (see review, Collier and Sortwell, 1999 and Chapter 14). However, the clear front-runner in terms of potency in in vivo models is GDNF. This consistent neuroprotective effect with direct GDNF protein infusions has provided a compelling rationale for the investigation of effects of GDNF gene transfer.

As our understanding of the mechanisms at work in the developing, adult, and aging nervous system has evolved, an important role for GDNF in the formation and stabilization of DA neuron structure and function has emerged. GDNF is a member of the transforming growth factor-β superfamily of molecules with two receptors required to mediate GDNF signal transduction, RET, and GDNFα. (Takahashi 2001). Nigral DA neurons express GDNFα. and ret receptors in great abundance (Trupp et al., 1996, 1997; Aroujo et al., 1997; Treanor et al., 1996). Type-1 astrocytes residing in the ventral midbrain express high to moderate levels of GDNF with GDNF expression peaking during early postnatal development and then decreasing to low levels in the adult (Schaar et al., 1993; Choi-Lundberg et al., 1995). In the striatum, expression levels of GDNF follow this same pattern of high expression in the early postnatal period followed by a steady decline to low levels in adulthood (Schaar et al., 1993; Choi-Lundberg et al., 1995; Stromberg et al., 1993; Trupp et al., 1997; Springer et al., 1994; Blum et al., 1995). Glial fibrillary acidic protein (GFAP) may modulate GDNF in vivo as GFAP knockout mice display increased levels of striatal GDNF, but not nerve growth factor (NGF) or CNTF (Hanbury et al., 2003). Lastly, GDNF levels are altered in response to experimental manipulations with increases in GDNF observed in the striatum following status epilepticus and 6-OHDA lesions (Schmidt-Kastner et al., 1994; Yurek et al., 2001). This finding indirectly suggests that perturbation of the nigrostriatal system by injury or

disease may trigger compensatory responses resulting in alterations in GDNF expression and secretion and provides a justification for gene delivery of GDNF for PD.

There have been numerous studies employing different vectors that illustrate the potency of GDNF gene delivery for neuroprotection (Table 16.3). Bohn and coworkers (Choi-Lundberg et al., 1997) provided the groundbreaking data set that illustrated the potency of this procedure. In this study, adenoviral (AV) delivery of GDNF to the SN of rats one week prior to striatal 6-OHDA lesions was the first approach used. Prior to the lesion, the striatum was backfilled with fluorogold (FG) to unequivocally identify striatally-projecting nigral neurons. This study demonstrated a threefold increase in large, FG-labeled nigral neurons suggesting significant neuroprotection by GDNF. Although this experiment suggested great promise for this approach, no examination was made to determine whether these FG cells maintained their DA phenotype and lack of behavioral analysis and striatal biochemistry similarly could not provide information regarding the functionality of the protected nigral neurons. Subsequent studies utilizing AV-mediated gene transfer of GDNF to the striatum of 6-OHDA-lesioned rats demonstrated significant protection of THir nigral neurons (Bilang-Bleuel et al., 1997), increases in striatal THir fibers (Bilang-Bleuel et al., 1997), and behavioral improvements (Bilang-Bleuel et al., 1997; Choi-Lundberg et al., 1998). Similar increases in THir neuron number using AV GDNF injections to the SN or striatum prior to striatal 6-OHDA lesions to aged rats were reported, as well as behavioral and striatal THir fiber density improvements in the case of striatal injections specifically (Connor et al., 1999). Lastly, injection of AV GDNF to the striatum of rats one week prior to striatal MPP+ injections failed to provide neuroprotection for THir nigral neurons but was the only study using an AV vector to demonstrate partial protection of DA, HVA, and DOPAC levels in the striatum (Eberhardt et al., 2000). These first studies utilizing AV delivery of GDNF demonstrated proof of principle for this approach; however, the host immune response and often limited transgene expression associated with AV vector delivery (Byrnes et al., 1996) has directed studies to investigate GDNF gene transfer using AAV and lentiviral (LV) vectors.

To improve the transgene expression and minimize toxicity, the field then moved to AAV-GDNF delivery (see Chapter 14). These initial studies examined the effects of AAV GDNF injected to the SN of rats either 3 weeks prior or immediately after a striatal 6-OHDA lesion (Mandel et al., 1997, 1999). In both studies, significant preservation (60–73%) of nigral THir neurons was reported; however, striatal biochemistry and behavioral effects were not examined. More modest protection of nigral DA neurons were reported utilizing HSV GDNF injections to the SN, with significant reductions in amphetamine-induced rotations observed (Natsume et al., 2001). In order to determine what the optimal site for AAV-GDNF gene delivery (SN vs. striatum), the vector was injected to either site or both in striatal 6-OHDA-lesioned rats and were

TABLE 16.3. Gene Transfer of Neurotrophic Factors for Neuroprotection

| Factor | Vector/ injection Site/timing | Species/ lesion type | Morphological outcome | Striatal biochemistry | Behavioral outcome | % Transduced/ duration of expression | Toxicity | Reference |
|---|---|---|---|---|---|---|---|---|
| **GDNF** | AV/SN/1 week prior | Rats/Striatal 6-OHDA | 200% ↑ FG cells No TH cell counts | Not conducted | Not conducted | Not conducted/ 7 weeks | No difference from no virus control = None | Choi-Lundberg et al., 1997 |
| **GDNF** | AV/Striatum/ 1 week prior | Rats/Striatal 6-OHDA | 74% ↑ FG cells No TH cell counts | Not conducted | Amph:96% ↓ Apo:0% ↓ ↓ forelimb asymmetry | Not conducted/ 7 weeks | No difference from no virus control = None | Choi-Lundberg et al., 1998 |
| **GDNF** | AV/Striatum/ 6 days prior | Rats/Striatal 6-OHDA | 100% ↑ SN (THir) ↑ TH+ fibers | Not conducted | Amph:90% ↓ | Not conducted/ 4 weeks | 37% fewer THir cells in SN due to vector, no toxicity in 6-OHDA rats | Bilang-Bleuel et al., 1997 |
| **GDNF** | AV/SN/ 1 week prior | Aged Rats/ Striatal 6-OHDA | 86% ↑ SN (THir) No ↑ TH+ fibers | Not conducted | Amph: 85% ↑ No significant effects on forelimb asymmetry | Not conducted/ 6 weeks | 18% fewer THir cells than no virus control | Connor et al., 1999 |
| **GDNF** | AV/Striatum/ 1 week prior | Aged Rats/ Striatal 6-OHDA | 64% ↑ SN (THir) ↑ TH+ fibers | Not conducted | Amph: 160% ↓ ↓ forelimb asymmetry | Not conducted/ 6 weeks | 52% fewer THir cells than no virus control | Connor et al., 1999 |
| **GDNF** | AV/Striatum 1 week prior | Rats/Striatal $MPP^+$ | 0% ↑ SN (THir) | Partial protection of DA, HVA, DOPAC | Not conducted | Not conducted/ 1 week | No difference from PBS control = None | Eberhardt et al., 2000 |
| **GDNF** | AAV/SN/ 3 weeks prior | Rats/Striatal 6-OHDA | 73% ↑ SN (THir) | Not conducted | Not conducted | Not conducted/ 10 weeks | No PBS or no virus control | Mandel et al., 1997 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **GDNF** | AAV/SN/ 10–20 mins after 6-OHDA | Rats/Striatal 6-OHDA | 60% ↑ SN (THir) | Not conducted | Not conducted | Not conducted/ 2 weeks | No PBS or no virus control | Mandel et al., 1999 |
| **GDNF** | AAV/SN/ 4 weeks prior | Rats/Striatal 6-OHDA | 600% ↑ SN (THir) | ↑ DA turnover (intact rats) | Amph/Apo 0% ↓ No effects on forelimb asymmetries or paw reach | Not conducted/ 6 months | No difference from PBS control = None | Kirik et al., 2000 |
| **GDNF** | AAV/Striatum/ 4 weeks prior | Rats/Striatal 6-OHDA | 300% ↑ SN (THir) ↑ THir fibers | ↑ DA turnover (intact rats) | Amph: 53% ↓ Apo: 0% ↓ Improvements in paw reach and asymmetry (cylinder) | Not conducted/ 6 months | No difference from PBS control = None | Kirik et al., 2000 |
| **GDNF** | AAV/Striatum and SN/ 4 weeks prior | Rats/Striatal 6-OHDA | 500% ↑ SN (THir) | ↑ TH enzyme activity ↑ DA turnover (intact rats) | Amph/Apo 0% ↓ No effects on forelimb asymmetries or paw reach | Not conducted/ 6 months | No difference from PBS control = None | Kirik et al., 2000 |
| **GDNF** | HSV/SN/ 1 week prior | Rats/Striatal 6-OHDA | 30% ↑ SN (THir) | Not conducted | Amph: 100% ↓ | Not reported/ 3 weeks | No difference from PBS control = None | Natsume et al., 2001 |
| **GDNF** | LV/SN/ 1 week prior | Rats/MFB axotomy | 100% ↑ SN (THir) | No DA increase | Not conducted | 40.1%/1 week | No PBS or no virus control | Deglon et al., 2000 |
| **GDNF** | LV/SN/ 2 weeks prior | Mice/Striatal 6-OHDA | 100% ↑ SN (THir) | No DA increase | Apo: 52% ↓ | 20% THir/β galir | No PBS or no virus control | Bensadoun et al., 2000 |
| **GDNF** | LV/SN Striatum/ 1 week prior | Rats/Striatal 6-OHDA | 126% ↑ SN (THir) | Not conducted | Not conducted | Not conducted/ 4 weeks | No PBS or no virus control | Rosenblad et al., 2000; Rosenblad et al., 2001 |

*(Continued)*

TABLE 16.3. Gene Transfer of Neurotrophic Factors for Neuroprotection—cont'd

| Factor | Vector/ injection Site/timing | Species/ lesion type | Morphological outcome | Striatal biochemistry | Behavioral outcome | % Transduced/ duration of expression | Toxicity | Reference |
|---|---|---|---|---|---|---|---|---|
| **GDNF** | LV/Cd, Pt and SN/1 week prior | Rhesus Monkeys/ Intracarotid MPTP | 88% ↑ SN (THir) (32% ↑ intact) ↑ THir fibers ↑ TH mRNA/cell | 300% ↑ FD Uptake | Handreach task: 56% ↓ latency CRS: 36% ↓ | Not conducted/ 8 months | No PBS or no virus control | Kordower et al., 2001 |
| **GDNF** | LV/Cd, Pt and SN/NA | Aged Rhesus Monkeys/ None | 85% ↑ SN (THir) ↑ THir fibers ↓ TH mRNA/cell | 27% asymmetry FD Uptake Cd: DA 140% ↑ HVA 207% ↑ Pt: DA 47.2% ↑ HVA 128% ↑ | Not conducted | Not conducted 8 months | No PBS or no virus control | Kordower et al., 2001 |
| **NBN/ART** | LV/Striatum and SN/ 1 week prior | Rats/Striatal 6-OHDA | 28% ↑ SN (THir) | Not conducted | Not conducted | Not conducted/ 4 weeks | No PBS or no virus control | Rosenblad et al., 2000; Rosenblad et al., 2001 |

*ir = immunoreactive; ↑ = increase; ↓ = decrease; FG = fluorogold; Apo = apomorphine; Amph = amphetamine; No PBS or no virus control = only expression plasmid and reporter plasmid groups examined, which does not allow for assessment of possible cytotoxicity due to viral infection.

assessed on neuroanatomical (number of THir neurons) and a series of behavioral outcome measures (Kirik et al., 2000). In addition, intact rats were assessed for effects on striatal biochemistry. A critical concept emerged from this carefully designed study. While all delivery sites afforded significant neuroprotection of THir neurons, behavioral recovery and significant THir striatal reinnervation was only observed in rats that had received injections of AAV GDNF to the striatum. In addition, striatal combined with nigral delivery of AAV GDNF to intact rats yielded optimal effects on striatal TH enzyme activity and DA turnover.

More recent experiments have turned to lentiviral-mediated delivery of GDNF (see Chapter 14). Lentivirus (LV) is a HIV-based vector with a large cloning capacity and display virtually no toxicity or immunogenicity following in vivo delivery to rats or monkeys (Kordower et al., 2000a). LV vectors have demonstrated long-term transgene expression at high levels. When LV GDNF was injected to the SN of rats or mice prior to medial forebrain bundle axotomy or striatal 6-OHDA, respectively, significant neuroprotection of THir SN neurons was observed (Bensadoun et al., 2000; Deglon et al., 2000) and apomorphine-induced rotations were significantly reduced (Bensadoun et al., 2003). Simultaneous nigral and striatal lentiviral GDNF delivery to rats prior to striatal 6-OHDA lesion yielded an even greater neuroprotection of nigral DA neurons (Rosenblad et al., 2000, 2001). Gene transfer of another GDNF family member, neublastin/artemin (NBN/ART) has also been investigated with much more modest neuroprotection of nigral DA neurons reported (Rosenblad et al., 2000, 2001).

On the basis of the success reported with GDNF transduction in rodents and other emerging data illustrating the safety and efficacy of lentiviral vectors, our group began a collaboration with Aebischer and coworkers to test the safety, expression, and functional effects of LV GDNF in nonhuman primate models of PD. (Kordower et al., 2000b). Initially, we examined the efficiency of lentiviral delivery of the marker gene ß-galactosidase (ßGal). Three rhesus monkeys were injected unilaterally into the caudate, putamen, and SN. One or three months later, there were 187,000 ßGal-positive cells in the substantia nigra and between 900,000 and 1.5 million cells in the striatum. Initially, just the cytoplasm of the cell soma expressed the transgene. As survival time progressed, the transgene could be seen filling both the cytoplasm and dendritic arbors of infected striatal cells. The rate of transfection was similar in both loci with the different numbers of surviving cells being due to different volumes of vector injected (40 $\mu$l in the striatum and 5 $\mu$l in the nigra). Double-label confocal microscopy showed that the vast majority (84–88%) of the transfected cells were neurons. Furthermore, there was no evidence of an immune response or inflammation.

Once the infectability of the LV was established in nonhuman primates, we set out to evaluate the ability of therapeutic genes to modify the degenerative nigrostriatal system (Kordower et al., 2000b). The first model used was aged rhesus monkeys. Our choice of nonlesioned aged monkeys was based in the

finding that monkeys display a slow progressive loss of striatal dopamine (Irwin et al., 1994) and varying degrees of THir neuron loss within the SN (Emborg et al., 1998; Irwin et al., 1994; Gerhardt et al., 2002). However, the crucial point in using this model is that these cells lose TH immunoreactivity, but they do not die. (Emborg et al., 1998). Aged monkeys thus demonstrate changes within the nigrostriatal system that mimic some of the earliest cellular changes seen in early PD (Kastner et al., 1993). We also wanted to use aged monkeys because, despite these degenerative changes, aged monkeys still display sufficient integrity of the host nigrostriatal system, providing a substantial substrate for the LV-GDNF to work. We realized from the onset that this experiment would have only neuroanatomical and neurochemical end points because in our hands aged monkeys, as with aged humans, (Quinn et al., 1986) fail to demonstrate improved motor function after augmentation of the dopaminergic system (e.g., with L-DOPA). In this experiment, aged (>25 years old) female rhesus monkeys received injections of lentiviral vectors encoding GDNF or ßGal (as a control) targeted for the striatum and SN and were killed 3 months later (Kordower et al., 2000b). All aged monkeys receiving LV GDNF displayed robust GDNF immunoreactivity within the injected striatum and SN. In contrast, no monkeys receiving LV-ßGal displayed specific GDNF immunoreactivity in the striatum indicating that needle penetrations into the striatum are not sufficient to upregulate endogenous GDNF to within detectable levels. LV-GDNF injections resulted in marked anterograde transport of the trophic factor. In this regard, intense GDNF immunoreactivity was observed within fibers of the globus pallidus and SN pars reticulata after striatal injections. In contrast, anterograde transport of the nuclear form of ßGal was not observed in LV ßGal-treated monkeys. This suggests that secreted GDNF, and not the virus per se, was anterogradely transported. The significance of this anterograde transport of GDNF remains to be elucidated.

Aged monkeys underwent fluorodopa positron emission tomography (PET) before surgery and again just before sacrifice 3 months following the LV injections. Before treatment, all monkeys displayed symmetrical fluorodopa uptake in the caudate and putamen bilaterally. Similarly, there was symmetrical fluorodopa uptake in all LV-ßGal-treated monkeys. In contrast, significant asymmetries in fluorodopa uptake (27%) were seen in LV-GDNF-treated monkeys with greater uptake on the side of the GDNF expression. In support of the imaging data, post-mortem biochemical analyses showed significant increases in dopamine and homovanillic acid levels in the right caudate nucleus (140% dopamine, 207% homovanillic acid), and putamen (47.2% dopamine, 128% homovanillic acid) in LV-GDNF-treated aged monkeys compared with the LV ßGal group.

Morphologically, lentiviral delivery of GDNF clearly increased TH immunoreactivity within the striatum on the side of the LV GDNF injection (Fig. 16.1). In contrast, LV ßGal-treated monkeys displayed a symmetrical THir staining pattern with no increase in comparison with the

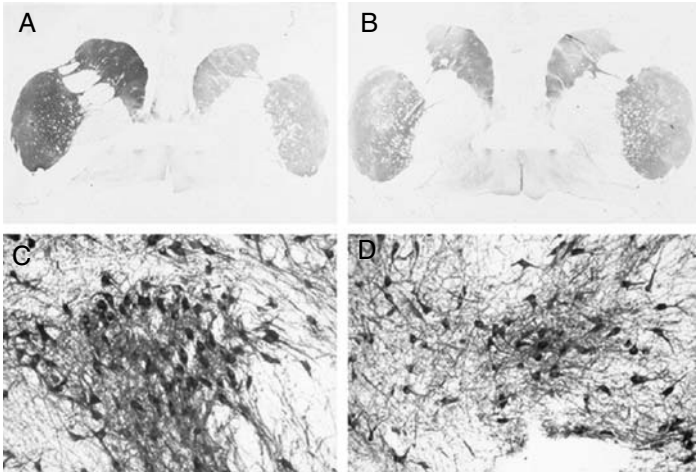


FIGURE 16.1. **A:** Section stained for TH immunoreactivity through the anterior commissure illustrating the increase in TH immunoreactivity within the right caudate nucleus and putamen after LVGDNF delivery to aged monkeys. **B:** Symmetrical and less intense staining for TH immunoreactivity in a monkey injected with LV βGal. **C:** There were greater numbers and larger TH-immunoreactive neurons within the substantia nigra of a LV GDNF-treated animal relative to (**D**) a LV βGal-treated monkey. (See color insert.)

control side. Quantitatively, LV GDNF increased the relative optical density of TH staining within the caudate nucleus and putamen by 44.1% and 38.9%, respectively, relative to LV ßGal-treated animals. Similarly, stereological counts showed an 85% increase in the number of THir nigral neurons on the side receiving LV GDNF relative to LV ßGal-treated animals. This increase in THir cell number likely reflects a phenotypic upregulation of TH in existing neurons that previously had downregulated TH production that was below detectable levels. A similar pattern of effects was seen when the volume of THir neurons was quantified. Nigral neurons on the side of the LV-GDNF injections were not only bigger than the atrophic residual nigral neurons seen in LV ßGal-treated animals, but even hypertrophic relative to the intact side. In addition, there was a significant increase in the optical density of TH mRNA-containing neurons within the right SN in LV GDNF-treated monkeys (21.5%) compared with LV ßGal-containing animals, indicating that LV GDNF increased dopamine production within individual nigral neurons.

These data, in the absence of any behavioral side effects or neuroanatomical toxicity, gave us great optimism for the potential role of this type of gene therapy as a treatment approach for PD. However, the preservation and upregulation of dopaminergic function seen in aged animals is immaterial if the therapy of choice cannot restore motor function from the parkinsonian state

or prevent the progression of symptoms. To achieve this goal, we used the best animal model of PD available, namely, the MPTP-treated monkey. In this experiment (Kordower et al., 2000b), young adult rhesus monkeys initially were trained on a hand-reach task in which the time to pick up food treats out of recessed wells was measured. Once per week, monkeys were also evaluated on a modified parkinsonian clinical rating scale (CRS). All monkeys then received an injection of MPTP into the right carotid artery, initiating a parkinsonian state. One week later, monkeys were evaluated on the CRS. Only monkeys displaying severe hemiparkinsonism with the classic crooked arm posture and dragging leg on the left side continued in the study. It is our experience that monkeys with this behavioral phenotype have the most severe lesions and do not spontaneously recover (Kordower et al., 1995). On the basis of CRS scores, monkeys were matched into two groups that received either LV ßGal or LV GDNF treatment. Monkeys received injections of either LV GDNF or LV ßGal into the caudate nucleus, putamen, and SN on the right side. One week later, monkeys were retested on the hand-reach task and the CRS. After LV treatment, significant differences in CRS scores were seen between the two groups (Fig. 16.2). CRS scores of monkeys receiving LV ßGal did not change over the 3-month period after treatment, demonstrating the stability of the model. In contrast, CRS scores of monkeys receiving LV GDNF diminished significantly. LV GDNF-treated animals

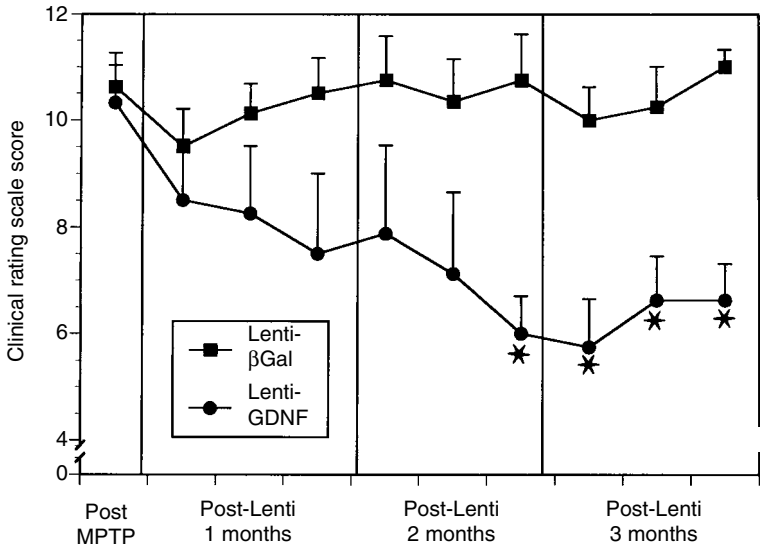


FIGURE 16.2. After MPTP-treatment, LV-GDNF-injected monkeys displayed functional improvement on the clinical rating scale (CRS). All tests were performed 3 weeks per month. On the CRS, monkeys were matched into groups based upon the post-MPTP score. $^{*}P < 0.05$ relative to LV βGal.

also improved on the operant hand-reach task. After MPTP, all LV ßGal-treated animals were severely impaired, requiring more than the maximally allowed 30 seconds. In contrast, only one LV GDNF monkey remained impaired and performed this task in a manner similar to the LV ßGal-treated animals. All other LV GDNF-treated monkeys performed the task at near-normal levels. Consider also that in addition to the elimination or marked reduction of parkinsonian symptoms in these animals, no behavioral or other toxic side effects were seen.

Three months after LV injections, all monkeys underwent fluorodopa positron emission tomography (PET) scans (Fig. 16.3). Quantitatively, no differences in fluorodopa uptake were observed between groups within the untreated striatum. In contrast, there was a significant (300%) increase in fluorodopa uptake in LV GDNF-treated animals in the right striatum relative to LV ßGal-treated animals. Immediately after fluorodopa PET scanning, all animals were killed. Post-mortem, a strong GDNF-ir signal was seen in the caudate nucleus, putamen, and substantia nigra of all LV GDNF-treated but not in any of the LV ßGal-treated animals. In support of the fluorodopa PET scan data, post-mortem analyses showed that LV GDNF provided substantial protection of striatal dopamine at the level of the striatum (Fig. 16.4). All LV ßGal-treated monkeys displayed a comprehensive loss of TH immunoreactivity within the striatum on the side ipsilateral to the MPTP injection (see Fig. 4A). In contrast, all LV GDNF-treated monkeys displayed enhanced striatal TH immunoreactivity relative to LV ßGal controls (see Fig. 16.4B).

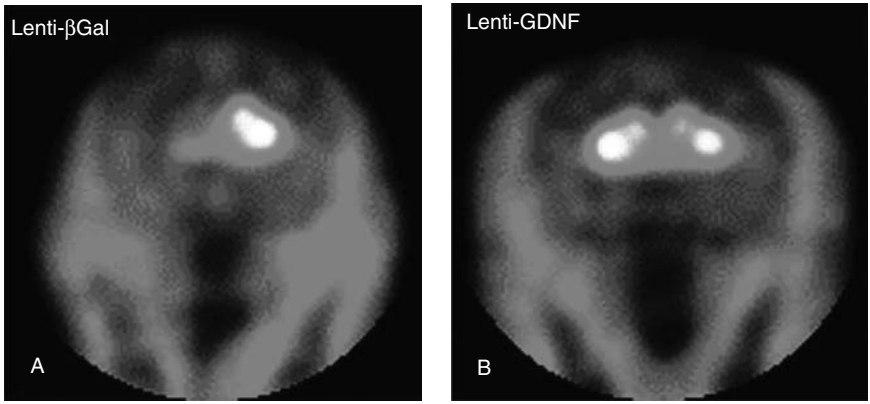


FIGURE 16.3. Positron emission tomography (PET) scan data evaluating the influence of LV GDNF on fluorodopa (FD) uptake in young adult MPTP-treated monkeys. **A:** After MPTP lesions, a comprehensive loss of FD uptake was seen within the right striatum of LV βGal-treated young adult monkeys. **B:** In contrast, FD uptake was enhanced in LV GDNF-treated monkeys. *K*i values (per minute) for the striatum are as follows: LV βGal left, 0.0091 ± 0.0004; LV βGal right, 0.0017 ± 0.0005; LV GDNF left, 0.0084 ± 0.0004; LV GDNF right, 0.0056 ± 0.0018. (See color insert.)

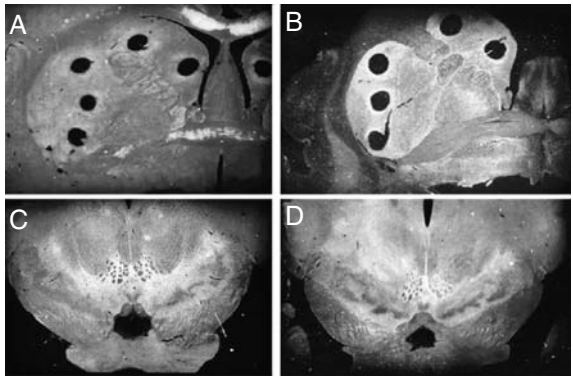


FIGURE 16.4. Low-power dark-field photomicrographs through the right striatum of TH-immunostained sections of MPTP-treated monkeys treated with (**A**) LV βGal or (**B**) LV GDNF. A: There was a comprehensive loss of TH immunoreactivity in the caudate and putamen of LV βGal-treated animal. In contrast, near normal level of TH immunoreactivity is seen in LV GDNF-treated animals. Low-power (**C** and **D**) photomicrographs of TH-immunostained section through the substantia nigra of animals treated with LV βGal (C) and LV GDNF (D). Note the loss of TH-immunoreactive neurons in the LV βGal-treated animals on the side of the MPTP-injection. TH-immunoreactive sprouting fibers, as well as a supranormal number of TH-immunoreactive nigral perikarya are seen in LV GDNF-treated animals on the side of the MPTP injection. (See color insert.)

When analyzed as a group, TH optical density in the right caudate nucleus and putamen of LV GDNF-treated monkeys was significantly greater than that seen in LV ßGal-treated monkeys and was similar to that seen on the intact side of these animals. Interestingly, the one monkey that did not recover on the hand-reach task displayed only minor protection of striatal TH. The level of TH immunoreactivity within the striatum of this monkey was greater than controls, but far less than in the other LV GDNF cohorts. The absence of functional benefits in this animal supports the concept that significant protection of striatal innervation is required for functional recovery.

Substantial neuroprotection was also seen at the level of the SN (see Fig. 16.4C, D). All LV ßGal-treated monkeys displayed a comprehensive loss of THir neurons within the substantia nigra on the side ipsilateral to the MPTP injection (see Fig. 16.4C). In contrast, the nigra from all LV GDNF-treated displayed complete neuroprotection, regardless of the degree of functional recovery with some sprouting of THir fibers extending into the midbrain of these animals (see Fig. 16.4D). In LV ßGal-treated monkeys, intracarotid injections of MPTP resulted in an 89% decrease in the number, and an 81.6% decrease in the density of THir nigral neurons on the side ipsilateral to the toxin injection. In contrast, not only was there complete preservation of THir nigral neurons in lenti-GDNF-treated animals, but these monkeys displayed

32% more THir nigral neurons and an 11% increase in THir neuronal density relative to the intact side. The supranormal number of TH-ir neuronal number in the LV GDNF-treated animals was surprising. We hypothesize that there is some crossover of MPTP after the unilateral intracarotid injection and that a small lesion occurs on the intact side. Thus, we believe that LV GDNF was protecting all of the THir neurons on the lesioned side but did not protect the small population of neurons on the side contralateral to LV GDNF that degenerate after unilateral MPTP injections. Other morphological parameters of cell integrity and function were also protected and augmented by LV GDNF. In LV ßGal-treated animals, MPTP significantly reduced (32%) the volume of residual THir nigral neurons on the lesion side relative to the intact side. Not only was this prevented by LV GDNF, these cells had hypertrophied and were increased in size by 44% relative to the intact side. The optical density of TH mRNA within nigral neurons was quantified bilaterally in all animals. In LV ßGal-treated animals, there was a significant decrease (24.0%) in the relative optical density of TH mRNA within residual neurons on the MPTP-lesioned side relative to the intact side. In contrast, LV GDNF-treated animals displayed a significant increase (41.7%) in relative optical density of TH mRNA relative to intact side or LV ßGal-treated animals. These benefits were found without any inflammatory or immune response as determined by the relative absence of staining using CD8, CD3, and CD45 antibodies, probes which are directed against immune and inflammatory cells.

Using three different model systems (normal monkeys, aged monkeys, and MPTP-treated monkeys), we demonstrated that delivery of LV to transfer the marker gene ßGal or the therapeutic gene GDNF to be safe and effective. To date, over a million striatal cells have been successfully transfected with the ßGal and GDNF transgenes without any evidence of any immune or inflammatory response as measured by multiple histological markers. So, what further needs to be considered before initiating clinical trials? First, robust long-term gene expression needs to be established. To accomplish this, we took two normal monkeys and injected them in the striatum with LV GDNF using the same injection schema that we used in the previous experiments (Kordower et al., 2000b). Eight months later, we killed these animals. Robust GDNFir was seen in both animals at this longer survival time point. In these animals, punches were taken for GDNF enzyme-linked immunosorbent assay analysis (ELISA). We found that LV GDNF delivery resulted in the in vivo expression of between 2.5 and 3.5 ng/mg GDNF protein in the caudate nucleus and putamen, respectively. When one considers the area of GDNF immunoreactivity that was seen it is clear that for at least 8 months, we were delivering a total of microgram doses of GDNF to the primate striatum. Thus, our goal of having long-term high-level transgene expression has been achieved. Again, note that we did not observe any evidence of behavioral side effects following LV GDNF delivery in any of the animals we have studied to date.

### *4.2. Augmenting Functionality of Injured DA Neurons*

Progress has also been made with regard to GDNF gene transfer eliciting positive effects on dopaminergic phenotype when delivered after stable lesions of the nigrostriatal system (Table 16.4). Injection of AV GDNF to the SN of rats 10 weeks *after* 6-OHDA lesions of the MFB demonstrated increases in nigral DA and DOPAC levels and modest but significant reductions in apomorphine-induced rotations (Lapchak et al., 1997). Four to five weeks *following* AAV GDNF injections to the striatum, significant increases were observed in the number of nigral neurons displaying TH immunoreactivity (Wang et al., 2002); the density of striatal THir fibers (Wang et al., 2002); and the striatal levels of DA, DOPAC and HVA (Wang et al., 2002) in conjunction with significant reductions in apomorphine-induced rotations (Wang et al., 2002; McGrath et al., 2002). Collectively, these studies using delayed delivery of GDNF illustrate its potential to rescue DA neurons after disease progression ensues. Substantial numbers of DA neurons succumb to the disease process before obvious symptoms bring patients to the clinic. Further experiments utilizing delayed GDNF gene transfer in different experimental parkinsonian models will clarify whether this rescue strategy will offer further hope for PD patients

## 5. Gene Transfer of Glutamic Acid Decarboxylase

Degeneration of nigral DA neurons of the pars compacta (SNpc) leads to depletion of DA in the striatum which in turn results in disinhibition of the subthalamic nucleus (STN) due to a decrease in inhibitory input from external segment of the globus pallidus (GPe). The outcome of this disinhibition is an overactive STN that leads to increased excitatory input to the internal segment of the globus pallidus (GPi) and the substantia nigra pars (SNpr) reticulata. Overactivity of GPi and the SNpr leads to overinhibition of the motor projections to the brainstem and thalamus thereby inhibiting the initiation of voluntary movements. Thus, overactivity of the STN is an fundmental feature of PD (Obeso et al., 2000). In animal models of PD, lesions of the GPi and the STN result in significant improvements in motor function (Bergman et al., 1990; Wichmann et al., 1994). These results have led to the development of surgical procedures directed at the STN as a clinical treatment strategy for PD. These surgical procedures include deep brain stimulation (DBS), which has now become commonplace in the treatment of PD and to a lesser extent lesion of the STN. Both procedures, especially DBS (Limousin et al., 1995; Benazzouz et al., 2000; The Deep-Brain Stimulation for Parkinson's Disease Study Group, 2001) have resulted in marked improvement of the motor symptoms of PD. The rationale underlying this approach of suppressing the firing activity of the STN has also provided a novel gene therapy strategy; gene transfer of glutamic acid decarboxylase (GAD) to the

TABLE 16.4. Gene Transfer of Neurotrophic Factors for Rescue of Injured DA Neurons*

| Factor | Vector/ injection site/timing | Species/ lesion type | Morphological outcome | Striatal biochemistry | Behavioral outcome | % Transduced/ duration of expression | Toxicity | Reference |
|---|---|---|---|---|---|---|---|---|
| **GDNF** | AV/SN/ 10 weeks *after* | Rats/MFB 6-OHDA | Not examined | No ↑ DA or DOPAC | Apo: 28% ↓ | Not conducted/ 3 weeks | No PBS or no virus control | Lapchak et al., 1997 |
| **GDNF** | AAV/Striatum/ 4 weeks *after* | Rats/Striatal 6-OHDA | 200% ↑ SN (THir) ↑ THir fibers | DA: 330% ↑ DOPAC: 280% ↑ HVA: 220% ↑ | Apo: 75% ↓ Normalization of sponta-neous forelimb use | 13.1%/ 20 weeks | No PBS or no virus control | Wang et al., 2002 |
| **GDNF** | AAV/Striatum/ 5 weeks *after* | Rats/MFB 6-OHDA | Inconclusive | Not conducted | Apo: 49% ↓ | Not conducted/ 4 weeks | No PBS or no virus control | McGrath et al., 2002 |

*ir = immunoreactive; ↑ = increase; ↓ = decrease; Apo = apomorphine; Amph = amphetamine; No PBS or no virus control = only expression plasmid and reporter plasmid groups examined, which does not allow for assessment of possible cytotoxicity due to viral infection.

glutamatergic neurons of the STN which would transduce this normally excitatory nucleus, overactive in the parkinsonian state, to a structure that would release the inhibitory neurotransmitter (γ-aminobutyric acid (GABA) at its terminals in the SNpr.

Only one study to date has reported on gene transfer of GAD to the STN (Luo et al., 2002). AAV vectors that separately encode two different isoforms of GAD were stereotactically injected into the STN of rats receiving complete 6-OHDA lesions and unlesioned rats. Transgene expression of both GAD enzyme isoforms within the STN was confirmed up to 5 months after vector injection. In lesioned rats, GAD65 gene transfer generated a fourfold increase in release of GABA within the SNpr after STN stimulation. In addition, rats injected with GAD65 AAV 3 weeks prior to lesion exhibited a 65% reduction in apomorphine-induced rotations, near normalization of head position bias, and significant improvements in forelimb use at 8 and 16 weeks after lesioning. Most surprisingly, 35% of nigral DA neurons were protected from degeneration with GAD65 gene transfer. The authors provide a mechanism for this GAD65-mediated gene transfer protection by referring to previous work demonstrating the involvement of excitatory inputs from the STN in nigral DA neuron death induced by neurotoxin (Nakao et al., 1999). Therefore, as is the case with all neuroprotection gene transfer strategies, it is unclear whether the GAD gene transfer approach will confer protection of nigral DA neurons in the parkinsonian brain or whether this protection is specific to the 6-OHDA neurotoxin. Nontheless the authors propose that their GAD gene transfer strategy will not only moderate parkinsonian symptoms by inhibiting STN activity, as with DBS, but that the conversion of excitatory STN projections to inhibitory projections may provide an additional advantages over DBS. Despite no published reports utilizing this strategy in non-human primates, a clinical trial utilizing this GAD gene transfer strategy in Parkinson's patients is currently underway (Oransky, 2003; During et al., 2001).

## 6. Conclusions

In summary, in vivo gene delivery of DA biosynthetic enzymes, antiapoptotic factors, neurotrophic factors, and GAD represents exciting opportunities for the treatment of PD. Comparative analysis to determine the relative efficacy of differing approaches is difficult due to the variety of viral vectors and titers used. Nonetheless, some general conclusions can be drawn from the studies conducted to date.

Gene transfer of DA biosynthetic enzymes attempts to facilitate symptomatic relief resulting from striatal DA depletion without endeavoring to impact nigral DA neuron degeneration. AADC gene delivery paired with peripheral L-DOPA administration nearly normalizes dopaminergic biochemistry in transduced striatal areas. In addition, the recent development of

viral vectors with large cloning capacities allows for triple transduction with TH, AADC, and GTPCH1, an approach that may lead to more efficient replacement of striatal DA. However, critical work with gene delivery of DA biosynthetic enzymes remains to be performed to demonstrate normalization of functionality. Antiapoptotic gene transfer strategies provide neuroprotection for the existing nigral DA neuron population that may prevent or slow further degeneration. Unfortunately, the state in which these protected DA neurons remain may not truly recapitulate DA neuron functionality. Neurotrophic factor gene therapy with GDNF provides the unique opportunity to both prevent nigral degeneration (neuroprotection) and restore function to injured dopaminergic neurons. In particular, lentiviral delivery of GDNF to parkinsonian rodents and non-human primates has proven to be extremely effective in protecting nigral DA neurons and striatal dopaminergic terminals from degeneration, replenishing striatal DA and eliminating motor deficits. Demonstration of long-term and regulatable transgene expression will be critical to the future success of the GDNF gene transfer approach. Lastly, limited preclinical data suggest that gene transfer of GAD to the STN may simulate the effects of surgical lesions of the STN. Although controversial, it appears that this approach will comprise the first clinical trial utilizing gene transfer in PD patients.

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