Toward Full Restoration of Synaptic and Terminal Function of the Dopaminergic System in Parkinson's Disease by Stem Cells

Ole Isacson, DrMedSci,[1,2,3] Lars M. Bjorklund, MD, PhD,[1,2] and James M. Schumacher, MD[4]

New therapeutic nonpharmacological methodology in Parkinson's disease (PD) involves cell and synaptic renewal or replacement to restore function of neuronal systems, including the dopaminergic (DA) system. Using fetal DA cell therapy in PD patients and laboratory models, it has been demonstrated that functional motor deficits associated with parkinsonism can be reduced. Similar results have been observed in animal models with stem cell-derived DA neurons. Evidence obtained from transplanted PD patients further shows that the underlying disease process does not destroy transplanted fetal DA cells, although degeneration of the host nigrostriatal system continues. The optimal DA cell regeneration system would reconstitute a normal neuronal network capable of restoring feedback-controlled release of DA in the nigrostriatal system. The success of cell therapy for PD is limited by access to preparation and development of highly specialized dopaminergic neurons found in the A9 and A10 region of the substantia nigra pars compacta as well as the technical and surgical steps associated with the transplantation procedure. Recent laboratory work has focused on using stem cells as a starting point for deriving the optimal DA cells to restore the nigrostriatal system. Ultimately, understanding the cell biological principles necessary for generating functional DA neurons can provide many new avenues for better treatment of patients with PD.

Ann Neurol 2003;53 (suppl 3):S135–S148

Experiments demonstrate that while the mammalian adult brain is structurally stable with limited neurogenesis, it is a very plastic system, capable of incorporating transplanted embryonic or endogenous stem, progenitor, or fetal xenogeneic primary neurons into functional neurotransmission.[1] Such new neurons can grow to replace damaged or degenerated neuronal pathways. In clinical trials, despite technical shortcomings, human fetal dopamine (DA)–specified phenotypic ventral mesencephalic (VM) neurons have shown functional capacity in Parkinson's disease (PD) patients. Recently, it has been suggested that unregulated production of DA from grafted fetal neurons is the cause of unwanted dyskinesias in transplanted PD patients.[2] However, preclinical and clinical transplantation work demonstrates that striatal DA terminal release from grafted fetal DA neurons is highly regulated by both intrinsic and extrinsic synaptic and autoreceptor mechanisms, and therefore, at least theoretically, the risk for dyskinesias should be less than with drug treatments.[1,3] In grafted DA neurons, presynaptic DA autoreceptors regulate excess DA release and in vivo infusion of the full DA agonist apomorphine blocks spontaneous DA release.[1,4,5]

The restoration of appropriate cellular and biochemical characteristics of transplanted DA cells can also be shown by behavioral experiments. In a rodent model of parkinsonism, recovery from movement asymmetry is correlated with the rate of cellular maturation of the *donor* species.[1,6] Embryonic stem (ES) cells generating DA neurons also abide by such biological principles.[1,7] Multiple anatomical analyses have demonstrated that specific axon guidance and cell differentiation factors remain in the adult and degenerating brain, providing growth and axonal guidance cues for fetal or ES cells.[8–10]

Potentially of great significance for treatments, the pathology of PD shows a relatively selective loss of DA neurons in the substantia nigra pars compacta (SNc; A9) with a relative sparing of ventral tegmental area (VTA; A10) neurons. Our preliminary studies and reasoning suggest that selective repair by A9 DA cells in the putamen is more likely to contribute to symptomatic relief and prevent motor side effects compared

From the [1]Udall Parkinson's Disease Research Center of Excellence, and [2]Neuroregeneration Laboratories, McLean Hospital/Harvard Medical School, Belmont; [3]Program in Neuroscience, Harvard Medical School, Boston, MA; and [4]Department of Neurosurgery, University of Miami, Miami, FL.

Published online Mar 24, 2003, in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/ana.10482.

Address correspondence to Dr Isacson, Neuroregeneration Laboratories, McLean Hospital/Harvard Medical School, 115 Mill Street, Belmont, MA 02478. E-mail: isacson@helix.mgh.harvard.edu

with implantation of the A10 DA cell group.[6,10] For example, transplanted A9 (AHD2+ and DA D2 receptor+) neurons selectively reinnervate the appropriate motor regions of the affected putamen and correlate better with improved behavioral function in rodent models[10] than non-A9 SNc DA neurons. Because the A9 DA neurons have a better capacity to downregulate DA release than A10 DA neurons by synaptic mechanisms, the A9 neurons are also a priori less likely to have unregulated DA release, that may result in high, low, or unstable DA levels, thereby provoking motor side effects such as dyskinesia that are typically seen in PD patients after long-term L-dopa or DA agonist treatment.[11–13]

Development of a New Treatment Modality: The Conceptual and Technical Hurdles of Cell versus Neurotransmitter Replacement

Clinical transplantation studies in the 1980s involved autologous transplantation of catecholamine-containing adrenal medulla cells.[14,15] The lack of objective improvement in PD signs, the low adrenal medulla graft survival, and the reported morbidity of patients lowered enthusiasm for this procedure and led to trials of fetal neural mesencephalic donor cells instead of adrenal medullary cells (that produces minute nonsynaptic DA even after NGF stimulation). Since 1990, some success has been achieved with fetal DA prototype cell replacement methodology, but the quality of cell implantation technology and cell preparations using fetal DA cells have so far been variable and highly experimental.

The development of brain cell transplantation with embryonic neurons and glia is innovative both from a technical and biological standpoint and thus will require much work to optimize the transplant protocol. The scaling up of this method from rodents to primates has proved very challenging, particularly in obtaining an acceptable, abundant, and reliable donor cell source and tissue preparation. Early studies indicated some motor improvement associated with increased fluorodopa uptake after transplantation of cell suspensions containing fetal nigral DA neurons.[16] Longitudinal data from Lindvall and colleagues indicate stable DA cell survival and function in patients for almost a decade after surgery and a substantially reduced need for pharmacological treatment with dopaminergic drugs.[3] The transplantation of nondissociated human ventromesencephalic (VM) tissue pieces also has been reported to provide benefits to advanced PD patients.[17,18] In a series of pilot transplantation studies conducted by Olanow and colleagues in the United States, autopsy from two bilaterally transplanted (6.5–9-week human fetal VM) patients who died 18 to 19 months after surgery from unrelated causes, showed more than 200,000 surviving DA neurons.[19] Implanted tissue *partially* reinnervated the right putamen (approximately 50%) and the left putamen (only approximately 25%). Electron microscopy showed axodendritic and occasional axoaxonic synapses between graft and host, and analysis of tyrosine hydroxylase (TH) mRNA showed higher expression within the fetal neurons than within the residual host nigral cells.[19] Autopsy of another patient in this study group showed more than 130,000 surviving DA neurons reinnervating almost 80% of the putamen.[20] Notably, both patients experienced major improvement in motor function and increased fluorodopa uptake in the putamen on positron emission tomography (PET) scanning. Nevertheless, these and other detailed observations indicate that many specific regions of the human putamen may not be innervated by such nonspecific VM grafts unless they contain the appropriate number and type of the A9 DA neuronal phenotype (O. Isacson, unpublished observations).

Methodological Developments of a New Technology

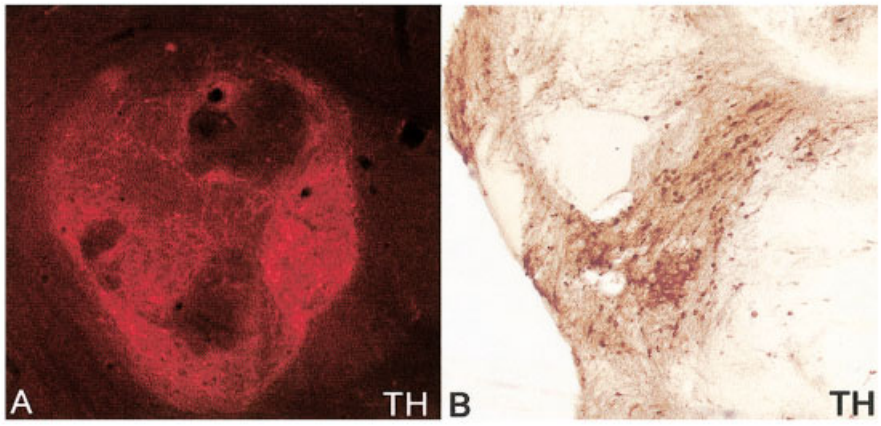
There are many variables that influence the survival of implanted DA neurons, and the specific protocol used for transplantation of donor cells and associated cell-based procedures is very important and likely to influence the clinical result. For example, there are major differences in the way cells are prepared in the pilot clinical trials that have been performed for PD. Freshly dissected fetal tissue pieces (minute cubic millimeter pieces) can be treated with proteolytic enzymes and then dissociated into a cell suspension for implantation.[21] In contrast, other approaches such as the so-called "noodle" technique[2] utilize an intermediate cell culture step. The pretransplantation growth in a cell culture dish may alter the cells and select for cell types that are different than the populations present in fresh preparations. In addition, current PD transplantation studies use rather crude cell preparations derived from fetal mesencephalon. This tissue contains only approximately 10% newborn DA neurons; the rest are cells of types not generally relevant to PD degeneration.[21] In addition, whereas there is selective degeneration of SNc (A9) neurons with a relative sparing of VTA (A10) neurons in PD,[22–25] both of these DA cell groups currently are transplanted as a mixture.[2,19,26,27] These two subpopulations of DA neurons within the SNc have very different functions and project to different brain areas (importantly also within the SN through dendritic release). The midline positioned DA neurons (known as area A10)[28] selectively send axons to limbic and cortical regions,[29] whereas the adjacent A9 DA neurons (primarily affected in PD) grow selectively to putamenal motor areas.[30] Thus, understanding differences and selecting the correct population of DA A10 and A9[10,31] is potentially very important for under-

standing how to optimize cell replacement strategies. Storing or cell culture of DA neurons before transplantation may cause in vitro–based selection favoring the A10 neurons because they are less sensitive to oxidative stress than A9 cells, but possibly less relevant to PD.[32] Topographical placement of the tissue strands, continued L-dopa treatment with free radical generation, and phenotypic DA cell differences are other potentially important variables that might influence the likelihood of obtaining maximal benefits for PD patients. In summary, whereas current work demonstrates a clear capacity of fetal dopamine cell transplants to repair PD brains,[3,33,34] there are technical limitations with available prototypes utilizing this methodology. Both animal model experiments and clinical trials are limited by today's mixed cell preparations of low DA neuronal yield, and potentially the loss of DA cell subpopulations of therapeutic interest during the tissue incubation, cell culture, and preparation steps. Furthermore, it is neither practical nor perhaps ethical to use fetal tissue as a source for transplantation for PD in more than rare experimental situations. For example, to replace a sufficient number of DA neurons, one needs six to eight fetal donors per patient, primarily because of the low posttransplantation survival rate of grafted fetal DA neurons. It is, however, encouraging that recent research in stem cell biology may provide a solution to this problem of low cell access and yield.[7]

Embryonic Stem Cells

Previous work has shown that DA neurons can be expanded in a cell culture dish from both growth factor expanded embryonic day 12 (E12) VM precursor cells[35] or ES cells,[36,37] as well as from Nurr-1–transfected mouse progenitor clones cultured with midbrain type 1 astrocytes.[38] Remarkably, undifferentiated ES cells injected directly into living tissue (brain or kidney capsule) spontaneously differentiate into neurons[39] (Fig 1). We have further demonstrated that ES cells implanted in low numbers into a DA-depleted striatum can develop and function as replicas of the DA neurons lost in PD (Fig 2). These new cells restore changes in amphetamine-induced motor behaviors and cortical activation[7] in animal models of PD (see Fig 2). Such findings suggest that ES cells are a reasonable cell source for transplantation in PD and could overcome the problem associated with using fetal primary neurons (predifferentiated) or fetal VM-expanded precursor cells,[35] both of which show low survival rates after transplantation (approximately 3–5% of the grafted DA neurons survive transplantation).[35,40] In contrast, the direct ES cell transfer to the brain has a high in vivo yield with an up to fourfold expansion after implantation of low numbers of ES cells (approximately 1,000) and differentiation of a portion of them into DA neurons (see Fig 2).[7] Vigorous basic research that defines the developmental sequences and repair mechanisms involved in fetal and stem cell–derived DA neuron cell therapy therefore has the potential to provide a future modality for the treatment of PD. Nonetheless, the growth potential of the implanted fetal nigral and ES cells needs to be tempered and controlled, and the risk associated with the growth of nonneural tissues from the endodermal and mesodermal germ layers needs to be eliminated.[7] This can be accomplished by blocking activation or influence of mesodermal and endodermal cell fate inducers.[7]

Fig 1. Midbrain dopamine neuronal development from embryonic stem (ES) cells after transplantation to (A) thalamus or (B) kidney capsule. Tyrosine hydroxylase (TH) staining showing ES cell–derived dopaminergic (DA) neurons after transplantation into non-DA target areas such as thalamus (A) or the kidney capsule (B). This indicates that the target site is not instructive toward DA phenotype. However, significant DA fiber outgrowth from grafted ES cells will occur only in normal target areas as seen by the very sparse (A) TH fiber density outside the thalamic grafts (A) compared with a ES cell graft in the striatum (see Fig 2B).



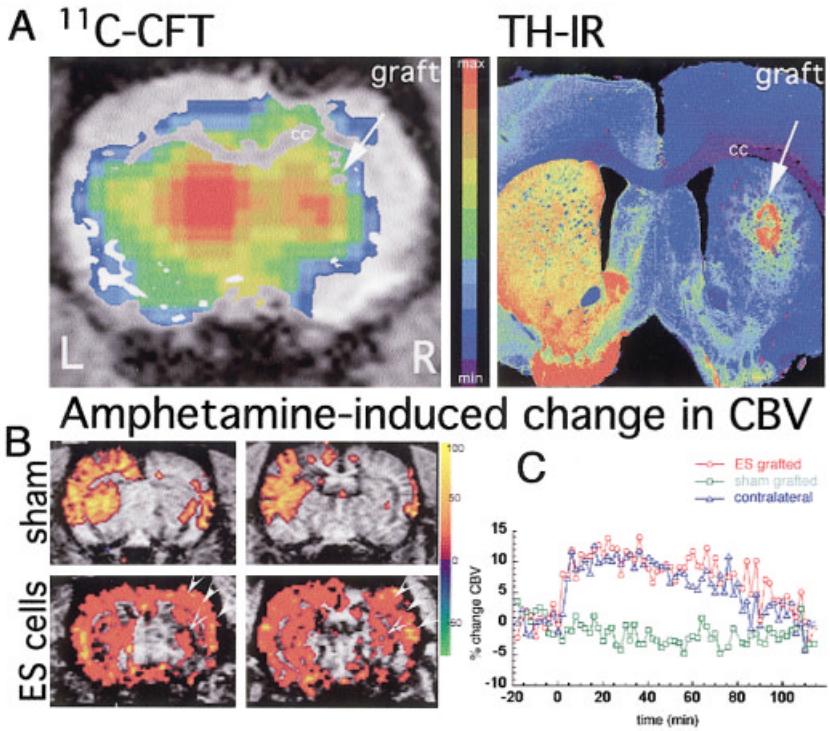


Fig 2. In vivo imaging of dopamine neurons after transplantation of mouse embryonic stem cells to the adult dopamine dennervated rat striatum (A) positron emission tomography (PET) imaging using the specific dopamine transporter (DAT) ligand carbon-11–labeled 2β-carbomethoxy-3β-(4-fluorophenyl) tropane (^{11}C-CFT) showing specific binding in the right grafted striatum, as shown in this brain slice (A, left panel) acquired 26 minutes after tail vein injection of the ligand. Color-coded (activity) PET images were overlaid with magnetic resonance imaging images for anatomical localization. The increased ^{11}C-CFT binding in the right striatum correlated with postmortem presence of TH-immunoreactive (IR) neurons in the graft (A, right panel). (B) Animals receiving embryonic stem (ES) grafts showed restored DA release mediated neuronal activation in response to amphetamine (2mg/kg). Color-coded maps (percentage change) in relative cerebral blood volume (rCBV) in an animal with an ES cell–derived DA graft are shown in three slices spanning the striatum. Only ES cell–grafted animals showed recovery of signal change in motor and somatosensorial cortex (arrows)*, and this was also seen, although to a minor extent, in the striatum. (C) Graphic representation of signal changes over time in the same animal as shown in B. The response in grafted* (red line) *and normal* (blue line) *striata was similar in magnitude and time course, whereas no changes were observed in sham-grafted dopamine-lesioned animals* (green line)*. Baseline was collected for 10 minutes before and 10 minutes after MION injection, and amphetamine was injected at time 0. cc = corpus callosum. (From Bjorklund et al.[7] with permission).*

Obtaining Structural Reconstitution of Terminal Synaptic Function and Regulated Dopamine Release by New Dopamine Neurons

The most important factor to consider in trying to obtain optimal functional effects (and minimal side effects) in PD by brain repair is probably the establishment of new synapses and DA transmission that adequately adapts to the local milieu and provides physiologically appropriate DA release in the host nigrostriatal system. Transplanted fetal DA neurons have been shown to produce new connections with mature host striatal neurons. Normal-appearing synaptic connections between transplanted fetal DA cells and host cells, as well as afferents from host neurons to transplanted cells, have been extensively documented.[41,42] Of both theoretical and practical relevance are analyses showing that pharmacological delivery of dopamine into the striatum may not be as effective in ameliorating the motor symptom of PD, as regulated, synaptic dopamine release that can be obtained with transplanted DA neurons.[43] Nonregulated dopamine release can be associated with serious problems. When DA is directly administered into the ventricle of PD patients, psychosis and motor abnormalities can develop.[44] In addition, differential display experiments show abnormal upregulation of more than 10 genes within the striatum after abnormal DA exposure in vivo.[45] Complications associated with unregulated DA levels are apparent with long-term L-dopa administration: as PD progresses and DA neurons and synapses continue to degenerate, nonphysiological delivery of DA to the striatum is associated with abnormal downstream activity in the basal ganglia and severe motor abnormalities such as dyskinesias and motor fluctuations. Current evidence indicates that DA-mediated regulation of striatal neuronal and network interactions is of critical impor-

tance for normal motor control, and that if DA release is not regulated, glutamatergic synapses are less able to provide normally control of striatal GABAergic output neurons.[46,47] Indeed, intermittent stimulation of dopamine receptors such as may occur with L-dopa treatment in the dopamine denervated state could be the cause of the induction of dyskinesia. Normally, there can be phasic increases in the firing rate of dopamine neurons in several situations such as anticipation of reward, at least in animal models, but these changes in firing rate do not normally raise or increase the extracellular concentrations of dopamine because the synaptic network and terminals work to reduce fluctuations in DA concentration through reuptake mechanisms and possibly other autoreceptor mediated functions.[47] In neurophysiological recordings, it is clear that dopamine provides a modulatory role for the glutamate-mediated transmission, so that it appears to have a gating function at that important striatal synapse.[46–52] Physiologically appropriate DA functions can be achieved by normal DA terminal and synapse activity or, alternatively, by implanted cells that express the complete set of feedback elements required to regulate the release and uptake of DA.[4,6,53] Indeed, fetal DA transplants have been shown to reduce the incidence of L-dopa–induced dyskinesias in 6-OHDA–lesioned rodents.[54] Similarly, L-dopa–induced dyskinesias in nonhuman primates also are reduced after fetal DA cell transplantation.[1]

Several studies also have shown normalized metabolic activity throughout the basal ganglia after DA neural transplantation. Using cytochrome oxidase histochemistry as an indicator of neuronal metabolism in the 6-OHDA–lesioned rat, Nakao and colleagues have shown intrastriatal VM grafts to normalize the lesion-induced increases in cytochrome oxidase activity of the entopeduncular nucleus and SN reticulata, whereas the lesion-induced increases in globus pallidus and subthalamic nucleus were not changed by grafting.[55] Similarly, in MPTP-treated monkey receiving VM transplants, DA cell implants increased the metabolic activity of the implanted striatum, particularly in the region of grafts containing greater numbers of DA neurons and higher levels of DA innervation.[56]

PET and carbon-11–labeled 2B-carbomethoxy-3B-(4-fluorophenyl)tropane (11C-CFT) also can be used to visualize and quantify striatal presynaptic DA transporters in PD patients and PD models. In one study, a unilateral lesion of the nigrostriatal system in rodents, reduced the binding ratio to 15 to 35% of the intact side. After fetal DA neuronal transplantation, behavioral recovery occurred gradually and only after the 11C-CFT binding ratio had increased to 75 to 85% of the intact side. This suggests that there is a threshold for functional recovery in the lesioned nigrostriatal system after neural transplantation consistent with our understanding of the normal DA system requirements.[57] Importantly, autoregulation of DA release and metabolism by intrastriatal DA cell–containing grafts has been shown by in vivo microdialysis in the striatum. Infusion of a nonselective DA agonist (apomorphine) almost abolished endogenous DA release in the DA neuron–grafted striatum,[58,59] showing a normal autoregulation of DA levels by the implanted cells. Further proof for the formation of functional DA terminals and synapses after transplantation of fetal DA neurons has been observed in rodents.[54,60] In these studies, appropriate DA release and regulation were observed even after repeated L-dopa injections.

These data indicate that DA levels within the transplanted striatum can be regulated in a functional manner by correctly transplanted DA neurons if they appropriately innervate and form functional cellular communications with their normal target areas.[1,6] Below, we describe some of these important variables in detail as they have been evaluated in animal model experiments and the early observations that have been made in clinical applications to date.

Subpopulations of Midbrain Dopaminergic Neurons Perform Different Functions and Reach Different Targets

An important issue to consider in neural transplantation is the capacity for specific neuronal cell types to selectively reinnervate specific denervated host target regions.[8,9,61,62] Transplanted neurons developed from fetal or embryonic stem cell stages display a relative specificity in axonal outgrowth such that they tend to reinnervate regions that are typical of their mature phenotype.[8,9,61,62] Midbrain DA neurons can be clearly categorized into subpopulations based on differences in their expression of certain proteins such as the dopamine transporter, TH, calbindin and cholecystokinin (CCK), and their specific target neuron selection.[61,63,64] The biochemical markers expressed reflect the propensity of the different categories of dopamine neurons to project to specific target areas. Schultzberg and colleagues[61] have demonstrated that subsets of SN DA neurons derived from embryonic VM tissue and implanted into the dorsal deafferented striatum show distinct patterns of axonal terminal networks. CCK-negative/TH (A9) cells extend their axons into the striatum. In contrast, CCK-positive/TH neurons (A10) did not but rather extended their axons in cortical areas typical of their normal patterning. Another interesting protein is the retinoic acid–generating enzyme, aldehyde dehydrogenase 2 (AHD2), which is expressed only in a subset of A9 DA neurons in the SN.[65] These neurons project to the dorsal and rostral regions of the striatum and have a reduced DA terminal density gradient in more ventral regions. Transplanted DA neurons appear to extend fibers in a nonspecific heliocen-

tric fashion around the graft, whereas the subset of transplanted DA fibers expressing AHD2 provide preferential reinnervation to the dorsolateral part of striatum.[10] Histological observations of postmortem patient tissue (O. Isacson, unpublished data) also indicate that transplanted human DA neurons only seek their normal targets (and actively avoid innervation of regions of the caudate-putamen that their specific phenotype would not normally innervate). Interestingly, the AHD2 enzyme is very sensitive to oxidation, which may relate to the increased vulnerability of A9 neurons seen in PD.[65] Because only 2 to 10% of transplanted VM neurons are of a DA phenotype, hypothetically they may compete for trophic support with the majority of transplanted cells which are non-DA neurons. It is well known that midbrain DA neurons are positively influenced by growth factors such as basic fibroblast growth factor[66] and GDNF.[67, 68] Such trophic dependency may vary between midbrain DA cell subpopulations, and it is likely that a limited supply of trophic molecules influences DA cell survival and growth. In this regard, it is noteworthy that 20 to 80% of the transplanted SN cell population normally undergoes programmed cell death between days P2 and P14.[69]

Transplanted fetal VM-derived DA neurons develop extensive axonal terminal networks in the host striatum and nucleus accumbens in a normal appearing, dense, homogeneous fashion.[70,71] By contrast, identical phenotypic DA neurons from fetal or stem cell derivation transplanted into nontarget areas such as cortex, thalamus, and hypothalamus only extend a few axons into the host brain[72] (see Fig 1). Ultrastructural data show that grafted DA neurons are able to form appropriate and abundant synaptic connections with medium-sized spiny striatal neurons, which are the primary target of the mesencephalic DA afferents.[73] The molecular mechanisms defining anatomical specificity of the DA projections have not been fully elucidated, but they are likely to involve target-derived trophic signals and recognition of target-specific cell surface molecules by growth cones.

Does the Graft Tissue Type, Surgical Cell Preparation, or Specific Surviving Dopaminergic Neuronal Phenotype Influence the Degree of Functional Recovery?

We have demonstrated[10] that a nonlinear regression function exists between DA neuron survival and extent of functional motor recovery. For example, in the unilateral dopamine-lesioned rodent, approximately 590 rat-derived DA cells in the graft were necessary to obtain a 50% reduction in motor asymmetry using solid VM transplants. A plateau in motor recovery was reached with implantation of approximately 1,200 TH-positive neurons. Interestingly, using pieces (nondissociated grafts), the number of cells required for a 50% reduction in rotation is higher than comparable values in the literature for cell suspension grafts.[10] There may be several reasons for differences in efficacy between cell suspension and solid grafts. Single cell suspension grafts become more vascularized than multiple-fragment transplants.[74] Furthermore, Nikkah and colleagues[75] showed that smaller suspension grafts provide relatively better axonal coverage of the striatum compared with larger grafts. Large grafts, whether transplanted as cell suspensions or as tissue pieces, actually may limit direct access to host tissue cues causing grafted cells to have less interaction with the host and integrate more within the actual transplanted tissue. These data suggest that single fetal cells transplanted in small deposits or possibly endogenous progenitor cells are more likely to be better integrated into the host brain and to elicit behavioral recovery more efficiently.[21,76,77] Cografting of embryonic striatal tissue with fetal VM show that such grafts result in enhanced DA neuronal survival and function through trophic support.[78] Thus, graft–host interaction and graft–graft interaction are both factors that regulate fetal VM transplant development. Interestingly, we have established that the degree of reduction in rotations 10 weeks posttransplantation is primarily associated with the proportion of AHD2-positive A9 DA neurons in the grafts.[10] An inverse tendency was also apparent with the presence of A10 neurons. This observation suggests that the greater the proportion of A9 neurons in cell transfer or repair, the greater the degree of symptomatic recovery of motor signs one might be able to obtain.

The importance of proper development of appropriate molecular and phenotypic cell characteristics can be illustrated by studies of the functional effects of transplanted xenogeneic DA neurons. Thus, in a rat model of parkinsonism, recovery from amphetamine-induced motor asymmetry is tightly linked to the normal developmental rate of the species from where the grafted DA neurons are derived.[6,7] Similar correlations can be noted for the size of the brain of the species from which the xenografts is derived. For instance, Galpern and colleagues[79] reported that between 80 and 120 TH-positive neurons derived from larger species were required to see the same as was obtained using 120 to 140 rodent DA neurons. This observation is consistent with the concept that axonal growth areas are more extensive with dopamine neurons derived from animals with larger brains.

Can We Produce Better Therapies with Fewer Side Effects by Accomplishing Specific Cellular and Synaptic Replacement?

Recently, it has been postulated that excess and unregulated production of DA from grafted fetal neurons could be responsible for some unwanted side effects

seen in transplanted PD patients.[2] However it has been convincingly demonstrated that striatal DA release is normally tightly regulated by both intrinsic and extrinsic mechanisms. Under normal circumstances, presynaptic dopamine autoreceptors regulate excess DA release, and administration of the DA agonist apomorphine can inhibit spontaneous DA release up to 100%.[5] Half of this inhibition has been attributed to a direct effect on DA autoreceptors and the other half is thought to involve postsynaptically mediated short and long distance feedback circuits.[5] It has now been shown that grafted fetal DA neurons also display similar autoinhibition mechanisms as demonstrated by a 40% reduction in spontaneous DA release when grafted animals are treated with apomorphine.[1,4] These basic studies are important as they provide evidence contrary to current theories, suggesting that implanted DA neurons may release excess amounts of DA in an unregulated manner, thereby leading to side effects such as dyskinesias.[2] Such side effects as have been observed may have several alternative explanations.[11] DA released from nerve terminals into the synaptic cleft is normally rapidly taken up and transported back into the terminals by the dopamine transporter (DAT). This mechanism is important for appropriate temporal regulation of DA concentration in the synaptic cleft. When the DAT is blocked by agents such as cocaine, DA will remain in the synaptic cleft in higher concentrations and for longer time periods than normal, thus allowing increased binding and activation of postsynaptic receptors. When fetal ventral midbrain is dissected before transplantation, most published protocols do not make any distinction between the DA neurons residing in the VTA (A10) and those in the SNc (A9).[2,19,26,27] These two midbrain subpopulations of DA neurons express different levels of DAT,[64] project to different areas of the brain,[80] and show different response to growth factors.[68,81] In addition, PD patients show a relative sparing of DA neurons in the VTA compared with the SNc indicating that the VTA DA neurons are less vulnerable than their SNc counterparts.[22–25] One possible explanation for the reported dyskinesias in 5 of 37 patients in the study reported by Freed and colleagues[2] could be that culturing the fetal VM dopamine neurons for 2 to 4 weeks before implantation may result in selective survival of the less sensitive VTA DA neurons.[11] When such neurons are implanted into the putamen of PD patients, they may form inappropriate connections with or avoid the projection neurons of the putamen, because these are not their normal targets.[6,11] In addition, differences in DAT expression between subpopulations of DA neurons[64,82,83] also may result in abnormal dendritic DA release[84] and uptake patterns that could cause suboptimal DA transmission. Another possibility for uncontrolled motor response after transplantation is that the implantation procedure may in some instances create a small lesion in the putamen with subsequent dysregulation of the GABAergic output neurons. This theory is supported by the fact that small lesions in the striatum of primates make these animals dyskinetic in response to DA agonist treatment.[85–87]

How Can Stem Cell Biology Research Help Parkinson Patients?

Most living systems undergo continuous growth. In humans, bone marrow stem cells are capable of dividing into most of the cells necessary for blood and immune systems. Even entire tissues or organs, such as the liver, can be regrown. Cells in the lining of the gut are shed and replaced on a daily basis, and, in the skin, the basal cell layers of the dermis provide a continuous supply of growth. In adult mature mammalian cell systems, however, it is typical that only organ-specific and specialized progenitor cells divide to maintain growth of organ systems in the body, whereas pathological processes can limit such replenishment. In contrast, a fertilized cell is capable of cell divisions that grow logarithmically, and in the early cluster of cells (in the range of 250 cells) each cell is capable of forming any germ layer and any part of the body plan.[88] This type of cell therefore is denoted a stem cell, or in this case, an ES cell. Recently, such divisible (yet nonmalignant and noncarcinogenic) cells have gained increased attention. In a more restricted scientific context, the concept and methodology for producing dopamine cells derived from stem cells has intrigued both neurobiologists and clinically oriented scientists, insofar as this information could help to explain the biology of developing dopamine cells and what controls their fate, as well as providing an abundant source of optimal dopamine cells for clinical application in conditions such as PD (Fig 3).

Several genes are known to relate to the development or control of dopaminergic identity and specialization (eg, sonic hedgehog protein, Pitx3, and Nurr-1) and to act in concert with other transcription factors to activate specific transmitter enzymes (eg, TH, DAT, and dopa-decarboxylase) in dopaminergic neurons[89–91] (Fig 3). Upregulation of these genes in ES cells could lead to the development of dopamine cells. An alternate or different route to cell repair is the possibility of manipulating inherent neurogenesis in the adult brain.[92] For example, in the adult brain neural precursor cells are embedded in the subventricular zone that are capable of migration and differentiation into different neural cell types.[93] These neural progenitor cells can be expanded and studied, possibly even as a neuronal repair tool.[94] The expansion of subventricular zone neuroprecursor cells is stimulated by delivery of basic fibroblast growth factor, brain-derived neurotrophic factor (BDNF),[95] noggin,[96] ciliary neurotrophic factor,[97] or

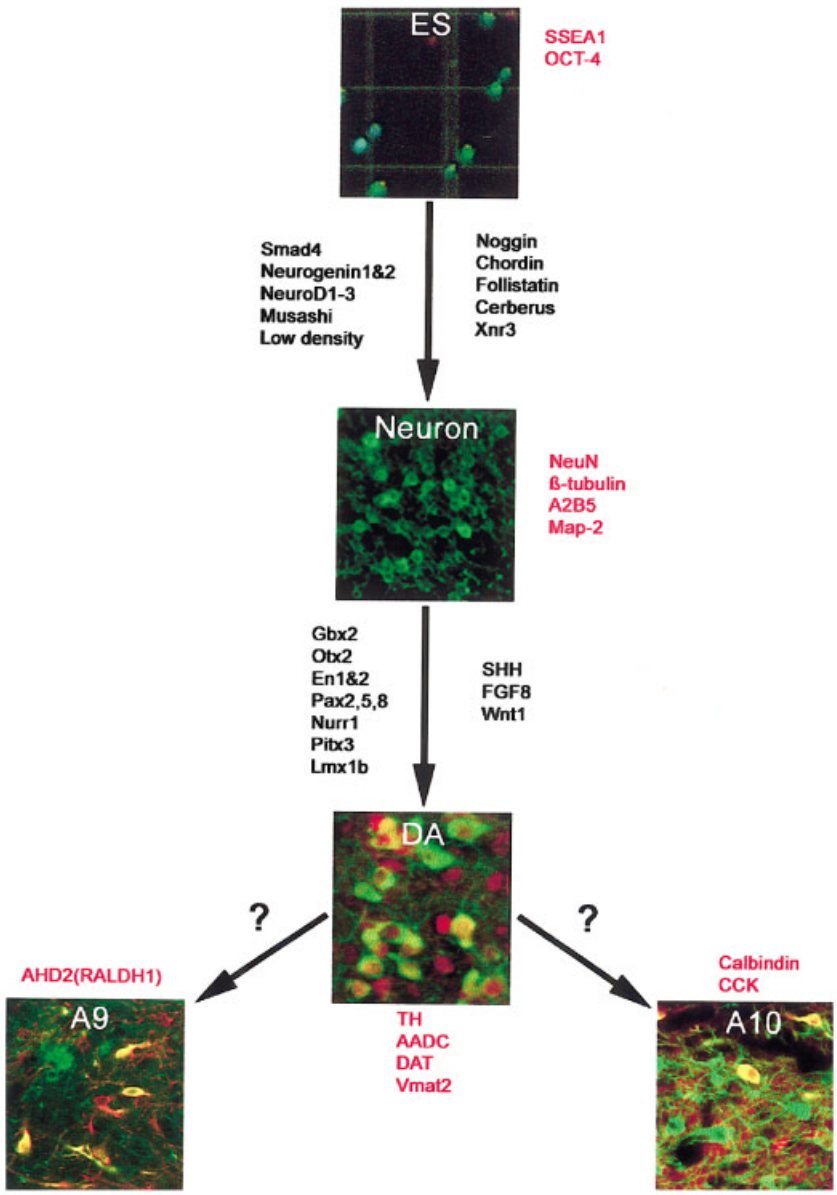


Fig 3. Midbrain dopamine neuronal development from embryonic stem (ES) cells. Schematic illustration of known developmental factors involved in the identification and generation of midbrain dopamine (DA) neurons from mouse ES cells. ES cells can be identified by expression of embryonic markers such as stage specific embryonic antigen 1 (SSEA-1) or OCT-4. ES cells can adopt neuroectodermal fate through a "default" mechanism involving inhibition of transforming growth factor (TGF)–β related molecules such as activin and bone morphogenetic proteins (BMPs) as well as TGF-β downstream targets like smad4. Factors known to be of importance for "default" neuralization are the so-called "BMP inhibitors" Noggin, Chordin, Follistatin, Cerberus, and Xnr3 as well as culturing or transplanting ES cell in low density to avoid autocrine and paracrine TGF-β signaling. Cells of neuroectodermal lineage are believed to adopt a neuronal fate under the influence of basic helix-loop-helix (bHLH) factors such as NeuroDs and Neurogenins or other factors such as Musashi 1 and 2. Neuronal phenotype can be identified by expression of β-tubulin, neuronal nuclear antigen (NeuN), A2B5 antigen, or microtubule-associated protein 2 (MAP-2). Midbrain DA neurons are generated the intersection between midbrain and hindbrain in response to a ventral-dorsal gradient of floor plate–derived sonic hedgehog (SHH) and a anterior-posterior gradient of fibroblast growth factor 8 (FGF-8). Factors known to be of importance for proper development of midbrain DA neurons are Gbx-2, Otx-2, Pax 2, 5, 8, Wnt-1, Nurr 1, Pitx-3, and Lmx1b. Midbrain DA neurons that express tyrosine hydroxylase (TH), L-aromatic amino acid decarboxylase (AADC), the dopamine transporter (DAT), and the vesicular monoamine transporter 2 (Vmat-2), will, through yet unknown mechanisms, develop into functionally and anatomically distinct subpopulations such as the A9 (aldehyde dehydrogenase 2, also known as retinaldehyde 1 [Raldh1] expressing) and A10 (calbindin and cholecystokinin [CCK]) expressing.

epidermal growth factor[98] to the cerebrospinal fluid. Migration into the parenchyma can be stimulated by infusion of transforming growth factor (TGF)–α into a target region.[99] Neuronal differentiation from precursors is enhanced by GDNF, BDNF,[100] and natural BMP receptor antagonists.[96] Expression of a dopaminergic phenotype can be driven by transcription factors such as Pitx3[101] and Nurr1, which drive genes of the full DA neuronal phenotype such as TH, DAT, and components of trophic factor receptors[102–104] (see Fig 3). Furthermore, release of GDNF or BDNF by genetically modified cells in the caudate-putamen can increase survival of precursor cells and of dopaminergic neurons in the lesioned SN,[105] and the programmed cell death process also can be temporarily suspended by antiapoptotic factors, for example, XIAP.[106] Interestingly, genetically modified cells also can serve as biological pumps to produce growth factors that sustain neurons on site in the striatum or projecting dopaminergic neurons from the SN.[105,107,108]

In summary, stem cell biology is valuable as a means of understanding the mechanisms leading to dopamine neuronal formation and as a potential new treatment for PD. For example, research on ES cells may demonstrate the genetic transcription factors that control the specific genes participating in DA orchestrated neuronal function and cell development (see Fig 3). Genetic programs have dynamic components; for example, concerted actions of growth factors or neurotrophic factors that act as molecular switches are required for initiating and maintaining the function of a DA neuron. The knowledge of how DA neurons can be formed might provide a means for industrially producing many such cells. These cells could be used for effective cell transplantation or cell therapy in which replacement cells could be implanted under local anesthesia to brain regions that have lost greater than 60 to 80% of their normal human set (500,000 to 1,000,000 dopamine cells in the human brain, SN region).[109] Stem cell biology related to brain development and repair can be approached using either adult or ES cells that are potentially capable of generating new neurons after selective expansion either in cell culture systems or in the living brain. The investigation of ES cells also provides a system in which transcription factors responsible for directing the typical dopaminergic cell fate in the nigrostriatal systems can be determined (see Fig 3). This allows a sophisticated understanding of the factors controlling the specialization and health of such cells. All of these types of investigations could help to clarify mechanisms responsible for pathological stress, toxic events, or genetically induced cell dysfunction.

In conclusion, recent discoveries elucidating the cell biology of dopaminergic neurons allow for the development of both sequential and parallel strategies for treating and restoring function in Parkinson's patients[110] (see Fig 2). The rapidly developing understanding of pathological mechanisms in PD and the life cycle of the dopamine neuron from stem cells, via progenitor cells, to adult and later aging dopamine neurons provides opportunities for new interventions to reverse the effects of this disease.

This work is supported by the following US federal grant awards to O.I.: Udall Parkinson's Disease Research Center of Excellence (P50 NS39793), DAMD17-01-1-0762, RO1-NS-41263, and RO1-NS-30064. Support from the Kinetics Foundation and the Parkinson Foundation of the National Capital Area is also gratefully acknowledged.

References

1. Bjorklund LM, Isacson O. Regulation of dopamine cell type and transmitter function in fetal and stem cell transplantation for Parkinson's disease. Prog Brain Res 2002;138:411–440.
2. Freed CR, Greene PE, Breeze RE, et al. Transplantation of embryonic dopamine neurons for severe Parkinson's disease. N Engl J Med 2001;344:710–719.
3. Piccini P, Brooks DJ, Bjorklund A, et al. Dopamine release from nigral transplants visualized in vivo in a Parkinson's patient. Nat Neurosci 1999;2:1137–1140.
4. Strecker RE, Sharp T, Brundin P, et al. Autoregulation of dopamine release and metabolism by intrastriatal nigral grafts as revealed by intracerebral dialysis. Neuroscience 1987;22: 169–178.
5. Zetterstrom T, Ungerstedt U. Effects of apomorphine on the in vivo release of dopamine and its metabolites, studied by brain dialysis. Eur J Pharmacol 1984;97:29–36.
6. Isacson O, Deacon TW. Neural transplantation studies reveal the brain's capacity for continuous reconstruction. Trends Neurosci 1997;20:477–482.
7. Bjorklund LM, Sánchez-Pernaute R, Chung S, et al. Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model. Proc Natl Acad Sci USA 2002;99:2344–2349.
8. Isacson O, Deacon TW, Pakzaban P, et al. Transplanted xenogeneic neural cells in neurodegenerative disease models exhibit remarkable axonal target specificity and distinct growth patterns of glial and axonal fibres. Nat Med 1995;1: 1189–1194.
9. Isacson O, Deacon TW. Specific axon guidance factors persist in the mature rat brain: evidence from fetal neuronal xenografts. Neuroscience 1996;75:827–837.
10. Haque NS, LeBlanc CJ, Isacson O. Differential dissection of the rat E16 ventral mesencephalon and survival and reinnervation of the 6-OHDA-lesioned striatum by a subset of aldehyde dehydrogenase-positive TH neurons. Cell Transplant 1997;6:239–248.
11. Isacson O, Bjorklund L, Pernaute RS. Parkinson's disease: interpretations of transplantation study are erroneous. Nat Neurosci 2001;4:553.
12. Olanow CW, Obeso JA. Preventing levodopa-induced dyskinesias. Ann Neurol 2000;47:167–178.
13. Olanow CW, Tatton WG. Etiology and pathogenesis of Parkinson's disease. Annu Rev Neurosci 2000;22:123–144.
14. Madrazo I, Leon V, Torres C. Transplantation of fetal substantia nigra and adrenal medulla to the caudate putamen in two patients with Parkinson's disease. N Engl J Med 1988; 318:51.

15. Backlund E, Granberg, P., Hamberger, B. Transplantation of adrenal medullary tissue to striatum in parkinsonism. J Neurosurg 1985;62:169–173.
16. Widner H, Tetrud J, Rehncrona S, et al. Bilateral fetal mesencephalic grafting in two patients with parkinsonism induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. N Engl J Med 1992;327:1556–1563.
17. Freeman TB, Olanow CW, Hauser RA, et al. Bilateral fetal nigral transplantation into the postcommissural putamen in Parkinson's disease. Ann Neurol 1995;38:379–388.
18. Freed CR, Breeze RE, Rosenberg NL, et al. Survival of implanted fetal dopamine cells and neurologic improvement 12 and 46 months after transplantation for Parkinson's disease. N Engl J Med 1992;327:1549–1555.
19. Kordower JH, Rosenstein JM, Collier TJ, et al. Functional fetal nigral grafts in a patient with Parkinson's disease: chemoanatomic, ultrastructural, and metabolic studies. J Comp Neurol 1996;370:203–230.
20. Kordower J, Freeman T, Chen E, et al. Fetal nigral grafts survive and mediate clinical benefit in a patient with Parkinson's disease. Mov Disord 1998;13:383–393.
21. Brundin P, Isacson O, Gage F, Bjorklund A. Intrastriatal grafting of dopamine-containing neuronal cell suspensions: effects of mixing with target or non-target cells. Dev Brain Res 1986;24:77–84.
22. Iacopino AM, Christakos S. Specific reduction of calcium-binding protein (28-kilodalton calbindin-D) gene expression in aging and neurodegenerative diseases. Proc Natl Acad Sci USA 1990;87:4078–4082.
23. Yamada T, McGeer PL, Baimbridge KG, McGeer EG. Relative sparing in Parkinson's disease of substantia nigra dopamine neurons containing calbindin-D28K. Brain Res 1990; 526:303–307.
24. Ito H, Goto S, Sakamoto S, Hirano A. Calbindin-D28k in the basal ganglia of patients with parkinsonism. Ann Neurol 1992;32:543–550.
25. Gibb WR. Melanin, tyrosine hydroxylase, calbindin and substance P in the human midbrain and substantia nigra in relation to nigrostriatal projections and differential neuronal susceptibility in Parkinson's disease. Brain Res 1992;581: 283–291.
26. Hauser RA, Freeman TB, Snow BJ, et al. Long-term evaluation of bilateral fetal nigral transplantation in Parkinson disease. Arch Neurol 1999;56:179–187.
27. Lindvall O, Rehncrona S, Gustavi B, et al. Fetal dopamine-rich mesencephalic grafts in Parkinson's disease. Lancet 1988; 2:1483–1484.
28. Dahlstrom A, Fuxe K. Localization of monoamines in the lower brain stem. Experientia 1964;20:398–399.
29. Gerfen CR, Herkenham M, Thibault J. The neostriatal mosaic. II. Patch- and matrix-directed mesostriatal dopaminergic and non-dopaminergic systems. J Neurosci 1987;7: 3915–3934.
30. Damier P, Hirsch EC, Agid Y, Graybiel AM. The substantia nigra of the human brain. I. Nigrosomes and the nigral matrix, a compartmental organization based on calbindin D(28K) immunohistochemistry. Brain 1999;122:1421–1436.
31. Costantini LC, Lin L, Isacson O. Medial fetal ventral mesencephalon: a preferred source for dopamine neuron grafts. Neuroreport 1997;8:2253–2257.
32. German DC, Manaye KF, Sonsalla PK, Brooks BA. Midbrain dopaminergic cell loss in Parkinson's disease and MPTP-induced parkinsonism: sparing of calbindin-D28k-containing cells. Ann N Y Acad Sci 1992;648:42–62.
33. Piccini P, Lindvall O, Bjorklund A, et al. Delayed recovery of movement-related cortical function in Parkinson's disease after striatal dopaminergic grafts. Ann Neurol 2000;48:689–695.
34. Mendez I, Dagher A, Hong M. Simultaneous intraputaminal and intranigral fetal dopaminergic grafts in Parkinson's disease: first clinical trials. Exp Neurol 2000;164:464.
35. Studer L, Tabar V, McKay RD. Transplantation of expanded mesencephalic precursors leads to recovery in parkinsonian rats. Nat Neurosci 1998;1:290–295.
36. Lee SH, Lumelsky N, Studer L, et al. Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. Nat Biotechnol 2000;18:675–679.
37. Kawasaki H, Mizuseki K, Nishikawa S, et al. Induction of midbrain dopaminergic neurons from ES cells by stromal cell-derived inducing activity. Neuron 2000;28:31–40.
38. Wagner J, Akerud P, Castro DS, et al. Induction of a midbrain dopaminergic phenotype in Nurr1-overexpressing neural stem cells by type 1 astrocytes. Nat Biotechnol 1999;17: 653–659.
39. Deacon T, Dinsmore J, Costantini L, et al. Blastula-stage stem cells differentiate into dopaminergic and serotonergic neurons after transplantation. Exp Neurol 1998;149:28–41.
40. Brundin P, Bjorklund A. Survival of expanded dopaminergic precursors is critical for clinical trials. Nat Neurosci 1998;1: 537.
41. Mahalik T, Finger T, Stromberg I, Olson L. Substantia nigra transplants into denervated striatum of the rat: ultrastructure of graft and host interconnections. J Comp Neurol 1985;240: 60–70.
42. Doucet G, Murata Y, Brundin P, et al. Host afferents into intrastriatal transplants of fetal ventral mesencephalon. Exp Neurol 1989;106:1–19.
43. Bjorklund A. Better cells for brain repair. Nature 1993;362: 414–415.
44. Venna N, Sabin T, Ordia J, Mark V. Treatment of severe Parkinson's disease by intraventricular injection of dopamine. Appl Neurophysiol 1984;47:62–64.
45. Gerfen C, Keefe K, Steiner H. Dopamine-mediated gene regulation in the striatum. Adv Pharmacol 1998;42:670–673.
46. Onn S-P, West AR, Grace AA. Dopamine-mediated regulation of striatal neuronal and network interactions. Trends Neurosci 2000;23:S45–S56.
47. Nutt JG, Obeso JA, Stocchi F. Continuous dopamine-receptor stimulation in advanced Parkinson's disease. Trends Neurosci 2000;23:S109–S115.
48. Strecker RE, Jacobs BL. Substantia nigra dopaminergic unit activity in behaving cats: effects of arousal on spontaneous discharge and sensory evoked activity. Brain Res 1985;361: 339–350.
49. Ljungberg T, Apicella P, Schultz W. Responses of monkey dopamine neurons during learning of behavioral reactions. J Neurophysiol 1992;67:145–163.
50. Freeman AS, Meltzer LT, Bunney BS. Firing properties of substantia nigra dopaminergic neurons in freely moving rats. Life Sci 1985;36:1983–1994.
51. Johnson SW, Seutin V, North RA. Burst firing in dopamine neurons induced by N-methyl-D-aspartate: role of electrogenic sodium pump. Science 1992;258:665–667.
52. Grace AA. Phasic versus tonic dopamine release and the modulation of dopamine system responsivity: a hypothesis for the etiology of schizophrenia. Neuroscience 1991;41:1–24.
53. Zetterstrom T, Brundin P, Gage FH, et al. In vivo measurement of spontaneous release and metabolism of dopamine from intrastriatal nigral grafts using intracerebral dialysis. Brain Res 1986;362:344–349.
54. Lee CS, Cenci MA, Schulzer M, Bjorklund A. Embryonic ventral mesencephalic grafts improve levodopa-induced dyskinesia in a rat model of Parkinson's disease. Brain 2000;123: 1365–1379.

55. Nakao N, Ogura M, Nakai K, Itakura T. Intrastriatal mesencephalic grafts affect neuronal activity in basal ganglia nuclei and their target structures in a rat model of Parkinson's disease. J Neurosci 1998;18:1806–1817.
56. Collier T, Redmond DJ, Roth R, et al. Metabolic energy capacity of dopaminergic grafts and the implanted striatum in parkinsonian nonhuman primates as visualized with cytochrome oxidase histochemistry. Cell Transplant 1997;6: 135–140.
57. Brownell AL, Livni E, Galpern W, Isacson O. In vivo PET imaging in rat of dopamine terminals reveals functional neural transplants. Ann Neurol 1998;43:387–390.
58. Strecker R, Sharp T, Brundin P, et al. Autoregulation of dopamine release and metabolism by intrastriatal nigral grafts as revealed by intracerebral dialysis. Neuroscience 1987;22: 169–178.
59. Galpern WR, Burns LH, Deacon TW, et al. Xenotransplantation of porcine fetal ventral mesencephalon in a rat model of Parkinson's disease: functional recovery and graft morphology. Exp Neurol 1996;140:1–13.
60. Gaudin D, Rioux L, Bedard P. Fetal dopamine neuron transplants prevent behavioral supersensitivity induced by repeated administration of L-dopa in the rat. Brain Res 1990;506: 166–168.
61. Schultzberg M, Dunnett SB, Bjorklund A, et al. Dopamine and cholecystokinin immunoreactive neurones in mesencephalic grafts reinnervating the neostriatum: evidence for selective growth regulation. Neuroscience 1984;12:17–32.
62. Nilsson OG, Clarke DJ, Brundin P, Bjorklund A. Comparison of growth and reinnervation properties of cholinergic neurons from different brain regions grafted to the hippocampus. J Comp Neurol 1988;268:204–222.
63. Haber SN, Ryoo H, Cox C, Lu W. Subsets of midbrain dopaminergic neurons in monkeys are distinguished by different levels of mRNA for the dopamine transporter: comparison with the mRNA for the D2 receptor, tyrosine hydroxylase and calbindin immunoreactivity. J Comp Neurol 1995;362: 400–410.
64. Blanchard V, Raisman-Vozari R, Vyas S, et al. Differential expression of tyrosine hydroxylase and membrane dopamine transporter genes in subpopulations of dopaminergic neurons of the rat mesencephalon. Brain Res Mol Brain Res 1994;22: 29–38.
65. McCaffery P, Drager UC. High levels of a retinoic acid-generating dehydrogenase in the meso-telencephalic dopamine system. Proc Natl Acad Sci USA 1994;91:7772–7776.
66. Mayer E, Fawcett JW, Dunnett SB. Basic fibroblast growth factor promotes the survival of embryonic ventral mesencephalic dopaminergic neurons. II. Effects on nigral transplants in vivo. Neuroscience 1993;56:389–398.
67. Mott JL, Eken S, Bowenkamp K, et al. Effects of glial cell line-derived neurotrophic factor on dopaminergic transplants to the 6-OHDA denervated striatum. Abstr Soc Neurosci 1996;22:1492.
68. Meyer M, Zimmer J, Seiler RW, Widmer HR. GDNF increases the density of cells containing calbindin but not of cells containing calretinin in cultured rat and human fetal nigral tissue. Cell Transplant 1999;8:25–36.
69. Janec E, Burke RE. Naturally occurring cell death during postnatal development of the substantia nigra pars compacta of rat. Mol Cell Neurosci 1993;4:30–35.
70. Bjorklund A, Schmidt RH, Stenevi U. Functional reinnervation of the neostriatum in the adult rat by use of intraparenchymal grafting of dissociated cell suspensions from the substantia nigra. Cell Tissue Res 1980;212:39–45.
71. Dunnett SB, Bunch ST, Gage FH, Bjorklund A. Dopamine-rich transplants in rats with 6-OHDA lesions of the ventral tegmental area. 1. Effects on spontaneous and drug-induced locomotor activity. Behav Brain Res 1984;13:71–82.
72. Abrous N, Guy J, Vigny A, et al. Development of intracerebral dopaminergic grafts: a combined immunohistochemical and autoradiographic study of its time course and environmental influences. J Comp Neurol 1988;273:26–41.
73. Clarke DJ, Brundin P, Strecker RE, et al. Human fetal dopamine neurons grafted in a rat model of Parkinson's disease: ultrastructural evidence for synapse formation using tyrosine hydroxylase immunocytochemistry. Exp Brain Res 1988;73: 115–126.
74. Leigh K, Elisevich K, Rogers KA. Vascularisation and microvascular permeability in solid versus cell-suspension embryonic neural grafts. J Neurosurg 1994;81:272–283.
75. Nikkah G, Cunningham MG, Jodicke A, et al. Improved graft survival and striatal reinnervation by microtransplantation of fetal nigral cell suspensions in the rat Parkinson model. Brain Res 1994;633:133–143.
76. Hernit-Grant CS, Macklis JD. Embryonic neurons transplanted to regions of targeted photolytic cell death in adult mouse somatosensory cortex re-form specific callosal projections. Exp Neurol 1996;139:131–142.
77. Sotelo C, Alvarado-Mallart RM. Embryonic and adult neurons interact to allow Purkinje cell replacement in mutant cerebellum. Nature 1987;327:421–423.
78. Costantini LC, Snyder-Keller A. Co-transplantation of fetal lateral ganglionic eminence and ventral mesencephalon can augment function and development of intrastriatal transplants. Exp Neurol 1997;145:214–227.
79. Galpern WR, Burns LH, Deacon TW, et al. Xenotransplantation of porcine fetal ventral mesencephalon in a rat model of Parkinson's disease: functional recovery and graft morphology. Exp Neurol 1996;140:1–13.
80. Graybiel AM, Hirsch E, Agid Y. The nigrostriatal system in Parkinson's disease. In: Streifler MB, Korczyn AD, Melamed E, Youdim MBH, eds. Advances in neurology. New York: Raven Press, 1990:17–29.
81. Johansson M, Friedemann M, Hoffer B, Stromberg I. Effects of glial cell line-derived neurotrophic factor on developing and mature ventral mesencephalic grafts in oculo. Exp Neurol 1995;134:25–34.
82. Sanghera MK, Manaye K, McMahon A, et al. Dopamine transporter mRNA levels are high in midbrain neurons vulnerable to MPTP. Neuroreport 1997;8:3327–3331.
83. Ciliax BJ, Drash GW, Staley JK, et al. Immunocytochemical localization of the dopamine transporter in human brain. J Comp Neurol 1999;409:38–56.
84. Falkenburger BH, Barstow KL, Mintz IM. Dendrodendritic inhibition through reversal of dopamine transport. Science 2001;293:2465–2470.
85. Hantraye P, Riche D, Maziere M, Isacson O. Intrastriatal transplantation of cross-species fetal striatal cells reduces abnormal movements in a primate model of Huntington disease. Proc Natl Acad Sci USA 1992;89:4187–4191.
86. Hantraye P, Riche D, Maziere M, Isacson O. A primate model of Huntington's disease: behavioral and anatomical studies of unilateral excitotoxic lesions of the caudate-putamen in the baboon. Exp Neurol 1990;108:91–104.
87. Burns LH, Pakzaban P, Deacon TW, et al. Selective putaminal excitotoxic lesions in non-human primates model the movement disorder of Huntington disease. Neuroscience 1995;64:1007–1017.
88. Hemmati-Brivanlou A, Melton D. Vertebrate embryonic cells will become nerve cells unless told otherwise. Cell 1997;88: 13–17.

89. Mandel R, Rendahl K, Spratt S, et al. Characterization of intrastriatal recombinant adeno-associated virus-mediated gene transfer of human tyrosine hydroxylase and human GTP-cyclohydrolase I in a rat model of Parkinson's disease. J Neurosci 1998;18:4271–4284.
90. Marsden CD. Basal ganglia disease. Lancet 1982:1141–1147.
91. Montgomery R, Warner M, Lum B, Spear P. Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. Cell 1996;87:427–436.
92. Rakic P. Adult neurogenesis in mammals: an identity crisis. J Neurosci 2002;22:614–618.
93. Alvarez-Buylla A, Garcia-Verdugo JM. Neurogenesis in adult subventricular zone. J Neurosci 2002;22:629–634.
94. Flax JD, Aurora S, Yang C, et al. Engraftable human neural stem cells respond to developmental cues, replace neurons, and express foreign genes. Nat Biotechnol 1998;16: 1033–1039.
95. Benraiss A, Lerner K, Chmielnicki E, et al. Adenoviral transduction of the ventricular wall with a BDNF expression vector induces neuronal recruitment from endogenous progenitor cells in the adult forebrain. Mol Ther 2000;1:S35–S36.
96. Lim DA, Tramontin AD, Trevejo JM, et al. Noggin antagonizes BMP signaling to create a niche for adult neurogenesis. Neuron 2000;28:713–726.
97. Shimazaki T, Shingo T, Weiss S. The ciliary neurotrophic factor/leukemia inhibitory factor/gc130 receptor complex operates in the maintenance of mammalian forebrain neural stem cells. J Neurosci 2001;21:7642–7653.
98. Craig CG, Tropepe V, Morshead CM, et al. In vivo growth factor expansion of endogenous subependymal neural precursor cell population in the adult mouse brain. J Neurosci 1996; 16:2649–2658.
99. Fallon J, Reid S, Kinyamu R, et al. In vivo induction of massive proliferation, directed migration, and differentiation of neural cells in the adult mammalian brain. Proc Natl Acad Sci USA 2000;97:14686–14691.
100. Zurn AD, Widmer HR, Aebischer P. Sustained delivery of GDNF: towards a treatment for Parkinson's disease. Brain Res 2001;36:222–229.
101. Lebel M, Gauthier Y, Moreau A, Drouin J. Pitz3 activates mouse tyrosine hydroxylase promoter via a high-affinity binding site. J Neurochem 2001;77:558–567.
102. Saucedo-Cardenas O, Quintana-Hau JD, Le WD, et al. Nurr1 is essential for the induction of the dopaminergic phenotype and the survival of ventral mesencephalic late dopaminergic precursor neurons. Proc Natl Acad Sci USA 1998;95: 4013–4018.
103. Wallen AA, Castro DS, Zetterstrom RH, et al. Orphan nuclear receptor Nurr1 is essential for Ret expression in midbrain dopamine neurons and in the brain stem. Mol Cell Neurosci 2001;18:649–663.
104. Tornqvist N, Hermanson E, Perlmann T, Stromberg I. Generation of tyrosine hydroxylase-immunoreactive neurons in ventral mesencephalic tissue of Nurr1 deficient mice. Brain Res Dev Brain Res 2002;133:37–47.
105. Akerud P, Canals JM, Snyder EY, Arenas E. Neuroprotection through delivery of glial cell line-derived neurotrophic factor by neural stem cells in a mouse model of Parkinson's disease. J Neurosci 2001;21:8108–8118.
106. Eberhardt O, Coelin RV, Kugler S, et al. Protection by synergistic effects of adenovirus-mediated X-linked chromosome-linked inhibitor of apoptosis and glial-derived neurotrophic factor gene transfer in the 1-methyl-4-pheyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease. J Neurosci 2002;20:9126–9134.
107. Schumacher JM, Short MP, Hyman BT, et al. Intracerebral implantation of nerve growth factor-producing fibroblasts protects striatum against neurotoxic levels of excitatory amino acids. Neuroscience 1991;45:561–570.
108. Frim DM, Uhler TA, Galpern WR, et al. Biologically delivered BDNF increases dopaminergic neuronal survival in a rat model of Parkinson's disease. Proc Natl Acad Sci USA 1994; 91:5104–5108.
109. Mufson EJ, Kroin JS, Sobreviela T, et al. Intrastriatal infusions of brain-derived neurotrophic factor: retrograde transport and colocalization with dopamine containing substantia nigra neurons in rat. Exp Neurol 1994;129:15–26.
110. Check E. Parkinson's patients show positive response to implants. Nature 2002;416:666.

Discussion

Kordower: Ole, are you convinced that the striatal innervation in your model is derived from the implanted stem cells and is not host derived.

Isacson: Yes. In the animals I described, there were no remaining dopamine neurons in the SNc.

Kordower: There was staining in the nucleus accumbens.

Isacson: But that is in the A-10 region which is quite a bit away from the graft. The graft creates its own halo of innervation and the two never meet. If there was a gradual transition between them I might agree with you.

Olanow: A-10 neurons do not tend to grow to the striatum and colocalize for CCK. You should be able to prove there is no innervation from A10 by staining for CCK.

Isacson: You are correct; the A-10 dopamine axons do not grow into the striatum. Rather, they pass through the accumbens lateral, medial striation and fan out to layer 6 of the cortex. Although human cells do not exactly express the same markers as the mouse and rat, I believe there is an old literature in humans indicating that A-10 neurons do not express D2 autoreceptors. What we are doing now is using in situ hybridization to look for signals that will help us to be sure which types of dopamine terminals we are seeing.

Olanow: The importance of stem cell transplant lies in its potential to be translated into clinical benefit. Currently, it remains to be established that stem cells can provide benefits in animal models that are as good as can be obtained with fetal nigral dopaminergic cells. And, whereas open-label studies suggest benefit after implantation of fetal nigral cells in PD patients, these studies are subject to placebo effect and bias. The double-blind, controlled trials with human and porcine cells that have been reported to date have more-or-less failed. What does this mean for stem cells and how can you do better? In addition, sooner or later we are going to have to deal with this phenomenon of off-period dyskinesia described by Stan Fahn and his colleagues, which could be a major limitation of transplantation strategies. What do you think is the mechanism re-

sponsible for these dyskinesia and what can be done to prevent them?

Isacson: The first issue is survival of the implanted dopamine cells. If there is no cell survival, then there should be no improvement over placebo. With porcine transplantation, there was minimal cell survival in animal models, and only 100 cells survived in the first PD patient. Furthermore, there was no change on PET. When you have no cell survival and no PET signal, it is not surprising that your clinical trial fails.

Olanow: So the porcine study failed because they did not get adequate cell survival?

Isacson: Yes. When you have no dopamine innervation, you don't expect to get recovery. The Freed trial has two components to it. The first is the observation of off-period dyskinesia. I think there are ways of getting dyskinesia in every patient. If I transplant A-10 dopaminergic cells into small foci in the putamen of L-dopa–primed monkeys, I think I would get dyskinesia.

Olanow: Why? What do A-10 neurons have to do with dyskinesia?

Isacson: Well, A-10 doesn't grow into the motor system, but the dendrites do and you essentially get an infusion of dopamine which I don't think is optimal.

Olanow: So you get a little pulse of dopamine at the site?

Isacson: Yes, I think infusing dopamine by pump into regions of the striatum would create a similar situation. If you create that kind of innervation or lack of innervation in the motor regions you don't get good motor results. Also, the Freed trial used a protocol that was different from that used by Lindvall, and this may have made a big difference. I believe that with the protocol used by Lindvall, there is less risk of developing this really severe dyskinesia.

Olanow: I am not sure this is correct. Lindvall recently published that his group has seen off-period dyskinesia, although they claim this was less severe than what was observed in the Freed study. Aside from this, why do you think the patients in the Freed study did not get better from the parkinsonian point of view.

Isacson: That was at the one-year point. The patients did get better at the 3-year time point, and they are improving at the same rate as seen in the prototype trials in Europe.

Brooks: Freed/Fahn also used a very strange primary end point. If you look at the UPDRS motor score, there was significant improvement. They also put in about half the amount of fetal tissue that everyone else has been using and the cells were stored for up to 4 weeks before implantation. So, he did not use any of the recognized protocols that have been reported to have good clinical and PET benefits.

Isacson: My answer is that Freed's patients did improve somewhat, using less than optimal transplant variables, and some of the patients had off-period dyskinesia. Those are the facts.

Olanow: Transplanted patients younger than age 60 years showed significant improvement over controls for total UPDRS off score, but there was no significant change for those older than 60 years over the group as a whole, and the statistical analyses were not adjusted for multiple comparisons. Our patients and the Lindvall patients showed greater benefits, but these were open-label studies and no control group.

Brooks: I am concerned about this arbitrary young/old differentiation. Both groups showed a similar increase in fluorodopa PET and both responded at 3 years.

Olanow: I think it is important to differentiate that part of the study that was double blind in which they failed to meet the primary endpoint from the part that was open label. The porcine trial makes the magnitude of the placebo and physician bias effect very clear.

Isacson: Part of the problem is that the transplant protocols used are not optimal. This doesn't tell you if a better protocol might not have worked more effectively. Your focus on the double blind disregards the differences between the Freed and Lindvall procedure.

Olanow: No, I was hoping that you would address the differences. The double-blind question addresses the reliability of your observation and data obtained in a double-blind study is more reliable than that obtained in an open-label trial. Now if you look at the double-blind data that currently exists, what it tells you is that the porcine trial did not work and the Freed study worked a little, although the primary end point was not met, meaning it was a failure by statistical standard. That is the only double-blind data we have.

Isacson: In my opinion, the current double-blind studies were badly designed from a biological perspective. Using a more optimal protocol, Lindvall described one patient who has been followed up for 10 years and is so improved that he does not require L-dopa. This goes well beyond a placebo effect. So despite the Freed study, I think the treatment is still capable of doing something profound. However, the cell preparations and surgical methodologies are sub-optimal.

Rascol: It is interesting to see one patient with such a strong benefit, but if the procedure is that effective, we should be able to confirm these benefits in a double-blind controlled trial.

Olanow: I think the data from the Freed study has to stand as it is, and for better or worse, the study did not meet its primary end point. Anything you look at after that is secondary and has much less significance. Now I certainly don't think that based on this single study we should throw out our interest in fetal nigral or stem cell transplantation. What I want you to explain, though, is what we can learn from that study

and how can we design a better study in which we might see a more profound difference.

Isacson: I would say it seems very clear from all the data now that we need to have a better defined preparation of the tissue as not all dopamine neurons are the same. And, in the best case scenario, dopamine neurons need at least 24 months to establish their effect, so patients should be followed for at least that long.

Olanow: Although in both the Lindvall studies and our own, we saw benefits within 3 to 6 months, so you would expect that Freed would have seen the same by 12 months.

Isacson: But, you maintained your patients on a constant L-dopa dosage. This is a good thing to do from a clinical trial perspective, but when transplanted cells kick in their own dopamine system, you may essentially be overdosing the patient. In Freed's study, the best responders were the ones that were most likely to go on and have off-period dyskinesia. So, the excess dopamine may be the cause of the dyskinesia.

Olanow: I think that interpretation is also very much open to question. Fabrizio Stocchi and I have evidence suggesting very strongly that it is not the amount of L-dopa that causes dyskinesia, it is how the L-dopa is administered. In other words, small amounts of L-dopa delivered incorrectly (ie, in a pulsatile manner) are more likely to induce dyskinesia than large amounts of L-dopa delivered correctly (in a continuous manner). So, our studies suggest that the concentration per se is not that important and that intermittent release may be more critical.

Brooks: Lindvall has gone back and analyzed off-period dyskinesias in their patients and what they correlate with. They did not find that off-period dyskinesia correlated with graft size, with clinical status before surgery or with presence of on-period dyskinesia before surgery. What did correlate was severity of dyskinesias in the on state after grafting and prolonged storage of cells before grafting.

Olanow: I think that transplantion is an extremely promising strategy for PD, but I think we have to approach it clinically with as much dispassion as you approach it in the laboratory. We can't allow ourselves to become angry or aggravated because we know transplantation works in the laboratory but are not able to make it work clinically. We have to be able to sit back and apply as much rigor clinically as you do scientifically in evaluating and solving this problem.

Isacson: I just want to make the point that negative results with some transplant studies do not mean that positive results will not be attained in another study using a different transplant protocol and method. I think it is important to remember that all transplant protocols are not the same. Some of the clinical trials that have been performed to date have used transplant protocols that do not make any sense to me. But, with the appropriate protocol, I appreciate your points about the importance of using a well-designed clinical trial in evaluating the intervention.

Stocchi: What type of stem cells do you believe will be best for generating dopaminergic neurons and reinnervating the striatum in a condition like Parkinson's disease?

Isacson: The most desirable age of a stem cell would be just before it differentiates into a fetal dopaminergic neuron. That would be optimal. Reality is slightly different. When we take cells at that stage, they tend to differentiate into glial cells, possibly because they already express receptors for receiving other signals. This may be particularly relevant in the setting of a neurodegenerative disorder. So the answer is I don't know, and I think we need to try cells that are derived from different levels in the developmental chain. We have used the embryonic stem cell, and that has worked for us. We chose it because you have a better chance of guiding it down the right pathway.

Olanow: What is the difference between embryonic stem cells, umbilical stem cells, and adult stem cells such as those derived from bone marrow?

Isacson: To make a complicated answer simple, the primary difference is in how they are genetically programmed. The embryonic stem cell has no instructions; when you deal with stem cells derived from the umbilical cord or neuroprogenitor cells, they have a lot of their destiny already designed into them. Some have argued that there can be trans-differentiation, but no one is really sure of what is happening. Cells may be able to flip between different fates, but usually only for a little while. Some umbilical and hematopoetic cells can become neurons, but of the 240 to 250 known neuronal phenotypes they may be capable of differentiating into only 3 or 4 of them because they are already more differentiated than the embryonic stem cell. So, I would look at it from the perspective of generating dopamine A9 neurons, which are the preferred cell for transplantation in PD. Although umbilical or adult stem cells can differentiate into neurons, no one has been able to demonstrate that they can yield dopamine A9 neurons. It doesn't mean that somebody won't be able to figure out how to do that, but for right now they are more limited in their phenotype.

Stocchi: So the embryonic stem cells are the best?

Isacson: I would say the embryonic stem cell is the best for right now because you have the best chance of generating appropriate dopamine neurons and preventing all other forms of embryological development. Ideally, the cell that you would like to transplant is the fetal A9 neuron with appropriate glial support, but we don't yet have that.