

Long-Term Health of Dopaminergic Neuron Transplants in Parkinson's Disease Patients

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SUMMARY

To determine the long-term health and function of transplanted dopamine neurons in Parkinson's disease (PD) patients, the expression of dopamine transporters (DATs) and mitochondrial morphology were examined in human fetal midbrain cellular transplants. DAT was robustly expressed in transplanted dopamine neuron terminals in the reinnervated host putamen and caudate for at least 14 years after transplantation. The transplanted dopamine neurons showed a healthy and nonatrophied morphology at all time points. Labeling of the mitochondrial outer membrane protein Tom20 and α -synuclein showed a typical cellular pathology in the patients' own substantia nigra, which was not observed in transplanted dopamine neurons. These results show that the vast majority of transplanted neurons remain healthy for the long term in PD patients, consistent with clinical findings that fetal dopamine neuron transplants maintain function for up to 15–18 years in patients. These findings are critically important for the rational development of stem-cell-based dopamine neuronal replacement therapies for PD.

INTRODUCTION

There is a need to understand how transplanted neurons can survive despite ongoing disease processes in the brains of patients with Parkinson's disease (PD). Currently, there is some controversy surrounding the neural transplantation field and neuroscience research regarding interactions between potentially pathological toxic proteins as a cause of neurodegeneration, and the concept of "disease spread" from cell to cell (Desplats et al., 2009; Isacson and Mendez, 2010). The accumulation of Lewy-body-like inclusions in some transplanted fetal dopamine neurons after long-term survival (over a decade) in the PD brain has been described (Cooper et al., 2009; Kordower et al., 2008; Kurowska et al., 2011; Li et al., 2008). Such pathology is a rare occurrence, with only a very low frequency (~1%) of

grafted neuromelanin-containing neurons in cell suspension grafts exhibiting signs of α -synuclein pathology even 22 years after grafting (Kurowska et al., 2011). These isolated cell inclusions are not observed in all patients (Mendez et al., 2008) and are usually found in less than 1%–5% of transplanted neurons depending on the transplantation method used, and clinical and postmortem data indicate that this rare pathology does not affect overall graft function (Cooper et al., 2009; Isacson and Mendez, 2010). It has been suggested that such Lewy-body-like pathology is a product of protein transfer from the parkinsonian host brain to the transplanted fetal cells (Kurowska et al., 2011). However, α -synuclein pathology is not definitive for PD, and incidental α -synuclein pathology has also been reported in the normal aging brain, with frequencies of 8%–22.5% in normal aging and up to 34.8% in centenarians (Ding et al., 2006; Klos et al., 2006; Mikolaenko et al., 2005; Saito et al., 2004; Wakisaka et al., 2003). Experimental paradigms of oxidative stress (e.g., rotenone exposure) or neuroinflammation can also induce α -synuclein accumulation in dopamine neurons (Gao et al., 2008; Sherer et al., 2003).

Recent results from postmortem examinations of fetal ventral mesencephalic grafts in PD patients suggested that dopamine transporters (DATs) are downregulated in the transplanted dopamine neurons (Kordower et al., 2008; Kurowska et al., 2011), and that such changes (which also include reduction of the dopamine neuron phenotypic marker tyrosine hydroxylase [TH]) are indicative of neuronal dysfunction and PD pathophysiological changes in the transplanted neurons. Since a cell therapy approach holds considerable promise as a therapeutic strategy for PD (C.R. Freed et al., 2013, Soc. Neurosci., conference; Kefalopoulou et al., 2014; Ma et al., 2010; Mendez et al., 2005; Politis et al., 2010, 2012), it is important to address the status of transplanted fetal dopamine cells in more detail. In previous studies, we reported surgical, clinical, and histopathological data obtained in five patients with advanced idiopathic PD who had received intracerebral transplantation of fetal dopaminergic cell suspension grafts 4–14 years earlier (Cooper et al., 2009; Mendez et al., 2005, 2008). In those studies, therapeutic improvements were seen without clinical side effects, such as off-period dyskinesias. Postmortem examinations demonstrated that grafted dopaminergic neurons survived for up to 14 years posttransplantation. In the current study, we examined DAT expression as a measure of neuronal function, and the mitochondrial marker Tom20 (translocase of outer mitochondrial membrane 20 kDa)

to assess mitochondrial morphology, to further understand the long-term phenotypical characteristics of the transplanted dopamine neurons and potential effects of the aging of transplants.

RESULTS

Dopamine Transporter Localization and Expression in Transplanted Fetal Dopamine Neurons

In the present study, we assessed DAT immunostaining in 4- to 14-year-old grafts in five patients from our previously published series (Mendez et al., 2005, 2008) in order to further understand the long-term phenotypical characteristics of the transplanted dopamine neurons and potential effects of the aging of transplants.

We conducted immunofluorescence staining for DAT using a monoclonal antibody that recognizes the N terminus of DAT (Miller et al., 1997), and performed colabeling with a TH antibody to label dopaminergic neurons and fibers. A general assessment of the integrity of the grafted TH-immunoreactive neurons in all patients revealed cells with a healthy appearance, including a robust cell soma and absence of signs of atrophy (Figures 1A–1C, 1F–1H, 1K–1M, and 1P–1R). In two independent patients at 4 years posttransplantation (Figures 1A–1J), an examination of DAT/TH immunostaining at low magnification (Figures 1A and 1F) showed dense DAT and TH expression in the reinnervated putamen and caudate in areas both near to and farther away from the graft. Although DAT was also expressed in the grafted cell soma, the intense punctate staining pattern in the reinnervated areas was most striking (Figures 1B and 1G). This expression, consistent with that of synaptic proteins, was easily observed at high magnification (Figures 1C, 1D, 1H, and 1I) where DAT was localized along TH-immunoreactive fibers.

To determine whether DAT expression was maintained in the long term, we examined DAT immunolabeling in transplanted neurons at 9 years and 14 years posttransplantation (Figures 1K–1T). As also seen at the younger time points, a robust punctate expression in the reinnervated striatum was observed (Figures 1K–1M and 1P–1R) and higher-magnification imaging verified the coexpression of DAT puncta along TH-immunoreactive dopaminergic fibers (Figures 1N and 1S). The intensity of DAT immunofluorescence was quantified in the reinnervated putamen at 4–14 years after transplantation (Figure S1) and compared with DAT labeling intensity in the contralateral (non-transplanted) putamen from subject 2. As expected, very low levels of DAT labeling in the nontransplanted parkinsonian putamen were observed, consistent with the severe loss of DAT expression in the putamen in PD (Miller et al., 1997). In contrast, at 4, 9, and 14 years after transplantation, DAT expression was significantly increased within the grafted putamen.

Parallel control immunostainings in which the primary antibodies were omitted showed no immunoreactivity of DAT or TH (data not shown). To further confirm the specificity of the DAT labeling observed in the reinnervated putamen and caudate, we also examined DAT immunolabeling in adjacent anatomical regions on the same tissue sections (see Figure 1). As expected, in the lateral and medial globi pallidi, which are regions that receive comparatively little dopaminergic innervation

and normally exhibit little DAT expression in the human brain (Ciliax et al., 1999), we observed weak DAT immunolabeling and a sharp boundary from high to low DAT and TH expression (Figures 1E, 1J, 1O, and 1T).

Mitochondrial Localization and Expression in Transplanted Fetal Dopamine Neurons

Tom20 was used to label mitochondria in grafted neurons and also in the host substantia nigra and globus pallidus. In the remaining substantia nigra TH-immunoreactive neurons from PD patients (subjects 2, 5, and 6; Figures 2A, 2A', 2D, 2D', 2G, and 2G'), Tom20 labeling often appeared intensely labeled in the cell soma, with accumulation in the perinuclear area and little immunostaining in the axon and processes. In neurons costained with Tom20 and α -synuclein, the host patient's substantia nigra showed Lewy bodies and variable or reduced distribution of Tom20-stained mitochondria (Figure 3A). In grafted TH-immunoreactive neurons at 4 years posttransplantation (Figures 2B and 2B'), Tom 20 immunostaining was robust in the perikarya and neuronal processes, similar to what was observed in the normal brain. At 9 and 14 years posttransplantation, Tom20 labeling was generally less intense in the grafted TH-immunoreactive neurons (Figures 2E, 2E', 2H, and 2H') compared with the Tom20 staining pattern observed in subject 2 at 4 years posttransplantation; however, there was no abnormal accumulation of mitochondria in the cell soma as was observed in the host substantia nigra. The localization of Tom20 in neurons within the host medial globus pallidus (Figures 2C, 2F, and 2I) exhibited a homogeneous localization throughout the cell soma and processes, and showed no evidence of abnormal perinuclear accumulation as was observed in the patients' own substantia nigra. In neurons within the transplants costained with Tom20 and α -synuclein, a normal distribution of Tom20 staining was observed in the absence of Lewy bodies (Figures 3B–3D).

DISCUSSION

Efficacious fetal ventral mesencephalic grafts can reduce both PD motor symptoms and levodopa-induced dyskinesia for many years, and can reduce or negate the requirement for dopamine replacement therapy. Months to years are required for the newly replaced dopaminergic neurons to mature, integrate into the host brain, and function (Barker et al., 2013), and most fetal ventral mesencephalic cell transplants provide improvement in PD motor symptoms starting at ~1 year after transplantation (Evans et al., 2012). However, successful transplants can survive and function for many years. Recent studies by Kefalopoulou et al. (2014) and Politis et al. (2010, 2012) described two patients who were still improving (as shown by PET neuroimaging of dopamine uptake and reduction of the Unified Parkinson's Disease Rating Scale score) more than 18 years after they had undergone transplantation of fetal ventral mesencephalic cells.

The study presented here shows long-term graft survival in PD patients with maintained DAT localization along TH-immunoreactive axons in the reinnervated striatum, indicating functional dopaminergic neurons. Abnormalities in mitochondrial localization, as indicated by accumulation in the cell soma in dopaminergic neurons in the host substantia nigra, were not observed

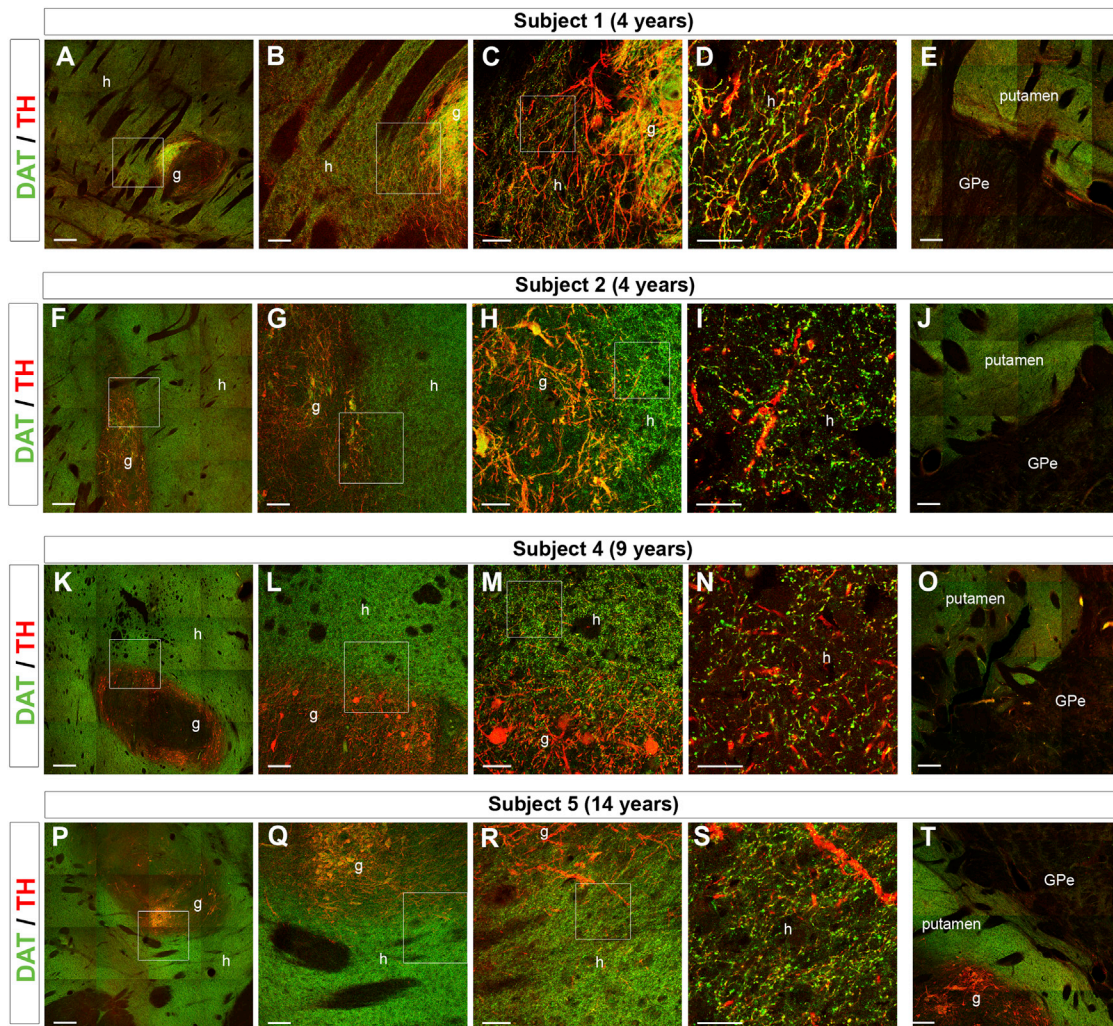


Figure 1. Dopamine Transporter Expression in the Reinnervated Putamen at 4–14 Years Posttransplantation

(A–T) Double immunolabeling for DAT (green) and TH (red) in subjects 1 (A–E; graft survival of 4 years), 2 (F–J; graft survival of 4 years), 4 (K–O; graft survival of 9 years), and 5 (P–T; graft survival of 14 years). Panels (A), (F), (K), and (P) show a low-magnification composite for each subject to illustrate the grafted cell bodies and adjacent reinnervated putamen. Successively higher-magnification images are illustrated in (B)–(D), (G)–(I), (L)–(N), and (Q)–(S). Boxed areas represent the image shown in the subsequent panel. Panels (E), (J), (O), and (T) show a low-magnification composite of the patient’s putamen and adjacent external segment of the globus pallidus. DAT immunostaining shows a robust punctate localization along dopaminergic (TH-immunoreactive) fibers in the reinnervated putamen and caudate in all grafts, even up to 14 years posttransplantation. In addition, the transplanted dopamine neurons show a healthy and nonatrophied morphology. Parallel control immunostainings in which the primary antibodies were omitted showed no immunoreactivity of DAT or TH (data not shown). To further confirm the specificity of the DAT labeling observed in the reinnervated putamen and caudate, we also examined DAT immunolabeling in adjacent anatomical regions on the same tissue sections. As expected, in the lateral and medial globus pallidi, which are regions that receive comparatively little dopaminergic innervation and normally exhibit little DAT expression in the human brain (Ciliax et al., 1999), we observed weak DAT immunolabeling and a sharp boundary from high to low DAT and TH expression (E, J, O, and T). We also performed immunostaining in the substantia nigra of each patient and confirmed the loss of both DAT and TH immunoreactivity (data not shown). g, graft; h, host; GPe, globus pallidus externa. Scale bars, 400 μ m in (A), (E), (F), (J), (K), (O), (P), and (T); 100 μ m in (B), (G), (L), and (Q); 50 μ m in (C), (H), (M), and (R); and 20 μ m in (D), (I), (N), and (S). See also Figure S1.

in grafted neurons. These data are consistent with clinical and neuroimaging data showing stable dopamine cell survival and function more than 18 years after surgery (Kefalopoulou et al., 2014; Politis et al., 2010, 2012).

DAT is a plasma membrane protein located on presynaptic dopamine nerve terminals, where it is responsible for the termination of dopamine transmission and reuptake of dopamine released into the synaptic cleft back into the presynaptic dopa-

mine neurons (Nirenberg et al., 1997). DAT is highly concentrated in the striatum in nigrostriatal dopaminergic projections and is localized to the plasma membranes of axonal varicosities and terminals containing synaptic vesicles (Nirenberg et al., 1997), consistent with its involvement in dopaminergic synaptic transmission in the striatum. Recent studies have suggested that in human PD patients, DAT expression in transplanted fetal dopamine ventral mesencephalic neurons is downregulated over

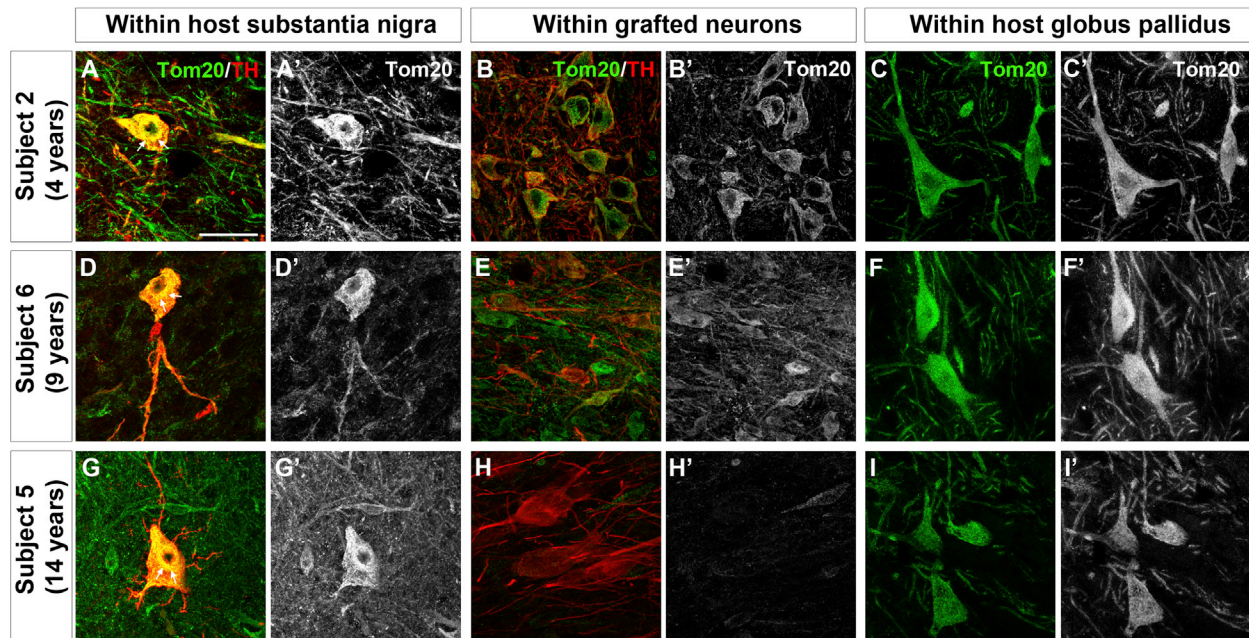


Figure 2. Mitochondrial Phenotype in Transplanted Fetal Dopamine Neurons at 4–14 Years Posttransplantation

(A–I) Double immunolabeling for translocase of outer mitochondrial membranes 20 kDa (Tom20, green) and TH (red) in the host substantia nigra (left panels), grafted dopamine neurons (middle panels), and host globus pallidus (right panels) in subject 2 (A, A', B, B', C, and C'; graft survival of 4 years), subject 6 (D, D', E, E', F, and F'; graft survival of 9 years), and subject 5 (G, G', H, H', I, and I'; graft survival of 14 years). Panels (A')–(I') show single-channel Tom20 labeling from corresponding panels (A)–(I). In dopamine (TH-immunoreactive) neurons from the patients' own substantia nigra, Tom20 labeling often appeared intensely labeled in the cell soma, with accumulation in the perinuclear area (arrows) and little immunostaining in the dopaminergic axons and processes (A, A', D, D', G, and G'). In grafted TH-immunoreactive neurons at 4 years posttransplantation, Tom20 labeling was robust in the perikarya and neuronal processes (B and B'). At 9 and 14 years posttransplantation, Tom20 labeling was generally less intense in the grafted TH-immunoreactive neurons (E, E', H, and H') compared with the Tom20 staining pattern observed in subject 2 at 4 years posttransplantation; however, there was no abnormal accumulation of mitochondria in the cell soma as was observed in the host substantia nigra. No perinuclear accumulation or fragmentation of Tom20-labeled mitochondria was observed in the host globus pallidus (C, C', F, F', I, and I'). Scale bar, 50 μ m.

time, and that this is indicative of PD processes within the graft and loss of function of the grafted dopamine neurons (Kordower et al., 2008; Kurowska et al., 2011). In these studies, the authors used immunolabeling for DAT and subsequent light microscopy. Surprisingly, in older grafts, these studies described only the expression of DAT in the grafted cell soma, with no reported analysis or discussion of the expression and localization of DAT in the dopamine neuron fibers reinnervating the putamen. In the current study, we also noted a qualitative reduction in the intensity of the DAT signal in the cell soma of the grafted dopamine neurons over time when we compared grafts at 4, 9, and 14 years posttransplantation, but it was striking to us that the punctate DAT expression in the reinnervated putamen and caudate was maintained, even over a decade posttransplantation. We previously showed that the extent of DAT labeling in the caudate and putamen of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned monkeys, as detected by [11 C]CFT PET neuroimaging, is congruent with the number of surviving dopaminergic fibers (Hantraye et al., 1992). DAT is a marker of mature dopamine synapse function, and thus our findings of robust DAT labeling in the putamen of patients analyzed in our series are indicative of the long-term, continued health and function of transplanted dopamine neurons. Therefore, based on DAT expression, we do not find evidence of PD pathophysiological

processes in grafted dopaminergic neurons. Clearly, using the current transplantation methodology, fetal grafts can result in dopamine neurons that are clinically functional for the long term (Barker et al., 2013; Mendez et al., 2005). A previous analysis of α -synuclein pathology in the same transplant cases described here (Cooper et al., 2009; Mendez et al., 2008) revealed only one Lewy body in a neuron of a fetal graft in one of these cases (Cooper et al., 2009). In fetal ventral mesencephalic transplant cases from other transplant-method series, in which we have also observed more frequent Lewy body and α -synuclein pathology (albeit still <5% of grafted neurons), the overall morphology in the vast majority of the surviving transplanted dopaminergic neurons was also unchanged (Cooper et al., 2009; Kordower et al., 2008). Indeed, it was also reported that there is no alteration in the expression of VMAT2, another marker of dopamine presynaptic nerve terminals, in grafted dopamine neurons over time (Kordower et al., 2008). The current study provides important additional evidence against the relevance of a postulated prion-like α -synuclein mechanism for disease propagation. Instead of the concept of α -synuclein spreading from host to graft to cause dysfunction, the opposite may be true, i.e., the healthy transplanted cells provide a clearance mechanism for exogenous unfolded proteins. Our present study unequivocally demonstrates positive markers of dopamine neuron

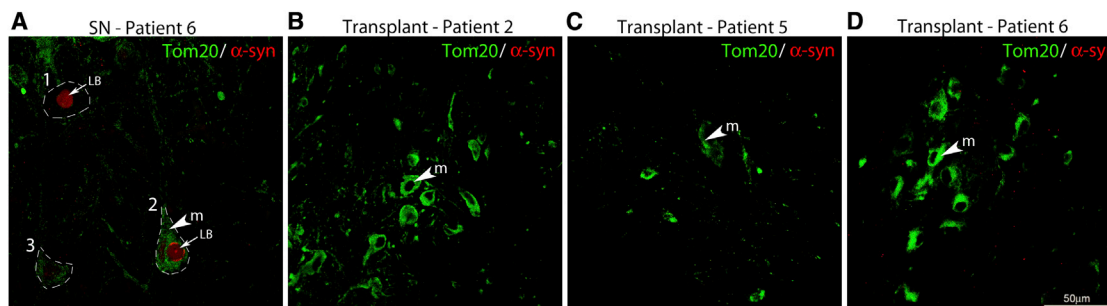


Figure 3. Mitochondria Abnormalities Occur Independently of Lewy Body Pathology in Host Neurons and Neither Lewy Body Pathology nor Mitochondrial Abnormalities Were Found in Transplanted Neurons

(A) Coimmunofluorescence labeling of α -synuclein (red) and the mitochondrial import receptor subunit Tom20 (green) was observed in neurons within the substantia nigra of PD patient 6. Mitochondria in neurons containing accumulated α -synuclein in the host were either diffusely distributed throughout the cytoplasm (cell 1) or were dense in appearance (cell 2). Mitochondria were also diffusely distributed in the cytoplasm of cells without Lewy bodies (cell 3). (B–D) No Lewy body pathology was found in the transplanted neurons of PD patients 2 (B), 5 (C), and 6 (D), and mitochondria were dense throughout the cytoplasm. m, mitochondria; LB, Lewy body. Scale bar, 50 μ m.

function and thus provides a further rationale for clinical cell therapy for PD using stem cell-derived dopamine neuron equivalents.

We also examined the localization and expression of mitochondria in grafted dopaminergic neurons as a possible indicator of increased aging and readout of neuronal function. Mitochondrial dynamics, including fission, fusion, and transport, are crucial for the maintenance of bioenergetic function and prevention of apoptosis (Detmer and Chan, 2007). Mitochondrial dysfunction is a prominent feature of PD and dopaminergic substantia nigra neurons are particularly vulnerable to mitochondrial dysfunction due to their dependence on a high metabolic rate (Exner et al., 2012). We frequently observed that the remaining dopaminergic neurons in the host substantia nigra pars compacta contained Tom20-labeled mitochondria that were accumulated in the cell soma, with very little staining or only a fragmented staining pattern in neurites. Such a redistribution of mitochondria has also been observed in pyramidal neurons in postmortem brains of patients with Alzheimer's disease, using antibodies against COX I and mitochondria fusion/fission proteins (Wang et al., 2009). Intracellular mitochondrial distribution is critical for the functioning of neurons and mitochondrial fragmentation, and a reduced localization of mitochondria in neurites may reflect disruptions in axonal transport and/or disrupted mitochondrial fusion/fission. In contrast, in grafted TH-immunoreactive neurons at 4 years posttransplantation, the Tom20-labeled mitochondria displayed a more uniform distribution throughout the cell soma and neurites, with no indication of fragmentation or accumulation in the cell soma. The overall reduced expression of Tom20-labeled mitochondria that we observed in grafted dopamine neurons at 9 and 14 years posttransplantation may reflect an overall reduced mitochondrial biogenesis or increased mitophagy as a function of accelerated aging in these neurons. By all current cell-biological accounts, the distribution of the mitochondrial network we describe is likely more informative than a total mitochondrial count, which is practically difficult (if not impossible) to obtain in regular postmortem material. Furthermore, even if such a method existed, a similar mitochondrial mass or number between populations of neurons would not

necessarily equate with physiologically functional mitochondria due to heteroplasmy (the coexistence of both faulty and functional mitochondria in the same cell) (Kraytsberg et al., 2006; Larsson, 2010; Sanders et al., 2014).

The observations of continued DAT expression and normal mitochondrial cellular distribution at 4–14 years posttransplantation are consistent with the clinical data and show that implanted dopamine neurons remain healthy and functional for decades (C.R. Freed et al., 2013, Soc. Neurosci., conference; Kefalopoulou et al., 2014; Politis et al., 2010, 2012). In summary, our post-mortem examination of tissue from PD patients who had received fetal ventral mesencephalic cells transplanted as a cell suspension shows that following long-term graft survival, DAT localization along TH-immunoreactive axons and fibers is maintained in the reinnervated striatum, indicating functional dopaminergic neurons. Abnormalities in mitochondrial localization, as indicated by accumulation in the cell soma in dopaminergic neurons in the host substantia nigra, were not observed in grafted neurons. Our data are not consistent with the suggestion that these grafts degenerate over time, and instead show that there is no cell-biological evidence for such an assertion. These data support clinical and neuroimaging findings of the long-lasting functional benefit of dopamine neuron transplantation in PD patients (C.R. Freed et al., 2013, Soc. Neurosci., conference; Kefalopoulou et al., 2014; Ma et al., 2010; Mendez et al., 2005; Politis et al., 2010, 2012), and provide an important foundation for the development of future fetal and stem cell-derived dopamine replacement therapies for PD.

EXPERIMENTAL PROCEDURES

Patient Selection

Caudate putamen, globus pallidus, and substantia nigra tissues from five patients (referred to as subjects 1, 2, 4, 5, and 6) with advanced idiopathic PD who had undergone fetal tissue transplantation for 4–14 years were examined in this study. All protocols were approved by institutional review boards. We previously reported the surgical procedures, neuroimaging data, clinical outcome, and postmortem histological assessments of the phenotypical characteristics and PD pathophysiological markers in transplanted neurons in this series of patients (Cooper et al., 2009; Mendez et al., 2005, 2008).

Tissue Preparation

At postmortem examination (after a delay of 3–4 hr), the brains were infused with 2 l of cold 0.1 M phosphate buffer (pH 7.4), followed by 2 l of ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were subsequently blocked in the coronal plane in 3-cm-thick slabs. The slabs were cryoprotected in 30% sucrose in PBS at 4°C.

Immunohistochemistry

Sections (40 μm) were stained using immunofluorescence techniques. In order to maintain consistency of DAT or TOM20 labeling between subjects, immunostainings were performed at the same time and using identical reagents. DAT labeling was enhanced using a fluorescent streptavidin conjugate as described below. Sections were rinsed three times for 10 min in PBS, incubated in 10% normal donkey serum (Vector Laboratories) and 0.3% Triton X-100 in PBS for 60 min, and then incubated with gentle agitation for 48 hr at 4°C in primary antibody (rat anti-DAT, 1:200 [MAB369; Millipore]; sheep anti-TH, 1:300 [P60101-0, Pel-Freez]; rabbit anti-Tom20, 1:200 [sc-11415; Santa-Cruz]). For α-synuclein staining, the LB509 antibody (Invitrogen/Life Technologies) was used at 1:500 dilution. After an additional three 10 min rinses in PBS, the sections were incubated in fluorescent dye-conjugated secondary antibodies in PBS for 60 min at room temperature for detection of TH and Tom20 (Alexa Fluor donkey anti-rabbit/sheep 488/568; 1:500 [Molecular Probes]). For detection of DAT and α-synuclein, sections were incubated in a biotinylated secondary antibody (donkey anti-rat/mouse; 1:250; Vector Laboratories) in PBS for 60 min at room temperature, followed by three 10 min rinses in PBS, and incubation in streptavidin Alexa Fluor 488 (1:500, Molecular Probes) for 60 min. After rinsing in PBS (three times for 10 min), sections were mounted onto Superfrost Plus slides and an autofluorescence eliminator reagent was applied (Millipore) prior to coverslipping in Mowiol mounting media. The specificity of DAT, Tom20, TH, and α-synuclein labeling was confirmed using stainings in parallel tissue sections from each subject in which the primary antibody was omitted.

Confocal Microscopy

Immunofluorescence staining was examined with the use of a confocal microscope (LSM510 Meta; Carl Zeiss) at 10, 25, or 100× magnification. Single or z-stack images were acquired using a sequential scanning mode with a frame size of 1,024 × 1,024 pixels, and averaging of four frames. The laser intensity, confocal aperture, photomultiplier voltage, scan speed, image size, filter, and zoom were kept identical while all images were acquired. For quantification of DAT labeling, confocal images at 25× magnification (at least five per subject) were obtained in the reinnervated putamen or in the denervated, nontransplanted putamen. The average optical density of DAT immunofluorescence intensity in each subject was analyzed using ImageJ software (ImageJ 1.44i; National Institutes of Health).

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2014.05.027>.

AUTHOR CONTRIBUTIONS

P.J.H. designed and performed research, analyzed and interpreted data, and wrote the paper. O.C. designed research and analyzed data. D.S. performed research. H.R. evaluated data and wrote the paper. I.M. designed and performed research, evaluated data, and wrote the paper. O.I. designed and performed research, analyzed and interpreted data, and wrote the paper.

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