J Neurol (2009) 256 (Suppl 3):S310–S316
DOI 10.1007/s00415-009-5242-z

Lack of functional relevance of isolated cell damage in transplants of Parkinson's disease patients

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Abstract Postmortem analyses from clinical neural transplantation trials of several subjects with Parkinson's disease revealed surviving grafted dopaminergic neurons after more than a decade. A subset of these subjects displayed isolated dopaminergic neurons within the grafts that contained Lewy body-like structures. In this review, we discuss why this isolated cell damage is unlikely to affect the overall graft function and how we can use these observations to help us to understand age-related neurodegeneration and refine our future cell replacement therapies.

Keywords Cell transplantation · Lewy body · Therapy · Dopaminergic

Introduction

Parkinson's disease is a neurodegenerative disorder characterized by the progressive loss of dopamine (DA) neurons in the substantia nigra pars compacta and by the development of eosinophilic inclusions in surviving neurons, so called Lewy bodies (LBs) [8]. The clinical manifestations of Parkinson's disease (PD) become evident only after the pathology has reached an advanced stage with approximately 60% of the DA neurons of the SN lost, with a consequent depletion of roughly 70% of striatal DA (Fig. 1) [8, 16, 31]. Given the critical reduction of DA levels in the nigro-striatal system, effective pharmacological treatments elevate DA levels by L-dopa or DA agonists to produce meaningful clinical improvements for years [8]. The DA based drug therapies, in particular the use of L-dopa, produce long-term side-effects, including severe dyskinesias [8]. To reduce L-dopa induced dyskinesia and produce a motor activation similar to DA drugs, deep brain stimulation (DBS) of the pallidum or subthalamic nucleus is a surgical alternative/complement to drug therapies [27, 28, 43]. However, perhaps not surprising, given it is simply an electrical interference of abnormal circuitry, a reduced efficacy occurs after a few years of DBS treatment (Fig. 1) [24, 38, 42]. DBS is also associated with a suicide risk after surgery [46]. Another important surgical approach is cell-based replacement therapy.

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Novel restoration therapy for Parkinson's disease: fetal ventral midbrain transplantation

This relatively novel methodology has many current challenging biological and technical limitations, but the potential therapeutic benefit of physiological DA release over a sustained period of time is highly desirable and DA neurons transplanted at fetal-stage into the caudate-putamen or substantia nigra of patients with PD can grow, connect and release DA with long-term remarkable and neurologically clear benefits to PD patients [9, 12, 20, 21, 23, 26, 29, 30, 32–34, 37, 39, 40, 44, 48]. Given the relative

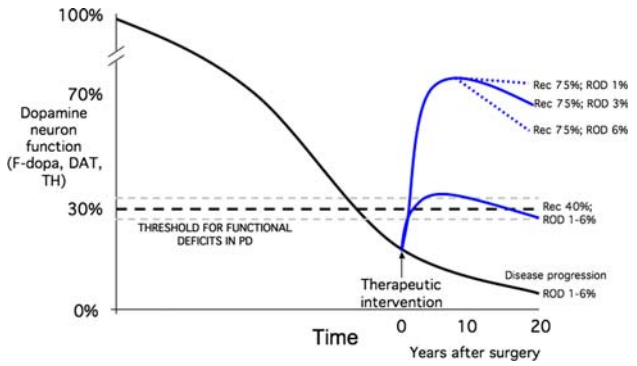

Fig. 1 *Comparison of the rates of PD patient recovery with neural transplantation.* Patients lose midbrain DA neurons for many years prior to the development of symptoms (threshold for functional deficits). At this threshold, approximately 70% of midbrain DA neurons have degenerated. Patients that receive neural transplantation after passing this threshold improve over a long period with a modest rate of decline (ROD 1–6%). Even poorly functioning grafts (40% recovery) may improve patient symptoms for several years. Also, we believe that isolated protein aggregates within the graft may only increase the rate of decline from 1 to 6%. In a patient that responds well to neural transplantation, a rate of decline of 6% would improve symptoms for more than 20 years.

novelty of these techniques, a number of different methods have been utilized for fetal ventral midbrain (VM) transplantation. One of the first clinical neural transplantation trials utilized dissociated fetal VM tissue transplanted into the striatum of patients who had developed PD after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) exposure [48]. These young patients exhibited marked motor improvement and an increase of striatal ^{18}F-fluorodopa uptake in positron emission tomography (PET) studies. Indirect evidence of graft survival from ^{18}F-fluorodopa uptake and postsynaptic raclopride DA receptor occupancy PET studies has indicated that fetal VM tissue transplanted into the striatum can survive for up to a decade and provide sustained motor benefits in PD [39]. However, few postmortem studies of the survival and integration of grafted DA neurons exist. Recently, we and several other groups have shown that DA neurons implanted into the PD brain can survive for over a decade as demonstrated by postmortem analysis [21, 26, 34]. In a meta-analysis of all these patients, three patients had grafted DA neurons that contained LB-like inclusions and Lewy neurites pathognomonic for PD [21, 26], whereas the majority of these reported postmortem cases contained no such pathology [34]. One of these reported patients had received "solid" pieces of human fetal VM tissue, and this patient had the most marked LB-like pathology in the grafts [21], confirmed by us independently. For detailed information about these patients, see Table 1. Although this series of reports provided a certain insight into aging of transplants and possibly corroborated known factors involved in the pathogenesis of PD [4], most importantly for therapies; the vast majority of transplanted neurons remained healthy and continued to provide substantial clinical benefits for over a decade (Fig. 2) [21, 26, 34].

Clinical correlates of postmortem data in PD patients receiving fetal ventral midbrain transplants as a surgical therapy

We have recently reported the clinical outcome and postmortem histopathological analyses of a series of patients with advanced PD who received fetal VM cell suspension transplants into the striatum and in one case also into the ventral midbrain [33, 34]. Importantly, histological evidence identified many fetal VM DA neurons that had reinnervated the host putamen while the untransplanted caudate remained denervated (Fig. 2). For all but one of these patients the fetal VM tissue was incubated in glial derived neurotrophic factor (GDNF) and hibernation medium for 6 days prior to surgery, before being dissociated and stereotaxically transplanted as cell suspensions (Table 1) [33, 34]. The first two patients in

Table 1 Summary of 20 grafted PD post-mortem cases

Patient	1, 2, 4–6 Canada [33, 34]	3, 8 Sweden [26]	A, B, C, D United States [21, 22]	9–17 United States Personal communication, [9]
Years that patient survived after transplantation	4, 4, 9, 14, 9	12–16, 11–13	14, 14, 4, 4	1–14
Cell preparation method	Hibernation, cell suspension	Fresh tissue, small cellular aggregate suspension	Small tissue pieces	Tissue strands
Total number of surviving DA neurons in grafts per hemisphere	20,000–>100,000	~20,000	Numerous 30,000/100,000	Numerous
Ubiquitin$^+$/alpha-synuclein$^+$ inclusions in graft?	No	Few isolated cells	Few isolated cells, 2/4 patients	No
Microglia around graft	Resting	Resting	Activated	Unknown

Patients 1, 2, 4, 5 and 6 are from our own Canadian series [33, 34]. For comparison, patients 3 and 8 are from the Swedish series [26], patients A, B, C and D are from the American series [21, 22] and patients 9–17 are from the NIH double-blind study ($n = 4$) and open label clinical trial ($n = 5$) [9]

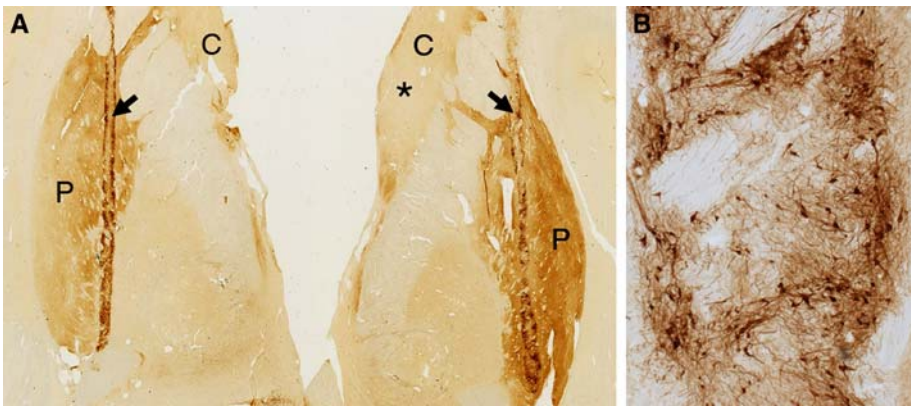

Fig. 2 Human fetal VM DA neurons 9 years after transplantation into PD patients reinnervate the host putamen. **a** TH immunostaining demonstrates bilateral long, slender grafts extending the length of the putamen. There is extensive reinnervation of the putamina bilaterally, in contrast to the denervated caudate (*). **b** High power image of TH staining showing DA cell bodies with axonal processes. *P* putamen, *C* caudate

this series displayed marked clinical improvement with a 50% reduction of Unified PD Rating Scale (UPDRS) scores. Dyskinesia scores also improved and, importantly, no off-period dyskinesias were observed. Three years after transplantation, PET studies showed an increase in ^{18}F-DOPA uptake bilaterally indicative of functioning DA grafts [33]. These two patients died of unrelated causes 3.5–4.5 years after transplantation. The first patient had bilateral graft survival in the putamina. Total number of DA neurons on the right side was found to be 127,189. On the left side, a total of 98,913 DA neurons were found. The second patient was transplanted unilaterally and was found to have a total of 202,933 TH$^+$ cells in the right putamen, in addition to 4,289 TH$^+$ cells in a graft in the right ventral midbrain [33]. Subsequently, we have described the histopathological findings in the brains of three additional patients who died of unrelated causes 9–14 years after transplantation [34]. Clinical benefits were less marked in the three patients than the previously two reported but importantly, no side-effects (off-period dyskinesias) were observed. PET studies of two of these subjects showed increased ^{18}F-DOPA uptake 2 years after transplantation. The less marked clinical benefits correlated with smaller graft sizes observed in these patients. The first of these patients had a total of 11,100 and 11,687 TH$^+$ cells in the left and right putamina respectively, the second patient had 10,917 and 21,552 TH$^+$ cells in the left and right putamina, respectively, while the last of these patients, who was unilaterally transplanted, had a total of 9,861 TH$^+$ cells in the right putamen. The smaller graft sizes observed, likely were due to fewer cells transplanted and less advanced transplantation techniques utilized in these early transplantation cases (Table 1) [34].

Cell source, tissue preparation and surgical cell infusion technology may influence patient immune response

Postmortem analysis of all five patients showed well-integrated grafted DA neurons with extensive neuritic outgrowth with partial or full re-innervation of the host putamen without host tissue displacement. In all grafts, the majority of TH$^+$ neurons were located at the graft periphery and contained small amounts of neuromelanin but no extracellular neuromelanin was observed. A six-month postoperative course of Cyclosporin immunosuppression was given to all of these patients. CD45$^+$ microglial cells were found to be mildly increased around grafts, but activated microglia were present mainly along needle tracks. Interestingly, cell suspension grafts had much less microglial activation than that observed for solid tissue pieces grafts (Fig. 3) [9, 37]. This reduced host response observed for cell suspension grafts is most likely a result of the relative lack of MHC-I containing donor blood vessels in suspension grafts, compared with solid

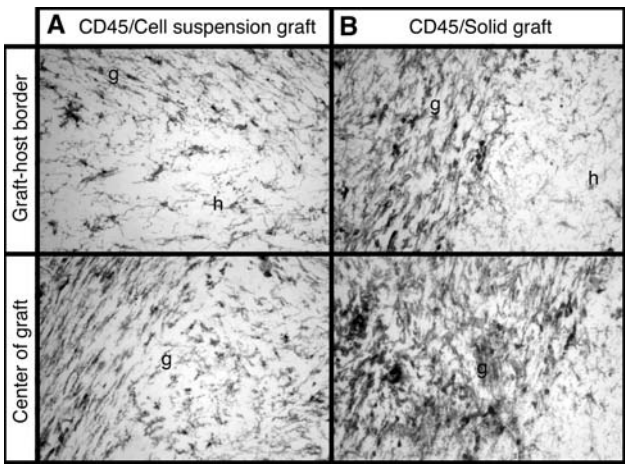

Fig. 3 *Immune response to cell suspension and solid grafts of fetal VM in PD patients.* Representative images of CD45 expression in a cell suspension graft (**a**) and a solid tissue graft (**b**) (Collaboration, Dr. J. Kordower). **a** At the graft-host border of the cell suspension graft (*upper panel*) and in the center of the graft (*lower panel*), CD45$^+$ microglia exhibited an inactive morphology. **b** In contrast, CD45 immunoreactive cells exhibited amoeboid morphology consistent with active microglia in the solid tissue graft. Furthermore, numerous small round immunoreactive cells were observed, consistent with leukocyte morphology

tissue grafts, and the fact that host-derived angiogenic processes dominate in cell suspension grafts [14].

Organotypic distribution of grafted fetal DA and non-DA neurons

Coexpression of TH and G-protein coupled inward rectifying current potassium channel type 2 (Girk-2) defines the most vulnerable population of DA neurons in PD [7, 33]. This group of DA neurons is located in the ventral tier of adult SN pars compacta (A9 SNc), projecting axons and terminals to motor areas of the putamen. In the putaminal cell suspension grafts, approximately 70% of DA neurons coexpressed Girk-2 with and without calbindin coexpression. In contrast, approximately 40% of TH^+ neurons coexpressed calbindin [33]. Additionally, Girk-2 coexpressing TH^+ cells were predominantly located at the periphery of the grafts while the center of the grafts contained TH^+ neurons that were more often Girk-2 [33, 34]. We also demonstrated the presence of serotonin neurons in grafted human fetal VM tissue in all five patients [34]. While we did not observe graft-induced dyskinesias in any subject, a recent report using a rodent model of PD describes the role of serotonin neurons in the development of L-dopa induced dyskinesias when DA neurons are depleted [6]. Given the potential role of serotonin neurons in the development of L-dopa-induced dyskinesia, our novel finding highlights the need for controlling cell composition in clinical neural transplantation [14].

Cell preparation techniques and patient recipient status may contribute to Lewy body-like inclusions in patients' grafts

We searched for neuropathological evidence of LBs in our patients. In the five subjects, triple immunostaining for TH, alpha-synuclein and ubiquitin demonstrated the presence of LBs in all substantiae nigrae, the pathological hallmark of PD. In all but one patient, grafted neurons did not contain alpha-synuclein or ubiquitin inclusions and there were no other morphological signs of neurodegeneration in the graft neuropil (Fig. 4a–c) [34]. However, in one of the patients with the youngest graft, one or two grafted neurons out of several tens of thousands appear to contain neuromelanin and LB-like pathology (Dr. D. Dickson, personal communication). These isolated cells may represent stochastic events and are unlikely to affect graft function.

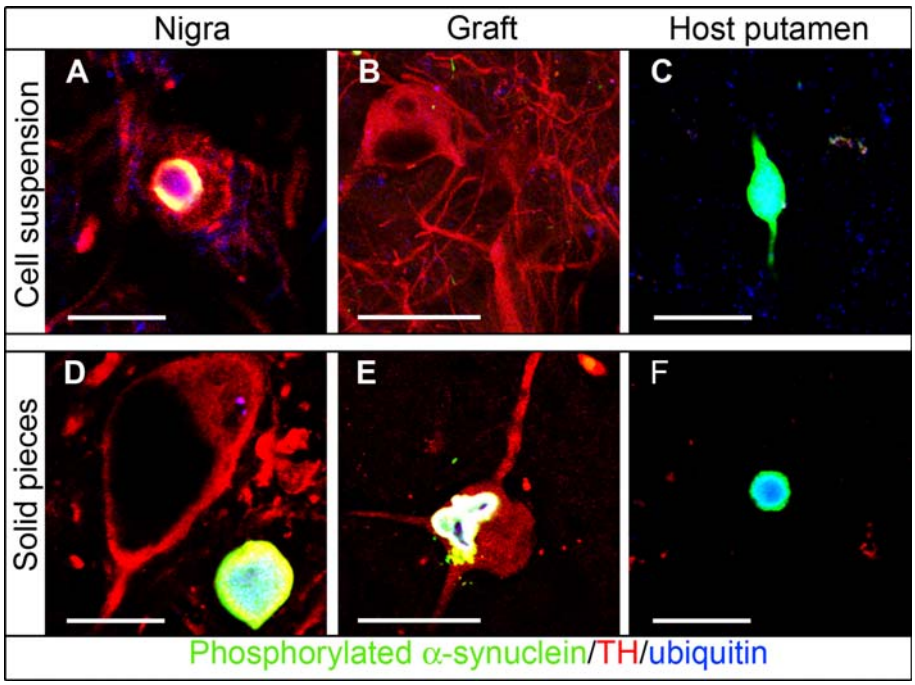

Fig. 4 *In PD patients, transplanted DA neurons from solid tissue pieces but not cell suspensions contain phosphorylated alpha-synuclein immunolabeled inclusions.* **a**, **d** In the patients' nigra, DA neurons (TH, *red*) contained inclusions typical of LBs (phosphorylated alpha-synuclein (81A antibody)/green and ubiquitin/blue), consistent with the diagnosis of PD. **b** In contrast, cell suspension grafts of VM DA neurons did not contain LBs but inclusions were observed in the host's non-DA neurons of the adjoining putamen **c**. **e** However, using identical immunohistochemical methods, LB-like inclusions were observed in 5–10% of grafted DA neurons in a patient that received a solid tissue transplant of fetal ventral midbrain. **f** Similar inclusions were observed in host putaminal cells. *Scale bars* A, D, C and F = 20 μm; B, E = 50 μm

To address the effect of different transplantation techniques on graft fate and outcome, we performed immunohistochemical staining of tissue sections from a PD patient grafted with solid tissue, not cell suspensions, provided by Dr. Jeff Kordower, Rush University (Fig. 4d–f). This patient died 14 years after transplantation. On postmortem analysis using our standard triple immunofluorescence staining for TH, alpha-synuclein and ubiquitin demonstrated numerous LB-like inclusions as well as Lewy neurites within the grafts and host putamen, in marked contrast to that observed for our series with cell suspension transplants [34]. Interestingly, the solid graft was also associated with marked microglial activation [21], in contrast to mild to moderate microglial activation observed for the cell suspension grafts [26, 34]. This is in agreement with a recent transplantation study in non-human primates, where increased host gliosis was related to solid grafts [41]. The reduced host inflammatory response observed for cell suspension grafts compared with solid tissue grafts is most likely due to the presence of major histocompatibility complex Class I, which is expressed by *donor* blood vessels that predominate in solid tissue grafts. In contrast, host-derived angiogenic processes predominate in cell suspension grafts [11, 25]. It is conceivable that an increased host inflammatory response could have contributed to the development of PD-like pathology in some of these patients [21, 26]. Other contributing factors could be oxidative stress or even an accelerated aging process (grafts are placed ectopically in a neuroinflammatory environment). Differences in cell preparation and injection techniques are likely to play a major role in patient responses and the susceptibility to degeneration of grafted cells in some patients. This highlights the importance of adequate control and improvements in fetal dissection, cell preparation and implantation techniques [2, 14]. Two controlled double blind clinical trials in the US of fetal VM tissue transplantation for PD utilized solid tissue pieces for grafting [9, 37]. Although some objective clinical improvement was observed on standard rating scales for a subset of patients, mainly younger patients, and those with less severe disease, both studies failed to meet a primary endpoint of clinical improvement at 1 year after transplantation [9, 37]. Given our clinical data [33, 34], it is likely that differences in cell preparation techniques (including hibernation and growth-factors) and final cell composition, especially between solid and cell suspension techniques, play a major role in the differential development of off-period dyskinesias. With fetal midbrain tissue, inadvertent cotransplantation of other cell types not relevant for ventral midbrain neural grafting may contribute to the development of dyskinesias. For example, the A9 DA neurons have a better capacity to control the physiological release and synaptic availability of DA than the ventral tegmental area (VTA) A10 neurons because of the higher presynaptic DA autoreceptor and DA transporter levels that occur in A9 DA neurons [14, 15]. Furthermore, the presence of non-DA neurons, such as serotoninergic neurons, has recently been implicated in the evolution of dyskinesias in animal studies [5, 6]. We feel that stem cell technology will provide a more uniform and desirable cell source than fetal cells for clinical transplantation.

In conclusion, the vast majority of the implanted DA neurons appear normal and healthy, having survived, grown and functioned in a PD brain for a very long period, relative to other non-neurological patient groups of clinical cell and organ transplantation cases [2, 16]. The potential clinical benefits obtained by improved technology (cell and transplant) provide the impetus for continued work on this relatively novel cell therapy paradigm. Although rare LB and neuritic pathology is sometimes seen [3, 34], we feel it is misleading to suggest that the occasional LBs or neuritic pathology teach us something profoundly "new" about PD pathogenesis. In fact, the most reasonable explanation is that the occasional LBs found (usually less than 1–5% of the neurons) are caused by accelerated aging in some (but not all) more than decade old implanted fetal brain tissue transplants in PD patients. Such tissue changes could obviously be related to the (1) ectopic placement of the VM transplant, lacking normal substantia nigra local neural growth-factor support, (2) evident local tissue inflammation probably caused by known mismatched donor-host tissue combinations, which over time produce (3) accelerated age related processes known to precipitate and simulate PD like pathology (for example by oxidative stress, growth factor loss and neuroinflammation) [1, 10, 17–19, 35, 36, 45]. A reasonable future focus is testing the functional outcome of various cell transplantation innovations, including the use of PD patient individualized induced pluripotent stem (iPS) cells as a source for donor cells. Such new technology could possibly improve both transplant quality and content, by providing cell sorted pure DA populations [13, 47] compared to elective abortion dissected, mixed fetal tissue including tissue with blood-vessel donor antigens and inflammatory reactions [21]. In particular, breakthroughs in the use of human stem cells are necessary to provide a reliable cell source for a large-scale clinical application. It is critical that necessary experiments for PD stem cell transplantation and technology move this therapeutic field of PD forward.

Summary

In summary, grafted DA and serotonin neurons can survive without or with minimal signs of neurodegeneration for up to 14 years despite ongoing degeneration of midbrain DA

neurons and other DA structures in the host parkinsonian brain. Overall, these findings are very encouraging and offer support to the further development and use of fetal and stem cell-derived DA neurons for the treatment of PD.

Conflict of interest statement The authors declare no conflict of interest.

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