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REVIEW

# Historical perspective of cell transplantation in Parkinson's disease

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## Abstract

Cell grafting has been considered a therapeutic approach for Parkinson's disease (PD) since the 1980s. The classical motor symptoms of PD are caused by the loss of dopaminergic neurons in the substantia nigra pars compacta, leading to a decrement in dopamine release in the striatum. Consequently, the therapy of cell-transplantation for PD consists in grafting dopamine-producing cells directly into the brain to reestablish dopamine levels. Different cell sources have been shown to induce functional benefits on both animal models of PD and human patients. However, the observed motor improvements are highly variable between individual subjects, and the sources of this variability are not fully understood. The purpose of this review is to provide a general overview of the pioneering studies done in animal models of PD that established the basis for the first clinical trials in humans, and compare these with the latest findings to identify the most relevant aspects that remain unanswered to date. The main focus of the discussions presented here will be on the mechanisms associated with the survival and functionality of the transplants. These include the role of the dopamine released by the grafts and the capacity of the grafted cells to extend fibers and to integrate into the motor circuit. The complete understanding of these aspects will require extensive research on basic aspects of molecular and cellular physiology, together with neuronal network function, in order to uncover the real potential of cell grafting for treating PD.

**Key words:** Parkinson's disease; Cell replacement; Animal models; Nigrostriatal pathway; Striatum; Dopaminergic loss



**Core tip:** The first studies on cell transplantation for Parkinson's disease were published during the early 80s. Since then, it has been shown that different cell types induce functional benefits but with high variability among subjects. Here, we first provide a general overview of the field during its early years. Then, we discuss some factors associated with the functionality of the graft based

on the latest findings, and highlight the importance of understanding basic aspects (*e.g.*, factors influencing graft integration) which ultimately could contribute to reducing the variability of the functional outcome-an important requirement for its application in the clinic.

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## INTRODUCTION

The transplantation of different tissues into the brain began as an experimental approach for understanding fundamental aspects of the development and function of the central nervous system. The first transplant in an animal model of Parkinson's disease (PD) was performed in 1979 with the objective of determining whether grafted dopamine-producing cells were able to reduce the motor alterations in the animal model[1,2]. These and other initial reports of graft tissue survival in the brain, and its beneficial effects on a PD animal model, contributed to the beginning of cell grafting era in PD, including both basic and clinical research approaches. Nearly 40 years after the first studies in this field, there is continuing interest in the development of cell-replacement therapies for treating PD, with a particular focus on the search for optimal cell-sources for grafting. The objective of this review is to perform a general description and a critical evaluation of our current understanding of the mechanisms underlying the success of cell-replacement therapy in animal models of PD. We will mainly focus on the mechanisms underlying the functionality of the grafts when evaluated using pharmacological tests, and on the comparison of the results obtained principally with fetal ventral mesencephalic cells (FVM) and embryonic stem cells (ESCs)-derived midbrain dopaminergic neurons. Ultimately, the purpose of this review is to provide a perspective of what has been gained relative to the prevailing knowledge during the starting point of this research area: Basically, that in order to provide a long-term benefit in PD motor symptoms, functional integration of the transplanted cells into the host brain circuit is essential.

## EARLY YEARS OF CELL GRAFTING INTO THE BRAIN

The earliest known report of neural tissue transplantation into the brain was conducted by Thompson[3] in 1890. He published a brief description of the transient survival of grafted cat cortical tissue into the brain of a dog, in a work entitled "Successful brain grafting"[3]. In 1907, in another attempt to prove that brain grafting was possible, Del Conte[4] implanted fetal cortex tissue into an adult mammalian brain, showing similar results to those reported by Thompson. In 1909 Ranson provided evidence that suggested that postnatal nervous tissue, the cervical ganglion obtained from 1-wk-old rats, survived when grafted into the adult cortex[5]. Later, in 1917 Dunn found that rat neonatal cerebral cortex tissue transplanted into the adult rat brain was able to survive, grow, and even exhibited myelinated fibers[6]. Other studies were performed during the following years (*e.g.*, Ref[7,8]), which together with those described thus far, constitute the earliest antecedents for cell transplantation.

The functional consequences of brain transplants were not evaluated until 1979[1,2] using the 6-hydroxydopamine (6-OHDA) animal model of PD, which was developed 10 years before[9]. This model allows the selective destruction of dopaminergic neurons in the substantia nigra pars compacta (SNpc) of only one hemisphere of a rat's brain[9]. The motor asymmetry observed in this toxin-based model is characterized by a turning behavior contralateral or ipsilateral to the side of the lesion, and is induced by the systemic administration of dopaminergic agonists (amphetamine or apomorphine) (Figure 1A and B)[9,10]. These experimental approaches allowed to test the functional consequences of cell transplantation by grafting dopamine-producing cells[1,2,11]. The general assumption was that, since motor asymmetry is a consequence of a decrement in dopamine in the striatum, then that asymmetry could be reversed by grafting dopaminergic cells, as long as they release dopamine in the host (Figure 1C and D).

## CELL GRAFTING IN PD: THE PIONEERING STUDIES (1979-1990)

### *FVM grafts in pre-clinical studies*

Cells derived from FVM tissue were the first type of cells used for brain grafting in the 6-OHDA rat model of PD[1,2] (for a timeline of pre-clinical studies see Figure 2). This tissue was selected because it contains dopaminergic neurons[12]. In 1979 and 1980, two independent studies confirmed that FVM cells were able to survive (from few to approximately 4000 surviving grafted cells observed 1-7 mo after transplantation), to extend projections into the host striatum after being grafted into the lateral ventricle (Figure 3)[1] or in a cavity at the surface of the striatum (Figure 3)[2], and to reduced circling behavior induced either by apomorphine[1] or amphetamine[2] by approximately 50%, when compared to measurements of motor asymmetry before transplantation. These results were encouraging as they were the first demonstration of a functional outcome induced by grafting exogenous cells in the brain.

The mechanism underlying the functional effects of the grafts was proposed to be the dopamine released from FVM cells (Figure 1C and D). However, the first studies found that some animals with surviving grafts did not exhibit any improvement in turning behavior. Several authors using either the same cell type[13-19] or a different cell source[11,20] have replicated these observations, which has not received a complete explanation to date. However,

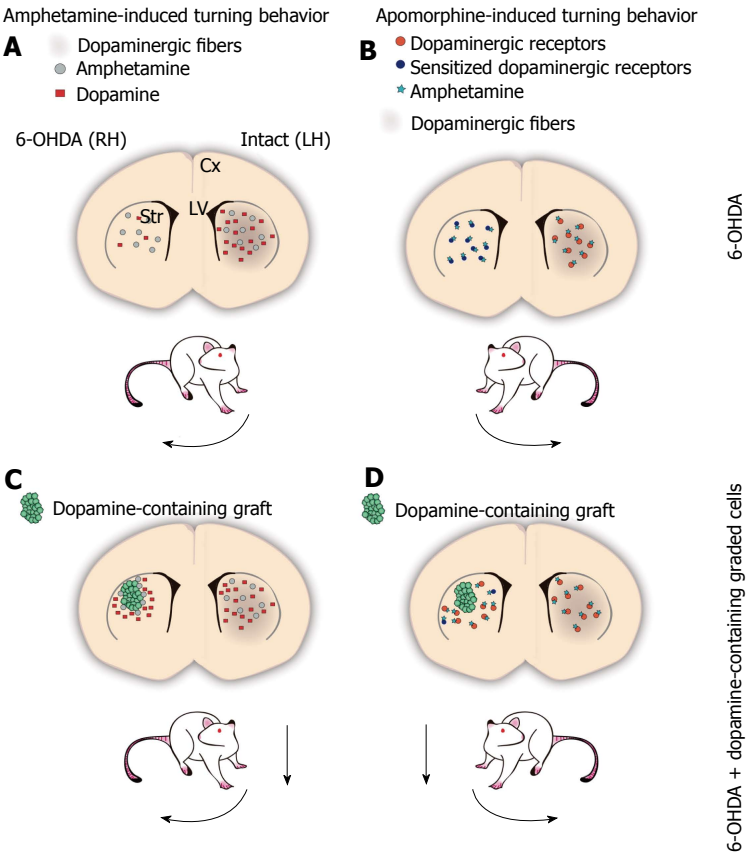


**Figure 1 The 6-OHDA rat model of Parkinson's disease.** A-D: Schemes of a coronal representation of the rat brain. Dopaminergic fibers are depicted with brown shadowing, which is lacking in the 6-OHDA-lesioned hemisphere; A: Amphetamine (grey circles) administration promotes the release of dopamine (red squares) from the intact dopaminergic terminals of the striatum, disproportionally increasing dopamine concentration in the non-lesioned side relative to the lesioned side, as the latter contains fewer (or none at all) dopaminergic terminals. The asymmetry in extracellular dopamine levels between both hemispheres induces the stereotypical behavior known as circling or turning behavior, ipsilateral to the lesioned side (curved arrow next to the rat); B: Apomorphine is a dopaminergic receptor agonist that can activate postsynaptic dopamine receptors in the striatum (orange circles). 6-OHDA-induced dopaminergic denervation in one hemisphere of the striatum, results in postsynaptic supersensitivity to dopamine in the lesioned side (sensitized dopamine receptors are represented as dark blue circles), such that apomorphine (teal stars) stimulation increases the activity in the lesioned side to a greater extent than in the non-lesioned side. The supersensitivity effect promotes that lesioned animals turn contralateral to the lesioned side after apomorphine administration (curved arrow); C: Amphetamine stimulates dopamine-containing cells (green circles) grafted into the denervated striatum increasing extracellular dopamine concentration in the lesioned side, which leads to a decrement in motor asymmetry (dashed arrow); D: Grafted cells that release dopamine decrease the supersensitivity effect on the lesioned hemisphere, normalizing the response to dopamine or agonists relative to the non-lesioned side. Thus, after apomorphine administration, grafted animals decrease their turn number (dashed arrow). Cx: Cortex; LH: Left hemisphere; LV: Lateral ventricles; RH: Right hemisphere; Str: Striatum.

by that time, Björklund and Stenevi[2] proposed that fiber ingrowth from grafted cells into the striatum was, together with the release of dopamine, the determining factors for producing a reduction in circling behavior. Subsequently, a correlation between the reduction in amphetamine-induced turning behavior and the degree of fiber ingrowth was reported[21]. The observations on the variability in the motor improvement in grafted animals with surviving transplants was also found to correlate with the degree of dopaminergic lesion[1,22] and graft survival[23].

One year after the first reports of cell grafting in an animal model of PD, evidence confirmed that dopamine was present in the lesioned striatum of FVM grafted animals[14]. Dopamine tissue-content was found to correlate with the reduction of circling behavior induced by amphetamine. It was also found that a restoration of at least 3% of normal dopamine levels in the striatum was sufficient to reduce the motor asymmetry[24]. However, these observations only demonstrated that mesencephalic transplants contain the neurotransmitter, but not that they release it. In 1983, Freed *et al*[25] provided more direct evidence for the role of dopamine on motor improvement in the 6-OHDA model of PD. The authors suggested that the graft can release dopamine spontaneously on a tonic basis, reversing the supersensitivity effect caused by dopaminergic denervation by directly quantifying the binding of dopamine to its receptors using a dopamine-receptor binding assay. A few years later, Zetterström *et al*[26], conducted a study using an *in vivo* dialysis assay, where they corroborated that mesencephalic transplants release dopamine spontaneously, and after amphetamine administration. One-year later, the same group observed that dopamine release was higher in animals with more surviving grafted cells and more fiber ingrowth, reaching about 85% of normal dopamine levels under basal conditions[23].

In addition to the reduction in turn number induced either by amphetamine or apomorphine, FVM grafts were shown to reduce some aspects of spontaneous abnormal behaviors observed in the PD animal model, such as sensorimotor orientation deficits and asymmetric limb use[15,27]. However, other studies failed to replicate these results[28,29].

Nowadays, FVM-derived cells remain as one of the most promising sources for cell grafting[30], and much more information has been obtained by using this cell source compared with other cell types. However, a major problem related to the use of FVM tissue as a source for cell grafting was the ethical concern due to the use of fetal-derived tissue, which led to the search for alternative cell-sources.

### *Adrenal medulla grafts in pre-clinical studies: Dopamine vs neurotrophic effects*

Chromaffin cells are neuroendocrine cells that synthesize and release catecholamines from the adrenal medulla (AM) into the bloodstream in response to sympathetic stimulation, triggering the fight-or-flight response. This cell source was chosen for use in cell replacement therapy mainly due to the capacity of chromaffin cells to produce dopamine (for review[31]). The first published report using AM tissue grafted in a PD animal model was conducted by Freed *et al*[11]. They demonstrated that AM

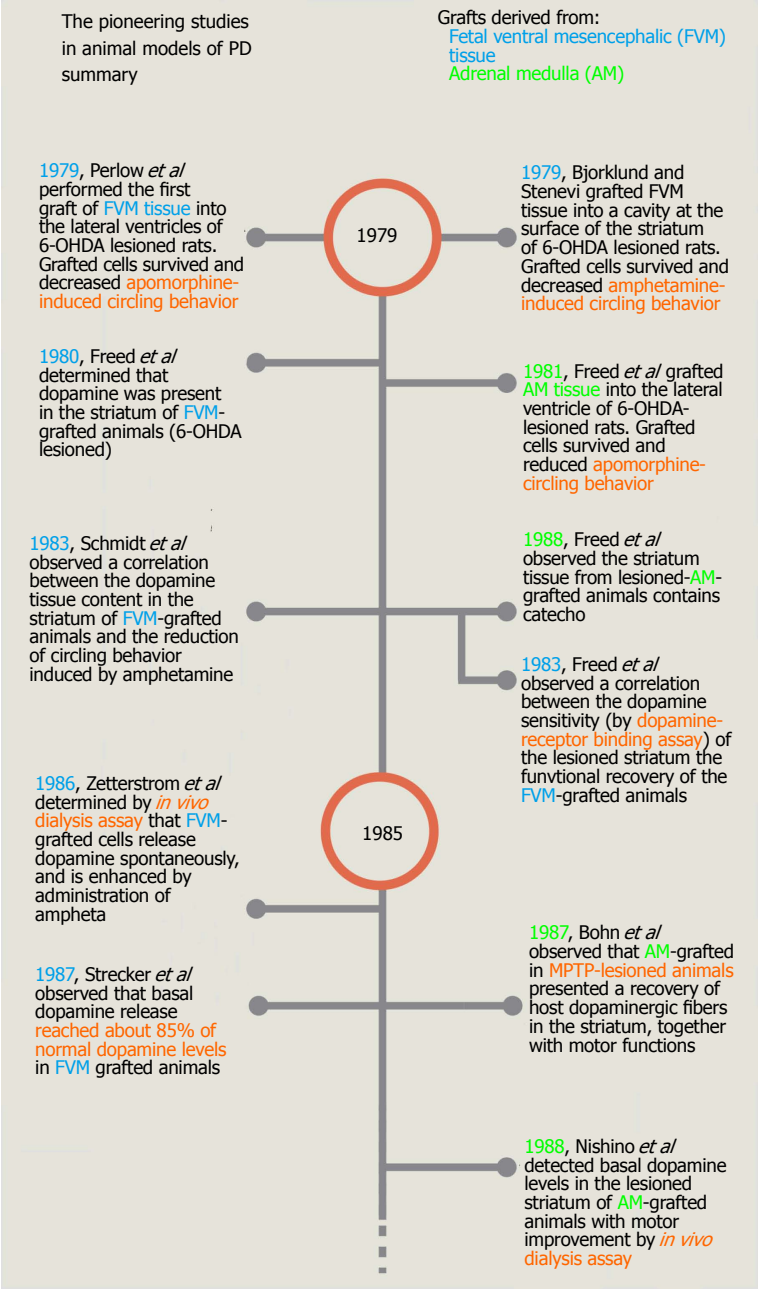


**Figure 2 Timeline of the pioneering studies on cell transplantation in animal models of Parkinson's disease.** This timeline shows only a few of the studies performed during the first 10 years of cell grafting in animal models of PD. Most of these studies were selected because they were the first published reports of either the use of a new animal model of PD, a site of grafting, a type of cell or a specific technique. PD: Parkinson's disease; FVM: Fetal ventral mesencephalic cells; AM: Adrenal medulla.

grafted into the lateral ventricle of 6-OHDA-lesioned rats reduced apomorphine-circling behavior by 20%-50% relative to the initial values before grafting, and this effect lasted for at least 2 mo[11]. However, the cells extended only very few fibers into the host tissue and the mean number of surviving cells was approximately 1535 two months post-grafting[11]. In addition, the animal with the highest number of surviving grafted cells (approximately 4000) did not reduce its circling behavior.

During the eighties it was assumed that the mechanism of action of AM grafts was similar to FVM cells, consisting of the diffusion of high concentrations of dopamine spontaneously released by the graft[11]. Later, it was demonstrated that AM grafts contain high concentrations of adrenaline and noradrenaline, but low concentrations of dopamine[32], mirroring their native characteristics in the AM. When the release of these catecholamines by AM grafts was evaluated by *in vivo* dialysis assays, the authors of the study detected basal dopamine levels only in those animals with motor improvement. Surprisingly, the dopamine levels found were only 50% lower than normal values in the non-lesioned striatum, despite the low survival of grafted chromaffin cells (approximately 50-600 cells)[20]. However, other authors found that the results obtained from chromaffin cell grafts were highly variable and unpredictable in terms of survival and functional outcome, especially when grafts were placed into the striatum (intraparenchymal)[33].

The discrepancies observed when AM-tissue was grafted in the 6-OHDA model of PD, together with results derived using a different model of PD, the 1-methyl-1,2,3,6-tetrahydropiridine (MPTP)[34], led the scientific community to suggest a different mechanism of action for chromaffin cell grafts: A neurotrophic effect. In this

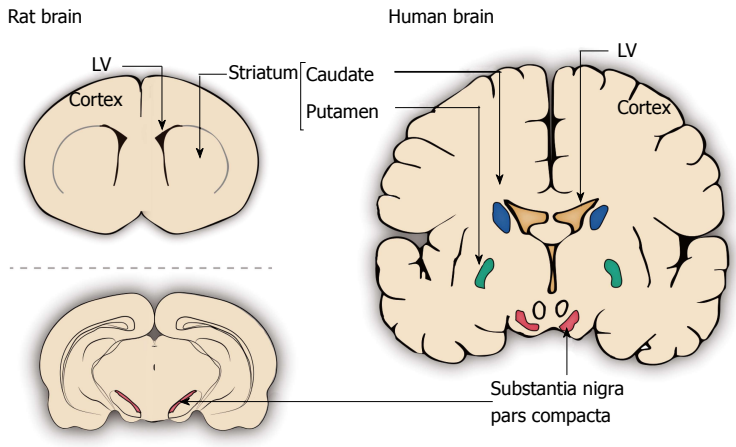


**Figure 3 Schematic representation of different sites in the rat and human brains used for grafting in Parkinson's disease.** The depicted grafting sites include the lateral ventricles (LV), the striatum (in rat) or caudate nucleus and putamen (in human) and the substantia nigra pars compacta. The above schemes are coronal sections of the rat striatum and human caudate (blue) and putamen (green) together with the substantia nigra pars compacta (red). The scheme below is a coronal section at the level of the rat substantia nigra pars compacta (red).

regard, different authors observed that MPTP-lesioned animals with chromaffin cell grafts presented an enhanced recovery of host dopaminergic fibers in the grafted striatum of mouse[35] and monkeys[36], together with a transient functional recovery[37]. These studies suggested a neuroprotective action of the chromaffin cells, which induced the reappearance of tyrosine hydroxylase (TH) immunoreactivity (THir) or the sprouting of surviving host fibers, leading to an increment of dopamine released by the endogenous cell (for review[31]). However, a direct comparison of AM grafts to FVM-derived cells in 6-OHDA lesioned rats demonstrated that AM grafts were less effective in terms of functionality and in their long-term survival, even when AM grafts were placed in the lateral ventricles[38], a site which was assumed to induce a better survival of AM grafts. Thus, despite some studies showing transitory and modest recovery of motor function, AM-derived cells were shown to induce variable and unpredictable results, probably derived from their different mechanism of action compared to FVM grafts.

### *Clinical studies: A brief description*

Although this review is focused in studies using animal models of PD, it is also important to provide at least a general overview of the clinical trials that have been done using both cell sources described above (FVM- and AM-derived cells) (for a timeline of the pioneering studies see Figure 4). There are several extensive reviews aimed at describing critically and in a deeper way the results derived from clinical studies (*e.g.*, Ref[30]).

AM-derived cells were the first to be tested in human patients with PD, with similar results as those observed in animals: Variable and transitory restoration of some motor function[39-41]. Autologous chromaffin cells were first grafted in three different places: The caudate nucleus (Figure 3)[39], the putamen (Figure 3)[40], or in a cavity made at the interface between the caudate nucleus and the lateral ventricles (Figure 3)[41]. In the two first studies the patients showed only moderate recovery that did not last longer than a few months[39,40]. However, by placing the grafts in proximity to the lateral ventricles, other authors reported that one of their two patients showed motor improvements that persisted for at least 10 mo after the grafting procedure[41]. As a result, many clinical studies were done worldwide (*e.g.*, Ref[42-46]) despite the fact that the original articles only reported transitory and modest improvements. As described in a comprehensive review on the topic by Barker *et al*[30], the scientific community started to be concerned about the clinical trials that were taking place, due to the poor or absent functional outcome induced by the AM grafts, the frequent complications from the surgery (*e.g.*, psychiatric alterations), and the fact that post-mortem studies revealed a poor survival of the grafted cells. This led to the abandonment of the use of AM tissue for transplantation.

FVM tissue was the second cell source to be grafted in patients with PD. The grafted tissue was placed into the caudate nucleus[47], the putamen (*e.g.*, Ref[48,49]), both sites (*e.g.*, Ref[50]), in a cavity made at the interface between the caudate nucleus and the lateral ventricles[51] and even directly into the SNpc (Figure 3)[52]. Unfortunately, the results varied from clear benefits to poor or none, but there were also promising results showing improvements by [$^{18}$F]-DOPA uptake by positron emission tomography (PET) imaging[47].

One of the most controversial issues with these studies was the lack of control groups to discard a placebo effect. In 2001, Freed *et al*[53] performed the first double-blind study that included a placebo control group, in which some patients received FVM cell-grafts bilaterally implanted in the putamen, and observed a modest recovery compared with the sham group. Other double-blind studies were done during subsequent years with a similarly variable symptomatic outcome[54]. Another important issue that became apparent several years after the surgery was that some of the grafted patients started to develop dyskinesias (involuntary movements) as a side effect of the transplant (see[55] for review).

Many clinical studies were subsequently done using FVM cells, chromaffin cells or other types of cell sources including retinal pigmented epithelial cells attached to microcarriers[56,57], adult neural stem cells[58] and autologous bone marrow-derived mesenchymal stem cells[59], all with similar results: Some patients showed moderate recovery,

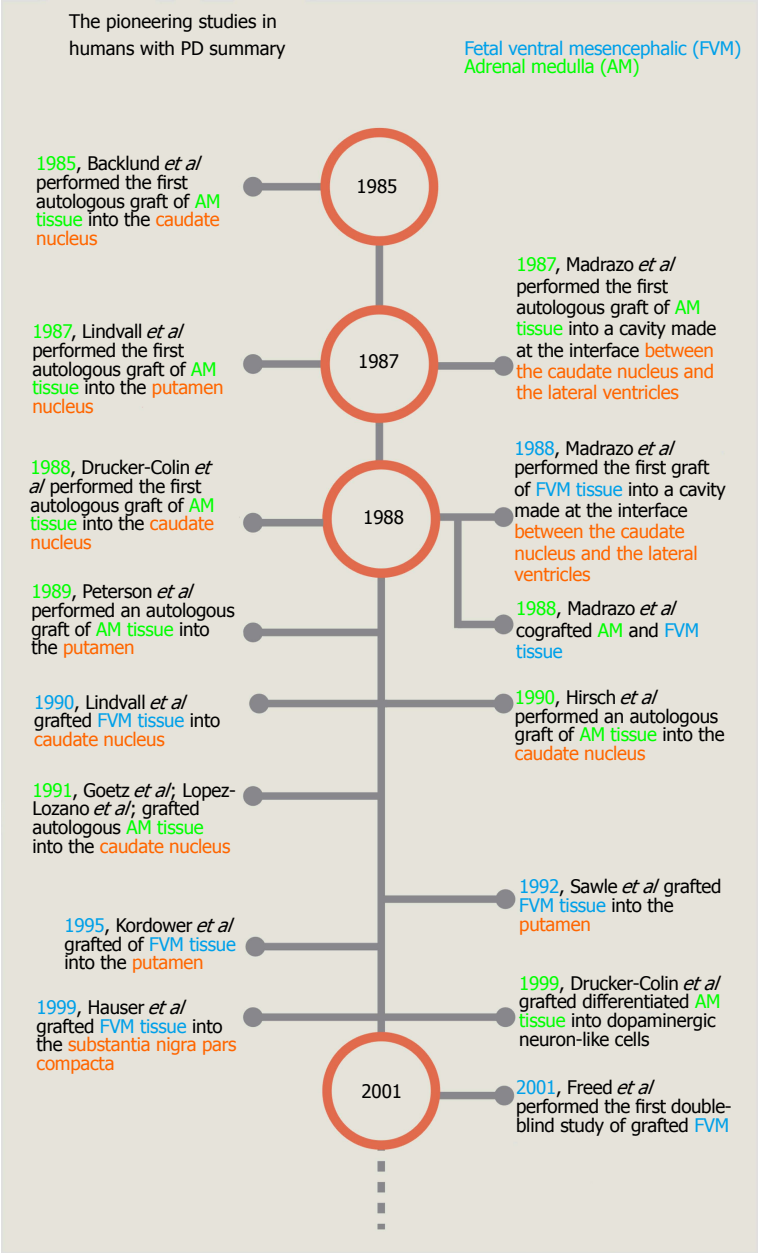


**Figure 4 Timeline of the pioneering studies on cell transplantation in human patients with Parkinson's disease.** This timeline shows only a few of the studies performed during the first 15 years of cell grafting in patients with PD. Most of them are the first published reports in which, a new site of grafting or a new type of cell were used. PD: Parkinson's disease.

whereas others showed poor or no recovery at all (for review see[60,61], visit http://clinicaltrials.gov for clinical NIH-funded trials currently underway, Table 1). The highly variable results obtained even to date strongly argue that some of the key requirements for this type of therapeutic option to work are still unknown.

## WHAT DO WE KNOW NOW?

The study of graft-associated mechanisms producing motor improvements in animal models of PD has been largely done using experimental paradigms with a strong bias towards the role of dopamine. However, actually we know that several additional factors also somehow influence the functional motor recovery. These include the degree of survival of the graft, the capacity of the graft to extend fibers into the host, the ability of these fibers to establish functional connections with host cells and the extrinsic factors that influence all the previously mentioned aspects. The next section of this review focuses on comparing the facts that we knew in the early years with the latest advances in the field. We will describe the results derived using two cell types, which have been widely demonstrated to possess the greatest capacity to survive and to decrease the circling behavior and improve other motor functions in animal models of PD: FVM-derived cells and ESC-derived dopaminergic neurons.

### *Graft survival and the effects of grafting into the striatum and the SNpc*

The survival of the grafted cells is modified by different factors including the age of the donor tissue, the graft composition and the location of the graft.

**Table 1 Current clinical trials (2013-2016)**

| | Type of cells | Site of procedure | Age of patients | No. of patients | Control group(s) | Phase[2] | Current status and notes |
|---|---|---|---|---|---|---|---|
| The University of Texas Health Science Center, United States. NCT02611167[1] | Allogeneic bone marrow-derived mesenchymal stem cell | Delivered intravenously | 45-70 | 20 | No | I and II | Nov 2017. Starts on May 2016 |
| ISCO-Florey. Cyto Therapeutics Pty Limited. Australia. NCT02452723[1] | Human parthenogenetic stem cells-derived neural stem cells | Striatum and SNpc | 30-70 | 12 | No | I and II | Approved from the TGA of Australia (received on December 2015) |
| University of Saskatchewan and Manitoba, Canada. NCT02538315[1] | Fetal dopaminergic grafts | NS | 18 and older | 30 | NS | NS | Study type: Observational. Using [$^{18}$F]FDOPA PET/CT to monitor the effectiveness of grafts. Started on December 2015 |
| University of Kentucky, United States. NCT01833364[1] | Autologous peripheral nerve | SNpc | 40-75 | 16 | No | NS | Started on 2015. |
| TRANSEURO, Europe. NCT01898390[a] | FVM Tissue | NS | 30-68 | 40 | Yes (no surgery) | I | No updates. Patients undergoing deep brain stimulation surgery Enrolling participants. |
| CHA University, South Korea. NCT01860794[1] | Mesencephalic neural precursor cells | NS | 18-70 | 15 | NS | I and II | No updates since December 2014 Started on 2013. No updates |
| Living cell technologies. Auckland City Hospital, New Zealand. NCT01734733[a] | NTCELL [immunoprotected (alginate-encapsulated) choroid plexus cells] | NS | 40-70 | NS | NS | I and II | Started on 2013. No updates |

[1]Is the ClinicalTrials.gov identifier. For more information and other trials visit the website; [2]Clinical phases: I: Test a new treatment in a small group to evaluate its safety, dosage range and side effects; II: Treatment in a small group to see its effectiveness and to further, evaluate its safety. NS: No specified; TGA: Therapeutic Goods Administration.

The relation between survival and functional recovery has been studied by different authors[17,19,23,62,63]. FVM tissue is usually obtained from 12.5-d-old mouse embryos or 14-d-old rat embryos. However, it has been observed that the survival of intra-striatal grafts of FVM-derived dopaminergic neurons is higher when 12-d-old rat embryos are used[64]. Interestingly, the increment in survival of grafted FVM cells (derived from rat embryos of 12 d *vs* 14 d) is not necessarily accompanied by an equivalent improvement in the functional outcome. This suggests that a critical number of cells is required for improvement, above which a higher survival does not contribute to further improvement[65]. Sauer *et al*[19] estimated that approximately 2000 surviving cells were necessary for complete recovery of turning behavior, whereas only 600 cells were necessary for a moderate level of recovery. It is important to note that, in that study, the improvement observed in four animals with 600-1500 surviving cells ranged from negligible to low[19]. In other reports, it has been observed that an even smaller number (100-200) of surviving $TH^+$ cells was sufficient to obtain a 50% reduction in turning behavior (*e.g.*, Ref[17,62]). More recently, using human FVM cells, it was observed that at least 657 $TH^+$ surviving cells were necessary to induce a significant reduction (50% relative to the initial circling behavior before grafting) in apomorphine- induced circling behavior[66]. Similarly, using human ESC-derived midbrain dopaminergic neurons, a complete recovery of amphetamine-induced circling behavior was achieved with approximately 986 $TH^+$ surviving cells[67]. Therefore, in general, the studies that have correlated survival of the grafted cells with behavioral improvement have, surprisingly, found that a very small number of cells are sufficient to produce a robust motor improvement.

An additional factor to be considered in the case of FVM-derived cells is that the age of the donor tissue in turn influences the composition of the grafted cells. The developing mesencephalon contains two major sub-populations of neurons: A9 and A10 neurons[12,68]. The A9 sub-population in particular corresponds to dopaminergic neurons that will form the SNpc, whereas the A10 neurons are dopaminergic neurons that form the ventral tegmental area. Each subtype differs in multiple characteristics, including their morphology, their protein-expression profile and their target areas in the brain (SNpc in the dorsal striatum and ventral tegmental area in the ventral striatum). Since FVM grafts contain a mix of these two sub-populations[69,70], researchers started to elucidate the role of each subtype on the functional outcome induced by the graft. A9 neurons were found to be critically important for a major functional recovery, due to these grafted-cells innervating the regions of the striatum corresponding to the areas normally innervated by dopaminergic neurons from the SNpc[71].

Thus far, we have only discussed ectopic sites (*i.e.*, located outside the SNpc) for grafting as a therapeutic

approach to reverse the motor alterations observed in PD. However, we have to consider that dopaminergic cells from the nigrostriatal pathway are part of a complex circuit that receives regulatory inputs from other structures (*e.g.*, SN pars reticulata). In agreement with this, it has been observed that intrastriatal grafts do not ameliorate all the symptoms associated with degeneration of the nigrostriatal pathway, since the proper function of the basal ganglia circuitry is far from being restored[16,28,65,72]. Current approaches on this front focus on the possibility of reconstructing the nigrostriatal pathway, by grafting cells into the SNpc (Figure 3) and directing their fibers to reestablish the lost dopaminergic circuitry in the striatum[73]. The first studies that attempted this procedure succeeded in demonstrating that FVM grafts survive when placed into the SNpc and that, in some cases, the neurons extended projections into the striatum and induced some reduction in drug-induced circling behavior[74-79]. However, the survival of FVM cells grafted into the SNpc was less prominent as compared to intra-striatal grafts[74,78,80].

### *Fiber ingrowth and dopamine release*

The occurrence of fiber ingrowth from the graft into the host depends in part on the type of cell used. Intra-striatal grafts of FVM cells[67], ESC-derived dopaminergic neurons[67] and induced pluripotent stem cells (iPSC)-derived dopaminergic neurons[81] have been shown to extend fibers into the host striatum. It has been suggested that the extension of projections is important for mesencephalic grafts[13,16,21,23,27], although FVM-grafts have been shown to produce motor improvement without any detectable projections[1,82]. However, it is reasonable to consider that the greater the extension of the graft projections, the further the molecules they release can diffuse. In addition, with more and longer projections, the establishment of synaptic contacts between the host cells and the graft becomes more likely.

Certainly, an ideal scenario for intra-striatal grafts is one in which dopamine release and clearance are regulated by the necessities of the host circuit. Different authors have shown that FVM grafts release dopamine under basal conditions, and that the release can be enhanced by stimulation with amphetamine[18,26] or high extracellular potassium[83,84]. This has also been demonstrated for ESC-derived dopaminergic neurons[67,85]. Notably, these two types of cells have been shown to deliver sufficient dopamine into the striatum to restore its concentration to normal levels[67,85]. Interestingly, a recent study showed that grafts of FVM cells placed into the SNpc increased striatal dopamine levels to 77% compared to lesioned animals[86]. This study also observed extensive axonal growth from the grafted cells (confirmed by grafting cells from transgenic mice overexpressing green fluorescent protein, GFP) that reached the striatum, together with a significant behavioral recovery in the apomorphine-induced rotation of 94% relative to the initial rotation numbers before grafting[86]. Another study published the same year showed similar results[87], and demonstrated that over-expression of glial cell-derived neurotrophic factor (GDNF) enhanced survival and axonal growth from the grafted cells positioned in the SNpc. The authors also observed a reduction in turn number induced by amphetamine of approximately 75% relative to the initial values before grafting in GDNF-treated animals, which lasted for at least 12 wk[87]. In a more recent study, Grealish *et al*[67] demonstrated that human ESC-derived dopaminergic neurons (A9 and A10 phenotypes) can restore dopaminergic transmission in the transplanted striatum, as occupancy of D2/D3 receptors by dopamine measured using PET showed dopamine binding levels that were similar to the non-lesioned side. More importantly, the study demonstrated that human ESC-derived midbrain dopaminergic neurons grafted into the SNpc provided widespread innervation that extended more than 10 mm throughout the forebrain, with dense innervation in the striatum (A9 subtype), as well as nucleus accumbens, amygdala and frontal cortex (A10 subtype), which are normally innervated by endogenous dopaminergic fibers from the SNpc. In addition, they obtained similar results using human FVM, with an average axonal number of 2169 for the FVM cells and 2453 for the human ESC-derived cells[67]; although, the functional effects of the nigral grafts were not determined in this study. Taken together, these findings are encouraging, suggesting that the reconstruction of the dopaminergic pathway is a plausible approach. However, more research is necessary, to determine whether normal connectivity and physiology are established by the grafted cells into the SNpc. In this regard, it seems that the projections extended by the grafted cells are highly specific, as they connect exclusively to targets that are normally innervated by dopaminergic fibers from the SNpc (for a review on this topic see[73]).

### *Establishment of connections*

A property of central importance for the grafted cells is their capacity to integrate into the host circuit by establishing functional synaptic connections with other cells. This feature marks a difference between grafted cells that function only as release-pumps for dopamine and trophic factors, and those that integrate into the circuit and respond to the physiological needs of the site.

Different sources of evidence support the idea that some types of grafted cells, especially FVM cells and human ESC derived-dopaminergic neurons, establish synapsis with the host cells[88-93]. Electrophysiological studies were initially difficult to perform, as no direct method existed for differentiating the graft from the host cells. Hence, in early electrophysiological studies the recorded cells were chosen blindly, and later identified by THir or by their electrophysiological properties[88,89]. These electrophysiological recordings showed that host striatal cells close to THir fiber projections of FVM cells decreased their firing rates to levels normally observed in a healthy striatum[89]. In contrast, cells located far from the graft or graft-projections presented altered firing

rates[89]. Additionally, Freund *et al*[90] demonstrated by using electronic microscopy that FVM cell grafts establish synapses with the dendritic shafts and spines of the striatal neurons, including medium spiny neurons and giant cholinergic interneurons. However, they failed to track reciprocal afferent connections to the graft from the host striatum[90]. Evidence of synaptic connections, both from graft to host and from host to graft, was later observed by other authors using immunostaining for postsynaptic and presynaptic markers and electron microscopy[91]. These results confirmed that some FVM cell grafts have the capacity to integrate into the host circuit and induce changes in host cell firing rates. Concurrently, to identify electrically active afferent and efferent connections of the graft to the host cells, Arbuthnott *et al*[88] grafted FVM cells in the striatum and implanted stimulating electrodes under the grafts in the striatum but also in the frontal cortex, locus coeruleus or dorsal raphe nuclei of 6-OHDA-lesioned animals. They found that grafted cells fired action potentials after striatal stimulation in a similar manner as naïve SNpc dopaminergic neurons, but remarkably, only in those animals in which rotational behavior was compensated and had longer antidromic latencies[88]. They also observed that some grafted cells were activated after stimulation in the frontal cortex, locus coeruleus or raphe nuclei[88].

More direct evidence supporting electrical activity and connectivity of grafts has been recently obtained using FVM grafts derived from transgenic mice expressing GFP under the control of the TH gene promoter, and measuring their electrical activity with whole-cell patch clamp recordings[92]. They observed that a higher proportion of grafted cells in the lesioned striatum fired spontaneous action potentials than grafted cells in the non-lesioned striatum. However, the firing frequency was similar for both[92]. Furthermore, they measured lower frequency of inhibitory and excitatory postsynaptic currents in cells grafted into lesioned, as compared to non-lesioned, animals[92]. Based on these data, the authors suggested that dopamine levels in the striatum could modulate the activity of grafted cells by the activation of $D_2$ autoreceptors in FVM cells. Another possibility is that the grafts in non-lesioned animals received more GABAergic synaptic inputs[92].

The evidence presented thus far did not confirm that dopamine release was regulated by electrical activity, and that the release was responsible for the functional recovery observed in behavioral experiments. Interestingly, Dell'Anno *et al*[94] were able to control the electrical properties and neurotransmitter release of grafted reprogramed dopaminergic neurons by using designer receptors exclusively activated by designer drug technology. The authors demonstrated that the functional outcome is higher when the neural activity of the striatal-grafted cells is stimulated by clozapine-N-oxide (the pharmacologically inert molecule that activates the designed receptor expressed by the cells), achieving similar results to those observed using FVM tissue[94]. *In vitro*, stimulation of the reprogramed cells resulted also in an increment in neural activity (number of spikes per second) together with an increment of dopamine release[94].

Using a different approach to control the neuronal activity of the grafted cells in order to understand its relation to the functional outcome, Steinbeck *et al*[93] grafted differentiated mesencephalic dopaminergic neurons derived from human ESC that expressed the inhibitory light-activated chloride pump halorhodopsin (eNpHR3.0-EYEP, also known as HALO). After corroborating the functionality of the cells *in vitro*, they were grafted into the striatum of 6-OHDA lesioned immunodeficient mice. The authors observed that transplanted animals gradually decreased their amphetamine-induced turning behavior for a period of 4 mo[93]. Electrophysiological recordings on brain slices showed that the grafted cells produced action potentials that ceased after illumination (*i.e.*, activation of the HALO-mediated chloride conductance). It was also corroborated that grafted cells are able to modulate the activity of spiny medium neurons, and that they receive functional glutamatergic inputs from the host cells[93]. *In vivo* studies performed in freely moving grafted animals showed that the reduction of spontaneous rotations and sensorimotor deficits evaluated with the corridor test is dependent on graft activity, as optogenetic silencing of the cells reversed the recovery[93]. To test the dependence of recovery on dopamine release by grafted cells, the animals were injected with apomorphine before optogenetic silencing. The authors observed that after illumination the recovery of the behavior was still present, as host dopaminergic receptors were expected to be occupied by apomorphine. This study provides an appropriate strategy to interrogate the mechanisms underlying the functionality of grafted cells. In general, grafted cells have been proven to be able to integrate into the host tissue but more experiments are necessary for a complete understanding of their role in the population dynamics of the striatal circuit.

## GENERAL DISCUSSION: LOOKING INTO THE FUTURE, BACK TO BASICS

After the studies by Perlow[1] and Björklund and Stenevi[2], several authors have replicated their results with the same type of cells as well as different dopamine-containing cells. As laid out in the preceding sections, there are several different cell sources that have demonstrated a capacity to survive and reverse motor alterations in animal models of PD. However, the clinical benefits of brain grafting in PD patients have not yielded the expected results. A look back in history indicates that some questions related to basic aspects of molecular and cellular physiology, as well as neuronal network function, remain unanswered.

One important issue is to identify the factors that determine whether a graft will induce motor recovery or not. Independently of the cell type used, the available evidence shows that, in animal models or human subjects, some graft recipients exhibit no recovery despite having

equivalent levels of graft survival to the individuals that presented striking motor improvement. The reason for this variability is still unknown. Some results have shown that electrical activity of the grafted cells is a common feature of those animals with compensated behavior[88]. But how is this electrical activity or integration of the grafted cells achieved? The question remains unanswered. One possibility is that the host needs to have one or more individual-specific traits to provide a permissive microenvironment for the correct integration of the graft into the host tissue. These traits may involve molecular and cellular signaling pathways and communication between the endogenous and exogenous cells. What are these traits? Are there genetic or immunologic factors involved? Knowing the answer to these questions would allow clinicians to predict who can be a candidate for cell-replacement therapy, or even adjust the microenvironment of a host or the nature of the grafted cells to successfully treat all PD patients in an individualized manner. Current technology can be used for answering these questions. For example, current genome engineering technology such as CRISPR-Cas (see[95] for review) and TetR-, Cre- or Flp-mediated DNA recombination (see[96] for review) could allow us to delete, insert, reverse, silence or enhance the expression of different genes in order to elucidate the factors involved in the permissibility of the host. This technology would also contribute to understanding the mechanisms and molecules involved in the communication between the cells from the graft and those from the host. Additionally, regarding the influence of the microenvironment on the grafted cells, it has been shown that uncommitted ESC-derived cells grafted into different areas of the brain are capable of sensing the host site, and respond by modifying their survival and differentiation into a specific cell type[97].

Another important aspect is to understand the mechanisms related to the functionality of the graft. The unanswered questions in this regard are more related to systems-biology aspects concerning the consequences of the graft on the basal ganglia circuit. Further studies are necessary to determine the physiological consequences of grafting over the altered basal ganglia connections during natural behavior, as opposed to the use of pharmacological tools. By combining current approximations such as *in vivo* electrophysiological recordings or optogenetic activation and calcium imaging, it would be possible to determine whether grafts have differential effects on the activity of the direct and indirect pathways of the basal ganglia, and in general over the dynamics of the striatal microcircuit. These technologies have been used for the study of the normal function of the basal ganglia circuit and have also been applied to animal models of PD (*e.g.*, Ref[98-100]). Additionally, by coupling *in vivo* pharmacology experiments with optogenetics[101], we can understand more about the mechanisms underlying the functionality of the grafts in PD, as has been done recently[93].

Survival of grafted dopaminergic neurons remain as a limitation; only 1% to 20% of FVM-derived cells are able to survive in animal models of PD[102]. Different cellular stress responses occurring by the dissection of the cells and after the graft procedure are part responsible for the observed cell death[102]. The majority of the studies that follow graft survival and behavior in animal models focus on analyzing short and medium periods of time (*e.g.*, Ref[64,102]). However, despite the low survival of grafted cells, clinical trials have shown cases with significant motor improvements that last for varying time periods (*e.g.*, over some years to 20 years after grafting of human mesencephalic tissue[103]). Thus, as long as the underlying mechanisms related to the variability observed between subjects is comprehended, controlled and reduced, transplantation of dopaminergic-containing cells could be a potential treatment for motor symptoms in PD.

Finally, we have to remember that PD is a very complex disease that affects other systems in addition to the dopaminergic pathway[104]. Thus, the aim of cell replacement therapy in PD is merely symptomatic, and focused exclusively on the motor symptoms associated with the degeneration of the nigrostriatal pathway. An important concern related to the pathology *per se* is the fact that some PD-grafted patients have shown Lewy-body inclusions in the grafted cells[105]. Lewy-bodies are aggregates of normal, misfolded and truncated proteins and ubiquitin enzymes mainly composed of $\alpha$-synuclein, and constitute the histological hallmark of PD (see[106] for review). This discovery is part of the evidence that supports the idea that PD spreads as a prion-like pathology (see[107] for review). Thus, it is probable that independently of the site of grafting, striatum or SNpc, the grafted cells will eventually develop the pathology. However, as Petit, Olsson and Brundin[108] have argued, the observation of Lewy-body inclusions does not necessarily invalidate the cell replacement therapy approach, based on the following arguments: Some patients have demonstrated motor improvements for up to 18 years; only a small proportion of grafted cells present Lewy-body inclusions; and finally we have to examine the cost-effectiveness relationship. Despite the logic of the arguments, on which we agree, we still have to remember that cell replacement therapy is not a cure for the disease, but rather a symptomatic relief. Thus, understanding the mechanisms related to the pathophysiology of PD is of fundamental importance if we wish to provide a more definitive strategy to face this disease (for review see[109]).

## CONCLUSION

Important progress has been made since the first demonstration of a functional effect of dopaminergic-cell grafts in an animal model of PD. After the first decade of cell grafting in PD, it was clear that FVM-derived cells were a better cell source for grafting in comparison to chromaffin cells derived from the AM. To date FVM-derived cells are considered as the most promising source for cell therapy in PD. After all these years of extensive efforts, it has been demonstrated that striatal

FVM grafts survive, extend projections, release dopamine and more importantly, alleviate motor alterations in both animal models and in human subjects with Parkinson's disease. Cell integration is also important for achieving a positive functional outcome in other cell sources such as ESC-derived dopaminergic neurons. In addition, midbrain dopaminergic neuron grafts placed directly into the SNpc have also been shown to survive, to extend projections into the striatum, to increase striatal dopamine content, and to induce functional recovery. These observations are important and encouraging as they point to the possibility of reconstructing the nigrostriatal dopaminergic pathway.

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