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# Towards stem cell replacement therapies for Parkinson's disease

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ABSTRACT

Current therapeutic approaches for Parkinson's disease (PD) provide symptomatic relief but none of them change the course of disease. There is therefore a clear need for regenerative and cell replacement therapies (CRT). However, CRT faces several important challenges. First, the main symptoms of PD result from the loss of midbrain dopamine (DA) neurons, but other cell types are also affected. Second, transplantation of human ventral midbrain tissue from aborted fetuses has lead to proof of principle that CRT may work, however, it has also pointed out to important patient-, surgery- and cell preparation-related variables, which need to be improved. Third, while some patients have developed dyskinesias and, with time, Lewy bodies in the grafted cells, other patients have experienced remarkable improvement and have been able to stop their medication. Is there a case for PD CRT today? What is the possible contribution of stem cells to CRT? In this review, I will discuss what we learned from clinical trials using fetal tissue grafts, recent progress in the development of human stem cell-derived-DA neurons for CRT, and some of the issues that need to be solved in order to develop stem cells as tools for PD CRT.



## 1. Introduction: Parkinson's disease

Parkinson's disease (PD) is one of the most common neurodegenerative diseases. It affects about 2% of the population over 65 years and is characterized by a triad of classical symptoms: resting tremor, rigidity and hypokinesia. These symptoms are later followed by disturbances in gait, balance, and they can be accompanied by autonomic disturbances, depression and dementia.

At a pathological level, PD is characterized by a progressive loss of midbrain substantia nigra dopaminergic (DA) neurons that project to the striatum, the neurons responsible for the main motor symptoms in the disease. However, other cell types in the peripheral and central nervous system are also affected. Notably, Braak has proposed [1] that neuronal degeneration may first start in the olfactory and enteric nervous system, followed by alterations in the brainstem, including the vagus nerve, pedunculopontine nucleus, locus coeruleus and dorsal raphe nucleus. Only then, alterations in midbrain DA neurons would appear and, later on, forebrain neurons, such as cortical neurons, would be affected. These pathological findings are also accompanied by the presence of intracellular inclusions, known as Lewy bodies and Lewy neurites, which are widespread, distributed in the peripheral and the central nervous system.

The cause of PD is largely unknown, with 95% of the cases being sporadic. However, several causative genes and susceptibility factors have been identified in the last decade (for review see [2]). These studies have suggested that abnormally increased oxidative stress and mitochondrial dysfunction, together with protein misfolding and impairments in the ubiquitin–proteasome and autophagy–lysosomal systems, contribute to PD.

PD patients are currently treated with a variety of pharmacological tools, including L-DOPA, dopaminergic agonists and inhibitors of dopamine degrading enzymes. The current surgical treatment of PD is mainly based on the use of deep brain stimulation of the internal segment of the globus pallidus, the subthalamic nucleus or the pedunculopontine nucleus. While these treatments provide symptomatic relief, none of them change the course of the disease. There is therefore a clear need for restorative and regenerative approaches, including neuroprotection, neurogenesis and CRT. In this review, I will exclusively focus on the latter.

## 2. CRT: lessons from mesencephalic tissue grafts

Fetal mesencephalic tissue has been used for CRT in order to replace the DA-producing neurons lost during the course of PD. These cells have been grafted in the target of midbrain DA neurons, the striatum, in order to facilitate reinnervation. In the last 20 years, between 300 and 400 PD patients have so far been grafted with human fetal mesencephalic tissue, and as expected from a new experimental therapy, results have ranged from failure to success. While success provided proof of principle that CRT can work

*Abbreviations:* CRT, cell replacement therapy; DA, dopamine; PD, Parkinson's disease; TH, tyrosine hydroxylase; hES, human embryonic stem; iPS, induced pluripotent stem; SDIA, stromal derived inducing activity; AA, ascorbic acid.

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in PD [3], failures pointed out to very important issues [4,5] that need to be improved in order to develop a viable and competitive CRT for PD.

CRT has been a challenging therapeutic strategy from the beginning. The first obstacle was the nature of the donor tissue itself: ethical issues surrounding the use of fetal tissue obtained from legal abortions, limited tissue availability and variable tissue quality, which made difficult to standardize donor cells. In addition, 6–7 ventral midbrain tissues from 6 to 9 week-old human embryos are needed in order to treat one single patient of PD. It thus became rapidly apparent that alternative sources of donor cells had to be identified. Stem cells, for their capacity to expand indefinitely and give rise to large numbers of specialized cells, are nowadays thought to be main cell source candidates for CRT.

A second challenge for fetal tissue grafting was the poor survival of DA neurons after transplantation. It has been estimated that in the best of the cases as few as 10% of the human grafted cells survive after transplantation [6]. Multiple variables related to tissue processing prior to grafting are suspected to contribute to variability in cell composition and performance of the grafts. These include (i) dissection technique. (ii) Cell cultivation with growth factors prior to grafting [4], which may have an impact on cell composition. (iii) Cell preparation and grafting. For instance, cell suspensions [3,6] survive better than tissue blocks [5] or tissue strands [4]. These results may be related to limited availability of nutrients/survival factors and/or enhanced immune response due to the presence of immunogenic cells such as endothelial vascular cells in solid grafts. The innate immune system, and in particular microglia, has been long known to be involved in the pathogenesis of PD [7]. More recently, $CD4^+$ and $CD8^+$ T lymphocytes have been found in the substantia nigra of PD patients and in experimental models of PD [8]. Moreover, patients transplanted with fetal tissue in which immunosuppression had been withdrawn 6 months after grafting, activation of microglia was observed [5] and a trend for a functional improvement disappeared. These data suggested that an additional immune response is triggered in PD patients by the breaking of the brain–blood barrier during the grafting procedure. Thus, it is nowadays thought that PD patients receiving CRT could benefit from immunosuppressive therapies for periods longer than 6 months.

Third, adverse effects such as worsening of L-DOPA-induced dyskinesias and off-medication dyskinesias have been reported in all the different clinical trials [4,5,9]. Interestingly, the incidence of dyskinesias in these trials varied from 15% to 56% of the patients receiving fetal tissue grafts. The most severely affected patients were those with older age, advanced PD, and L-DOPA-induced dyskinesias prior to grafting. These findings have suggested that young patients, with a short history of typical PD disease, that respond to L-DOPA, but do not show L-DOPA-induced dyskinesias, may benefit the most from CRT. It thus seems that CRT may need to focus on certain forms of PD and that patient selection may be needed. Other factors that have been linked to higher incidence of dyskinesias are grafts containing more serotonin than DA neurons [10]. Cell composition is also important for functional outcome, as grafts containing substantia nigra (A9) DA neurons provide greater behavioral recovery than grafts rich in ventral tegmental (A10) DA neurons [11]. Thus, a major task for the future will be to standardize cell composition in the grafts and enrich cell preparations in A9 neurons, in order to improve CRT.

Fourth, recent studies have shown that grafted DA neurons survive for as much as 16 years in the brains of PD patients after transplantation [12–14]. However, Lewy bodies and α-synuclein aggregates have been found in few cells in patients grafted for 11–16 years [12,13]. Lower levels of tyrosine hydroxylase (TH) and dopamine transporter have also been found in some of the grafted PD patients [12]. For the moment, it is unclear what is the functional significance of these findings, but even if we consider that the grafts may start declining after the first 10 years, the therapeutic window for CRT would still be wide enough to allow a significant clinical intervention.

Fifth, since the best results in CRT were obtained in open label clinical trials [3,6], a placebo effect has been suspected. However, these improvements were not due to a placebo effect since they were paralleled by improvements in $^{18}$F-dopa uptake, long-term DA neuron survival, profuse reinnervation, and functional integration, as assessed by synaptic inputs to the host striatum [6]. Moreover, in the best cases, the effects lasted for at least 10 years and allowed withdrawal of L-DOPA [3].

## 3. Generation and transplantation of midbrain DA neurons derived from stem cells

Excellent reviews have recently addressed the potential of stem cells in PD and the way that induced pluripotent stem (iPS) cells are changing the landscape of CRT [15–17]. Thus, in this section, I will exclusively focus on some of the most recent advances in the DA differentiation of human ES (hES) and iPS cells, as well as their transplantation in models of PD.

### 3.1. hES cell-derived-DA neurons

Human ES cells are thought to be one of the most promising tools for CRT today. These cells together with recent developments in the understanding of midbrain development have allowed the development of novel protocols for the DA differentiation of hES cells. These include the administration of morphogens and/or survival factors, as well as the overexpression of transcription factors. In some protocols, these interventions are complemented by the use of feeder cells, which together with genetic manipulation are less preferred interventions in a clinical grade CRT, but they are certainly useful tools for its development.

One of the most successful feeder-based protocols for the DA differentiation of hES cells was developed by Perrier et al. [18]. This protocol is based on co-cultivation of hES cells with bone marrow stromal cells, the source of a "stromal derived inducing activity" (SDIA) [19], and a sequential treatment with morphogens and growth factors, including Shh, Fgf8, bFGF, BDNF, GDNF, TGFB3, dcAMP and ascorbic acid (AA). This method yields 65–80% TH+ cells of the total neurons (TuJ1) in vitro. DA neurons obtained by this method express several of the typical midbrain markers, but do not survive well after transplantation. Interestingly, survival was improved in another feeder-based protocol in which midbrain astrocytes were used to promote DA differentiation [20], a strategy previously used in mNSCs [21]. These cell preparations were also treated with Shh and FGF8, and when transplanted in the adult striatum of hemiparkinsonian rats, they induced a significant behavioral recovery. In this case, DA neurons survived for 8 weeks, but neural tissue overgrowth and neuroepithelial tumors were found. These two protocols have thus underlined the need of identifying the differentiation and survival factors produced by the feeders, as well as the need of eliminating the factors that cause excessive proliferation, in order to move forward with CRT.

In recent years, particular attention has been paid to understanding midbrain DA neuron development and to the identification of novel DA-differentiation factors that could be used to improve DA differentiation protocols for hES cells. Efforts in this area have focused both on transcription factors and diffusible factors. With regard to transcription factors, overexpression of Lmx1a, an important factor for midbrain DA neuron development [22], in conjunction with Shh, FGF8 and bFGF treatment, increased from 25% to 50% the percentage of hES-derived neurons that acquire a

midbrain DA phenotype in vitro [23]. In vivo experiments with Lmx1a-differentiated hES cells have so far not been performed, but results obtained with mES cells indicate that cells in these cultures retain their capacity to proliferate and form tumors. This problem was solved in mES cells by performing positive cell sorting with PSA-NCAM antibodies [23], a strategy previously shown to enrich in postmitotic cells [24]. However, survival was impaired in the sorted preparations, indicating that additional factors provided by cells in the negative fraction may have been required for DA neuron survival. Thus, current results in this area indicate that strategies to simultaneously improve differentiation, eliminate undifferentiated tumor-forming cells, and enhance in vivo survival of DA neurons still need to be implemented.

With regard to secreted factor-based protocols for DA neuron differentiation of hES cells, several studies have focused on the identification of factors derived from either midbrain astrocytes [25], stromal cells [26], or from the developing midbrain [28]. Interestingly, Wnt5a is one such factor that was found in midbrain astrocytes and is known to promote DA neuron differentiation of mouse cells both in vitro and in vivo [25,28–30]. In another study [26], CXCL12, pleiotrophin, insulin growth factor 2 and ephrin B1 were found to reconstitute part of the SDIA activity in hES cell cultures on matrigel. On the other hand, Sacchetti et al., [27] reported a novel function of oxysterols, the ligands of liver X receptors, in promoting midbrain DA neurogenesis during development in vivo, as well as in m/hES cell cultures. Indeed, oxysterols increased the percentage of TH+ neurons from 20% to 60% (5–25% of the total cells) in the hES cultures co-cultured with PA6 cells and treated with Shh, FGF8, bFGF, BDNF, GDNF, TGFB3, dcAMP and AA [27]. However, none of these factor-based protocols for hES cells described above have been tested in animal models of PD.

Other studies have focus on the development of growth factor-based protocols for the propagation of hES-derived neural stem cells and their subsequent differentiation into DA neurons. Long-term hES-derived neural stem cells (lt-hESNSCs) can be propagated as adherent cultures by using EGF and FGF2 [31]. These cells differentiate into a default hindbrain GABAergic interneuron phenotype, but when treated with Shh, retinoic acid, BDNF and GDNF, they give rise to TH+ cells with a midbrain phenotype (30–35% of the total neurons). While lt-hESNSC-derived GABA neurons integrate very well in the telencephalon after grafting, the potential of lt-hESNSC-derived-DA neurons in animal models of PD remains to be examined. In another study, hES cells were propagated as spherical neural masses, and upon treatment with Shh and FGF8, they also gave rise to cultures highly enriched in TH+ neurons (86% of the total cells) [32]. When these cultures were transplanted in hemiparkinsonian rats, a significant number of TH+ cells were found. However, these cells did not re-innervate the striatum and only a partial behavioral recovery was detected, suggesting that their phenotype and/or functionality was not optimal. Histological analysis of the grafts revealed no teratoma formation, but some of the cells continued to proliferate after 3 months, indicating a poor differentiation. Finally, a fast and efficient protocol for neural induction and DA differentiation of hES/iPS cells was recently described [33]. This protocol is based on blocking BMPs, Lefty, Activin and TGFβ signaling in hES/iPS cells using noggin and SB431542 for neural induction. These cells can be subsequently differentiated into DA neurons by sequential treatment with Shh, FGF8, BDNF and AA, followed by GDNF, TGFβ3 and dcAMP, in the absence of Shh and FGF8. So far, cells derived by this method have not yet been tested in animal models of PD. In the future, it will thus be important to examine whether this and other protocols, alone or in combination with some of the recently identified differentiation factors, will allow researchers to improve midbrain DA differentiation, survival, functionality and safety in parkinsonian rodents.

### 3.2. iPS cell-derived-DA neurons

Recent success on the generation of human iPS cells has triggered considerable excitement in the stem cell field for the possibilities these cells offer for disease modeling and personalized CRT (for review see [17]). Particularly relevant for PD CRT have been two studies in which human iPS cells were isolated from patients suffering from sporadic PD [34,35]. Compared to other stem cells, iPS cells offer the advantage that being derived from somatic cells, they rise no ethical concern, and since they are autologous, no immunosuppressive therapy would be necessary. An important and necessary step in order to materialize all their potential has been the verification that PD iPS cells generate midbrain DA neurons as efficiently as iPS cells generated from healthy individuals [35]. Moreover, these cells did not generate any specific feature of PD in vitro. These results indicate that sporadic PD iPS cells may not be easy tools for modeling PD, but they may be appropriate tools for CRT. However, the differentiation and functionality of human PD iPS-derived-DA neuron in vivo, after transplantation in animal models of PD, remains to be examined. A study by Wernig et al. [36] has shown that mouse fibroblast-derived iPS cells pre-differentiated into midbrain DA neurons with Shh, FGF8, FGF2 and AA can functionally integrate in the host striatum of parkinsonian rats and lead to behavioral improvements. However, these cells also lead to the formation of neural overgrowths similar to those seen in ES cell grafts. Caution should thus be exercised, as iPS cells are expected to face at least the same challenges as hES cells, plus additional challenges related to the fact that they are PD-patient derived, the way they are generated, and their performance compared to human ES cells. Indeed, iPS cells derived from PD patients may carry mutations, polymorphisms or epigenetic marks that could make them more susceptible to develop PD-like features than fetal tissue after grafting in PD patients [12,13]. A second important issue is the heterogeneity of iPS cells, which is in part contributed by the genetic modification, variable transgene expression levels, incomplete reprogramming and reactivation/lack of inactivation of the transgenes [17]. So far all of the reprogramming genes, except for Oct4, have been found to be replaceable by either choosing a cell that endogenously expresses the reprogramming factor or by administration of a drug or small molecule [17,37]. A third important issue is the risk of tumor formation by iPS cells, that is expected to be greater than that of ES cells, as the reprogramming process involves the regulation of p53 [38]. A fourth issue is whether the phenotype and in vivo functionality of human iPS-derived midbrain DA neurons is as complete and stable as that of hES-derived midbrain DA neurons. Finally, the elevated cost of generating patient-specific iPS has lead to the suggestion of creating iPS cell banks, as previously proposed for ES cells. While this step would make iPS more competitive from cost and regulatory perspectives, it would also make them less of the personalized tool for regenerative medicine that was initially envisioned.

We should, however, not underestimate the fast pace and creativity of the reprogramming field in order to find new solutions to these problems and new strategies for CRT. One example of that is a recent report showing a method for the direct reprogramming of fibroblasts into a neurons, avoiding thus the creation of undifferentiated cells and their differentiation [39]. The method to create induced neurons (iN) involves the transduction of fibroblasts with Ascl1, Brn2 and Myt1l. iN cells clearly offer some advantages compared to iPS cells as they avoid the generation of pluripotent and undifferentiated cells that can give rise to tumors. This method gives rise to GABAergic neurons but their regional identity, from a developmental perspective, is unclear. In the future, it will be very important to determine whether appropriate regional identity can be acquired by these cells, whether other phenotypes such as

midbrain DA neurons can be generated, and whether critical functional properties, such as target innervation after grafting, will be identical to that of endogenous neurons. There is therefore a long way to go, but it is certainly a very exciting one.

## 4. The road ahead for stem cells in cell replacement therapy

As summarized above, progress in the last years has significantly improved our capacity to produce large quantities of standardized human stem cell-derived midbrain DA neurons in vitro. However, their efficient and safe application in animal models of PD has not yet been achieved. In order to advance CRT, we need to (i) improve protocols for the generation of midbrain A9 substantia nigra DA neurons. (ii) Identify markers and develop protocols for separation of transplantable cells. (iii) Eliminate any chance of tumor formation/neural outgrowth. (iv) Prevent excessive inflammatory response from the host. (iv) Improve imaging methods to monitor graft and DA cell function in vivo. (v) Improve animal models of PD to recapitulate more features of the disease and increase predictability. (vi) Eliminate the response from the host to xenografts or increase tolerance. (vii) Comply to GMP conditions and increasing regulatory requirements.

What is the level of performance currently required from a stem cell-derived-DA preparation in order to be a valid candidate for a clinical trial? First, no undifferentiated stem cell or highly proliferative progenitor should remain in the preparation. The preparation should consist of neurogenic DA progenitors, and postmitotic DA cells expressing the appropriate repertoire of midbrain DA transcription factors and preferably those markers present in substantia nigra DA neurons. Such cell preparations should be able to release DA in a regulated manner and exhibit appropriate electrophysiological properties both in vitro and in vivo. Moreover, a sufficient number of DA cells (>100,000 cells) should survive, reinnervate the host striatum, establish functional contacts, reverse motor deficits in animal models of PD over a period of several months and should not lead to tumor formation. Indeed, the expectation is that stem cells need to be more competitive than existing therapies and should have less adverse effects than fetal tissue grafts and/or offer some additional advantages such as stable and reproducible reconstitution of striatal neurotransmission at a synaptic level.

How will the next clinical trial look like? A new double blind, placebo-control multicentric clinical trial using fetal tissue, has been recently proposed as a way to determine whether we have indeed learned the appropriate lessons from previous clinical trials. If this is the case, and a positive outcome is reached, we will then be in the right position to start incorporating novel stem cell biology techniques with proven efficacy and safety in animal models of PD. For the moment, stem cell-based CRT should be considered as a strictly experimental therapeutic approach. This should thus be taken in account when providing advice to PD patients and when confronted to scientifically and clinically unproven practices (www.isscr.org/clinical_trans/index.cfm). It should be noted that a huge scientific effort and continuous progress are constantly being made. We thus remain hopeful that the promise of this field will continue to materialize and will have a positive impact on the development of CRT for PD.

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