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Cell Therapy for Parkinson's Disease: Failure or Success?

Magdalena Guerra-Crespo[1,2], Alberto K. De la Herrán-Arita[1],
Arturo Hernández-Cruz[1,2], José Bargas[1,2] and René Drucker-Colín[1,2]
[1]Departamento de Neuropatología Molecular, Instituto de Fisiología Celular
[2]Grupo Células troncales adultas, regeneración neuronal y enfermedad de Parkinson
(IMPULSA-02), Universidad Nacional Autónoma de México
*México*

1. Introduction

The mature central nervous system (CNS) is probably the most complex structure known in nature. This fact and the irreversibility of most forms of clinical brain damage are the basis for the long-held belief that the adult brain cannot restore itself and cannot be repaired. Parkinson's disease (PD) is a chronic and progressive neurodegenerative disorder characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNPc) with a concomitant loss of the catecholamine called dopamine (DA), the neurotransmitter released at the axon terminals of the SNPc neurons that project to the striatum (caudate nucleus and putamen) (Fig.1).

Historically, the therapy for PD is aimed at reinstalling proper stimulation of the dopamine receptors in striatum. The dramatic breakthrough was the introduction of L-DOPA treatment in 1969 (Cotzias *et al.*, 1969). L-DOPA, the precursor of dopamine, passes the blood-brain barrier (BBB) and is converted to dopamine, which becomes available for dopamine receptors in striatum, thereby improving the balance between excitatory and inhibitory influences in this brain region. Together with L-DOPA treatment, dopamine reuptake inhibitor, dopaminergic agonists and muscarinic antagonists also have a clinical effect. Despite this pharmacologic advance in treatment, there remains no cure for PD.

Because PD is a neurodegenerative process and long term therapy is necessary, development of severe side effects such as dyskinesias (movement disorder), limits the usefulness of L-DOPA therapy over time and progressively becomes less effective; consequently, patients become more troubled by freezing or akinesias. In addition, L-DOPA will not only reach the striatum, but the entire CNS as well as the rest of the body, where it can develop unwanted side effects.

Additionally, surgical treatment is being used to treat people with advanced PD for whom drug therapy is no longer sufficient. The more frequently employed techniques are thalamotomy, lesion of the internal globus pallidus or subthalamic nucleus and chronic implantation of electrodes for deep brain stimulation, amongst others. Even though there is a clinical recovery in PD patients after surgical therapy, as seen with pharmacological therapy, the progression of the disease cannot be avoided. Hence, the basic principle of

neural transplantation for PD is to provide DA from the graft in a stable fashion directly into the striatum where the intrinsic dopaminergic system has been degenerated (Fig.1). This procedure attracted the attention of the entire scientific community over the past decades.

In this chapter we will describe the experimental work and clinical trials that provided the basis for the development of cell therapy in PD. Afterward, this chapter will depict the suitability and the therapeutic potential of stem cells from different origin that could be employed for regeneration therapy in human clinical trials. Finally, this chapter will discuss the current strategies for the assessment of tissue integration after grafting and our proposal of an alternative method for evaluating the effectiveness of the transplants in rodent models of PD.
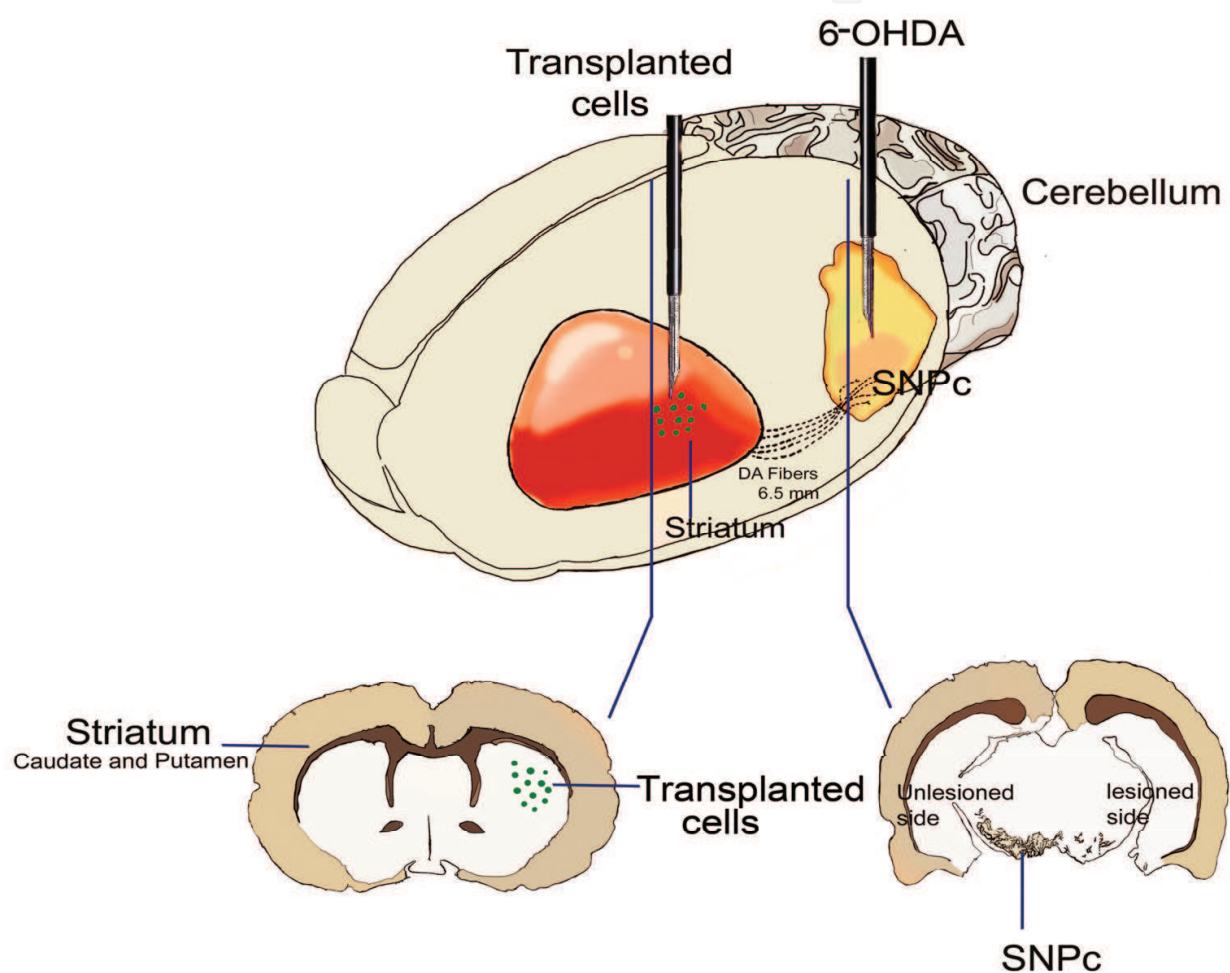
Fig. 1. Transplant therapy in a rodent model of PD. Grafted cells are located in the denervated striatum of the 6-hydroxydopamine (6-OHDA) lesioned rat.

2. The experimental approach in animal models of PD: the foundation of transplant therapy

Even though PD is mostly idiopathic, it is well known that loss of a specific and highly specialized neuronal subpopulation underlie the process of the disease. The optimal tactic to evaluate the benefits and/or drawbacks of different treatments, devoid of using human

patients, is to develop an animal model that resembles the pathology observed in PD. To this point, animal models of PD are the best method to evaluate the success or failure of transplant therapy.

Section 2 depicts the evolution of transplant research done in animal models. A summary of the trials can be found in Table 1.

2.1 Animal models of PD

The most commonly used PD model is the 6-OHDA unilateral lesioned rat. 6-OHDA is a specific neurotoxin for catecholaminergic neurons; uptake of 6-OHDA by these neurons is performed in a similar fashion to that for intrinsic catecholamines (Sachs & Jonsson, 1975). Because 6-OHDA is not able to cross the BBB, unilateral direct injection into the substantia nigra or into the medial forebrain bundle is sufficient to destroy >95% of the midbrain dopaminergic neurons. Unilateral lesioned rats will rotate contralaterally in response to DA agonists such as apomorphine, which is the result of the supersensitivity of striatal DA receptors in the lesioned side. On the opposite, unilateral lesioned rats will rotate ipsilaterally to the lesion in response to amphetamine. Circling behavior can be analyzed by gross visual observation, video recording or rotometer.

Another neurotoxin commonly employed for the development of animal models of PD is 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). This toxin was discovered in young drug addicts who used illicit drugs contaminated with meperidine, causing symptoms very similar to idiopathic PD (Langston et al., 1983). Sensitivity to MPTP varies considerably among animal species. Non-human primates are most sensitive in a manner similar to humans; mice, cats, and dogs are rather sensitive, whereas rats are resistant to MPTP (Kopin & Markey, 1988).

2.2 Fetal substantia nigra transplants

With the extensive variety of animal models of PD, experimental transplant therapy for PD swiftly began in the late 70's; fetal substantia nigra was the initial choice for transplants, since fetal tissue was considered to have lower possibility of rejection from the host's immune system than adult cells.

The firsts fetal substantia nigra experimental transplant performed in a PD model, reported a reduction of the motor abnormalities in a unilaterally DA-denervated rat (Björklund & Stenevi, 1979; Perlow et al., 1979). Since then, the technique has been improved in several ways and established that DA-containing grafts can alleviate some of the symptoms in different experimental models of PD, including mice and rats (Aguayo et al., 1984; Annett et al., 1994; Brundin et al., 1988; Di Porzio & Zuddas, 1992; Dunnett et al., 1981; Hefti et al., 1985; Herman et al., 1991; Zhu et al., 1992).

Once the first experiments using fetal substantia nigra grafts in rodents were concluded and giving preliminary and promising results, the next rational step was to extrapolate these experiments to a higher member of the evolutionary chain, the non-human primates. Morihisa et al., were the first ones to explore the potential of this therapeutic approach, transplanting fetal substantia nigra into the denervated caudate nucleus of the rhesus monkey with unilateral lesion of the striatum secondary to 6-OHDA direct administration. For their surprise, under the specific conditions of their experiment, fetal substantia nigra graft did not survive in either of two animals tested. The complete loss of the transplanted dopaminergic graft could be due to an immunologic incompatibility; which placed the notion that the brain is an immunologically privileged site under inquiry and questioned the

idea that rejection processes would be less severe inside the brain. In order to circumvent graft rejection, they employed grafts from autolog adrenal medulla tissue; of particular interest is that adrenal medulla tissue from four animals tested did survive (Morihisa *et al.*, 1984). Even though they did not report improvement after adrenal medullary chromaffin transplant, they were the pioneers in the usage of autologous transplants for PD treatment, one that took notoriety in the years to come.

2.3 Sympathetic neuron transplants

The potential therapeutic value of autologous transplants was also explored using sympathetic neurons for cell transplant in PD. Sympathetic ganglion cells mainly release noradrenaline whereas a minute amount of DA is also synthesized and secreted by a small subpopulation of the ganglion cells (Lyon *et al.*, 1987). With this taken into consideration, autologous sympathetic neurons derived from the superior cervical ganglion were grafted in animal models of PD, monkey and rat. Tissue pieces of the sympathetic ganglion were grown in serum-free medium supplemented with nerve growth factor; short neurites were found to emanate from sympathetic ganglion tissue after 6 days in culture. These studies have revealed that sympathetic neuron autografts placed in the DA-denervated striatum survive and improve motor deficits such as drug induced circling behavior and hypokinetic disorders, seen in rodent and non-human primate models of PD (Itakura *et al.*, 1988; Nakao *et al.*, 1995).

2.4 Human retinal pigment epithelial (hRPE) cells transplant

With the arrival of new and more advanced cell culture procedures, innovative sources were available for the acquisition of alternative DA-producing cells. Amongst them, the hRPE cell was one of the most promising ones; the study of hRPE cells demonstrated that these cells express vesicular monoamine transporter and the D1 receptor. Moreover, hRPE cells secrete L-DOPA and small quantities of dopamine. This alternative graft was transplanted in a rat and monkey model of PD with significant improvement of circling behavior and motor task, respectively. Nevertheless, a thorough depiction of dopaminergic transplanted cell survival and function was not provided (Doudet *et al.*, 2004; Subramanian *et al.*, 2002).

2.5 The dim truth about experimental transplant in animal models of PD

Regardless of the improvement reported in these animal models of PD after transplant, several factors were not taken into account in order to make an adequate evaluation of the enhancement produced by the graft. Even though there is evidence of a decrease in circling behavior, the survival of DA-producing transplanted neurons is not fully described in many of the experiments, and for that reason, a correlation between neuronal graft survival and behavior improvement cannot be supported.

With the purpose of making an un-doubtful conclusion that a functional recovery is clearly undergoing, a clear correlation between the extent and location of dopaminergic re-innervation within the striatum and recovery of dopaminergic function should be done. The remission of circling behavior may relate to the fact that DA receptors on the denervated side are supersensitive and/or that striatum is normally supplied with a far greater innervation than is needed for minimal maintenance of function. It is also possible that a low number of neurons and terminals may increase their synthesis and turnover of DA to help compensate for the deficit.

Cell Source	Donor	Recipient	Result	Time period (after transplant)	TH neuron count	Reference
Fetal 19 days Nigra	Rat	6-OHDA unilateral lesion Rat	Decrease in 33% of ipsilateral rotation (amphetamine)	3.5 months	300 neurons	Björklund & Stenevi, 1979
Fetal Nigra	Rat	6-OHDA unilateral lesion Rat	Decrease in 70% of contralateral rotation (apomorphine)	4 weeks	NA	Perlow et al., 1979
Fetal 19 days Nigra	Rat	6-OHDA bilateral lesion Rat	Decrease in 50% of spontaneous akinesia	20 days	NA	Dunnett et al., 1981
Fetal 16-17 days Nigra	Rat	6-OHDA unilateral lesion Rat	Augmentation of DA fiber density	10 months	NA	Aguayo et al., 1984
Adrenal medulla autografts	Rhesus macaque (non-human primate)	6-OHDA unilateral lesion Rhesus macaque	NA	8 months	150 cathecolamine neurons	Morihisa et al., 1984
PC12 cells (rat adrenal medulla pheochromocytoma)	Rat PC12 cell culture	6-OHDA unilateral lesion Rat	Decrease in 27% of ipsilateral rotation (amphetamine)	2 weeks	NA	Hefti et al., 1985
Superior cervical ganglia autografts	Rhesus macaque (non-human primate)	MPTP lesion Rhesus macaque	Increase of 60% of homovanillic acid content in cerebrospinal fluid.	3 months	NA	Itakura et al., 1988
Fetal 6.5-8 weeks Nigra	Human	6-OHDA unilateral lesion Rat	Decrease in 94% of ipsilateral rotation (amphetamine)	19 weeks	1, 600 neurons	Brundin et al., 1988
Fetal 3 weeks Nigra	Pig	6-OHDA unilateral lesion Rat	Abolishment of circling behavior (amphetamine)	17 weeks	Up to 12, 000 neurons	Huffaker et al., 1989
Fetal 14 days Nigra	Rat	6-OHDA unilateral lesion Rat	Decrease in 50% of contralateral rotation (apomorphine)	9 months	3, 400 neurons	Herman et al., 1991
Fetal 12-13 days Nigra	Mouse	MPTP bilateral lesion Mouse	NA	4 months	2, 600 neurons	Di Porzio & Zuddas, 1992
Fetal 14 days Nigra expressing PKCβ1 cDNA (second messenger)	Rat	6-OHDA unilateral lesion Rat	Decrease in 64% of ipsilateral rotation (amphetamine)	8 weeks	NA	Zhu et al., 1992
Fetal 13-16 days Nigra	Rat	6-OHDA unilateral lesion Rat	Decrease in 70% of contralateral rotation. (apomorphine)	3 months	NA	Chung et al., 1993
Fetal 74 days Nigra	Marmoset (non-human primate)	6-OHDA unilateral lesion Marmoset	Ipsilateral/contralateral rotations per minute ratio of 0.63 vs 5.15 of control lesion	6 months	1, 800 neurons	Annett et al., 1994
Superior cervical ganglia (4-week-old culture)	Rat	6-OHDA unilateral lesion Rat	Decrease in 40% of contralateral rotation (apomorphine)	4 weeks	NA	Nakao et al., 1995.
Fetal 14 days Nigra mixed with cultured bFGF-producing cells	Rat	6-OHDA unilateral lesion Rat	Total reversal of ipsilateral rotation (amphetamine)	8 weeks	2, 800 neurons	Takayama et al., 1995

Cell Source	Donor	Recipient	Result	Time period	TH neuron count	Reference
				(after transplant)		
Engineered grafts						
Fetal 17 weeks retinal pigment epithelial cells attached to microcarriers	Human	6-OHDA unilateral lesion Rat	Decrease in 50% of contralateral rotation (apomorphine)	12 weeks	NA	Subramanian *et al.*, 2002
Fetal retinal pigment epithelial cells attached to microcarriers	Human	MPTP lesion Rhesus macaque	Improvement of 38% in motor tasks (personalized scale)	8 weeks	NA	Doudet *et al.*, 2004

Table 1. Cell transplant therapy in animal models of PD.

In spite of the methodological scarcity, these investigations gave the stepping stone towards a clinical approach in PD transplantation and supported the idea that it could be extrapolated to human clinical trials.

3. Transplant therapy in PD patients: A leap forward to a potential successful treatment

Transplantation of dopaminergic cells into the denervated striatum is an experimental approach that was brought to practice to overcome the disadvantages of medications used in the treatment of PD in an attempt to provide the brain with an unlimited source of DA synthesized by the grafted cells. On section 3 we depict the clinical trials that have been taking place until nowadays (Table 2).

The results of the first human trials were published in 1980's. More than 30 years ago, the major difficulty for performing a transplant was the shortage of dopaminergic cells suitable for transplant and cell-based manipulation techniques were not sophisticated enough at that time. The most logical selection was to employ autologus dopaminergic tissue. Several of the technical and ethical problems, and probably all of the immunologic difficulties, would be solved if catecholamine-producing cells from the patient's own body were used; this reasoning led two independent groups (Backlund *et al.*, 1985; Drucker-Colín *et al.*, 1988) to investigate if adult adrenal medullary chromaffin cells were able to function as an alternative DA source for transplant. Because the systemic administration of L-DOPA or DA agonist has been successfully applied in the treatment of PD, the logical inference was to expect that adrenal medullary chromaffin cells might act as a chronic pump for delivering DA into the denervated striatum of PD patients.

The first attempt to use autologus medullary chromaffin cell transplant in PD patients was done in Sweden (Backlund *et al.*, 1985), though, the clinical improvement was over a period of one week and one of the patients developed a resistance to L-DOPA treatment.

The first report of significant improvement of symptoms in PD patients after autologous medullary chromaffin cell transplant was done by our (Drucker-Colín) group in México (Drucker-Colin et al., 1988; Madrazo, 1987, 1988). Our team chose a microsurgical transcortical intraventricular approach and implanted autologous adrenal medulla directly into the unilateral caudate nucleus. Although the grafts were unilateral, the improvement was bilateral and symmetric. The direct contact with the caudate nucleus (without the use of a stainless-steel tissue holder that the Swedish group used), exposure to cerebrospinal fluid, and the shorter interval between adrenal dissection and transplantation almost certainly

made the difference. However, there were significant risks and disadvantages associated with autologous adrenal transplants; surgical time in a double operation is considered perilous for a PD patient (since most of them are over 60 years) and DA producing cells obtained from adrenal medulla might be not sufficient to compensate the neural DA deficit. These trials were a breakthrough result in the context of transplant therapy in neurodegenerative diseases; the notion that peripheral tissue would be able to survive and integrate within the host's CNS as well as to induce clinical improvement was not considered to be possible before these studies.

Given the promissory results obtained using adrenal chromaffin cells, which showed variability and technical difficulties; tissue from fetal substantia nigra became to be considered a better option as source of DA cells. Madrazo and Drucker-Colín performed the first transplant of fetal substantia nigra using tissue from cadaveric consent donation (Madrazo et al., 1988). The two transplanted patients showed an improvement of the UPDRS score at 8 weeks post-surgery.

Some years later, in another attempt to improve autolog transplantation of adrenal medullary chromaffin cells, our group took advantage that cultured adrenal chromaffin cells differentiate into neurons in a higher rate when they are stimulated by a low frequency magnetic field (Drucker-Colín et al., 1999). This study proved that adult differentiated adrenal chromaffin cells can be successfully transplanted into the caudate nucleus of a patient with PD and substantially mitigate the clinical symptoms and reduce the intake of L-DOPA medication (see reference video of pre and post-transplanted stages of the surgically treated patient). Postoperative clinical assessments revealed a significant amelioration of visuospatial deficits and a progressive fading of rigidity and akynesia, as well as an improvement in memory tasks; furthermore, a decrease in approximately 70% of L-DOPA intake to ameliorate parkinsonian symptoms was reported.

In spite of the political controversy involving this pioneer studies, the clinical improvement obtained in some patients set off the interest of transplant therapy in PD patients around the world (Deacon et al., 1997; Freed 1990, 2001; Hirsch et al., 1990; Itakura et al., 1997; Kordower 1995, 1996; Lindvall et al., 1990; Peterson et al., 1989; Sawle et al., 1992; Watts et al., 2003) (See table 2).

However, the employment of human fetal dopaminergic neurons as a DA source for transplant is a complicated approach, due to its availability, which is imposed by ethical affairs; for this reason, they have been only employed in some human cases (Freed et al., 1990; Hauser et al., 1999; Kordower et al., 1995; Lindvall, et al., 1990; Madrazo et al., 1988; Mendez et al., 2008; Sawle et al., 1992).

All these initial trials were performed as "open label" (no placebo group as a control), which arose the debate regarding the inclusion of a control placebo group, which is envisaged in general as unethical.

Freed's group published in 2001 the first double-blind placebo trials using embryonic tissue. Their results were considered disappointing by some researchers and promising by others (Dunnet et al., 2001; Isacson et al., 2001). The controversy is based in the modest recovery observed in the grafted patients compared with the sham group; moreover, the serious dyskinetic side effects observed after several years of transplantation, places this procedure under a doubtful perception of acceptability. However, the fact they found improvement in subjects younger than 60 years, leads to consider better methodologically controlled approaches.

One of the disadvantages in employing this cell source (fetal nigral tissue) is the variability of the results; improvements of parkinsonian symptoms occur in patients who received the

largest amounts of fetal mesencephalic tissue (Hauser *et al.*, 1999; Sawle *et al.*, 1992), whereas in other cases, the transplanted cells did not survive for more than a few months and the patients usually return to preoperative state (Goetz *et al.*, 1991; Hirsch *et al.*, 1990). Additionally, to maintain the fetal tissue viable after transplantation, long-term immunosuppressive treatment is needed. Nonetheless, without a doubt the major limiting factor of this approach is the difficulty in obtaining a sufficient amount of human viable fetal tissue.

Dejectedly, as has been observed in those studies, there are some patients that clearly demonstrate improvement and others that do not show any recovery; however, it is still not possible to unravel this mystery of selectivity. Whitin the possible mechanisms accounting for these changes, there is patient age, transplant technique, cell graft source and as the most probable candidate the immune system; which remains the most formidable barrier to transplantation as a routine medical treatment (Freed *et al.*, 2004). The immune system has developed elaborate and effective mechanisms to combat foreign agents and we are now in pursuit of elucidating them.

The successes of human to human organ transplantation, coupled with severe limitations in the availability of human organs, propelled research into the use of non-human organs in the 1990's. The domesticated pig was the preferred donor at the time. In an audacious attempt to replace fetal tissue, surgery to transplant brain tissue from pig fetuses into the caudate-putamen of individuals diagnosed with PD was performed. They reported a clinical improvement of rigidity in a period of 7 months and that autoimmune processes did not become established in the patients (Deacon *et al.*, 1997). Unfortunately, safety and efficacy have not yet been clearly demonstrated, and information is still preliminary to warrant this therapy. Current opinion is that the risk of transfer of a significant infectious microorganism from the pig to the human recipient of a xenograft is small and ethically acceptable if the transplant will be highly beneficial to the host. However, pig specific pathogens are considered to be of potential risk, thus, limiting the use of pig xenografts. Clinical xenotransplantation is becoming feasible and attractive as a routine therapy, nonetheless, some attention should be set on this matter.

In another attempt to evade the host immune response after transplantation, human retinal pigment epithelial (hRPE) cells were taken into consideration. As mentioned previously, experimental studies suggested that RPE cells were a suitable cell type to serve as a potential enduring source of L-DOPA for implantation into the striatum of animal models of PD (Subramanian *et al.*, 2002). These implants ameliorated the motor deficits in rodent and non-human primate models of PD and immune suppression was not required even when transplants were made from one species to another (Doudet *et al.*, 2004; Subramanian *et al.*, 2002).

Those results were taken into account in a novel tissue engineering strategy; hRPE cells were attached to gelatin microcarriers (spheramine™) and transplanted into the putamen contralateral to the more symptomatic side of patients with Parkinson disease (Watts *et al.*, 2003). They reported an average improvement of 48% in motor score 12 months after implantation evaluated through the Unified Parkinsons's Disease Rating Scale (UPDRS) with the patient in the "off" state, which was sustained for 24 months. Improvement was also observed in activities of daily living, quality of life, and motor fluctuations.

However, in a more recent work, a post-mortem study of a patient enrolled in a similar clinical trial reported that only 118 cells from the transplant endured (estimated 0.036% survival) and that clinical improvement was not observed (Farag *et al.*, 2009). This

engineered tissue approach is still an emerging therapy alternative for PD and the existing body of literature is still inadequate to allow conclusions regarding this procedure. Concerns related to small sample sizes and a limited number of controlled trials are joined with a wide array of methodological issues with the procedure itself. We have no doubt that better procedures will arrive alongside the advances in tissue development.

The leading inquiry raised by transplant therapy hitherto is the variability in functional recovery either in animal models and human patients. In human studies, this unevenness is not only seen between different trials, but also within groups of PD patients transplanted in the same assay. In order to attend such matters, a comprehensive clinical trial must be done, one that includes a meticulous analysis of patient selection (e.g., age, level of PD), knowledge of the number of DA neurons transplanted, type of surgery, follow up of the patient before surgery, monitoring of the clinical improvement after grafting, analysis of cell survival and neuron functionality. A successful cell therapy must settle advantages over current treatments for lessening motor symptoms in PD patients. Cell replacement should provide long-lasting, major improvements of mobility and no manifestation of dyskinesias without the need of further therapeutic interventions, an uneasy task that most likely will not be achieved by using solid fetal ventral midbrain grafts. This is suggested in the recent report from Mendez (Mendez *et al.*, 2008), who transplanted fetal ventral midbrain as cellular suspension. The analysis did not reveal development of diskynesias in the transplanted PD subjects in a period spanning from 9 to 14 years; moreover, the postmortem analysis of the transplanted patients' brains did not show any sign of degeneration (alpha synunclein, ubiquitin), and only a minimum of microglial reaction from the host was found. In contrast, in the two double blind placebo trials, where solid pieces of fetal midbrain tissue were used, severe microglial reaction was found and diskynesias were also observed (Freed *et al.*, 2001; Olanow *et al.*, 2003). Mendez's results supports a new "hope" in medical treatment for PD patients based in stem cell tissue procedures, after the setback given by the report from Kordower of transplant analysis following 14 years after the procedure (Kordower *et al.*, 2008), in which the postmortem tissue revealed that the grafted cells in the striatum were affected by a neurodegenerative mechanism, suggesting that the neurodegenerative process was not exclusive of the SNPc. Taken together, the present observations suggest that the methodological procedures and not the cell source, are directly involved in the limited success of transplant therapy that employs solid portion of fetal ventral tissue.

Currently, there are various ongoing stem cell based clinical trials registered by the National Institutes of Health (NIH): (http://www.clinicaltrials.gov/ct2/results?term=transplant +parkinson), and new assessments are being developed by the Transeuro consortium (Allan *et al.*, 2010) (http://www.transeuro.org.uk/index.html). The European group coordinated by Dr. Roger Barker is planning highly controlled studies in the near future (2012). Interestingly, the cells to be employed in these trials will be obtained from the midbrain of human fetuses, the same tissue we used the first time more than 3 decades ago. Again, as in past, the idea still creates controversy between those who concur with such methods and those who are unfavorable to it due to the limited success percentage (Holden, 2009).

We expect that the new trails should provide better motor recovery to the ones observed in previous assays. Even though behavioral improvement is seen in experimental transplant trials, going from 30% to total reversal of circling behavior (Tables 1 and 3), the motor symptoms observed in grafted human subjects when analyzed by UPDRS do not surpass 60% of clinical improvement (Table 2 and Dunnet *et al.*, 2001).

Cell Source	Donor	Implant Zone	Result	Time period (after transplant)	TH neuron count	Reference
Autologous adrenal medullary tissue	Human	Striatum (Putamen)	Improvement of rigidity	1 week	NA	Backlund *et al.*, 1985
Autologous adrenal medullary tissue	Human	Lateral Ventricle	Disappearance of rigidity and akinesia	5 months	NA	Madrazo *et al.*, 1987
Autologous adrenal medullary tissue	Human	Striatum (Caudate)	Decrease in 50% of L-DOPA dose	1 year	NA	Drucker-Colín *et al.*, 1988
Autologous adrenal medullary tissue/fetal 13 weeks substantia nigra tissue	Human	Lateral Ventricle	Disappearance of rigidity and akinesia. Decrease in 70% of L-DOPA dose	2 years	NA	Madrazo *et al.*, 1988
Autologous adrenal medullary tissue	Human	Striatum (Putamen)	Improvement of rigidity	2 weeks	Necrotic tissue	Peterson *et al.*, 1989
Autologous adrenal medullary tissue	Human	Striatum (Caudate)	None	4 months	Necrotic tissue	Hirsch *et al.*, 1990
Fetal 45-55 days Nigra tissue	Human	Striatum (Caudate and Putamen)	Improvement of Hoehn –Yahr scale from 3.71 to 2.5	4 years	N.A.	Freed *et al.*, 1990
Fetal 8-9 weeks Nigra tissue	Human	Striatum (Caudate)	Increase in 130% of [18F]-DOPA intake in PET scan. Increase of L-DOPA effectiveness from 2 to 14 hours	5 months	NA	Lindvall *et al.*, 1990
Autologous adrenal medullary tissue	Human	Striatum (Caudate)	Clinical improvements of 19% in UPDRS score	2 years	NA	Goetz *et al.*, 1991
Autologous adrenal medullary tissue	Human	Striatum (Caudate)	Decrease in 60% of L-DOPA dose	18 months	NA	López-Lozano *et al.*, 1991
Fetal 8-9 months Nigra tissue	Human	Striatum (Putamen)	Increase in 200% of [18F]-DOPA intake.	1 year	NA	Sawle *et al.*, 1992
Fetal 6.5-9 weeks Nigra tissue	Human	Striatum (Putamen)	Improvement of UPDRS from 78 to 49.5	18 months	210, 000 neurons	Kordower *et al.*, 1995/1997
Superior cervical ganglia autografts (stellate ganglion)	Human	Striatum (Putamen)	Reduction of 33% in the time taken to perform motor task. No changes in UPDRS score	3 years	NA	Itakura *et al.*, 1997
Fetal 9 weeks Nigra tissue	Pig	Striatum (Caudate and Putamen)	Improvement of rigidity	7 months	NA	Deacon *et al.*, 1997
Adrenal medullary tissue differentiated to DA cells in 60Hz magnetic field	Human	Striatum (Caudate)	Decrease in 70% of L-DOPA dose	1 month	NA	Drucker-Colín *et al.*, 1999

Cell Source	Donor	Implant Zone	Result	Time period	TH neuron count	Reference
				(after transplant)		
Fetal 6-9 weeks Nigra tissue	Human	Substantia Nigra Pairs compacta	Clinical improvements of 32% in UPDRS score	20 months	80, 000 neurons	Hauser *et al.,* 1999
Fetal Nigra tissue	Human	Striatum (Putamen)	Increase in 40% of [18F]-DOPA intake in PET scan. Clinical improvements of 30% in UPDRS score.	1 year	30, 000 neurons	Freed *et al.,* 2001
Fetal Nigra tissue	Human	Striatum (Putamen)	Clinical improvements of 15% in UPDRS score	2 years	NA	Olanow *et al.,* 2003
Suspended fetal ventral midbrain cells from 6-10 weeks	Human	Striatum (Putamen)	Increase in 185% of [18F]-DOPA intake in PET scan. Clinical improvements of 60% in UPDRS score	1 year	NA	Mendez *et al.,* 2008
Adult neural stem cells grown as neurospheres	Human	Striatum (Putamen)	Increase in 30% of [18F]-dopa intake in PET scan. Clinical improvements of 80% in UPDRS score	1 year	NA	Lévesque *et al.,* 2009
Autologous bone marrow-derived mesenchymal stem cells	Human	Sublateral ventricular zone	Clinical improvements of 22% in UPDRS score	3 years	NA	Venkataramana *et al.,* 2010
Engineered grafts						
Retinal Pigment Epithelial cells attached to microcarriers	Human	Striatum (Putamen)	Clinical improvements of 48% in UPDRS score	1 year	NA	Watts *et al.,* 2003
Retinal Pigment Epithelial cells attached to microcarriers	Human	Striatum (Putamen)	None	6 months	118 cells	Farag *et al.,* 2009

Table 2. Cell and stem cell transplant therapy in patients with PD.

4. Stem cell transplantation: Is it the idyllic approach?

Early research gazed at ventral mesencephalic fetal dopaminergic tissue for transplantation in animal models of PD and PD patients with partial success. Nonetheless, while fetal primary tissue showed promise, the widespread clinical application of this approach is considered limited due to the matter of rejection and low cell survival. The search for

alternative sources able to circumvent at least some of the problems inherent to fetal tissue is actually undergoing.

Since the advent of stem cells, they have been proposed as potential candidates to generate dopaminergic mesencephalic neurons and replace the cell loss that takes place in PD.

Stem cells are of an undifferentiated nature and possess an extend capacity to proliferate, as well they are capable to endow cells with the same sort of undifferentiated state. These cells are sorted in accordance to their intrinsic capacity to generate different cellular types, ability that diminishes throughout embryonic development.

They are considered totipotent when they are able to originate any kind of embryonic or extra-embryonic cell (few days embryo) (Thomson & Odorico, 2000). Further in embryonic development, in the blastocyst stage, cells of the inner cell mass (ICM) have the potential to contribute to the three embryonic germinal layers, ectoderm, mesoderm and endoderm, and they are classified as pluripotent. Finally, when the embryo evolves to the gastrula stage, the ICM cells have already differentiated and compromised to a specific lineage, based on the elapsed time and location within the embryo. These cells are multipotent and they are present in the embryo as well as in the adult organism; an example is the neural stem cells (NSC) discussed below.

We should classify stem cells in three broad groups: Adult neural stem cells (neural stem cells and mesenchymal), embryonic stem (ES) cells and the recently obtained induced pluripotent stem (iPS) cells. All of them have been considered in greater or lesser degree accordingly to their specific characteristics as a potential source of dopaminergic cells. With this wide diversity of cells available for transplant, the question directly arising is, "is there an idyllic stem cell to be employed as source of dopaminergic neurons?" said it in other way, "is there a cell capable of long-term survival, steady release of DA, integration into the host brain and therefore induce functional benefits without side effects?" In this section we will describe the properties and capacity of stem cells from different origin to become dopaminergic neurons, with the purpose of elucidating that question. Additionally, we will describe the results of the first stem cell transplant experiments performed in animal models of PD and the few trials implemented in humans.

4.1 Adult neural stem cells

Several studies indicated the existence of dividing cells in the CNS (Altman, 1969) but the discovery and isolation of a subtype of multipotent cells, the NSC, in specific regions of the mice adult brain (Reynolds & Weiss, 1992) and later in the human brain (Eriksson *et al.*, 1998) were the events that clearly revealed that the adult CNS posses an inherent plastic capability. This instigated the relentless pursue to replace the cellular loss that takes place in the CNS after injury or neurodegenerative diseases.

NSC are self-renewing progenitors specified to give rise only nervous tissue-specific cell types, neurons, glia and oligodendroglia (Reynolds and Weiss, 1996). In adult rodent brain, the SVZ and the dentate gyrus of the hippocampus contain NSC population with permanent capacity of proliferation (Doetsch *et al.*, 1999; Gage, 2000). These brain areas with potential to generate new neurons are defined as neurogenic niches. The advantage of containing an adult source of stem cells is the possibility of avoiding the use of human fetal tissue and embryonic derived stem cells for replacement therapy in neurodegenerative diseases.

4.1.1 NSC from adult subventricular zone (SVZ)

Neural stem cells from the SVZ move in chain migration along the rostral migratory stream (RMS) and differentiate into periglomerular interneurons at the olfactory bulb throughout

the rat lifespan (Doestch *et al*., 1999). The functional relevance of permanent replacement of periglomerular neurons in the rodent brain is attributed to the olfactory adjustment to odor changes in the environment.

Noteworthy, stem cells in the SVZ are able to respond to adverse damage in the brain. An increase in proliferation is observed under traumatic conditions such as acute stroke (Arvidsson *et al*., 2002; Parent *et al*., 2002) or in chronic stroke after being stimulated by transforming growth factor alpha (TGFa) (Guerra *et al*., 2009). Conversely, in animal models of PD, decreased proliferation of the progenitors in the SVZ takes place (Hoglinger *et al*., 2004). Interestingly, a simultaneous increase of Paired box gene 6 (Pax6) dopaminergic interneurons in the periglomerular layer is also occurring (Winner *et al*., 2006). The same increase of TH+ cells is observed in the olfactory bulb of postmortem tissue in PD patients (Huisman *et al*., 2004).

The reasons to consider the adult multipotent progenitors of the SVZ as an option to regenerate the dopaminergic population in the affected striatum of PD patients are their commitment to differentiate into dopaminergic neurons in the olfactory bulb and that they remain responsive to different signals.

However, it is well known that different set of transcription factors determine the correct dopaminergic fate in the olfactory bulb and SNPc. In the olfactory bulb, transcription factors such as the ETS transcription factor Er81 (Er81), Pax6 and Distal-less homeobox 2 (Dlx2), regulate the terminal differentiation of periglomerular dopaminergic neurons (Brill *et al*., 2008; Cave and Baker 2009; Hack *et al*., 2005; Kohwi *et al*., 2005). Instead, the initial specification of dopaminergic mesencephalic neurons from the SNPc is regulated by the LIM homeobox transcription factor 1 (Lmx1a) and the homeobox transcription factor 1 (Msx1) (Andersson *et al*., 2006). This evidence suggests that the neuroblasts arising from the SVZ do not develop into A9 dopaminergic neurons, the cells mainly affected in PD (German *et al*., 1992). Additionally, when NSC are expanded in culture as floating cellular aggregates in the neurospheres assay they mainly generate glial cells (Storch *et al*., 2004). Together, these results on animal models suggest that adult NSC arising from the SVZ should not be an optimal selection for cell therapy in PD.

4.1.2 Neural stem cells-fetal derived transplants.

Different protocols *in vitro* have been developed to generate dopaminergic neurons from NSC (for review see Deirborg *et al*., 2008). In spite the expansion process of NSC, it decreases their potential to differentiate into dopaminergic neurons (Ptak et al., 1995); in rats, it was demonstrated that fetal ventral mesencephalic (VM) precursor cells have the potential to proliferate and differentiate into dopaminergic neurons in culture when stimulated by the growth factor FGF-2 (Studer *et al*., 1998). An important fact involving the use of the expanded fetal neural stem cells is the low survival observed after grafting the lesioned striatum (around 3-5%), therefore, the improvement of behavioral deficits is not great compared to the one obtained with primary fetal cells (not amplified) (Brundin *et al*., 1988). Even thought fetal neural stem cells are not the most adequate source for transplant, they could be considered a better option than NSC from the SVZ.

4.1.3 Neural stem cells-adult derived transplant

NSC from adult tissue have been employed in a relevant clinical trial, neural stem cell-derived neurons were isolated from cortical and subcortical tissue and expanded *in vitro* for

several months. Nine months after harvesting, autologous cell suspensions containing differentiated dopaminergic and GABAergic neurons were microinjected unilaterally in a patient with advanced Parkinson's disease. Over the next 3 years, the overall UPDRS improved by 80%. However, at five years post-operatively, clinical motor scores returned to baseline (Table 2) (Lévesque *et al.*, 2009).

Even though this result is not the most promising one, it laid the foundation for the development of autologous neural stem cell based therapy in PD patients and pointed out that this source represents another option for cell replacement therapy, avoiding the employment of embryonic tissue.

4.1.4 Mesenchymal stem cells (MSCs)

Efforts in employing non-fetal stem cells for transplant therapy in PD are also currently undergoing. Venkataramana *et al.* performed a unilateral transplantation of autologous bone marrow-derived mesenchymal stem cells (BM-MSCs). The BM-MSCs were transplanted into the sublateral ventricular zone by stereotaxic surgery. The transplanted patients showed a clinical improvement of 23% in their UPDRS score. Moreover, a subjective improvement was found in symptoms like facial expression, gait, and freezing episodes (Venkataramana *et al.*, 2010). These results indicate that this new protocol seems to be safe, and no serious adverse events occurred after stem-cell transplantation in PD patients, additionally, no fetal tissue was needed to obtain human stem cells. However, more efforts should be done to improve these stem cell-based techniques in experimental models of PD before using them in PD patients.

As far as we know, this is the last report of a clinical trial in PD patients. Taking this and all of the previous works done for transplant therapy in PD patients, we can state that the international quest for the best transplant therapy should not have been taken with such haste; a more detailed analysis of graft survival and long term clinical improvement are considered necessary. Relevant concerns prevail about methodology aspects, and also about the most debated complication of cell therapy in PD, the occurrence of post-operative graft-induced dyskinesias in the majority of the clinical protocols.

4.2 Embryonic stem cells: The most promising source of dopaminergic neurons

The information described above shows that even thought fetal midbrain precursors have capacity to proliferate and differentiate into dopaminergic neurons, the efficiency of this process in culture is low, therefore, restricting their potential as donor tissue. ES cells instead have the unique ability to self-renew indefinitely while maintaining the potential to give rise to all cell types in the human body. These two properties of ES cells make them gain a remarkable interest as promising tools for regenerative medicine, specially in PD transplant therapy. Another advantage that propelled the use of stem cells is that genetic manipulations has became easily practicable in the last few years.

Nevertheless, currently there are three major aspects limiting the success of stem cell therapy; one being the low number of stem cells sources with the potential to differentiate into the mesencephalic dopaminergic phenotype; the limited survival of grafted cells transplanted in both animal models and humans; and the most important, the danger of teratomas.

In this section, we summarize the different attempts to increase the number of stem cells using basically 3 different culture systems (feeder stromal cells, embryoid bodies and neural

rossetes) to induce differentiation towards a dopaminergic phenotype. Evaluation of survival, integration and function of ES-dopaminergic cells after transplantation in animal models of PD is also described.

4.2.1 Mouse Embryonic stem (mES) cells

In 2000, Kawasaki *et al*. performed the first grafted study of mice ES-dopaminergic derived cells into the mouse striatum that had been previously treated with 6-OHDA. The system used to derivate TH mesencephalic-type neurons consisted in culturing ES cells onto the bone marrow stromal cell line PA6, which they identified to have the feature (by unknown mechanism) of promoting specific dopaminergic differentiation. The number of TH neurons obtained by this co-culture method is significantly higher (~16% of the total cells) to the five stages protocol reported in the same year by Lee et and collaborators (Lee *et al*., 2000). Two weeks after transplantation, 22% of the grafted TH-positive neurons survived and even though the improvement of rotational behavior after transplant was not analyzed, they confirmed that ES-dopaminergic cells are capable of being transplanted into a parkinsonian model and survive, which laid the interest to explore further possibilities for therapeutic application (Kawasaki *et al.,* 2000).

In continuance work, other research groups have been improving culture protocols in order to increase the number of dopaminergic neurons to be grafted. In 2002, the first transplant of mES cells took place, Kim *et al*. reported that ~78% of dopaminergic neurons could be derived from mES cells *in vitro*. The highest number of TH cells reported until now was promoted by the overexpression of the nuclear receptor related-1 (Nurr1), a transcription factor relevant in the induction of mesencephalic precursors into dopamine neurons (Wallen *et al.*, 1999). Interestingly, a remarkable behavioral improvement after grafting was observed; the transplanted parkinsonian rats presented a total reversal of amphetamine-induced rotational behavior 8 weeks after transplant (Kim *et al.*, 2002). The behavioral enhancement correlates with the evaluated release of dopamine and functional synapses of the trasplanted cells. Additionally, no tumor formation was found.

That same year, Bjorklund *et al.* followed an opposite transplant approach. The group grafted ES cells on embryoid body (EB) stage. It is important to mention that EBs are aggregated of cells with spherical shape that differentiate stochastically from ES cells under specific *in vitro* conditions, they have the inherent property of recapitulate embryonic development and were developed the first time by Lee in a 5 stage protocol (Lee *et al.*, 2000). Rats transplanted with EB presented a decrease in 46% of amphetamine-induced rotation 9 weeks after transplantation (Bjorklund *et al.,* 2002). The reasoning of the improvement in the motor asymmetry is that the ES cell-derived neurons released dopamine in sufficient amount when they were stimulated by amphetamine. However, the number of TH+ neurons produced after grafting was not studied. They instead analyzed the functional activity of ES cells by PET imaging, finding high similarity to that observed in transplanted dopaminergic midbrain fetal neurons. Unfortunately, in contrast to the study realized by Kim *et al.*, 25% of the animals developed tumors, a result expected given the fact that not all of the grafted cells were in a differentiated state.

To improve the therapeutic potential of ES cells Barberi *et al.* implemented a faster and simpler culture method. Their protocol consisted in the induction of the differentiation of mice ES cells into dopaminergic neurons using the feeder stromal cell line (MS5). They found that nuclear transfer-derived ES dopaminergic neurons following this protocol and

transplantation into the striatum of parkinsonian mice provoked a decrease in 80% of circling behavior; additionally, some animals contained up to 40,000 transplanted TH+ neurons 8 weeks after transplant (Barberi *et al.*, 2003).

In another effort to obtain functional DA neurons derived from ES cells but without any genetic modification, Rodríguez-Gómez *et al.* generated CNS progenitor populations from mES cells that were later expanded and promoted to differentiate into dopaminergic neurons in the presence of mitogen and specific signaling molecules. Mitogen withdrawal from the growth medium was done after expressing Engrailed 1 (En1), Paired box gene 2 (Pax2), and Orthodenticle homeobox 2 (Otx2) specific neuronal transcription factors, hence, achieving differentiation (Rodríguez-Gómez *et al.*, 2007). They also described that in the grafted animals, PET imaging showed that the number of postsynaptic DA D2 receptors was normalized in the host striatum. Additionally, microdialysis in grafted animals displayed that dopaminergic transplanted neurons release was induced by depolarization and pharmacological stimulants. Their data suggest that ES cell-derived neurons release DA in a physiological manner and that reuptake postsynaptic responses remains after implantation.

On the other hand, to optimize the procedure for generation of mesencephalic dopaminergic neurons from ES cells, Jönsson et al. elaborated a protocol to determine the optimal stage of development of cells used for grafting. By means of fluorescence-activated cell sorting procedures, they isolated DA precursors from mouse ventral mesencephalon in two defined stages of differentiation, at 10.5 and 12.5 embryonic days, when the dopaminergic mesencephalic neurons are arising. After transplantation into the striatum of 6-OHDA-lesioned rats, the histological analysis showed that TH-expressing cells poorly survived sorting and transplantation; however, this study demonstrated that the differentiation state of the progenitors is important. The transplanted neuroblasts originate more TH+ cells when obtained from an early state of differentiation (Jönsson *et al.*, 2009). These outcomes have inferences for recent efforts to develop well-characterized stem cell-derived mesencephalic DA progenitor cell preparations for future cell therapy.

Even though the 6-OHDA lesioned rat is a great model of PD for analyzing ES cell transplant therapy, before considering preliminary transplantation trials in PD patients, experiments on non-human primates are quintessential. With this in mind, Takagi *et al.* generated neurospheres composed of neural progenitors from monkey ES cells cultured on PA6 stromal feeder cells and transplanted them into the putamen of MPTP lesioned monkeys. They reported a significant behavioral recovery and an average survival of 2,100 TH+ transplanted neurons 14 weeks after transplantation; additionally, PET imaging revealed a 50% increase in 18F-fluorodopa uptake. This study demonstrated that the transplanted ES cells functioned as DA neurons and alleviated the motor symptoms in a parkinsonian non-human primate (Takagi *et al.*, 2005); furthermore, they demonstrated that ES cell transplant therapy might be an appropriate treatment alternative to human PD patients.

4.2.2 Human ES cells

After Thomson isolated stem cells from human embryo (Thomson *et al.*, 1998), the next boundary to overcome was to generate neural progenitors derived from human ES (hES) cells. In an innovating experiment, undifferentiated hES cells were plated on fresh mitotically inactivated feeders and cultured for 8 days to induce neural differentiation, these progenitors were transplanted into the striatum of parkinsonian rats. The grafts survived for at least 12 weeks, managed to differentiate *in vivo* to DA neurons and improved circling

behavior with a 45% reduction of amphetamine-induced ipsilateral rotation (Ben-Hur *et al.*, 2004). Induced dopaminergic differentiation from hES was shortly addressed by other groups with different culture approaches but with similar relatively successful outcomes (Brederlau *et al.*, 2006; Chiba *et al.*, 2008; Cho *et al.* 2008; Roy *et al.*, 2006).

Roy used a combined strategy to improve dopaminergic neuron amounts. Sonic hedgehog (Shh) and fibroblast growth factor 8 (FGF-8) were co-cultured with telomerase-immortalized hES cells derived from human fetal midbrain astrocytes in order induce a dopaminergic neuronal fate. After achieving a high number of TH+ cells specific for the A9 dopaminergic lineage (colocalizing with the G protein–gated inwardly rectifying K+ channel, Girk2), they were transplanted into the striatum of 6-OHDA lesioned parkinsonian rats. The authors reported that the dopaminergic implants generated a significant, substantial and long-lasting restitution of motor function. Nonetheless, the grafts displayed increasing cores of undifferentiated mitotic neuroepithelial cells, which can turn tumorigenic (Roy *et al.*, 2006). These data dictate the need for extreme caution in developing a clinical application of hES cell-derived grafts, given their potential for undifferentiated expansion, yet they also proved that TH+ neurons can be obtained and/or induced from a diverse source of cells.

Chiba *et al.* followed a different method to improve hES cell transformation to phenotypically stable DA neurons. They blocked the effect of the neuronal differentiation promoter Noggin by means of the bone morphogenic protein (BMP) antagonist. With this strategy they found that BMP inhibitor Noggin increased production of DA neurons from hES cells differentiated on PA6 stromal cells. In addition, these DA neurons survived transplantation and led to behavioral improvement in parkinsonian rats 4 weeks after grafting (Chiba *et al.*, 2008). These neurons derived from hES cells may not be suitable for eventual transplantation into PD patients (due to a triple trisomy created by continuous passaging), but with this work, they demonstrated that Noggin has a critical role for determining midbrain dopaminergic phenotype at an early stage in ES cell differentiation, which will be of great value for future research in dopaminergic cell engineering.

The recent generation of dopaminergic mesencephalic neurons obtained from human ES cells by employing spherical neural masses (SNMs), also called neural rossete cells, is considered the protocol that allows to yield the higher number of dopaminergic neurons in cell culture (roughly 66%) (Cho *et al.*, 2008). SNMs are columnar epitelial structures that represent a novel NSC type, distinct and more primitive than those previously characterized from other NSC stages (Elkabetz *et al.*, 2008; Pankratz *et al.*, 2007). NSC in neural rossetes mimic the neural plate stage and can be directed towards a dopaminergic mesencephalic lineage by employing the signaling molecules Shh and FGF-8 (Elkabetz *et al.*, 2008; Cho *et al.*, 2008). However, whether the fully differentiated DA cells obtained from this novel protocol have an ameliorative effect on parkinsonian rodent models or PD patients still remains to be determined. Cho and collaborators decided to transplant cells originated from neural rossetes in the initial stage of dopaminergic neuronal specification into the lesioned striatum of parkinsonian rats in order to avoid a low survival rate. In this specific time point, approximately 50% of the cells were TH+ and immunohistochemical analysis demonstrated that only 2.7% of the grafted neurons were TH+ 12 weeks after transplantation. Despite this low cell number, a significant behavioral recovery was observed in 3 different motor behavioral tests. Although this stem cell source is promissory due to the high number of yield DA cells without the need of genetic modifications or employment of feeder cells; this technique stills needs to circumvent the problem of low survival ratio, which is generally

observed when terminal differentiated cells are grafted. Additionally, in order to generate an efficient long-term culture (30 to 40 days), experimented handling and dedicated efforts are required. These results also point out the necessity of better and rational procedures to direct cell specification to a desired phenotype *in vitro*.

The bulk of results suggest ES cell transplantation is approaching the point of technical practicability toward clinical therapy for PD. hES cell transplantation has the potential to be considered one of the most promising therapies for PD treatment; however, this excitement is gradually fading as a result of political and ethical concerns. Amongst the limiting methodological factors, is the difficulty to maintain normal karyotype and to avoid tumor formation. Furthermore, the differentiation rate of ES cells towards a dopaminergic lineage is not high enough to obtain sufficient dopaminergic cells for transplantation.

4.3 Induced pluripotent cells: The most recent breakthrough in stem cells field

Induced pluripotent (iPS) cells are derived from somatic differentiated cells by overexpression of specific transcription factors, which induces cell reprogramming. They are fairly similar to ES cells in terms of self-renewal and pluripotency, which provides them with the potential to differentiate into any cell type in the organism.

iPS cells were obtained for the first time in 2006 by Yamanaka´s group; they were acquired from embryonic and adult mouse fibroblasts reprogrammed to a pluripotent-like state by viral transduction of four transcription factors: Klf4, cMyc, Oct3/4 and Sox2. The obtained cells demonstrated to possess typical ES cells properties (Takahashi *et al.,* 2006); however, they did not form effective fertile chimeras, meaning that the ability to transmit genotype through the germinal line was not achieved, this being one of the most important properties that defines a pluripotent cell. This was later rein-vindicated in 2007 in a parallel work by Jaenisch´s and Yamanaka´s groups, they successfully reprogrammed fibroblasts and obtained viable chimeras (Okita *et al.,* 2007; Wernig *et al.,* 2007); nonetheless, they emphasized that the transduction of c-Myc should be avoided in iPSC reprogramming, due to its tumorigenic capacity.

At the end of 2007, and almost simultaneously, Takahashi and Yu were able to manufacture iPS cells from human dermal fibroblast. Takahashi enforced the expression of the same transcription factors employed in the previous study (Klf4, cMyc, Oct3/4, Sox2); while Yu *et al.*, demonstrated that other two transcription factors, Nanog and LIN28 (besides Oct3/4, Sox2) were also relevant in cell reprogramming (Takahashi *et al.,* 2007; Yu *et al.,* 2007). One year later, iPS cells were derived from human fetal tissue, neonatal and adult somatic cells by employing the same combination of genes used by the pioneer team, they also reported that c-Myc only increases the efficiency of reprogrammation, but it is not essential for the establishment of a pluripotency state, suggesting that c-Myc could be nonessential for reprogrammation (Park *et al.,* 2008). Taken together, these results reveal that iPS cells are an attractive resource for replacement therapy, given the fact that its use would overcome the technical and ethical difficulties of ES cells by avoiding the use of human embryos, circumventing transplant rejection and therefore avoiding the use of immune suppressants. However, concerns for the transduction of the oncogen c-Myc were still latent and attempts to remove it from the transcription factor cocktail were in progress.

Later in the same year, it was demonstrated that c-Myc is absolutely expendable to obtain viable iPS cells, either from mice and human samples (Nakagawa *et al.,* 2008). With the removal of this specific proto-oncogen, the possibility of tumor formation was reduced, and therefore, research on possible clinical applications achieved important notoriety.

iPS cell lines obtained from patients afflicted with different diseases have been developed with the purpose of investigating the therapeutic potential of autologous transplants, but also to offer an unprecedented opportunity to recapitulate pathological conditions *in vitro* (Gunaseeli *et al.,* 2010; Park *et al.,* 2008). Neurodegenerative diseases are particularly taken into consideration, since they are secondary to a relatively selective loss of neurons, for that reason, the quest for replacing the neuronal loss of a specific population employing iPS cells is currently undergoing in a similar way that occur for ES cells.

The iPS cells neuronal differentiation *in vitro* assays have been successful and were achieved by employing established ES cell differentiation protocols. The first dopaminergic differentiation was obtained from neural precursors derived from mice iPS cells. These cells were grafted into the mice fetal encephalon (chimerical brain) and revealed a high capacity for migration, differentiation and integration within different brain areas. The iPS-derived dopaminergic neurons generated in this study were grafted in the 6-OHDA rat model of PD, such transplants improved significantly circling behavior stimulated by amphetamine (Wernig *et al.,* 2008). However, it was reported that the presence of undifferentiated cells in the graft promoted tumor formation; this fact contributed to the idea that obtaining a purified population is essential for a satisfactory transplant outcome.

It has been proved that pluripotent cell production can be obtained from patients suffering from other neurodegenerative disease. *Dimos et al.* developed iPS cells from patients with amyotrophic lateral sclerosis (ALS), which was effectively differentiated *in vitro* to motoneurons, the degenerated cells that are accountable for the development of ALS (Dimos *et al.,* 2008). This corroborated that iPS cells patient-specified manufacture is a viable option and possess certain advantages over ES cells since iPS cells evade the ethical and methodological difficulties presented in ES cells production.

A new study performed in 2009 widened the possibility of inducing patient-specified neural differentiation *in vitro* into iPS cells engineering. Jaenisch´s group managed to generate fibroblast-derived iPS cells from 5 idiophatic PD patients, with the advantage that once the cellular reprogrammation employing three transcription factors (Oct4, Klf4 y Sox2) was achieved, they were able to remove all viral vectors using Cre-recombinase (iPS cells factor-free). These cells maintained all the properties of a pluripotent cell and were capable to induce neuronal differentiation towards a dopaminergic phenotype *in vitro*; additionally, they have the advantages of being a free viral transcription factor cell line and that overall gene expression patterns were more similar to ES cells than carrying-factor iPS cells (Soldner *et al.* 2009).

Different approaches have been developed in order to analyze the latent benefits of human iPS cells in animal models of PD. Cai and collaborators employed a commercially available iPS cell line, the IMR90 clone 4, to induce its differentiation towards a mesencephalic dopaminergic lineage in culture. Once they achieved the dopaminergic phenotype, they were transplanted into the striatum of 6-OHDA unilateral lesioned rats. Six weeks after the transplant, they analyzed survival and integration of the transplanted cells within the host brain. They reported that several transplanted cells survived, but some of them expressed nestin and Ki67 which are expressed in dividing cells, such as tumoral cells (Cai *et al.,* 2010). This work however, does not allow conclusions regarding physiological effects, since behavioral outcome was not analyzed.

Later in that year, Hargus *et al.* employed dopaminergic neurons derived from factor-free iPS cells and transplanted them into the striatum of 6-OHDA unilateral lesioned rats; they later analyzed development, growth, survival and degeneration in an *in vivo* longitudinal study. They observed a high level of cell survival with no apparent degeneration; they also detected axonal outgrowth in different areas, this can be translated to a soaring capacity of the adult brain to keep axonal guidance instructions that allow the transplanted cells to integrate within the adult brain. In addition, a progressive reduction of motor asymmetry (about 70%) was observed in the transplanted animals over a period of sixteen weeks, 50% of the transplanted DA neurons were identified as SNPc mesencephalic cells and presence of tumoral cells was null (Hargus *et al.,* 2010). However, synaptic function analysis and a longer examination of transplant survival still remain to be done. Besides, the improvement of motor asymmetry was similar to the one reported in other experimental trials that employed different cells, in that aspect, there is no greater advantage of iPS cells over ES cells.

In 2010, reprogramming mice fibroblast directly into a neuronal phenotype directly to a pluripotency stage *in vitro* was achieved. A combination of 3 genes specifically expressed in neural tissue, Ascl1, Brn2 y Myt1l, was transduced by means of a lentiviral vector. These kind of cells were called induced neurons (iN), since they expressed neural proteins, generate action potentials and form functional synapses; most of these cells express an excitatory phenotype that was later confirmed by cortical neural markers and only a low number of these cells belong to the GABAergic phenotype (Vierbuchen *et al.,* 2010). Yet, it still remains to be determined if iN can be derived from fibroblasts obtained from patients afflicted with a neurodegenerative disease, and which transcription factors must be overexpressed in order to induce different neuronal phenotypes.

Regardless of all of the possibilities that iPS cells might offer, the uncertainty about the latency of tumor formation by being employed in experimental trials still remains. Albeit, better methods to induce reprogrammation are currently being developed, but even with viral vector removal, this possibility cannot be totally excluded.

Additionally, recent results demonstrate that production of aberrant methylation sites are originated during the epigenomic reprogrammation of iPS cells. Methylation of human iPS cells was observed recurrently at different rates throughout the reprogrammation process. Furthermore, the variability of the methylated sites is often seen in iPS cell lines, which indicates that there are some regions more prone to insufficient or aberrant reprogrammation. These methylated sites are categorized as "hotspots" for incorrect epigenomic reprogrammation, this alteration is not a simple anomaly exclusive of the pluripotent state, it can also be transmitted through iPS cells differentiation (Lister *et al.,* 2011).

So far, iPS cells cannot be employed as a feasible resource for cellular replacement therapy and further work needs to be developed before a clinical trial could take place; however, without a doubt, they possess great potential to be employed as tools for patient-specific disease modeling.

Finally, if we were to choose the type of cell that would bestow the best results after transplantation, taking into consideration the information aforementioned; we believe that the most promising candidate today is the ES cell. In order to provide this personal opinion, we are leaving aside the ethical issues that embrace them, albeit this kind of concerns are of the outmost importance, they are out of the scope of this chapter.

Cell Source	*In vitro* protocol for TH cells production	Recipient	Result	Time period	TH neuron count	Reference
					(after transplant)	
Mouse Embryonic Stem Cells						
mES cells-derived dopamine neurons	ES cultured on PA6 stromal cell	6-OHDA unilateral lesion Mouse	N/A	2 weeks	13, 000 (~22% of the original graft)	Kawasaki *et al.,* 2000
mES cells (no differentiated)	Embryoid body formation stage	6-OHDA unilateral lesion Rat	Decrease in 46% of ipsilateral rotation (amphetamine)	9 weeks	NA	Bjorklund *et al.,* 2002
mES cells-derived dopamine neurons	Genetically-engineered to over express Nurr1	6-OHDA bilateral lesion Rat	Total reversal of ipsilateral rotation (amphetamine)	8 weeks	20, 000	Kim *et al.,* 2002
ntES cell-derived dopamine neurons	Nuclear transfer-derived ES (ntES). Coculture with stromal cells	6-OHDA unilateral lesion Mouse	Decrease in 80% of ipsilateral rotation (amphetamine)	8 weeks	~22,000 (some reaching 40,000)	Barberi *et al.,* 2003
mES cells-derived dopamine neurons	Mice transfected with GFP differentiated using 5 stages protocol	6-OHDA unilateral lesion Mouse	Decrease in 50% of ipsilateral rotation (amphetamine)	2 weeks	NA	Nishimura *et al.,* 2003
mES cells-derived dopamine neurons	Differentiated using 5 stages protocol	6-OHDA unilateral lesion Rat	Total reversal of ipsilateral rotation (amphetamine) Starting at 4 weeks	32 weeks	5, 000	Rodriguez-Gomez *et al.,* 2007
ntES cell-derived from parkinsonian mice	Nuclear transfer-derived ES (ntES). Coculture with stromal cells	6-OHDA unilateral lesion mouse	Total reversal of ipsilateral rotation (amphetamine)	10 weeks	Avg 8,784 ± 4,293 cells in the group with better results	Tabar *et al.,* 2008
Mesencephalic DA neurons	Ngn2-GFP mice Pitx3-GFP mice Nestin-GFP mice Sox2-GFP mice	6-OHDA unilateral lesion Rat	Total reversal of ipsilateral rotation (amphetamine) Starting at 3 weeks	6 weeks	440	Jönsson *et al.,* 2009
Non-human primates Embryonic Stem Cells (monkey ES cells)						
Non-human primates ES cells	Neural progenitors expanded as neurospheres. FGF2 addition	MPTP bilateral lesion Cynomolgus monkeys	Behavioral improvement in neurological score	14 weeks	2, 100 per side	Takagi *et al.,* 2005
Human Embryonic Stem cells						
hES cells	Culture of hES cells enriched with neural progenitors	6-OHDA unilateral lesion Rat	Decrease in 45% of ipsilateral rotation (amphetamine)	12 weeks	389 (0.18% of the graft)	Ben-Hur *et al.,* 2004

Cell Source	*In vitro* protocol for TH cells production	Recipient	Result	Time period	TH neuron count	Reference
					(after transplant)	
			Decrease in 31% of contralateral rotation (apomorphine)			
hES cells-derived dopamine neurons	Culture on PA6 cells (stromal cells)	6-OHDA unilateral lesion Rat	No improvement (amphetamine)	13 weeks	10-50	Brederlau *et al.,* 2006
hES cells-derived dopamine neurons	Cocultured with human telomerase-immortalized midbrain astrocytes	6-OHDA unilateral lesion Rat	Total reversal of ipsilateral rotation (apomorphine)	Since 6 weeks	26,000 per mm^3	Roy *et al.,* 2006
hES cells-derived dopamine neurons	Generation of spherical neural masses (SNMs) (rossetes)	6-OHDA unilateral lesion Rat	Decrease in 49.43% of contralateral rotation (apomorphine) Decrease in 58.37% of ipsilateral rotation (amphetamine)	12 weeks	10,700 (2.7% of transplanted cells)	Cho *et al.* 2008
Induced Pluripotent Stem cells						
iPS clone O9	Reprogrammed fibroblasts (mouse)	6-OHDA unilateral lesion Rat	Decrease in 60% of ipsilateral rotation (amphetamine)	8 weeks	29, 000	Wernig *et al.,* 2008
iPS cells IMR90 clone 4	Reprogrammed fibroblasts (human)	6-OHDA unilateral lesion Rat	Decline in rotation score	6 weeks	NA	Cai *et al.,* 2010
iPS cells excisable virus	Human PD (patients)	6-OHDA unilateral lesion Rat	Decrease in 70% of ipsilateral rotation (amphetamine)	16 weeks	122 neurons per mm^3	Hargus *et al.,* 2010

Table 3. Stem cell therapy in animal models of PD.

5. Evaluating the functional effectiveness of Stem Cell Therapy for PD

One of the major difficulties in restoring the motor functionality in PD through transplant therapy is the correct integration of grafted cells into the nigrostriatal pathway; they are required to improve motor symptoms and circumvent the appearance of undesirable side effects, such as dyskinesia (Politis *et al*., 2010). Grafted cells should not only provide a DA source for the correct modulation of the basal ganglia, but also be able to integrate the nigrostriatal pathway and receive the regulatory input that allows compensatory mechanisms, such as feedback regulation. In addition, the complexity of the adult brain and the lack of a permissive environment to neuronal regeneration make it particularly complicated for even the best non-modified or engineered stem cell to integrate into the basal ganglia motor loop.

Cell grafts might be able to compensate the loss of modulatory inputs from the SNPc by forming local neural circuits within the striatum (Barker *et al.*, 1999; Kreitzer *et al.*, 2008; Lindvall *et al.*, 2004). Ideally, these circuits should be capable to modulate basal ganglia pathways through interaction with D2 and the D1 dopamine receptors, as well as to be subject to feedback regulation in order to prevent variations of DA availability.

In order to evaluate these neural circuits, the need of joined functional and behavioral tests to assess improvement is one of the main objectives. The use of positron emission tomography (PET) coupled to magnetic resonance imaging (MRI) techniques has diminished the gap between behavioral and functional data.

5.1 MRI assessments

Conventional MRI reveals brain structural changes as reductions in volume (atrophy) and alterations in water-proton relaxation T1 and T2 signals. Water normally flows along neural tracts in the brain. Diffusion-weighted or diffusion tensor MRI can be used to quantify loss of anisotropy (directionality) or increase in amplitude of water diffusion in order to demonstrate disruption of neural tracts.

Conventional T1 and T2 weighted MRI show normal nigral structure in idiopathic PD and therefore is not diagnostically helpful. Volumetric T1 weighted MRI studies have also failed to detect a reduction in nigral volume in PD, possibly because of difficulties in accurately defining the border of the SNPc (Geng *et al.*, 2006) and thus it would be the most appropriate technique for evaluating the survival of dopamine-derived transplants.

MRI inversion recovery sequences can be designed to suppress either gray or white matter signal. Segmented inversion recovery ratio imaging generates ratio images of gray matter and white matter suppressed signal at a voxel level. With the segmented inversion recovery ratio approach, PD patients were reported to show a gradient of altered nigral signal that was absent in healthy controls (Hutchinson & Raff, 2000). Although the use of gray matter and white matter suppressing inversion recovery sequences can detect changes in nigral structures in PD, it is a complicated approach to implement and currently not sensitive enough to be of diagnostic value in transplant therapy.

Regardless of the limitations previously mentioned, MRI is a non-invasive, real-time cellular imaging modality that has no radiation, and for these reasons, several attempts have been made in order to take advantage of these qualities. New MRI techniques currently employ magnetic labeling using superparamagnetic iron oxide particles (SPIOs) coupled to stem cells transplanted in the rat striatum with the purpose of assessing the survival time of transplanted tissue (Berman *et al.*, 2011; Obenaus *et al.*, 2011). Nevertheless, this innovative technique is only useful for quantifying cell survival and does not provide data to evaluate transplanted cell function.

5.2 PET assessments

Terminal dopa decarboxylase activity and dopamine turnover can be assessed using radioactive markers such as 6-[18F]-fluoro-L-DOPA (18F-DOPA), which could be helpful for the evaluation of PD progression *in vivo* (Brooks *et al.*, 2008) and graft survival in transplanted PD patients (Mendez *et al.*, 2002; Sawle *et al.*, 1992). Early hemiparkinsonian patients show a bilateral reduced putamen dopaminergic terminal function. Clinical parkinsonism occurs when PD patients have lost 40%–50% of posterior putamen-dopamine terminal function (Morrish et al., 1995). Levels of 18F-dopa uptake in the putamen and DAT

binding correlate inversely with bradykinesia and rigidity of PD patients, and could be valuable for estimation of cell therapy functional recovery.

However, this type of analysis has limitations; ^{18}F-DOPA is not specific for evaluation of DA re-uptake in small animal PET scanning, which is an essential feature for the assessment of emergent cell therapy in animal models of PD. Other radiotracers of DAT binding, such as [^{11}C]-(+)-α-dihydrotetrabenazine ([11]C-(+)DTBZ), have better specificity and spatial resolution when evaluating animal models of PD (Collantes *et al.*, 2008) and could be used to examine the survival of DA neurons.

Still, in order to correctly evaluate DA release, it is necessary to use radioactively labeled dopamine receptor antagonists, such as [^{11}C]-raclopride, which can be displaced from DA receptors by DA released from nerve terminals located in situ or exposed to amphetamine (Piccini *et al.*, 2000). ^{11}C-raclopride PET is able to indirectly detect synaptic dopamine fluxes by monitoring changes in striatal D2 receptor availability (Laruelle, 2000). The higher the extracellular dopamine level, the lower the dopamine D2 site availability to the tracer. Animal microdialysis studies suggest that a 25% fall in putamen 11C-raclopride uptake after amphetamine equates to a 10-fold rise in synaptic dopamine levels (Breier *et al.*, 1997). In PD patients, when given L-DOPA treatment, they show a fall in striatal 11C-raclopride binding; the response of bradykinesia and rigidity to L-DOPA in PD correlates with the resulting increases instriatal dopamine levels detected with 11C-raclopride PET (Pavese *et al.*, 2006).

By means of ^{18}F-DOPA and [^{11}C]-raclopride PET scans, it has been shown that grafted (human embryonic mesencephalic and fetal dopaminergic tissue) cells remain active and form connections with neurons residing in the host striatum when transplanted in human brains (Mendez *et al.*, 2008; Piccini *et al.*, 1999; Spencer *et al.*, 1992). The major drawback is that the spatial resolution achieved with the current available PET scanners (2×10^{-3} m) is insufficient to evaluate single cell events, which can only be resolved with 100-fold higher resolutions (10–20 μm).

So far, the information already described shows that current MRI and PET analysis have some limitations for a correct cell graft evaluation of survival and integration within the host, specifically at the cellular level.

5.3 Microcircuits on brain slice preparations: the need for functional studies

It is clear that better protocols for cell replacement therapy would benefit from a better understanding of normal basal ganglia circuit functions. One of us (Bargas' group) designed a brain slice preparation in which individual cell activity can be observed and evaluated, using calcium-imaging techniques (Carrillo-Reid *et al.*, 2008). When corticostriatal slices are loaded with a calcium-sensitive fluorescent dye, it is possible to analyze single-cell activity in a widespread area of the striatum. Additionally, it is possible to examine the activity of cell clusters that have an associated firing pattern (neural ensembles) and represent functional microcircuits within the striatum (Carrillo-Reid, 2008, 2009). Different cells within the striatum belong to different ensembles, and there are some cells that belong to most of the ensembles present in a given slice. These cells serve as central pattern generators (CPGs), which are considered to represent memory traces in the nervous system (Carrillo-Reid, 2008, 2009; Grillner, 2005, 2006). CPG activity within the striatum might be related to the encoding of motor programs that are activated secondary to cortical stimulation. In this regard, it is worth of notice that unstimulated corticostriatal slices mostly show silent cells, with some uncoordinated activity.

Ensemble activity is triggered by stimulation of cortical fibers or after exposure to glutamatergic agonist NMDA. These results suggest the existence of intrinsic microcircuits in the striatum, which could be the actual modulators of motor activity in this brain area.

Thus, it would be necessary to determine if the segregated pathways represented by neurons expressing either the D1 or the D2 dopaminergic receptors, belong to distinct activity ensembles; this would be of major interest, since deregulation of the D1 receptor pathway has been proposed to be responsible for the dyskinesias observed in PD patients after prolonged exposure to L-DOPA (Dupre *et al.*, 2008; Santini *et al.*, 2008; Taylor *et al.*, 2005). Of particular interest would be to demonstrate if there is a difference between ensembles in the intact brain and those in the affected striatum of PD patients.

We propose that the use of this brain slice preparation will allow us to study if transplanted dopaminergic cells can integrate into local microcircuits within the striatum, and if formation of new modulatory connections could compensate for the loss of the dopaminergic input from the SNPc and reestablish control of motor function.

6. Conclusion

This chapter highlights that although almost three decades have passed since the Backlund-Lindvall and Drucker-Colín *et al.* work was published, transplant therapy for PD is still retained as a potential clinical approach; stem cell research is currently providing a more adequate source of cells. So far, the large body of evidence appears to validate the use of manipulated stem cells in the coming years.

Although stem cell therapy shows promise, it is still in development, and even with a wide range of methods, currently there is no ideal scenario for clinical transplantation and perhaps it will not arrive in the near future. ES cell therapy still remains elusive and is burdened with social and logistical concerns. In contrast, iPS generation circumvents some previous limitations, since it does not require embryonic material. iPS cell technology might have a significant impact on regenerative medicine in the near future.

In order for cell therapy to become a viable option of treatment, a pure source of dopaminergic neurons is essential. However, the poor survival of transplanted differentiated cells limits transplant therapy; ergo a pure population would not be the best option, since cell survival decreases after transplantation. Additionally, the combination with other cell populations could also be necessary for a successful transplant, given the fact that primary fetal cells that provide behavioral improvement are only ~20% of the total implanted cells.

Finally, it should be particularly noted, that aside of a couple of studies, all report incomplete motor improvements. The search for the basis of this result is today still in its embryonic stage. As a final point, research with better surgical procedures that provides controlled assessments consequently will lead to a more clear understanding of the phenomena involved grafting in PD.

In sum, despite several failures, we believe that cell replacement therapy has a viable future.

7. Acknowledgments

8. References

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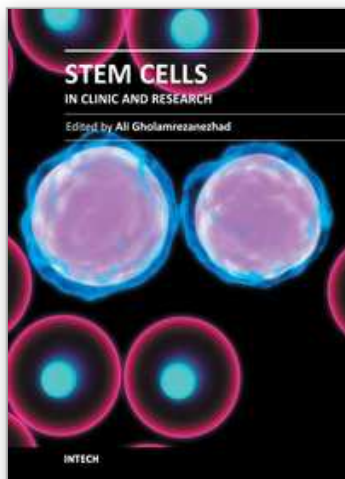
Stem Cells in Clinic and Research

Edited by Dr. Ali Gholamrezanezhad

Based on our current understanding of cell biology and strong supporting evidence from previous experiences, different types of human stem cell populations are capable of undergoing differentiation or trans-differentiation into functionally and biologically active cells for use in therapeutic purposes. So far, progress regarding the use of both in vitro and in vivo regenerative medicine models already offers hope for the application of different types of stem cells as a powerful new therapeutic option to treat different diseases that were previously considered to be untreatable. Remarkable achievements in cell biology resulting in the isolation and characterization of various stem cells and progenitor cells has increased the expectation for the development of a new approach to the treatment of genetic and developmental human diseases. Due to the fact that currently stem cells and umbilical cord banks are so strictly defined and available, it seems that this mission is investigationally more practical than in the past. On the other hand, studies performed on stem cells, targeting their conversion into functionally mature tissue, are not necessarily seeking to result in the clinical application of the differentiated cells; In fact, still one of the important goals of these studies is to get acquainted with the natural process of development of mature cells from their immature progenitors during the embryonic period onwards, which can produce valuable results as knowledge of the developmental processes during embryogenesis. For example, the cellular and molecular mechanisms leading to mature and adult cells developmental abnormalities are relatively unknown. This lack of understanding stems from the lack of a good model system to study cell development and differentiation. Hence, the knowledge reached through these studies can prove to be a breakthrough in preventing developmental disorders. Meanwhile, many researchers conduct these studies to understand the molecular and cellular basis of cancer development. The fact that cancer is one of the leading causes of death throughout the world, highlights the importance of these researches in the fields of biology and medicine.

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