Cell therapy in brain neurodegenerative movement disorders – a clinical perspective

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Abstract

Neurodegenerative movement disorders such as Parkinson´s disease or Huntington´s disease share the same progressive and relentless course with increasing motor disability coupled with neuropsyquiatric impairment and have been, over these last few decades, a passionate topic of experimental and clinical research due to their lack of therapeutic options. Restorative therapies have brought both enthusiasm with the prospect of its potential to restore brain damage and also disbelief for the less than compelling results from clinical trials. The aim of this review is to discuss the cell replacement therapies applied to both Parkinson´s and Huntington´s diseases since their beginning, following their natural sequence from animal studies into the early human trials, addressing fetal neural tissue transplantation and other more recent prominent cell sources.

Nowadays, stem cells became at the forefront of cell therapy. Embryonic, neural and induced pluripotent stem cells were successfully able to generate a desired neuron phenotype and/or provide growth factors to the vulnerable or degenerating host cells allowing researchers to enter a new pre-clinical era. There are still many barriers to overcome before clinical application is possible, it must be assured that the stem cell source has an optimal differentiation potential with full integration and functional enhancement, bears measurable clinical benefits with minimum impact on hosts immune system and absence of tumor formation. The future prospects for stem cell therapy are overwhelming but steady and solid basic and pre-clinical progresses must be held before clinical application becomes eligible.

Key Words

Cell differentiation; Embryonic stem cells; Fetal neural tissue; Growth factors; Huntington´s disease; Induced pluripotent stem cells; Neural stem cells; Parkinson´s disease; Stem cells; Transplantation;

Contents

1. Introduction

Research on chronic neurodegenerative disorders of the central nervous system (CNS) is a very important branch of medical science due to their increasing prevalence worldwide.

Neurodegenerative movement disorders, such as Parkinson's disease (PD) and Huntington's disease (HD), share the property of neuronal damage caused by the accumulation of aggregation-prone proteins that have toxic effects, as is the case of alpha-synuclein and mutant huntingtin, respectively.

PD represents the movement disorder with the higher incidence and prevalence, affecting about 0.5 to 1% of the aging population, being the majority of cases of sporadic origin. HD is an uncommon hereditary autosomal dominant disorder caused by expanded polyglutamine repeats at the N-terminal of huntingtin. HD is the most studied genetic movement pathology.

The estimated prevalence of HD is of 0.01%, but it varies depending on the geographical area. Presently, the causes and molecular mechanisms of these brain diseases are not completely understood. Furthermore, for both of these disorders there is still no cure or symptomatic relief with long lasting effects, being the neurodegenerative process irreversible and inevitable.

The basal ganglia once thought solely as part of the "extrapiramidal" motor system, is now recognized as a brain area whose function relies on receiving and sending back signals to cortical areas, which also play an important and integrating role in the cognitive and emotional sphere. Therefore, we can not acknowledge PD and HD as only mere "movement" diseases as they represent complex and multifunctional neurodegenerative disorders.

In this review we discuss the cell replacement therapies concerning these diseases as well as the various cell resources that are currently being investigated. For both of them, we will

address the early trials involving the grafting of fetal brain tissue, their best results and disappointments, which laid the foundations for stem cell research, as well as the scientific breakthroughs that were accomplished so far.

2. Parkinson´s disease

Parkinson´s disease (PD) was first described in 1817 by James Parkinson in his essay about the "Shaking palsy" as an *involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forward, and to pass from walking to a running pace: the senses and intellects being uninjured* (Parkinson 2002).

Nowadays, PD is the second most common neurodegenerative disease, after Alzheimer´s disease, affecting between 0.3 to 1% of population in the range of 65 to 69 years old and being the estimated prevalence of 1 to 3% in people over 80 years old (Lau et al. 2006). About 90% of the cases are of sporadic origin, known as idiopathic PD, being the remaining 5 to 10% caused by inheritable genetic mutations.

The exact pathological mechanisms underneath PD are not completely understood. It is believed that a combination of environmental (exogenous toxins and inflammation among others) and genetic factors leads to mitochondrial dysfunction with oxidative stress increase, decreased activity of the ubiquitin-proteasome system and activation of glial cells leading to neuronal death (Lau et al. 2006).

In this chronic degenerating illness there is known to be a progressive loss of dopaminergic and non-dopaminergic neurons particularly in the mesencephalon (figure 1). The degeneration of dopaminergic neurons in the substantia nigra (SN) results in severe loss of dopamine (DA) in the striatum with disorganization of the basal ganglia circuits thereby

playing a major role in PD´s cardinal symptoms – bradykinesia, resting tremor and rigidity. Current criteria also include gait disturbance with postural instability and freezing as main symptoms.

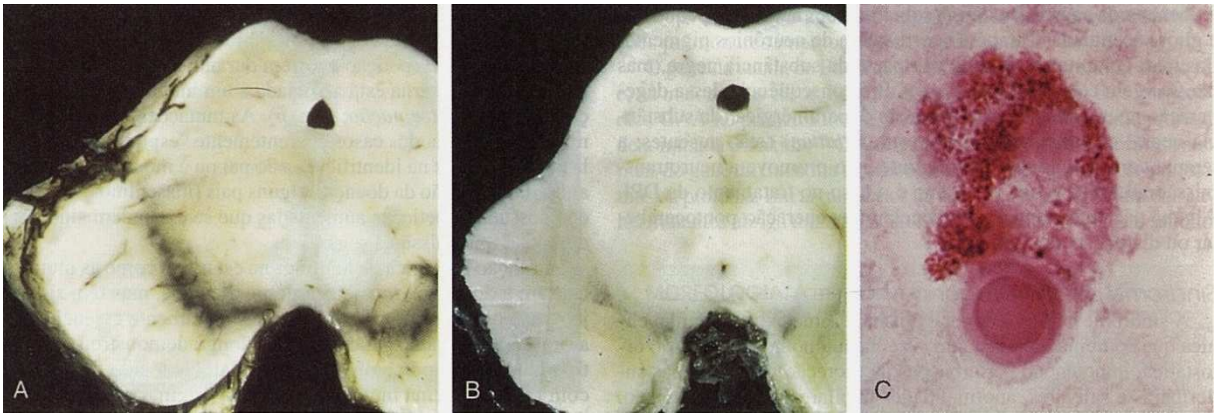

Figure 1: Comparison between SN from a PD patient and a non-PD patient;
Legend: A – SN from a non-PD patient; B – SN from a PD patient showing loss of pigmented dopamine neurons; C – Lewy body; Image from de Girolami et al. 1999;

It is well known the existence of more-widespread neuronal changes that cause complex and variable symptoms in the cognitive and psychiatric spectrum as well as autonomic and sensory disturbances. Another pathological hallmark of PD is the presence of Lewy bodies, intracellular fibrillar inclusions consisting of abnormal proteins, including alfa-synuclein that can exist in both central and peripheral autonomic nervous systems. Current treatment approaches include symptomatic relief with DA precursor, L-DOPA (L-3,4-dioxyphenylalanine) and DA receptor agonists, but beneficial effects tend to lessen with chronic use and severe non-motor and motor fluctuations (on-off, wearing-off, ...) as well as diskynesias (abnormal involuntary choreiform or dystonic movements) appear as detrimental side effects. There are also other drugs which include monoaminoxidase inhibitors (selegiline, rasagiline) amantadine, catechol-O-methyl transferase inhibitors and anticholinergics, but older patients might be highly sensitive to them and symptoms like confusion, hallucinations, orthostatic hypotension and fatigue may emerge, making them inadequately effective in later PD stages.

Another current approach is surgical therapy. The first surgical procedures, based on surgical ablation of deep brain structures, such as Thalamotomy or Pallidothomy, are almost completely abandoned. Nowadays, Deep Brain Stimulation of the subthalamic nucleus is the preferred procedure but several other surgical targets are under investigation.

These treatment options, however, can neither detain nor reverse the degenerating course of the disease, which is why the extensive investigation is now turning to discovering newer and more effective therapeutic strategies, either pharmacological or cell replacement therapies based on stem cells, as well as developing vaccines and various surgical techniques for the management of PD.

2.1. The beginning of cell therapy in PD

Selective lost of dopaminergic neurons is one of the key features of PD. Thus, cell therapy has been considered as a potential therapeutic approach in PD since the mid-1980s. For this therapy´s success, neurons must integrate into local host circuits, establish new synapses, synthesize, release and take up DA in a similar manner to healthy host cells. We will briefly address the studies performed in animal models and the trials involving the grafting of fetal brain tissue in human patients.

2.2.1. Transplantation of fetal dopaminergic neurons – what we have learned from animal models

Several animal studies conducted in the late 70s and early 80s transplanted human fetal dopaminergic tissue to replace the lost of DA in PD rodent animal model based on the idea that it would induce long lasting clinical improvement. It is beyond the range of this analysis to describe them in detail as they have been previously reviewed (for review see Dunnet et al. 1990).

Pre-clinical experimental data have demonstrated that intrastriatal grafted DA neurons, obtained from human fetal ventral mesencephalon, display many of the morphological and functional characteristics of normal DA neurons: they reinnervate the dennervated striatum and form synaptic contacts being spontaneously active and releasing DA. Successful reinnervation provided by the grafts is accompanied by a significant amelioration of Parkinson-like symptoms in animal models (Goren et al. 2005; Dunnet et al. 1990).

To reassure the survival of neuronal transplants several issues must be taken into consideration, such as the optimal donor age, or the number and main neuronal subtype present in the grafts, amongst others.

Early studies in experimental animals established a crucial developmental time window in which immature DA neurons could be harvested and were able to survive a subsequent grafting. Animal studies also revealed that the transplantation technique influenced the donor age window. Mature donor tissue was found to be highly sensitive to mechanical trauma due to their long extended processes but, conversely, immature tissue was even more difficult to adopt a dopaminergic phenotype after grafting (Freeman et al. 2006). The vast majority of experiments usually employs embryos around 13-15 embryonic day (E13-15), but a recent paper reported better survival of dissociated grafts when donor age was E12 (Torres et al. 2007). These questions are essential once even when using optimal donor age tissue only around 5-20% of grafted dopaminergic neurons survive due to harvesting, dissection or transplantation procedures (Brundin et al. 2000a; Sortwell 2003). It is also important to remember that nigral grafts are only made up of 5–10% neurons destined to become dopaminergic neurons, being the remaining from other neuronal and glial cellular subtypes.

Several attempts were done to enhance neuronal dopaminergic survival, including testing different grafting techniques, neurotrophic support, antioxidant therapies, increasing

graft vasculature, promoting caspase inhibition, Bcl-2 overexpression along with others, whose main purpose was to reduce cell oxidative stress (Sortwell 2003).

A recent work has drawn attention to the existence of two major dopaminergic neuron subtypes that stain for tyrosine hydroxylase (TH) within transplants of fetal ventral mesencephalic tissue: the A9 neurons of the SN *pars compacta* and the A10 neurons of the Ventral Tegmental Area (VTA). These TH positive cells can be identified based on their expression of a G-protein-regulated inward rectifier potassium channel subunit (Girk2) that marks A9 neurons or Calbindin that labels A10 neurons. The same work also illustrates that striatum´s dopaminergic innervation in rats derives almost exclusively from the A9 subtype, whereas the Calbindin-positive VTA neurons project to the frontal cortex and probably also other forebrain areas. These results are in agreement with the existence of axon regulation and target detection mechanisms that can guide the growing axons to their appropriate targets (Thompsom et al. 2005). These facts may imply that better outcomes may emerge when using neurons with the molecular properties of A9 subtype that reinnervate the striatum and also synthesize and release DA in the host brain.

Nigrostriatal lesions in animal models create motor behaviors PD-like that can be ameliorated by transplantation of dopaminergic neurons. Some of the latter can be evaluated with motor performance tasks such as the amphetamine-induced rotation, the cylinder test and the stepping test in rodents (Breysse et al. 2007; Olsson et al. 1995). Breysse and colleagues reported that behavior tests outcomes were compromised when the dopaminergic lesion was extended to include also the medial and ventral striatum as well as the cortical and limbic dopaminergic projections (Breysse et al. 2007). Furthermore, the data implicates that there might be no valuable effects when transplantation occurs in advanced stages of the disease.

2.1.2. Transplantation of human fetal dopaminergic neurons – clinical trials

The earliest transplantation attempts for PD patients involved the use of readily available cell sources such as autologous adrenal medullary cells, surprisingly it was devoid from the clinical benefits expected, if one takes into account the previous animal data. The clinical benefit could not be replicated in subsequent open-label trials and the procedure was associated with considerable morbidity related to the need for both intra-abdominal and intracranial operations with only a few surviving cells detected post-mortem. Hence, this procedure was abandoned (Olanow and Fahn 2006).

Studies involving the grafting of human embryonic tissue started in the mid-1980s and the initial transplants were able to demonstrate the feasibility of performing this procedure as well as beneficial clinical effects on motor behaviour. Since then, about 400 patients have been transplanted with embryonic dopaminergic neurons in several clinical trials, which provided proof-of-principle that these cells survive grafting, restore DA release and ameliorate some PD motor features. The trials mentioned above have been reviewed extensively elsewhere (e.g. Hagell and Brundin 2001; Olanow and Fahn 2006).

Clinical outcomes (table 1) in the best of cases displayed striking symptomatic relief with a decrease of the “off” periods and also lessening of medication needs, with a clear impact on the quality of life of PD patients who had undergone grafting procedure (Hagell et al. 1999; Hauser et al. 1999; Brundin et al 2000b). In some patients the impact was so significant that they were able to return to work in full time or to leave completely the anti-parkinsonian medication (Brundin et al. 2000b; Hagell et al. 1999).

The majority of studies focused mainly on reinnervating the striatum alone and their outcomes have not yet provided solid evidence favoring neural transplantation as a routine therapeutic measure for PD patients. To investigate the benefits of target and, based on previous animal data, Mendez and coworkers held a pilot study to demonstrate the feasibility

of reinnervating the SN and striatum using a double transplant strategy in humans. Their results (table 1) suggested that this double reinnervation could be crucial to improve clinical outcomes in PD patients, but this method still requires further investigation (Mendez et al. 2002).

One other major concern of cell transplantation surpassed with positron emission tomography (PET) with uptake of [^{18}F]-fluorodopa (FD was graft survival and function assessment. FD-PET scan is an *in vivo* study that allows measurement of the number of viable striatal DA terminals using pre-synaptic uptake. Usually PD patients display diminished FD uptake with increased motor impairment in the putamen and caudate nucleus. The use of FD-PET scan provided evidence to support graft survival and function for as long as 10 years (Freed et al. 2001; Hauser et al. 1999; Piccini et al. 1999). Post-mortem neuropathological examinations from patients who have died added more evidence concerning graft survival and fiber outgrowth in the host brain (Freed et al 2001; Kordower et al. 1995; Mendez et al. 2005).

However encouraging, all previous open-label trials were unblinded, displaying variability in the processing protocols as well as on surgical methods. Moreover, there was a requirement for a more standardized, large and placebo controlled studies (table 2). For that purpose, two NIH-sponsored studies were performed in a double blind controlled-trial design (Freed et al 2001; Olanow et al. 2003).

Freed and coauthors enrolled forty PD patients in a study where they either received tissue from two embryos in each putamen or sham surgery and where the source of the human embryonic mesencephalic tissue consisted of aborted embryos seven to eight weeks after conception. The tissue was stored as solid tissue strands and maintained in culture for up to eight weeks prior to implantation. There was no postoperative immunossuppression given.

Table 1: Results from four open-label trials concerning implantation of human fetal mesencephalic tissue in PD patients.

Reference	Hagell et al. 1999	Hauser et al. 1999	Brundin et al. 2000b	Mendez et al. 2002
Number of patients	5	6	5	3
Age (mean)	48.8 (43-53)	55.5 (39-63)	53 (41-68)	50 (48-59)
PD stage	Advanced	Advanced	Advanced	Advanced
Number of grafts and location	Unilateral in the putamen in previously grafted patients	Bilateral in the putamen	Bilateral in the putamen and caudate nucleus	Bilateral in the putamen and SN
Number of donors Donors age	4-8 donors 6-8 weeks	6-8 donors 6.5-9 weeks	7-9 donors 5-7 weeks	6 donors 6-9 weeks
Graft preparation	Dissociated VM tissue	Solid grafts VM tissue	Dissociated VM tissue	Dissociated VM tissue
Immunossupression	Cyclosporin, azathioprine and prednisolone*	Cyclosporin 6 months	None	Cyclosporin 6 months
Follow-up	18-28 months	24 months	18-24 months	3-13 months
Viability assessment				
PET-FD - Pre/Post	31%/52% + 69%	34%/55% + 61%	31%/48% + 55%	NA + 84.9%
Motor score (mean) – “off” - UPDRS				
Preoperative	32.4 (25-41)	58.8	41.7 (23-67)	97.3 (75-139)**
Postoperative	33.2 (20-45)	35.4	25.1 (16-42)	61.3**
L-DOPA dose (mean)				
Preoperative	640mg (200-950)	854mg (200-1200)	825mg (225-1500)	65% and 45% decrease in two patients respectively; 1 patients without dose change
Postoperative	430mg (0-800)	717mg (250-1000)	455mg (0-900)	
Relevant events	1 patient deteriorated motor score after grafting and multiple system atrophy was suspected; 1 patient died 25months after second procedure due to massive intracerebral haemorrhage ipsilateral to the first graft	2 patients died 18 months after from unrelated causes and post-mortem analysis showed graft survival	1 patient without UPDRS improvement	1 patient with intracerebral haemorrhage ipsilateral to the graft probably due to hypertension
Side-effects	1 cortical dementia	1 asymptomatic cortical hemorrhage	No major side effect detected	No major side effect detected
Dyskinesias	Variable	Increased “on” period without	Variable	NR

Legend: NR- not reported * 18-24 months **UPDRS total score

The results were based on a subjective global rating scale (diaries) which has shown no statistically significant differences between treatment groups, even though in the group under sixty years old better results were described. The Unified Parkinson’s Disease Rating Scale (UPDRS) and the Schwab and England scale were used as secondary outcome measures and, in a similar manner, no statistically significant differences were found between groups;

nonetheless, the patients under sixty years old displayed better results. PET scans with FD performed before and twelve months after the procedure showed significant increase in radionucleotide uptake in the putamen among patients from the transplantation group with no significant changes compared to those submitted to sham surgery. Post-mortem examination on two deceased patients from unrelated causes revealed modest neuronal survival and fiber outgrowth (Freed et al. 2001). Despite modest clinical benefit held by younger patients in this trial, there was a failure to reach statistic results. Several of this trial´s features were severely criticized and considered responsible for the lack of results, such as the absence of immunossupression, the storage method or the fact that the tissue was used as solid grafts but also the rostro-caudal trajectory chosen instead of dorsoventral which was more frequently selected.

Olanow and colleagues, in a similar double-blind design, had thirty four PD patients randomized to receive bilateral grafting in the putamen of human embryonic mesencephalic tissue from one or four donor compared to sham surgery (table 2). The tissue contained dopaminergic neurons recovered from fragments of embryos aborted six to nine weeks after conception. All patients were treated with cyclosporin at a higher dose before the procedure and during six months after with a lower amount. The primary outcome measure was the UPDRS motor score change. From the results, one interesting feature was that four donor´s transplanted patients demonstrated a trend to improve motor scores between six-nine months in comparison with what was reported in open-label trials, and worsened in the following months, showing in overall no significant differences. This deteriorating period matched the end of immunossupression which probably gave rise to graft rejection held by host´s immune system (Olanow et al. 2003). PET studies established considerable increases in striatal FD metabolism in grafted regions, with more pronounced changes in the four donor group. Post-mortem studies, from patients who died of unrelated causes, demonstrated robust graft

survival with normal-appearing reinnervation of the striatum, once more with pronounced effects in the four donor group (Olanow et al. 2003). In this study transplantation failed to meet its primary outcome and provide significant clinical benefits despite autopsy and PET evidences of high numbers of implanted cells survival (Olanow et al. 2003).

Table 2: Results from two double-blind placebo controlled trials concerning implantation of human fetal mesencephalic tissue in PD patients.

Reference	Freed et al. 2001		Olanow et al. 2003		
Number of patients	Grafted 20	Placebo 20	1 donor 11	4 donor 12	Placebo 11
Age (mean)	54.5 (34-75)		58.5		
PD stage	Severe		Advanced		
Number of grafts and location	Bilateral on the putamen or sham surgery		Bilateral on the putamen or sham surgery		
Number of donors Donors age	4 donors 7-8 weeks		1 or 4 donor per side or placebo 6-9 weeks		
Graft preparation	Solid VM tissue		Solid VM tissue		
Immunossupression	None		Cyclosporin 6 months		
Follow-up	12 months		24 months		
PET-FD - Pre/Post	NR + 40%	NR -2%	NR *		
Motor score (mean) – “off” - UPDRS					
Preoperative	66**	58.5**	47.9	48.6	51.5
Postoperative	66**	51**	+3.5%	-0.72%	+9.4%
L-DOPA dose (mean)					
Preoperative	NR		1257mg	1427.7	1399mg
Postoperative	NR		-20%	-11%	NR
Relevant events	2 patient died from unrelated causes, 1 from an automobile accident and 1 from myocardial infarction; post-mortem analysis revealed graft survival and striatum reinnervation		2 patients died from unrelated causes, 1 from myocardial infarction and 1 from drowning; post-mortem analysis revealed graft survival and striatum reinnervation		
Side-effects	1 subdural hematoma		No major side effect detected		
Dyskinesias	15% with “off” medication dyskinesia		56.5% with “off” medication dyskinesia		

Legend: NR – not reported; * Increase in fluorodopa uptake on PET on one and four donor group was reported; ** UPDRS total score shown; UPDRS motor score decreased 18% for the transplantation group;

Taken together, the results from the two double-blind placebo-controlled trials failed to meet their primary end point despite increased striatal FD uptake on PET scans and post-mortem evidence of surviving transplanted neurons. An interesting difference is that Freed and coworkers reported motor benefits in a subpopulation of patients under sixty years old,

whereas Olanow and coauthors did not detect selective benefits in younger patients but noted significant improvements in those with milder disease (Olanow et al. 2003).

There is no plausible explanation for why these double-blinded were not as successful as their predecessors open-label trials. We cannot exclude doctor/patient/caregiver bias in open label trials, but this can not provide the only explanation. Other protocol divergences may include the immunossupression, the technique of tissue preparation or surgical and patient selection methods.

An additional concern to both studies was the development of graft-induced dyskinesias. Freed and coworkers reported 5 out of 33 grafted patients with these involuntary movements in the range of 2-3 years after surgery, whereas Olanow and colleagues reported the same problem in about 56% of grafted patients. Though in the latter there was an increase in the number of patients, Freed and coworkers reported the higher severity in graft-induced dyskinesias. In both cases, however, dyskinesias persisted after therapeutic discontinuation and were considered a major drawback in the transplantation field.

Twenty years have passed since the beginning of neuronal transplantation and many lessons have benn learned from pre-clinical and clinical trials. We must now and for the future recognize the importance of cellular developmental stage as well as the number, main phenotype and purity of cells needed for each procedure as well as optimize patient selection criteria and adopt standard protocols which allow comparison between studies.

Despite the initial reported clinical benefit from neural grafting in PD patients, only about 400 patients have been transplanted worldwide, partly due to the shortage of embryonic donor tissue. Hence, there is a need for an alternative cell source with the ability to be manipulated in order to expand indefinitely, constituting an unlimited and homogenous standardized pool of cells. These cells should then be able to differentiate, extend axons, form synapses, produce and release DA in a regulated manner similarly to normal neurons.

2.1.3. Graft-induced dyskinesias and immunossupression in clinical trials

Long-term treatment with L-DOPA causes the development of motor complications, known as dyskinesias and motor fluctuations, they are normally related to the “peak of dose” or “wearing off” phenomenons, respectively, and are often severely disabling. The development of severe “off”-medication dyskinesias was observed in nearly 15% patients after the first double-blinded clinical trial and reached 56% in the subsequent trial (Freed et al. 2001; Olanow et al. 2003). In both cases dyskinesias affected only the grafted patients suggesting they were caused by the graft itself.

Hagell and colleagues made a retrospective analysis but were not able to find the same phenomenon described in the double-blind trials (Hagell et al. 2002; Freed et al. 2001).

There are several hypotheses regarding the origin of this mechanism, they can be caused by an uneven pattern of reinnervation induced by the grafts, or by changes in its physiology due to immune rejection as well as insufficient or excessive DA released from the graft (Hagell and Cenci 2005).

Presently, investigators are putting their efforts in trying to mimic these graft induced dyskinesias in PD animal models, not only to learn their mechanism but also to find a way of avoiding them. However, there is so far no reports from studies in which these graft-induced “off” stereotyped movements were induced in rodents and very few studies involved animals who received L-DOPA treatment first and transplant afterwards, as occurs in humans.

Maries and colleagues identified a new type of motor behavior - facial-forelimb stereotypy – in rats receiving single-site grafts in the ventrolateral striatum, the same was not reported in animal receiving implants in multiple sites or sham surgery, however they were still dependent on L-DOPA therapy (Maries et al. 2006). Two other groups revealed similar motor behaviors from rats that were first put on L-DOPA therapy and then received nigral implants and to whom was given amphetamine (Carlsson et al. 2006; Lane et al. 2005).

According to Lane and coworkers, the movements begun to appear several weeks after procedure, were more severe in animal with larger grafts, depended on DA release and were less severe in those animal that were not submitted to L-DOPA therapy before grafting (Lane et al. 2005).

In clinical trials it seemed that all patients who experienced graft-induced dyskinesias also displayed similar behavior during prior L-DOPA treatment, however, there is still no apparent relation between these events (Hagell et al. 2002). Interestingly, "off" dyskinesias were not observed in two patients who received fetal midbrain grafts as a cell suspension in the striatum or SN, respectively. These patients had a good clinical and imagiological outcome and did not develop motor complications (Mendez et al. 2005). Thus we should now focus more on animal models to offer an answer for the graft-induced dyskinesias, despite de overwhelming differences between basal ganglia anatomy among species.

The human brain, once thought to be an immunologically privileged site, is now known as a place where there can be immune rejection, like elsewhere in the body, in both allografts and xenografts. However, the rejection of autografts is also possible. Glial cell activation and immunologic marker up-regulation, which might be detrimental to both graft survival and function, may result from major histocompatibility complex antigen expression differences between graft and host, leading to an inflammatory reaction (Olanow and Fahn 2006). Despite the immune reaction, grafts are still able to survive in the absence of immunossuppressive therapy.

There was no sustained immunological therapy in both double-blind clinical trials previously described (Freed et al. 2001; Olanow et al. 2003), which may have contributed to the negative results obtained. Furthermore, it can be implied that multiple donor tissue is partly to blame due or even that solid tissue grafts, used in the first study (Freed et al. 2001), could induce an immune response due its richness in major histocompatibility complex

antigen 1, known to be highly immunogenic. However, in both studies there was evidence of immune reactivity in the grafts. Indirect evidence of immune rejection can be presumed from modest dopaminergic neuronal survival obtained in the first double-blinded trial, in which no immunossuppressive treatment was used. In the second placebo-controlled trial immunosuppressive therapy was given for 6 months and the initial benefits were lost after its suspension (Olanow et al. 2003).

2.1.4. Recent data

Kordower and coworkers reported recent data from a post-mortem analysis, regarding a patient who died 14 years after grafting. In this patient it was shown neuronal survival with positive immunostaining for dopaminergic neurons and classical morphology with extensive innervation of host´s striatum (Kordower at al. 2008). In a similar study from Li and coauthors, regarding two patients who had undergone grafting for 8-16 years before death, it was also reported long dopaminergic neuronal survival with dense fiber network in the grafts and surrounding striatum (Li et al. 2008). These findings were in agreement with previous port-mortem analysis (Kordower et al 1995; Mendez et al 2005). It was then proven that survival of grafted neurons can reach beyond a decade after transplantation.

In both studies, nevertheless, there was an unexpected event, a few grafted cells displayed similar neurophatological changes as the ones detected in standard PD patients which were not shown in any previous post-mortem analysis. These grafted neurons had cytoplasmic inclusions of ubiquitinated alfa-synuclein which resembled Lewy bodies. The inclusions were positive for antibody recognizing alfa-synuclein phosphorylated at Ser129, similarly to Lewy bodies in regular PD. Another interesting finding by Li and colleagues was that in the patient who received the left graft 16 year before death and the right graft 4 years later, there were 80% and 40%, respectively, TH cells with detectable amounts of alfa-

synuclein, suggesting that the increase in alfa-synuclein aggregates are time and/or age dependent (Li et al. 2008).

Altogether, these results reveal that typical PD pathological features can be found in human embryonic grafts and that young age is not a protective factor. Also they suggest that "responsible mechanisms" are still present in advanced stages of disease; whether they are transmitted cell-to-cell or from extracellular environment to healthy cells it is not known yet. The exact impact these aggregates have on neuronal function is still unclear and previous studies have shown that grafted neural function can be maintained for at least 10 years after grafting (Piccini et al. 1999). These data further suggest that regarding the future of cell therapy, investigators must also be able to prevent disease spreading into the new grafted cells.

2.1.5. Ethical concerns and future perspectives

There are several ethical issues concerning the use of embryonic cell grafts in human patients. Despite the fact that tissue is derived from women who underwent abortion, there are other concerns regarding the number of fetuses required per patient due to their low dopaminergic neuron content as well as the purity level and reduced cell viability. The tissue preparation must also be prompt, as these cells are pos-mitotic and not able to expand or be kept in culture for more than a few days. For these reasons we must consider that the time window for neurosurgical procedure is very narrow. Due to inconsistent outcomes between grafted patients, low availability of donor cells and the existence of graft-induced dyskinesias one can presume that this method will never be a reliable treatment for PD patients Therefore, an alternative cell source is currently needed for true restorative treatment.

Tthere are no currently ongoing trials concerning the use of fetal neural tissue, nonetheless; there is still a great interest in grafting trials. Data suggest that better outcomes

can be obtained from younger patients in less advanced stages of disease that still respond to L-DOPA therapy. However, grafting younger patients that can still benefit from medical therapy is troublesome.

Another limiting factor was the modest neuronal survival observed after implantation procedure, which led to several attempts to enhance the pool of surviving dopaminergic neurons. Improvement of tissue collecting and grafting technique, calcium channel agonist (nimodipine, flunarizine) agents that counteract oxidative stress and its consequences (superoxide dismutase overexpression, lazaroids), caspase inhibitors and neurotrophic factors have shown to increase neuronal survival (Brundin et al. 2000a; Brundin et al. 2000b). Simultaneous transplantation in both SN *pars compacta* and striatum that could provide more extensive reinnervation was tested in both animals and humans with reported clinical improvement, tolerability and without graft induced dyskinesias (Mendez et al. 2002; Mendez et al. 2005).

Although restorative therapy has come a long way, there is still much to be done to allow cell therapy to be one of PD greatest allies.

2.1.6. Porcine neural xenotransplantation

Studies involving xenotransplantation of porcine embryonic tissue started in the mid 1980s, approximately at about the same time human embryonic tissue was starting to grab hold of interest. Porcine neural cells, staged at embryonic day 26-27, were held as a suitable alternative cell for several reasons, the resemblance between human and pig brain size and development, the physiological similarity between the two neuronal tissues, the feasibility of generating a large number of animals for research, easily to breed in controlled conditions and collect (Shumacher et al. 2000). The porcine tissue can also be genetically modified. Despite these reasons, other facts remain as potential threatening blockages in this field of

investigation, which are the existence of an immuno-mediated rejection process and the likely hazard of human infection with porcine endogenous retroviruses (PERVs).

The first studies were held on animal models and it soon became clear that xenografted tissue was rapidly rejected over a period from days to weeks and that the immune process was a mix of cellular and humoral host responses (Barker and Sayles 2006).

Apart from these uncertainties surrounding potential immune rejection or PERVs infection, a small-scale clinical trial was conducted by Shumacher and coworkers in 2000. The trial involved 12 PD patients with unilateral striatal grafts of porcine ventral midbrain tissue in the form of cell suspension that were collected at embryonic day 25-28. Half of the patients received cyclosporin immunossuppression and the other half received tissue treated with a monoclonal antibody directed against major histocompatibility complex class I.
One year later, the data obtained with PET scan did not show significant signal increase on the grafted side and motor scores were inconsistent between patients. It remained no doubts the grafts were well tolerated and that was no PERVs infection (Schumacher et al. 2000).

A second trial with porcine embryonic tissue grafted in PD patients was held, however, the results still remain unpublished. Eighteen PD patients were involved, 10 of them received embryonic porcine tissue and 8 of them sham surgery. The trial showed modest motor score improvement in both groups after 18 months, but complete written data must be published before any more considerations are made (Barker and Sayles 2006).

In the field of xenotransplantation, there is still a lot that can be done to assure the safety issues concerning immune reaction and retroviral infection. Genetically modified animals can be employed in order to decrease antigenicity and also suppress the risk of infection. Perhaps one day we will be able to see these unique cells no more as a threat and can start believing in their possible role in a future restorative therapy for PD patients.

2.2. The quest for the perfect cell – stem cell research

The quest for the perfect cell started years ago, as many ethical, safety and political concerns have held up the use of embryonic fetal cells, scientists sought for a new cell type, capable of unlimited self renewal and differentiation into multiple cell types or all cells of the body – the stem cell. However, in order to be able to optimize cell survival, integration and function, we must also understand CNS developmental biology and learn how a stem cell can turn into a neuron. In the following section we aim to briefly address the complex differentiation of dopaminergic neurons, the promising sources of stem cells and what has already been accomplished using these extraordinary new cells.

2.2.1. Development and differentiation of dopaminergic neurons

There are nine dopaminergic neuronal subtypes differently located in the mammalian brain, classified from A8-A16, all TH positive and able to synthesize and release DA. The A9 subtype from SN, as mentioned earlier, is the most affected in PD and their normal task is to innervate the putamen and caudate nucleus. So, there is a need to understand more about the A9 neuronal subtype, their specification, migration and maturation. Mesencephalic dopaminergic neurons, whose degeneration is a main feature of PD, are derived from precursors located in the ventral midline of the midbrain while other progenitors nearby give rise to motor or different interneuron subtypes (Hynes and Rosenthal 1999).

During embryonic CNS development, neuronal fate is controlled by local inductive cues that control gene expression in precursor cells and give rise to neuronal specification (Jessel et al. 2000).

There are two main signaling systems of the neural tube, the rostrocaudal (or antero-posterior) responsible for dividing the CNS in forebrain, midbrain, hindbrain and spinal cord and the dorsoventral system (figure 2), whose key role is to establish cell type diversity in the

above mentioned subdivisions (Jessel et al. 2000). For the development of mesencephalic ventral neurons these patterns of signaling are both necessary. The major molecule involved in dorsoventral signaling is sonic hedgehog (Shh) and the anteroposterior pattern results from a more intricated interaction of genes that form the isthmic organizer. Shh is a ventral morphogen secreted by floor plate cells, responsible for inducing a dopaminergic phenotype, while the isthmic organizer is involved on the rise of midbrain-hindbrain structures (Hynes et al. 1995; Placzek and Briscoe 2005). The isthmic organizer (figure 2) is located at the midbrain-hindbrain edging, its accurate location is controlled by the expression of two homeodomain transcription factors, the orthodenticle homologue 2 (Otx2) in the midbrain and gastrulation brain homeobox 2 (Gbx2) in the hindbrain (Arenas 2002).

Two other important molecules, wingless related 1 (Wnt1) and fibroblast growth factor 8 (FGF8), are expressed at the same location (Castelo-Branco et al. 2003; Liu and Joyner 2001). When Shh and FGF8 are mutually present, dopaminergic neurons are specified establishing a midbrain identity in the early neuronal development (figure 2). The Wnt family consists in secreted glicoproteins that control cell proliferation and fate decision, as in the case of ventral dopaminergic neurons, Wnt 1 and 5 have shown to increase the amount of rat dopaminergic neurons throughout different mechanisms while Wnt-3a promoted the proliferation of progenitor cells expressing the orphan nuclear receptor-related factor 1 (Nurr1) but without increasing the number of TH positive neurons (Castelo-Branco et al. 2003).

The neurons are generated in the dorsal segment of ventral mesencephalon from where they travel along the radial glial cells to reach the ventral component of the midbrain where they will form the VTA and SN (Kawano et al. 1995).

Dopaminergic neurons are generated from the midline of ventral midbrain initially occupied by Shh-expressing glial-like floor plate cells. Therefore, this neuronal creation must

be preceded by a switch of floor-plate cells into neuronal precursors, however, how such a conversion occurs is yet unknown (Placzek and Briscoe 2005; Andersson et al. 2006).

Other transcription factors that interfere with dopaminergic neuron determination have been identified. In the mouse, the expression of two Engrailed genes – En1 and En2 – at E8 is known to be mediated by FGF8 in the isthmic organizer. This En1 and En2 expression is essential as it induces continuous production of FGF8, allowing proper survival and neuronal development (Liu and Joyner 2001; Simon et al. 2001). Studies in mice with null mutations for either En1 or En2 have shown a paired action in neuronal dopaminergic survival by compensating for the loss of one another while double null mutated mice exhibited complete loss of TH positive midbrain neurons (Simon et al. 2001). Yet, little is known about how En1 and En2 are involved in specification and survival of dopaminergic neurons.

While the genes mentioned above are involved in the midbrain/hindbrain regional development, recent studies have identified genes required for neuronal subtype specification. Nurr 1, an orphan member of steroid/thyroid hormone receptor superfamily, is expressed at the ventral mesencephalon prior to the birth of dopaminergic neurons, at E 10.5. It was shown that Nurr 1 is expressed in developing dopamine neurons before the appearance of their characteristic phenotypic markers and that mice lacking Nurr1 failed to produce midbrain dopaminergic neurons showing TH immunoreactivity absence, displayed hypoactivity and died shortly after birth (Zetterstrom et al. 1997). These data suggest that Nurr 1 is decisive for midbrain dopaminergic differentiation. A second gene found to be important for final dopaminergic differentiation is the paired-like homeobox transcription factor-3 (Pitx3) which is expressed at E 11.5 in the mouse midbrain matching the appearance of mesencephalic dopaminergic neurons (Smidt et al. 1997). In both rodent and human brain Pitx3 is strictly expressed in mesencephalic dopaminergic neurons and its expression persists throughout adult life being severely impaired in PD patients and completely absent from 6-

hydroxydopamine (6-OHDA) lesioned rats (Smidt et al. 1997). Mice lacking Pitx3 expression have shown to develop dopaminergic neurons in lower numbers and different location in the SN (Smidt et al. 2004). Thus, it seems that Pitx3 is not required for inducing cell fate, but instead, is a key member for terminal differentiation and final location (figure 2).

Andersson and coworkers suggested that two other genes: LIM homeobox transcription factor 1 (Lmx1a) and the muscle segment homeobox transcription factor 1 (Msx1) function as midbrain dopaminergic neuron determinants. Both Lmx1a and Msx1 are expressed in the midbrain when Shh signaling is present. Lmx1a is expressed before Msx1 and first identified at E9, its expression is sufficient to induce dopaminergic differentiation from ventral midbrain neurons and continues in post-mitotic cells functioning as a specific activator of downstream genes, including Nurr 1. Altogether, the findings suggest that Lmx1a functions as a transcriptional activator. Msx1 is induced by Lmx1a and functions by suppressing alternative cell fates at the same time that restrains floor-plate characteristics. It induces panneural differentiation through the induction of proneural basic helix-loop-helix protein Ngn2 triggering glial-to-neural switch (Andersson et al. 2006).

Another group of transcription factors is forkhead/winged helix transcription factors Foxa1 and Foxa2. They act in the specification of midbrain dopaminergic neurons by regulating Nurr1 expression, En1 in non mature neurons and Ngn2 which regulates the extent of neurogenesis in dopaminergic precursors. They also regulate the expression of aromatic-L-amino acid decarboxilase and TH in mature neurons in late stages of differentiation (Ferri et al. 2007).

In this quest for the perfect cell, full knowledge about intrinsic and external factors that influence the generation of a midbrain identity must be gathered in order to achieve stem-cell derived dopaminergic neurons.

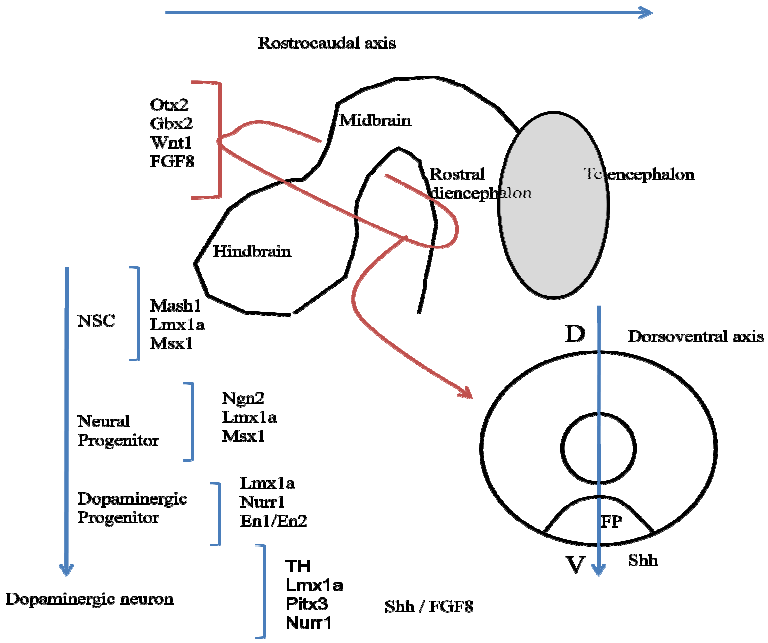

Figure 2: Schematic development of midbrain dopaminergic neurons. Legend: D- dorsal V-ventral.

2.2.2. Embryonic stem cells

Since human Embryonic stem cells (hESC) were first derived in 1998 by Thompson and colleagues they have been considered as a major potential source in the cell therapy field. Their ability to unlimited self-renewal both *in vivo* as *in vitro*, while maintaining the potential to differentiate into the three main embryonic layers makes them an attractive alternative source to obtain dopaminergic neurons (figure 3).

These cells represent a unique model that allows us to gain access to the early stages of human development. These pluripotent cell lines are isolated from the inner mass of the human blastocyst prior to implantation (E5) and display high levels of telomerase activity which makes them less susceptible to senescence (Thomson et al. 1998). Despite its interest there was a significant delay between the finding of mouse ESC (mESC) and hESC which was due to sub-optimal culture media.

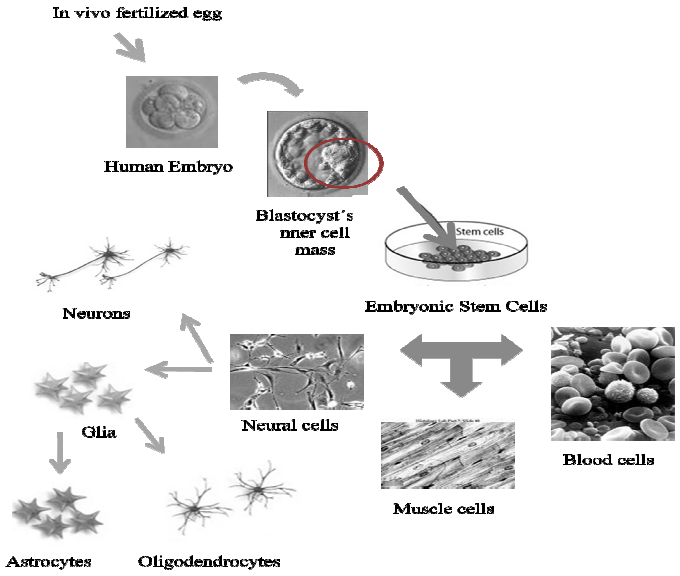

Figure 3: Differentiation of embryonic stem cells derived from the inner cell mass of the blastocyst.

Before we can turn hESC into a safe restorative method, we must be able to generate sufficient amounts of differentiated cells and transplant them safely, ensure their survival as well as the absence of tumor formation. The cells must form new synapses, reinnervate the striatum successfully and release DA.

Nowadays, the main hurdle to overcome is the possibility of tumor formation. There are several reports concerning transplantation of dopaminergic neurons derived from hESC into a PD rat model which resulted in teratoma formation (Brederlau et al. 2006; Roy et al. 2006; Sonntag et al. 2007) leading to scientific efforts on the way to develop means of avoiding it.

2.2.2.1. Embryonic stem cells differentiation

To obtain dopaminergic neurons from hESC they must be efficiently differentiated into the desired phenotype in both amount and "quality", meaning there is a need for the right amount of neurons without the interference from other undesirable cell types. Several protocols have been developed to obtain dopaminergic neurons from hESC. The first studies involved the use of mouseESC and non-human primate ESC (pESC), but soon after scientists

started to apply those same protocols to hESC. There are nowadays three main strategies for neuronal induction, the use of embryoid body-based protocols, stromal feeder-mediated or default neural differentiation protocols (table 3 for different TH positive yields obtained after neural induction from mESC, pESC and hESC through the use of different methods).

In the year 2000, Lee and colleagues described a five step technique for mESC induction which relied on the expression of the same genes usually expressed in CNS stem cells and neurons *in vivo* (Lee et al. 2000). It consisted of (1) expanding undifferentiated mESC with generation of Embryoid Bodies (EBs) in suspension cultures; (2) plating the cells onto an adherent culture surface; (3) selection of nestin-positive cells; (4) further culture in the presence of additional factors (SHH, FGF8) for induction of TH positive cells and (5) final differentiation by withdrawing bFGF and adding ascorbic acid (AA). These neurons could be depolarized and release DA representing functionally active mature neurons. After the success of differentiating mESC, Zhang and coworkers demonstrated that the same outcome could be obtained from hESC (Zhang et al. 2001). After the formation of EBs, this group added to the medium insulin, transferrin, progesterone, putrescine, sodium selenite and heparin in the presence of FGF-2. The use of immunofluorescence revealed expression of neural marker antigens such as nestin and Musashi-1 as well as the neuronal precursor protein polysialylated neuronal cell adhesion molecule (PSA-NCAM). After differentiation by removal of bFGF the precursors obtained were able to form all three major cell types *in vitro*, despite the small amount of TH positive cells achieved (Zhang et al. 2001).

More recently, Cho and coauthors reported a more efficient generation of DA neurons from hESC obtaining 60.2% TH positive neurons out of the total hESC derived-neurons (Cho et al. 2008). These new data regarding ESC threw away the idea that differences between species were behind the low numbers of DA neurons obtained and hESC reemerged as potential targets for regenerative medicine.

Stromal feeder mediated neuronal induction of mESC was first described by Kawasaki and coworkers in the year 2000. The possibility of inducing ESC into neuronal precursors after co-culture with stromal cells is a property known as stromal-derived inducing activity (SDIA). These cells are frequently derived from connective tissue such as skull bone from which the PA6 cell line derives (Kawasaki et al. 2000). Other stromal cells commonly used are MS5 or S2 and all stromal subtypes can be genetically altered to overexpress neuron inducing genes such as Wnt1 (Perrier et al. 2004). Perrier and coauthors used a three step protocol which involved (1) culture of hESC on mitotically inactivated mouse embryonic fibroblasts; (2) neural induction by co-culture with S2, MS5 and MS5 stably overexpressing Wnt1 stromal cells in the presence of certain factors such as Shh, FGF8, brain derived neurotrophic factor (BDNF), glial cell derived neurotrophic factor (GDNF), transforming growth factor type β3, dybutril cAMP and AA added at certain time points and (3) differentiation in the absence of Shh and FGF8. Another study used stromal PA6 cell line with similar efficiency (Brederlau et al. 2006; Zeng et al. 2004). The mechanism by which stromal cells promote neuronal induction is still unclear but it is thought that both secreted soluble factor and cell-to-cell contact play important roles (Kawasaki et al. 2000). An intriguing feature from PA6 cell line is that although they provide hESC neuronal induction they do not work in the same way with neural stem cells meaning this yet unknown mechanism is also specific for ESC (Roybon et al. 2005). A recent report regarding the use of co-culture with stromal cells demonstrated that treatment with fibroblast growth factor-20 (FGF-20) improved neuronal survival with a fivefold increase in the yield of domapminergic neurons partly due to reduced cell death (Correia et al. 2007). There are several other differentiation protocols for ESC, some involve the combination of both co-culture and suspension culture methods (Takagi et al. 2005), other feeder cell types such as Sertoli cells (Yue et al. 2006), telomerase-immortalized midbrain astrocytes (Roy et al. 2006) or even

human amniotic membrane matrix (Ueno et al. 2006). The genetic manipulation of ESC to overexpress certain transcription factors, such as Nurr1, is another method to obtain a dopaminergic fate (Chung et al. 2002; Kim et al. 2002).

2.2.2.2. Transplantation of dopaminergic neurons derived from ESC - outcomes

For the ultimate goal of developing clinical trials based on embryonic stem cell therapy first it must be demonstrated adequate *in vivo* cell survival and function as well as the absence of adverse effects or immune rejection. Some of the earliest encouraging results came from Barberi and coworkers that grafted mESC-derived neurons into the striatum of parkinsonian rats obtaining 80% reduction in amphetamine-induced rotation around 8 weeks after the procedure. Before this paper, Kim and colleagues working with mESC-derived dopaminergic neurons transplanted into parkinsonian rodents obtained functional recovery enhanced by Nurr1 overexpression in the ESC-derived cells (Kim et al. 2002). The same promising results have been harder to obtain with neurons derived from hESC. Ben-Hur and coauthors have shown survival of TH positive neurons derived from hESC in immunosuppressed rats but with unpretentious effects on drug-induced rotation behavior (Ben-Hur et al. 2004).

A later study employing hESC-derived neurons from co-culture with PA6 stromal cells also demonstrated graft survival in the striatum of immunossuppressed parkinsonian rats, but without motor improvement and with only small numbers of surviving TH positive neurons. A serious concern came from teratoma formation seen in those animals where transplants have been submitted to a short hESC differentiation *in vitro* (Brederlau et al. 2006). A recent work, showed generation of a high yield of dopaminergic, neurons reported significant behavioral improvement after transplantation into a PD rodent model with no tumor formation observed during a period of 12 weeks (Cho et al. 2008). One other paper

demonstrated significant and long lasting motor function recovery on parkinsonian rats with grafts obtained from hESC co-culture with telomerase-immortalized human fetal midbrain astrocytes in the presence of Shh and FGF8 (Roy et al. 2006). Despite the fact that a high number of TH positive neurons were found in the grafted rats 10 weeks after procedure the motor tasks suffered a rapid amelioration with complete recovery. Some other differences in terms of procedure can be noted, such as the method for creating the nigrostriatal lesion that was done by injecting 6-OHDA into the lateral ventricle instead of the brain parenchyma, which is the standard procedure in many of the previous works, but also the drug dosage employed to induce rotation was unusually high (Christophersen and Brundin 2007).

In the meanwhile currently face the need for standard protocols with precise determination of optimal lesion processes, graft size and insertion site, as well as improved interpretation of the motor behavioral tests to better understand and establish comparisons between studies.

Nowadays, important work is being done towards an increased survival of dopaminergic neurons derived from hESC.

Table 3 : Different protocols to differentiate ESC from mouse, non-human primate and human sources into dopaminergic neurons.

ESC source	Culture conditions	Soluble factors	Genetic manipulation	Protocol duration (d)	TH^+ neurons out of total	Graft survival	Reference
mESC	EBs formation	bFGF, FGF8, Shh, AA	/	24-37 d	7% out of 72% TuJ^+ cells (5%)	ND	Lee et al. 2000
mESC	Co-culture with PA6 cells	/	/	14 d	16% of total cells	4% of total grafted cells	Kawasaki et al. 2000
mESC	EBs formation	bBGF, FGF8, Shh	Nurr1 overexpression	ND	78% of total cells	3% of total grafted cells	Kim et al. 2002
mESC	Co-culture with MS5 cells	FGF8, Shh, AA	/	14 d	ND	10-20% of total grafted cells	Barberi et al. 2003
mESC	EBs formation	bFGF, FGF8, Shh, AA, BDNF	Pitx3 or Nurr1 overexpression	26-30 d	25% of TuJ^+ cells	3.4% of total grafted cells	Chung et al. 2005
mESC	Co-culture with PA6 cells	bFGF, FGF8, Shh, AA	Nurr1 overexpression	14 d	90% out of 62% TuJ+ cells (56%)	312 TH^+/mm^3	Kim et al. 2006
mESC	Co-culture with matrix layers of human amniotic membrane	/	/	13 d	26% out of total	ND	Ueno et al. 2006

mESC	/	bFGF, FGF8, Shh	Lmx1a overexpression	ND	TH+ cells in 87% colonies	ND	Andersson et al. 2006
mESC	EBs formation	bFGF, Shh, FGF8,	/	26-30	11% out of total	ND	Rodríguez-Gómes et al. 2007
pESC	Co-culture with PA6 cells	bFGF, BDNF, neurotrophin 3	/	28 d	2.9-12.8%	2130 TH^+ cells/graft	Takagi at al. 2005
pESC	Co-culture with sertoli cells	/	/	21 d	24.6% of total cells	ND	Yue et al. 2006
hESC	EBs formation	FGF2, insulin, transferrin, heparin, progesterone, putrescine	/	> 14 d	ND	ND	Zhang et al. 2001
hESC	Co-culture with PA6 cells	/	/	21 d	TH+ cells in 60% colonies	9 TH^+ cells/section	Zeng et al. 2004
hESC	EBs formation	bFGF, FGF8, Shh	/	ND	67% TuJ+ cells (40% of total)	A few TH^+ cells	Schultz et al. 2004
hESC	Spheres formation	MEF, Noggin, bFGF, EGF	/	> 23 d	0.56% of total cells (29% TuJ^+ cells)	389 TH cells (0.18% of grafted cells	Ben-Hur et al. 2004
hESC	Co-culture with MS5 cells	Shh, FGF-8, BDNF, GDNF, TGF-β3, dbcAMP, AA	/	> 50 d	19-39.5% of total cells (64% -79% TH^+ out of 30%-50% TuJ^+ cells)	ND	Perrier et al. 2004
hESC	EBs formation	Transferrin, selenium, fibronectin, laminin	/	39 d	20% of total cells	ND	Park et al. 2004
hESC	EBs formation	bFGF, FGF-8, Shh, BDNF, GDNF, AA	/	35-42 d	31.8% of total cells	ND	Yan et al. 2005
hESC	Co-culture with human amniotic membrane	/	/	40-42 d	12.4% of total cells (31% TH+ out of 40% TuJ^+ cells)	ND	Ueno et al. 2006
hESC	EBs formation with human fetal midbrain astrocytes	bFGF, BDNF, GDNF	/	24-36 d	75% TuJ+ cells	27 000 TH^+ neurons/mm^3	Roy et al. 2006
hESC	Co-culture with PA6 cells	/	/	16-23 d	7.4% of total cells	10-50 TH^+ cells/graft	Brederlau et al. 2006
hESC	EBs formation	bFGF, dbcAM	/	21-42 d	56-81% of all colonies	ND	Iacovitti et al. 2007
hESC	Co-culture with PA6 cells	bFGF, FGF-20	/	21 d	85% of TuJ^+ cells	ND	Correia at al. 2007
hESC	Co-culture with MS5 cells	Shh, FGF-8, BDNF, GDNF, TGF-β3, AA	Wnt1 overexpression	42-49 d	23.6% of total cells	160 TH^+ cells/graft	Sonntag et al. 2007
hESC	EBs formation	bFGF, FGF-8, Shh, BDNF, TGF-β3, GDNF, AA, Wnt3a	/	52 d	43% TuJ^+ cells	1273 TH^+ cells/graft	Yang et al. 2008
hESC	EBs formation	bFGF	/	> 40 d	60.2% of total cells (86% TH^+ out of 77% TuJ^+ cells)	2.7% of the surviving h ESC derived cells	Cho et al. 2008

Legend: d – days; ND – not determined; TuJ1 is an antibody directed against the neuron-specific β-III tubulin which bounds cells with a clear neuronal morphology (TuJ^+).

Nowadays, important work is being done towards an increased survival of dopaminergic neurons derived from hESC.

Taken as a whole, the several studies have established the proof-of-concept that it is possible to derive dopaminergic neurons from ESC and investigators are now on the road for a large-scale generation of pure and functional dopaminergic neurons, envisaging its possible future clinical application.

2.2.3. Neural stem cells

The first crossroad in the attempt to create a stem cell based regenerative therapy is to be able to find a renewable source of dopaminergic neurons with midbrain identity. This new and unlimited cell source could minimize methodological and ethical concerns associated with the usage of fetal tissue or ESC. Neural Stem Cells (NSC), neural progenitors or precursors exist in both developing and adult nervous system of all mammalian organisms and can comprise such a cell source (Gage 2000). NSC are capable of differentiating into all neural lineage cells *in vivo* and *in vitro*, as well as to give rise to other multipotent counterparts and can be isolated from two different neurogenic regions, the subgranular zone in the hippocampus and the subventricular zone near the ventricles (Doetch et al. 1999; Gage 2000; Temple 2001).

NSC can be grown in culture as free floating aggregates called neurospheres or be expanded as monolayers (Conti et al. 2005; Ostendfeld et al. 2002).

There are several works regarding dopaminergic differentiation from NSC due to genetic manipulation and researchers have shown that transcription factor Nurr1 plays an important role in regulating dopaminergic identity as it can directly activate TH promoter as well as other genes involved in DA uptake and storage. (Kim et al. 2003).

In a recent study where Nurr1 overexpression was combined with Ngn2, TH-expressing neurons were generated; nonetheless, other mesencephalic markers were found to be expressed. Ngn2 by itself increased neuronal differentiation without promoting the desired dopaminergic phenothype but, in co-transduction with Nurr1, synergistic effects were shown and up to 4% of the transduced cells became TH positive. Hovewer, there was no significant numerical difference between TH positive neurons with co-transduction of Nurr1 alone or in combination with Ngn2, the major difference resides in the fact that dual delivery TH cells displayed longer and more elaborated projections. Due to this experiment, the role of Ngn2 is

now clearer at the level of neuronal maturation (Andersson et al. 2007). Kim and coauthors used dual transgene delivery of either Nurr1-Ngn2 or Nurr1-Mash1 on neural progenitor cells from ventral midbrain or the striatum. After dual transduction with Nurr1-Ngn2 or Nurr1-Mash1 on striatal neurons few numbers of dopaminergic neurons were obtained; when applied the same method was applied to ventral mesencephalic progenitors there were even fewer TH positive cells out of the total. These data, despite of complex interpretation, suggest that NSC from different origins, even though cultured in the same conditions *in vitro*, give rise to the same controlled amount of neurons as if they were functioning *in vivo* (Kim et al. 2006). Both studies demonstrated that NSC can be differentiated into TH positive neurons if added with certain midbrain determinants. A later study reported a more efficient NSC transduction following Nurr1 overexpression in combination with Mash1 and evidences the hypothesis that, in contrast to Mash1, Ngn2 expression may inhibit dopaminergic differentiation through repression of Nurr1-induced dopaminergic differentiation. It was hypothesized that even though Mash1 and Nurr1 appear to work together, they seem to have different roles, whereas Mash1 induces neural differentiation and maturation, Nurr1 mediates the acquisition of a dopaminergic fate. The effect mediated by Ngn2 was thought to be related with cell cycle exit and is reinforced by the similarity that exists in its late manifestation and apparent inhibitory action in the ventral midbrain during development. (Park et al. 2006).

When mESC are under effect of Lmx1a overexpression, Shh and a nestin enhancer a high percentage of dopaminergic neurons bearing midbrain identity can be obtained; conversely, Lmx1a has not reported as playing a part in NSC dopaminergic differentiation (Andersson et al. 2006);

In a recent study, Roybon and coworkers investigated the potential of Lmx1a, Msx1, Ngn2 and Pitx3 in rat derived neurospheres from embryonic day 14.5. Remarkably, none of those genes expressed by themselves or in dual combinations was enough to enhance

dopaminergic neural differentiation in rat derived NSC, which could indicate that at E14.5 progenitor cells have lost their potential to become dopaminergic neurons (Roybon et al. 2008). When Lmx1a and Nurr1 were co-expressed in NSC there was still no dopaminergic differentiation enhancement compared to Nurr1 alone (Roybon et al. 2008). Altogether, it seems that younger cells may become better sources for obtaining a dopaminergic phenotype from NSC and that multiple sequential expression of several transcription and/or soluble factors, which may include some yet not known to us, might be needed to obtain larger numbers of desired cell type. One other recent report from Parish and colleagues presented a new method for generating a larger quantity of dopaminergic neurons. They expanded mouse NSC with FGF2, differentiated with Shh and FGF8 and then transfected the cells with Wnt5a, a ventral midbrain soluble factor, obtaining a significant increase in the yield of TH positive neurons (Parish et al. 2008).

An emerging theme in developmental neurobiology is the existence of a combination of different factors orquestrated in some yet unknown and crucial manner for the development and differentiation of all neural lineages subtypes.Therefore, before NSC can play a role in transplantation procedures development of differentiating strategies is needed, in order to make possible the their brain implantation.

2.2.4. Induced pluripotent stem cells

The cloning of Dolly established that adult cell nuclei can be reprogrammed into a previous undifferentiated stage under the action of molecules present in the oocyte (Wilmut et al. 1997). These findings led investigators in the search for the identity of such molecules so similar reprogramming could be done without nuclear transfer. In 2001, Tada and coauthors reproduced the same nuclear reprogramming of somatic cells using a fusion method of mature thymocytes with hESC which proved that the same transforming molecules existed on both

non-fertilized oocyte and ESC. With this major breakthrough, it became possible to dream about generating a patient-specific pluripotent cell line without the use of nuclear transfer.

Byrne and colleagues recently described somatic cell nuclear transfer (SCNT) in nonhuman primate material using adult skin fibroblasts and oocytes. They obtained blastocysts with subsequent isolation of two primate ESC lines (Byrne et al. 2007). Although it was proven that SCNT is possible in primate material, ethical and practical issues attest that other approaches may be easier to apply to human material, such as uncovering the nature of such reprogramming molecules. There are two major groups of investigators leading this research field, the Yamanaka group and Yu and colleagues.

The Yamanaka group employed mouse somatic cells and was able to find 4 factors sufficient for their reprogramming into undifferentiated pluripotent stem cells (termed induced pluripotent stem cells) which are very similar to mESC: Oct4; Sox2; c-Myc and Klf4 (Takahashi and Yamanaka 2006). Other investigators verified and replicated the same results in mouse cells (Okita et al. 2007; Maherali et al. 2007) and rapidly there was a move on to human material (Takahashi et al. 2007; Park et al. 2008).

The other group, Yu and coworkers, developed their research on human material providing evidence that hESC have the capacity to reprogram differentiated hematopoietic cell nuclei through cell-cell fusion (Yu et al. 2006). In 2007 the same group identified 4 factors sufficient for somatic nuclei reprogramming into a pluripotent stage: Oct4; Sox2; Nanog and Lin28. Oct4 and Sox2 were presented as essential factors for the process while the remaining molecules appeared to have interest in increasing process efficiency. Reprogram by Oct4, Sox2, Nanog and Lin28 was not reported for mouse cells (Yu et al. 2007).

Although mouse induced pluripotent stem cells (iPS) cells and mESC display high similarity regarding their morphology and proliferation they are also alike in terms of tumor formation. Is was observed that iPS cells can function as germline competent cells but tumor

formation in chimeric mice was high, probably due to c-Myc transgene reactivation in the somatic cells originated fom iPS cells (Okita et al. 2007; Maherali et al. 2007). Recently, a modified protocol allowed generation of iPS cells without c-Myc expression, even though with a lower efficiency in cell generation, demonstrated absence of tumor formation (Nakagawa et al. 2007).

Human iPS cells fulfill the criteria proposed by Thomson and coauthors for embryonic stem cells with the exception that they are not derived from embryos. These iPS cells are extremely similar to hESC in terms of cell surface marker expression, karyotype, ability to differentiate into all the three primary germ layers *in vitro* as well as regarding teratoma formation. It was shown they can be produced either by expressing Oct4, Sox2, c-Myc and Klf4 or Oct4, Sox2, Nanog and Lin28.

There is innumerous research ongoing and extensive literature showing Oct4, Sox2 and Nanog are the main pluripotency regulators, but it is still unclear how they are able to manage it. From previous publications, the POU transcritption factor Oct4, expressed in early mouse embryogenesis, is fundamental for ESC derivation and pluripotency maintenance (Pesce et al. 1998). Similar to Oct4, Sox2 also plays a key role in ESC self-renewal and pluripotency maintenance and its expression must be kept within a critical range. Nanog, as Oct4, is considered a core transcription factor with rapid decrease as ESC differentiate, it is found in mammalian pluripotent and developing germ cells and its deletion causes early embryonic death (Mitsui et al. 2003). Its expression is now known not to be absolutely necessary for ESC pluripotency, as ESC maintain their self-renewal ability in its permanent absence (Chambers et al. 2007). The three factors above mentioned are held as key regulators of pluripotency as they are thought to be able to trigger or suppress many other genes; however, little is known about their interaction. The same uncertainty gathers around the roles of c-Myc, Klf4 and Lin28 in pluripotency maintenance and regulation.

Nowadays it is important that iPS cell research does not replace ESC investigation as they can complement each other and together they may have a tremendous potential to achieve the must expected clinical benefit in the beginning of a truly regenerative medicine (figure 4).

2.2.5. Growth factor delivery

Many growth factors have been evaluated as potential neurotrophic agents, namely epidermal growth factor (EGF), BDNF, conserved dopamine neurotrophic factor (CDNF), ciliary neurothophic factor (CNTF) and the glial cell line derived neurotrophic factor subfamily which contains GDNF, nerturin (NTN), persephin and artemin/neuroblastin amongst others. In this following section we will focus mainly on GDNF as it is the most studied growth factor in terms of pre-clinical and clinical accomplishments in PD.

GDNF is known for its neurotrophic activity displayed in dopaminergic neurons both *in vivo* as *in vitro* when retrogadely transported from the striatum to the nigra (Sauer et al. 1995).

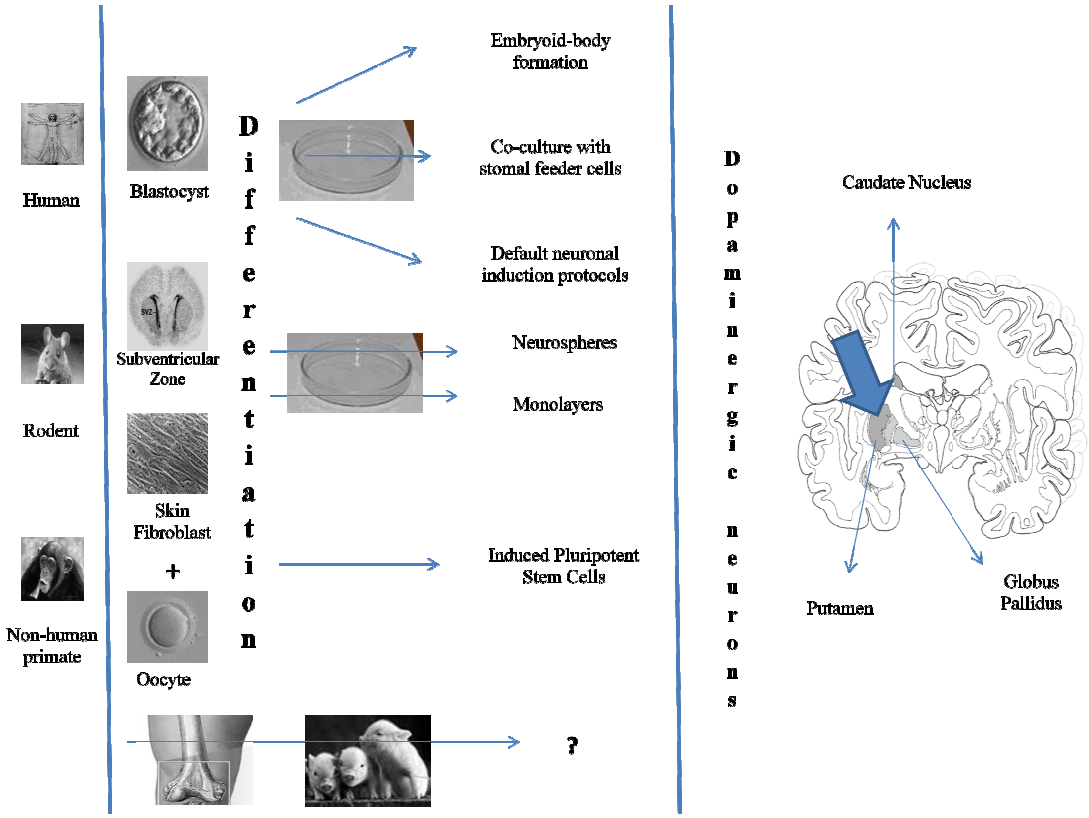

Figure 4: Stem cell sources and differentiation.

GDNF protects nigral dopaminergic neurons in both non-human primate and rodent lesion models of PD, which led to its application on clinical trials (Alexi et al. 2000). Besides the cytoprotective effect that GDNF exerts on dopaminergic neurons, it also increases the excitability and promotes axonal sprouting (Yang et al. 2001; Rosenblad et al. 2000). For GDNF to become part of the therapeutic arsenal safe and reliable delivering techniques have been developed over the years.

Nowadays, direct brain delivery is held as main delivery option and several strategies are being investigated such as direct stereotaxic injection, delivery by minipump, in microspheres and transduction with recombinant virus. There was one attempt to overcome brain delivery by means of generating a fusion protein Tat-GDNF to allow GDNF to cross the blood-brain-barrier but the results were disappointing (Dietz et al. 2006).

Several intracranial administration techniques have been tried over the years such as intraventricular, intrastriatal and intranigral. When implanting an intracerebroventricular catheter to administer GDNF, Nutt and colleagues observed that there was no symptomatic relief in PD patients and assumed it was probably due to the inability of GDNF to reach the target – putament and nigra (Nutt et al. 2003). In animal models, GDNF delivery into the lateral ventricle or striatal parenchyma was proven effective. When GDNF is delivered intranigrally at the site of toxin damage (6-OHDA) neuroprotection is observed in a time dependent manner (Kearns et al. 1997). After proven its efficiency and safeness, clinical studies started with a phase 1 clinical trial in which GDNF was directly delivered into the putamen of five PD patients. After one year there were no significant side effects reported and some clinical improvement was observed on motor scores. Medication induced dyskinesias were reduced by 64% (Gill et al. 2003). Nevertheless, there is some degree of uncertainty of how far will GDNF diffuse away from the catheter tip. Moreover, it remains an open possibility that the rostral portion of the putamen continues to degenerate if GDNF is unable to reach it. Besides the risks of catheter implantation, site infection, limited diffusion and the need for infusion system maintenance, there are also advantages regarding the optimal dose control or its proven value on improving the quality of life of PD patients. GDNF-releasing spheres emerged as a viable alternative to GDNF infusion. In one study biodegradable drug-releasing microspheres stereotaxically implanted into the brain of parkinsonian rats were well tolerated, induced sprouting and preservation of dopaminergic fibers in the striatum with functional improvement on motor behavior tasks (Jollivet et al. 2004). Microspheres have the advantage to allow *in vivo* sustained release of GDNF (which is preferable to single high dose) with a lower risk of side effects and can also be implanted into several sites compensating for its low diffusion rate and non-constant drug release. Microspheres have already been validated for brain tumor treatment but still require technological optimization to

embrace larger volumes of brain tissue without detrimental effects on neighboring cells before clinical trials on PD patients become eligible.

Due to the long and degenerating course of PD, an efficient mean of providing constant and local GDNF or GDNF production could offer patients many advantages over the single or repeated administrations systems, pumps or microspheres which eventually will need to be refilled or reinjected, respectively. The alternative GDNF source came in form of a virally mediated expression via adenovirus (Ad), Adeno-associated virus (AAV) or Lentivirus (LV) granting gene transfer into the dopaminergic cells. Recombinant Ad encoding GDNF delivered into rat striatum after unilateral 6-OHDA lesion offered neuronal protection and motor function recovery (Bilang-Bleuel et al. 1997). For random clinical use of viral vectors there are some parameters needed to be ensured, such as maximal safety, minor toxicity, genetic stability and absence of immunogenicity. Ad have been widely used for *in vivo* gene delivery for its ability to be transduced in both dividing and non-dividing cells with high expression effectiveness, but on the other hand, its capacity for triggering an immune response shortens the probability of ever reaching widespread clinical use. For that reason, recombinant AAV were created and their lack of apparent neurotoxicity, long term expression and absence of immune reaction are some of their most interesting features. GDNF delivery through AAV vector has been tested in animal models of PD with the same degree of success of its predecessor Ad (Kirik et al. 2000b). Kordower and colleagues injected LV-GDNF one week after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in non-human primates rescuing nigrostriatal degeneration and recovering its function (Kordower et al. 2000). Other works were able to validate lentiviral vectors as a GDNF delivery system (Brizard et al. 2006). LV vectors are HIV-derived and provide a broad and sustained transduction with lack of immune response in both rodents and non-human primates. Nevertheless, in some reports LV did not enhance the pool of dopaminergic neurons higher than AAV and bared the cost of

possible mutagenesis. By using different viral vectors, with their advantages and drawbacks, several works provided solid evidence that GDNF gene deliver is efficient in protecting the nigrostriatal system, especially when administered before the degeneration process.

To overcome the need for viral vector direct brain injection alternative gene therapy methods were created to achieve cellular transduction *in vitro* prior to transplantation. The development of this *ex vivo* gene therapy involving engineered GDNF as a transgene was reported as a potential successful method to treat PD patients. In one study, bone marrow stromal cells expressing GDNF were intravenously transplantated into MPTP-lesioned mice. Motor behavioral improvement was reported along with the expression of GDNF (Park et al. 2001). Åkerud and coauthors used a similar approach by producing GDNF-secreting NSC whose engraftment in the striatum of 6-OHDA lesioned mice prevented dopaminergic neuronal death and reduced behavioral impairment in experimental animals (Åkerud et al. 2001). Genetically engineered GDNF-expressing astrocytes also offered marked protection of nigral dopaminergic neurons and partial protection of striatal dopaminergic fibers when transplanted into the nigra of a parkinsonian mouse model with 6-OHDA lesion, leading to favorable motor effects on mice behavior (Cunningham et al. 2002). Nonetheless, these engineered cells may suffer rejection due to immune host response. In order to surmount this problem, protection capsules were developed to contain the modified cells. Engineered baby hamster kidney cells that held the ability to produce GDNF, encapsulated in a polymer fiber, resulted in nigral dopaminergic neuronal protection with subsequent motor improvement when grafted closely to SN in a PD rat model (Tseng et al. 1997).

It is yet unknown which GDNF delivery vehicle is the most effective and safe, whether is preferable a short or long term delivery method, how long the beneficial effects after treatment is withdrawn last and how can we sustain these favorable outcomes without inducting detrimental side effects. There are several many other questions related to gene

dosing, the possibility of insertional mutagenesis and other immune related issues. These, among others, are some of the questions investigators are now trying to answer. However, despite the uncertainty surrounding growth factor delivery, the encouraging results held at this point stimulated the beginnig of clinical trials. After an initial negative clinical trial with unfavorable side effects, followed by two other subsequent open label trials withholding more optimistic results, a double-blind placebo-controlled design with direct infusion of GDNF at a lower dose was held, although showing as no improvement of motor symptoms in PD patients (Nutt et al. 2003; Gill et al. 2003; Slevin et al. 2005; Lang et al. 2006). The lack of clinical efficacy and safety issues brought up from parallel studies regarding GDNF delivery, despite controversial and emotional debate, led to withdrawal of GDNF therapy from clinical tests.

Other clinical approach now on a phase II trial involves NTN delivery into the striatum of PD patients via AAV delivery with already successful results regarding safety, tolerability and potential efficacy (Marks et al. 2008). Despite all that was said, growth factor delivery survived its major setbacks still being a flaming field of cell therapy and maybe, in a nearby future, this will become a true clinical option.

3. Huntington´s Disease

Huntington´s Disease (HD) had its first complete description in 1872 by George Huntington at *The Medical and Surgical Reporter* with a detailed description of a progressive movement disorder associated with neuropsychiatric and cognitive impairment. HD is an uncommon hereditary autossomal dominant disorder with complete penetrance caused by expanded polyglutamine repeats at the N-terminal of huntingtin protein (The Huntington´s Disease Collaborative Research Group 1993). Nowadays, HD is the most studied genetic movement pathology. Its estimated prevalence is of 5-10 per 100.000, varying accordingly to

the geographical area (Bradley et al. 2008). Onset typically occurs at middle age, between 30-55 years, but can also arise less commonly in juveniles and in old age (Bradley et al. 2008). Due to its relentless progressive course, the outcome is unvaryingly fatal, within approximately 15-20 years of onset (Bradley et al. 2008).

The clinical symptoms of HD comprehend involuntary movement disorders such as choreiform and sometimes athetotic limb or oro-facial movements, reduced coordination, motor impersistence, bradykinesia and gait disturbance. The uncontrollable movements or "chorea" once thought as the major hallmark of the disease are now understood as a part of HD´s behavioral profile. There are also psychiatric symptoms that may appear before the beginning of motor disturbances, such as affective disorders – depression, mania, hypomania - or violent behavior. Besides motor and psychiatric disability other important feature is the cognitive impairment with appearance of dementia and executive dysfunction over the years, revealing an intellectual decline. The course of the disease is both progressive and unremitting leading to an inexorably death (Bradley et al. 2008).

Although identified for more than a decade, the exact function of the huntingtin protein is not yet fully understood due to complex cellular interactions and underlying pathological mechanisms, hence the answer to why this cellular destruction is so selective and sustained is currently unclear. Until we know how this mutated protein provokes neuronal death, there is no way to prevent, slow the course or even to stop the progressive disability.

Despite all uncertainty surrounding the degenerative process, neuropathological studies have provided solid evidence of its existence and extension outward from the striatum. It was found that expanded and unstable "CAG repeats" within the huntingtin gene on the short arm of human chromosome 4 lead to progressive degeneration of basal ganglia, cerebral cortex, brainstem, spinal cord, thalamus and hypothalamus. Neuropathological findings show

progressive loss of small to medium spiny GABAergic projection neurons within the striatum (caudate nucleus and putamen) as well as degeneration of cortical and hipoccampal neurons, with progressive cell loss, gliosis and atrophy (Bradley et al. 2008). In the advanced disease cell loss and atrophy also involve widespread areas of the forebrain (figure 5).

After the discovery of the HD´s mutation in 1993 and its mutated protein huntingtin, a preclinical test has been available for clinical practice as well as the possibility for pre-natal screening with all the complex ethical issues that the awareness of the presence of an inheritable disease entails. (The Huntington´s Disease Collaborative Research Group 1993).

Once there is still no cure available at the present or means of halting the progressive disability, symptomatic relief is the primary goal to achieve in HD patients as well as genetic counseling and palliative care. There is some therapeutic potential in managing the movement disorder with low dose dopamine receptor antagonists such as classical or atypical neuroleptics and anti-dopaminergic drugs. The psychiatric symptoms appear to benefit from selective serotonin reuptake inhibitors. As more we learn about the toxicity mechanisms of mutant huntingtin, additional and novel strategies will sure become available. In the meantime, investigation is turning towards disease modifying and restorative therapies.

The latter has the ultimate goal of promoting brain cells self-repair through supported neurogenesis while protecting vulnerable or dying nervous cells. As in PD, cell therapy may have the power to bring HD patients a better and brighter future.

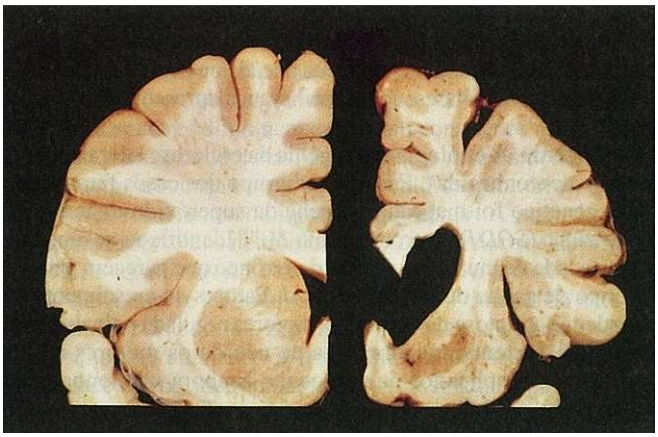

Figure 5: Comparison between cerebral cortex from a HD patient with degeneration of the striatum and global atrophy (on the right side) and a non-HD patient (on the left side). From de Girolami et al. 1999;

3.1. The beginning of cell therapy in HD

Cellular therapy has been a blossoming field of science with intensive clinical research and constant debate over the last few decades. Investigators have headed their main attention towards cell replacement therapy in neurodegenerative disorders. This particular field of science has been guided by pre-clinical studies in animal models with the ambition to achieve results that may lead to human clinical trials. After reaching considerable success concerning PD, and moving towards new cell sources, HD has been at the forefront of interest, not so much for its complex and challenging task of basal ganglia circuit repair but for the urgent need to be able to offer immediate and long term answers to HD patients. In order to cell therapy succeeds in HD, grafted tissue must survive transplantation, differentiate into GABAergic striatal cells and establish connections within host brain in a physiological manner.

We will first describe the studies concerning the use of animal models that provided proof of principle for the beginning of clinical trials with fetal tissue and offered HD patients hope in a future healing treatment.

3.2. Animal models for the study of HD

In a similar way to PD, the development of reliable pre-clinical studies rely firstly on developping an accurate transplantation technique and afterwards a lesion method that mimics HD symptoms in experimental models.

Since HD genetic mutation was identified as an unstable expansion of trinucleotide CAG repeats, researchers drove their efforts to generate transgenic animal models to enable the study of molecular, pathophysiological and cellular mechanisms underlying the disease, in addition to test potential therapeutic approaches in an attempt to halt its progression.

Studies in the mid 1970s started by using cellular excitotoxins to replicate the human malady and one of the initial choices was kainic acid administered directly into the striatum where it induced focal striatal cell loss. However, this toxin was found to be epileptogenic and was soon replaced first with ibotenic acid and afterwards with quinolinic acid due to their toxicity´s profile resemblance to the striatal cell loss found in HD (Schwarcz et al. 1979). These excitotoxic amino acids act not only in the motor function but also in the cognitive sphere of HD´s animal models and spare the cortical pathways to and from the striatum. Quinolinic acid became the toxin of choice due to its selectivity on neuronal loss within the striatum close resembling HD´s degenerating process (Döbrössy et al. 2009).However, these toxins could not mimic the slow and progressive feature of the human illness. Thus a second type of lesion model was used by peripheral delivery of metabolic toxins, such as 3-nitropropioinic acid or malonate, which target more accurately striatal neurons. These molecules disrupt mitochondrial respiratory chain leading to neuronal loss with a high pattern of similarity to HD metabolic defects, providing neuropathological validity. Nonetheless, the excitotoxins provide more convenient and reproducible lesions then metabolic toxins as the latter require slow and chronic titration of drug dose and display interindividual variability (Palfi et al. 1996). After finding the genetic mutation, several researchers embraced the

production of genetically modified animal models using rodents as a base and giving rise to several lines. One of the first accomplished models involved the introduction of an exon 1 fragment of the human gene with expanded CAG repeats, but several others followed (Mangiarini et al. 1996). From all the animal lines, the R6/2 line has been the most studied until now and its natural course of malady resembles the human disease with its main features of cognitive and motor impairment. The R6/2 model exhibit disease onset symptoms between 9-11 weeks, displaying a complex motor behavior involving tremor, chorea, ataxia, with progressive decrease in body weight, death usually occurs between 10-13 weeks (Mangiarini et al. 1996). Subsequent studies on motor and cognitive behavior as well as neuropathological findings from R6/2 mice show that these animals display several features resembling HD first stages (Carter et al. 1999). Nevertheless, the rapid disease progression of transgenic mice leaves insufficient time for observing progressive brain atrophy as well as for obtaining a detailed analysis on motor and cognitive behavior. Therefore, other transgenic models were produced bearing different human gene fragment insertion or full-length mutant huntingtin or even mouse gene with CAG repeats, among others. Each new model reveals different settings from HD cellular pathology and behavior profile (reviewed by Menalled et al. 2002). Another interesting mouse line is HD94, which contains a 94 CAG repeat terminal fragment of huntingtin gene under controlled expression. This animal model exhibits progressive motor impairment, cellular inclusions and other neuropathological features that strictly resembles HD. When gene expression is switched off, cellular inclusions retract and motor behavior is ameliorated (Yamamoto et al. 2000). The same paper provides further evidence on huntingtin´s toxicity profile, revealing the need for continuous gene expression to sustain disease symptoms and inclusions, thus helping to uncover uncover the possibility of a reversible HD pathology.

3.3. Striatal repair – what we have learned from animal models

There are few reports on mouse embryonic neural tissue grafting in excitotoxic animal models of HD, however there is still sufficient amount of data that demonstrate cell survival and function with alleviation of behavioral features after striatal transplantation as a result of broad mutual connections with the host brain. Intrastriatal grafts receive afferent dopaminergic inputs from the nigra as well as from the cortex and thalamus and send their efferent outputs into the host brain, in great extent to the globus pallidus and in lesser extent to the entopeduncular nucleus. The results suggest that the connections mentioned above are responsible for functional integration of the grafts and symptom amelioration (Nakao et al. 1999). Isacson and coworkers in 1986 transplanted fetal striatal cell suspension either into the striatum or globus pallidus in rats bilaterally lesioned with iboteinic acid. They observed amelioration of learning ability and motor behavior. Better results were obtained after striatal grafting by the striatum grafts compared to the group who received transplants into the globus pallidus. Nonetheless, the latter showed similar significant improvements to the control group. These results demonstrate that functional recovery and neuronal replacement is possible in HD even after destruction of an important telencephalic structure (Isacson et al. 1986). Other experiments with excitotoxic lesions describe motor and cognitive improvements, being the last of great importance due to its complexity and need for a viable cortical-subcortical pathway net integrating the striatum (Dunnet et al. 1995). Nakao and colleagues investigated the effect of mouse embryonic grafts derived from lateral and medial ganglionic eminence in the globus pallidus activity in rats with quinolinic acid-induced striatal lesions. They found the transplants derived from the lateral ganglionic eminence, but not from the medial ganglionic eminence, were able to repair the striatopallidal pathway, attenuating altered motor behavior (Nakao et al. 1999). A recent work by Dunnet and coworkers assessed the amelioration of cognitive impairment in rats following quinolinic acid bilateral striatal lesion

and mouse embryonic tissue graft. They reported partial though considerable alleviation of cognitive deficits in an operant delayed alternation learning task and, since the latter is dependent on the integrity of the corticostriatal pathway, their results are in agreement with the hypothesis that embryonic grafts can restore corticostriatal circuits (Dunnet et al. 2006).

Despite the existence of few reports on this particular area, there is still considerable clinical, electrophysiological and neurochemical evidence indicating the feasibility of striatal grafting in HD animal models with functional benefits and repair of damaged neuronal circuits. Striatal cell grafting was largely applied after the use of excitotoxic lesion models, thus there are few studies describing cell transplantation in the transgenic mice model which is explained by the fact that few animal models show striatal cell death. The toxic lesions are restricted to a particular focus and despite reproducing certain features of HD, fail to replicate the true degenerative process of human disease. Another problem concerning toxin studies is the widespread pathology involving cortical, neostriatal and other basal ganglia areas in HD, and the uncertainty of their underlying symptoms, which may not be entirely alleviated by cell transplantation. The use of transgenic mice has provided relevant information regarding whether and how the disease influences graft survival and function. Dunnet and coauthors grafted dissociated cell suspensions prepared with striatal tissue from normal mouse embryos in the transgenic R6/2 mouse line. They observed good graft survival and a similar internal organization, as described in the excitotoxic model studies; however, despite the apparent integration, the behavioral impact of the grafts was modest and devoid of clinical noteworthy benefits (Dunnet et al. 1998). In another report, the transgenic R6/1 mice line was submitted to cortical grafts in the anterior cingulated cortex, revealing the existence of a specific delay of deficit onset; conversely, there was no significant postponement in the development of other important motor behavior features in the grafted rodents (van Dellen et al. 2001). In the same experimental work a group of wild-type animals that underwent anterior cingulated

cortex resection were found to develop motor impairment closely resembling HD mice, revealing not only the importance og this brain region in this pathology but also the need for therapeutic measures to reach beyond the striatum (van Dellen et al 2001).

Both animal models have benefits and disadvantages. The excitotoxic lesion model does not include the cortical pathology developed in the later HD stages, but mimics the motor behavior and the first phases of the disease at the time striatal degeneration begins to emerge. On the other hand, transgenic mice models are able to show more widespread pathology even though grafting benefits are yet not fully established. Notwithstanding the results observed in transgenic mice, cell transplantation in HD should not be abandoned. Indeed, striatal repair was already made possible in toxin animal models. Moreover, in the near future further improvements will provide the solid evidence in favor of the existence of the so called effective restorative therapy. Whether we must concentrate our efforts on repairing the striatum alone, hoping to affect the subsequent degenerative process, or repair the striatum simultaneously with other cortical/subcortical structures still remains uncertain. To answer these questions and based on the successful striatal brain grafting in rat HD models, as well as on the evidence for the clinical potential of human fetal brain grafting in PD, clinical trials with HD patients were set in motion.

3.4. Transplantation of human fetal tissue – clinical trials

In order to develop clinical transplantation trials in HD patients, the first step is to create and validate standard procedures and technique protocols that may be able to ensure human fetal tissue accurate handling, as well as determine optimal donor age, safe transplantation methods and proper efficacy assessment. Fetal tissue optimal dissection has been a reason for concern as it is known that the striatum develops from lateral and medial ridges of the ganglionic eminence; therefore traditional striatal grafts used to comprise the

entire ganglionic eminence. Early studies suggested that the lateral ganglionic eminence contained the higher amount of striatal like-cells and hence the optimal source for transplantation in HD patients. Nonetheless, several striatal interneurons derive from medial ganglionic eminence and it appears that a combined proportion of these two compartments may contribute to higher and improved functional recovery (Watts et al. 1997). Other worries result from the possibility of tissue overgrowth with tumor formation and the need for long-lasting immunossupression.

Clinical trials in HD commenced early in 1990 with the reports from Cuba, Czechoslovakia and Mexico City. They involved grafting of human fetal tissue collected from within 1-2 hours of spontaneous abortions and reported no major complications from the implantation procedure. However, the tissue source in the former studies raised serious ethical concerns amongst the scientific community in a way that it became established that in subsequent studies only freely donated tissue from elective abortions could be used as a cell source (reviewed in Dunnet and Rosser 2007).

Kopyov and coauthors in 1998 engraved the first report on clinical safety in three moderately advanced HD patients who underwent bilateral grafts from 5-8 donors in the caudate nucleus and the putamen (table 4). One year after the procedure, graft survival was observed showing no significant side effects from the surgery or the immunosupression given, and there was no noticed general state deterioration. This work provided proof of principle that fetal tissue grafting can be performed in HD patients with safety (Kopyov et al 1998a). The same research group, with reference to a small number of HD patients and maintaining the initial trial design, revealed neuropsychological and motor benefits with increased scores on measuring tasks, as well as resonance imaging studies showing graft survival and neuronal differentiation (Kopyov et al. 1998b; Philpott et al. 1997). Freeman and coauthors reported the first autopsy case from Kopyov´s initial clinical trial, a patient who died 18 months after

grafting due to non-related causes and whose neurophatological findings confirmed graft survival with differentiation into mature striatal-like cells. There was no histological evidence of immune rejection despite the fact that immunossupression was maintained only within the first 6 months, and even though the integration on host circuits was limited, the grafts were not affected by the disease process (Freeman et al. 2000). In the year 2007, additional clinical trial results were made public from two other patients who died 79 and 74 months, respectively, after procedure. One patient developed bilateral subdural hematomas two months after transplantation requiring surgical intervention after which he abandoned the follow up study. The second subject did not suffer from complications in the postoperative period and even though reporting improved ambulation the assessment scores continued to show deterioration as in the classic HD. In both patients, autopsy study showed surviving grafts as well as striatal neuronal differentiation and viability, nonetheless there was poor integration in the host striatum (Keene at al. 2007).

From the same trial, one other paper was published concerning autopsy findings in a patient who died 121 months after transplantation. This patient received ten intrastriatal human fetal transplants and a co-graft from autologous sural nerve, after which was reported as clinically stable for 2 years, and after that time the patient showed clinical worsening in motor and non-motor features. Five years after the patient appeared to aggravate motor behavior markedly on the right side of the body, the MRI study which showed a 3.1 cm cyst in the left putamen and a similar sized nodular mass in the right putamen. Neuropathological findings revealed survival of grafted tissue and multiple mass lesions as a result of tissue overgrowth. Even though other masses were found, the largest one was in close proximity with the sural nerve co-graft. Once again there was a confirmation of long-graft survival and proper differentiation into mature neuronal cells, however, the price paid for these long

lasting grafts may have been high and once more, there was scarce evidence of integration and connectivity with the host brain (Keene at al. 2009).

A second major clinical trial was performed by Bachoud-Lévi and colleagues. In this human embryonic cells were grafted in five HD patients that underwent bilateral striatal surgery in two sessions with 1 year interval (table 4). Three patients have shown either increased or steady metabolic activity throughout the striatum by PET-scan analysis suggesting graft survival and function as well as stable or improved cognitive and motor behavior scores. Opposite results were seen in the remaining two patients, one without any positive response and the other showing deterioration after an acute fever, raising the possibility of graft rejection (Bachoud-Lévi et al. 2000; Gaura et al. 2004). The same research group reported a follow up study of the same five patients 6 years after procedure whereas they found a clinical improvement plateau after the first 2 years with progressive motor decline afterwards. Surprisingly chorea and cognitive performance did not worsen. The two patients lacking clinical benefits in the previous study showed progressive decline similarly to the control group (Bachoud-Lévi et al. 2006). Altogether, these results provide useful information concerning neuronal transplantation in HD as a period of remission and stability were found but still without permanent results.

In one other study from Hauser and coworkers, embryonic tissue from 2-8 fetuses was grafted into each side of the striatum of seven HD patients in a two staged procedure (table 4). In the follow up study six patients showed scarce improvement on motor scores, while the remaining suffered a significant decline after an incident causing bilateral subdural hemorrhage and from which he never again returned to the baseline. After a 12 month period, no significant clinical benefit was seen and, in the overall, three subjects developed subdural hemorrhages two of which required surgical drainage. This problem was thought to be related with a more advanced stage of the disease in comparison to other clinical trials. The results

suggested a higher morbidity risk for transplantation on HD patients with increased level of degeneration of the basal ganglia and cerebral atrophy (Hauser et al. 2002).

In the year 2002 another report came to public, from Rosser and coauthors, using unilateral graft of human fetal tissue into the striatum of four mild to moderate staged HD patients (table 4). The subjects received a triple immunosuppressive therapy during 6 months associated with reversible disturbances of routine blood tests and no other adverse event was reported (Rosser et al. 2002). Once again fetal grafting safety and feasibility was demonstrated in HD patients.

A recent study reporting two patients with moderate HD who received bilateral fetal striatal grafts aimed to study synaptic metabolism and activity with PET scan assessment with ^{11}C-raclopride (RAC), a D2 receptor binding, over a five year designed follow up study (table 4). In one patient long clinical improvement and increased striatal receptor D2 binding was observed suggesting long-term survival and function of the graft. Nevertheless, in contrast, the second patient continued to deteriorate as in HD non-grafted controls, presumably as a result of infectious complications following a fall; moreover the lack of clinical or metabolic positive results indicate that the graft failed to survive or differentiate (Reuter et al. 2008). It is difficult to draw any conclusions based on such small number of patients and further studies with PET RAC in a bigger cohort of patients regarding a blind study design are needed (Reuter et al. 2008).

Due to ethical and practical concerns regarding transplantation of fetal tissue and despite proven safety and feasibility, in a similar approach as in PD, porcine fetal tissue was grafted in a phase I trial on twelve HD patients. Though safety was assured, there was no graft survival detected or functional improvement despite immunosupression therapies (Fink et al. 2000). Even though long follow up results or autopsy findings were not yet published, it may

be of interest to compare this approach to allografting in correlation with disease progression, immune rejection and graft integration, as the latter represents the major goal for the success of cell transplantation.

Table 4: Results from clinical trials concerning implantation of human fetal tissue in HD patients

Reference	Kopyov et al. 1998	Bachoud-Lévi et al. 2000	Hauser et al. 2002	Rosser et al. 2002	Reuter et al. 2008	
					Grafted	Control
Number of patients	3	5	7	4	2	8
Age	37 (25-48)	51 (43-61)	50.1 (28-64)	NR	52.5	48.6
Number of grafts and location	Bilateral on the putamen and caudate nucleus	Bilateral on the putamen and caudate nucleus	Bilateral on the putamen	Unilateral on the putamen and caudate nucleus	Bilateral on the putamen and caudate nucleus or none	
Number of donors	5-8	2-4	2-8	1	2-3	
Donors age	8-10 weeks	7.5-9 weeks	8-9 weeks	8.5-12 weeks	9-10 weeks	
Graft preparation	Dissected LGE	Dissected WGE	Dissected LLGE	Dissected WGE	Dissected WGE	
Immunossupression	NR	Cyclosporin 6 months	Cyclosporin 6 months	Cyclosporine, azathioprine, prednisolone 6-12 months	Cyclosporin 3-12 months	
Follow-up	12 months	24 months	12 months	6-60 months	36 months	
UHDRS motor score						
Preoperative	42.3	47.6	32.9	44	51	30.7
Postoperative	24	49.6	29.7	30	26	48.4
Imaging	MRI demonstrated appropriate growth with no tumor or cyst formation	PET scan with increased or stable metabolism in three patients and decreased in two patients	PET scan with decreased metabolism	MRI showed no signs of tissue overgrowth	PET scan showed increased metabolism in 1 patient during the first 6 months with a decrease afterwards similar to the second patient and the control group	
Side effects	No major side effect detected	Mild psychiatric	2 patients developed 3 subdural haematomas	None	None	
Relevant events	None reported	None reported	1 patient died from sudden cardiac arrhythmia	None	None	

Legend: LGE – lateral ganglionic eminence; LLGE – lateral half of the lateral ganglionic eminence; NR – not reported; UHDRS - Unified Huntington's Disease Rating Scale; WGE – whole ganglionic eminence;

The transplantation field regarding fetal tissue in HD has provided proof of principle that it can be a safe method when applied within a certain timing and that it can function during a defined period. However, there is a limited amount of fetal tissue for transplantation and relevant ethical issues concerning this human cell source. One other interesting matter is the unethical need for sham surgery to measure the placebo effect, as well as the need for patient randomization and proper blinded placebo-controlled studies. There are still many unanswered questions as how much striatum can be replaced with cell transplantation and whether striatal grafting only can have a major impact on disease progression, since HD degeneration also occurs elsewhere. Whether some improvements can be made by determining optimal donor age and number of fetuses, refining cell preparation or implantation techniques, or even by developing better assessment tools to evaluate efficacy, the battle for cell transplantation is now being held with the new stem cell sources emerging.

3.5. Stem cell therapy and growth factor delivery in HD

As mentioned above, the future for restorative therapies may lie within stem cell research as these cells hold the ability to self renewal as well as to differentiate in some (NSC) or all cell lines of the body (ESC, iPS cells). Researchers are now trying to answer some of the questions surrounding stem cell therapy, as how can we produce neurons with a striatal-like phenotype and whether they can survive transplantation and function to restore lost function.

3.5.1. Embryonic stem cells

Cell therapy has now become a viable possibility for HD patients as clinical trials have shown feasibility and encouraging data regarding motor benefits, nonetheless the challenge

met its difficulties regarding the limited availability of fetal cells as well as the ethical concerns they imply. Nowadays, to overcome such problems, new cell sources are being put up to the test, including ESC. These cells derive from the inner mass of the human blastocyst prior to implantation (see above for more detailed information). Animal studies have already shown that ESC can turn into specific cell lines of a desired type by manipulation of culture conditions and exposing the cells to extrinsic signals in a similar way to the early stages of neural patterning. Nevertheless, in contrast to what happens with PD where there have been major breakthroughs, until this date there have been few reports on clear demonstration of the possibility to differentiate ESC into a striatal phenotype. Aubry and coworkers designed a multistep *in vitro* protocol for human ESC (hESC) regarding the derivation of striatal progenitors. They begun by expanding hESC within a supplemented media followed by co-culture with bone marrow-derived stromal feeder cells after which growth factors were added (Shh, DKK1 – a Wnt pathway inhibitor and BDNF). They obtained approximately 22% MAP2 (terminal striatal differentiation marker) positive postmitotic neurons, 53% of which expressed DARPP32 (key striatal marker). The cells were transplanted into the right striatum of quinolinic acid lesioned rats and showed a three month survival with a significant yield of DARPP32 positive cells (21%). However, despite promising results, human xenografts were found to overgrow in the rat brain over time and these results have shown to be in agreement with similar data obtained from other reports regarding hESC (Roy et al. 2006). Apart from the disappointing results, this report opened the door to the world of hESC therapy in HD (Aubry et al. 2008).

3.5.2. Neural stem cells

One other source for cell replacement therapy is the NSC. These cells exist in both developing and adult nervous system of all mammalian organisms and even though displaying

a more restrict fate than ESC, they have the potential to give rise to the major cell types in the CNS with a decreased risk of tumor formation (see above for more detailed information). While previous studies on HD models have focused on establishing cell survival, stable integration and absence of tumor formation, McBride and coauthors investigated the neuroanatomical and behavioral effects of NSC grafting into the striatum of quinolinic acid lesion models and observed significantly enhanced motor performances as well as cell integration and differentiation into the host striatum with extensive migration of transplanted cells to nuclei that normally receive striatal projections (Svendsen et al 1996; Lundberg et al 1997; McBride et al. 2004). More recently it has been suggested that NSC injected intravenously may have the ability to migrate and integrate ischemic brain undergoing proliferation due to mitotic signals (Chu et al. 2004). These findings suggest that the invasive technique of stereotaxic surgery may no longer be necessary and based on these same results Lee and coworkers injected NSC intravenously in an adult rat model of HD to investigate the feasibility and the benefits of such delivery method. This report has shown that NSC migrate into the striatum, reduced striatal atrophy, differentiated into neurons and glia and induced functional improvement in the quinolinic acid lesion model (Lee et al. 2005). Despite the reported success of NSC surgical or intravenously engrafment in the quinolinic acid lesion model, it is widely known that the latter does not reproduce the entire human pathogenic process. Intrastriatal lesions created by amino acids may interfere with engraftment and/or NSC migration due to signaling messages and further experiments using the transgenic model are warranted as they may have the potential to provide an additional optimal way to investigate the ability of NSC regenerate damaged cells and/or to offer behavior benefits in HD.

Johann and colleagues investigated the graft development in different hosts, comparing the quinolinic acid lesion model with the transgenic R6/2 line and also

determining the weight of the delay between lesion and graft procedure as well as the method of tissue preparation. They found a correlation between graft preparation and transplantation timing, NSC showed improved survival when transplanted as intact spheres rather than single cell suspensions at an early stage after excitotoxic lesion, avoiding detrimental host´s acute glial reaction. The transplantation of intact spheres circumvents the mechanical trauma which may lead to better outcomes. When comparing the two HD models, they found it hard to study the R6/2 trangenic line due to early death and lack of neuronal degeneration. Even though donor cells seemed to survive in a similar way to quinolinic acid model long term survival could not be assessed since these animals tend to die after 12-15 weeks (Johann et al. 2007).

Experiments with NSC into the striatum lesioned by quinolinic acid have shown that growth conditions *in vitro* are a crucial factor to influence graft survival *in vivo* and that environmental enrichment and behavioral experience also play important roles concerning neuronal plasticity and functional recovery (see for a review Döbrössy and Dunnet 2001).

The potential of NSC regarding cell replacement therapy in HD is still very unclear, raising more questions than answers at the present moment. There is an urging need for improved animal models that mimic more closely the disease´s features and simultaneously offer a similar and fully neurodegenerative process. The path to clinical trials using NSC depends on obtaining safe and reliable results from enhanced representative HD´s animal models

3.5.3. Growth factor delivery

Delivering of growth factors into the brain aims to protect neurons against damage and cell death, thereby yielding a neuroprotective effect. Trophic factors are large proteins that do

not readily cross the blood-brain barrier and thus must be delivered directly into the brain, via viral carriers or via stem cell transplantation (see above for more detailed information on growth factors and viral vectors).

Three growth factors yield a particularly interest regarding HD therapy namely BDNF, CNTF and GDNF. BDNF was found to play a role in the survival and activity of medium-sized spiny striatal neurons, the main cells that degenerate in HD, but there are also evidence of reduced endogenous neurotrophic support being involved in the development of the disease, thereby making BDNF a potential therapeutic target (Zuccato et al. 2001). The majority of BDNF is produced in cortical neurons that project into the striatum. Data suggest that wild-type huntingtin, but not the mutated form, stimulates BDNF production by acting on its gene transcription and axonal transport, which favors the hypothesis that the reduction in BDNF production reaching the striatum might cause the preferential susceptibility of these neurons to cell death in HD (for review see Cattaneo et al. 2005). Striatal damage or BDNF transport blockade was found to increase the level of BDNF, suggesting that its upregulation may constitute a protective mechanism against neurodegeneration that might be used in HD treatment (Canals et al 2001). Using embryonic striatal neurons growing in culture Nakao and coauthors found that BDNF enhanced survival and morphological differentiation (Nakao et al. 1995). Zuccato and coworkers evaluated the levels of BDNF transcription at different disease stages in the R6/2 mice demonstrating a correlation between BDNF reduction and disease progression (Zuccato et al. 2005). Based on these facts, Bemelmans and colleagues injected intrastriatal BDNF encoding adenovirus in rats and after two weeks lesioned the animals with quinolinic acid. One month after the lesion histological studies revealed neuronal protection with 55% smaller lesions and increased survival of striatal GABAergic neurons in the animals that received BDNF (Bemelmans et al. 1999). Kells and coauthors obtained similar results with adeno-associated viral gene delivery of BDNF in a quinolinic acid rodent model of HD

(Kells et al. 2004). Alternative approaches developed in parallel with viral delivery of BDNF involved growth factor secreting engineered cells. The first studies with BDNF cell delivery revealed only modest or even discouraging results, but subsequent attempts reported better accomplishments due to probably lower and safer doses of BDNF released. An interesting study from Ryu and coauthors where human neural stem cells were grafted into the adult rat striatum one week prior to the administration of 3-nitropropionic acid (an irreversible inhibitor of succinate dehydrogenase on mitochondrial complex II) that showed significant motor improvement and reduced striatal damage in the animals that received BDNF secreting cells compared to those that received sham surgery or were transplanted only 12 hours before the lesion treatment (Ryu et al. 2004). Even though BDNF has shown promising results in animal models in terms of viral or cell deliver, there is still a lot to overcome regarding the release method, the lack of gene expression control or the possible vector toxicity, as well as the invasiveness of the procedure. New research aims to regulate gene expression and increase BDNF endogenous levels.

CNTF is a neuroprotective cytokine found to offer significant protection against neurodegeneration in the neurotoxic rodent model of HD whether stereotaxically or lentiviral delivered into the striatal neurons with reduction of motor impairment (Anderson et al. 1996; de Almeida et al. 2001). Cellular delivery of CNTF in rodent and nonhuman primate models of HD also revealed a positive trophic influence on striatal neurons as well as on critical non striatal regions (Emerich et al. 2004; Emerich et al. 1997). In another other study, baby hamster kidney cells previously engineered to secrete CNTF were implanted bilaterally into the striata and offered neuronal protection against degeneration with restoration of cognitive and motor functions in a primate model of HD (Mittoux et al 2000). After the proven efficacy in animal models, a phase-I study was held to evaluate the safety of CNTF administration in HD patients. Six patients with mild HD received one capsule of baby hamster kidney cell line

engineered to produce CNTF exchangeable every 6 months during a two year period. The results demonstrated safety and feasibility with tolerance to the procedure, but the capsules retrieved contained a variable number of surviving cells which raised the need for further technique improvements (Bloch et al. 2004). Indeed, CNTF may likely an advantageous neuroprotective agent, but additional assessment on its effect and impact on HD patients is needed, as well as an optimization of technical procedures, among others.

GDNF is present in the striatum and its expression can be selectively regulated by excitotoxic insults which promote its release from astrocytes. After being delivered to rodent models of HD through viral vectors, GDNF was found to protect striatal GABAergic projection neurons from toxic lesion and improve motor behavior (Kells et al. 2004; McBride et al 2006). Genetically modified neural stem cells and fibroblasts where used for GDNF deliver administered before quinolinic acid toxine lesion, resulting in protection of striatal neurons and motor behavior improvement in a rodent model of HD (Pérez-Navarro et al. 1996; Pineda et al. 2007).

Most reports regarding growth factor deliver feasibility and efficacy through grafted engineered cells or viral vectors provided crucial information to reinforce the idea of the potential of this method to grant protection to striatal cells. Despite promising results in HD animal models, there are still ethical concerns regarding the type of cell source and the possibility of an outspread from an uncontrolled viral vector. There was, however, a phase I clinical trial held, but unfortunately without any significant clinical improvement reported, which added even more complexity to the already intricate issue of growth factor delivery in neurodegenerative diseases (Bloch et al. 2004).

4. Concluding remarks

Research on neurodegenerative disorders, due to their magnitude worldwide, is a rapidly growing field in Neuroscience. In the last decades we observed major breakthroughs in cell therapy and neurodegenerative research areas. Investigation on the cellular sources for cell replacement strategies in the brain has gained significant importance after the recent development of stem cell-based neuronal therapies, including the control of their differentiation potential. The aim is to obtain a specific neuronal cell fate to repair the lost cells and regain their function. Several cell-based therapeutic approaches which seemed promising on animal models of PD and HD have not managed similar success in human patients. Despite its shadowy future, fetal transplantation in HD and PD patients was the key that opened the door to what seems to be a novel and dazzling world of restorative therapy.

Stem cell therapy, in particular, still did not manage to gather enough conditions for their eligibility for clinical trials. New cellular, molecular and pharmacological approaches may contribute to improve the neuronal survival of grafted cells and thus the treatment of these debilitating brain diseases.

This new and overwhelming world still carries great uncertainties within itself. Still, there is hope that in nearby future stem cells can fulfill their clinical promise.

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6. List of abbreviations

6-OHDA, 6-hydroxydopamine; AA, ascorbic acid; AAV, adeno-associated virus; Ad adenovirus; BDNF, brain derived neurotrophic factor; CDNF, conserved dopamine neurotrophic factor; CNS, central nervous system; CNTF, ciliary neurotrophic factor; DA, dopamine; DARPP32, key striatal marker; En1, engrailed gene 1; En2, engrailed gene 2; EGF, epidermal growth factor; FGF8, fibroblast growth factor 8; EBs, embryoid bodies; E, embryonic day; FD, [^{18}F]-fluorodopa; Gbx2, gastrulation brain homeobox 2; GDNF, glial cell derived neurotrophic factor; Girk2, G-protein-regulated inward rectifier potassium channel subunit; HD, Huntington's disease; hESC, human Embryonic stem cells; iPS, induced pluripotent stem cells; LGE, lateral ganglionic eminence; LLGE, lateral half of the lateral ganglionic eminence; Lmx1a, LIM homeobox transcription factor 1; LV, lentivirus; MAP2, terminal striatal differentiation marker; m ESC, mouse embryonic stem cell; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; Msx1, muscle segment homeobox transcription factor 1; NR, not reported; NSC, neural stem cells; NTN, nerturin; Ngn2, proneural basic helix-loop-helix protein; Nurr1, orphan nuclear receptor-related factor 1; Otx2, orthodenticle homologue 2; pESC, non-human primate embryonic stem cell; PD, Parkinson's disease; PERVs, porcine endogenous retroviruses; PET, positron emission tomography; Pitx3, paired-like homeobox transcription factor-3; PSA-NCAM, neuronal precursor protein polysialylated neuronal cell adhesion molecule; SCNT, somatic cell nuclear transfer; SDIA, stromal-derived inducing activity; Shh, sonic hedgehog; SN, substantia nigra; TH, tyrosine hydroxylase; UHDRS, Unified Huntington's Disease Rating Scale; UPDRS, The Unified Parkinson's Disease Rating Scale; VTA, ventral tegmental area; WGE, whole ganglionic eminence; Wnt1, wingless related 1;

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