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Titolo

DEVELOPING A REGENERATIVE MEDICINE APPROACH FOR THE TREATMENT OF PARKINSON'S DISEASE

Settore Scientifico Disciplinare: BIO/14 FARMACOLOGIA
Coordinatore del Corso di Dottorato: Chiar.mo Prof. Alfredo Gorio
Docente Guida: Chiar.ma Prof. Anna Maria Di Giulio

Dottoranda: Stefana Carelli
Matricola n° R09272

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"Doctors are men who prescribe medicines of which they know little, to cure diseases of which they know less, in human beings of whom they know nothing"

François-Marie Arouet-Voltaire

Ad Alessandro e Anastasia

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1. Summary

Parkinson's disease (PD) is the second most common neurodegenerative disease, after Alzheimer's disease, and the most common movement disorder. Drug treatment and deep brain stimulation can ameliorate symptoms, but the progressive degeneration of dopaminergic neurons in the substantia nigra eventually leads to severe motor dysfunction. While some effective treatments for patients with PD exist, these treatment strategies are mainly symptomatic and aimed at increasing dopamine levels in the degenerating nigrostriatal system. Existing drugs are limited in their relief and decrease in effectiveness as PD progresses.The transplantation of stem cells has emerged as a promising approach to replace lost neurons in order to restore dopamine levels in the striatum and reactivate functional circuits. Post mortem neural precursor cells (PM-NPCs) are a subclass of sub ventricular zone (SVZ)-derived neural progenitors, capable of surviving many hours (16 hours) after donor death. The *in vitro* differentiation yields more neurons (about 30-40%) compared to regular NPCs (Marfia et al., 2011). Recently from the SVZ of a transgenic mouse strain expressing green fluorescent protein (GFP) under the promoter C of the ubiquitin gene [(C57BL/6-Tg(UBC-GFP)30Scha/J)] we isolated GFP PM-NPCs, from mice at 6 hours after the donor death. These cells were characterized and their potential of in terms of replacement therapy was investigated in a mouse model of Parkinson disease. The degeneration of dopaminergic neurons was obtained through the administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) at the dosage of 36 mg/kg intraperiteoneally (i.p.). After 1 week the lesion was stabilized by a second administration (i.p.) of the neurotoxin at the dosage of 20 mg/kg. 1x 10^5 of PM-PCs-GFP were injected unilaterally into the striatum of C57/BL mice by using specific stereotaxic coordinates 3 days after the second MPTP administration. The effects of transplanted cells were determined by means of performance tests aimed at detecting behavioral improvements. Moreover, the neurochemical changes were also studied by high performance liquid chromatography (HPLC). In order to study the *in vivo* fate of grafted GFP PM-NPCs animals were perfused 2 weeks after transplantation and immunohistochemistry studies were performed. Our results show that animals grafted with GFP PM-NPCs determined a remarkable improvement of behavioral parameters measured by means of both horizontal and vertical grid tests (forepaw fault and time required to grab on the grids while turning and climbing down) since the third day after transplantation. These improvements were very significant and the average values were close to control animals. This was maintained during all the two weeks of experimental observation. By means of immunofluorescence staining we observed that the majority of transplanted GFP-PM-NPCs were vital and able to migrate ventrally and caudally from the injection site lengths as far as 1000 microns into the striatum,

and could reach the ipsilateral and contralateral *substantia nigra pars compacta*. Moreover, morphological analyses revealed that transplanted cells in the striatum are able to differentiate into tyrosine hydroxylase (40%), cholinergic (40%), and gabaergic neurons (25%). This study provides new evidences that PM-NPCs will be useful for developing cellular PD therapies. Future studies should further explore the clinical potential role of the investigated post mortem neural precursors cells in order to provide new perspectives for PD treatment.

2. Introduction

2.1 Regenerative medicine

Regenerative medicine is an emerging field that combine the knowledge and skill of many disciplines with the aim of healing impaired function in the body (Haseltine 2003; Gutmann et al. 2005). The regeneration of tissues and organs offers a radical new approach for the treatment of injury and disease, but the main goal of regenerative medicine is not only the replacement of what is malfunctioning in the body, but to provide the biological requirements fundamental for in vivo repair, to devise replacements that seamlessly interact with the living body, and to stimulate the body's intrinsic capacities for regeneration (Greenwood et al, 2006). Regenerative medicine take advantage of the combination of several technological approaches that moves it beyond traditional transplantation and replacement therapies. These approaches may include, but are not limited to, the use of stem cells, soluble molecules, genetic engineering, tissue engineering, and advanced cell therapy (Greenwood et al, 2006). The United States National Academies of Science report, Stem Cells and the Future of Regenerative Medicine, estimates that the potential patient populations in the US for stem cell–based therapies include more than a hundred million patients affected by diseases such as cardiovascular conditions, autoimmune diseases, diabetes, cancer, neurodegenerative diseases, and burns (Petit-Zeman et al, 2001). The following table shows some of pathologies in which the potential application of a regenerative medicine could improve the patients' health.

Applications of Regenerative Medicine	Examples Identified by the Panellists
Novel methods of insulin replacement and pancreatic islet regeneration for diabetes	Bone marrow stem cell transplantation for pancreatic regeneration Microencapsulation (e.g., poly-lactide-co-glycolide) for immunoisolation of transplanted islets Cultured insulin-producing cells from embryonic stem cells, pancreatic progenitor cells, or hepatic stem cells Genetically engineered cells to stably express insulin and contain a glucose-sensing mechanism
Autologous cells for the regeneration of heart muscle	Myocardial patch for cardiac regeneration Direct injection of autologous bone marrow mononuclear cells for cardiac repair Stromal cell injection for myocardial regeneration Localized angiogenic factor therapy through controlled release systems or gene therapy
Immune system enhancement by engineered immune cells and novel vaccination strategies for infectious disease	Genetically engineered immune cells to enhance or repair immune function Single-injection DNA vaccines
Tissue-engineered skin substitutes, autologous stem or progenitor cells, intelligent dressings, and other technologies for skin loss due to burns, wounds, and diabetic ulcers	Bilayered living skin constructs (e.g. Apligraf) Engineered growth factors (e.g. rbbFGF, rhEGF) applied in conjunction with topical treatments (e.g. SD-Ag-Zn cream) Intelligent dressings composed of a slow-releasing growth hormone polymer Epithelial cell sprays
Biocompatible blood substitutes for transfusion requirements	Polyhemoglobin blood substitutes for overcoming blood shortages and contamination issues
Umbilical cord blood banking for future cell replacement therapies and other applications	Preserved umbilical cord blood stem cells to provide future cell replacement therapies for diseases such as diabetes, stroke, myocardial ischemia, and Parkinson disease Pooled cord blood for the treatment of leukemia
Tissue-engineered cartilage, modified chondrocytes, and other tissue engineering technologies for traumatic and degenerative joint disease	Matrix-induced autologous chondrocyte implantation for cartilage repair Tissue-engineered cartilage production using mesenchymal stem cells
Gene therapy and stem cell transplants for inherited blood disorders	Genetically engineered hematopoietic stem cells to restore normal blood production in β-thalassemic patients
Nerve regeneration technologies using growth factors, stem cells, and synthetic nerve guides for spinal cord and peripheral nerve injuries	Synthetic nerve guides to protect regenerating nerves Embryonic stem cell therapy for spinal cord regeneration Growth factor–seeded scaffolds to enhance and direct nerve regeneration
Hepatocyte transplants for chronic liver diseases or liver failure	Microencapsulation of hepatocytes to prevent immunological reaction Derivation of hepatocytes for transplantation from embryonic stem cells Transdifferentiation of hepatocytes for transplantation from bone marrow cells

Figure 1. Top Ten Regenerative Medicine Applications for Improving Health in Developing Countries (Greenwood et al, 2006).

The current approaches are influenced by our understanding of embryonic development, of tissue turnover and replacement in adult animals (Alonso et al., 2003, Harada et al, 2003, Radtke et al., 2005), and by tissue engineering and stem cell biology (Stocum, 2004). The regeneration of organs and appendages after injury occurs in diverse animal groups and provides another important viewpoint, in addition to the demonstration that complex adult tissues can be rebuilt.

Regenerative medicine currently is based on three approaches (Figure 2) (Stocum, 2004): the transplantation of stem cells to build new structures, the transplantation of cells pre-primed (genetically or chemically) to develop in a given tissue, and the "induction" of endogenous cells to replace missing structures. Each of the different aspects identified in the first two examples—the generation of an appropriate cohort of regenerative cells, their regulated division and differentiation, and the restoration of the appropriate part of the structure—must be stimulated from endogenous cells in the third approach.
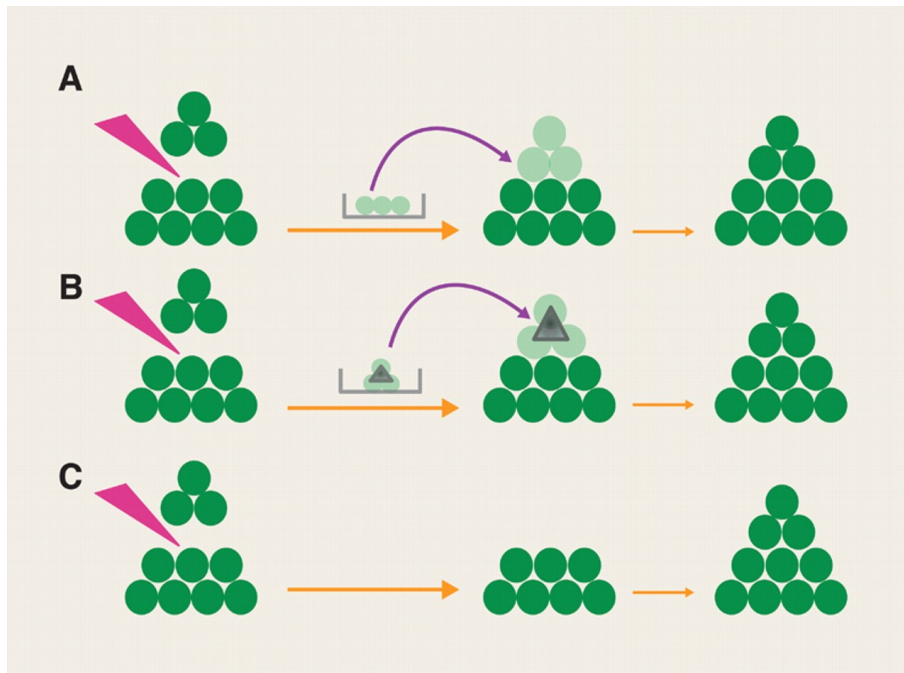
Figure 2. Schematic of three approaches on which is based regenerative medicine. (**A**) Implantation of stem cells (light green) from culture leads to the restoration of the structure. (**B**) Stem cells are provided with a scaffold (triangle) in order to guide restoration. (**C**) The residual cells of the structure are induced to make a regenerative response (from Stocum et al 2004).

Stem cell therapy has only been established as a clinical standard of care for diseases of the blood system. However, there are a growing number of clinics throughout the world that are testing stem cell interventions and some that are claiming to offer stem cell treatments for a variety of conditions without clear evidence of safety or efficacy. For the successful application of novel stem cell-based approaches, it will be necessary to define the scientific and clinical advances, as well as the associated regulatory, ethical, and societal issues, that need to be addressed in order to deliver treatments safely, effectively, and fairly (Hyun et al., 2008).
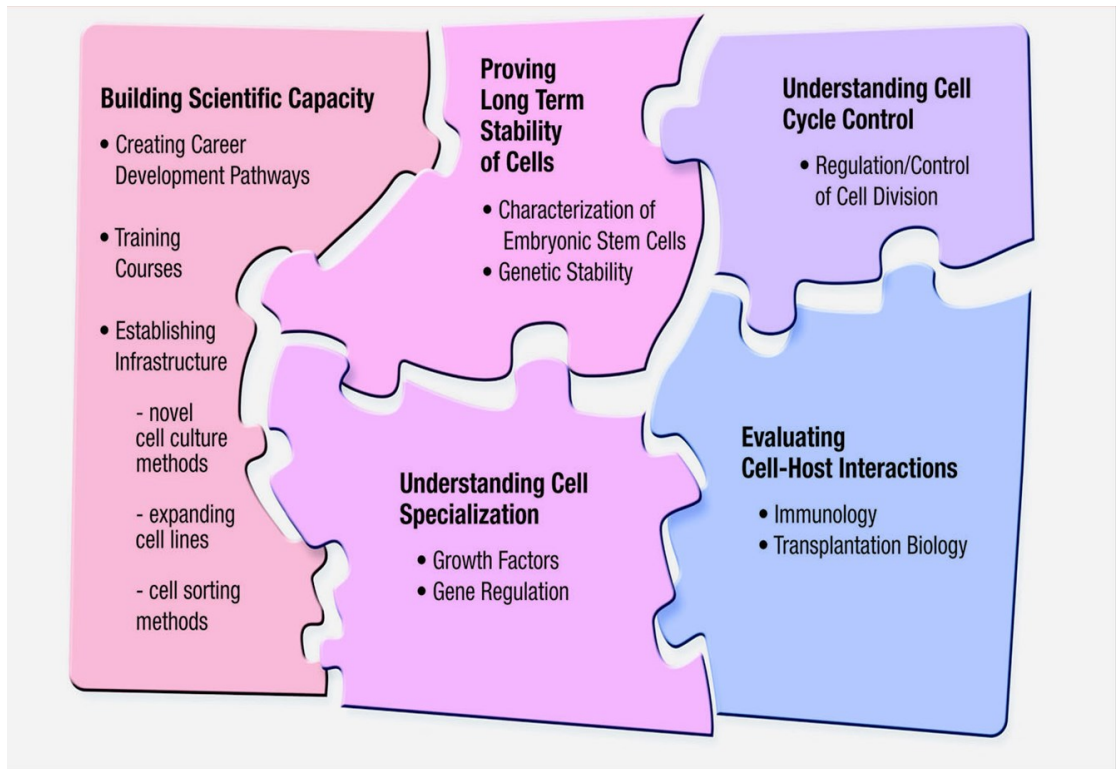
Figure 3. The scientific challenges of stem cells research in regenerative medicine.

2.2 Parkinson's disease

Parkinson's disease (PD) is a common neurodegenerative disorder—a synucleinopathy—with a prevalence of 160/100 000 in Western Europe rising to ~4% of the population over 80 (http://www.epda.eu.com). With an ageing population, the management of PD is likely to prove an increasingly important and challenging aspect of medical practice for neurologists and general physicians. Our understanding of the pathogenesis of the disease has been advanced in the last decade with the identification of several gene mutations which may shed light on the mechanisms of pathogenesis in sporadic cases of PD.

The diagnosis of PD remains essentially a clinical one, and it is important to recognize the early features together with symptoms and signs suggesting other causes of parkinsonism. There has also been a rapid expansion in the treatment options both in the early and in the later stages of the illness together with a greater awareness of non-motor complications.

The most unique and obvious sign of parkinson is the hand tremor, often described as "pill rolling". Uncontrollable shaking of a hand or arm occurs on one or both sides of the body. Tremors can also occur in the legs, feet, or chin. Shaking lessens as the affected area is used (it is also called a resting tremor) and stops completely during sleep (Poewe et al., 1998).
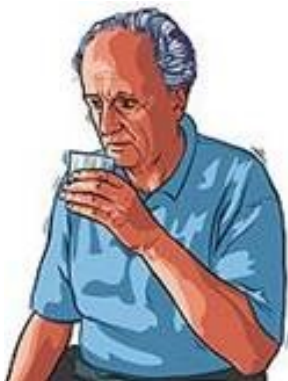
Muscle rigidity:

Muscles can become tight and rigid as they fail to receive messages from the brain to relax. Thus the resulting muscle spasm further slows movement. This can cause muscle aches, a stooped posture, and slow movement. Walking may

be limited to short, shuffling steps. Climbing stairs or getting out of a chair or a bed may take extra effort.

Often people with Parkinson's disease become "frozen" - unable to continue movement at all. In this case, help may be needed to resume movement by "putting a foot in front of the patient to step over" or suggesting that they are "stepping over lines" (Bhat and Weiner 2005).

Postural instability:

Parkinson's disease can give problems with balance, causing the individual to fall overset of movements. Muscles simply do not work as rapidly as they should. It's as if the messages from the brain take a detour, sometimes even getting lost before arriving at their destination. Rapid, coordinated movements like writing, speaking, typing or dancing are most affected.

Maintaining posture requires rapid adjustments in response to changing forces on the body, adjustments that are not possible due to the slowed movement and stiffened muscles of parkinsonism (Scott L. 2006).

Bradykinesia:

The word bradykinesia means simply slowed movements (Scott L. 2006)

Other symptoms:

Other symptoms may include speaking softly in a monotone voice, and difficulty with swallowing and writing.

Constipation is also a common problem.

Depression, feelings of insecurity and fear often bring distress to the patient and can make it difficult to cope with the illness, both for the patient and for relatives (Scott L. 2006).

Combination of symptoms: picture of the patient

These disabilities combine to produce a number of specific characteristics in a patient with Parkinson's which; taken together, make up the disease also known as *paralysis agitans*.

The full picture of the disease includes (Scott L., 2006):

- The typical hand tremor
- A stooped posture
- A short, shuffling gait with no associated arm movements
- A tendency to fall over, either forwards or backwards
- Difficulty both in starting to walk and in stopping
- Difficulty rolling over in bed and in getting in and out of a car or chair
- Poorly coordinated hand use
- Small handwriting
- A face that is empty of expression -- the so-called "Parkinson's Mask"
- Soft speech
- Drooling and difficulty swallowing, caused by uncoordinated movements of the throat and mouth.

2.3 Pathology, aetiology and pathogenesis of Parkinson's Disease

The pathological hallmark of PD is cell loss within the substantia nigra particularly affecting the ventral component of the pars compacta. By the time of death, this region of the brain has lost 50–70% of its neurons compared with the same region in unaffected individuals.

The earliest documented pathological changes in PD have been observed in the medulla oblongata/pontine tegmentum and olfactory bulb. In these early stages—

Braak stages 1 and 2—patients are pre-symptomatic. As the disease advances—Braak stages 3 and 4—the substantia nigra, areas of the midbrain and basal forebrain become involved. Finally, the pathological changes appear in the neocortex (Davie CA, 2008).
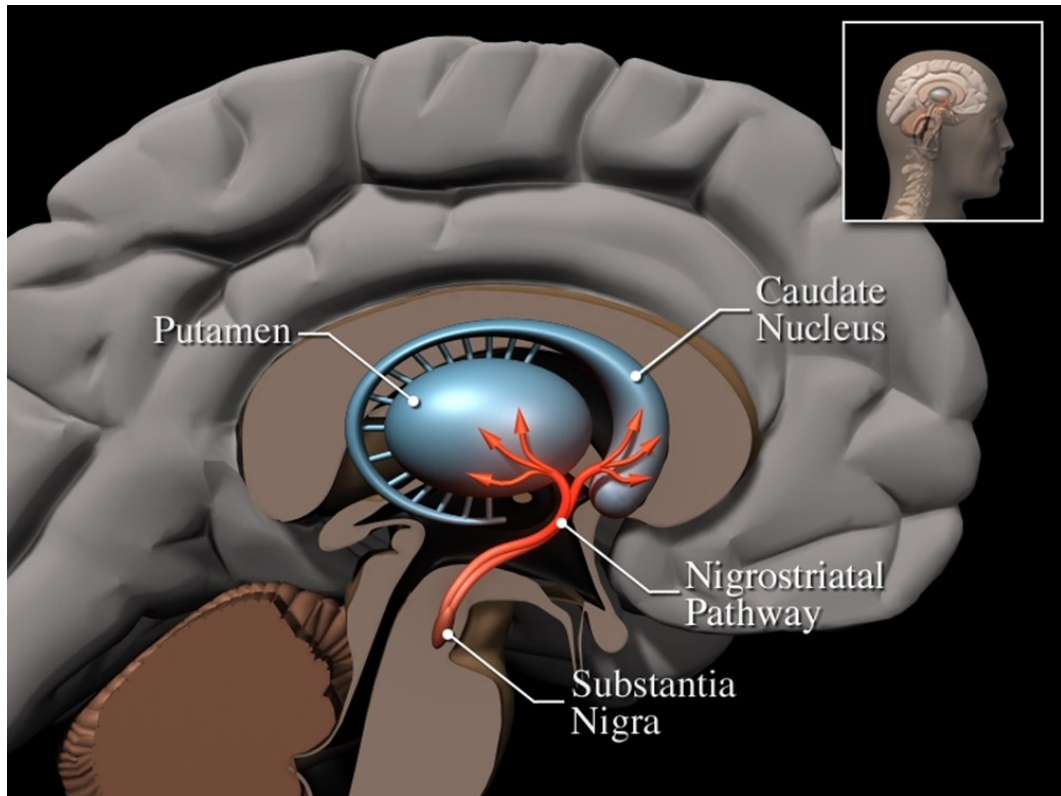
Figure 4. This figure shows neuronal pathways that degenerate in Parkinson's Disease. Signals that control body movements travel along neurons that project from the substantia nigra to the caudate nucleus and putamen (collectively called the striatum). These "nigro-striatal" neurons release dopamine at their targets in the striatum. In Parkinson's patients, dopamine neurons in the nigro-striatal pathway degenerate for unknown reasons.

This pathological staging is based on the distribution of lewy bodies. Lewy bodies are the pathological hallmark of PD. They are α-synuclein-immunoreactive inclusions made up of a number of neurofilament proteins together with proteins responsible for proteolysis. These include ubiquitin, a heat shock protein which plays an important role in targeting other proteins for breakdown.

Figure 5. Lewy bodies. Brown spots are immunostaining using an antibody specifically recognizing an abnormal form of alpha-synuclein (http://www.uphs.upenn.edu)

Mutations in the α-synuclein gene are responsible for some familial forms of PD in which lewy bodies are also seen. Mutations in the parkin protein produce a parkinsonian syndrome without lewy bodies in juvenile cases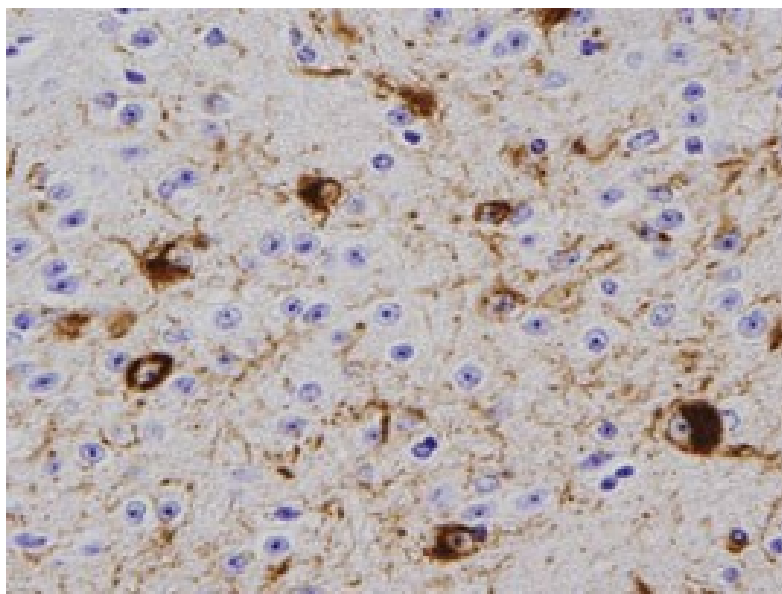 suggesting that the parkin protein plays an important role in the development of the lewy body. It has been shown that parkin facilitates the binding of ubiquitin (ubiquination) to other proteins such as the α-synuclein interacting protein synphilin-1 leading to the formation of lewy bodies. Lewy bodies are found in PD and Dementia with lewy bodies (DLB), but are not a pathological hallmark of any other neurodegenerative disease.

The identification of single gene defects in PD has focused interest on the ubiquitin-proteasome system (UPS) as one potential candidate in the development of cell death. The UPS is important for intracellular proteolysis and a large number of intracellular processes that maintain the viability of cells. It does this by removing unwanted proteins that are no longer required by the cell. Failure of the UPS leads to the abnormal aggregation of proteins including α-synuclein which are a major component of lewy bodies. One of the first sites for LB deposition in early PD is the olfactory bulb. It is, therefore, of interest that a disturbance in smell and taste is often one of the earliest clinical features in PD raising the possibility that LB formation may be integral for the activation of pathways leading to neuronal dysfunction and death.
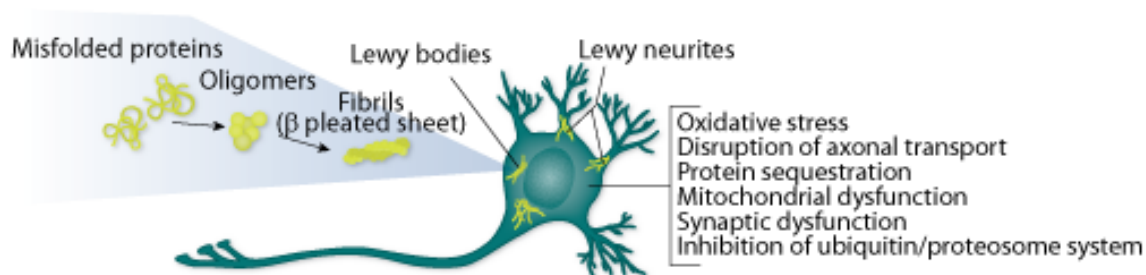
Figure 6. Schematic showing the role of a-synuclein misfolding in aetiology of PD. Misfolded alpha-synuclein proteins are converted into pathological oligomers and higher order aggregates that form fibrils and deposit into Lewy bodies and Lewy neurites in affected neurons of the PD brain. Several consequences of these fibrillar deposits of alpha-synuclein have been proposed and are indicated (Lee et al., 2006).

The link between UPS and neurodegeneration has been strengthened by the discovery of mutations in genes which code for several ubiquitin-proteasome pathway proteins in PD.

2.4 Genetic aspects of Parkinson's disease

Although PD is usually a sporadic disease, there are a growing number of single gene mutations which have been identified. At the time of writing, 11 genes have been mapped by genetic linkage with six genes identified: α-synuclein (SNCA), ubiquitin C-terminal hydrolase like 1 (UCH-L1), parkin (PRKN), LRRK 2, PINK 1 and DJ-1 genes. These single gene defects with the notable exception of LRRK 2 are responsible for only a small number of patients with PD, though more importantly their identification and the proteins that they encode for are providing significant insight into the disease mechanisms that may be responsible for PD and other neurodegenerative diseases. A point mutation of the SNCA gene leads to the early onset of PD in affected members in an autosomal dominant pattern. Of interest, duplication or triplication of the SNCA gene in affected members leads to PD symptoms developing at a later age in the fourth or fifth decades raising the possibility that overexpression of SNCA may be a factor in sporadic disease.

The LRRK 2 gene (PARK8) is the most common cause of familial or the so-called 'sporadic' PD to date.8 The frequency of LRRK2 mutations in patients with a family history of PD is 5–7%. The heterozygous mutation, 2877510 g → A, produces a glycine to serine amino acid substitution at codon 2019 (Gly2019

ser). This LRRK2 G2019S mutation is the most commonly described, accounting for the majority of familial cases and up to 1.6% of cases of idiopathic PD, though the prevalence seems to be very variable. The LRRK2 gene encodes for a protein named dardarin (derived from the basque word for tremor; the original families described came from Spain and England). Lewy bodies have been identified in some LRRK 2 cases. Many of the LRRK2 patients reported have typical features of PD with onset in middle or late onset. Symptoms at onset may be typical of idiopathic PD characterized by unilateral bradykinesia and rigidity, with tremor present in some but not all patients.

A number of single gene mutations, e.g. parkin and DJ-1 with an autosomal recessive pattern of inheritance, may have a clinical pattern of earlier age of onset, a more benign course with good response to levodopa and the presence of dystonia. However, it is not possible to identify parkin positive young onset PD patients from parkin negative patients on clinical features alone.

There has been a great deal of research into mitochondrial genetics and function in PD. Abnormalities in Complex 1 of the oxidative phosphorylation enzyme pathway is the most consistent finding, having been detected in PD brains, blood platelets and skeletal muscle, although defects in other complexes have also been reported.

It appears that the cells of the pars compacta are particularly susceptible to oxidative damage. Mitochondrial DNA studies have as yet failed to identify a convincing gene mutation to explain the oxidative phosphorylation defects in PD. However, it seems likely that a mitochondrial defect may play a role in the pathways leading to cell dysfunction and death. The PINK1 gene codes for a mitochondrial complex and has been shown to be responsible for an autosomal recessive form of PD, though is not a major risk factor for sporadic disease.
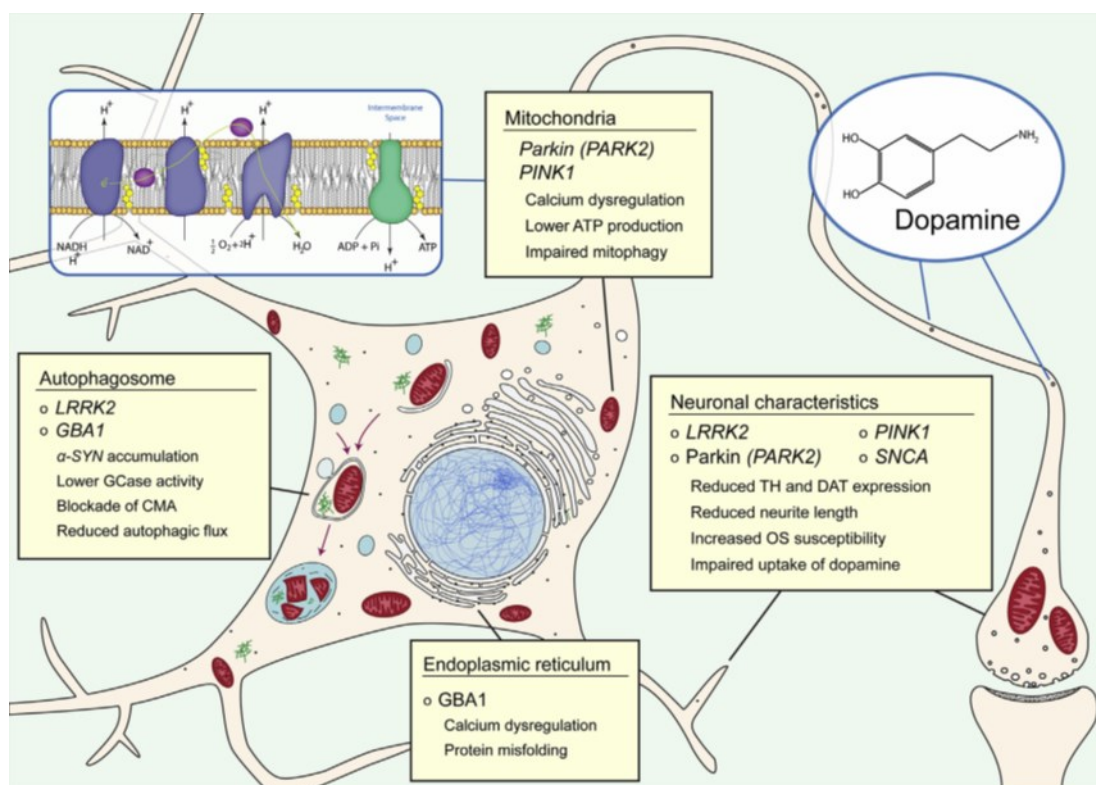
Figure 7. The cellular pathways causing cell death in PD. Summary of PD-associated genes that can cause pathway dysfunction, and ultimately cell death. Mutations to the genes LRRK2, GBA1, SNCA, PINK1 and PARK2 affect different cellular processes, but all cause PD (from Badger et al. 2014).

2.5 Environmental factors and Parkinson's disease

Besides age and family history, a number of potential contributing factors, such as comorbidities (e.g., diabetes, hypertension) and lifestyle habits (e.g., dietary pattern, smoking), have been identified (Barichella et al 2009; Cereda et al 2011; Qiu et al 2011, Riz et al 2007; Sofi et al. 2010; Hernan et al 2002). Also, the role of living and working environments has been considered to be of great significance. The first demonstration that the active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine can cause a sub-acute form of parkinsonism (Langston et al 1983) aroused considerable interest in the role of some organic pollutants, such as pesticides. Identifying environmental factors that predispose to the development of PD has proved elusive. Living in a rural environment appears to confer an increased risk of PD, and perhaps causally linked to this some but not all epidemiological studies have shown a correlation between exposure to pesticide use and wood preservatives. The only consistent environmental factor is a strong negative correlation between cigarette smoking

and the development of the disease. It is also possible that mitochondrial dysfunction in PD is triggered by one or more environmental toxins. A recent published meta-analysis shows that epidemiologic data generally support an association between pesticides and the risk of PD (Pezzoli and Cereda, 2013). It is well known that some pesticides are toxic to humans after acute exposure to a very high amount (poisoning). However, the effects of chronic, low-dose exposure to this diverse group of chemicals are not so clear. An analysis of over 100 epidemiologic studies establishes that pesticide exposure (in the absence of poisoning) is indeed linked to PD (Pezzoli and Cereda 2013). Perhaps one of the most important unanswered questions is which pesticides are associated with PD. Of several specific pesticides or chemical classes of pesticides evaluated in the meta-analysis, only the herbicide paraquat was significantly associated with PD. But what is needed is detailed information on the nature of exposure—which pesticides, at what dose, and for how long—to help design policies and practices that prevent the relevant exposures. Genetic susceptibility is one of the factors that may modify effects of pesticides on the risk of PD. Studies of gene variants related to pesticide metabolism and transport, to mitochondrial dysfunction and oxidative stress, and to familial forms of PD suggest that associations of pesticides with PD are stronger in genetically susceptible individuals (Cannon and Greenamyre 2013). Again, most studies have assessed effects of pesticides as a group; studies of genetic susceptibility will prove most fruitful when they focus on specific pesticides. Also needed are informations on the cellular and molecular mechanisms that, over time, lead from pesticide exposure to neurodegeneration and ultimately to PD.
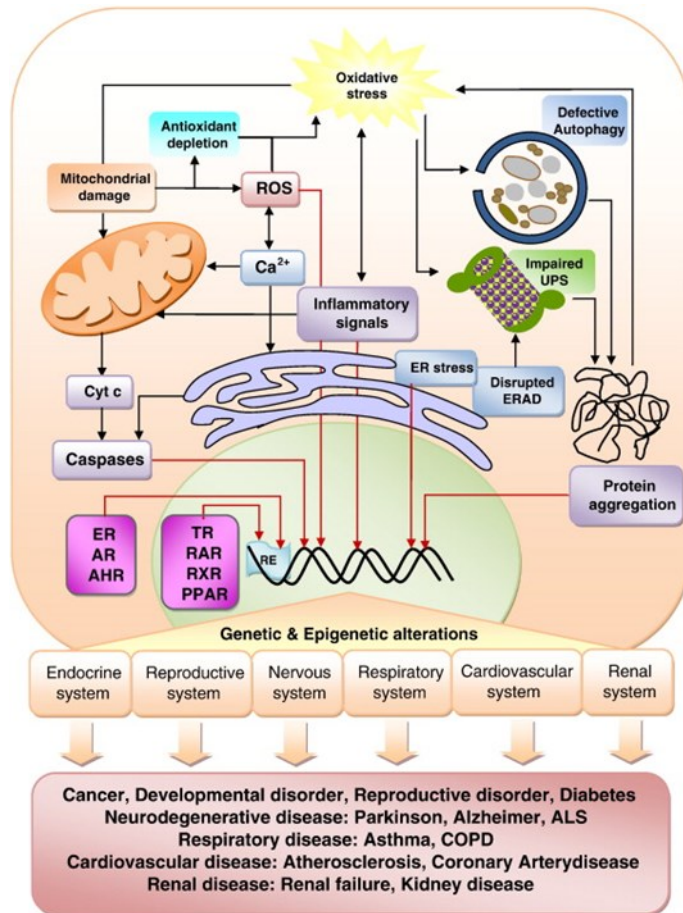
Figure 8. Simplified model for mechanisms by which pesticides induce and develop chronic disease.

2.6 Synthesis, Metabolism and Dopamine transmission
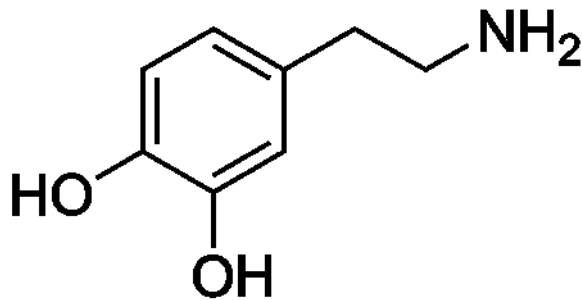
Figure 9. Structure of dopamine, the principal neurotransmitter involved in Parkinson's Disease.

The amino acids phenylalanine and tyrosine are the precursors of Dopamine (Figure 9). For the most part, mammals convert dietary phenylalanine to tyrosine by phenylalanine hydroxylase (predominantly located in the liver). Tyrosine crosses readily into the brain through uptake; however, unlike tryptophan, normal brain levels of tyrosine are typically saturating. Conversion of tyrosine to L-DOPA (3,4-dihydroxyphenylalanine) by the enzyme tyrosine hydroxylase is the rate-limiting step in the synthesis of DA. Tyrosine hydroxylase is a mixed-function enzyme that requires iron and a biopterin co-factor; enzyme activity is regulated by phosphorylation and by end-product inhibition. Four alternatively spliced isoforms of tyrosine hydroxylase have been identified in humans, which is in contrast to many non-human primates (two isoforms) and rat (one isoform). At present, it is unclear if these various isoforms play different roles. Once generated, L-DOPA is rapidly converted to DA by L-aminoacid aromatic decarboxylase (AADC), the same enzyme that generates 5-HT from L-5-hydroxytryptophan. In the CNS and periphery, AADC activity is very high, and basal levels of L-DOPA cannot be readily measured.
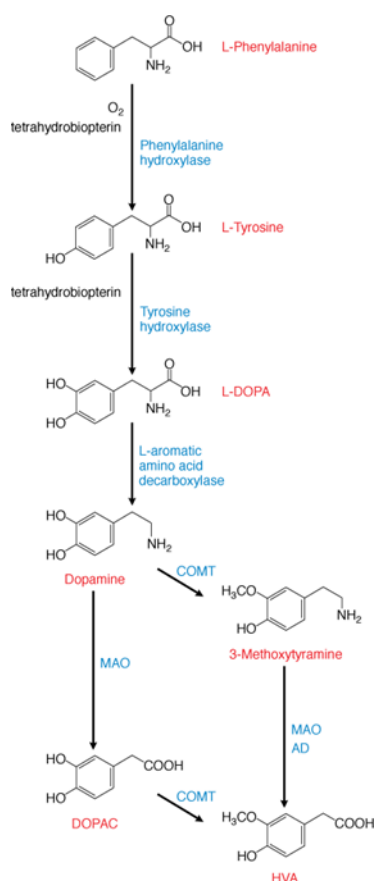
Figure 10. Schematic of Dopamine Synthesis and Metabolism (from Goodman & Gilmans, Edition 12).

Metabolism of DA occurs primarily by the cellular MAO enzymes localized on both pre- and postsynaptic elements. MAO acts on DA to generate an inactive aldehyde derivative by oxidative deamination (Figures 5), which is subsequently metabolized by aldehyde dehydrogenase to form 3,4-dihydroxyphenylacetic acid (DOPAC).

DOPAC can be further metabolized by catechol-O-methyltransferase (COMT) to form homovanillic acid (HVA). Both DOPAC and HVA, as well as DA, are excreted in the urine. Levels of DOPAC and HVA are reliable indicators of DA turnover; ratios of these metabolites to DA in cerebral spinal fluid serve as accurate representations of brain dopaminergic activity. In addition to metabolizing DOPAC, COMT also utilizes DA as a substrate to generate 3-methoxytyramine, which is subsequently converted to HVA by MAO (Goodman & Gilman's Ed. 12).

Dopamine neurotransmission

The neurochemical events that underlie DA neurotransmission are summarized in Figure 6. In dopaminergic neurons, synthesized DA is packaged into secretory vesicles (or into granules within adrenal chromaffin cells) by the vesicular monoamine transporter, VMAT2. This packaging allows DA to be stored in readily releasable aliquots and protects the transmitter from further anabolism or catabolism. Synaptically released DA is subject to both transporter clearance and metabolism. The DA transporter (DAT) is not selective for DA; moreover, DA can also be cleared from the synapse by the NE transporter, NET. Reuptake of DA by the DA transporter is the primary mechanism for termination of DA action, and allows for either vesicular repackaging of transmitter or metabolism. The DA transporter is regulated by phosphorylation, offering the potential for DA to regulate its own uptake.
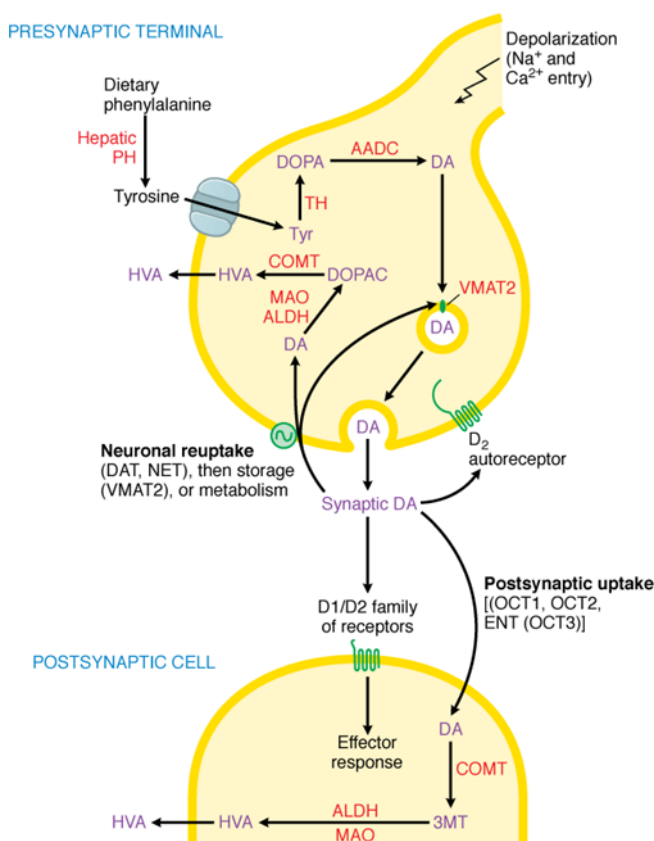
Figure 11. Dopaminergic nerve terminal. Dopamine (DA) is synthesized from tyrosine in the nerve terminal by the sequential actions of tyrosine hydrolase (TH) and aromatic amino acid decarboxylase (AADC). DA is sequestered by VMAT2 in storage granules and released by exocytosis. Synaptic DA activates presynaptic autoreceptors and postsynaptic D1 and D2 receptors. Synaptic DA may be taken up into the neuron via the DA and NE transporters (DAT, NET), or removed by postsynaptic uptake via OCT3 transporters. Cytosolic DA is subject to degradation by monoamine oxidase (MAO) and

aldehyde dehydrogenase (ALDH) in the neuron, and by catechol-O-methyl transferase (COMT) and MAO/ALDH in non-neuronal cells; the final metabolic product is homovanillic acid (HVA).

2.7 *Clinical diagnosis of PD*

The characteristic features of PD are bradykinesia, rigidity and rest tremor. These may not all be present. Postural instability may be a feature, though early postural instability backwards particularly with a history of falls is more suggestive of progressive supranuclear palsy (PSP). The clinical findings are usually asymmetrical in PD. The clinical diagnosis may often appear straightforward, though it is worth noting that post-mortem studies have shown an alternative diagnosis in up to a quarter of patients with PD diagnosed by general neurologists. Of note, there is substantially less diagnostic error in patients diagnosed in expert movement disorder clinics which strengthens the argument for early referral of patients to specialists expert in movement disorders.

There are a number of other clinical signs that are worth highlighting. A change of handwriting with micrographia is often an early feature as is reduced facial expression. A loss of arm swing on one side is also an early and useful diagnostic feature. A glabellar tap does not seem to be particularly sensitive or specific.

A reduced sense of smell is, however, worth asking about since this may be one of the first symptoms in early PD. As the disease becomes more advanced, hypophonia, drooling of saliva (from reduced swallowing) and impairment of postural reflexes may develop. Non-motor complications of the disease often become more troublesome as the disease progresses. It is helpful to enquire about symptoms of depression which occurs in ~40% of PD patients.

The diagnosis of essential tremor should be considered when a patient presents with a symmetrical limb tremor, worse with posture and is suppressed by alcohol. Head or voice tremor may also be present. In this condition, there may be an autosomal dominant inheritance, suppression of the tremor with alcohol and there should be no evidence of rigidity or bradykinesia on examination. Adult onset dystonia may also present with asymmetrical rest tremor and may explain some patients previously labelled as 'benign tremulous PD' who have scans with no evidence of dopaminergic deficit. Although the diagnosis of PD is a clinical one, there are certain situations where investigations can prove useful.

Conventional brain imaging with MRI or CT is usually not required unless an alternative diagnosis is suspected such as normal pressure hydrocephalus or vascular parkinsonism.

Single photon emission computerized tomography (SPECT) imaging using a dopamine transporter (DAT) can be helpful in differentiating PD from a number of conditions, including essential tremor and dystonic tremor, neuroleptic-induced parkinsonism and psychogenic parkinsonism all of which demonstrate normal DAT scans. Uptake within the basal ganglia is reduced in PD, the parkinsonian syndromes and DLB.

2.8 Existing pharmacological therapies for Parkinson's Disease

While these are some effective treatments for patients with PD but treatment strategies are mainly symptomatic and aimed at increasing dopamine levels in the degenerating nigrostriatal system.

Existing drugs are limited in their relief and decrease in effectiveness as PD progresses. Nevertheless, these treatments do not halt the progressive nature of PD and are ineffective against gait freezing and postural instability, which are more disabling than tremors and rigidity. Further, as PD progresses, drug-induced motor complications, such as "wearing off" and dyskinesia appear and notes that PD dementia is not responsive to dopaminergic therapy and is considered the most important factor influencing the daily activities and survival of Parkinson's patients.

The main families of drugs useful for treating motor symptoms are levodopa (usually combined with a dopa decarboxylase inhibitor or COMT inhibitor), dopamine agonists and MAO-B inhibitors. The most logical approach to correcting this problem is to replace the dopamine (DA). It cannot be taken orally as it does not cross the blood brain barrier (BBB). L-dopa therapy is the gold standard treatment for Parkinson's disease. L-dopa is a naturally occurring substance. It is in the chemical family of compounds known as amino acids. Amino acids are the building blocks of proteins. Its absorption can be reduced if taken with a high protein meal. Once absorbed, L-dopa is converted to DA in the blood, liver and kidneys. A compound called carbidopa is given with L-dopa. Carbidopa inhibits the enzyme in the liver, kidneys, and blood that converts L-dopa to DA. This allows more L-dopa to pass into the brain. Carbidopa and benserazide are peripheral dopa decarboxylase inhibitors, which help to prevent

the metabolism of L-DOPA before it reaches the dopaminergic neurons, therefore reducing side effects and increasing bioavailability. Levodopa has been related to dopamine dysregulation syndrome, which is a compulsive overuse of the medication, and punding. Tolcapone inhibits the COMT enzyme, which degrades dopamine, thereby prolonging the effects of levodopa. It has been used to complement levodopa; however, its usefulness is limited due to its side effects such as liver damage. Dopamine agonists were first introduced in the 1970s. Dopamine agonists have the capacity to directly stimulate brain dopamine receptors. The first generation of dopamine agonists were all ergot derivatives, including bromocryptine, cabergoline, dihydroergocriptine, lisuride, and pergolide., Also the non-ergot agonist is apomorphine which is a potent mixed D1-type and D2- type dopamine agonist receptor agonist with L-dopa-like antiparkinsonian effects. Most of the currently used nonergot dopamine agonists include pramipexole, ropinirole, rotigotine, and piribedil. Rotigotine is available as a once-daily transdermal patch formulation while apomorphine is used as subcutaneous infusion treatment for patients with refractory response oscillations or as rescue medication for sudden unpredictable off periods. Selegiline and rasagiline are both irreversible inhibitors of MAO-B producing dopaminergic effects. They inhibit monoamine oxidase-B (MAO-B) which breaks down dopamine secreted by the dopaminergic neurons. The reduction in MAO-B activity results in increased L-DOPA in the striatum. Selegiline is metabolized to methamphetamine and to a lesser extent, to amphetamine which blocks dopamine reuptake and releases dopamine and may account for some of the dopaminergic effects. Rasagiline even highly potent and does not give rise to amphetamine like metabolites, but degrades to aminoindan and selegeline, rasagiline and aminoindan have neuroprotective properties. Other drugs such as amantadine and anticholinergics may be useful as treatment of motor symptoms. Amantadine's exact mechanism of action remains uncertain, its NMDA receptor–blocking activity is generally believed to play a major role in producing its anti parkinsonian effects. In the 1860s Ordenstein and Charcot were the first to treat (PD) by using extracts from Hyscyamus niger, Atropa belladonna, and Datura stramonium. With the development of synthetic anticholinergic drugs, they were the gold standard for treating PD in the 1940s.

2.9 Animal models of Parkinson's disease: classical toxin-induced rodent models

Over the last two decades, significant strides has been made toward acquiring a better knowledge of both the etiology and pathogenesis of Parkinson's disease (PD). Experimental models are of paramount importance to obtain greater insights into the pathogenesis of the disease.

Model	Gradual loss of dopamine neurons in adulthood	Easily detectable motor deficits	Development of Lewy bodies	Based on a single mutation	Short timecourse
6-hydroxydopamine	No	Yes (quantifiable rotation deficit)	No	N.A.	Yes
Rotenone	Yes, but variable individual susceptibility	Yes	Yes	N.A	Yes
Acute MPTP*	No	Yes	No	N.A.	Yes
Drosophila α-synuclein overexpression	Yes	Yes	Yes	Yes	Yes
Mouse α-synuclein overexpression	Yes, but not in the substantia nigra	Yes	Nuclear as well as cytoplasmic inclusions	N.A.	No (1 year)

*Chronic MPTP administration produces slow evolution of a parkinsonian syndrome and might produce Lewy bodies. (MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; N.A., not applicable.)

Table 1. Characteristics of Animal Models for Parkinson's disease

Thus far, neurotoxin based animal models have been the most popular tools employed to produce selective neuronal death in both in vitro and in vivo systems. These models have been commonly referred to as the pathogenic models.

The two most widely used rodent models of PD are the classical 6-OHDA-treated rat and the MPTP-treated mouse.
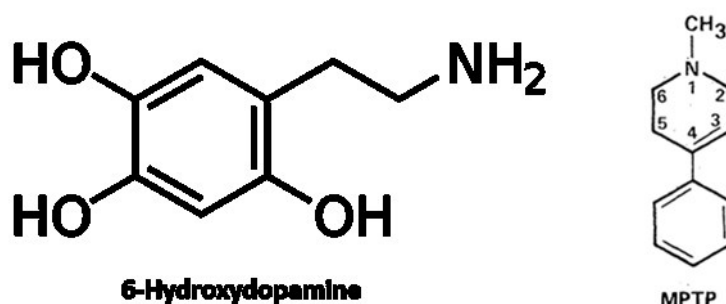

Figure 12. Chemical structure of 6-hydroxidopamine (6-OHDA) and MPTP.

Of these, the 6-OHDA model has been extensively used as a test bed for novel symptomatic agents as well as providing a means for assessing neuroprotective and neurorepair strategies. Although unlikely to be the first model of choice for testing symptomatic agents, since its behavioural phenotype is less robust than the 6-OHDA rat, the MPTP-treated mouse provides a useful secondary screening model and has the added advantage of being relatively easy to construct compared with the 6-OHDA rat. The characterization of the hydroxylated analogue of dopamine, 6-OHDA, as a toxin-inducing degeneration of dopaminergic neurons in the nigro-striatal tract (Ungerstedt, 1968) has led to it being a widely used tool to induce Parkinsonism in rodents. Unlike MPTP, 6-OHDA does not efficiently cross the blood–brain barrier and so requires direct injection into the brain. 6-OHDA is injected into the nigro-striatal tract at one of three locations: into the substantia nigra pars compacta (SNpc) where the A9 dopaminergic cell bodies are located; into the median forebrain bundle (mfb), through which the dopaminergic nigro-striatal tract ascends; or into the terminal region, the striatum. Following its injection, 6-OHDA is taken up into the dopaminergic neurons via the dopamine transporter, DAT (Figure 13). Given that 6-OHDA also shows high affinity for the noradrenaline transporter, NET (Luthman et al., 1989). Although the exact mechanism behind 6-OHDA toxicity is still subject to investigation, current understanding is that, once inside dopaminergic neurons, 6-OHDA initiates degeneration through a combination of oxidative stress and mitochondrial respiratory dysfunction. Certainly, 6-OHDA readily oxidizes to form reactive oxygen species (ROS) such as H_2O_2 (Mazzio et al., 2004), to reduce striatal levels of antioxidant enzymes [total glutathione (GSH) or superoxide dismutase] (Perumal et al., 1992; Kunikowska and Jenner, 2001), to elevate levels of iron in the SN (Oestreicher et al., 1994) and to interact directly with complexes I and IV of the mitochondrial respiratory chain, leading to subsequent respiratory inhibition and further oxidative stress (Glinka et al., 1997). Many of these effects are thought to mirror events occurring in PD brain (Jenner, 1989), thereby supporting a high degree of construct validity for the 6-OHDA model. The 6-OHDA model also mimics many of the biochemical features of PD, including reduced levels of striatal dopamine and tyrosine hydroxylase (TH; rate-limiting step of DA biosynthesis). The 6-OHDA model shares a common failing with many other animal models of PD as it does not lead to the formation of the pathological hallmark of PD, the Lewy body. Lewy bodies are eosinophilic inclusions that contain ubiquitinated proteins such as a-synuclein (Spillantini *et al*., 1997 and 1998) and are associated with lipofuscin-containing lysosomes that

have also been shown to accumulate α-synuclein in PD brain stem (Braak *et al*., 2001). The exact role of Lewy bodies remains to be established, but drugs to reduce aggregate formation are considered a potential future strategy for treating PD. A recent report of parkin-containing aggregate formation in 6-OHDA-lesioned rat (Um *et al*., 2010) is therefore an exciting advance but requires confirmation. The one pathological feature of PD robustly displayed by the 6-OHDA model is degeneration of the nigro-striatal tract. The extent of degeneration can be established post-mortem by assessing the reduction in various parameters in the lesion (ipsilateral) hemisphere, compared with the intact (contralateral) hemisphere including number of nissl-stained cells or TH-positive neurons in the SNpc; levels of TH or DAT immunoreactivity in the striatum and levels of [3H]mazindol binding to DAT in the striatum. Behavioural indices can also be taken as a potential pre-screen for predicted lesion size.

MPTP is a commonly used toxin for inducing both rodent and primate models of PD based on its ability to induce persistent Parkinsonism in man (Davis *et al*., 1979; Langston *et al*., 1983). Subsequent investigations in non-human primates identified that selective destruction of dopaminergic neurons of the nigro-striatal tract was the pathological basis

behind the motor deficits observed (Burns *et al*., 1983; Jenner *et al*., 1984; Langston *et al*., 1983), and out of this came the most relevant animal model of PD that persists today. The

impact of the MPTP-treated primate model in the PD field is second to none, but first we will focus attention on the use of MPTP in non-primate species. Many species, including rats, are insensitive to the toxic effects of MPTP, possibly due to the relatively rapid clearance of MPP+, the toxic metabolite of MPTP (Figure 13; Johannessen *et al*., 1985). However, specific strains of mice, notably black C57, and Swiss Webster are sensitive to MPTP (Sonsalla and Heikkila, 1988) and have enabled development of the MPTP mouse model of PD. The mechanism behind the neurotoxic action of MPTP has been the subject of intense investigation and is relatively well understood (see also Materials and Methods paragraph). MPTP is a lipophilic pro-toxin that, following systemic injection (usually i.p. or s.c.), rapidly crosses the blood–brain barrier (Riachi *et al*., 1989). Once inside the brain, MPTP is converted by MAO-B (principally in glia and serotonergic neurons) into the intermediary, 1-methyl-4-phenyl-2,3,dihydropyridinium (MPDP+) before its rapid and spontaneous oxidation to the toxic moiety, 1-methyl-4-phenylpyridinium (MPP+) (Chiba *et al*., 1984). Following

its release into the extracellular space, MPP+ is taken up via DAT into dopaminergic neurons where cytoplasmic MPP+ can trigger the production of ROS, which may contribute to its overall neurotoxicity (Javitch *et al*., 1985). However, the majority of MPP+ is eventually accumulated within mitochondria where the key toxic mechanism occurs. Once inside mitochondria, MPP+ impairs mitochondrial respiration via inhibition of complex I of the electron transport chain. This action impairs the flow of electrons along the respiratory chain, leading to reduced ATP production and the generation of ROS, such as superoxide radicals. The combined effects of lowered cellular ATP and elevated ROS production are most likely responsible for initiation of cell death-related signalling pathways such as p38 mitogen-activated kinase (Karunakaran *et al*., 2008), c-jun N-terminal kinase (JNK) (Saporito *et al*., 2000) and bax (Hassouna *et al*., 1996; Vila *et al*., 2001), all of which have been demonstrated *in vivo* following MPTP treatment and may contribute to apoptotic cell death (Jackson-Lewis *and* Przedborski S, 2007). The MPTP-treated mouse has some clear advantages over the 6-OHDA lesion model, not least of all economic benefits in terms of the cheaper costs associated with purchasing and housing mice. Being systemically active, MPTP administration does not require the type of skilled stereotaxic surgery that production of a 6-OHDA lesion requires. The systemic injection also produces a bilateral degeneration of the nigrostriatal tract, more reflective of that seen in PD. The MPTP model also mimics many of the known biochemical features of PD. For example, in addition to the well-known reductions in striatal dopamine and TH, there are also elevated levels of both striatal PPE-A (Gudehithlu et al., 1991) and ACh (Hadjiconstantinou et al., 1985). Further downstream in the basal ganglia, extracellular glutamate levels have been shown to be elevated in the SN of MPTP-treated mice, a rise associated with the induction of programmed cell death (Meredith et al., 2009), also glutathione (GSH) levels are significantly reduced (Ferraro et al., 1986) as in PD itself. Finally, in further support of the face validity of this model, inflammatory markers are elevated in the striatum and SN of MPTP-treated mice (Kurkowska-Jastrzebska et al., 1999; Hebert et al., 2003), which occurs as a result of reactive microgliosis in PD. The MPTP model does, however, have some clear disadvantages over the 6-OHDA model, particularly in terms of reproducibility and the range of behavioural readouts that can be obtained. Mice are far less sensitive to MPTP than primates, and the higher doses required can be acutely lethal as a result of the peripheral neuro- or cardiotoxicity induced (see Jackson-Lewis and Przedborski, 2007). Given that the risk of mortality usually occurs

within 24 h of the first dose of MPTP and is dose-dependent, the high rates (up to 50%) seen following acute bolus dosing in the earlier studies (see, e.g., Ferger et al., 2000) can be reduced to acceptable levels (<20%) with reductions in the dose administered and can be almost completely avoided using alternative protocols in which the same or even higher total dose is given in multiple doses (e.g. Gibrat et al., 2009). The various dosing regimens used to generate the MPTP mouse model have been extensively reviewed in Jackson-Lewis and Przedborski, 2007. In many cases, MPTP is given with probenecid (250 mg·kg-1), a uricosuric agent that reduces the renal clearance of MPTP, thereby prolonging its action. The most common protocols and the degree of nigrostriatal tract denervation produced by these can be summarized as follows: acute bolus, 1 x 30–40 mg·kg-1 giving 80–90% striatal DA depletion; acute multiple, 2 x 40 mg·kg-1 or 4 X 12.5–25 mg·kg-1 given at 2 h intervals producing variable 60–90% striatal DA loss; sub-acute, 25–40 mg·kg-1/day for 5 days plus probenecid giving 76% loss striatal DA and 60% SNpc cell loss; chronic intermittent, 25 mg·kg-1 twice weekly for 5 weeks+ probenecid, giving 95% loss around 1 week, but reducing to a stable 70–80% loss by 12 weeks post treatment (Pothakos et al., 2009). Chronic infusion can be performed by administration of 20–40 mg·kg-1/day for up to 28 days given via osmotic minipumps, giving most variable degree of cell loss so far ranging from 25% to 80% loss of cells in the SNpc and 28–90% loss of striatal dopamine (Fornai et al., 2005; Alvarez-Fischer et al., 2008). The pattern of cell death produced is similar to that seen in humans, with the SNpc affected more than the VTA , and chronic infusion may also induce loss of noradrenergic cells in the locus coeruleus, further resembling the clinical picture (Fornai et al., 2005). However, in all cases, the cell death is rapid in onset, with first signs appearing within 12–72 h, and is maintained for up to 28 d (Novikova et al., 2006), although striatal dopamine depletion may show signs of recovery when using acute or sub-acute MPTP dosing paradigms (Lau and Meredith, 2003). As noted for the 6-OHDA model, this rapidity of cell death is not reflective of the disease itself and is an obvious weakness of this model.
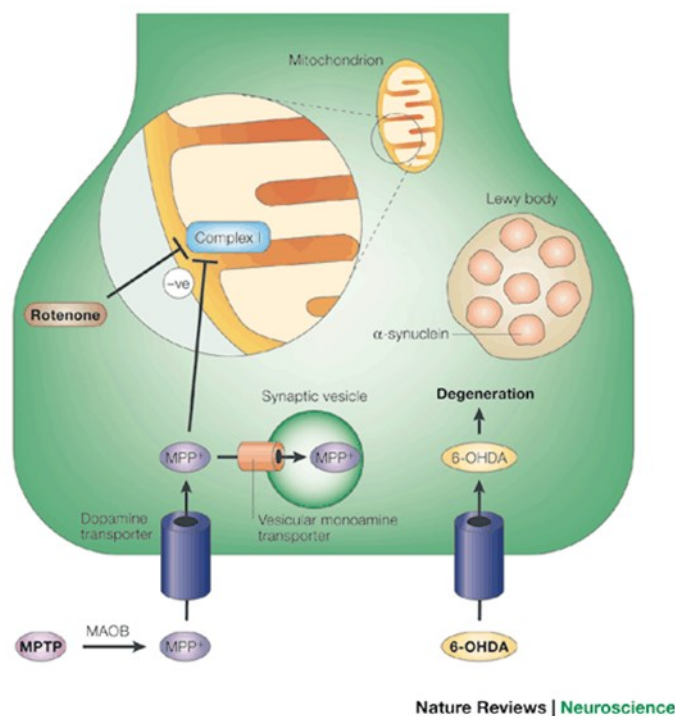
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Figure 13. The mechanisms by which neurotoxins kill dopamine neurons involve mitochondrial dysfunction and oxidative damage. 6-hydroxydopamine (6-OHDA) is taken up by the dopamine transporter and it then generates free radicals. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is converted by monoamine oxidase B (MAOB) to 1-methyl-4-phenylpyridinium (MPP+). MPP+ is taken up by the dopamine transporter and can then be accumulated by mitochondria, leading to complex I inhibition and the generation of free radicals, or by the vesicular monoamine transporter, thus reducing toxicity. Rotenone is a direct inhibitor of complex I, which also leads to free-radical generation. MPTP and rotenone treatment increase the expression of alpha-synuclein and, in the latter case, this leads to the formation of Lewy bodies.

2.10 Cell therapy for Parkinson's Disease

Cell replacement therapy for treatment of PD is based on the idea that immature dopaminergic neurons, following transplantation and functional integration, can restore dopaminergic neurotransmission and exert long-lasting effects on motor symptoms. Based on data from animal experimentation, the current main focus is on elevating striatal dopamine levels rather than trying to reconstruct the relatively long nigrostriatal pathway.

Particular emphasis was attributed to the transplantation of fetal ventral mesencephalic tissue, which has resulted in the greatest clinical benefits in open labelled clinical trials. This approach was followed more recently by a discussion of future perspectives of cell replacement for PD, with particular focus on stem cell based strategies.

Stem cells to treat Parkinson's disease

Stem cell research has the potential to significantly impact the development of disease-modifying treatments for Parkinson's disease, and considerable progress has been made in creating dopamine-producing cells from stem cells. The development of new cell models of Parkinson's disease is a particularly promising area of stem cell research, as the current lack of progressive, predictive models of Parkinson's disease remains a major barrier to drug development. Stem cells can help researchers as they can be utilized as cellular model to study pathogenesis of the disease particularly when it is possible to use cells taken from people living with the disease. Moreover, cellular models of Parkinson's disease generated from stem cells could help the drug screening.
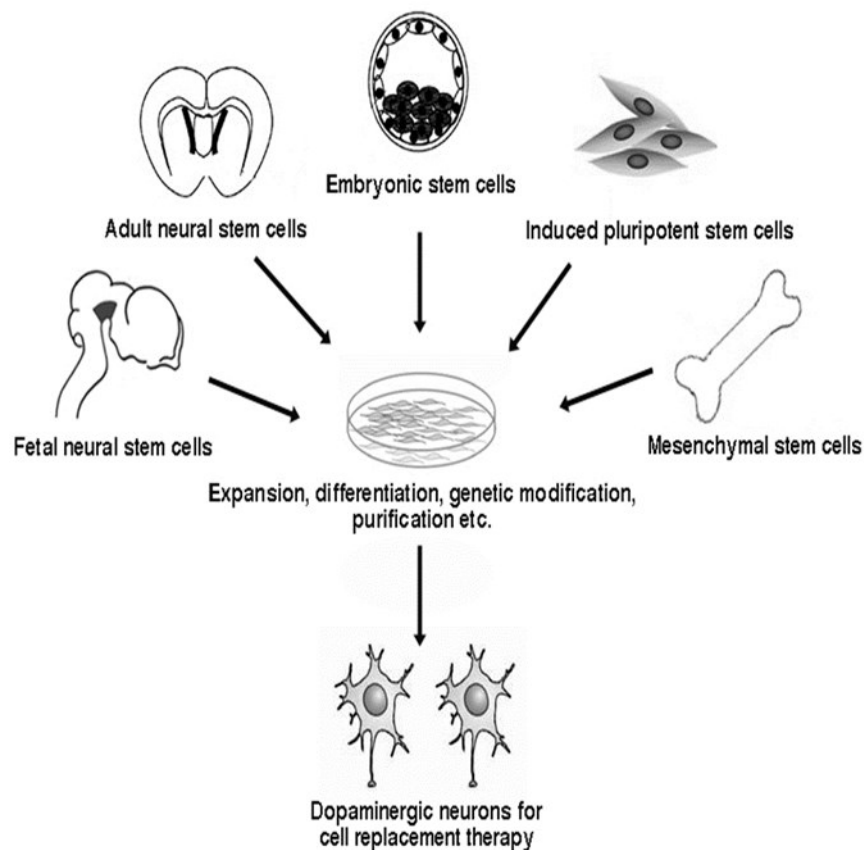

Figure 14. Sources of stem cells for cell therapy approach in PD (from Jensen et al., 2011)**.**

However, there are many challenges that need to be overcome before stem cell-based cell replacement therapies for Parkinson's disease become a reality. Work

is still needed to generate robust cells, in both quality and quantity, that can also survive and function appropriately in a host brain. Although ES (and more recently induced pluripotent stem cells, iPS) cells hold great potential, we do not yet know which stem cell type ultimately holds the greatest promise. Numerous studies using expanded and/or induced bone marrow-derived mesenchymal stem cells have been reported for animal models and yet only three clinical studies with intracerebral or intravasal application of these cells have been reported for PD patients. In two open label studies, subventricular application of both allogenic and autologous bone marrow-derived mesenchymal stem cells showed improvement of motor behavior as reflected by reduction of UPDRS ON and OFF scores in most but not all PD patients (Venkataramana et al 2010 and 2011). Furthermore, generation of iPS cells provides a new avenue for transplantation therapy as it can avoid immunorejection, a major complication in current transplantation medicine.

Neural tissue transplants

This cell therapy approach was pioneered in the 1970ies to develop methods for cell transplantation to the mammalian brain. Important contributions during this dynamic period included studies on the survival, integration and functional connectivity of fetal neuroblasts grafted to the CNS of adult rats, functional cell replacement in rodent models of Parkinson's disease.

In the mid-1980ies was performed a series of preclinical experimental studies that provided the experimental and methodological basis for the first open-label clinical trials that were initiated at Lund University Hospital in 1987. The results obtained in the Lund PD transplantation program have provided proof-of-principle that human fetal midbrain dopamine neurons can survive and function for many years (more than a decade) after transplantation to the striatum in patients with advanced Parkinson´s disease (1990-2005). The Lund PD transplantation program has over the ensuing decades been one of the leading programs in the development of restorative therapies in Parkinson´s disease. In 2008 were published results obtained from three independent groups reporting findings obtained from post-mortem tissue analyses in a total of eight subjects transplanted with transplanting fetal nigral dopaminergic nerve cells, with the intention of replacing striatal dopamine. These studies showed that long-term clinical benefits in six individuals who survived 9–16 years after surgery is limited.

Moreover three of these subjects showed brain damages typical of Parkinson's disease at autopsy, such as α-synuclein

inclusions (Lewy bodies) (Mendez, I. et al. 2008; Kordower, J.H. et al. 2008; Li, J.-Y. et al. et al. 2008). The results se studies provide insights into the extent to which pathogenic factors in the host can influence the transplanted cells.

The transplantation of stem cells or fetal nerve cells in the hopes of re-innervating the striatum in individuals with severe Parkinson's disease was initially heralded as a breakthrough. But the results, which depend on a wide variety of factors—age, disease duration, site of implantation, graft size and administration of immunosuppressive therapies—have been generally mixed. Moreover, graft recipients can experience adverse effects, including dyskinesias (Freed et al. 2001; Snyder, B.J. & Olanow, C.E. 2005). The additional risk factor of developing Lewy pathology open a strong issue about surgical replacement options, especially because we know that transplantation of fetal nerve cells does not alleviate neuronal dysfunction or neuronal loss at extranigral sites (Snyder, B.J. & Olanow, C.E. 2005)

Embryonic stem cells

For a long time ESCs have been considered the most promising source of cells for future cell replacement therapy in PD. However, ethical concerns regarding the use of human embryos and the possibility of immune rejection due to immunological incompatibility between patient and donor ESCs may limit their use. To overcome these problems scientists have worked on the development of pluripotent stem cells from adult somatic cells, involving genetic changes (reprogramming) of the somatic cell nucleus. Until recently, reprogramming of cells involved cloning of cells by somatic cell nuclear transfer, where the nucleus in an oocyte is replaced with the nucleus from an adult somatic cell (Wilmut et al. 1997) or by fusion of an adult somatic cell with an ESC (Cowan et al. 2005). However, besides being both time consuming and technical difficult this kind of reprogramming still requires the use of human oocytes or ESCs and hence do not solve the ethical problems.

Induced pluripotent stem cells

In 2006, Takahashi and Yamanaka reported for the first time that it was possible to reprogram adult mouse fibroblasts to pluripotent stem cells by retroviral transduction with the four transcription factors Oct3/4, Sox2, c-Myc and Klf4,

which are highly expressed in ESCs (Takahashi and Yamanaka 2006). The resulting so-called induced pluripotent stem (iPS) cells share several properties with ESCs, including morphology, growth properties and pluripotency, defined by their ability to form teratomas in vivo and differentiate into distinct cell types of all three germ layers, but they are not identical to ESCs when it comes to epigenetic status (Deng et al. 2009) and gene expression (Chin et al. 2009). In addition, the original iPS cells failed to generate postnatal chimeras and contribute to the germline (Takahashi and Yamanaka 2006), but refinement of the method for selection of iPS cells has already solved these issues and moreover, the resulting iPS cells are even more similar to ECSs at the epigenetic level (Okita, Ichisaka, and Yamanaka 2007; Maherali et al. 2007; Meissner, Wernig, and Jaenisch 2007). Shortly after these initial studies in mice, iPS cells were derived from human fibroblasts using slightly different combinations of genes (Takahashi et al. 2007; Yu et al. 2007) and since then, iPS cells have been generated from a number of different cell types and species (Masip et al. 2010).

The similarities of iPS cells to ESCs, and the fact that these cells can be generated from the patient's own cells, make them very attractive candidates for cell-based therapy. Wernig and collegues reported upon transplantation into the fetal mouse brain, iPS derived neural precursor cells extensively differentiate into glia and neurons (Werning et al 2001). Functional recovery was observed after transplantation of iPS-derived midbrain dopamine neurons into the adult brain of Parkinsonian rats. Risk of tumor formation from grafted cells was minimized by the separation of contaminating pluripotent cells and committed neural cells using fluorescence-activated cell sorting. Encouraging data from rodent studies then prompted subsequent assessment in a primate model. Kikuchi and co-workers observed that human iPS-derived neural progenitor cells grafted in the brain of a primate PD model survived as dopaminergic neurons for as long as six months, implying the therapeutic potential of human iPS cells (Kikuki et al. 2012). Direct reprogramming of mouse and human fibroblasts into induced neural stem cells (iNSCs) has been proven feasible with a single factor, Sox2. iNSCs express NSC markers and resemble wild-type NSCs in their morphology, self-renewal, ability to form neurospheres and differentiate into several types of mature neurons as well as astrocytes and oligodendrocytes, indicating multipotency. Importantly, implanted iNSCs can survive and integrate in mouse brains without tumorigenic potential. Moreover, the reprogramming method used in this work allowed

shortening of the duration for neuronal cell creation from fibroblasts (Ring et al 2012).

However, before iPS cells can be used in the clinic there are several issues that need to be resolved. First, it is extremely important to optimize the procedure for reprogramming of the somatic cells. As described above, many groups have generated iPS cells by use of retroviral vectors. Since retrovirus integrates randomly in the DNA it may alter the differentiation potential or even induce malignant transformation of the iPS cells. Moreover, most research groups have used c-Myc as one of the reprogramming factors and since c-Myc is a proto-oncogene, its reactivation could give rise to transgene-derived tumor formation. In one study 20% out of 121 mice derived from an iPS cell line developed tumors. In these tumors, retroviral expression of c-Myc, but not Oct3/4, Sox2, or Klf4 was reactivated (Okita, Ichisaka, and Yamanaka 2007). Hence, recent studies have focused on developing safer reprogramming strategies, for example, by generation of iPS cells without using the c-Myc oncogene. In 2008, Nakagawa et al. generated iPS cell lines from both mouse and human fibroblasts without the use of c-Myc. They found that it was possible to generate cells expressing ESC markers, and that the induction of the fibroblasts to iPS cells was more specific, since a lower number of non-iPS cells were generated. However, efficiency of the generation process was substantially reduced with the omission of c-Myc. They also examined the tumourigenicity in mice derived from these cells and found that omission of the c-Myc retrovirus significantly reduced the risk of tumorigenicity in chimeras (Nakagawa et al. 2008). Other studies have also examined different combinations of transcription factors, and of the original four transcription factors, Oct3/4 is the only one that cannot be replaced by other transcription family members and have been required in every reprogramming strategy in both mouse and human cells. Other strategies have been to generate iPS cells using integration systems such as plasmids (Kaji et al. 2009) and transposons (Woltjen et al. 2009), which allow removal of these systems after reprogramming. Moreover, iPS cells have also been generated without genomic integration using nonintegrative adenoviral vectors (Zhou and Freed 2009; Stadtfeld et al. 2008), repeated plasmid transfection with or without the use of small molecules (Okita et al. 2010; Okita et al. 2008; Yu et al. 2009) and just addition of recombinant proteins (Zhou et al. 2009; Kim et al. 2009). Although, these methods are not yet as efficient as the retroviral approach, they have clearly shown that random DNA mutagenesis is not a requirement for

reprogramming and give hope for the development of clinically applicable iPS cells free of viruses and transgenic integration.

Interestingly, a recent publication have shown a method for direct reprogramming of fibroblasts into neurons, avoiding generation of undifferentiated cells and their following differentiation (Vierbuchen et al. 2010). They started from a pool of nineteen neural-lineage specific transcription factors and identified a combination of only three factors (Mash1, Brn2 and Myt1l) that were sufficient to convert mouse fibroblasts into functional neurons in vitro. Such induced neurons clearly have some advantages to iPS cells, since they avoid generation of pluripotent cells that can give rise to tumors. In this study the induced neurons mainly gave rise to GABAergic neurons after differentiation, but their regional identity is still unclear (Vierbuchen et al. 2010). This strategy offers new perspectives to the reprogramming field and future studies need to determine if certain cell types are more useful in generation of specific cells for replacement strategies and if it is necessary to reprogram cells all the way back to pluripotent stem cells.

	Advantages	Disadvantages
ESCs	• Unlimited proliferation capacity • Potential to generate any cell type (pluripotent) • Generation of stem cell banks	• Risk of teratomas (heterogenous composition) • Time demanding/complex differentiation into dopaminergic neurons • Risk of immune rejection • Ethical issues
NSCs	• Restricted differentiation potential (multipotent) • Low risk of tumor formation	• Limited proliferation capacity • Reduced neuronal differentiation capacity following long-term proliferation • Risk of immune rejection • Ethical issues (fetal NSCs)
MSCs	• Easily harvested • Possibility of generating patient specific cells • No ethical concerns	• Differentiation into "truly" functional dopaminergic neuron still uncertain
iPS cells	• Unlimited proliferation • Potential to generate any cell type (pluripotent) • Generation of stem cell banks • Possibility of generating patient specific cells • No risk of immune rejection • No ethical concerns	• Risk of teratoma (heterogenous composition) • Risk of tumor formation (viral transduction) • Time demanding/complex differentiation into dopaminergic neurons • Expensive procedure

Table 2. Overview of advantages and disadvantages of different types of stem cells for cell replacement therapy for Parkinson Disease (from Jensen et al 2011).

Therapeutic molecules released by stem cells

Instead of acting as a source of cells for neuronal replacement an alternative use of stem cells for transplantation is as so-called "cell factories" releasing neurotrophic and neuroprotective factors. Several neurotrophic factors have been shown to stimulate survival and/or differentiation of nigral dopaminergic neurons in vitro and in vivo (Evans and Barker 2008; Levy et al. 2005). Among neurtrophic factors, GDNF and some of the GDNF family members have shown the greatest promise as therapeutic agents for PD. Several studies have shown that GDNF can protect against nigrostiatal lesions in animal models. For instance, intravenous administration of MSCs overexpressing GDNF in mice before lesioning with MPTP has been shown to protect dopaminergic neurons in the substantia nigra as well as their striatal fibers (Park, Eglitis, and Mouradian 2001). In agreement with this observation, another study has shown that GDNF-producing mouse NSCs transplanted into the mouse striatum 16 days prior to 6-OHDA lesioning could prevent death of dopaminergic neurons and resulted in a reduced behavioral impairment (Akerud et al. 2001). A similar protective effect was found by overexpressing Neurturin, another member of the GDNF family, in the same NSC line (c17.2 NSCs derived from the mouse cerebellum) (Liu et al. 2007). NSCs are considered very suitable for ex vivo gene delivery, since they can be cultured, differentiated into specific neuronal subtypes, transduced and selected for subsequent transplantation to the brain where they can migrate and extend projections. Human NCSs genetically modified to overexpress GDNF in an inducible way have been shown to express GDNF for up to 3 month in the brains of rodents and aged primates (Behrstock et al. 2006).

Furthermore, transplantation of these cells after a partial lesion of the dopamine system resulted in increased host dopamine neuron survival and fiber outgrowth. MSCs are also very suitable as neuroprotective cells through secretion of neurotrophic factors and furthermore, these cells can easily be harvested from the patient's own bone marrow in order to avoid immune rejection. Along this line, Sadan et al. (2009) have recently developed a protocol to induce MSCs into neurotrophic factor-secreting cells, especially producing GDNF and BDNF without genetic modification of the cells.

Another way to overcome problems of host immunoresponses when using allografts, is the development of new materials to encapsulate transplanted cells, thereby serving as a biological shield to prevent immune rejection and eliminate the need for immunosuppression. The encapsulating material should allow

inward diffusion of nutrients to the transplanted cells and at the same time permit outward secretion of trophic factors. Besides protection from the host immune system, these capsules have the advantage that they can be removed for safety reasons or for renewal of cells. Several studies have reported successful delivery of GDNF from encapsulated cells (Kishima et al. 2004; Tseng et al. 1997; Ahn et al. 2005). Kishima et al. (2004) found a transient recovery in motor function after intraventricular infusion of encapsulated GDNF-producing cells in MPTP lesioned baboons, however no neuroprotection against the toxin was found. The lack of neuroprotective effect may be due to the intraventricular placement of the capsules resulting in inefficient passage of GDNF to the striatum, thus better graft sites should be used. Furthermore, they reported GDNF release and survival of encapsulated cells for more than 1 year after transplantation (Kishima et al. 2004).

Delivery of neurotrophic factors by co-grafted genetically modified stem cells or cell lines may also serve as a useful tool for stimulation of functional integration and protection of grafted dopaminergic neurons from any source. In one study, Ahn et al. (2005) tested such a strategy by co-grafting an encapsulated cell line overexpressing GDNF with human fetal mesencepahlic tissue into 6-OHDA lesioned rats, leading to increased fiber outgrowth in areas between the capsules and the grafts. This result indicates that continuous delivery of GDNF or other important neurotrophic factors via encapsulated genetically modified cells could further optimize neural transplantation as a therapy for PD.

2.11 Post- Mortem Neural Precursors Cells

In 2010 we were able to isolate and characterize adult neural stem cells from the mouse subventricular zone (SVZ) at different time after animal killing (Marfia et al., 2011). The isolation of ischemia-resistant neural precursors supplied cells with properties different from NPCs, and able to survive in the not favorable environment of the traumatically injured central nervous system (Molcanyi et al., 2007; Popovich et al., 2003; Marfia et al 2011). This new type of neural stem cells were called Post-Mortem Neural Precursor Cells (PM-NPCs). Their proliferation is similar to that of NPCs obtained at killing time (T0) (Gritti et al., 1996; Marfia et al., 2011) and was investigated by population analyses. Post-mortem NPCs (PM-NPCs) proliferate in response to the exposure to bFGF and EGF, and formed typical neurospheres identical to those formed by NPCs

obtained from fresh tissue (Figure 15). Under these conditions, less than 1% of the total cells plated survived, and began to divide, until a spherical cluster of cells was formed. We observed continuous growth over 1 year in presence of EGF and FGF-2, and the rate between the different types of PM-NPCs groups and T0 NPCs was not significantly different (Figure 15). The capability to generate neurospheres was also investigated. The total number of neurospheres generated within 7 days of cell seeding was calculated, and the average was plotted (Figure 15).
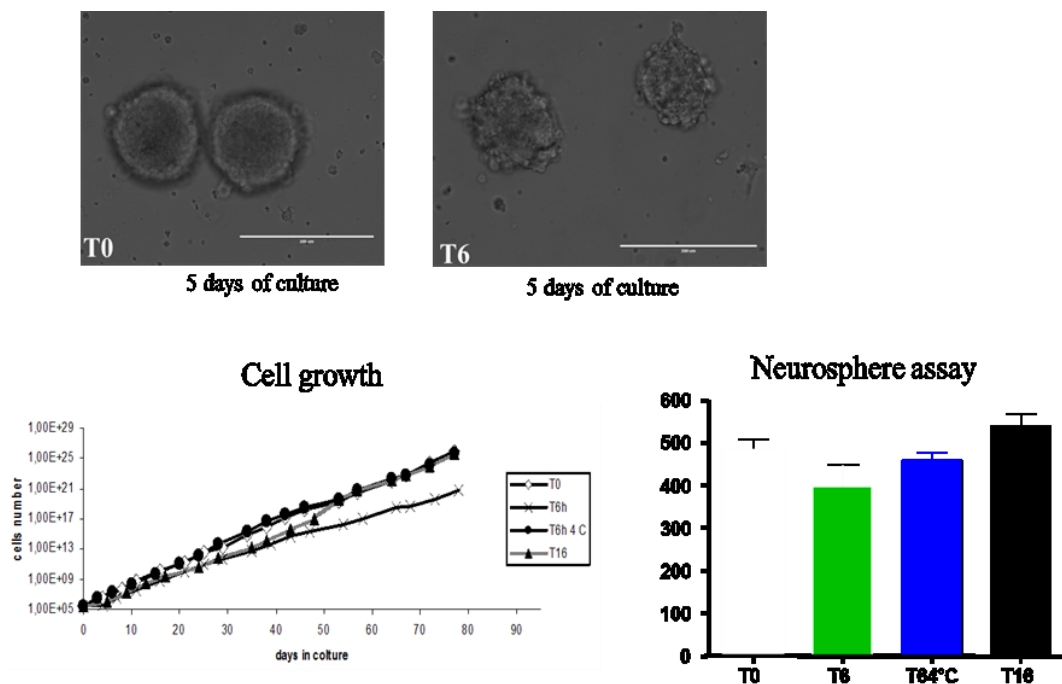

Figure 15. PM-NPCs perpetuation and population analysis. The perpetuation and continuous growth properties of PM-NPCs are comparable of T0 NPCs. Cells were plated in growth medium at the density of 10,000 cells/cm2. PM-NPCs population analysis was performed by counting the number of neurospheres generated after 10 days of culture. It is represented the number of neurospheres generated by 3000 cells plated in a single well.

After removal of growth factors and addition of serum and adhesion molecules, PM-NPCs and NPCs attach to the dish and begin the differentiation process into neurons, astrocytes, and oligodendrocytes. The extent of the differentiation was evaluated by monitoring the number of cells expressing β-tubulin III, MAP-2, GFAP, and O4 antigens 7 days after differentiation induction (Figure 16)
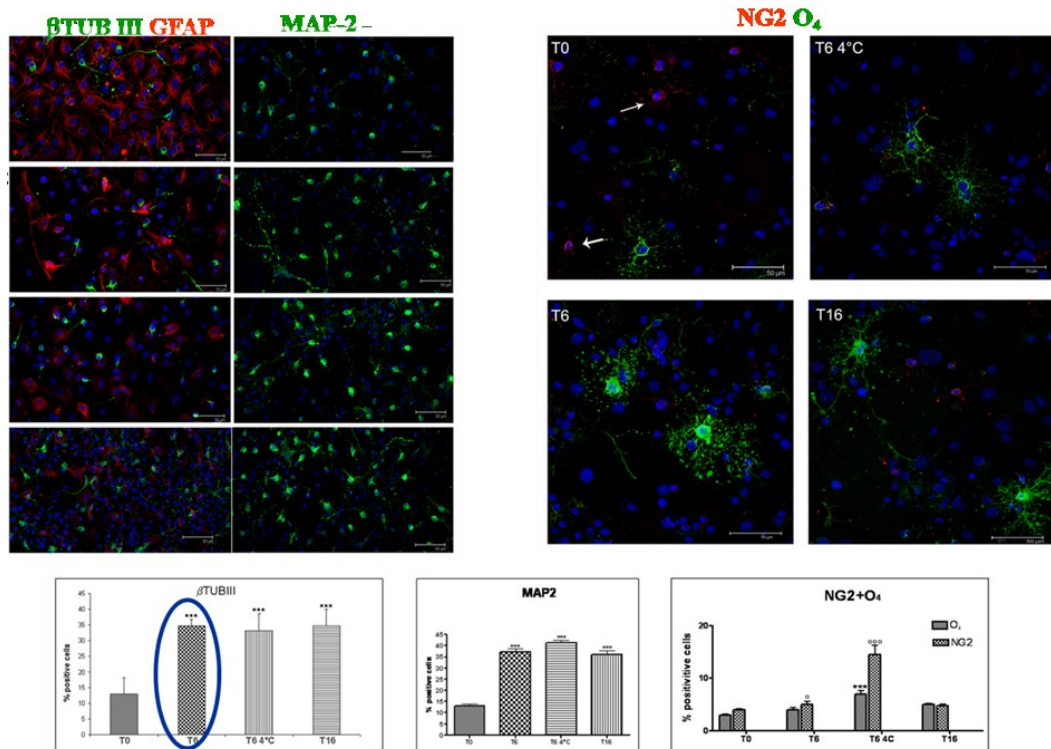
Figure 16. Differentiation features of PM-NPCs. After removal of growth factors, PM-NPCs and NPCs neurospheres readly differentiates into β-tubulin III - and MAP2 positive neurons, and O4 labelled oligodendrocytes with NG2- positive precursors.

The differentiation of PM-NPCs yields about 33 % β tubulin III- and 36% of MAP2- positive cells compared to 10-12% of classical neural precursors. Moreover it has been shown that differentiated PM-NPCs show higher HIF-1α activation, express both EPO and EPO-R, and active voltage-dependent Ca^{++} channels (Marfia et al, 2011). Such a higher differentiation requires the functionality of mTOR and MAPK systems and is prevented by exposure to anti-EPO and anti- EPO-R antibodies (Figure 17; Marfia et al 2011).
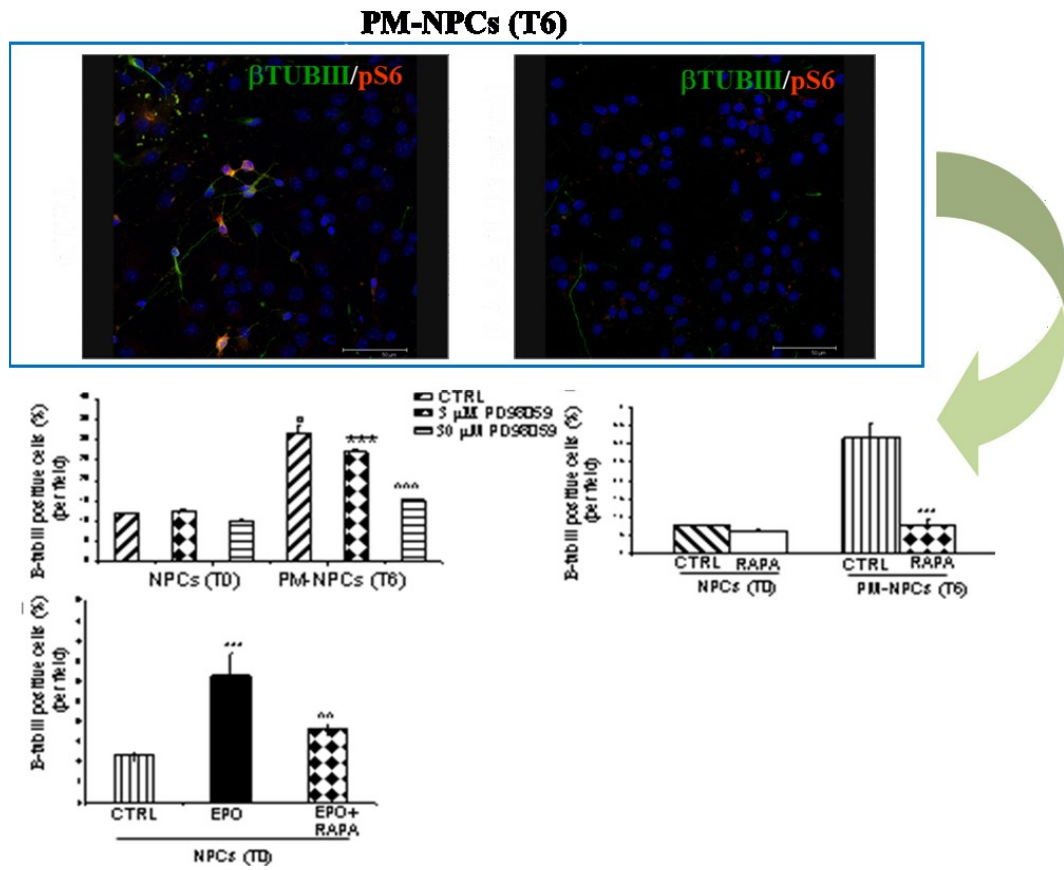
Figure 17. EPO-dependent differentiation of SVZ-neural progenitors and PM-NPCs into neurons and MAP-kinase ERK and mTOR involvement.

3. Aim of the Study

Parkinson's disease (PD) is a common neurodegenerative disorder—α synucleinopathy—with a prevalence of 160/100 000 in Western Europe rising to ~4% of the population over 80 (http://www.epda.eu.com). With an ageing population, the management of PD is likely to prove an increasingly important and challenging aspect of medical practice for neurologists and general physicians. The pathological hallmark of PD is cell loss within the substantia nigra particularly affecting the ventral component of the pars compacta. While there are some effective pharmacological treatments for patients with PD these strategies are mainly symptomatic and aimed at increasing dopamine levels in the degenerating nigrostriatal system. Existing drugs are limited in their relief and decrease in effectiveness as PD progresses.

Cell replacement therapy for treatment of PD has been focused on the hypothesis that immature dopaminergic neurons, after transplantation and functional integration into the striatum, can restore dopaminergic neurotransmission and exert long-lasting effects on motor symptoms. Based on data obtained from pre-clinical research approaches, the current main focus is on elevating striatal dopamine levels rather than trying to reconstruct the relatively long nigrostriatal pathway. Particular emphasis was attributed to the transplantation of fetal ventral mesencephalic tissue, which has resulted in the greatest clinical benefits in open labelled clinical trials. This approach was followed more recently by a discussion of future perspectives of cell replacement for PD, with particular focus on stem cell based strategies. **The principal aim of this thesis work was to test the potential role of post mortem neural precursors cells (PM-NPCs) in terms of replacement therapy in a mouse model of Parkinson's disease.** PM-NPCs are a subclass of Sub Ventricular Zone (SVZ)-derived neural progenitors, capable of surviving many hours after donor death (Marfia et al., 2011). The *in vitro* differentiation of these cells yields more neurons (about 30-40%) compared to NPCs isolated immediately after the animal sacrifice. With the aim to improve the monitoring of cells after transplantation, PM-NPCs was isolated from the SVZ of mice constitutively expressing the green florescent protein (GFP).

Detailed Aims:

- ***Isolation and characterization of GFP- PM-NPCs.*** To this proposal we will focus our attention to the transgenic mouse strain expressing green fluorescent protein (GFP) under the promoter C of the ubiquitin gene [C57BL/6-Tg(UBC-GFP)30Scha/J]. Cells was isolated from the SVZ at 6 hours after the donor death. These cells was characterized for their growth features and capability to

aggregate into spheres (neuropheres). Moreover, the GFP PM-NPCs *in vitro* differentiation potential was investigated by means immunofluorescence approach, focusing on the expression of neural markers such as MAP-2 (neurons), GFAP (astrocytes) and NG2 (oligodendrocytes).

- ***GFP PM-NPCs transplantation in animal model of parkinson's disease.*** As experimental model C57BL/J mice will be utilized and the parkinsonism was induced by means of the administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) following a specific paradigm: a first i.p. injection at 36 mg/kg intraperiteoneally followed of a second injection of 20 mg/Kg i.p. after seven days. The effect of the neurotoxin on the motor behavior was evaluated by specific animals tests, such as horizontal, vertical grid tests and with stride length test. Furthermore, HPLC analyses was performed to measure catecholamines and their metabolites levels in order to corroborate the behavioral data.

- ***Evaluation of GFP PM-NPCs in vivo effects.*** During the two weeks after transplantation the PM-NPCs effects was assessed by specific behavioral tests (see above for details). At the end of this observation period (two weeks) the animals was sacrificed, their brains was removed and specific areas (striatum, midbrain and cortex) was dissected. By using HPLC approach the catecholamines and their metabolites levels were measured, in order to study the neurochemical changes.

- ***Fate and distribution of GFP PM-NPCs after transplantation.*** These part of the project was developed by means of immunohistochemistry approach associated to confocal microscopy and was performed at the end of the animal observation period (two weeks). Investigating the GFP distribution in brain areas was assessed whether grafted cells were able to migrate beyond the striatum and to overtake the striatum margins reaching other brain areas. Moreover, through the analysis of neural markers expression (Nestin, MAP-2, GFAP, NG2 and NEUN) the *in vivo* fate of grafted PM-NPCs after transplantation was assessed.

4. Materials and Methods

4.1 Post-Mortem Neural Precursor Cells Derivation

Post mortem neural precursors constitutively expressing green fluorescent protein were obtained from 2 month old C57 black mice carrying the transgene for green fluorescent protein under the control of ubiquitin promoter C (C57BL/6-Tg(UBC-GFP)30Scha/J; The Jackson Laboratories, Bar Harbor, Maine, USA).

Adult C57BL/6-Tg(UBC-GFP)30Scha/J mice weighing 25-30 g (Charles River) were anesthetized by intraperitoneal injection of 4% chloral hydrate (0.1 ml/10 gm body weight) and then killed by cervical dislocation. The cadavers were maintained at room temperature (25°C) for 6 hours. Their brains were removed after the indicated periods and the area encompassing the SVZ surrounding the lateral wall of the forebrain ventricle was dissected using fine scissors. Primary cultures and cultivation of NPCs, their differentiation and immunostaining were performed as previously described (Bottai et al 2008; Gritti et al 1999).

The cells were plated at 3500 cells/cm2 in the appropriate volume of the aforementioned medium in a 25 cm2 flask at 37 °C in a humidified 5% CO2 atmosphere.

4.2 PM-NPCs perpetuation, population analysis and differentiation

PM-NPCs Perpetuation of the culture

Neural Precursors were plated in growth medium containing FGF2 and EGF at the density of 10,000 cells/cm2, 4-5 days of cell division gave rise to neurospheres (Gritti et al 1996; 1999). The total number of viable cells was assessed by trypan blue exclusion. This procedure was repeated every 4-5 days for up to 6 months.

PM-NPCs Population Analysis

For population analyses primary cells were plated at the density of 3000 cells/cm2 in 500 ℓl and, then, cultured for 4-5 days at 37 °C in a humidified 5% CO2 atmosphere. The assay was carried out in a 48 -well plate coated with polylysine or polyornithine, and the spheres formed after 4-5 days were harvested and counted. At each passage neurospheres were harvested by centrifugation and mechanically dissociated by pipetting and cells were plated again. The logarithmic value of the total viable cell number was plotted against the day in vitro (days) since the beginning of the experiment. The experiment was performed three times in triplicate per each sample (Gritti et al 1996; 1999) and was repeated at passage 7, 15 and 30 without observing significant differences.

Differentiation of the PM-NPCs

Differentiation and expression of various epitopes were achieved within 7 days. Differentiation of PM-NPCs was achieved first of all plating the dissociated stem cells at the density of 40,000 cells/cm2 in presence of adhesion molecules (Matrigel™) and bFGF (10ng/ml) for 48 hours, for the

other 5 days the growth factor was removed and foetal bovine serum (1% vol/vol) was added to the medium. Cells were characterized by immunocytochemical staining.

The immunostaining was performed as follows, differentiated PM-NPCs were fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature, and, then, washed with PBS. The coverslips were incubated for 90 min at 37°C (or overnight at 4 °C) in PBS containing 10% normal goat serum (NGS), 0.3% Triton X-100, and the appropriate primary antibodies or antisera. After thorough washing with PBS and 10% NGS, cells were reacted for 45 minutes (room temperature) with secondary antibody Alexa Fluor 488 or 543 anti-mouse or rabbit (Invitrogen, Carlsbad, California). The cells were then counterstained with the DNA-binding dye 4'-6'-diamidino-2-phenylindole (DAPI) (2 μg/ml in PBS) for 10 minutes at room temperature, twice washed in PBS followed. Coverslips containing the stained differentiated cells were mounted onto glass slides and examined under a fluorescence microscope (Leica, Wetzlar, Germany). Acquisition of the stained cells was performed using the image-analysis software (Leica, Wetzlar, Germany) or by confocal microscope Leica SP2 microscope with He/Kr and Ar lasers (Leica, Wetzlar, Germany). Images were taken with a 40X objective lens and 1X zoom (Leica). In control experiments, primary antibodies were either omitted or replaced with equivalent concentrations of unrelated Ig of the same subclass. Moreover, in double labelling experiments, sections incubated with one primary antibody and two secondary anti-sera revealed no appreciable cross-reaction. Neural differentiation was assessed by immunocytochemistry with antibodies against: Nestin (monocl.1:100; Pharmingen, San Jose, CA, USA), β-tubulin III (monocl. 1:150; Sigma), microtubule-associated protein 2 (MAP2; monocl. 1:200; Sigma), Glial fibrillary acidic protein (GFAP; monocl. 1:400; Roche, Basel, CH), Tyrosine Hydroxylase (TH; monocl. 1:400; Chemicon),

4.3 Cell count

The quantification of positive cells to neural markers (see above) was performed, by confocal microscopy, after specific immunocytochemical staining following 7 days differentiation in culture. Briefly, cell counts were performed on a minimum of 9 independent fields (3 fields/3 coverslips/treatment) of photomicrographs captured with 40X objective. Total counts of each neural markers immunoreactive cells were performed and the number of positive cells per culture was expressed as the percentage to the total cells. DAPI supplied the total number of cells being a nuclear staining.

4.4 Experimental model

Male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) were used for these experiments. Five weeks old mice, weighing 22-24 g, were housed individually and handled daily for 1 week prior to MPTP injection, in order to make them amenable to behavioral testing. All mice were maintained on standard environmental conditions (12:12 light/dark cycle; room temperature +21 °C) and given access to lab chow and water ad libitum. MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is a

neurotoxin precursor to MPP+, which causes permanent symptoms of Parkinson's disease by destroying dopaminergic neurons in the substantia nigra of the brain. It has been used to study disease models in various animal studies.

Injection of MPTP causes rapid onset of Parkinsonism, hence users of MPPP contaminated with MPTP will develop these symptoms.

MPTP itself is not toxic, and as a lipophilic compound can cross the blood–brain barrier. Once inside the brain, MPTP is metabolized into the toxic cation 1-methyl-4-phenylpyridinium (MPP+) by the enzyme MAO-B of glial cells. MPP+ kills primarily dopamine-producing neurons in a part of the brain called the pars compacta of the substantia nigra. MPP+ interferes with complex I of the electron transport chain, a component of mitochondrial metabolism, which leads to cell death and causes the buildup of free radicals, toxic molecules that contribute further to cell destruction.

Because MPTP itself is not directly harmful, toxic effects of acute MPTP poisoning can be mitigated by the administration of monoamine oxidase inhibitors (MAOIs) such as selegiline. MAOIs prevent the metabolism of MPTP to MPP+ by inhibiting the action of MAO-B, minimizing toxicity and preventing neural death.

MPP+ has quite selective abilities to cause neuronal death in dopaminergic cells, it is presumed through a high-affinity uptake process in nerve terminals normally used to reuptake dopamine after it has been released into the synaptic cleft. The dopamine transporter moves MPP+ inside the cell.

The resulting gross depletion of dopaminergic neurons has severe implications on cortical control of complex movements. The direction of complex movement is based from the substantia nigra to the putamen and caudate nucleus, which then relay signals to the rest of the brain. This pathway is controlled via dopamine-using neurons, which MPTP selectively destroys, resulting over time in parkinsonism.

MPTP causes parkinsonism in primates including humans. Rodents are much less susceptible. Rats are almost immune to the adverse effects of MPTP. Mice were thought to only suffer from cell death in the *substantia nigra* (to differing degree according to the strain of mice used) but do not show Parkinsonian symptoms, however most of the recent studies indicate that MPTP can result in Parkinsonism-like syndromes in mice (especially chronic syndromes). It is believed that the lower levels of MAO-B in the rodent brain's capillaries may be responsible for this.
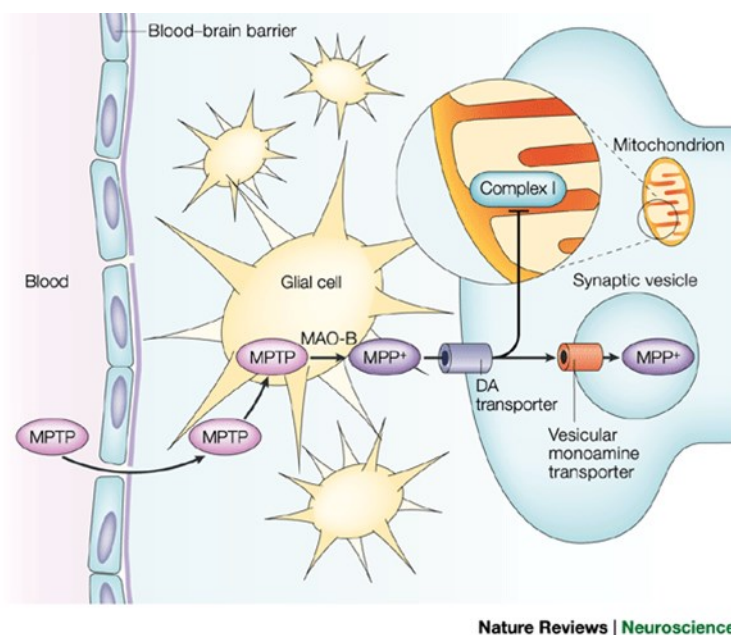
Figure 18. Scheme of MPTP toxicity in dopaminergic neurons

Six weeks old C5BL/6J mice were subjected of a first intraperitoneal injection of MPTP (36 mg/kg). Motor disfunctions were evaluated (see below) every 2 days for the following 7 days, then animals were subjected to a second injection (20mg/kg).

4.5 Cells injection

After 3 days from the second MPTP injection (day 10), cells were injected by stereotaxic surgery into the striatum ($1x10^5$ cells in 5 µL). Control group was injected with 5 µL of sterile PBS.

Coordinates, in relation to bregma, were: 0.1 mm posterior, 2.4 mm mediolateral, and 3.6 mm dorsal (Cui et al., 2010).

4.6 Animal care and behavioral evaluation.

After transplantation, animals were looked after for 5 days, subjected to antibiotic therapy (Gentamicin 1mg/ml, SIGMA) and rehydrated with saline solution and Vitamin C. Animals weight and urine pH were checked periodically until the end of the experiments. The recovery of motor dysfunctions, before and after cells transplantation, was investigated by means of horizontal and vertical grid tests.

Horizontal grid test. Once the mouse firmly grabbed on the grids (12 cm^2) with all four paws, the apparatus was inverted and the animal was videotaped for a maximum duration of 30''. With each

weight-bearing step, the paw may fall or slip between the wire. This was recorded as a foot fault. The total number of steps (movement of each forelimb) that the mouse used to cross the grid was counted, and the total number of foot faults for each forelimb were recorded *(*Tillerson et al., 2002, Tillerson and Miller, 2003 and Meredith and Kang, 2006)*.

Vertical grid test. In this test the mouse was placed 3 cm from the top of the apparatus (8cm x 55cm x 5cm), facing upward, and was videotaped while it turned around and climbed down. The score reported was the time required to climb *(Kim S. T. et al., 2010).*

4.7 HPLC analysis of catecholamines

1. Homogenize tissue samples in 600 μL of ice-cold 0.1 mol/L perchloric acid containing 10 pg/μL of dihydroxybenzylamine (DBA) as the internal standard;

2. Keep an aliquot of homogenate for the protein assay by using Bradford method *(Bradford, 1976)*

3. Centrifuge homogenates and use the supernatant for the determination of levels of monoamines and their metabolites by reverse-phase HPLC coupled with an electrochemical detector.

One liter of mobile phase contained 10.35 g (75 mol/mL) of sodium dihydrogen orthophosphate, 0.505 g (2.5 mol/mL) of heptane sulfonic acid, 25 mol/μL EDTA, 100 μL of triethylamine, and 200 mL of acetonitrile, and the final pH was adjusted to 3.00 with phosphoric acid. We used a C18 reverse-phase column (Inertsil ODS-3, 4.6 × 250 mm, 5 μm; Beckman, San Ramon, CA, USA). The mobile phase (filtered and degassed) was delivered at a flow rate of 1.2 mL/min; the applied potential was set to −0.10 V for detector 1 and +0.30 V for detector 2.

For catecholamine assays, a standard curve was prepared using known amounts of DA and its metabolites dissolved in 0.1 mol/L perchloric acid containing a constant amount (10 pg/μL) of the internal standard (DBA), as used for tissue samples. The standard curve for each compound (DA or its metabolites) was calculated using regression analysis of the ratios of the peak areas (compound area/DBA area) for various concentrations of each compound recorded at the reducing electrode. An analogous regression analysis was performed for the oxidizing electrode. For DA and its metabolites, results are expressed as the mean ± SEM of eight animals per group. (*Viaggi et al.,2009*)

4.8 Real Time RT-PCR analyses

Real-time RT-PCR quantities the initial amount of the template most specifically, sensitively and reproducibly, and is a preferable alternative to other forms of quantitative RT-PCR that detect the amount of final amplified product at the end-point (Freeman WM, 1999). Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle as opposed to the endpoint detection (Higuchi R et al., 1993), eliminating post-PCR processing of PCR products. This helps to increase throughput and to reduce the chances of carryover contamination. In comparison to conventional RT-PCR, real-time PCR also offers a much wider dynamic range of up to 10^7-fold (compared to 1000-fold in conventional RT-PCR). The real-time PCR system is based on the detection and quantitation of a fluorescent report (Livak KJ et al., 1995). This signal increases in direct proportion to the amount of PCR product in a reaction. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. Currently four different chemistries, TaqMan®, Molecular Beacons, Scorpions® and SYBR® Green (BIO-RAD), are available for real-time PCR. All of these chemistries allow detection of PCR products via the generation of a fluorescent signal.
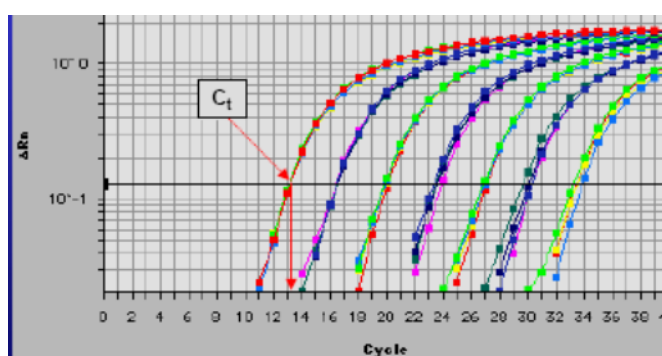

Figure 19. Real time RT-PCR curves and Ct values.

TaqMan probes, Molecular Beacons and Scorpions depend on Förster Resonance Energy Transfer (FRET) to generate the fluorescence signal via the coupling of a fluorogenic dye molecule and a quencher moiety to the same or different oligonucleotide substrates. SYBR Green is a fluorogenic dye that exhibits little fluorescence when in solution, but emits a strong fluorescent signal upon binding to double-stranded DNA. The threshold cycle or C_t value (Figure 19) is the point when the system begins to detect the increase in the signal associated with an exponential growth of PCR product during the log-linear phase. This phase provides the most useful information about the reaction and the slope of the log-linear phase is a reflection of the amplification efficiency. The efficiency (Eff) of the reaction can be calculated by the formula: $Eff = 10^{(-1/slope)} - 1$. The efficiency of

the PCR should be 90 - 100% (–3.6 > slope > –3.1). A number of variables can affect the efficiency of the PCR. These include length of the amplicon, secondary structure and primer quality. The C_t is an important parameter for quantitation. The higher the initial amount of genomic DNA, the sooner accumulated product is detected in the PCR process, and the lower is the C_t value. The threshold should be placed above any baseline activity and within the exponential increase phase. Some software allows determination of the cycle threshold (C_t) by a mathematical analysis of the growth curve. This provides better run-to-run reproducibility. Two strategies are commonly employed to quantify the real-time RT-PCR data: the **standard curve method** (absolute quantification) and the **comparative Ct method** (relative quantification).

In the **standard curve method**, a standard curve is first constructed from an RNA of known concentration. This curve is then used as a reference standard for extrapolating quantitative information for mRNA targets of unknown concentrations, thus generating absolute copy number data. In addition to RNA, other nucleic acid samples can be used to construct the standard curve, including purified plasmid dsDNA, *in vitro* generated ssDNA or any cDNA sample expressing the target gene. Spectrophotometric measurements at 260 nm can be used to assess the concentration of these DNAs, which can then be converted to a copy number value based on the molecular weight of the sample used. However, cDNA plasmids are the preferred standards for standard curve quantitation. The **comparative C_t method** involves comparing the C_t values of the samples of interest with a control or calibrator such as a non-treated sample or RNA from normal tissue. The C_t values of both the calibrator and the samples of interest are normalized to an appropriate endogenous housekeeping gene. The comparative C_t method is also known as the $2^{-\Delta\Delta Ct}$ method [Livak and Schmittgen, 2001], where $\Delta\Delta C_t = \Delta C_{t,sample} - \Delta C_{t,reference}$. $\Delta C_{t,sample}$ is the C_t value for any sample normalized to the endogenous housekeeping gene and $\Delta C_{t, reference}$ is the C_t value for the calibrator also normalized to the endogenous housekeeping gene. For the $\Delta\Delta C_t$ calculation to be valid, the amplification efficiencies of the target and the endogenous reference must be approximately equal. This can be established by looking at how ΔC_t varies with template dilution. If the plot of cDNA dilution versus delta C_t is close to zero, it implies that the efficiencies of the target and housekeeping genes are very similar. This quantification method is described in the Applied Biosystems User Bulletins #2 and #5. Relative gene expression comparisons work best when the expression of the chosen internal control is abundant and remains constant among the samples. By using an invariant endogenous control as an active reference, quantitation of an mRNA target can be normalised for differences in the amount of total RNA added to each reaction. For this purpose, the most common choices are 18S RNA, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and β-actin.

 Real-time PCR requires an instrumentation platform that consists of a thermal cycler, a computer, optics for fluorescence excitation and emission collection, data acquisition, and analysis software. These machines, available from several manufacturers, differ in sample capacity (some are 96-well

standard format, others process fewer samples or require specialized glass capillary tubes), method of excitation (some use lasers, others broad spectrum light sources with tuneable filters), and overall sensitivity.

RNA Extraction

Brain areas were obtained from C57black mice and the experiments were performed on frontal cortex, striatum and midbrain. Total RNA was isolated by using TRI Reagent® (SIGMA) in accordance with the manufacturer's instructions. In briefly, the procedure used was the following:

- Add 1 ml of TRI Reagent directly on the culture dish. After addition of the reagent, the tissue homogenate should be passed several times through a pipette to form a homogenous lysate.

- Phase Separation: To ensure complete dissociation of nucleoprotein complexes, allow samples to stand for 5 minutes at room temperature. Add 0.2 ml of chloroform per ml of TRI Reagent used. Cover the sample tightly, shake vigorously for 15 seconds, and allow to stand for 15 min at room temperature. Centrifuge the resulting mixture at 12,000 x g for 15 minutes at 4°C.

  Centrifugation separates the mixture into 3 phases: a red organic phase (containing protein), an interphase (containing DNA), and a colourless upper aqueous phase (containing RNA).

- Transfer the aqueous phase to a fresh tube and add 0.5 ml of isopropanol per ml of TRI Reagent used in Sample Preparation and mix. Allow the sample to stand for 10 min at room temperature. Centrifuge at 12,000 x g for 10 min at 4°C. The RNA precipitate will form a pellet on the side and bottom of the tube.

- Remove the supernatant and wash the RNA pellet by adding 1 ml of 75% ethanol per 1 ml of TRI Reagent used in Sample Preparation. Vortex the sample and then centrifuge at 7,500 x g for 5 min at 4°C.

- Briefly dry the RNA pellet for 5–10 minutes by air-drying. Do not let the RNA pellet dry completely, as this will greatly decrease its solubility. Add an appropriate volume of 0.5% SDS water, or to the RNA pellet. To facilitate dissolution, mix by repeated pipetting with a micropipette at 55–60 °C for 10 min.

  The ratio between A260/A280 was calculated to verify RNA purity. 100 ng/sample was used for cDNA synthesis.

Degradation of genomic DNA and reverse transcription-PCR (RT-PCR)

The degradation of contaminating genomic DNA from RNA samples was performed with DNase I (RNase-free) (New England BioLabs) according to manufacturer's instructions. In briefly, the procedure used was the following:

- Resuspend 1 μg RNA in 1X DNase I Reaction Buffer to a final volume of 10 μ.

- Add 1 unit of DNase I, mix thoroughly and incubate at 37^{0}C for 10 min.

- Add 0.5 M EDTA to a final concentration of 5 mM. EDTA should be added to a final concentration of 5 mM to protect RNA from being degraded during enzyme inactivation.

- Heat inactivate at 75^{0}C for 10 min.

The synthesis of single-strand cDNA was carried out on 1 μg of RNA template, using iScript™ Reverse Transcription Supermix for RT-qPCR (BIO-RAD) following the manufacturer's instructions.

Component	Volume per reaction
5X iScript reverse transcription supermix	4 μl
RNA template (1 μg total RNA)	11 μl
Nuclease-free water	5 μl
Total volume	20 μl

Reaction protocol:

Priming	5 min at 25^{0}C
Reverse transcription	30 min at 42^{0}C
RT inactivation	5 min at 85^{0}C

Retrotrascription samples are stored at -20°C, until their use for real time RT-PCR.

In our experimental conditions, Real-time PCR was performed in an MJ Opticon 2 using iQ™ SYBR Green supermix (BIO-RAD) following the manufacturer's instructions. 18S rRNA was used as reference housekeeping gene for normalization. We performed an analysis using the ΔΔCt, this procedure can be used since we have determined previously that the replication efficiencies (slopes of the calibration or standard curves) for the genes of interest and housekeeping gene are very close. All the amplification reactions were performed in duplicate. The primers were designed using Oligo Perfect® Designer Software (INVITROGEN). The nucleotide sequences of the primers were:

Gene Name	Forward primer	Reverse primer
18S	TTTCGGAACTGAGGCCATGATTAAG	AGTTTCAGCTTTGCAACCATACTCC

IL1beta	GGCAACTGTTCCTGAACTCAACTGTGAAAT	CAGGTAGCTGCCACAGCTTCTCCACAGCCA
IL1alpha	ATGGCCAAAGTTCCTGACTTGTTTGAAGAC	GTTGCTTGACGTTGCTGATACTGTCACCCG
IL6	TCCAGTTGCCTTCTTGGGACTGATGCTGGT	AGTTTCAGATTGTTTTCTGCAAGTGCATCA
IL8	GCTCCTGCTGGCTGTCCTTAACCTAGGCAT	ATTGGGCCAACAGTAGCCTTCACCCATGGA
IL10	CCTGGCTCAGCACTGCTATGCTGCCTGCTC	AAGTAACCCTTAAAGTCCTGCATTAAGGAG
IL15	CCATCTCGTGCTACTTGTGTTTCCTTCTAA	GAAAGCAGTTCATTGCAGTAACTTTGCAAC
TNFalpha	GACGTGGAACTGGCAGAAGAGGCACTCCCC	GAGGCCATTTGGGAACTTCTCATCCCTTTG
NGF	CTCAGCAGGAAGGCTACAAGA	TACAGGCTGAGGTAGGGAGG
BDNF	ACTACCAAAGCCACAAGGCA	GCTGATCCTCATGCCAGTCA
GDNF	GGGATGTCGTGGCTGTCTGCCTGGTGTTGC	GCATATTGGAGTCACTGGTCAGCGCGAAGG

Table 3. **The nucleotide sequences of the primers used for the experiments**

Real Time RT PCR conditions

Amplification reactions were performed in 96-well standard plate. Each sample was analysed in duplicate. Reaction mix for all primers was prepared adding 1 µl of cDNA sample (dilution 1:10) to the following mix:

- 0.6 µl of *primer forward* (10 µM)

- 0.6 µl of *primer reverse* (10 µM)

- 5.3 µl of Dnase and Rnase free water

- 7.5 µl of 2X iQ™ SYBR Green supermix

Amplification conditions were:

- 95°C for 40 sec
- 58°C for 40 sec
- 72°C for 1 min

40 cycles

4.9 Immunohistochemistry

Confocal microscopy

Confocal microscopy offers several advantages over conventional optical microscopy, including controllable depth of field, the elimination of image degrading out-of-focus information, and the ability to collect serial optical sections from thick specimens. The key to the confocal approach is the use of spatial filtering to eliminate out-of-focus light or flare in specimens that are thicker than the plane of focus. In recent years, there has been a tremendous explosion in the popularity of confocal microscopy, due in part to the relative ease with which extremely high-quality images can be obtained from specimens prepared for conventional optical microscopy. In a conventional wide field microscope, the entire specimen is bathed in light from a mercury or xenon source, and the image can be viewed directly by eye or projected onto an image capture device or photographic film. In contrast, the method of image formation in a confocal microscope is fundamentally different. Illumination is achieved by scanning one or more focused beams of light, usually from a laser or arc-discharge source, across the specimen. This point of illumination is brought to focus in the specimen by the objective lens, and laterally scanned using some form of scanning device under computer control. The sequences of points of light from the specimen are detected by a photomultiplier tube (PMT) through a pinhole (or in some cases, a slit), and the output from the PMT is built into an image and displayed by the computer (Figure 23). Although unstained specimens can be viewed using light reflected back from the specimen, they usually are labelled with one or more fluorescent probes.
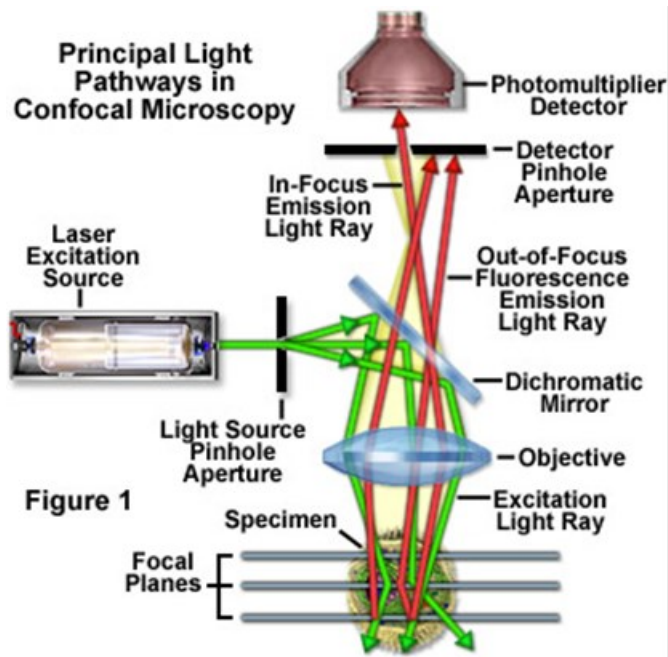
Figure 20. principles of confocal microscopy

Specimen Preparation and Imaging

The procedures for preparing and imaging specimens in the confocal microscope are largely derived from those that have been developed over many years for use with the conventional wide field microscope. In the biomedical sciences, a major application of confocal microscopy involves imaging either fixed or living cells and tissues that have usually been labelled with one or more fluorescent probes. A large number of fluorescent probes are available that, when incorporated in relatively simple protocols, specifically stain certain cellular organelles and structures. Among the plethora of available probes are dyes that label nuclei, the Golgi apparatus, the endoplasmic reticulum, and mitochondria, and also dyes such as fluorescently labelled phalloidins that target polymerized actin in cells. Regardless of the specimen preparation protocol employed, a primary benefit of the way in which confocal microscopy is carried out is the flexibility in image display and analysis that results from the simultaneous collection of multiple images, in digital form, into a computer.

Critical aspects of Confocal microscopy

Quantitative three-dimensional imaging in fluorescence microscopy is often complicated by artefacts due to specimen preparation (*e.g.* autofluorescence problems, retractile structures presence, presence or absence of highly stained structures, immersion oil, coverslip thickness etc.), controllable and uncontrollable experimental variables or configuration problems with the microscope (*e.g.* optical component alignment, objective magnification, bleaching artefacts, aberrations, quantum efficiency, and the specimen embedding medium).

In our experimental conditions, the immunofluorescence experiments were assessed by confocal microscopy TSC SP2 Leica.

Tissue collection and processing, histology and immunohistochemistry

At the end of the experimental period, animals were anesthetized by i.p. injection of cloralium hydrate 4% in distilled water, and perfused with 4% paraformaldehyde in phosphate buffer (PB) 0.1 M pH 7.4 by transcardial perfusion. Brains were post-fixed overnight in the same fixative, cryoprotected with 30% sucrose, quickly frozen, stored at -80°C and sectioned by means of a cryostat (Leica). Cryostat sections (10 µm) were collected onto glass slides and processed for immunocytochemistry. Sections were rinsed with PBS, treated with blocking solution and incubated with primary antibodies overnight at 4°C. After treatment with primary antibodies, the sections were washed with PBS and incubated with appropriate secondary antibodies (Alexa Fluor® 546 or/and Alexa Fluor® 488, Molecular Probes®, Invitrogen, Life Technologies Italia, Monza, Italy) for 2 hours at room temperature. Sections were washed in PBS, nuclei were stained with DAPI (Hoechst 1/1000) and then mounted using the FluorSave Reagent (Calbiochem, Merck Chemical, Darmstadt, Germany) and analyzed by confocal microscopy. In control determinations, primary antibodies were omitted and replaced with equivalent concentrations of unrelated IgG of the same subclass. The following primary antibodies were used: Microtubule-Associated Protein 2 (MAP2) (1:300; Chemicon), Glial Fibrillary Acidic Protein (GFAP) (1:400; Covance), β-Tubulin III (1:150; Covance), Choline Acetyltransferase (ChAT) (1:1000; Chemicon. For immunofluorescence, the following secondary antibodies were used: 488 goat-anti-mouse IgG (1:200; Alexa), 488 donkey-anti-rabbit IgG (1:200; Alexa), 546 goat-anti-rabbit IgG (1:200; Alexa), 546 goat-anti-chicken IgG (1:200; Alexa). Images were taken using Leica SP2 confocal microscope with He/Kr and Ar lasers (Heidelberg, Germany). In negative control experiments, primary antibodies were replaced with equivalent concentrations of unrelated IgG of the same subclass. The quantification of positive cells was performed by considering on a minimum of 9 independent fields (3 fields/3 coverslips/treatment) of photomicrographs captured with 20X objective. Total count of each markers immunoreactive cells was performed and the number of positive cells expressed as the percentage to the total cells. DAPI supplied the total number of cells being a nuclear staining.

4.10 SDS Page, Western blotting and protein detection

Protein sample preparation for electrophoresis

50-75 µg of protein samples (cell lysate or tissue homogenate), previously TCA precipitated or not, are combined with Novex Tris-Glycine SDS sample buffer 2X (Invitrogen) containing 5% 2-β mercaptoethanol (70mM) in a microcentrifuge tube. In the case of TCA precipitation, Tris buffer is sometimes added to adjust pH if TCA residues are still present in sample resuspension. Many proteins have significant hydrophobic properties and may be tightly associated with other molecules, such as lipids, through hydrophobic interaction. Heating the samples to at least 60°C shakes up the molecules, allowing SDS to bind the hydrophobic regions and completing the denaturation. Thus, the samples are boiled for 5-10' to fully denature the proteins and kept at room temperature until they are ready to load onto the gel. Molecular weight marker (5µl) (Bio-Rad) is aliquoted in a separate tube and boiled for 1 min.

SDS Page

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is probably the most common analytical technique used to separate and characterize proteins, using a discontinuous polyacrylamide gel as support medium and SDS to denature the proteins. SDS (also called lauryl sulfate) is an anionic detergent bound by a polypeptide chain in proportion to its relative molecular mass. The negative charges on SDS destroy most of the complex structure of proteins and are strongly attracted toward an anode in an electric field. Polyacrylamide gels restrain larger molecules from migrating as fast as smaller molecules. Because the charge-to-mass ratio is nearly the same among SDS-denatured polypeptides, the final separation of proteins is dependent almost entirely on the differences in relative molecular mass of polypeptides. In a uniform density gel, the relative migration distance of a protein (Rf) is negatively proportional to the log of its mass. The relationship between Rf and mass can be plotted running proteins with an unknown mass and a known mass simultaneously, so that the masses of unknown proteins can be estimated. Protein separation by SDS-PAGE can be used to estimate relative molecular mass, to determine the relative abundance of major proteins in a sample and to determine the distribution of proteins among fractions. Acrylamide is used for preparing electrophoretic gels to separate proteins by size. Acrylamide mixed with bisacrylamide forms a cross-linked polymer network when the polymerizing agent, ammonium persulfate, is added. The ammonium persulfate produces free radicals faster in the presence of TEMED (N,N,N,N'-tetramethylenediamine). The size of the pores in a gel is inversely related to the amount of acrylamide used. Very large polypeptides cannot penetrate far into a gel and thus their corresponding bands may be too compressed for resolution. Polypeptides below a particular size are not restricted at all by the gel, and regardless of mass they all move at the same pace along with the tracking dye. Gel concentration (%T) should be selected to resolve precisely proteins of interest. A typical gel of 7% acrylamide composition separates polypeptides with molecular mass between 45 and 200 kDa. There are two types of buffer systems used in

PAGE electrophoresis: continuous and discontinuous systems. A continuous system has only a single separating gel and uses the same buffer in the tanks and the gel. In a discontinuous system, a non-restrictive large pore gel, called stacking gel, is layered on top of a separating gel called resolving gel. Each gel is made with a different buffer, and the tank buffers are different from the gel buffers. The stacking gel has a lower concentration of acrylamide (larger pore size), lower pH and a different ionic content. This allows the proteins to be concentrated into a tight band before entering in the resolving gel. The resolving gel may consist of a constant acrylamide concentration or a gradient of acrylamide concentration (high percentage of acrylamide at the bottom of the gel and low percentage at the top). A gradient gel is prepared by mixing two different concentrations of acrylamide solution to form a gradient with decreasing concentrations of acrylamide. As the gradient forms, it is layered into a gel cassette. This latter gel is recommended for separation of a mixture of proteins with a greater molecular weight range.

In our experimental conditions, continuous gel was running under reducing and denaturant conditions. Gel preparation procedures and running conditions used are described in the following protocol:

- Assemble glass plate sandwich using two clean glass plates and two 75 mm spacers. Assemble the casting frame and stand as described by manufacturer.

- In a clean falcon, prepare the resolving gel solution, mixing the ingredients as described in Table 4, remembering that AP and TEMED must be added at the end. Be careful not to generate bubbles.

- Pour the resolving gel to a level about 1 cm from the top. Wait 1 min, then layer the top of the gel with saturated butyl alcohol (this will help the gel to solidify quickly in the absence of air).

- Wait another 15-20' for the gel to polymerize. Pour off the top layer of butyl alcohol and rinse with distilled water. Dry off as much of water as possible, using absorbent paper.

- In a clean falcon, prepare the stacking gel as described in Table 4, being careful not to generate bubbles.

- Pour the resolving gel till the space is full, then put in the appropriate comb. (The difference of pH in the two layers causes the stacking of the proteins. Thus, if the proteins are not stacking properly check the buffers pH).

- Allow the top portion to solidify and then carefully remove the comb or store at 4°C in humidified condition, until use.

- Place gel in opposite side of apparatus (Mini-PROTEAN 3-Cell, Bio-Rad).

- Slowly add 1X Running buffer.

- Load samples and molecular weight marker.

- Run the gel at 60V until the blue dye overpass stacking phase, and then run it at 110 V until the blue dye reaches the bottom or runs off. At this time, the run is stopped, cables removed. The gel is removed from the cassette and can be processed for western blotting.

Table 4. Resolving and Stacking gel solution recipes.

Reagents	Running gel (1x)				Stacking gel (1x)
Acrylamyde percentage	**7%**	**10%**	**12%**	**15%**	**3%**
dH$_2$O (ml)	7.2	15.3	12.3	10.2	3.075l
30%/0.8% Acrylamide/Bis (ml)	7.5	7.5	7.5	7.5	1.25
4x Lower Tris Buffer (ml)	0.15	0.15	0.15	0.15	0.025
10% SDS (ml)	15.0	6.9	9.9	12.0	0.67
APS (ml)	0.15	0.15	0.15l	0.15	0.025
TEMED (ml)	0.02	0.02l	0.02	0.02	0.005

Western blotting and protein detection

The term "blotting" refers to the transfer of biological samples from a gel to a membrane and their subsequent detection on the surface of the membrane. Western blotting was introduced by Towbin and collegues [1989] and is now a routine technique for protein analysis. The specificity of the antibody-antigen interaction enables a single protein to be identified in the midst of a complex protein mixture. Western blotting is commonly used to identify a specific protein in a complex mixture and to obtain qualitative and semiquantitative data about that. The first step in a western blotting procedure is to separate the macromolecules using gel electrophoresis. Following electrophoresis, the separated molecules are transferred onto a second matrix, generally a nitrocellulose or polyvinylidene fluoride (PVDF) membrane. Next, the membrane is blocked to prevent any nonspecific binding of antibodies to the surface of the membrane. The transferred protein is complexed with an enzyme-labeled antibody as a probe. An appropriate substrate is then added to the enzyme and together they produce a detectable product such as a chromogenic or fluorogenic precipitate on the membrane for colorimetric or fluorimetric detection, respectively. The most sensitive detection methods use a chemiluminescent substrate that, when combined with the

enzyme, produces light. This output can be captured using film, a CCD camera or a phosphorimager designed for chemiluminescent detection. Whatever substrate is used, the intensity of the signal correlates with the abundance of the antigen on the blotting membrane. In our experimental procedure, western blotting and protein detection protocols are the following:

- Cut a piece of nitrocellulose membrane (Hybond-ECL, Amersham Biosciences). Wet the membrane in dH$_2$O. Soak membrane in 1X Transfer buffer for 10'.
- Wet sponges, filter papers (slightly bigger than gel) in 1X Transfer buffer. Assemble sandwich (sponge-paper-gel-membrane-paper-sponge) for the electroblotting apparatus (Mini Trans-Blot Electrophoretic Transfer Cell, Bio-Rad).
  - Transfer for 1 h at 100 V with a cold buffer and frozen pack.
- turn off power supply and remove cathode plate of blotter.
- Staining the membrane with Ponceau's dye and the gel with Coomassie Blue dye to verify the correct transfer.
  - Remove Ponceau's staining washing the membrane with dH$_2$O.
- Block the nitrocellulose membrane in T-TBS (TBS with 0.05% Tween-20 V/V) containing 5% skim milk (w/v) for at least 1h on a rocking platform.
- Wash 3 times the nitrocellulose membrane in T-TBS for 10' each on a rocking platform.
- Incubate the nitrocellulose membrane on a rocking platform with primary antibody diluted in T-TBS, overnight at 4°C.
- Wash 3 times the nitrocellulose membrane in T-TBS for 10' each on a rocking platform.
- Incubate with secondary antibody horseradish peroxidase-conjugate diluted in 5% milk T-TBS for 60-90' on a rocking platform at room temperature.
- Wash 3 times the nitrocellulose membrane in T-TBS for 10' each on a rocking platform.
- Wash the nitrocellulose membrane in TBS to remove the excess of tween.
- Detect proteins incubating the membrane for 5' with an enhanced chemiluminescence detection system (ECL^{TM}, Amersham).
- Place the blots in a sheet protector and exposed to Kodak X-Omat Blue Film (Blue X-ray Film) (Perkin Elmer) for different times. Several exposures allow better densitometric analysis. Autoradiography films are automated processed with an Automated x-ray film developer (Kodak M35A X-OMAT processor).

Nitrocellulose membrane stripping

Once visualized the proteins of interest, it is possible to strip off the first set of protein probes so that different proteins on the blot may be detected using a second set of specific probes. Stripping generally involves soaking the blot in a buffer that is sufficiently harsh to dissociate the affinity

interactions between antibodies and the target sample protein that was transferred to the membrane. The goal is to find a stripping condition that is efficient in removing the probes without also removing or damaging the target proteins on the blot. Because every antibody-antigen affinity interaction and target protein is unique, no one stripping condition is appropriate for all situations. Empirical testing and some optimization are necessary to determine appropriate stripping conditions for every particular western blot system. In most cases, conditions can be optimized to make at least one round of stripping and reprobing possible. In some cases, several rounds of stripping and reprobing are possible.

In our experimental condition, the stripping protocol consists in:

- Wash abundantly the nitrocellulose membrane with T-TBS on a rocking platform.
- Incubate the membrane with Stripping Buffer at 50°C for 30'. Add 0.5 M NaCl to the stripping buffer slightly reduces the background from multiple probings.
  - Wash abundantly the nitrocellulose membrane with TBS on a rocking platform.
- Return to membrane blocking and primary antibody incubation as previously described.

Densitometric analysis

Exposures of the same membrane for different times were acquired using GelDoc™ image capture system (Bio-Rad), following Manufacturer's instructions. The autoradiograms were quantified using Quantity One™ and Image J software. The data were then analyzed with suitable statistic methods.

Polyacrylamide gel staining

After western blotting procedure, gel staining allows to verify the correct loading of protein samples and the correct protein transfer to nitrocellulose membrane. A commonly used stain for detecting proteins in polyacrylamide gels consists in 0.1% Coomassie Blue dye in 50% methanol and 10% glacial acetic acid. Acidified methanol precipitates the proteins. Staining is usually performed overnight on a rocking platform. The dye penetrates the entire gel, and sticks permanently to the proteins. Excess dye is washed out by destaining procedure with acetic acid/methanol washes on a rocking platform. Destaining in two steps (first using 50% methanol, 10% acetic acid for 1-2 h and then using 7% methanol, 10% acetic methanol) provided a better efficiency. Finally, gels can be dried or photographed for later analysis.

4.11 Statistical Analysis Data are expressed as means ± SEM. The two way analysis of variance (Anova) and Bonferroni's post-test were applied using Prism 5 software (GraphPad Software Inc, La Jolla, USA) assuming a *p* value less than 0.05 as the limit of significance.

5. Results

5.1 Derivation of GFP positive Post-Mortem Neural Precursor Cells and Characterization of their Self-Renewal capability

Adult C57BL/6-Tg(UBC-GFP)30Scha/J mice were anesthetized and then killed by cervical dislocation. The cadavers were maintained at room temperature (25°C) for 6 hours, and after this period of time their brains were extracted, the area encompassing the SVZ surrounding the lateral wall of the forebrain ventricle was dissected and neural precursors were obtained as described in material and methods and according to Gritti et al 1996 and 1999.

Post-mortem Neural precursors cells expressing green fluorescent protein (GFP PM-NPCs) proliferate in response to FGF-2 and EGF exposure, and formed typical spheroids similar to those formed by NPCs obtained from CD1 mice and defined as neurospheres (Figures 21 and 22; Marfia et al 2011).

PM-NPCs from C57BL GFP + mouse
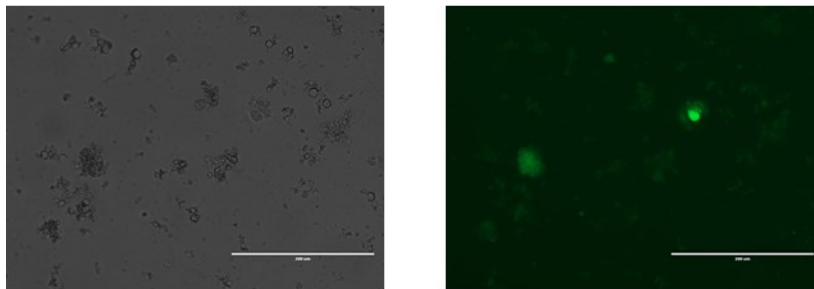
48 hours after isolation

Figure 21. Live images isolated precursors from SVZ of Adult C57BL/6-Tg(UBC-GFP)30Scha/J mice. Pictures were taken 48 hours after cells isolation. Scale bars 200 μm.

PM-NPCs from CD1 Mouse   PM-NPCs from C57BL GFP + mouse

5 days in culture
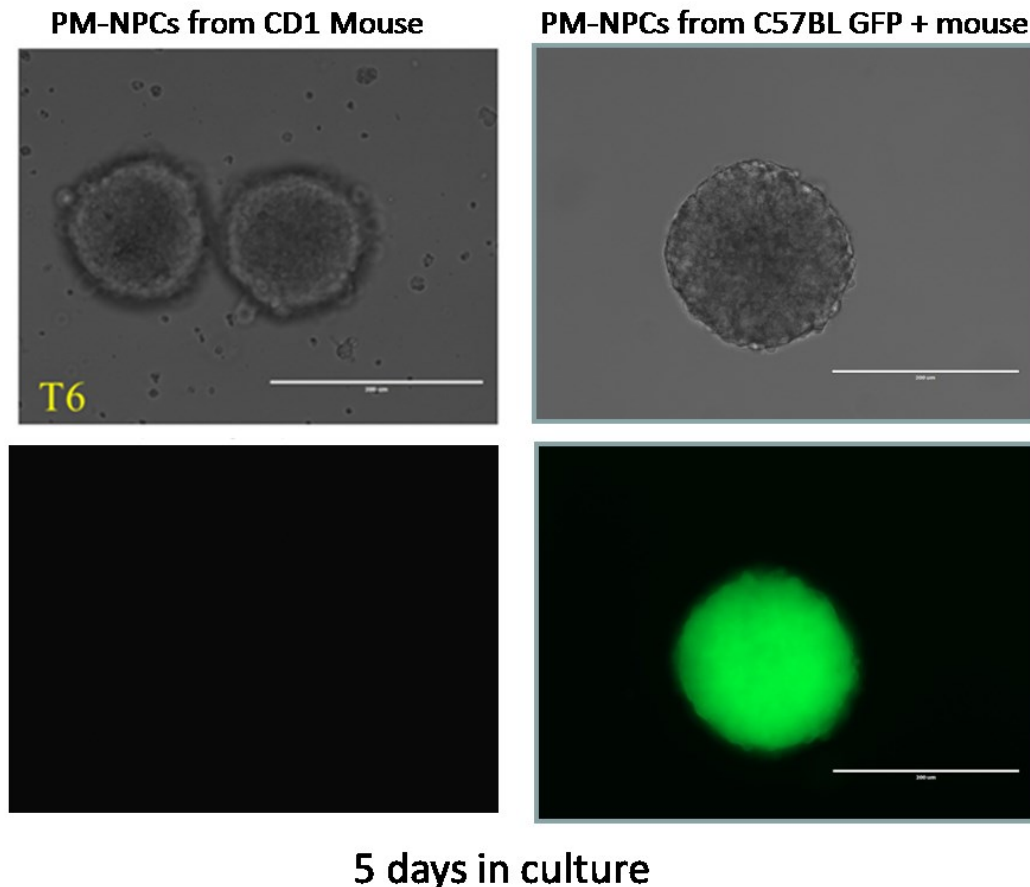
Figure 22. GFP positive PM-NPCs are able to form neurospheres. Undifferentiated post mortem neural precursors cells isolated from the Sub Ventricular Zone (SVZ) of adult mice that constitutively expresses the green fluorescent protein (GFP) proliferate in response to growth factors (EGF and FGF-2) and form neurospheres. Scale bars 100 μm.

To assess for self-renewal, individual primary spheroids were mechanically dissociated, and cells were plated in growth medium at the density of 3000 cells/cm2 in 500 ml and, the cultured for for 4-5 days at 37 °C in a humidified 5% CO2 atmosphere. The spheroids formed after 4-5 days were harvested and counted (Figure 3). The number of neurosphers formed by GFP-positive PM-NPCs was 169 ± 7,63/well and was similar to the number of spheres formed by both classical neural stem cells (NPCs) and post mortem neural precursors cells from CD1 mice (Figure 23 and Marfia et al 2011). At each passage neurospheres were harvested by centrifugation and mechanically dissociated by pipetting and cells were plated again. This assay was performed at passage 7, 17 and 30 without observing any significant difference.
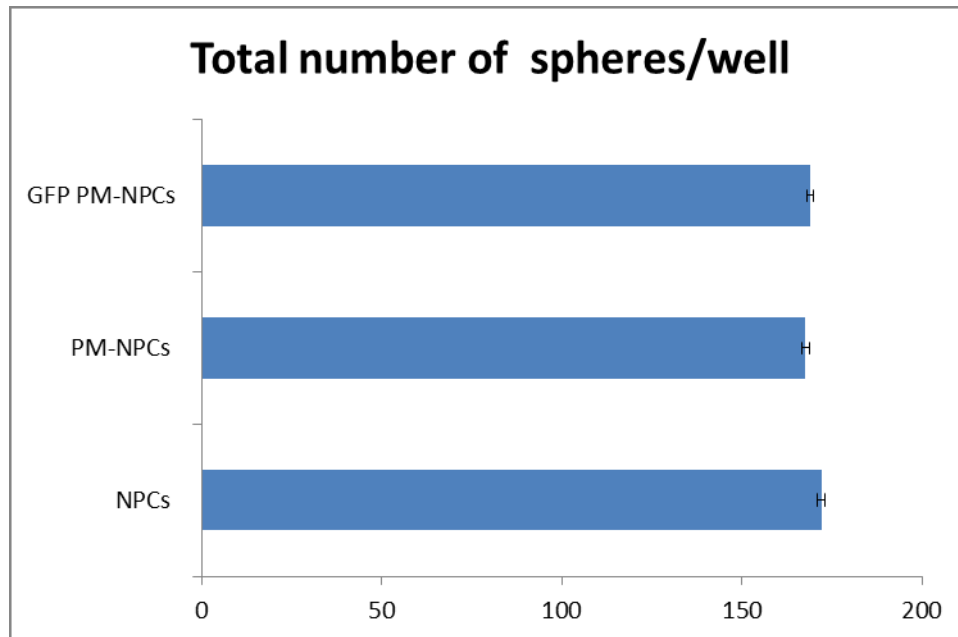
FIGURE 23. Neurosphere forming assay. PM-NPCs population analysis was performed by counting the total number of neurospheres generated in 10 culture days. Classical neural stem cells from SVZ of adult CD1 mouse (NPCs; Gritti et al 1996), post mortem neural precursors cells from SVZ of adult CD1 mouse (PM-NPCs, Marfia et al 2011) and post mortem neural precursors cells from Adult C57BL/6-Tg(UBC-GFP)30Scha/J mouse (GFP+ PM-NPCs) were cultured in the presence of EGF, and FGF-2; the number of spheres formed in each well was counted after 7–12 days. It is represented by the number of neurospheres generated by 3000 cells plated in a single well. The experiment was performed three times in triplicate for each cell source and was repeated at passage 7, 15 and 30 without observing significant differences in growth and markers expression (data not shown). Data are expressed as the mean of three independent experiments with similar results reporting ± standard error.

5.2 GFP positive PM-NPCs differentiation features

In order to assay for multipotency GFP PM-NPCs were induced to differentiate as briefly reported below . Neurospheres were mechanically dissociated and cells (1x 104 cells/cm2) were seeded onto Matrigel®-coated glass coverslips (12 mm diameter) in the presence of FGF-2. After 48 hours of growth culture in presence of growth factors cells were shifted into the differentiation medium in which FGF-2 and EGF were substituted with serum (1%; FBS) (Invitrogen). After growth factors removal and serum supplement, PM-NPCs attach to the dish and begin the differentiation process into neurons, astrocytes, and oligodendrocytes in a typical cell linage ratio (as reported in Marfia et al 2011). The differentiation capability of PM-NPCs was evaluated by monitoring the percentage of cells expressing MAP-2, GFAP, and NG2 markers, after 7 and 15 days growth in differentiation medium without growth factors (Figure 24). The quantification of neuronal-like differentiation after 7 days is shown in panel B of Figure 4. MAP-2

positive neurons were 39,3 ± 0,77 %; GFAP (astrocytes) and NG2 (oligodendrocyte precursors) -positive cells were 60,9 ± 2,81and 12,86 ± 0,48 respectively. The extent of the GFP PM-NPCs differentiation confirmed that these precursors cells have similar features of that previously obtained and described (Marfia et al 2011).
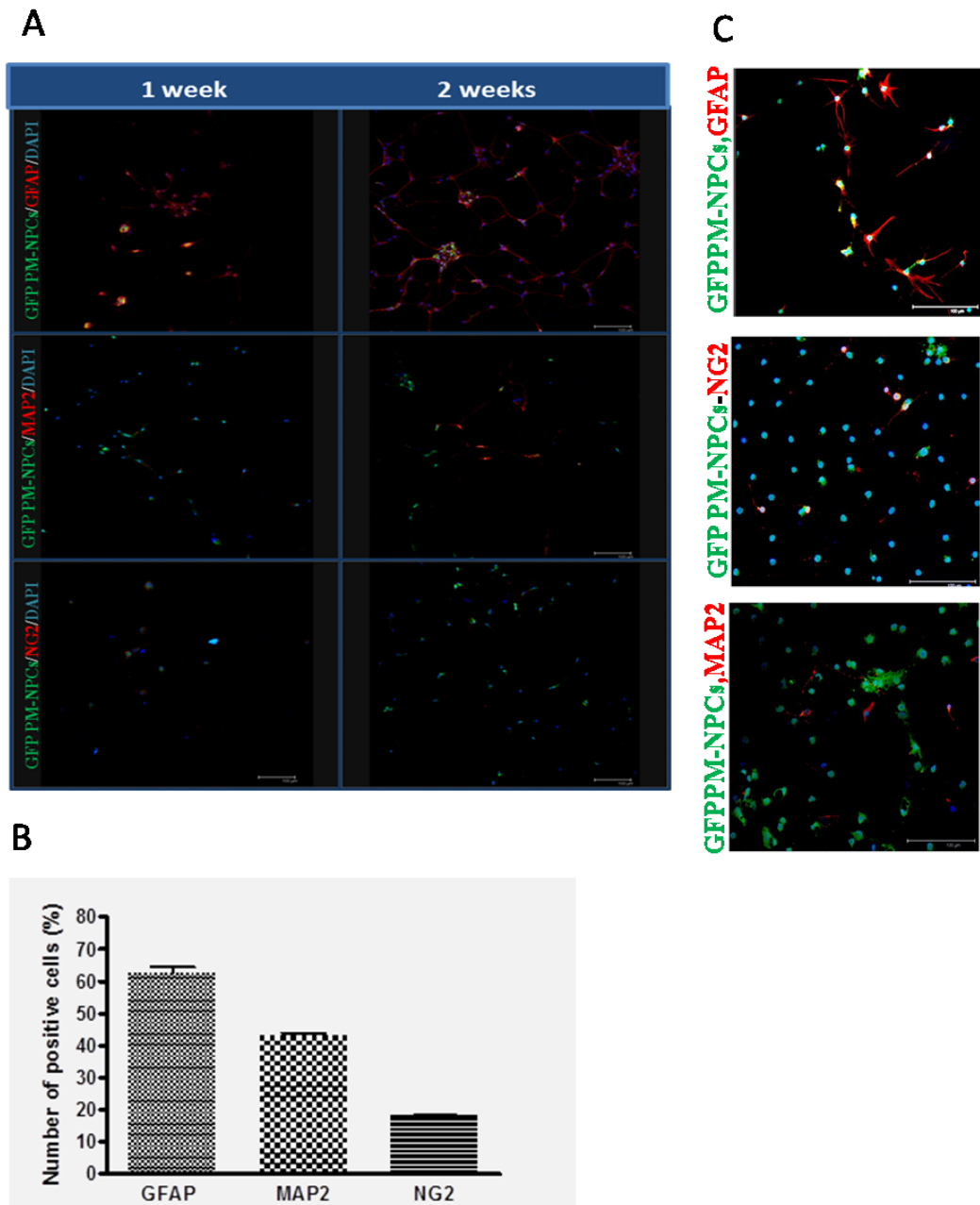

Figure 24. Neural marker expression after differentiation induction. After removal of growth factors, PM-NPCs neurospheres readly differentiates into MAP2 positive neurons, NG2 labelled oligodendrocytes precursors and astrocytes (GFAP). Staining with specific markers is showed in red. DAPI was used as counterstaining, in blue. Bars are 100 m. (B) Quantification was performed by counting the number of cells positive for each marker staining after 15 days of differentiation. Marker positivity was expressed as a percent of the total cells. The quantification indicates that

about 40 % of PM-NPCs differentiates into neurons. Data are expressed as the mean of three independent experiments with similar results ± standard error.

5.3 PM-NPCs Striatal Transplants Ameliorate the Behavioral Symptoms of MPTP-Lesioned Mice

The flow chart reported below describes the experimental plan followed to investigate the effects of GFP positive PM-NPCs transplanted in an experimental model of Parkinson's disease.
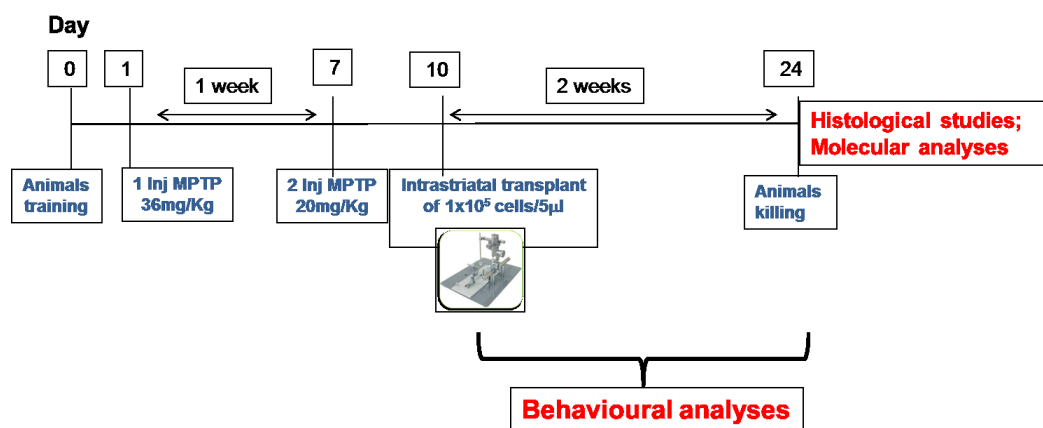
As control, before transplantation the expression of tyrosine hydroxylase (TH), the key enzyme in the synthesis of dopamine and markers of dopaminergic neurons, was investigated by immunocytochemistry assay in GFP PM-NPCs neurospheres. As showed in figure 25 these neurospeheres were not stained by specific antibody against TH; instead, as expected, were positive to the expression of nestin, the typical stem cells marker.
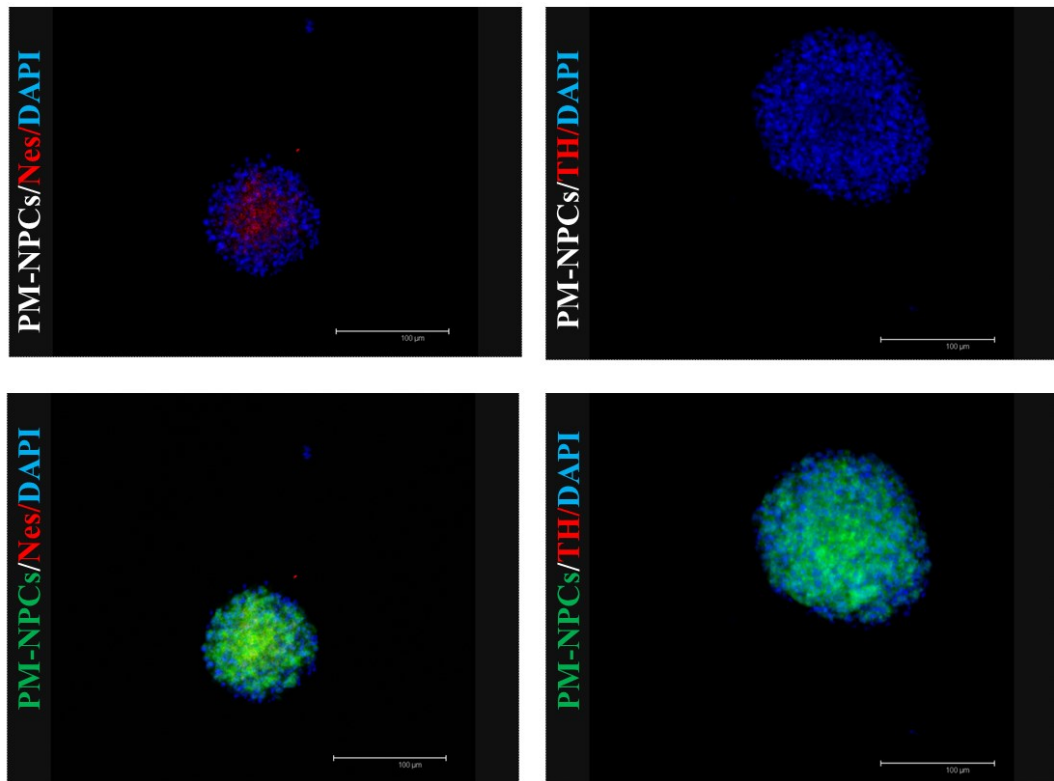
Figure 25. PM-NPCs do not express tyrosine hydroxylase. PM-NPCs cositutively expressgreen fluorescent protein and for that reason are showed in green. The labelling for nestin and tyrosine hydroxylase are showed in red. Nuclei were stained with DAPI and are showed in blue. The experiment was performed in duplicate with similar results and the picture showed here are representative. Scale bars are 100 microns.

For transplantation GFP PM-NPCs neurospheres were mechanically dissociated, cells were counted by trypan blue exclusion and then were suspended in PBS at the concentration of $1x10^5$ cells/5μl. Cells were injected into the striatum of MPTP mice. To test the effect of transplanted PM-NPCs on the behavior of MPTP-lesioned mice, we performed a series of behavioral tests on each mouse before and after transplantation or treatment.

Figure 26 shows the Image of the horizontal grid test apparatus, inverted.
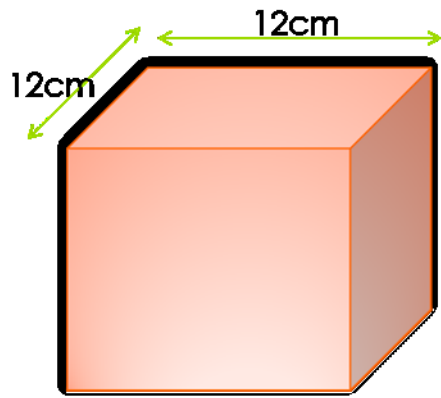
Figure 26. Scheme of horizontal grid used in the behavior evaluation (modified from Tillerson JL 2002 and Tillerson et al 2003)

Once the mouse firmly grabbed on the grids with all four paws, the apparatus was inverted upsidedown and the animal is videotaped for a maximum duration of 30 seconds (Figure 27).

Figure 27. Position of the animal for the horizontal grid test at the starting of videotaping.

With each weight-bearing step, the paw may fall or slip between the wire. This was recorded as a foot fault. The total number of steps (movement of each forelimb) that the mouse used to cross the grid was counted, and the total number of foot faults for each forelimb were recorded. This test was performed every 2 days before and after the transplants. Healthy animals tend to move along the grid with great ease exploring the whole territory for which the residence time in the vicinity of the side walls, that damage their security is reduced compared to that of animals that following the lesion with MPTP have coordination problems motor. This reduced safety of lateral displacement reduces their ability exploratory and increase the time of stay at the safe haven represented by the side wall. This behavioral problem is the direct consequence of the reduction of dopamine levels in the striatum following the lesion with the neurotoxin MPTP. As showed in the histogram reported in figure 8 the behavior impairment due to the first injection of MPTP start to be significant 3 days after the administration of the drug, that correspond to a 20% percent of forepaws fault in MPTP injected animals ($p < 0.05$ vs control mice, CTRL). Then the percent of forepaws fault reach the 30% at day 9 ($p < 0.01$ vs CTRL). The effect of transplant of PM-NPCs is rapid and determines a significant improvement of animal behavior by decreasing the percent of forepaws fault from 45% in PBS treated animals to about the 25% in PM-NPCs treated animals ($p < 0.05$ PM-NPCS injected group vs PBS injected group). The improvement in transplanted animals group is maintained for all the period of observation reaching values similar to the control animals (healthy mice) after two weeks (14,5 % of forepaw fault at days 21 and 23) (Figure 28).
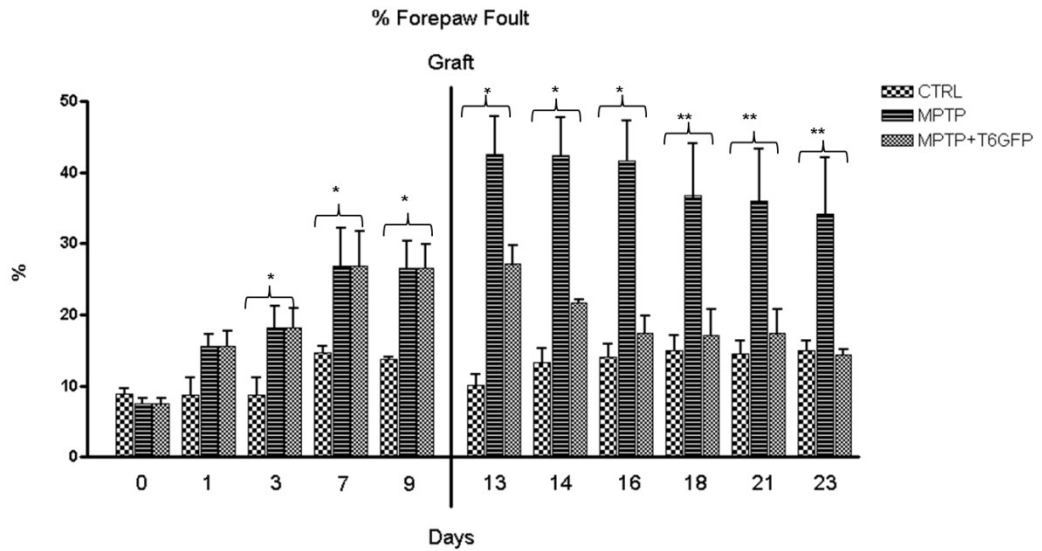
Figure 28. PM-NPCs reduce the percent of forepaw fault in transplanted animals. The table under the graphs show the number of animals evaluated in each group. Data are expressed as the mean of two independent quantification (two independent observers in blind) with similar results ± standard error (* $p<0.05$ MPTP and vs CTRL from day 0 to day 9; * $p<0.05$, ** $p<0.01$ MPTP vs PM-NPCs treated animals).

The Vertical Grid Test, whose technique has been widely described in materials and methods, has enriched our results confirming the data described above. The schematic of the vertical grid apparatus is showed the figure 29.
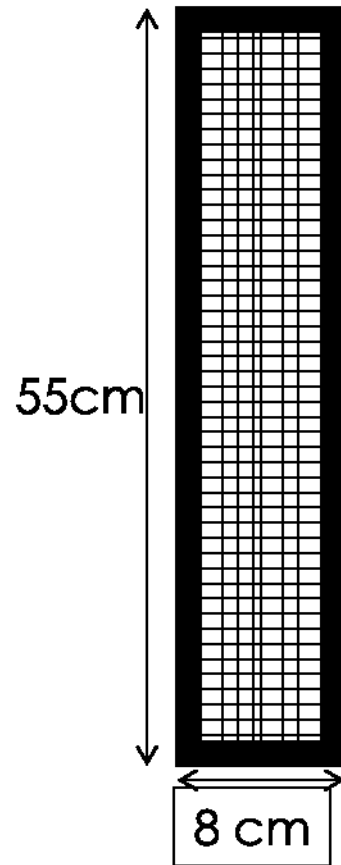
Figure 29. Scheme of vertical grid used in the behavior test (from Kim et al 210).

In vertical grid test mouse is placed 3 cm from the top of the apparatus, facing upward, and is videotaped while it turned around and climbed down (Figure 30). The score that was reported was the time required to climb down. MPTP-treated mice took longer to make a turn on the vertical grid.
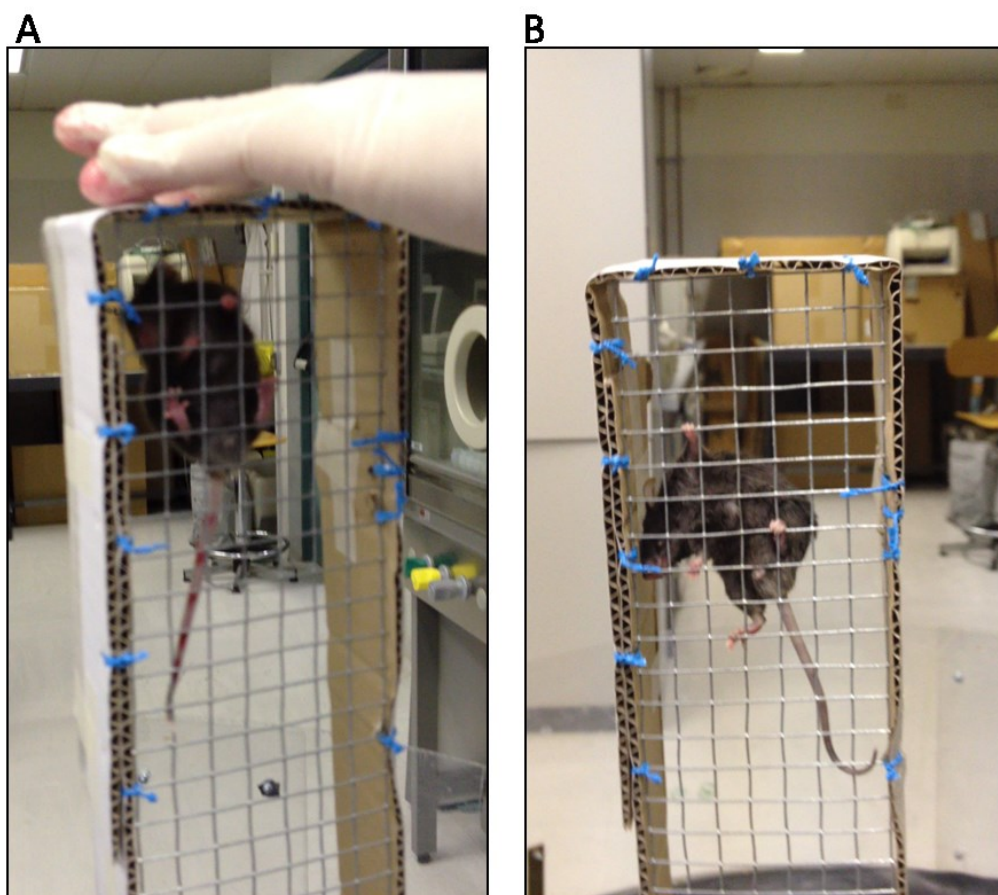
Figure 30. Position of the animal for the vertical grid test at the starting of videotaping. The mouse is placed 3 cm from the top of the apparatus, facing upward (A); then it has to turn around and climb down (B).

As showed in the histogram reported in figure 11 significantly longer time to turn head down and to climb down was observed in the MPTP-treated mice compared to the control animals. In normal conditions, healthy animals (CTRL) took approximately 27 seconds (as a mean of the analyzed animals) to turn and to climb down from the top of the grid (Figure 31). Figure 11 shows how the lesion strongly modify the animals ability to control their actions when hung on the grid and their ability to turn around and climb down. The impairment due to the MPTP injection become significantly evaluable in the same day of injection and the animals of this group (n=20) employed a mean of 75 seconds to perform the test (p<0.01 MPTP vs CTRL). The impairment increases during time and it is stabilized by the second injection of MPTP (20mg/kg i.p.) performed at day 7 (see experimental plan showed at the beginning of the Results paragraph).
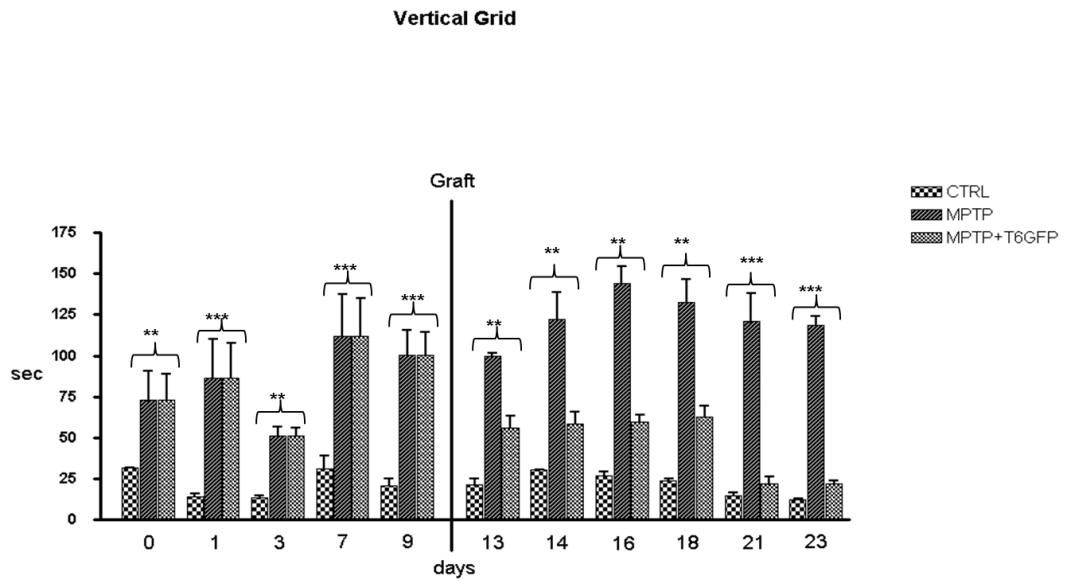
Vertical Grid

CTRL	MPTP	MPTP+PM-NPCs
11	9	11

Figure 31. PM-NPCs reduce the time required to climb down from the vertical grid in transplanted animals. The table under the graph shows the number of animals evaluated in each group. Data are expressed as the mean of two independent quantification (two independent observers worked in blind) with similar results ± standard error (** $p < 0.01$, *** $p < 0.001$ MPTP vs CTRL from day 0 to day 9; ** $p < 0.01$ and $p < 0.001$ MPTP vs PM-NPCs treated animals).

The application of the cells counteracts the effect of the drug. As showed in horizontal grid test also in the vertical grid test animals treated with PM-NPCs showed an significant improvement starting at day 3 after injection, with a reduction of the time to perform the test at 50 seconds (Figure 31). As showed for horizontal grid test the behavioral recovery is stable for the following 10 with an improvement at days 21 and 23 reaching levels performed by healthy animals (Figure 31).

The recovery of behavioral deficits was also investigated by means of the forepaw stride length test. The mice feet were dipped in red ink and they were placed in a 1 meter long runway that was covered with paper to capture the mice' footsteps. The length of stride between experimental and control groups was measured and was plotted in the histogram showed below. At two weeks after transplantation we found that the stride length of the PM-NPCs treated mice

increased after cell transplantation (n = 4) and was significantly longer than that of the MPTP group mice (n = 4) (Figure 32).
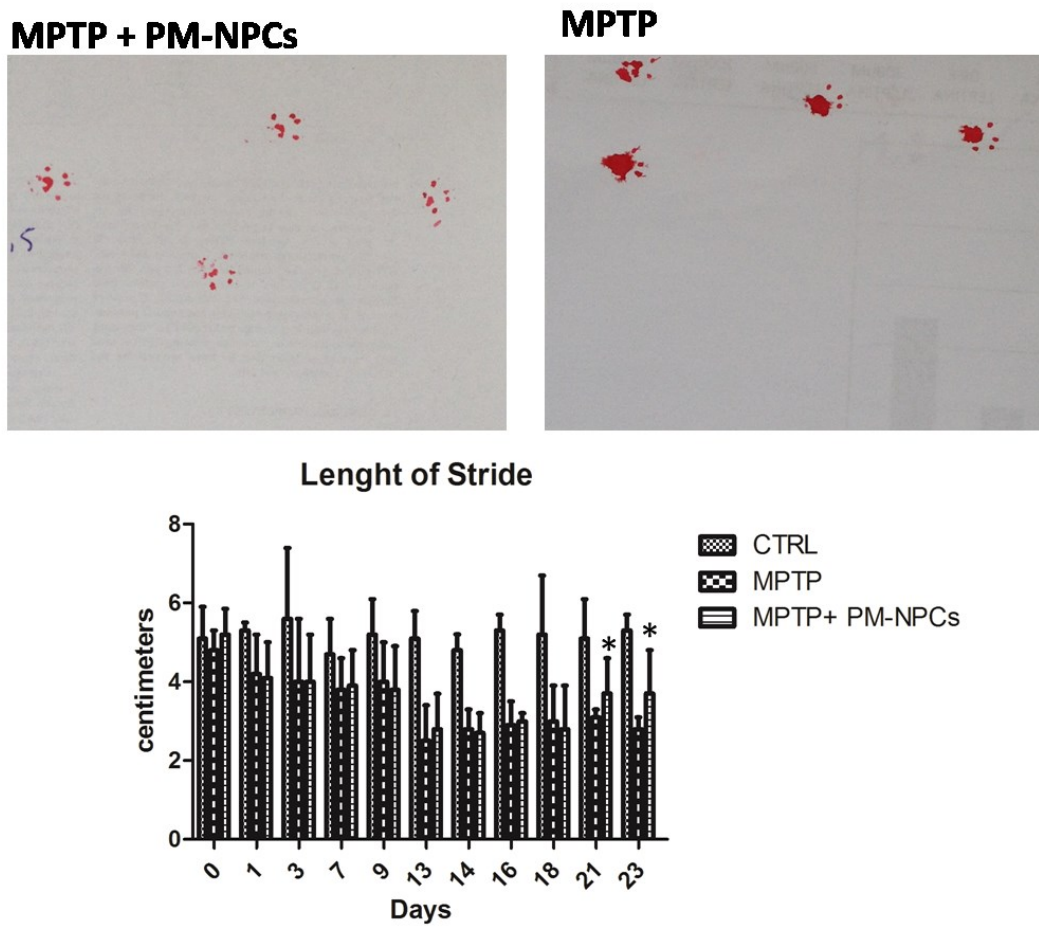

Figure 32. Stride length of Forepaw. Representative runs of MPTP lesioned mice untreated and treated with PM-NPCs at day 21. At days 21 and 23 The length of stride was significantly higher in mice injected with PM-NPCs cells (n = 4) than in MPTP mice (n=4). Data are expressed as the mean ± standard error (*p<0.05 MPTP vs PM-NPCs treated animals).

5.4 PM-NPCs transplantation does not affect MPTP-mediated dopamine loss

At the end of the observational period (2 weeks after PM-NPCs engraftment, day 24) some of the animals were killed by cervical dislocation, brains were removed and striatal areas (left and right striatum) were dissected-out separately and immediately frozen in liquid nitrogen. After homogenization the individual intrastriatal content of dopamine (DA) and related metabolites (DOPAC, 3MT and HVA) was determined by High Performance Liquid Chromatography (HPLC) following the procedure described in Materials and Methods. The effect of MPTP administration on DA relative metabolites content after 2 weeks from the neurotoxin administration is shown in figure 33. In agreement with previous reports by other authors (Corsini et al. 1985, 1987 and Viaggi et al. 2009) levels of DA ranged around to 100ng/mg of proteins in normal striatum. MPTP treatment causes a decrease of DA to 51,2 ng/mg proteins (about 50% decrease in striatal DA content compared with control levels) (Figure 33A). These results consolidate the observations obtained in behavioral tests (Figures 28, 31 and 32). PM-NPCs transplantations improve recovery of function without promoting the recovery of DA content, both into the left and right striatum (Figure 33A). Also the content of DA metabolites into the striatum of cells-grafted animals remains similar to that of MPTP-saline treated animals.

In the same tissue extracts 5-hydrossitriptamine, noradrenaline and their metabolites were also determined. Panel B of figure 33 shows that MPTP and PM-NPCs-treated groups do not differ significantly.
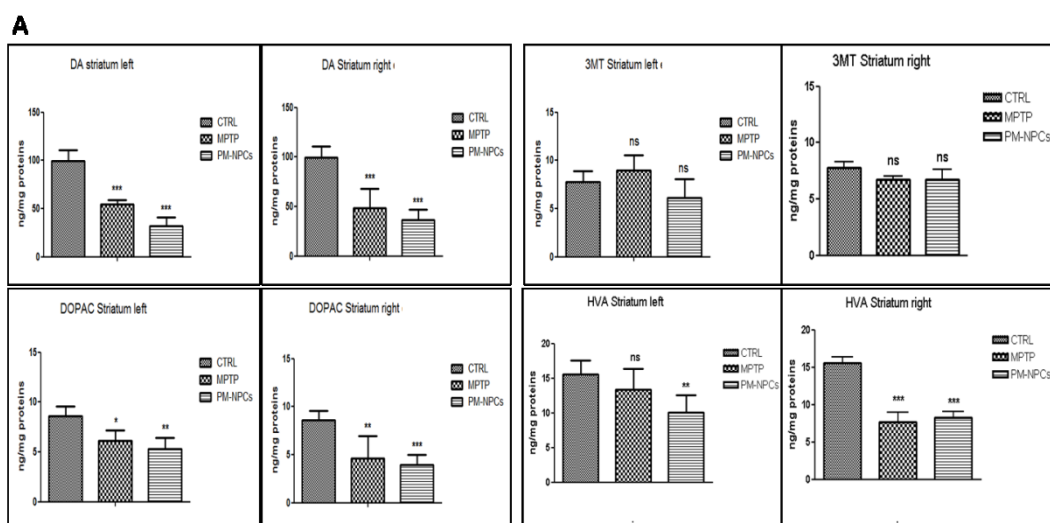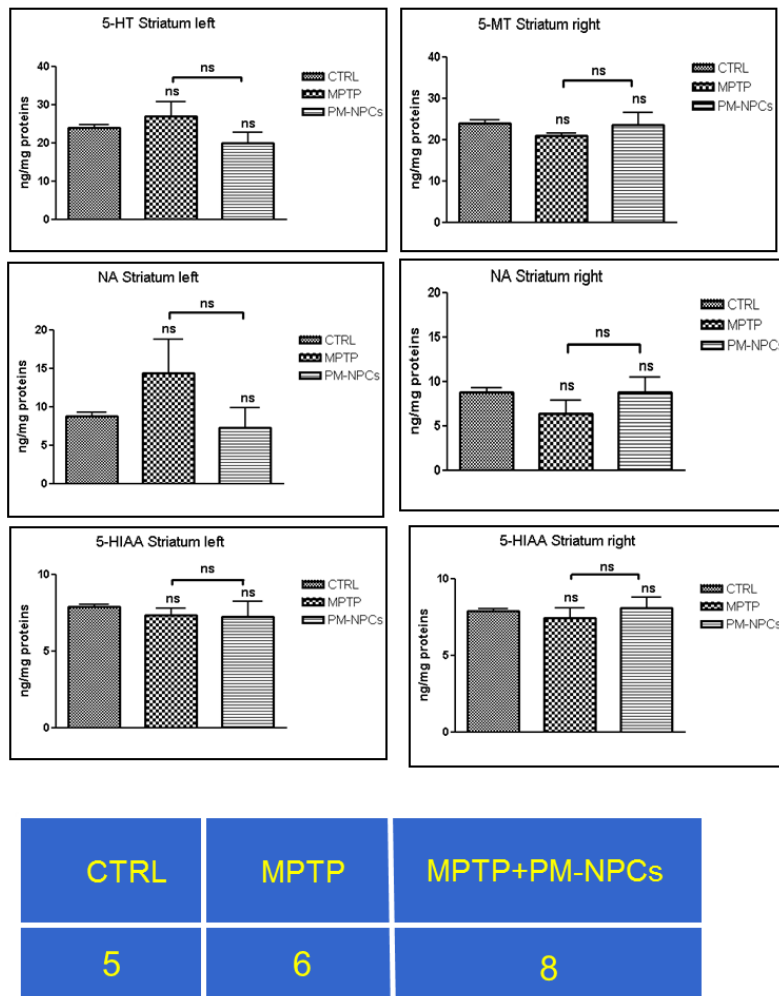
Figure 33. Effect of PM-NPCs transplantation on striatal tissue levels of DA and its metabolites. The levels of DA in the striatal areas (left and right, individually evaluated) were assessed by HPLC. The graph shows a significant decrease in DA content in MPTP group compared to the aged-matched controls. The cells graft does not determines a recovery of DA content. The number of animals utilized for the determination are reported in the table below the graphs. DA content was measured relative to the amount of proteins in the samples (ng/mg proteins). Data are expressed as mean ± SEM . *p< 0.05, ** p<0.01, ***p<0.001 versus control group (CTRL).
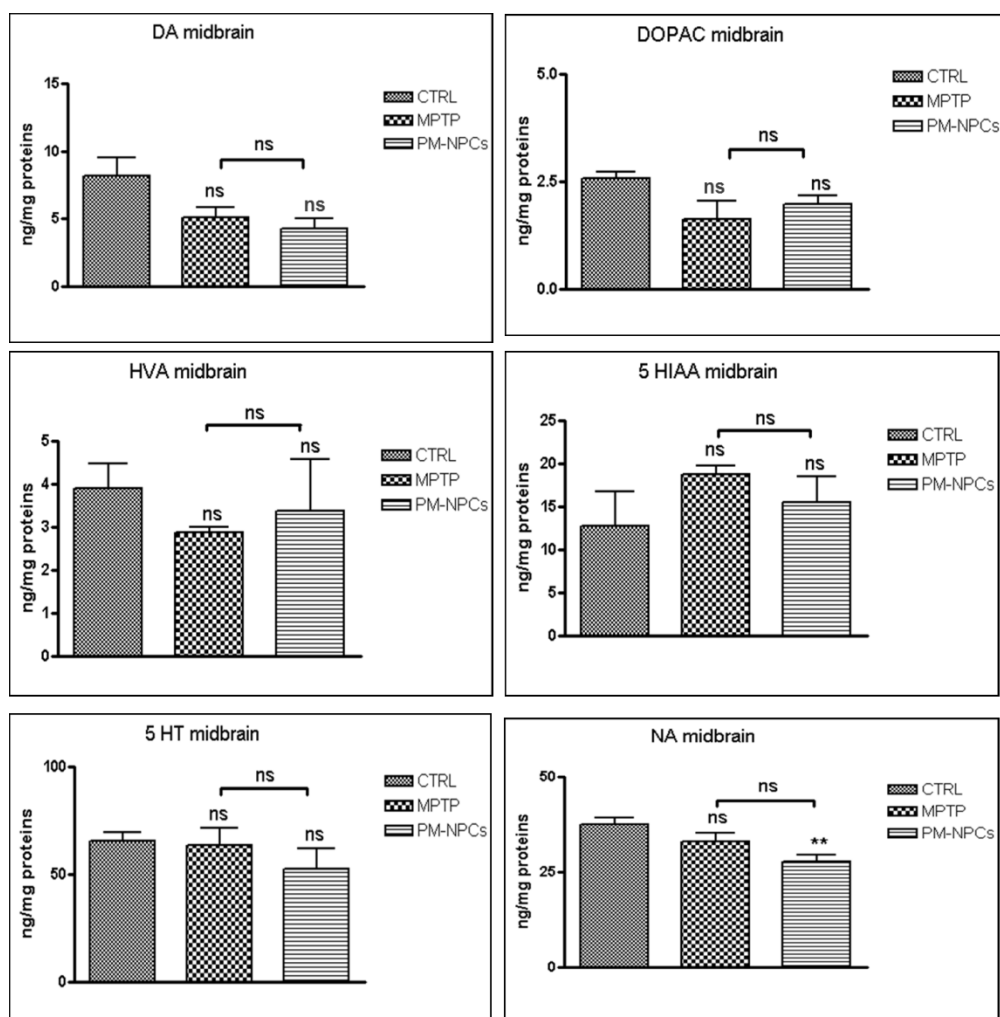
Figure 34. Effect of PM-NPCs transplantation on midbrain levels of catecholamines and their metabolites. The levels of neurotransmitters in midbrain were assessed by HPLC. Catecholamines content was measured relative to the amount of proteins in the samples (ng/mg proteins). Data are expressed as mean ± SEM . ns= not significant. **p<0.01 vs CTRL.

5.5 Intrastriatal localization of transplanted PM-NPCs

Approximately 100,000 cells were injected into the striatum of each animal and I found that many of the transplanted cells did survive 2 weeks after the transplantation. The vast majority of the GFP+ cells were found in the striatum. The morphology of the surviving PM- NPCs cells and at 2 weeks post-transplantation, the cells appeared more mature with an oval soma and abundant neuritic processes extending 30 μm from the cellular soma (Figure 35). Healthy animal brains and lesioned animal brains injected or not with PM-NPCs were sectioned by means of cryostat. Coronal sections containing the striatum, were immunodecorated with anti- tyrosine hydroxilase (TH) antibody. As expected the immunoreactivity to the TH significantly decreased into the striatum of MPTP

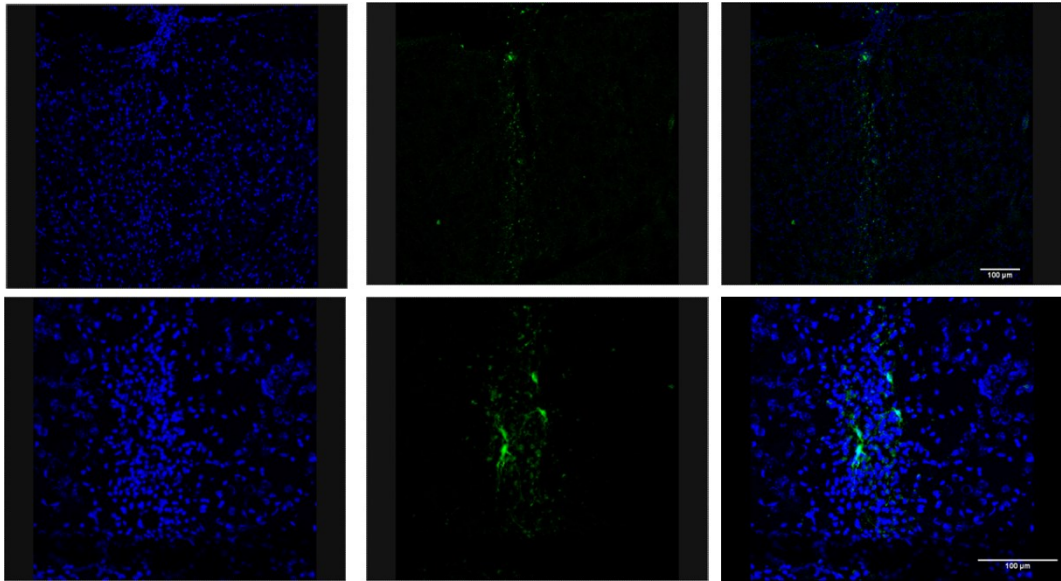animals, while it resulted lightly increased into the striatum of cells transplanted animals (Figure 36).

Figure 35. Intrastriatal localization of transplanted PM-NPCs.
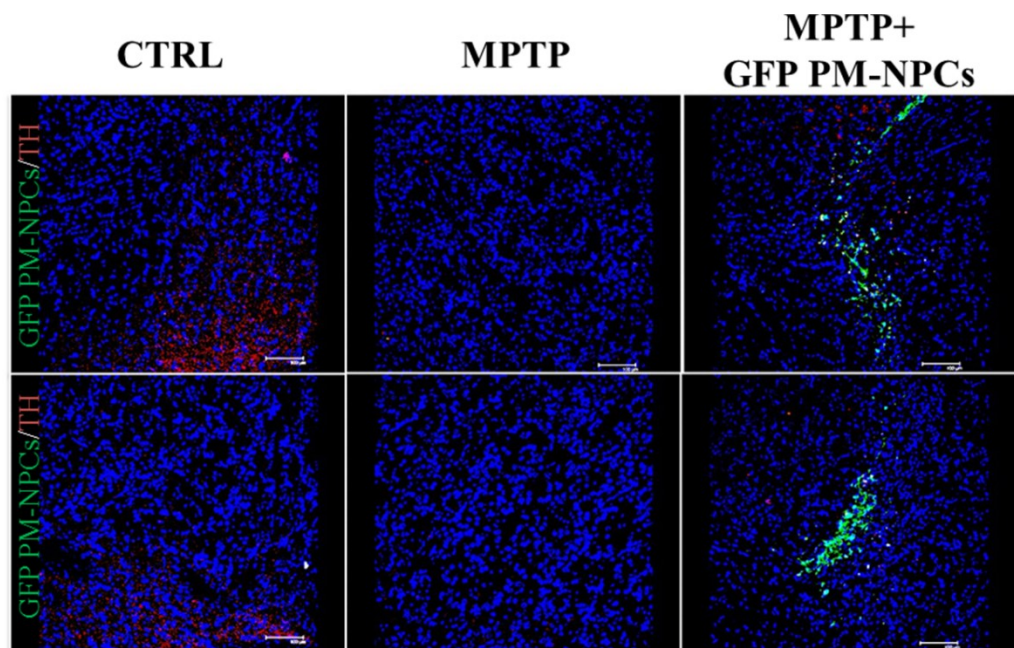

Figure 36. Tyrosine hydroxylase expression analyses into the striatum after PM-NPCs transplantation.

Moreover, considering the details showed in the figure 37 it is possible to appreciate that many PM-NPCs resulted positively decorated by the anti-TH antibody. Thus, this suggests that 2 weeks after transplantation a part of grafted

PM-NPCs were differentiated. This is also confirmed by the morphology of the cells (see yellow arrows in the enlargement of figure 37)
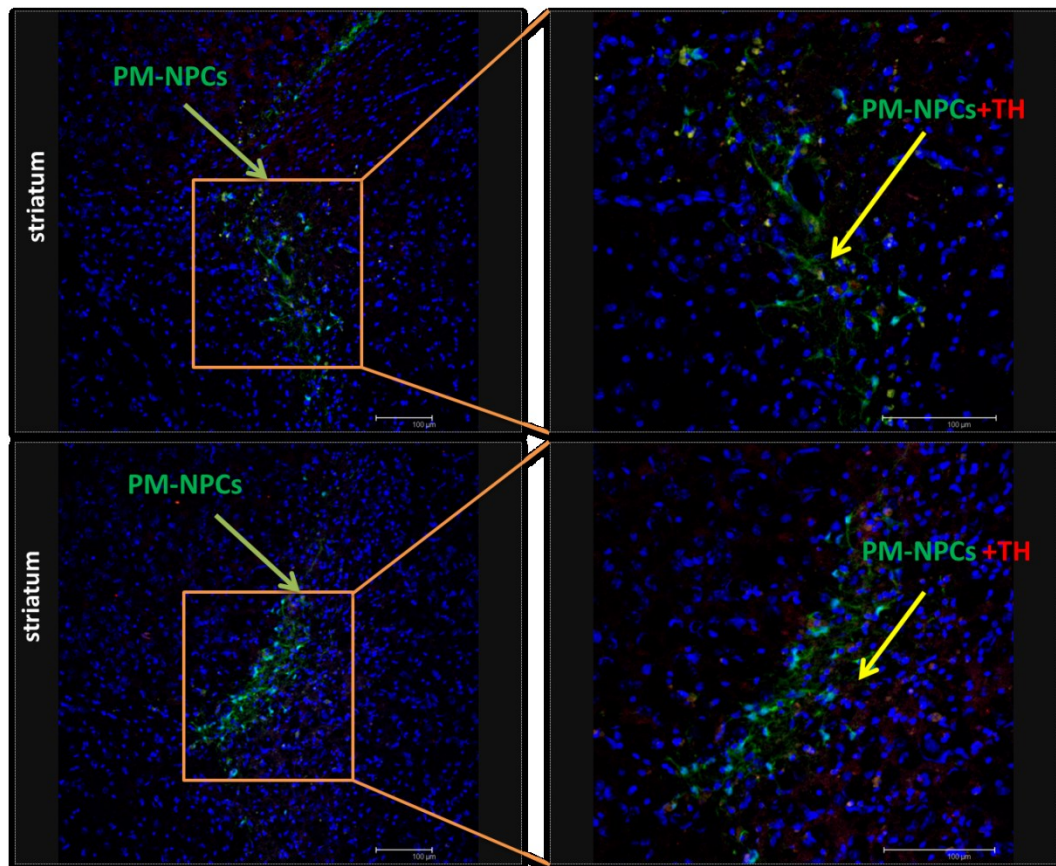

Figure 37. PM-NPCs expresses TH two weeks post-transplantation.

5.6 Most Transplanted PM-NPCs Develop into neurons

The fate of transplanted cells was investigated by means of immunoistochemical analyses into the striatum coronal sections by investigating the expression of cell specific differentiation markers. As showed in figure 18 most of transplanted PM-NPCs resulted positively decorated by the anti-MAP2 antibody (a marker of neuronal differentiation) and some cells were positive to the NG2 (the marker of oligodendrocytes precursors). The quantification showed that the grafted cells differentiate mostly in neurons: 71,88 % ± 2,3 % of GFP positive cells resulted positive for MAP-2 expression, instead 38,77% ± 3,8 % of GFP positive cells were positive for NG2 expression (Figure 38).

MERGE

DAPI

PM-NPCs

NG2

MERGE

DAPI
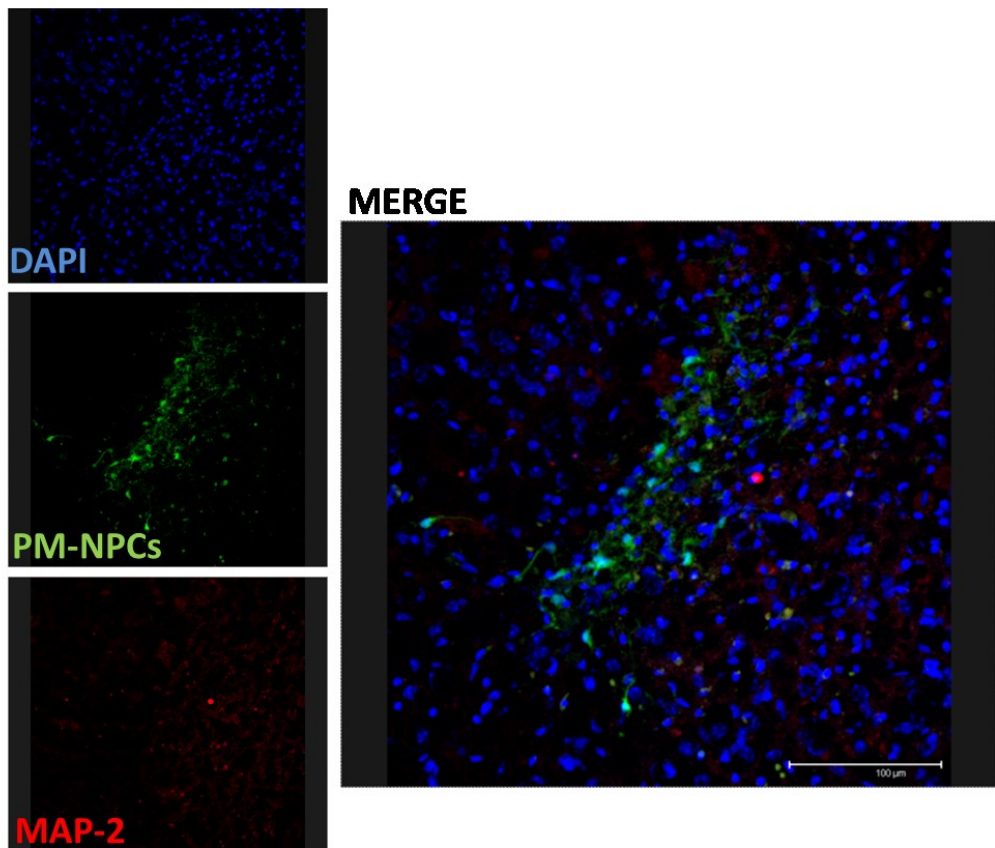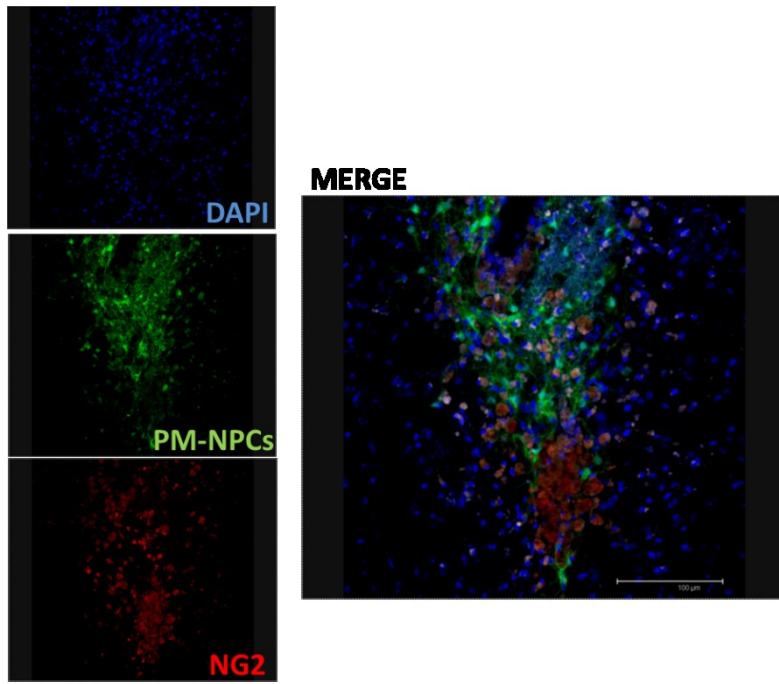
PM-NPCs

MAP-2

**Striatum
coronal sections**
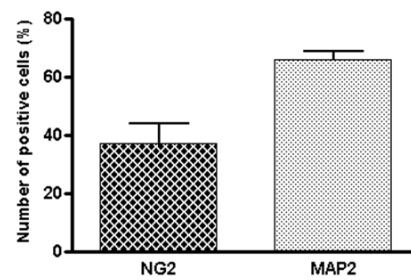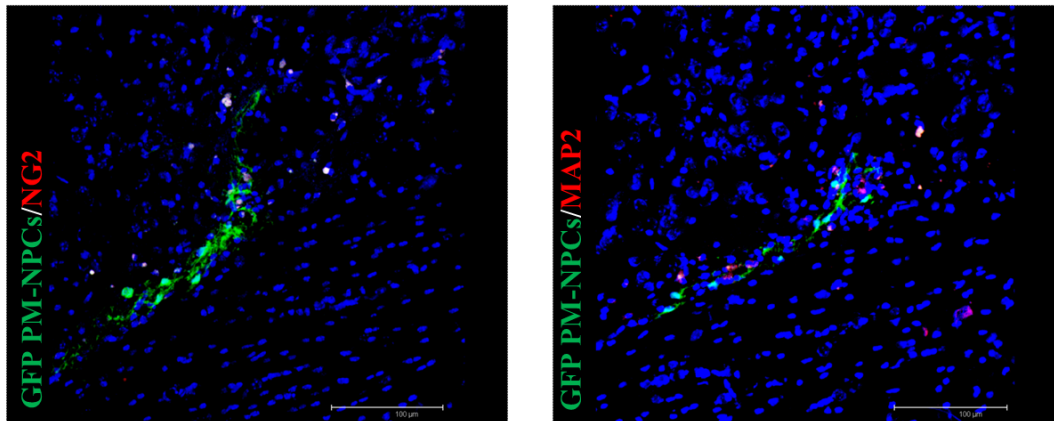
Figure 38. Most PM-NPCs adopted a neuronal Fate when Transplanted into the Adult Striatum. Two weeks after transplantation, most PM-NPCs expressed the neuronal markers MAP-2 (71,88% ± 2,3%). Scale bar represents 100 μm.

Two weeks post-transplantation some grafted cells into the striatum resulted also positive to the labelling with anti-NeuN, the mature neuronal marker (Figure 39).
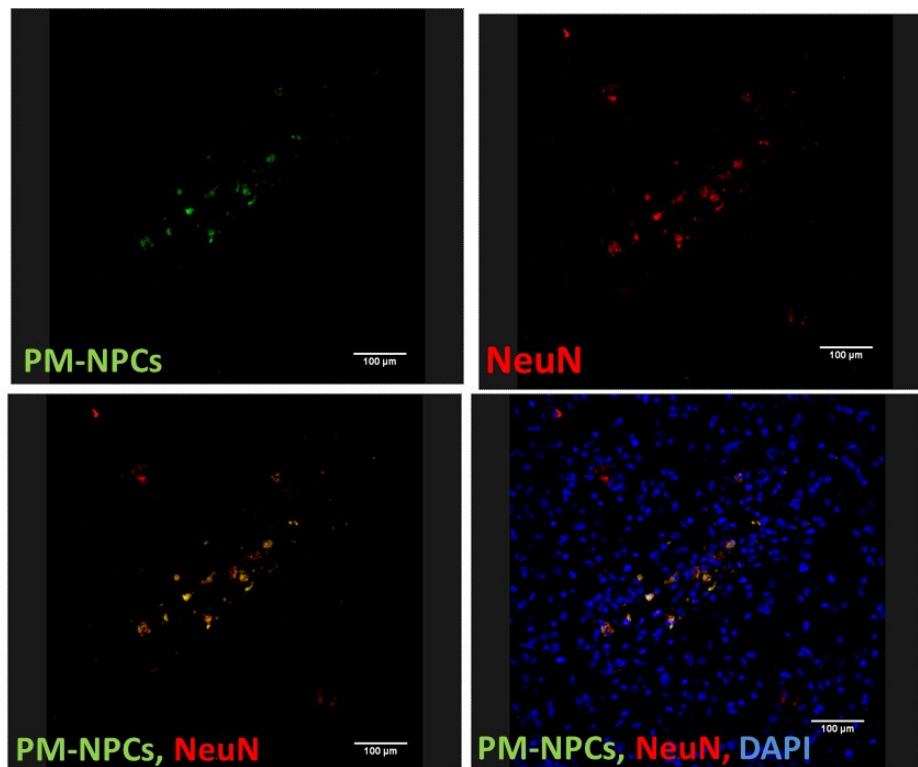
Figure 39. Transplanted PM-NPCs express NeuN. Two weeks after transplantations some intrastriatal grafted cells were decorated by anti-NeuN antibody. Scale bar represents 100 μm

We also found that most of the transplanted cells expressed the marker related to dopamine synthesis such as tyrosine hydroxylase (TH) (47,88% ± 11%, n= 311 cells), the majority of transplanted cells resulted to express the cholinergic interneuron marker ChAT, which is expressed by striatal excitatory interneurons (58,33%± 8%, n= 289 cells), and a relative lower amount of cells expressed GABA a marker of GABAergic neurons (35% ± 4%, n= 254 cells) (Figure 40).
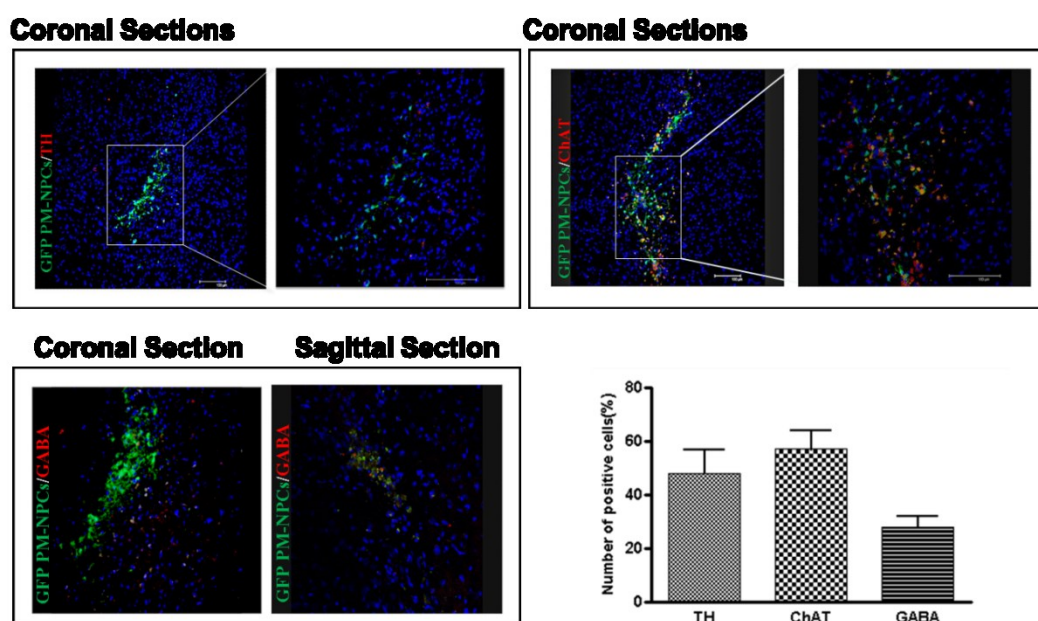
**Figure 40. Most PM-NPCs express cell specific markers after transplantation into the Adult Striatum.**

5.7 Transplanted PM-NPCs Migrate throughout the Striatum and in Substantia Nigra pars compacta

By means of confocal analyses on coronal, longitudinal and transversal sections of transplanted animal brains we investigated the migration features transplanted neural precursor cells. At 2 weeks after transplantation we observed that grafted PM-NPCs were able to move away from the injection site through the impaired striatum (Figures 41 and 42). Differently from what it was observed for other authors with other grafted cells, such as the one isolated from the embryonic medial ganglionic eminence (MGE cells; Martinez- Cerdeno et al 2010), 2 weeks post-transplantation many GFP-PM-NPCs had migrated to 2.0 to 2.5 mm following the ventral direction (Figure 41).

After re-assembly longitudinal sections obtained by confocal acquisition we were able to observe that 2 after transplantation PM-NPCs moved away from the site of injection following rostro-caudal and rostro-ventral pathways, that seems to be specific (Figure 42) for distance longer than 1 mm.
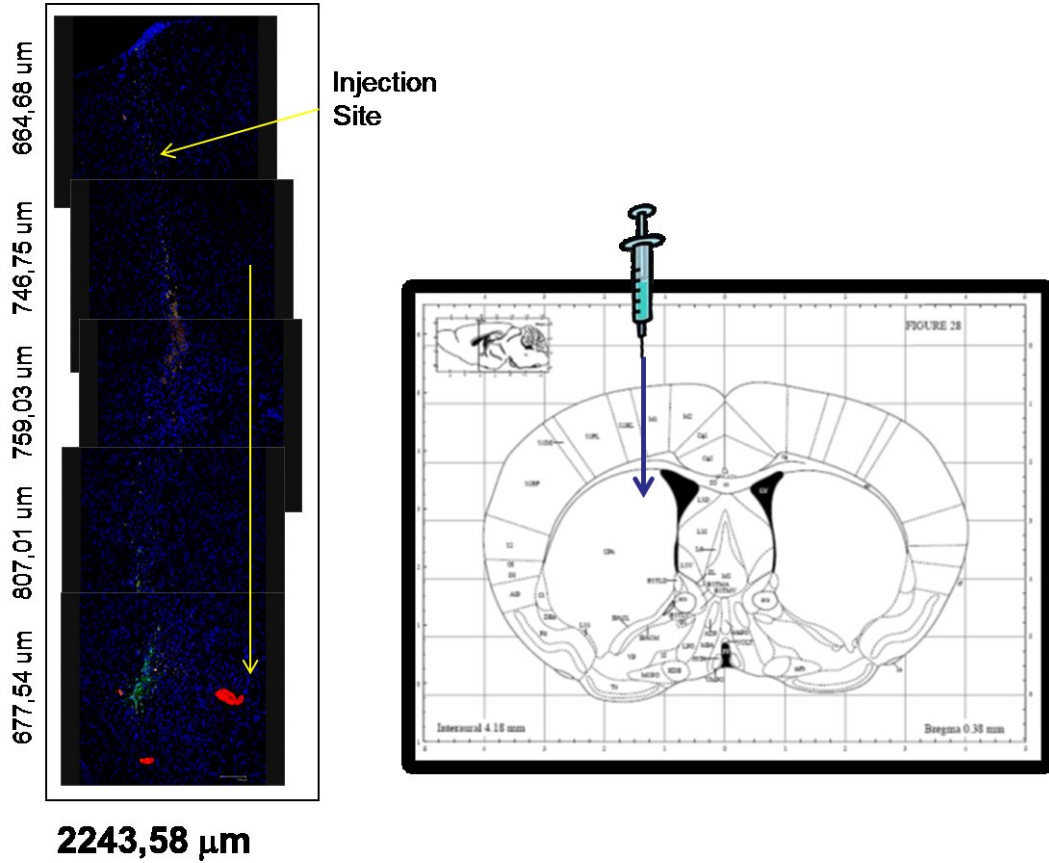
Figure 41. Coronal sections assembly showing the intrastriatal migration of PM-NPCs grafted cells.

Longitudinal Sections; GFP PM-NPCs -TH

1141,09 µm
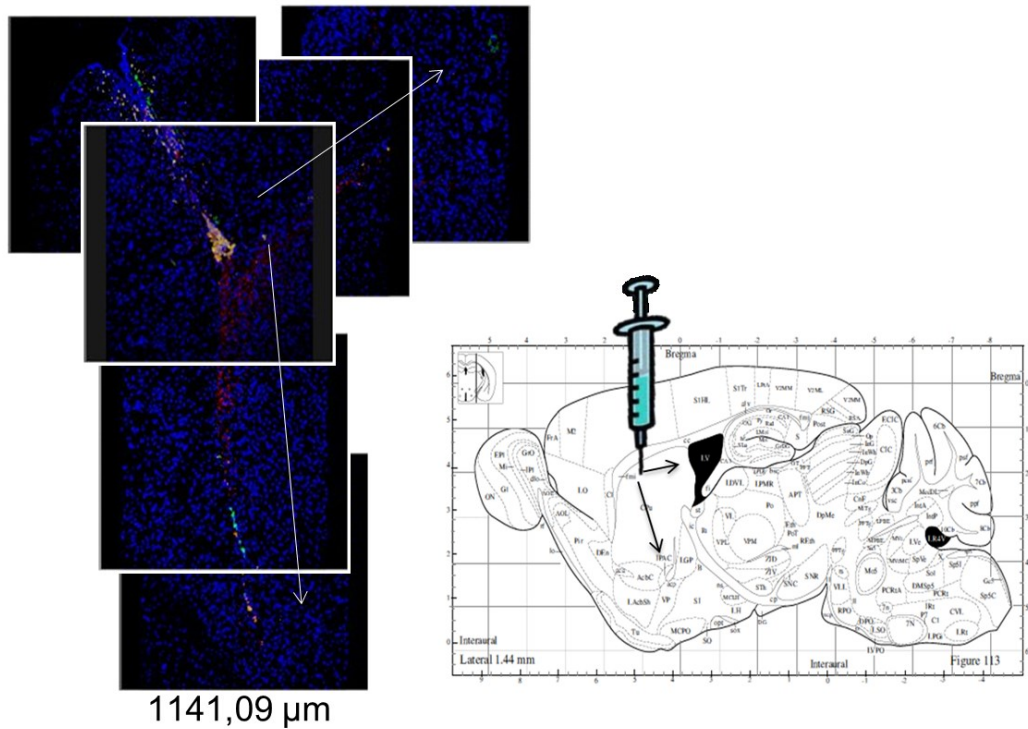
Figure 42. Sagittal sections assembly showing the intrastriatal migration of PM-NPCs grafted cells.

Further by means of confocal analyses performed on transversal brain sections of PM-NPCs grafted animals we assessed that two week after transplantation cells were able to migrate beyond the borders of the striatum and some of them were found into the *Substantia Nigra pars compacta* ipsilateral and contralateral to injection site (Figure 43).
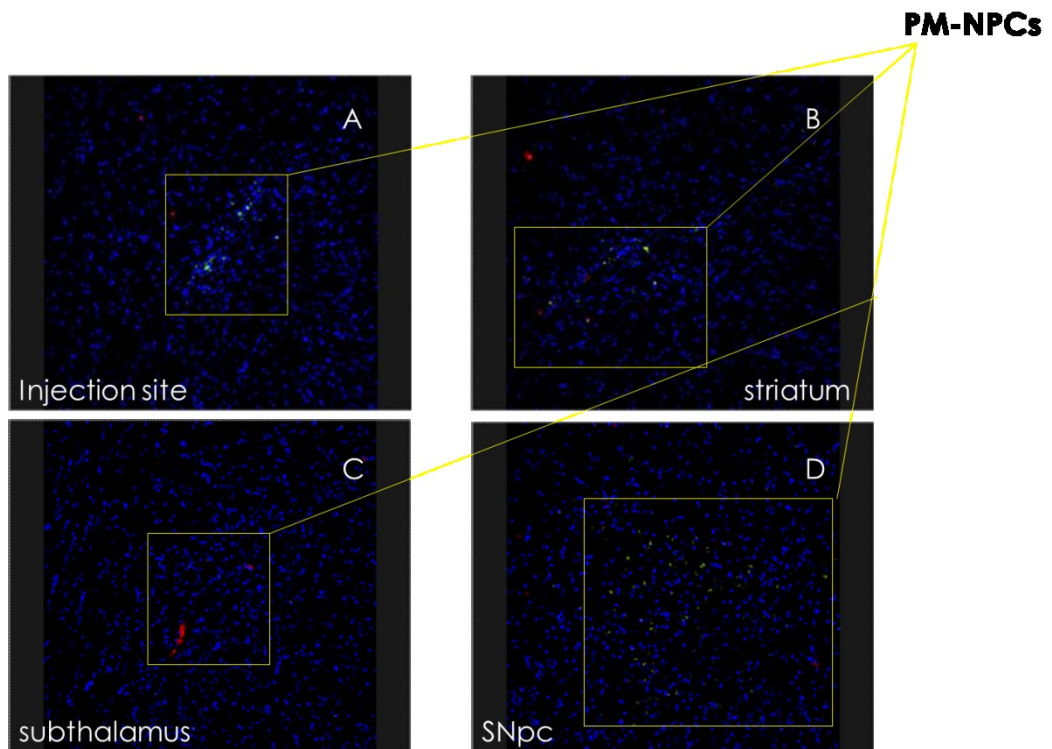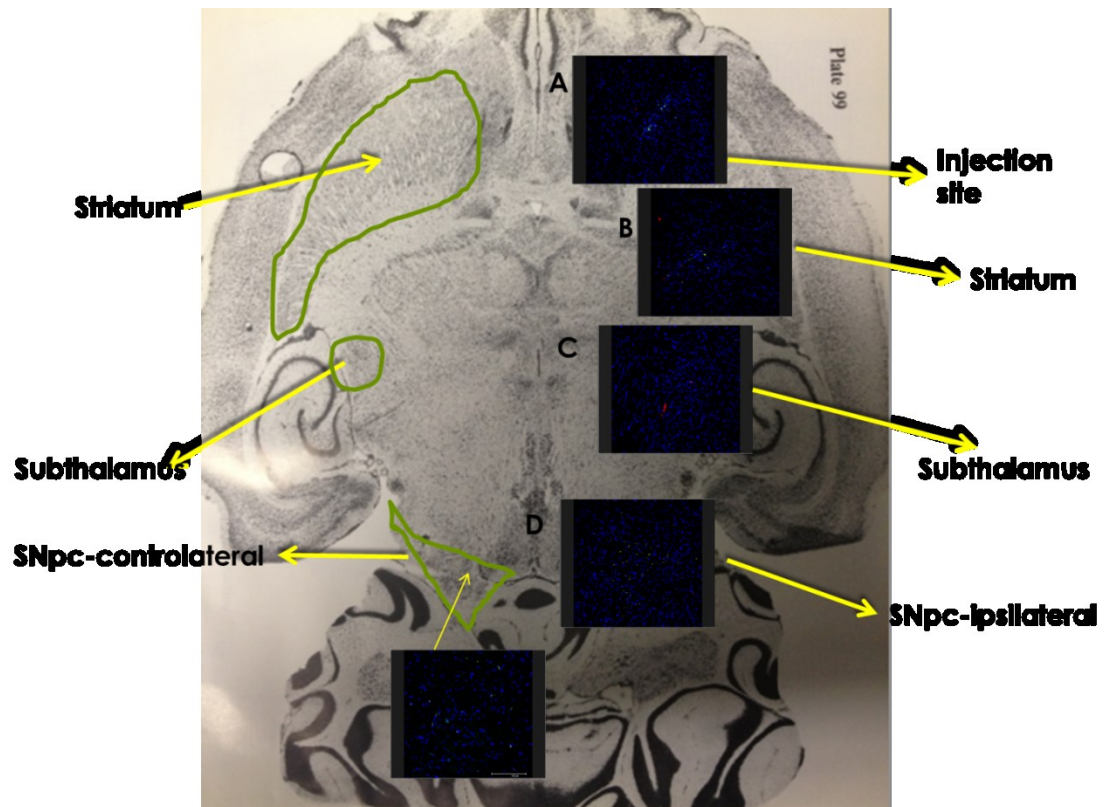
Fig 43. Transplanted PM-NPCs migrate beyond the borders of the striatum. (A) Picture showing the mouse brain transversal sections with the site of localizations of PM-NPCs two weeks after transplantation. (B) Enlargement of pictures showed in the upper panel. PM-NCs are green and in red is showed the immunoreactivity to NeuN antibody. Nuclei are stained with DAPi (blue).

5.8 Transplanted PM-NPCs Migrate in the Ventricular Zone

In rodents and in non-human primate the lateral ventricle is the larger area of adult neurogenesis; that is called, the subventricular zone (SVZ). This region lines the lateral ventricles of the forebrain and is comprised of three main cell types. The multipotent, type B astrocytes, that have been identified as the bona fide SVZ stem cells, give rise to fast-cycling transiently proliferating precursor cells that are called type C precursors and that, in turn, generate mitotically active type A neuroblasts. The type A cells, while dividing, migrate tangentially towards the olfactory bulbs where they integrate as new interneurons. In addition adult neurogenetic region is found in the subgranular zone (SGZ), which is located within the dentate gyrus of the hippocampus. A number of studies have shown that dopamine receptors (D2 and D3) are expressed on neurospheres derived from reodents SVZ precursors, suggesting that dopamine can act directly on these cells (Kippin et al 2005 and Coronas et al 2004). Moreover, in vivo studies have shown the expression of D2-like, D1-like and D3 receptors in the SVZ of both rats and mice (Diaz et al 1997, Hoglinger et al 2004, Kim et al 2010). It has been shown that in adult mice the administration of MPTP reduces the number of proliferating cells in the SVZ (Hoglinger et al., 2004). With the aim to investigate the role of transplanted PM-NPCS we found that these cells are able to reach the SVZ of MPTP lesioned mice and are positive to the staining of Nestin antibody suggesting that in this area these are maintain the state of precursors cells (Figure 44).
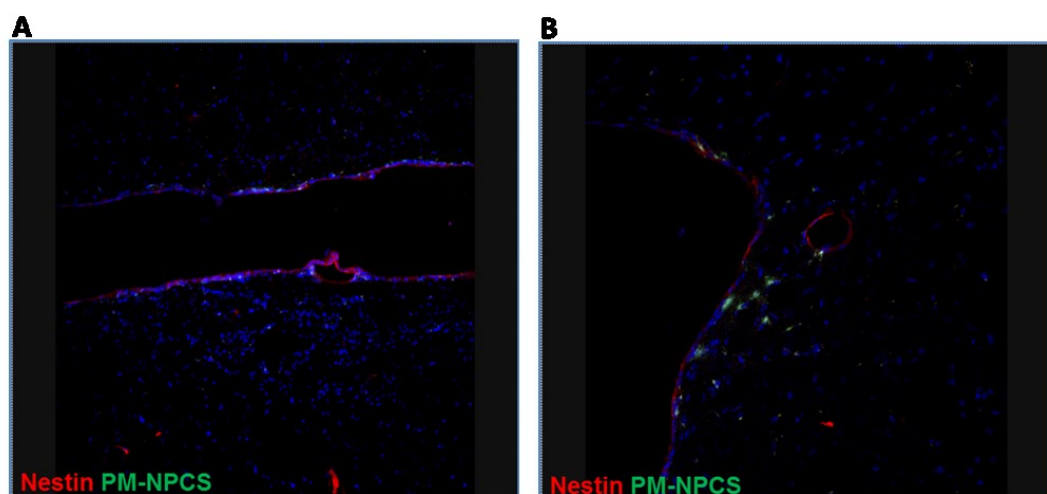
Figure 44: Localization of intrastriatal injected PM-NPCs in Lateral Ventricle of SVZ.

5.9 PM-NPCs and inflammation

After PM-NPCs transplantation in animal model of spinal cord injury in which we observed that grafted stem cells were able to influence the expression both inflammatory and anti-inflammatory some cytokines (Carelli et 2013 submitted). Moreover, a considerable literature suggesting that a local immune reaction occurs in affected regions of the brain in Parkinson's disease (PD) is available (for review see Hirsh et al. 2012 and Collins et al. 2012). Therefore we aimed to attempt whether one of the actions of transplanted PM-NPCs in our experimental model of Parkinson disease would be the regulation of neuroinflammatory process. Accordingly, we performed a new set of PM-NPCs injections was performed and animals were killed by cervical dislocation 24, 48 hours and 7 days after grafting. Brains were rapidly removed and some area such as left and right striatum, frontal cortex and mesencephalon were dissected and immediately frozen in liquid nitrogen (see materials and method paragraph for detailed protocol). The mRNA levels of cytokines was quantitatively assayed by means of real time RT-PCR. The comparison was made between the treated with PBS or PM-NPCs. As additional controls, we assayed the cytokines in the same brain regions of healthy animals. This part of the work is still in progress and figure 45 shows some results obtained until now relative to the striatal areas investigated individually. As expected the assayed cytokines, namely IL-6, IL-10 and IL-15 were increased in the striatum of lesioned animals 7 days after the PM-NPCs transplantation.
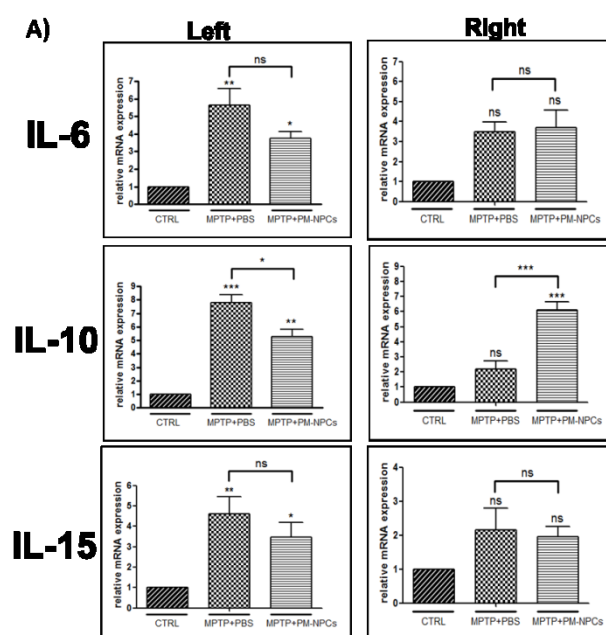
B)

CTRL	MPTP	MPTP+ PM-NPCs
3	3	5

C)
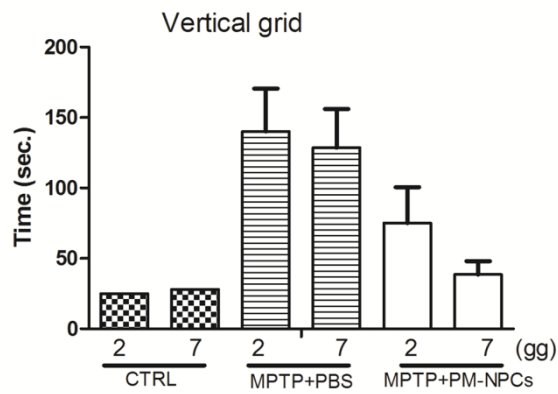
Figure 45. Transplanted PM-NPCs do not modify the expression of inflammatory cytokines. (A) mRNA levels of indicated cytokines were investigated 7 days after cells transplantations. Data are expressed as the mean of at least three independent quantifications with similar results ± standard error (** $p<0.01$, *** $p<0.001$ are vs CTRL; ns= not significant). (B) number of animals evaluated in each group. (C) Behavioral test performed as control.

6. Discussion

The aim of this study was the evaluation of the repair ability of post mortem neural precursors (PM-NPCs) in a model of Parkinson's disease created by the administration of the neurotoxin MPTP.

We described that following their administration PM-NPCs survived and differentiated in neural like cells in the injection area (about 72 % were MAP-2 positive). Part of them even migrated to very distal area such as the ispilateral and contralateral S*ubstantia Nigra* (SN). Differentiation was specific for the area of injection, most of the cells become tyrosine hydroxylase (TH) positive (47,88±11 %), and some of them become cholinergic (58,33±8% were ChAT positive) and gabaergic (35±4 were GABA positive). There was also the co-expression of more than one neurotransmitter related enzyme. In detail, 20% of TH positive cells were also ChAt positive.

From the behavioral stand point the effect of PM-NPCs was rather quick. After 10 days of MPTP administration mice were unable to properly place their forepaw and hind limbs on the horizontal grid test and unable to properly descend when positioned on top of the vertical grid. Such a behavioral deficit was maximal at 10 days after lesion and maintained unmodified thereafter in the group of cell untreated mice. Differently, the behavioral recovery occurred within 72- 96 hours after PM-NPCs administration. Within 7 days behavioral parameters were totally comparable with the control not lesioned animals. Also the walking parameters evaluated by measuring the stride length was normalized within 4 days following cells administration. The behavioral effects are likely unrelated to the changes of neurotransmitters loss caused by MPTP intoxication. The HPLC analyses have unequivocally shown that MPTP administration caused about 50% loss of dopamine content in the striatum, and such a decrease is not modified in the lesioned animals that were subsequently treated with PM-NPCs. Similar results were obtained for 5-HT and noradrenaline. Thus this suggests that the repair action of PM-NPCs may not be mediated by the restoration of dopamine levels in the striatum but rather in a novel mechanism that may be represented by an enhanced efficacy in the synaptic transmission of the surviving dopaminergic axon terminals, perhaps through an improved signal transduction mechanism. This is also supported by our finding that lesion causes only a mild change in inflammatory parameters and neurotrophic factors and that such parameters are only modestly affected by cellular transplantation.

Regenerative medicine is an emerging interdisciplinary field of life sciences with the aim of healing impaired function in the body (Haseltine 2003; Gutmann et al. 2005). The regeneration of tissues and organs offers a radical new approach for the treatment

of many resulting from any cause, including congenital defects, disease, and trauma. It uses a combination of several technological approaches that moves it beyond traditional transplantation and replacement therapies that may include the use of stem cells.

Originally described in 1817 by James Parkinson (Parkinson, 1817), Parkinson's Disease (PD) is a neurodegenerative disease that afflicts more than one million people in the United States, with over 60,000 new cases being diagnosed each year (XXX). In Italy the prevalence of PD was 230.000 cases in 2012 and it has been estimated that the number of cases will double in 2030 (Abruzzese et al. 2013). PD causes the nerve cells that generate dopamine to degenerate and die, leaving patients with limited muscle control. PD generally affects the elderly with the average onset occurring around age 60, although early onset cases do occur. Patients suffer from numerous side effects that have a strong and continued impact on their quality of life. These include uncontrollable muscle tremors and twitching, loss of facial expression, poor balance, trouble swallowing, pain and loss of movement control. While PD is not lethal and patients can live with the disease for over 20 years, the burden on patients and their families is significant. There is currently no cure for Parkinson's and treatments are limited to managing symptoms rather than addressing the underlying cause of the disease. Patients have to cope with frequent medication changes and side effects to manage (and not eliminate) the primary symptoms of this disease, resulting in a condition that is extraordinarily difficult to control on a chronic basis. PD can be caused by inherited genetic mutations (recently revised in Badger et al 2014), or be idiopathic with no known cause. There is also likely a strong contribution of environmental factors in PD. For example, the pesticide rotenone can be used to induce Parkinsonism in animal models, and some links have been shown between rural living or agricultural work and PD (Pezzoli and Cereda, 2013).

Stem cells are defined as undifferentiated cells endowed with continuous self-renewal ability and pluri- or multipotentiality (Hall and Watt, 1989). Moreover, in response to proper stimuli stem cells are able to produce more specific progenitor cells that can further differentiate into one or more functional cell types and could consequently give rise to a wide panel of cell types, including neural cells. Research on adult stem cells has generated a great deal of excitement. Scientists have found adult stem cells in many more tissues than they once thought possible. This finding has led researchers and clinicians to ask whether adult stem cells could be used for transplants. Regenerative medicine currently take advantage of the stem cells usage following three different approaches (Stocum, 2004): the implantation of stem cells to build new

structures, the implantation of cells pre-primed to develop in a given direction, and the stimulation of endogenous cells to replace missing structures. However, stem cell research has been controversial and has raised ethical dilemmas primarily concerning the creation, treatment, and destruction of human embryos inherent to research involving ES cells. There has been a long history of fetal tissue transplantation for the treatment of patients with advanced PD. Despite the wake of a long series of encouraging open-label studies, initial enthusiasm for cell replacement therapy by grafting fetal neuronal precursor cells into the striatum has vanished after two double-blind placebo controlled clinical trials showing only moderate symptomatic improvement and the occurrence of severe disabling dyskinesia (revised in Barker et al., 2013). Moreover, adult stem cells have great potential because of their self-renewal and self-replication abilities as well as their pluripotency. Furthermore, the use of adult stem cells is not limited only by ethical considerations. Several protocols have been described to generate TH-positive neurons from ESCs (Cai et al. 2009, Friling et al., 2009), and several studies have demonstrated the differentiation of NSCs and MSCs into neurons, astrocytes and oligodendrocytes (Barzilay et al., 2009; Jiang et al, 2010); the transplantation of these induced cells into animal models of PD has resulted in some therapeutic effects improving the animals impairment. In view of such results few years ago we aimed at the isolation of adult neural stem cells capable of surviving in a highly unfavorable environment. We have isolated adult neural stem cells from SVZ several hours after death of the mouse donor (Marfia et al. 2011). This procedure provides a population of NSCs, called post mortem neural precursors (PM-NPCs), that differentiate *in vitro* preferentially in neurons and such process is dependent on the autocrine EPO release (Marfia et al 2011).

Data reported in this study were obtained by performing analyses two weeks after cells transplantation and can be considered a part of a more detailed investigation in which the effects of such transplants will be investigated after longer time period from the cells implantation such as 30 and 70 days. These evidence will allow to make a comparison with other stem cells type that have been previously transplanted by other groups, such as classical embryonic neural stem cells, embryonic medial ganglionic eminence (MGE) cells (Campbell et al., 1995; Olsson et al., 1997; Wichterle et al. 1999; Alvarez-Dolado et al. 2006; and Martınez-Cerdeno et al 2010).

Considering MGE cells, the effect of their transplantation in the striatum of 6-hydroxydopamine (6-OHDA) rat has been explored by many research groups in the past. For example, Campbell et al. (1995) injected MGE cells into the lateral ventricle of mouse embryos and found that grafted cells survived, and were not able to migrate

throughout the striatum. Olsson et al. (1997) transplanted mice MGE cells into the striatum of postnatal day 1, 7, and 21 rats and reported that the cells distributed widely he striatum. Wichterle et al. (1999) transplanted mouse neuronal precursor cells from the MGE, lateral ganglionic eminence (LGE), and cortical ventricular zone into the adult striatum and found that only MGE cells possessed a unique capacity to migrate and differentiate into neurons. Alvarez-Dolado et al. (2006) showed that MGE cells transplanted into postnatal day 3 and 4 rats striatum differentiated into GABA positive neurons. More recently, Martınez-Cerdeno et al. (2010) showed that MGE cells transplanted in the striatum of adult 6-OHDA rats were able to survive for more than 1 year after the injection; these cells were mainly localized into the striatum and differentiate in inhibitory interneurons that functionally integrate into the striatum. In our experimental conditions we found that PM-NPCs transplanted cells two weeks after from the injection were found distributed in specific directions into the striatum (2,5 mm ventrally to the injection site). Moreover, we also found that these PM-NPCs overtake the striatum margins reaching the SNpc were they show a mature neuronal phenotype (NeuN expression). These evidences suggest that these cells are characterized by interesting migratory capabilities that allow them to reach damaged brain area. Concerning preclinical cell therapy experiments on PD animals models, neural differentiation-based therapy protocols were performed using stem cells from Wharton's Jelly (Fu et al., 2006), dental pulp (Wang et al., 2010) and bone marrow (Offen et al., 2007) that underwent various culture conditions before being transplanted in 6-OHDA-parkinsonian rats. Although behavioral and pathological enhancements were observed in most of the above cited studies on stem cells transplantation, the underlying mechanisms responsible were not sufficiently detailed, and there is not clear evidence for an appropriate integration into the lesioned central nervous system (CNS). Conversely, significant improvements were observed in PD animal models that were transplanted with bone marrow stem cells (BMSC) without any pre-differentiation step. In those conditions, no sign of neural differentiation was properly observed. Still, beneficial effects and rescue of dopaminergic neurons were noticed and mainly associated with neuroprotection (Park et al. 2011 and 2012), trophic support (i.e. glial cell line-derived neurotrophic factor (GDNF) or epidermal growth factor (EGF) secretion (Park et al., 2012; Baldini et al., 2010) or anti-inflammation (attenuation of blood-brain barrier damages, microglia inactivation) (Chao et al. 2009). Moreover, BMSC graft induced proliferation and migration of endogenous SVZ neuroblasts in two PD animal models (Park et al 2012; Cova et al. 2010).

PM-NPCs were obtained after donor death and show high differentiation capability compared to classical NPCs. These cells differentiate in vitro mostly in neurons and fewer cells show GFAP positive differentiation. This appears to be a specific process for several reasons: one is the number of floating spheres as parameter of neural features. We observed that there was not differences in neurospheres formation between PM-NPCs and NPCs (Figure 23). The second reason stems from the peculiar properties of PM-NPCs that is the ability to produce and release erythropoietin (EPO). We had shown that PM-NPCs (differently form NPCs) synthetize EPO that once released is the factor that allow their neuronal differentiation. Since the blockade of its receptor or the elimination of EPO release by specific antibody blocks the differentiation completely. Indeed it is well known that EPO promotes proliferation of NPCs *in vitro* and *in vivo* (Bernaudin et al 1999; Sasaki et al. 2000; Brines et al. 2000; Shingo et al. 2001; Gorio et al 2002, 2005) and that is related to the fact that all stem cells express the receptor for erythropoietin. Thus it is not surprising that the transplantation of PM-NPCs may have a favorable impact on behavioral recovery after their transplantation into the striatum dopamine depleted by means of MPTP. We must consider the enormous literature showing that in vivo administration of EPO promotes recovery of function after CNS lesionig (revision in Leist et al. 2004; Grasso et al. 2004; Brines and Cerami 2005; Carelli et al 2011) and mobilization of neural stem cells from the site of origin into the affected area. Thus, we suggest that the local release of EPO by PM-NPCs may have a dual effect, one is related to the local neurotrophic action on neurons and neural processes affected by MPTP and the other may be the migratory response of endogenous stem cells to the area of PM-NPCs accumulation. This may be supported by the highly effective and rapid reparatory effect of transplantation occurring within four days. This is highly coherent with a local release of EPO while the migratory action of endogenous stem cells may be a secondary effect that may promote the reparatory action of transplants.

The migratory pathway of PM-NPCs into the mouse brains is also suggestive of a protective action by the transplanted cells that may modify the environment allowing such a distal migration. We must remind that cells within two weeks migrate more than 2 mm, this is a very long distance in a very short period. Thus this suggest that the injection of PM-NPCs promotes the recovery of function in a model of Parkinson's disease via a modification of the microenvironment in the striatum and in the connected brain structures. Such a modifications allow both an improved DA signal transduction that is the only explanation for behavioral recovery and molecules changes that allows stem cells to migrate through the brain. One of the major points for the future

investigations will be the evaluation of the locally released EPO by PM-NPCs. The silencing of EPO in PM-NPCs will be performed in the next future.

This study provides new evidences that will be useful for developing cellular PD therapies. Future studies should further explore the clinical potential role of the investigated post mortem neural precursors cells in order to provide new perspectives on PD treatment.

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8. Publications

Publications during the phD (period 2011-2013)

Full papers

1. Giovanni Marfia, Laura Madaschi, Francesca Marra, Mauro Menarini, Daniele Bottai, Alessandro Formenti, Carmelo Bellardita, Anna Maria Di Giulio, **Stephana Carelli** and Alfredo Gorio. Erythropoietin-Dependent Differentiation of Death Resistant Neural Progenitors. *Neurobiology of Disease* 43 (2011) 86–98.

2. **Carelli S.,** Marfia G., Di Giulio A. M. and Gorio A. Erythropoietin: recent developments in the treatment of spinal cord injury. *Neurology Research International* 2011, 2011:453179. Epub 2011 Jul 4.

3. Carmelo Bellardita, Francesco Bolzoni, Melissa Sorosina, Giovanni Marfia, **Stephana Carelli**, Alfredo Gorio, Alessandro Formenti. Voltage-Dependent Ionic Channels in Differentiating Neural Precursor Cells Collected From Adult Mouse Brains Six Hours Post-Mortem. *J Neurosci Res.* 2012 Apr;90(4):751-8. doi: 10.1002/jnr.22805. Epub 2011 Dec 20. ISSN 1097-4547. -

4. **Carelli S**, Hebda D, Traversa M, Messaggio F, Giuliani G, Marzani B, Benedusi A, Di Giulio AM, Gorio A. A specific combination of zeaxanthin, spermidine and rutin prevents apoptosis in human dermal papilla cells. *Exp Dermatol.* 2012 Dec;21(12):953-5. doi: 10.1111/exd.12029. Epub 2012 Oct 23.

5. Ghilardi Giorgio; Rubino, Federico Maria; Pitton, Marco; Massetto, Nicoletta; Bianciardi, Paola Rosanna; Samaja, Michele; **Carelli, Stephana**. Glutathionyl-haemoglobin levels in crotid endarterectomy: a pilot study on 12 cases clinically uneventful. *J Cardiovasc Surg* (Torino). 2013 Apr 18. [Epub ahead of print].

6. Pertici V, Amendola J, Laurin J, Gigmes D, Madaschi L, **Carelli S**, Marqueste T, Gorio A, Decherchi P. The use of poly(N-[2-hydroxypropyl]-methacrylamide) hydrogel to repair a T10 spinal cord hemisection in rat: a behavioural, electrophysiological and anatomical examination. *ASN Neuro.* 2013 May 30;5(2):149-66. doi: 10.1042/AN20120082.

7. Caracciolo L, Fumagalli F, **Carelli S**, Madaschi L, La Via L, Bonini D, Fiorentini C, Barlati S, Gorio A, Barbon A. Kainate Receptor RNA Editing is Markedly Altered by Acute Spinal Cord Injury. *J Mol Neurosci.* 2013 Nov;51(3):903-10.

Abstracts

1. **S. Carelli**, D. Merli, G. Marfia, A. Raspa, F. Messaggio, L. Madaschi, A. M. Di Giulio, and A. Gorio. Adult Mouse Post Mortem Neural Precursors regenerate neuronal tissue and promote functional recovery after transplantation in a spinal cord injury model. 9th Annual meeting, International Society Stem Cell Research Society-ISSCR, Toronto, 14-18 June 2011.

2. G. Marfia, D. Bottai, L. Madaschi, D. Merli, M. Menarini, A.M. Di Giulio, **S. Carelli** and A. Gorio. Death resistant Neural Stem Cells regenerate neuronal tissue and promote functional recovery after transplantation in a spinal cord injury model. 21st Meeting of the European Neurological Society (ENS) May 28 - 31, 2011; Lisbon, Portugal.

3. Marfia, G; Campanella, R ; **Carelli, S**; Messaggio, F; Gorio, A. Death-resistant neural progenitors yield mostly neurons: an erythropoietin-dependent process. 21st Meeting of the European Neurological Society (ENS) May 28 - 31, 2011; Lisbon, Portugal.

4. Marfia, G ; Campanella, R; Andrea, R ; **Carelli, S** ; Gorio, A. Death-resistant neural stem cells regenerate neuronal tissue and promote functional recovery after transplantation in a spinal cord injury model. 21st Meeting of the European Neurological Society (ENS) May 28 - 31, 2011; Lisbon, Portugal.

5. F. Messaggio, **S. Carelli**, A. Raspa, G. Marfia, G. Tremolada, A. M. Di Giulio, and A. Gorio, Phenotype plasticity of mesenchymal stem cells from Lipostem™-derived human adipose tissue, CTS-IXA, Joint Congress of International Transplant Society and International Xenotransplant Association, Miami 23-26 October 2011.

6. Andrea Raspa, **Stephana Carelli**, Fanuel Messaggio, Giovanni Marfia, Carlo Tremolada, Anna Maria Di Giulio and Alfredo Gorio. Expression of surface and intracellular specific markers by human stem cells derived from Lipostem™-treated adipose tissue. CTS-IXA, Joint Congress of International Transplant Society and International Xenotransplant Association, Miami 23-26 October 2011.

7. **Stephana Carelli,** Fanuel Messaggio, Toniella Giallongo, Filippo Caremoli, Hebda Danka, Mattia Colli, Carlo Tremolada, Emilio Trabucchi, Anna Maria Di Giulio, and Alfredo Gorio. Fresh and Frozen Lipogems-derived micro-fractured human adipose tissue generates mesenchymal stem cells with higher differentiation potential and in

vivo repair efficacy. International Society of Plastic Regenerative Surgery (ISPRES); Berlin, June 7-9, 2013. *Oral Presentation

8. Carlo Tremolada, Carlo Ventura, Alfredo Gorio, **Stephana Carelli,** Camillo Ricordi, Mattia Colli, Michela Bosetti. A new device (Lipogems®) to obtain micronized fat tissue with a highly preserved SVF for autologous /allogenic use in Regenerative Medicine: clinical use and three years' experience. International Society of Plastic Regenerative Surgery (ISPRES); Berlin, June 7-9, 2013. *Oral Presentation

9. **Stephana Carelli**, Andrea Raspa, Toniella Giallongo, Anna Maria Di Giulio, and Alfredo Gorio. Exogenous Adult Mouse Post Mortem Neural promote functional recovery in a mouse model of Parkinson disease and differentiate in TH-positive neurons. 11th Annual meeting, International Society Stem Cell Research Society-ISSCR, Boston, 13-17 June 2013.

10. **Stephana Carelli**, Toniella Giallongo, Cristina Viaggi, Andrea Raspa, Giovanni Umberto Corsini, Anna Maria Di Giulio, and Alfredo Gorio. Exogenous Adult Mouse Post Mortem Neural promote functional recovery in a mouse model of Parkinson disease and differentiate in TH-positive neurons. 36° Congresso della Società Italiana di Farmacologia (SIF), Torino, 23-26 Ottobre 2013.

11. **Stephana Carelli,** Fanuel Messaggio, Toniella Giallongo, Filippo Caremoli, Hebda Danka, Mattia Colli, Carlo Tremolada, Emilio Trabucchi, Anna Maria Di Giulio, and Alfredo Gorio. Fresh and Frozen Lipogems-derived micro-fractured human adipose tissue generates mesenchymal stem cells with higher differentiation potential and in vivo repair efficacy. 36° Congresso della Società Italiana di Farmacologia (SIF), Torino, 23-26 Ottobre 2013.

12. **Stephana Carelli***, Giovanni Marfia*, Davide Merli, Luisa Ottobrini, Michelle Basso, Anna Maria Di Giulio, and Alfredo Gorio. Exogenous Adult Post Mortem Neural Precursors attenuate secondary degeneration, and promote myelin sparing and functional recovery following experimental spinal cord injury. 36° Congresso della Società Italiana di Farmacologia (SIF), Torino, 23-26 Ottobre 2013. * Equal contribution