

Parameters impacting the outcome of cell replacement therapy for Parkinson's disease: a preclinical study.

This thesis is submitted for the degree of Doctor of Philosophy at
Cardiff University.

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Summary

Parkinson's disease (PD) is the most common neurodegenerative movement disorder, currently affecting 6.3 million people worldwide. Although it is associated, in the long term, with severe complications (dyskinesias), L-DOPA remains the gold standard treatment. An alternative approach to the treatment of PD is the replacement of the lost striatal dopaminergic innervation by transplantation of foetal ventral mesencephalon (VM) dopaminergic precursor cells. Opened trials have provided the proof of concept that intrastriatal VM transplant can survive, integrate and in some cases, restore motor functions. Nevertheless, later double blind studies reported inconsistent benefit of the therapy and the development of dyskinesias remaining after withdrawal of L-DOPA medication.

The failure of the animal models in predicting these problems raises concern about their reliability. Therefore, the global aim of this PhD work was to identify some of the critical factors that can influence the functional outcome of cell therapy for PD, and on the basis of this, to develop an improved 6-OHDA unilaterally lesioned rat model for transplantation. The first step was to determine the most reliable method to assess dyskinesias in rats. The second part of this thesis was set out to determine the effect that chronic L-DOPA treatment, administered at different time could have on the survival and function of immunologically incompatible foetal VM transplant. The results demonstrated that L-DOPA administered chronically post-grafting increases the host immune response around the xenogeneic transplant. Therefore, the last set of experiments were designed to create a model of mixed donors graft to better reproduce the patient situation, where each transplant required up to 8 donors from unknown immunological background. All of these experiments come together to help to develop a rat model that more accurately represents all aspects of patients undergoing transplantation for PD.

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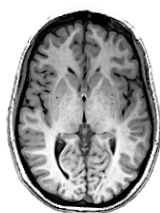
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PARKINSON'S^{UK} CHANGE ATTITUDES. FIND A CURE. JOIN US.



ABBREVIATIONS

3-OMG: 3-O-methyl dopa
5-HT: 5-hydroxytryptamine or serotonin
6-OHDA: 6-hydroxydopamine
 α -syn : alpha-synuclein
AADC: aromatic L-amino acid decarboxylase
ABCB1: ATP-Binding Cassette sub-family B member 1
AIM: abnormal involuntary movement
AIMS: abnormal involuntary movements scale
BDNF: brain-derived neurotrophic factor
BH4: tetrahydrobiopterine
COMT: Catechol-O-methyltransferase
CR: control release
DA: dopamine
GABA: γ -amino butyric acid
GCH-1: guanosine diphosphate cyclohydrolase-1
GDNF: glial cell line-derived neurotrophic factor
GIDs: graft induced dyskinesias
GPe: globus pallidus external
Gpi: globus pallidus internal
HVA: Homovanilic acid
IL: interleukin
INF- γ : interferon-gamma
I.P.: intraperitoneal
L-DOPA: L-3,4-hydroxyphenylalanine
LIDs: L-DOPA induced dyskinesias
LRRK2: leucine-rich repeat kinase 2
MAO: Monoamine oxydase
MFB: medial forebrain bundle
MPP⁺: 1-methyl-4-phenylpyridinium
MPTP: 1-methyl-1,4-phenyl-1,2,3,6-tetrahydropyridine
NGF: neural growth factor

NIH: national institute of health (US)
PD: Parkinsons's disease
PET: positron emission tomography
PINK 1: PTEN-induced putative kinase 1
PTEN: phosphatase and tensin homolog
S.E.M.: standard error of the mean
S.C.: subcutaneous
SNpc, r: substantia nigra pars compacta, reticulata
STN: subthalamic nucleus
Th1,2: T helper lymphocyte phenotype 1, 2
TNF: tumor necrosis factor
UDysRS: unified dyskinesia rating scale
UPDRS: unified Parkinson's disease rating scale

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Chapter 1: General Introduction

1.1 Parkinson's disease

Parkinson's disease (PD) is the most common neurodegenerative movement disorder. It affects 1% of the population at 65 years of age, and reaches 5% for those over 85 years (Lang and Lozano, 1998). It is a progressive disease that typically appears in the 6th decade of life, although up to 20% of people affected are classified as “young-onset” and develop the disease before they are 50. PD has a higher incidence in men than in women, with a risk 1.5 times higher in men (Wooten et al., 2004). Currently, 6.3 million people suffer from PD worldwide. In “The pledge for Parkinson's disease”, the European Parkinson's disease association stated that the annual cost of the disease in Europe reached 13.9 billion Euros in 2012 for approximately 1 200 000 patients affected (EPDA, 2012).

The following section traces the main scientific discoveries on PD and cell therapy, providing a global view of the timeline in context (Figure 1). It is succeeded by a more in-depth discussion of the literature.

1.1.1 A brief overview of the history of Parkinson's disease

Although evidence of PD has been found in the remains of ancient civilisations, it was first formally described in 1817, by the English physician James Parkinson (Manyam and Sanchez-Ramos, 1999, Raudino, 2012). In his publication entitled *Paralysis Agitans* (Shaking Palsy), James Parkinson described 6 cases of individuals presenting similar symptoms: “*Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend forwards, and to pass from a walking to a running pace*” (Parkinson, 1817). The disease was named after him, 60 years later, now referred to as “Parkinson's disease”. The second man to have his name associated with this disorder is the German neurologist Fritz Heinrich Lewy, who performed the autopsy of a patient affected with PD in 1912 and reported the presence of neuronal inclusions, which later bore his name (Lewy, 1912). These aggregates were the first pathological hallmark of the disease to be described.

In 1926, Bell and Clark reported that PD inheritance in these families follows a Mendelian distribution, therefore confirming the familial aggregation of the disease described by some of the clinicians in the early 1880s (Bell and Clark, 1926). It is worth highlighting that the only information available for Bell and Clark regarding DNA was

that it is a string of nucleotides residing in the nucleus of cells (Levene, 1919). The concept of DNA as a hereditary molecule emerged the following year, thanks to the work of Nikolai Koltsov (Koltsov, 1927).

More than 25 years after the discovery of Lewy bodies in the brain of PD patients, the second main pathological feature of the disease was unveiled by German pathologist Rolf Hassler. In 1938, he described a dramatic loss of the pigmented neurones of the substantia nigra pars compacta (SNpc) in the brains of patients presenting with PD (Hassler, 1938). Nowadays, the confirmation of diagnosis is still based on these two hallmarks: presence of Lewy bodies and degeneration of the SN. In the mid-50s, dopamine (DA) was recognised as having an effect on the central nervous system, the basal ganglia nuclei in particular, which led Herbert Ehringer and Oleh Hornykiewicz to believe that it might be a key player in PD (Bertler and Rosengren, 1959, Holzer and Hornykiewicz, 1959, Sano et al., 1959). Their *post mortem* analysis of the brain of patients, who were diagnosed with basal ganglia symptomatology, demonstrated that all and only PD patients showed a severe loss of DA in the striatum (Ehringer and Hornykiewicz, 1960). This discovery marks a giant step forward in the understanding of the disease, but fundamentally, it led to the development of the first pharmacological treatment.

The amino acid 3,4-dihydroxyphenylalanine was firstly synthesized as an L, D racemate 50 years before the role of DA in PD was discovered (Funk, 1911). The purified DA precursor L-3,4-dihydroxyphenylalanine (L-DOPA) was originally extracted from fava beans (*Vicia Faba*) but is now synthetically manufactured. Soon after the publication evidencing the lack of striatal DA in PD patients, clinical trials using intravenous injections of L-DOPA (Birkmayer and Hornykiewicz, 1961, Birkmayer and Hornykiewicz, 1962) or oral administration (Barbeau et al., 1962) were initiated. Although some improvement was observed with the motor symptoms, particularly akinesia, the results were controversial (Barbeau, 1969). At the end of the 60s, the administration of a peripheral aromatic L-amino-acid decarboxylase (AADC) inhibitor, reducing the peripheral side effects and increasing the amount of DA precursor reaching the brain, established L-DOPA as an effective treatment for PD (Cotzias et al., 1969). The same paper published by Cotzias and colleagues, however, also highlights the appearance of severe abnormal motor movements, called dyskinesias, which rapidly

limited the effect of the treatment.

Around the same period, Urban Ungerstedt performed studies in rats, using intracerebral injection of the neurotoxin 6-hydroxydopamine (6-OHDA) and induced depletion of the dopaminergic and noradrenergic neurons (Ungerstedt, 1968). The use of the neurotoxin 6-OHDA became very popular among researchers working on PD as it was the only way to produce permanent lesions in the dopaminergic system, until the discovery of 1-methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine (MPTP) in the 80s. In 1982, young drug addicts developed severe parkinsonian symptoms after injecting themselves with desmethyprodine (1-methyl-4-phenyl-4-propionoxypiperidine, MPPP), a potent opioid analgesic. Unfortunately, the home-made drug was not purified and contained a fair proportion of MPTP, which was later proven to be responsible for the accelerated degeneration of their SN, leaving them unable to move, completely “frozen” (Langston et al., 1983). MPTP was rapidly seen as a new way to create better animal models of PD and is still currently widely used in a variety of non-human primates (Chiueh et al., 1984, Langston et al., 1984, Bezard and Przedborski, 2011, Blandini and Armentero, 2012).

In the 80s, it was well established that, although L-DOPA was the best treatment available to relieve akinesia in PD patients, it could not stop disease progression. Scientists strived to develop new strategies to “heal the brain” and restore motor functions, using the newly developed MPTP-lesioned animal models. The 20th century was marked by the advent of organ transplants, beginning with the first successful corneal graft performed by Eduard Zirm in, what was at the time the Austro-Hungarian Empire (Zirm, 1906). It was followed (2 World Wars later) by pancreas, kidney and liver transplants and the first heart transplant in South-Africa (Lawler et al., 1950, Starzl et al., 1963, Kelly et al., 1967, Barnard, 1967). Continuing along that path, the interest in cell therapy grew quickly among the scientific community. The introduction of stereotaxic surgery in 1945, allowing the precise targeting of specific brain regions (Spiegel et al., 1947), followed by the development of the powerful immunosuppressive drug cyclosporine (cycloA) in the 70s allowed the start of clinical trials for transplantation (Borel et al., 1976a, Borel et al., 1976b). The first clinical study performed in 1982 in Stockholm by Erik-Olof Backlund and co-workers reported transient and limited alleviation of symptoms in the 2 patients who received the first

striatal implantation of autologous adrenal medulla (Backlund et al., 1985). Ignacio Madrazo and colleagues claimed rapid and drastic improvement of the motor symptoms, using the same approach, although these results could have never been reproduced by any other scientific teams (Madrazo et al., 1987). Indeed, systematic comparisons of the outcome of adrenal medulla transplantation for PD performed in the US demonstrated that improvements, if any, were limited and transient and that the procedure was associated to serious risk of mortality (Quinn, 1990, Goetz et al., 1990b, Goetz et al., 1990a). In parallel, in 1988, another Swedish group initiated clinical trials for the transplantation of dopaminergic precursors obtained from foetal tissue (Lindvall et al., 1989, Lindvall et al., 1990). Transplanted patients exhibited significant clinical benefit, marking the first milestone in the history of foetal transplant. 10 years later, the American double-blind randomized studies reported severe dyskinesias in some of the patients, who received intrastriatal foetal graft (Freed et al., 2001, Olanow et al., 2003). It then took over a decade before the next trial was initiated (TransEUro).

The end of the 20th century also saw the advent of genetics. In 1997, Michael Polymeropoulos and co-workers identified a mutation associated with autosomal dominant inheritance of PD in the alpha-synuclein gene (α -syn). α -syn has been shown to be the main component of the Lewy bodies discovered 85 years earlier (Polymeropoulos et al., 1997, Spillantini et al., 1997). The number of genetic studies frenetically increased following the mapping of the human genome at the beginning of the 21st century. To date, 25 genes have been identified as involved in PD (Puschmann, 2013).

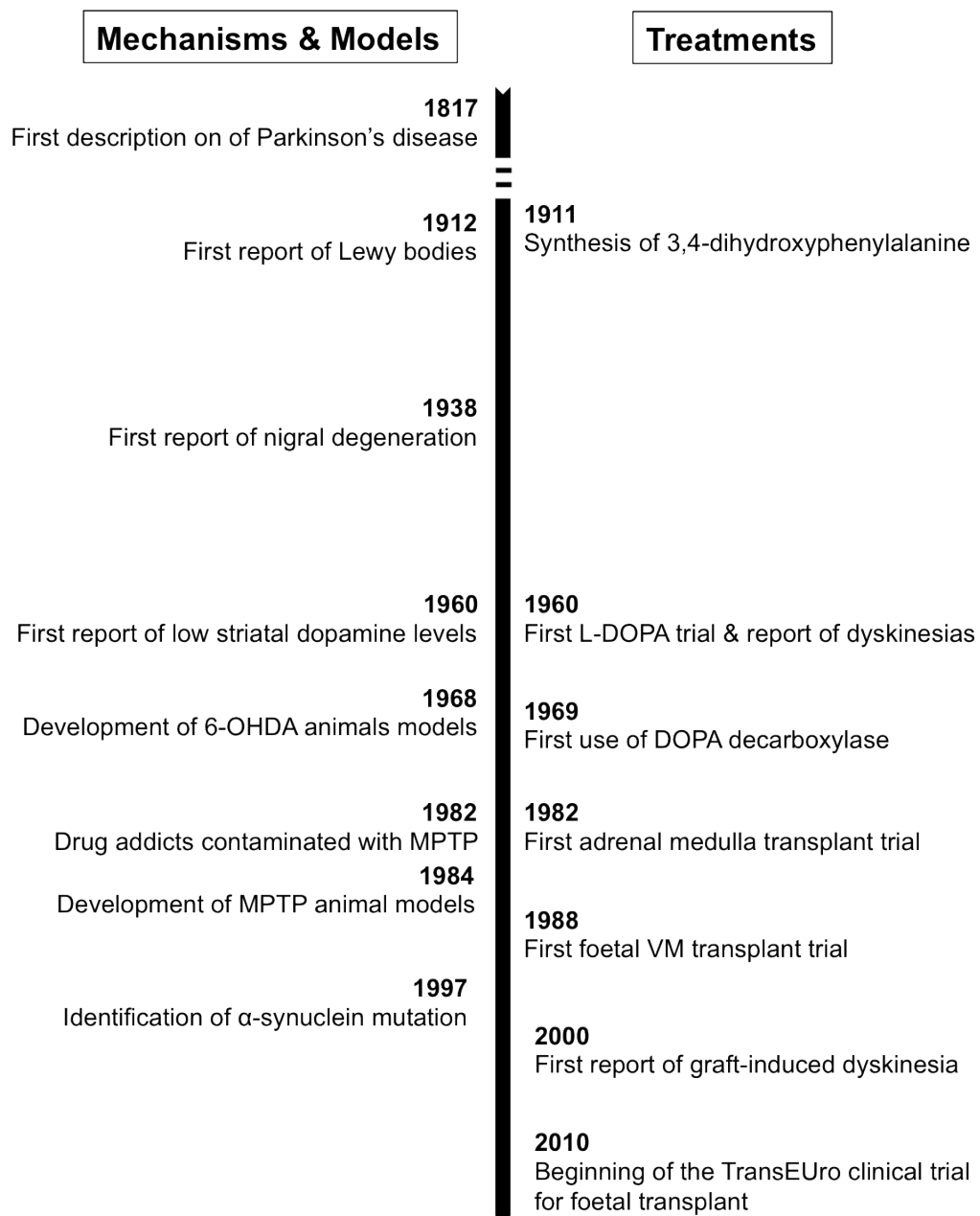


Figure 1: Key discovery timeline of PD research.

1.1.2 Symptoms & diagnosis

Currently, PD diagnosis is based on the observation of the motor symptoms that were described by James Parkinson, nearly 200 years ago, and autopsy remains the only definitive proof of the disease. PD is clinically recognised by 4 cardinal motor dysfunctions: bradykinesia or akinesia, tremor, rigidity and postural instability. Bradykinesia manifests as slowness followed by complete loss of spontaneous movements (akinesia), including facial expressions, gesturing, impaired swallowing and blinking, and one of the most easily recognised signs of PD, reduced or absent of arm swing while walking (Jankovic, 2008). Although bradykinesia represents the first step

of the diagnosis, it is not a definite proof of PD as it can be associated with other diseases (Chang et al., 1992, Kim, 2001, Staekenborg et al., 2008). PD is also characterised by unilateral tremors disappearing with activity or during sleep. Often the hands are affected with supination–pronation (or “pill-rolling”) movements but it can also involve the lower limbs; characteristically in young-onset patients. Facial tremors involving the jaw, tongue, chin etc. tend to be observed in older patients; over 70 (Lees et al., 2009). Rigidity, defined as involuntary muscle stiffness, is one of the most frequent initial symptoms of PD but is often misdiagnosed as arthritis. Finally, postural instability is a late stage manifestation, which commonly appears after the onset of other features. It is, with “freezing periods”, one of the main causes of falls. Other characteristic motor disturbances or abnormalities can help to diagnose PD, including micrographia (Rao et al., 2003) or anosmia (Doty et al., 1995). It is important to note that separately, these symptoms can be the manifestation of other diseases. It is usually the association of unequivocal bradykinesia and at least one of the other 3 cardinal symptoms, combined with the effectiveness of L-DOPA treatment that defines the disease (Rao et al., 2003, Jankovic, 2008).

When James Parkinson depicted PD, he described it as a motor disorder, affecting the motor functions: “*the senses and intellects being uninjured*” (Parkinson, 1817). One of the most significant deviations from this original description is the growing body of evidence that PD patients suffer from a diverse range of other symptoms, all grouped under the designation: “non-motor symptoms”. These symptoms include: neuropsychiatric problems (such as depression, anxiety and/or cognitive impairment), sleep disorders (insomnia, restless-leg syndrome, REM sleep disturbance), autonomic dysfunctions (bladder and sexual dysfunctions) and have been recently reviewed by others in more details (Simuni and Sethi, 2008, Chaudhuri et al., 2011, Lima et al., 2012). Some of these symptoms appear at a very early stage, before the first motor manifestation of the disease (Goldstein et al., 2011). Although most of the research performed during the past century focused on treating the motor symptoms of PD, the past 10 years have witnessed a growing interest in the other aspects of the disease as demonstrated by the increasing number of scientific publications on the non-motor symptoms of PD (Figure 2).

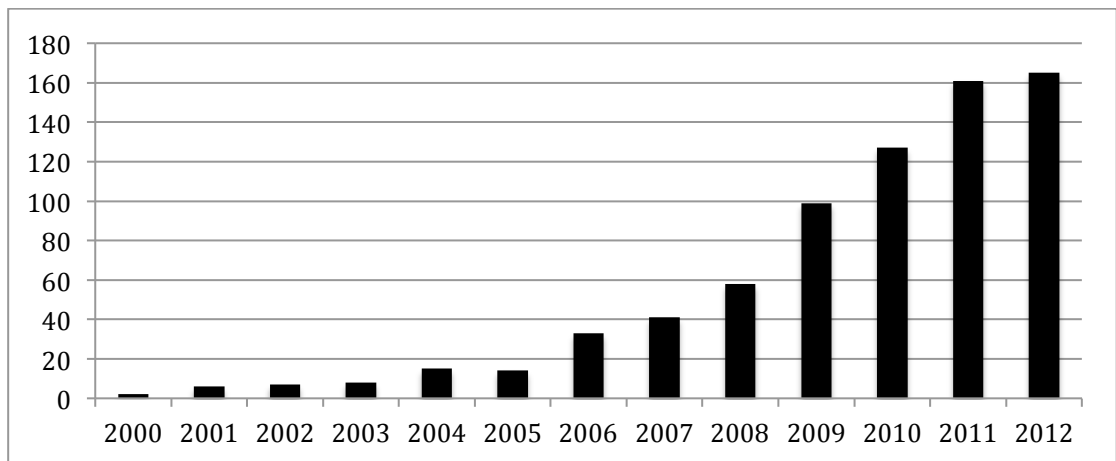


Figure 2: Number of scientific publications on “non-motor symptoms of Parkinson’s disease from 2000 to present (Corlan, 2004).

1.1.3 Pathology

1.1.3.1 *Cell loss and basal ganglia dysfunction*

A major pathological hallmark of PD is the extensive degeneration of the melanised dopaminergic A9 neurons in the SNpc, which project and release DA in the caudate nucleus and the putamen, the two basal ganglia nuclei that form the striatum in primates (Figure 3) (German and Manaye, 1993). The putamen is implicated in planning and modulation of movements through the stimulation of the motor cortex while the caudate nucleus plays an important role in other cognitive processes (Haber, 2003). A general deregulation of the basal ganglia has been observed in PD patients. The basal ganglia are a group of subcortical nuclei composed of the caudate nucleus and the putamen, the SN, the subthalamic nucleus (STN) and the globus pallidus (internal (entopeduncular nucleus in rodents) and external: GPi and GPe respectively).

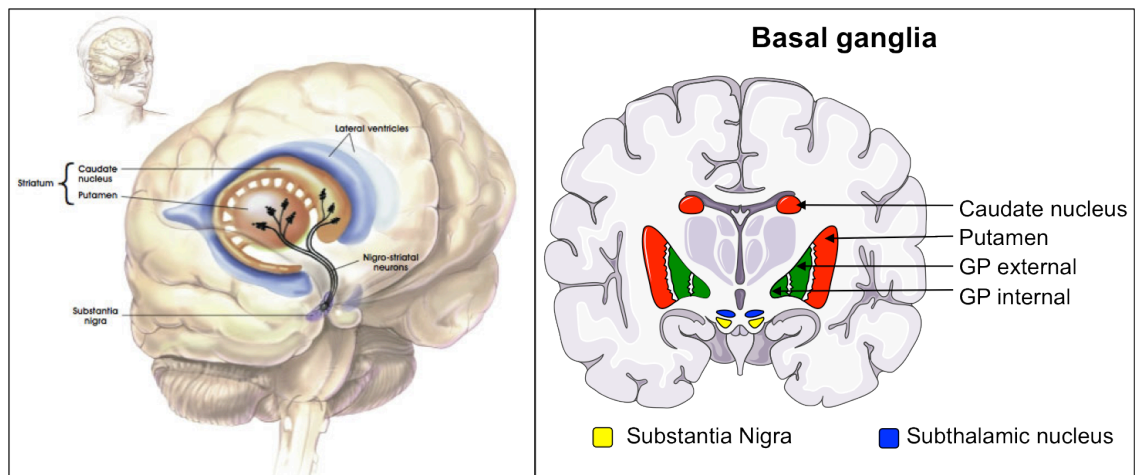


Figure 3: The nigrostriatal pathway, affected in PD (left) (Bethesda, 2001) and the basal ganglia nuclei (right, modified from www.servier.co.uk/medical-art-gallery).

An oversimplification of the interactions occurring in the basal ganglia had been described in the 80s (Chevalier et al., 1985, Deniau and Chevalier, 1985). The post-commissural putamen receives glutamatergic cortical input for all the basal ganglia, in a topographic manner. Under normal conditions, (Figure 4a) the striatum also receives dopaminergic input from the SNpc, leading to the activation of the striatal γ -amino butyric acid (GABA)-ergic medium spiny neurons. DA binding on D_1 -family G-protein coupled receptors activates the excitatory pathway, also called the direct pathway. Direct pathway neurons, expressing substance P and dynorphin, send inhibitory GABAergic efferents to the ventrolateral internal part of the globus pallidus (GPi) (Gerfen et al., 1990). This inhibition of the GPi causes a decrease in its GABAergic output to the thalamus, disinhibiting the latter. Conversely, the thalamus also receives an increased GABAergic signal from the SN pars reticulata (SNr) through the indirect pathway. Indeed, the DA binding on D_2 -family DA receptors inhibits the indirect pathway neurons, which contain enkephalin, leading to a decrease in the GABAergic signal reaching the GPe (Gerfen et al., 1990). This lack of inhibition of the GPe leads to a reduction of the STN's activity, decreasing the glutamatergic signal reaching the caudolateral part of the GPi and the SNr (Kita et al., 1983). The dual and opposing effects of DA stimulation on the striatal GABAergic neurons, through activation of both the direct and indirect pathway, is necessary to ensure an appropriate balance of stimulation of the motor cortex and the execution of normal motor function. The reduced dopaminergic drive occurring in PD affects both the direct and indirect pathways leading to hyperactivity of the output nuclei (GPi and SNr) and resulting in a strong inhibition of the thalamus and the output from the cortex, and thus an overall

decrease in motor drive (Figure 4b) (Albin et al., 1989). Since the first description of the basal ganglia communication model, other reciprocal pathways have been identified and are believed to play a role in the regulation of motor functions (Gerfen et al., 1990).

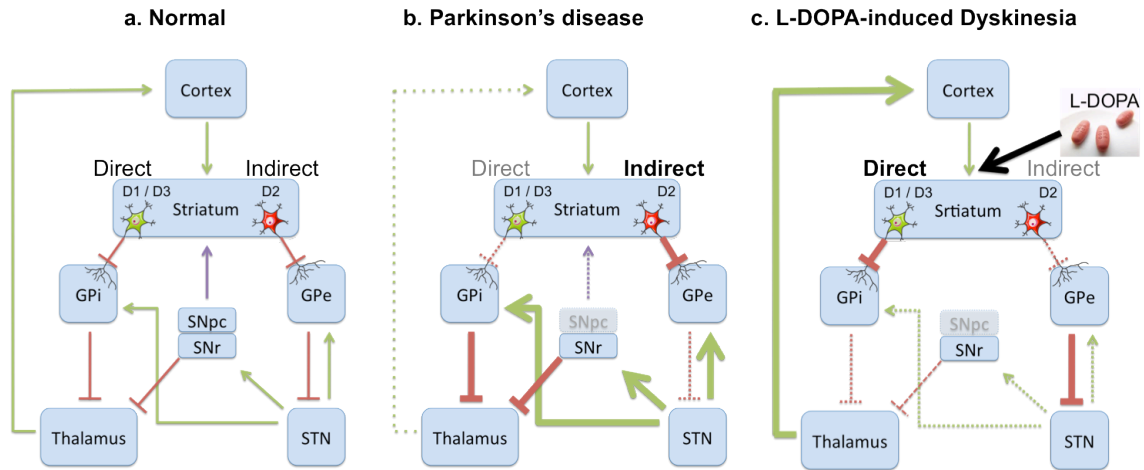


Figure 4: Simplified schematic of the basal ganglia communication pathways in normal brain (a), PD brain before (b) or after (c) the development of L-Dopa-Induced dyskinesia modified from Jenner *et al.* (2008). Representation of the dopaminergic (purple), glutamatergic (excitatory, in green) and GABAergic (inhibitory, in red) pathways between the cortex, the substantia nigra (SN), the striatum, the globus pallidus internal (GPi) and external (GPe), the subthalamic nucleus (STN) and the thalamus. Arrows represent activating signals while flat lines are for inhibition, the line weight represent the modifications occurring: increase (thick line) or decrease (dotted lines) of the signal.

Although degeneration mainly occurs in the SN, cell loss has also been observed in other structures of the brainstem, namely in the: raphe nucleus, locus coeruleus, dorsal nuclei of the vagus and nucleus basalis of Meynert (Mann and Yates, 1983). PD is also associated with a significant depletion of serotonin (5HT) levels in the striatum (mainly in the caudate nucleus), hypothalamus and the frontal cortex, due to serotonergic cell loss in the raphe nucleus. The noradrenergic and cholinergic pathways are also affected. The degeneration of the locus coeruleus leads to a decrease of noradrenaline in the hypothalamus, the cortex and the hippocampus (Fahn et al., 1971). Degeneration of cholinergic neurons occurs in the pedunculopontine nucleus and is believed to play a role in gait and posture disorder (Hirsch et al., 1987).

1.1.3.2 *Lewy bodies*

Lewy bodies are associated with various diseases, such as depression, multiple system atrophy, Alzheimer's disease etc. (Kahle, 2008). However, they are one of the characteristic features of PD and the confirmation of the diagnostic, post mortem, is established based on extensive cell loss in the SNpc combined with the presence of these cytoplasmic inclusions. These insoluble phosphorylated protein aggregates are mainly composed of phosphorylated α -syn, a small protein abundant in the brain, but also contain other proteins, such as ubiquitin (Lowe et al., 1988, Maroteaux et al., 1988, Spillantini et al., 1997). Several studies reported the presence of α -syn at the presynaptic membrane, in smaller aggregates than Lewy bodies, which suggests that α -syn could also play a role in the modulation of neurotransmitter release at the synapse (Sidhu et al., 2004, Lundblad et al., 2012). Interestingly, α -syn inclusions are usually found at the location of major cell loss, in particular in the SNpc and locus coeruleus but also in the cortex as the disease progresses and could explain some of the non-motor symptoms. Post-mortem studies performed on patients at various stages of the disease revealed that Lewy bodies first appear in the dorsal motor nucleus of the vagus nerve and anterior olfactory bundle. They then spread to the raphé nucleus and the locus coeruleus before reaching the amygdala, the basal forebrain (pedunculopontine nucleus and magnocellular nucleus) and the SN, which corroborates with the appearance of motor symptoms. Finally, Lewy bodies affect the cortex, starting in the antero-medial-temporal mesocortex and gradually extending to the neocortex (Braak et al., 2006). It has been proposed that the presence of Lewy bodies in the vagus at the early stage of the disease may contribute to autonomic symptoms such as constipation, which precede the motor disorder. Following the same train of thought, the spread of Lewy bodies to the locus coeruleus and raphé is believed to contribute to depression while their presence in the frontal cortex, during late stages of the disease, could explain dementia. Nonetheless, attempts established a correlation between Lewy body pathology and extent of cell death or severity of clinical symptoms has failed (Schulz-Schaeffer, 2010).

1.1.4 Aetiology of Parkinson's disease

The exact causes of PD remain unknown, however, different risk factors have been identified such as age and family history. Nevertheless, less than 20% of PD patients

come from a family where other members are affected and where a genetic link could have been established (Nuytemans et al., 2010). PD is believed to be due to a combination of genetic and/or environmental factors, especially for the 80% of sporadic cases.

1.1.4.1 Genetic factors

Familial forms of PD are generally associated with young onset of the disease (Latourelle et al., 2009). During the last 2 decades, several molecular genetic analyses in PD families have been carried out to identify genes responsible for the disease. Mutations in seven genes have been shown to be implicated in the autosomal dominant forms of PD (Table 1): α -syn (Polymeropoulos et al., 1997), leucine-rich repeat kinase 2 (LRRK2, dardarin) (Paisan-Ruiz et al., 2004, Zimprich et al., 2004), Vacuolar protein sorting-associated protein 35 (VPS35) (Wider et al., 2008, Vilarino-Guell et al., 2011), eukaryotic translation initiation factor 4 gamma 1 (EIF4G1) (Chartier-Harlin et al., 2011), parkin (Kitada et al., 1998), phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (PINK1) (Valente et al., 2004) and DJ-1 (Bonifati et al., 2003) (Table 1). Gain-of-function mutations in LRRK2 have been identified as the most common cause of familial PD (Horowitz and Greenamyre, 2010). However, the link between these genetic factors and the cell death observed in the SN of PD patients remain to be unveiled. However, there is a growing body of evidence that impaired lysosomal function could be involved in Lewy body formation and nigral degeneration as the α -syn, LRRK2 and PINK1 have been associated with these alterations (Dehay et al., 2013).

Gene	Role	Transmission	Lewy bodies
α -syn	Presynaptic protein	Dominant	Always
LRRK2	Kinase	Dominant	Usual
VPS35	Involved in the transport of proteins from endosomes to the Golgi	Dominant	Unknown
EIF4G1	Involved in the recruitment of mRNA to the ribosome	Dominant	Usual
Parkin	Subunit of the E3 ubiquitin ligase	Recessive	Usual
PINK1	Mitochondrial kinase	Recessive	1 case reported
DJ1	Ubiquitin ligase	Recessive	Unusual

Table 1: Main genes implicated in Parkinson's disease (Puschmann, 2013) .

Although PD has been shown to follow a Mendelian distribution in family cases, most forms are sporadic. Some of the genes implicated in the familial forms of PD also play a role in idiopathic PD such as α -syn (Kruger et al., 1999, Kay et al., 2008), PINK1 (Valente et al., 2004) and DJ-1 (Choi et al., 2006) and mostly LRRK2 (Pirkevi et al., 2009, Horowitz and Greenamyre, 2010). Mutation or over-expression of α -syn leads to the formation of Lewy bodies resulting in the death of the dopaminergic cells, mainly in the SNpc (see 1.1.3.2. Lewy bodies). PINK1 and DJ-1 are implicated in oxidative stress regulation mediated by the mitochondria. Mutations in these genes can also lead to cell death. Finally, mutations in the LRRK2 gene are the most frequently known cause of both familial and sporadic PD, and are usually associated with a late-onset slow progression form of PD. LRRK2 is a 2527-amino acid cytoplasmic protein thought to be implicated in aggregate formation, oxidative stress and increased level of reactive oxygen species, resulting in neuronal autophagy and apoptosis (Pirkevi et al., 2009, Horowitz and Greenamyre, 2010). The cause of idiopathic PD still awaits elucidation but probably involves a mix of genetic predispositions and environmental components. It has been suggested that idiopathic PD may be more a spectrum of conditions characterised by parkinsonism resulting from the degeneration of the SNpc, rather than a single entity. This hypothesis is supported by the heterogeneity of the disease (Ramsden et al., 2001).

1.1.4.2 Environmental factors

PD cannot be explained only by genetic factors, exposures to certain environmental agents have been consistently associated with this disorder. Repeated exposure to MPTP induced rapid and extensive degeneration of the SN. MPTP, crosses the blood-brain barrier and is converted in MPP⁺, a potent inhibitor of complex I of the mitochondrial respiratory chain. MPP⁺ exposure results in selective death of the dopaminergic neurons and the development of PD (Langston et al., 1983, Langston et al., 1984, Barbeau et al., 1985). Although MPTP exposure is highly unlikely in the general populations, it illustrates how a toxin of this nature could manifest as neuronal degeneration. Several epidemiological studies have shown that some agro-chemicals, such as Paraquat, a widely used herbicide with a similar chemical structure to MPP⁺, is associated with an increase risk of developing PD, also its ability to cross the blood-brain barrier remains highly debated (Naylor et al., 1995, Corasaniti et al., 1998, Shimizu et al., 2001, Widdowson et al., 1996). Other agents, such as Maneb, a common

fungicide (Ferraz et al., 1988, Hertzman et al., 1990) or rotenone, a mitochondrial complex I inhibitor commonly used as insecticide (Betarbet et al., 2000) have also been incriminated in as potential causes of PD. Similar compounds can be found in solvents such as paints, varnishes and glues, or even simply in household products, might increase the risk of developing PD (Goldman et al., 2012).

1.1.5 Animal models of Parkinson's disease

In recent decades, animal models have used different methods to mimic the specific loss of dopaminergic neurones in the SN observed in PD. The first animal models of PD used pharmacological agents, such as the alkaloid reserpine, to induce reversible loss of DA. Reserpine blocks the vesicular monoamine transporter which prevents the reuptake of neurotransmitters, including DA, thus inducing akinesia in rabbits and rats (Carlsson et al., 1957). The models moved on to use neurotoxins, due to the improvement of our understanding of the aetiology of PD and now includes genetically modified models of PD.

The use of toxins to induce cell loss comparable to the depletion observed in PD patients is the most commonly used. Many different toxins are used but the majority produce degeneration of dopaminergic neurones by inhibition of the Complex I of the mitochondrial respiratory chain and enhancing the production of reactive oxygen species (Meredith et al., 2008). First described in 1968 (Ungerstedt, 1968), the 6-OHDA rat model is currently one of the most extensively used. The neurotoxin 6-OHDA has a structure similar to DA and noradrenaline and is able to penetrate dopaminergic neurons through their catecholamine reuptake transporters (Breese and Traylor, 1971). Once inside neurons, 6-OHDA inhibits mitochondrial complexes I and IV and produces two highly toxic components: hydrogen peroxide and paraquinone (Glinka et al., 1997). 6-OHDA is not able to cross the blood-brain barrier but can be injected directly in the SN, in the terminal fields of the striatum or in the nigro-striatal dopaminergic tracts of the medial forebrain bundle (MFB). Bilateral 6-OHDA lesions produce severe akinesia compromising animal health (Ungerstedt, 1971). Consequently, the unilateral dopaminergic depletion model is commonly preferred, as it does not alter feeding and drinking ability of the animals. Moreover, the ipsilateral side of the animal can be used as a control for behaviour testing and the intact side of the brain as a control for brain analyses. For example, the extent of the dopaminergic depletion can be assessed by

various motor and sensory tests (e.g. cylinder test, vibrissae-elicited limb placing) comparing the rat's performances between the side of the body contralateral to the lesion and the other side (Torres and Dunnett, 2012). Despite the absence of Lewy bodies and the fact that the model creates severe lesions without progression, it has been extensively used in the development of new therapies for PD (e.g. cell transplant or gene therapy) (Dunnett et al., 1981, Choi-Lundberg et al., 1997, Neto et al., 2012, Back et al., 2013).

MPTP can induce severe parkinsonism in human by causing rapid nigral cell death (as previously described in section 1.1.4.2. Environmental Factors). Following that discovery, MPTP has been widely used in animals, mainly in mice (Sonsalla and Heikkila, 1986) and non-human primates (Burns et al., 1983) to mimic the human condition. For reasons that are not yet clear, rats are not sensitive to this toxin. It has been hypothesised that they cannot convert MPTP to MPP⁺ in adequate quantities due to a lower expression of monoamine oxidase B. However, intraventricular delivery of MPP⁺ produces dopaminergic cell loss, comparable to what has been observed in mice and monkeys (Yazdani et al., 2006). Similarly to the 6-OHDA toxicant models, MPTP-induced parkinsonism is not associated with Lewy bodies although it is associated with an increased in α -syn immunoreactivity (Chiueh et al., 1984, Kowall et al., 2000, Purisai et al., 2005, Halliday et al., 2009). Nevertheless, exposure to MPTP is still currently the best way to create a non-human primate model of PD, characterized by all of the cardinal symptoms and responsive to anti-parkinsonian drugs (Blandini and Armentero, 2012).

Environment toxins, as they were suspected to play a role in the development of PD, could be used to generate chronic PD models (Betarbet et al., 2000, Sherer et al., 2003). Others models based on the inflammation observed in PD patients are also used but they are inconsistent and could be associated with high death rates in animals. In those models an endotoxin: lipopolysaccharide is used to induce inflammation in the SN which again produces the death of dopaminergic neurons (Meredith et al., 2008). Lastly, recent human genetic linkage studies led to the development of various genetically modified animal models for PD (drosophila, nematode, mouse etc.) (Feany and Bender, 2000, Tabrizi et al., 2000, Vartiainen et al., 2006). Unfortunately, none of the current PD models completely recreates the key clinical and neuropathologic features of PD.

They usually present with α -syn pathology but either: do not express the motor phenotype, or do not present dopaminergic cell loss. Furthermore, the models that have motor symptoms appear to do so because the spinal cord is more vulnerable. I will not develop this topic here but genetic animal models of PD have been recently reviewed (Dehay and Bezard, 2011, Lee et al., 2012)

1.2 Current treatments for Parkinson's disease

Despite centuries of research, there is currently no cure for Parkinson's disease and L-DOPA remains the most effective treatment available. Nonetheless, because of its long-term adverse effects, every effort is made to postpone its usage. Pharmacological alternatives, such as DA agonists, monoamine oxidase B (MAO-B) inhibitors or amantadine, can be used at the early stages of the disease. However, as the disease progresses, these treatments will become ineffective and all patients will eventually receive L-DOPA treatment (Antonini and Barone, 2008).

1.2.1 Early pharmacological treatments

In the early stage of the disease, as long as there are enough dopaminergic terminals remaining, the level of DA available at the synaptic cleft can be stabilised by blocking the metabolism of DA. The enzyme MAO-B, responsible for DA deamination, is found at the outer mitochondrial membrane of the serotonergic neurons of the basal ganglia (Youdim et al., 1972). Lazabemide had very limited effect on PD motor symptoms and has therefore been dropped as a treatment (Riederer et al., 2004). Contrarily, selegiline and rasagiline are commonly used as mono-therapy in the treatment of motor symptoms in early PD (Figure 5). Selegiline generates amphetamine or methamphetamine metabolites, while rasagiline does not. MAO-B inhibitors are also used in later stages of PD, as an adjunct to L-DOPA therapy for 'end-of-dose' fluctuations. Indeed, by limiting the conversion of DA, they prolong the anti-kinetic effect of L-DOPA, therefore reducing sudden "wearing off". Finally, MAO-B inhibitors might have a protective effect and slow down the progression of the disease (Zhu et al., 2008, Maruyama et al., 2002).

DA receptor agonists were developed during the 1970s. Initially, they were used as an adjunctive therapy to L-DOPA in the treatment of advanced PD patients exhibiting

fluctuating motor responses or dyskinesias (Braham et al., 1970, Calne et al., 1974). Although apomorphine is still exclusively used to help even out the L-DOPA response (discussed later in section 1.2.2.1 Motor fluctuations), most DA agonists are now used as mono-therapy in the early stage of the disease, to delay L-DOPA treatment. Indeed, they can cross the blood-brain barrier easily and stimulate directly post-synaptic DA receptors in a selective manner (Perachon et al., 1999). Various DA agonists are currently available and fall into two major classes, the ergolines: bromocriptine, pergolide, lisuride, alpridone, and the non-ergolines: ropinirole, pramipexole, rotigotine and apomorphine. DA agonists are associated with side effects such as dizziness, hallucination, psychosis, nausea (Thomas et al., 2006). Ergot-derived DA agonists are also associated with a risk of pulmonary and cardiac fibrotic reactions, although it is limited (Lledo, 2000). Despite having different physical and chemical properties, they all present the advantage of acting directly on DA receptors and most of them have longer half-lives than L-DOPA, providing a more physiological and continuous stimulation of the receptors, with the exception of apomorphine (Brooks, 2000, Bonuccelli et al., 2009, Martinez-Martin and Kurtis, 2009).

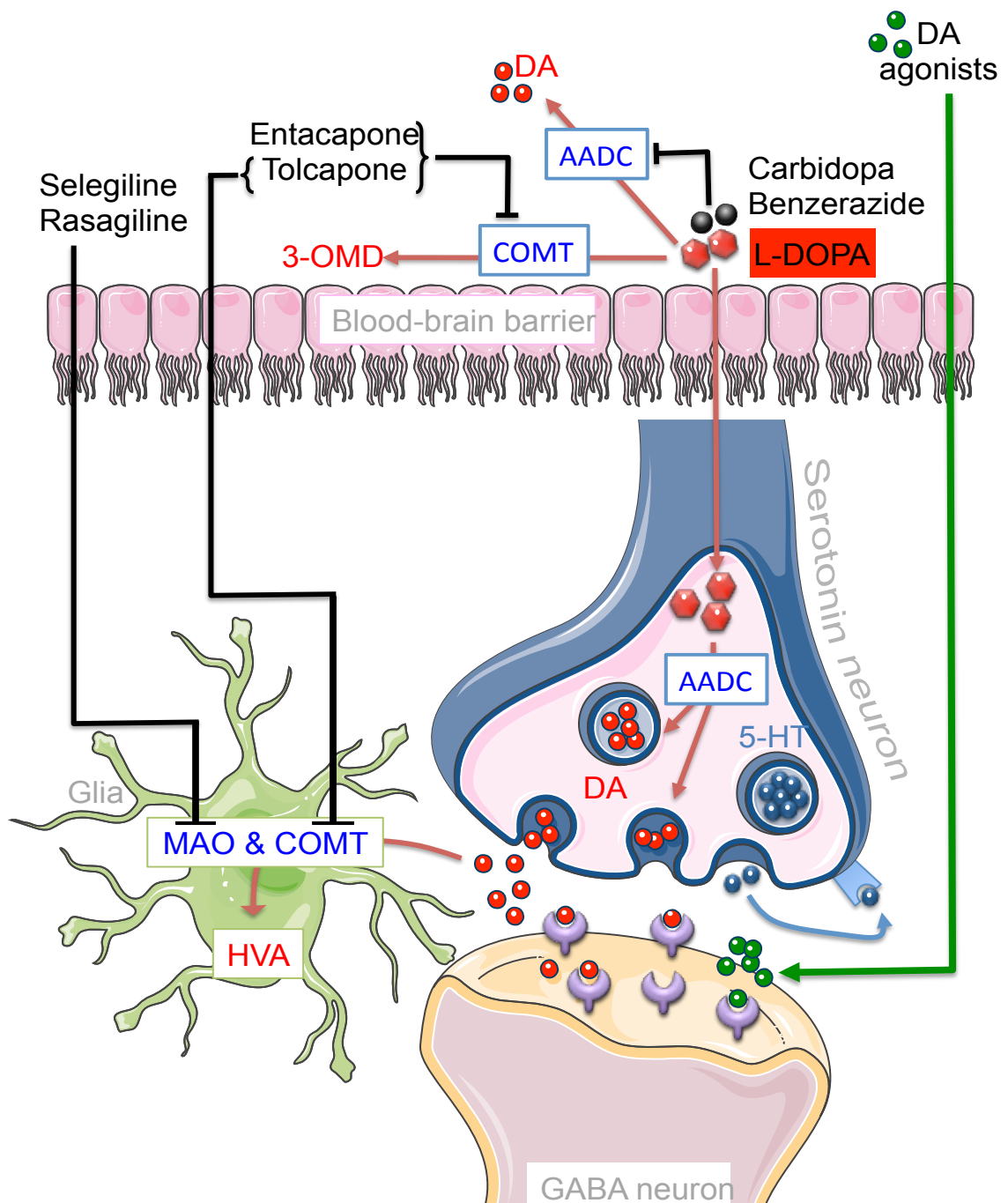


Figure 5: Pharmacotherapy for PD and mechanisms of action. Current pharmacological treatments are written in black, enzymes in light blue. As the disease progresses, dopaminergic neurons degenerate and L-DOPA is taken into 5-HT neurons and converted into dopamine (see section 1.2.2.2 L-DOPA-induced dyskinesia). 3-OMG: 3-O-methyl dopa, 5-HT: serotonin, AADC: aromatic L-amino acid decarboxylase, DA: Dopamine, COMT: Catechol-O-methyltransferase, HVA: Homovanilic acid MAO: Monoamine oxydase (inspired by Youdim et al. 2006).

1.2.2 L-DOPA

Currently, no curative treatment for PD exists. The administration of the DA precursor L-3,4-dihydroxyphenylalanine (L-DOPA), introduced in the 1960s (Barbeau et al., 1962, Birkmayer and Hornykiewicz, 1962), is highly effective at replacing the missing dopaminergic drive and therefore alleviate motor symptoms, in particular bradykinesia, in the early stages of the disease. It is widely used and still represents the “gold standard” treatment for PD (Worth, 2013). L-DOPA, in contrast to dopamine, is able to cross the blood-brain barrier. Once transported to the brain through the large amino acid transporters, L-DOPA is converted into DA by the aromatic AADC present in the few dopaminergic neurones still intact and in the serotonergic terminals, before being released at the synapse (Wade and Katzman, 1975b). Indeed, serotonin (5-HT) neurons contain the enzymatic machinery required for the conversion of L-DOPA to DA and can store the neurotransmitter in synaptic vesicles (Carta et al., 2007). Serotonergic neurons are very efficient at releasing this dopamine, but lack the auto-receptors allowing feedback mechanisms. This uncontrolled delivery leads to the over stimulation of the postsynaptic DA receptor, which plays a role in the development of L-DOPA-induced long term side effects, discussed in the following paragraph. L-DOPA is available as oral tablets containing a combination of L-DOPA and the AADC inhibitor: carbidopa (Sinemet, Parcopa, Atamet) or benserazide (Madopar, Prolopa), in order to prevent the conversion of L-DOPA into DA in the periphery. The co-administration of L-DOPA with AADC inhibitors, unable to cross the blood-brain barrier, prevents peripheral side effects such as oedema, nausea or vomiting, and increases the quantity of L-DOPA reaching the brain, making the treatment more effective at lower doses (Greenacre et al., 1993). Although L-DOPA remains the best medication currently available, long-term treatment is associated with severe and disabling motor complications (Curzon, 1967, Cotzias et al., 1969).

1.2.2.1 *Motor fluctuations*

After a few years of chronic treatment and as the neurodegeneration progresses, patients are subject to “motor fluctuations” and they oscillate between 2 phases: 1) “on”-phase: when L-DOPA provides therapeutic benefit, reducing the PD motor symptoms and 2) “off”-phase: associated with poor drug response and increase of PD-related disability

(Quinn, 1998). These fluctuations can become progressively more severe leading to “freezing” behaviours, when patients suddenly switch into the “off” state. This narrowing of the therapeutic window can be improved with the use of control-release (CR) formulation to ensure constant release of L-DOPA over 4 to 6 hours (e.g. Sinemet CR, Madopar CR). Continuous L-DOPA delivery can be accomplished by surgical implantation of a duodenal probe releasing constant dose of L-DOPA gel (Duodopa) and has been proven effective in reducing “off-state” (Nyholm, 2006, Antonini et al., 2007, Honig et al., 2009, Antonini et al., 2010a). However, this strategy presents some major drawbacks, as it requires invasive surgery and constant carrying of a portable duodenal infusion system (Antonini et al., 2010b).

Alternatively, catechol-O-methyl transferase (COMT) inhibitors can be prescribed in order to prevent peripheral conversion of L-DOPA into 3-methoxy-4-hydroxy-L-phenylalanine (3-OMD), either as an additional treatment (e.g. Tolcapone and Entacapone), or combine with L-DOPA and carbidopa (Stalevo). The COMT inhibition also limits peripheral production of 3-OMD, which competes with L-DOPA for transport through the blood-brain barrier (Wade and Katzman, 1975a). Prolongation of the “on-phase” can be obtained by administration of DA agonists and apomorphine, pergolide, pramipexole, ropinirole and rotigotine have demonstrated some efficacy in clinical trials (Olanow et al., 1994, Lieberman et al., 1998, Dewey et al., 2001, Holloway et al., 2004, LeWitt et al., 2007). Apomorphine delivered as continuous infusion reduces “off-time” by 50% in most patients and could also have anti-dyskinetic properties (Dewey et al., 2001, Katzenschlager et al., 2005). Moreover, it is now available in prefilled syringes, pen or ampoules, allowing patients to self-administered a “rescue injection” when L-DOPA starts to wear out. Patches of rotigotine, a non-ergoline DA agonist, had proven efficacy in smoothing out motor fluctuations (LeWitt et al., 2007, Poewe et al., 2007).

1.2.2.2 L-DOPA-induced dyskinesia

The most striking and disabling side effect of L-DOPA is the development of abnormal movements; named L-DOPA-induced dyskinesias (LIDs) appearing after prolonged treatment. A significant proportion of patients, estimated 40%, develop dyskinesias after 4-6 years of repeated pulsatile treatment and this risk increases by 10 % with every additional year on medication (Ahlskog and Muenter, 2001). The risk of developing

dyskinesias is highly increased with young-onset of the disease. Indeed, more than 90% of the patients who were diagnosed before the age of 40 develop dyskinesia within 5 years of L-DOPA treatment (Golbe, 1991, Kostic et al., 1991). Other parameters, such as genetic factors might play a role in LID development, such as mutations in LRRK2 gene (Lesage et al., 2008). Genes coding for DA transporters (Kaiser et al., 2003) or receptors (Oliveri et al., 1999) and more recently identified, brain-derived neurotrophic factor (BDNF) (Foltynie et al., 2009) might also play a role in the development of LIDs.

LIDs are described as hyperkinetic and dystonic abnormal movements and postures, which are irregular, purposeless and uncontrollable. Although the manifestation of LIDs is heterogeneous and varies between patients, they most commonly arise in facial muscles before rapidly spreading to the head and neck, eventually affecting the limbs. LIDs are mainly characterised by hyperkinetic movements, included chorea and dystonic movements, presented as sustained muscle contraction (Goetz et al., 2008, Armand et al., 2009). Dyskinesias fluctuate with L-DOPA dosing, usually associated with high plasma and brain concentrations of the drug, so called “peak-dose” or “on phase” dyskinesias, they are predominantly choreiform and choreo-dystrophic movements. LIDs can also be related to changes in L-DOPA concentration, during the increasing or decreasing phases (diphasic dyskinesia) or to very low levels of L-DOPA (“off state”) and are, in both cases, mostly characterized by dystonic, sometime painful, movements. These three patterns of dyskinesias can be exhibited in the same patient (Thanvi et al., 2007). In clinical practice, dyskinesias are assessed using subjective rating scales based on clinical examination, patient historical self-evaluation or a combination of both. The variability in evaluation techniques made clinical studies impossible to compare and at the end of the 2000s, the Movement Disorders Society commissioned a task force to compare the different clinical rating scales (Goetz et al., 2007, Colosimo et al., 2010). Moreover, recent clinical trials aiming at evaluating the effect of potential anti-dyskinetic drugs, such as fipamezol or levetiracetam, showed contradictory results, depending on the scale used and despite significant effect on the time spent “on” without dyskinesia (Zesiewicz et al., 2005, Wolz et al., 2010, Lewitt et al., 2012). This observation raises important questions concerning the relevance of these scales and their sensitivity for the detection of an improvement in the patients’ condition. Validating LID scales, in term of their sensitivity regarding anti-dyskinetic drug properties is not an easy task since the only medication available for the treatment

of dyskinesia is the anti-viral amantadine, a weak glutamate N-Methyl-D-aspartic acid (NMDA) antagonist. Indeed, amantadine has very limited effects and only works for a few years in some patients (Godwin-Austen et al., 1970, Elahi et al., 2012).

If the causes of abnormal movements remain elusive, 3 main factors have been identified as risk factors in the development of LIDs: disease progression, duration of L-DOPA treatment and medication dosage. These 3 parameters are closely linked and the relative contribution of each toward LIDs development is hard to establish. Indeed, as the disease progresses, more DA neurons are lost; therefore, a higher dose of L-DOPA will be required to alleviate motor symptoms. Not to mention that, over the years, the duration of medication will increase in parallel of disease progression. The only exception is the MPTP-intoxicated drug addicts who developed the disease over few days and within months, presented motor complications (motor fluctuations and dyskinesia) similar to the one normally encountered in patients following years of L-DOPA chronic treatment (Langston and Ballard, 1984, Ballard et al., 1985). Moreover, some case studies have sporadically reported dyskinesia appearance after the first exposure to dopaminergic drugs (Onofri et al., 1998). From these reports, it is apparent that the extensive loss of dopaminergic neurons is sufficient to induce motor complication when the patients are exposed to L-DOPA. Although many animal studies have showed that lesions in the dopaminergic pathway are required to observe dyskinesia, some studies performed in non-primates have reported contradictory results (Boyce et al., 1990b, Pearce et al., 2001, Togasaki et al., 2001, Paille et al., 2007). In both cases, with or without lesions, dyskinesias appear in a dose dependent manner but occur earlier, more consistently and with lower doses of L-DOPA in severely lesioned animals (Schneider, 1989, Di Monte et al., 2000, Pearce et al., 2001).

Pulsatile discharge of DA in the striatum is thought to be the main actor in LID development (Carta et al., 2008, Lindgren et al., 2010). Animal studies have demonstrated that intrastriatal infusion of DA is sufficient to induce dyskinesia in 6-OHDA lesioned rats (Carta et al., 2006). While given systematically, L-DOPA crosses the blood-brain barrier and enters dopaminergic and serotonergic neurons. As the disease progresses, less dopaminergic neurons remain. In the absence of dopaminergic cells, L-DOPA is converted into DA by the 5-HT neurons and released in an uncontrolled manner (Hollister et al., 1979). The striatum is therefore flooded with DA,

which leads to an over stimulation of postsynaptic DA receptors. Lesions of the serotonergic pathway or administration of a combination of 5-HT_{1A/1B} auto-receptor specific agonists (8-OH-DPAT and CP94253) have been proven effective at abolishing established dyskinesias in the rat and non-human primate models (Carta et al., 2007, Munoz et al., 2008). Similar results have been reported recently with the mixed 5-HT_{1A/1B} receptor agonist eltoprazine in the same 2 animal models (Bezard et al., 2013). Finally, there is evidence that chronic L-DOPA treatment elicits sprouting of 5-HT terminals in the striatum of dyskinetic animals, which correlates with AIM's severity, a phenomenon that is believed to exacerbate aberrant DA release even more (Rylander et al., 2010, Lindgren et al., 2010). In dyskinetic patients, positron emission tomography (PET) scans revealed a correlation between the severity of LID and the amplitude of changes observed in striatal DA level (Pavese et al., 2006).

As the disease progresses, compensation mechanisms occurring in the striatum lead to maladaptive plasticity of the medium spiny neurons. The density of D₂-like DA receptors increases, while D₁ DA receptors are over-expressed and become hypersensitive (Creese et al., 1977, Lee et al., 1978, Aubert et al., 2005, Guigoni et al., 2007, Berthet et al., 2009). Thus, the rapid increase in striatal DA levels following L-DOPA administration leads to hyper-activation of the thalamus through the over-activation of the direct pathways and inhibition of the indirect pathway. This unbalanced striatal response drives abnormal stimulation of the basal ganglia, resulting in hyperactivity of the motor cortex and therefore, development of involuntary and uncontrolled movements (Figure 4c). To elaborate, the activation of D₁ DA receptors by DA converted from L-DOPA, leads to inhibition of the GPi through the direct pathway. In parallel, D₂ DA receptors stimulation decreases glutamatergic striatal output to the GPe, which releases more GABA, thus inhibiting the STN. The STN inhibition results in a lack of activation of the GPi and SNr, lifting the inhibition on the thalamus. The over activation of the thalamus results in the development of abnormal and involuntary movements (AIMs) (Albin et al., 1989, Jenner, 2008).

After years of chronic treatment with L-DOPA important post-synaptic modifications occur that correlate with the severity of LIDs. In greater details, the aberrant stimulation of sensitized D₁ DA receptors leads to hyper-activation of the cAMP-signalling cascade. The increased activity of the cAMP-dependent protein kinase (PKA) and of the

dopamine- and cAMP-dependent phosphoprotein of 32 kDa (DARPP-32), has been shown to correlate with LID in animal models (Picconi et al., 2003, Santini et al., 2007, Santini et al., 2010). Moreover, genetic depletion of DARPP-32 reduces AIMs in mice, whether the protein was knock out in striatonigral neurons or in striatopallidal neurons (Santini et al., 2007, Bateup et al., 2010). Furthermore, stimulation of the PKA and DARPP-32 pathway is accompanied by an increased phosphorylation of the extracellular signal-regulated kinases (ERK) correlating with LID in animal studies (Bezard et al., 2005, Pavon et al., 2006, Santini et al., 2007). Finally, ERK signalling is involved in the regulation of the mammalian target of rapamycin complex 1 (mTORC1) and FosB-like proteins, which have also been reported, to be increased in dyskinetic rodent and primate models (Andersson et al., 1999, Francardo et al., 2011). FosB has been shown to be up-regulated in dyskinetic patients (Lindgren et al., 2011). Moreover, animal studies have demonstrated that striatal over-expression of Δ -FosB gene is sufficient to induce AIMs in lesioned rats, highlighting the causative role played by the Fos-B family genes in the development of LIDs (Andersson et al., 1999, Cao et al., 2010).

1.2.3 Animal models of L-DOPA-induced dyskinesia

The first animal models of LIDs used non-human primates treated with L-DOPA after MPTP lesion (Crossman et al., 1987). MPTP-lesioned monkeys exhibit choreiform and dystonic movements very similar to the adverse effects observed in patients after chronic treatment with L-DOPA, which can be assessed using subjective rating scales adapted from the clinic, as recently reviewed by Fox and colleagues (e.g. the Abnormal Involuntary Movements Scale (AIMS), similar to the patient's scale bearing the same name, or the Dyskinesia Disability Scale resembling the Unified Dyskinesia Rating Scale (UDysRS)) (Fox et al., 2012). Non-human primates represent a reliable model for the study of LID as they exhibit phenomenological similarities to peak-dose dyskinesias experienced by PD patients but also display other features associated with long-term treatment with L-DOPA such as fluctuations in the response to the treatment inducing “wearing off” phases or diphasic dyskinesia (Boyce et al., 1990a, Petzinger et al., 2001, Jenner, 2003). For these reasons, non-human primates have been used to study the mechanisms underlying the development of LID, as well as test new potential anti-dyskinetic drugs (Grondin et al., 1999, Guigoni et al., 2007, Berton et al., 2009, Porras et al., 2012, Berthet et al., 2012, Bezard et al., 2013).

As a result of its resemblance to the human manifestation of LID, the MPTP-lesioned non-primate model was rapidly and widely accepted. In contrast, the adaptation of the LID primate scale to the assessment of AIMs in the 6-OHDA-lesioned rats was at first highly debated as it was believed that rodents could not express the full panel of LIDs movements observed in humans and monkeys (Cenci et al., 1998, Cenci et al., 2002). Furthermore, it has been argued that motor effects induced by L-DOPA in dopamine-denervated rats were simply repetitive behaviour, resembling stereotypies observed with other psychostimulants such as apomorphine, amphetamine or cocaine (Randrup et al., 1963, Fog, 1969, Ervin et al., 1977). The first dyskinesia rodent scale used a 0-4 scoring methods (similar to the human Abnormal Involuntary Movement Scale) based on the duration of the 3 different subtypes of dyskinesias and the evaluation of the rotational behaviour. Due to its ethical, practical and financial advantages over the primate model, the 6-OHDA-lesioned rat, and more recently mouse, became rapidly widely used in the study of LIDs and new scales were developed (Winkler et al., 2002, Steece-Collier et al., 2003, Lundblad et al., 2005, Lane et al., 2006, Maries et al., 2006). Among these new scoring methods, very few have been validated and their sensitivity toward the detection of new potential anti-dyskinetic agents has never been assessed. Moreover, one of these scales included the scoring of facial stereotypic behaviour where the boundary between dyskinesias and stereotypy induced by L-DOPA remains blurry. The first part of this thesis is therefore dedicated to the comparison of the most common rodent rating scales as well as defining the difference between LID, stereotypic and rotational behaviour.

1.3 Alternative therapeutic approaches

1.3.1 Ablative therapy and deep brain stimulation

In 1952, an accidental ligation of the anterior choroidal artery was performed on a PD patient and showed beneficial effect on motor symptoms (Cooper, 1953). Earlier studies had already demonstrated some improvement following lesion in the pallidal or sub-pallidal structures (Meyers, 1942, Fenelon, 1950, Guiot and Brion, 1953). Later, neurosurgeons refined the technique and demonstrated a more consistent benefit by specifically destroying the medial part of the pallidus or the ventral part of the thalamus (Svennilson et al., 1960, Krayenbuhl and Yasargil, 1960). More rigorous study has

found that pallidotomy alleviates tremor, rigidity, bradykinesia, and dyskinesias in PD patients, whilst thalamotomy does not improve bradykinesia or akinesia but has a beneficial effect on rigidity and tremor (Krayenbuhl et al., 1961, Paine, 1962, Scott et al., 1970, Kelly et al., 1987, Vitek et al., 2003). However, electrolytic lesions were not well tolerated when performed bilaterally and were associated with high morbidity. In the 1960s, surgeons performing ablative surgeries on PD patients reported rhythmic cellular discharges synchronous with the patients' tremors. The observation later led to the development of deep brain stimulation (DBS), which was introduced in the 1980s and slowly replaced ablative procedures (Albe Fessard et al., 1963, Benabid et al., 1987, Vitek et al., 1998, Benabid and Torres, 2012). This surgical technique uses electrical electrodes attached to an impulse generator of 2-5 V, placed under chest skin, offering a finer control and the possibility of being turned off, while there is no way back with ablative surgeries. DBS typically uses high frequency stimulations around 130 Hz, with pulse of 60 to 120 μ sec (Benabid et al., 1987, Siegfried and Lippitz, 1994, Schuurman et al., 2000, Wooten et al., 2004). The loss of the nigro-striatal dopaminergic pathway occurring in PD is associated with an over activity of the GPi and the STN, making these 2 nuclei the most commonly targeted (see section 1.1.3.1. Cell loss and basal ganglia deregulation) (Benabid and Torres, 2012). PD motor symptoms respond well to both targets, while dyskinesia is most commonly alleviated with GPi stimulation. A reduction in LID can be observed following STN DBS, however it is more the result of a decrease in L-DOPA medication enabled by the alleviation of PD symptoms than direct effect of DBS (Mentzel et al., 2012). Although the exact changes induced by DBS remain unclear, it is believed to modify the firing rate of individual neurons and act at the synapses, inducing the release of neurotransmitters, such as adenosine and glutamate (Lee et al., 2004, Tawfik et al., 2010).

1.3.2 Gene therapy

The idea of using genetic material as a therapy emerged in the 1960s following the developing understanding of the viral cycle and the transduction mechanism (Tatum, 1966). PD mainly affects a well-defined area of the basal ganglia, which can be effectively targeted since the development of stereotaxic surgery. However, because PD is not a monogenic disorder, there is no obvious gene candidate for this therapeutic approach. Different strategies have been investigated, among them: the restoration of DA synthesis, or the production of neurotrophic or neuroprotective factors. They will be

examined in further detail below. (Other approaches have been used and have been reviewed elsewhere (Porrás and Bezard, 2008, Coune et al., 2012)).

L-DOPA is synthesized from tyrosine by the tyrosine hydroxylase (TH) enzyme. It is then converted to DA by the AADC. The activity of TH, and therefore the efficiency of the production of L-DOPA, requires the co-factor tetrahydrobiopterine (BH₄) synthesized by the enzyme GTP-cyclohydrolase-1 (GCH-1). The “enzymes replacement” gene therapy strategy aims to restore the level of striatal DA by inducing ectopic synthesis of DA in the GABAergic medium spiny neurons. One possible approach is to deliver the genes encoding for TH, AADC, and GCH1 in the striatum, using a viral vector. The transfer of these 3 genes produces a significant increase in striatal DA level, accompanied by some improvement of motor functions in the 6-OHDA-lesioned rat and the MPTP-lesioned non-human primate models (Azzouz et al., 2002, Shen et al., 2000, Muramatsu et al., 2002, Sun et al., 2003, Jarraya et al., 2009). This approach is currently undergoing a clinical trial (Wirth and Yla-Herttuala, 2013).

The glial cell line-derived neurotrophic factor (GDNF) is believed to have a protective effect on dopaminergic neurons. The delivery of the GDNF gene in 6-OHDA-lesioned rat and the MPTP-lesioned non-human primate models of PD showed a significant improvement in dopaminergic neurons survival and motor functions (Engele et al., 1996, Bilang-Bleuel et al., 1997, Choi-Lundberg et al., 1998, Kordower et al., 2000, Rosenblad et al., 2000). Although open label trials provided encouraging results, double blind studies failed to demonstrate significant improvement following GDNF intraputamenal infusion (Gill et al., 2003, Patel et al., 2005, Lang et al., 2006, Slevin et al., 2007, Marks et al., 2010). It is difficult to draw any conclusions on the benefit that patients could derive from GDNF treatment as the inconsistencies of the results between double blind trials and positive open-label studies may be due to technical differences.

1.3.3 Cell therapy

One of the major challenges in the research for new treatments lies in the impossibility of diagnosing PD before the occurrence of widespread degeneration and establishment of long-term changes in the basal ganglia circuitry. At the beginning of the 20th century, scientists were seeking ways to regenerate the tissue or replace it with transplanted neurons, in order to reverse the damage and restore normal function. Using cell therapy

to repair brain tissue is a very old idea, which presents its fair share of significant challenges. First, reaching a discrete area of the brain without affecting the surrounding nuclei is not an easy task and could not be achieved without the development of stereotaxic apparatus. Secondly, mature neuronal tissue does not survive transplantation well, possibly due in part to the severing of the axonal projections (Dunn, 1917). Nevertheless two schools of thought emerged, using an autograft of the patient's own catecholamine producing adrenal gland cells, or using an allograft of embryonic dopaminergic neurons precursors collected from surgical terminations of pregnancy. The refinement of transplantation techniques for the use of embryonic neurons allowed an excellent rate of survival of the grafted cells in animals models (Le Gros Clark, 1940, Stenevi et al., 1976). Based on this data, and the poor results emanating from clinical trials of adrenal graft in PD patients, scientists started to consider foetal ventral mesencephalon (VM) tissue as a promising source of dopaminergic cells (Backlund et al., 1985, Brundin et al., 1986). The use of human foetal cells, obtained from elective abortions, as the "donor" raised important ethical questions and was, and still is, subject to active debate (Boer, 1994). Furthermore, 5-8 embryos of 6-10 weeks old are required in order to transplant a patient unilaterally, a timeline that can cause practical issues, as the tissue cannot be kept for more than few days, added to the changing protocols for terminations of pregnancy from surgical to medical in some countries. Notwithstanding the ethical and practical hurdles, the first open clinical trial for foetal VM transplant for PD went on in Sweden in 1989 with 2 of the drug abusers left "frozen" by MPTP consumption. The patients transplanted exhibited clinical benefit, which correlated with increased of 18 F-fluorodopa binding in PET scans. For the following 2 decades several open-label trials were performed and provided the proof of concept that foetal VM cells survive and innervate the striatum of PD patients, providing, an alleviation of PD motor symptoms and allowing a decrease in L-DOPA medication (Lindvall et al., 1990, Kordower et al., 1995, Hauser et al., 1999, Hagell and Brundin, 2001). Between 1980 and 2002, over 100 patients received intrastriatal VM transplants but the outcomes have been extremely variable. Although the European trials demonstrated a clear clinical benefit in most of the grafted patients, the two double-blind studies funded by the American National Institute of Health (NIH) in the mid-1990s failed to show a significant improvement in the transplanted group when compared to the placebo group (Freed et al., 2001, Olanow et al., 2003). However, further detailed analysis revealed a significant clinical benefit in younger patients (less than 60) reaching a maximum 4

years post transplantation. This highlights the importance of the choice of study end-point, as the graft needs time to mature (Ma et al., 2010). If one can argue that the design of the trials were inappropriate, the development of severe abnormal and uncontrollable movements, which persisted after the withdrawal of L-DOPA medication, raised significant concerns. Indeed, 9 out of the 70 patients transplanted in the NIH trials had to undergo subsequent DBS to alleviate these so called graft-induced dyskinesias (GID) (Greene et al., 1999).

1.3.3.1 Graft-induced dyskinesia

The first report of these severe motor side effects following intrastriatal transplantation of foetal dopaminergic neurons emanated from the first US NIH sponsored double-blind control trial performed by Freed and co-worker in the early 2000s. These abnormal movements were described as stereotypic and dystonic, mainly affecting the lower parts of the body, similar to diphasic LID (Olanow et al., 2003). In total, 30 PD patients received human embryonic VM tissue grafts and 15% of them developed severe dyskinesias during the second and third year post grafting. These dyskinesias were directly linked to transplantation and persisted after reduction or complete withdrawal of dopaminergic drug therapy (Greene et al., 1999). Similar adverse effects of the transplantation were described after the second US NIH sponsored double blind study involving 23 patients. More than half (13, 56%) of these patients developed mild off-medication dyskinesias between the 6-12 months following transplantation (Olanow et al., 2003). Lastly, a retrospective analysis of the video-based evaluation of GIDs performed on 14 grafted patients during the Swedish trials 11 years ago, underlined 8 cases of mild GIDs, which reached their peak severity after 3 years (Hagell et al., 2002). Interestingly, patients in all of these studies continued to derive benefit from the grafts, which gradually improved for up to 13-16 years post transplantation, based on the reduction of their Unified Parkinson's Disease Rating Scale (UPDRS) scores (Hagell et al., 2002). This was a feature, which echoed through all of the studies.

1.3.3.2 Animal models of graft-induced dyskinesia

After the development of transplantation side effects in patients and the variable lack of efficacy, scientists went back to the bench. Transplantation studies in non-human primates and rodents were performed and carefully monitored for dyskinesia in the absence of anti-parkinsonian drugs. Only infrequent, spontaneous GIDs were reported

in the MPTP-treated primates (Redmond et al., 2008). Similarly, in the 6-OHDA-lesioned rat model, very few spontaneous dyskinesias were observed in the absence of medication and were considered too inconsistent and unreliable to constitute a good model of GID (Lane et al., 2006, Vinuela et al., 2008). Although, it has been established that VM dopaminergic grafts are capable of reducing AIMs induced by L-DOPA in the 6-OHDA-lesioned rat, a few studies have described the appearance of a novel stereotypic behaviour limited to the forelimb and facial region described as a specific GID-type behaviour. It is however important to note that this forelimb-facial stereotypy appears under L-DOPA treatment and are associated with single site 'hot-spot' VM transplants (Steece-Collier et al., 2003, Maries et al., 2006, Steece-Collier et al., 2009). These facial-forelimb and forelimb stereotypies were not observed in rats receiving multiple sites deposition of the same number of foetal cells producing a more evenly innervating graft. This observation was consistent with the proposal from the Denver-Columbia clinical trial, which identified 'hot spots' of DA in their GID patients (Ma et al., 2002, Maries et al., 2006). However, this movement is hard to evaluate as it can be easily masked by mild LIDs.

An alternative approach, which still relies on drug stimulation, is that of amphetamine-induced abnormal movements. The amphetamine-induced rotation test is commonly used to assess the severity of DA depletion following 6-OHDA lesions and as a crude estimate of foetal VM transplantation success. Closer observation, following description of GIDs in the American trials, demonstrated that dyskinetic movements, generally resembling mild to moderate LIDs, were evoked by amphetamine administration in 6-OHDA lesioned rats, transplanted with VM tissue (Carlsson et al., 2006, Lane et al., 2006). Typically, the amphetamine-induced dyskinesias are hyperkinetic limb and/or orofacial dyskinetic movements, reaching their peak severity at 12-16 weeks post transplantation and disappearing if the graft is completely rejected by the host brain (Carlsson et al., 2006, Lane et al., 2006, Lane et al., 2008). Although this model is now commonly used to study the parameters influencing the development of GIDs (Carlsson et al., 2006) it is not fully representative of the clinical situation. Indeed, transplanted patients receiving amphetamine for ^{11}C raclopride PET imaging scans did not show the appearance or worsening of GIDs (Piccini et al., 1999, Carlsson et al., 2006, Smith et al., 2012). Despite the efforts made to reproduce some important clinical conditions (i.e. extremely severe DA depletion mimicking late stage patients, and long-term, high dose

of L-DOPA to induce severe LID prior to grafting), in neither the rodent, nor the primate, are we able to establish true spontaneous GIDs. One hypothesis that will be addressed in this thesis is that these models have still failed to recreate a situation close enough to the clinical experience, omitting specific critical factors. For instance, the impact of L-DOPA treatment post-graft and non- fully immunologically compatible transplants have never been rigorously assessed in the context of GIDs (Steece-Collier et al., 1990, Steece-Collier et al., 1995, Maries et al., 2006, Lane et al., 2008, Soderstrom et al., 2008, Steece-Collier et al., 2009, Garcia et al., 2011).

1.4 Aims of the thesis

Although neuronal transplantation represents a promising way to treat PD, previous clinical trials have reported very inconsistent results in terms of clinical efficacy. Furthermore, the appearance of unpredicted dyskinesias following transplantation raised numerous questions about the safety and the efficacy of this therapeutic approach. The global aim of this thesis was to identify some of the critical factors that can influence the functional outcome of neurotransplantation for PD.

The fact that, neither the heterogeneity of the results of the clinical trials, nor the development of GIDs, was predicted pre-clinically raised concern about the reliability of the animal models currently used in transplantation studies. This thesis focuses on the 6-OHDA unilaterally lesioned rat model of PD and first addresses the question of the accuracy of the most commonly used rat dyskinesia rating scales in a systematic manner. Once this was established, the model itself required adapting to consider clinically relevant factors. To this end, I assessed the effect that chronic L-DOPA treatment, administered either pre and/or post-transplantation, had on the survival and function of foetal transplantation. Furthermore this was considered in transplants of different level of immune compatibility. A final clinically applicable condition to be considered was the use of multi-donor grafts and preliminary work to define this model was undertaken.

The main aims of this PhD work were:

1. To compare the different methods of AIM scoring in the 6-OHDA unilaterally lesioned rat model and identifying the most reliable (Chapter 3)
2. To determine the effect of chronic L-DOPA treatment can have on the survival and function of foetal transplant of different level of immune compatibility (Chapter 4)
3. To create a model of multi-donor transplantation with mixed level of compatibility (Chapter 5)

Chapter 2: General Methods

Declaration

Details of the compounds used for the experiments can be found in the table provided in Appendix I.

2.1 Animal husbandry

Adult female Sprague Dawley rats (200-250 g body weight at the start of the experiments) were housed 2-4 per cage (L: 54 cm, W: 37 cm, D: 21 cm) containing hygienic animal bedding (Lignocel), a cardboard tube and wood stick. The animals had *ad libitum* access to food (14% protein, Harlan) and water, and were maintained under 14 hours light-10 hours dark cycle with an environment of 45-65% humidity and temperature of 20-22° C. Time-mated pregnant female Wistar rats and CD1 mice were also kept in this environment (mice and rats were housed in separated rooms), until embryo collection for transplantation. Animals were purchased from Charles River UK (Chapter 3) and Harland UK (Chapter 4 and 5). All of the experiments were carried out in accordance with the Animals (Scientific Procedures) Act 1996 Home Office regulations (Project Licence 30/2498, Personal Licence 30/9044).

2.2 Surgical procedures

For all surgeries anaesthesia was induced in an induction chamber with 3-4% Isoflurane in oxygen and then maintained on 2–3% Isoflurane, in a 2:1 mixture of oxygen and nitrous oxide. Surgery was performed on a Kopf stereotaxic frame and the rat mounted with blunt ear bars. After the intervention, the wound was cleaned and sutured using Vicryl 4-0 sutures. The animals received subcutaneous (s.c.) injection of 5 ml of 0.18% sodium chloride, 4% glucose for hydration and 10 µl of analgesic (Metacam) before being placed in a recovery chamber at 30° C until they were fully righting themselves. Health and weight checks were carried out for the 3 days following the surgical procedures and twice weekly intervals thereafter.

2.2.1 Unilateral 6-hydroxydopamine lesion

For the unilateral DA denervation, the rats received 6-OHDA injection into the ascending DA fibres of the MFB in the right hemisphere (MFB, Figure 6) according to a previously established protocol, first developed by Ungerstedt and then refined by Torres and colleagues (Ungerstedt, 1968, Torres et al., 2011). 6-OHDA (hydrobromide salt, premixed with ascorbic acid) was dissolved in 0.9% saline to obtain a 30 mM solution containing 0.03% of ascorbic acid. 3 µl of this solution were infused stereotactically using a 30-gauge stainless steel cannula connected via polyethylene tubing to a 10 µl glass syringe mounted on a Harvard micro-drive infusion pump. 6-

OHDA was delivered at a rate of 1 $\mu\text{l}/\text{min}$. Injection into the MFB was performed at the following coordinates: -4.0 mm on the anterior-posterior axis from bregma (AP); +1.3 mm laterally from the midline (ML); -7.0 mm deep from dura (DV) with the incisor bar set at -4.4 mm below the intra-aural line. The cannula was left in place for a further 3 min before being withdrawn to allow better diffusion of the toxin.

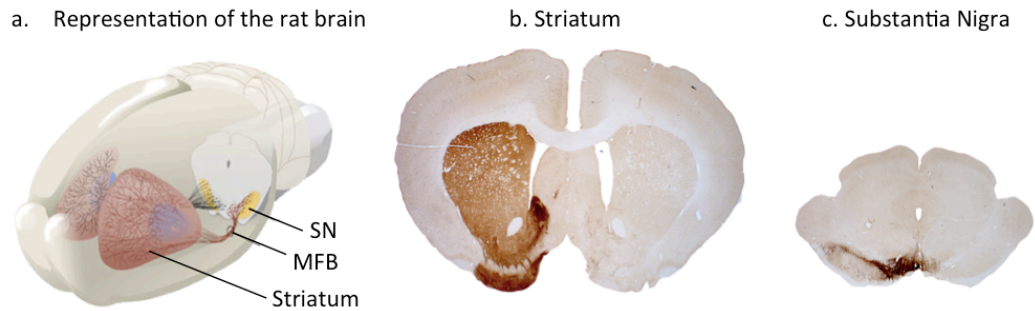


Figure 6: 6-OHDA unilateral lesion. The neurotoxin was injected in the right MFB leading to retrograde death of the dopaminergic cell bodies in the substantia nigra and loss of their projections in the striatum. a: representation of the SN, MFB and striatum (modified from Kirik *et al.* (2004)). b and c: TH⁺ labelling showing a typical absence of dopaminergic projection and cells bodies in the right striatum and substantia nigra respectively.

2.2.2 Ventral mesencephalon transplantation

Time-mated pregnant female (Sprague Dawley or Wistar rats, CD1 mice) were obtained commercially (Harlan, UK) and terminally anesthetized by intra-peritoneal (i.p.) injection of 500 mg/kg sodium pentobarbitone (Euthatal). According to a previous study, the optimal embryonic stage for mouse VM transplantation is 12 days post-copulatory (E12) (Torres *et al.*, 2007). The mouse embryos correspond to Carnegie stage 16, which in rats is achieved 14 days post-copulation (E14) rats (Butler and Juurlink, 1987). Mouse and rat embryos were collected at 12 days and 14 days post-copulation respectively in order to maintain comparable developmental stages (Table 2). The crown rump length (CRL) of the embryos was measured before decapitation and the brains were removed and dissected in Hanks' balanced salt solution (HBSS) (Figure 7, 8). Each VM was cut as indicated in figure 7 and the meningeal layer was removed. The dissected pieces were pooled in trypsin solution (0.1% trypsin and 0.05% DNase in Dulbecco's minimum Eagle medium (DMEM) at 37° C. When using a common dissociation protocol typically used in rats (Bjorklund *et al.*, 1983, Torres *et al.*, 2007),

mouse tissue became rather sticky, preventing proper dissociation and consequently, transplantation. The protocol was therefore adapted with a reduced duration of trypsin incubation from 30-20 min to 10 min and the addition of trypsin inhibitors to stop the enzymatic reaction (37° C for 10 min). These small procedural changes significantly improved the usability of the tissue. After one wash in DNase (0.1% in DMEM medium), VM were broken into small cells clusters by mechanic dissociation using gentle triturations using a 1,000 μ l and 200 μ l Gilson pipette. The solution was centrifuged at 2,000 rpm/min for 3 min and the number of cells was counted using a haemocytometer slide. After centrifugation, the pellet was suspended in adequate volume of DNase to obtain the desired concentration (between 100 000 and 400 000 cell/ μ l, as specified in each chapter) and loaded into a 10 μ l Hamilton syringe. 2 μ l of the cell suspension were injected over 2 min in the depleted striatum of anaesthetized animals, at the following coordinates: AP: +0.5 mm; ML: -2.5 mm; DV: -5.0 mm and -4.0 mm (1 μ l at each depth) with the nose-bar set at -4.5 mm allowing a flat head. In the last experiment of chapter 5, two deposits were performed at AP: +1.2 mm and -0.3 mm; ML: -3 mm; DV: -5.0 mm (note: after realising that the graft were too medial in chapter 4, the ML coordinates were changed to -3 mm). The syringe was left in place for an additional 3 min for diffusion before being retracted. Animals transplanted with xenogeneic tissue received cyclosporine A i.p. injections (50 mg/kg) daily from the day preceding surgery as an immunosuppressant treatment. The health and weight of rats receiving cycloA was monitored weekly, and particular attention was paid to tumour development and the growth of teeth and claws.

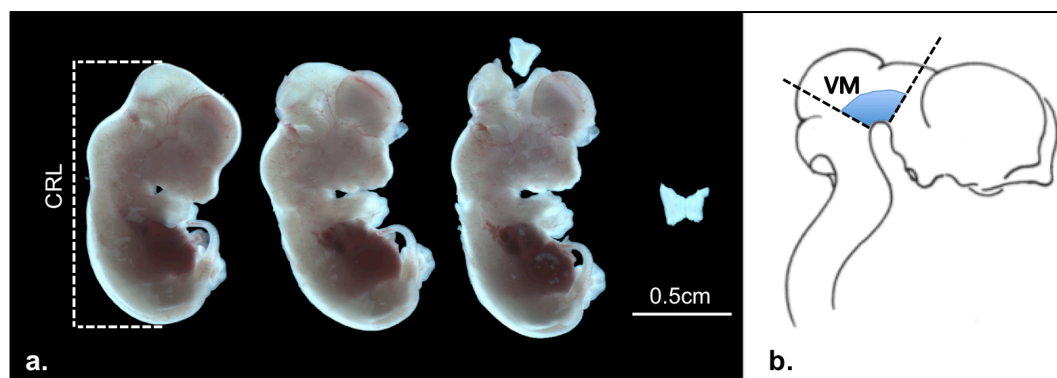


Figure 7: Ventral mesencephalon (VM) dissection (E14 rat embryo). For all VM dissections, the crown rump length (CRL) of the mouse and rat embryos was measured, the skin and skull were pulled out and VMs were cut (a). A representative drawing of the embryonic brain and the cutting lines is shown in b (modified from Bjorklund et al. (1983)).

	Species & Strain	Bred	Days post-copulatory
Syngeneic graft	Rat - Sprague Dawley	Outbred	14
Allogeneic graft	Rat - Wistar	Outbred	14
Xenogeneic graft	Mouse - CD1	Outbred	12

Table 2: Animals used in transplantation studies

Embryos were used if measuring: 10.5-12.5 mm for the E14 rats and 8.5-10.5 mm for E12 mice (Torres et al., 2008). Wistar rat embryos were bigger than the Sprague Dawley ($t(97)=4.529$, $p<0.0001$), however no significant difference was observed in the number of cell per VM (data not shown). As expected, E14 rat embryos were significantly bigger than mouse E12 and had more cells (Mann Whitney test, $p<0.001$) (Figure 8).

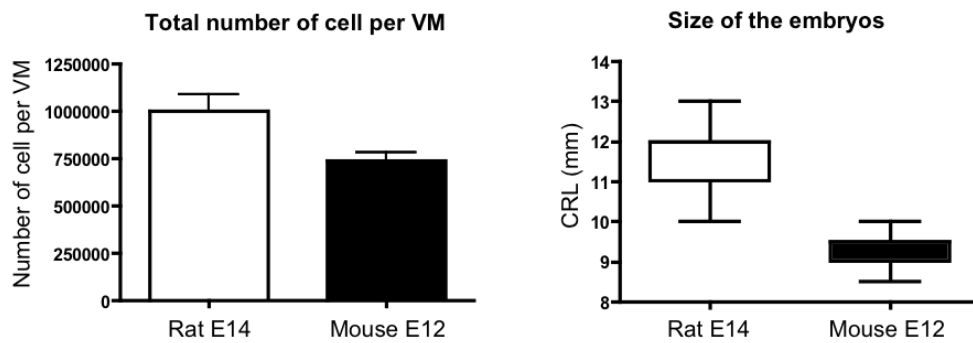


Figure 8: Average number of cells per VM dissected and size of the embryo of E14 rats (n=100) and E12 mouse (n=39)

2.3 Spleen cells injections

The peripheral injection of spleen cells obtained from the same strain as the VM donor has been shown to trigger an immune response against the allograft that would otherwise be well-tolerated by the host immune system (Soderstrom et al., 2008). To this end, male Wistar rats were terminally anesthetised with i.p. injection of 0.5 ml sodium pentobarbital (Euthatal) and the spleen was removed and placed in HBSS medium. The tissue was forced through a 100 μ m sterile cell strainer. The cell suspension obtained was then centrifuged (1500 rpm for 3 min) and the pellet was suspended in fresh HBSS. Sprague Dawley female rats, which have been transplanted

with Wistar VM (allogeneic transplant), recipients were then injected s.c. with 50,000,000 cells.

2.4 Behavioural assessments

2.4.1 Amphetamine-induced rotations

The extent of the 6-OHDA lesions was evaluated 2 and 4 weeks post-surgery, based on the ipsilateral (toward the lesioned side) rotational behaviour induced by the i.p. administration of *D*-Amphetamine (2.5 mg/kg in 0.9% saline). The animals were placed in perspex bowls and harnessed to automated rotometers (Figure 9) based on the design of Ungerstedt and Arbuthnott (Ungerstedt and Arbuthnott, 1970) and the net rotations were recorded over 90 min. Rats were deemed adequately lesioned and used for further study if they performed at least the equivalent of an average of 6 full turns per minute. This is reported to correspond to more than 90% depletion of the dopaminergic terminals (Torres et al., 2011). The same process was used post-transplantation in order to monitor the functional efficacy of the graft (typically successful transplantation will lead to reduced amphetamine rotations or reversal). During these sessions the rats were rated for amphetamine-induced dyskinesia using Scale B (adapted from Winkler et al (2002) as detailed later in section 2.3.3.2.1 Scale B and later discussed in chapter 3).

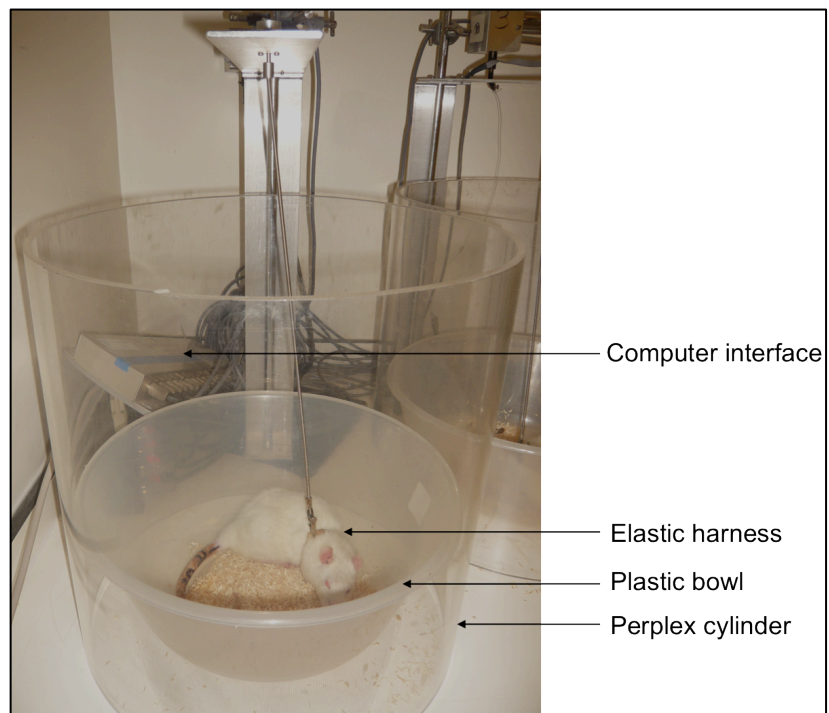


Figure 9: Rotometer apparatus

2.4.2 Motor tests

All behavioural assessments were carried out during daytime hours, between 8am-7pm. Rats were not deprived of food and water except for the duration of the test.

2.4.2.1 Stepping test

This task evaluates the ability of the rat to adjust a weight bearing forepaw in response to movement along a flat surface. The rats were held above a flat bench allowing only one forelimb to touch the surface of the table bearing weight (the body weight was largely supported by the experimenter but some weight is given to the paw). The rat was moved across 1 meter of bench over a period of 10 s and the number of steps made by the paw was counted for each forelimb when moved in the forehand and backhand directions (Figure 10). This test was repeated 3 times at each time points (before lesion, after lesion, after grafting). A practice session was performed at the beginning of the experiment to ensure that the rats were comfortable handled this way.



Figure 10: Stepping test: the animals are able to perform adjusting steps when the paw on the ipsilateral side of the lesion is dragged along the bench surface (a,b), but they are unable to move the contralateral forelimb (c, d).

2.4.2.2 *Vibrissae-elicited limb placing - Whisker test*

The rats were held in a way that allowed the forelimbs to move freely when the torso and hind limbs were supported (Figure 11). The rats were moved upward from under the bench, allowing the whisker on the tested side to gently brush the edge of the table. This induced an automatic limb placing response ipsilaterally to the side of the whiskers that had been stimulated (Fleming and Schallert, 2011). This test was performed 10 times on each side in order to obtain an accurate percentage of response on each side. At each time point (before lesion, after lesion and after grafting) the test was repeated 3 times for each animals and the mean number of accurate placements calculated.

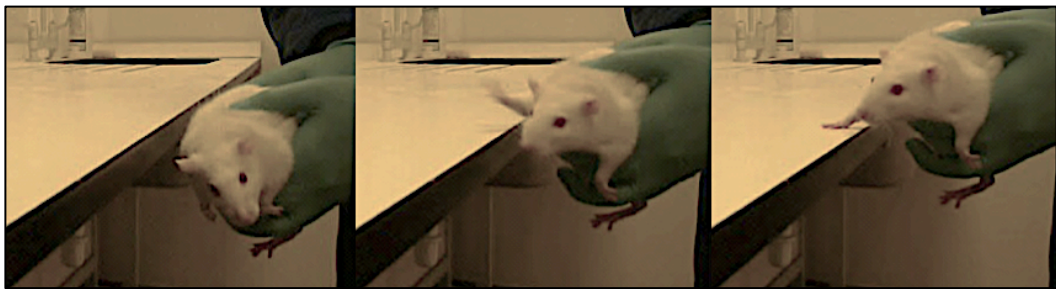


Figure 11: Whisker test: Animal whiskers are brushed against the edge of the bench, inducing automatic response of limb placement.

2.4.2.3 *Cylinder test*

The rats were put in a perspex cylinder (height: 33.5 cm, diameter: 19 cm) and video recorded. The cylinder was placed between 2 mirrors, creating a 45° angle to allow scoring when the rats were back to the camera (Figure 12). The number of each forelimb touches made by each paws, out of the first 20 touches performed on the cylinder wall was counted. Results are given in percentage of right or left touches out of the total.

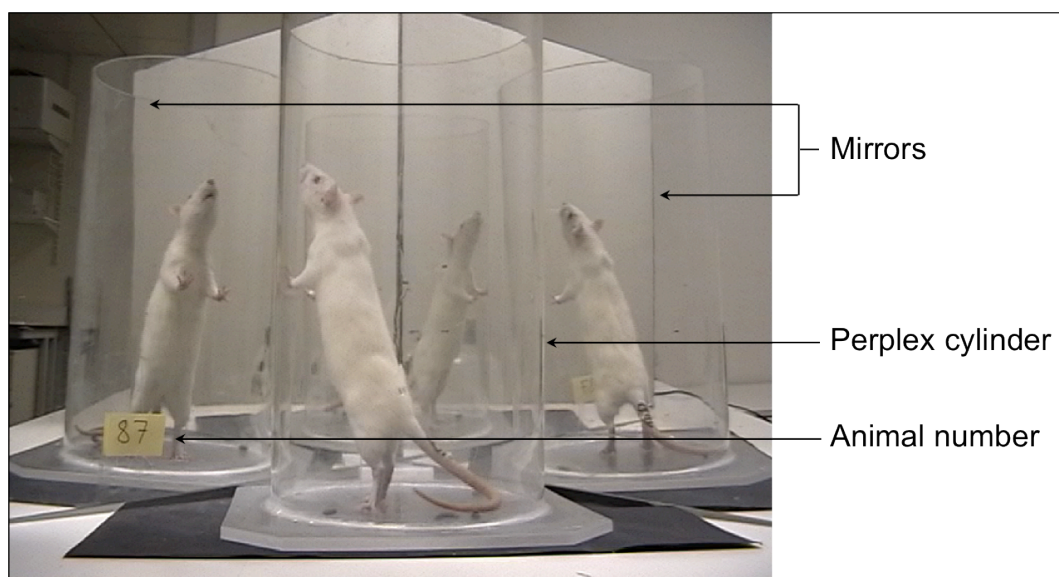


Figure 12: Cylinder test.

2.4.3 L-DOPA-induced motor dysfunctions

2.4.3.1 Drugs

The dose of L-DOPA used is specified in each chapter but always between a range considered therapeutically relevant (6-12 mg/kg). L-DOPA solutions were prepared in 0.9% saline by mixing the appropriate amount of L-3,4-Dihydroxyphenylalanine methyl ester hydrochloride and 15 mg/ml of benserazide hydrochloride. Benserazide is a peripheral AADC inhibitor, unable to cross the blood brain barrier, which blocks the conversion of L-DOPA into DA in the periphery. This permits a higher concentration of L-DOPA to reach the brain and limiting peripheral side effects. It has been shown that a dose of 12-15 mg/kg of benserazide a day gives an optimal effect, blocking most of the peripheral AADC without affecting the enzyme activity in the brain. L-DOPA solution was injected s.c. (1 ml/kg) as this route of administration gives a more homogeneous response than i.p. (Lindgren et al., 2007). For the dyskinesia rating sessions, L-DOPA injections were performed such that a constant interval of 1 min between each animal so that they were all rated precisely at the same time following L-DOPA administration.

D₁ and D₂ DA receptor antagonists, R(+)-SCH-23390 hydrochloride (0.2 mg/kg) and R(-)-Raclopride(+)-tartrate (2 mg/kg), and amantadine (40 mg/kg) were all prepared in 0.9% sodium chloride and injected i.p. 20 min before L-DOPA administration (Smith et al., 2012).

Bromocriptine was mixed with 2.5% acetic acid and 3 drops of 95% ethanol. Sonication was used to dissolve the powder and distilled water was added to allow a final concentration of 2 mg/ml (animals were injected i.p. with 2 mg/kg of bromocriptine).

2.4.3.2 *AIMs rating scales*

This section describes the different rating scales used to assess L-DOPA-induced motor dysfunction in unilateral 6-OHDA lesioned rats. These methods have been compared for reproducibility, variance and time dependent changes in chapter 3. Based on these results, the subsequent experiments (Chapter 4 and 5) were performed using Scale B to assess AIMs and stereotypies were evaluated separately, according to the scale developed by Creese and Iversen (Creese et al., 1977).

2.4.3.2.1 *Scale A*

Animals were individually observed and rated for 1min every 20 min for a total duration of 180min following L-DOPA injection. This method, based on the scale developed by Cenci *et al.* (Cenci et al., 1998), assesses 4 subtypes of AIMs for duration (Table 3). The duration scores are summed together to obtain the total AIMs score. Hind limb dystonia has been added as a 5th subtypes and the results with or without this extra parameter are discussed in chapter 3.

AIMs subtype	Definition	Duration score
Locomotive	Increased locomotion toward the contralateral side of the lesion	0 = absent 1 = occasional 2 = frequent 3 = continuous but interrupted by sensory distraction 4 = continuous, severe, not interrupted by sensory distraction
Axial dystonia	Axial dystonia, i.e. contralateral twisted posturing of the neck and upper body	
Orolingual dyskinesia	Stereotyped jaw movements and contralateral tongue protrusion	
Forelimb dyskinesia	Repetitive rhythmic jerks or dystonic posturing of the contralateral forelimb, and/or grabbing movement of the contralateral paw	
Hind limb dystonia	Abnormal extension of the hind limb	

Table 3: Scale A

2.4.3.2.2 Scale B

Scale B is based on the method described by Winkler and co-workers (2002) and evaluates 4 different AIMs subtypes and allocates a duration score (identical to the one described in the Cenci method) but also an amplitude score to each subtype (Table 4). The AIMs severity score is obtained by multiplying the duration score to the associated amplitude score. The total AIMs score of each rating session is obtained by summing all the severity scores.

AIM subtype	Amplitude Score	Duration score
Locomotive	None	0 = absent 1 = occasional 2 = frequent 3 = continuous but interrupted by sensory distraction 4 = continuous, severe, not interrupted by sensory distraction
Axial dystonia	1 = lateral deviation of head and neck (<30°) 2 = lateral deviation of head and neck (30°- 60°) 3 = lateral deviation or torsion of head, neck and upper trunk (60°- 90°) 4 = torsion of neck and trunk at (>90°), causing the rat to lose balance	
Orolingual dyskinesia	1 = jaw movements, facial grimacing 2 = tongue protrusion.	
Forelimb dyskinesia	1 = tiny oscillatory movements of the paw and the distal forelimb around a fixed position 2 = movements of low amplitude but causing visible translocation of both distal and proximal limb 3 = translocation of the whole limb with visible contraction of shoulder muscles 4 = vigorous limb and shoulder movements of maximal amplitude.	
Hind limb dyskinesia	1 = abnormal posturing of limb 2 = sustained posturing of limb, mildly extended in abnormal posture 3 = severely hyperextended in abnormal position 4 = hind limb fully extended, causing the rat to lose balance	

Table 4: Scale B

2.4.3.2.3 *Scale C*

The scale is based on the methods used by the US group led by Dr Steece-Collier. Due to the extensive number of AIMs subtypes assessed, the animals had to be recorded for 1min, every 30 min of 180 min. The AIMs were then scored, based on recording. The forelimb dystonia subtypes had to be excluded because wrist and digits movements cannot be assessed accurately on a video, especially in the animals that rotate a lot. Similarly to Scale B, the severity score of each AIM was obtained by multiplying the duration and the amplitude score (Table 5). This scale traditionally considers only 1 time point at 30 min (Steece-Collier et al., 2003, Maries et al., 2006). In order to study the importance of multiple time points, the score at 30 min (Scale C₃₀) were compared with the total severity score (Scale C) obtained by summing all severity scores obtained at each time point.

AIM subtype	Amplitude score	Duration score
Neck dystonia	0.5 = mild tilt of head 1 = mild displacement of head posture but returns to neutral position 1.5 = mix of mild and moderate posture 2 = more notable displacement (>45°) no return to normal position 2.5 = mix of moderate and severe posture 3 = severe torsion of neck musculature	0 = absent 1 = intermittent, <50% of observation period 2 = intermittent, 50% of observation period 3 = persistent throughout entire observation period without stopping
Trunk dystonia	1 = mild dystonia; <45° between upper and lower torso; 1.5 = mix of mild and moderate dystonia 2 = moderate dystonia; (>45°) some loss of balance when ambulating 2.5 = mix of moderate and severe dystonia 3 = severe dystonia, twisted (“corkscrew”) or unable to ambulate	
Forelimb dyskinesia	1 = abnormal posturing of limb with returns to neutral position 2 = mix of mild and severe extension of hind limb 3 = sustained posturing of limb; severely hyperextended in abnormal	
Hind limb dystonia	1 = small amplitude side to side, up & down wiping/tapping cage wall 2 = same as 1 + downward hyperextension (<50%) 3 = severe downward hyperextension, pulls opposite of neck	
Orolingual dyskinesia	1 = chewing 2 = chewing + tongue protrusion and/or open mouth chewing	
Head bobbing	1 = small amplitude 2 = large amplitude	
Forelimb-facial stereotypy	1 = present	None

Table 5: Scale C

2.4.3.3 *Stereotypy rating scale*

This scale assesses stereotypic movements. Animals were rated for 1min every 10 min, from 10 min post-injection to 180 min. The scores were given as follows: 0= asleep or stationary, 1= active, 2= predominantly active but with bursts of stereotyped sniffing or rearing, 3= stereotyped activity such as sniffing along a fixed path, 4= stereotyped sniffing or rearing maintained in one location, 5= stereotyped behaviour in one location

with bursts of gnawing or licking, 6= continual gnawing or licking of the bowl or cage. The total score is obtained by summing of all the scores over the 180min (Creese and Iversen, 1973).

2.4.3.4 *L-DOPA-induced contralateral rotations*

In all experiments, involving AIMs and stereotypy scoring, rotations were recorded simultaneously using automated rotometers (Figure 9) (Ungerstedt and Arbuthnott, 1970). The system records the number of quarter of turns performed in each direction every min. The relative number of full contra-lateral turns, toward the side opposite to the lesion, was calculated as follow: $(c-i)/4$, where c = number of quarter turns performed contralaterally and i = number of quarter of turns performed ipsilaterally.

2.5 Perfusion

At the end of each experiment the animals were terminally anaesthetized with intra-hepatic injection of 200 mg/kg of Euthatal before being perfused transcardially with 50 ml of 0.1M TRIS buffer saline (TBS) pH 7.4 in order to remove all the blood. They were then perfused with a solution of 1.5% paraformaldehyde (PFA, in TBS): 100 ml over 4 min. The brains were removed and placed into 1.5% PFA over night before being immersed in a TBS solution containing 25% sucrose until they sank. They were then cut on a freezing microtome into 40 μ m thick coronal sections and kept at -20° C in a cryoprotective solution (0.1 M TBS, 25% sucrose, 0.1% sodium azide) for further analysis.

2.6 Immunohistology

All immunohistochemistry straining was performed on fixed sections following the same protocol at room temperature on an automated shaker. A 1 in 12 series was used for each staining. After 3 washes of 10min in TBS, the sections were placed in 10% hydrogen peroxide and 10% Methanol in 80% distilled water for 15 min in order to quench endogenous peroxidase enzyme activity. 3 washes were then performed before putting the sections into a block solution of 0.2% triton in TBS (Tx-TBS) containing 3% normal goat serum for 1 hour. The sections were then placed in the primary antibody solution over night (Table 6). After 3 washes of 10 min in TBS, the brain slices were transferred in the secondary biotinylated antibody solution for 1-2 hours.

After 3 wash of 10 min, the sections were incubated in the Vectastain Elite ABC solution (an biotin/avidin system linked to the horse radish peroxidase). Tissue sections were washed in TRIS non-saline buffer (TNS) 4 times for 10 min each and incubated in TNS 0.03% H₂O₂, 0.8% 3,3'-Diaminobenzidine tetrahydrochloride hydrate, substrate of the peroxidase, in order to obtain a specific dark brown labelling of the tissue. Finally, the sections were rinsed 4-5 times in TBS before mounting on gelatine coated microscope slides. The slides were allowed to dry at room temperature over night or for 2 hours at 37° C before dehydration in successive alcohol baths (75%, 95% and 100%, 5 min each). The slides were then transferred into xylene for delipidisation before coverslipping with distyrene plasticizer and xylene (DPX).

Labelling	Serum	Primary antibody against	Secondary antibody against
Dopaminergic neurons	Goat	Tyrosine Hydroxylase 1/2 000 (Rabbit, Millipore)	Rabbit IgG 1/200 (Goat, Vector)
Blood vessels	Horse	Reca-1 1/2 000 (Mouse, AbD Serotec)	Mouse IgG (Rat IgG absorbed) 1/200 (Horse, Vector)
Leukocytes		CD45 1/500 (Mouse, AbD Serotec)	
T-lymphocytes (CD4)		CD4 1/500 (Mouse, AbD Serotec)	
T-lymphocytes (CD8)		CD8 1/500 (Mouse, AbD Serotec)	
Microglia		Ox42 (CD11b) 1/2 000 (Mouse, AbD Serotec)	

Table 6: List of the antibodies used for immunohistochemical analysis of the tissue. Prepared in TBS, 1% serum.

2.7 Quantification and microscopy

All quantifications were carried out by keeping the experimenter blind to the animal grouping. TH⁺ cell bodies were counted on a Leica light microscope (20x). SN TH⁺ counts were performed at the level of the medial terminal accessory nucleus of the optic tract (separating the SN from the ventral tegmental area (Figure 13) and the results are expressed as a percentage of the intact SN (left side).

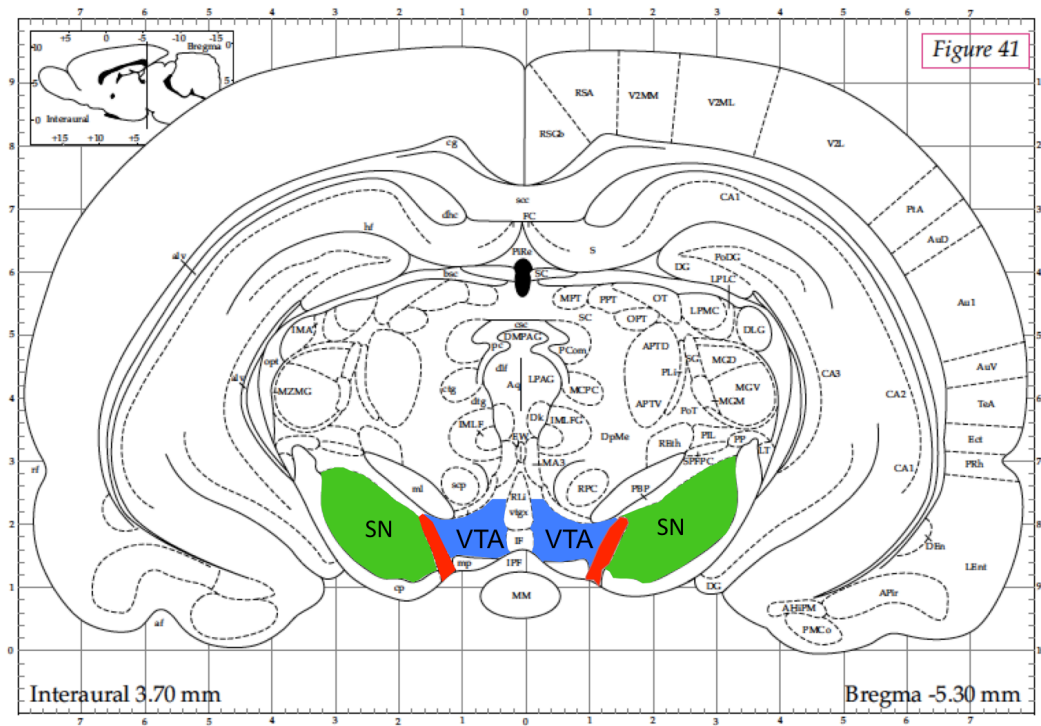


Figure 13: Schematic representation of the accessory optic tract (red), substantia nigra (SN, green) and ventral tegmental area (VTA, blue) in the rat brain (Paxinos and Watson, 1998)

In the transplantation experiments, TH^+ cell bodies were counted for all sections from a 1 in 12 series through the rat striatum and the total number of dopaminergic cells was estimated using the Abercrombie method: $N = \Sigma \{ n \times F \times T / (T+H) \}$ where n =number of cells counted, F =frequency of the sections (12), T =thickness of the sections ($40\mu m$) and H =mean diameter of the cells (Hedreen, 1998). $CD45^+$ quantification was carried out using an automated stereology microscope (Olympus BX50) and a PC-based image analysis software (Olympus C.A.S.T grid system version 1.6). The whole striatum was outlined (4x) and the enclosed area was measured by the software. Sections within the selected striatal area were sampled randomly and cells were counted within a $3862\mu m^2$ window (40x). The total number of cells was estimated using the following formula: $N = \Sigma \{ n \times (A/a) \} \times F \times T / (T+H)$ where n =number of cells counted, A = inclusion area (striatum), a = total sampling area, F = frequency of the sections (12), T =thickness of the sections ($40\mu m$) and H =mean diameter of the cells.

The surface area of the blood vessel was estimated by taking pictures of the sections, stained for Recla-1 (x10), on a Leica DMRBE microscope and analysing them with ImageJ software (version 1.45, National Institutes of Health, USA). The pictures were

converted in 8-bit black and white pictures and the threshold was adjusted to avoid background noise, before the stained area was measured by the software and used as a measure of blood vessel area.

2.8 Analysis

Statistical analyses were performed using Prism5 in chapter 3 and then SPSS20 in the subsequent chapters (Chapter 4 and 5) as Prism5 does not allow the consideration of so many factors. The nature of dyskinesia rating data is ordinal rather than interval, therefore strictly a non-parametric analysis should be considered. However, non-parametric analyses do not permit combining scores between different limbs or analysis interaction between the different parameters studied (times, type of treatment, transplantation...) as is required for the factorial design used in all experiments. Therefore, I have used throughout parametric ANOVA analysis as the only practical option for analysis of this type of data, taking into account that: 1) the categories of dyskinesia rating are strictly interval and monotonic 2) inspection of the data indicated that the scores were well distributed between the different categories and 3) analysis of variance is recognised to be extremely robust to derivation of the data from the normality of distribution, that is the basis of the underlying mathematics (Box, 1953). Details of the test used are given in each chapter, briefly, Univariate analysis of the variance (One-way ANOVA) or Multivariate analysis of the variance (Two-way ANOVA) were used to analyse immunohistochemistry staining data and the repeated measure ANOVA analysis was used for the behaviour tests. T-tests were also used when appropriate. Post-hoc tests were implemented to specific significance to 95% (* $p < 0.05$), 99%(* $p < 0.01$), 99.9% (* $p < 0.001$).

Chapter 3: Reliability of different approaches used in assessing L-DOPA-induced motor dysfunctions

Summary

Establishing a sensitive and robust method to evaluate L-DOPA-induced motor impairment in rodents is crucial for the study of the mechanisms underlying LID as well as for the development of potential treatments. In this chapter, 3 different AIMs rating scales, 1 stereotypy scale and automated recording of L-DOPA-induced contralateral rotations are compared. The reliability of these different methods in the evaluation of the development of motor dysfunction induced by L-DOPA over time and their sensitivity in revealing anti-dyskinetic effects have been assessed in the 6-OHDA-lesioned rat model in this chapter. The results showed a good correlation between the 3 AIMs scales but clearly demonstrated that AIMs and stereotypies are two different components of the motor dysfunctions induced by L-DOPA, which do not follow the same pattern of development. Neither do the two behaviours respond to drugs with anti-dyskinetic properties in the same manner. Interestingly, the number of amphetamine-induced rotations performed by the animals after unilateral 6-OHDA-lesion was proven non-predictive of the development of AIMs, stereotypy and rotational behaviour induced by L-DOPA. Finally, the specific test environment was found to have no impact on the results obtained with the different methods.

Declaration

Most of the data presented in this chapter have been published in *Neurobiology of Disease* (Breger et al., 2013).

3.1 Introduction

In PD patients, L-DOPA-induced dyskinesias are assessed using subjective rating scales based on clinical examination, patient historical self-evaluation, or a combination of both. Over 8 different methods have been used since the 1970s and significant effort is currently being put in the establishment of a unified, reliable and widely accepted dyskinesia rating scale (Colosimo et al., 2010). This is particularly important to measure the effectiveness of potential anti-dyskinetic treatments in clinical trials (Colosimo et al., 2010, Wong et al., 2011). Preclinical studies are currently facing the same challenge. Indeed, the scales vary not only from one laboratory to another, but the shape of the testing environment in which the animals are tested (circular bowls or rectangular cages), as well as the method applied (single versus multiple time points assessment, video based rating or direct scoring) can also differ. For practical reason, rodents are usually tested in hemispherical bowls while rotations are recorded in rectangular activity cages. Pinna and colleagues have performed the only comparisons of environment but different techniques were applied to the two groups, making this inconclusive (Pinna et al., 2006).

The first animal model of LID was the MPTP treated non-human primate (Burns et al., 1983, Bedard et al., 1986, Schneider, 1989). When chronically exposed to L-DOPA, MPTP-lesioned primates develop LID that can be evaluated using a rating scale adapted from the human yardstick (Petzinger et al., 2001, Jenner, 2003). Since its introduction in the 1980s, the MPTP model has been used extensively to determine the value of potential anti-dyskinetic therapies. However, the results obtained with this model have been sometime over interpreted and led to less successful clinical outcome than expected. Indeed agents, such as the adenosine A2a antagonist KW-6002 or the α -2 noradrenergic antagonist idazoxan, that had a promising effect in rodent and primates failed to show significant and consistent improvements in patients (Manson et al., 2000, Rascol et al., 2001, Hauser et al., 2003, Linazasoro, 2004, Hauser et al., 2008, Knebel et al., 2012). As with patient studies, primate experiments have used a variety of scales to evaluate motor improvements and LID, and this lack of consensus on the LID scoring method to be used in preclinical studies is believed to play a critical role in the reliability of the animal models and how well preclinical results transfer to the clinic (Fox et al., 2012). Whilst in primates studies work has been undertaken to compare and develop new reliable scales for non-human primates, rodents have been left out of this

debate with different laboratories adapting scales as they wish without the validation undertaken by the Cenci group during the original scales development (Imbert et al., 2000, Petzinger et al., 2001).

The major complications associated with the use of primates in research, raised interest in cheaper and less ethically challenging models of PD, the most common being the unilaterally 6-OHDA-lesioned rat. However, for many years since its introduction, lesioned rats were thought to be unable to show the full repertoire of the L-DOPA-induced side effects observed in human and non-human primates (Cenci et al., 2002). Much earlier studies had focused on stereotypic behaviours, showing a causal link between over-stimulation of dopaminergic system and stereotypy in rodents. Indeed, stimulation of DA receptors with the mixed DA agonist apomorphine, exacerbation of endogenous DA release with amphetamine or inhibition of monoamine reuptake with cocaine, all lead to the development of normal but purposeless and repetitive movements which exemplify stereotypy (Randrup et al., 1963, Fog, 1969, Hall et al., 1984). Because of the association between dopaminergic pathway stimulation and appearance of these stereotypic movements, motor side effects induced by L-DOPA in DA depleted rats were first simply described as stereotypies (Ervin et al., 1977, Blunt et al., 1991) and were not recognised and scored as abnormal movement until the late 90s when Cenci and colleagues published the first dyskinesia rating scale for rats (1998).

In 1998 Cenci and colleagues demonstrated that lesioned rats also exhibit abnormal dystonic and choreic movements when repeatedly exposed to L-DOPA, which are considered comparable to the dyskinesia observed in patients or non-human primates. Moreover, these movements are alleviated by drugs that have known anti-dyskinetic effects in humans and non-human primates, establishing the clinical relevance of the hemi-parkinsonian rat model for the study of LID (Lundblad et al., 2002). In the original method described by Cenci and co-workers, forelimb movements, orolingual and jaw movements, axial contortion and rotational locomotion were rated depending on the duration of time that each subtypes was present during the observation phases (Cenci et al., 1998). In 2002, an adapted version was published, which included an amplitude score as a second parameter to each movement subtype. This new parameter considered the extent of the movement, differentiating, for example, between small but clearly present movements of the forelimb and wrist and larger, more dystonic-like

movements involving the whole shoulder (Winkler et al., 2002). Not long after that, Steece-Collier and colleagues developed a new scoring method, which considered fewer time points but more behavioural characteristics (Steece-Collier et al., 2003, Maries et al., 2006). Thus far, these three different scoring methods have been used to follow the development of AIMs and test new agents aiming to reduce LIDs (Lundblad et al., 2002, Monville et al., 2005, Taylor et al., 2005, Rylander et al., 2010). Nevertheless, arbitrarily modified versions of these rating scales are also commonly used, without prior validation, which has heightened the need for well-defined and validated rating scales (Steece-Collier et al., 2003, Carta et al., 2006, Maries et al., 2006, Monville et al., 2009, Bido et al., 2011).

There have been no controlled studies, which systematically compare the different scoring approaches. Moreover, for practical reason, scientists often assess animals in different environments, when it has been suggested that the shape of the testing environment can influence the expression of L-DOPA-induced motor side effects (Pinna et al., 2006). This chapter, therefore, critically discusses the reliability of 4 behavioural rating scales (3 AIM scales and 1 stereotypic scale) used to assess motor dysfunction induced by chronic L-DOPA treatment in the 6-OHDA-unilaterally-lesioned rats (Creese and Iversen, 1973, Cenci et al., 1998, Winkler et al., 2002, Maries et al., 2006). In order to compare these methods in a systematic manner, all scales were used to follow the development of L-DOPA-induced motor dysfunctions and assess the effect of amantadine and DA receptor antagonists in the all animals. The animals were also tested in 2 different environments, oblong cages or perplex bowls, to study the effect that the shape of the environment can have on the AIM and stereotypic scores.

3.1.1 Aims of the chapter

The aims of this study were to evaluate the validity of the 3 published AIMs rating scales alongside a stereotypy rating scale:

- 1) Throughout the development of LID and at different doses of L-DOPA
- 2) In different testing environments
- 3) In the presence of agents known to reduce LIDs in the primate model or clinical setting

All of which will define the protocols to be used in further experiments in this thesis.

3.2 Experimental design

28 female Sprague Dawley rats received 6-OHDA infusions into the right median forebrain bundle. The extent of the lesion was assessed using the amphetamine-induced rotations test and later confirmed by immunohistochemistry (TH⁺ cell depletion greater than 90% in the right SNpc). The rats received daily s.c. injections of L-DOPA 6mg/kg for 5 weeks then the dose was increased to 12 mg/kg for a further 5 weeks, always co-administered with 15 mg/kg benserazide (Figure 14). All animals were assessed for motor dysfunction induced by L-DOPA twice a week. On the first session, half of the animals were rated using Scale A and B, the other half using Scale C and the stereotypic scale. The groups were then interchanged for the second weekly session. The 4 scales are described in details in chapter 2 and the main characteristics have been summarised in table 7. Rotation was automatically recorded in each of the test environments at the same time as the rats were rated for dyskinesia and stereotypy.

In order to assess the reliability of these methods in detecting anti-dyskinetic effects, D₁ and D₂ DA receptor antagonists and the weak NMDA receptor antagonist amantadine were administered i.p. 20 min prior to L-DOPA injection (12 mg/kg + 15 mg/kg benserazide). These agents are known to decrease LID in MPTP-treated primates and humans. All drugs were dissolved in 0.9% sterile saline at the following concentration D₁ DA receptor antagonist: R(+)-SCH-23390 hydrochloride (0.2 mg/kg), D₂ DA receptor antagonist: R(-)-Raclopride(+)-tartrate (2 mg/kg) and amantadine (40 mg/kg). After 10 weeks of chronic L-DOPA treatment, the 3 “anti-dyskinetic” drugs and saline were administered to the animals following a 4x4 Latin square randomisation. 3 days washout intervals were allowed between each session. At that stage 2 animals had to be

excluded because of tumour development. Therefore, for this part of the study: n=26.

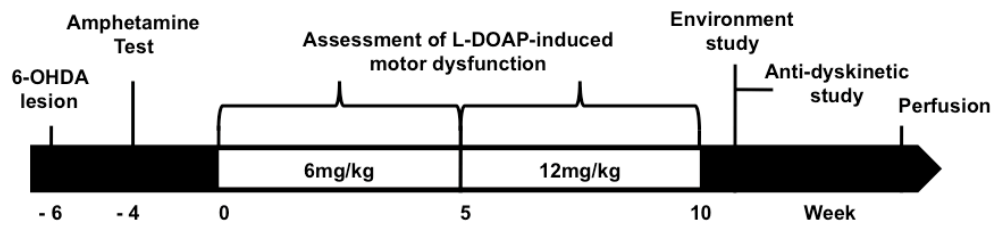


Figure 14: Time line of the experiments

Scales	Observation	Time course	Subtypes rated (Amplitude scores)	Duration score	Total score
Scale A	Direct For 1 min	Every 20 min From 20 to 180 min	Trunk (none) Forelimb (none) Orolingual (none)	0: Never 1: <50% 2: >50%	Min: 0 Max: 144
Scale B			Trunk (0-4) Forelimb (0-4) Hind limb (0-4) Orolingual (0-2)	3: Stop with sensory stimuli 4: Never stop	Min: 0 Max: 478
Scale C	Video Recording For 2 min	At 30 min Or Every 30 min From 30 to 180 min	Neck (0-3) Trunk (0-3) Hind limb (0-3) Forelimb(0-3) Orolingual (0-2) Head bobbing (0-2) FFS (0-1)	0: Never 1: <50% 2: >50% 3: continuous	Min: 0 Max: 306
Stereotypy	Direct For 1 min	Every 10 min From 10 to 180	Stereotypic behaviour (0-6)	None	Min: 0-18* Max: 108

Table 7: Summary of the main characteristics of the 3 different AIMs scales and stereotypic scale. (*18 can correspond to an animal active during the entire session)

On completion of the scale comparison, half of the animals were retested in bowls (height: 14.5cm; width: 33.5cm (top), 15.5cm (bottom)), the other half in rectangular cages (height: 18.5cm; length: 36cm; width: 25.5cm). The following day, the testing was repeated in the other environment. The rotational behaviour was assessed using an automated rotometer to measure the number of contralateral rotations for a total of 180 min in both the round and rectangular environments.

Statistical analyses of the performance on each scale over time were undertaken by repeated measures ANOVA with significant changes over time evaluated by comparison against the scores recorded on the first week of the treatment as the baseline value (Dunnett post-hoc test). Correlational analyses were performed using linear

regression. For the study on the impact of the environment shape, the statistical analysis was performed using a two-tailed Student's t-test. Results were considered to be significant if $p < 0.05$.

3.3 Results

3.3.1 Development of L-DOPA-induced motor impairments

The 3 dyskinesia rating scales (AIMs scales) showed an increase in the total AIMs scores as a function of time ($F_{9,243}=62.38, 42.45, 54.00$ and 31.29 for Scales A, B, C, C_{30} respectively, all $p < 0.0001$). AIMs scores gradually increased and reached significance after 4-5 weeks of 6mg/kg chronic L-DOPA treatment ($p < 0.05$, relative to the first day of treatment) (Figure 15). A dose dependent increase was observed with scales A and B when the treatment was enhanced from 6mg/kg to 12mg/kg ($p < 0.001$). However, scale C did not change significantly when the L-DOPA dose was doubled, regardless of the use of single or multiple time points (C and C_{30}). Although the stereotypic scores displayed an increasing trend, this scale did not show any significant difference over time during the first phase of the treatment. Moreover, the mean score remained below 18, which can correspond to a normal but constantly active animal (i.e. 18 scoring sessions each scoring 1 for an active animal would give a score of 18). Animals were generally active but displayed none or very few stereotypies when treated with 6mg/kg L-DOPA. To the contrary, when the dose was increased to 12 mg/kg L-DOPA the animals exhibited severe stereotypic behaviour ($F_{9,243}=6.919, p < 0.0001$). With all scales, a ceiling effect was observed after the first injection of the higher dose of L-DOPA (12mg/kg) and no significant change was observed within the second phase of treatment (Figure 15, a-e). Finally, no difference was observed in the total contralateral rotations during the first phase of treatment (6 mg/kg) but the increased dose led to a significant worsening of the rotational behaviour ($F_{9,243}=40.52, p < 0.0001$). Contrary to the AIMs and stereotypy scores, the total of rotations did not reach a plateau until the 3rd week following the increase in L-DOPA dosage (Figure 15, f).

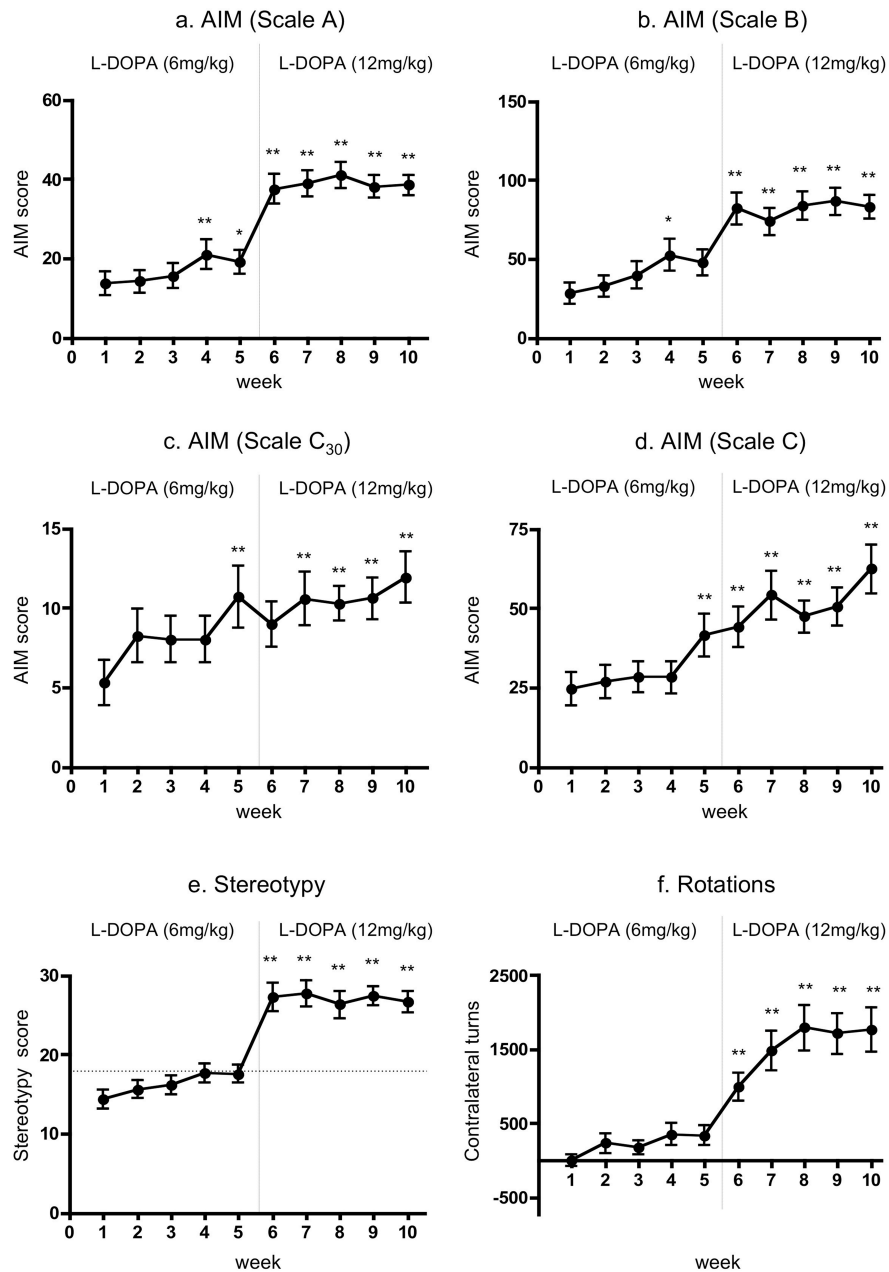


Figure 15: Development of motor side effects induced by chronic treatment with L-DOPA. AIMS scores obtained with scale A, B, C₃₀ and C (respectively a, b, c and d) and stereotypy scores (e) following injection of L-DOPA 6 mg/kg (left) and 12 mg/kg (right) are presented as mean score of all the animals \pm SEM. The vertical dotted line at 18 corresponds to the maximum score animal will obtain while being just active (e). The total of contralateral turns performed by the animals over 180 min is presented as the mean of all animals \pm SEM (f). n=28, One-way ANOVA and Dunnett post-hoc test *p<0.01, **p<0.001 compared to week 1.

3.3.2 Time course of L-DOPA-induced motor impairments

To better characterise the evolution of L-DOPA induced motor dysfunction, the time course of motor responses within the sessions were compared for each method, for the

first and final weeks of each dose (week 1 and 5 at 6 mg/kg and weeks 6 and 10 at 12 mg/kg) (Figure 16). The maximum AIMS score increased between the first and last scoring sessions with 6 mg/kg L-DOPA when measured by Scales A and B (46.9% and 76.7% increase respectively) but not with Scale C. With all AIMS scales, the time for the response to peak varied depending on the scale used. In week 1 (6 mg/kg), the AIMS response as measured by Scales A and B peaked at 80 min, while with Scale C this was recorded at 60 min. Chronic treatment with L-DOPA for 5 weeks shifted the peak response from 80 min down to 60 min as identified with Scale A and B and from 60 to 30 min with Scale C (Figure 16, a-c).

None of the scales showed a significant difference in the maximum AIMS score obtained after the original increase of the L-DOPA dose from 6 mg/kg to 12 mg/kg, between week 5 and week 6. The increase observed in the total score is therefore not due to an augmentation of the severity but was only produced by an extension in the duration over which the animals exhibited these movements (Scale A: 120–180 min, $p < 0.001$; Scale B: 120–140 min, $p < 0.05$, Scale C: 100–160 min, $p < 0.001$). Prolonging treatment with L-DOPA 12 mg/kg, however, did not affect AIMS scores and no significant difference was observed between week 6 and week 10, with the exception of changes in time-to-peak which were delayed back to 60 min with Scales A and B, and 80 min with Scale C (Figure 16, a-c).

The recording of rotational behaviour over time showed that the animals rotate toward the side ipsilateral to the lesion for approximately 20 min, until the L-DOPA takes effect, inducing contralateral rotations (Figure 16, e). Both speed and duration of the rotational behaviour increased in a dose dependant manner (week 5 to week 6). Interestingly, the speed continued increasing after chronic treatment with L-DOPA 12 mg/kg (between week 6 and week 10).

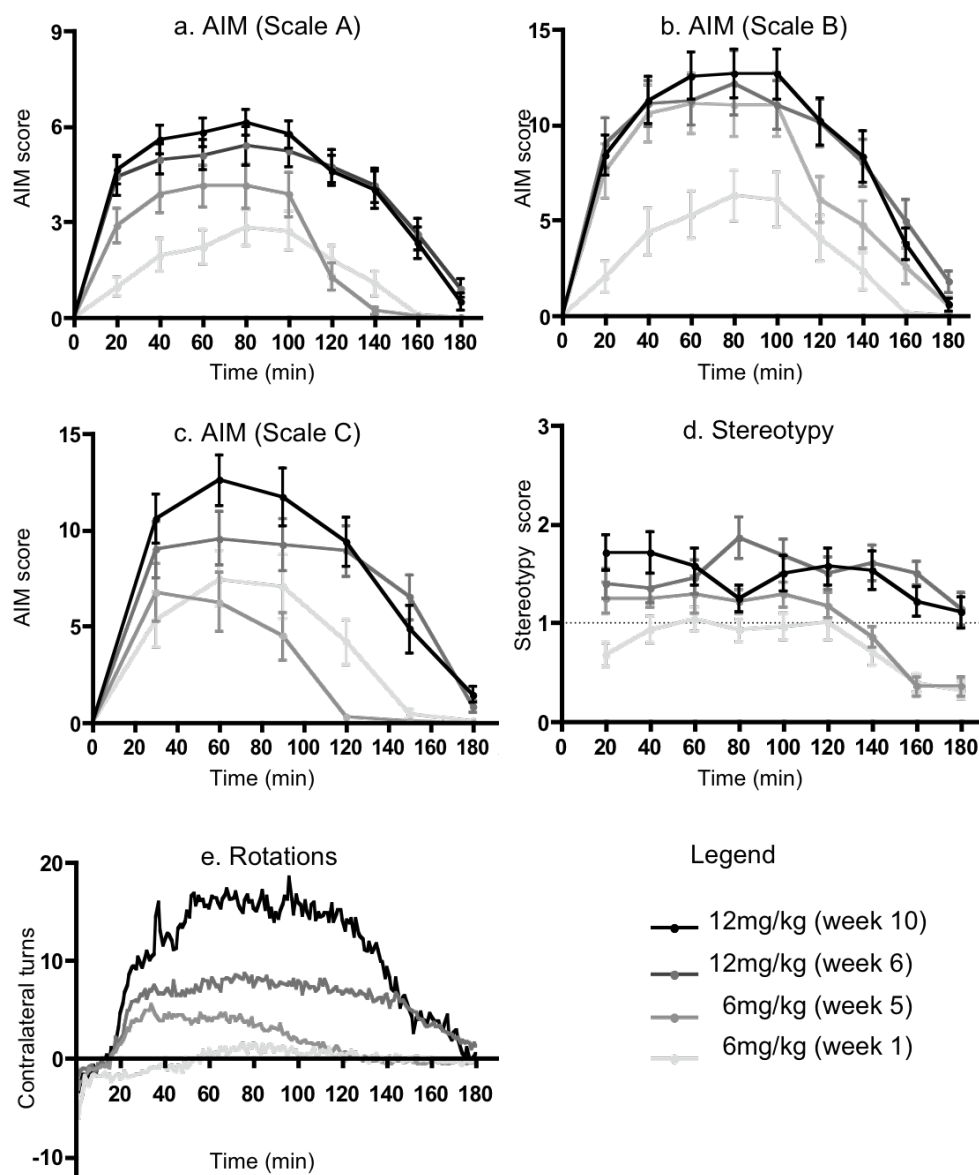


Figure 16: Evolution of L-DOPA induced motor side effects over the 180 min following drug administration. AIMS scores obtained with scale A, B and C (respectively a, b and c) and stereotypy score (d) following injection of L-DOPA 6 mg/kg (week 1 and 5) and 12 mg/kg (week 6 and 10) are presented as mean score of all the animals \pm SEM. The vertical dotted line on the stereotypy graph (e) corresponds to the score of a normal active animal. The total of contralateral turns performed by the animals is presented as the mean of all animals \pm SEM. n=28.

3.3.3 Pharmacological validation of the scales

In order to validate the sensitivity of the different rating scales in assessing the potential of new anti-dyskinetic therapies, we co-administered L-DOPA with agent known to reduce dyskinesia in humans or non-human primates: D₁ receptor antagonist SCH-23390, or D₂ receptor antagonist raclopride, or the NMDA antagonist amantadine. Interestingly, the 3 different components of L-DOPA-induced motor dysfunctions studied in this experiment: AIMs, stereotypies and rotational behaviour, did not respond the same way to the drugs. All three multiple time points dyskinesia scales (Scale A, B and C) and the stereotypic scale illustrated a significant reduction of AIMs scores following administration of SCH-23390 and raclopride (Figure 17); $F_{3,75} = 20.71$; 19.70; 11.16 and 23.57 for scales A, B, C, and stereotypies respectively, all $p < 0.01$). Importantly, when considering a single time point, the Scale C₃₀ failed to show an effect of D₂ receptor antagonism (Figure 17, c; $F_{3,75} = 5.39$; $p > 0.05$). On closer inspection it was noted that the reduction induced by raclopride was found mainly during the last hour of observation (Table 8). Finally, rotational behaviour was only significantly reduced by D₁ antagonists only ($F_{3,75} = 8.67$, $p < 0.05$).

Amantadine had no significant effect on the AIMs scores rated with scale A (Figure 17 & Table 8). In contrast, Scale B, C and C₃₀ showed a significant decrease of AIMs scores when L-DOPA was co-administered with amantadine ($F_{3,75} = 8.67$, $p < 0.01$). Interestingly, amantadine significantly increased the total score of stereotypy ($p < 0.01$). Indeed, if it decreased AIMs scores significantly around peak time, it prolonged the temporal course of stereotypy and rotations (Table 8).

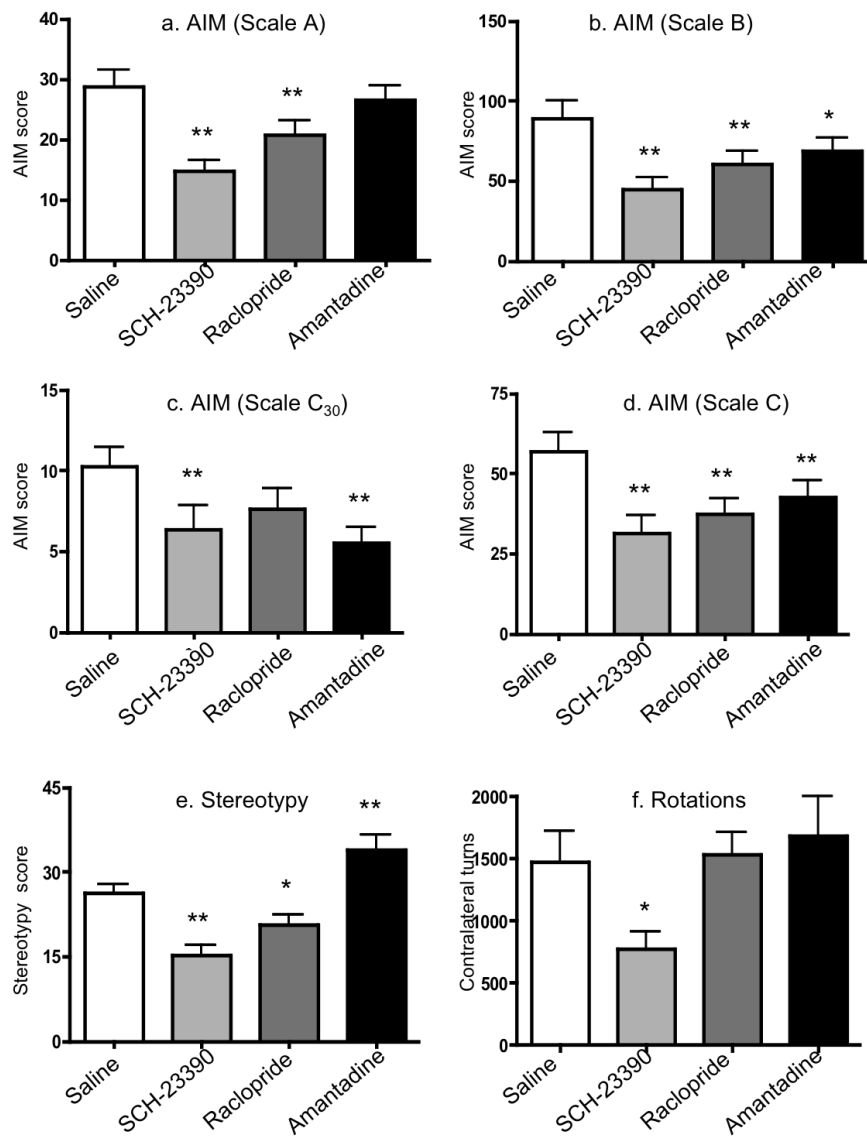


Figure 17: L-DOPA induced motor side effects following administration of drugs known to reduce dyskinesia. The AIM scores obtained with a) Scale A, b) Scale B, c) Scale C₃₀, d) Scale C, e) stereotypy scores and f) total number of contralateral rotation over 180 min following co-administration of L-DOPA (12 mg/kg) and: saline (white), SCH-23390 (light grey), raclopride (dark grey) (D₁ and D₂ receptor antagonists) or amantadine (black) are presented as mean score of all the animals \pm SEM. n=26, One-way ANOVA and Dunnett post-hoc test: *p<0.05, **p<0.01 (compared to L-DOPA co-administer with saline score).

	Saline	SCH-23390	Raclopride	Amantadine
AIM - Scale A				
1h	13.8 (±1.1)	10.0 (±1.1)**	12.6 (±1.2)	10.9 (±1.1)
2h	14.7 (±1.2)	8.9 (±1.1)***	12.8 (±1.2)	13.2 (±1.1)
3h	8.2 (±1.4)	1.5 (±0.5)***	4.4 (±0.8)**	10.3 (±1.2)
AIM - Scale B				
1h	28.0 (±3.4)	18.7 (±3.3)**	24.5 (±2.8)	22.1 (±3.3)
2h	31.7 (±3.7)	17.6(±3.7)***	24.4 (±3.1)*	24.8 (±3.3)*
3h	17.0 (±3.1)	5.0 (±2.1)***	6.0 (±1.2)***	15.8 (±2.6)
AIM - Scale C				
1h	23.7 (±2.3)	15.8 (±3.0)***	18.1 (±2.6)*	12.6 (±1.9)***
2h	22.1 (±2.3)	12.7 (±2.3)***	16.9 (±2.0)*	16.7 (±2.0)*
3h	11.2 (±2.2)	2.9 (±1.3)***	13.5 (±1.8)***	13.5 (±1.8)
Stereotypy				
1h	9.5 (±0.5)	6.5 (±0.8)**	8.3 (±0.8)	11.5 (±1.0)
2h	9.5 (±0.6)	6.7 (±0.7)*	8.2 (±0.7)	12.2 (±1.0)**
3h	8.5 (±0.7)	2.5 (±0.6)***	4.8 (±0.6)***	11.4 (±0.9)**
Rotation				
1h	445.9 (76.5)	269.7 (42.8)	568.9 (68.8)	276.5 (74.6)
2h	699.1 (113.3)	398.8 (73.2) **	860.5 (112.2)	718.7 (132.6)
3h	360.2 (85.5)	132.1(45.0) *	230.9 (46.0)	665.8 (135.0)**

Table 8: Evolution of L-DOPA induced motor side effects over the 3 hours following administration L-DOPA (12 mg/kg) and either saline, or SCH-23390 or Raclopride, or Amantadine. Data are presented as mean score or rotations ± SEM of all animals (n=26). *p<0.05, **p<0.01, ***p<0.001 compared to saline. Significant increases from saline are presented in bold.

3.3.4 Correlation between the different methods

When considering the scores of each rat individually after 10 week of chronic treatment with L-DOPA, there was a good correlation between the 3 AIMS scales (Figure 18). Understandably, given that they share similar roots, the strongest correlation was found between Scale A and B (Linear regression, p<0.0001). The inclusion of the hind-limb variable did not alter this correlation (Figure 19). There was a good correlation between the single time point scoring method and the other AIMS scales. However, the stereotypy score did not correlate with any of the dyskinetic scales (Figure 20), or with the severity of rotational behaviour induced by L-DOPA (Table 9). Finally, amphetamine-induced rotations performed after the 6-OHDA lesions did not relate to any of the L-DOPA-induced motor dysfunction components assessed in this experiment (Table 9).

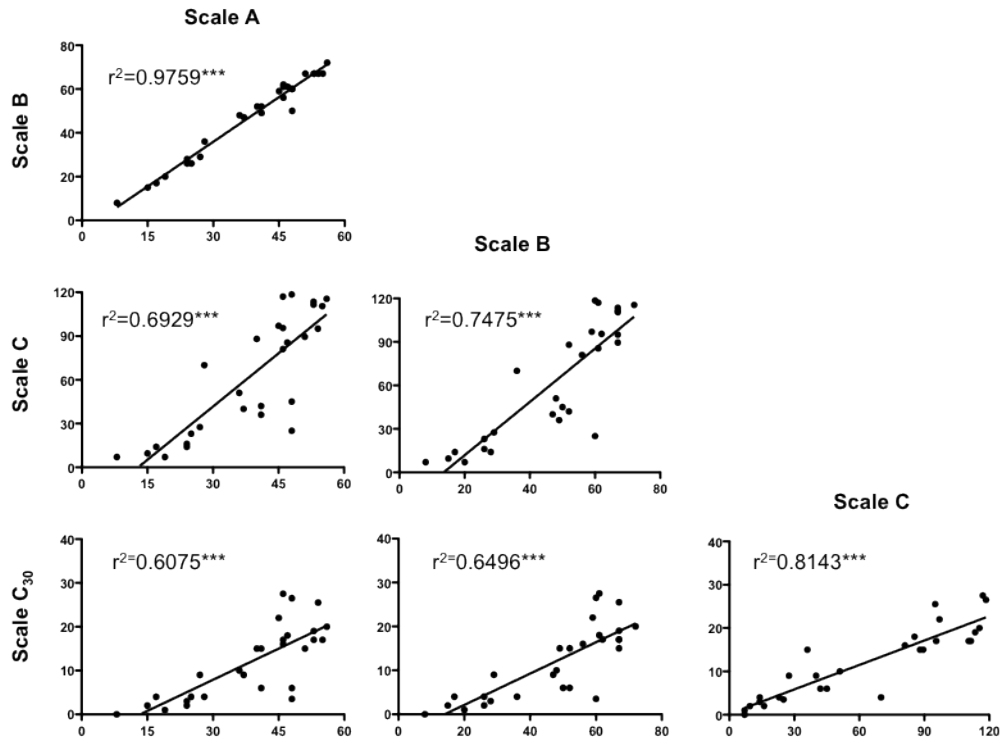


Figure 18: Correlation between the different AIMs scales (L-DOPA 12 mg/kg). ** $p < 0.01$, *** $p < 0.0001$, $n=28$.

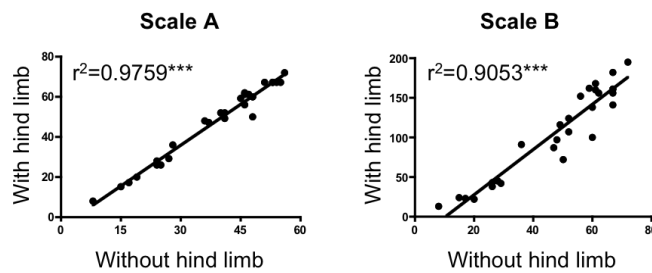


Figure 19: Impact of adding hind limb subtype to the AIMs scale A and B. $n=28$, *** $p < 0.0001$

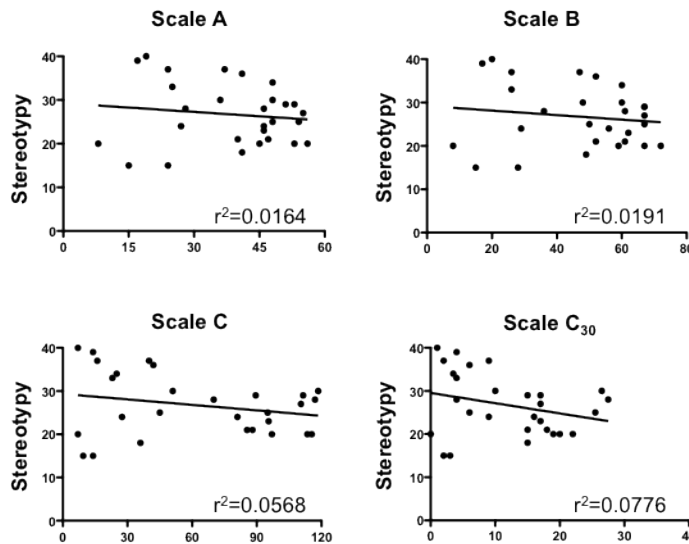


Figure 20: Correlation between stereotypic score (y) and the different AIMs scores (x). n=28, p>0.05.

r^2	Scale A	Scale B	Scale C ₃₀	Scale C	Stereotypy	L-DOPA rotations
Amphetamine rotations	0.0112	0.0261	0.0432	0.0214	0.0234	0.0476
L-DOPA rotations	0.2710**	0.2998**	0.2778**	0.3157**	0.0072	-

Table 9: Correlation between the number of amphetamine-induced ipsilateral rotations performed post-lesion, the number of L-DOPA-induced contralateral rotations and AIMs and stereotypic scores after chronic treatment with L-DOPA 12 mg/kg. The table presents r^2 value, n=28, **p<0.01.

3.3.5 Impact of the shape of the environment

No difference in AIMs score was found, regardless of the testing method used, when the animals were tested in rectangular cages or bowls (Figure 21) (Scale A: $t(27)=1.844$, $p=0.0763$; Scale B: $t(27)=0.4840$, $p=0.6323$, Scale C₃₀: $t(27)=1.147$, $p=0.2615$; Scale C: $t(27)=0.3974$, $p=0.6942$; Stereotypy: $t(27)=1.058$, $p=0.2995$; Rotations: $t(27)=0.4912$, $p=0.6272$)).

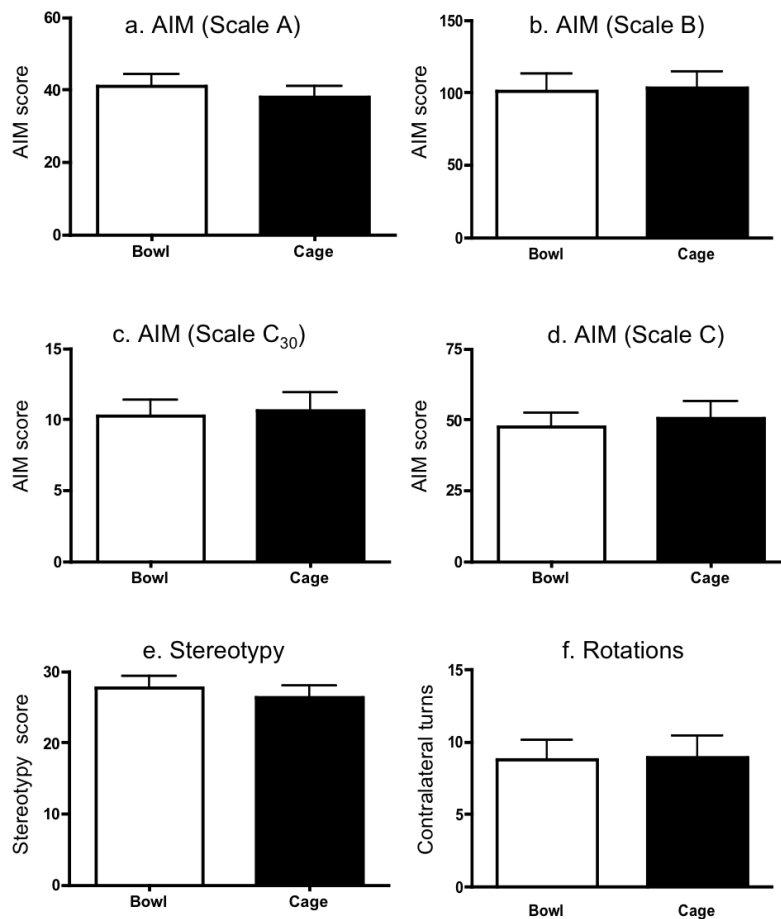


Figure 21: Impact of the shape of the environment on the AIM score obtained with Scale A, B, C₃₀ and C (a-d), on stereotypic (e) and on rotational behaviour (f) induced by L-DOPA. Data presented as mean \pm S.E.M. No significant difference has been found between the two testing environments $p > 0.05$. $n = 28$.

3.4 Discussion

The unilaterally 6-OHDA-lesioned rat is an affordable and easily constructed model that is broadly used for the study of LID. However, the lack of well-validated and systematic way of assessing the severity and development of dyskinesia could hinder its reliability and translatability. The work performed in this chapter is an important step toward establishing a more standardised approach to study of L-DOPA-induced motor dysfunction in the hemi-parkinsonian rat model.

3.4.1 Stereotypic and rotational behaviour

One of the main findings emanating from this work is that LID, stereotypies and rotational behaviour are three distinct motor complications induced by L-DOPA in this

model, with different profiles of development. The fact that dyskinetic, stereotypic and rotational behaviours do not respond to agents known to have anti-dyskinetic properties similarly indicates that these 3 types of motor dysfunctions might be driven by different mechanisms. It has been shown previously that rotational behaviour does not respond as well to anti-dyskinetic compounds but it is the first time that stereotypic behaviour has been assessed in parallel (Lundblad et al., 2005, Monville et al., 2005). Interestingly, amantadine worsened stereotypy. Since no correlation was found between stereotypy and AIMS scores, this effect cannot be attributed to the reduction of AIMS, which could have masked these repetitive movements. It is reasonable to think that the distinction between AIM and stereotypy will become even more crucial in the study of graft-induced side effects. Indeed, patients transplanted with foetal dopaminergic cells who developed “off drug” adverse effect have been described as more stereotypic than actual dyskinesia (Olanow et al., 2003). Similarly, post-grafting dyskinesia in rodents, induced by L-DOPA or amphetamine, are also more stereotypic in nature (Maries et al., 2006).

3.4.2 Dyskinesia rating scales

Out of the 3 main rodent AIMS methods commonly in used, Scale A, which was the original scale described by Cenci and co-workers, is the only one that has been validated pharmacologically and pathologically (Lundblad et al., 2002, Lundblad et al., 2005, Dekundy et al., 2007). Studies have indeed shown a good correlation between striatal FosB protein, prodynorphin expression and the AIMS score (Andersson et al., 1999, Lindgren et al., 2011). Moreover, Scale A has been proven effective in showing a reduction in AIMS score with compounds such as amantadine, clozapine or buspirone, which have a known anti-dyskinetic efficacy in human and non-human primates (Lundblad et al., 2002, Lundblad et al., 2005, Dekundy et al., 2007). On the contrary, Scale B and C have never been validated with known anti-dyskinetic agents, let alone compared with the original Scale A.

As expected, all AIMS scales were effective at following the development of AIMS over time. However, Scale A and B better reflected the increase in L-DOPA dose. All the scales showed a reduction in dyskinesia scores when animals were treated with DA receptor antagonists or amantadine. Nevertheless, none of the 2 DA receptor antagonists tested here were able to completely abolish AIMS in the rats. These results corroborate previous findings showing that dyskinesias and the rotational behaviour are not

mediated by a single DA receptor subtype (Lane et al., 2005, Taylor et al., 2005). While Scale A failed to show an effect of amantadine on AIMs in this study, it has been shown to be effective by others. This discrepancy may be due to a lower dose of L-DOPA being used in these studies (Lundblad et al., 2002, Lundblad et al., 2005, Dekundy et al., 2007). This then introduces the importance of dose in the assessment of anti-dyskinetic agents, even within the therapeutic range used in this study and by others. Finally, although Scale A and B showed a good correlation between scores with or without the addition of the hind limb subtype, adding this parameter increased the significance of the amantadine effect with Scale B. Therefore, although it is not a mandatory element, adding hind limb rating is recommended as it improves the sensitivity of the scale.

The description of the rating criteria for intensity in Scale C is very subjective and open to interpretation and, although the authors provided video clips in the supplementary data, they did not associate with the corresponding score. Generally speaking, it would be advisable that rating scales are published along with video material providing the corresponding final intensity score as well as the break down for each subtype of dyskinesia. Moreover, Scale C presents important drawbacks from a practical point of view. Indeed, the number of different AIMs rated and the complexity of the scale necessitates video recording of the animals. Indeed, the original scale considers 8 different subtypes of AIMs and uses different scoring scales (e.g. neck dystonia is ranked from 0.5 to 3 with steps of 0.5 when hind limb dystonia is scored from 1 to 3 with steps of 1, other are simply scored from 1 to 2). Moreover, it is difficult to score wrist dystonia accurately and to separate it from the forelimb dyskinesia due to the small size of the rats. This scale would be almost impossible to apply to mice in such detail. Finally, whereas video recording provides an easy way to ensure blind assessment, it disproportionately increases the amount of work (i.e. time to record the rats and time to score the video tape at low speed to allow accurate rating) without contributing significantly to a greater sensitivity. This is a major limitation of this method, especially when considering multiple time points of recording, which is likely to be why single or limited time points are considered by users of this scale. Moreover, scoring small movements on video recording can be challenging, in particular for animals displaying a strong rotational behaviour or facing away from the video camera.

3.4.3 Single versus multiple time points

The AIMs scale described by Steece-Collier (Scale C) is generally used to rate the animals at a single time-point, 30 min, considered as the time-of-peak response to L-DOPA (Steece-Collier et al., 2003, Steece-Collier et al., 2009). Nevertheless, considering a single time point could be detrimental, as it does not faithfully reflect the worsening of the AIMs severity. Moreover, prolonged L-DOPA treatment induces a shift of the time-of-peak severity and increases the duration during which the animals exhibit severe dyskinesia. Therefore, animals should be assessed at multiple time points to ensure that the effect observed translates to a global reduction of dyskinesia instead of a shift in time-to peak response. Furthermore, the single time point method, Scale C₃₀, failed to show an effect of D₂ DA receptors antagonism when the multiple time points version, Scale C, highlighted a significant reduction of AIMs scores with raclopride. The use of a single time point method to test the potential of anti-dyskinetic therapies can be misleading and it is likely to be important to consider the global effect of new treatments on the full duration of LID expression. Indeed, a short acting drug providing a significant effect at the time-of-peak response might not confer any real benefit for the patients. In contrast, the effect of an agent which could reduce the overall duration of LID, would not be sufficiently detected using this type of methodology and could fail in pre-clinically studies, despite having real potential.

3.4.4 Impact of the testing environment

In this study, the shape of the environment has been found to have no effect on AIMs and stereotypy scores or on rotational behaviour. By contrast, previous study reported an increase in the duration of some AIMs subtypes, as well as an increase in the amplitude score of limb dyskinesia and axial bias, when the animals were tested in cages comparing to the assessment in bowls (Pinna et al., 2006). In parallel, these authors showed an enhanced number of contralateral rotations when the animals were placed in a hemispherical environment. In this chapter, 26 female rats were tested successively in the 2 environments, whereas Pinna and colleagues used 8 males. It has been found, in our lab, that contrary to what is observed in patients, gender has little influence on the development of LID in rats (Lane EL, unpublished data) so the difference in the results is unlikely to be due to the animal gender. Moreover, another study performed in males did not report any difference in AIMs score between the two environments (Papathanou, 2010). Finally, the effect described by Pinna *et al.* on

rotational behaviour is highly questionable as the method used to count the number of contralateral rotations varies between the 2 environments (recorded by an automated rotometer in hemispherical bowls but evaluated visually in the cage). The experiment described in this chapter measured the number of rotations using the same automated rotometer, in the same testing room, for both environments, which allows a more accurate comparison. It is important to note that the size and probably more importantly the steepness of the bowl itself may have an impact and could explain the divergence in results.

3.4.5 Validating dyskinesia rating scales

This chapter highlighted the fact that small modifications, such as adding time points or a new subtype of abnormal movement, can change the sensitivity of the scale. Consequently, any modifications from the original scales should be tested and fully validated before using the modified method to assess potential new anti-dyskinetic drugs. Furthermore, given that the use of multiple time points significantly improves the sensitivity of the scale in detecting anti-dyskinetic effects of amantadine or DA agonists, one could suggest that patients diary based evaluation dyskinesia might provide a more accurate measurement of potential anti-dyskinetic drugs efficacy (Hauser et al., 2000, Hauser et al., 2004). Most of the scales currently available only assess patients for few minutes (2-15 min) and might not reflect the full effect of the treatments (Colosimo et al., 2010). This idea is supported by the one of the levetiracetam (Keppra) clinical trials, where a significant reduction of “on-time” with troublesome dyskinesias was reported using a patients diary based method, yet there was no improvement on the AIMS, the Unified Parkinson's Disease Rating Scale IV (UPDRS) or the UDysRS scores (Zesiewicz et al., 2005, Wolz et al., 2010). Of course, patients based evaluation has serious drawback as it could be easily affected by mood swings and depression, which are common in PD. The results obtained in this chapter highlighted the importance of pharmacologically validating the dyskinesia rating scales. This is mirrored by the effort beginning in 2010, when the Michael J. Fox Foundation for Parkinson's Research founded a multi-centres trial aiming at evaluating the responsiveness of the different clinical LID rating scales to amantadine treatment in order to validate them pharmacologically (Colosimo et al., 2010, Goetz, 2010).

3.5 Conclusion

In this chapter, I have demonstrated the importance of using well-characterised methods to study L-DOPA-induced motor dysfunction. To be complete, a study of the motor effects induced by L-DOPA in rodent should assess in parallel: AIMs, stereotypic behaviour as well as the measuring of the contralateral rotations, which is the approach that will be used throughout the remainder of this thesis. Scale B was proven to be a reliable and practical scale and was therefore chosen to rate AIMs in the subsequent studies (Table 10).

	Cost effectiveness	Objectivity	Practicality	Sensitivity to L-DOPA dose increase	Sensitivity to “anti-dyskinetic” agents
Scale A	+++	-	++	+++	++
Scale B	+++	-	++	+++	+++
Scale C	++	-	+	+	+++
Scale C ₃₀	++	-	+	-	++
Stereotypies	+++	-	++	+++	-
Rotations	+	+++	+++	++	+

Table 10: Summary of the main advantages of each method.

Chapter 4: Effect of L-DOPA treatment on the efficacy of cell therapy

Summary

There are discordant views on the potential toxic effect of long term L-DOPA treatment on dopaminergic neurons, particularly in the context of transplantation. The aim of this chapter was therefore to determine the effect of chronic L-DOPA administered during different phases of the transplantation process on the survival and function of non-compatible grafts. To that end, 6-OHDA unilaterally lesioned rats received sham surgery, allogeneic transplant or xenogeneic transplant, while being treated with L-DOPA before and/or after transplantation. The results showed that both types of graft survived and alleviated amphetamine-induced rotations. L-DOPA was found to have no detrimental effect on the graft, despite significantly promoting immune response around the xenograft (as demonstrated by the infiltration of peripheral immune cells). However, a critical finding was the differing immune response between the groups. Indeed, chronic L-DOPA administered post-transplantation increases the number of microglia and infiltrated peripheral immune cells around the graft.

Declaration

Part of the leukocytes immunostaining was performed by Korbinian Kienle during his Master's degree training.

4.1 Introduction

Transplantation of foetal dopaminergic precursor neurons into the striatum of PD patients showed encouraging results in restoring dopamine production at a level enabling improvement of motor symptoms in PD patients (reviewed by Barker and Kuan (2010)). However, a number of patients failed to receive benefit from the graft, and many that did improve also developed the motor side effects of GID (Hagell et al., 2002, Olanow et al., 2003) (see 1.3.3 Cell therapy). Typically, patients selected for these transplantation trials were at an advanced stage of disease progression. They had therefore been treated with L-DOPA for several years and in most cases had already developed LID. The dopaminergic neurons take time to mature post transplantation and most patients needed to remain under the dopaminergic medication regimes. Some patients were then able to reduce or even cease their anti-parkinsonian medications, but for many the improvement was only marginal. There have been previous studies exploring how L-DOPA could potentially impact upon the transplanted neurons but these have not been systematic and have not taken into account L-DOPA pre- and post-transplantation (Blunt et al., 1991, Steece-Collier et al., 1995, Steece-Collier et al., 2009).

To date, there has been little agreement on whether L-DOPA could hasten or prevent nigral degeneration in PD patients. In an early study, Diamond and colleagues showed that life expectancy was increased in PD patients who had taken L-DOPA soon after PD symptoms appeared (1987). This idea was supported by autopsy of non-PD patients, who have been treated with L-DOPA for years (4 to 26 years) and displayed normal SN (Quinn et al., 1986, Rajput et al., 1997). A double-blind study reported a significant reduction in UPDRS scores of patients treated with L-DOPA for 40 weeks, when compared to placebo the group, suggesting that L-DOPA might slow down the progression of the disease. However, it has been argued that, although a washout period of 2 weeks was observed before assessing the patients “off medication”, the symptomatic relief from L-DOPA might still be present (Fahn et al., 2004). A more recent imaging trial, taking PET-scan measurements 2 years apart, of patients taking L-DOPA or ropinirole showed a reduction in ^{18}F -DOPA signal in the L-DOPA treated group (Whone et al., 2003). Similarly, the Parkinson Study Group reported a significant decrease of the DAT probe β -CIT binding in the striatum of patients treated with L-DOPA for 4 years when compared the pramipexole treated group (2002). These 2

imaging studies used striatal DAT binding as an indirect measurement of the number of remaining DA neurons. Arguably, L-DOPA treatment could directly affect DAT level of expression and/or availability at the membrane without having any effect on dopaminergic cell survival. Moreover, due to the absence of placebo control, it is impossible to distinguish between a detrimental effect of L-DOPA and/or a protective effect of ropinirole and pramipexole. The effect that L-DOPA might have on the survival of the remaining dopaminergic cells is too delicate to address in patients because of the impossibility of directly evaluating neuronal survival.

Many *in vitro* studies have tried to answer the question L-DOPA toxicity. Most of them have reported a toxic effect of DA and L-DOPA on human and rodent immortalized neuronal cells (Michel and Hefti, 1990, Tanaka et al., 1991, Mena et al., 1992, Basma et al., 1995, Pardo et al., 1995, Ziv et al., 1997). L-DOPA and DA are easily autoxidised and produce oxygen reactive species and free radicals causing apoptosis (Graham, 1978, Cohen, 1987, Michel and Hefti, 1990), which could explain the detrimental effect of L-DOPA on neuronal cultures. These *in vitro* studies however have to be interpreted with caution, as they do not necessarily accurately reflect the environment of the neurons. For example, the concentration of L-DOPA used in culture is 100 to 1,000 times higher than the concentration measured in the cerebrospinal fluid of patients under L-DOPA treatment (Olanow et al., 1991, Tanaka et al., 1991, Basma et al., 1995, Pardo et al., 1995, Ziv et al., 1997). Moreover, the lack of glial cells, which play a crucial role in defending the brain against quinones involved in L-DOPA-induced toxicity, has also raised concerns regarding the relevance of the neuronal cell culture model (Miyazaki et al., 2011). Indeed, the culture of foetal rat midbrain in the presence of astrocytes or simply glia-conditioned medium, can rescue DA neurons from L-DOPA toxicity (Mena et al., 1996, Mena et al., 1997).

In vivo studies have suggested that L-DOPA is not toxic for the nigral DA neurons. 18 months of L-DOPA treatment in mice had no effect on the survival of nigral neurons (Hefti et al., 1981). Similarly, L-DOPA treatment did not affect remaining cells in the SN of 6-OHDA-lesioned rat model of PD, and may even have had a trophic effect (Murer et al., 1998, Mytilineou et al., 2003). However, data regarding the effect of L-DOPA upon the survival of foetal dopaminergic transplants are controversial. VM cells have been shown to be particularly vulnerable to dopamine L-DOPA *in vitro* (Blunt et

al., 1991, Pardo et al., 1995, Alexander et al., 1997). Nevertheless, other studies have reported both an absence of, or a beneficial effect of L-DOPA treatment on foetal neurons in cultures, depending on the presence of glial cells (Mytilineou et al., 1993, Mena et al., 1996, Mena et al., 1997). *In vivo* studies looking at the survival of VM transplant in rats also gave mixed messages. For instance, studies performed by Blunt and colleagues showed no significant effect of oral L-DOPA treatment (even at a very high dose: 200 mg/kg/day), on the survival and the function of VM transplant in 6-OHDA lesioned rats (Blunt et al., 1990, Blunt et al., 1991, Blunt et al., 1992b). In contrast, Steece-Collier and co-workers, reported a morphological impairment of the foetal neurons when the rats received i.p. injections of L-DOPA following transplantation (Steece-Collier et al., 1990). Moreover, they have reported a reduction of behavioural improvement post-grafting in rats pre-treated with L-DOPA, although no cell loss has been observed (Steece-Collier et al., 2009). The question of the potential toxic effect of L-DOPA treatment on foetal dopaminergic transplant therefore remains unsolved.

Direct neuronal toxicity is not the only way L-DOPA treatment could influence dopaminergic graft survival. Prolonged L-DOPA treatment has been shown to affect the blood-brain barrier, which could have knock on effects on the immune system. Micro vessels, present in the brain, express DA receptors (Choi et al., 2006) and it has been shown previously that chronic L-DOPA treatment is associated with alteration of microvasculature of the basal ganglia nuclei, predominantly in the striatum and output nuclei (GP and SNr), in the 6-OHDA-lesioned rat. Interestingly, the enhanced endothelial proliferation observed in these areas was associated with an increased permeability to bromodeoxyuridine and correlated with the severity of LID exhibited by the animals (Westin et al., 2006). Considering that good vascularisation of the transplant is necessary to ensure good graft survival, L-DOPA may in fact support the generation of the new vasculature and therefore enhance graft survival. The flip side of the coin is that the increased blood-brain barrier permeability associated with the neovascularisation could alter the “immune privileged” status of the brain and expose the graft to greater host immune challenges. This is further complicated by the fact that patients receive VM tissue collected from 5-8 embryos of unknown immunologic background. It is therefore reasonable to think that the combination of non-matching grafts with a permeable blood-brain barrier due to L-DOPA treatment could trigger

immune response against the transplant and therefore affect how the graft matures and functions.

4.1.1 Aims of the chapter

The question of the impact that prolonged L-DOPA could have on the survival, implantation or function of foetal VM transplant has yet to be resolved. In order to generate a clinically relevant model, this experiment uses allograft and xenograft to assess the effect of L-DOPA on VM grafts of different level of immune compatibility. The different transplant groups were then subdivided in sub-groups receiving: no L-DOPA, L-DOPA only prior to the transplant, only post transplant or both prior to and post-transplant (see details in 4.2. Experiment design). This chapter specifically aims to determine whether chronic L-DOPA treatment can affect the success of intrastriatal allogeneic and xenogeneic foetal transplantation of dopaminergic cells, depending on when it is administered, by assessing:

- 1) Cell survival and graft function
- 2) Motor function recovery
- 3) AIMs severity
- 4) Host immune response

4.2 Experimental design

All methods are detailed in the 2nd chapter. In this study, 140 female Sprague Dawley rats were unilaterally lesioned by injection of 6-OHDA in the right MFB and were assessed for motor deficits using assessment of amphetamine-induced rotation, lateralized stepping, vibrissae-elicited limb placement and cylinder tests before and after lesion. The 122 animals presenting most impairment (based on the amphetamine test) were kept for this experiment, while the 18 remaining rats were used for pilot studies on graft rejection (Chapter 5). Out of the 122, 2 had to be put down before the end of the experiment due to poor health (unrelated to the experiment: internal tumour and broken teeth); and 2 were excluded following histology due to the presence of brain tumour or pituitary gland tumour. The data presented here correspond to 118 rats for the behaviour experiments. TH cell counts are based on the original 118, further immunohistochemistry could only be carried out on 115 due to fungal contamination of 3 samples.

The rats were distributed into 12 homogenous groups based on their performances on the 4 motor tests, in order to ensure that no group was statistically different from another using a one-way analysis of variance (Table 11, amphetamine test: $p=0.9564$; Vibrissae test $p=0.9999$; Stepping test $p=0.9978$; cylinder test $p=0.6114$). The same tests were later to be used to assess motor recovery 12 weeks post-transplantation, with the exception of the cylinder test as the rats refused to explore the environment a 3rd time.

Surgery Group	Treatment Group	Amphetamine Mean (\pm SEM)	Vibrissae Test Mean (\pm SEM)	Stepping Test Mean (\pm SEM)	Cylinder Test Mean (\pm SEM)
Sham	SS	951.7 (\pm 206.5)	1.5 (\pm 0.2)	13.8 (\pm 4.6)	17.0 (\pm 4.8)
	SL	961.6 (\pm 115.0)	1.5 (\pm 0.5)	15.3 (\pm 5.8)	14.4 (\pm 4.3)
	LS	942.0 (\pm 136.8)	1.4 (\pm 0.4)	19.5 (\pm 9.0)	14.3 (\pm 5.1)
	LL	1117.0 (\pm 103.2)	1.4 (\pm 0.4)	13.8 (\pm 3.6)	8.1 (\pm 2.7)
Allograft	SS	925.9 (\pm 180.1)	1.5 (\pm 0.3)	15.0 (\pm 2.8)	16.3 (\pm 4.4)
	SL	943.3 (\pm 70.0)	1.5 (\pm 0.3)	14.3 (\pm 4.8)	11.7 (\pm 3.5)
	LS	997.5 (\pm 118.2)	1.4 (\pm 0.4)	13.4 (\pm 3.2)	13.0 (\pm 2.4)
	LL	834.4 (\pm 58.9)	1.6 (\pm 0.3)	17.7 (\pm 3.4)	9.4 (\pm 2.6)
Xenograft	SS	925.9 (\pm 180.1)	1.4 (\pm 0.3)	13.0 (\pm 2.4)	21.0 (\pm 7.2)
	SL	943.3 (\pm 70.0)	1.5 (\pm 0.2)	14.2 (\pm 3.0)	15.3 (\pm 2.8)
	LS	997.5 (\pm 118.2)	1.8 (\pm 0.3)	14.8 (\pm 4.8)	15.5 (\pm 3.8)
	LL	834.4 (\pm 58.9)	1.5 (\pm 0.3)	16.7 (\pm 3.3)	19.2 (\pm 4.3)

Table 11: Group allocation. Animals were distributed evenly in 12 groups based on their performances on motor tests following 6-OHDA lesions. Animals were balanced based on results from amphetamine-induced ipsilateral rotations, contralateral deficit on the vibrissae-elicited limb placing test (vibrissae test: expresses in percentage of left limb response), contralateral deficit on the lateralized stepping test (expresses in percentage of left steps taken while the animal were pulled to the right side) and contralateral deficit on the cylinder test (express in percentage of left touches). One-way ANOVA analysis of variance, $p>0.05$.

The animals were administered L-DOPA (10 mg/kg) or saline for 8 weeks (daily for 4 weeks then every other day for 4 weeks), corresponding to the first phase of the treatment (Phase I, Figure 22). The rats received intrastriatal transplant of 200 000 foetal VM cells obtained from E14 Wistar rats (allograft) or E12 CD1 mice (xenograft) or had sham surgery (DNase). The second phase of the treatment (Phase II) began on the first day following surgery and lasted for another 8 weeks (again, daily injections for

4 weeks and then every other day for an extra 4 weeks, Table 12). After the second phase of treatment there was a rest period of drug abstinence for 2 weeks to allow complete wash out of the drug prior to evaluation of motor function repeating the amphetamine-induced ipsilateral rotations, vibrissae-elicited limb placing and lateralized stepping. Finally, a last challenge with L-DOPA was performed a week before perfusion (Figure 22). Brains sections were stained for dopaminergic cells, immune cells and blood vessels markers. The statistical methods used to analyse the results are specified in the text.

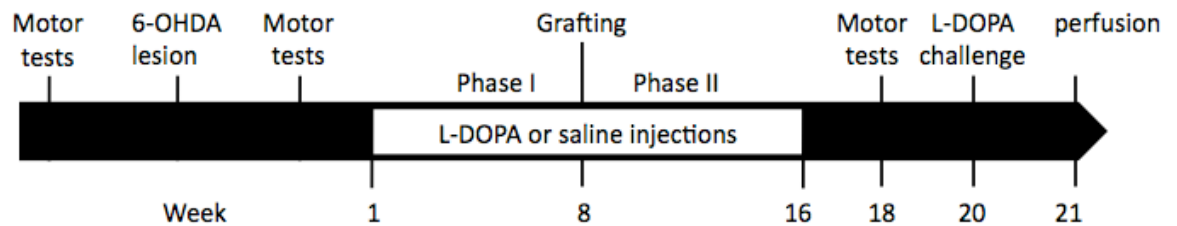


Figure 22: Time line of the experiment.

	Group	Number of animals	Phase I (pre-graft)	Graft	Phase II (post-graft)
Sham	SS	6	Saline	Sham	Saline
	SL	8*	Saline	Sham	L-DOPA
	LS	8	L-DOPA	Sham	Saline
	LL	8	L-DOPA	Sham	L-DOPA
Allograft	SS	10	Saline	Rat VM (E14)	Saline
	SL	11*	Saline	Rat VM (E14)	L-DOPA
	LS	12*	L-DOPA	Rat VM (E14)	Saline
	LL	12	L-DOPA	Rat VM (E14)	L-DOPA
Xenograft	SS	10	Saline	Mouse VM (E12)	Saline
	SL	10	Saline	Mouse VM (E12)	L-DOPA
	LS	11	L-DOPA	Mouse VM (E12)	Saline
	LL	12	L-DOPA	Mouse VM (E12)	L-DOPA

Table 12: Treatment and transplantation received by the animals for each group. The group were named after the treatment they received in treatment phase 1 and 2: S = saline, L= L-DOPA thus, SS group received saline in treatment phase 1 and 2, SL group received saline in treatment phase 1 and L-DOPA in treatment phase 2, LS received L-DOPA in Treatment phase 1 and saline in treatment phase 2 and LL received L-DOPA in treatment phase 1 and 2.*loss of animal brain due to contamination.

4.3 Results

4.3.1 Nigral lesion

Retrospective analyse of the dopaminergic neurons of the SN demonstrated that all rats had lesions greater than 95%. No significant difference was found between the groups ($F_{11,106}=1.009$, $p=0.4439$), confirming the results obtained with the different motor tests (Table 13).

Surgery Group	Treatment Group	TH ⁺ cell survival on the lesioned SN (in % of intact side) Mean (\pm SEM)
Sham	SS	1.00 (\pm 0.51)
	SL	0.91 (\pm 0.45)
	LS	2.69 (\pm 1.25)
	LL	0.85 (\pm 0.36)
Allograft	SS	0.64 (\pm 0.25)
	SL	1.23 (\pm 0.99)
	LS	1.22 (\pm 0.63)
	LL	1.50 (\pm 0.75)
Xenograft	SS	0.98 (\pm 0.37)
	SL	3.23 (\pm 1.50)
	LS	1.23 (\pm 0.37)
	LL	2.40 (\pm 0.95)

Table 13: TH⁺ survival in the SN. Percentage of dopaminergic cells remaining in the lesioned substantia nigra (in % of the non-lesioned side). One-way ANOVA analysis of variance, $p>0.05$.

4.3.2 Survival and functionality of the graft

Graft survival was estimated using TH immunohistochemistry as a marker of dopaminergic cells. As expected a significant difference was observed between the sham surgery group, which did not receive any cell transplant, and the 2 grafted groups ($F_{2, 106}=37.103$; $p<0.0001$). However, no difference was found between the different treatments groups ($F_{3, 106}=0.979$; $p=0.406$), suggesting that chronic treatment with L-DOPA pre and/or post graft did not affect the survival of dopaminergic neurons (Figure 23).

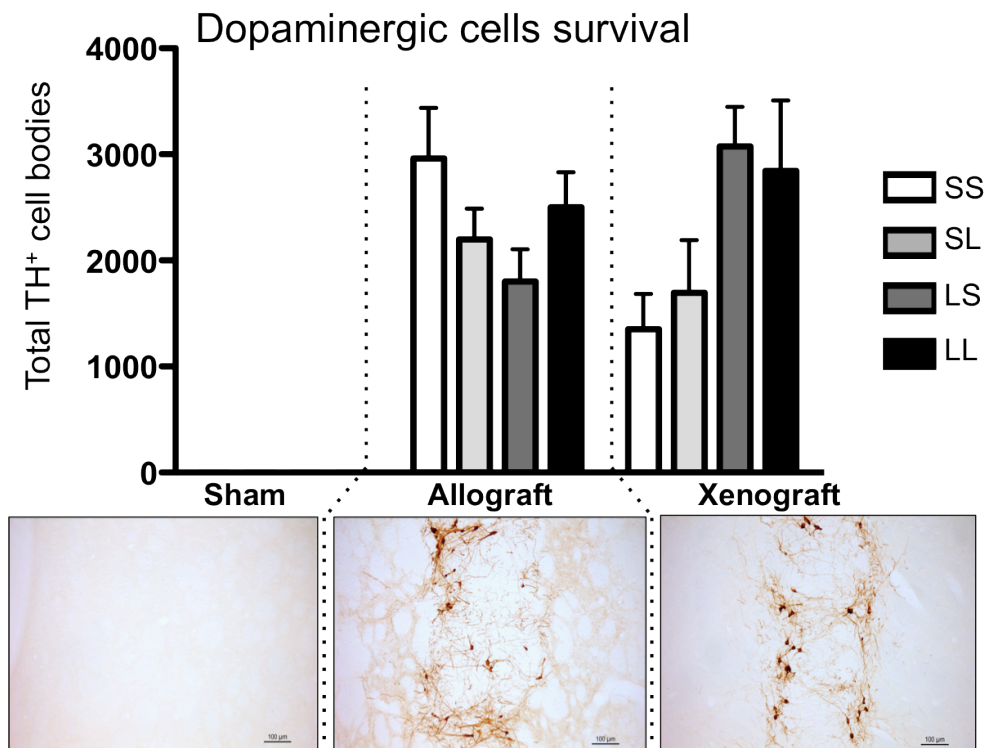


Figure 23: Survival of intrastriatal VM graft. Dopaminergic cells were labelled by immunohistochemistry, using an antibody against TH. The total number of TH⁺ cells for each group is presented as mean \pm SEM. Representative pictures of each transplant group are presented under the graph.

The ability of the graft to release dopamine was assessed by recording the total of amphetamine-induced rotations performed by the rats 3 months post-transplantation. The animals receiving sham surgery did not show any reduction in the total rotations performed over 90 min. Conversely, both groups of animals transplanted with VM tissue had a significant reduction in rotational behaviour ($F_{2,106}=32.60$; $p<0.0001$). There was no difference between the groups grafted with allogeneic (rat) or xenogeneic (mouse) tissue ($F_{3,106}=0.32$; $p=0.807$), nor between the different treatments within each transplantation group ($p>0.05$) (Figure 24). Importantly, no amphetamine-induced dyskinesia was observed in any group.

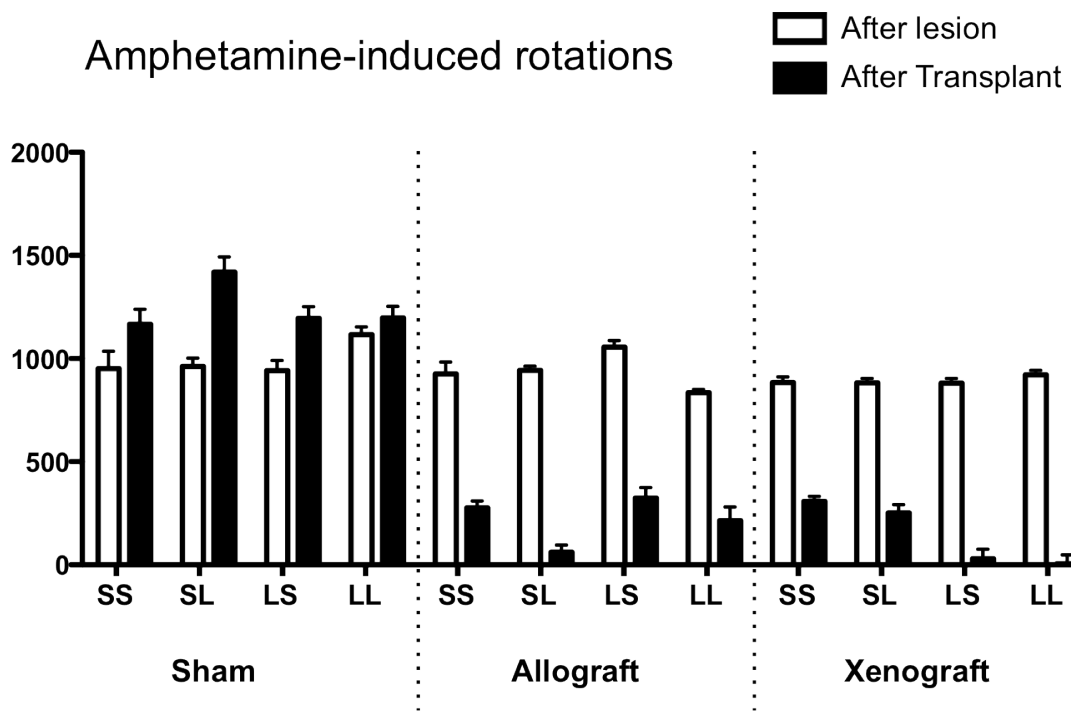


Figure 24: Amphetamine-induced ipsilateral turns performed over 90min following 3months after transplantation, before (white) and after (black) transplantation.

Linear regression analysis was used to see if a correlation existed between the number of TH⁺ surviving from the transplant and the reduction of cycling behaviour induced by amphetamine. No significant correlation was found (Figure 25).

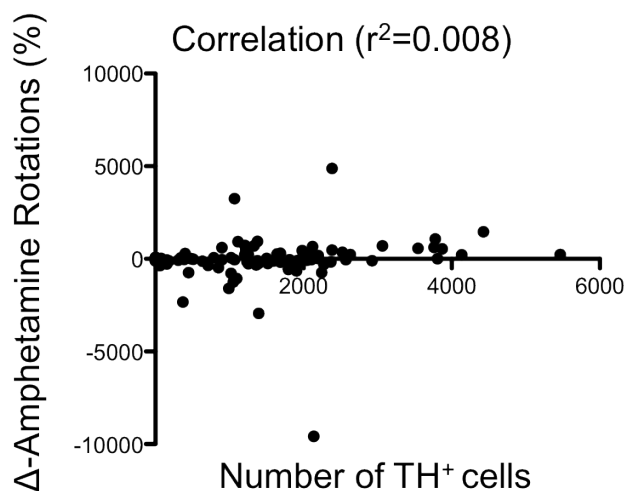


Figure 25: Correlation between the percentage of reduction in amphetamine-induced ipsilateral rotations and the number of dopaminergic cells present in the graft. No significant correlation has been found (linear regression).

4.3.3 Motor Function Recovery following Transplantation

The impact of dopaminergic striatal graft on the improvement of the motor function was assessed by counting the number of lateralized steps and vibrissae-elicited limb placement performed on the contralateral side to the lesion (Figure 26, 27). The animals performed more steps while moved in the forehand direction compared to backhand direction. However, no motor recovery was observed in any of the group: the rats either showed no difference (vibrissae test $F_{1,106}=4.98$; $p=0.085$, stepping test forehand $F_{1,106}=2.95$; $p=0.089$) or a significant worsening after transplant (stepping test backhand $F_{1,106}=7.23$; $p=0.008$).

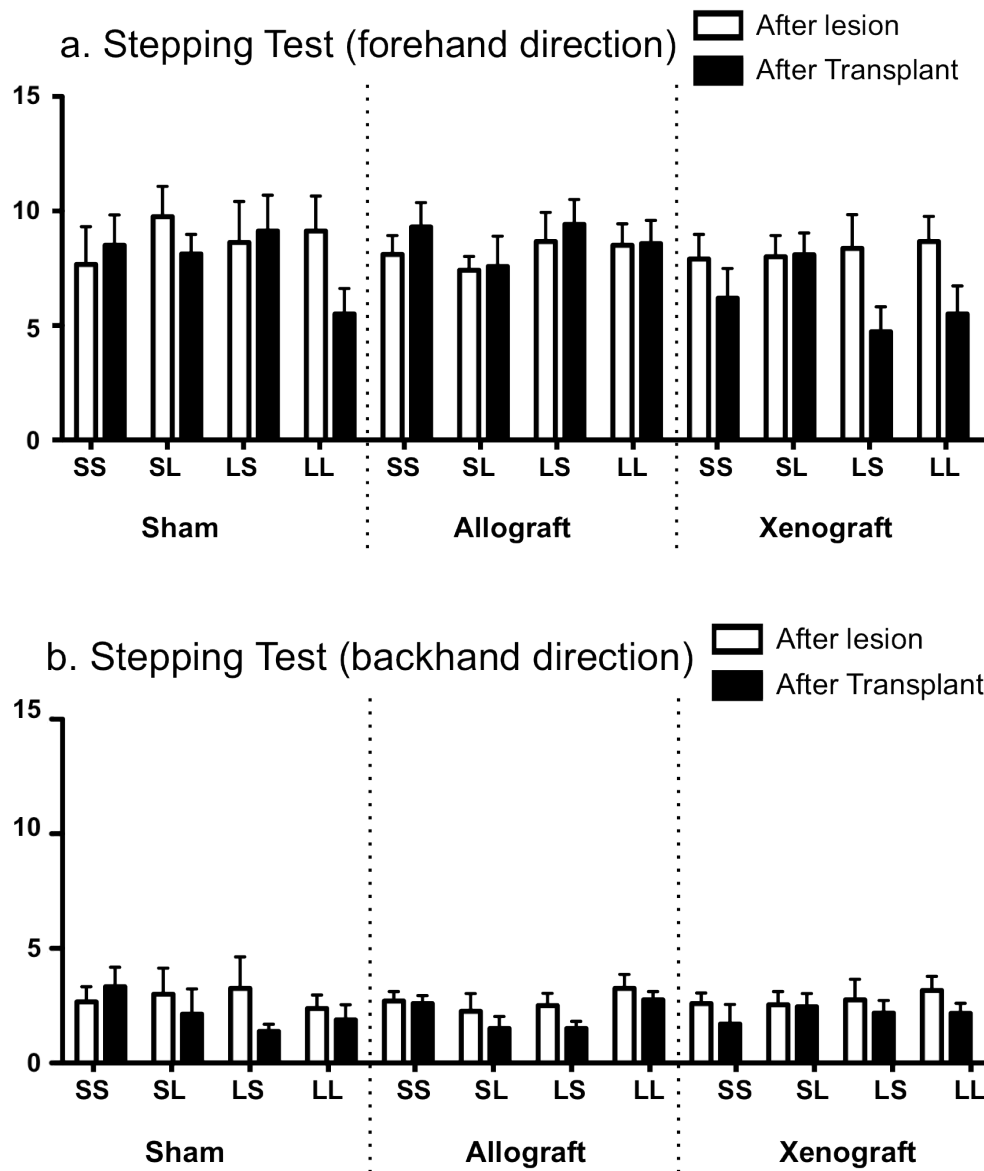


Figure 26: Mean number of adjusting steps performed on the contralateral side (left) while moved sideways on the forehand (a) and backhand (b) direction, before (white) and after transplantation (black). Two-way ANOVA analysis showed no significant difference between the rats' performance before and after transplantation, nor between the different treatments ($p > 0.05$)

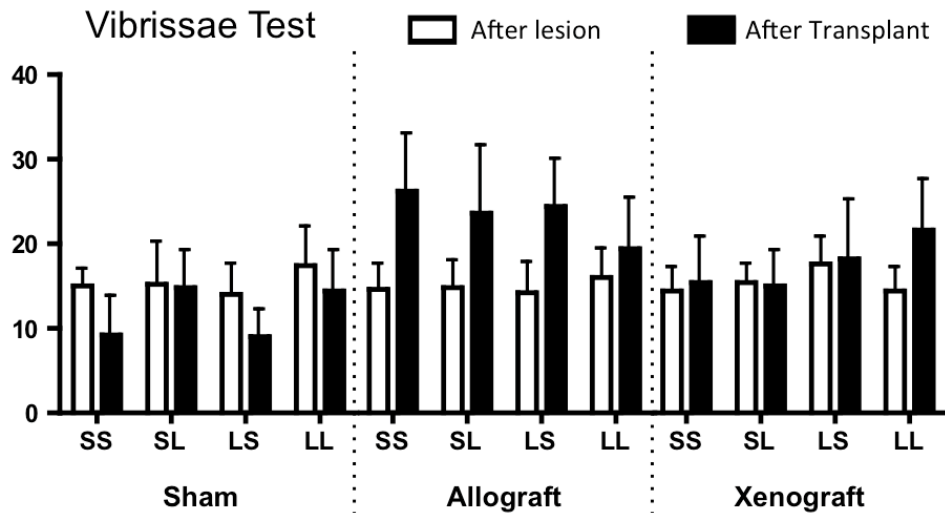


Figure 27: Mean number of vibrissae-elicited limb placement performed by the animals on the contralateral side of the lesion (left) expressed in percentage of response \pm SEM, before (white) and after (black) transplantation. Two-way ANOVA analysis showed no significant difference between the rats' performance before and after transplantation, nor between the different treatments ($p > 0.05$).

4.3.4 L-DOPA-induced motor dysfunction

In order to evaluate the impact of L-DOPA treatment before and after transplantation, rats were scored using a stereotypy scale and AIMs scale (Scale B, see Chapter 3). As expected, all the control groups (SS), which received only saline during the two treatment phases never developed any AIMs, nor stereotypic or rotational behaviour. The SS AIMs results are not shown as all animals obtained a consistent null score.

Rats that only received saline injections (SS) had a significantly lower stereotypy score when compared to L-DOPA treated animals ($F_{3,106}=23.97$, Tukey post-hoc comparison, $p < 0.0001$). In fact, their mean score was 1, reflecting basal activity rather than stereotypies, as this score is given to active but not stereotypic animals. In the other treatment groups (SL, LS, LL), stereotypic scores gradually increased over the weeks of L-DOPA treatment, however, neither the treatment regime, nor the type of graft had an impact on L-DOPA-induced stereotypic behaviour in these 3 groups, as observed in the last L-DOPA challenge (Figure 28, Treatment: $F_{3,106}=23.97$, $p < 0.0001$, Tukey post-hoc comparison between SL, LS, LL $p > 0.05$; Graft: $F_{2,106}=0.64$, $p = 0.528$).

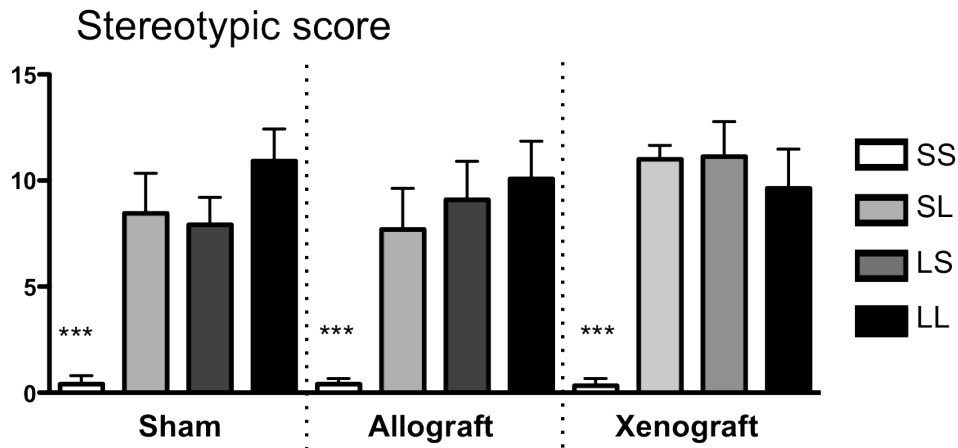


Figure 28: Stereotypic score obtained during the final L-DOPA challenge (week 12 post-grafting). The SS group was injected with saline while the 3 other groups, which have received L-DOPA previously during at least 1 of the treatment phases, were injected with L-DOPA. Data presented as mean \pm SEM, Univariate analyse of variance, Tukey post-hoc comparison, *** $p < 0.0001$.

To evaluate the impact of the different L-DOPA regimes on the development and persistence of dyskinesia after transplantation, L-DOPA-induced AIMs were rated weekly. All L-DOPA treated groups (SL, LS, LL) progressively developed AIMs from the first day of L-DOPA treatment and rapidly increased to reach a ceiling after 2-3 weeks. Animals treated with L-DOPA during the first phase of the experiment only (LS) did not show any reduction in AIMs score, whether they had been grafted or not, when tested 12 weeks post transplant (Figure 29, b, e, h). The rats receiving chronic treatment with L-DOPA during the entire experiment (before and after grafting, LL, Figure 29, c, f, i) showed a decreasing trend in AIMs scores following VM transplantation, although this was only significant in the xenografted group $p < 0.05$ from week 14. Interestingly, the sham group with delayed L-DOPA treatment had a higher mean of AIMs scores than the corresponding treatment groups, which had received VM transplant (SL, Figure 29, a, $F_{2,28} = 27.95$; $p < 0.0001$) (Dunnett 2-sided post-hoc, Allograft $p = 0.16$; Xenograft $p = 0.49$, Figure 29, b, c).

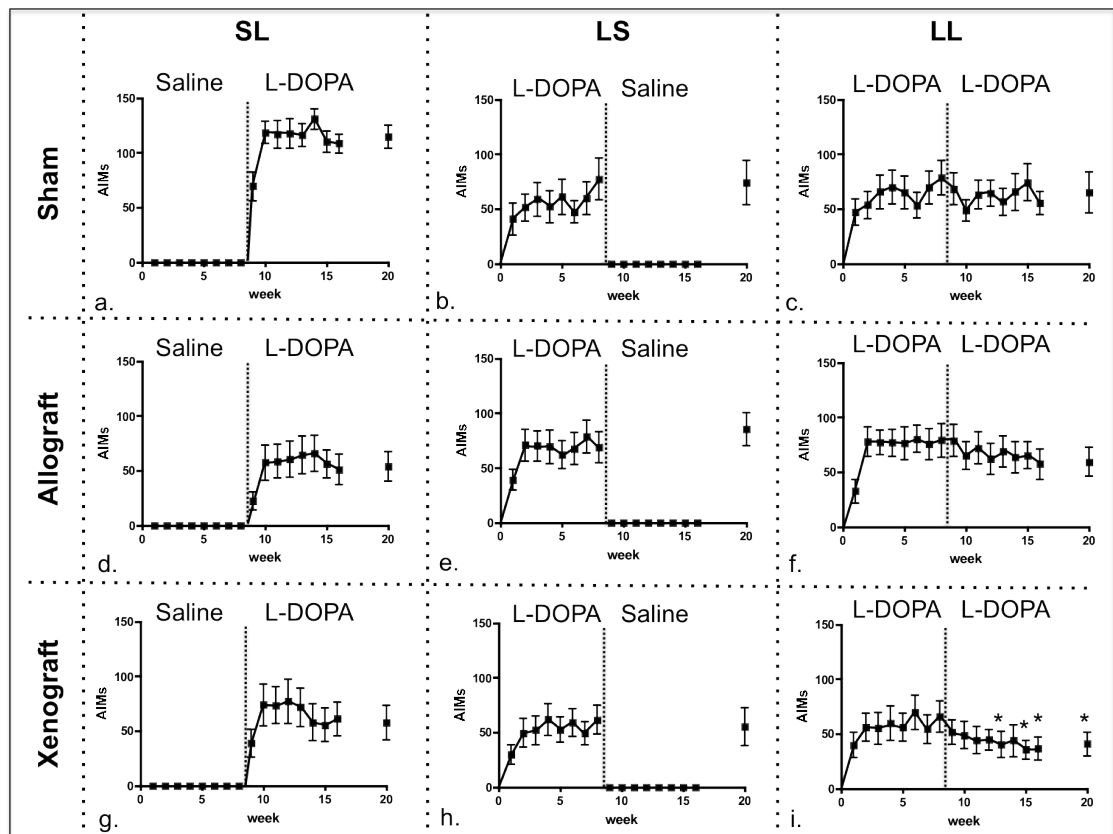


Figure 29: Means of AIMs scores rated before and after striatal transplantation (dotted line) of rat VM (left panel) mouse VM (central panel) or sham surgery (right panel). The different treatments for each phase (I and II) are indicated on each graph: a, b and c, received L-DOPA in phase I and saline in Phase II; d, e, f, received saline in phase I and L-DOPA in phase II; g, h, I were treated with L-DOPA all the way through. The arrows indicate the last L-DOPA challenge performed 12 weeks post-transplantation. Data presented as mean ± SEM, *p<0.05.

In order to better characterise the reduction in AIMs score observed in the xenogeneic group treated with L-DOPA in phase I and II, the score of each rat was plotted against the number on TH⁺ found in their striatum. Once again, no correlation was found between the number of TH⁺ surviving from the transplant and the decrease observed in the xenografted group that received L-DOPA all the way through this experiment (Figure 30).

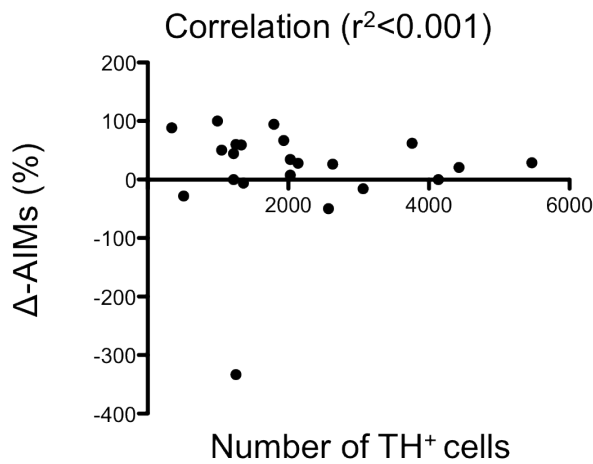


Figure 30: Correlation between the percentage of reduction in amphetamine-induced ipsilateral rotations and the number of dopaminergic cells present in the graft. No significant correlation has been found (linear regression).

The last L-DOPA challenge (12 weeks post-grafting) showed a reduction in rotational behaviour in grafted rats when compared to the sham surgery groups (Figure 31, Univariate analyse of the variance ($F_{2,106}=11.346$, $p < 0.001$, allogeneic graft: $p < 0.002$, xenogeneic graft: $p < 0.0001$). As expected, the rats that received saline instead of L-DOPA during that challenge (SS) did not exhibit any L-DOPA-induced rotational behaviour ($F_{3,106}=8.493$, $p < 0.001$, SS: $p < 0.01$ when compare to the rats that received L-DOPA (SL, LS, LL)). No significant difference was found between the 3 other treatment groups (SL, LS, LL).

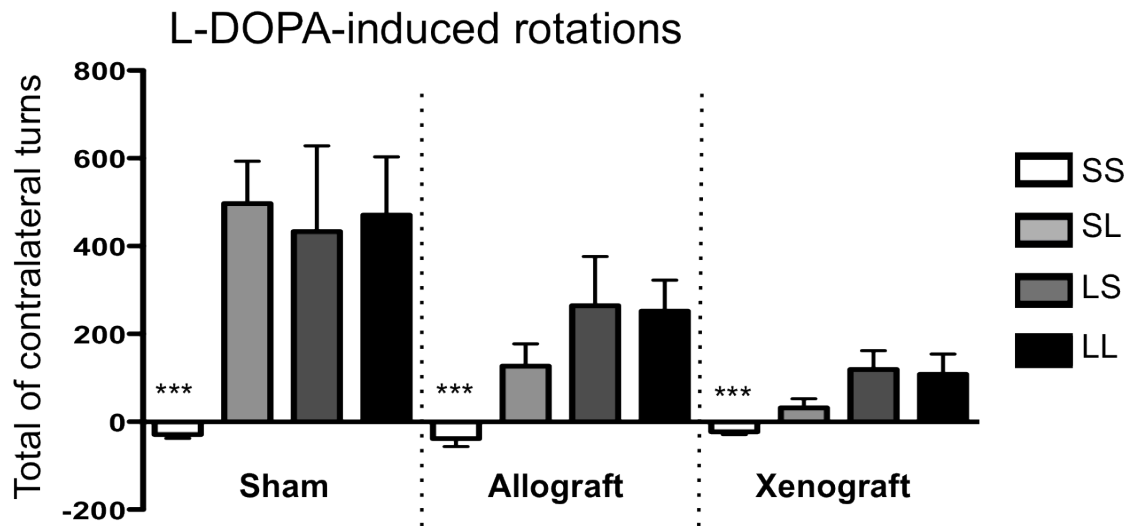


Figure 31: Number of net rotations performed toward the left side during the 3h following saline (SS) or L-DOPA administration (SL, LS, LL), 12 weeks post-transplantation. Data presented as mean \pm SEM. Univariate analyse of the variance, Bonferroni post-hoc test *** $p < 0.0001$ when compared to other treatment within the same graft group.

4.3.5 Immune markers in the striatum

The immune response against the graft was evaluated by immunohistochemistry using a leukocyte marker (CD45) and microglia marker (Ox42). The presence of the graft, whether it was an allogeneic graft or a xenogeneic graft, generally increased the number of microglia in the grafted area (Figure 32, $F_{6,103}=9.890$, $p < 0.0001$). However, an interaction was found between the type of graft that the rats received and the treatment they had been administered ($F_{6,103}=3.365$, $p = 0.005$). Bonferroni post-hoc comparison showed no significant difference between the 4 treatment groups, in the rats that received sham surgery or allograft. In contrast, the treatment's regime had a significant impact on the number of Ox42⁺ cells in the xenograft group. Indeed, an increase in microglia was found in the group treated with L-DOPA in both treatment phases (LL) when compared to the other 3 treatment groups (SS and LS: $p < 0.0001$, SL: $p = 0.020$). Representative pictures of the microglia staining in each group are presented (Figure 33).

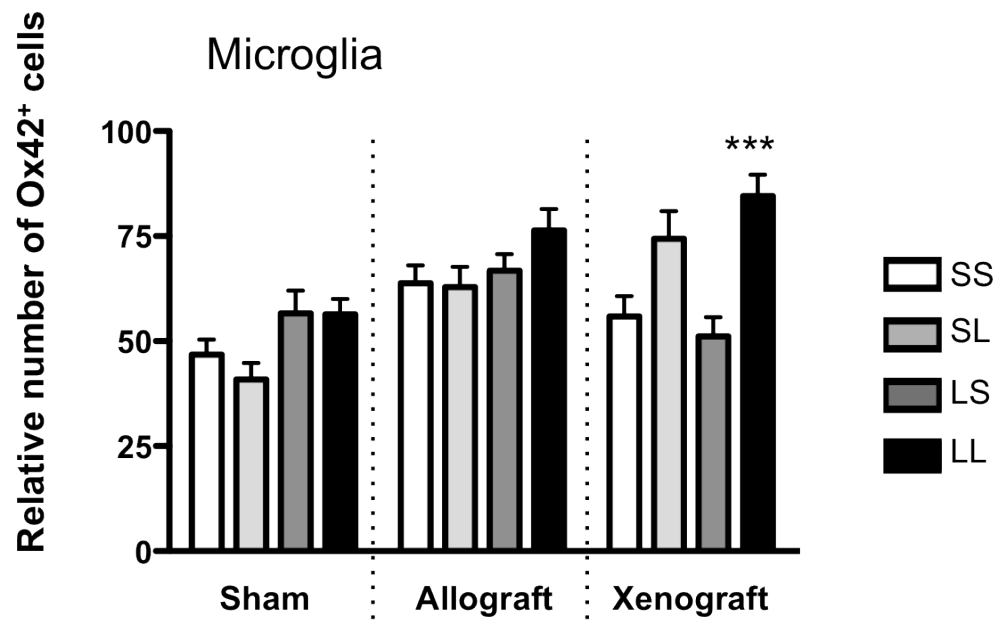


Figure 32: Relative number of microglia counted in the grafted area (285.94x214.46 μm window). Data are presented as Mean \pm SEM. *** p <0.0001 relative to the corresponding SS group.

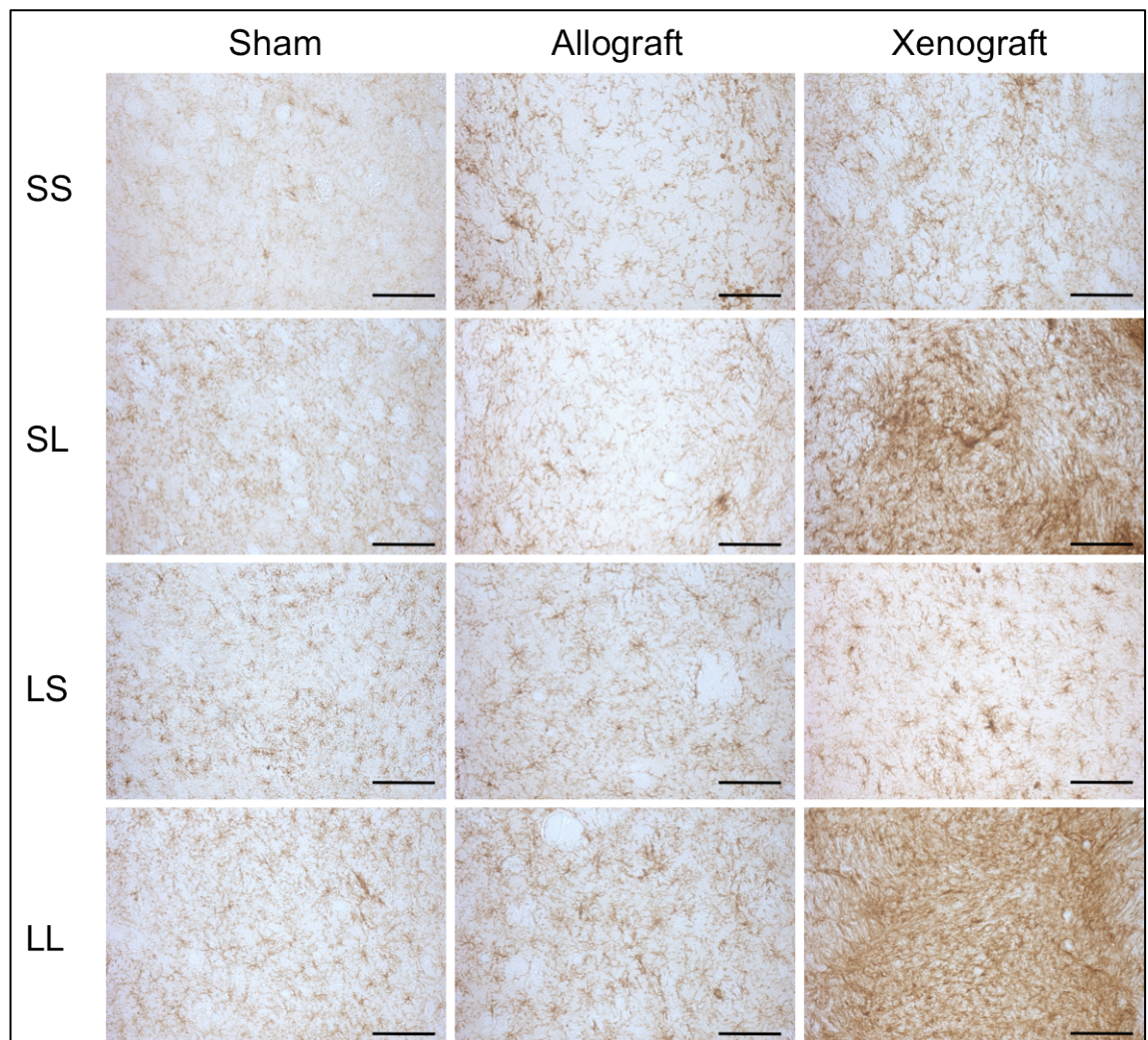


Figure 33: Microglia immunocytochemistry staining (Ox42) of the grafted area. Scale bar: 100 μm .

The number of infiltrated leukocytes in the transplanted striatum was significantly higher in the xenogeneic graft group (Figure 34, $F_{6,103}=8.887$, $p=0.0001$, Bonferroni post-hoc analysis $p=0.001$) but no difference was found between the sham and the allograft groups ($p=0.827$). No effect of treatment was found overall ($p=0.149$). To better understand the effect that the different L-DOPA regimes had on the xenograft group, data were analysed separately. As they did not have a normal distribution, a non-parametric test was used. The mean number of leukocytes infiltrated in the 2 groups that received L-DOPA post-grafting was 3 to 6 times higher than in the saline group (Kruskal-Wallis test $p=0.0052$, Dunn's multiple comparison test: SL $p<0.05$, LL $p<0.01$ when compared to the SS group).

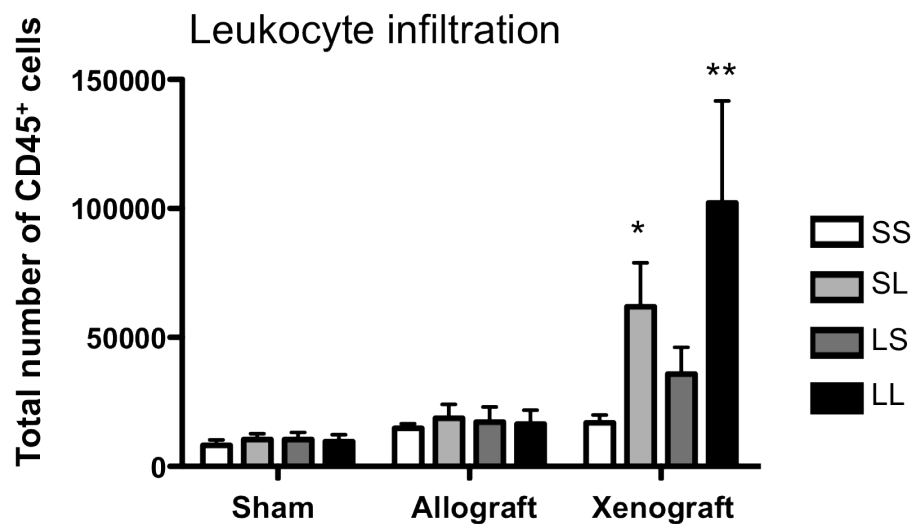


Figure 34: Total number of leukocytes infiltrated in the striatum.

In the group of rats that had never been exposed to L-DOPA, very few CD45⁺ cells were observed (SS, Figure 35, a). In contrast, in the groups that had been treated with L-DOPA, a consistent increase in infiltrated cells was found throughout the striatum. The most striking observation was the presence of agglomerated leukocytes along the blood vessels, close to the grafted area, which was only seen in some of the rats treated with L-DOPA post-grafting (SL and LL, Figure 35, b, d). Interestingly, in the same animals, the Ox42 staining revealed the presence of dense and aligned microglia, resembling gliosis (microglia scarring).

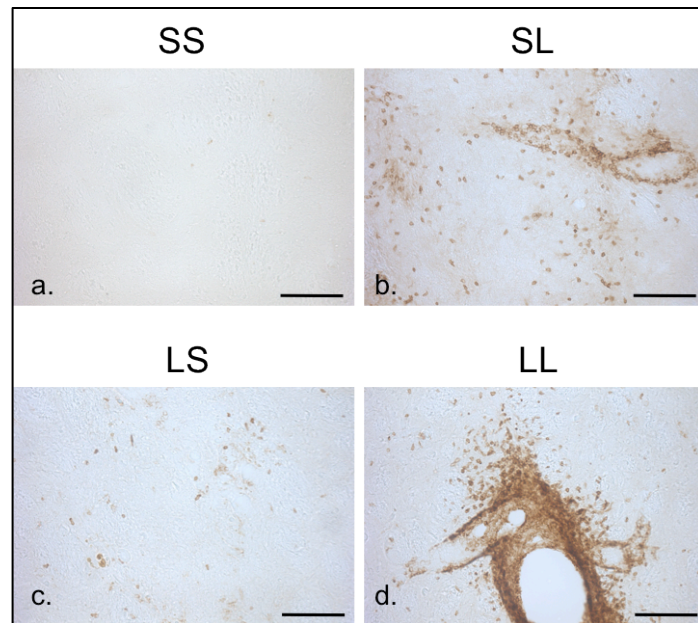


Figure 35: Leukocytes immunochemistry staining (CD45⁺) of the grafted area. Scale bar: 100μm.

There was a good correlation between the 2 immune markers studied, i.e. number of microglia and the number of leucocytes infiltrated in the striatum (Figure 36, $p < 0.001$).

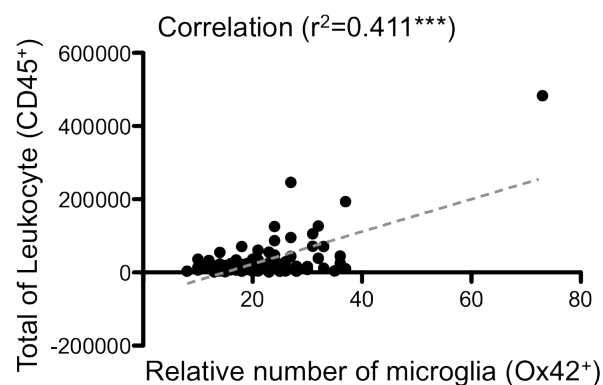


Figure 36: Correlation between the total number of leukocytes and the relative number of microglia.

To further investigate the leukocyte infiltration, the number and sub-type of lymphocytes involved in the immune response was evaluated using CD4⁺ and CD8⁺ markers. A complementary staining was performed on the group showing the most immune reaction, i.e. xenogeneic graft treated with L-DOPA during the 2 treatment phases (LL). The lymphocytic response was dominated by T helper cells (CD4⁺, Figure

37, Mann Whitney test, $p=0.0351$). (Note: the number of $CD45^+$ has been added as a reference only, showing the total number of white blood cells infiltrated in the striatum, including lymphocytes. The number of $CD4^+$ and $CD8^+$ was obtained by double staining immunochemistry and therefore has been analysed as a non-parametric T-test, as the data did not follow a normal distribution).

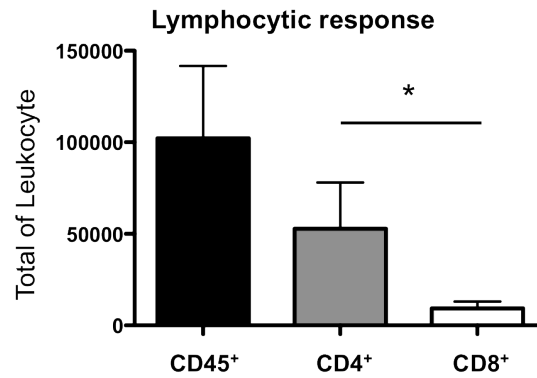


Figure 37: Lymphocytes population presents in the striatum of the xenograft rats treated with L-DOPA in phase I and II. Total number of $CD4^+$ and $CD8^+$ cells is presented as Mean \pm SEM. * $p<0.05$

To evaluate the effect of prolong exposure to L-DOPA on the blood vessels and angiogenesis, the sections were labelled immunohistochemically using a pan-vascular endothelium marker (RECA-1, Figure 38, 39). No significant difference was found between the contralateral (left) and ipsilateral (right) hemisphere to the graft (Multivariate analysis of the variance, $F_{1,40}=0.732$, $p=0.397$). As no difference was found between groups receiving saline throughout and the group receiving L-DOPA throughout ($F_{1,40}=0.105$, $p=0.748$), the other groups were not analysed as it reasonable to expect that L-DOPA treatment administered only during 1 of the 2 treatment phases would not have shown a different outcome. The xenograft group had a significantly reduced blood vessel surface area when compared to the allograft ($F_{1,40}=39.141$, $p<0.0001$).

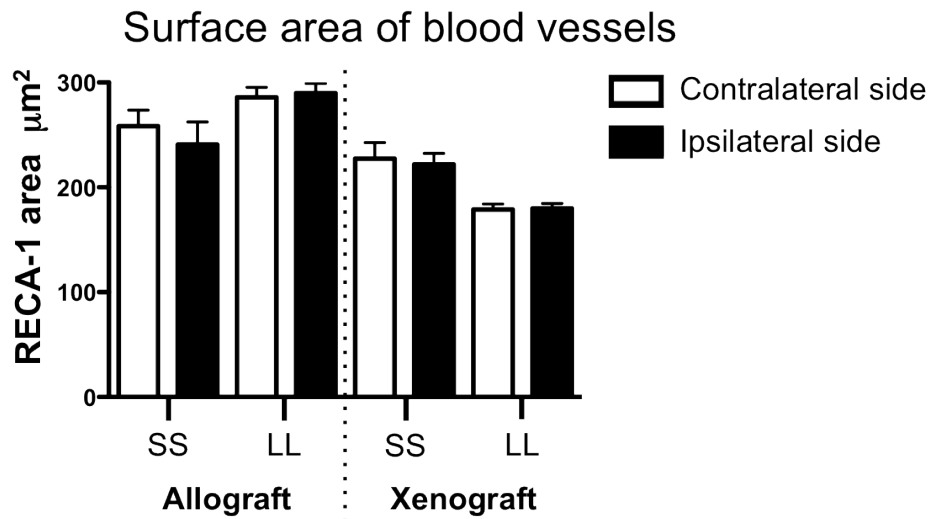


Figure 38: Relative surface of the blood vessel in the striatum. Mean \pm SEM.

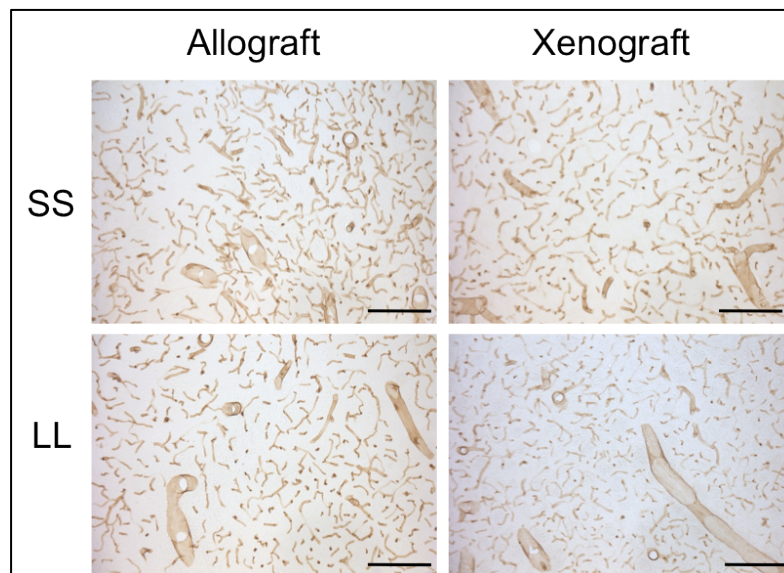


Figure 39: Immunohistochemistry of the blood vessel in the striatum, ipsilateral side to the graft (RECA-1). Scale bar: 100 µm.

4.4 Discussion

Preclinical studies for foetal dopaminergic transplants in 6-OHDA rats have traditionally used VM tissue harvested from the same strain as the hosts, in order to avoid graft-induced immune response. Furthermore, most studies focused on transplantation only and overlooked the importance of using a chronic L-DOPA treatment, when trying to mimic the human condition. This type of model therefore suffers from major drawbacks as the use of syngeneic tissue does not trigger an immune

response, but post-mortem analysis performed on transplanted patients' brains have demonstrated the presence of infiltrated immune cells (Kordower et al., 1997). To provide full consideration of these issues, the current study assessed the survival and function of allogeneic and xenogeneic VM grafts as well as host tolerance of the transplant, when being subject to different L-DOPA regimes. The original aim of this chapter was to evaluate how the timing of chronic L-DOPA treatment could affect the development of post-transplant dyskinesia. This question could not be addressed, as the animals did not develop spontaneous AIMs, or amphetamine-induced AIMs.

4.4.1 Allogeneic versus xenogeneic graft

Clinically, foetal VM transplants are allogeneic, human-to-human. However, because human transplants require multiple donor embryos, each with a different immunological background, the use of a xenogeneic graft constitutes a more realistic model. Additionally, it is generally held that the diversity of the human genome is much higher than that found between different rat strains or between rats and mice, again making the xenograft model more applicable (Sanchez-Mazas, 2007). Correspondingly, concordant xenograft are used in non-human primates to model human allogeneic transplantation (Sanchez-Mazas, 2007). The results of this study showed that VM allogeneic and xenogeneic transplant can survive equally well in the striatum of 6-OHDA-lesioned rats and produce some functional effects, enabling a reduction of drug-induced motor asymmetry. Nevertheless, the beneficial effect of the transplantation was limited and no improvement of motor functions has been observed. Transplant studies in 6-OHDA rats usually graft a higher number of cells (2-10 times more cells) (Dunnett et al., 1981, Nikkhah et al., 1994, Goren et al., 2005, Torres et al., 2007). Although it has been shown that transplantation of as little as 50 000 cells is enough to obtain reduction on the drug-induced rotation test, however, motor function recovery appears to require larger grafts (Bartlett et al., 2004). In the absence of correlation between the number of surviving dopaminergic cells and the reduction in amphetamine-induced cycling behaviour, it is reasonable to think that other factors might limit behavioural recovery. The location of the transplant was found to be more medial than expected, in the associative and limbic area of the striatum instead of the dorsolateral motor striatum as originally aimed for. Moreover, in light of previous work, the graft might have been too rostral to alleviate AIMs (Carlsson et al., 2006). This location issue also explains the fact that, contrarily to what has been reported previously, the rats never developed post-

transplant AIMs “off-drug” (neither during light-cycle or during dark-cycle), or following amphetamine injection (Carlsson et al., 2006, Lane et al., 2006, Lane et al., 2009, Garcia et al., 2011).

Over the past 25 years, several studies have demonstrated that intrastriatal VM transplants in 6-OHDA lesioned rats could reduce L-DOPA-induced motor dysfunction, including AIMs and rotational behaviour (Blunt et al., 1990, Gaudin et al., 1990, Blunt et al., 1992b, Olsson et al., 1995, Lee et al., 2000, Carlsson et al., 2006, Steece-Collier et al., 2009). The change is believed to occur through the normalisation of the glutamate decarboxylase (GAD₆₇) and preproenkephalin (PPE) mRNA striatal level, as well as the normalisation of D₂ DA receptor availability in the striatum (Blunt et al., 1992a, Rioux et al., 1993, Lee et al., 2000). Furthermore, the graft does not only reverse the modifications observed following lesion of the dopaminergic nigro-striatal pathway, but also normalizes L-DOPA-induced molecular changes, namely the up-regulation of prodynorphin and c-Fos expression (Ishida et al., 1996, Lee et al., 2000). Thus, Steece-Collier and co-worker reported a decrease of 50-80% in AIMs scores when transplanting the same number of VM cells (200 000) (Steece-Collier et al., 2009). Interestingly, they have highlighted a decrease in AIMs score in L-DOPA naïve grafted rats compared to the sham group, when first exposed to the drug was 9 weeks post-transplant. The results presented in this chapter corroborate the idea that foetal dopaminergic transplant could prevent the development of AIMs. Moreover, both allogeneic and xenogeneic grafts were able to significantly decrease the development of dyskinesia, with drug treatment initiated as soon as 24h post-surgery. Taken together, these findings provide some support for the conceptual premise that dopaminergic transplant should be seen, not only as a “last chance therapy” to reduce PD symptoms and dyskinesia, but also as a potential preventive treatment for dyskinesia. Indeed, it is reasonable to think that VM transplantation could be more beneficial for early-stage and L-DOPA naïve patients, as the cells would be grafted in an environment of modest depletion, still containing some remaining endogenous dopaminergic neurons able to regulate the system. It is however important to acknowledge that the inclusion of L-DOPA naïve patients in transplantation clinical trials raises important ethical issues. Patients who can cope without L-DOPA treatment are usually at a very early stage of the disease and, although there is a strong rationale in believing that VM transplant will improve their condition and provide them with better future perspectives, the outcome

remains uncertain and the risks associated with brain surgery seem too high to justify the gamble.

4.4.2 L-DOPA-induced modifications in brain immune responses

The data collected in this chapter provide the first evidence that L-DOPA treatment post-graft increases the host immune response around mouse VM xenotransplant in immunosuppressed rats. This observation raises important questions regarding the mechanisms underlying this enhanced immune response. 3 main hypotheses can be formulated: 1) L-DOPA interferes with the action of cycloA immunosuppressive treatment, 2) L-DOPA acts on immune cells proliferation and/or activation, 3) L-DOPA treatment induced blood-brain barrier impairment.

4.4.2.1 L-DOPA & immunosuppression

CycloA was originally extracted from the fungus *Tolypocladium inflatum*. In the 1970s Borel and colleagues published a series of articles demonstrating the anti-lymphocytic properties of the cycloA peptide (Borel et al., 1976b, Borel et al., 1977, Borel, 1980, Wiesinger and Borel, 1980). It is still currently the preferred immunosuppressive treatment for most organ transplant, as well as for cell therapy clinical trials (Aliabadi et al., 2012). Although the full mechanism underlying cycloA actions remains unclear, it is known to reduce lymphocytic activity by inhibiting the production of cytokine, such as interleukine-2 (IL-2) and interferon-gamma (INF- γ). As cycloA, L-DOPA can also act on lymphocytes, but has an opposite effect promoting lymphocytic proliferation (this will be discussed in more detail in the following paragraph 4.5.2.2 L-DOPA & Immune cells). However, it has been shown that the effect of L-DOPA on lymphocytes is not mediated by an increase of IL-2 production, or by an alteration of IL-2 receptors, thus disproving a direct interaction between the two drugs at a lymphocytic level. Another possible way of interaction would be at the blood-brain barrier level. Indeed, cycloA can enter the brain through the ATP-Binding Cassette sub-family B member 1 (ABCB1) transporter, which can also be used by L-DOPA. It can therefore be hypothesised that L-DOPA might compromise the transport of cycloA through the brain. Because there is also a possibility that the reverse happens, and that cycloA interferes with L-DOPA access through the brain, the first experiment of the following chapter (Chapter 5) will investigate whether or not immunosuppression modifies L-DOPA-induced AIMs.

4.4.2.2 L-DOPA & immune cells

Another possible way by which chronic L-DOPA post-graft could have contributed to the increase of host leukocytic and glial response is by direct action on immune cells. There is a growing body of evidence showing that DA can play a role in the regulation of the immune system. Immune cells, such as lymphocytes, are known to express DA receptors (D_1 - D_5), (Santambrogio et al., 1993, Ricci and Amenta, 1994, Ricci et al., 1998, Amenta et al., 1999, Ricci et al., 1999, McKenna et al., 2002). In 1996, Josefsson and colleagues did an *in vitro* study demonstrating that murine lymphocytes can actively produce L-DOPA and DA. They hypothesised that DA was released in an auto/paracrine manner and intervened as a regulator of lymphocyte activity through induction of apoptosis (Josefsson et al., 1996). Similar results have been recognized by others scientists, who showed that human $CD4^+$ T lymphocytes and B lymphocytes also express DA and noradrenaline receptors and that *in vitro* exposure to these two catecholamines down-regulate proliferation and differentiation of the lymphocytes (Bergquist et al., 1994, Cook-Mills et al., 1995, Bergquist et al., 1998). In contrast, *in vivo* studies demonstrated that continuous infusion of L-DOPA, for 5 days, increased the number of lymphocytes present in the spleen of mice (Carr et al., 2003b, Carr et al., 2003a). This effect was abolished by co-administration of L-DOPA with the peripheral DOPA-decarboxylase inhibitor benserazide (Carr et al., 2003a). Similarly, intravenous injections of D_1 or D_2 DA receptor agonists led to an increase in splenic lymphocytes (Tsao et al., 1997). Taken together, the data suggests that L-DOPA is taken up by spleen cells, converted into DA, which acts on DA receptors and stimulates lymphocytic proliferation in nearby cells. Considering that L-DOPA is always co-administered with a peripheral DOPA-decarboxylase inhibitor in the treatment of PD, the increase host immune response induced by L-DOPA treatment post-graft is unlikely to be due to lymphocytic proliferation at the spleen level. However, L-DOPA might act on T and B cells already infiltrated in the brain. Moreover, the increase of microglia surrounding the xenograft observed in this chapter indicates that L-DOPA might have an effect on these immune cells. Microglia are important actors in brain immunity and constitute the main antigen presenting cells of the central nervous system. Although very little is known about possible effect of L-DOPA on glial cells, it has been shown that microglial cells express DA receptors and that DA could play a role in their migration (Farber et al., 2005, Chang and Liu, 2000). In the 6-OHDA-lesioned rat model, it has been shown that chronic L-DOPA treatment (in absence of graft) does not increase immune-

reactivity of astro- and microglia (Maeda et al., 2008). These results corroborate with the data obtained in this chapter that no difference has been found in the sham and allogeneic group, suggesting that L-DOPA had no effect on glial cells in a non-immunologically challenged environment. Based on these results, and considering the fact that microglia cells express dopaminergic receptors, one can speculate that L-DOPA might have a direct role on glial proliferation, in an immunologically challenging situation, for example a xenogeneic graft. However, further work is needed to assess this hypothesis.

4.4.2.3 L-DOPA & blood-brain barrier

Finally, L-DOPA treatment post-grafting might increase the immune response by compromising blood-brain barrier functions. The access to the brain is normally restricted by the tight microvascular endothelial cells, which form a wall of blood vessels and regulates cells, molecules and ions access to the brain. Other cells, such as astrocytes, pericytes and perivascular neurons, are also involved in the regulation of the selective diffusion toward the central nervous system. Although the brain is not exempt from the immune system, the number of leukocytes, in a non-diseased brain, is usually extremely low (Sallusto et al., 2012). This contributes towards a higher level of immune tolerance of intracerebral transplants. Indeed, early studies have shown that mice tumorigenic or skin cells survived in rat brains, whilst it was quickly rejected in the periphery (Murphy and Sturm, 1923, Medawar, 1948). These observations later led to the concept of the brain being an “immunologically privileged” site (Billingham and Boswell, 1953). Westin and colleagues reported that chronic L-DOPA treatment in 6-OHDA-lesioned rats increased blood-brain barrier permeability by promoting angiogenesis in the basal ganglia (Westin et al., 2006). This dose dependant effect is mediated by D₁ DA receptor stimulation and correlates with the severity of AIMs (Lindgren et al., 2009, Ohlin et al., 2011). Furthermore, chronic L-DOPA treatment has been shown to increase cerebral blood flow in the striatum, with greater effect in dyskinetic animals (Ohlin et al., 2012). Enhanced blood flow and blood vessel permeability, in the striatum of L-DOPA treated animals, are likely to facilitate the access of the brain to peripheral immune cells, such as leukocytes. Blood-brain barrier permeability might, therefore, have played a role in the increased number of CD45⁺ cells observed around the xenograft in L-DOPA treated animals. Interestingly, the angiogenesis observed in dyskinetic rats by Ohlin and co-worker was linked to an

increase in vascular endothelial growth factor (VEGF) released by astrocytes (Ohlin et al., 2011). It has been suggested that VEGF could induce proliferation and migration of microglia cells, which suggests an indirect effect of L-DOPA on microglia (Forstreuter et al., 2002). However, since no significant effect of L-DOPA has been found in the sham or the allogeneic group, this VEGF-mediated L-DOPA action on microglial cells is unlikely to be the primary mechanism involved in the L-DOPA-induced microglia proliferation phenomenon. Further work is required to establish the role played by, L-DOPA-induced blood-brain barrier modifications, on the increased host immune response observed in the xenogeneic transplanted group. Moreover, the question of the impact that this increased immune response could have on the outcome of cell therapy for PD still has to be determined.

4.5 Conclusion

The main finding of this chapter is that chronic L-DOPA treatment post VM transplant, in the context of xenogeneic transplant, increases glial and lymphocytic responses. However, this combined action on the innate and adaptive immune system did not affect graft survival or function. The next chapter will look at the development of a controlled method to trigger graft rejection, in order to provide the tools necessary to further characterise the relationship existing between chronic L-DOPA treatment and immune response, and its potential impact on the development of dyskinesias following transplantation.

Chapter 5: Establishment a model of multi-donor graft rejection

Summary

Cell therapy for PD requires the use of ventral mesencephalon obtained from several donors pooled together to provide enough to form a transplant capable of alleviating motor impairments. Given that these tissues may come from disparate genetic backgrounds, which will have varied immunogenic backgrounds, it is unclear how this could affect the success of cell therapy. I have hypothesized that the incompatibility between the patient immune system and one or more of the donors could either contribute towards global rejection of the transplant, or the inflammatory response around it. Despite the fact that patients receive multi-donor grafts, most experiments only ever explore single donor grafts. Therefore, to address the issue of multi donor grafts, I have aimed to develop a better model of heteroimmunogenic double grafts, using combinations of syngeneic, allogeneic and xenogeneic donors.

Declaration

The first half of this chapter is a series of pilot experiments, performed on small groups of rats, to gather information regarding the time course of rejection. Altogether, they informed the design of the final experiment of double donor grafts.

5.1 Introduction

As discussed previously in chapter 4, patients entering clinical trials for cell transplant would have been under L-DOPA treatment for years, and most of them have developed LID. They receive foetal mesencephalic tissue grafts, pooled from up to 8 donors of unknown immunologic background and remain under L-DOPA medication for months before the graft hopefully matures and provides some alleviation of the motor symptoms (Lindvall et al., 1990). In most of the trials, patients were immunosuppressed for 6-24 months (Winkler et al., 2005). The immunosuppression typically administered in VM transplantation procedures is cyclosporine A, (CycloA), a potent immunosuppressant, widely used in organ transplants to prevent rejection. It is administered alone or in combination with other drugs, such as azathioprine and/or prednisolone, but as with most issues in clinical trials of transplantation there is little consistency between studies and the duration of immunotherapy is similarly variable (some studies even decide not to give immunosuppressive treatment) (Winkler et al., 2005). Post-mortem studies have shown that grafts are able to survive years after the withdrawal of immunosuppression but also reported the presence of immune cells around the host-graft border (Kordower et al., 1997). Interestingly, an apparent relationship may exist between the withdrawal of immunosuppression and the development, or worsening, of the graft-induced abnormal movements observed in some of the transplanted patients, suggesting a link between immune response and GIDs (Piccini et al., 2005). Taken together, these observations suggest that the host immune response could be a key player in failure of transplantation in many patients.

Chapter 4 demonstrated that prolonged L-DOPA treatment following non-immunologically compatible transplants increases the striatal immune response, despite immunosuppression, suggesting an interaction between the immune response and L-DOPA treatment. Moreover, regarding the multi-donors situation, questions can be raised as to how the rejection of part of the graft might affect the survival and function of the rest of the transplant. Preclinical studies have been carried out to address part of these questions but not in a patient relevant scenario. In order to investigate the role of L-DOPA in multi donor grafts, a model of mixed transplants presenting with different levels of immune compatibility is required. However, the design of such a model required intermediate experiments to define the following key parameters:

- 1) Does cycloA interfere with the action of L-DOPA or the development of AIMs?
- 2) Can slow and controlled rejection of allogeneic or xenogeneic tissue be engineered in a rat model and what is the time course of rejection?
- 3) In a double graft scenario, does the rejection of the “less compatible graft” affect the survival of the “more compatible” tissue

In addressing point 1, it has been shown that although L-DOPA uses the large amino acid transporter in order to cross the blood-brain barrier; it can also be a substrate at the ABCB1 transporter. Similarly, the dopamine agonist bromocriptine can interact with the ABCB1 transporter, also known as P-glycoprotein (Vautier et al., 2006, 2008). This efflux protein is used by cyclosporine to cross the blood-brain barrier and enter the brain (Tsuji et al., 1993, Sakata et al., 1994, Vautier et al., 2008). Competition at this transporter could lead to either the reduced level of L-DOPA or cycloA in the brain, causing either less immunosuppression or less effect of the L-DOPA.

It is necessary to address the second point in order to develop a well-characterised model of graft rejection; the research to date has produced a number of inconsistent and contradictory results regarding the survival of brain allo- and xenografts. Sprague Dawley rats have been used extensively for both VM transplant and dyskinesia studies and are therefore a logical choice as host for these experiments. In an allograft paradigm, Wistar rats VM transplants are well-tolerated and survive for a long period of time in Sprague Dawley rats without immunosuppression (as demonstrated in chapter 4) despite a lack of histological compatibility (Wistar rats express histocompatibility haplotype Ag-B2, while Sprague-Dawley rats express the Ag-B6 haplotype (Low et al., 1983)). Nevertheless, these compatible grafts can be rejected in a temporally controlled manner by peripheral stimulation of the immune system. For instance, orthotopic skin grafts, using the same donor strain as for the VM allograft induces a brutal rejection of the intra-striatal graft within 2 weeks of the challenge (Lane et al., 2008). A milder peripheral challenge can be achieved by the systemic injection of spleen cells, rich in immune cells (e.g. lymphocytes, macrophages and dendritic cells) (Soderstrom et al., 2008). It may be argued that human genetic diversity is such that, an allograft paradigm is insufficiently aggressive, therefore a more appropriate animal model would be a mouse to rat xenograft (Sanchez-Mazas, 2007). This idea has been confirmed in primate

in whom concordant xenografts are used to model human allotransplantation (Michler et al., 1996).

Most xenogeneic intra-cerebral neural transplants will be vigorously rejected in the complete absence of immunosuppressive treatment at the time of transplantation and even the later withdrawal of immunosuppression can also precipitate some form of rejection. The time course is highly variable and depends of a number of parameters, such as the host and donor species concordance (e.g. transplantation of mouse to rat is “concordant” whereas the transplantation of hamster or rabbit to rats is “discordant”), graft location, type of tissue and preparation (e.g. solid graft are rejected faster than the cells suspension) (Low et al., 1983, Brundin et al., 1985, Inoue et al., 1985, Mason et al., 1986, Finsen et al., 1990, Pakzaban and Isacson, 1994). With regard to immune compatibility between donor and host, mouse-to-rat transplant are considered as “easy” concordant graft, creating gradual rejection involving mainly T cell-dependent mechanisms (Dymecki and Freed, 1990, 1990, Murphy et al., 1996). Indeed some early studies have reported the long-term survival of mouse intra-cerebral neural transplant into rat, in the absence of immunosuppressive treatment (Daniloff et al., 1984, 1985a, 1985b, Brundin et al., 1985). Similarly, allogeneic graft survival highly depends on the host and donor immune compatibility. Some studies have therefore reported long term survival of neural rat allograft (over 6 months), when others found a strong immune response, leading to acute graft rejection (Low et al., 1983, Mason et al., 1986, Lawrence et al., 1990, Sloan et al., 1990, Isono et al., 1993).

This chapter sets out to define the experimental parameters of the allogeneic and xenogeneic rejection process, and the time course to set the scene for addressing the question of multi-donors grafts. Ultimately, grafts would be transplanted in a single pooled graft suspension made up from the tissue of multiple donors. However experimentally this is challenging, as there is no marker available to discriminate between the different types of tissue in the post-mortem analysis. Therefore, a more straightforward initial approach is to examine separate grafts implanted into the same striatum.

5.1.1 Aims of the chapter

The main aim of this chapter was to develop a reliable and well-characterised model of rejection of VM co-transplant of different level of compatibility (syngeneic, allogeneic and xenogeneic). To this end, 4 experiments have been carried out:

- Assessing possible interactions of cycloA treatment with the development of L-DOPA-induced dyskinesia.
- Creating a model of allogeneic graft rejection
- Establishing the time course of xenogeneic graft rejection
- Defining the impact that the rejection of the “less compatible” graft could have on the survival a “more compatible” one, when transplanted simultaneously.

5.2 Experimental design

Each of the following 4 experiments was performed separately on different groups of rats.

5.2.1 Effect of prolonged cyclosporine treatment on AIMs and rotational bias

16 female Sprague Dawley received unilateral 6-OHDA lesions and were selected to be included in the experiment based on the results of the amphetamine-induced rotation test. One group was treated daily with L-DOPA and cycloA (n=8), the other group with L-DOPA only (n=8). AIMs were scored twice a week, for 4 weeks (L-DOPA 6mg/kg) and then once a week, for 2 weeks (L-DOPA 12mg/kg, Figure 40). The rotational behaviour of the rats was recorded concomitantly. In addition, to evaluate any effect of cycloA on the behavioural response to the dopamine D₂ agonist bromocriptine (another substrate for the ABC transporter), the rats were injected with bromocriptine (2mg/kg) and AIMs and rotations were recorded 10 days after the last L-DOPA injection.

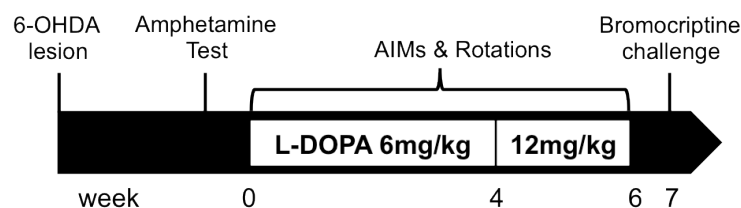


Figure 40: Time line of the experiment

5.2.2 Model of allogeneic graft rejection

18 female Sprague Dawley rats received unilateral 6-OHDA lesions and were subsequently assessed by amphetamine test. All rats received grafts of 400 000 cells E14 Wistar VM into the lesioned striatum. Spleen cells, obtained from male Wistar rats were then injected i.p. either: at day 1 and 14 (n=6), at day 14 and 21 (n=6) or at day 21 (n=4) following the VM graft. An additional group, which was used as a control cohort did not receive the spleen cell injections (n=3) (Figure 41). The animals were perfused at 60 days post transplantation and immunohistochemical analyses were performed using markers for dopaminergic cells (TH) and leukocytes (CD45).

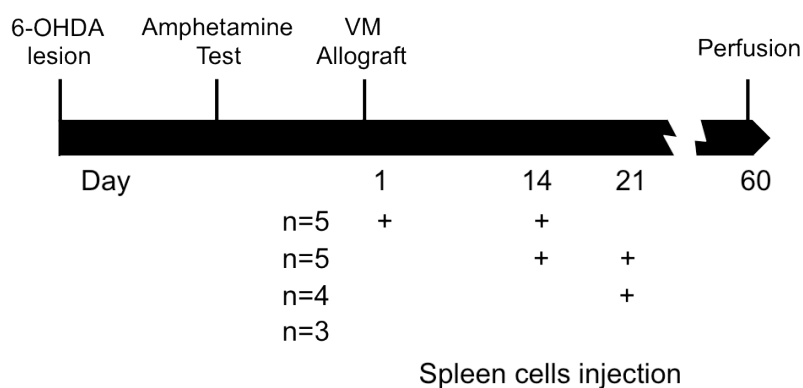


Figure 41: Time line of the experiment

5.2.3 Model of xenogeneic graft rejection

3 groups (each, n=4) of female Sprague Dawley rats received unilateral 6-OHDA lesions and were subsequently assessed by amphetamine test. All animals were grafted with VM tissues obtained from E12 CD1 mice (1 VM per transplant). Each group received cycloA for 4, 5 or 6 weeks. All rats were perfused 6 weeks post-transplantation (having either continuous treatment or 1 or 2 weeks of cycloA withdrawal) (Figure 42) and immunohistochemical analyses were performed using markers for dopaminergic cells (TH) and leukocytes (CD45).

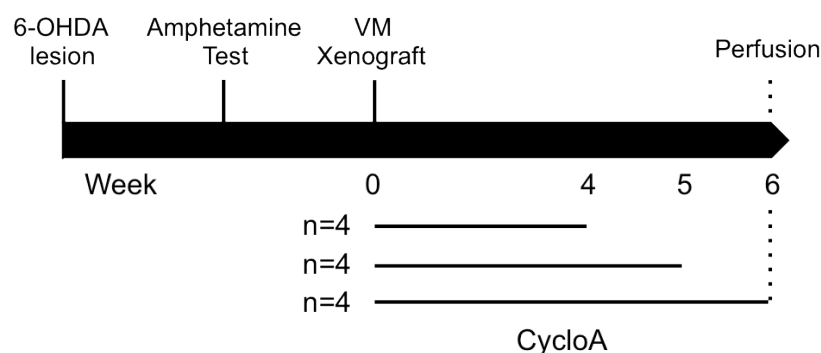


Figure 42: Time line of the experiment

Since no effect of cycloA treatment was seen on dopaminergic cell survival in the graft was found, a second experiment was performed using another 16 unilaterally lesioned Sprague Dawley. Two groups of 8 rats were grafted with CD1 mouse E12 VM and treated with cycloA for 2 weeks to allow closure of the blood-brain barrier, and then all animals were withdrawn from cycloA treatment for the rest of the experiment (Figure 43). According to a study published by Brundin and co-worker, minor leakage are observed 8 days after transplantation and the blood-brain barrier became completely impermeable to Evan's blue, a dye used to track blood-brain barrier leakage, after 12 days (Brundin et al., 1989). The rats were perfused 3 weeks (n=8) or 10 weeks (n=8) after the withdrawal from cycloA and immunohistochemical analyses were performed using markers for dopaminergic cells (TH) and leukocytes (CD45).

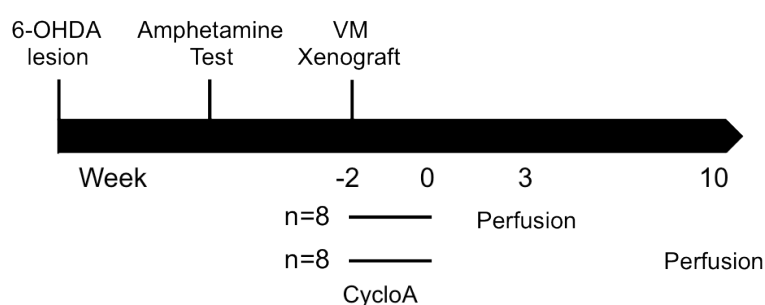


Figure 43: Time line of the experiment

5.2.4 Model of multi-donors graft

60 female Sprague-Dawley rats were unilaterally lesioned and a cohort of 56 rats was selected based on the number of spontaneous ipsilateral rotations that they performed over 10min (<10 full turns). And split into 8 groups. All the animals received 2 striatal grafts of VM tissue (200 000 cells/transplant): Syngeneic=E14 Sprague-Dawley rats, Allogeneic=E14 Wistar rats, Xenogeneic=E12 CD1 mice (Table 14) (Note: the

syngeneic-syngeneic group originally included 6 rats but 1 animal was removed from the study due to a blockage in the syringe during transplantation). All the animals were perfused 3 months after transplantation and immunohistochemical analyses were performed using markers for dopaminergic cells (TH) and leukocytes (CD45⁺).

n=	Graft 1 (rostral)	Graft 2 (caudal)	CycloA	Note
5	Syngeneic	Syngeneic	-	-
6	Syngeneic	Allogeneic	-	-
8	Syngeneic	Allogeneic	-	Spleen cell injections (day 7 and 14)
6	Syngeneic	Xenogeneic	Up to the end	-
8	Syngeneic	Xenogeneic	First 4 weeks	-
6	Allogeneic	Xenogeneic	Up to the end	-
8	Allogeneic	Xenogeneic	First 4 weeks	-
8	Allogeneic	Xenogeneic	-	-

Table 14: Summary of the different experimental groups.

5.3 Results

5.3.1 Effect of prolonged cyclosporine treatment on AIMs and rotational bias

As described in Chapter 3, AIMs and rotations increased with L-DOPA dose (two-way ANOVA: AIMs: $F_{9,126}=9.46$, $p<0.0001$; rotations: $F_{9,126}=16.38$, $p<0.0001$). However, chronic cycloA treatment neither interfered with the development of AIMs, nor the rotational behaviour induced by L-DOPA treatment, (regardless of the dose of L-DOPA used 6mg/kg or 12mg/kg; Figure 44-45; two-way ANOVA, effect of cycloA on AIMs: $F_{1,126}=0.03$, $p=0.8755$ and rotations: $F_{1,126}=0.01$, $p=0.9207$).

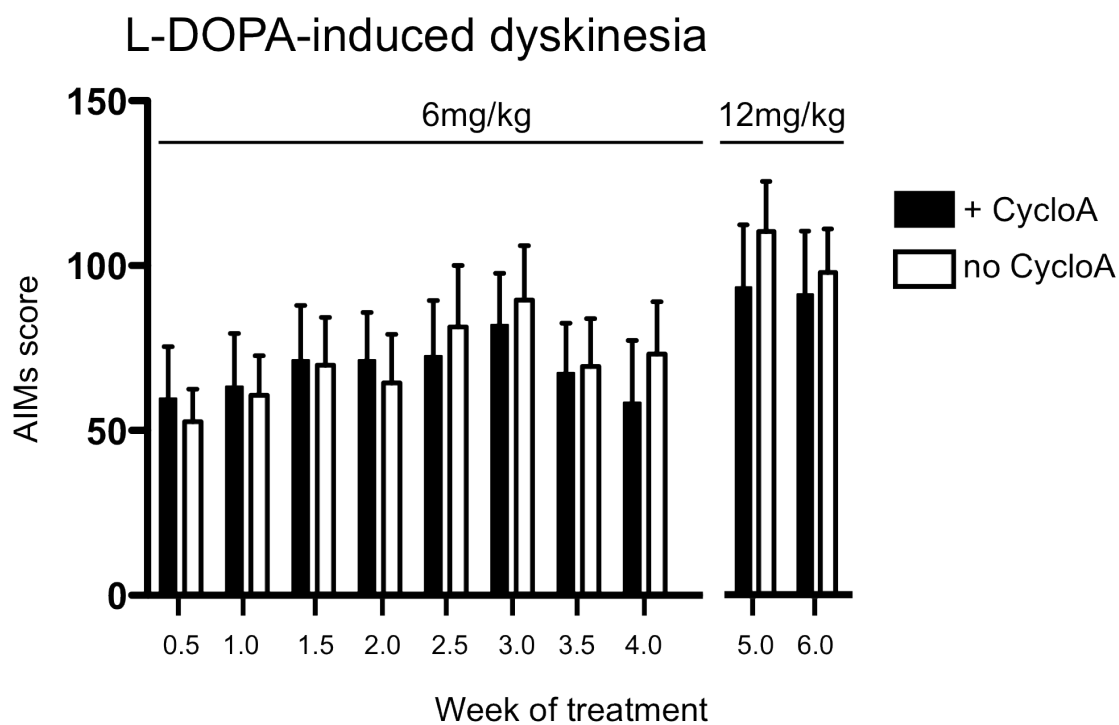


Figure 44: The effect of chronic injection of cycloA on the development of L-DOPA-induced AIMs, mean \pm SEM.

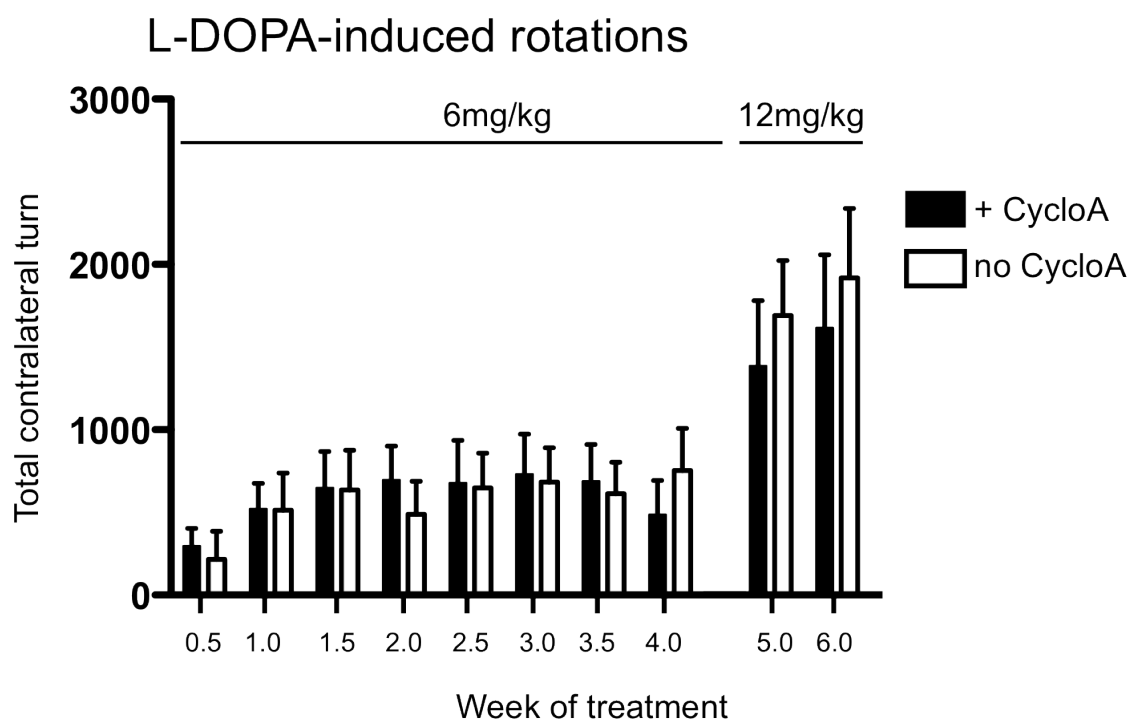


Figure 45: The effect of chronic injection of cycloA on the development of L-DOPA-induced contralateral rotations, mean \pm SEM.

Similarly, no difference was found between cycloA treated and non-treated animals in the severity of dyskinesia or extent of rotational behaviour following the administration of bromocriptine (Figure 46, two-tailed T-test: AIMs: $p=0.6208$; rotations: $p=0.4631$).

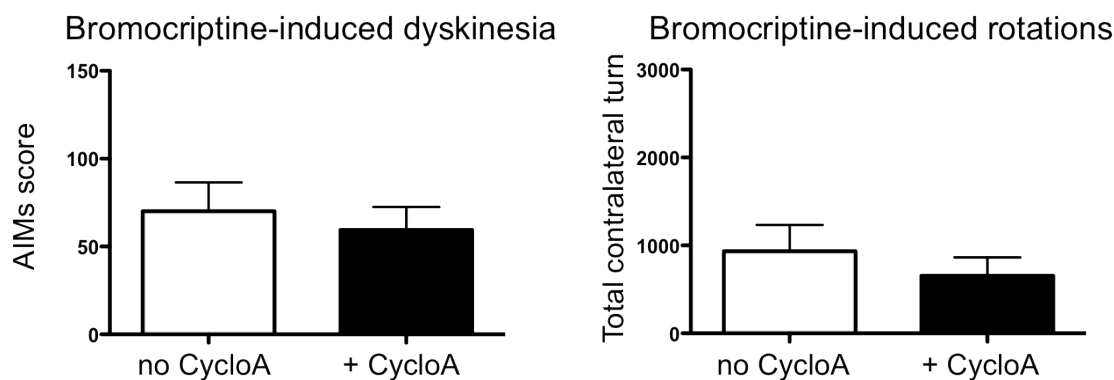


Figure 46: Effect of chronic injection of cycloA on the development of Bromocriptine-induced AIMs (left) and contralateral rotations (right), mean \pm SEM.

5.3.2 Model of allogeneic graft rejection

Peripheral injections of Wistar spleen cells into Sprague Dawley rats led to decreased survival of the allogeneic transplanted dopaminergic (Figure 47, Univariate analysis of the variance, $F_{3,16}=5.855$, $p=0.0093$). However, this reduction was only significant in the group that received 2 early injections of spleen cells (day 1 and 14) when compared to the non-injected control and the group receiving a single injection (day 21) (Dunnett post-hoc test: $p<0.05$, $p<0.001$ respectively).

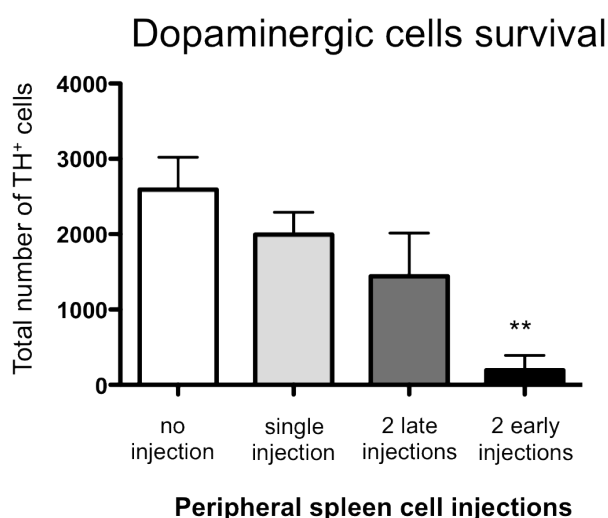


Figure 47: The effect of allogeneic spleen cell injections on the survival of dopaminergic neurons, (mean \pm SEM, ** $p<0.01$).

The rats that either did not receive spleen cells, or received late injection(s) showed a significant decreased in rotational behaviour (percentage of reduction relative to the number of amphetamine-induced rotations performed before transplant: no injection: 212.2% reduction, 1 injection: 107.3% reduction and 2 late injections: 101.2% reduction). In contrast, the early injections group showed an increase in the number of amphetamine-induced ipsilateral turns (24.5% increase). The difference in the percentage of change in rotations was significant in the 2 groups, which received 2 spleen injections when compared to the no injected control group (Univariate analysis of the variance, $F_{3,16}=11.70$, $p=0.0005$, Dunnett post-hoc test, late injections group: $p<0.05$, early injections group: $p<0.001$, Figure 48).

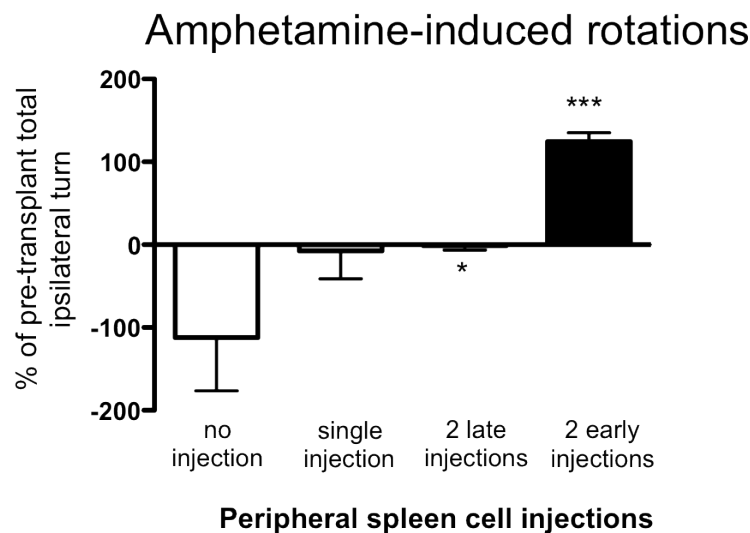


Figure 48: Effect of allogeneic spleen cell injections on amphetamine-induced ipsilateral rotations. Data presented as percentage change (mean \pm SEM) in full ipsilateral rotations between the test pre-graft and 4weeks post-transplant, *** $p<0.001$.

No significant difference was observed in the number of infiltrated white blood cells ($CD45^+$) in the grafted striatum between the different groups (Figure 49, Univariate analysis of the variance, $F_{3,16}=1.6990$, $p=0.2181$). However, it is interesting that the 2 groups receiving 1 or 2 late spleen cell injections showed a trend towards an increased $CD45^+$ response when compared not only to naïve animal, but also when compared to the group injected early after transplantation.

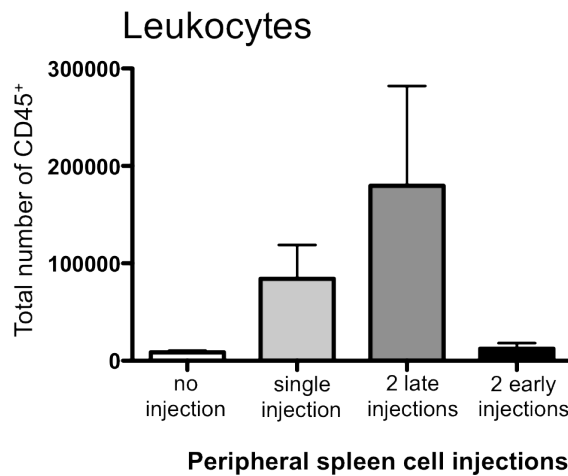


Figure 49: Effect of allogeneic spleen cell peripheral injections on striatal leukocytic response. Data shown as mean \pm SEM of the total of CD45⁺ present in the striatum.

5.3.3 Model of xenogeneic graft rejection

Only 62% (5 out of 8) and 50% (4 out of 8) of the transplanted animals still had TH positive cells present in the grafted striatum, 3 weeks and 10 weeks after the withdrawal of cycloA respectively. Despite a trend for decreased dopaminergic cell survival 3 and 10 weeks after the withdrawal of cycloA, no significant difference was found in the survival of xenogeneic dopaminergic cells when compared to the control group, which remained under cycloA treatment (mean=4227) (Figure 50, Univariate analysis of the variance, $F_{4,27}=2.787$, $p=0.0505$).

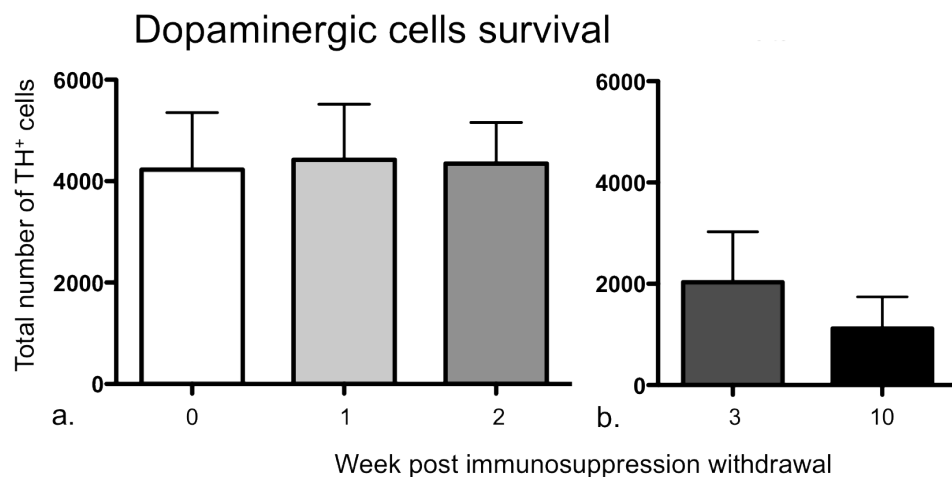


Figure 50: Effect of cycloA withdrawal following VM xenogeneic transplant (experiment 1: a and experiment 2: b) on dopaminergic cells survival, presented as mean \pm SEM.

No significant difference was observed in the amphetamine-induced rotation test, between the animals experiencing none, 1 or 2 weeks of cycloA withdrawal (Univariate analysis of the variance, $F_{2,11}=0.9513$, $p=0.4219$, data not shown). Although xenogeneic grafts significantly decreased the total number of ipsilateral rotations performed after amphetamine administration ($p<0.05$), the withdrawal of cycloA abolished this improvement after 7 weeks (Repeated measure ANOVA, $F_{8,71}=4.646$, $p=0.0002$, Tukey post-hoc test $p<0.05$ at week 7 and 8 of cycloA withdrawal, when compared to prior cycloA withdrawal, Figure 51).

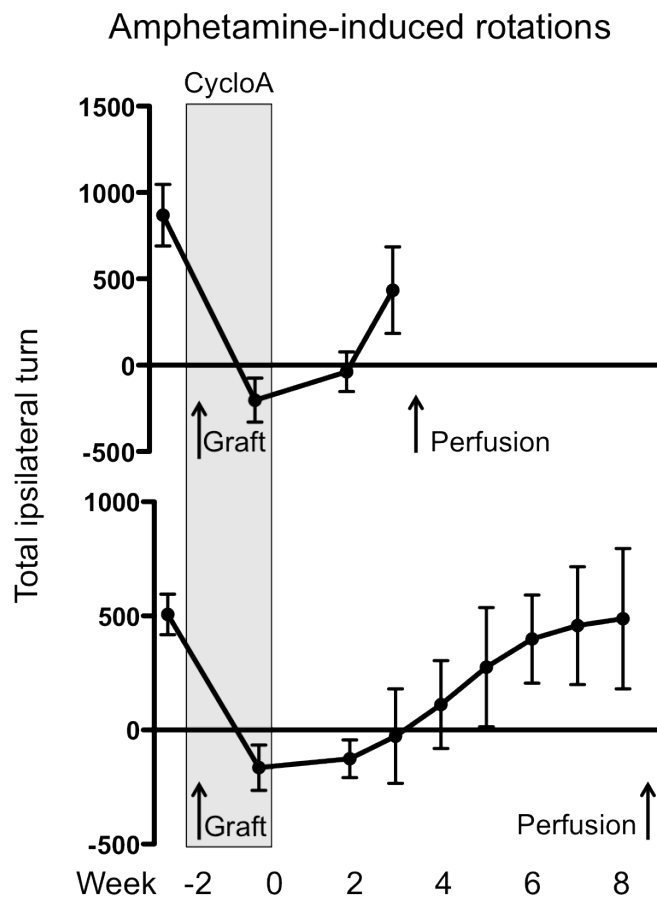


Figure 51: Effect of 3 (top) and 10 (bottom) weeks of cycloA withdrawal following VM xenogeneic transplant on rotational behaviour induced by amphetamine, presented as mean \pm SEM.

Despite a trend for increased numbers of infiltrated $CD45^{+}$ cells in the striatum of rats following 2 and 3 weeks of cycloA withdrawal when compare to the control group kept under immunosuppression (mean=1911 cells), no significant difference was detected (Figure 52, Univariate analysis of the variance, $F_{4,26}=1.889$, $p=0.1481$).

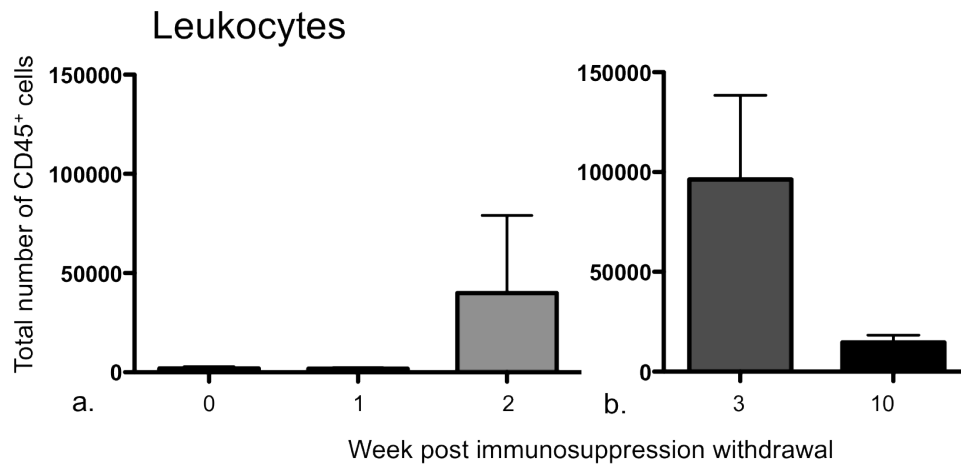


Figure 52: Effect of cycloA withdrawal following VM xenogeneic transplant (experiment 1: a and experiment 2: b) on leukocytes infiltration in the grafted striatum, presented as mean \pm SEM.

5.3.4 Model of multi-donors graft

The statistical analysis was first performed using multivariate analysis of the variance to compare all the groups together. The data are presented in subgroups. Each graph thus, represents a single group of rats (8 groups in total), and each bar on a graph represents the data for one of the double grafts: the rostral striatal graft (left bar on the graph) and the caudal striatal graft (right bar on the graph). Syngeneic grafts are represented in white bars, allogeneic in grey bars and xenogeneic in black bars.

5.3.4.1 Dopaminergic cells survival

Critically, there was no interaction between the two types of graft, suggesting that the grafts did not adversely affect each other (Multivariate analysis of the variance, $F_{1,47}=0.0001$, $p=0.992$) meaning that the presence of the rostral graft did not affect the survival of the caudal graft, and *vice versa*.

When the rats received 2 syngeneic grafts, no difference was observed in the number of dopaminergic cells that survived, whether the graft was placed in the rostral or caudal part of the striatum, confirming that the position of the graft does not play a role in survival of the dopaminergic cells (Figure 53, $t(8)=0.2021$, $p=0.8449$).

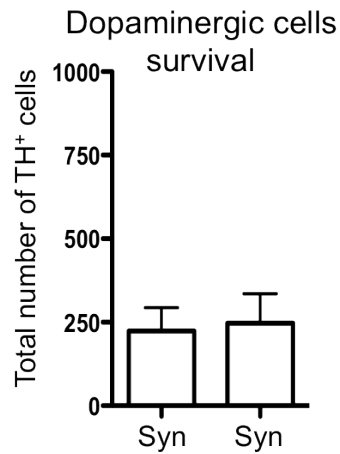


Figure 53: Double syngeneic grafts, number of TH⁺ cells present in the rostral striatal graft (left bar) and caudal striatal graft (right bar). Data shown as mean ± SEM.

No difference was found between the number of dopaminergic cells that survived from the allografts, despite immune peripheral challenge with spleen cells (Figure 54, $t(12)=1.165$, $p=0.2668$).

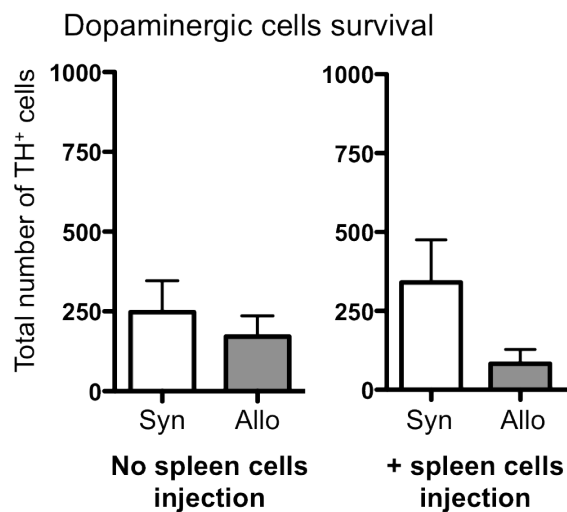


Figure 54: Syngeneic-Allogeneic double grafts, with (right graph) or without (left graph) immune challenge (spleen injection). Number of TH⁺ cells present in the rostral striatal graft (left bar) and caudal striatal graft (right bar). Data shown as mean ± SEM.

Similarly, no significant difference was found between the survival of TH⁺ cells in the xenografts, whether or not the rats were kept under cycloA for 4 weeks or the entire experiment (Figure 55, $t(12)=0.3535$, $p=0.7298$).

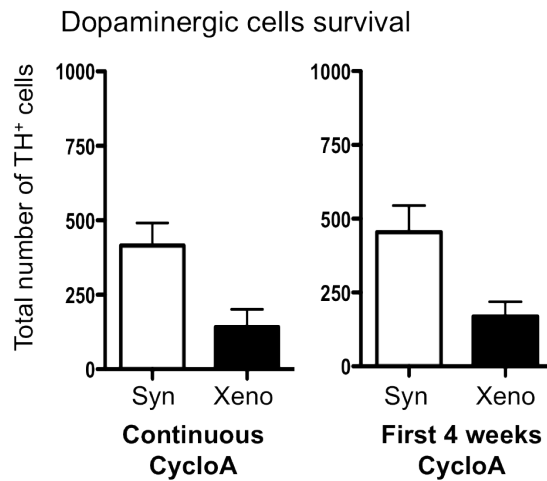


Figure 55: Syngeneic-Xenogeneic double grafts, with continuous (left graph) or brief (right graph) immunosuppressive treatment (cycloA). Number of TH⁺ cells present in the rostral striatal graft (left bar) and caudal striatal graft (right bar). Data shown as mean \pm SEM.

No significant difference was observed in term of TH⁺ survival in the xenogeneic grafts, despite the different immunosuppressive regime, (Figure 56, Univariate analyse of the variance: $F_{2,21}=1.576$, $p=0.2326$).

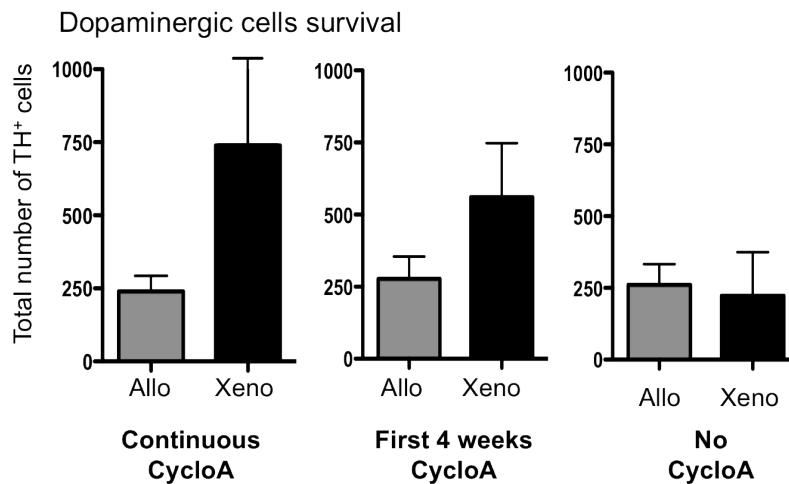


Figure 56: Allogeneic-Xenogeneic double grafts, with continuous (left graph), brief (middle graph) or no (left graph) immunosuppressive treatment (cycloA). Number of TH⁺ cells present in the rostral striatal graft (left bar) and caudal striatal graft (right bar). Data shown as mean \pm SEM.

5.3.4.2 Leukocytic response

No interaction was found between the number of CD45⁺ cells infiltrated around the rostral graft and the caudal graft, suggesting that the immune response against a “less compatible” graft does not impact on the leukocytic response against the “more compatible” graft, transplanted at the same time (Multivariate analysis of the variance, $F_{1,47}=0.346$, $p=0.559$).

In the double syngeneic graft group, no difference was observed in the number of infiltrated leukocytes found around the rostral graft and the caudal graft, confirming that the position of the graft does not influence the level of immune response (Figure 57, $t(8)=1.035$, $p=0.3307$).

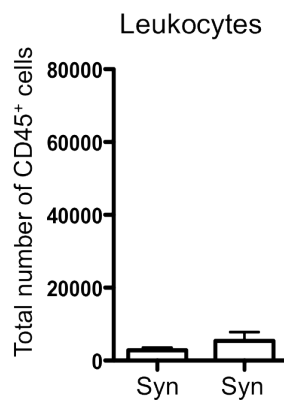


Figure 57: Double syngeneic grafts, number of CD45⁺ cells present around in the rostral striatal graft (left bar) and caudal striatal graft (right bar). Data shown as mean ± SEM.

No difference was found between the number leukocytes present around the allografts, whether or not the rats have been immunologically challenged by peripheral challenge with spleen cells (Figure 58, $t(12)=1.707$, $p=0.1136$).

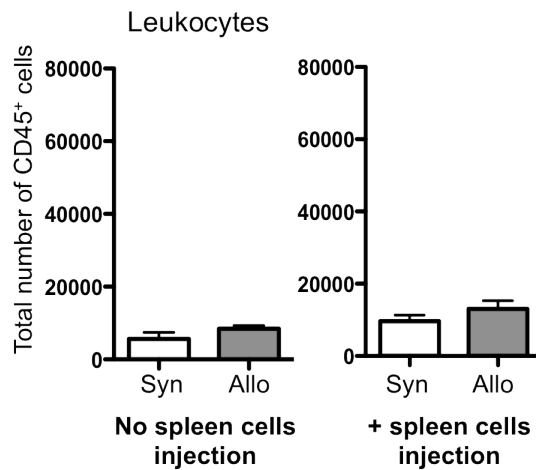


Figure 58: Syngeneic-Allogeneic double grafts, with (right graph) or without (left graph) immune challenge (spleen injection). Number of CD45⁺ cells present around the rostral striatal graft (left bar) and caudal striatal graft (right bar). Data shown as mean ± SEM.

No significant difference was found between the number of infiltrated leukocytes around the xenograft, whether or not the rats were maintained under cycloA for 4 weeks or the entire experiment (Figure 59, $t(12)=1.747$, $p=0.1062$).

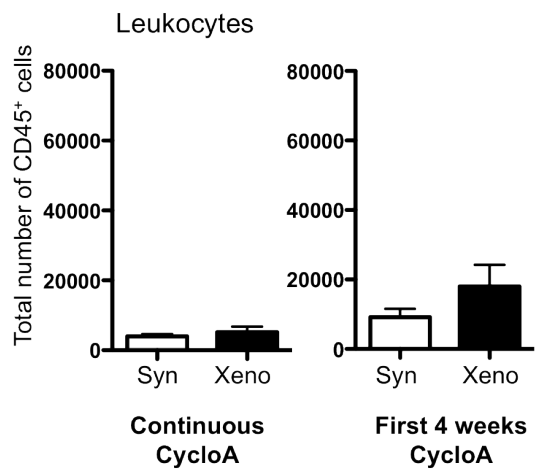


Figure 59: Syngeneic-Xenogeneic double grafts, with continuous (left graph) or brief (right graph) immunosuppressive treatment (cycloA). Number of CD45⁺ cells present around in the rostral striatal graft (left bar) and caudal striatal graft (right bar). Data shown as mean ± SEM.

No significant difference was observed in the number of CD45⁺ cells between the different xenografts, despite the different immunosuppressive regimes, (Figure 60, Univariate analyse of the variance: $F_{2,21}=0.6596$, $p=0.5285$)

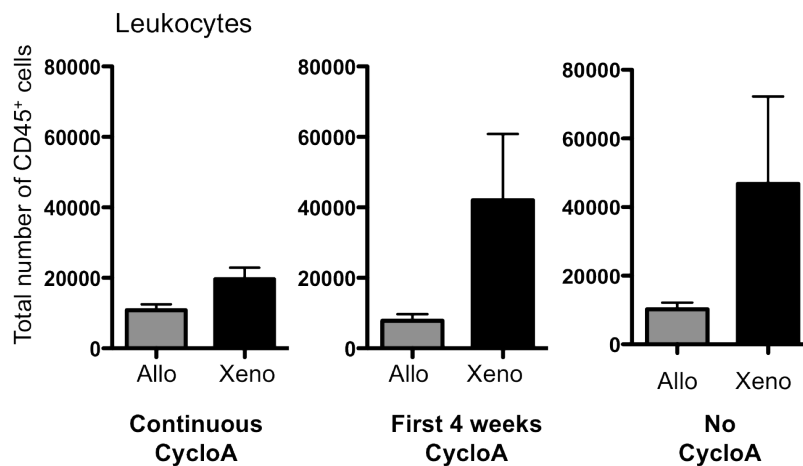


Figure 60: Allogeneic-Xenogeneic double grafts, with continuous (left graph), brief (middle graph) or no (left graph) immunosuppressive treatment (cycloA). Number of CD45⁺ cells present around in the rostral striatal graft (left bar) and caudal striatal graft (right bar). Data shown as mean \pm SEM.

5.4 Discussion

The fact that current animal models of VM transplant fail at evoking spontaneous graft-induced motor side effects could highlight incapacity at reproducing clinically important factors. A better rat model would create a multi-donors scenario with partial rejection and maintain the animals under L-DOPA prior and post-graft. Nevertheless, some important parameters needed to be refined in order to develop this model.

5.4.1 Effect of prolonged cyclosporine treatment on AIMs and rotational bias

Despite the fact that the commonly administered anti-parkinsonian drug L-DOPA and the immunosuppressant cycloA can both act on the ABCB1 efflux protein, no effect of their co-administration was found on LID or rotational behaviour. Similarly, cycloA did not affect AIMs or rotations induced by bromocriptine, which can also a substrate for ABCB1 transporter. In early clinical studies, bromocriptine was reported to induce dyskinesias, in late stage patients who previously suffered from LIDs (Calne et al., 1974, Teychenne et al., 1975, Godwin-Austen and Smith, 1977). Interestingly, Godwin-Austen and Smith reported the case of 2 patients (out of 18), who did not exhibit abnormal movements under L-DOPA treatment but developed dyskinesias when changing from L-DOPA to bromocriptine (dyskinesias disappeared when the patients were back on L-DOPA treatment). The dyskinesias involved dystonic movements of the limbs, which were troublesome and distressing in one of the patients (Godwin-Austen

and Smith, 1977). Bromocriptine does not induce AIMs in rat PD models, when administered *de novo* (Henry et al., 1999, Lundblad et al., 2002). However, as shown in the first experiment of this chapter, AIMs can be observed in rats that were previously chronically injected with L-DOPA. Although AIMs and rotations are crude measures, behaviour read-out could be a useful indicator of the effect of possible modifications of L-DOPA or bromocriptine action induced by co-treatment with cycloA and is clinically more relevant than simply looking at *in vitro* binding measures. Nevertheless, it is not sufficient to confirm whether or not cycloA can modify the entrance of these dopaminergic drugs into the brain. To my knowledge, no report of reduction of treatment efficacy in PD patients receiving cycloA has emerged, so it is unlikely that cycloA could heavily modify L-DOPA access into the brain. It is, however, important to confirm experimentally that the effect of anti-parkinsonian medications will not be compromised by the chronic cycloA treatment.

5.4.2 Model of allogeneic graft rejection

Allogeneic VM transplantation produced good grafts, comparable to syngeneic grafts, with a good survival of dopaminergic neurons in the absence of a peripheral trigger (Duan et al., 1995a, 1997a, 1997b, Lane et al., 2008, Soderstrom et al., 2008). Accordingly, in the previous chapter (Chapter 4), no significant difference was found between the syngeneic and allogeneic grafts. In contrast, a peripheral challenge of the immune system by spleen cell injections could compromise the survival of the allograft, normally well tolerated by the host, especially when administered early after transplantation (as showed in experiment 2). Again, this observation correlates with previous works, which demonstrated that intravenous, intraperitoneal or subcutaneous injections of spleen cells, either obtained from the donor itself or animals belonging to the same strain as the donor, could increase the host immune response toward allogeneic intraventricular neural graft (Freed et al., 1988, Soderstrom et al., 2008). In a previous study, Söderström and colleagues used subcutaneous injection of spleen cells to induce rejection of VM graft obtained from Lewis rats and transplanted into Sprague Dawley rats. The authors found it was an insignificant trend for reduced TH⁺ cell number in the rats injected at 10 and 18 weeks post-transplant with 1 000 000 spleen cells. Simultaneously, they reported a significant increase in MHC II expression around the graft. However, this result requires further clarification as an anti-mouse secondary antibody was used to detect the anti-MHC II primary antibody, which could just as well

bind infiltrating host antibodies (although an increase in infiltrated host antibodies would still indicate a sign of an enhanced immune response) (Soderstrom et al., 2008). In this chapter, I showed that the injection of spleen cells at day 14 and 21 post-graft increases the level of CD45⁺ cells infiltrated around the graft, highlighting an increase in the host immune response against the transplant. Interestingly, in the late 80s, Freed and Poltorak demonstrated that neural graft rejection induced by spleen cell injections depends, not only on the combination of rat strains, but also on which strain plays host. For instance, intraventricular transplants of embryonic cortex of F344 rats are not rejected when transplanted into Brown-Norway rats, despite spleen cell injections (Poltorak and Freed, 1991). In contrast, Brown-Norway rat tissue transplanted in the lateral ventricle of F344 rats will undergo severe rejection following spleen injections (Freed et al., 1988). Alternatively, some studies used more drastic ways to trigger the host immune system, by transplanting a mix of spleen cells and VM cells directly into the brain or by later performing orthotopic skin grafts using the same donor strain. These techniques induced rapid and violent rejection of the neural transplant and would not be appropriate as a model of human neuronal allograft, which is unlikely to induce a similarly extremely severe immune response (Carder et al., 1988, Duan et al., 1995b, 1997b, 1997a, Lane et al., 2008). Taken altogether, the data highlighted the importance of defining the time course of allograft rejection in pilot studies, before using a new model as it can be influenced by various factors, such as: the host and donor strains, the type of peripheral immune trigger (e.g. spleen cells injections, skin graft), the time between VM transplant and the immune challenge.

5.4.3 Model of xenogeneic graft rejection

Previous studies have proven that VM xenografts can restore motor functions in the lesioned rat model of PD, assuming that they are not rejected by the host brain (Bjorklund et al., 1982, Brundin et al., 1985, 1986, Dymecki and Freed, 1990). The literature differs greatly on the survival rate of xenogeneic grafts in absence of immunosuppression (Low et al., 1983, Brundin et al., 1985, Inoue et al., 1985, Mason et al., 1986, Finsen et al., 1990, Dymecki and Freed, 1990, 1990, Pakzaban and Isacson, 1994, Larsson et al., 1999). In the current experiment, the rats were kept under cycloA for at least 2 weeks post-transplantation in order to provide time for the closure of the blood-brain barrier following the invasive surgery. A slow rejection process was seen after 2 weeks following cycloA withdrawal, eventually leading to the abrogation of the

reduction observed in rotational behaviour induced by amphetamine (after 10 weeks). Interestingly, although it was not significant, the number of leukocytes infiltrated into the grafted striatum was higher 3 weeks after cycloA withdrawal than after 10 weeks where more of the graft has already been rejected, suggesting the end of the rejection process. The results of experiment 3 suggest that graft rejection could occur, even after closure of the blood-brain barrier, although confirmation of blood-brain barrier closure remains to be confirmed. This result corroborate with previous studies, which showed that cycloA cessation still results in rejection of some of the graft, even if the rats have been treated for more 42 days (Brundin et al., 1989).

5.4.4 Model of multi-donors graft rejection

Finally, the survival of syngeneic, allogeneic and xenogeneic grafts was assessed in the paradigm of multi-donors transplant. Although the pilot studies (experiments 2 and 3) showed a significant reduction of TH⁺ cells when rejection was induced (by spleen cells injections for the allograft or by withdrawal of cycloA for the xenograft) these results could not be reproduced in the double graft experiment. Indeed, despite a decreasing trend in graft survival, the degree of variability between the rats was very high and the results were not significant. Although it seems that the survival of syngeneic graft was not altered by the presence of allogeneic or xenogeneic transplant, neither was the survival of the allograft in the presence of a xenograft nearby. These results must therefore be taken with great caution and further experiments are required before any results could be confirmed. The general survival of the syngeneic and allogeneic transplant was extremely low comparing to what has been observed in the other experiments carried out for this thesis, as well as in the literature (Duan et al., 1995a, Lane et al., 2006, Torres et al., 2007, Soderstrom et al., 2008). Moreover, the mice embryos used for the generation of the 3 allogeneic-xenogeneic groups were much smaller than expected. The embryos measured 6-7mm (crown to rump length), which is the expected size of a E11 embryos (Butler and Juurlink, 1987). This might explain the increased number of xenogeneic dopaminergic cells observed in the 3 allogeneic-xenogeneic groups. Indeed, it has been shown that E12 rat VM transplant survive much better than the E14 (Torres et al., 2007). Thus, it is not surprising that mouse E11, which are equivalent to the rat E12 VM transplant, survives better than the mouse E12 (equivalent to the rat E14) (Butler and Juurlink, 1987). The question of the survival of heteroimmunogenic grafts has been partially addressed by Schwarz and colleagues, in

Sprague Dawley rats before, using Wistar rats and C57BL/6 mice foetal VM and striatum tissue, grafted either as separated deposits or pooled together. In this study, the authors reported a decrease in the graft volume when rat and mouse VM tissues were pooled together. However, they did not find any significant difference between pooled and non-pooled mixt grafts in term of TH⁺ cells survival (Schwarz et al., 1996). There is still a crucial need for a model of pooled mixed grafts with different level of immune compatibility with the host to better model the clinical paradigm of multi-donors transplants. Indeed, the potential immunogenic disparity of the donor embryos used in the clinical trials might have played an important role in the heterogeneity of the benefit derived by different patients from the transplant. In clinical practice, testing and selecting the foetal donors based on their immunogenic profile is impossible, it is therefore important to establish whether or not rejection of one of the donor cells might compromise the graft as a whole. To address this concern, it is important to create a model of multiple-graft enabling the analysis of each component of the graft separately. This could be challenging, as there is currently no good marker allowing the discrimination between mouse and rat tissues.

5.5 Conclusion

Establishing a reliable model of rejection, whether it is an allogeneic or xenogeneic model, is a complex task. The information available in the literature is quite limited in scope regarding the time course of rejection of allograft and xenograft in the rat. This could be due to the fact that most preclinical studies for transplantation do not publish results of rejection as it is seen as a failure of the experiment. The difficulty in interpreting results also derives from the fact that every study specifically investigating the host tolerance of the graft have used different protocols, not only to induce rejection, but also to quantify the level of rejection or the time course of rejection (e.g. number of TH⁺ cells, graft volume, changes in amphetamine or apomorphine-induced rotational bias). This chapter emphasizes the importance of better characterizing the multi-donors model in Sprague Dawley rats before further investigating the impact of L-DOPA treatment on double grafts.

Chapter 6: General Discussion

The future of cell therapy for PD faces numerous challenges. The aim of this PhD was to identify some critical factors that influence the functional outcome of neurotransplantation, and based on these findings, develop an improved 6-OHDA unilaterally lesioned/transplant rat model. Since dyskinesias represent the major side effect of both L-DOPA and cell transplant therapies, the first step was to assess and compare the reliability of the available dyskinesia evaluation techniques. This was to ensure the use of a comprehensive and adequate methodology throughout my PhD. The second part of this thesis was arranged to determine the effect of chronic L-DOPA treatment, administered at different times, pre and/or post-transplantation, had on the survival and function of foetal VM graft of different level of immune compatibility. Originally, this part was also intended to investigate how L-DOPA treatment and non-compatible grafts interacted and affected the severity of dyskinesia post-transplantation. However, since no spontaneous or amphetamine-induced dyskinesia have been observed, and given that, interesting results were obtained on the effect of L-DOPA on the immune response, the focus of this work focused toward assessing the impact of these factors on brain immunity. Indeed, L-DOPA treatment, when administered post-grafting had a clear impact on the host's immune response around the xenogeneic transplant. Therefore, the last set of experiments were arranged to create 2 separate models of allogeneic and xenogeneic controlled rejection, before attempting to develop a multi-donor model. All of these studies fill a critical niche: the lack of a model, which accurately represents all aspects of patients undergoing transplantation for PD.

6.1 L-DOPA-induced modifications of immunity

Due to the risks associated with cell therapy, patients eligible for foetal VM transplants are at a late stage of the disease, meaning that they have been treated with L-DOPA for several years and have consequently developed dyskinesias. The severity of late stage symptoms of PD dictates that their anti-parkinsonian medication cannot be halted or reduced until, hopefully, the graft matures and provides some functional improvement. However, most of the pre-clinical studies looking at graft survival and motor function recovery failed to take this into account, using either untreated animals or animals treated only prior to transplantation (Brundin et al., 1986, Nikkhah et al., 1994, Goren et al., 2005, Carlsson et al., 2006, Maries et al., 2006, Torres et al., 2007, Lane et al., 2009, Garcia et al., 2011). The work carried out in chapter 4 demonstrated that L-DOPA treatment post-transplant has an effect on the host immune response towards the

dopaminergic xenotransplant. Although the mechanisms involved remain to be determined, it is unlikely that the main effect relies on L-DOPA altering cycloA access to the brain, via the ABCB1 transporter. The fact that chronic cycloA administration did not affect the development of AIMs, as observed in chapter 5, only suggests that the immunosuppressive drug treatment does not modify L-DOPA action, but cannot exclude the possibility of a reversed effect. Considering that lymphocytes are formed in the bone marrow and are released in the blood stream (directly: B lymphocytes, or after maturation in the thymus: T lymphocytes), the effect of cycloA on lymphocytic proliferation is likely to take place principally in the periphery. Consequently, even if L-DOPA treatment was to compromise the access of cycloA into the brain, this may not have any impact on the overall immunosuppression. Nevertheless, the mechanisms underlying L-DOPA enhancement of the immune response needs to be fully addressed before removing the interaction between L-DOPA and cycloA out of the equation. Also, more needs to be done to investigate the effect that L-DOPA can have on the immune system of PD patients, which might be already altered by the disease.

Several studies have described changes in dopamine receptor expression on peripheral immune cells in PD patients, when compared to age matched healthy individuals. Indeed, an increased density of D1-like and D2-like receptors has been found on peripheral blood lymphocytes in L-DOPA-naïve patients, although DA transporter immunoreactivity was unchanged (in 10 patients out of 11 assessed) (Barbanti et al., 1999, Buttarelli et al., 2009). The density of some immune cells also seems to be altered. For instance, the percentage of natural killer lymphocytes present in the blood stream has been reported to be higher in PD patients (Niwa et al., 2012). In contrast, the proportion of T helper and B lymphocytes appeared to be reduced, with a greater decrease in T-helper cells (Baba et al., 2005, Bas et al., 2001, Niwa et al., 2012). In clinical study results it is hard to distinguish between modifications in immunity, associated with disease progression and neuroinflammation, or changes induced by pharmacologic treatments. Bas and colleagues published one of the rare studies directly comparing untreated and L-DOPA treated PD patients. The authors have found no difference between the two groups, which both showed comparable reduction of T and B lymphocytes when compared to the healthy controls group (Bas et al., 2001). In contradiction, Stevens and co-worker compared PD patients before and 3 months after the initiation of L-DOPA treatment and showed a 15% decrease in total of B cells with

L-DOPA (Stevens et al., 2012). Other scientists have also reported important modifications on T-cell proteome in patients undergoing long-term treatment with L-DOPA (Alberio et al., 2012). Taken together, these studies suggest that the modifications of the peripheral immune system observed in PD patients might be a manifestation of the disease, which may be further altered by dopaminergic stimulation. It has been suggested that α -syn may be one of the primary causes of the immune abnormalities observed in PD, inducing lymphocyte apoptosis (Kim et al., 2004). The question of the extent of the alteration of the immune system in PD patients, depending on: the disease stage, the dose and duration of L-DOPA treatment, is important and needs to be addressed. Furthermore, its potential impact on the outcome of neuronal transplantation for PD will also have to be determined.

6.2 L-DOPA enhancement of the immune response: friend or foe?

Although chronic L-DOPA treatment post-xenogeneic transplant enhanced the host immune response observed around the graft, the treatment did not affect the survival of the transplant or the rats' performances during the amphetamine test (chapter 4). This suggests that the increased immune response might not be detrimental to the graft. Moreover, the results in chapter 4 also demonstrated that the lymphocytic response observed was mainly composed of CD4⁺ lymphocytes helper T cells (Th). This observation corroborates the study performed by Boukhris and co-workers demonstrating that L-DOPA treatment reduced the proliferation and generation of cytotoxic T cells (CD8⁺ cells) in mice injected with sheep blood cells (Boukhris et al., 1987). By limiting the proliferation of cytotoxic CD8⁺ lymphocytes, L-DOPA could promote graft survival. Moreover, CD4⁺ helper T lymphocytes, which are the main actors in the lymphocytic response under L-DOPA treatment, can have various phenotypes and could either promote or reduce inflammation. Th1 CD4⁺ phenotypes release pro-inflammatory cytokines, including INF- γ , interleukin (IL)-2 and 6 and tumor necrosis factor (TNF), whilst in contrary, Th2-type lymphocytes produce IL-4, 5, 10, 13 and are associated with an anti-inflammatory response (Zhu and Paul, 2008). A shift in the balance of the helper lymphocytes population toward Th2 phenotype could have a beneficial effect on the survival of the graft. In 2003, Carr and colleagues demonstrated that repeated L-DOPA treatment in mice reduces the quantity of INF- γ produced by splenic lymphocytes, despite a global increase in lymphocytic cells, suggesting a specific inhibition of the Th1 subtype of CD4⁺ lymphocytes helper T cells (Carr et al.,

2003b). Interestingly, it has been shown that DA stimulation of inactivated lymphocytes tends to polarize the differentiation toward a Th2 phenotypes, while D₁ DA receptor antagonist SCH-23390 inhibits Th2 differentiation, increasing INF- γ production and reducing IL-4 concentration (Nakano et al., 2009, Mori et al., 2013). These studies suggest that DA could play a role in the determination between pro or anti-inflammatory phenotypes of lymphocytes. Finally, L-DOPA might reduce inflammation via other mechanisms, such as astrocytes stimulation as it has been suggested that astrocytic D₂ DA receptors stimulation could decrease neuroinflammation in mice treated with MPTP (Shao et al., 2013). Taken altogether, the data suggests that L-DOPA supports an anti-inflammatory response. However, great caution has to be taken in the interpretation of these studies, as most of them have not been performed in animal models for PD and might not reflect the effect that L-DOPA could have in a PD paradigm.

While immune activation within the brain is commonly viewed as a detrimental process in cell therapy, there has been evidence that, dependant on the form of the immune response, immune-reactive cells can support neural survival. For instance, the infiltration of cells as an adaptive response from the periphery can, in some conditions, be beneficial and support the graft through the release of neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) or insulin-like growth factor 1 (IGF-1) (Batchelor et al., 1999, Aberg et al., 2003). This phenomenon has been well studied in the modulation of hippocampal neurogenesis. For instance, microglia can acquire an alternative phenotype (M2), which is neuroprotective and plays a central role in regenerative processes. M2-type microglia produces anti-inflammatory cytokines, such as IL-10 or neural growth factor (NGF), and BDNF (Colton, 2009). It has been shown that anti-inflammatory cytokines such as IL-4 or INF- γ , released by CD4⁺ lymphocytes can push microglia toward an M2 phenotype (Butovsky et al., 2006). In agreement, Ziv and colleagues suggested that T lymphocytes could shift microglia towards M2 phenotype and contribute to neurogenesis in learning processes (Ziv et al., 2006). Since L-DOPA treatment seems to increase lymphocytes, in particular Th2 type T helper lymphocytes, and considering that these lymphocytes could promote M2 phenotype in microglia, it can be hypothesised that L-DOPA may support neuronal survival and proliferation. Importantly, the morphology of CD45⁺ infiltrated cells was different in chapter 4 and chapter 5. Indeed, in chapter 4, all CD45⁺ cells were spherical. In

contrast, in chapter 5, when rejection was voluntarily induced, either by withdrawal of cycloA in xenografted rats or peripheral spleen cells injection in the allograft model, round shaped cells CD45⁺ were found, as well as “microglia-like shape” cells (Figure 61). Considering their morphology, these cells are likely to be dendritic cells. This striking difference suggests that the type of immune response might differ between the leukocytic infiltration observed in L-DOPA treated and immunosuppressed rats, compared to the xenogeneic rejection processes. Therefore, it would be interesting to further study these 2 types of immune responses and establish whether or not L-DOPA-induced immune response can be beneficial and support neuronal survival.

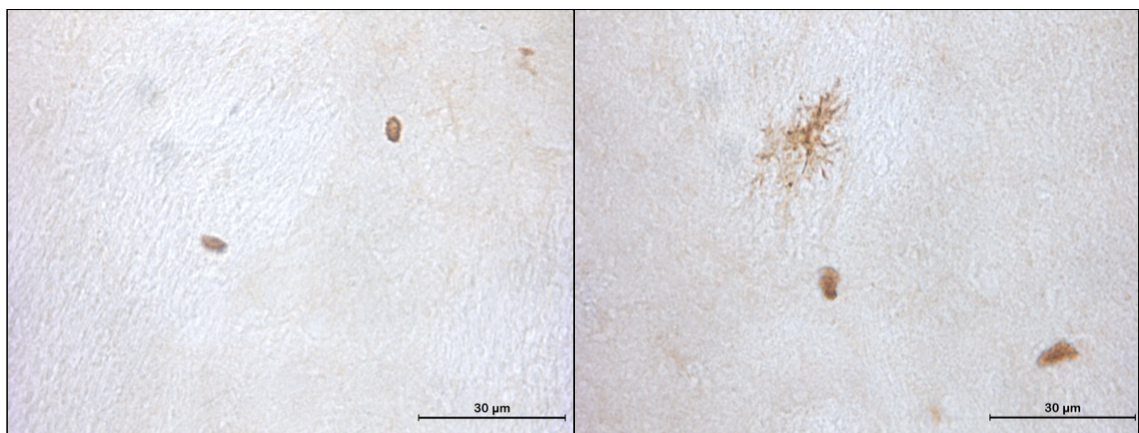


Figure 61: Difference in leukocytes (CD45⁺) shape between L-DOPA-induced immune response following xenograft (chapter 4, left) and xenograft rejection in untreated rats (chapter 5, right).

The withdrawal of immunosuppression in 6 of the transplanted patients, 2.5 years after the last surgery, did not seem to compromise graft survival. Indeed, no difference was found between the UPDRS motor scores collected before and after withdrawal of immunosuppressive treatment. Moreover, the ¹⁸F-DOPA uptake increased in the grafted putamen. However, all of these patients experienced a worsening of dyskinesias off-medication as compared to when they were receiving immunosuppression, suggesting that the immune response could play a role in the development of graft-induced dyskinesias (Piccini et al., 2005). It has been shown that immune cells, such as lymphocytes and dendritic cells contain L-DOPA and can release DA (Cosentino et al., 2007, Nakano et al., 2009). It can therefore be hypothesised that the presence of immune cells surrounding the dopaminergic graft could result in aberrant regulation of DA and might play a role in the development of GIDs. Moreover, gliosis was observed in some of the rats treated with L-DOPA post-xenograft (chapter 4) and it has been

shown that this phenomenon is associated with a 60% decrease in striatal DA following L-DOPA administration in rats. Indeed, Wang and colleagues, who used basic fibroblast growth factor infusion to induce gliosis in rats, reported an increased activity of MAO-A and MAO-B resulting in an increase in DA catabolism (Wang et al., 1994). In a transplantation scenario, this increased degradation of DA is likely to limit or even compromise functional recovery. It can therefore be hypothesized that, even if the enhanced immune response induced by L-DOPA treatment post-graft does not alter dopaminergic cells survival, it might compromise functional recovery and could play a role in the development of post-graft motor side effects.

6.3 Animal models of transplantation

Over the last 30 years, different clinical trials using foetal transplants for PD reported inconsistent benefits and adverse effects that were not predicted in the preclinical studies. Not only could they not be predicted, but, to date, no reliable animal model of spontaneous graft-induced dyskinesia has been developed. Part of the issue could be that a multitude of factors that could alter the clinical outcome of cell therapy for PD has not been considered in preclinical studies. Recently, it has been suggested that the severity of LIDs pre-transplantation, as well as the inclusion of 5-HT neurons in the graft could increase the risk of developing GIDs (Lane et al., 2009, Steece-Collier et al., 2009, Politis et al., 2010, Politis et al., 2011). Clinical trials are therefore taking this into account, now recruiting patients at earlier stages of the disease and presenting lower incidence of LIDs. Moreover, the VM dissections will be performed with a greater emphasis on minimising contamination with 5-HT cells. However, until now, other factors, such as L-DOPA treatment post-graft and non-immunologically fully compatible transplant, have not been considered with the same degree of rigour. The work presented here combined 3 factors for the first time, L-DOPA pre-graft, L-DOPA post-graft and immune incompatible of the donor tissue with the host. The data collected showed that the association of a non-immunologically compatible VM transplant with chronic L-DOPA treatment post-grafting has an effect on the host immune response. There was no suggestion of graft rejection resulting from this process, but larger grafts with greater functional effects need to be considered in the future and this potentially could reveal some effects of L-DOPA administration on the graft function. Moreover, the number of dopaminergic cells present in the graft is usually used as a measure of graft survival and behaviour tests as a way to evaluate

graft function. Although they provide useful information and are clinically relevant, these methods are fairly crude. The assessment of finer parameters, such as: measurement of dopaminergic fibres outgrowth, or evaluation of DA release from the graft, could provide a better insight of the effect that this newly discovered L-DOPA-induced immune response phenomenon could have on the efficacy of VM transplant.

The increased inflammation observed in rats treated with L-DOPA post transplant was limited to the animals receiving xenograft. Because the position of the graft was to medial, the experiment carried out in chapter 4 was inconclusive regarding the effect that this enhanced immune response could have on the development of amphetamine-induced post-graft dyskinesias. More work is required to unveil the mechanisms underlying the L-DOPA-induced enhancement of host immune response in incompatible grafts and determine the impact that this effect could have on the development of GIDs. The question of the impact that an increased inflammation could have on the development of GID has been addressed before, by Lane and colleagues; who showed that the induction of severe striatal inflammation using IL-2 inflammatory cytokine infusion did not worsen amphetamine-induced post-allograft dyskinesias. In a second experiment, the authors used a delayed orthotopic skin graft to induce complete rejection of the VM intra-striatal allograft, which abolished amphetamine-induced dyskinesias (Lane et al., 2008). These results suggest that severe striatal inflammation or graft rejection does not promote post-graft dyskinesia. However, this thesis provided the first evidence that immune response induced by L-DOPA differs from the one observed during graft rejection. Moreover, the immune response induced by L-DOPA treatment post-grafting seems to be biased toward Th2 lymphocyte types, which do not produce IL-2 and have anti-inflammatory effect. In light of this result, it is now reasonable to think that the IL-2 model would not be appropriate to assess the link between L-DOPA-induced immune response and dyskinesia post-graft. A further issue that has never has never been taken into account is the fact that patients received tissue collected from multi-donors. In chapter 5, I aimed to develop a model of multi-donor transplantation using the 6-OHDA-lesioned rat model to study how the rejection of a part of the graft could affect the other components of the transplant. Although the rejection of the less compatible graft did not seem to affect the second part of the co-transplant, it is important to bear in mind that patients are transplanted with a pool of the different embryos collected and that this question should be addressed in similar

conditions. A better model of transplant should therefore combine a mixed-pooled transplant, using tissues presenting different level of immune compatibility with the host, and chronic L-DOPA treatment pre and post-graft. Such a model could provide valuable data regarding the VM transplant and might help to improve the outcome of cell therapy. The question must be asked, would it be enough to accurately mimic the patient situation and create a reliable model, not only able to reproduce GIDs, but also able to predict side effects of new therapies?

The field of transplantation is moving toward the use of stem cells as a more sustainable, controlled and ethical source of dopaminergic precursors. As such, there are questions that need to be addressed as we transition from foetal cells to stem cells. Although the “multi-donor factor” will become obsolete and need to be taken out of the equation, the questions of incompatible allograft and continuous L-DOPA treatment remain valid. Since the 6-OHDA-lesion model failed to predict the side effect of foetal transplant, its validity in assessing the outcome of stem cell transplant is questionable. Although the genetic animal models of PD do not reproduce the extensive dopaminergic cell loss observed in the patients, they present the advantage of being progressive and are associated with protein inclusion. Moreover, they might better reproduce the alterations observed in the immune system of PD patients. Indeed, there is evidence that the 2 main genes involved in PD, α -syn and LRRK2 could play a role in the regulation of immune cells (Kim et al., 2004, Gardet et al., 2010, Kubo et al., 2010, Hakimi et al., 2011, Thevenet et al., 2011). They might therefore become a useful tool in the attempt to unveil the mechanisms underlying L-DOPA modifications of the immune system.

6.4 Conclusion

This PhD work highlighted the importance of using a model, which accurately reproduces most aspects of the patient’s situation. This promises to be a herculean task, as we know so little about certain aspects of PD. For instance, the modifications of the immunity, whether they are caused by the disease or by pharmacological treatment, are poorly understood and understudied. So are the alterations of the blood-brain barrier induced by L-DOPA and their implications, not only for cell therapy, but more broadly for the development of any new treatment for PD.

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Appendix I

6-OHDA	6-hydroxydopamine hydrobromide salt	Sigma
Acetic acid		Fisher
Amantadine	Adamantanamine hydrochloride	Sigma
Amphetamine	D-Amphetamine	Sigma
Benserazide	benserazide hydrochloride	Sigma
Bromocriptine	Bromocriptine mesylate	Sigma
Cyclosporine A	ciclosporin (250 mg/5ml)	Sandoz Sandimmun
DAB	3,3'-Diaminobenzidine tetrahydrochloride hydrate	Sigma
DMEM	Dubelcco's minimum Eagle medium	Gibco
DNAse	Deoxyribonuclease I from bovine pancreas	Sigma
DPX	distyrene plasticizer and xylene mounting medium	Fisher
Ethanol		Sigma
Euthatal	Pentobarbital sodium (200mg/ml)	Merial Animal Health Ltd.
Glucose-Saline	0.18% sodium chloride 4% glucose (Aquapharm, UK)	Aquapharm
HBSS	Hanks' balanced salt solution	Gibco
Hydrogen peroxide	Solution (30%)	VWR
Isofluorane		IVAK
L-DOPA	L-3,4-Dihydroxyphenylalanine methyl ester hydrochloride	Sigma
Metacam	Meloxicam 5mg/kg	Bohinger Hingelheim
Methanol		Fisher
Nylon Mesh	100µm Nylon Mesh	Fisherbrand
Paraformaldehyde		Fisher
PFA	Paraformaldehyde 96%	Fisher
R(-)-Raclopride(+)-tartrate	D2 DAR antagonist	Sigma
R(+)-SCH-23390 hydrochloride	D1 DAR antagonist	Sigma
Saline	0.9% sodium chloride	Aquapharm
Serum (goat)		Gibco
Serum (horse)		Gibco
Sodium azide		Fisher
Sodium pentobarbitone	Euthatal, Merial Animal Health	Harlow
Sucrose		Fisher
Triton	Triton-X 100	Sigma
Trizma base		Sigma
Trypan blue solution (0.4%)		Sigma
trypsin	Trypsine EDTQ 0.05%	Gibco
Trypsin inhibitor		Sigma
Vectastain Elite ABC		Vector
Vicryl 4-0 suture	Coated VICRYL® (polyglactin 910) Sutures, ETHICON	VWR
Xylene		Fisher

*An expert is a person who has made all the mistakes that can be made
in a very narrow field.*

Niels Bohr



To Meeny, Miny & Moe

(Drawing by nEVER-mor)