HUMAN RETINAL PIGMENT EPITHELIAL CELL TRANSPLANTATION FOR THE TREATMENT OF PARKINSON’S DISEASE

by

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ABSTRACT

Cell replacement therapies have been thoroughly investigated in the hope of finding a long-term, continuous dopaminergic (DAergic) source to treat motor dysfunctions in Parkinson’s disease (PD). However, mixed clinical results, safety and logistical concerns, and ethical issues have led to the interruption of these therapies in the clinic. Human Retinal Pigment Epithelial (hRPE) cells from fetal or neonatal origin have been proposed as a tissue transplant alternative for PD. HRPE cells are of neuroectoderm origin and play an integral part in normal retinal survival and function by providing nutritive, trophic, and anti-inflammatory support. HRPE cells are a potential cell therapy source for PD because of their DAergic properties. In the RPE, dopa is an intermediate product in the melanin biosynthetic pathway, catalyzed by the tyrosine hydroxylase analog tyrosinase. Since tyrosinase-produced dopa can exit the cell through plasma membrane amino acid transporters, RPE implantation into the parkinsonian brain could provide a continuous source of dopa to striatal DA terminals.

Previous reports have shown that hRPE cells attached to biocompatible gelatin microcarriers (hRPE-GM) can successfully ameliorate parkinsonian symptoms in PD patients. However, these observations are empirical in nature; indeed, little is known about long-term hRPE-GM survival or its underlying mechanism of action. The present thesis addresses the hypotheses that 1) hRPE-GM implants ameliorate behavioural deficits, 2) hRPE-GM survive long-term in the host striatum, and 3) the mechanism of action of hRPE-GM implants is not solely due to the in situ production of dopa and may involve alternate mechanisms of action, with an emphasis on anti-inflammatory factors. Using the rodent 6-OHDA model for PD, we investigated the qualitative survival and behavioural effects of hRPE-GM implants combining post mortem immunohistochemistry and non drug-induced behavioural paradigms. Next, we assessed the hypothesized reduction in inflammatory reactions to hRPE-GM implants (in the absence of immunosuppression) by quantifying the inflammatory response using stereological methods. Finally, we described a quantitative timeline of in vivo hRPE-GM survival using our recently developed superparamagnetic labeling techniques and MRI. These studies will provide further support for using hRPE cells as a therapeutic option for PD.
PREFACE

A version of Chapter 5 has been published: Cepeda IL, Flores J, Cornfeldt ML, O’Kusky JR, Doudet DJ (2007) Human Retinal Pigment Epithelial Cell Implants Ameliorate Motor Deficits in Two Rat Models of Parkinson Disease. J Neuropath Exp Neurol 66(7): 576-584. I am co-first author of this manuscript and contributed equally to all the published work. Specifically, I contributed to the design of all experimental surgical and behavioural paradigms. I prepared all cells that were used for transplantation and contributed equally to performing the surgical procedures, behavioural testing and analysis, and post-mortem autoradiography. I was involved with the data interpretation and co-wrote the submitted manuscript with IL Cepeda. All work was approved by the UBC Animal Care Committee (Certificate number A07-0229).

A version of Chapter 6 has been published: Flores J, Cepeda IL, Cornfeldt ML, O’Kusky JR, Doudet DJ (2007) Characterization and Survival of Long-Term Implants of Human Retinal Pigment Epithelial Cells Attached to Gelatin Microcarriers in a Model of Parkinson Disease. J Neuropath Exp Neurol 66(7): 585-596. I contributed to the design of all experimental paradigms with DJ Doudet, ML Cornfeldt, and JR O’Kusky. I performed the surgeries (with help from IL Cepeda) and performed all of the post-mortem processing, microscopy, and analyses by myself. ML Cornfeldt trained me on cell culturing procedures, and JR O’Kusky trained me on all post-mortem processing, immunohistochemistry, and microscopy. I was responsible for all the data interpretation and wrote all of the submitted manuscript myself. All work was approved by the UBC Animal Care Committee (Certificate number A07-0229).

Chapter 8 is based on collaborative work with the UBC’s High Field MRI Centre (Dr. P. Kozlowski) and the Hershey Brain Analysis Research Center, Penn-state University (Dr. G. Du and Dr. X. Huang). All MRI scans took place at UBC’s MRI Centre under the supervision of Dr. P. Kozlowski, and MR data was processed and converted by Dr. G. Du and Dr. X. Huang. I performed all cell preparations, surgical procedures, post-mortem histology, and quantitative analyses and data interpretation. All work was approved by the UBC Animal Care Committee (Certificate number A04-0150).
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<table>
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<th>Definition</th>
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<tbody>
<tr>
<td>5-HT</td>
<td>serotonin</td>
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<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
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<tr>
<td>$^{11}$C-RAC</td>
<td>$[^{11}C]$raclopride</td>
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<tr>
<td>$^{18}$F-DOPA</td>
<td>$[^{18}F]$-fluoro-Dopa</td>
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<tr>
<td>AADC</td>
<td>aromatic acid decarboxylase</td>
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<td>AAV</td>
<td>adeno-associate virus</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<td>APCs</td>
<td>antigen presenting cells</td>
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<td>AR-JP</td>
<td>Autosomal recessive juvenile parkinsonism</td>
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<td>BBB</td>
<td>blood brain barrier</td>
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<tr>
<td>β-CIT</td>
<td>$[^{123}]$-2-β-carbomethoxy-3-β-[4-iodophenyl] tropane</td>
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<td>BDNF</td>
<td>brain-derived growth factor</td>
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<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>CO-I</td>
<td>cytochrome oxidase I</td>
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<tr>
<td>COMT</td>
<td>catechol-O-methyl-transferase</td>
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<tr>
<td>COX</td>
<td>cyclo-oxygenase</td>
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<td>CSF</td>
<td>cerebrospinal fluid</td>
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<td>DA</td>
<td>Dopamine</td>
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<td>DAB</td>
<td>3,3'-diaminobenzidine</td>
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<td>DAergic</td>
<td>Dopaminergic</td>
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<tr>
<td>DAT</td>
<td>dopamine transporter</td>
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<td>DBS</td>
<td>deep brain stimulation</td>
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<td>DIC</td>
<td>differential interference contrast</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<td>dimethyl sulfoxide</td>
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<td>dopa</td>
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<td>DOPAC</td>
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<td>DTBZ</td>
<td>$[^{11}C]$dihydrotetabenazine</td>
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<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>EMMPRIN</td>
<td>extracellular matrix metalloproteinase inducer</td>
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<td>EM</td>
<td>electron microscopy</td>
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<td>ES</td>
<td>embryonic stem</td>
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<td>FBS</td>
<td>fetal bovine serum</td>
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<td>FDA</td>
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<td>FDG</td>
<td>$[^{18}F]$-fluorodeoxyglucose</td>
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<td>fibroblast growth factor</td>
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<td>Hanks Balanced Salt Solution</td>
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<td>high frequency stimulation</td>
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<td>hRPE</td>
<td>human Retinal Pigment Epithelium</td>
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<td>hRPE-GM</td>
<td>human Retinal Pigment Epithelial cells attached to gelatin microcarriers</td>
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<td>Hoehn and Yahr</td>
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<td>ICAM</td>
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<tr>
<td>ILBD</td>
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<td>inducible nitric oxide synthase</td>
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<td>induced pluripotent stem</td>
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<td>MFB</td>
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<td>MHC</td>
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<td>MP</td>
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<td>MPTP</td>
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<tr>
<td>MSCs</td>
<td>mesenchymal stem cells</td>
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<td>NGS</td>
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<td>positron emission tomography</td>
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<tr>
<td>PPN</td>
<td>pedunculopontine nucleus</td>
</tr>
<tr>
<td>Pro</td>
<td>protamine sulfate</td>
</tr>
<tr>
<td>PTP</td>
<td>permeability transition pore</td>
</tr>
<tr>
<td>RBD</td>
<td>rapid eye movement sleep behaviour disorder</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SN</td>
<td>substantia nigra</td>
</tr>
<tr>
<td>SNc</td>
<td>substantia nigra - pars compacta</td>
</tr>
<tr>
<td>SNr</td>
<td>substantia nigra - pars reticulata</td>
</tr>
<tr>
<td>SPECT</td>
<td>single photon emission computerized tomography</td>
</tr>
<tr>
<td>SPIO</td>
<td>superparamagnetic iron oxide</td>
</tr>
<tr>
<td>STN</td>
<td>subthalamic nucleus</td>
</tr>
<tr>
<td>TGF(\beta)</td>
<td>transforming growth factor (\beta)</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>TNF-(\alpha)</td>
<td>tumor necrosis factor-(\alpha)</td>
</tr>
<tr>
<td>UCH (L1)</td>
<td>ubiquitin carboxy-terminal hydrolase (L1)</td>
</tr>
<tr>
<td>UPDRS</td>
<td>Unified Parkinson’s Disease Rating Scale</td>
</tr>
<tr>
<td>UPS</td>
<td>ubiquitin-proteasome system</td>
</tr>
<tr>
<td>VMAT2</td>
<td>vesicular monoamine transporter type 2</td>
</tr>
</tbody>
</table>
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CHAPTER 1. INTRODUCTION TO PARKINSON’S DISEASE

1.1 EPIDEMIOLOGY OF PARKINSON’S DISEASE

Parkinson’s disease (PD) is a progressive neurodegenerative disorder associated with debilitating motor, autonomic, and cognitive disabilities. In his 1817 monograph “[a]n essay on the shaking palsy”, James Parkinson was the first to describe the syndrome in its entirety (Parkinson, 1817). He described six cases: three of which he personally examined and three which he observed on the streets of London, suffering from “involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellects being un-injured” (Parkinson, 1817). This disorder, originally referred to as “paralysis agitans”, was later credited to Parkinson and named “maladie de Parkinson”, or Parkinson’s disease, by the French neurologist Charcot in the 19th century.

PD is the second most common neurodegenerative disease (after Alzheimer’s disease), affecting an estimated 0.3% of the population in industrialized countries. Aging is the primary risk factor for developing PD: onset is rare before the age of 50, but prevalence increases sharply rising to 4% of the population over the age of 85 (de Lau and Breteler, 2006). Some have suggested gender differences in PD, with men reported to have a higher prevalence than women (Samii et al., 2004). The neuroprotective effects of estrogen have been suggested as a potential reason for these differences, but its role has yet to be clarified. There is still no consensus on whether there is any ethnic predisposition to PD. Some studies have suggested that PD is less common in African and Asian people than in Caucasian people, but results are conflicted (de Lau and Breteler, 2006). Moreover, these findings may be attributed to a variety of external factors, which include differences in survival rates, environmental exposure, or even applied methodology.

Although genetic links have recently been established in several familial forms of PD, the large majority of PD patients seen in the clinic are idiopathic (of spontaneous origin). The clinical and pathological features of PD are mostly similar in both idiopathic and genetic forms; the main differences lie in their major risk factors. As described, age is the primary risk factor for idiopathic PD. Occupational exposure to viruses (e.g. Influenza A), several pesticides and herbicides, and exposure to heavy metals such as iron, have also been reported as positive risk factors.
factors (Lai et al., 2002). In contrast, two of the most studied risk factors for a number of ailments, cigarette smoking and coffee consumption, seem to reduce the risk of PD.

Since the etiology of idiopathic PD is not yet known, there has been no intervention that has been shown to significantly prevent disease initiation. Similarly, no treatment has been shown to unequivocally stop or slow down the disease process and most therapies to date have focused on the symptomatic treatment of the “hallmark” motor symptoms of PD. Very few therapies alleviate the non-motor symptoms associated with PD. As a result, PD poses an increasing social and economic burden on society as the population ages.

1.2 CLINICAL FEATURES OF PD

1.2.1 Motor Dysfunction

PD is classically diagnosed by the onset of motor symptoms, and these are frequently the reason for which a patient seeks treatment. There are four cardinal symptoms of PD: resting tremor, rigidity, akinesia (or bradykinesia), and postural instability. Freezing and flexed posture have also been included among the main symptoms of parkinsonism. Disease subtypes can be identified based on the age of onset, predominant clinical features, and progression rate. In this regard, it is generally agreed that there are two main clinical subtypes: a tremor-dominant form, and a postural instability and gait disorder. Tremor-dominant patients are generally younger and progress more slowly; however, they have a poorer response to antiparkinsonian medication. Patients with early postural and gait dysfunction are usually older individuals, present with predominantly bradykinetic motor features (akinesia, rigidity), and generally have a worse prognosis of motor and cognitive function (Doder et al., 2003; Burn et al., 2006; Selikhova et al., 2009). Initially, symptoms often present unilaterally but progress to include both sides of the body.

1.2.1.1 Tremor

Resting tremor is the most common and easily recognized symptom of PD. Tremors are unilateral, present at a 4-6 Hz frequency, and are prominent in the patient’s distal extremities (Jankovic, 2008). It is typically one of the first motor symptoms to appear: one study reported 69% of PD patients had resting tremor at disease onset, which increased to 75% during the late stages (Hughes et al., 1993). It usually affects the lips, chin, jaw, and lower extremities, but rarely involve the neck or head. Hand tremors, or supination-pronation “pill-rolling” tremor, are
also commonly manifested. “Pill-rolling” tremor resembles hand movements that occur when rolling a pill between the thumb and forefinger, and typically spreads from one hand to the other. Tremors usually worsen with stress, contralateral motor activity, or during exercise, but disappear during sleep.

1.2.1.2 Rigidity

Muscular rigidity is defined as increased resistance to passive joint movement. It is characterized as having a “cogwheel” quality, particularly when associated with tremor. Initially, the movement force is resisted; however, a short movement eventually occurs. When this process is repeated, it results in a series of repeated “jerky” movements that resemble the movement of a cog. Rigidity can occur both proximally (in the neck, shoulder, and hips), or distally (in the wrist and ankles), and is most frequently clinically manifested as shoulder and joint pain. Axial rigidity, or rigidity in the neck and trunk, can also occur, which results in postural deformities and flexed posture. Flexed posture, however, generally occurs in later stages of the disease process.

1.2.1.3 Bradykinesia

Bradykinesia refers to the poverty or slowness of movement, and it is the most disabling clinical symptom of PD. Bradykinesia is the hallmark symptom of basal ganglia disorders, and it is manifested by extreme difficulties with planning, initiating, and executing movements, and with performing sequential and simultaneous tasks (Berardelli et al., 2001). Patients initially present with deficits in fine motor control, slower reaction times, and a general slowness in performing normal daily activities (e.g. buttoning, writing, using utensils). Other bradykinetic features include loss of spontaneous movements, drooling (because of impaired swallowing), monotonic and hypophonic dysarthria (difficulty articulating words), hypomimia (loss of facial expression), reduced blinking, and difficulty walking (Jankovic, 2008). Interestingly, bradykinesia depends on the emotional state of the patient; for example, a PD patient may be able to suddenly get up and run if somebody unexpectedly screams “fire”. This phenomenon, termed kinesia paradoxica, suggests that PD patients may still have intact motor systems but may have difficulty triggering them without an external stimulus.
1.2.1.4 Postural Instability

Bradykinesia is often exacerbated by postural instability in the late stages of PD. Postural instability, which is usually manifested after the onset of the other clinical features, is characterized by a loss of postural reflexes. PD patients have difficulty initiating and maintaining a normal gait, causing them to shuffle their feet. The pull test, in which the patient is quickly pulled backward and forward, is used to assess the degree of instability and the patient's ability to recover from an external force. PD patients either take more than two steps to compensate, or have no recovery response at all, which indicate marked postural dysfunction. With time, instability leads to poor balance, making it the most common cause of falling.

1.2.1.5 Freezing

Freezing, also referred to as motor blocks, is a form of akinesia that commonly affects the legs during walking (Jankovic, 2008). It usually manifests itself as a sudden (but transient) inability to move, and includes hesitation when initiating walking or a sudden inability to move the feet.

Although freezing is a feature of PD, it is not observed in all patients. In one study, 47% of PD patients exhibited freezing: freezing episodes were more frequent in men than in women and was less frequently observed in tremor-dominant patients (Macht et al., 2007). Notably, PD patients who initially presented with rigidity, bradykinesia, or postural instability had a higher risk of developing freezing compared to tremor-dominant patients (Giladi et al., 2001). Freezing typically occurs in the later stages of the disease and is normally not the predominant symptom. Unfortunately, freezing does not usually respond to treatment.

1.2.2 Non-Motor Symptoms

Idiopathic PD is generally considered a classical movement disorder; therefore, many of the current therapeutic strategies (see Chapter 2) have focused on the symptomatic treatment of motor dysfunction. However, non-motor symptoms of PD have long been recognized, and many of these symptoms can be as debilitating as the motor symptoms themselves. Importantly, research has gradually shifted towards viewing PD as a multisystem disease that affects a variety of brain structures at different times along the disease process. In this regard, the presentation of different non-motor symptoms at various stages of the disease has been suggested to be manifestations, or consequences, of the underlying pathophysiology at that specific stage of the
disease process. This section briefly describes some of the major non-motor symptoms in PD and, in combination with the following sections below (see section: Pathophysiological Hallmarks of PD), explains how studying these symptoms can give us insight into possible pathological correlates that can be used as preclinical markers for PD.

1.2.2.1 Olfaction and Hyponosmia

Initially studied by Ansari and Johnson (Ansari and Johnson, 1975), olfactory dysfunction affects over 90% of PD patients (Hawkes et al., 1997). It is present from the start of the disease, but it does not appear to progress over time. Interestingly, many patients complain of deterioration in their sense of smell long before they develop PD. Hyponosmia, which is the impaired ability to smell and detect odors, is commonly seen in genetic forms of PD and in undiagnosed elderly people with mild parkinsonism. However, many olfactory dysfunctions do not seem to be specific to PD and have been observed in Alzheimer’s disease and other synucleinopathies such as incidental lewy-body disease (ILBD) and multiple system atrophy.

Olfactory dysfunction has been pathophysiologically linked to the olfactory bulbs. Indeed, a recent report has suggested that PD may be etiologically linked to a pathogen originating from the olfactory bulb and spreading to olfactory structures, much like a prion disorder (Lerner and Bagic, 2008; Olanow and Prusiner, 2009). Accordingly, studies have reported olfactory deficits in asymptomatic relatives of PD patients, some of who developed PD (Siderowf et al., 2007), and olfactory deficits have been suggested to be a preclinical marker for PD.

1.2.2.2 Autonomic Dysfunction and Gastrointestinal Problems

Gastrointestinal (GI) symptoms, which include dysphagia (difficulty swallowing), esophageal dysmotility, and constipation, are common in PD. The origin of these dysfunctions is not entirely clear; however, evidence suggests that these symptoms are direct manifestations of abnormal pathology in the peripheral autonomic nervous system (see below). Several studies have reported an increased prevalence of constipation in PD. For example, constipation or prolonged intestinal transit time is seen in over 80% of PD patients (Jost, 1997). Further, constipation is a prominent symptom long before the onset of any overt motor dysfunction. As part of the Honolulu Heart Program prospective study (Abbott et al., 2003), over 8000 Japanese American men were followed from 1965 and given periodic health surveys. In the PD component of the study, researchers found that men who reported less than one bowel movement
per day in midlife were four times more likely to develop PD than men who reported more than two bowel movements per day. Moreover, there was a mean interval of ten years between initial complaints of constipation and initial signs of the disease (Abbott et al., 2001). Therefore, as with hyposomia, constipation may be one of the early predictors of PD.

### 1.2.2.3 Sleep Disorders – Rapid Eye Movement Behavioural Disorder (RBD)

Sleep disorders are among the most frequent non-motor symptom observed in PD (Tandberg et al., 1998). They include difficulty falling asleep, frequent awakening, dystonia, motor restlessness, restless legs syndrome, and excessive daytime sleepiness. PD patients also develop dysfunctional sleep structure, including sleep fragmentation, reduced sleep efficiency, and rapid eye movement sleep behaviour disorder (RBD). Only recently has there been increased awareness of the clinical implications of sleep dysfunction on the quality of life, which has prompted considerable research in this area.

RBD is characterized by the loss of normal skeletal muscle atonia during REM sleep, with phasic or tonic activity in the chin and extremity electromyography (Chaudhuri and Naidu, 2008). This leads to violent jerking and limb movements where a patient often enacts vivid and unpleasant dreams (Schenck et al., 1996). RBD often precede clinical motor manifestation of PD; in fact, recent studies have reported that RBD precedes motor symptoms in over 40% of PD patients (Chaudhuri et al., 2006), with an average of 13 years between the onset of RBD and clinical manifestation of PD (Schenck et al., 1996). Moreover, an imaging study in idiopathic RBD patients demonstrated significant pathophysiological changes that suggest pre-clinical PD (Eisensehr et al., 2000). Remarkably, in James Parkinson’s original monograph, he noted that “…sleep becomes much disturbed. The tremulous motion of the limbs occurs during sleep, and augments until they awaken the patient, and frequently with much agitation and alarm” (Parkinson, 1817). RBD is currently being investigated as a possible marker of pre-symptomatic PD.

### 1.2.2.4 Depression and Anxiety

Depression has a reported prevalence of 36-50% in PD patients (Shulman et al., 2001). It was initially suggested that most depressed PD patients met the DSM IV criteria for major depression (Starkstein et al., 1992); however, subsequent studies have indicated that most PD patients present depressive symptoms associated with minor depression or dysthymic disorder (Tandberg et al., 1996). PD patients with depression normally present with anxiety, panic
attacks, loss of initiative and assertiveness, anhedonia, and fatigue. In contrast, symptoms of self-blame, guilt, and thoughts of self-destruction and suicide are less frequent when compared to individuals diagnosed with major depression.

Studies have also suggested that depressive symptoms, particularly depressive episodes and panic attacks, precede the clinical manifestation of PD (Santamaría et al., 1986). One comparative study of PD and dystonia (Lauterbach et al., 2004) reported that PD patients more often had clinical diagnoses of simple phobia and atypical depression preceding their PD diagnosis. Moreover, a retrospective cohort study reported that PD patients had a significantly larger lifetime diagnosis of depression compared to controls (Schurmann et al., 2002).

1.2.2.5 Cognitive Dysfunction and Dementia

Population-based, longitudinal studies have suggested that cognitive impairment and dementia are common in the late stages of PD. The clinical profile of dementia in PD includes: psychomotor slowing; apathy and bradyphrenia; impairments in problem solving, planning, and organization of goal-directed behaviours; difficulties with set shifting; poor visuospatial functioning; and impairments in learning and memory. Eighty percent of PD patients develop some degree of cognitive impairment within 12-20 years of the initial diagnosis of PD (Aarsland et al., 2001; Hely et al., 2008). Post mortem studies of PD patients have shown several different pathological features that are associated with cognitive decline (Halliday et al., 2008), including Alzheimer-type pathology and Lewy body (LB) degeneration (described below) in subcortical and cortical areas. Furthermore, the development of dementia in PD has a significant impact on disease progression: it has been associated with a more rapid disease progression, increased nursing home placement, and increased mortality.

1.3 PATHOPHYSIOLOGICAL HALLMARKS OF PD

The etiology and pathogenic mechanisms that cause PD are still unknown. In recent years, there has been much debate over the clinical relevance of the “classical” basal ganglia model that outlines voluntary movement and PD dysfunction. With increasing awareness of non-motor symptoms in idiopathic PD, there has been a gradual shift in research to understanding the pathogenic predisposition of other cell and neurotransmitter types and functional systems outside the basal ganglia (Braak et al., 2003; Braak and Del Tredici, 2008a). Nevertheless, the progressive nature of PD and the onset of different symptoms (both motor and non-motor) at
different stages of the disease indicate that it is essential to study all systems within the context of a larger disease process.

1.3.1 Classical Model of Parkinson’s Disease

1.3.1.1 Dopamine (DA) Function

Dopamine (DA) is a catecholamine neurotransmitter that is present in many areas of the brain. It activates five different receptor types (D1-D5) and has many functions in the brain, including important roles in cognition, learning and memory, reward, and movement. Dopaminergic (DAergic) neurons are predominantly found in the ventral tegmental area (VTA) and substantia nigra (SN) of the midbrain and in the arcuate nucleus of the hypothalamus.

DA synthesis normally takes place intracellularly within DAergic terminals (Figure 1.1). The amino acid tyrosine is first converted to L-3,4-dihydroxyphenylalanine (dopa) by the rate-limiting enzyme tyrosine hydroxylase (TH). Dopa is then decarboxylated to DA by the enzyme aromatic acid decarboxylase (AADC). Synthesized DA enters pre-synaptic vesicles through the vesicular monoamine transporter type 2 (VMAT2), is released into the synaptic cleft upon activation of the nerve terminals, and interacts with both pre- and post-synaptic DA receptors. DA neurotransmission is terminated via the metabolism of extracellular DA by catechol-O-methyl-transferase (COMT) and monoamine oxidase (MAO)-B to homovanillic acid (HVA). Extracellular DA can also be transported back into the pre-synaptic terminals through the DA transporter (DAT), where it is either recycled back into pre-synaptic vesicles or metabolized by intracellular MAO-A into 3,4-dihydroxyphenylacetic acid (DOPAC). DOPAC can subsequently diffuse out of the terminals and be further metabolized to HVA (by COMT).

DA plays an important role in the pathophysiology of PD: the interaction between DA and post-synaptic DA receptors is essential in modulating motor function through different loops combining the basal ganglia, thalamus, and the motor cortex.
1.3.1.2 The Basal Ganglia – Direct and Indirect Pathways

PD was originally considered to be a single clinical entity with a simple pathological process. The “classical” basal ganglia model describes the regulation of movement (Figure 1.2A). It focuses on a complex set of feedback loops that involves the prefrontal cortex and extensive portions of the neocortex, thalamus, and basal ganglia, which together comprise the cortico-basal ganglia-cortical circuit (Albin et al., 1989; Alexander and Crutcher, 1990; Alexander et al., 1990; Parent and Hazrati, 1995; DeLong and Wichmann, 2007).

The basal ganglia are composed of four interconnected nuclei: the caudate and putamen (which, together, comprise the striatum), the internal (GPi) and external (GPe) portions of the globus pallidus, the SN (comprised of the pars reticulata, SNr, and the pars compacta, SNC) and the subthalamic nucleus (STN). Numerous studies that have examined the neuroanatomical,
physiological, and neurochemical characteristics of the basal ganglia have helped delineate a functional model that explains the initiation of normal and abnormal motor behaviour.

The basal ganglia model affirms that voluntary movement is mediated through two interdependent but opposing pathways that originate in the striatum. The striatal projection neurons associated with each pathway can be distinguished based on their DA-receptor and neuropeptide co-expression. The direct pathway, which consists of striatal GABAergic neurons that co-express the DA D1 receptor and the neuropeptides substance P and dynorphin, monosynaptically sends inhibitory projections to the GPi/SNr complex. The indirect pathway, which consists of a separate population of striatal neurons that co-express the DA D2 receptor and the neuropeptide enkephalin, sends its GABAergic projections to the GPe. The GPe, in turn, sends GABAergic projections to the STN. The STN then sends glutamatergic (excitatory) projections to the GPi/SNr complex. The GABAergic projection neurons in the GPi, which receive inhibitory and excitatory afferents from the striatum and STN, respectively, project to the ventrolateral (VL) and ventral-anterior (VA) portions of the thalamus. The VL/VA thalamus, which sends its projections to the supplementary motor and pre-motor areas, is responsible for the initiation of movement.

The equilibrium between the direct and indirect pathways, and ultimately the regulation of movement, is regulated by SNc DA projection neurons that synapse with striatal GABAergic neurons, making up the nigrostriatal pathway. Under normal physiological conditions, DA mediates direct (D1/substance P/dynorphin expressing) and indirect (D2/enkephalin expressing) GABAergic striatal neurons in opposing ways. D1-mediated GABAergic projection neurons are activated by DA activation, leading to an increased inhibitory effect on GPi neurons. GABAergic GPi neurons, in turn, are inhibited, leading to the “release” of their efferent targets, projection neurons of the VL/VA thalamus. In contrast, D2-mediated GABAergic projection neurons are inhibited by DA activation that, in turn, reduce the inhibitory tone on the GPe. The reduced inhibitory effect on GABAergic GPe neurons then leads to increased inhibition of the STN. The decrease in STN excitatory activity on the GPi leads to the reduction in GPi neuronal activity. The net result is a reduced inhibitory effect on the VL/VA thalamus, leading to increased activation of thalamic projection neurons to the supplementary motor cortex.
Figure 1.2. Schematic diagram of the classical basal ganglia model. The normal (A) and parkinsonian (B) states are depicted. Black arrows indicate inhibitory projections and white arrows indicate excitatory projections. Arrow thickness indicates the degree of activation of each projection. In the normal state (A), the striatum innervates output neurons of the GPi and SNr through the direct pathway, and synapses with the GPe and STN through the indirect pathway. DAergic input from the SNc is thought to inhibit neuronal input (through DA D2 receptors) in the indirect pathway and excite neuronal activity (through DA D1 receptors) in the direct pathway. In the parkinsonian state (B), the loss of DA leads to a significant increase in neuronal activity in the STN and GPi resulting in the disproportionate inhibition of the VL/VA thalamus. The net result is the over inhibition of the thalamo-cortical and brainstem motor centers which leads to parkinsonism. PPN = pedunculopontine nucleus. Adapted from (Lonser et al., 1999) as modified in Obeso et al. Physiology, 2002. Am Physiol Soc, used with permission.

1.3.1.3 Pathophysiology of Parkinson’s Disease

One of the pathophysiological hallmarks of PD is the degeneration of DAergic neurons in the SNc, leading to the significant loss of DA concentration in the striatum (Albin et al., 1989; Chesselet and Delfs, 1996; Parent and Cicchetti, 1998; Blandini et al., 2000; Wichmann and DeLong, 2003). The loss of striatal DA leads to a significant increase in activity in the STN and
GPi, leading to the disproportionate inhibition of the VL/VA thalamus, reducing its thalamocortical activity (Figure 1.2B). In the absence of striatal DA, striatal D1-mediated GABAergic neurons in the direct pathway (which are normally activated by DAergic activity) can no longer inhibit the GPi; therefore, the GPi can now inhibit the thalamus. In parallel, the indirect pathway (which is normally inhibited by normal DAergic functioning) exerts an inhibitory effect on the GPe. This leads to disinhibition of the STN, resulting in the over activation of the GPi. The net result is the increased inhibition of the VL/VA thalamic nuclei and subsequent cortical motor centers.

There are particular characteristics of midbrain DAergic degeneration in PD. DAergic systems do not all degenerate to the same degree. Within the midbrain, three DAergic cell groups exist: the A8 group in the lateral reticular formation, the A9 group in the SNc, and the A10 group in the medial and dorsal tegmentum (Dahlström and Fuxe, 1964). The SNc specific A9 group, which comprises the nigrostriatal system, is the most severely affected, while the neighboring SNr and the A8 and A10 groups are largely spared from degeneration (Halliday et al., 1996). Within the A9 group, neuronal loss also follows a specific pattern, preferentially affecting the ventral (as opposed to the dorsal) SNc (Halliday et al., 1996; Damier et al., 1999).

Striatal DA concentrations are also lost in a specific pattern: the greatest DA deficit is in the rostral striatum, particularly in the dorsolateral or post-commissural putamen. The caudate is mildly affected, while the ventral striatum is the least affected (Agid et al., 1987). Similarly, DAergic pathways arising from the VTA (A10), including the mesocortical and mesolimbic systems that project to the nucleus accumbens and limbic regions, are minimally affected.

There is a strong correlation between SNc neuronal degeneration, accompanying decrease in striatal DA concentrations, and symptom severity in PD patients (Damier et al., 1999). At least 80% of striatal DA and 60% of SNc neurons are lost before PD manifestations become symptomatic (Kish et al., 1988; Fearnley and Lees, 1991; Morrish et al., 1995). For example, in a single case report of a hemi-parkinsonian patient, there was an observed 80% loss of striatal DA in the hemisphere contralateral to the patient’s most affected side, whereas the asymptomatic side had less than 75% loss of striatal DA content (Agid et al., 1987). Although there is a gradual (and considerable) loss of SNc DAergic neurons with age, the extent of the degeneration in PD is substantially greater. The massive decrease in striatal DA content prior to any clinical manifestation has been explained by compensatory mechanisms in the nigrostriatal
system (Zigmond et al., 1989). In fact, increased DA turnover (due to altered activity of remaining DAergic neurons) has been shown to be an important compensatory mechanism (Javoy-Agid et al., 1982; Hornykiewicz and Kish, 1987; Doudet et al., 1998). However, once PD symptoms appear, nigrostriatal terminals progressively die at a rate of 10-12% per year (Morrish et al., 1995).

There is considerable evidence that supports the basal ganglia model in explaining motor dysfunction. TH and AADC enzymatic activity are severely reduced in both the SNc and the striatum (Agid et al., 1987). DA metabolites HVA and DOPAC are also reduced, albeit to a lesser extent, in the striatum. In situ hybridization for cytochrome oxidase I (CO-I) mRNA, a mitochondrial respiratory chain enzyme involved in cellular metabolism, is increased in the STN and Gpi/SNr of parkinsonian monkeys (Vila et al., 1997). In addition, mRNA expression of glutamic acid decarboxylase (GAD), an enzyme involved in the synthesis of GABA, is increased in the Gpi and SNr (Vila et al., 1996). Electrophysiological studies have also shown increased firing rates in the STN and Gpi, but reduced firing in the GPe of parkinsonian monkeys (Bergman et al., 1994). Notably, these effects can be reversed after lesioning the STN, which reduces hyperactivity (Wichmann et al., 1994) alleviating parkinsonian symptoms.

However, other experimental findings have cast doubt on the absolute role of the nigrostriatal DAergic system in PD. Hyperactivity in the STN actually precedes symptom manifestation and is not associated with GPe hypoactivity (Vila et al., 2000; Bezard et al., 2001). CO-I metabolic cell activity increases rather than decreases in the GPe of parkinsonian primates and rats (Vila et al., 1997; Vila et al., 2000), and GAD levels remain normal (not reduced) in the GPe (Herrero et al., 1996).

The basal ganglia model has contributed to the development of a plethora of pharmacological treatments and to the theoretical basis of stereotactic surgical procedures such as deep brain stimulation (DBS) (see Chapter 2). However, there are experimental findings that cannot be predicted by the basal ganglia model. More importantly, clinicians have become aware that certain symptoms (particularly non-motor dysfunctions) cannot be explained by DA deficiency and the basal ganglia model alone. In this regard, research has explored other CNS systems that have recently been shown to be involved in PD.
1.3.2 Lewy Body Pathology and Modeling in Parkinson’s Disease

1.3.2.1 Lewy Body (LB) Formation and PD

LB and lewy neurite (LN) formation constitute the other defining neuropathological feature of PD. First described by Friederich Lewy in 1912 (Lewy and Lewandowsky, 1912), Tretiakoff subsequently found that LBs were numerous in the SN and was the first to make a connection between LB formation, SN neuronal degeneration, and the onset of motor symptoms. There are two general types of LBs, the classical (brainstem) type and the cortical type. Brainstem and cortical LBs have numerous features in common with each other: they are eosinophilic, composed of filamentous structures, and contain large amounts of proteins including ubiquitin and α-synuclein (Gai et al., 2000). Structurally, brainstem LBs are spherical or elongated, possess a dense (protein rich) core, and are surrounded by a filamentous, peripheral halo. In contrast, cortical LBs are often poorly defined structures with no discernable central core or halo.

All forms of idiopathic and familial PD, with the exception of juvenile onset parkinsonism patients (see below), contain α-synuclein-rich LBs in neuronal cell bodies (Spillantini et al., 1997). The number of LBs in patients with mild to moderate SNc degeneration is higher than in patients with severe DAergic depletion, which implies that LB-containing neurons are dying neurons (Wakabayashi et al., 2007). However, LBs do not always accompany neurodegeneration: LB accumulation does not seem to increase the predisposition of SNc neurons to undergo degeneration anymore than the general population of LB-free SN neurons. In fact, most neurons that undergo cell death do not contain LBs (Tompkins and Hill, 1997). All SNc neurons are similarly affected (whether they contain LBs or not), which suggests that the disease process affects the neurons themselves.

Interestingly, despite the SN being regarded the principal site of degeneration and responsible for most of the motor abnormalities in PD, LB/LN accumulation is not limited to that brain region. LBs were also found in noradrenergic neurons of the locus coeruleus, cholinergic neurons in the nucleus basalis of the Meynert, and neurons in the dorsal motor nucleus of the vagus, olfactory bulb, autonomic nervous system, and cerebral cortex (Forno, 1996). LBs have also been reported in other neurodegenerative diseases, including dementia with LBs (a LB variant of Alzheimer’s disease) (Spillantini et al., 1998; Giasson et al., 2000). In this regard, LB formation has been shown to increase with age: LBs have been observed post mortem in elderly
individuals (over the age of 65) without clinical or pathologic evidence of parkinsonism (Parkkinen et al., 2001; Parkkinen et al., 2003). This suggests that LB accumulation is either part of the normal aging process, or precedes neurodegeneration and the clinical manifestation of the disease and is thought to represent a pre-symptomatic stage of PD, or ILBD (Dickson et al., 2008).

The etiology and molecular composition of LBs has largely remained a mystery for a number of years, and only recently have clues emerged with regards to its etiological connection to PD. The general consensus, however, is that LB formation is not the cause of neuronal death. In fact, studies have suggested that LBs may be part of a neuroprotective process. For example, LBs are formed in similar way to aggresomes (Olanow et al., 2004b), which are proteinaceous inclusions that are formed in response to proteolytic stress. Aggresomes segregate and facilitate the degradation of excess damaged, mutated, unwanted proteins (Kopito, 2000). In this regard, some proteins that are heavily expressed in LBs, which include α-synuclein, parkin, components of the ubiquitin-proteasome system (UPS), among others, have all been implicated in the pathogenic mechanisms underlying PD.

1.3.2.2 α-Synuclein: Evidence From Familial Forms of PD

Alpha-synuclein (α-synuclein) is an abundant, 140 amino acid long, soluble protein that is predominantly expressed in presynaptic terminals (Cookson, 2005). Although the exact functions of α-synuclein remain to be fully elucidated, studies in α-synuclein knockout mice have suggested that α-synuclein might be a pre-synaptic regulator of DA synthesis, storage, and release (Abeliovich et al., 2000).

Two seminal findings clearly linked α-synuclein to PD and have since changed the scope of PD research. The first was the discovery that point mutations in the genes that code α-synuclein were pathogenic in familial forms of PD (Polymeropoulos et al., 1997), and the second was the discovery that α-synuclein is a major component of LBs/LNs in idiopathic PD (Spillantini et al., 1997). Three point mutations in α-synuclein have been identified in familial PD: A53T in a Greek kindred (Polymeropoulos et al., 1997), A30P in a German kindred (Krüger et al., 1998), and E46K in a Spanish kindred (Zarranz et al., 2004). A triplication of the wild-type α-synuclein gene has also been identified in a large Iowa family (Singleton et al., 2003). The A53T kindred present several clinical features that distinguish them from idiopathic PD.
including earlier onset, more rapid disease progression, low prevalence of tremor, and earlier onset of nonmotor symptoms (such as dementia). The A30P kindred, in comparison, clinically resemble idiopathic PD (Lee and Trojanowski, 2006). Neuropathology from three of these kindreds (no neuropathology has been reported on the A30P kindred) has revealed SN degeneration and abundant α-synuclein-positive LBs/LNs (Kotzbauer et al., 2004).

Alpha-synuclein mutations increase their propensity to misfold, and misfolded α-synuclein appears to play a prominent role in cell death (Cookson, 2005; Lee and Trojanowski, 2006; Gupta et al., 2008). One theory for α-synuclein toxicity suggests that mutated α-synuclein monomers can form oligomeric β-pleated sheets (or protofibrils), which can coalesce into insoluble fibrils and aggregate in LBs (Conway et al., 1998; Wood et al., 1999). Recent evidence, however, has suggested that α-synuclein protofibrils may be cytotoxic to cells and that fibrillar aggregates may be cytoprotective. Both the A53T and E46K mutations accelerate fibril formation (Rochet et al., 2004; Savitt et al., 2006), but the A30P mutation slows the rate of fibril formation (Giasson et al., 1999; Conway et al., 2000). An increase in oligomeric α-synuclein deposits was found in patient brains with the triplication mutation, which suggests that oligomeric protofibrils (and not their conversion to fibrils) may be pathological in patients expressing these mutations (Miller et al., 2004). Further, Lansbury and colleagues (Lashuel et al., 2002) demonstrated that α-synuclein protofibrils can bind to lipid bilayers and form amyloid pores that can puncture cell membranes, resulting in the release of cellular contents and cell death.

PD is a disease composed of two parts: SNc DA degeneration, and α-synuclein-rich LB pathology. Interestingly, as described above, the clinical manifestation of the disease does not occur when there is significant (greater than 80%) loss of striatal DA levels, which implies that there is an ongoing pre-clinical degenerative process. In this regard, elderly individuals with α-synuclein-rich ILBD suggests that LB accumulation could identify predilection sites early in the disease process and represent a pre-clinical phase of PD (Dickson et al., 2008).

1.3.2.3 Braak Modeling – Staging and Susceptibility to LB Development

Since PD does not clinically manifest until there is a significant loss of striatal DA and, by that time, is likely close to the end phase of the disease, it would be beneficial to identify key stages of disease progression in pre-symptomatic individuals. Based on a semi-quantitative
assessment of α-synuclein inclusions in over 400 autopsy cases, a hypothetical staging system was developed to predict a sequence of LB and LN pathology throughout the brain (Braak et al., 2003).

Braak and colleagues compared three subsets of autopsied brains. The first group consisted of patient brains that were clinically diagnosed with PD, and pathologically exhibited a severe loss of SNc DAergic neurons and abundant LB accumulation. The second group (incidental cases) consisted of patient brains that were not clinically diagnosed with PD, but did demonstrate LN/LB accumulation throughout the brain. The third group (comparison cases) were patient brains that were neither clinically diagnosed with PD nor contained any LB/LN pathology.

Alpha-synuclein and LB pathology were divided into six successive stages. In the most mildly affected cases (stage 1), LB/LN accumulation were consistently seen in the anterior olfactory nucleus, dorsal IX/X motor nucleus of the vagus, and preganglionic vagal axons of the enteric nervous system. A subsequent study also identified LBs/LNs in the enteric nerve cell plexuses (Hawkes et al., 2007), which suggests that the pathological process simultaneously begins in the olfactory bulb/anterior olfactory nucleus and within enteric nerve cell plexuses. More severe lesions in the dorsal motor nucleus, and LN/LB formation in the caudal raphe nuclei, gigantocellular reticular nucleus (in the reticular formation), and locus coeruleus, were observed in stage 2. Notably, much of the midbrain, including the SN, remained unaffected. These initial stages (observed in 7% of the cohort) were pre-symptomatic and, in part, may help explain the non-motor symptoms that precede the motor dysfunction in PD (see below).

Stages 3 and 4 were highlighted by initial signs of LN/LB pathology in the SN. However, there was no indication of any depigmentation (at stage 3), and much of the pathology was restricted to the posterolateral and posteromedial SN. Extranigral areas, including the central nucleus of the amygdala, pedunculopontine nucleus (PPN), and cholinergic neurons in the basal forebrain (such as the nucleus basalis of Meynert), also began to develop LNs. By stage 4, marked continual damage was observed in the posterior SNc: there was pronounced depigmentation and increased signs of extraneuronal melanin accumulations from de-melanized SNc neurons. The anteromedial temporal mesocortex also became affected. These stages (which comprised 11% of the cohort) seem to parallel the first clinical manifestations of PD.
In stages 5 and 6, temporal mesocortical lesions became more pronounced and extended into adjoining association areas in the temporal and prefrontal cortex. By stage 6, cortical pathology extended further into the higher order sensory association and premotor areas, and later progressed into the primary sensory and motor cortex (and eventually the whole neocortex). These late stages, which comprised 6% of the cohort, frequently presented with a combination of cognitive deficits and severe motor disability seen in PD.

While there has been little objection to their observations, questions have arisen as to whether Braak’s staging method provides a reliable and accurate representation of the progression of PD from its early pre-clinical stages. For example, Parkkinen and colleagues (Parkkinen et al., 2008) found that over 55% of cases that corresponded to Braak’s stages 5-6 lacked clinical signs of dementia or extrapyramidal symptoms. Moreover, in another study that looked at a large cohort of 71 patient brains, over 47% of the cases did not fit the predicted spread of α-synuclein pathology and, conspicuously, 7% of the cases did not show any dorsal motor nucleus pathology despite finding α-synuclein inclusions in the SNc and neocortex (Kalaitzakis et al., 2008). Other studies have shown similar cases deviating from Braak’s original staging system (Braak et al., 2006a; Attems and Jellinger, 2008). Taken together, while there may be controversies associated with the use of the Braak’s system as a clinico-pathological predictor of PD, it has nevertheless identified key neuronal structures that are essential for establishing the etiology of PD. In combination with the basal ganglia model, we are one step closer to determining each process that contributes to this multisystem disorder.

1.3.2.4 Pre-Clinical Symptoms of Parkinson’s Disease – Olfactory, Gastrointestinal, Autonomic Dysfunction and Stages 1-2 of the Braak Model

Braak hypothesized that a patient is already in a more advanced stage of the disease (stages 4-6) when initially diagnosed and possibly before any clinical motor symptoms begin to manifest themselves (Braak et al., 2003). Therefore, by determining key pathological events prior to the degeneration of SNc DAergic neurons, and by deducing how this pathology is clinically manifested, we may be able to identify pre-symptomatic stages of the disease.

In the pre-clinical/pre-symptomatic stages of the disease prior to any observable SNc cell death (stages-1-2 of Braak’s model), one of the first signs of lesions and LB pathology are seen in the olfactory structures. LNs/LBs usually develop first in the anterior olfactory nuclei, and by stage 3, additional olfactory structures (such as the olfactory tubercle and olfactory portions of
It has long been reported that non-motor symptoms usually precede the typical motor dysfunction in PD by many years (Koller, 1992) and, in many cases, PD patients often complain of an impaired sense of smell years before any motor dysfunction appears (Hawkes et al., 1997; Mesholam et al., 1998). Taken together, olfactory impairments may be a risk factor for PD.

Another important area that has been identified to have abnormal pathology in the early stages of PD is the dorsal IX/X motor nucleus of the vagus and intermediate reticular zone. Both structures play important roles in autonomic nervous systems control: the parasympathetic pre-ganglionic projection neurons in the dorsal motor nucleus are involved in parasympathetic vagal output functions in the gastrointestinal tract, lungs, and abdominal cavity, while the intermediate reticular zone has autonomic control of heart rate, blood pressure, and respiration. In fact, inclusion bodies have also been observed in pre-ganglionic and postganglionic sympathetic neurons in the spinal cord (Bloch et al., 2006; Klos et al., 2006; Braak et al., 2007) and in the enteric nervous system (Braak et al., 2006b), areas that play important modulatory roles in gastrointestinal functioning. Therefore, it is not surprising that reports of impaired gastrointestinal functioning (Abbott et al., 2001; Pfeiffer, 2003; Kaufmann et al., 2004) have been observed in pre-clinical or early-diagnosed PD patients.

1.4 MECHANISMS OF DA DEGENERATION

Although there is no consensus regarding the etiology and underlying pathophysiological mechanisms responsible for PD, significant evidence suggests that DAergic neuronal death involves a cascade of “hallmark” events, which include (but are not limited to) oxidative stress, mitochondrial dysfunction, ubiquitin-proteasome dysfunction, and inflammation. The following section reviews genetic, post-mortem, and experimental evidence that provides support for the role of each process as a potential mechanism of DA degeneration. The alternative hypothesis, inflammatory reactions, will be discussed in more detail in Chapter 4.

1.4.1 Oxidative Stress

Oxidative stress is a deleterious condition that results from insufficient scavenging of “destructive” reactive oxygen species (ROS) and nitrogen radicals. ROS are normally eliminated by intracellular antioxidant systems; however, these systems are gradually impaired with age. Accordingly, there is a decline in the antioxidant glutathione (GSH) and an increase in protein
carbonyls (such as ketones and aldehydes) that correlate with the ageing process (Smith et al., 1991). All tissue, not just the CNS, progressively become more prone to oxidative stress and increasingly unable to scavenge ROS. However, this condition seems to be heightened in certain neurodegenerative diseases for which ageing is a risk factor.

SNc DAergic neurons are particularly susceptible to oxidative stress because DA metabolism gives rise to various potentially toxic species (Jenner, 2003). At normal pH, DA can auto-oxidize into DA-quinone species, superoxide radicals, and hydrogen peroxide \((\text{H}_2\text{O}_2)\). Alternatively, the degradation of DA by MAO leads to the production of DOPAC and \(\text{H}_2\text{O}_2\). \(\text{H}_2\text{O}_2\) is then converted by Fenton reactions to produce toxic hydroxyl radicals \((\text{OH}^-)\), superoxide radicals, and peroxynitrites. \(\text{OH}^-\) production is catalyzed by ferrous iron, of which there are high levels in the SNc, while peroxynitrite radicals are produced in a reaction catalyzed by nitric oxide. Peroxynitrites, which are strong oxidation and nitration agents, can further react with carbon dioxide to form nitrogen radicals. In the end, ROS and reactive nitrogen species can lead to abnormal alterations in proteins, DNA, and lipids.

The normal DA system, however, has intrinsic mechanisms that can cope with oxidative stress. Only cytoplasmic (unsequestered) DA has the ability to quickly undergo oxidation and form ROS. The rapid transport and storage into synaptic vesicles, where there is a low pH environment and absence of MAO, seems to protect DA from auto-oxidation. Nevertheless, there is a wealth of evidence that suggests heightened levels of oxidative stress in PD. Basal protein oxidation and protein carbonyls (Floor and Wetzel, 1998), and lipid peroxidation (Dexter et al., 1989) are increased in PD patients. DNA damage in PD also appear: increased levels of oxidized 8-hydroxyguanine or 8-hydroxy-2-deoxyguanosine and 4-hydroxy-2,3-nonenal are found in the SN (Yoritaka et al., 1996; Zhang et al., 1999). GSH is decreased in the PD brain, whereas oxidized GSH and superoxide dismutase, an enzyme that catalyzes the formation of \(\text{H}_2\text{O}_2\) from superoxides, are preferentially increased (Jenner, 2003). Lastly, iron levels, which are already higher in the SN than in other brain regions, are further increased (Dexter et al., 1992).

### 1.4.2 Mitochondrial Dysfunction

Mitochondrial dysfunction has long been implicated in the etiology of PD. Evidence of this first emerged when drug users were accidentally exposed to 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) – an unintended by-product of the synthesis of heroin that resulted in an acute and irreversible parkinsonian syndrome (Langston et al., 1983). It was subsequently
shown that the active metabolite of MPTP, 1-methyl-4-phenylpyridinium (MPP⁺), is an inhibitor of complex I of the mitochondrial transport chain. MPP⁺ is a substrate of DAT; therefore, it is able to accumulate in DAergic neurons where it induces neuronal death through complex I inhibition. Later work that indicated that rotenone, also a selective complex I inhibitor, produces a parkinsonian syndrome (Betarbet et al., 2000), cemented the theory of mitochondrial dysfunction in PD. Details about the effects of MPTP and Rotenone are discussed in later sections.

Complex I is the first enzyme in the mitochondrial transport, where it oxidizes NADH and transfers electron to ubiquinone. Complex I also translocates protons to the intermembrane space, where it helps establish the mitochondrial membrane potential needed for ATP synthesis. It has been suggested that electron flux through complex I regulates the mitochondrial permeability transition pore (PTP: a large Ca²⁺-dependent pore in the inner mitochondrial membrane) that, when open, causes mitochondria to de-energize, depolarize, and induce cell death (Fontaine et al., 1998; Chauvin et al., 2001; Greenamyre et al., 2001). If there is an impaired ability to maintain the mitochondrial membrane potential, then the mitochondria will be unable to handle Ca²⁺ influx, leading to a Ca²⁺ homeostatic imbalance and eventually Ca²⁺-induced stress (Chan et al., 2009). Mitochondria have also been implicated as a primary source of oxidative stress. There are several sites along the mitochondrial chain (proximal to complex I) where electrons can leak, and impairment in complex I may lead to the increased production of H₂O₂ and ROS. A combination of these effects may ultimately increase the probability of opening the PTP, leading to apoptotic death.

A number of observations have linked mitochondrial dysfunction to PD. The direct relation between mitochondrial dysfunction and PD came from post-mortem descriptions of complex I deficiency in the SN of PD patients (Mizuno et al., 1989; Schapira et al., 1989), revealing an estimated 35% reduction in activity compared to control populations (Mann et al., 1994). This deficiency was subsequently seen in skeletal muscles and platelets in PD patients (Bindoff et al., 1989; Parker et al., 1989). Studies on cytoplasmic hybrid (cybrid) cells also helped link mitochondrial dysfunction to PD (Swerdlow et al., 1996; Gu et al., 1998). Cybrids that were prepared using mitochondrial DNA from PD patients showed increased ROS production, increased antioxidant activity, and changes in pro- and anti-apoptotic genes (Cassarino et al., 1997; Veech et al., 2000). However, only about 40% of PD patients seem to
have a mitochondrial complex I defect (Olanow, 2007), and there has been no success in finding specific mutations in the mitochondrial genome that could account for the dysfunction (Howell et al., 2005).

Despite the abundant evidence linking mitochondrial dysfunction and oxidative stress to PD, it is still not known how these processes connect to the time course of neuronal degeneration: does oxidative stress initiate cell death in PD, or is it a secondary process triggered by an underlying mechanism? Nevertheless, it is clear that mitochondrial dysfunction and oxidative stress are present at the end stages of PD, and it can be assumed that these mechanisms are functionally linked that could accelerate or exacerbate the disease process.

1.4.3 Ubiquitin-Proteasome System (UPS) Dysfunction

The UPS is the major pathway that mediates the degradation of unwanted intracellular proteins (i.e. misfolded, mutant, denatured, or oxidatively damaged) in the cytoplasm, nucleus, and endoplasmic reticulum of all eukaryotic cells. The process whereby the UPS clears unwanted proteins involves the following sequence of events (McNaught et al., 2001):

1. **ATP-dependent activation of ubiquitin monomers.** Activated ubiquitin is generated by the ubiquitin-activating enzyme (E1), which hydrolyses ATP and forms a thiolester linkage between a cysteine residue and carboxy-terminal glycine in ubiquitin (Hochstrasser, 1996). The activated ubiquitin is then transferred to one of several ubiquitin-conjugating enzymes (E2) and ligated to protein substrates catalyzed by different ubiquitin protein ligases (E3). This process ensures selective protein targeting.

2. **Transport and recognition of the ubiquitin-protein conjugate to the proteasome.**
   Ubiquitin itself forms a substrate and expands into a polyubiquitin chain. A chain of four or more ubiquitin monomers then forms a recognition signal that allows the polyubiquitin chain-protein substrate to be shuttled. Chaperone molecules, such as heat shock proteins, transport the abnormal protein to the proteasome for degradation.

3. **ATP-dependent degradation of unwanted proteins by the 26S Proteasome.** The proteasome is a large, multicatalytic protease that degrades polyubiquitinated proteins. It is composed of 2 subcomplexes: the 20S core particle that carries out the catalytic activity, and the 19S regulatory particle. Proteasomal degradation yields short peptide fragments. These fragments can be further degraded into basic amino acids that can be recycled to form new proteins. At the same time, polyubiquitin chains are disassembled.
(by ubiquitin carboxy-terminal hydrolases [UCH]) to produce re-usable monomeric ubiquitin molecules.

Cells maintain a dynamic equilibrium between the production and degradation of unwanted proteins. The disruption of this balance, either by the excess formation of unwanted proteins (generated by oxidative stress and mitochondrial dysfunction) or by impaired protein degradation, will ultimately promote proteolytic stress and cell death.

1.4.3.1 α-Synuclein, Excess Protein Deposition and LBs in Idiopathic PD

Evidence of UPS dysfunction in PD first emerged when LBs were found in surviving DAergic neurons. LBs consist of a heterogeneous mixture of proteins, which includes ubiquitin and various proteasomal elements (McNaught et al., 2001). Despite being a pathological hallmark of PD, the mechanisms by which LBs are formed and their pathological significance in PD are still being debated. One theory is that aberrant proteins tend to aggregate if 1) the neuron’s ability to degrade unwanted proteins is exceeded or 2) proteasomal functioning is impaired. Another possible explanation is that the accumulation of ubiquitin (and other proteasomal elements) in LBs reflects a failed attempt by the UPS to remove abnormal proteins. In this regard, protein aggregates themselves could impair the UPS.

While the debate continues on whether UPS dysfunction is a cause or consequence, there has been a wealth of evidence that suggests that the UPS plays an important role in the pathophysiological mechanisms underlying PD. Selective proteasomal impairment and reduced proteasomal subunit expression has been demonstrated in postmortem SN tissue from idiopathic PD patients (McNaught et al., 2003). There was a reported 40% reduction in the α (but not β) subunit of the catalytic 20S subcomplex (McNaught et al., 2002; McNaught et al., 2003) in PD patients. Proteasomal enzymatic activity in the 20S subcomplex was also impaired: PD patients demonstrated a 45-55% reduction in chymotrypsin, trypsin, and peptidyl glutamyl peptide hydrolytic proteasomal enzymatic activity compared to controls (McNaught and Jenner, 2001; Furukawa et al., 2002; McNaught et al., 2003; Tofaris et al., 2003). Interestingly, proteasomal activity in regions outside the PD-affected areas, which including the hippocampus, cerebellum, and frontal cortex, has also been shown to increase (McNaught et al., 2003).

The sections below provide evidence of the possible role of the UPS in familial forms of PD, disease subtypes that share many clinical and pathological features with idiopathic PD. Recent studies have provided further proof by demonstrating that administration of proteasome
inhibitors can induce a PD model in rats, and is described in more detail below (Animal Models section).

1.4.3.2 Parkin Mutations: Protein Ubiquitination Dysfunction

Autosomal recessive juvenile parkinsonism (AR-JP), one of the most common familial forms of PD, was first described in a series of Japanese kindreds (Matsumine et al., 1997). Clinically, AR-JP resembles idiopathic PD, but differs greatly in that there is a very early age of onset (Lücking et al., 2000) and a slow disease progression. Pathologically, AR-JP slightly differs from idiopathic PD in that neurodegeneration is confined to the SNc and LC, and there are no signs of LB deposits (Mori et al., 1998), although late-onset AR-JP cases have been described with a more typical PD pathology and LB accumulation.

Several deletions, multiplications, and point mutations were identified, which encodes the 465-amino acid/52 kDa protein parkin (PARK2) (Kitada et al., 1998; Mizuno et al., 2001; Hattori and Mizuno, 2004). Normally, parkin is diffusely expressed in the cytoplasm, nucleus, golgi apparatus, and processes in neurons, but is absent in AR-JP patients (Shimura et al., 1999). It is now known that parkin is an ubiquitin-protein ligase (E3): Parkin acts with the ubiquitin-conjugating enzymes (E2) UbcH6, UbcH7, and UbcH8 to attach ubiquitin molecules to protein substrates (Olanow and McNaught, 2006).

The mechanisms that underlie parkin-induced neurodegeneration are still not firmly established; however, it is reasonable to consider that the loss of E3 activity could lead to the reduced ability to label unwanted substrates that are targeted for proteasomal degradation (Olanow and McNaught, 2006). Indeed, AR-JP parkin mutations impair its binding to UbcH7 and UbcH8 E2 enzymes, which leads to 1) reduced parkin activity, (Mori et al., 1998; Shimura et al., 2000) and 2) the accumulation of non-ubiquitinated parkin substrates in degenerating areas (Imai et al., 2001; Shimura et al., 2001). It would be important to identify parkin’s protein substrates on the assumption that a parkin mutation would lead to the abnormal accumulation of its substrates, which could be toxic to DAergic neurons. Some of parkin’s protein substrates include a synaptic vesicle-associated protein CDCrel-1, a G protein-coupled parkin-associated endothelin-like receptor (Pael-R), and an O-glycosylated isoform of α-synuclein (Zhang et al., 2000; Imai et al., 2001). Pael-R is present in exceptionally high levels in DAergic neurons in AR-JP patients (Dawson and Dawson, 2003) and, when overexpressed, it tends to misfold and aggregate, capable of inducing cell death (Imai et al., 2001). Indeed, parkin has been shown to
protect DAergic neurons from Pael-R-induced neurotoxicity in drosophila (Yang et al., 2003), which suggests that parkin may have a neuroprotective role in preventing cell death.

1.4.3.3 UCH-L1 Mutations: Deubiquitination Dysfunction

A PD syndrome was described in 2 German siblings with a missense mutation (I93M) in the gene encoding for UCH-L1 (Leroy et al., 1998). These patients clinically resembled idiopathic PD, but symptoms manifested themselves at relatively early age (49 and 51 years) (Olanow and McNaught, 2006).

UCH-L1 is a 230-amino acid/26 kDA ubiquitous protein that is expressed exclusively in neurons, and constitutes 1-2% of all proteins in the brain (Leroy et al., 1998). It belongs to a family of deubiquitinating enzymes that are responsible for the hydrolysis of polyubiquitin chains into monomeric ubiquitin. Mutations in the UCH-L1 gene (seen in these PD cases) results in a 50% decrease in deubiquitinating activity leading to reduced ubiquitin levels and impaired clearance of unwanted proteins (Leroy et al., 1998). Autosomal recessive UCH-L1 mutations in mice did not result in DAergic degeneration; instead, they developed gracile axonal dystrophy, a disorder pathologically characterized by axonal degeneration in the gracile tract (of the spinal cord) and medulla oblongata, and behaviourally develop sensory and motor ataxia (Saigoh et al., 1999). These mice had reduced deubiquitinating activity and neuronal degeneration with inclusions; however, these changes occurred in the cerebellum and not the SNc. The absence of parkinsonian features, both behaviourally and histopathologically, in UCH-L1 deficient mice suggests that this may not be the only factor involved in the pathophysiological process of the disease.

1.4.4 Inflammation

In their seminal paper, McGeer and colleagues (McGeer et al., 1988) revealed activated microglia in the SNc of PD patients post mortem. Since then, neuroinflammation has been hypothesized to be involved in the pathogenic mechanisms underlying PD. Substantial evidence from post mortem and in vivo studies has supported neuroinflammatory processes in PD (Orr et al., 2002; Whitton, 2007; McGeer and McGeer, 2008; Hirsch and Hunot, 2009). This evidence (along with some of the key neuroinflammatory mechanisms, which include, but are not limited to, microglial activation and astrogliosis) is discussed in more detail in Chapter 4.
1.5 ANIMAL MODELS OF PARKINSON’S DISEASE

An animal model is defined as an experimental preparation that is developed for the purpose of studying a disease condition in the same or often other species (Geyer and Markou, 1995; Shaw and Wilson, 2003). Animal models serve two important functions: 1) they offer the opportunity to study certain aspects of a disease, providing insight into the possible molecular, cellular, and systems mechanisms involved in its pathophysiology and progression, and 2) they provide a means to test new experimental treatments. Indeed, in PD and many other diseases, development and testing of new therapeutic strategies are always carried out in established animal models of PD.

In this regard, when assessing an animal model of a specific disease, four components of the model must be fulfilled to establish validity (Willner, 1986; Geyer and Markou, 1995):

1. **Face validity**, which is the phenomenological similarity between the behaviour represented in the animal model and the disease symptoms seen in the human condition;
2. **Construct validity**, which is most commonly defined as the accuracy with which an animal model measures what it is intended to measure;
3. **Etiological validity**, which is closely related to construct validity, is the degree of similarity with which the etiology of the disease model matches what is being modeled in the human condition; and
4. **Predictive validity**, or pharmacological similarity, is the ability of the model to identify drugs or other therapeutic strategies that are of value to the human condition.

Therefore, to successfully establish a valid animal model of PD, the following characteristics must be met:

1. Normal DAergic cell numbers in the SNc at birth, followed by the selective and progressive degeneration of DAergic neurons in late adulthood. (**Construct and Etiological validity**);
2. LB/LN accumulation in surviving neurons (**Construct and Etiological validity**);
3. Easily detectable motor deficits, including the cardinal symptoms of PD (rigidity, resting tremor, bradykinesia, and postural instability) (**Face validity**); and
4. Good response to pharmacological treatments that replace DA (**Predictive validity**).

In addition, if one were to update the model to take into account recent findings in PD, then additional criteria must be added, including:
5. Progressive degeneration of non-DAergic structures including the dorsal motor nucleus of the vagus, locus coeruleus, PPN, raphe nucleus, and anterior olfactory nucleus; and
6. Non-motor dysfunctions including autonomic dysfunction, cognitive disturbances, and olfactory deficits.

Since there is still no consensus on the etiology of PD, and it is often difficult to test for non-motor dysfunctions, most animal models of PD do not completely fulfill all four validity components. Therefore, it is important to carefully understand what the research is trying to address and choose the appropriate model. For example, many of the traditional animal models of PD (such as MPTP and 6-OHDA, see below) have great face and predictive validity, but may not necessarily be as useful in defining the etiological origins of PD. In contrast, many of the recently developed genetic models are aimed at understanding the etiology of the disease process; however, mixed results with regard to its pathology has diminished its construct validity. The following sections review the main animal models of PD and their advantages and disadvantages as they relate to the four validity components.

1.5.1 MPTP

In 1982, the meperidine (Demerol) analogue, MPTP, was accidentally discovered. When a small group of young drug addicts intravenously self-administered the synthetic heroin MPPP (1-methyl-4-phenyl-propion-oxy-piperidine), they developed a parkinsonian syndrome that closely resembles idiopathic PD (Davis et al., 1979; Langston et al., 1983; Langston and Ballard, 1983; Langton et al., 1999). It was later discovered that MPTP is the neurotoxic contaminant that was responsible for the effect. Since then, MPTP administration has been one of the most commonly used animal models for studying PD (Kopin and Markey, 1988; DeLong, 1990).

MPTP is highly lipophilic and, after systemic administration, it can easily cross the BBB. Once in the brain, MPTP is converted to 1-methyl-4-2,3-dihydropyridium (MPDP) exclusively in non-DAergic cells (particularly astrocytes and serotonergic neurons) by MAO-B, spontaneously oxidizes to MPP⁺, the active metabolite, and is released into the extracellular space (Przedborski and Vila, 2003). MPP⁺ is a polar molecule; therefore, it requires catecholaminergic plasma membrane transporters, particularly the DA transporter (DAT), to enter the cell. In fact, inhibiting or deleting DAT in transgenic mice prevents MPTP-induced degeneration (Bezard et al., 1999). Once inside DAergic cells, MPP⁺ can bind to VMAT2, translocating the toxin into synaptosomal vesicles containing DA. Alternatively, MPP⁺ can
accumulate within the mitochondria (by a mechanism dependent on the mitochondrial transmembrane potential) or can remain in the cytosol and interact with several cytosolic enzymes (Klaidman et al., 1993). MPTP induces toxicity by impairing mitochondrial respiration through inhibiting complex I of the mitochondrial electron transport chain, ultimately leading to ATP depletion and oxidative stress (Singer et al., 1987). Alternatively, MPP⁺ can trigger DA leakage from the synaptic vesicles into the cytosol, indirectly stimulating the ROS production.

MPTP exposure results in DAergic neurotoxicity in a number of different species (Zigmond and Stricker, 1989; Tipton and Singer, 1993); however, it is mainly used in non-human primates and mice. Interestingly, rodents are less sensitive to MPTP toxicity: this is likely due to higher intracerebral levels of MAO-B (needed to convert MPTP) or a higher DAT/VMAT2 ratio seen in mice. MPTP is usually administered systemically (subcutaneous, intraperitoneal, intravenous, or intramuscular), and it can be given either unilaterally (through intracarotid infusion), producing a hemiparkinsonian syndrome, or bilaterally. Different regimens of MPTP administration (acute versus chronic) are being used by different laboratories.

Acute MPTP exposure in primates causes specific degeneration of DAergic neurons in the nigrostriatal pathway: there is a 50-93% cell loss in the SNC (particularly in the ventral and lateral segments, with little loss in the VTA) and greater than 99% loss of DA in the striatum, with preferential degeneration of putaminal versus caudate DAergic terminals (Doudet et al., 1989; Hantraye et al., 1993). There are also decreased levels of DA and DA metabolites in the striatum (Doudet et al., 1998) and increased levels of lipid peroxidation suggesting oxidative stress (Rios and Tapia, 1987). Persistent inflammation, another feature of PD, is also observed after MPTP exposure (McGeer et al., 2003; Miklossy et al., 2006). Alpha-synuclein accumulation has also been observed in surviving SNC neurons in MPTP-treated primates; however, the aggregates did not resemble LB morphology (Kowall et al., 2000). Indeed, a recent study showed increased α-synuclein expression, but not LB formation in primates with over 10 years of MPTP-induced parkinsonism (Halliday et al., 2009). Behaviourally, MPTP-treated primates exhibit motor deficits (such as akinesia, bradykinesia, rigidity, and postural abnormalities) and myographic impairments that closely resemble those occurring in PD (Doudet et al., 1985; Doudet et al., 1990). In contrast, resting tremor is rarely observed.

The virtue of this model lies in its predictive value: the motor symptom responsiveness to all DAergic treatments known to be effective in PD. The administration of levodopa (see Chapter
2) or any other DA agonists can predictably reverse motor dysfunction in the MPTP model. Indeed, all new therapeutic strategies for PD have invariably been studied in the primate MPTP model prior to their induction into a human clinical trial.

Many variations of this model, such as multiple small-dose MPTP treatments (to produce a progressive neuronal loss), have been developed to help overcome one of its major shortcomings: DAergic loss in response to MPTP is rapid and complete resembling the late, rather than the early, stages of the disease process. In addition, MPTP produces an almost exclusive loss of nigral DAergic neurons and does not reflect the pathological changes seen in non-DAergic systems. To date, it is unclear whether the MPTP model can be used to investigate non-motor symptoms. MPTP-treated primates have been shown to develop autonomic dysfunction (Albanese et al., 1988) and sleep disturbances (Barraud et al., 2009). Additional symptoms include dry skin, seborrhea, and swallowing dysfunction (DJ Doudet, personal communication). Notably, MPTP-treated primates also develop cognitive deficits that can be reversed by DAergic medication (Fernandez-Ruiz et al., 1995; Fernández-Ruiz et al., 1999).

Many dosing regimens have also been developed to administer MPTP to mice (Przedborski and Vila, 2003; Jackson-Lewis and Przedborski, 2007). Each regimen has led to a variety of different models with distinct neuropathological features. MPTP administered acutely or sub-acutely (Jackson-Lewis et al., 1995; Tatton and Kish, 1997) has been shown to lead to a 40-90% loss of striatal DA. DAergic degeneration, however, seems to occur by a non-apoptotic form of death. Chronic models, such as MPTP infusion with osmotic pumps (Fornai et al., 2005) have also been developed, but have not yet been fully validated. As in primates, MPTP induces toxicity in mice by inhibiting complex I of the mitochondrial electron transport chain, leading to oxidative stress (Przedborski and Vila, 2003). MPP⁺-induced ROS production is highlighted in mice, as increasing ROS-scavenging enzymes (such as superoxide dismutase) in transgenic mice prevents MPTP-induced degeneration (Przedborski et al., 1992). Reactive microglia (and, to a lesser extent, reactive astrocytes) are also seen in response to MPTP intoxication (Vila et al., 2001).

1.5.2 6-Hydroxydopamine (6-OHDA)

The first demonstration of the biological effects of 6-OHDA was in the 1960s’ when Ungerstedt and colleagues demonstrated that the injection of 6-OHDA into the SNc caused the anterograde degeneration of the nigrostriatal DAergic pathway (Ungerstedt, 1968), thereby
creating the first animal model of PD. The 6-OHDA model is still one of the most widely used models to replicate the degeneration of SNC DAergic neurons in the SNC and accompanying motor dysfunction.

6-OHDA is a hydroxylated DA and norepinephrine (NE) analogue: it has structural similarities to both DA and NE and has a high affinity for catecholaminergic plasma membrane transporters such as DAT and the noradrenergic transporters. Since 6-OHDA is not DA specific, a noradrenergic transporter blocker (such as desipramine) must be co-administered to block the entry of 6-OHDA into noradrenergic neurons. 6-OHDA does not cross the BBB, and, therefore, cannot be administered systemically. The classical administration method for 6-OHDA is the stereotaxic injection into the SNC and/or medial forebrain bundle (MFB), which produces an almost complete degeneration of nigral DAergic cell bodies. SNC neurons begin to die within the first 12 hours after injection, followed by the anterograde degeneration of striatal DAergic terminals and marked striatal DA depletion within 2-3 days (Lee et al., 1996; Deumens et al., 2002).

6-OHDA neurotoxicity is mainly due to the production of ROS and quinones, resulting in massive oxidative stress (Schober, 2004). Once 6-OHDA accumulates intracellularly, it undergoes immediate oxidation and promotes the formation of H_2O_2 and para-quinones. Alternatively, 6-OHDA has a potent inhibitory effect on mitochondrial complex I, resulting in the formation of superoxide free radicals. Interestingly, the 6-OHDA-induced production of ROS is dependent on the presence of iron. Indeed, the intranigral injection of iron produces similar neurotoxic effects as 6-OHDA (Ben-Shachar and Youdim, 1991), which suggests a possible role for iron in 6-OHDA degeneration. Furthermore, 6-OHDA neurotoxicity can be partially or completely prevented by the administration of iron chelators, the antioxidant vitamin E (Cadet et al., 1989), or MAO-B inhibitors (Knoll, 1986), which further supports the role of oxidative stress in 6-OHDA neurotoxicity.

The unilateral 6-OHDA model is the most often used paradigm in preclinical PD research. A unilateral 6-OHDA lesion in the nigrostriatal system produces a hemiparkinsonian state, whereby one side of the body (contralateral to the lesioned side) becomes severely affected while the opposite (ipsilateral) side remains intact, acting as an intrinsic control. Phenotypically, there is an almost complete (>90%) loss of striatal DA levels and a denervation supersensitivity of post-synaptic DA receptors ipsilateral to the lesioned side. If a noradrenergic transporter
blocker is used, 6-OHDA does not pathologically affect other non-DAergic brain regions. Interestingly, despite the contralateral (control) side being unlesioned, there still seems to be alterations in its DA regulation compared to the pre-lesioned state of the animal, which suggests potential compensatory mechanisms that affects both (lesioned and non-lesioned) hemispheres. 6-OHDA toxicity has also been associated with a marked inflammatory response (Rodrigues et al., 2001; Cicchetti et al., 2002). However, LB and LN formation, one of the hallmarks of PD pathology, is not seen in anywhere in the brain.

Behaviourally, unilaterally lesioned animals, with an imbalance in DA activity between the two striata, will display motor stereotypies – asymmetric motor behaviour – in response to the systemic administration of a dopamine agonist (Ungerstedt and Arbuthnott, 1970). Animals challenged with amphetamine, a pre-synaptic DA agonist that will prompt the release of DA from the unlesioned intact side, will preferentially rotate ipsilateral to the lesioned side. Conversely, an animal that is challenged with a post-synaptic DA agonist such as apomorphine (that will act on the lesioned, denervated side) will rotate contralateral to the lesioned side. Spontaneous limb and body posture asymmetry, and deficits in spontaneous and skilled motor movements (Barnéoud et al., 1995; Kirik et al., 1998) are also manifested post-lesion and can be evaluated using various behavioural paradigms (Meredith and Kang, 2006).

Animals with bilateral 6-OHDA lesions have also been shown to exhibit motor disturbances that can be translated to clinical PD. Animals develop postural abnormalities and have a reduced capacity to maintain balance after a destabilizing challenge (Cenci et al., 2002). Reports of parkinsonian-like tremor have been rarely studied in 6-OHDA lesioned animals; however, Schallert and colleagues have observed resting tremor in the forepaws of lesioned animals (Cenci et al., 2002). Spontaneous movement is also greatly reduced, as demonstrated in tests for normal exploratory movement (Cepeda et al., 2007), and a footprint analysis of gait has shown that bilaterally lesioned animals take shorter steps compared to non lesioned animals (Cepeda et al., unpublished results). However, bilateral 6-OHDA lesion models are not commonly used. Even a partial bilateral lesion in rats leads to devastating neurological deficits, often requiring intensive post-operative care to avoid high morbidity and mortality rates.

Apomorphine and amphetamine-induced rotational behaviour has been used to test different transplantation and neuroprotective treatments, and new anti-parkinsonian medications (Schwarting and Huston, 1996). Accordingly, the normalization of rotational behaviour post
treatment and, thus, the restoration of symmetric DA levels, suggests good predictive value in using the 6-OHDA model to test new therapies. There are, however, caveats with using the unilateral 6-OHDA model with regards to other research questions. Although 6-OHDA lesioned animals develop marked degeneration of SNc DAergic neurons, the rate of progression is acute, and the lesion is permanent only when there is a complete (>95%) loss of nigral DAergic neurons. Indeed, partial recovery of function has been reported in the two to three months following 6-OHDA infusion and after incomplete lesions (Blandini et al., 2008). Perhaps most important, one of the defining features of PD, the accumulation of LBs, is absent. Nevertheless, the model is relatively easy to use with large sample sizes, is well characterized, and is an invaluable tool for studying specific research questions, particularly testing the efficacy of new strategies for PD.

1.5.3 Environmental Toxins

Two of the major issues with the classical PD models (described above) are their etiological and construct validity. Both models can accurately duplicate nigrostriatal DAergic loss and associated motor dysfunction, but they are at a disadvantage with regards to studying the etiological mechanisms of PD. Indeed, the etiology of PD has yet to be convincingly established: while a small percentage of PD is due to genetic mutations, the cause of the large majority of idiopathic PD cases has yet to be determined. Recently, there has been a surge of research looking at the role of environmental toxins and complex I dysfunction in PD (Ascherio et al., 2006; Schmidt and Alam, 2006; Hatcher et al., 2008; Cicchetti et al., 2009). The following section delineates some of the environmental pesticides that have been associated with the incidence of PD, which include Rotenone, the herbicide Paraquat, and the fungicide Maneb, and their use as chronic, systemic exposure models for PD.

1.5.3.1 Rotenone, Paraquat, and Maneb: Mechanisms of Action

Rotenone, a chemical that belongs to the family of isoflavones, is naturally occurring in the roots and stems of several plants. It is generally used as a broad-spectrum pesticide and, since it is a naturally occurring product, can surprisingly be used in organic food farming. However, rotenone rapidly breaks down in sunlight, soil, and water, and has a fairly short environmental half-life of one to three days. Rotenone is highly lipophilic: it can easily cross the BBB and pass through DAergic cell membranes without the need for DAT. Intracellularly, rotenone acts as a
potent mitochondrial systemic complex I inhibitor by inhibiting the transfer of electrons from iron-sulfur centers to ubiquinone, leading to increased ROS. More recently, rotenone has also been shown to inhibit proteasome activity in the UPS (Wang et al., 2006).

Paraquat (1,1’-dimethyl-4,4’-bipyridinium) is one of the most widely used herbicides. It is considered a quick-acting, non-selective viologen that specifically kills green plant tissue on contact. Paraquat is structurally similar to MPP⁺; the only difference is the additional N-methylpyridinium group instead of the phenyl group in MPP⁺ (Betarbet et al., 2002). Paraquat is suspected of entering the brain through amino acid transporter (Shimizu et al., 2001). However, it does not easily penetrate the BBB and does so at a much slower rate and in limited quantities. Once in the brain, paraquat undergoes redox cycling with NADPH before being oxidized to produce ROS and induce mitochondrial toxicity.

The fungicide Maneb (manganese ethyl-bis-dithiocarbamate) is used in overlapping geographical regions as paraquat. Though knowledge on its mechanism of action is limited, it has been suggested that maneb can, indeed, cross the BBB, and preferentially inhibit mitochondrial complex III (Cicchetti et al., 2009).

1.5.3.2 Chronic, Systemic Exposure Models for PD

Although the toxic effects of rotenone in the brain have been known for well over 20 years (Heikkila et al., 1985), it wasn’t until 10 years ago that it received the most attention when it was used as a chronic, systemic model for parkinsonism (Betarbet et al., 2000). In this study, rotenone infusion (via osmotic pumps) produced motor deficits reminiscent of many clinical features of PD including hypokinesia, unsteady movement (postural instability), and hunched posture. Rigidity and tremor were also observed, but the severity of these symptoms seemed to be dose related. These motor impairments were a result of complex I inhibition and the selective degeneration of SNc DA neurons. Degeneration (as determined by TH⁺ staining) was dose and duration dependent, and occurred in a retrograde manner. Indeed, only when there was a near complete striatal denervation was there obvious signs of SNc DAergic degeneration. Glutamic acid decarboxylase (indicative of GABAergic medium spiny projection neurons) and acetylcholinesterase (indicative of cholinergic interneurons) levels were unaffected, suggesting a DA specific process. Notably, α-synuclein and ubiquitin-containing inclusions suggestive of LBs were observed in SNc neurons, but it was not determined if these neurons had a DA phenotype.
Several issues have hindered the universal acceptance of the chronic systemic rotenone model for PD. The first is the high mortality rates (from adverse cardiac, stomach, and liver problems) due to marked systemic non-specificity. In this regard, the hunched posture and hypokinesia reported in these studies could conceivably be related to non-specific peripheral abnormalities or from severe treatment-induced illness (Lapointe et al., 2004). Another concern is the variability. In a subsequent study performed by the same group (Sherer et al., 2003), 36% of the rotenone-treated animal developed severe systemic toxicity and had to be removed from the study. Of the remaining animals, only 50% of them demonstrated distinct DAergic lesions. The final concern is the non-specificity within the CNS. Another group who used the same infusion protocol as Greenamyre’s group (Betarbet et al., 2000) revealed that rotenone infusion induced a multisystem degeneration more characteristic of atypical parkinsonism (Höglinger et al., 2003). To address these issues, other routes of administrations have been pursued. A recent study using daily intraperitoneal injections of rotenone (in a specialized fatty-acid based vehicle) reported high reproducibility of their lesions (Cannon et al., 2009). However, many of these observations come from single study reports with limited pathological evidence.

1.5.4 UPS Models

Due to strong evidence supporting the role of the UPS in PD, McNaught and colleagues (McNaught et al., 2004) published a report describing a novel model of PD that used systemic exposure to proteasome inhibitors. In this study, rats were subcutaneously administered with six doses of either 1) the naturally occurring proteasome inhibitor epoximicin, or 2) the reversible synthetic proteasome inhibitor PSI, over a two-week period. These animals developed progressive features suggestive of a parkinsonian syndrome, including hypoactivity, rigidity, abnormal posture, and tremor, all of which improved in response to DAergic medication. Positron emission tomography (PET) found a quantitative decrease in striatal DAT in these animals, which suggests a loss of DA terminals, and post mortem analyses demonstrated striatal DA depletion, SN neuronal death (by apoptosis), and microglial activation. Further, α-synuclein and ubiquitin-positive aggregates resembling LBs were seen accumulating within remaining neurons. Neurodegeneration and aggregate accumulation were also seen within the dorsal motor nucleus of the vagus, locus coeruleus, and nucleus basalis of Meynert, areas with known PD pathology (Braak et al., 2003). Subsequent work reported consistent findings using other UPS
inhibitors including lactacystin (McNaught et al., 2002; Matsui et al., 2010), MG-132 (Sun et al., 2006), or a lactacystin-epoxomicin combination (Fornai et al., 2003).

Despite these positive results, the UPS model for PD remains controversial. In a series of articles in the *Annals of Neurology* in 2006, a number of laboratories failed to replicate McNaught and colleagues’ initial findings (Bové et al., 2006; Kordower et al., 2006b; Manning-Boğ et al., 2006). These laboratories found no discernable differences in pathology, DA content, or motor activity in rats (Bové et al., 2006; Manning-Boğ et al., 2006) or primates (Kordower et al., 2006b). Only 2 studies were able to partially replicate McNaught and colleagues work. Zeng and colleagues (Zeng et al., 2006) reported a 50% reduction in SNC neurons and an increase in α-synuclein- and ubiquitin-positive inclusions. They also observed a qualitative decrease in DA β-hydroxylase-positive neurons in the locus coeruleus and choline-acetyltransferase-positive neurons in the dorsal motor nucleus of the vagus. Behavioural deficits were not detected at 2 weeks, but were seen in subsequent evaluations up to 10 months. However, motor decline was not progressive and could not be reversed by DA agonists. Schapira and colleagues (Schapira et al., 2006) also found significantly reduced DA neurons in the SNC. They were not, however, able to find any significant motor deficits at 4 and 8 weeks. In addition, they were unable to detect any α-synuclein positive aggregates.

These discordant results have been a topic of much criticism (Beal and Lang, 2006; McNaught and Olanow, 2006), and a number of confounding factors have been implicated in the failure of the systemic UPS model, including differences in 1) the properties (solubility, purity, potency) of the proteasome inhibitors from different suppliers, 2) dosage and brain bioavailability, and 3) environmental factors (diet, stress). It must be noted, however, that the discrepancy in results between laboratories described above were only seen after systemic administration of proteasome inhibitors. Indeed, direct stereotactic injections of proteasome inhibitors in the SNC or striatum have consistently induced DA degeneration and inclusion body accumulation in rats, mice, and fish (McNaught et al., 2002; Fornai et al., 2003). At this point, although appealing, the systemic UPS model has yet to be fully accepted as a valid animal model for PD. Taking into account the available evidence of UPS dysfunction in PD, if these confounding results can be resolved and other routes of administration (i.e. intracerebroventricular) can be studied in for longer periods, then the UPS model can have critical implications in future PD research.
1.5.5 Genetic Models

Several animal models have been developed to study the recently identified genetic causes of PD. These include autosomal-dominant models (such as α-synuclein and LRRK2) and autosomal-recessive models (such as parkin, PINK1, and DJ-1) (Fleming et al., 2005b; Dawson et al., 2010). Unfortunately, none of these genetic models have been able to recapitulate all of the defining features of PD. Indeed, the most defining feature of PD, DAergic neuron degeneration, which has been demonstrated in invertebrate genetic models (Drosophila and C. elegans), has generally failed to be replicated in transgenic mouse models of PD. This is well illustrated in α-synuclein models.

1.5.5.1 α-Synuclein

Alpha-synuclein models have been the most widely characterized genetic models of PD. Drosophila overexpressing wild-type or mutated α-synuclein have been shown to exhibit many PD characteristics, including age-dependent DA neuron loss, α-synuclein-containing inclusions, and DA-responsive locomotor deficits (Feany and Bender, 2000; Dawson et al., 2010). C. elegans overexpressing α-synuclein also demonstrated DA loss, but lacked α-synuclein-containing inclusions (Kuwahara et al., 2006). It is not clear, however, whether there is actual DA loss or just a loss of DA phenotype (Pesah et al., 2005). Also, DAergic neuron degeneration in these models was not progressive (Kuwahara et al., 2006).

Several α-synuclein transgenic mouse lines have also been generated. The overexpression of human mutated (A53T or A30P) α-synuclein has been the most widely studied transgenic line and has resulted in variable neuropathological and behavioural phenotypes. Unfortunately, none of these models seem to accurately represent PD. The majority of these models do not show extensive and progressive neurodegeneration of DAergic neurons (Chesselet, 2008). Alpha-synuclein pathology is widespread throughout the CNS but not along the nigrostriatal pathway (van der Putten et al., 2000), and these mice develop abnormal behavioural responses to DAergic agonists (Fleming et al., 2005b). Only a few α-synuclein transgenic mice have shown subtle abnormalities in the nigrostriatal DA system: mice overexpressing double mutated (A30P + A53T) or human wild-type α-synuclein have shown decreased DAT and VMAT density and reduced striatal DA and DA metabolite levels (Richfield et al., 2002; Fleming et al., 2005b). These mice also demonstrated a progressive loss of Nissl-
stained neurons in the SN, but subsequent analyses revealed that the number of TH* neurons remained the same (Thiruchelvam et al., 2004).

Interestingly, despite that lack of a “true” PD phenotype, α-synuclein-overexpressing transgenic mice seem to exhibit phenotypic signs of early PD, which include extra-nigral α-synuclein accumulation and impairments in olfaction and autonomic function (Fleming et al., 2005b; Chesselet et al., 2008). Therefore, these models may represent the early, pre-symptomatic stages of PD.

1.6 RODENT ANIMAL BEHAVIOUR PARADIGMS

The classic rotation test (Ungerstedt and Arbuthnott, 1970) has long been the gold standard for 6-OHDA lesions because it is reliable, objective, and is a direct measure of striatal DA receptor sensitivity in response to the unilateral loss of striatal DA. There are, however, caveats with using drug-induced rotational behaviour in studying both the disease process and new treatments. First, therapeutic improvement is indicated by the reduction in rotational behaviour, which is counterintuitive to the notion that symptomatic improvement in PD patients is obvious without drug induction and measured through the increase in voluntary movements (Meredith and Kang, 2006). Second, rotational behaviour does not linearly correlate with the degree of DA depletion or restoration. Amphetamine has been shown to elicit rotational behaviour with as little as a 55-70% depletion of striatal DA, whereas apomorphine requires a near complete (greater than 90%) depletion in striatal DA to induce rotation (Hefti et al., 1980). Third, it is not known how new treatments may interact with different drugs, as there could be potential adverse interactions between the acute, repeated administration of amphetamine or apomorphine and the new treatment regimen. Finally, the treatment procedures itself may induce false-positive effects due to the stimulation of alternative mechanisms. For example, Corti and colleagues (Corti et al., 1999) found a reduction in apomorphine-induced rotations in both their treated and untreated animals. Both groups of animals also returned to their pre-injection baseline by six weeks. They later concluded that the reduction in rotational behaviour observed in both their groups was due to the inflammatory response to their injection procedure.

In this regard, recent studies have begun to employ non drug-induced behavioural procedures that may have less compounding effects (Meredith and Kang, 2006). These tests have been shown to be independent of practice, past experiences, or food/drink-induced motivation.
The following is a description of the tests used in our laboratory (in place of the rotation test) that have been reported to detect nigrostriatal dysfunction in unilateral 6-OHDA rodent models.

1.6.1 Limb Use Asymmetry (Cylinder) Test

The Forelimb use asymmetry test (Cylinder test) has been previously shown to quantify hypokinesia (Schallert et al., 2000; Tillerson et al., 2001). Animals are placed in a plexiglass cylinder while their behaviour is recorded. Left and right forelimb movements (including rearing, stepping, and exploring) are scored to determine: 1) differences in contact against the cylinder between the unimpaired and impaired forelimb, 2) independent use of either limb during rearing, and 3) percentage use of each forelimb for landing after rearing.

Although this test is a sensitive measure of a unilateral lesion, it does not seem to be sensitive to subtle treatment-induced improvements. Furthermore, with time animals often develop a “preference” in the use of the unaffected forelimb.

1.6.2 Tapered Ledged Beam Walking Test

The Tapered ledged beam walking test measures gross alterations in limb use and coordination (Drucker-Colín and García-Hernández, 1991; Fleming et al., 2004). Each rat is placed on an ascending beam (sectioned off into wide, medium and narrow widths of increasing difficulty) with a wide transparent ledge spanning its entire length. As the animal traverses the beam towards their home cage, the beam narrows and becomes progressively more difficult. Normal animals will have no problems traversing the beam, whereas DA-depleted animals manifest their motor impairment by using the ledge as support for their lesioned hindlimb. Therefore, all lesioned rats gradually make more contralateral limb foot faults (errors) compared to non-lesioned rats.
CHAPTER 2. TREATMENT OPTIONS FOR PD

2.1 EVALUATION METHODS FOR PD SEVERITY AND TREATMENT EFFICACY

2.1.1 Clinical Rating Scales

There are a number of clinical rating scales that evaluate motor impairment and other disabilities in PD patients. However, most scales have not been fully evaluated for reliability and validity, and are subjectively based on a physician’s patient evaluation. Two of the most commonly used (and validated) scales are described below.

2.1.1.1 Hoehn and Yahr (HY) Scale

The HY scale, established in the 1960s, is commonly used to compare groups of patients and establish gross assessments of their disease severity (Hoehn and Yahr, 1967). It is composed of five different stages, each with its own set of criteria (Goetz et al., 2004):

- **Stage 1.** Unilateral involvement only, with minimal or no functional impairment.
- **Stage 2.** Bilateral or midline involvement with no impairment of balance.
- **Stage 3.** Bilateral involvement: disability is mild to moderate with first sign of impaired postural reflexes. Patients are physically capable of leading independent lives.
- **Stage 4.** Fully developed, severely disabling disease: patients are still able to walk and stand unassisted but are markedly incapacitated.
- **Stage 5.** Confinement to bed or wheelchair unless aided.

The HY scale has been used to enroll patients in clinical trials for new anti-parkinsonian treatments. Many new treatments seem to be beneficial only when a PD patient is at a specific HY stage (Goetz et al., 2004). For example, progression to stage 3 was the main reason for the initiation of levodopa treatment (see below) in the past (Goetz et al., 1987). Also, new treatments can be assessed based on its ability to delay the disease progression: treatments can prolong the latency between successive stages by as much as 5 years. Therefore, during any clinical trial, a PD patient can be assessed based on their clinical severity and disability before and during the course of the clinical trial.

2.1.1.2 Unified Parkinson’s Disease Rating Scale (UPDRS)

The Unified Parkinson’s Disease Rating Scale (UPDRS), originally developed in the 1980s, is the most established and clinically used scale for assessing disability and impairment...
It is a comprehensive inventory of PD symptoms, including mood and cognition, activities of daily living, motor performance, muscle rigidity, speech, and gait. The scale itself has four components, which include: I) Mentation, Mood, and Behaviour; II) Activities of Daily Living (ADL); III) Motor; and IV) Complications to therapy. Each component has a score that can be summed up to provide a total score ranging from 0 (normal) to 176 (worst possible).

The main advantage of the UPDRS is that it was developed as a compound scale to assess all the different aspects of the disease. Motor disability (with regards to daily life), and motor impairment (mUPDRS) are individually assessed (Part II and III, respectively), whereas non-motor and cognitive aspects of PD are assessed separately in Part I. The UPDRS has been easy to implement in the clinic: there is a short time requirement (10-20 minutes) for administration, good inter-rater reliability between patients (who self-complete Part I and II of the UPDRS) and the treating neurologists, and can be easily taught easily to other medical personnel. The mUPDRS (in the ON/OFF conditions) is often relied upon for the development of new drug treatments, and is the key component of the Core Assessment Programs for Intracerebral Transplantation and Surgical Interventional Therapies for PD (CAPIT/CAPSIT) for assessing surgical trials for PD.

Despite its long list of advantages, it was recently recommended that the UPDRS be revised to adapt to new advances and resolve problematic areas regarding sensitivity to mild impairment and disability, and to non-motor elements of PD (Goetz et al., 2007). In this regard, the UPDRS retained its four-part structure, but modified the parts to provide a section that integrates the non-motor elements of PD: I) Nonmotor Experiences of Daily Living; II) Motor Experiences of Daily Living; III) Motor Examination, and IV) Motor Complications.

2.1.2 Objective Assessment Methods: Brain Imaging and Positron Emission Tomography (PET) and Single Photon Emission Computerized Tomography (SPECT)

PET and single photon emission computerized tomography (SPECT) are useful tools for the in vivo assessment of nigrostriatal DAergic function. Since its introduction, it has provided valuable insight into some of the mechanisms involved in the disease progression and a means of evaluating new treatment options in PD. For example, $[^{18}\text{F}]$-fluorodeoxyglucose (FDG) imaging, which measure glucose metabolism, was one of the first applied neuroimaging techniques to study PD in vivo (Phelps et al., 1979; Eidelberg et al., 1990).
Currently, several DA-specific markers have since been developed (Au et al., 2005; Biju and de la Fuente-Fernández, 2009), and there are three frequently used types of markers that assess pre-synaptic DA function with PET (Figure 1.1):

1. $[^{18}\text{F}]$-fluoro-L-dopa ($^{18}\text{F}$-DOPA), which is used to estimate the activity of AADC (through the decarboxylation of $^{18}\text{F}$-DOPA to $^{18}\text{F}$-DA), and the subsequent storage into synaptic vesicles,

2. $[^{18}\text{F}],[^{11}\text{C}]$ PET and $[^{123}\text{I}]$CIT SPECT labeled DAT antagonists, such as $[^{11}\text{C}]$-d-threo-methylphenidate (MP), to determine DAT density, and

3. $[^{11}\text{C}]$dihydotetabenazine (DTBZ), to determine VMAT2 density.

There are also a variety of post-synaptic, DA receptor markers that have been used to study DAergic function:

1. $[^{11}\text{C}]$SCH 23390 and $[^{11}\text{C}]$NNC 112 that bind to DA D1 receptors, and

2. $[^{11}\text{C}]$raclopride ($^{11}\text{C}$-RAC) that bind to DA D2 receptors.

Each of these markers have important implications with regards to studying the disease progress, but is not without its shortcomings. For example, $^{18}\text{F}$-DOPA, which was first used in humans in 1983 (Garnett et al., 1983), was found to correlate well with SNc cell counts in humans (Snow et al., 1993); however, AADC is not DA specific and $^{18}\text{F}$-DOPA can undergo metabolism and storage in other monoaminergic systems. VMAT2, similar to $^{18}\text{F}$-DOPA, is often used as a DA marker in the striatum (where >90% of the terminals are DAergic) but is also not specific for DA. DAT markers also have some limitations. Most of the current DAT tracers exhibit some affinity for the serotonergic and noradrenergic transporter. Furthermore, although DAT is DA specific, DAT can be influenced by a number of external factors including striatal DA levels, compensatory mechanisms, and even DAergic medications.

$^{11}\text{C}$-RAC is a DA D2/D3 receptor competitive antagonist that can also be used as a surrogate marker for determining endogenous DA in competition or challenge studies. In these cases, $^{11}\text{C}$-RAC competes with DA for the D2 receptor binding site; therefore, any decrease in $^{11}\text{C}$-RAC binding suggests an increase in endogenous DA and vice versa. Two of the most commonly used D1 receptor ligands, $[^{11}\text{C}]$SCH 23390 and $[^{11}\text{C}]$NNC 112, have shown conflicting data in PD.

Notwithstanding these limitations, the use of the aforementioned PET ligands (in combination with clinical measures) has been fundamental to the study of the disease process.
and compensatory mechanisms and plays a role in early differential diagnoses. PET imaging was initially used as the gold standard for determining the functional efficacy of parkinsonian treatments; however, the discrepancies between in vivo PET data and clinical outcomes have shed doubt on the usefulness of these mentioned ligands for objective evaluations.

2.2 LEVODOPA (L-DOPA) AND OTHER DRUG THERAPIES – CURRENT GOLD STANDARD FOR TREATMENT

2.2.1 L-DOPA

Since its discovery in 1967, levodopa (L-DOPA) has revolutionized the treatment of PD. L-DOPA provides marked symptomatic improvement in virtually all PD patients, positively contributing to patients’ quality of life. To date, it is still the best available treatment for PD.

L-DOPA is an analog of the DA precursor dopa. It can pass through the BBB with ease (DA cannot pass through the BBB, which is why it is not used as a treatment), and it bypasses the rate-limiting enzyme TH, accelerating DA synthesis and replenishment of DA stores (through mass action). Since L-DOPA is administered orally, it must be combined with a peripheral AADC inhibitor (such as carbidopa or benserazide) to increase its bioavailability in the brain by minimizing peripheral decarboxylation in the intestinal walls, liver, kidneys, and brain endothelium.

L-DOPA has always been considered the first treatment option, irrespective of the patient’s age. Most patients can be maintained over the first 5 years of the disease with 300-600 mg/day of L-DOPA, resulting in a 20-70% reduction in motor symptoms (Lees et al., 2009). Bradykinesia, rigidity, and gait disturbance improve within the first few weeks of treatment and steadily ameliorate in the following 3 months (Fahn et al., 2004). Tremor is often more difficult to treat and disappears only in some patients after several years of treatment. Axial symptoms, which include speech disturbances, falling, freezing, standing, posture, and postural instability, may initially improve, but tend to be L-DOPA-unresponsive in the long-term (Hely et al., 2008; Katzenschlager et al., 2008). The most prominent side effects of L-DOPA treatment are nausea and vomiting, which occur through the peripheral decarboxylation of L-DOPA; however, they are generally well tolerated and only occur early in treatment.

Despite the known benefits of L-DOPA treatment, physicians have not always been comfortable prescribing it, often resulting in the delayed administration of L-DOPA. There are
two main areas of concern when initiating L-DOPA treatment. The first relates to whether L-DOPA is toxic to DA neurons, which can exacerbate (or accelerate) the disease process. In vitro studies have shown that high concentrations (>100 µM) of L-DOPA can induce DAergic neuronal death by undergoing auto-oxidation leading to the ROS production (Graham et al., 1978). However, low concentrations (<50 µM) of L-DOPA or cultures that include glial cells are unaffected by L-DOPA toxicity. Moreover, several in vivo studies were not able to confirm these results. Chronic L-DOPA treatment in 6-OHDA-lesioned rats resulted in a small loss of DA neurons in the VTA, but could not be replicated in subsequent studies (Blunt et al., 1993). In fact, in a subsequent study (Datla et al., 2001), chronic L-DOPA treatment was associated with significant recovery of DA neurons, which suggests a possible neuroprotective effect of L-DOPA therapy.

The key clinical trial that examined whether L-DOPA treatment has disease modifying (exacerbating the process) or neuroprotective effects is the “Earlier versus Later Levodopa Therapy in Parkinson Disease” (ELLDOPA) study (Fahn et al., 2004). In this randomized, double-blind clinical trial, 361 early-stage PD patients received 1 of 3 doses of L-DOPA/carbidopa (150/37.5 mg, 300/75 mg, or 600/150 mg) or placebo for 40 weeks followed by a 2-week washout period. Results revealed that L-DOPA treatment was significantly better than placebo: all 3 L-DOPA doses demonstrated behavioural improvement and significantly less worsening of symptoms from baseline to end point when compared to the placebo group. Interestingly, in an adjunct study that looked at the effect of L-DOPA treatment on DAT density, measured with \([^{123}I]\)-2-β-carbomethoxy-3-β-[4-iodophenyl] tropane (β-CIT) SPECT, L-DOPA-treated patients demonstrated a greater decline in DAT density compared to the placebo group. Interpreting these results, however, has proven to be difficult. Some have suggested that the 2-week washout may have been insufficient to eliminate all symptomatic benefits of the treatment, and that the difference between the L-DOPA and placebo groups may have disappeared if the subjects were followed for a longer period of time. It is also possible that DAT imaging may have been affected by prolonged pharmacological treatment through compensatory mechanisms (Au et al., 2005), invalidating DAT imaging as a measure of the disease process. Therefore, future studies are needed to clarify whether L-DOPA therapy is toxic.

The second, and most significant limitation, to chronic L-DOPA therapy is the development of motor complications in late stage PD. Approximately 50-80% of PD patients
develop motor fluctuations and dyskinesia after continuous L-DOPA treatment (Rajput et al., 2002), and this is historically why clinicians have believed that L-DOPA therapy should be delayed until it is really needed. With chronic treatment and disease progression, patients begin to experience a “wearing off” or “end of dose deterioration” of L-DOPA’s duration of symptomatic benefit. Over time, patients start to experience “on-off” phenomena; namely, patients will show sudden signs of tremor, freezing, or rigidity interspersed with phases of mobility in a pattern that cannot be predicted by the L-DOPA regimen.

Advanced-stage PD patients also develop L-DOPA-induced dyskinesia: involuntary choreiform-like movements that affect the head, neck, torso, upper and lower limbs, and respiratory muscles (Olanow et al., 2006). The most common L-DOPA-induced dyskinesias occur when plasma L-DOPA levels and clinical response are at their highest (peak-dose dyskinesia). Clinically, dyskinesias are mild and can be tolerated by the patient; however, they can restrict the amount of prescribed L-DOPA needed for an adequate antiparkinsonian effect. This, in combination with motor fluctuations, can ultimately confound the entire disease progression: L-DOPA’s ability to induce dyskinesia increases over time resulting in the gradual loss of L-DOPA’s therapeutic window (Mouradian et al., 1989) and further disabling the patient’s quality of life.

“Wearing off” effects are a predictable response of any sensitive pharmacological system when it is activated with a strong, but short acting, drug. In this regard, it is not surprising that L-DOPA, with a plasma half-life of 60-90 minutes and a strong antiparkinsonian effect, will eventually lead to changes in the DA system response. Although the mechanism has not yet been fully elucidated, evidence suggests that L-DOPA-induced motor fluctuations are due to L-DOPA’s short half-life and its pulsatile stimulation of DA receptors (Tedroff et al., 1996; Miller and Abercrombie, 1999). The firing pattern of DA neurons conveys information to the brain stem and other cortical regions about what movements to activate and what movements to inhibit. Under normal conditions, DA neurons tonically fire at 3-4 Hz independent of voluntary movement. In response to novel stimuli, their firing pattern changes: DA neurons develop a phasic (or bursting) firing pattern, leading to increased DA release into the synaptic cleft (Grace, 1991). Despite this, synaptic DA concentrations stay relatively constant due to the re-uptake capacity of the DAT. In PD, where this buffering capacity is severely diminished, fluctuations in plasma L-DOPA (due to L-DOPA’s short half life) lead to fluctuations in striatal DA.
concentrations. Therefore, administering repeated doses of L-DOPA results in marked oscillations in synaptic DA availability, leading to pulsatile stimulation of DA receptors.

Several strategies have been developed to combat this pulsatile effect, including long-acting L-DOPA preparations to produce “continuous DAergic stimulation” (Olanow et al., 2004a; Olanow et al., 2006; Nutt, 2007) and COMT inhibitors, which can minimize peripheral L-DOPA metabolism, extend its half-life, and prolong synaptic DA availability (Nutt et al., 1994; Marin and Obeso, 2010). These strategies, however, do not obviate the eventual need of L-DOPA therapy.

Recent reports have suggested that serotonin (5-HT) neurons play a role in L-DOPA-induced dyskinesias (Carlsson et al., 2007; Carta et al., 2007; Carlsson et al., 2009). For example, Carta and colleagues (Carta et al., 2007) demonstrated that serotonergic terminals in the denervated striatum of lesioned rats were responsible for dyskinesias, and that either 1) lesioning the serotonergic afferents or 2) administering 5-HT autoreceptor agonists reduced the dyskinesias. The same group also showed that implanting 5-HT neuron-rich grafts into the rat worsened L-DOPA-induced dyskinesias (Carlsson et al., 2007). Another hypothesis suggests that serotonergic neurons can release DA from synthesized from L-DOPA (Carta et al., 2010) into areas that are not regulated by the DAT. Therefore, it is plausible that the interaction between 5-HT and DA terminals may explain the onset of drug-induced motor fluctuations.

2.2.2 Other Pharmacological Treatments: DA Agonists, MAO-B Inhibitors

DA receptor agonists were developed in the 1970s to help reduce the incidence and severity of motor fluctuations and dyskinesias that resulted from long-term L-DOPA treatment. Bromocriptine, an ergoline D2-receptor agonist, was the first marketed DA agonist that demonstrated clinical benefits in PD patients. Since then, a number of first generation ergoline (pergolide, cabergoline), and more recently non-ergoline (pramipexole, ropinirole, rotigotine) DA agonists and MAO-B inhibitors (selegiline, rasagiline), have been developed as a monotherapy and first-line treatment for PD. Many of these drugs’ disease modifying and neuroprotective qualities have been widely studied and compared to L-DOPA therapy. These treatments, however, do not prevent the need of L-DOPA in the late stages of the disease.
2.2.2.1 Pramipexole

Pramipexole, a DA D2/D3 agonist, has been evaluated in 3 double-blind controlled trials (Hubble et al., 1995; Parkinson Study Group, 1997; Shannon et al., 1997). While the first trial (Hubble et al., 1995) did not report any significant improvements, the two subsequent larger studies revealed improved ADL and mUPDRS scores in patients administered pramipexole (when compared to placebo). The potential neuroprotective and disease modifying effects of pramipexole was evaluated in the large CALM-PD (Comparison of the Agonist Pramipexole With Levodopa on Motor Complications of Parkinson’s Disease) study using the β-CIT imaging to measure DAT density (Parkinson Study Group, 2002). The pramipexole treatment group revealed a significantly slower rate of decline in DAT density compared to the l-DOPA-treated group at 46 months. Although l-DOPA-treated patients had significantly better total and mUPDRS scores midway through the study, there was no difference in treatment efficacy between l-DOPA and pramipexole by the end of the trial.

2.2.2.2 Ropinirole

Two placebo-controlled clinical trials on ropinirole monotherapy have been reported (Adler et al., 1997; Brooks et al., 1998). Both trials reported significant improvement in mUPDRS scores compared to placebo. Furthermore, a large proportion of the placebo group required l-DOPA rescue therapy (Adler et al., 1997). The REAL-PET (Requip as Early Therapy versus l-DOPA-PET) study (Whone et al., 2003), which compared ropinirole to l-DOPA treatment over 2 years, reported a slower reduction in $^{18}$F-DOPA uptake in the ropinirole group, which suggests that ropinirole may be able to slow the progression of PD. However, mUPDRS scores significantly worsened over the 2 years when compared to l-DOPA treatment.

DA agonists are typically associated with better management of treatment-induced motor fluctuations and dyskinesias. For example, ropinirole-treated patients in the REAL-PET study reported less dyskinesia and longer time to dyskinesia onset. However, other studies have shown that early phase PD patients receiving ropinirole or pramipexole monotherapy developed motor fluctuations and “wearing-off” periods within 15-21 months of treatment initiation (Parkinson Study Group, 2000; Thomas et al., 2006).
2.2.2.3 Selegiline

MAO-B inhibitors have also been studied as an alternate drug therapy for PD. They are well tolerated, easy to use (administered only once daily), but are typically less efficacious than either L-DOPA or DA agonists. It was speculated, however, that MAO-B inhibitors could have neuroprotective properties. For example, Selegiline (deprenyl) has been reported to exert neuroprotective effects in various model systems (Mytilineou and Cohen, 1985; Wu et al., 1993; Wu et al., 2000). This was exemplified in the DATATOP (Deprenyl And Tocopherol Antioxidant Therapy of Parkinson’s Disease) study that revealed that selegiline could delay the need for L-DOPA in early PD by 9-12 months (Parkinson Study Group, 1989, 1993). Authors of the DATATOP study initially concluded that selegiline delayed the onset of disability, pointing to neuroprotection; however, it was later recognized that the clinical benefits was due to a confounding symptomatic and pharmacological effect exceeding the drug-washout period (Schulzer et al., 1992; Fowler et al., 1994). Although subsequent follow-up studies have questioned the disease-modifying effects of selegiline, the subject still remains controversial.

2.2.2.4 Rasagiline

Two, double-blind, delayed-start clinical trials that looked at the combined MAO-B inhibitor/iron chelator rasagiline also suggested possible neuroprotective or disease-modifying effects. The delayed-start clinical trial was designed to overcome confounding drug symptomatic effects (as seen in the selegiline trial). With this design, one group is assigned to receive the drug for the entire duration of the study (early-start group), while another group is assigned placebo for the first phase and the active drug for the second phase of the study (delayed-start group). The presumption is that the drugs’ symptomatic effects will stabilize and even out in both groups by the end of the study. However, if there is a disease-modifying (slowing) effect, then the delayed-start group (receiving the placebo) should never catch up to the early-start group.

The TEMPO (TVP-1012 in Early Monotherapy for Parkinson’s Disease Outpatients) trial, which compared 1 year to 6 months of rasagiline therapy, demonstrated that patients in the 1-year (early-start) rasagiline group had significantly better UPDRS scores than the 6-month (delayed-start) treatment group (Parkinson Study Group, 2004), which suggests a disease-modifying effect. In the second, larger rasagiline trial, designated ADAGIO (Attenuation of Disease progression with Azilect Givven Once-daily), PD patients were given either 1) 1 or 2 mg/day rasagiline for 72 weeks or 2) placebo for 36 weeks followed by 1 or 2 mg/day rasagiline
for the next 36 weeks (Olanow et al., 2009b). At the end of the 72 week study, significant improvements were seen in the 1 mg/day early-start group, but there were no significant differences between the placebo group and the 2 mg/day early-start group. Thus, the results of the 1 mg/day early-start group are consistent with a “possible” disease-modifying effect of rasagiline.

Although the authors of the ADAGIO study did a comprehensive job accounting for potential confounding variables, recent authors have commented on the potential rater bias in the latter part of the design (Ahlskog and Uitti, 2010; de la Fuente-Fernández et al., 2010). The “double-blind” label is true for only the first half of the study; for example, a rapid improvement in UPDRS scores at the beginning of phase 2 (as expected) can only mean that the patient was in the delayed-start group, and since clinical scoring at the end of the study is crucial, unblinding the clinician in the latter phase of the study could confound the results.

2.3 LESION TO STIMULATION: EMERGENCE OF DEEP BRAIN STIMULATION

The introduction of L-DOPA and DA agonists initially threatened to make surgical procedures obsolete. However, despite their effective control of motor symptoms in the early stages of the disease, these therapies eventually led to motor fluctuations and dyskinesias that may be as severe as the motor dysfunction of the disease itself. In this regard, due to the pioneering work on the basal ganglia circuitry (Albin et al., 1989; Alexander et al., 1990) and advancements in stereotactic surgical techniques, surgical therapy re-emerged as an additional treatment option for PD.

2.3.1 Surgical Ablation/Lesioning

Renewed interest in surgical treatment began with the use of thalamotomy to treat severe drug-resistant tremor (Tasker et al., 1983). A number of studies reported significant (75-86%) reductions in tremor (Fox et al., 1991; Linhares and Tasker, 2000), with one study reporting tremor reduction in patients who were followed up to 10 years post-thalamotomy (Diederich et al., 1992).

Pallidotomy, which is the destructive lesioning of the Gpi, was subsequently demonstrated to provide marked improvement in tremor, rigidity, bradykinesia, and dyskinesia (Laitinen et al., 1992; Baron et al., 1996; Uitti et al., 1997). For example, significant (65%) improvement in UPDRS scores was seen in 18 pallidotomy patients (Dogali et al., 1995), while
Lang and colleagues (Lang et al., 1997) reported a 28% and 29% improvement in “off” mUPDRS and ADL subscores, respectively, in 40 late-stage patients who underwent posteroventral medial pallidotomy. l-DOPA-induced contralateral dyskinesia also improved and was maintained up to 2 years post-surgery. Ipsilateral dyskinesia also improved, albeit to a lesser degree and only up to 1 year post-surgery.

Subthalamotomy, which lesions the STN, has also been considered, but little data has been published. Gill and Heywood (Gill and Heywood, 1997) were the first to report their results on bilateral subthalamotomy: they observed a 35-50 point reduction in “off” mUPDRS scores in 2 late stage PD patients. Another study reported significant reductions in “off” mUPDRS and ADL scores in 11 patients up to 12 months post-surgery (Alvarez et al., 2001). Four of these patients had prolonged improvement up to 24 months post-surgery; however, dyskinesia did not improve in any of the patients.

Lesion therapies are associated with a number of non-reversible complications. Thalamotomy patients exhibit transient complications with surgery, including contralateral weakness, confusion, aphasia, dysarthria, ataxia, and dystonia (Hallett and Litvan, 1999). Furthermore, thalamotomy does not seem to be effective in treating bradykinesia or gait dysfunction (Tasker et al., 1983). Pallidotomy patients also demonstrate transient side effects, including drowsiness, confusion, and memory deficits. Indeed, a small subset of pallidotomy patients developed permanent memory deficits and mental deterioration (Laitinen, 2000), which has hindered the clinical use of pallidotomy. Subthalamotomy patients have a high risk of developing hemiballismus, which are involuntary “jerking” movements normally seen in the extremities. Although hemiballismus is absent in a large majority of subthalamotomy patients (Alvarez et al., 2001), its potential (as a detrimental side effect) has typically outweighed the beneficial effects of subthalamotomy surgery, and it has minimized subthalamotomy surgery in the clinic.

2.3.2 Deep Brain Stimulation (DBS)

In 1987, Benabid and colleagues (Benabid et al., 1987) discovered that high frequency stimulation (HFS) to the ventral intermediate (VIM) nucleus of the thalamus was able to mimic the effects of surgical ablation and provide marked, long-term benefits reducing tremor. This fueled the development of DBS: a surgical technique where an electrode, connected to an internal pulse generator (much like a pacemaker), is implanted in a target area where it emits
high frequency electrical stimulation that can be telemetrically controlled. Although no formal studies have compared the efficacy of DBS to lesion therapy, DBS has virtually replaced all ablative surgeries because 1) DBS does not involve creating a destructive lesion, 2) the effects are reversible, and 3) the stimulation parameters can be adjusted post-operatively to tailor to the patient’s specific needs and adapt to the ongoing disease process. Because of this, the Food and Drug Administration (FDA) approved DBS as a treatment option in 2002, and it has since been the “gold standard” for surgical interventions in PD.

2.3.2.1 Mechanisms of Action

Attempting to understand the mechanisms of action of DBS has proven to be complex: there is a paradox concerning the manner in which stimulation (traditionally thought to activate neurons) can mimic the effects of ablative surgery on “overactive” basal ganglia regions. Accordingly, there is evidence that supports several potential mechanisms of action of DBS:

1. Inducing a “functional lesion” by inhibiting neuronal cell bodies in close proximity to the electrode. Support for this has mainly come from in vitro slice preparations (Beurrier et al., 2001; Magariños-Ascone et al., 2002). Nonetheless, in vivo cell recordings in the STN and GPi of parkinsonian primates also revealed a time-locked reduction in firing rates in response to HFS (Bar-Gad et al., 2004; Meissner et al., 2005). This was hypothesized to be due to a “depolarization block” from an increase in potassium currents or sodium channel inactivation. However, further in vivo experiments have shown that HFS only reduces (and not completely blocks) neuronal activity and can increase the firing rate of a small subpopulation of STN neurons (Johnson et al., 2008). In this regard, the depolarization block hypothesis has fallen out of favour with most experts in the field.

2. Indirect modulation of neuronal output by activating pre-synaptic terminals that synapse neurons in close proximity to the electrode. McIntyre and colleagues (McIntyre et al., 2004) found that HFS was able to suppress thalamocortical projection neurons by exciting the axonal (inhibitory) terminals innervating them. Similarly, reduced activity was reported in the GPi after HFS in PD patients (who were undergoing exploratory stereotactic surgery to localize the optimal placement of DBS electrodes), which suggests that HFS of the GPi preferentially activates GABAergic afferents, leading to the release of GABA on GPi neurons (Dostrovsky et al., 2000).
3. Alterations in neurotransmitter release. Microdialysis studies have shown significant increases in striatal DA concentrations after STN HFS in 6-OHDA lesioned rats (Bruet et al., 2001). Changes in glutamate and GABA release within the basal ganglia after HFS have also been reported (McIntyre et al., 2004).

4. Activation of fiber tracts that pass through the stimulation field. The STN, for example, is a small structure that is surrounded by several major fiber tracts (Johnson et al., 2008). Incidentally, HFS can sufficiently spread beyond the STN and activate many of these adjacent tracts, including the pallidothalamic fibers within the lenticular fasciculus (Forels’ field H2) (Miocinovic et al., 2006), zona incerta (Plaha and Gill, 2005), and cerebrothalamic fibers (Stover et al., 2005b), all of which have been hypothesized to contribute to the clinical effects of DBS.

5. Stimulation-induced modulation of the basal ganglia network. Typically referred to as an “informational lesion” (Grill et al., 2004), this theory proposes that DBS can override the altered, disruptive pattern of neuronal activity associated with PD with a more regular, “informational” neuronal pattern. It is widely accepted that the firing pattern, rather than the firing rate, is responsible for the pathological state of PD: motor disabilities are associated with the development of pulsatile, oscillatory neuronal activity (Olanow et al., 2006). Thus, it has been suggested that the therapeutic effect of DBS is due to its ability to replace this altered pattern with a more tonic pattern of activity (Hashimoto et al., 2003; Johnson et al., 2008).

These mechanisms are not mutually exclusive, and the therapeutic effects of DBS are likely due to a combination of mechanisms described above. Nevertheless, many studies remain at odds with one another, and there is still significant controversy over the putative mechanisms of action of DBS. It is not until there is a complete understanding of the mechanisms that DBS will reach its full clinical potential.

2.3.2.2 Clinical Effects

There are three current DBS targets for PD: 1) the VIM nucleus of the thalamus, 2) the ventrolateral GPi, and 3) the STN. While DBS of the VIM nucleus has largely been specific to treating tremor, DBS of the STN and GPi have been used to treat the cardinal symptoms of PD as well as reduce drug-induced motor fluctuations and dyskinesia. However, STN and GPi DBS
are less effective in treating gait dysfunction and postural instability; in fact, effectively treating these symptoms seems to depend on how they responded pre-operatively to L-DOPA treatment.

GPi DBS has been shown to improve motor symptoms in a number of studies. GPi DBS seems to be most beneficial in reducing dyskinesia: patients have shown a 50-76% improvement in dyskinesia (Ghika et al., 1998; Volkmann et al., 1998; Kumar et al., 2000) that was maintained up to 4-5 years post surgery (Deep-Brain Stimulation for Parkinson's Disease Study Group, 2001; Volkmann et al., 2004). Cardinal motor symptom improvement has varied between studies, with a reported range of 26-56% improvement in mUPDRS scores. As such, UPDRS scores significantly improved within the first year in PD patients who received bilateral GPi DBS (Ghika et al., 1998; Volkmann et al., 1998; Kumar et al., 2000). However, clinical improvement seemed to decline after the first year (Deep-Brain Stimulation for Parkinson's Disease Study Group, 2001; Volkmann et al., 2004), with some studies reporting a gradual (but sustained) decline in improvement up to five years post-surgery (Jens Volkmann et al., 2004). Some studies reported an improvement in the quality of life after surgery (Rodrigues et al., 2007); however, this effect was likely due to the reduction in dyskinesia.

STN DBS has been the preferred treatment in many centers. Studies have reported a 40-60% improvement in “off” period motor symptoms (Limousin et al., 1998; Herzog et al., 2003; Krack et al., 2003; Deuschl et al., 2006; Wider et al., 2008). In the first patients that received STN DBS (Benabid et al., 1994; Limousin et al., 1995), tremor, rigidity, and limb bradykinesia all improved significantly, and it allowed a 60% reduction in L-DOPA dosage (Limousin et al., 1998). Axial symptoms (particularly gait difficulty, balance, and freezing) also improved with STN DBS, but only if the symptoms were L-DOPA responsive pre-surgery. Dyskinesia also improved over time, with some patients reporting a greater than 50% reduction symptoms (Limousin et al., 1998; Herzog et al., 2003; Krack et al., 2003; Wider et al., 2008).

A meta-analysis of 37 cohorts (comprising 921 patients) (Kleiner-Fisman et al., 2006) reported a 50% and 52% decrease in UPDRS II and III scores, respectively, in the “stimulation-on”, “medication-off” state. There was a 68% reduction in daily “off” periods and a 34% improvement in quality of life (assessed using the Parkinson’s Disease Questionnaire – PDQ-39). L-DOPA medication was reduced by an average of 56%, and dyskinesia was reduced by 69%. In two randomized, clinical trials comparing DBS to ‘best medical therapy’ (Deuschl et al., 2006; Weaver et al., 2009), pairwise comparisons indicated that 71% of DBS patients versus
32% of ‘best medical therapy’ patients had significantly improved UPDRS II and PDQ-39 scores. These improvements were maintained up to five years post-surgery (Krack et al., 2003). It is important to note that not all patients improved: 23 of the 78 pairs (in the pairwise comparison) did not show any significant improvement after DBS when compared to the ‘best medical therapy’ group (Deuschl et al., 2006; Weaver et al., 2009), and 3% of patients in another study (Weaver et al., 2009) progressively became worse. In fact, most long-term studies have reported some decline in improvement over time. Axial symptoms (such as speech, freezing, and postural stability) typically declined in both the “on” and “off” medication state (Krack et al., 2003), and a number of patients developed dementia (Wider et al., 2008).

Although the majority of centers have preferentially targeted the STN with DBS, few studies have actually compared STN and GPi DBS. In a recent study, unilateral STN and GPi DBS had similar effects on mood and cognition, but GPi DBS resulted in an improved quality of life (Okun et al., 2009). The most definitive answer on this subject came from the recently published Co-op study (Follett et al., 2010): 299 patients were randomly assigned to receive either STN or GPi DBS and blindly assessed in the “stimulation-on”, “medication-off” state. Both groups reported similar improvements in UPDRS III scores at the two-year follow-up. The STN group was able to reduce its DAergic medication more than the GPi group. Depression worsened after STN DBS but improved after GPi DBS, and visuomotor processing declined less after GPi DBS. Both groups experienced a similar number of adverse events.

Side effects are frequently reported after DBS. Temporary side effects after STN DBS include dysarthria, changes in mood (confusion and apathy), and weight gain. More severe side effects include increased falls, gait disturbance, depression, and dystonia (Weaver et al., 2009). Many studies have also reported changes in cognitive function: DBS patients declined in verbal fluency, verbal learning and memory, and executive function (Limousin and Martinez-Torres, 2008). The biggest shortcoming associated with DBS is the increased number of adverse effects that are inherent to the procedure. Approximately 40% of DBS patients experienced serious adverse events, the most common being surgical site infection and surgical site pain (Weaver et al., 2009). Most of these infections resulted in antibiotic therapy and lead removal. Moreover, each of the aforementioned double-blind DBS clinical trials has reported a death due to cerebral hemorrhage from lead implantation. In this regard, DBS is a surgical alternative that has
demonstrated symptomatic relief and increased quality of life; however, these benefits need to be carefully weighed against the potential risk of complications for each patient.

2.3.2.3 Future of DBS – “Low” Frequency Stimulation of the PPN

Gait and postural disturbances (and consequent falling) are some of the most disabling symptoms of late stage PD (Hamani et al., 2007). Unfortunately, these symptoms are also some of the most difficult to treat: they only moderately improve after STN or GPi DBS (and only if they were L-DOPA responsive pre-surgery), and they continue to decline even after surgical treatment.

The PPN is a locomotor center that plays a key role in gait modulation and postural control. It is composed of a wide variety of cell types including cholinergic, glutamatergic, GABAergic, and DAergic projection neurons. Interestingly, the PPN is more interconnected with the basal ganglia than any other region of the brain and has reciprocal connections with the GPi, GPe, SNr, DAergic neurons in the SNc, and the STN (Pahapill and Lozano, 2000; Mena-Segovia et al., 2004; DeLong and Wichmann, 2007).

DBS of the PPN can elicit motor behaviour that depends on stimulation frequency. For example, Nandi and colleagues (Nandi et al., 2002) demonstrated that HFS of the PPN in normal primates at high frequencies (>60 Hz) elicited severe akinesia and loss of postural control. Low frequency stimulation (<10 Hz) of the PPN increased motor activity in a parkinsonian monkey (Jenkinson et al., 2004). In light of this evidence, clinical trials were initiated to examine the potential therapeutic effects of PPN DBS (Mazzone et al., 2005; Plaha and Gill, 2005).

A recent study that compared bilateral PPN to STN DBS (Stefani et al., 2007) in six patients found that bilateral PPN DBS (at 25 Hz) produced a 45% improvement in “off” UPDRS score immediately after surgery, which declined to a 32% improvement at 3 and 6 months post-surgery. PPN DBS was most effective on gait and postural control. The combination of PPN and STN DBS in the “on” state provided significant improvement in ADL scores when compared to the benefit of DBS on either target alone. If these findings can be replicated in a larger number of patients assessed over a longer period of time, PPN DBS may be an alternative therapeutic option to alleviate treatment-resistant symptoms in advanced PD.
2.4 CELL THERAPY AND NEURAL TRANSPLANTATION

Cell replacement therapies have been thoroughly investigated in the hope of finding a long-term, continuous DAergic source to treat motor dysfunctions in PD (For recent reviews, refer to Astradsson et al., 2008; Lindvall and Kokaia, 2009; Olanow et al., 2009a). There are numerous reasons why cell replacement therapies are a suitable alternative for treating PD. The pathophysiology of PD has been anatomically well-defined and is surgically accessible. Also, since neuronal loss is predominantly DAergic-specific, cell replacement therapy has an easier task of replacing only a single cell type. For these reasons, several studies have been conducted to test the efficacy of fetal cell transplants. Many of these studies resulted in significant behavioural improvements with evidence of graft survival and striatal reinnervation. Therefore, many important clinical trials were conducted in the 1990s and early 2000s (referenced below) to determine the safety and efficacy of fetal cell transplants in human PD patients.

2.4.1 Fetal Ventral Mesencephalic (FVM) Tissue Transplantation

2.4.1.1 FVM Transplantation: Proof of Principle

Fetal ventral mesencephalic (FVM) tissue transplantation is the most widely studied transplant procedure for PD. Three hundred to four hundred PD patients worldwide have been grafted with FVM tissue; however, long-term follow-up and post-mortem analyses have only been conducted in a small portion of these patients.

Several open-label clinical trials have reported variable clinical improvement in several PD patients (Lindvall et al., 1990; Freed et al., 1992; Lindvall et al., 1992; Sawle et al., 1992; Spencer et al., 1992; Lindvall et al., 1994; Freeman et al., 1995a; Wenning et al., 1997; Hagell et al., 1999; Hauser et al., 1999; Piccini et al., 1999; Brundin et al., 2000; Mendez et al., 2000; Mendez et al., 2005). Spencer and colleagues (Spencer et al., 1992) reported moderate bilateral improvement in four patients who received unilateral FVM transplants (gestational age 7-11 weeks). Three of the four patients were able to lower their L-DOPA dose, but their UPDRS and HY scores were still markedly affected one year post-transplant. Freed and colleagues (Freed et al., 1992) reported moderate behavioural improvement 3-12 months post-transplant in 7 patients receiving unilateral FVM transplants (gestational age 7-8 weeks). These patients demonstrated a reduction in “off” period dyskinesia, a 38% reduction in L-DOPA dosage, and improvement in “on” ADL scores. Increased \(^{18}\)F-DOPA uptake (within the implant site) was also observed in 2
unilaterally implanted patients at 12-13 months and in 1 patient 33 months post-implant (Freed et al., 1992; Sawle et al., 1992). Similar results were reported in bilaterally transplanted patients (Freeman et al., 1995a; Hagell et al., 1999; Hauser et al., 1999; Brundin et al., 2000). These results should be interpreted with caution. Many of these studies had small sample sizes, and their open-label design could potentially be influenced by clinician bias or a patient-induced placebo response.

The open-label studies demonstrated variable, but overall positive, results and have provided proof of principle that cell replacement can work in PD patients. Accordingly, two double-blind, sham surgery-controlled clinical trials were initiated to investigate FVM transplants (Freed et al., 2001; Olanow et al., 2003). In the first study (Freed et al., 2001), 40 patients were randomly assigned to undergo either sham surgery (drill holes without penetrating the dura mater) or bilateral FVM transplantation (gestational age 7-8 weeks) in the posterior tip of the putamen. None of these patients received immunosuppression, and all patients were assessed at 4, 8, and 12 months post-transplant. FVM-transplanted patients showed an 18% reduction in mUPDRS scores at 12 months but were not significantly different from the sham-control patients. Only a subset of younger patients (who were 60 years of age or younger) demonstrated any clinically significant improvement. Notably, 16 of the 19 FVM transplant recipients demonstrated a significant increase in $^{18}$F-DOPA uptake. In two patients who died shortly post-transplant (independent of the treatment), a total of 40,000 and 24,000 grafted DAergic neurons in one patient, and 7,000 and 40,000 grafted neurons the other patient, were counted. These numbers were significantly less than what was found in two patients reported in one of the open-label studies (Kordower et al., 1995; Kordower et al., 1996; Kordower et al., 1998).

In the second sham surgery-controlled clinical trial (Olanow et al., 2003), 34 advanced stage PD patients were assigned to one of three groups: 1) bilateral FVM transplantation in the postcommissural putamen using one donor per side, 2) bilateral FVM transplantation in the postcommissural putamen using four donors per side, or 3) bilateral placebo (partial burr holes that did not penetrate the inner table of the skull). FVM tissue was 6-9 weeks gestational age, and patients received immunosuppressive treatment for 6 months (post-surgery). The timeline of improvement was similar to that observed in the open-label studies: FVM-transplanted patients progressively improved between 6-9 months post surgery; however, after 9 months, the one-
donor group started to deteriorate behaviourally, and the four-donor group improved only slightly. There were no group differences in mUPDRS scores at 24 months. As seen in the previous clinical trial, transplanted recipients (in both the one-donor and the four-donor groups) demonstrated significant increases in $^{18}$F-DOPA uptake.

2.4.1.2 Contributing Factors to Transplant Survival and Function

Collectively interpreting the results of the aforementioned studies has proven to be difficult: variations in patient selection, surgical technique, transplant target selection, and the quality, quantity, and preparation of FVM tissue has made it difficult to formulate direct comparisons across studies. There has been no attempt to standardize the way in which transplantation is carried out. For example, both solid tissue and cell suspension grafts were used in the open-label studies. The two double-blind clinical trials used different types of solid tissue grafts: the Freed study (Freed et al., 2001) used fragmented FVM tissue “strands” that were maintained in culture for up to 4 weeks, whereas the Olanow study (Olanow et al., 2003) used solid FVM grafts that were stored for no more than 48 hours after harvesting. These subtle differences may explain why overall functional outcome differed between the studies.

Tissue immunogenicity may also contribute to graft function. Cell suspension grafts, as the one chosen in the Freed study (Freed et al., 2001), survive by receiving most of their blood supply from the patient. In contrast, solid FVM tissue grafts (Olanow et al., 2003) receive much of their blood supply from their own (donor) blood capillaries. A donor blood supply will significantly induce the expression of human leukocyte antigens, which increases the risk of a prolonged immune response compromising graft survival and function. Interestingly, patient deterioration in the Olanow study coincided with the withdrawal of immunosuppressive treatment six months after surgery. Furthermore, there were prominent signs of an inflammatory response (surrounding the graft) in two patients who were autopsied (Olanow et al., 2003).

In the most successful cases, patients demonstrated major clinical improvement for several years and were able to withdraw l-DOPA (Hagell et al., 1999; Piccini et al., 1999; Brundin et al., 2000). However, other transplant patients have developed atypical behavioural characteristics suggestive of a Parkinson’s-plus syndrome (Spencer et al., 1992; Wenning et al., 1997). Perhaps more worrisome, 15% (Freed et al., 2001; Hagell et al., 2002) and 56% (Olanow et al., 2003) of patients developed “off” period dyskinesia. Three of these patients developed dyskinesia that was so severe that DBS was necessary after the clinical trial was completed.
(Herzog et al., 2008). There are a number of theories as to why FVM transplant recipients developed dyskinesia. Some noticed that “off” phase dyskinesia resembled biphasic dyskinesia, which suggests intermediate DA levels (Hagell and Cenci, 2005). Others hypothesized that small FVM grafts can lead to re-innervating “hot spots” that are surrounded by supersensitive, postsynaptic striatal neurons (Maries et al., 2006). Another theory, in line with recent findings regarding 5-HT influences on L-DOPA-induced dyskinesia, suggests that “off” phase dyskinesia could be due to the diverse cell composition, particularly serotonergic neurons, in tissue grafts (Carlsson et al., 2007; Carta et al., 2007; Carlsson et al., 2009). Finally, there has been debate as to whether graft-induced dyskinesia could depend on chronic inflammatory responses. “Off” phase dyskinesia did not seem to develop until well after patients stopped immunosuppressive treatment. Piccini and colleagues (Piccini et al., 1999) found that immunosuppressive-drug withdrawal at 29 months led to increasingly severe dyskinesia. Interestingly, two immunosuppressed FVM transplant recipients who did not report any “off” phase dyskinesia during their four-year survival had few signs of inflammation post-mortem (Mendez et al., 2005). These findings suggest that immunological mechanisms may be involved in the development of dyskinesia.

2.4.1.3 Pathologic Changes in Grafted DAergic Neurons

Recently, three independent studies reported autopsy findings in eight FVM-transplanted patients (Kordower et al., 2008; Li et al., 2008a; Mendez et al., 2008). Three of the four patients who died 11-16 years after surgery revealed α-synuclein-rich LBs in grafted DAergic neurons (Kordower et al., 2008; Li et al., 2008a). No signs of LBs were seen in the other patient who survived for 14 years after transplantation (Mendez et al., 2008). In contrast, there were no signs of LB pathology in the other four patients who survived for only four or nine years after transplantation (Kordower et al., 2008; Mendez et al., 2008). This suggests that PD pathology is progressive and that at least one decade is required for LBs to develop in young, healthy neurons. Indeed, one patient who received bilateral implants several years apart developed LBs that were more advanced in the older graft (transplanted 16 years ago) than in the newer graft (transplanted 4 years later) (Li et al., 2010). These observations have raised a number of important questions as to how PD pathology propagates from diseased to young, healthy transplanted neurons. These recent findings suggest that the pathophysiological process of PD is not cell autonomous and that disease-affected neurons (or glia) in the surrounding microenvironment may be involved in the
propagation of the disease process to healthy neurons. In particular, the ongoing inflammatory process or the loss of neurotrophic support in PD are two of the suggested mechanisms involved in the pathological spread of PD.

At this point, it is unlikely that FVM transplantation will become a routine treatment for PD. The unfavorable results from the double-blind placebo-controlled clinical trials and the recent discovery of LB pathology in grafted DA neurons suggest that many issues still need to be resolved. Furthermore, the large number of fetal donors that are needed for one patient and the ethical considerations involved in obtaining fetal tissue has hindered the utility of FVM transplantation. Nevertheless, the lessons learned from these studies will be invaluable in devising novel therapeutic strategies for PD.

2.4.2 Embryonic Stem (ES) Cell Transplantation

Despite disappointing results, the FVM clinical trials still provide proof of principle that neuronal replacement can work in PD patients. One of the greatest logistical challenges of FVM transplantation is the limited availability and variable quality of FVM tissue. Moreover, due to the inherent nature of acquiring FVM tissue (from aborted fetuses), tissue availability will always be sporadic. Accordingly, the main interest has shifted to producing DA neuroblasts from human embryonic stem (ES) cells.

2.4.2.1 Stem Cell Generation From Different Sources

Stem cell-derived DA neuroblasts have been generated *in vitro* from several different sources, including:

1. ES cells grown from embryoid bodies and differentiated with various trophic and transcription factors (Lee et al., 2000; Bjorklund et al., 2002; Kim et al., 2006; Cho et al., 2008).
2. Therapeutically cloned ES cells using somatic cells nuclear transfer (SCNT). SCNT is a process whereby the cell nucleus and genetic material is transferred from one cell to another (i.e. unfertilized egg), generating an ES cell line that is genetically tailored to the recipient (Byrne et al., 2007; Tabar et al., 2008).
3. Neural stem cells (NSCs) and progenitors from embryonic or fetal ventral mesencephalic tissue and expanded with different trophic and transcription factors (O'Keeffe et al., 2008; Parish et al., 2008).
4. Adult NSCs from the subventricular zone (Shim et al., 2007).
5. Bone marrow-derived mesenchymal stem cells (MSCs) (Dezawa et al., 2004).
6. Fibroblast-derived induced pluripotent stem (iPS) cells (Wernig et al., 2008).

The differentiation of the aforementioned cells to DA neuroblasts has followed numerous experimental protocols with various combinations of growth and transcription factors. However, there are certain mainstay transcription factors that need to be overexpressed in order to increase the yield of DA neuroblasts with the correct phenotype, including \textit{Nurr1}, \textit{Pitx3}, and \textit{Lmxla} (Kim et al., 2002; O'Keeffe et al., 2008; Friling et al., 2009). Recently, it was demonstrated that mouse ES cell-derived DA neuroblasts could be further enriched to greater than 90% purity using fluorescent-activated cell sorting (Hedlund et al., 2008). These cells were able to re-innervate the striatum of 6-OHDA-lesioned animals; however, enrichment resulted in fewer surviving cells.

A recent and promising development is the demonstration that somatic cells can be reprogrammed to a pluripotent state (Takahashi and Yamanaka, 2006). In 2008, Wernig and colleagues (Wernig et al., 2008) were the first to generate DA neurons from iPS cells (derived from mouse fibroblasts), implant them into 6-OHDA lesioned rats, and improve rotational behaviour. These studies have important implications on the future use of iPS cells in the clinic: the potential generation of patient-specific DA neuroblasts for implantation would eliminate all ethical concerns associated with ES cells and would avoid immune/inflammatory reactions.

\textbf{2.4.2.2 ES Cell Transplantation in PD}

Various studies have demonstrated that implanted ES cell- or NSC-derived DAergic cells can survive and ameliorate behavioural deficits in animal models of PD. For example, mouse ES cell-derived DA neurons were able to survive and partially re-innervate the striatum of 6-OHDA lesioned rats (Bjorklund et al., 2002; Kim et al., 2002). These cells expressed DA-specific markers including TH, DAT, AADC, and calretinin (which is normally co-expressed with TH in the A9 and A10 regions). DA levels increased, and amphetamine-induced rotational behaviour normalized, after grafting. Tabar and colleagues (Tabar et al., 2008) reported similar results using therapeutically cloned ES cells: ES cell implants in parkinsonian mice resulted in improvements in rotational behaviour and the cylinder test and an increase in TH$^+$ neurons at the graft site. Human stem cell-derived DA neuroblasts have also been shown to survive in 6-OHDA lesioned rats (Sánchez-Pernaute et al., 2001; Dezawa et al., 2004; Roy et al., 2006; Cho et al., 2008).
Translation of these therapeutic approaches to the clinic, however, has been slow. To date, no clinical trial has tested ES cell transplantation as a treatment option for PD. Indeed, the very first clinical trial of ES cell transplantation (using ES cell-derived oligodendrocytes to treat acute spinal cord injury), which was approved in January 2009, has been put on hold (Sharp and Keirstead, 2009).

2.4.2.3 Potential Shortcomings of ES Cell Transplantation

One of the biggest concerns that has hindered the use of ES-cell transplantation in the clinic is the risk of tumor formation, which has been observed in animal models of PD (Roy et al., 2006). Several factors contribute to tumor formation, including the presence of undifferentiated cells and chromosomal abnormalities after DA differentiation. This is particularly important in PD: since the life expectancy of PD patients is normal, even a small risk of tumor formation is unacceptable.

The amount and availability of ES cells needed for differentiation could also be a potential drawback. An estimated 200,000 DA neurons per brain are needed for any behavioural recovery (Hagell and Brundin, 2001); considering that the estimated cell survival after transplantation is 5-20% (Olanow et al., 1996; Sortwell, 2003), a large number of DA neuroblasts would be needed for transplantation. The origin of ES cells would also spark ethical debate, as ES cells for clinical use would require human embryonic donors. The use of patient-specific iPS cells could circumvent this issue. However, one potential shortcoming of iPS cells is that in certain disorders (such as PD) in which there is a genetic component to etiology, a patient’s genetic profile might make grafted DA neurons particularly susceptible to the disease process.

Other shortcomings of ES cell therapies are inherent to the manufacture of ES cells. All differentiation protocols use various growth factors and signaling molecules that are of animal origin. The use of animal products in these cultures will create a problem if the cells are to be used in a clinical setting. The specific differentiation into DA precursors/neuroblasts also requires the genetic over-expression of certain transcription factors, which means that genetic modification is needed. This raises both safety and ethical issues. Finally, due to the public reaction to the genetic cloning of Dolly the sheep in 1997, there is a negative connotation and general misconception associated with stem cell research. To this day, stem cell research has sparked considerable ethical debate that has made it difficult for the field to progress.
2.5 Trophic Factor Implantation

Neurotrophic factors are important to the survival and function of several specific cell populations. The potential of these proteins was discovered in 1954, when Cohen and colleagues (Cohen et al., 1954) isolated nerve growth factor (NGF), the first trophic factor shown to exert a protective effect on neurons. Since then, a large number of trophic factors have been investigated in PD, which include (but are not limited to) basic fibroblast growth factor (bFGF), brain-derived growth factor (BDNF), and glial-derived neurotrophic factor (GDNF) (Siegel and Chauhan, 2000).

2.5.1 Potential Mechanisms of Action of PD

Researchers have exploited the neuroprotective and neurorestorative effects of neurotrophic factors as a potential therapeutic option for many degenerative disorders. Some of the therapeutic mechanisms that may be involved in treating PD include (but are not limited to) the following:

1. Neuroprotective effect on degenerating neurons. Although many in vitro studies have demonstrated a reduction in neuronal loss induced by specific toxins (such as MPTP or 6-OHDA), and sometimes an increase in neurite outgrowth from culture neurons, in vivo findings in animal models of PD have been more variable. For the most part, GDNF has been the only trophic factor to consistently demonstrate significant neuroprotection in vivo (Chiocco et al., 2007).

2. Restoration of functional DAergic phenotype. Some have suggested that SNc DA neurons may not necessarily degenerate; rather, they lose their specific phenotype so that they no longer have the capacity to synthesize and release DA. In this regard, the observation that there is a preceding (and greater) loss of striatal DA concentration when compared to SNc DA neurons suggests that SNc neurons may initially be dysfunctional and that trophic factor restores this by altering gene expression and/or protein synthesis (Peterson and Nutt, 2008).

3. Trophic factor loss is a direct cause of PD; therefore, specifically replacing the missing trophic factor would directly treat PD.
The following sections address three of the most studied neurotrophic factors in PD: BDNF, GDNF, and neurturin (which belongs to the GDNF superfamily), with the latter two being studied for their therapeutic potential in human clinical trials.

### 2.5.2 BDNF

BDNF is a member of the large family of neurotrophins (which also includes NGF, neurotrophin 3, and neurotrophin 4/5) that are critical for the development and survival of SNc DAergic neurons (Baquet et al., 2005). BDNF is highly expressed by DA neurons in both the SN and VTA (Seroogy et al., 1994), but in lesser quantities in the striatum (Zhang et al., 2007b). Post-mortem studies have demonstrated decreased BDNF expression in PD brains (Mogi et al., 1999a); however, it is not known whether reduced BDNF is the cause of degeneration or simply represents the loss of BDNF-containing DAergic neurons.

BDNF has been shown to protect DA neurons from 6-OHDA and MPTP-induced toxicity (Hyman et al., 1991; Spina et al., 1992). Fibroblasts engineered to secrete BDNF and implanted in MPTP-lesioned rats markedly increased DAergic neuron survival (Frim et al., 1994). Similarly, astrocytes transduced to produce BDNF ameliorated rotational behaviour in 6-OHDA-lesioned rats (Yoshimoto et al., 1995). Intrastriatal BDNF injections prior to 6-OHDA lesioning produced similar effects (Shults et al., 1995).

There was no significant increase in DA neuron density or detectable expression of BDNF (Yoshimoto et al., 1995) in many of these studies. Furthermore, neuroprotection was more effective on SNc neurons than on striatal nerve terminals and was observed only at areas closest to the site of injection, which suggests that the therapeutic approaches used may not deliver a sufficient amount of BDNF. Despite evidence that BDNF is neuroprotective, no studies suggest that BDNF has a restorative action on the DA system. Consequently, there has not been sufficient evidence to warrant a clinical trial.

### 2.5.3 GDNF and Neurturin

#### 2.5.3.1 GDNF

GDNF belongs to the transforming growth factor (TGF)β superfamily, which includes neurturin, artemin, neublastin, enovin, and persephin (Chiocco et al., 2007). GDNF has important implications in PD: it is highly expressed in the striatum (predominantly on medium spiny neurons that receive DAergic input from the SNc), and it is essential for the postnatal survival of
DAergic neurons (Oo et al., 2005). Increased GDNF mRNA has been shown in PD patients (Bäckman et al., 2006); however, there are no differences in GDNF protein expression between PD patients and control subjects (Mogi et al., 2001). This suggests that GDNF loss is not directly responsible for PD.

Interest in GDNF stemmed from in vitro studies that found that GDNF enhanced DA cell survival and differentiation (Lin et al., 1993) and promoted neuritic outgrowth (Beck et al., 1995; Sauer et al., 1995). Soon after, it was demonstrated that GDNF injected in 6-OHDA lesioned animals prevented DA cell death (Kearns and Gash, 1995; Sauer et al., 1995). Studies of MPTP-lesioned primates also demonstrated GDNF’s antiparkinsonian effects: GDNF infusions in the SN resulted in improved bradykinesia, rigidity, and postural instability (Gash et al., 1996). Post mortem analyses revealed increased SN neuronal size and fiber density and increased DA levels. However, these effects were not sustained after GDNF infusion was discontinued. The withdrawal of GDNF induced a slow deterioration, to the point that the primates returned to their behavioural baseline deficit (Zhang et al., 1997). This suggests a need for a continuous GDNF infusion to maintain beneficial effects.

Elevating GDNF in the brain with gene therapy using adeno-associate virus (AAV) or lentiviral vectors has also been studied (Kirik et al., 2000). GDNF gene transfer in MPTP-lesioned primates has consistently been shown to prevent DAergic degeneration and promote behavioural recovery (Kordower et al., 2000; Eslamboli et al., 2005). Notably, lentiviral-delivered GDNF resulted in an increase in TH+ striatal neurons (Palfi et al., 2002), which suggests that GDNF has the ability to re-convert DA neurons to a DA phenotype.

2.5.3.2 GDNF Clinical Trials

Based on the aforementioned results that suggest that GDNF has neuroprotective and neurorestorative properties, Amgen (Newbury Park, CA, USA) sponsored a randomized, double-blind, placebo-controlled clinical trial that administered recombinant GDNF intracerebroventricularly using mechanical pumps (Nutt et al., 2003). Fifty PD patients received either placebo or GDNF (ranging from 25-4000 µg once a month) over eight months. Sixteen of these patients received an extra 4000 µg of GDNF and were observed for an additional 20 months (using an open-label design). Results demonstrated no improvement in total or mUPDRS scores with any dose. Acute side effects such as nausea, anorexia, and vomiting were common for hours to several days after GDNF injections. Weight loss and hyponatremia was seen in the
higher (>75 µg) dose groups. Parasthesias (described as “electric shocks”) were common in
GDNF-treated patients, independent of the dose received. The open-label extension
demonstrated similar therapeutic efficacy and adverse effects.

Around the same time, an open-label study was conducted that administered GDNF
(through implanted catheters) directly into the posterior putamen of five PD patients (Gill et al.,
2003). These patients showed rapid and marked improvement over the first three months: there
was a 39% and 61% improvement in mUPDRS and ADL scores, respectively, at one year. These
results initiated another randomized, double-blind, placebo-controlled clinical trial administering
GDNF intraputamenally (Lang et al., 2006), the only difference being that the double-blind
clinical trial used a slightly larger catheter diameter. Unfortunately, the double-blind clinical trial
did not find any significant differences in “off” mUPDRS scores when compared to the placebo
group. Adverse effects, this time relating to the device, required the surgical repositioning of
catheters in two patients and removal of the entire device in another patient. Notably, GDNF-
binding antibodies appeared in the serum of one-half of the patients, and GDNF-neutralizing
antibodies developed in a subset of these patients (Tatarewicz et al., 2007). As a result, the
clinical trial was discontinued in 2004. However, many aspects of the aforementioned trials,
particularly trial design, methodology, and analyses, are still being debated in the literature.

2.5.3.3 Neuturin

Neuturin is 42% homologous to GDNF and, like GDNF, it has been shown to enhance
DAergic neuron survival and improve motor behaviour in rodent and primate models of PD
(Hoane et al., 1999; Oiwa et al., 2002; Kordower et al., 2006a). As seen in the GDNF trials, the
route of administration seemed to contribute to neuturin’s efficacy. In a study that compared
GDNF and neuturin (Rosenblad et al., 1999), GDNF provided an almost complete (90-92%)
protection of lesioned SN neurons after striatal or ventricular administration. The neuturin group,
however, provided a 72% protection after striatal administration but no DAergic neuron
protection after ventricular administration. Another desired property of neurotrophic factor
treatments is the ability to promote neuronal regeneration and fiber sprouting. There has been
conflicting evidence with regard to whether neuturin could promote regeneration and sprouting:
while neuturin did not induce sprouting in vitro (Akerud et al., 1999), Oiwa and colleagues
(Oiwa et al., 2002) found thicker and more abundant TH+ fibers in the striatum of neuturin-
treated 6-OHDA lesioned rats.
Based on the aforementioned studies, Ceregene (San Diego, CA, USA) initiated a phase I, open-label study in which AAV-associated neuturin was administered bilaterally into the putamen of 12 PD patients (Marks et al., 2008). The patients showed no serious adverse effects and improvements in “off” mUPDRS scores. Unfortunately, a subsequent double-blind, randomized clinical trial in 58 PD patients found no significant improvement in neuturin-treated patients (Marks et al., 2010). In addition, serious adverse effects were observed in 34% of patients, with three of the patients developing tumors.
CHAPTER 3. HUMAN RETINAL PIGMENT EPITHELIAL CELLS: AN ALTERNATIVE CELL SOURCE FOR THE TREATMENT OF PD

3.1 FUNDAMENTAL PROPERTIES OF HRPE CELLS

Human retinal pigment epithelial (hRPE) cells are neuroepithelial-derived cells that form a monolayer on Bruch’s membrane between the choroid capillary bed and photoreceptor outer segments (Marmor and Wolfensberger, 1998; Strauss, 2005; Bharti et al., 2006). The RPE is essential in maintaining retinal homeostasis: it provides nutritional support, forms the blood-retinal barrier, regulates ion movement (between the choroid and photoreceptors) and oxidative stress, phagocytizes dead or detached photoreceptor organelle components, and helps renew or reassemble these organelles (Strauss, 2005). Indeed, the RPE and retina form a functional unit in which the loss or atrophy of the RPE results in both photoreceptor degeneration and choriocapillary abnormalities (Korte et al., 1984). Apart from their numerous responsibilities, hRPE cells are also extremely robust. Under normal conditions, hRPE cells, once fully differentiated, do not renew themselves and remain viable throughout their lifetime. That is, hRPE cells must perform all of their functions during the lifetime of the individual (which may last over 100 years). For these reasons, it is an understatement that RPE functioning is critical to retinal functioning and sight. Accordingly, its importance explains why RPE transplantation has been closely investigated as a potential treatment option for numerous retinal diseases (da Cruz et al., 2007).

Melanogenesis, the formation of melanin (pigment), has important physiological functions relating to hRPE cells. For example, Ocular albinism, a genetic disease that leads to low vision, is due to the loss of pigment in the RPE (Lopez et al., 2008). Melanosomes in the RPE begin to mature between 8-14 weeks gestation and are viable throughout life (Boulton et al., 2004). Melanin exerts a protective effect on the eye: it absorbs and blocks light coming through the sclera and protects the retina from light-induced damage. In addition, melanin has been hypothesized to act as an antioxidant capable of scavenging ROS (Ostrovsky et al., 1987; Rózanowska et al., 1999). There are, however, age-related changes in melanosomes within the RPE, which include a gradual decline of melanosomes, melanin loss, and lipofuscin accumulation (Boulton et al., 2004). This leads to reduced protection from phototoxicity and diminished antioxidant capacity, which suggests that melanosomes undergo age-related changes.
as a result of oxidative stress (Sarna et al., 2003). Importantly, studies have shown that aged melanosomes are highly photoreactive and can lead to RPE dysfunction, whereas young melanosomes appear to confer protection (Boulton et al., 2004).

More recently, the concept of RPE cells as a therapeutic cell replacement strategy has extended more towards their trophic supporting function. HRPE cells secrete various trophic factors, which include (but are not limited to) pigment epithelial-derived factor (PEDF), BDNF, FGF, and vascular endothelial growth factor (VEGF) (Kociok et al., 1998; Kolomeyer et al., 2011). Trophic factor secretion, however, has only been studied \textit{in vitro} or in its native environment; its expression and release after transplantation has not been fully established. Nevertheless, the co-expression of different trophic factors is an added benefit of hRPE cells (along with the cells’ other mechanisms of action, discussed below).

The immune privilege of the anterior chamber of the eye may also contribute to the potential beneficial effects of hRPE cells when used in transplantation. Under normal conditions, hRPE cells constitutively express low levels of major histocompatibility complex (MHC) class I molecules and do not express MHC class II molecules (Rezai et al., 1997); this suggests that hRPE cell transplants can be immune-privileged tissue. Indeed, RPE grafted onto histoincompatible tissue has been shown to survive (Streilein et al., 2002); further, this effect seems to be related to Fas ligand (CD95L) expression (Wenkel and Streilein, 2000).

Much evidence suggests that hRPE transplantation can be an effective treatment strategy for a number of retinal diseases. The “supportive” capacity of hRPE has not gone unnoticed, and it has garnered interest with respect to treatment modalities other than the eye. The sections below describe how our knowledge of hRPE cells can further the cells’ use as a potential cell replacement alternative for the treatment of PD.

3.2 RPE-CELL PROPERTIES RELEVANT TO THE TREATMENT OF PD

HRPE cells are a potential cell therapy source for PD because of their DAergic properties. Since hRPE cells are melanized cells, they contain the necessary machinery needed to produce melanin. In the RPE, dopa is an intermediate product in the melanin biosynthetic pathway; it is catalyzed by the TH analog tyrosinase (Asanuma et al., 2003) (Figure 3.1). Since tyrosinase-produced dopa can exit the cell through plasma membrane amino acid transporters, hRPE implantation in the parkinsonian brain could provide a continuous alternative source of
dopa to striatal DA terminals (Jimbow et al., 1997; Asanuma et al., 2003). Notably, this is in contrast to FVM and ES-cell transplants that were designed to continuously provide DA.

Although dopa seems to be reoxidized immediately (to dopaquinone) in normal adult functioning, embryonic RPE contains a more stable (and higher) dopa concentration that has been suggested to be the dopa source for retinal development (Kubrusly et al., 2003). There are, however, few published reports on dopa concentrations in fetal RPE cells in situ. A PET study performed in our laboratory provided indirect evidence of dopa release from implanted hRPE cells (Doudet et al., 2004; explained in more detail below). Further, high performance liquid chromatography (HPLC) by Titan Pharmaceuticals showed that hRPE cells used in our studies had dopa concentrations that varied significantly across different hRPE-cell donors (ML Cornfeldt, personal communication).

It has also been shown that hRPE cells possess the molecular machinery involved in regulating DA production and release, including the DA D2 autoreceptor and VMAT2 (Marmor and Wolfensberger, 1998). Recent in vitro work has also suggested that hRPE cells express AADC and are capable of producing DA (and its metabolites) (Zhang et al., 2007a; Ming et al., 2009). However, the production of DA (and its metabolites) is significantly less than the production of dopa (Kubrusly et al., 2003). RPE cells do not express DAT or the DA D1 receptor.

A few laboratories have developed in vitro model systems to study the potential neurotrophic support that hRPE cells can exert in PD. Using a Ca\(^{2+}\)-switch protocol (Rak et al., 2006) that alters the phenotypic properties of hRPE cells, McKay and colleagues (McKay et al., 2006) demonstrated that hRPE-conditioned media can increase neuritic outgrowth and survival of midbrain DAergic neurons in vitro. However, this effect was not due to tyrosinase activity; rather, it was due to the induced production of PEDF. Indeed, PEDF was later shown to be protective in an in vitro model of PD (Falk et al., 2009). Importantly, the neurotrophic potential of hRPE cells depended on the premise that hRPE cells were fully differentiated and pigmented (which was accomplished with the Ca\(^{2+}\)-switch protocol) (McKay et al., 2006). HRPE cells have also been reported to produce BDNF and GDNF (Zhang et al., 2007a; Ming et al., 2009). Accordingly, hRPE-conditioned media, which contained significant levels of BDNF and GDNF, was able to protect FVM DAergic slice cultures from 6-OHDA or rotenone toxicity.
Figure 3.1. Metabolic pathways leading to eumelanin and pheomelanin production in hRPE cells. Adapted from (Ito, 2003) with permission from Pigment Cell and Melanoma Research (John Wiley and Sons©).

Well-established cell culturing techniques are another advantage of hRPE cell use. HRPE cells used in transplantation are isolated from postmortem eye tissue acquired from human banks and can be expanded in tissue culture on a permeable substrate (Figure 3.2) (Davis et al., 1995; Hernandez et al., 1995). There are several advantages to using hRPE cells by expanding primary tissue. First, hRPE cells from a single donor can be expanded to produce the number of cells needed for multiple surgeries: since fetal tissue is always in short supply, expanding primary cultures can treat a large number of patients and eliminate donor availability issues. Second, hRPE cells can be maintained in culture for an extended period of time with minimal loss of viability (Hu et al., 1994). Previous FVM transplantation studies have shown that differences in storage time may impact cell survival and function post-transplantation. For example, storing FVM tissue for longer periods of time in culture resulted in both reduced cell viability and survivability (Olanow et al., 1996). In contrast, HRPE cells maintain excellent viability over time. Moreover, the availability of a large number of cells, and the ease of maintaining hRPE
cells in culture, provides sufficient tissue and time to extensively test each donor for the presence of viral or bacterial contamination.

Figure 3.2. Cell culture characteristics of hRPE-cell lines. Slight differences in growth rate were observed between the 0802 (A-C) and 1105 (D-F) hRPE-cell lines. Photomicrographs were taken at two days (A, D), four days (B, E) and six days (C, F) post-seeding. Scale bars = 25 µm (Note: scale bar in C applies to A-C; scale bar in F applies to D-F).

3.3 HRPE-GM IMPLANTATION AND PD

3.3.1 HRPE Cell Attachment to Gelatin Microcarriers (hRPE-GM)

Gelatin microcarriers (GMs) have been used in cell culturing procedures to enhance cell viability and survival of multiple cell types. Accordingly, cells that are passively attached to biocompatible GMs and transplanted in the brain have demonstrated prolonged survival even in the absence of immunosuppressive treatment (Cherksey et al., 1996; Saporta et al., 1997; Borlongan et al., 1998). FVM cells attached to GMs survived remarkably better than FVM cells alone when transplanted in the rat (Saporta et al., 1997). Similarly, chromaffin cells attached to GMs resulted in significantly better cell survival and improved behavioural recovery (when compared to chromaffin cells alone) when implanted in 6-OHDA lesioned rats (Borlongan et al., 1998). In this regard, GMs appear to circumvent survival issues that are typically observed in cell transplantation studies.
The studies described in this dissertation utilize the following technology: hRPE cells are attached to biocompatible GMs (hRPE-GM) prior to surgery (refer to methods section in Chapter 5). GMs are 40-60 µm diameter crosslinked porcine microcarriers. Since hRPE cells are anchorage-dependent and undergo apoptosis in the absence of a support matrix, GMs provide a way of preventing cell death and enhancing cell survival.

3.3.2 HRPE-GM Implantation in 6-OHDA Model of PD

Few studies have looked at hRPE-GM implants in the rodent 6-OHDA model of PD (Subramanian et al., 2002; Cepeda et al., 2007; Flores et al., 2007; Zhang et al., 2007a; Wang et al., 2008; Ming et al., 2009). Subramanian and colleagues (Subramanian et al., 2002) were the first to demonstrate that hRPE-GM implants can ameliorate behavioural symptoms in parkinsonian rats. In this study, rats were assigned to one of four groups: hRPE-GM group, hRPE-cells alone, GM alone, or tract alone. All four treatment groups initially demonstrated a reduction in apomorphine-induced rotational scores over the first eight weeks post-implant. The hRPE-cells alone, GM alone, and needle-tract alone groups gradually returned to pre-implant baseline levels. Only the hRPE-GM treatment group demonstrated a significant reduction in rotational behaviour. Similar results were reported in other studies (Zhang et al., 2007a; Ming et al., 2009).

Post-mortem analyses revealed surviving cells that “resembled hRPE cells” in the hRPE-GM group. A qualitative analysis also demonstrated minimal inflammatory reactions: few GFAP-positive reactive astrocytes surrounded the implant site, but no MHC class I, MHC class II, or activated microglia staining was observed. There was no evidence of cell survival in the RPE-cell only group.

3.3.3 Primate hRPE-GM Safety and Efficacy Studies

A handful of studies have tested the safety and efficacy of hRPE-GM implants in MPTP-lesioned primates (Subramanian, 2001; Watts et al., 2003). In one study, three MPTP-treated primates were implanted with 10,000 hRPE-GMs per site into five different targets in the left striatum. Two of the primates were periodically examined up to seven months post-implant, and the third monkey was imaged using $^{18}$F-DOPA PET pre- and post-implantation. None of the primates developed any adverse effects. UPDRS motor scores improved for both primates: the first primate improved from 4.75 (at baseline) to 1.75 (at 3 months) and 1 (at 8 months), while
the second primate improved from 9.75 (as baseline) to 4 (at 3 months) and 4.5 (at 6 months). PET (at 6 weeks post-implant) revealed areas of high \(^{18}\text{F}\)-DOPA uptake that co-registered with implant location. A post-mortem histological analysis revealed morphologically characterized hRPE cells attached to GMs at the implant site with minimal inflammatory response to the implants.

Based on these results, in an expanded blinded, placebo-controlled, preclinical trial, 16 MPTP-treated hemiparkinsonian primates were implanted with hRPE-GM (Subramanian, 2001). Each primate was assigned to one of four different groups, receiving hRPE-GM (10,000 cells/target), hRPE-GM (50,000 cells/target), GM alone, or needle tract alone. Each primate received five implant tracts (two in the caudate nucleus, three in the putamen) and was examined for up to 12 months post-implant. All animals tolerated the surgery well without any post-operative complications. A behavioural assessment revealed a 56% improvement in mUPDRS scores in the hRPE-treated group, compared to 16% improvement in the control group (GM alone and needle sham).

Supplementary studies have demonstrated the potential mechanisms that underlie hRPE-GM-induced behavioural improvements. In our laboratory, five MPTP-lesioned primates implanted with hRPE-GM showed a significant increase in \(^{18}\text{F}\)-DOPA uptake and a concomitant decrease in \(^{11}\text{C}\)-RAC binding (Doudet et al., 2004), which suggests increased DA release over the first two months post-implant.

Although a behavioural change was not the primary endpoint of this study, there was a 39% improvement in clinical scores over the first eight weeks: primates showed improvement in bradykinesia, balance and coordination, and daily activities.

In all of the aforementioned studies, primates fully recovered from surgery, and hRPE-GM implants were well tolerated despite the absence of immunosuppressive treatment. Based on these results, the FDA approved an open-label clinical trial of intraputamenal hRPE-GM implantation in PD patients.

3.3.4 HRPE-GM Clinical Human Studies

In 2000, the FDA approved the first open-label clinical trial to study the safety and tolerability of hRPE-GM implants in 6 PD patients (Watts et al., 2003; Bakay et al., 2004; Stover et al., 2005a; Stover and Watts, 2008). All 6 patients were in HY stages 3-4, had bilateral but asymmetric PD, and presented with moderate to severe motor symptoms and varying degrees of
motor fluctuations and dyskinesia. Using MRI-guided stereotactic surgical procedures, each patient received approximately 325,000 fetal hRPE cells attached to GMs. Five tracts, spaced 5 mm apart, were implanted in the postcommissural putamen contralateral to the patient’s most affected side. Each tract was injected with two 25 µl deposits of the hRPE-GM suspension. None of the patients received any immunosuppressive treatment.

All patients were evaluated at 1, 3, 6, 12, 36, and 48 months post implantation. There was a significant 48% and 41% improvement in “off” mUPDRS scores at 12 and 24 months, respectively. Five of six patients maintained improvement (43%) at 48 months; the sixth patient refused to cease taking PD medication the night before. Mean “off” mUPDRS motor scores (± SD) improved from 52 ± 9 (at baseline) to 27 ± 7 (at 12 months), 31 ± 7 (at 24 months), and 28 ± 5 (at 48 months). Motor improvement was more evident contralateral to the implanted side. “Off” state time decreased from 41% at baseline to 30% (at 12 months), 28% (at 24 months), and 35% (at 48 months). “On” state time improved from 44% at baseline to 55% (at 12 months), 65% (at 24 months), and 53% (at 48 months). Total UPDRS scores decreased from 118 ± 14 at baseline to 69 ± 10 at 48 months (Stover et al., 2005a; Stover and Watts, 2008).

ADL scores also improved for up to 24 months post-implant, and the PDQ-39 scores improved from 41.8 ± 11.3 at baseline to 29.2 ± 11.2 (at 6 months), 26 ± 12.2 (at 12 months), 28.6 ± 10.6 (at 18 months), and 25.5 ± 11.5 (at 24 months). Dyskinesia also improved, but the UPDRS IV and Dyskinesia Rating Scale pairwise comparison scores were not significant.

All patients tolerated the surgery well; further, there were no serious adverse effects related to the treatment. The most frequent side effect was headache immediately after surgery; however, it improved on its own in one to two weeks. One patient developed a small hemorrhage after surgery, but it was asymptomatic and subsequently resolved. Another patient developed depression with suicidal ideation 14 months post-implantation. This patient was subsequently treated and the episode resolved after a few weeks. Moderate adverse effects that seemed to be related to treatment were 1) transient increase in peak-dose dyskinesia, and 2) visual hallucinations. Both symptoms, however, improved after the patient’s dosage of DA medication decreased.

Based on the aforementioned results, a multicenter, randomized, double-blind, placebo-controlled clinical trial was initiated. In the STEPS trial (Study of the Safety, Tolerability, and Efficacy of Spheramine® Implanted Bilaterally Into the Postcommissural Putamen of Patients
With Advanced Parkinson’s Disease), 71 patients (separated into three cohorts) received either hRPE-GM (bilaterally implanted with 325,000 cells/side) (N=35) or sham surgery (N=36) (Gross et al., 2011). Each cohort received hRPE cells derived from post-natal donors and none of the patients received adjunct immunosuppression. Patients were assessed at 12 and 24 months post-implant and the primary outcome measure was the mean change in “off” mUPDRS score at 12 months. Unfortunately, there were no significant differences in “off” motor scores between hRPE-GM implanted and sham-surgery patients. Interestingly, both the hRPE-GM and sham groups demonstrated similar (~20%) reductions in “off” mUPDRS scores 12 months post-implant. Mean “off” mUPDRS motor scores (± SD) decreased from 48.8 ± 7.74 (hRPE-GM) and 48.7 ± 9.44 (sham) at baseline to 38.3 ± 10.41 (hRPE-GM) and 38.7 ± 11.38 (sham) 12 months post-implant. A slight reduction in “on” UPDRS ADL subscore was also seen in the hRPE-GM group, but was not significantly different from the sham group. The reduction in “off” mUPDRS scores in both the implanted and sham groups were sustained up to 48 months post-implant. It is important to note that the placebo effect has been suggested to “mask” the clinical effect during the 24-48 months (Goetz et al., 2008). Accordingly, continued observation of these patients is needed.

The laboratory of Bronstein and colleagues (Farag et al., 2009) recently reported post-mortem findings of a single patient who was enrolled in the STEPS trial mentioned above. The patient was a 68-year-old man with an 18-year history of PD who underwent bilateral hRPE-GM implantation. The patient did not report any motor improvement post-implantation and died after a fall leading to pulmonary empyema. The patient’s brain was sagittally cut, separating the two hemispheres. The left hemisphere was processed by Bronstein and colleagues (Farag et al., 2009) and was the purpose of this report. The right cerebral hemisphere was processed and analyzed by Dr. John O’Kusky (University of British Columbia). A post-mortem examination identified three implant tracts; the remaining two tracts seemed to fuse together. HRPE cell survival was very low: a stereological analysis found 118 RPE cells (less than 1% survival), which the authors suggested contributed to the lack of post-implant benefit. An inflammatory reaction, characterized by CD68+ macrophages and activated microglia, was observed infiltrating the implant site; however, the authors noted that there was less inflammation in areas containing hRPE cells.
It must be emphasized that these results are from a single hemisphere and that variable cell survival has been well established in previous cell implantation trials. There have been large differences in cell survival between tracts; for example, cell survival ranging from zero survival to 100,000 cells per tract within the same hemisphere has been reported (Kordower et al., 1996; Mendez et al., 2005). Therefore, more patients must be examined before hRPE-GM cell survival can be adequately assessed.

3.4 RATIONALE AND OBJECTIVES

Unfortunately, even though hRPE-GM implants have already been studied in double-blind, placebo-controlled clinical trials, few reported studies demonstrate the potential beneficial effects of hRPE-GM treatment. Furthermore, although studies have indicated that hRPE-GM implants can ameliorate behavioural deficits (Subramanian et al., 2002; Stover et al., 2005a), there has been no direct indication of in vivo hRPE-GM survival post implant. The only connections that have been made between behavioural improvement and hRPE-cell survival have been through increased $^{18}$F-DOPA uptake with PET in primates (Doudet et al., 2004) and one hemisphere of one patient (Farag et al., 2009). Therefore, it was important to identify specific hRPE-cell markers in order to adequately study cell survival.

When this research commenced, another limitation was the lack of morphological characterization of hRPE-GM. HRPE cells are known to alter their phenotypic characteristics depending on their host microenvironment. Indeed, changing culture conditions was shown to significantly alter the hRPE-cell production of different trophic factors (McKay et al., 2006; Rak et al., 2006). There was also a possibility that hRPE cells could change in response to GM attachment, and since hRPE-GM implantation in the brain has not been studied in detail, we decided to specifically address some of these questions.

Chapter 5 describes the behavioural efficacy of hRPE-GM implants in a rodent model of PD. HRPE-GM was implanted into unilateral 6-OHDA lesioned rats that were tested with non-drug induced behavioural tests to determine whether hRPE-GM implants can ameliorate parkinsonian deficits. The studies described in Chapter 6 were aimed at characterizing hRPE-GM implants in vivo. Our first step was developing immunohistochemical methods of identifying hRPE-GM by testing various hRPE-cell markers in vitro. Once these hRPE specific markers were characterized, 6-OHDA lesioned animals were implanted with hRPE-GM, and we
determined their 1) qualitative survival, and 2) morphological characteristics at different time-points post-implant. We hypothesized that hRPE-GM implants could survive long-term \textit{in vivo} and ameliorate lesioned-induced behavioural deficits in the rat.
CHAPTER 4. INFLAMMATION AND PD

4.1 “IMMUNE PRIVILEGE” AND THE CNS

Sir Peter Medawar, a pioneer in organ transplantation and immunology research, was the first to coin the term “immune privilege”, a term he used to describe a phenomenon where tissue grafts were able to overcome immune rejection when placed in specialized sites in the body (Medawar, 1948). This concept continued to develop in the ensuing years, and numerous sites in the body were identified to be immunologically privileged; that is, exempt from the continual immune surveillance that normally occurs throughout the body. These sites include the eye, gonads (testis and ovaries), placenta (during pregnancy), and the CNS (Barker and Billingham, 1977; Streilein et al., 2002).

The CNS has evolved into an immune privileged site due to a number of anatomical and physiological factors, which include the following:

1. The CNS is protected by the blood-brain barrier (BBB): a network of microvascular endothelial cells (interconnected by tight junctions) at the vasculature, choroid plexus, and meningeal surfaces that restrict the passage of most peripheral blood borne cells into the CNS (Prat et al., 2001).
2. The absence of professional antigen presenting cells (APCs) (such as dendritic cells) in the brain, which normally assist immune surveillance (Aloisi et al., 2000).
3. The lack of a lymphatic system and perivascular space (except in the arachnoid and subarachnoid space, and the Virchow-Robin spaces) that would normally facilitate antigen delivery to the lymph nodes (Kreutzberg, 1996).
4. The low constitutive expression of MHC molecules, particularly MHC class-II, in the brain parenchyma, which are required to present both self and foreign antigens to CD4+ T cells in order to initiate an immune response (Darnell, 1998).

These traits imply that the CNS has no intrinsic immune system and, assuming an intact BBB, is separate from the general immune system that surveys the rest of the body. It is understandable why the CNS is an immune privileged site: the inflammatory process is potentially much more damaging in the brain when compared to everywhere else in the body. Conventional inflammation (as seen in the periphery) involves rapid immune cell recruitment, the production of pro-inflammatory cytokines, and inflammatory edema. These processes would
undeniably lead to a disruption in neuronal functioning and the death of post-mitotic, irreplaceable neurons (Orr et al., 2002). Accordingly, the brain has developed in such a way as to limit the frequency of potentially deleterious inflammatory reactions.

**4.2 INFLAMMATORY PROCESSES IN THE BRAIN: HOW IS THIS ACHIEVED?**

Until relatively recently, the general consensus was that the CNS benefited from “absolute” immunological protection and bypassed peripheral immune surveillance. However, this concept has changed: the immune system and the CNS do interact, albeit in a much more tightly regulated matter. For example, Cserr and colleagues (Harling-Berg et al., 1989; Cserr et al., 1992) were able to induce a robust immune response to a protein antigen microinjected into the cerebrospinal fluid (CSF) (with an intact BBB). Furthermore, Aloisi and colleagues (Aloisi et al., 2000) demonstrated that activated T cells continuously circulated, albeit slowly, throughout a healthy CNS parenchyma and traveled back towards the cervical lymph nodes. These observations suggest that 1) the BBB is not completely impenetrable, 2) the CNS has access to a lymphatic system that can present CNS antigens to the lymph nodes, and 3) the CNS does give way to regular scrutiny from the immune system (Perry, 1998; Orr et al., 2002). However, the CNS does not contain any professional APCs; therefore, what cell types mediate the inflammatory process in the CNS?

Microglia and astrocytes are two of the major cell types that contribute to the inflammatory process within the CNS. These glial cells have been traditionally thought to have different and seemingly opposing roles: while astrocytes were thought of as passive support cells that provided general homeostatic control of the extracellular environment, microglia are generally known to act as scavenger cells actively involved in the removal of cellular debris. Recent studies, however, now show that both microglia and astrocytes are involved in a much wider range of neuronal function. The following sections describe the roles of microglia and astrocytes in CNS inflammation and inflammatory processes in PD.

**4.2.1 Microglia and CNS Inflammation**

Microglia are the resident immunocompetent cells in the CNS. First described by Del Rio Hortega (del Rio-Hortega, 1932), microglia constitute approximately 10% of the cell population in the brain. Microglia are heterogeneously distributed within the CNS: they are predominantly found in the grey matter, with the highest concentrations found in the hippocampus, olfactory
telencephalon, basal ganglia, and SN (Lawson et al., 1990). The current consensus is that microglia are derived from mesodermal cells. Microglial precursors (or blood monocytes) enter the developing CNS (from the bloodstream, ventricles, and meninges) during embryonic development and differentiate into resident microglia (Cuadros and Navascués, 1998; Raivich et al., 1999). Indeed, microglia express many of the same surface antigens found on bone marrow-derived macrophages (Flaris et al., 1993; Kim and de Vellis, 2005).

Microglia mediate innate immune responses in the CNS. They mount a graded response to any form of CNS disturbance (trauma, infection, invading pathogens, stroke, neurodegeneration) by undergoing a series of phenotypic changes to become activated (Kreutzberg, 1996; Raivich et al., 1999; Wojtera et al., 2005). In their resting state (in the healthy brain), microglia take on a downgraded phenotype: they possess a ramified morphology (small cell body with numerous slender processes) and show very little expression of macrophage membrane-associated ligands and receptors including leukocyte common antigen (LCA) (also known as complement receptor CD45), CD14, Mac-1 (CD11b/CD18), and Fcγ (Ford et al., 1995; Kreutzberg, 1996). MHC class-II expression is also very low and seems to be restricted to some microglia (particularly around the white matter) (Aloisi et al., 2000). Even in their “resting” state, microglia are highly sensitive to changes in the surrounding microenvironment. For example, Nimmerjahn and colleagues (Nimmerjahn et al., 2005), using time-lapse microscopy, demonstrated that microglia constantly extended and retracted their ramified processes randomly sampling their extracellular space. This enables them to react at a very early stage in response to injury, and their activation often precedes any action from any other neuronal cell types (Streit et al., 1988; Perry et al., 1993).

Microglial activation is observed as early as 24 hours in response to any CNS disturbance (Kreutzberg, 1996; Raivich et al., 1999). Their cytoskeletal structure changes: their cell body enlarges and their ramified processes withdraw, developing more of a macrophage-like appearance (at this point they are now considered as amoeboid microglia). Microglia can also undergo mitosis, which increases their CNS numbers (Raivich et al., 1999). Functionally, they have elevated immunoglobulin (IgG) reactivity and upregulated expression of complement receptors and cell adhesion molecules including lymphocyte function associated antigen 1 (LFA-1), intercellular adhesion molecule (ICAM)-1 (CD54), vascular adhesion molecule (VCAM)-1 (CD106), Mac-1, and CD1. At this early stage of activation, microglia are thought to play a
homeostatic role by scavenging cellular debris and releasing trophic factors that promote neuronal survival (Aloisi, 1999; Nakamura, 2002; Orr et al., 2002). With continued activation, activated microglia adhere to neurons by means of their attraction to chemokines such as monocyte chemoattractant protein-1 and interferon (IFN)-inducible protein-10 expressed on the neurons themselves (Aloisi et al., 2000; Aloisi, 2001). Microglial transformation continues with the upregulation of inflammatory glycoproteins such as CD40, B7.1 (CD80) and B7.2 (CD86), which are potent stimuli for inflammatory cell activation (Aloisi et al., 2000). At this stage, microglia possess an immunological capacity that is similar to that of professional APCs.

Microglia can lose their activation properties and revert back to their resting state at any stage of their response if the pathologic stimulus disappears (Orr et al., 2002). However, under certain circumstances, microglia can become overactivated and mature into macrophage-like cells and become phagocytic. Indeed, at this stage, microglia begin to express large amounts of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), IFN-γ, and interleukin (IL)-1β (Raivich et al., 1999), and other cytotoxic factors including ROS and nitric oxide (Hirsch and Hunot, 2009). In addition, microglia can also recruit passing T-cells and other monocytes that express receptors for LFA-1 and ICAM-1, which themselves release further pro-inflammatory cytokines. The combination of these factors leads to even greater microglial activation and additional inflammatory cell (leukocyte) recruitment, perpetuating a cycle of sustained neuroinflammation and eventual neuronal death.

4.2.1.1 Perivascular Macrophages

Perivascular macrophages (or pericytes) comprise the other population of bone marrow-derived microglia (Hickey and Kimura, 1988; Graeber and Streit, 1990). They are located in between the neural parenchyma and endothelial cell layer in the Virchow-Robin spaces (Orr et al., 2002). There are two general characteristics that make pericytes distinct from microglia. First, unlike microglia that are permanent and scattered throughout the parenchyma, pericytes are continuously re-populated with circulating monocytes in the perivascular spaces (Williams et al., 2001). Second, pericytes constitutively express MHC class II molecules (which is upregulated in response to injury) (Streit et al., 1989) and possess significant phagocytic capacity compared to the relatively immature microglia. Indeed, perivascular cells, which are efficient APCs for intracerebral antigens, are critical in graft rejection within the CNS (Hickey, 2001; Barker and Widner, 2004).
4.2.2 Astrocytosis

Astrocytes are highly differentiated, neuroectodermal-derived, glial cells that play a key role in normal CNS functioning (Sofroniew, 2009). In the healthy CNS, astrocytes have a spongiform morphology with several fine processes that give rise to many other finely branched processes. They tile the entire CNS in a contiguous, non-overlapping manner, developing boundaries with adjacent astrocytic domains. (Sofroniew and Vinters, 2010). Similar to microglia, astrocytes are heterogeneously distributed. In fact, within the midbrain, astrocytic density is low in the SNc but high in the VTA or A8 DAergic cell group (Damier et al., 1993).

There are two main subtypes of astrocytes: protoplasmic astrocytes, which are found in the grey matter and envelop neuronal cell bodies and synapses; and fibrous astrocytes, which are found in the white matter and wrap their fine processes around the nodes of Ranvier (Sofroniew and Vinters, 2010).

One of the hallmark cellular responses to CNS neuropathologies is reactive astrogliosis, which is, by definition, the proliferation and hypertrophy of astrocytes in response to injury. However, astrogliosis is not an all-or-none response; similar to microglia, astrocytes mount a progressive response that varies with both the severity and type of insult (Sofroniew and Vinters, 2010). In mild to moderate astrogliosis, reactive astrocytes undergo variable changes in molecular expression and functionality. Morphologically, they become hypertrophied but maintain their non-overlapping domains (Wilhelmsson et al., 2006). In severe cases of astrogliosis, astrocytes become hypertrophied and proliferate, and scar formation occurs along the borders of extreme tissue damage and inflammation. Also, astrocytes no longer occupy separate domains and begin to overlap their hypertrophied processes with each other (Sofroniew, 2009). Importantly, the molecular and functional changes associated with moderate astrogliosis offers the potential for full normal restoration if the triggering insult is resolved. In contrast, the changes associated with severe astrogliosis and scar formation are persistent (even after the insult has been resolved). These differences highlight the importance of the degree of injury and its impact on full CNS recovery.

Mature astrocytes are characterized by their expression of the 8-9 nm intermediate filament glial fibrillary acidic protein (GFAP). Antibodies raised against GFAP are the most frequently used marker for immunohistochemical preparations (Eng et al., 2000). However, GFAP is not an absolute marker for non-reactive astrocytes. GFAP is often not
immunohistochemically detectable in astrocytes in the healthy CNS, nor is it a reliable marker for determining an astrocyte’s true cellular morphology. It is also important to note that rodent astrocytes are smaller and less structurally complex than human astrocytes. Nevertheless, relevant to this dissertation, reactive astrocytes exhibit prominent increases in GFAP immunoreactivity. An increase in GFAP expression has been classically interpreted as cellular hypertrophy and is necessary for reactive astrogliosis and scar formation (Pekny and Nilsson, 2005).

4.3 INFLAMMATION IN THE ETIOLOGY OF PD

4.3.1 Patient Studies

4.3.1.1 Reactive Microgliosis

The first indication of a neuroinflammatory process in PD came from post-mortem studies. McGeer and colleagues (McGeer et al., 1988) reported an increased number of activated microglia (human leukocyte antigen [HLA]-DR positive) in the SN of PD patients. Immunoreactive staining using additional markers for activated microglia, including β2-microglobulin (the light-chain of MHC) (Mogi et al., 1995), EBM11 (CD68) (Banati et al., 1998), and ferritin (Mirza et al., 2000), also revealed microgliosis in the striatum and SN. Increased levels of pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-1β, and IL-6, as well as inducible nitric oxide synthase (iNOS) and cyclo-oxygenase (COX)-2 (enzymes involved in the production of ROS), have also been found in the striatum, SN, and CSF of PD patients (Mogi et al., 1994; Blum-Degen et al., 1995; Hunot et al., 1996; Müller et al., 1998; Nagatsu et al., 2000).

Further support comes from in vivo PET imaging studies that looked at microglial activation with the peripheral benzodiazepine receptor binding ligand [$^{11}$C]-PK11195 (Ouchi et al., 2005; Gerhard et al., 2006). Elevated ligand binding was shown in the pons, basal ganglia, and the frontal and temporal cortical regions in PD patients (Gerhard et al., 2006). Unfortunately, the limited PET resolution prevented the SN from being analyzed. However, there were no changes in [$^{11}$C]-PK11195 binding when these patients were longitudinally followed for two years. This suggests that microglial activation occurs earlier in the disease process (either in parallel with DAergic loss or at clinical manifestation) and remains activated throughout the disease (Gerhard et al., 2006). Therefore, the production of pro-inflammatory cytokines and
chemoattractant molecules (that leads to the continuous infiltration of activated microglia) may contribute to disease progression.

The most intriguing evidence that inflammation may contribute to PD comes from epidemiological studies of the effects of anti-inflammatory drugs on PD (Chen et al., 2003; Chen et al., 2005; McGeer and McGeer, 2008). In a large prospective study of hospital workers, there was a 46% decrease in the incidence of PD in those who chronically used over-the-counter non-steroidal anti-inflammatory drugs (NSAIDs) compared to non-NSAID users (Honglei et al., 2003). Similar results (a 35% lower risk) were reported in a follow-up study that looked at the selective COX inhibitor ibuprofen (Chen et al., 2005). This, combined with evidence of the potential beneficial effects of anti-inflammatory drugs in animal models of PD (described below), has garnered increasing interest (Esposito et al., 2007) and raises the possibility that early intervention with NSAIDs or other anti-inflammatory therapies may be neuroprotective in PD.

4.3.1.2 Reactive Astrogliosis

The role of astrocytes in PD etiology has been a little more difficult to elucidate. Astrocytes have been shown to be neuroprotective because they (1) detoxify ROS and (2) secrete neurotrophic factors such as GDNF (Vila et al., 2001); therefore, it is thought that astrocytes would help alleviate the disease process. Interestingly, reactive astrogliosis has been reported to be largely absent in PD (Banati et al., 1998; Mirza et al., 2000). This has been met with some controversy, as Damier and colleagues found a 30% increase in astroglial density in PD patients (Damier et al., 1993). Furthermore, ICAM-1 was overexpressed in reactive astrocytes in the SN of PD patients (Miklossy et al., 2006). Recent findings have also suggested that astrocytes may be associated with the increased accumulation of PD gene products α-synuclein and parkin (Halliday and Stevens, 2011).

4.3.2 Animal Models

The intimate connection between microglial activation and DAergic degeneration in animal models of PD was demonstrated using the bacterial endotoxin and potent microglial cell activator lipopolysaccharide (LPS). LPS-induced microglial activation resulted in specific DAergic loss in the SN and a parkinsonian-like syndrome in experimental animals (Castaño et al., 1998; Herrera et al.). Since then, several animal models of PD have been shown to present
activated microglia. The microglial response in two of the most commonly used animal models of PD, MPTP and 6-OHDA, are described below.

4.3.2.1 MPTP

Langston and colleagues (Langston et al., 1999) found a marked microglial response in MPTP-induced parkinsonian patients several years after exposure. Increased activated microglia was also confirmed in MPTP models of PD in mice (Liberatore et al., 1999) and primates (McGeer et al., 2003; Barcia et al., 2004). Increased expression of MHC class I and II antigens (Kurkowska-Jastrzebska et al., 1999), pro-inflammatory cytokines IL-1β and Il-6 (Nagatsu et al., 2000), and iNOS (Liberatore et al., 1999), were found in the striatum and SN of MPTP-treated mice. ICAM-1 and LFA-1, which are important in sustaining inflammation (Collins et al., 1994), were also elevated (Miklossy et al., 2006). Microglial activation however, seems to vary with the method of administration. Indeed, microglial activation is predominantly seen in response to acute MPTP exposure but is often more difficult to induce after a subchronic dosing schedule. However, there are some exceptions to this: reactive microgliosis was observed in primates that received MPTP subchronically (over several months) (McGeer et al., 2003).

Microgliosis seems to play a direct role in MPTP-induced neuronal toxicity rather than as a by-product or late response to the neurotoxin itself. MPTP toxicity is significantly decreased in transgenic mice deficient in pro-inflammatory mediators, including COX-2 (Feng et al., 2002), iNOS (Wu et al., 2002), and TNF-α (Sriram et al. 2002). Minocycline, a tetracycline derivative that inhibits microglial inhibition, has been shown to reduce (in a dose-dependent manner) DAergic neuronal loss after MPTP exposure in mice (Wu et al., 2002). Similar results were seen after co-treatment with other anti-inflammatory medications including dexamethasone (Kurkowska-Jastrzebska et al., 1999) and the COX-2 inhibitor meloxicam (Teismann and Ferger, 2001).

4.3.2.2 6-OHDA

Microglial activation is also believed to contribute to 6-OHDA toxicity. The stereotaxic administration of 6-OHDA has been shown to increase the number of activated microglia in the SN (He et al., 2001). PET using [11C]-PK11195 revealed increased binding in the striatum and SN after 6-OHDA lesioning and was confirmed post-mortem up to 4 weeks post-injection (Cicchetti et al., 2002). Elevated levels of TNF-α, IL-1β, and Il-6 were also detected in the
striatum and SN (Mogi et al., 1999b; Nagatsu and Sawada, 2006). As seen with MPTP, 6-OHDA-induced toxicity can also be attenuated with minocycline (He et al., 2001) and other anti-inflammatory drugs.

However, results from this model must be interpreted with caution: the intracerebral injection of 6-OHDA can cause a transient opening in the BBB which can lead to an influx of peripheral inflammatory cells. Indeed, a peak microglial response typically occurs at 6-10 days but gradually subsides and disappears by 30 days post-injection (Akiyama and McGeer, 1989).

4.4 THE BRAIN AS A TRANSPLANTATION SITE: IMMUNE SYSTEM CONSIDERATIONS

A combination of factors (some of which were explained above) contribute to neural graft survival and the intensity of the inflammatory (rejection) response. Some of these include the immunological disparity between the donor tissue and the recipient, the type of graft and the location of the graft, and the possible neurotrophic and/or anti-inflammatory factors that the donor tissue may express. The importance of each of these factors is described below.

4.4.1 Immunological Disparity

The immunological disparity between the donor tissue and recipient plays an important role in immune response and rejection (Barker and Widner, 2004; Chidgey et al., 2008). Cells that are targeted by the immune system express MHC molecules (also known as HLA molecules in humans). MHC class I molecules are present in almost all nucleated cells, whereas MHC class II molecules are expressed mainly on APCs. Both classes of protein can initiate an immune response if their antigens are presented to T lymphocytes. This can be achieved in two ways. Donor-derived APCs can directly present their self-antigen and activate the host immune response. Alternatively, host APCs (such as pericytes or activated microglia) can take up donor antigens (either whole or fragmented molecules) and indirectly present them to T lymphocytes (Gould and Auchincloss, 1999). However, it is not known which form of antigen presentation is likely used in neural transplantation.

Other antigens that may be involved in immune rejection include ABO blood-group antigens, H antigens, and fetal antigens. ABO (and other related blood-group) antigens can elicit strong immune responses in FVM and hRPE-GM implantation; however, there is less of a concern with ES cell transplantation because ES cell lines can be selected from a universal (O
blood group) donor (Chidgey et al., 2008). H antigens, although less immunogeneic, have also been shown to induce rejection (Robertson et al., 2007). Similarly, fetal antigens that are expressed by donor cells may also initiate a response when presented to the adult immune system. However, it is important to note that the presentation and matching of MHC class I/II antigens is the rate-limiting step in graft rejection.

The phylogenetic difference is also important in neural transplantation studies. Allogeneic transplant immune responses, where tissue is taken from a donor of the same species as the recipient (i.e. human donor to human recipient), are mediated mainly by MHC I/II expression. Similarly, in concordant transplants between two closely related species (i.e. human donor to primate recipient), immune reactions are mediated through a combination of MHC molecules and xenogeneic peptides (Barker and Widner, 2004). Graft rejection in both allogeneic and concordant transplants is mediated mainly by T-cell activation and microglia. Conversely, in discordant xenografts between distantly related species (i.e. human donor to rodent recipient), the innate immune response involves the complement system and natural killer T cells (in addition to microglial activation), which often leads to hyper-acute rejection (Barker and Widner, 2004). In this “worst-case” scenario, graft survival is often very low.

4.4.2 Graft Type and Graft Location

Two main graft types have been used in neural transplantation: cell suspension grafts and solid tissue grafts. Cell suspension grafts are typically revascularized by host endothelial cells and blood vessels. Solid tissue grafts, which are implanted with intact blood vessels, form connections with the host vasculature. Solid tissue grafts do not survive as well as suspension grafts due to the increase in MHC presentation from the donor vasculature.

Graft location is also an important factor that contributes to graft survival. Graft survival is poor in regions close to the cerebral ventricles where antigens can be transported (via CSF-draining pathways) along the ventricular system and drain to cervical lymph nodes (Aloisi et al., 2000). Grafts implanted deep in the brain parenchyma (such as the striatum) and relatively distant from the ventricular system seem to improve survival (Barker and Widner, 2004).

4.5 RATIONALE AND OBJECTIVES

It has been suggested that hRPE-GM implants induce a significantly reduced immune response in the brain (Subramanian, 2001). However, few studies have addressed this. The
studies described in Chapter 7 were designed to quantify the host inflammatory response to hRPE-GM implanted in unilateral 6-OHDA lesioned rats. We assessed cellular inflammation by 1) quantifying ED1\(^+\) macrophages/activated microglia, and 2) measuring the reactive astroglial depth in response to implantation. We hypothesized that hRPE-GM implants can induce a significantly reduced host inflammatory response when compared to GM-alone implants.
CHAPTER 5. HRPE-GM IMPLANTS AMELIORATE MOTOR DEFICITS IN ANIMAL MODELS OF PD

5.1 INTRODUCTION

HRPE cells have been investigated as a potential alternative source of cell therapy. Early studies of hemiparkinsonian primates demonstrated significant improvements in UPDRS scores over a 12-month period (Subramanian, 2001), while hRPE-implanted PD patients continued to show behavioural improvement 4 years post-implant (Stover and Watts, 2008). In our laboratory, behavioural improvements in parkinsonian primates were still observed 4-5 years after unilateral hRPE-GM implants (DJ Doudet, unpublished observations).

Only few studies have demonstrated the behavioural effects of hRPE-GM implants on rats. Using the unilateral 6-OHDA animal model and the drug-induced rotation, Subramanian and colleagues (Subramanian et al., 2002) demonstrated a significant improvement in apomorphine-induced rotations after hRPE-GM implants over an 18-week period. This model, although widely used for pharmacological testing, is suggested to be at a disadvantage: the repeated intermittent exposure to the drug may lead to unknown effects of the drug on hRPE cells which possess a DA D2 receptor (Marmor and Wolfensberger, 1998). Further, questions have been raised as to the reliability of the model (Meredith and Kang, 2006). In order to avoid some of the uncertainties associated with the drug-induced rotation model, we elected to use two tests of spontaneous sensorimotor function that are independent of learning, practice, handling, or motivation and that evaluate normal spontaneous rodent behaviours such as rearing and walking (Drucker-Colín and García-Hernández, 1991; Schallert et al., 2000; Tillerson et al., 2001; Fleming et al., 2004).

The studies described in the following chapter investigated the behavioural effects of hRPE-GM implants on forelimb-use asymmetries and hindlimb motor deficits in a severe unilateral and moderate bilateral 6-OHDA-lesion model of PD in rats. We report here that hRPE-GM implants ameliorated contralateral forelimb disuse and hindlimb deficits in 6-OHDA lesioned rats.

5.2 MATERIALS AND METHODS

5.2.1 Animals

Adult male Sprague-Dawley rats (N=24) (University of British Columbia Animal Facility) weighing 275-300 g were used for these experiments. Animals were housed in pairs in Plexiglas cages with ad libitum access to food and water. Animals were maintained under a 12:12 h light-dark cycle (lights off from 12:00 to 24:00 h) with constant temperature (22°C) and relative humidity (55%).

Rats were assigned to two groups. Group 1 (N=13) consisted of rats that were unilaterally 6-OHDA lesioned. Of these animals, seven were implanted with hRPE-GM and six were implanted with GM-alone. Group 2 (N=11) consisted of rats that were bilaterally 6-OHDA lesioned (see protocol below). Of those, seven were implanted with hRPE-GM, and four with GM-alone. Based on test-retest variability in previous (pilot) studies, a minimum group of six animals would have been sufficient to detect a biologically meaningful (30%) lesion size (compared to baseline) with 80% power (5% two-sided significance).

A subset of animals was also used to assess lesion severity with autoradiographic binding (see below). The majority of animals from Group 1, plus additional animals that also underwent 6-OHDA lesioning and implantation, were used to study the post-mortem histological identification of surviving hRPE cells (described in the next chapter).

5.2.2 6-OHDA Lesioning

After completing baseline behavioural testing (see below), animals underwent either unilaterally or bilateral intracerebroventricular (ICV) 6-OHDA lesioning. All surgical operations were performed under isoflurane anesthesia (Aerrane; Baxter, Mississauga, ON). Isoflurane was set at 4% with a 60 mL/min oxygen flow for induction and 1.5-2% for maintenance. Atropine sulfate (0.05 mg/kg) (Rafter, Calgary, AB) was injected subcutaneously to decrease respiratory secretions. Rats were placed in a Kopf stereotaxic frame with the skull flat between bregma and lambda.

Specific details of our lesioning procedure can also be found elsewhere (Cepeda et al., 2007). Briefly, 6-OHDA (10 µg/4 µl in 0.05% ascorbic acid in sterile 0.9% saline) (Sigma-Aldrich) was stereotaxically infused at a rate of 1µl/min into the SNC and MFB at the following coordinates: AP: -2.8; ML: -1.8 (from bregma) DV: 8.0, and AP: -4.7; ML: -1.5 (from midline);
DV: 7.9 (Paxinos and Watson, 1997). The injection cannula was left in place for an additional 4 min to allow for diffusion.

For bilateral ICV 6-OHDA lesions, animals received 175 µg of 6-OHDA in 3.5 µl (infused at 1 µl/min) in each of the lateral ventricles at the following coordinates: AP: -0.8; ML: +/-1.4 (from bregma); DV: 3.8 (Paxinos and Watson, 1997). Each animal was administered desipramine HCl (24 mg/kg, dissolved in sterile H₂O, injected intraperitoneally) (Sigma-Aldrich, Oakville, ON) 30-60 minutes prior to surgery to protect noradrenergic terminals.

At the end of each surgical procedure, each rat received subcutaneous normal saline or lactated ringer’s solution (for rehydration), analgesics (Anafen; Merial, 5 mg/kg, injected subcutaneously) and antibiotics (Duplocillin; Intervet, 90,000 I.U./kg, injected intramuscularly). All animals were monitored daily and allowed to recover for a minimum of 3 weeks prior to surgical implantation. All animals typically lose weight during the first few days (for up to a week) post-lesion. Animals that did not regain their weight (compared to pre-surgical levels) before implantation were euthanized.

Animals that were bilaterally lesioned were allowed to recover up to 10 weeks. This recovery time was longer than usual and was needed to accommodate the longer recovery times of bilaterally lesioned animals.

5.2.3 HRPE-GM Preparation and Surgical Implantation

The development of in vitro hRPE-cell culturing and in vivo implantation procedures was part of the focus of our adjunct study and is described in more detail in the following chapter. Briefly, cryopreserved hRPE cells were rapidly thawed, resuspended in fresh cell culture medium and then centrifuged and transferred to an un laminated T-25 cell culture flask. To maximize cell viability, hRPE cells were grown to confluence in an incubator at 37°C prior to attachment to GM and implantation. Dry GMs were hydrated and autoclaved for sterility. The prepared hRPE cells were added to the GM suspension and the mixture was placed in an incubator at 37°C overnight. Immediately prior to implant, cell viability was assessed using the trypan blue exclusion method (minimum required cell viability: 80%; 2000-3000 cells/µl). GM-alone suspensions were treated in a similar manner. All hRPE-GM and GM-alone slurry preparations were kept on an ice bath during the implantation procedure for a maximum storage time of five hours.
Surgical implantation procedures are described in detail in the next chapter. Briefly, unilaterally lesioned animals were implanted in the lesioned striatum. Bilaterally lesioned animals were implanted in the striatum contralateral to the most affected side of the body (as assessed behaviourally). Animals were unilaterally implanted with hRPE-GM or GM-alone in the striatum. Each animal received two tracts (6 µl volume injected into each tract) at the following coordinates: AP: +1.6; ML: -2.5 (from bregma) (tract 1); and AP: -0.4; ML: -3.5 (from bregma) (tract 2) (Paxinos and Watson, 1997). HRPE-GM and GM-alone implants were injected at two depth levels in each tract: -6.0 and -4.0 mm below the skull. All animals in this study were implanted with the 0802 hRPE-cell lot (Table 5.1).

<table>
<thead>
<tr>
<th>hRPE-Cell Line</th>
<th>Donor Age</th>
<th>Cell Concentration (hRPE cells/µl)</th>
<th>Cell Viability Pre-Implant</th>
<th>Cell Viability Post-Implant*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0802 hRPE</td>
<td>22 weeks</td>
<td>2200</td>
<td>94%</td>
<td>82%</td>
</tr>
<tr>
<td>Male donor</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Harvested: 2001</td>
<td></td>
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<tr>
<td></td>
<td>3232</td>
<td>86%</td>
<td>81%</td>
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<td></td>
<td>2300</td>
<td>88%</td>
<td>80%</td>
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<td></td>
<td>2508</td>
<td>91%</td>
<td>N/A</td>
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<tr>
<td></td>
<td>2290</td>
<td>90%</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

*Table 5.1. HRPE-cell lines used in Chapters 5 and 6. HRPE cell concentrations and pre-implant cell viability were assessed immediately prior to surgical implantation (minimum required cell viability: 80%; 2000 cells/µl). Cell viability of the remaining hRPE-GM suspension (after surgery) was calculated at the end of the surgical day. *HRPE-cell lots with no post-implant viability were used for in vitro purposes.

We elected not to implant hRPE-GM in normal (control) rats for a few reasons. First, previous work (by Titan Pharmaceuticals) found no behavioural effects in normal primates implanted with hRPE-GM (ML Cornfeldt, unpublished observations). This is consistent with expected effects: since the primary mechanism of action of hRPE cells is the release of dopa, one would expect no hRPE-induced effect in a normal animal. Indeed, studies in normal animals and human subjects have shown no behavioural clinical response aside from the peripheral effects (e.g. nausea) of large doses of L-DOPA. Second, our own MRI study (described in Chapter 8),
which was conducted in normal rats, showed no behavioural effect in response to hRPE-cell implants.

5.2.4 Behavioural Testing

Behavioural testing was performed within the first six hours of the dark cycle when animals were more active. Group 1 (unilaterally 6-OHDA lesioned rats) were tested pre-lesion, 3-10 weeks post-lesion (before implant) and at 8-10 and 18-20 weeks post-implant. Bilaterally lesioned rats (Group 2) were tested pre-lesion, 10 weeks post-lesion (before implant) and at 2-4 and 8-10 weeks post implant. Bilateral animals were part of our initial methodological development study and were sacrificed 10 weeks post implant. Due to the significant amount of post-operative care needed (post-lesion) to maintain bilaterally lesioned animals, we decided to alter the study design and performed the remainder of the study in unilaterally 6-OHDA lesioned animals (Group 1). In addition, our preliminary analysis of the bilaterally 6-OHDA lesioned animals (Group 2) led us to extend the survival time of Group 1 to 20 weeks post-implant. Behavioural testing was also performed 10 weeks post implant in the unilaterally lesioned animals for data comparison between the 2 groups.

5.2.4.1 Forelimb Function - Forelimb Use Asymmetry Test (Cylinder Test)

The forelimb use asymmetry test was performed as previously described (Schallert et al., 2000; Tillerson et al., 2001). Testing was performed under red lighting conditions. This was done so that the experimenters could see without compromising the animal’s dark cycle (as Sprague-Dawley rats cannot see red light). Rats were placed in a transparent cylinder and exploratory motion was videotaped for 3-5 minutes per session for a total of 2-4 sessions (a maximum of 2 sessions per week on non-consecutive days). Scoring was performed at a later time by an experimenter blind to the animals’ condition. Each particular forelimb movement (contralateral, ipsilateral or both) was expressed in terms of percentage use (mean ± SEM) of that limb relative to the total number of movements (i.e. % contralateral forelimb use = 100 x [contra independent movements] /[(contra + ipsi + both)]. A single score representing forelimb use asymmetry was obtained by calculating the difference between [contra % use] (impaired/implanted) and [ipsi % use] (non-impaired/non-implanted); therefore, the greater the negative score, the greater the asymmetry and vice versa.
5.2.4.2 Hindlimb Function - Ledged Tapered Beam-Walking Test

The hindlimb Ledged Tapered Beam Walking Test has been previously described in detail (Schallert et al., 2002; Schallert and Woodlee, 2005). Briefly, the apparatus consists of a tapered beam with underhanging ledges on each side. The ledges allow the measurement of ‘foot faults’ (steps on the ledge) without the risk of falling for the rat. The beam is divided in 3 sections: wide, medium and narrow, each of which represents progressive levels of difficulty. The addition of the ledge reduces the learned compensation mechanisms reported in early studies in PD and stroke rodent models. Rats with unilateral 6-OHDA lesions use the contralateral ledge as a crutch while bilaterally lesioned animals may even straddle the beam. Training (which consisted of 10 trials) was done only once on the day prior to animal’s first testing day (pre-lesion): each animal was allowed to walk along the beam to their darkened home cage. Each test consisted of 5 consecutive trials (spaced 2-3 minutes apart). The animals remained in the home cage between each trial. Testing was done with the lights on during the dark part of the animals’ light/dark cycle so that the animals could be videotaped. All trials were scored at a later date by an investigator blind to the experimental groups. The rats’ performance was calculated as the slip percentage of the impaired hindlimb (contralateral to the implant and/or the lesion): the \[
\frac{\text{total number of slips (foot faults)}}{\text{total number of steps}} \times 100\%
\] over the 5 trials for each beam section.

5.2.5 Post-Mortem Autoradiography

Lesion severity was determined in a small sample of each group using \[^{3}\text{H}]\text{WIN 35,428}\ binding to the DAT in the striatum. For unilaterally lesioned animals, percent lesion was measured with respect to the non-lesioned hemisphere. Bilaterally lesioned animals percent lesion was compared to a vehicle-infused sham lesion group. The animals were sacrificed by decapitation and their brains removed and frozen in isopentane cooled to -70°C in dry ice. The brains were then stored at -80°C until sectioning. Binding to the DAT using \[^{3}\text{H}]\text{WIN35,428}\ was performed on 20 µm sections as previously described using phosphor imaging autoradiography (Strome et al., 2005). Striatal binding was measured on at least six sections per animal, and after subtraction of non-specific binding, the average specific binding data for each striatum was converted to a percent lesion.
5.2.6 Data Analysis

One-way repeated measures analysis of variance (ANOVA) with planned comparisons was conducted to compare post-implant behavioural test performance to pre-implant test performance. When significant F values were found, planned comparisons using Bonferroni's comparison test were used to determine implant effect (post-lesion compared to each of the two post-implant time points) with $p$ values < 0.05 considered to be statistically significant.

Pre-lesion data was not included in the repeated ANOVA analysis as the main goal of this study was to determine the effects of hRPE-GM implants in a lesioned striatum. When compared to the lesion effect, the implant effects would be expected to be discrete and overshadowed by the lesion effect. By analogy, data from the pre-clinical condition are rarely included in the analysis of therapeutic effects in clinical studies. The lesion effect was, however, evaluated separately performing a t-test between pre-lesion and pre-implant.

5.3 RESULTS

All unilaterally lesioned animals recovered well after 6-OHDA lesions. There were no signs of morbidity, mortality rate was low (<10%), and minimal post-operative care was needed. Bilaterally lesioned animals demonstrated different recovery characteristics. There was increased morbidity and mortality rate was higher (20-25%) compared to the unilateral lesion group. Bilaterally lesioned rats needed considerable post-operative care; each rat was given daily subcutaneous fluids, and the most severe rats were manually (tube) fed with a high protein liquid diet. However, there were no differences in mortality rate in unilaterally- and bilaterally-lesioned animals in response to the implant. The shortened experimental period of the bilaterally lesioned group (8-10 weeks versus 18-20 weeks in the unilaterally-lesioned group) was due to the initial study design, and not due to changes in mortality rate due to the implant.

5.3.1 Behavioural Effects of hRPE-GM Implants in a Unilateral Rat Model of PD

5.3.1.1 Forelimb Function

Prior to implant, both hRPE-GM and GM-alone implanted animals displayed a significant lesion effect in the cylinder test demonstrated by a decrease in contralateral forelimb use ($p < 0.001$), an increase in ipsilateral forelimb use ($p < 0.002$), and a decrease in simultaneous (both) limb use ($p < 0.003$) during vertical exploration (Figure 5.1).
A repeated measures ANOVA revealed a significant implant effect on the mean percentage use of the contralateral forelimb in hRPE-GM implanted animals \((F_{2,12} = 4.64; p = 0.03)\). No effect was seen in GM-alone implanted animals \((F_{2,10} = 0.81; p = 0.47)\). Post hoc planned comparisons revealed a significant increase in mean percentage use of the contralateral forelimb in hRPE-GM implanted animals at 18-20 weeks post implant \((p < 0.05, \text{Figure 5.1A})\), but no significant differences were found at 8-10 weeks post implant, or between pre-lesion and post-implant scores.

HRPE-GM implanted animals showed a slight improvement in mean percent \((100 \times \frac{\text{post-implant %use} - \text{post-lesion %use}}{\text{pre-lesion %use} - \text{post-lesion %use}})\) simultaneous forelimb use at 8-10 weeks \((7.20 \pm 6.77\%)\) and 18-20 weeks \((35.57 \pm 14.88\%)\) post-implant. However, these scores did not reach significance \((p = 0.11)\). No improvement in mean simultaneous forelimb use were seen in GM-alone implanted animals: -11.77 ± 7.08% and -4.37% ± 10.63% at 8-10 weeks and 18-20 weeks, respectively (Figure 5.1B). There were no differences in ipsilateral forelimb use in both the hRPE-GM and GM-alone groups.

Forelimb use asymmetry scores increased (towards the negative side) post-lesion in all unilaterally lesioned animals in favor of the unimpaired (ipsilateral) forelimb \((p < 0.001)\). Forelimb use asymmetry scores went from -14.22 ± 11.22% pre-lesion to -93.58 ± 4.61% post-lesion. There was a progressive decrease in forelimb use asymmetry post-implant in hRPE-GM implanted animals (scores became less negative). This decrease did not reach statistical significance at 8-10 weeks \((6.96\% \text{ mean improvement from base line})\), but was statistically significant at 18-20 weeks post-implant \((19.98\% \text{ mean improvement from baseline, } F_{2,12} = 4.68; p = 0.031)\). GM-alone implanted animals did not show any significant improvement between post-lesion and any of the post-implant time-points \((-3.0\% \text{ mean improvement from baseline at 8-10 weeks; and } -4.49\% \text{ at 18-20 weeks})\).

### 5.3.1.2 Hindlimb Function

All animals displayed a strong lesion effect: there was a significant increase in the mean percentage of errors/steps of the contralateral hindlimb on the narrow section of the beam post lesion \((p < 0.002)\) in unilaterally lesioned rats. However, no significant lesion effect was seen on the wide or the medium beam sections (Figure 5.2). There was a progressive decrease in the mean percentage of errors/steps of the contralateral limb in all beam sections in the hRPE-GM
group post-implant (Figure 5.2A). A significant decrease in errors was observed at 18-20 weeks post-implant, where animals made significantly less errors/steps with the contralateral hindlimb on the narrow section, compared to post-lesion ($p < 0.05$, Figure 5.2A). No significant differences were found between post-lesion and the earlier (8-10 weeks) time point. GM-alone implanted animals did not show any significant changes on the contralateral hindlimb percentage of errors/steps in any of the beam sections post implant (Figure 5.2B).

![Graph A](image)

**Figure 5.1.** Forelimb use asymmetry of hRPE-GM (N=7) (A) and GM-alone (N=6) (B) implanted animals with severe unilateral 6-OHDA lesion. A significant increase in the independent use of the contralateral forelimb was seen at 18-20 weeks post-implant. No behavioural recovery was seen in the GM-alone group. Bars represent mean percentage use ($\pm$ SEM) during vertical exploration in the cylinder. * $p < 0.05$; *** $p < 0.001$. Reprinted with permission from the Journal of Neuropathology and Experimental Neurology.
Figure 5.2. Hindlimb function of hRPE-GM (N=7) (A) and GM-alone (N=6) (B) implanted animals with severe unilateral 6-OHDA lesion. There was a trend in improvement (reduced number of errors) at all levels of the ledged beam test; however, a significant decrease in errors was only found at the narrow section at 18-20 weeks post-implant. Bars represent mean percentage of errors/steps (± SEM) on the tapered ledged beam-walking test. * p < 0.05; ** p < 0.01. Reprinted with permission from the Journal of Neuropathology and Experimental Neurology.

The percentage of improvement from baseline (pre-lesion) was calculated for both hRPE-GM and GM-alone implanted rats, using the contralateral hindlimb percentage of errors/steps for each beam section and time point in the following formula: 100 x [post-lesion – post-implant]/[post-lesion – pre-lesion] (Table 5.2).
5.3.2 Behavioural Effects of hRPE-GM Implants in a Bilateral Rat Model of PD

5.3.2.1 Forelimb Function

HRPE-GM implanted animals showed an implant effect on the mean percentage independent use of the forelimb contralateral to implant ($F_{2,12} = 3.77; p = 0.05$). No effect was seen with the ipsilateral forelimb ($F_{2,12} = 0.983; p = 0.40$). Post hoc planned comparisons revealed no significant difference in contralateral forelimb use 2-4 weeks post-implant, but a significant increase in contralateral forelimb use 8-10 weeks post-implant compared to post-lesion values ($p < 0.05$; Figure 5.3A). No significant implant effects were seen on the independent use of the contralateral forelimb ($F_{2,6} = 0.15; p = 0.86$) or ipsilateral forelimb ($F_{2,6} = 0.04; p = 0.96$) of GM-alone implanted animals (Figure 5.3B).

There was an implant effect on forelimb use asymmetry scores of hRPE-GM implanted animals ($F_{2,12} = 3.84; p = 0.05$), but not of GM-alone implanted animals ($F_{2,6} = 0.04; p = 0.96$). HRPE-GM implanted animals showed a gradual shift in forelimb use asymmetry post-implant in
favor of the contralateral forelimb. This effect did not reach statistical significance at 2-4 weeks, but became significant at 8-10 weeks post-implant ($p < 0.05$) compared to post-lesion values. Forelimb use asymmetry scores of hRPE-GM implanted animal at the four different time-points were: 6.95 ± 7.88% (pre-lesion), -30.24 ± 13.74% (post-lesion), -17.57 ± 14.86% (2-4 weeks post-implant), and 8.4 ± 19.56% (8-10 weeks post-implant). Corresponding values for the GM-alone implanted animals were: 16.64 ± 1.01% (pre-lesion), 10.33 ± 23% (post-lesion), 13.72 ± 24.21% (2-4 weeks post-implant), and 10.42 ± 33.89% (8-10 weeks post-implant).

5.3.2.2 Hindlimb Function

Bilaterally lesioned animals revealed a moderate increase in the percentage of errors/steps with both hindlimbs, predominantly in the medium and narrow sections of the beam. HRPE-GM implanted animals showed a progressive decrease in the percentage of errors/steps with both limbs post-implant, but was primarily seen in the contralateral hindlimb. This effect reached statistical significance 8-10 weeks post-implant ($p < 0.05$, Figure 5.4A). GM-alone implanted animals did not show a decrease in the contralateral hindlimb percentage of errors/steps (Figure 5.4B). The percentage improvement from baseline (pre-lesion) is shown in Table 5.2.

It should be noted that, in both unilaterally- and bilaterally-lesioned animals, there was only partial behavioural recovery post-implant; that is, the mean motor performance of hRPE-GM implanted animals did not return to pre-lesion levels in either behavioural test.

5.3.3 Post-Mortem Analysis of Lesion Severity

Unilaterally lesioned animals developed a > 90% lesion. Bilaterally lesioned animals showed a 50-60% reduced binding to DAT bilaterally in the dorsal striatum, following a medial-lateral gradient (Figure 5.5).
Figure 5.3. Forelimb use asymmetry of hRPE-GM (N=7) (A) and GM-alone (N=4) (B) implanted animals with a moderate bilateral 6-OHDA-lesion. A significant increase in independent use of the contralateral forelimb was seen at 8-10 weeks post-implant. No behavioural recovery was seen in the GM-alone group. Bars represent mean percentage use (± SEM) during vertical exploration in the cylinder. * $p < 0.05$. Reprinted with permission from the Journal of Neuropathology and Experimental Neurology.
Figure 5.4. Hindlimb function of hRPE-GM (N=7) (A) and GM-alone (N=4) (B) implanted animals with a moderate bilateral 6-OHDA lesion. A significant decrease in errors (compared to post-lesion scores) was seen at 8-10 weeks post implant. Bars represent mean percentage of errors/steps (± SEM) on the tapered ledged beam-walking test. * p < 0.05. Reprinted with permission from the Journal of Neuropathology and Experimental Neurology.
Figure 5.5. $[^3]H$ WIN 35,428 striatal DAT binding in unilateral (A) and bilateral (B) 6-OHDA-lesioned rats, compared to vehicle-infused controls (C). Reprinted with permission from the Journal of Neuropathology and Experimental Neurology.

5.4 STUDY SUMMARY

Using validated tests of motor function that are sensitive to striatal DA depletions, we demonstrated that: 1) hRPE-GM implanted in unilaterally-lesioned rats ameliorated forelimb asymmetry and hindlimb deficits, and 2) hRPE-GM implanted in bilaterally-lesioned rats increased independent use of the forelimb contralateral to the implant, and decreased forelimb asymmetry and hindlimb deficits of the hindlimb contralateral to the implant. These results are consistent with previously reported data in PD patients (Stover et al., 2005a), MPTP-treated monkeys (Doudet et al., 2004), and rodents (Subramanian et al., 2002).
CHAPTER 6. CHARACTERIZATION AND LONG-TERM SURVIVAL OF HRPE-GM IMPLANTS IN THE 6-OHDA MODEL OF PD

6.1 INTRODUCTION

The previous studies revealed that hRPE-GM implants could ameliorate motor deficits in 6-OHDA lesioned rats. The next step in our assessment of hRPE-GM implants was to determine whether hRPE-GM implants could survive long term in the brain.

There is sparse data on hRPE-cell survival post-implant: there has only been one histological report suggesting continued survival of hRPE cells. Subramanian and colleagues (Subramanian et al., 2002) revealed hRPE-like cells using TH immunohistochemistry (IHC). However, TH is not a specific hRPE marker, and more evidence of the long-term survival of hRPE cells is needed.

The following study was aimed at characterizing a variety of hRPE-specific markers in vitro and using these markers to investigate the long-term survival of hRPE-GM implants. Unilaterally 6-OHDA lesioned rats were implanted with hRPE-GM without immunosuppression. At specific time points post implant (48 hours, 7 days, 4 weeks, and 5 months), rats were euthanized and immunohistochemically analyzed with the following antibodies: 1) human-specific NuMA-Ab2, 2) epithelial-specific EMMPRIN, 3) RPE cell-specific RPE65, and the inflammation markers: 4) GFAP, and 5) ED1 (rat CD68). Our analysis revealed hRPE-immunoreactive cells at different times post-implant. The morphological features of hRPE cell implants (at 48 hours and 5 months) were confirmed by electron microscopy (EM). Despite evidence of an inflammatory reaction in response to the implants, there seemed to be an appreciable number of surviving hRPE cells.

6.2 MATERIALS AND METHODS

Sprague-Dawley Rats (N=22) (University of British Columbia Animal Facility) weighing 275-300 g were used for these experiments. All animals were housed either in pairs or in groups of three in Plexiglas cages with ad libitum access to food and water. A 12-hour light-dark cycle

was maintained (where the animals’ dark (active) cycle was 12:00 noon to 12:00 midnight). Animal housing, animal care, and all experimental procedures were approved by the UBC Animal Care Committee.

6.2.1 6-OHDA Lesioning

The surgical procedure for unilateral 6-OHDA lesioning was described detail in the previous chapter. Briefly, rats were anesthetized with isoflurane and received a unilateral stereotaxic 6-OHDA injection (10 μg/4 μl in 0.05% ascorbic acid in saline infused at 1μl/min) into the SNc and MFB.

6.2.2 HRPE Cell Culturing

All experiments in this chapter (and previous chapter) used the 0802 hRPE cell lot (Table 5.1). HRPE cells were isolated and expanded at Titan Pharmaceuticals Inc. (Somerville, NJ, USA) and frozen after two or three passages; each passage represents the subdivision of hRPE cells into two new culture flasks with fresh culture medium. We received hRPE cells frozen in storage medium containing 7.5% dimethyl sulfoxide (DMSO), 20% fetal calf serum (FCS), and Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA). Upon arrival, hRPE cells were stored in vapor phase liquid nitrogen until use.

Approximately four to six days prior to implantation, cryopreserved hRPE cells were rapidly thawed in a 37°C water bath and re-suspended in high glucose DMEM (with L-glutamine, 25 mM HEPES buffer, pyridoxine hydrochloride; Invitrogen) containing 10% heat inactivated fetal bovine serum (FBS; Biomed, Foster City, CA). This medium will be further referred to as “complete hRPE medium”. The cell suspension was centrifuged and the remaining cell pellet was seeded into non-laminated polystyrene T-25 cell culture flasks containing complete hRPE medium. Initial seeding densities ranged from 200,000-300,000 cells per flask with greater than 85% viability. HRPE cells were grown to 100% confluence in an incubator (37°C, 5% CO₂) prior to GM attachment and implantation. The complete hRPE medium was exchanged every 48 hours or if a color change was noted in the hRPE medium. HRPE cells were examined regularly for proper cell growth and health.
6.2.3 HRPE Cell Attachment to GM

Twenty-four hours prior to intended use, 40-60 µm-diameter dry GMs (proprietary, Theracell Inc. Menlo Park, CA) were hydrated in a 1.5 ml microcentrifuge tube with calcium/magnesium-free phosphate-buffered saline (PBS; Invitrogen) for a minimum of 90 minutes and autoclaved (121°C, 15 psi, 15 minutes) for sterility. Sterile GMs were re-suspended and washed in fresh PBS, and stored in complete hRPE medium until cell attachment time.

The 100% confluent hRPE cells were removed from the incubator, washed with PBS, and harvested by trypsinization and mechanical agitation. The hRPE cell suspension was transferred to a sterile polypropylene-coated 15 ml tube containing complete medium and centrifuged. After centrifugation, the hRPE cell pellet was re-suspended with fresh complete medium, and a small (10 µl) sample of hRPE cells was taken and assessed for cell viability and vial concentration using the trypan blue exclusion method (minimum criteria: ≥80% cell viability; ≥1x10^6 cells/ml solution) to stain dead or dying hRPE cells blue. Cell viability (calculated as: live cells [no trypan blue inclusion] / total number of hRPE cells x 100%) and the calculated mean number of hRPE cells per vial was then used to determine the volume of cell suspension needed for a final concentration of 1x10^6 cells per 10 mg GM suspension. HRPE cells were passively adsorbed or adhered to GMs, and the hRPE-GM suspension was stored in complete medium in a 1.7 ml tube and placed on its side in a 37°C incubator for 15-18 hours until immediately pre-implant.

On the morning of implantation, the hRPE-GM suspension was gently washed with sterile Hanks Balanced Salt Solution (HBSS, without phenol red; Invitrogen) to remove any unattached hRPE cells. Prior to use, a slurry was created by removing the HBSS until a liquid meniscus formed just above the hRPE-GM suspension. A small (10 µl) sample was taken, treated with a dispase solution to break down GMs, and assessed for cell viability and dose concentration using the trypan blue exclusion method. HRPE-GM suspensions not meeting the minimum required cell viability (≥80%) or dose concentration (≥2000 cells/µl hRPE-GM suspension) were re-assessed and/or discarded. GM suspensions were treated in a similar manner. All preparations were kept cool in a beaker with shaved ice during the implantation procedure. The maximum storage time for the hRPE-GM suspension was five hours.
6.2.4 HRPE-GM Surgical Implantation

Animals were unilaterally implanted with either hRPE-GM or GM-alone. Immediately prior to implant, 6 µl of the hRPE-GM slurry was drawn into a sterile 20 µl Hamilton syringe (preloaded with sterile HBSS) attached to a 22-gauge micropolished beveled needle (to facilitate the passage of beads). Each rat received 2 striatal hRPE-GM (or GM-alone) implants on the same side as the lesion at the following coordinates: AP: +1.6, ML: -2.5, DV: -6.0, -4.0 (tract 1); and AP: -0.4, ML: -3.5, DV: -6.0, -4.0 (tract 2) (Paxinos and Watson, 1997). A “pocket” was created by advancing the needle another 0.5 mm below the deepest DV coordinate (to a depth of DV: -6.5) and then brought back up to implant level. The hRPE-GM slurry was divided in half: 3 µl was injected (using a “pulse-like” injection technique) in each of the two DV locations (DV: -6.0, -4.0). Another 6 µl of the hRPE-GM suspension was drawn into another syringe and subsequently injected into the second tract. The needle was held in place for an additional 5 minutes to allow diffusion. Each animal was injected with a set volume (12 µl) of hRPE-GM suspension with hRPE-cell concentrations dependent of pre-implant calculations (see results). GM-alone implants were performed following the same procedure.

6.2.5 Histology and IHC

Rats were sacrificed at several time points post-implant: 48 hours (N= 4), 1 week (N= 4), 4 weeks (N= 4) and 5 months (N= 4). Rats sacrificed at the later time points underwent behavioural testing as described in the previous chapter. The additional implanted animals that were used for early (48 hours, 1 week) histological time points were not behaviourally tested due to the time consuming nature of testing and the sufficient recovery time needed post implant.

Rats were euthanized by administration of a ketamine HCl:xyalazine overdose, and perfused with one of two fixatives, depending on the antibody used. Some of the rats were perfused through the ascending aorta under systolic pressure with 0.9% cold saline followed by 70% EtOH in distilled water or 4% paraformaldehyde in 0.1 M PB. The brain was removed, cut into two thick coronal blocks, and immersed in additional 70% EtOH (at 4°C) for 24 hours. The remaining brains, fixed with 4% paraformaldehyde, were immersed in additional fixation medium for 24 hours, and then stored in 70% EtOH until paraffin processing. Coronal blocks were paraffin embedded and coronal sections (4-6 µm) were made through the implant site,
placed on Superfrost-plus charged slides (Fisher Scientific, Pittsburgh, PA), and placed in a 37°C oven overnight.

To identify the implant site, representative coronal sections through the striatum were deparaffinized and stained with Gill’s hematoxylin solution #2 (Fisher Scientific) and ethanolic eosin (H&E), dehydrated in ascending grades of EtOH, cleared in xylene, and mounted with permount. Adjacent sections were processed for IHC.

*In vitro* hRPE-GM pellets for histological analyses were prepared in a similar manner. After the 15-18 hour attachment time, the hRPE-GM suspension was washed in HBSS, and a small sample hRPE-GM was dispase-treated and assessed for cell viability and attachment efficiency. The hRPE-GM suspension was then fixed with a sterile 10% formalin solution, washed in additional HBSS, and stored in 70% EtOH until the pellet was paraffin embedded. Sections (4-6 µm) were made through the hRPE-GM pellet for IHC.

### 6.2.5.1 HRPE-Cell Identification

Coronal sections were deparaffinized in three washes of xylene and hydrated in descending concentrations of EtOH. After deparaffinization, *in vitro* hRPE-GM sections and *in vivo* sections fixed with 4% paraformaldehyde underwent 20 minutes of microwave heat-induced antigen retrieval in 10 mmol/L citric acid buffer (pH 6.0; Vector Laboratories, Burlingame, CA). EtOH-fixed sections did not require antigen retrieval. After cooling, *in vitro* sections and 70% EtOH-fixed brain sections were treated with a blocking solution for 1 hour (at room temperature) containing 10% normal goat serum (NGS), 2% bovine serum albumin (BSA), and 0.25% Triton X-100 (Sigma Aldrich). EtOH fixed sections were incubated overnight at 4°C with the following primary antibodies diluted in blocking solution: 1) nuclear mitotic apparatus protein (NuMA-Ab2) (1:500; Oncogene Research Products, San Diego, CA), a mouse monoclonal antibody against a 240 kDA nuclear matrix protein derived from the human cervical carcinoma cell line; 2) extracellular matrix metalloproteinase inducer (EMMPRIN) (1:500; Zymed Laboratories, South San Francisco, CA), a rabbit polyclonal antibody against the cell membrane of normal epithelium.

Sections treated with anti-NuMA primary antibody were washed with 0.1 M PBS (pH 7.4) and incubated with the fluorochrome-conjugated secondary antibody Alexa Fluor 488 goat anti-mouse IgG (1:500; Molecular Probes, Eugene OR). Polyclonal EMMPRIN treated tissue was incubated with either Alexa Fluor 488 or Alexa Fluor 555 goat anti-rabbit IgG (1:500,
Molecular Probes) fluorescent secondary antibodies. Secondary antibody incubation was for one hour at room temperature. Sections were coverslipped and sealed using an antifade mounting medium (Molecular Probes). Sections were examined using an Olympus Fluoview 500 confocal laser scanning microscope. Differential interference contrast (DIC) images of sections were taken with each scan and viewed using Olympus Fluoview software. Negative control sections were incubated with no primary Ab.

Tissue sections from a subsequent set of animals (4% paraformaldehyde fixed) at 3 and 5 months (N=5) underwent IHC using RPE-65 (Chemicon, Temecula, CA), a mouse monoclonal antibody that specifically reacts to a 65kDA protein on RPE-cell membranes, or DA specific mouse monoclonal TH (Sigma Aldrich). RPE65 IHC has been previously described for flat-mounted, retinal tissue sections (Znoiko et al., 2002). Therefore, we describe here our development of RPE-65 IHC for fixed, coronal, paraffin embedded brain sections. Paraformaldehyde-fixed sections underwent antigen retrieval, quenched in 3% hydrogen peroxide in PBS for 15 minutes and subsequently treated with a blocking solution containing 0.5% Tween-20 and 10% NGS. Sections were incubated overnight at 4°C with anti-RPE65 (1:10) diluted in blocking solution. Following primary antibody incubation, sections were washed and treated using a goat anti-mouse secondary antibody pre-adsorbed to rat IgG (1:300; Jackson Immunoresearch, West Grove, PA), and then visualized using a Vector elite ABC kit (Vector Laboratories) and 3,3’-diaminobenzidine (DAB; MP Biomedicals, Solon, OH). In addition, to confirm TH-positive findings in previous studies (Subramanian et al., 2002), sections taken from a set of animals at 10-12 weeks (3 months) were blocked with a solution containing 10% normal horse serum, 2% BSA, and 0.1% Triton X-100, and incubated overnight with anti-TH (1:400). Following primary antibody incubation, sections were treated with a horse anti-mouse secondary antibody, visualized using a Vector elite ABC kit and Vector red (Vector laboratories), and counterstained with hematoxylin. Sections were then dehydrated, coverslipped, and examined by light microscopy.

6.2.5.2 Inflammation

Additional tissue sections were stained for inflammatory markers GFAP and ED1 (rat CD68) to identify reactive astrocytes and macrophages/activated microglia, respectively. After deparaffinization and hydration, ED1-treated sections were digested with proteinase-K (5 µg/ml, Sigma Aldrich) in a 37°C oven for 15 minutes. Sections were then blocked in a solution
containing 10% normal horse serum (NHS), 2% BSA, and 0.1% Triton X-100, and subsequently incubated with mouse monoclonal anti-ED1 (1:500; Serotec, Raleigh, NC) overnight at 4°C. GFAP-treated sections underwent microwave heat-induced antigen retrieval in citric acid buffer (pH 6.0; Vector Laboratories), blocked in 10% NGS, 2% BSA, and 0.1% Triton X-100, and were then incubated rabbit polyclonal anti-GFAP (1:1000; Chemicon) overnight. Tissue sections were then processed and visualized using a Vector elite ABC kit (Vector Laboratories) and DAB (MP Biomedicals), dehydrated, coverslipped and examined.

6.2.6 Electron Microscopy (EM)

For ultrastructural analyses, rats were euthanized and perfused through the ascending aorta with a fixative solution containing 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M PB fixative for 60 minutes. Afterwards, the brain was removed and placed in additional fixative solution at 4°C for 24-48 hours. Ultrastructural analyses were made on in vitro hRPE-GM and in vivo implanted hRPE-GM at 48 hours (N=2) and 5 months (N=1) post-implant. Four-six blocks of whole brain tissue (measuring approximately 5.0 x 5.0 x 10.0 mm) through the implant site were sectioned, and each block was cut with the long axis oriented in the dorsal-ventral direction. Individual tissue blocks were washed in 0.1 M PBS and post-fixed overnight in 1% buffered osmium tetroxide. Blocks were then washed in acetate buffer (pH 3.7) and stained with 2% aqueous uranyl acetate. Tissue blocks were dehydrated in ascending grades of EtOH, equilibrated in propylene oxide, and embedded in Epon. Each block was trimmed, and ultrathin (65-70 nm) sections were collected, individually mounted on Formvar-coated slot grids and stained with lead citrate. Electron micrographs were photographed with 3.25 x 4.00 inch Kodak 4489 plate film, and negatives were scanned on an Epson Expression 1680 scanner using Epson Twain-Pro software. All images were taken as TIFF files and edited with Adobe Photoshop.

6.3 RESULTS

All lesioned rats demonstrated behavioural deficits manifested by a lack of use of the contralateral forelimb and a significant increase in hindlimb contralateral step errors during the ledged beam test (Cepeda et al., 2007). Using previously described phosphor-imaging autoradiography (Strome et al., 2005) a random subset of animals underwent [³H] WIN 35, 428 (Perkin Elmer, Boston, MA) binding to the striatal DAT to quantify 6-OHDA lesion severity.
[³H] WIN 35, 428 binding revealed a >90% lost of striatal DA terminals compared to the unlesioned striatum.

All hRPE cell cultures appeared healthy and presented unaltered morphology during the 5-day incubation period prior to cell attachment. The cells displayed typical elliptical-shaped nuclei and elongated cell shape. They reached 100% confluence in T-25 flasks within 4-6 days after seeding, arranging in a monolayer with a characteristic cobblestone appearance. Confluent hRPE cells produced consistent results: cell numbers ranged from 2-2.5x10⁶ cells per flask, with cell viabilities between 80%-92% (Table 5.1). Only after cells were 100% confluent (and before they started to proliferate beyond a monolayer) were they trypsinized and prepared for GM attachment. HRPE-GM dose estimates prior to implantation consistently resulted in greater than 83% (89% ± 4.5%) viability with a mean calculated dose concentration of 2,453 ± 369 cells per µl of hRPE-GM solution (total of 24,000-30,000 hRPE cells injected). At the end of each surgical day (which was no more than five hours after the end of the attachment procedure to remain within the clinical surgical parameters) cell assessments were made to determine changes in cell viability and concentration during storage. Cell viability slightly decreased to 80-82% (Table 5.1).

6.3.1 In Vitro Characterization of hRPE Cells

All of the primary antibodies used were previously assessed for hRPE-cell specificity in human RPE cultured monolayer and RPE-choroid eye tissue. NuMA-Ab2 demonstrated good reactivity to hRPE cells with minimal or no staining of GMs or rat tissue. EMMPRIN and RPE65 also demonstrated good specificity to human epithelial structures in hRPE cells but not to rat tissue or GM components. GFAP and ED1 (CD68) showed typical staining in vivo in rat tissue, but were negative for hRPE cells in vitro.

The morphological features of in vitro hRPE-GM are illustrated in Figure 6.1. GM profiles were spherical or ovoid in shape with characteristic surface grooves and pores, with a maximum diameter of approximately 60 µm (Figures 5.1A, 5.1B). GMs were moderately eosinophilic (Figure 6.1A). The majority of hRPE cells were attached to the outer edges of the GMs, exhibiting an elongated cell profile with large, irregularly shaped, eosinophilic cytoplasm. In some cases, hRPE cells appeared to have penetrated the GM grooves and pores. Relatively few hRPE cells were isolated and not attached to GMs. The morphology of attached hRPE cells did not resemble the typical cobblestone-like appearance characteristic of hRPE cells in
monolayer during cell culture (refer to Figure 3.2). RPE cells attached to the GMs were immunoreactive for NuMA-Ab2 (Figure 6.1C), EMMPRIN (Figure 6.1D), and RPE65 (Figure 6.1E) but not for GFAP (Figure 6.1F). GMs displayed little or no immunoreactivity to any of the antisera.

**Figure 6.1. In vitro characterization of hRPE-GM.** H&E stained (A) and DIC photomicrograph (B) of hRPE cells attached to GMs. HRPE cells were characterized in vitro by NuMA-Ab2 (C), EMMPRIN (D), and RPE65 (E). No GFAP⁺ reactivity was seen hRPE-cell cultures (F). DIC photomicrograph in B corresponds to the EMMPRIN fluorescence shown in D. Scale bars = 25 µm (Scale bar in F applies to B-D, F). *Reprinted with permission from the Journal of Neuropathology and Experimental Neurology.*
6.3.2 In Vivo Characterization of hRPE-GM Implants

Histological sections stained with H&E were used to verify the striatal placement of hRPE-GM implants (Figure 6.2). All implants were localized within the striatum. Individual implants contained a variable number of GMs at all post-surgical time points examined. In a few rats, some of the GMs were observed in the subcortical white matter and corpus callosum, adjacent to the injection tract. At 48 hours and 1 week after surgery, the implant sites were characterized by edema, focal hemorrhage and an accumulation of inflammatory infiltrates within and immediately surrounding the implant site (Figures 6.2A, 6.2B, 6.2E), suggestive of polymorphonuclear leukocytes. At 1 month and 5 months post-implant, the edema and hemorrhage had resolved as the adjacent neuropil of the striatum gradually closed over the implant site (Figures 6.2C, 6.2D, 6.2F). Gliosis was largely limited to the neuropil immediately surrounding the implant, and macrophages and activated microglia were observed in and around the implant site.

IHC (using a number of different antibodies) demonstrated the survival of implanted hRPE cells. NuMA-Ab2+ cells were found attached to the GMs at all time points post-implant (Figure 6.3). Although there was some heterogeneity among animals, there appeared to be a gradual loss of immunoreactive hRPE cells within the implant. As expected, NuMA-Ab2+ cells were most numerous at 48 hours post-implant, but there was a marked decrease in their numbers at later time points. Positive staining was more homogeneous at late time-points, which allowed us to make gross visual estimates of the number of cells per bead of 4-5 cells/bead. This estimate was consistent with the calculated hRPE cell/bead ratio detailed in a previous study (Subramanian et al., 2002). No NuMA-Ab2+ cells were observed outside the implant site.

Figure 6.4 illustrates EMMPRIN+ cells at the different time points post-implant. EMMPRIN+ cells were observed attached to GMs and infiltrating the surface pores at all time points. The morphology of EMMPRIN+ cells was similar to that observed in hRPE-GM in vitro. At 48 hours post-implant, EMMPRIN+ cells were most numerous, although smaller in size than at later time points. The number of immunoreactive cells decreased noticeably from 48 hours to 1 week after implantation, and then decreased more gradually at later time points. Implanted hRPE cells with confluent cytoplasm were observed attached to the GMs as long as 5 months after the surgery. EMMPRIN+ cells were seen only in the implant site with no staining observed in the surrounding neuropil.
Figure 6.2. Morphological characteristics of *in vivo* hRPE-GM. H&E staining of hRPE-GM at 48 hours (A), 1 week (B), 1 month (C) and 5 months (D) post-implant. Edema, hemorrhage and acute inflammatory infiltrates were observed at 48 hours post-implant (E). By 5 months post-implant (F) macrophages and activated microglia were observed, while the surrounding neuropil had gradually closed over the implantation site. Scale bar in (D) = 200 µm, (F) = 30 µm (Note: scale bar in D applies to A-D; scale bar in F applies to E-F). *Reprinted with permission from the Journal of Neuropathology and Experimental Neurology.*
Figure 6.3. NuMA-Ab2⁺ hRPE-GM implants in vivo at 48 hours (A), 1 week (B), 4 weeks (C), and 5 months (D) post implant. NuMA-Ab2⁺ cells were seen both infiltrating and on the outer edges of GMs. Scale bar = 25 µm. Reprinted with permission from the Journal of Neuropathology and Experimental Neurology.

Figure 6.4. EMMPRIN⁺ hRPE-GM implants in vivo at 48 hours (A), 1 week (B), 4 weeks (C), and 5 months (D) post implant. Scale bar = 25 µm. Reprinted with permission from the Journal of Neuropathology and Experimental Neurology.
IHC using antibodies against RPE65 confirmed the survival of implanted hRPE cells as late as 5 months post-implant (Figure 6.5). RPE65+ cells were observed attached to the surface and within the pores of GMs, although occasional isolated cells could be seen in the space between the GMs (Figure 6.5A). These cells may have been attached to GMs beneath or above the plane of section. No detached immunoreactive cells or cells outside the implant area were observed.

Figure 6.5. RPE65+ staining in hRPE-GM implanted animals 5 months post-implant, illustrating the distribution of hRPE cells attached to GMs (A). At higher magnification (B) RPE65+ immunoreactivity seemed to be localized to the cytoplasm of hRPE cells. Scale bar in (A) = 50 µm, (B) = 30 µm. Reprinted with permission from the Journal of Neuropathology and Experimental Neurology.
**Figure 6.6.** TH\(^+\) staining in hRPE-GM implanted animals 3 months post-implant. Photomicrograph (A) reveals prominent asymmetric TH\(^+\) staining of the right striatum with a near complete depletion of the contralateral, hRPE-GM implanted, striatum. Note the presence of the implant (containing visible GMs) near the center of the lesioned striatum. At high magnification (B, C) TH\(^+\) immunoreactivity was localized to the cytoplasm surrounding presumptive (hematoxylin-stained) hRPE-cell nuclei. Scale bar in (A) = 1000 µm, (C) = 20 µm (Note: scale bar in C applies to B-C). Reprinted with permission from the *Journal of Neuropathology and Experimental Neurology*.

TH IHC revealed a prominent unilateral lesion in all animals studied (Figure 6.6A). Striatal sections from rats 3 months post-implant revealed TH\(^+\) cells within the GMs (Figures 6.6B, 6.6C) on the lesioned side. TH-immunoreactivity was largely cytoplasmic surrounding
distinct, round nuclei (stained with hematoxylin) and was predominantly found within the
grooves (not the outer surface) of GMs. This was presumed to be TH⁺ presumptive hRPE cells as
no other TH immunoreactivity was found anywhere else in the lesioned striatum (Figure 6.6A).

6.3.3 EM

The identification and survival of implanted hRPE cells up to 5 months post-implant was
confirmed by EM (Figure 6.7). In vitro preparations of hRPE-GM displayed the ultrastructural
characteristics of hRPE cells (Figure 6.7A), including evenly distributed dense nuclear chromatin
and dark cytoplasm containing an abundance of rough endoplasmic reticulum. Electron dense
pigment granules and melanosomes were observed in the cytoplasm of these cells, although the
relative size and number of these inclusions varied considerably among individual cells (Figure
6.7A). At 48 hours post-implant numerous cells with similar morphology were observed attached
to the GMs (Figure 6.7B). These cells displayed the characteristic dark cytoplasm with abundant
rough endoplasmic reticulum and occasional pigment granules. At 5 months post-implant
relatively fewer hRPE cells were observed, but they contained a greater volume of confluent
cytoplasm and more abundant pigment granules and melanosomes (Figure 6.7C).

6.3.4 Inflammatory Response to hRPE-GM Implants

Initial evidence of the inflammatory response to hRPE-GM implants at 5 months post-
implant are illustrated in Figure 6.8. ED1⁺ macrophages and activated microglia were observed
in the neuropil (at the edge) and within the implant site (Figure 6.8A). GFAP⁺ immunoreactivity
revealed hypertrophied astrocytes (comparable to a glial scar) adjacent to the implant site (Figure
6.8B), which encapsulated the implant to a depth of approximately 50-100 µm. This observation
was confirmed by electron microscopy (not shown). A morphologically characterized glial scar
(as seen in Figure 6.8B) was seen in some, but not all, of the animals that were examined. The
striatal neuropil immediately adjacent to the layer of hypertrophied astrocyte processes appeared
relatively normal.

Interestingly, there seemed to be a qualitative difference in inflammatory response
between hRPE-GM and GM-alone implants, as seen by both ED1⁺ staining and astroglial depth
(not shown). These observations will be further pursued, and are described in detail in Chapter 7.
Figure 6.7. EM of hRPE-GM in vitro (A) and at 48 hours (B) and 5 months (C) post-implant. Note the relative electron density of the GMs in (B) and (C). Scale bars = 5 µm. Reprinted with permission from the Journal of Neuropathology and Experimental Neurology.
**Figure 6.8.** Inflammatory response to hRPE-GM implants. ED1⁺ macrophages and activated microglia (black arrows) were observed in and around the implant site at 5 months post implant (A). GFAP⁺ hypertrophied astrocyte processes were also seen encapsulating the implant site at 5 months post-implant (B). Scale bar in (A) = 100 µm, (B) = 50 µm. *Reprinted with permission from the Journal of Neuropathology and Experimental Neurology.*

### 6.4 STUDY SUMMARY

This is the first study to assess the specificity of a number of immunohistochemical markers to identify hRPE cells both *in vitro* and implanted in the rat brain. HRPE-GM implanted in the lesioned striatum of rats can be identified up to 5 months post-implant as shown by NuMA-Ab2, EMMPRIN, RPE65, and TH immunoreactivity. This indicates the relatively long-term survival of hRPE cells despite the absence of immunosuppression. This is consistent with
previous studies that described the survival of presumptive hRPE-cell implants in hemiparkinsonian rats (Subramanian et al., 2002).

Notably, hRPE-GM implants seemed to elicit a decreased inflammatory response when compared to GM-alone implants despite the absence of any immunosuppressive treatment. The inflammatory response was greatest at the early time-points but subsided by 5 months. Knowing the endogenous immunosuppressive properties of hRPE cells, we thought it would be prudent to quantitatively determine whether hRPE cells are, in fact, able to reduce the inflammatory response after implantation.
CHAPTER 7. QUANTITATIVE ANALYSIS OF CELLULAR INFLAMMATION IN RESPONSE TO HRPE-GM IMPLANTS IN THE RAT BRAIN

7.1 INTRODUCTION

The long-term survival and function of cell transplants seems to depend, in part, on the host inflammatory response and other related mechanisms (Barker and Widner, 2004). It has been suggested that hRPE cells induce a reduced immune response after implantation (Subramanian, 2001; Eve and Sanberg, 2008). In spite of this, few studies have addressed this. Subramanian and colleagues (Subramanian et al., 2002) demonstrated that hRPE-GM implants were well tolerated in the rat and provided qualitative evidence of a reduced inflammatory response. Our laboratory also confirmed this (Flores et al., 2007); however, there is no quantitative data that measures the extent of this inflammatory reduction.

The objective of the present study was to quantify the host inflammatory response to hRPE-GM implanted in unilateral 6-OHDA-lesioned rats. In this study, lesioned animals were implanted with hRPE-GM or GM-alone, and the host inflammatory response was assessed at various times post-implant by counting the number of ED1+ macrophages/activated microglia and measuring the reactive astroglial depth in response to the implant. Our results demonstrate that there is a reduced inflammatory reaction in response to hRPE-GM (when compared to GM-alone) implants, even in the absence of immunosuppression. This suggests that hRPE cells may have “natural” immunosuppressive properties that contribute to their survival and function.

7.2 MATERIALS AND METHODS

A total of 120 Sprague-Dawley rats (weighing 225-275 g) were used for these experiments. Rats were received either from the University of British Columbia Animal Facility or from Charles River Laboratories (Sherbrooke, QC). The animal supplier was changed because the UBC Animal Breeding Unit shut down halfway through our experiments. However, all animals underwent pre-behavioural testing paradigms (as explained in Chapter 6) to ensure that behavioural characteristics (such as learning and lesion-induced deficits post-lesion) were as similar as possible. The behavioural results of these animals are described elsewhere (K. Russ,
PhD dissertation, 2011). All animal housing, animal care, and experimental procedures were approved by the UBC Animal Care Committee.

7.2.1 6-OHDA Lesioning

The surgical procedure for unilateral 6-OHDA lesions has been previously described in Chapter 5. Briefly, rats received a unilateral stereotaxic 6-OHDA injection (10 µg/4 µl in 0.05% ascorbic acid in saline) into the SNc and MFB.

7.2.2 HRPE Cell Culturing and GM Attachment

In addition to the 0802 cells used in the previous studies, two other hRPE-cell donor types (1105, 0010) were used for these experiments. Please refer to Table 7.1 for details on donor age and pre- and post-surgical cell viabilities.

The isolation and expansion protocols for HRPE cells were detailed previously in Chapter 6. Briefly, hRPE cells were received frozen in storage medium and stored in vapor phase liquid nitrogen upon arrival. Prior to intended use, hRPE cells were removed from cryopreservation, rapidly thawed in a 37°C water bath, and re-suspended in complete hRPE medium. The cell suspension was then centrifuged, the remaining cell pellet was re-suspended with fresh complete medium, and a small (10 µl) sample of hRPE cells was taken and assessed for cell viability and vial concentration using the trypan blue exclusion method. The calculated concentration of hRPE cells per vial was then used to determine the volume of cell suspension needed for a final concentration of 1x10⁶ cells per 10 mg GM suspension. HRPE cells were then passively adsorbed or adhered to sterile GMs, and the hRPE-GM suspension was stored (in complete medium) in a 37°C incubator for 15-18 hours until immediately pre-implant.

The morning of implantation, the hRPE-GM suspension was gently washed with HBSS, and afterwards a slurry of the hRPE-GM suspension was created. A 10-µl sample was taken and treated with a dispase to assess cell viability and dose concentration using the trypan blue exclusion method. HRPE-GM suspensions not meeting the minimum required cell viability or dose concentration were re-assessed and/or discarded. GM suspensions were treated in a similar manner. All preparations were kept cool in a beaker surrounded by shaved ice during the implantation procedure for a maximum storage time of five hours.
7.2.3 HRPE-GM Implantation

Briefly, 6 µl of the hRPE-GM slurry was drawn into a sterile 20 µl Hamilton syringe attached to a 22-gauge micropolished beveled needle. Each rat received two striatal hRPE-GM implants on the same side as the lesion. Each animal was injected with a set volume (12 µl) of hRPE-GM suspension with hRPE-cell concentrations dependent on pre-implant calculations. An estimated total of 24,000-36,000 hRPE cells were implanted into each rat. GM-alone implants were performed following the same procedure. Immunosuppression was not used in any of the animals.

7.2.4 Post-Mortem Histology

At 2-4 weeks (N=37), 10 weeks (N=43), and 20 weeks (N=40) post-implant, rats were euthanized. Approximately half of these animals were sacrificed by decapitation and used for additional analyses not part of this dissertation. An additional group of rats (N=10) previously implanted with the 0802 hRPE-cell donor group were also used in these studies. The animals (used in the current studies) were sacrificed by pentobarbital overdose, followed by transcardial perfusion with 4% paraformaldehyde in PB. After the brains were removed, the striatum was dissected out and immersed in additional fixative for 24 hours. Tissue blocks were paraffin-embedded and coronally sectioned at a thickness of 5-7 µm through the striatal implant site. At least half the animals were serially sectioned throughout the entire span of the implant site, while the remaining animals were sectioned at a periodicity of every 5-10 sections. SN sections were also taken at a thickness of 7 µm and a periodicity of every 5 sections.

7.2.4.1 IHC

ED1 (rat CD68) and GFAP staining were used to evaluate the host inflammatory response to hRPE-GM and GM-alone implants. Initially, every 10-20th section through the implant site and the SN was stained for ED1, and every 20-40th section through the implant site was stained for GFAP. Protocols for each antibody staining was described previously. In brief, after deparaffinization and tissue rehydration, tissue undergoing ED1 IHC were digested with proteinase-K (5 µg/ml) at 37° for 15 minutes. After PBS washes (to stop digestion), sections were blocked with a solution containing BSA, Triton X-100, and NHS in PBS. Sections were then incubated overnight with anti-ED1 (1:500) (Serotec). Sections undergoing GFAP underwent citrate buffer antigen retrieval (Vector Laboratories), blocked with BSA, Triton X-100, and
normal goat serum, and incubated overnight with anti-GFAP (1:1000) (Chemicon). The following day, all sections were incubated with the appropriate secondary antibody (1:200; Vector Laboratories), followed by visualization using Vector ABC alkaline phosphatase and Vector Red chromagen (Vector Laboratories), prepared according to the manufacturer’s recommendations. All sections were lightly counterstained with hematoxylin to identify cell nuclei, then dehydrated and coverslipped.

7.2.5 Stereological Analyses

7.2.5.1 Area and Volume Analysis

A Nikon Eclipse E800 microscope (Nikon Instruments, Melville, NY), QImaging Retiga Exi camera (QImaging, Surrey, BC), and NIS Elements software (Nikon Instruments) were used for all stereological estimates described in this chapter.

To determine implant volume, a representative series of sections (i.e. every 10th section) through the striatum were stained with H&E. Starting from a random section within this series, the region of interest, e.g. implant sites on each section defined by the external surface of the GMs, was traced using a 4x objective and the areas were measured. We used a total of 8-12 sections to make an appropriate estimate. The total volume of the implant was calculated using the formula: \( V = \Sigma A \times T \times P \), where \( \Sigma A \) is the sum of the area measurements, \( T \) is the section thickness, and \( P \) is the section periodicity. Total volume estimates were based on Cavalieri’s principle. Since each animal received 2 implant tracts (approximately 2 mm apart), sections taken between the two implant tracts (where there was no observable implant) were not included in the analyses and the reported volumes is the combined volume of both implants. Animals with no visible implant site were excluded from analyses.

Animals in the 0802 hRPE-GM group were sectioned throughout the implant site; however, the section periodicity (\( P \)) of the samples was unknown; therefore, we were unable to make the appropriate estimates of implant volume.

In typical animals studies, a large sample size (greater than 200) would be needed to detect small effects with high (95%) statistical power. This number is neither justifiable nor viable for in vivo animal experiments. Accordingly, it was estimated that the number of animals presented in this chapter (with a target sample size of approximately 6 animals per group as
determined by post-hoc analyses collected from previous studies in our laboratory) was sufficient to detect a small (but biologically meaningful) effect with 80% power.

7.2.5.2 ED1 Quantitative Analyses

The numerical density \([N_V] \) number of cells per \( \text{mm}^3 \) of ED1\(^+\) macrophages/activated microglia was measured and compared between hRPE-GM and GM-alone implanted animals. The \( N_V \) was measured using the previously described optical dissector method (Gundersen et al., 1988a, Gundersen et al., 1988b). Evenly spaced ED1-stained sections were selected from the section series, and square counting grids (50 \( \mu \text{m} \times 50 \mu \text{m} \) were randomly placed within the implant site. Using a 60x objective, the number of ED1\(^+\) cells with a visible hematoxylin-stained nucleus was counted on the pre-determined dissector volume. An object was counted in a dissector when the identifiable profile (combination of hematoxylin-stained nuclei with red cytoplasm) was observed in the second (sampling) depth but not in the first (lookup) depth of the dissector pair, which corresponds to the leading edge of a cell. The dissector counting rule was to count the number of leading edges that come into focus as the objective lens moves through the section. A minimum total of 150-200 cells per animal were needed to make a reliable estimate. Additional sections were counted (e.g. every 10\(^{\text{th}}\) section) if we were unable to reach our minimum number. The \( N_V \) of ED1\(^+\) macrophages and/or activated microglia within the implant site was calculated using the formula \( N_V = \frac{\sum Q}{\sum V_{\text{ref}}} \), where \( \sum Q \) is the sum of counted cells and \( \sum V_{\text{ref}} \) is the sum of the reference volume. The reference volume was calculated from \( \sum V_{\text{ref}} = a_{\text{ref}} \times h \), where \( a_{\text{ref}} \) is the counting frame area (50 \( \mu \text{m} \times 50 \mu \text{m} \) and \( h \) is the dissector height. Cell counts were taken blinded to the treatment group of the animal.

7.2.5.3 Astroglial Depth

Astroglial depth was used as a marker to assess reactive astrogliosis in response to hRPE-GM and GM-alone implants. While GFAP expression is typically widespread and not limited to reactive astrocytes, in our studies, GFAP\(^+\) staining in areas outside the immediate implant area was either sparse or absent. Accordingly, GFAP\(^+\) reactive astrocytes were morphologically defined as having hypertrophied cell bodies and processes with either overlapping or non-overlapping cellular processes (depending on the level of activation) (Sofroniew, 2009). To measure astroglial depth, 6-8 sections that contained a visible implant site were selected from the series of GFAP immunostained sections. As an indication of overt tissue damage and
inflammation (astrocytosis), reactive astroglial depth was measured from the outer edges of the implant (where the surface of the GMs end) as it extended out into the neuropil. Astrocytosis was defined by our morphological characterization of reactive astrocytes and by astrocytic proliferation. Areas that showed no GFAP staining, or astrocytic areas that were devoid of hypertrophied cell bodies and processes were defined as spared, healthy tissue. A minimum of 5 measurements was taken around the implant site in each section and averaged per animal. Measurements were taken blinded to each animal’s treatment group.

### 7.2.6 Statistical Analyses

Two-way [treatment (hRPE-GM vs. GM-alone) × time] ANOVA with planned comparisons was used to compare inflammatory responses between hRPE-GM and GM-alone implanted animals using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). When significant $F$ values were found, Bonferroni’s comparison test was used to determine individual differences between different cell types and across time.

### 7.3 RESULTS

HRPE-GM dose estimates and viability pre- and post-implant are listed in Table 7.1. HRPE-GM dose estimates prior to implantation consistently resulted in greater than 80% cell viability with a mean calculated dose concentration range of 1,840-3,036 cells per µl of hRPE-GM solution. This resulted in approximately 22,000-36,000 hRPE cells injected into each animal. Post surgical cell viability of the hRPE-GM suspension was slighter lower than expected in this set of experiments (compared to those reported in Chapter 5), ranging 59-78%.

All animals responded well to the surgical implantation procedure. Most animals did not lose weight or need any post-operative care. However, 6 animals (in the 10 and 20 weeks post implant group) had to be euthanized prematurely due to complications not associated with the treatment. Most of these animals were in the GM-alone group (particularly the 20 weeks post-implant group). Since all GM-alone animals were implanted with the same GM lot, we determined whether it was possible to group together the GM-alone animals implanted with the 1105 group with those implanted with the 0010 group. A statistical analysis made prior to each study revealed no significant differences between the two GM-alone groups; therefore, each GM-alone group will be combined as a single GM-alone group in our analyses.
Table 7.1. HRPE-cell lines used in Chapter 7. HRPE cell concentrations and pre-implant cell viability were assessed immediately prior to surgical implantation. Cell viability of the remaining hRPE-GM suspension (after surgery) was calculated at the end of the surgical day.

Animals in the 0802 hRPE-GM group detailed in this chapter are remaining animals from the group described in Chapter 5. Although these animals were sectioned throughout the implant site, the section periodicity of the samples was unknown; therefore, we were unable to make the appropriate estimates of implant volume. We were, however, able to carry out stereological analyses to determine the ED1⁺ \( N_V \) and measure astroglial depth.

7.3.1 HRPE-GM Implant Characterization

Figures 7.1, 7.2, and 7.3 represent H&E reconstructions of hRPE-GM implants 2-4 weeks, 10 weeks, and 20 weeks post-implant, respectively. Implants were localized to the striatum. Implant were heterogeneous: the number of GMs varied between sections and implant volumes differed between animals (see below). In a couple of rats, some of the GMs extended dorsally into the corpus callosum and cortex along the implant tract. In most animals, both implant tracts could be distinguish from each other. However, in some animals (particularly in
the early 2-4 week post-implant group) there seemed to be one continuous implant tract with no separation between the two. Edema, focal hemorrhage, and inflammatory cell infiltration was seen within and immediately surrounding the implant site. Increased cellular infiltration was observed in the corpus callosum immediately surrounding the implant tract in some animals (not shown), but these cases were restricted to only the 2-4 weeks post-implant group.

**Figure 7.1.** H&E reconstruction of an hRPE-GM implant site 2-4 weeks post-implant. GMs were heterogeneously distributed anterior (A) to posterior (H) within the implant tract. Increased cellular accumulation was seen surrounding the implant site (D). The two implant tracts were difficult to distinguish (and seemed continuous) in this animal. Photomicrographs were taken ≈ 250 µm apart. Scale bar = 100 µm.
Figure 7.2. H&E reconstruction of an hRPE-GM implant site 10 weeks post-implant. By 10 weeks post-implant, inflammatory infiltration seemed to have subsided. Both implant tracts (tract 1: A-D; tract 2: E-H) were distinguishable in this animal. Photomicrographs were taken ≈ 200 µm apart. Scale bar = 100 µm.
Figure 7.3. H&E reconstruction of an hRPE-GM implant site 20 weeks post-implant. Minimal inflammatory infiltration was seen surrounding the implant site. Both implant tracts were distinguishable in this animal. The anterior implant tract (A-C) seemed smaller than the second posterior tract (D-F). GMs seemed to be relatively evenly distributed anterior (A) to posterior (H), but implant areas were qualitatively smaller at 20 weeks compared to the earlier time points. Photomicrographs were taken ≈ 200 µm apart. Scale bar = 100 µm.

Two animals (one animal from the 20-week group and one from the 2-4 week post-implant group) contained discernable implant tracts (as characterized by H&E), but did not contain any GM material. These animals were subsequently removed from the subsequent analyses. The implant volumes of each animal in two of the cell groups (1105 and 0010) are plotted in Figure 7.4. As expected, implant volume was greatest at 2-4 weeks post-implant, but gradually decreased with time. A two-way ANOVA revealed an overall significant interaction ($F_{4,39} = 3.40; p = 0.0178$) between treatment ($F_{2,39} = 8.35; p = 0.0010$) and time ($F_{2,39} = 12.56; p < 0.0001$) in implant volume between hRPE-GM and GM-alone implants. Post-hoc planned
comparisons revealed significant differences in implant volumes between the 1105 and 0010-hRPE-GM implanted groups \( (p < 0.001) \) and between the 0010-implanted and GM-alone group at 2-4 weeks post-implant \( (p < 0.01) \) (Figure 7.4). Mean (± SEM) implant volumes for the 1105 hRPE-GM implanted group were: 0.568 ± 0.030 mm\(^3\) (at 2-4 weeks), 0.532 ± 0.039 mm\(^3\) (at 10 weeks), and 0.371 ± 0.053 mm\(^3\) (at 20 weeks). The 0010 hRPE-GM group mean implant volumes were 1.326 ± 0.264 mm\(^3\) (at 2-4 weeks), 0.623 ± 0.066 mm\(^3\) (at 10 weeks), and 0.571 ± 0.127 mm\(^3\) (at 20 weeks). Mean volumes for GM-alone implanted animals were 0.761 ± 0.099 mm\(^3\) (at 2-4 weeks), 0.380 ± 0.030 mm\(^3\) (at 10 weeks), and 0.548 ± 0.052 mm\(^3\) (at 20 weeks post-implant).

- **Figure 7.4.** Scatter plot of hRPE-GM and GM-alone implant volumes of each animal. As expected, implant volumes gradually decreased with time in all groups. The 0010 hRPE-GM implanted group had significantly larger implant volumes than both the 1105 \( (p < 0.001) \) and GM-alone group \( (p < 0.01) \) at 2-4 weeks post-implant.

- **7.3.2 Post-Mortem Stereological Analysis of Cellular Inflammatory Response to hRPE-GM Implants**

\( ED1^+ \) (CD68) staining was used to determine cellular inflammation and macrophagic activity in response to hRPE-GM and GM-alone implants (Figure 7.5). There were observable qualitative differences in \( ED1^+ \) staining between hRPE-GM implanted (Figures 7.5A and 7.5C) and GM-alone (Figures 7.5B and 7.5D) animals. Widespread staining of macrophages and activated microglia was seen across all groups at all time-points. The early (2-4 weeks post-implant) time-point revealed the most staining but gradually decreased with time. \( ED1^+ \) staining...
was most frequently found within the implant site (Figure 7.5D). Occasional ED1\(^+\) staining was also found in the corpus callosum and in the cortex along the implant tract. No staining was found in remote striatal areas away from the implant site. Due to the intracellular localization of the CD68 receptor, it was not possible to assess the morphological features of ED1\(^+\) cells with its staining pattern (Figure 7.5E). However, knowing the response pattern of activated microglia (see Chapter 4), it is safe to assume that ED1\(^+\) cells were predominantly composed of over-activated “phagocytic” microglia.

A stereological analysis revealed differences in the inflammatory response between hRPE-GM and GM-alone implanted animals (Figure 7.6). A two-way ANOVA of ED1\(^+\) \(N_V\) (Figure 7.6A) revealed an overall interaction effect \((F_{6,46} = 3.41; p = 0.0073)\) between treatment (hRPE-GM vs. GM-alone) \((F_{3,46} = 34.95; p < 0.0001)\) and time \((F_{2,46} = 141.54; p < 0.0001)\). Post hoc planned comparisons revealed significant differences in the \(N_V\) of ED1\(^+\) cells between the 1105 hRPE-GM and GM-alone implanted groups at 2-4 weeks \((p < 0.001)\) and 10 weeks \((p < 0.001)\) post-implant, the 0010 hRPE-GM and GM-alone implanted groups at 2-4 weeks \((p < 0.001)\) and 10 weeks \((p < 0.001)\) post-implant, and between the 0802 hRPE-GM and GM-alone implanted groups at 2-4 weeks \((p < 0.001)\), 10 weeks \((p < 0.01)\) and 20 weeks \((p < 0.05)\) post-implant. No significant differences \((p > 0.05)\) were found between the 1105 or 0010 hRPE-GM groups and the GM-alone group at 20 weeks post-implant.

Comparable results were seen using total estimated numbers of ED1\(^+\) macrophages (Figure 7.6B). A two-way ANOVA revealed an overall interaction effect \((F_{6,41} = 278; p = 0.039)\) between treatment \((F_{2,41} = 13.34; p < 0.0001)\) and time \((F_{2,41} = 63.66; p < 0.0001)\). Post hoc comparisons revealed significant differences in ED1\(^+\) total cell numbers between the 1105 hRPE-GM and GM-alone implanted groups at 2-4 weeks \((p < 0.001)\) and 10 weeks \((p < 0.05)\) post-implant, and the 0010 hRPE-GM and GM-alone implanted groups 10 weeks \((p < 0.05)\) post-implant. The non-significant differences between the 0010 hRPE-GM and GM-alone implanted groups at 2-4 weeks post-implant were likely due to the large outlying volume implants of 2 animals in the 0010 hRPE-GM group (see Figure 7.4). No significant differences \((p > 0.05)\) were found between the 1105 or 0010 hRPE-GM groups and the GM-alone group at 20 weeks post-implant.
Figure 7.5. ED1⁺ staining at the implant site of hRPE-GM (A, C) and GM-alone (B, D) implanted animals. The morphological features of ED1⁺ activated microglia (E) were difficult to establish due to the internalization of the CD68 receptor. Scale bars in (B) and (D) = 50 µm, (E) = 10 µm (Note: scale bar in B applies to A-B; scale bar in D applies to C-D).
Figure 7.6. \( N_V \) of ED1\(^+\) macrophages/activated microglia in hRPE-GM and GM-alone implanted animals (A). All three hRPE-GM cell groups induced a significantly reduced inflammatory response (compared to the GM-alone group) at 2-4 weeks and 10 weeks post-implant. Only the 0802 hRPE-GM group revealed a significantly reduced inflammatory response at 20 weeks post-implant. Total number of ED1\(^+\) macrophages (B) revealed similar results. Due to the unknown section periodicity of the 0802 hRPE-cell group, we were unable to make estimates of total numbers. *** \( p < 0.001 \); ** \( p < 0.01 \); * \( p < 0.05 \).

An attempt was also made to determine any differences in ED1\(^+\) staining in the SN (Figure 7.7). Surprisingly, of all the animals analyzed, only six animals exhibited ED1\(^+\) staining in the SNc. Further, all six animals that did demonstrate ED\(^+\) staining were all from the 2-4 weeks post-implant group (Figure 7.7; top). No ED1\(^+\) staining was seen in animals at 10 weeks (Figures 7.7; middle) or 20 weeks (Figure 7.7; bottom) post-implant. ED1\(^+\) staining was also seen in dorsal areas that seemed to be due to inflammation surrounding the cannula tract from 6-
OHDA lesioning (not shown). This observation was unexpected as previous researchers observed an activated microglial response to 6-OHDA lesioning. However, most microglial responses were observed within 4 weeks post-lesioning (Akiyama and McGeer, 1989, Cicchetti et al., 2002). In our studies, a minimum of 8-10 weeks has passed between 6-OHDA lesioning and analysis. This result will be discussed further in the conclusions chapter.

**Figure 7.7.** ED1\(^+\) staining in the SN of unilateral 6-OHDA-lesioned animals. Left SNC of each image was lesioned. ED1\(^+\) macrophages/activated microglia was observed in some animals at 2-4 weeks post-implant (top; black arrow), but no staining was seen in subsequent time points at 10 weeks (middle) or 20 weeks (bottom) post-implant. SNC = substantia nigra pars compacta; SNR = substantia nigra pars reticulata. Scale bar = 1000 μm.
7.3.3 Post-Mortem Analysis of Reactive Astroglial Depth

Reactive astrogliosis is a dynamic process that can persist for weeks (or months) following surgery. This process includes morphological changes to astrocytes (hypertrophied cell bodies and processes), astrocyte proliferation, and increased GFAP expression. Therefore, we examined the degree of response by measuring the reactive astroglial depth as it extended out from the implant site (to the surrounding parenchyma) using the aforementioned characteristics. We observed qualitative differences in astroglial responses between hRPE-GM and GM-alone implanted animals Figure 7.8. There was increased proliferation and GFAP expression in the surrounding neuropil (Figures 7.8A and 7.8E) compared to areas with a minimal response (Figure 7.8B and 7.8D). A high magnification photomicrograph also illustrates the morphological changes associated with activated astrocytes (Figure 7.8B, inset). Very little GFAP expression was observed in the neuropil away from the implant site (Figure 7.8C).

Mean differences in astroglial depth between the hRPE-GM and GM-alone implanted groups are illustrated in Figure 7.9. The depth measurements were variable both within each animal (between sections analyzed) and between animals in the same group. Therefore, a minimum total of 30 measurements were made within each animal and the mean astroglial depth of each animal was calculated and compared. A two-way ANOVA revealed overall significant effects in treatment (hRPE-GM vs. GM-alone) \((F_{3,37} = 8.52; \ p = 0.0002)\) and time \((F_{2,37} = 10.02; \ p = 0.0003)\). Post hoc planned comparisons revealed significant differences between the 1105 hRPE-GM and GM-alone group at 2-4 weeks post-implant \((p < 0.05)\), the 0802 hRPE-GM and 0010 hRPE-GM group \((p < 0.01)\) at 2-4 weeks post-implant, and between the 0802 hRPE-GM and GM-alone group \((p < 0.001)\) at 2-4 weeks post-implant. The reactive astroglial depths in response to the 1105 hRPE-GM implants were \((\text{mean} \pm \text{SEM}): 111 \pm 11 \mu m \text{ (at 2-4 weeks)}, 77 \pm 18 \mu m \text{ (at 10 weeks), and } 41 \pm 9 \mu m \text{ (at 20 weeks). The astroglial depths in response to the 0010 hRPE-GM implants were } 152 \pm 26 \mu m \text{ (at 2-4 weeks)}, 90 \pm 16 \mu m \text{ (at 10 weeks), and } 66 \pm 15 \mu m \text{ (at 20 weeks). For the 0802 hRPE-GM group, the astroglial depths were: } 44 \pm 20 \mu m \text{ (at 2-4 weeks), } 38 \pm 5 \mu m \text{ (at 10 weeks), and } 29 \pm 3 \mu m \text{ (at 20 weeks). The GM-alone group had astroglial depths of } 182 \pm 29 \mu m \text{ (at 2-4 weeks), } 108 \pm 8 \mu m \text{ (at 10 weeks), and } 96 \pm 26 \mu m \text{ at 20 weeks post-implant.}
Figure 7.8. GFAP* staining in the surrounding neuropil of hRPE-GM and GM-alone implanted animals. Qualitative differences in astrogliosis were observed between hRPE-GM (A, D), and GM-alone (B, E) implants. The striatal neuropil away from the implant site (C) revealed little GFAP expression. Black arrow in (B) is an example of measured reactive astroglial depth based on the morphological features of reactive astrocytes (B, inset). GM = gelatin microcarriers; STR = striatum. Scale bars in (B) and (E) = 50 µm, (B, inset) = 10 µm (Note: scale bar in B applies to A-B; scale bar in E applies to C-E).
Figure 7.9. Reactive astrogliosis in response to hRPE-GM and GM-alone implants. Significant differences in astroglial depth were found between the 1105 and 0802 hRPE-GM implanted and GM-alone implanted animals. There was a qualitative reduction in astrogliosis in the 0802 hRPE-GM (compared to the GM-alone) group at 10 and 20 weeks post-implant; however, no statistical significance was found (which was likely due to the small number of animals (N=2) in the 0802 hRPE-GM group at each time point). * $p < 0.05$; *** $p < 0.001$.

7.4 STUDY SUMMARY

This is the first study to quantitatively compare long-term inflammatory reactions to hRPE-GM and GM-alone implants in the rat. Along with the data presented in Chapter 5, we hypothesize that hRPE-GM implants can survive long-term and endogenously reduce the host-inflammatory response despite the absence of immunosuppression. Our results revealed a decrease in both ED$^+$ macrophage/activated microglial response and reactive GFAP$^+$ astrocytosis to hRPE-GM implants when compared to GM-alone implants. This was particularly evident at the earlier time points, which is the critical period during which the interaction between host-inflammatory cells and implanted cells can determine the implants’ long-term survival. The implications of these results and the mechanisms of action that may reduce the inflammatory response are discussed in the conclusions.
CHAPTER 8. LONGITUDINAL TRACKING OF HRPE-GM IMPLANTS WITH FEPRO LABELING AND MRI

8.1 INTRODUCTION

Only one study has attempted to longitudinally track hRPE-GM implants in the brain. In our laboratory, Doudet and colleagues (Doudet et al., 2004) demonstrated an increase in $^{18}\text{F}$-DOPA (and concomitant decrease in $^{11}\text{C}$-RAC) binding with PET in hRPE-GM implanted parkinsonian primates. However, the PET signal resolved by six months (despite long-lasting behavioural improvements). We hypothesized that this was due to PET’s relatively poor spatial resolution which is particularly problematic when assessing a small volume of transplanted cells. Therefore, we attempted to find an alternative, non-invasive, method to longitudinally follow the fate of implanted hRPE cells in the brain.

Several advances in magnetic resonance imaging (MRI) have led to a range of alternative approaches to in vivo imaging. MRI has deep brain imaging capabilities and is continually improving its spatial resolution. Accordingly, high-field (>7 Tesla) MR scanners are able to assess relatively small volumes of tissue or cell implants, which makes them an attractive alternative for in vivo small animal studies of cell transplant survival (Shapiro et al., 2006; Kim et al., 2007; Kuhlpeter et al., 2007). Furthermore, MR requires no radiation exposure and is non-invasive, making it useful for longitudinal studies of animals and humans.

In order to use MRI in cell transplantation studies, cells need be pre-labeled with an MR-compatible contrast agent that is 1) non-toxic and 2) can be intracellularly incorporated (in sufficient quantities) for MRI detection (Figure 8.1). Accordingly, the use of magnetic iron particles such as superparamagnetic iron oxide (SPIO) nanoparticles has been shown to be viable. SPIOS such as ferumoxides (Fe) have been extensively used as an ex vivo MRI contrast agent (Frank et al., 2003; Arbab et al., 2004a; Arbab et al., 2004c; Dunn et al., 2005) and approved for the in vivo use of imaging blood flow and volume in humans. Dextran-coated Fe suspensions such as Feridex (Clément et al., 1998) have also been used for intracellular labeling. When Fe is electrostatically complexed, in vitro, to a transfer vehicle such as protamine sulfate (Pro; an FDA approved, low molecular weight, naturally-occurring polycationic peptide that has been used as a transfection agent in gene therapy studies (Sorgi et al., 1997)), the FePro complex can be efficiently and effectively incorporated into cells with minimal adverse effects (Frank et
al., 2003; Arbab et al., 2004b). FePro clusters intracellularly and shortens the nuclear magnetic resonance T₂ relaxation time, leading to dark (hypointense) spots when viewed with T₂- or T₂*-weighted imaging methods. These characteristics make these agents attractive for potential use in \textit{in vivo} cell labeling and tracking and for future clinical use. Therefore, it is critical to validate the use of these MR-based cell tracking methods.

\textbf{Figure 8.1}. Schematic diagram of FePro-labeling of hRPE cells.

One aspect of SPIO labeling that has often been overlooked is the potential effect that the MR label itself might have on the host. As detailed in the previous chapters, the evaluation of cell-based therapeutic strategies is confounded by inflammatory reactions to the implant. These responses are believed to play a major role in the significant cell loss consistently reported in transplantation studies (Brundin et al., 1988; Olanow et al., 1996). While several studies have looked at the ability of FePro labeling to longitudinally monitor labeled cells \textit{in vivo}, few studies have been done on the brain. Further, the effects that exogenous iron (released from dying cells) may have on both the implant itself and on the host (particularly in areas that are sensitive to iron
accumulation) has been poorly investigated. It is believed that SPIO complexes released after cell death are taken up by activated macrophages present in the transplant surrounding area, potentially confounding quantification. Of more concern, the accumulation of free SPIO (i.e. iron) in the surrounding neuropil may lead to an exacerbated inflammatory reaction. The use of intravenous SPIO as a contrast agent and for intracellular labeling appears to be safe. On the other hand, the accumulation and breakdown of iron in the proximity of native cells may lead to an increased load of free iron and subsequent inflammatory response and/or oxidative stress. Increased iron concentrations in areas of neuronal loss are routinely reported in most neurodegenerative diseases (Berg and Youdim, 2006), and whether this is a cause or a consequence of the neurodegenerative process is still being debated.

Several features of hRPE cells make them an attractive source for studying SPIO labeling. First, hRPE cells are easily expanded and maintained in culture with minimal loss of viability, providing an extended opportunity to monitor cell viability and toxicity in response to SPIO labeling. Second, hRPE cells are fully differentiated epithelial cells, which minimizes potential complications associated with fetal or stem cell differentiation patterns in response to cell labeling and transplant. Finally, hRPE cells do not proliferate when in a stable monolayer or when attached to GMs for *in vivo* implantation, and they do not migrate away from the implant site (Flores et al., 2007), eliminating transfer of the MR label and dilution issues.

The aim of the following study was to 1) determine the feasibility of using MR and SPIO labeling to non-invasively track implanted hRPE cells *in vivo* over time, and 2) evaluate the potential effects of the SPIO label on the host. HRPE cells were labeled with different concentrations of FePro, and the labeling efficiency, cell viability, proliferative capacity, and MRI detection threshold were determined post-labeling. Then, using pre-determined concentrations of FePro, FePro-labeled hRPE-GM were implanted into rats and monitored with MRI at different times post-implant. Finally, a post-mortem histological analysis was done to estimate cell survival and intensity of the host inflammatory response to FePro-labeled and non-labeled hRPE-GM implants.
8.2 MATERIALS AND METHODS

8.2.1 HRPE Cell Culture

The 0802 hRPE cell lot (Table 8.1) was used throughout these experiments. HRPE cells were grown in complete cell culture medium, and maintained in non-laminated T-25 cell culture flasks under 5% CO₂ at 37°C. The flasks were left alone for 3-4 days to allow the cells to grow to 80-90% confluence. The culture medium was exchanged 1-2 times during incubation, or if a color change was noted in the culture medium. The cells were examined regularly under the microscope for proper cell growth and contamination.

<table>
<thead>
<tr>
<th>hRPE-Cell Line</th>
<th>Donor Age</th>
<th>Cell Concentration (hRPE cells/µl)</th>
<th>Cell Viability Pre-Implant</th>
<th>Cell Viability Post-Implant</th>
</tr>
</thead>
<tbody>
<tr>
<td>0802 hRPE</td>
<td>22 weeks</td>
<td>2992</td>
<td>91%</td>
<td>88%</td>
</tr>
<tr>
<td>Male donor</td>
<td>Harvested: 2001</td>
<td>2904</td>
<td>80%</td>
<td>83%</td>
</tr>
</tbody>
</table>

Table 8.1. HRPE-cell lines used in Chapter 8.

8.2.1.1 FePro Cell Labeling and Preparation

FePro labeling was carried out using preparations previously described (Arbab et al., 2004b) (Figure 8.1). The contrast agent Feridex IV (Berlex Laboratories, Wayne, NJ) is a dextran-coated SPIO particle approximately 80-150 nm in size with a stock iron content of 11 mg/mL. The transfection agent Pro (American Pharmaceutical Partners, Schaumburg, IL), supplied at 10 mg/mL, was prepared as a stock solution of 1 mg/mL in sterile dH₂O. FePro labeling solutions were prepared fresh immediately prior to labeling: 50 µg/mL Fe and varying concentrations of Pro (0.5-4.5 µg/mL) was added to the cell culture medium and intermittently mixed for 5-10 minutes by hand.

Three to four days after seeding, culture medium was aspirated from the hRPE-cell containing flasks and replaced with fresh FePro-containing culture medium. HRPE cells were incubated overnight for approximately 16 hours. The in vitro cell viability and MR experiments were conducted over a range of Fe (0-50 µg/mL) and Pro (0-4.5 µg/mL) concentrations. The final concentrations of FePro used in our in vivo animal studies were 50:1.0 µg/mL, respectively.

After incubation, the FePro-containing culture medium was aspirated, the hRPE cells
were washed with PBS and then stored in fresh culture medium for a minimum of 24 hours to allow the culture to equilibrate and to ensure that FePro complexes were fully endocytosed.

8.2.1.2 HRPE-GM Attachment

The same attachment procedure described previously was used in these experiments. Briefly, confluent hRPE cells, either FePro-labeled or non-labeled, were harvested by trypsinization (with 10x trypsin for 1-2 minutes) and mechanical agitation, re-suspended in fresh culture medium, and passively adhered to sterile GMs. The hRPE-GM suspension was maintained in an incubator overnight under 5% CO₂ at 37°C. Just prior to use, the hRPE-GM suspension was gently washed with sterile HBSS, and a small sample was treated with a dispase solution to assess cell viability and dose concentration using the trypan blue exclusion method. HRPE-GM suspensions that did not meet our required cell viability or dose concentration (as described previously) were re-assessed and/or discarded.

8.2.2 In Vitro Analyses of FePro-Labeled hRPE Cells

8.2.2.1 Cell Viability and Growth Assessment

To determine changes in cell viability in response to FePro transfection, FePro-labeled hRPE cells were seeded in 8-well chamber slides and trypsinized at 2, 4, 6, and 8 days post-seeding. HRPE cells were assessed for cell viability and concentration using the trypan blue exclusion method. HRPE-cell viability (calculated as live cells [no trypan blue inclusion]/total number of hRPE cells x 100%) and mean concentrations were compared to concurrently growing non-labeled hRPE cells.

8.2.2.2 Staining of Intracellular Iron and Labeling Efficiency

FePro-labeled and non-labeled hRPE cell suspensions were fixed with 4% paraformaldehyde, paraffin embedded, and sectioned at a thickness of 6 µm. Sections were deparaffinized and rehydrated, and prussian blue (PB) staining was performed to visualize SPIOs in FePro-labeled cells. HRPE cells were stained with Perl’s Prussian blue (5% potassium ferrocyanide/ 10% HCl) (Sigma-Aldrich) for 20 minutes, washed with dH₂O, and counterstained with nuclear fast red. Sections were then dehydrated and coverslipped for analysis.
8.2.2.3 EM

FePro-labeled hRPE cells (50:4.5 µg/mL) were processed for EM ultrastructural morphological analyses. Details of the preparation are described elsewhere in Chapter 6. Briefly, FePro-labeled hRPE cells were fixed with 4% paraformaldehyde plus 1% glutaraldehyde, and post-fixed overnight in 1% buffered osmium tetroxide. Cell suspensions were then washed in acetate buffer, stained with 2% aqueous uranyl acetate, then dehydrated and equilibrated in propylene oxide, and embedded in Epon. EM blocks were sectioned ultrathin (65-70 nm), mounted in Formvar-coated slot grids, and stained with lead citrate. Electron micrographs were photographed with 3.25 x 4.00-inch Kodak 4489 plate film and negatives were scanned on an Epson Expression 1680 scanner using Epson Twain-Pro software.

8.2.2.4 In Vitro MRI of FePro-Labeled hRPE Cells

HRPE cell suspensions (of equal cell concentration and volume) labeled with varying concentrations of FePro (0-50:0-4.5 µg/mL, respectively) were suspended between two gelatin layers, and MR was carried out to determine differences in signal intensity in relation to total SPIO concentration loaded. HRPE cells preparations were scanned with a 7T Bruker BioSpin system (Bruker, Etlingen, Germany), with data acquired using a FLASH sequence (TR/TE = 500/6).

8.2.3 In Vivo Analyses of Implanted FePro-Labeled HRPE-GM

8.2.3.2 FePro-Labeled hRPE-GM Surgical Implantation

Sprague-Dawley rats (N=8) (University of British Columbia Animal Facility) weighing 280-325 g were used for these experiments. All experimental procedures were approved by the University of British Columbia Animal Care Committee.

Surgical details of hRPE-GM implantations in rodents are previously described in Chapter 5. Briefly, normal (non-lesioned) rats received two FePro-labeled and two non-labeled hRPE-GM implants in opposite hemispheres at the following striatal coordinates: 1) AP +1.6, ML ±2.5, DV -6.0, -4.0; and 2) AP -0.4, ±3.5, DV -6.0, -4.0 (Paxinos and Watson, 1997). HRPE cells were deposited through a to 22-gauge spinal needle connected to a Hamilton syringe. A total of 6 µL of the hRPE-GM suspension was injected into each site. Immunosuppression was not used in any of the animals.
8.2.3.3 In Vivo MRI of Implanted FePro-Labeled hRPE-GM

For *in vivo* MRI, each rat was anesthetized with isoflurane and imaged at 1 week and 1, 2, 3, 4 and 5 months post-implant. MR studies were performed with a 7T Bruker BioSpin animal scanner using a quadrature volume coil for excitation and a 4-element phased array coil for signal reception. T₂-weighted images were acquired using a fast-spin-echo sequence with the following parameters: TR/TE = 2000/17, FOV = 40 x 40 mm, matrix = 256 x 256, slice thickness = 1 mm, slice number = 10.

8.2.3.4 MRI Image Analysis

All images were converted to analyze7.5 format from the scanner’s private formats. Intensity inhomogeneities that were introduced by non-uniform radiofrequency coil transmission and gradient-induced eddy currents were corrected by employing a homomorphic unsharp masking (Brinkmann et al., 1998) and a histogram-based standardization in-group level (Nyul et al., 2000). After images were processed and corrected, rectangular ROIs were manually placed around the regions of interest e.g. observable hypointense sites and the means of signal intensity were calculated.

8.2.4 Post-Mortem Stereological Analyses

8.2.4.1 PB⁺ Cell Counts

After each MRI session at 1 week and 1, 2, 3, 4 and 5 months post implant, one rat was randomly selected, euthanized by pentobarbital overdose, and transcardially perfused with 4% paraformaldehyde as previously described. After the brains were removed, the striatum was dissected out and immersed in additional fixative for 24 hours. Tissue blocks were paraffin-embedded and coronally sectioned at a thickness of 6 μm through the implant site. Tissue from the 4 months post implant rat was damaged during processing and could not be used for any quantitative analyses.

A stereological analysis was performed to determine the number of PB⁺ cells (to identify Fe³⁺-containing cells) in the implant site using the optical disector method previously described (Chapter 7). A Nikon Eclipse E800 microscope (Nikon Instruments), QImaging Retiga Exi camera (QImaging), and NIS Elements software (Nikon Instruments) were used for the analyses. Briefly, 50 μm × 50 μm disector counting grids were randomly placed within the implant site across sections. Using a 60x objective, the number of PB⁺ cells with a visible red-stained nucleus
was counted, until a total of 150-200 hits were obtained across sections. The \( N_V \) of PB\(^+ \) cells in the implant was calculated from: \( N_V = \frac{\sum Q}{\sum V_{ref}} \), while the reference volume, \( \Sigma V_{ref} \), was calculated as \( \Sigma V_{ref} = \alpha_{ref} \times h \). Using the sampled PB-stained sections, the implant area was measured on each section and the total volume was calculated from: \( V = \Sigma A \times T \times P \). Cell counts were taken on both the FePro-labeled and non-labeled side. We were unable to blind our counts since it was visually obvious which hemisphere received what treatment. We were, however, blinded to the time-point at which each animal was assigned to.

### 8.2.4.2 Stereological Inflammatory Analyses

The inflammatory response to FePro- non-labeled hRPE-GM implants was measured in the same way as described in Chapter 7. Briefly, series of sections were treated with ED1 (1:500) and GFAP (1:1000) to visualize macrophages/activated microglia and astrocytosis, respectively. The \( N_V \) of ED1\(^+ \) cells within the implant site was estimated by dividing the cell numbers, \( \Sigma Q \), by the total reference volume analyzed, \( \Sigma V_{ref} \) as described above. To measure astrocytosis, reactive astrocytes were morphologically defined (as GFAP\(^+ \) reactive cells that contained hypertrophied cell bodies and processes), and astroglial depth was measured from the outer edges of the implant as it extended out into the healthy parenchyma. Areas that were either devoid of GFAP staining or contained astrocytes that did not show hypertrophied cell bodies and processes were considered healthy tissue.

### 8.2.5 Statistical Analyses

Since these experiments were designed as part of a “proof of concept” study, no prior power analysis was done. Therefore, two-way ANOVAs were used to determine overall interactions between treatment (FePro-labeled vs. non-labeled) and time. Exponential regression was used to model hRPE-cell survival, and a multiple regression analysis was used to compare MR hypointensity values with our post-mortem stereological analysis.

### 8.3 RESULTS

#### 8.3.1 In Vitro Characteristics and Cell Viability of FePro-Labeled hRPE Cells

HRPE cell cultures tolerated the FePro labeling procedure well. Regular microscopic inspections revealed no premature death or contamination in any FePro-labeled hRPE cell
cultures, and FePro-labeled cells adhered to non-laminated surfaces as well as non-labeled hRPE cells. FePro-labeled cells, however, were significantly darker in color, presumably from the intracellular uptake of Fe. Labeled hRPE cells also seemed “heavier”; FePro-labeled cells sank to the bottom of a microcentrifuge tube faster than unlabeled cells when taking aliquots (for cell counts) forcing us to gently agitate the slurry more often to keep the cells in suspension. This, however, did not seem to affect growth or viability (see results below).

PB staining was used to visualize SPIO uptake in vitro in FePro-labeled hRPE cells (Figure 8.2). PB$^+$ staining was seen intracellularly in hRPE cells labeled with 50 µg/mL Fe and varying concentrations (0-4.5 µg/mL) of Pro. The accumulation of SPIO complexes was heterogeneous and dependent on the Pro concentration added. Manual counting of random samples of PB$^+$ hRPE cells yielded close to 100% labeling efficiency at 50 µg/mL Fe combined with 1.0-4.5 µg/mL Pro. However, there were qualitative differences in PB$^+$ staining between hRPE cells cultured with low (1.0-1.5 µg/mL) (Figures 8.2A, 8.2B) and those cultured with high (4.5 µg/mL) (Figure 8.2C) Pro concentrations: hRPE samples incubated with higher Pro concentrations seemed “overloaded” with SPIO complexes. Indeed, the FePro label occupied almost all of the cell’s intracellular space and made them look swollen. HRPE cells labeled with Fe only (and no Pro) showed no intracytoplasmic PB$^+$ granules (Figure 8.2D).

EM of hRPE-GM FePro-labeled with 50:4.5 µg/mL confirmed the endocytosis of electron dense, intracytoplasmic SPIOs (Figures 8.2E, 8.2F). FePro complexes seemed confined to cytoplasmic or endosomal regions with little accumulation in the extracellular space or GM matrix. There were no observable morphological differences between FePro-labeled and non-labeled hRPE-GM (data not shown). Cell viability and proliferative capacity of hRPE cells were analyzed using the trypan blue exclusion assay (Figure 8.2G). There was no significant difference in cell viability between FePro-labeled and non-labeled hRPE cells ($F_{3,88} = 1.09; p = 0.357$) using our highest Pro concentration (4.5 µg/mL) and maximum intracellular labeling (Figure 8.2C). Furthermore, there were no observable differences in growth rate, cell phenotype, and morphology of FePro-labeled hRPE cells when compared to control (data not shown).
Figure 8.2. In vitro characterization of FePro-labeled hRPE cells. Photomicrographs of PB\(^+\) staining of hRPE cells treated with different concentrations of FePro. PB\(^+\) staining of hRPE cells (A) and hRPE-GM (B) treated with 50:1.5 \(\mu\)g/mL FePro were qualitatively different than hRPE cells treated with 50:4.5 \(\mu\)g/mL FePro (C). HRPE cells treated with 50:0 \(\mu\)g/mL FePro (D) showed minimal intracellular iron accumulation. EM of FePro-labeled (50:4.5 \(\mu\)g/mL) hRPE-GM (E) and hRPE cells (F) confirmed intracellular uptake of SPIOs (dark spots). Graph comparing cell viability between FePro-labeled (50:4.5 \(\mu\)g/mL) and non-labeled hRPE cells (G) suggests no deleterious effects from FePro labeling in vitro. Scale bar in (D) = 10 \(\mu\)m, (E) = 5 \(\mu\)m, (F) = 2 \(\mu\)m (Note: scale bar in D applies to A-D).
8.3.2 MR Imaging of FePro-Labeled hRPE Cells

8.3.2.1 MRI of In Vitro FePro-Labeled hRPE-GM

MRI was performed on FePro-labeled hRPE cells to establish phantom data and determine MR sensitivity to detecting different concentrations of SPIOs (Figure 8.3). MR demonstrated hypointense areas from most FePro-labeled hRPE cells. Signal intensity depended on the varying concentrations of FePro (25-50:0.5-1.5 µg/mL) that were used. Based on these results and the in vitro histological observations described above, we decided to use a final concentration of 50:1.0 µg/mL FePro for our in vivo animal studies. This helped us maximize MR imaging sensitivity while minimizing any potential adverse effects on the host.

![Figure 8.3](image)

**Figure 8.3. In vitro MRI of hRPE cells.** MR images of hRPE cells (approximately 10^5 cells) using the 7T animal system. The following FePro concentrations were used: unlabeled (A); 25:1.0 µg/mL (B); 50:0.5 µg/mL (C); 50:1.0 µg/mL (D); 50:1.5 µg/mL (E). A final concentration of 50:1.0 µg/mL FePro (C) was subsequently used for our in vivo animal studies.

8.3.2.2 MRI of FePro-Labeled hRPE-GM Implanted Into the Rat Brain

All animals tolerated the surgery well. There were no observable complications associated with FePro labeling, and all animals survived until their primary endpoint. As expected in normal animals, there were no observable behavioural effects in response to hRPE-GM implants (not shown). T2-weighted MR images of FePro-labeled and non-labeled hRPE-GM implants in the rat are shown in Figure 8.4 (A, B). FePro-labeled hRPE-GM implants were easily identified as hypointense areas surrounded by a characteristic “blooming” artifact (Figure 8.4; right [R] hemisphere). Several “dark” areas were also observed along the implant tract in the white matter and corpus callosum. Hypointense areas were also seen on the contralateral, non-labeled hemisphere (Figure 8.4; left [L] hemisphere), albeit qualitatively less than that seen on the FePro-labeled side. A quantitative analysis showed a significant effect with time (F subscript 5,30 = 3.99; p = 0.0068) but not treatment (F subscript 1,30 = 0.10; p = 0.76) on mean image intensity between
FePro-labeled and non-labeled hRPE-GM implants (Figure 8.4C). Mean intensity values were significantly different at 1 week post implant (p < 0.05), but not significant at any time point thereafter.

Figure 8.4. *In vivo* MRI of hRPE-GM implants. T2-weighted coronal (A) and transverse (B) MR images of hRPE-GM implants 2 months post-implant. Typical hypointense spots and “blooming” artifact were observed on the right [R] implanted side spanning through the striatum. Minimal hypointense regions were also seen in the contralateral, non-labeled hemisphere ([L] left side). Graph (C) shows mean image intensity and exponential regression curves for FePro-labeled and non-labeled hRPE-GM implants.

8.3.3 Post Mortem Histological Analysis of Implanted FePro-Labeled hRPE-GM

PB staining was seen in both FePro-labeled and non-labeled hRPE-GM implants (Figure 8.5). PB⁺ staining on the FePro-labeled side (Figures 8.5; top) was most intense at the early and intermediate (1 week and 1, 2 months) time points, but tapered off at the later (3, 5 months) time points. Consistent with MR, there was minimal PB⁺ staining in the non-labeled hemisphere (Figure 5; bottom), subcortical white matter, and corpus callosum.
As expected, the number of PB\(^+\) cells steadily decreased with time (Figure 8.6), indicating a gradual loss in presumptive PB\(^+\) hRPE cells. PB\(^+\) staining was also quantified on the non-labeled side despite the absence of FePro complexes, but the cell counts on the non-labeled side was much smaller and relatively constant with time compared to the FePro-labeled side. A quantitative analysis revealed an overall treatment effect \((F_{1,4} = 9.41; p = 0.0374)\) of the PB \(N_Y\) between the FePro-labeled and non-labeled sides. If we take the PB\(^+\) staining on the non-labeled side as a hypothetical baseline of non-specific PB\(^+\) staining due to inflammatory infiltrates and hemosiderin, then the number of PB\(^+\) cells at each time-point post-implant are the following (Figure 8.6; grey open symbols): 35,516 (1 week), 19,972 (1 month), 18,448 (2 months), 6816 (3 months), 8400 (5 months).

![Figure 8.5](image.png)

**Figure 8.5.** Post mortem analyses of hRPE-GM implants. PB\(^+\) staining of FePro (top) vs. non-labeled (bottom) hRPE-GM implants in the brain. FePro-labeled cells were consistently identified at all time points post-implant. PB\(^+\) staining was also observed on the non-labeled side. Scale bar = 50 \(\mu\)m.
Figure 8.6. Estimated number of PB$^{-}$ cells in the FePro-labeled and non-labeled implant sites. Grey dotted line and open symbols (○) represent a hypothetical baseline subtraction of the non-specific signal to the actual signal generated in the FePro-labeled striatum (as discussed in Chapter 9).

8.3.3.1 Histological Analyses of Host Immune Response to FePro-Labeled hRPE-GM Implants

The host inflammatory response was compared between FePro-labeled and non-labeled hRPE-GM implants (Figure 8.7). A basic morphological analysis revealed focal edema and the accumulation of red blood cells and inflammatory infiltrates within and immediately surrounding the implant site.

Astrocytosis, which involves astrocytic proliferation, cell hypertrophy, proliferation, and ultimately a glial scar, occurs in response to trauma and inflammation. An analysis of astroglial depth that contained dense GFAP$^{+}$ staining (indicative of moderate to severe reactive astrogliosis) (Figures 8.7A and 8.7B) revealed an overall effect in treatment ($F_{1,4} = 8.95; p = 0.0403$) and time ($F_{4,4} = 7.87; p = 0.0353$) in the astroglial response comparing FePro-labeled and non-labeled hRPE-GM implant (Figure 8.7D). Astroglial responses seemed most prominent at the early and intermediate time points (up to 3 months post implant) that correspond to the time when the inflammatory response is greatest. This astroglial response gradually subsided by 5 months with no discernable difference between the two implants. The astroglial depths in response to FePro-labeled and non-labeled hRPE-GM implants, respectively, were 385 µm and
214 µm (at 1 week), 273 µm and 150 µm (at 1 months), 171 µm and 97 µm (at 2 months), 109 µm and 62 µm (at 3 months), and 106 µm and 96 µm (at 5 months).

Consistent with our previous observations (refer to Chapter 7), ED1⁺ macrophages and activated microglia were observed within and in the surrounding neuropil of both FePro labeled and non-labeled hRPE-GM implants. A quantitative analysis revealed overall differences in treatment ($F_{1,4} = 14.93; p = 0.0189$) and time ($F_{4,4} = 108.03; p = 0.0003$) in ED1⁺ $N_{V}$ between FePro-labeled and non-labeled hRPE-GM implants (Figure 8.7D). The inflammatory response to FePro-labeled implants was consistently larger at the early and intermediate time-points and peaked at 1 month. ED1⁺ staining diminished by 5 months post implant with no difference between the two implants.

8.4 STUDY SUMMARY

The purposes of this study were 1) to evaluate the feasibility of using a FePro labeling method to track and assess the in vivo survival of transplanted cells with MRI, and 2) to assess the potential effects of FePro-labeled cells on the host. SPIO labeling has been widely described and has been successfully used in many acute preparations; however, only a few studies have fully detailed the longitudinal, in vivo use of SPIO labeling in cell therapies. To the best of our knowledge, this study is unique because: 1) it combined histological evidence of FePro-labeled implants with each MRI time point up to five months post implant, 2) it attempted to quantify differences in MR signaling over time and between non-labeled and FePro-labeled implants, and 3) it described inflammatory reactions and potential consequences of the exogenous release of SPIOs.

A major finding of this study was the significant difference in host inflammatory response between FePro and non-labeled hRPE-GM implants, which was supported by the following observations. First, reactive astrocytosis in the surrounding neuropil was more pronounced on the FePro (when compared to the non-labeled) side. This effect seemed to be maintained up to 3 months post implant. Second, we observed an overall increase in ED1⁺ macrophages/activated microglia in the FePro labeled implant. These two factors support our hypothesis that SPIO labeling can induce an increased inflammatory reaction in the host tissue in response to the implant.
Figure 8.7. Host inflammatory response to hRPE-GM implants. Photomicrographs demonstrate GFAP$^+$ staining surrounding FePro-labeled (A, B) and non-labeled (C) hRPE-GM implants. As an indication of inflammation, overall treatment and time effects in reactive astroglial depth (D) and ED1$^+$ cell density (E) were seen between FePro-labeled and non-labeled hRPE-GM implants. Scale bar in (A) = 1000 µm, (C) = 100 µm (Note: scale bar in C applies to B-C).
CHAPTER 9. DISCUSSION

9.1 BEHAVIOURAL EFFECTS AFTER HRPE-GM IMPLANTATION

The experiments described in Chapter 5 (Cepeda et al., 2007) were designed to assess whether hRPE-GM implants can ameliorate motor deficits of PD. Using validated behavioural tests that are sensitive to striatal DA depletions (Meredith and Kang, 2006), we were able to demonstrate that hRPE-GM implants improved lesion-induced forelimb and hindlimb deficits in both the unilateral and partial bilateral 6-OHDA lesion models. These results are consistent with behavioural data previously reported in rodents (Subramanian et al., 2002), MPTP-treated primates (Doudet et al., 2004), and PD patients (Stover et al., 2005a).

9.1.1 Validation of Non Drug-Induced Behavioural Models to Study Treatment Efficacy

Striatal implantation of hRPE-GM has been previously shown to reduce apomorphine-induced rotation in a unilateral 6-OHDA model of PD (Subramanian et al., 2002). Results of this behavioural paradigm must be interpreted with caution, particularly when evaluating the effects of a potential clinical therapy. For example, DAergic treatments that can reverse rotational behaviour may not necessarily improve motor function. Metz and colleagues (Metz et al., 2001) demonstrated that L-DOPA, which is known to induce contralateral rotation, had no effect on skilled forelimb movements in unilaterally lesioned rats. Additionally, they found no correlation between rotation intensity and performance in skilled motor tests (Metz and Whishaw, 2002), which suggest that rotational behaviour and skilled movements should be considered independent effects of 6-OHDA lesioning. Treatment procedures can also induce “false-positive” effects depending on their mode of action. Lane and colleagues (Lane et al., 2006) tested the effects of DA uptake inhibitors and L-DOPA, both of which are anti-parkinsonian drugs, on rotational behaviour when administered alone or together. As expected, the DA uptake inhibitor and L-DOPA induced ipsilateral and contralateral rotation, respectively. Interestingly, both drugs administered in combination produced the same contralateral rotation response as L-DOPA administered alone, which indicates the predominance of post-synaptic supersensitivity on the denervated side. Finally, it is not known how new treatments may interact with different drugs.

In fact, hRPE cells express the DA D2 receptor, and the effects of repeated apomorphine stimulation on hRPE cells are unknown: apomorphine could either enhance the response, overestimating its positive effects, or diminish the response, underestimating its efficacy. Because of these aforementioned limitations, more clinically relevant measures of behavioural deficits have recently been explored (Castaneda et al., 2005; Meredith and Kang, 2006).

Two appropriate sensorimotor tests were used in this study: the forelimb use asymmetry test and the Ledged Tapered Beam Walking Test (Drucker-Colín and García-Hernández, 1991; Tillerson et al., 2001; Schallert et al., 2002). Both tests are sensitive to unilateral and bilateral lesions in rodent models of PD. In addition, these tests do not need aversive motivation or food deprivation and are not sensitive to the effects of practice or weight gain when performed repeatedly over an extended period of time. Previously, we conducted a pilot study to determine test stability and the effect of practice over time on normal and unilateral 6-OHDA-lesioned animals. In our experiment, neither normal nor 6-OHDA lesioned animals demonstrated any significant improvement in performance or spontaneous recovery in either test when tested multiple times up to 20 weeks post-lesion (unpublished observations). This suggests that hRPE-GM-induced motor improvement after a severe (> 97% striatal DA depletion) unilateral 6-OHDA lesion was unlikely due to learning and practice.

9.1.2 HRPE-GM Implants, Behavioural Improvement, and the “Dopa Pump” Hypothesis

In our study, behavioural improvement in response to hRPE-GM implants also differed between moderately and severely lesioned animals and deserve further discussion. The timing of post-implant recovery depended on the severity of the original motor impairment and consequently striatal DA loss. Moderate bilaterally lesioned rats demonstrated significant motor improvements by 10 weeks post-implant. In contrast, motor improvement in severely impaired animals was not significant until 20 weeks post-implant. A similar phenomenon was observed in unilaterally hRPE-GM implanted MPTP-treated primates (DJ Doudet, unpublished observation). Comparable effects were also observed in the human FVM clinical trials: patients who were more severely disabled at the time of transplantation (Olanow et al., 2003) improved less when compared to transplanted patients who were either younger or less disabled (Wenning et al., 1997; Brundin et al., 2000). In this regard, if the principal mechanism of action of hRPE cells is the continuous release of dopa in the striatum, then the degree and speed of behavioural recovery likely depends on the health and number of remaining DA terminals in the implant vicinity.
HRPE cells have been shown to release dopa and (to a lesser extent) DA \textit{in vitro} (Subramanian, 2001; Kubrusly et al., 2003; Zhang et al., 2007a; Ming et al., 2009). Accordingly, Doudet and colleagues (Doudet et al., 2004) reported a significant increase in $^{18}$F-DOPA uptake and concomitant decrease in $^{11}$C-RAC binding (which indicates the presence of hRPE cells and increased DA release, respectively) in hRPE-GM implanted primates in the first 6-8 weeks post-implant. This suggests that the beneficial effects of hRPE-GM can be attributed, in part, to a DAergic process. However, the increase in $^{18}$F-DOPA uptake was modest (at 10%) and is significantly less than what was reported after FVM cell transplants. This may be due to 1) the relatively small number of implanted cells, and 2) the lack of adequate DA storage in hRPE cells. The main metabolic product of $^{18}$F-DOPA in DA neurons is $^{18}$F-DA, and since hRPE cells do not produce significant quantities of DA, it is more likely that the increase in radioactivity in the hRPE-GM implanted putamen is due to other $^{18}$F-DOPA metabolites. Accordingly, dopa is an intermediate by-product (in the eumelanin synthesis pathway) of hRPE cells and is rapidly metabolized; therefore, the increased accumulation of $^{18}$F-DOPA in response to hRPE-GM implants is likely due to $^{18}$F-tagged dopa metabolites that remain in the cells.

However, the increase in $^{18}$F-DOPA (and decrease in $^{11}$C-RAC) binding resolved by six months (up to four years) post-implant, despite continued clinical improvement (DJ Doudet, unpublished results). This was likely due to 1) the loss of hRPE cells from an already small number of implanted cells, and 2) the possible increase in the turnover of $^{18}$F-tagged dopa metabolites in the eumelanin synthesis pathway. Importantly, this also suggests that dopa production (and its release in the host striatum) may not be the only mechanism involved in behavioural improvement. Other putative mechanisms of action of hRPE cells are discussed later in this chapter.

Although hindlimb and forelimb motor recovery were demonstrated in both animal models tested, these improvements were not complete. This is consistent with earlier reports in patients and non-human primates. HRPE-GM implanted patients demonstrated a 45-50% improvement at 48 months post-implant (Stover et al., 2005a; Stover and Watts, 2008). In our laboratory, none of our primates have shown full recovery, and their improvement ranged from 30-60% at 2-3 years post-implant (DJ Doudet, unpublished observation). However, patients, primates, and rats only received \textit{unilateral} implants. This raises the possibility that a bilateral implant procedure may be able to further improve clinical recovery. Notably, hRPE-GM
implanted patients were maintained on DAergic medication (albeit reduced) throughout the 48-month observation period. None of our animals received supplemental L-DOPA since it has been shown that spontaneous forelimb movement is sensitive to both the acute beneficial effects and delayed side effects of L-DOPA treatment (Lundblad et al., 2002; Fleming et al., 2005a). In other words, behavioural performance declined when abnormal involuntary movements appeared.

9.2 CHARACTERIZATION AND LONG-TERM SURVIVAL OF HRPE-GM IMPLANTS

The experiments described in Chapter 6 (Flores et al., 2007) were designed to assess the in vivo survival of hRPE-GM implants. Our results demonstrate that hRPE-GM can be identified up to five months post-implant. This indicates the potential for the long-term survival of hRPE cells, despite the absence of immunosuppression, and it is consistent with previous studies that have shown the survival of presumptive hRPE cells in hemiparkinsonian rats (Subramanian et al., 2002). These results, together with the findings described in Chapter 5, confirm the efficacy of hRPE-GM implantation as a therapeutic option for PD.

9.2.1 In Vitro Characterization of hRPE Cells

The antibodies used (NuMA-Ab2 and EMMPRIN, which are human and epithelial-specific markers, respectively) were previously shown to be hRPE-specific when tested in hRPE-cell monolayers in vitro and in ex vivo RPE/choroid tissue (Titan Pharmaceuticals, personal communication). Our in vitro observations validated these results: NuMA-Ab2\(^+\) and EMMPRIN\(^+\) hRPE cells were stretched out and extended along the edges of GMs, while some cells seemed to penetrate the GM grooves. The morphologic features of NuMA-Ab2\(^+\) and EMMPRIN\(^+\) were identical to those of hRPE cells stained with H&E or examined by DIC.

RPE65 is a protein that is abundantly expressed in fully differentiated hRPE cells (Znoiko et al., 2002), and it is expressed in the RPE/choroid, normal human epidermis, and other whole tissue preparations (Hamel et al., 1993; Ma et al., 2001). Unfortunately, hRPE cells alter their phenotype depending on the host microenvironment, and many of the factors that hRPE
cells express *in situ* may be lost (or expressed in low, undetectable quantities) in cultured hRPE cells, resulting in de-differentiated cells that lack an epithelial phenotype (Zhao et al., 1997; Nicoletti et al., 1998; Rak et al., 2006). Our preliminary results revealed no RPE65\(^+\) staining on isolated hRPE cells that we grew in a monolayer (unpublished results). These changes could be attributed to the artificial cell culture environment and trypsin dissociation (von Recum et al., 1999); however, different culturing conditions and substrates, or increased cell passaging could lead to the re-expression of some of these traits (Davis et al., 1995; Rak et al., 2006).

Accordingly, the hRPE-cell monolayers we cultured that were RPE65-negative were only grown in culture for a short period of time; therefore, there may not have been a significant enough opportunity for hRPE cells to re-express certain proteins.

Importantly, RPE65 seemed to be re-expressed once hRPE cells were attached to GMs. RPE65\(^+\) staining revealed the same characteristic morphology of hRPE cells extended along the outer surface of GMs, indicating that RPE65 was localized at the RPE cell membrane. Western blot analyses in our laboratory confirmed RPE65 expression in different fetal hRPE cells and ARPE19 cells (an immortalized adult RPE cell line) attached to GMs. This suggests that hRPE cells (when attached to GMs) can re-establish a fully differentiated epithelial phenotype.

### 9.2.2 HRPE-GM Implant Survival

A number of factors contribute to implant survival and will be discussed throughout this chapter. One of these factors relates to tissue preparation and cell culturing. HRPE cells are epithelial cells and, as such, can be grown in culture and survive for extended periods of time. The ability to grow and expand hRPE cells from primary tissue with minimal risk provides a number of advantages (previously described in Chapter 3) for the use of hRPE cells in transplantation (Hu et al., 1994; Davis et al., 1995).

The hRPE-cell lots used in our experiments were originally harvested and expanded (using a bioreactor) at Titan Pharmaceuticals for pre-clinical and/or clinical investigations. We received hRPE cells frozen at passage 2-3. Our cell culturing consistently resulted in viable hRPE cells throughout most of our experiments. Cell viabilities (pre-implantation) were in the clinically acceptable range (>80%), and most dose concentrations were within the recommended clinical range of 2000-3500 cells/\(\mu\)l of hRPE-GM solution. Post-surgical viability counts (which were taken at the end of a routine surgical day to determine any changes in viability during
surgery) were relatively consistent and rarely dropped below 80%. These viabilities suggest that the majority of implanted cells were healthy, viable hRPE cells.

However, we encountered subtle drops in cell viability and concentration with older cell lots. The last sets of experiments we performed (see Table 7.1) still maintained good cell viability pre-implant (>80%), but cell concentrations dropped slightly (particularly in our 0010 hRPE cell group). Furthermore, post-surgical viability counts dropped to the low 70% range. In one instance, we were unable to determine a post-implant viability count due to the low number of remaining hRPE cells. The reasons for this are unclear; however, taking into account the lessons that we learned from the FVM clinical trials, we suspect that two major factors that contribute to cell viability are 1) length of cell storage, and 2) donor age and origin. These issues are discussed later in this chapter.

Our in vivo results are the first to demonstrate that an appreciable number of hRPE cells implanted into a 6-OHDA lesioned rat can survive up to 5 months post-implant. NuMA-Ab2 and EMMPRIN immunofluorescence revealed numerous presumptive hRPE cells attached to GMs. HRPE cells were restricted to the implant site with no observable migration in the surrounding neuropil. Detached hRPE cells were rarely observed. This was expected, as hRPE cells not attached to a suitable substrate will undergo apoptosis (or, more specifically, anoikis of the cells) (Tezel and Del Priore, 1997; Tezel et al., 2004). Previous studies have shown that animals implanted with hRPE-cells alone did not show any behavioural improvements or cell survival (Subramanian et al., 2002); therefore, our studies only utilized hRPE cells attached to GMs as a way to protect against an immune attack (Chen et al., 1997) and prevent cell death (Saporta et al., 1997; Borlongan et al., 1998).

RPE65+ cells were also observed within the implant site, and their antigenic profile was more favorable than that observed with NuMA-Ab2 immunofluorescence. RPE65 appeared more compartmentalized with distinct reactivity in the cytoplasm of hRPE cells. As previously described, we were not certain whether hRPE cells would retain some of their phenotypic properties when implanted into a foreign tissue environment. Our results, which demonstrate RPE65+ cells within the implant site, suggest that hRPE cells (when attached to GMs) can express at least some of their typical characteristics.

Each antibody we assessed possesses both advantages and disadvantages for future use. For example, hRPE cell identification using NuMA-Ab2 fluorescence, which is human-specific,
will not be useful for identifying hRPE cells in human (or even primate) tissue because of its cross-reactivity to host antigens. In addition, our inability to restrict NuMA-Ab2 labeling to cell nuclei will restrict its use in any quantitative measure. Similarly, TH labeling is an indirect measure of hRPE cell survival, as it may cross-react with surviving striatal DA terminals. It is also unclear whether hRPE cells constitutively express TH. HRPE cells have been shown to possess “TH-like” activity (Smith et al., 1998; Kubrusly et al., 2003). However, we are unsure if our TH staining represents true TH expression from hRPE cells, or if it is cross-reacting with a “TH-like” protein instead (such as tyrosinase). Accordingly, a recent genetic study has shown that certain tyrosinases exhibit high TH activity (Hernández-Romero et al., 2006). Although TH has been previously used to demonstrate hRPE cell survival (Subramanian et al., 2002), it is unlikely to be useful for any quantitative measure. RPE65 and EMMPRIN possess good specificity for RPE and epithelial cells, respectively, with minimal cross reactivity to antigens of other species. However, due to the phenotypic changes associated with hRPE cells (which have yet to be fully characterized), our findings that RPE65 can be used as a definitive marker for hRPE cells need to be carefully considered, as there may be surviving hRPE cells that do not express the protein.

9.3 INFLAMMATION AND NEURAL TRANSPLANTATION

One of the main concerns with cell transplantation (with regard to any disease) is the possibility of immune rejection. Despite the brain being an “immune privileged” site, strong immune responses can still occur, which result in reduced transplant efficacy and possible rejection. The acute inflammatory response (as described in Chapter 6) is likely responsible for the hRPE-cell loss that is observed over time. However, alternative transplant procedures have reported similar cell losses post-implant (Olanow et al., 1996; Sortwell, 2003). For example, a small fraction of FVM neurons survived when xenotransplanted in the rat (Brundin et al., 1988); further, they only survived in immunosuppressed rats. In our studies, it is remarkable to find surviving human RPE cells five months post-implant despite the lack of immunosuppression.

Immunosuppressive treatments were used to inhibit immune rejection in some, but not all, previously reported FVM clinical trials (Freed et al., 2001; Olanow et al., 2003). Interestingly, it was suggested that transplant efficacy was, in part, related to immune activation. Olanow and colleagues (Olanow et al., 2003) noticed that motor improvement started to
deteriorate at the same time immunosuppression ceased in patients (6 months post-implant). Immunosuppressive drugs are useful for reducing the short-term risk of rejection, but they do not prevent chronic rejection. Further, they can increase the risk of opportunistic infections, which is problematic for an already fragile population (such as the elderly) (López et al., 2006; Li et al., 2008b). In this regard, it is important to determine how the host immune response might affect the survival and function of hRPE-GM implants.

### 9.3.1 Cellular Inflammatory Response to hRPE-GM Implantation

The studies described in Chapter 7 are the first to quantitatively assess long-term inflammatory reactions in response to hRPE-GM and GM-alone implants in the rat. Our results demonstrated a significant reduction in both ED1⁺-characterized cellular inflammatory reactions and reactive astrocytosis in response to hRPE-GM when compared to GM-alone implants. This was particularly evident at the earlier time points, which is the critical period during which acute inflammation occurs and can ultimately determine the implant’s long-term survival.

Subramanian and colleagues (Subramanian et al., 2002) performed the only other study that has characterized the inflammatory response to hRPE-GM implants in the rat. In this study, they qualitatively demonstrated a minimal immune response to hRPE-GM implants characterized by reduced staining for activated microglia and MHC class II molecules. In combination with the data presented in Chapter 6, we hypothesize that hRPE-GM implants can survive long-term and endogenously reduce the host-inflammatory response despite the absence of adjunct immunosuppressive treatment.

Certain trends in our quantitative analyses require further discussion. Interestingly, a closer inspection of our data suggests that the 0802 hRPE-GM group can maintain anti-inflammatory effects longer than either of the 1105 or 0010 hRPE cell groups. The 0802 hRPE-GM group was the only cell group that maintained a significant decrease in inflammatory response at 20 weeks post-implant. Reactive astrocytosis in the 0802 group also seemed to be considerably reduced at 20 weeks post-implant. However, there was variability in measuring astroglial depth, and since the animals in the 0802 hRPE-GM group were remaining animals from a previous group (described in Chapter 5), we were only able to study a reduced sample size of N=2 for the 0802 hRPE-GM group at each time point. Therefore, our study lacked sufficient statistical power to determine whether or not hRPE cells in the 0802 group exhibit a “greater” anti-astrocytic response when compared to both the 1105 and 0010 hRPE cell groups.
Nevertheless, these observations suggest that there may be donor-dependent differences in “anti-inflammatory function” that can influence long-term hRPE-cell survival.

Surprisingly, minimal ED1+ staining was observed in the SN. Activated microglia was only found at our earliest time point (2-4 weeks post-implant) and seems confined to the cannula tract. Microgliosis is a mechanism that has been proposed to be involved in 6-OHDA neurotoxicity; however, many of these studies have only presented short-term (4 weeks) evidence of an activated microglial response in 6-OHDA-lesioned animals (Akiyama and McGeer, 1989; Cicchetti et al., 2002). There was a 4-6 month period between 6-OHDA lesioning and post mortem analyses in our longer-term animals which suggests that microglial activation in response to 6-OHDA lesioning may have subsided during this time.

HRPE cells may express certain immunosuppressive factors that can alter the immune response to the implantation procedure itself. The immunosuppressive effect seems to persist long-term, and all three hRPE-cell donors we tested produced the same effect (but not to the same degree). Furthermore, the reduced inflammatory response is not likely due to the influx of trophic factors and cytokines from the breakdown of the BBB (in response to the implantation procedure itself), as one would expect to see the same degree of response in the GM-alone implanted animals. In this regard, future studies should be aimed at determining putative mechanisms that could contribute to the immunosuppressive effect.

9.3.2 Donor-Expressed Anti-Inflammatory Factors – the Concept of “Natural” Immunosuppression

Endogenous immunosuppressive properties of grafted cells are an aspect of graft survival that is commonly overlooked. This is exemplified by the growing field studying stem cell-based therapies, in which there is a constant search for alternative approaches to suppress the immune system and reduce rejection (Chidgey et al., 2008). For example, the immunosuppressive properties of mesenchymal stem cells (MSCs) have rapidly attracted attention. MSCs have been shown to express anti-inflammatory factors such as TGFβ and inhibit T-cell proliferation (Di Nicola et al., 2002). Even though MSCs express MHC class I/II molecules, they do not seem to elicit an immune response in vitro (Le Blanc et al., 2003). The clinical potential of these “natural” immunosuppressive properties is subject to debate, as they still need to be tested in

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Indeed, a recent study that implanted MSCs into 6-OHDA lesioned rats (Camp et al., 2009) suggests that MSCs can survive despite a marked immune response.

CNS functioning depends on an intricate balance of constitutively expressed pro- and anti-inflammatory factors that differentiate a healthy cell from an unhealthy cell requiring removal. When a graft is placed in the brain, this balance is disrupted and the up-regulation of pro-inflammatory factors leads to rejection. If this balance can be restored (preferably by endogenous immunosuppressive properties of the grafted cells themselves), then this might facilitate the increased survival of neural transplantation therapies.

HRPE cells have been shown to express various pro- and anti-inflammatory factors, cytokines, and neurotrophic factors (see below) that may be able to restore this “balance”. However, since hRPE-GM implantation in PD is a relatively new concept, it is not known whether these putative mechanisms still function when hRPE cells are transplanted in the brain. The following section describes some of the potential mechanisms that may be involved in hRPE-cell function as a treatment for PD.

9.3.3 Putative Mechanisms of Action of hRPE-GM Implants

“Immune privilege” is thought to contribute to the beneficial effects of transplanted hRPE cells. Under normal conditions, hRPE cells constitutively express low levels of MHC class I molecules and do not express MHC class II molecules (Rezai et al., 1997), which is important in reducing antigen presentation in the immune system. HRPE cells express the anti-inflammatory factor TGF-β (Kociok et al., 1998; Nagineni et al., 2007), which has been shown to suppress T-cell activation (Horie et al., 2010). Interestingly, hRPE cells can also induce the apoptosis of activated T-cells through Fas ligand expression (Bellgrau et al., 1995; Griffith et al., 1995; Jorgensen et al., 1998).

HRPE cells express many antioxidant enzymes, including superoxide dismutase, catalase, and vitamin E (Liles et al., 1991; Boulton et al., 2004). These antioxidant enzymes can minimize oxidative stress and the formation of ROS that is commonly seen in PD patients.

HRPE cells produce numerous neurotrophic factors that have been pathogenically linked to PD (Siegel and Chauhan, 2000). HRPE cells have been recently shown to produce BDNF and GDNF (Zhang et al., 2007a; Ming et al., 2009). In fact, it was demonstrated that conditioned media from hRPE cells, which contained significant levels of BDNF and GDNF, was able to...
protect FVM DAergic cultures from 6-OHDA neurotoxicity (Ming et al., 2009). BFGF is another trophic factor produced by hRPE cells that may contribute to hRPE-cell functioning by promoting self-survival (Faktorovich et al., 1990).

HRPE cells have also been found to produce large amounts of PEDF (Tombran-Tink and Barnstable, 2003). PEDF is a neurotrophic factor that promotes photoreceptor survival (Cayouette et al., 1999) and has been shown to protect retinal neurons (Cao et al., 1999), motor neurons (Bilak et al., 2002), cerebellar granule neurons (Taniwaki et al., 1997), and hippocampal neurons (DeCoster et al., 1999) against neurotoxicity or oxidative stress. PEDF has also recently been shown to be neuroprotective in in vitro models of PD (McKay et al., 2006; Falk et al., 2009). Interestingly, PEDF can also increase mRNA expression of other neurotrophic factors including GDNF and BDNF (Yabe et al., 2004).

Although it is not the scope of this dissertation, our laboratory is currently quantifying the trophic potential of each of the hRPE-cell lots we investigated to determine whether neurotrophic factors contribute to the mechanism of action of hRPE cells. However, based on the trends we observed when we quantified the anti-inflammatory potential of each hRPE-cell lot, we can speculate that different hRPE-cell donors will express different amounts of trophic factors, cytokines, and pro- and anti-inflammatory molecules. Interestingly, the trophic potential of hRPE cells, in vivo, seems to depend on the level of differentiation of hRPE cells (Rak et al., 2006). The morphological characteristics, expression of epithelial markers, and level of pigmentation (melanogenesis) all correlate with the ability of hRPE cells to provide trophic support. The section below describes how these phenotypic characteristics may relate to differences in the efficacy between different hRPE-cell lots.

9.4 EXPERIMENTAL LIMITATIONS

One of the limitations of these studies is the lack of stereological cell counts to quantitatively assess hRPE-cell survival. Based on our aforementioned results (described in Chapter 6), we were optimistic that we would be able to perform a stereological analysis of cell survival.

A pilot study that was done on a small subset of animals (to set parameters for future stereological studies) using hRPE cells of a similar gestational age to the 1105/0010 cell lots revealed no EMMPRIN$^+$ staining in hRPE-GM implanted rats. Similar results were seen in
primates (JR O'Kusky, personal communication) and humans (Farag et al., 2009). Indeed, in the human study, stereological cell counts were based on morphological features (i.e. neuromelanin-containing profiles), rather than on immunohistochemical detection, to identify hRPE cells. HRPE-GM implanted primates in our laboratory underwent the same type of stereological assessment. Although the reasons for this are unclear, the most plausible explanation is that a significant number of hRPE cells do not survive for an extended period of time (i.e. over six months). This is consistent with previous PET studies that revealed hRPE-cell induced changes in $^{18}$F-DOPA and $^{11}$C-RAC binding in primates (Doudet et al., 2004) and rats (Wang et al., 2008) at 6-8 weeks post-implant, but disappearing after six months. Our rats described in Chapter 6, which showed (using the appropriate controls) EMMPRIN$^+$ presumptive hRPE cells, were only observed up to five months post-implant. There are, however, other plausible reasons for this discrepancy that warrant further discussion.

9.4.1 Long-Term Storage of hRPE Cell may be Detrimental to Viability

Cryopreservation may contribute to hRPE-cell survivability. Cryopreservation was initially used to store FVM tissue prior to transplantation (Redmond et al., 1988), but it was later found to significantly decrease cell viability (Collier et al., 1993). Since then, FVM tissue intended for transplantation has been kept either in culture medium (Freed et al., 2001) or transplanted immediately after harvesting (Olanow et al., 2003). HRPE cells used in our experiments were cryopreserved for varying periods of time, but the differences in cell viabilities and overall “health” of these cells seemed to correlate with time spent in cryopreservation. All three hRPE-cell lines used (0802, 1105, and 0010) were harvested from 2001 to 2003. The 0802 hRPE cell line was used from 2005 to 2007, which is approximately 3-5 years of storage. The 1105 and 0010 hRPE cells lines were used from 2008 to 2010, which is approximately 6-8 years of cryopreservation. Although it is remarkable that hRPE cells can maintain decent viability after many years of storage, extended time in storage may contribute to the overall function of hRPE cells in vivo.

9.4.2 Donor Age

Another factor that plays an important role in hRPE-cell survivability and function is donor age. This is exemplified by the FVM studies. The optimal donor age for FVM tissue transplantation was thought to be from the time at which DA cells appeared in the mesencephalic
subventricular zone to the time at which they developed neuritic processes (Björklund et al., 1983), which equated to 5-9 weeks gestation. Fetal grafts harvested before or after this optimal period and implanted into immunosuppressed rats did not survive (Freeman et al., 1995b). In this regard, we cannot rule out the possibility that there are donor-dependent differences associated with hRPE-cell survival and functioning.

Based on what we know about the embryonic development and physiology of the RPE, it was determined that the optimal period for harvesting hRPE cells (for implantation) is 16-24 weeks gestation. At this stage of development, RPE cells rapidly develop cadherin adhesions (facilitating cell-cell adhesion) and begin to form a non-mitotic, fully differentiated hRPE-monolayer (Rak et al., 2006). Further, tyrosinase expression (and melanogenesis) is stable and is at its peak (Boulton et al., 2004; Julien et al., 2007). With age, hRPE cells quickly undergo a variety of structural and biochemical changes: hRPE cells gradually lose their cadherin properties, their antioxidant efficiency declines, trophic factor and cytokine expression decreases, and tyrosinase expression and melanogenesis is absent (Julien et al., 2007; Kolomeyer et al., 2011).

In our limited experience with culturing hRPE cells, we noticed subtle variations between the different hRPE-cell lots (Figure 3.2). For example, all three hRPE-cell lots displayed distinct RPE morphologies (Burke et al., 1996). While the 0802 hRPE cells grew in a monolayer, the 1105 and 0010 hRPE-cell lots were multilayered, fusiform-shaped cells that grew in a “basket-weave” type pattern. With time, these (multilayered) hRPE cells formed large masses. Fully differentiated hRPE cells display a more epithelioid-type morphology (i.e. “cobblestone”-like appearance) and, with continued cell-to-cell adhesion, do not proliferate beyond the monolayer.

Another difference we observed between the hRPE-cell lots was their level of pigmentation. The 0802 hRPE cells were more pigmented and maintained their pigmentation with multiple cell passages. In contrast, both the 1105 and 0010 hRPE cells were less pigmented (after initial seeding) and lost their pigmentation over time (unpublished observations). Melanin pigmentation (and accompanying tyrosinase expression) in the RPE naturally occurs mainly during the early fetal stages (Smith-Thomas et al., 1996) and can only be induced in the late fetal stages or in the adult (Julien et al., 2007). Further, the neurotrophic potential of hRPE cells seems to parallel the level of pigmentation in hRPE cells; that is, highly pigmented, differentiated hRPE cells have enhanced neurotrophic properties (McKay et al., 2006).
These aforementioned traits have important implications with regard to hRPE-cell functioning. In our study, the 0802 hRPE-cell group was harvested from a 22-week fetal donor, while the 1105 and 0010 hRPE-cell groups were harvested from 37- and 38-week neonatal donors, respectively. Accordingly, we hypothesize that the differences in the donor ages of our hRPE cells, which lead to differences in the cells’ tyrosinase expression and neurotrophic potential, play a pivotal role in their survival and efficacy as a treatment option for PD.

We speculate that this hypothesis may also help to explain the results of the two existing hRPE-GM clinical trials. Although the initial open-label Phase I clinical trial was highly successful (Stover et al., 2005a), the recently completed double-blind placebo controlled STEPS trial failed to meet its primary end-point, and patients showed no improvement (Gross et al., 2011). In the open-label Phase I trial, 6 patients received unilateral hRPE-GM implants that were harvested from a 22-week fetal donor. Despite the open-label design (and potential biases associated with it), these patients continued to show improvement after five years (Stover and Watts, 2008), which, at this point, is not likely due to a continued “placebo” response. For the Phase II STEPS trial, Schering Pharma (Berlin, Germany) decided to use hRPE cells harvested from neonatal donors (i.e. neonates that had been born and have a birth certificate) to avoid ethical issues associated with fetal tissue. This has important implications with regards to choosing an appropriate hRPE-cell donor. Some have suggested that the birth and death process can induce changes in cell physiology. For example, the RPE is sensitive to environmental insults, so that exposure to light (resulting in phototoxicity) will increase the oxidative load on the RPE that may affect cell survival and function (Boulton et al., 2004). The differences in donor characteristics are a disadvantage for many cell therapies: as each donor is different, every donor must be characterized each time. This also suggests that not every donor may be viable for cell transplantation.

9.4.3 HRPE-Cell Passaging Leads to Different Phenotypic Characteristics

The number of cell passages may also contribute to hRPE-cell efficacy. The hRPE cells studied in our laboratory were harvested and expanded at Titan Pharmaceuticals, and our laboratory received these cells at passage 2-3. It is widely accepted that hRPE cells can alter their phenotype with continuous cell passages (Davis et al., 1995; Rak et al., 2006). When we expanded hRPE cells in culture, we noticed less pigmentation and a gradual decline in hRPE-cell
viability with increasing cell passages. Consequently, hRPE-cell expansion does not likely produce absolute quantities of cells with the same phenotypic characteristics.

**9.5 FEPRO LABELING OF HRPE CELLS FOR LONGITUDINAL TRACKING OF CELL SURVIVAL IN VIVO**

One of the major challenges of studying cell transplantation is the difficulty in assessing longitudinally, *in vivo*, the characteristics and survival of transplanted cells. Post-mortem histological evaluations can help us answer these questions; however, they provide only a single time point per animal (or patient) and a limited view of function *in vivo*. Therefore, the goal of the studies described in Chapter 8 was to 1) determine the feasibility of using MR and SPIO labeling to longitudinally track hRPE-GM implants in the brain, and 2) evaluate the potential effect the MR label itself might have on the host brain.

**9.5.1 Feasibility of Assessing hRPE-GM-Implant Survival With FePro**

HRPE cells are ideal cells for testing FePro labeling. When implanted, hRPE cells are fully differentiated, they do not proliferate (when attached to GMs), and they do not migrate from the implant site. HRPE cells were successfully labeled using the FDA approved drugs Feridex and Protamine sulfate. Even when high concentrations of FePro (50:4.5 µg/mL) were used which resulted in extensive intracellular accumulation of Fe, hRPE-cell viability remained high, and FePro labeled cells were not significantly different from non-labeled cells. Microscopy and EM analyses confirmed that there were no differences in hRPE-cell morphology and phenotype: FePro labeled hRPE monolayers appeared healthy and retained much of their typical morphological appearance. Care must be taken with every cell type when optimizing FePro concentrations. Others have demonstrated that Fe concentrations similar to those used in our study can severely impair survival and proliferation of other cell types (Hu et al., 2009). Furthermore, although hRPE cells are fully differentiated cells, there have been mixed results with regard to alterations in stem cell differentiation with labeling (Kostura et al., 2004; Arbab et al., 2005; Farrell et al., 2008; Cohen et al., 2010). This suggests that an ongoing evaluation of cell viability and morphology must be performed for each cell type in order to optimize FePro concentrations.
SPIO accumulation in hRPE cells seemed to depend on iron and transfecting agent concentrations. There were qualitative differences in PB staining between the low (1.0 µg/mL) and high (4.5 µg/mL) Pro concentration groups. However, Pro was necessary to effectively label hRPE cells as Fe-only labeled hRPE cells revealed very little SPIO staining. A 50 µg/mL Fe concentration was the minimum concentration needed for effective SPIO loading: lower Fe concentrations (25 µg/mL) complexed with higher Pro concentrations (4.5 µg/mL) revealed little PB+ staining and a diminished MR signal hypointensity.

FePro-labeled hRPE-GM implants were detected in the rat up to 5 months (20 weeks) post-implant. PB+ staining was abundantly seen within the implant site. Sporadic PB+ staining was also observed in the cortical areas and corpus callosum (which lined the implant tract), and no staining was seen in the striatal parenchyma away from the implant site. HRPE-GM implant survival followed an expected trend seen in cell transplantation studies. The Nf of FePro-labeled hRPE-GM implants were initially high but steadily decreased to numbers similar to those observed on the non-labeled side. In this regard, it is widely accepted that as much as 95% of the cells transplanted into the recipient die due to the inflammatory process (Brundin et al., 1988; Olanow et al., 1996).

The number of hRPE cells implanted into these animals, which was approximately 36,000 cells attached to GMs distributed among 2 implant tracts, was adequate for detection in a 7T MR scanner. T2-weighted MRI matched our post-mortem observations: hypointense areas (surrounded by a characteristic “blooming” artifact) were seen within the implant site, along the implant tract in the cortex, and in the corpus callosum. Unfortunately, our quantitative MR analyses did not match what we observed with post mortem histology. MR revealed no significant differences in mean image intensity when comparing FePro and non-labeled implants. Mean intensity values were significantly different at 1 week post implant, which corresponds to the time at which hRPE cell survival is likely at its maximum, but were not different at any subsequent time point. This can be attributed to a number of factors. First, because of study design, the lack of significant difference of mean intensities (between FePro-labeled and non-labeled hRPE cells) may be due to the small sample of animals that we could statistically analyze on a longitudinal basis since only animals with data at ALL time points (i.e. N=1) was considered. Also, the mean intensity scores at 3 months post-implant were exceedingly similar, and we suspect that there may have been issues with image acquisition at that time.
MR revealed hypointense areas at the implant site on the non-labeled side (albeit less than that seen on the FePro labeled side). Histology also demonstrated a similar (although sporadic) distribution of PB\(^+\) deposits along the implant and cannula tract on the non-labeled side. However, the \(N_V\) remained relatively small and constant at all of the time points. PB\(^+\) deposits on the non-labeled side could not be distinguished from PB\(^+\) SPIOs and were more prevalent at the early (1 week) and intermediate (1, 2, and 3 month) time-points. This suggests that this non-specific PB\(^+\) staining was likely due to the surgical procedure itself, which induced focal hemorrhage and the accumulation of hemoglobin-rich hemosiderin, and inflammatory infiltrates. Interestingly, in one primate study we concurrently performed, we observed more specific PB\(^+\) staining within the FePro-labeled implant with little PB\(^+\) staining seen on the non-labeled side (unpublished results). This qualitative difference can be attributed to a few important factors. First, similar gauge (22 gauge) cannulae were used in both procedures, and the surgical procedure in the rat, which comparatively affects a larger tissue volume, induces a much larger physical trauma than in the primate. Furthermore, a rat xenotransplanted with human cells is likely to induce a much more severe immune response compared to human cells implanted into a primate.

**9.5.2 Host Inflammatory Reactions in Response to FePro-Labeled hRPE-GM Implants**

The major finding of this study was the significant difference in host inflammatory response between FePro and non-labeled hRPE-GM implants. This was supported by two observations: 1) Morphologically defined reactive astroglial depth was significantly larger on the FePro (when compared to the non-labeled) side. This effect was maintained up to 3 months post-implant. 2) ED1\(^+\) activated microglia and macrophages increased in areas surrounding the FePro-labeled implant. In addition, the volume of FePro-labeled hRPE-GM implants was consistently larger than that of non-labeled hRPE-GM implants. We hypothesized that this was attributed to either “swollen” FePro-labeled cells or an increased accumulation of inflammatory cells.

It was not possible to determine whether SPIOs were transferred to resident macrophages. However, a recent study using an *in vitro* model of inflammation found a 10-20\% uptake of SPIOs into activated macrophages (Pawelczyk et al., 2008). Alternatively, the increased inflammatory response we observed could be due to released SPIOs in the surrounding neuropil, which raises the issue of a potential adverse reaction to SPIOs. The potential consequences of released SPIOs on native tissue are unknown. SPIO complexes are composed of
ferric (Fe$^{3+}$) and ferrous (Fe$^{2+}$) iron coated with a protective dextran coating, which make them theoretically inactive and unable to react with surrounding molecules. Nevertheless, a breakdown of the protective coating and the ferric molecule can potentially lead to the release of free iron, which can induce ROS-induced toxicity. Conditions like this, particularly in PD where iron has been connected to oxidative stress and mitochondrial dysfunction, could exacerbate the disease process and lead to an apparent ineffectiveness of the cell therapy treatment in the preclinical model (Berg and Youdim, 2006).

9.5.3 Limiting Factors of FePro Labeling and MR for Cell Implantation Studies

A number of limiting factors could influence the potential outcome of SPIO labeling in our studies. One factor was the small sample size. This study was originally designed as a pilot study, and having only a small number of animals at each time point may have contributed to the unexpected lack of statistically significant correlation between our MR and post mortem analyses. Another variable was our choice of slice thickness (1 mm) with MR. MR signal quantification was difficult to perform with larger slice thicknesses: signal intensity varied with respect to whether the implant site could be identified in one slice or “spread” across sections. Finally, the nature of quantifying SPIO labeling, which is measured by the “absence” of signal with $T_2$ or $T_2^*$ MR sequences, makes it more difficult to quantify than a “positive” signal. These factors, and how they relate to contributing variables including cell numbers and implant volume, need to be further addressed if these studies are to continue.

9.5.4 How Can FePro Labeling and MR be Useful for Cell Implantation Studies?

Although we did not fully obtain the results we were expecting, we were able to demonstrate differences in the rate of change between FePro-labeled and non-labeled hRPE-GM implants over the course of several months. From our observations, we can infer that the signal (in both our MR and post mortem histology) from the FePro-labeled hemisphere originated from two sources. The first, seen as the constant PB$^+$ staining seen on the non-labeled side, represents non-specific accumulation of hemosiderin and macrophages due to hemorrhage and exacerbated inflammation. The second, larger component is that of FePro-labeled cells. Assuming that the signal generated by the non-labeled implant is strictly due to hemorrhage and inflammation, is non-specific, and is likely to have a similar time course in both striata, then it may be feasible to perform a baseline subtraction of the non-specific signal to the actual signal generated in the
FePro-labeled striatum (Figure 8.6K; grey dotted line). This method may not be optimal for quantifying survival in absolute cell numbers; however, it may be useful for tracking key stages at which cell death occurs and plateaus, and it can provide us with insight into strategies (i.e. the administration of anti-inflammatory medication) that may increase transplant survival.

9.6 CONCLUSIONS

Cell replacement therapies have been thoroughly investigated in order to find a long-term, continuous DAergic source to treat motor dysfunctions in PD. However, these therapies have not yet met our expectations: mixed clinical results, safety and logistical concerns, and ethical issues have interrupted the use of these therapies in the clinic. HRPE cells of fetal or neonatal origin have been recently proposed as a tissue transplant alternative for PD. These cells are a potential cell therapy source for PD because of their DAergic properties: hRPE cells produce dopa in their eumelanin biosynthetic pathway, catalyzed by the TH analog tyrosinase. Therefore, hRPE cells implanted in the parkinsonian brain could provide a continuous source of dopa for striatal DA terminals.

Using the 6-OHDA rodent model of PD, this dissertation has demonstrated that the striatal implantation of hRPE-GM can 1) ameliorate parkinsonian deficits and 2) survive in the host striatum for up to 20 weeks (5 months) post-implant. However, the limited survival of certain hRPE-cell lots led us to determine the specific donor characteristics that make certain hRPE cells more viable than others when used in transplantation studies. All the hRPE-cell lots used in our experiments were originally harvested for pre-clinical and/or clinical use; however, not all hRPE cells may be optimal for implantation. Donor ages (fetal vs. neonatal), morphological characteristics, and phenotype differences (i.e. levels of pigmentation) are all important factors that contribute to hRPE cell function. Further, additional studies are needed to determine alternative methods of cell culturing that may better preserve hRPE cell quality and maintain the cells’ epithelial phenotype.

Most importantly, our studies suggest that the beneficial effect of hRPE-GM implants is not solely due to dopa production and may involve an alternative mechanism of action. HRPE-GM provoke a minimal inflammatory response, which suggests that hRPE cells can express immunosuppressive factors that may not only help with their self-survival in vivo, but may also slow neuroinflammation in PD.
These studies have important implications in the treatment of PD. The most advantageous treatment options for PD may be those that combine symptomatic relief (i.e. dopa production) with the ability to slow the disease progress (through anti-inflammatory mechanisms). Despite the unfavorable results of the double-blind hRPE-GM clinical trials, if we are able to identify the specific mechanisms that increase hRPE cell survival and efficacy, then hRPE cells may be a valuable cell therapy option for PD.
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