

BEHAVIOURAL ASSESSMENT OF THE EFFECTS OF HUMAN RETINAL PIGMENT
EPITHELIAL CELL IMPLANT ON MOTOR DEFICITS OF A RODENT MODEL OF
PARKINSON'S DISEASE

by

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ABSTRACT

Parkinson's disease (PD) is the most common neurodegenerative movement disorder. PD is a progressive disorder characterized pathologically by preferential degeneration of the dopaminergic neurons in the substantia nigra pars compacta that project to the striatum, and the appearance of intracytoplasmatic inclusions known as Lewy bodies. Progressive loss of the nigro-striatal pathway results in decrease in the concentration of dopamine in the striatum. Clinically, PD is characterized by resting tremor, rigidity, bradykinesia and postural instability. To this day, L-dopa associated with a dopa decarboxylase inhibitor is the most effective symptomatic therapy for PD. However, L-Dopa long-term therapeutic limitations have imposed the need for searching alternative treatments for PD. Neural transplantation offers the possibility to place dopamine-producing cells into the striatum of patients with PD.

Retinal pigment epithelial (RPE) cells produce the DA precursor L-dopa, are readily isolated from eyes obtained from eye banks, and can be cultured and expanded in to allow implant in multiple patients from a single donor, and when attached to a gelatin microcarrier (GM) and implanted into the striatum, cause long-term amelioration of parkinsonian motor deficits in human and non-human primates without adverse effects. The advantages of RPE cell implants over other cell options for transplantation as a promising therapy for patients with PD makes it deserving of further study.

This study provides evidence that the cylinder test and the tapered ledged beam-walking test are sensitive, reliable, clinically relevant and useful for longitudinal studies of the effects of new therapeutic strategies for PD, and reports the first evidence that intrastriatal implantation of RPE-GM ameliorate deficits in spontaneous forelimb and hindlimb motor behaviour, in both a bilateral and a unilateral 6-OHDA rat model of PD.

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LIST OF ABBREVIATIONS

6-OHDA	6-hydroxydopamine
CPu	Caudate Putamen
DA	Dopamine
DMEM	Dulbecco's modified eagle medium
EMMPRIM	Extracellular matrix metalloproteinase inducer
FBS	Fetal bovine serum
FD	Fluorodopa
GABA	γ -amino-butiric acid
GM	Gelatin microcarrier
HBSS	Hank's balanced salt solution
hRPE	Human retinal pigment epithelial cells
ICV	Intracerebroventricular
IF	Immunofluorescence
I.U.	International Units
i.m.	Intramuscular
i.p.	Intraperitoneal
i.v.	Intravenous
L-dopa	Levodopa
MFB	Medial forebrain bundle
NuMA	Nuclear mitotic apparatus protein
PBS	Phosphate-buffered saline
PD	Parkinson's disease
PET	Positron emission tomography
RPE	Retinal pigment epithelium
RPE-GM	Human retinal pigment epithelial cells attached to gelatin microcarrier
s.c.	Subcutaneous
SNc	Substantia nigra pars compacta
SNr	Substantia nigra pars reticulata
UPDRS	Unified Parkinson's disease rating scale

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CHAPTER I Overview

1.1 Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease and the most common neurodegenerative movement disorder (Forman et al., 2004). First described in 1817 by the British clinician James Parkinson in his classical publication titled "*An Essay on the Shaking Palsy*" (Critchley, 1955), PD is a progressive disorder characterized pathologically by preferential degeneration of the dopaminergic neurons in the substantia nigra pars compacta that project to the striatum, and the appearance of intracytoplasmatic inclusions composed of protein aggregates, first described in 1912 by Friederich Lewy, known as Lewy bodies (Holdorff, 2002). Progressive loss of the nigro-striatal pathway results in decrease in the concentration of dopamine (DA) in the caudate nucleus and more predominantly in the putamen (Hornykiewicz, 2001), two of the basal ganglia nuclei that in the human brain constitute the striatum and in the rodent brain the caudate-putamen (CPu) and modulate motor output.

In most cases the exact cause of PD is unknown, making the development of etiological therapy unavailable at the present time. Various pharmacological and surgical options are being used to control most of PD symptoms, however no ideal therapy has been found. This situation imposes the challenge of developing new and more effective therapeutic strategies. However, the effectiveness of every new therapy needs to be initially tested in appropriate animal models using reliable behavioural measures.

Developing such behavioural tests adapted to the type of therapy being tested may be a challenge. For example, if the mechanism and site of action of the drug to be tested is already known and the behavioural assessment is aimed at evaluating only efficacy, the rotational model in the 6-hydroxydopamine (6-OHDA) unilaterally lesioned rat, in response to a pharmacological

challenge may be an adequate measure. However, if the exact mechanism of action is unclear, as is often the case in surgical procedures and especially in neural transplantation, then the use of behavioural tests without the added confounding effects of drugs or practice offers additional advantages.

Our ultimate goal is to understand the mechanism of action of a new type of cell transplant procedure, using human retinal pigment epithelial (hRPE) cells as the source of the implant. Although studies in human and non-human primates have demonstrated good efficacy, to date, very little data exist on the effects and mechanism that underlies the significant motor improvements seen after implant.

The main focus of the work reported in this thesis was to develop and validate the use of behavioural tests for sensitive, accurate, and reliable detection of motor impairments in the 6-OHDA rodent model of PD, and also for detection of subtle improvements in response to hRPE cell transplant therapy. Two tests were chosen, one to assess upper limb function and another to assess lower limb function. After evaluating these tests in normal animals, pilot studies were performed in 6-OHDA lesioned animals and in lesioned animals after hRPE implants to assess the sensitivity of the tests to 1) the effects of lesion and 2) the effects of therapy.

The two sensorimotor tests chosen rely on animals' spontaneous motor activity, require neither pharmacological challenges nor motivational incentives, are insensitive to the effect of learning, require little or no training and are useful in established bilateral and unilateral rodent models of PD. The usefulness of the tests was tested in a pilot study of the effects of hRPE cell implants in a unilateral and a bilateral rat model of PD. The present study tested the hypothesis that the forelimb use asymmetry test (cylinder test) and the tapered ledged beam-walking test

(TLBWT) are reliable tests to assess nigrostriatal function and sensitive to the long-term effects of RPE-GM implants on motor deficits of a 6-OHDA rat model of PD.

The remainder of the chapter that follows reviews the main aspects of PD, including functional organization of the basal ganglia, epidemiology, etiology, clinical manifestations and current treatment options with especial emphasis on the use of hRPE cells as a new promising option for neural transplantation. Additionally, evidence for the relevance of using the cylinder test and the TLBWT to assess the effects of hRPE cell implants on parkinsonian motor deficits of a 6-OHDA rodent model of PD will be presented.

1.2 The Basal Ganglia: Functional Organization

The term basal ganglia refers to a group of nuclei strategically located deep in the brain beneath the cerebral cortex surrounding the thalamus and the hypothalamus (Kiernan, 1998). The best understood functions of the basal ganglia are the modulation and facilitation of movements, but extensive connections with the temporal and anterior frontal cortex indicate involvement in memory, emotion and other cognitive functions (Graybiel et al., 1994; Parent and Hazrati, 1995).

The basal ganglia structures important in controlling movement include the caudate nucleus, the putamen, the globus pallidus (both internal and external segments), the subthalamic nucleus and the substantia nigra (pars compacta and pars reticulata) (Fig. 1.1). Extensive research has been carried out to elucidate the connections among these structures and between them and other brain regions however, despite this extensive knowledge, questions about how these regions function remain unanswered. As a result of these studies, many hypotheses concerning the mechanism by which the basal ganglia influence motor function have been tested, and models about how various pathologic states can disrupt motor behaviour have been proposed (Penney, Jr. and Young, 1983; Parent et al., 2000a).

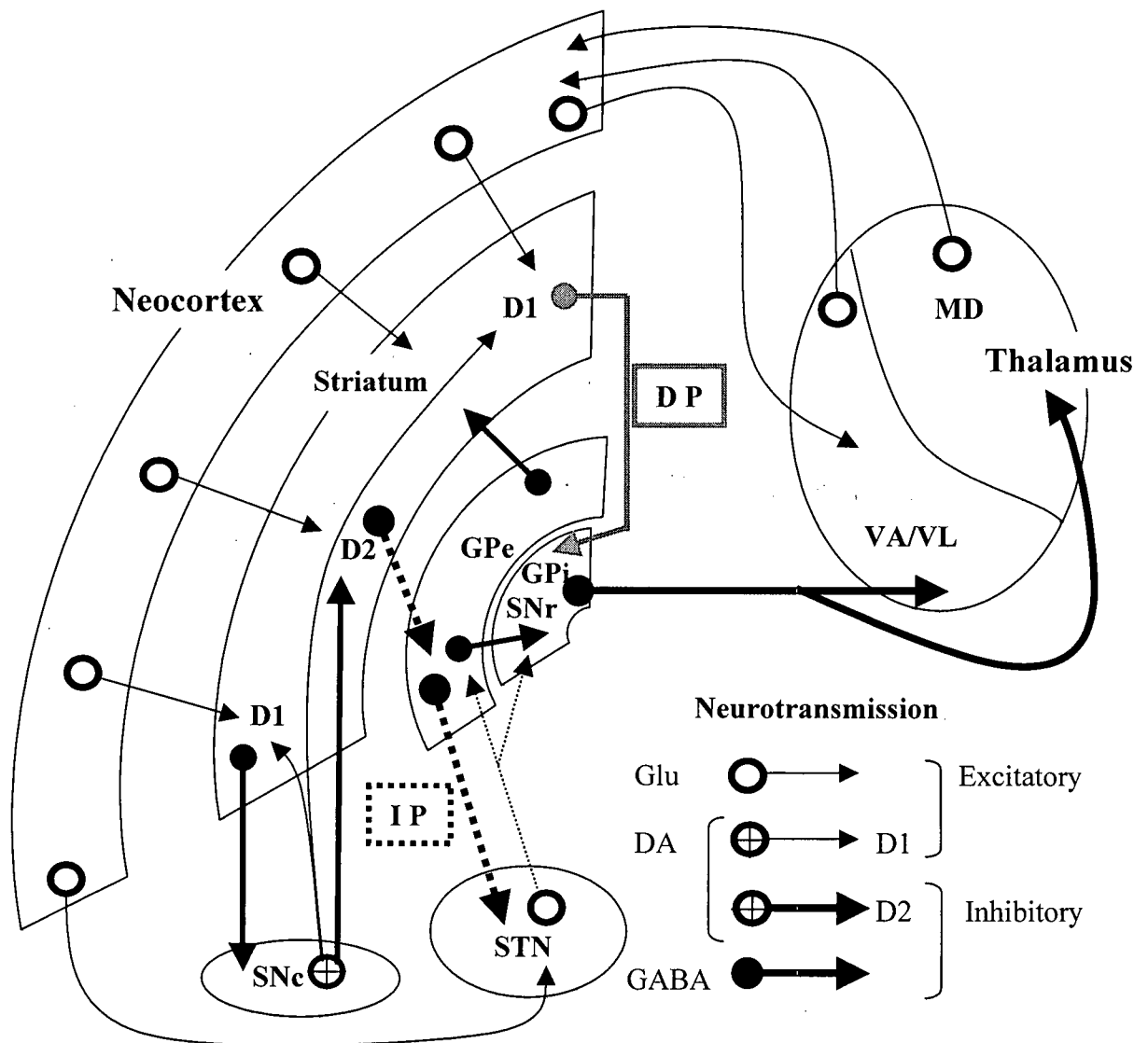


Figure 1.1 Diagram of the cortico-basal ganglia-thalamocortical loop. Thin arrows represent excitatory pathways and thick arrows represent inhibitory pathways. DA, dopamine; GABA, γ -aminobutyric acid; Glu, glutamate; D1 and D2 are dopamine receptors. GPe and GPi, globus pallidus externa and interna; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; STN, subthalamic nucleus; Thalamic nuclei are the mediodorsal (MD), ventroanterior (VA), and ventrolateral (VL); DP, direct pathway (grey arrow); IP, indirect pathway (dashed arrows). Adapted from Elble, (2002).

Diseases of the basal ganglia result in a variety of abnormal movements ranging from extreme hypokinesia to hyperkinesia. PD is considered the prototypical hypokinetic syndrome. The current model describes two pathways from cortex to basal ganglia to thalamus and back to cortex (the cortico-basal ganglia-thalamo-cortical loop) (Parent et al., 2000b; Young and Penney, Jr., 2002).

In the first pathway (direct pathway) glutamatergic cortical inputs excite striatal GABAergic neurons, which project to and inhibit the internal (medial) segment of the globus pallidus (GPi) and the substantia nigra pars reticulata (SNr); GPi and SNr neurons in turn send their GABAergic projections to the ventrolateral nucleus of the thalamus (Fig. 1.1). When a particular motor behaviour is selected, the appropriate thalamic neurons are disinhibited, facilitating execution of the planned motor program. Therefore, this pathway serves to facilitate motor programs. Activity in the striatal neurons involved in this direct pathway is enhanced by dopaminergic input from the substantia nigra pars compacta (SNc) because these striatal neurons express predominantly dopamine D1 receptors. In PD, loss of dopaminergic input to this pathway results in difficulty facilitating or maintaining motor programs (Elble, 2002). The clinical result is the parkinsonian hypokinetic state.

In the second pathway (indirect pathway) glutamatergic corticostriatal projections excite GABAergic inhibitory outputs to the external (lateral) segment of the globus pallidus (GPe); GPe neurons in turn inhibit the subthalamic nucleus (STN), the GPe and the SNr (Fig. 1.1). Activation of striatal neurons in this pathway therefore disinhibits subthalamic, GPi and SNr neurons. Subthalamic neurons are excitatory and project to the GPi and the SNr, meaning that disinhibition of a select set of these neurons further increases the activity of certain GPi and SNr neurons. GPi and SNr neurons inhibit the thalamic neurons that would otherwise facilitate

unwanted motor programs. Therefore, this parallel pathway through the GPe and STN and back to the cortex may be responsible for suppressing undesired movements (Alexander and Crutcher, 1990). The striatal neurons involved in this indirect pathway are inhibited by dopamine because they express mainly dopamine D2 receptors. As a result, in PD, decreased inhibition of the indirect pathway causes excessive subthalamic activity resulting in excessive suppression of unwanted movements and difficulty stopping an ongoing motor program and switching to another (Young and Penney, Jr., 2002).

This, and other models have increased our understanding on the complex functional organization of the basal ganglia, and have served to test potential new therapeutic interventions. For example, the finding that in fact MPTP-treated monkeys improve their parkinsonian symptoms after STN lesions (Bergman et al., 1990) gives support for the revival of pallidotomy (Gildenberg, 2003; Okun and Vitek, 2004a) and the use of subthalamic nucleus stimulation (Iansek et al., 2002; Kleiner-Fisman et al., 2004) as treatments for PD. However, new models that incorporate dynamic circuits that continually change in response to perturbations are needed to explain some responses of particular pathways to drugs, lesions and disease that current models are not able to explain.

1.3 Epidemiology of Parkinson's disease

The epidemiology of PD has been systematically studied in a wide variety of populations providing important clues about the cause of the disease.

1.3.1 Age and sex

PD is rare before age 50 years. However the number of people afflicted with the disease increases steadily by age group, affecting 1% of the population at age 65 and up to 5% of the

population by age 85 (de Rijk et al., 1997). PD is nearly twice as common in men than in women irrespective of geographic location or race (Tanner and Goldman, 1996b).

1.3.2 Prevalence and incidence

Although the prevalence rates of PD vary between 18 and 418 per 100,000 worldwide, the variations are much less marked when the differences between study methodology are considered (Schrag, 2002). Age-adjustment and restriction to studies using similar methodology reduce the variation between prevalence rates to between 102 and 190 cases per 100,000 population in Western countries. Reported annual incidence rates of PD range from 4.9 to 26 cases per 100,000 (MacDonald et al., 2000).

1.4 Etiology of Parkinson's disease

Despite intensive research during the past several decades, the cause of PD remains unknown. Nevertheless, significant progress has been made in elucidating genetic and environmental risk factors, as well as neurodegenerative processes contributing to PD.

1.4.1 Genetic factors

Family history has consistently been reported to be associated with an increased risk of PD compared with controls, suggesting that genetic factors play a role in the etiology of PD (Tanner and Goldman, 1996a; Rybicki et al., 1999). However, it is uncertain whether this reflects an increased genetic susceptibility, pure genetic inheritance, shared familial exposure, or biased recall in families of patients with PD (Tanner and Aston, 2000). Twin studies are of particular value to address this question. If genetic factors play an important role in the etiology of a disorder, the concordance rates in monozygotic twins are higher than in dizygotic twins. However, most twin studies have not confirmed an overall increased risk of PD in monozygotic twins compared with dizygotic twins (Tanner, 2003). A large twin study conducted by Tanner et

al. (1999b) in a cohort of World War II veterans in the United States found no overall difference in concordance rates in monozygotic twins compared with dizygotic twins, arguing for the lack of genetic causation of PD. However in twins with age of onset below 50 years, the concordance rate in monozygotic twins was significantly increased with 1.0 compared with 0.167 in dizygotic twins. These results suggest that genetic factors contribute significantly to the development of PD in those with young onset but do not play a major role in causing typical PD beginning after age 50 years.

A very important discovery in the last years in PD research has been the recognition that there are families in whom PD is genetically determined. Whereas mutations in the *α -synuclein* gene on the long arm of chromosome 4, inherited in an autosomal dominant pattern (Polymeropoulos et al., 1997), account for only a small number of cases (Chan et al., 2000), the autosomal recessively inherited *parkin* gene mutation on chromosome 6 (Kitada et al., 1998), on the other hand, appears to account for a substantial minority of cases of sporadic PD (Lucking et al., 2000). Another mutation was identified in the ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) gene on chromosome 4p, and associated with a form of young-onset autosomal recessive parkinsonism (Leroy et al., 1998). More recently, additional gene loci have been linked with inherited parkinsonism (Huang et al., 2003a). Both *parkin* and UCH-L1 function in the ubiquitination of proteins, possibly including *α -synuclein*, thus suggesting a general defect in the ubiquitin-mediated proteasome function in PD (Giasson and Lee, 2003).

These results suggest that there is a genetic contribution to PD, but whether this contribution is restricted to a small number of cases or a smaller contribution relevant to the majority of cases is unclear. Because it has been found that familial aggregation does not

necessarily imply genetic causation, it is thought that most PD cases including most familial cases, are due to shared exposure to environmental factors (Fuente-Fernandez and Calne, 2001).

1.4.2 Environmental factors

Results of epidemiological studies suggest that environmental factors play an important role in the occurrence of PD in populations of similar genetic background (Calne and Langston, 1983; Schrag A, 2002). As mentioned before, a large study comparing monozygotic and dizygotic twins failed to reveal a higher concordance rate in the monozygotic group when the disease began after age 50 years suggesting environmental causation in most cases of PD (Tanner et al., 1999a).

The few reports on clustering of PD (Herishanu et al., 1989; Goldsmith et al., 1990; Kumar et al., 2004) give support to the idea that a brief infective and/or toxic exposure in the shared environment may be responsible for the resulting PD cluster several years later. Infectious disease as a cause of PD has been implicated since the endemic encephalitis lethargica (von Economo encephalitis) leading to postencephalic parkinsonism (Duvoisin R.C. and Yahr M.D., 1965). More recently, studies suggest that exposure to influenza A may also lead to damage to the DA nigral neurons (Takahashi and Yamada, 2001). Additionally, the discovery that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a street drug contaminant, can cause human parkinsonism similar to that in PD has provided strong support to the hypothesis that PD may be caused by a single environmental toxin (Langston et al., 1983).

Case-control studies in patients with PD, which have attempted to identify potential risks factors for the development of PD, have despite their abundance not identified strong evidence for a single environmental cause. Some life exposures that have been found to be associated with PD are working on a farm or having a job that involves head trauma (Taylor et al., 1999),

exposure to metals such as copper, lead, iron and manganese (Gorell et al., 1997) or pesticides like bipyridyl (paraquat), (Seidler et al., 1996), well-water drinking, and rural living (Kuopio et al., 1999). Additionally, it has been reported that individuals working as teachers or those occupied in health care in Greater Vancouver had 2 to 2.5 fold risk of PD compared to controls (Tsui et al., 1999).

On the other hand, smoking (Tanner et al., 2002), frequency of bowel movements and alcohol consumption (Benedetti et al., 2000), coffee drinking (Ross et al., 2000), and even hypertension (Paganini-Hill, 2001) have been reported to be inversely related to PD.

Finally, it has been proposed that specific environmental and genetic factors, separately or synergistically contribute to the development of PD (Di Monte, 2003;Huang et al., 2003c).

1.4.3 Mechanisms of cell death

Numerous factors have been implicated in the pathogenesis of PD, including oxidative stress, excitotoxicity, mitochondrial dysfunction, apoptosis and inflammation (Olanow et al., 2002;Huang et al., 2003b).

Within nigral cells, DA normally undergoes oxidative metabolism and has the potential to generate cytotoxic free radicals and other reactive oxygen species (Jenner and Olanow, 1996). A pathological excess of toxic radicals might be formed as a result of (1) increased DA metabolism with excess hydrogen peroxide (H_2O_2) formation; (2) glutathione deficiency with failure to clear H_2O_2 (Jha et al., 2000); or (3) increased ferrous iron (Fe^{2+}) which can interact with H_2O_2 to form the hydroxyl radical ($OH\cdot$) (Jenner and Olanow, 1998).

Excitotoxicity is an established cause of neurodegeneration (Choi, 1988) occurring in PD as a response to a rise in glutamate transmission (Beal, 1998). Excessive glutamatergic activity results in an increase in calcium influx with a rise in cytosolic free calcium and subsequent

activation of calcium-dependent proteases, lipases and endonucleasas which cause damage to cytoskeletal proteins, membrane lipids, and DNA respectively.

Mitochondrial dysfunction can produce neuronal degeneration by accumulation of reactive oxygen free radicals generated during oxidative phosphorylation (Golden and Melov, 2001). Studies of post-mortem brain material from PD patients have found decrease in complex I activity in the SNc (Schapira et al., 1990). This biochemical defect is the same as that produced in animal models of PD by MPTP or 6-OHDA and adds further support to the proposition that PD may be due to an environmental toxin with actions similar to those of these selective neurotoxins.

Apoptosis is a slow cellular degradation process consisting of shrinkage, nuclear chromatin condensation, nuclear DNA fragmentation, cytoskeletal digestion and the formation of membrane-wrapped cytoplasmic and nuclear bodies, thought to occur in several neurodegenerative disorders as well as in response to toxins that are relevant to PD (Tatton and Kish, 1997).

Inflammatory responses as well as reactive gliosis with activation of microglia have been found in the substantia nigra in subjects with PD and other degenerative diseases (McGeer et al., 2001;McGeer and McGeer, 2004). However is not clear if inflammation is the cause or a consequence of the underlying neurodegeneration. The ability of antiinflammatory agents to delay the onset and slow down degenerative diseases of the nervous system is an interesting possibility that needs to be studied further.

1.5 Clinical Features of Parkinson's disease

James Parkinson's clinical descriptions, based on six persons (three of his patients and three he had observed on the street) in 1817, represent the first attempt, not to announce new

findings, but to organize the clinical manifestations of the disease. As the major characteristics of the disease he included: “involuntary tremulous motion”, “lessened muscular power” and “a propensity to bend the trunk forward and to pass from a walking to a running pace” with “the senses and intellects being uninjured” (Critchley M., 1955). It was the French neurologist Jean Martin Charcot in 1862 who added muscular rigidity to the list, and insisted on changing the name “shaking palsy” to “Parkinson’s disease” to honour Dr. Parkinson’s careful observations (Goetz C.G., 1987).

Four cardinal signs are now considered to characterize the clinical manifestations of PD: resting tremor, rigidity, bradykinesia and postural instability. In general, two of the first three are required to make the clinical diagnosis, since the fourth one emerges late in the disease (Sethi, 2003). Typically, PD manifestations are asymmetric and progressive (Calne et al., 1992; Toth et al., 2004). Idiopathic PD is the most common cause of parkinsonism (Hughes et al., 1993), but because there is no known biological marker, the challenge facing the clinician is to make an accurate diagnosis of PD. The accuracy of clinical diagnosis of idiopathic PD has been found to increase when the United Kingdom Parkinson’s Disease Society Brain Bank Clinical Diagnostic Criteria (UKPDBBCDS) is applied by the movement disorder specialist, requiring bradykinesia and at least one other feature, including rigidity, resting tremor or postural instability, and focusing on clinical progression, asymmetry of onset, and L-dopa response (2001a; 2001b; Hughes et al., 2002). To address the plurality of clinical rating scales, a concerted effort was made to produce a “gold standard” measure resulting, in 1987, with the Unified Parkinson’s disease rating scale (UPDRS) (Fahn et al., 1987). To date, is the most commonly used disease-specific scale.

The characteristic PD tremor is present and most prominent at rest, has a frequency of 4-6 Hz and may appear as a “pill-rolling” motion of the hand or simple oscillation of the hand or arm (Romanov et al., 2004).

Rigidity refers to an increase in resistance to passive movement, and it can be either smooth (lead pipe) or intermittent (cog-wheeling). Recent studies suggest the involvement of spinal circuits in the generation of parkinsonian rigidity (Le Cavorzin et al., 2003).

Bradykinesia refers to not only slowness of initiation of voluntary movement, but also to paucity of spontaneous movements and decreased amplitude of movement. Bradykinesia also is expressed as micrographia (small handwriting), hypomimia (decreased facial expression), decreased blink rate, and hypophonia (soft speech). Bradykinesia is considered the clinical sign that best reflects the nigrostriatal dopaminergic deficit in PD (Vingerhoets et al., 1997).

Postural instability makes reference to impaired balance and loss of righting reflexes. It appears usually after eight years or more, it is poorly amenable to treatment and is a common source of disability in late disease (Sethi, 2003; Nova et al., 2004).

Other motor manifestations include freezing when starting to walk (start-hesitation), during turning or while crossing a threshold. Non-motor symptoms include dementia (approximately 40% of patients experience cognitive decline with a progressive dementia syndrome) (Serrano and Garcia-Borreguero, 2004), depression (reports range from 4 to 90%, with an average of 25 to 40% of PD patients) (Leentjens, 2004), other neuropsychiatric manifestations (i.e. anxiety and psychosis) (Thanvi et al., 2003), sleep disorders (in more than 66% of PD patients) (Thorpy, 2004) and autonomic dysfunction (Visser et al., 2004). All these symptoms are believed to be related to involvement of non-dopaminergic pathways affecting brain neuronal populations that utilize other neurotransmitters such as serotonin (raphe nuclei),

norepinephrine (locus ceruleous) and acetylcholine (pedunculopontine nucleus), explaining why these symptoms respond poorly to dopaminergic medication (Bozi and Bathia, 2003).

1.6 Treatment of Parkinson's Disease

Advances in basic and clinical research have generated the development of improved therapeutic strategies in PD during the last decades. Important events during the 1960's are an example of this progress. Based on the observations of the parkinsonism-like DA-depleting effects of reserpine in rats and encouraged by the finding of Carlsson and his group (1959), who discovered that about 80% of the DA in the brain is found in the basal ganglia, Ehringer and Hornykiewicz tested the hypothesis that PD patients would have abnormal levels of DA in the basal ganglia. As a results of these neurochemical analyses, severe reduction of the concentration of DA and of its metabolite homovanillic acid in the basal ganglia region of PD patients' brain specimens was found for the first time in their laboratory in Vienna in 1960 (translated into English in Ehringer and Hornykiewicz, 1998). These discoveries led to the development of specific therapies for PD, intended to correct this biochemical defect.

1.6.1 Therapeutic options

The various therapeutic options in PD can be classified into different approaches according to their aim: modified from (Jankovic J.J., 2002)

I. Enhancing dopaminergic transmission by

A. Increasing DA synthesis:

1. Enhancing precursor availability (L-dopa + a dopa decarboxylase inhibitor such as carbidopa or benserazide) (Olanow et al., 2004d)
2. Stimulating tyrosine hydroxylase i.e., tetrahydrobiopterin (Werner-Felmayer et al., 2002)

B. Enhancing DA release:

1. Amantadine (Crosby et al., 2003)
2. Nicotine (Quik, 2004)
3. Electroconvulsive therapy (Kennedy et al., 2003)

C. Blocking DA reuptake:

1. Tricyclics, bupropion, mazindol, benztropine

D. Inhibiting DA degradation

1. Catecholamine *O*-methyltransferase (COMT) inhibitors (entacapone) (Poewe, 2004)
2. Monoamine oxidase B (MAO-B) inhibitors i.e., deprenyl (Magyar and Szende, 2004)

E. Administering DA agonists (bromocriptine, pergolide, lisuride, apomorphine, pramipexole, ropinirole, carbegoline) (Calne, 1999; Tan and Jankovic, 2001)

II. Manipulating non-DA neurotransmitters (Johnston and Brotchie, 2004)

- A. Acetylcholine (anticholinergics, tricyclics, amantadine)
- B. Norepinephrine (L-theodihydroxyphenylserine (DOPS))
- C. 5-hydroxytryptamine (clonazepam, lisuride)
- D. γ -aminobutyric acid (GABA) (progabide)
- E. Glutamate receptor blockers (NMDA and AMPA antagonists)
- F. Glutamate release blocker (riluzole)

III. Attempting neuroprotection (Clarke, 2004)

- A. Antioxidants
- B. Free radical trappers

C. Iron chelators

D. Gutamate antagonists

E. Trophic factors i.e., glial cell line–derived neurotrophic factor (GDNF) (Kirik et al., 2004)

F. GM1 gangliosides

IV. Surgical procedures:

A. Causing ablation: (Hariz, 2003;Okun and Vitek, 2004b)

1. Pallidotomy (posteroventral)

2. Thalamotomy (ventralis intermedius)

B. Using deep brain stimulation (Lozano and Mahant, 2004)

C. Brain grafting (Freed, 2000;Subramanian, 2001;Roitberg et al., 2004;Drucker-Colin and Verdugo-Diaz, 2004)

For the purpose of this thesis, further review of the therapeutic strategies in PD will focus on levodopa (L-dopa) and neural transplantation.

1.6.2 L-Dopa

To this day L-dopa is the most effective symptomatic agent in the treatment of PD and the “gold standard” against which new agents must be compared (Olanow et al., 2004c). L-dopa’s antiparkinsonian activity was first demonstrated in 1961 by Birkmayer and Hornykiewicz in Vienna, who administrated L-dopa intravenously to PD patients (Cotzias and McDowell, 1971). At the same time in Montreal, Barbeau (1962) and his colleagues begun the first real oral trials with L-dopa. In 1967 Cotzias, credited with establishing effective L-dopa therapy, removed the last doubts about L-dopa’s efficacy by introducing the successful regimen of high oral dose treatment (Cotzias et al., 1967).

The amino acid L-dopa is dopamine's immediate precursor substance. When taken orally, L-dopa is almost entirely absorbed at the level of the small intestine, but the majority of the compound is metabolized in the liver, kidneys and the blood and only 1% of the ingested L-dopa dose is transported by the neutral amino acid transport system across the blood-brain barrier into the brain where it is decarboxylated to form DA (Mannisto and Kaakkola, 1990). The benefits of L-dopa have been markedly enhanced by the addition of carbidopa or benserazide due to their peripheral dopa decarboxylase inhibitory effect (Opacka-Juffry and Brooks, 1995).

The surviving striatal dopaminergic terminals are primary responsible for conversion of L-dopa to DA, but glial cells may also participate in this process (Poewe and Wenning, 2002). With progression of the disease and concomitant neuronal loss, the long-term capacity for conversion of L-dopa to DA is reduced. This leads to a shortening of the striatal half-life, and loss of the brain's ability to store and buffer the shifts in striatal concentration of L-dopa; changes that associated with long-term use of L-dopa, result in the development of motor complications and symptomatic deterioration (Poewe and Wenning, 2002; Kumar et al., 2003; Olanow et al., 2004b). The development of these motor complications (motor fluctuations and dyskinesias) constitute the major limitation to the chronic use of L-dopa, reported to occur in 50 to 80% of PD patients who have received L-dopa for more than 5 to 10 years (Marsden and Parkes, 1977; Rajput et al., 2002).

The precise cause of motor complications is not known, but increasing evidence suggests that they are related to a combination of abnormal pulsatile stimulation of DA receptors (Olanow et al., 2004a), changes at the presynaptic level (changes in DA turnover, in DAT or in presynaptic DA receptor regulation) (Kumar et al., 2003), and other alterations at different

postsynaptic levels and further downstream, suggesting that these phenomena are more complex than previously thought.

The most common form of clinical fluctuation is the wearing-off effect, characterized by end-of-dose deterioration and recurrence of parkinsonian symptoms as a result of a shorter (sometimes only 1 to 2 hours) duration of benefit after a given dose of L-dopa (Rajput et al., 2004).

There are various types of L-dopa-induced dyskinesias, such as peak-dose (the most common type, usually consisting in choreiform or stereotypic movements involving the head, trunk and limbs), diphasic (occurring in about 15 to 20% of chronically treated PD patients), and wearing-off dyskinesias (Luquin et al., 1992; Fahn, 2000) which are subject to individual differences. Besides chorea, other L-dopa-related dyskinesias include various stereotypies, dystonia, and myoclonus.

Finally, even though L-dopa strikingly improves PD patients' quality of life initially, in the long run more research aimed at clarifying the controversies on whether L-dopa is toxic and whether it is the main cause of motor complications, is of paramount importance to provide optimal holistic care to PD patients, especially because patients with PD may be affected for 20 years or more.

L-Dopa long-term therapeutic limitations have imposed the need for searching alternative treatments for PD. While the development of effective neuroprotective agents will need to wait until the cause of idiopathic PD is unveiled, the promising field of neural transplantation offers the provocative possibility of brain function restoration.

1.6.3 Neural transplantation

The earliest published report on brain tissue transplantation appeared in 1890, when W. Gilman Thompson described attempts to transplant pieces of cortex from adult dogs or cats into cavities in the cortex of adult dogs, even almost 50 years before Medawar established that graft rejection is an immunological phenomenon (Freed, 2000). Multiple subsequent studies followed, until Lund and Hauschka in 1976 found that transplanted neurons could develop connections with the host brain, and Rosenstein and Brightman in 1978 described the favourable properties of the ventricles as site of transplantation; studies that formed the basis for the development of modern neural transplantation as a technique capable of being used to repair circuits in the brain (Freed, 2000).

The basic principle underlying neural transplantation in PD is the possibility to transplant dopamine-producing cells into the striatum of patients with PD. In the 1970's, Bjorklund et al, (1976;1976) demonstrated that transplanted fetal catecholaminergic and cholinergic neurons can survive, extend processes, establish synaptic connections, and enhance the release of neurotransmitters. Since then, more than 300 patients have undergone cell transplantations under various protocols (Encarnacion and Hauser, 2003). To date, new research is focusing on ways to improve the methodology to provide meaningful clinical benefit for PD patients.

Early reports describing results in PD patients who received human fetal mesencephalic cell transplants demonstrated no major therapeutic benefit in most outcome measures, but a small yet significant improvement in motor performance during off time (Lindvall et al., 1989). Patients received fetal grafts aged 7-9 weeks postconception and required immunosuppression, but there was no increase in the duration of L-dopa benefit and there was also no significant increase in fluorodopa (FD) uptake by positron emission tomography (PET) at the graft site.

Freed et al., (2001) performed the first double-blind, placebo-controlled trial using embryonic grafts aged 7-8 weeks postconception, transplanted into the putamen without immunosuppression. Evaluation at one year revealed improvement in off UPDRS motor scores in subjects 60 years or younger, while the older group did not show any significant improvement as compared to the sham-surgery control group. At 5.5 years post surgery, patients who demonstrated a good response to L-dopa preoperatively also experienced significant improvement during off time postoperatively, regardless of age. Nonetheless, in spite of the symptomatic benefit that has been observed, the improvement has been incomplete, both in the degree and pattern of functional recovery and dyskinesias postoperatively have been reported in 15 % of patients. Additionally issues regarding the use of large quantities of human fetal tissue limit the use of fetal mesencephalic cell transplants.

Alternative cells have being considered as options for neural transplantation in PD. Implantation of embryonic porcine mesencephalic grafts have not shown clinical efficacy in PD patients (Schumacher et al., 2000). Research have shown that it will be possible to generate DA neurons from stem cells for transplantation purposes, however there are still many unresolved issues including differentiation, long-term survival, migration and uncontrolled growth (Lindvall and Hagell, 2002).

It is generally agreed that neural transplantation is a promising therapy for PD, however new approaches are required to overcome the current issues and improve clinical efficacy (Bjorklund et al., 2003).

1.7 Retinal Pigment Epithelial Cells as a Source for Cell Therapy in Parkinson's Disease

The retinal pigment epithelium (RPE) is a melanin-containing cellular layer that lies between the neural retina and the choroids (Marmor and Wolfensberger, 1998) (Fig. 1.2). In the

eye, the RPE is a vital tissue for the maintenance of photoreceptor function. Embriologically, the RPE is derived from the same neural tube tissue that forms the neural retinal, but the cells differentiate into a monolayered transporting epithelium which main function is to metabolically insulate and transport the overlying neural retina.

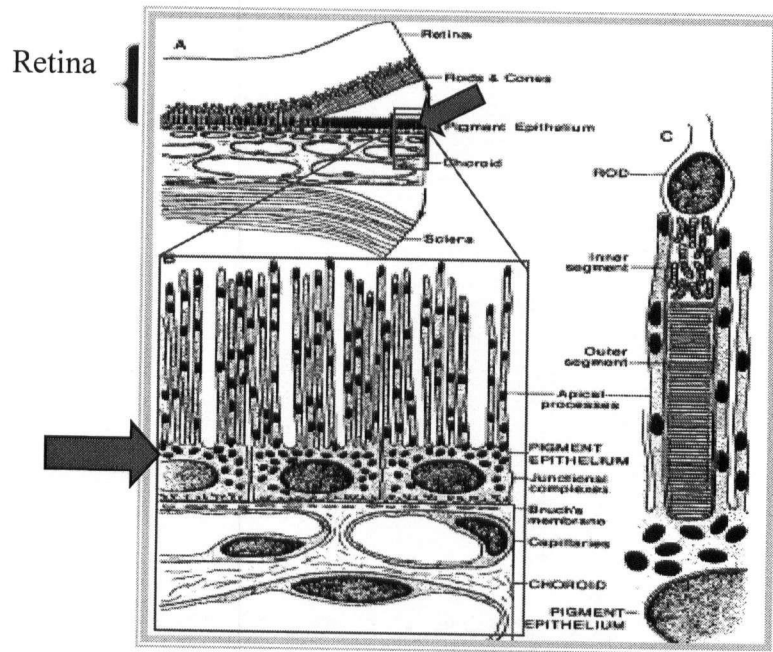


Figure 1.2 Retinal pigment epithelial cells *in situ*. Modified from (Marmor and Wolfensberger, 1998)

RPE cells appear cuboidal in cross-section and hexagonal when viewed from above. This monolayer of cells is joined by tight junctions (zonula occludens), which block the free passage of water and ions. This junctional barrier is the equivalent of the blood-retinal barrier formed by the capillary endothelium of the intrinsic retinal vasculature. Although cells of the RPE vary in size, shape and density across the retina, since the density of photoreceptors also vary, the number of photoreceptor that overlie each RPE cell remains roughly constant to about 45 photoreceptor per RPE cell. On the apical side (facing the photoreceptors) long microvilli reach up between and around the outer segments of photoreceptors (Fig. 1.2). Melanin granules are

concentrated in the apical end of the cell. The midportion of the cell contains the nucleus, synthetic machinery, and lysosomes. The basal membrane lacks microvilli, but has convoluted infolds to increase the surface area for the interchange of material through ion channels and pumps.

The study of RPE cells is relevant to PD because RPE cells synthesize the pigment melanin from the amino acid tyrosine, used by DA neurons to synthesize dopamine (Fig. 1.3).

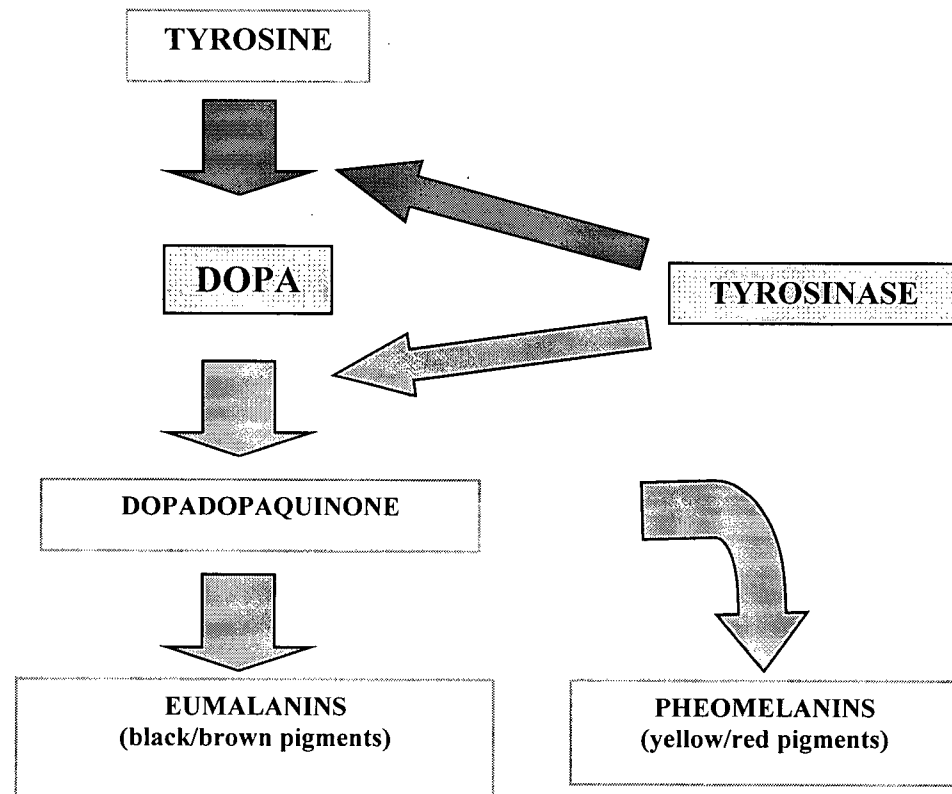


Figure 1.3 Metabolic pathways leading to eumelanin and pheomelanin in RPE cells.

Additionally, and although the role of melanin in the eye remains unclear, it is known that it serves as a free-radical stabilizer and as an agent that can bind toxins. The RPE also contains antioxidant enzymes superoxide dismutase and catalase, which minimize the formation of free radicals that can damage lipid membranes, processes that have been implicated in cell damage in

PD. RPE cells also take very active part in repair processes through secretion of growth factors and immunologic interaction.

RPE cells appear to be a suitable cell therapy option for transplantation in patients with PD for several reasons. RPE cells produce the DA precursor L-dopa, as part of the metabolic pathway leading to melanin synthesis (Pawelek and Korner, 1982a; Boulton, 1998). These cells contain tyrosinase (Dryja et al., 1978), the rate-limiting enzyme in melanin biosynthesis, and have tyrosine hydroxylase (TH)-like activity (Orlow et al., 1990; Varela et al., 1995; Smith et al., 1998). RPE cells are readily isolated from eyes obtained from eye banks, and can be cultured and expanded in quantities sufficient to allow implant in multiple patients from a single donor. Furthermore, microcarrier-attached RPE cells implanted into the striatum, have shown to cause long-term amelioration of parkinsonian motor deficits in human (Watts et al., 2003; Bakay et al., 2004b) and non-human primates (Subramanian, 2001; Doudet et al., 2004c) without adverse effects.

The advantages of RPE cell implants over other cell options for transplantation as a promising therapy for patients with PD makes it deserving of further study in established animal models of the disease, such as the 6-OHDA rat PD model.

1.8 Behavioural Assessment of the 6-OHDA Rat Model of Parkinson's Disease

It is generally agreed that behavioural assessment is essential for the development of new and better interventions aimed at recovering neurological deficits. However, there is little agreement about which tests should be used causing that test selection continues to be the subject of considerable debate. Tests that are not dependent on pharmacological challenges, and are not subject to practice improvement are especially important to perform *in vivo* longitudinal

assessment of the effects of a new cell therapy, when little is known about the mechanisms of action involved and potential adaptive processes.

Two convenient sensorimotor tests sensitive to impairment in the nigrostriatal pathway in rodent models have been recently developed that are not altered by repeated testing, require little or no training, do not include aversive motivation or food deprivation, and appear to be very useful in assessing established unilateral and bilateral rat models of PD. The tests are the forelimb use asymmetry test (cylinder test) and the tapered ledged beam-walking test (TLBWT). These tests are also easy to perform for the investigator, do not require elaborate equipment, are not stressful for the animals and give a reliable evaluation of different degrees of nigrostriatal dysfunction. An additional advantage of these tests is that the behavioural assessment involved is based on observing specific aspects of spontaneous motor activity rather than drug-induced behaviours.

1.8.1 Forelimb use asymmetry test (Cylinder test)

This test was developed by Schallert and Tillerson (2000) to measure spontaneous forelimb use during vertical exploration in a clear cylindrical chamber, based on a motor test of forelimb asymmetry described first by Schallert and Linder (1990). It has since been shown to be highly reliable, easy to score, does not require pharmacological intervention, and can detect a wide range of nigrostriatal lesion both acutely and chronically. This test has been very useful to evaluate the effects of new therapeutic interventions after partial loss of dopaminergic neurons caused by unilateral infusions of the neurotoxin 6-OHDA into the striatum or nigrostriatal projections, which are well established models of important neurochemical characteristics of PD (Ungerstedt, 1968a; Ungerstedt, 1971; Schallert et al., 1978b; Schallert et al., 1979b; Lee et al., 1996b; Zigmond and Keefe, 1998). The extent of asymmetry in the forelimbs during exploratory

movements, is determined by videotaping rats in a clear Plexiglas cylinder and later analyzing a slow motion version of the tape. In the cylinder, rats use their forelimbs to initiate movements that require weight shifting. Difficulty in initiating such movements is one of the primary signs of extensive degeneration of dopaminergic neurons in the substantia nigra. Thus, the test is considered to have clinical relevance (Schallert and Tillerson, 2000).

Forelimbs are observed during the first contact against the wall after rearing and during lateral exploration of the wall. Independent use of a forelimb and simultaneous use of both forelimbs are calculated. Each particular forelimblimb movement is then expressed in terms of the percentage of (1) use of that limb relative to the total number of limb-use movements, and (2) the percentage of use of both limbs relative to the total number of limb-use movements. The difference in percentage independent use between forelimbs is the forelimb use asymmetry score. These parameters are sensitive behavioural measures that are significantly correlated with striatal DA content (Schallert and Tillerson, 2000;Schallert et al., 2000a;Tillerson et al., 2001a).

Movements evaluated in the cylinder test are those spontaneously used by the animals in their home cage; therefore repeated testing does not influence the asymmetry score.

Pharmacological validation of this test demonstrated that spontaneous forelimb use in 6-OHDA-lesioned rats was sensitive not only to the initial beneficial effect of L-dopa treatment, but also to its delayed side effects (Lundblad et al., 2002). That is, unilaterally-6-OHDA-lesioned rats improved soon after L-dopa therapy was administered, but test performance levels declined when drug-induced abnormal involuntary movements appeared. Interestingly, rotational behaviour could not discriminate between dyskinetic and antiakinetic effects of L-dopa. The test has been used successfully by many laboratories on 6-OHDA treated animals to determine the effects of various interventions, including casting (Cohen et al., 2003), infusion of GDNF and

GDNF gene therapy (Tillerson et al., 2001b; Cenci et al., 2002b), deep brain stimulation (Shi et al., 2004), and neural grafting (Johnston and Becker, 1997).

1.8.2 Tapered ledged beam-walking test

Standard beam-walking tasks have been used to make evident motor deficits after central nervous system injury in rats, but it was not until studies made by Drucker-Colin and Garcia-Hernandez that a beam-walking test was found to be useful in studying aging and dopaminergic function (Drucker-Colin and Garcia-Hernandez, 1991c)

Their innovative experiments used a series of 2-meter wooden strips clamped together to form beams of increasing width (3, 6, 12, 18 and 24mm) supported by two pedestals at each end. The pedestals were of different heights in order to create a 15° inclination. This uphill slope prevented animals from crawling over the beam. Not surprisingly, they found that young rats (3 months of age) easily crossed the beam (even the narrowest one) and took less time to do it than aged rats (23-34 months old). They also determined that increasing weight load to the group of young rats (by wearing a 100 g lead belt) did not change their performance significantly, implying also that weight increase over time (alone) do not worsen the performance of this task.

More interestingly, they found that pharmacological manipulations targeting the dopaminergic system significantly changed their performance. Young rats injected with the D2-D3 DA antagonist haloperidol, severely slowed down beam-walking in all beam widths or the rats became unable to perform the task depending on the dose administrated. On the other hand, old rats receiving the DA precursor L-dopa orally, or when injected with the DA releasing agent amphetamine, dramatically improved their overall performance (decrease in latency to cross the beam and increase in the number of old rats that were able to cross at all widths). This

pharmacological evidence supported the idea that a beam-walking task is sensitive to nigrostriatal function.

There is recent additional evidence that performance in a beam-walking test is sensitive to rodent nigrostriatal impairment. Goldberg et al (2003) found that mice with a mutation of the parkin gene (parkin^{-/-}) (a rodent model of early-onset familial PD) performed significantly worse than wild-type controls with higher numbers of slips and slips per step in a beam traversal task. Dluzen and others, (2001) using two beams of different widths found that mice carrying a mutated brain derived neurotrophic factor (BDNF) gene (+/-) (shown to affect the nigrostriatal dopaminergic system of young adult BDNF mice) required a significantly greater amount of time to traverse both the wide and the narrow beams compared with non-mutant (+/+) BDNF mice. This study also indicated enhanced sensorimotor difficulty involved with a narrow versus a wide beam as previous studies have also reported (Drucker-Colin and Garcia-Hernandez, 1991a).

In a study designed to assess motor deficits in the rat following a high-dose regimen of methamphetamine (a psychomotor stimulant which produces neurodegenerative changes in the dopamine and serotonin systems) Walsh and Wagner, (1992) noted that after treatment and 1 week of recovery, treated rats were impaired to cross the 2 cm wide beam compared to control rats, as measured by footslips per trial, deficit that was recovered with L-dopa. Interestingly no significant differences were found in the latency to traverse a beam after treatment. The same beam-walking test designed by Drucker-Colin and Garcia-Hernandez has been used to assess the effects of intrastriatal adrenal medulla transplants in aged rats (Garcia-Hernandez et al., 1993). In this study, sham-grafted rats showed no spontaneous improvement in performance suggesting that the test is not subject to practice improvement over extended periods of time in aged rats.

The fact that the beam-walking tasks mentioned above focus on quantifying the number of foot slips and latency to traverse the beam has some implications. The main implication is that injured rats may develop compensatory strategies to complete the task. The development of compensatory mechanisms (in this task) after brain injury has been demonstrated (Schallert et al., 2000b). For instance, it has been consistently found that rats with unilateral infarcts after middle cerebral artery occlusion can gradually learn to keep the impaired limbs from slipping as they traverse a tapered beam (Schallert et al., 2003). This limits the use of such tests to detect chronic deficits and be sensitive to real recovery. Current behavioural tests still have difficulty differentiating these compensatory mechanisms which mask the motor impairment (Schallert et al., 2000c).

Fortunately, an original approach to this problem has been proposed recently. In order to unveil the otherwise masked limb impairment; a novel beam-walking test has been developed to allow manifestation of the deficit without inducing the development of compensatory mechanisms. Only now we can start distinguishing nervous system repair strategies from learned motor compensation (Schallert et al., 2003).

This behavioural test, the tapered ledged beam-walking test (TLBWT) uses a beam that is 165 cm in length, with a width that varies from 6.5 cm at the wide end and tapers evenly down to 1.5 cm at the narrow end that conduces to the rat's darkened home cage (Fleming et al., 2002). The innovation consists on the presence of ledges along both sides of the beam, 2 cm below the upper surface of the beam. These ledges are 2.5 cm wide and provide an auxiliary support surface for the animal to use by stepping down, rather than slipping, when there is a deficit. This specific task provides a more refined index of sensorimotor performance, which may better reflect nigrostriatal dopaminergic function.

This beam-walking test permits rapid measurement of a complex motor ability linked to coordinated movement. Normal and Sham-lesioned control animals keep all four limbs on the top surface of the beam, rarely stepping down on the ledges as they walk except sometimes at the narrowest, highest last part. Rats infused with 6-OHDA into the MFB, on the other hand, consistently use the ledges for stepping with the hindlimb contralateral to the lesion side to compensate for hindlimb dysfunction (Fleming et al., 2002).

Without the ledge, used as a “crutch”, the animals would be forced to adopt weight altering and postural strategies to compensate, masking the deficit and thus any improvements in outcome associated with brain repair treatments. As the beam narrows, rats with unilateral striatal dopamine depletion increasingly step with the impaired hindlimb on the ledge as they walk, with their intact limb on top of the beam. Rats with partial dopamine depletions show fewer use of the ledge by the impaired hindlimb, showing deficits only at the narrow third section of the beam. This tapered ledged beam permits the animal to manifest its deficit and makes feasible to detect improvement related to nervous system recovery associated with therapeutic interventions.

CHAPTER II Methodology

2.1 Animals and Surgical Procedures

A total of 45 male Sprague-Dawley rats (Animal Care Centre Breeding Unit, South Campus, UBC) weighing 275-300 g at the beginning of the experiments were used. They were housed in pairs in Plexiglas cages with free access to rat chow and water under a 12:12 h light-dark cycle (lights on at 1200 h) in a room with constant temperature (22°C) and relative humidity (55%). While some rats were kept as normal controls, the others received two successive surgical procedures over several weeks: 1) Unilateral medial forebrain bundle (MFB) 6-hydroxydopamine (6-OHDA) or bilateral intracerebral ventricular (ICV) 6-OHDA lesions and 2) Unilateral implants of either human Retinal Pigment Epithelial (hRPE) cells attached to Gelatin Microcarriers (RPE-GM) or Gelatin Microcarriers (GM) alone.

All surgical operations were performed under general anaesthesia using isoflurane (AErrane®, Baxter) and a Stephens vapourizer (Cenvet) (Fig. 2.1). For induction of anaesthesia, 4% isoflurane at a 60 mL/min flow of oxygen was used and 1-1.5% of the anaesthetic at the same flow was used for maintenance. During induction of anaesthesia, atropine sulphate

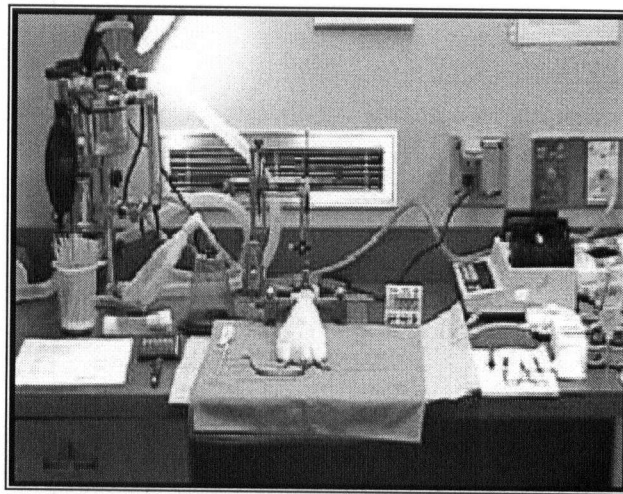


Figure 2.1 Stereotaxic rat surgery setup

(Atro-Sa®, Rafter) 0.05 mg/kg was injected s.c. to decrease respiratory secretions. Once the rats were anaesthetized, they were placed in a Kopf stereotaxic frame and kept warm using a warm water heating blanket (T/Pump®, Gaymar) beneath them. Chloramphenicol 1% ophthalmic ointment (Pentamycetin®, Sabex) was applied on the rats' eyes to prevent their eyes from drying out. At the end of each surgical procedure all animals were injected with ketoprofen (Anafen®, Merial, 10 mg/ml) 5 mg/kg s.c. to provide analgesia, procaine penicillin G/benzathine penicillin (Duplocillin®, Intervet, 300,000 I.U./ml) 90,000 I.U./kg i.m. to decrease the risk of infection, and 0.9% saline 5 ml s.c. to replace lost fluids and prevent dehydration during recovery. After surgery animals were placed in an incubator where they were kept warm and closely monitored until they were fully awake.

2.1.1 Unilateral medial forebrain bundle 6-OHDA lesion

Ten animals were injected with desipramine hydrochloride (Sigma) 25 mg/kg i.p. one hour prior to the infusion of 6-OHDA (Sigma) to prevent noradrenergic cell damage. Next, they received two unilateral stereotaxic injections of 10 µg 6-OHDA/4 µl in each of two sites along the right MFB. Coordinates were: AP: -2.8; ML: -1.8; DV: 8.0 and AP: -4.7; ML: -1.5; DV: 7.9, from Paxinos and Watson (1997) (Fig. 2.2).

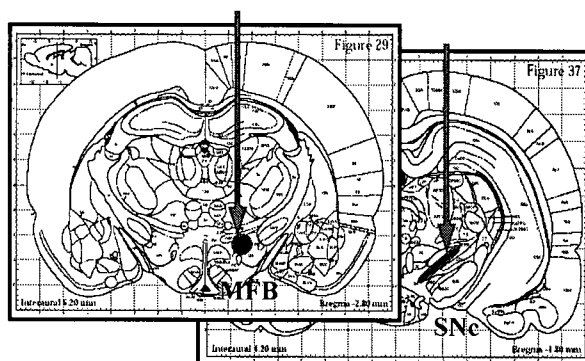


Figure 2.2 Unilateral right MFB double lesion. Modified from Paxinos and Watson (1997).

The 6-OHDA was dissolved in 0.05% ascorbic acid in 0.9% saline, and then kept chilled and protected from light to slow down oxidation. An infusion cannula attached to a 50 μ l Hamilton syringe mounted on a Harvard infusion pump set at 1 μ l/min. was used to infuse at a constant rate for 4 minutes. After infusion the cannula was held in place for 4 extra minutes to facilitate 6-OHDA diffusion.

2.1.2 Bilateral intracerebr ventricular (ICV) 6-OHDA lesion

Five animals were injected with desipramine hydrochloride (Sigma) 25 mg/kg i.p. one hour prior to the infusion of 6-OHDA (Sigma) to prevent noradrenergic cell damage. Next, they received 2 stereotaxic injections of 175 μ g 6-OHDA/3.5 μ l, one in each of the lateral ventricles. Coordinates were: AP: -0.8; ML: \pm 1.4; DV: 3.8 from Paxinos and Watson (1997) (Fig. 2.3).

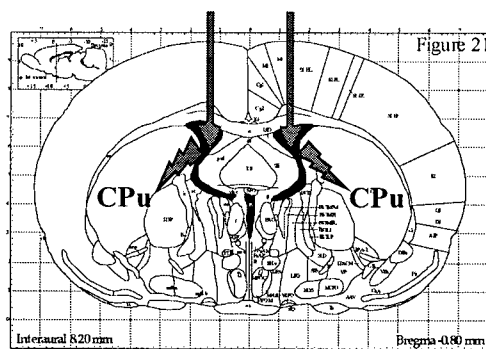


Figure 2.3 Bilateral ICV 6-OHDA lesion.
Modified from Paxinos and Watson (1997).

The 6-OHDA was dissolved in 0.05% ascorbic acid in 0.9% saline, and then kept chilled and protected from light to slow down oxidation. An infusion cannula attached to a 50 μ l Hamilton syringe mounted on a Harvard infusion pump set at 1 μ l/min. was used to infuse at a constant rate for 3.5 minutes. After infusion the cannula was held in place for 2 extra minutes to facilitate 6-OHDA diffusion.

2.1.3 Human retinal pigment epithelial cell implantation

2.1.3.1 Human RPE cells

Cryovials containing the frozen hRPE cells (Titan Pharmaceuticals, Inc) from eye bank tissue (Castillo, Jr. et al., 1995), were kept in liquid nitrogen storage until use. One week prior to implant, the hRPE cells were rapidly thawed in a 37°C water bath and resuspended in fresh complete medium containing Dulbecco's Modified Eagle Medium (DMEM) and 10% fetal bovine serum (FBS), then centrifuged and transferred in an unlaminated T-25 cell culture flask with DMEM-10% FBS. Subsequently, the hRPE cells were grown to confluence in an incubator at 37°C prior to attachment to microcarriers and cell implantation.

2.1.3.2 Cell Attachment

A total of 10 mg of dry gelatin microcarriers (GM) (18-34 µm diameter, Cytodex 3, Sigma) were hydrated in a 1.5 ml centrifuge tube with 1 ml of phosphate-buffered saline (PBS) for a minimum of 1.5 h then autoclaved at 120°C for 15 min (15 psi). Sterile GM were resuspended and washed in 1 ml of PBS two times, and then stored in 1 ml of DMEM-10%FBS until the time of attachment. The hRPE cells were washed with PBS, then harvested by trypsinization and mechanical agitation and transferred to a 15 ml polypropylene tube containing DMEM-10%FBS. The suspension was then centrifuged, washed with medium to give a final concentration of $\geq 1 \times 10^6$ cells/ml solution. These cells were added to the 1.5 ml polypropylene tube containing the GM. Once mixed, the tube was filled to the top with DMEM-10%FBS, sealed with parafilm, and placed on its side in an incubator at 37°C for 15-18 h. Non-attached cells were separated from the hRPE cell/GM mixture by gentle washing with Hank's Balanced Salt Solution (HBSS). After removing the supernatant, cell viability was assessed by using the trypan blue exclusion method; the hRPE cells attached to GM viability was

>80%. Microcarrier-alone suspensions were treated in a similar manner, except that no cell assessment was needed. All preparations were kept in an ice bath during the implantation procedure to a maximum storage time of 4 h. Hematoxylin and eosin (H&E) stained hRPE cells attached to a GM (RPE-GM) *in vitro* are shown in Fig. 2.4.



Figure 2.4 100x H&E stained RPE-GM *in vitro*. Courtesy of Joe Flores

2.1.3.3 RPE-GM Implant Procedure

The 10 unilaterally lesioned animals were implanted in the right Caudate-Putamen (CPu) and the 5 bilaterally lesioned animals were implanted in the CPu contralateral to the most affected side of the body. All animals were unilaterally implanted with two tracks of either ~15,000 microcarrier-attached hRPE cells each (n=10; 5 unilaterally and 5 bilaterally lesioned) or the equivalent volume of GM only (n = 5; unilaterally lesioned) ten weeks post 6-OHDA lesion. Coordinates were: for track #1 AP: +1.6; ML: -2.5 and for track #2 AP: -0.4; ML: -3.5, from Paxinos and Watson (1997). RPE-GM and GM-alone implants were injected at two depth levels for each track at DV: 6.0 and 4.0 mm below the skull (Fig. 2.5). Once the animals were anaesthetized and placed with their skulls flat in the stereotaxic frame, two holes were drilled on

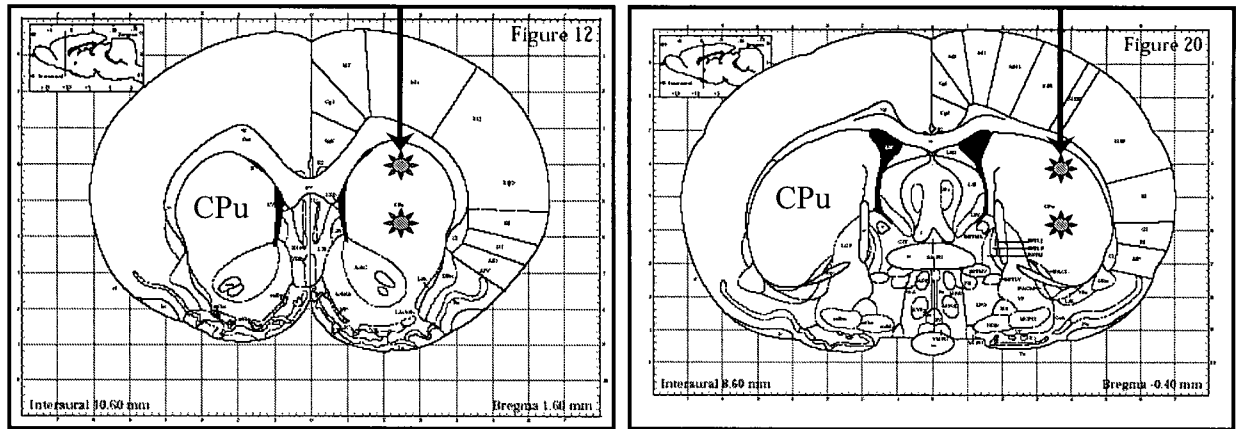


Figure 2.5 Unilateral (right) intrastriatal two-track RPE-GM or GM-alone implants. Track #1, rostral (left) and track #2, caudal (right) are 2 mm apart. Each track consisted of two injection sites (ventral and dorsal) that are also 2mm apart. Modified from Paxinos and Watson (1997).

their skull at the coordinates mentioned above, and the dura mater pierced with a sterile 25-gauge needle.

A sterile Hamilton syringe with a micro-polished needle was rinsed with DMEM and then with HBSS keeping the syringe held vertically at all times. The syringe was then filled with 20 μ l of HBSS avoiding air bubbles. The first 10 μ l were disposed of. The syringe's needle was then lowered into the eppendorf tube containing the RPE-GM and held at the level between the two upper thirds and the lower third. A circling motion of the needle was applied to gently agitate the contents of the eppendorf tube and then the syringe was loaded to 16 μ l. After taking the needle out of the tube, the sides of the needle were cleaned with sterile gauze.

The loaded Hamilton syringe was attached to the stereotaxic frame and gently lowered until the needle entered the brain tissue. The needle was advanced 0.5 mm below the exact first (deepest) implant site level (6.0 mm below the skull) and immediately brought back up to the implant site level to create a miniature pocket. Using a "pulse-like" injection technique 3 μ l were injected in the first site. The needle was then slowly brought up 2 mm and the last 3 μ l were

injected at the second implant site (4.0 mm below the skull). After the second injection, the needle was held in place for five minutes and then slowly brought back up out of the brain. The same procedure was repeated for the second track at the corresponding coordinates.

GM-alone implants were performed following the exact same procedure described above except with no hRPE cells.

2.2 Behavioural Tests

Rats were transported in their home cages from the colony room to a quiet room beside the testing room 30 minutes before testing. Testing was consistently done within the first 6 hours of the dark part of the light/dark cycle (1200 – 1800 h).

Prior to any intervention, 45 normal rats were evaluated with the forelimb use asymmetry test (cylinder test); of those, 32 were additionally tested with the tapered ledged beam-walking test (TLBWT) to measure the normal parameters of animal performance for both tests and to investigate the direction and magnitude of correlation between forelimb function measured with the cylinder test and hindlimb function measured by the TLBWT.

A total of 19 animals were then divided into 4 experimental groups: 1) normal ($n = 4$), 2) unilaterally 6-OHDA-lesioned + GM alone implants ($n = 5$), 3) Unilaterally 6-OHDA-lesioned + RPE-GM implants ($n = 5$), and 4) Bilaterally 6-OHDA-lesioned + unilateral RPE-GM implants ($n = 5$). The timeline and purpose of behavioural testing for these groups is outlined below.

To assess the variability and reproducibility of both tests, as well as the presence of a multiple exposure effect under normal conditions, Group 1 rats (normal) were tested in both tasks six times within a five-month period, at weeks 2, 4, 6, 8, 10, and 20 after arrival to the animal care facility. Group 2 rats were tested prior to lesion and four times after right-MFB-6-OHDA infusion over a period of 10 weeks, to evaluate the development of motor deficits over

time, and to assess the variability and reproducibility of the tests, as well as the presence of a multiple exposure effect, under lesioned conditions.

To assess the behavioural effects of hRPE cell implants on a unilateral rat model of PD, 10 animals were severely lesioned on the right MFB with 6-OHDA as described before (Groups 2 and 3). Ten weeks after lesion, animals were implanted with either GM alone (Group 2) or with RPE-GM (Group 3). Behavioural testing was done prior to lesion, 10 weeks after lesion (before implant) and two times after implant at weeks 2-4 and 8-10.

To assess the behavioural effects of hRPE cell implants on a bilateral rat model of PD, 5 animals were moderately ICV lesioned with 6-OHDA as described before (Group 4). Ten weeks after lesion, animals were unilaterally implanted with RPE-GM in the striatum contralateral to the most affected side of the body. Behavioural testing was done prior to lesion, 10 weeks after lesion (before implant) and two times after implant at weeks 2-4 and 8-10.

2.2.1 Forelimb use asymmetry test (Cylinder test)

For this test a clear Plexiglas cylinder 20 cm in diameter and 30 cm high was used (Fig. 2.6). The cylinder was placed on a glass surface (60 cm x 50 cm) 60 cm off of the bench top, and a mirror was placed at an angle underneath the cylinder to allow detection of limb use at all times (Fig. 2.6 D). A Canon ZR85® digital video camera mounted on a tripod was placed 70 cm away from the cylinder's mirror image. The zoom of the video camera was adjusted and the auto-focus function turned on, so the image of the cylinder bottom filled the screen (Fig. 2.6 C). The speed setting on the video camera was "standard play".

Testing was performed under red lighting in a room at 22°C during the dark part of the animals' light/dark cycle when they are more active. One lamp with a reflector-type 100W red

light bulb illuminated the mirror image of the cylinder, thus illuminating the lower surface of the animal during the test (Fig.2.6 D).

Two to four 3-5 minute sessions were carried out on different days, two sessions per week. For normal rats two 3-5 minute sessions corresponded to one complete test, whereas for lesioned or implanted rats four sessions of the same length constituted a complete test. No habituation sessions were given; data collection began with the first exposure to the cylinder.



Figure 2.6 Forelimb use asymmetry test (cylinder test)
A: General set-up; B: Cylinder, lateral view; C: Cylinder, lower view; D: Ongoing test.

Test scoring was carried out a later time by the same experimenter using a video camera with slow motion and frame-by-frame capabilities and a scoring sheet (APPENDIX I). To ensure that the experimenter was blind to the animal condition, each rat was assigned a unique code number, different from the identification number written on its tail. This code and the test date

were the only information available to identify each test when the video recordings were analyzed.

Scoring involved counting the number of times the rat used either or both forelimbs for weight support on the cylinder wall after rearing and during lateral exploration according to the original descriptions from Schallert and Tillerson (2000). In short, during a rear, the first limb to contact the wall with clear weight support (without the other limb contacting the wall within 0.4s) is scored as an independent wall placement for that limb. After the first limb contacts the wall, a delayed (later than 0.4 s) placement of the other limb on the wall while the first limb remains anchored on the wall is counted as an additional movement and scored as simultaneous (both). For example, if an animal places its left limb on the wall, followed by delayed contact with both forelimbs, the animal would receive a score of “one left” and “one both” for that sequence. If only one forelimb contacts the wall, all lateral movements thereafter are each scored as independent movements of that limb until the other forelimb contacts the wall with weight support, at which point one “both” is scored. If the rat continues to explore the wall laterally in a rearing posture while alternating both limbs on the wall (wall stepping) a “both” is recorded, and every additional combination of two-limb movements would receive a “both” score. If one limb remains stationary but in contact with the wall while the other makes small adjusting steps, this is scored only as one “both”. Thus, both paws must be removed from the wall surface before another movement can be scored. If the animal removes both forelimbs from the wall during a rear and then immediately resumes wall exploration, the movements are again scored as independent (left or right) or simultaneous (both) as described previously.

For each test session, the total number of independent movements of each forelimb and simultaneous use of both forelimbs were calculated. Each particular forelimb movement was

then expressed in terms of the percentage of use of that limb relative to the total number of movements as follows:

- 1). %Use of LEFT forelimb = $100 \times \text{Left independent movements} / [\text{left} + \text{right} + \text{both}]$
- 2). %Use of RIGHT forelimb = $100 \times \text{Right independent movements} / [\text{left} + \text{right} + \text{both}]$
- 3). %Use of BOTH limb use = $100 \times \text{Both movements} / [\text{left} + \text{right} + \text{both}]$

A single score indicative of forelimb use asymmetry was obtained by calculating the difference between RIGHT %Use and LEFT %Use under normal conditions or between CONTRALATERAL %Use and IPSILATERAL %Use after a unilateral intervention (lesion or implant) was performed.

2.2.2 Tapered ledged beam-walking test

For this test a 165 cm long Plexiglas beam was used. The beam was 6.5 cm wide at one end and tapered evenly down to 1.5 cm at the narrow end, which led into the rat's home cage. Ledges along both sides of the beam, 2.5 cm wide and 2 cm below the upper surface of the beam provided an auxiliary support surface for the animals to use by stepping down, rather than slipping, when there was a deficit. These beam dimensions were appropriate for rats weighing 275-700 g (Fig. 2.7).

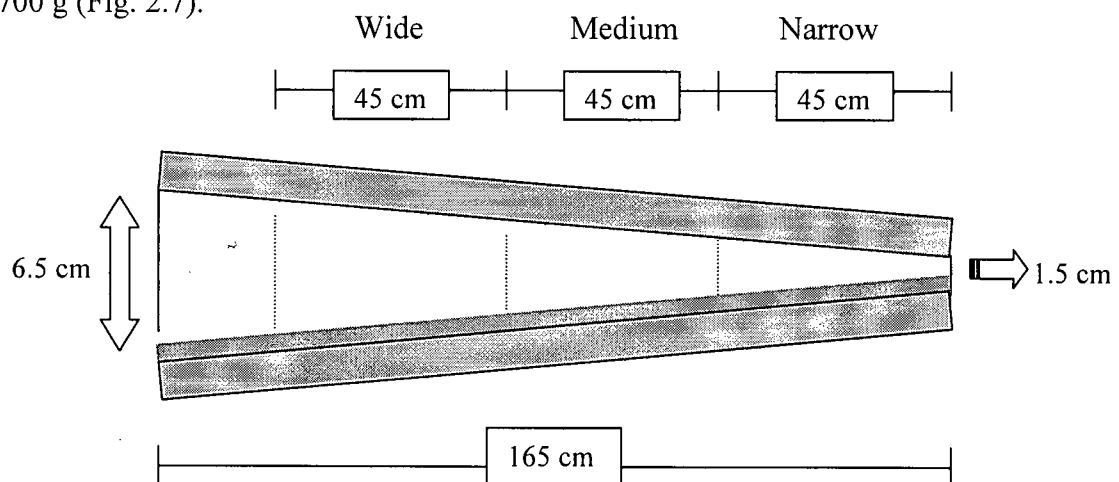
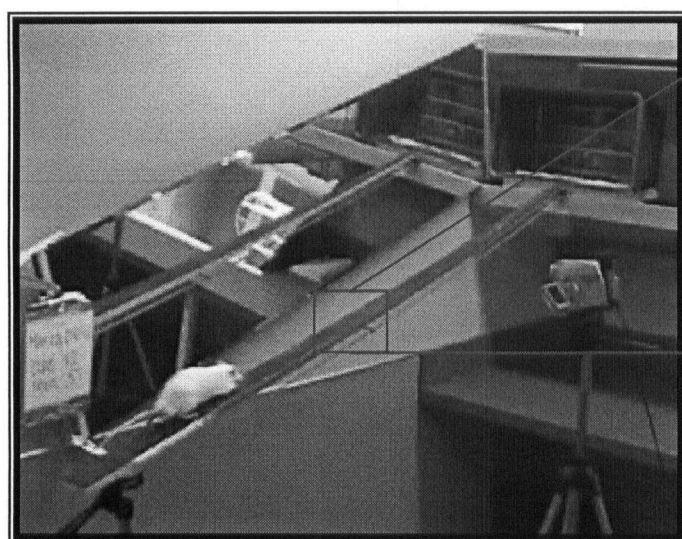


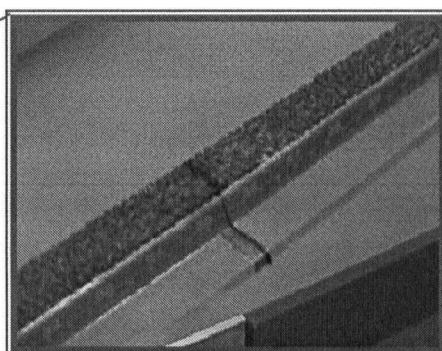
Figure 2.7 Tapered ledged beam dimensions

The beam was divided into three 45 cm segments (wide, medium and narrow) of increasing difficulty so that the location of a foot fault (stepping on the ledge for weight support) could be noted for each limb (Fig. 2.8B). The beam was placed at a 15-degree inclination, 90 cm above the ground, and its surface covered with rubber matting to prevent slipping (Fig. 2.8A). A mirror was attached to the wall on one side of the beam to allow simultaneous viewing of all limbs (Fig. 2.8C).

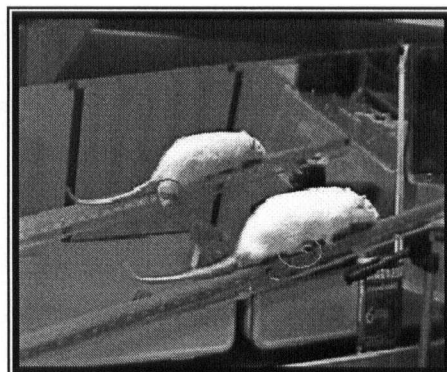
All animals were trained to walk from the widest, lowest part of the beam, to the narrowest, highest part of it, which leads directly into the animal's home cage. Training was done only once on the day prior to the first testing day (pre-lesion) and consisted of exactly 10 trials. During the training trials, subjects were placed on the beam at points successively further away from the goal home cage until they traversed the entire length of the beam.



A. General test set-up



B. Beam & ledge close-up



C. Left hindlimb foot fault

Figure 2.8 Tapered ledged beam-walking test

At both training and testing trials, each time the animals walked along the beam into their home cage, darkened relative to the area of the beam and containing the animals' own wood-chips, the lights were turned off and they were allowed to stay in there for one minute for reinforcement purposes. The home cage was wrapped with black plastic and placed on its side at the same level of the upper surface of the end of the narrowest section of the beam, resting on a darkened platform.

Testing was performed in a room at 22°C with the lights on during the dark part of the animals' light/dark cycle when they were more active. Five consecutive trials corresponded to one complete test. All trials were videotaped from the side using a digital Canon ZR85® video camera 120 cm from the middle of the beam and scored at a later date.

Test scoring was performed using the same video camera connected to a computer monitor. Scoring involved counting the number of steps and "errors" (stepping on the ledge during a forward movement) for both hindlimbs within each beam section, using the video camera's slow motion and frame-by-frame capabilities. The use of a scoring sheet form facilitated accurate scoring of the test (APPENDIX II). Data were then entered into a spreadsheet designed to calculate the total 5-trial errors per step ratio for each hindlimb and section of the beam.

2.3 Post-mortem Analysis

Lesion severity was determined using [³H]WIN 35,428 binding to the dopamine transporter in the striatum. For unilaterally lesioned animals, % lesion was measured with respect to the non-lesioned hemisphere, whereas with bilaterally lesioned animals, % lesion was determined with respect 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 dopamine transporter was performed on 20 µm sections as described (Van Kampen and Jon Stoessl, 2003). The slides were then allowed to dry overnight, and then placed in a vacuum dessicator with paraformaldehyde powder as the dessicant for 2 d (Liberatore et al., 1999) to prevent contamination of the tritium-sensitive storage phosphor screens (Fuji Medical Systems, Inc.) used for autoradiographic detection of the radioligand binding. The slides were placed against the phosphor screens for 3 d, and then scanned in a Cyclone phosphor imaging system (Perkin Elmer) at a resolution of 600 dpi. Striatal binding was measured on at least 6 sections per animal, and after subtraction of non-specific binding, the average specific binding data for each striatum was converted to a percent lesion score by the formula:

$$\% \text{ lesion} = 100 - [(\text{binding in lesioned striatum} / \text{binding in intact striatum}) \times 100]$$

Only some animals were used to determine the severity of the lesion while others were used to identify the presence of hRPE cells in the implanted striatum 5 months after implant as part of a separate research project.

2.4 Data analysis

2.4.1 *Cylinder test in normal rats*

The Kolmogorov-Smirnov statistic was calculated on the forelimb use asymmetry score to determine if the scores followed normal distribution. Relevant descriptive statistics were also calculated. Data was analyzed using a one-way repeated measures analysis of variance (ANOVA). Where significant *F*-values were found, Tukey's multiple comparisons test was made. A two-way analysis of variance (ANOVA) was conducted to examine the effects of TREATMENT (RPE-GM implant or GM-alone implant) and TIME on the forelimb use asymmetry score. $p < 0.05$ were considered statistically significant.

2.4.2 Tapered ledged beam-walking test in normal rats

A one-way analysis of variance (ANOVA) was conducted to compare the number of steps in the three different beam sections for each hindlimb. Where significant F values were found, post-hoc comparisons using the Tukey's multiple comparison test were made. The Kolmogorov-Smirnov statistic was calculated on the hindlimb asymmetry score in the narrow section to determine if the scores followed normal distribution

A two-way analysis of variance (ANOVA) was conducted to examine the effects of SIDE (left or right hindlimb) and BEAM SECTION (wide, medium or narrow), on the number of errors and % errors/steps, made by rats in the TLBWT. Also, a two-way analysis of variance (ANOVA) was conducted to examine the effects of TREATMENT (RPE-GM implant or GM-alone implant) and TIME for each beam section (wide, medium or narrow). Where significant F values were found, post-hoc comparisons using Tukey's multiple comparison test were made.

2.4.3 Correlation between forelimb function and hindlimb function

A Pearson correlation coefficient was calculated to investigate the direction and magnitude of the correlation between forelimb use asymmetry scores measured with the cylinder test and hindlimb errors/steps ratio asymmetry scores measured with the narrow section of the TLBWT under normal conditions.

CHAPTER III Results

3.1 Normal Behaviour

Prior to any intervention 45 normal rats were evaluated with the forelimb use asymmetry test (cylinder test); of those, 32 were additionally tested with the tapered ledged beam-walking test (TLBWT) to determine the normal parameters of animal performance for both tests and to investigate the degree of correlation between forelimb function measured with the cylinder test and hindlimb function measured by the TLBWT.

3.1.1 Forelimb Use Asymmetry Test (Cylinder test)

Normal rats used both forelimbs simultaneously during vertical exploration to lean on the cylinder in about half of the times they contacted the wall (51.38%), whereas in the other half, they used independently and in similar proportion, either the left (23.05%) or the right (25.57%) forelimb (Fig. 3.1).

Even though rats used the right forelimb more than the left (right % use – left % use = 2.52%), no marked preference was found in these rats as a population. However, at the individual level, calculating the forelimb use asymmetry score for each subject (right % use – left % use), the mean difference between preferred and non-preferred forelimb was 14.20%. This mean difference went up to 17.18% when rats with an exact score of 0.0% (no left/right asymmetry) (N=8; 17.77% of the rats) were excluded, leaving only lateralized rats (N=37; 82.23% of the rats). The percentage of rats with an asymmetry score between 10 and –10 (no forelimb preference) was 35.55%, while rats that displayed a limb preference corresponded to 24.44% (left) and 40% (right) (Fig. 3.2). A histogram and a normality test of the forelimb use asymmetry scores confirmed normally distributed values (Fig 3.3).

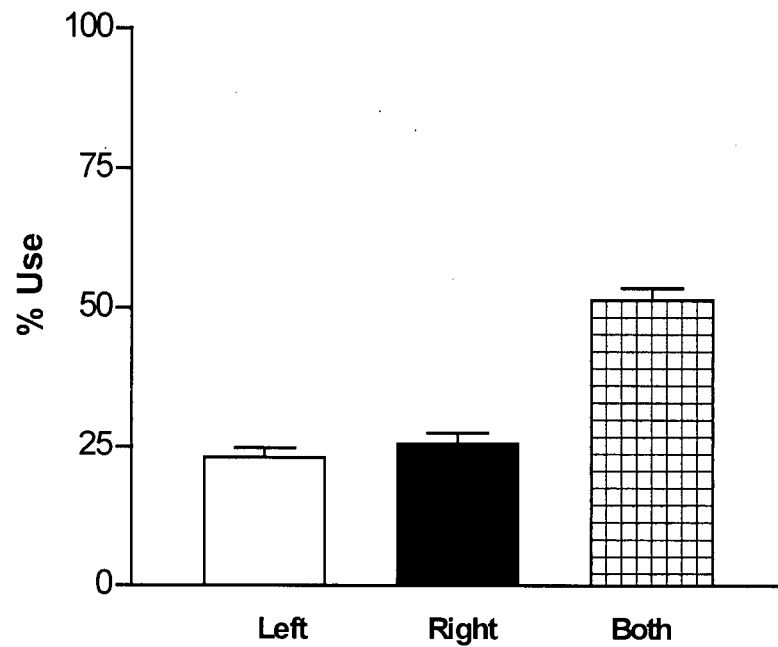


Figure 3.1 Forelimb use percentage in normal rats. Bars represent the mean percent (\pm SEM) forelimb use for left, right and both movements in the cylinder test. Independent forelimb use showed no strong left/right preference. Limb use was calculated as (1) Left forelimb use (Left/ left+right+both), (2) Right forelimb use (Right/ Left+right+ both), and (3) Simultaneous (both) use (Both/ Left+right+both) (N=45).

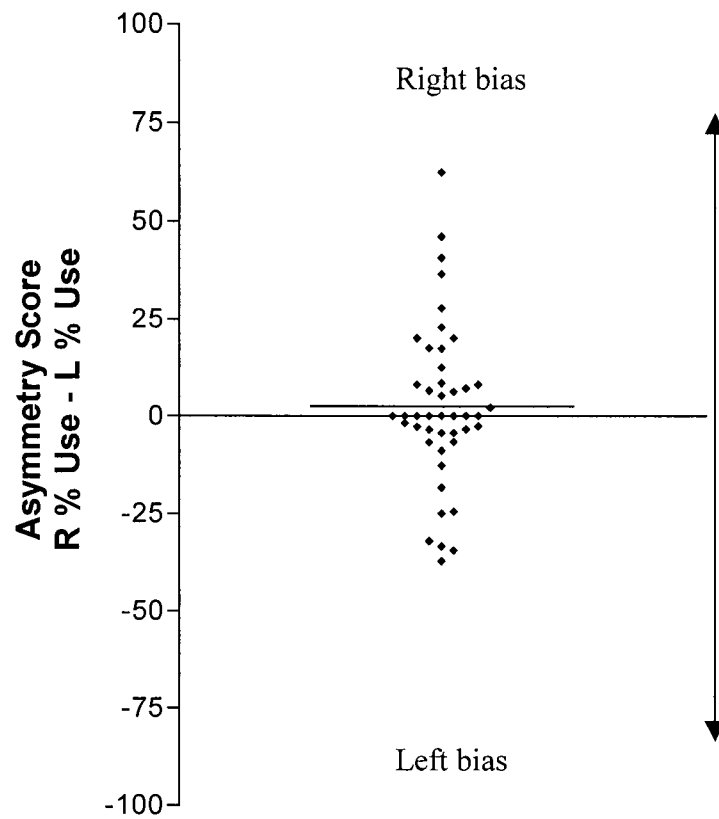


Figure 3.2 Scatter plot of individual forelimb use asymmetry scores in normal rats. Scores were clustered around 0 (no right/left difference). Rats without forelimb preference represented 35.55%, left biased rats 24.44%, and right biased rats 40%. Mean forelimb use asymmetry score was 2.53%, SD=20.60, range: -37.14% to 62.50% (N=45).

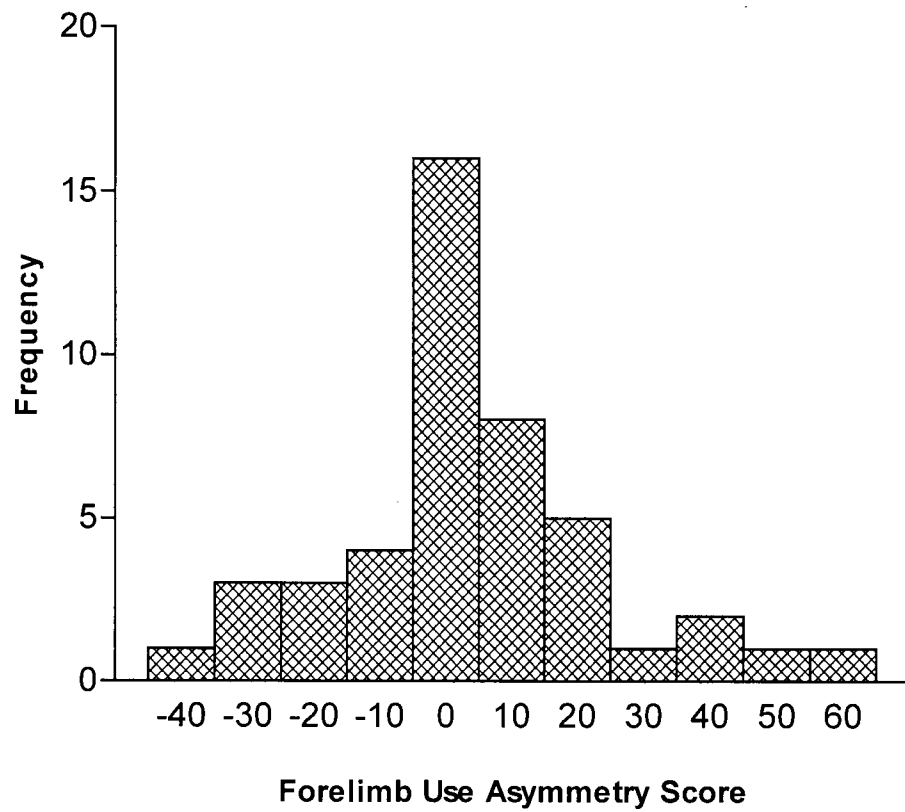


Figure 3.3 Histogram of individual forelimb use asymmetry scores of normal rats.

Limb asymmetry scores followed normal distribution demonstrated by the Normality test:

Kolmogorov-Smirnov statistic = 0.14, $p > 0.10$ (N=45).

3.1.2 Tapered Ledged Beam-Walking Test

Characterization of the test included analysis for both hindlimbs of (1) number of steps per beam section (wide, medium and narrow), (2) number of errors (using the ledge with a hindlimb for weight support) per beam section and (3) percentage of errors/steps per beam section. Additionally, hindlimb function asymmetry scores were obtained by calculating the difference between the left hindlimb percentage of errors/steps and the right hindlimb percentage of errors/steps.

From the TLBWT data obtained, the first parameter analyzed was number of steps. A one-way analysis of variance (ANOVA) was conducted to compare the number of steps in the three different beam sections for each hindlimb. Even though the three sections of the beam had the same length (45 cm), there was a statistically significant difference in the number of steps for the three beam sections made with both the left ($F_{2,93} = 3.40, p < 0.04$) and the right ($F_{2,93} = 5.64, p < 0.005$) hindlimb. Post-hoc comparisons using the Tukey's multiple comparison test indicated that the mean number of steps made with the left hindlimb in the narrow section of the beam ($\underline{M}=16.47, \underline{SD}=2.03$) was significantly different (higher) than in the medium section ($\underline{M}=14.91, \underline{SD}=2.40$) ($p < 0.05$), but not significantly different than in the wide section ($\underline{M}=15.53, \underline{SD}=2.79$) ($p > 0.05$) (Fig. 3.4). Similarly, the mean number of steps made with the right hindlimb in the narrow section ($\underline{M}=16.94, \underline{SD}=2.73$) was significantly different (higher) than in the medium section ($\underline{M}=14.69, \underline{SD}=2.53$) ($p < 0.01$), but not significantly different than in the wide section ($\underline{M}=15.81, \underline{SD}=2.76$) ($p > 0.05$) (Fig. 3.4). There were no statistically significant differences in the mean number of steps between the wide and the medium sections for neither the left ($p > 0.05$) nor the right hindlimb ($p > 0.05$).

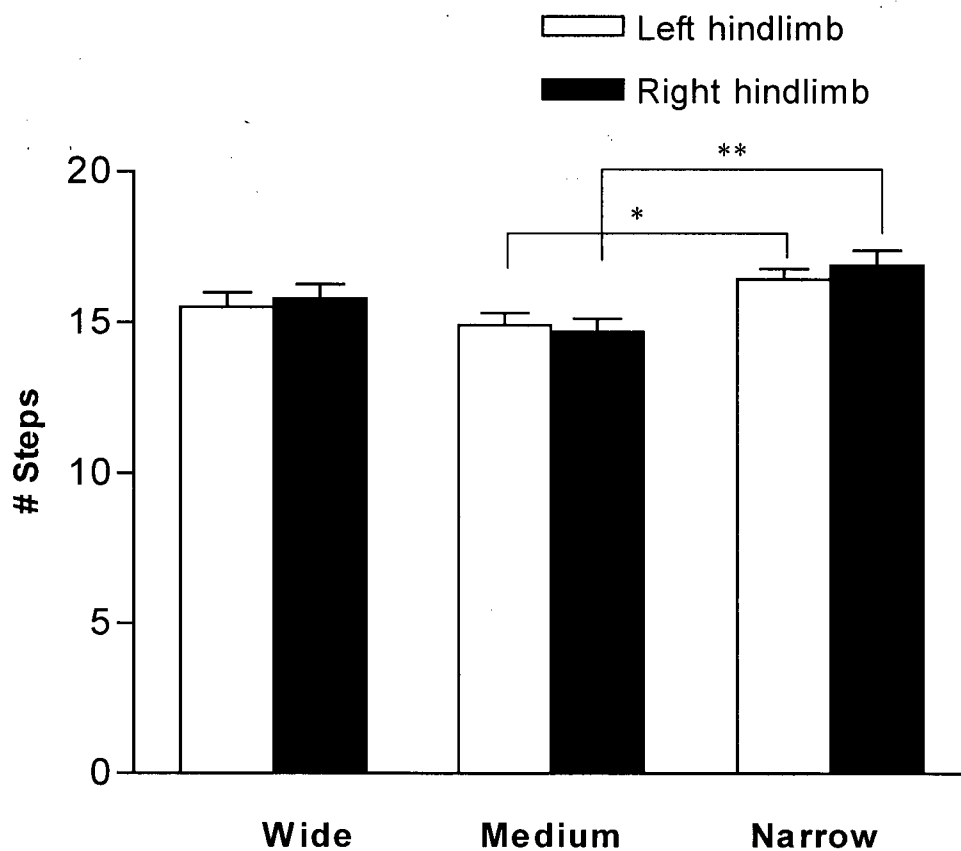


Figure 3.4 Mean (\pm SEM) of left and right hindlimb number of steps per beam section in normal rats. In the narrow section of the beam rats made with both the left and the right hindlimbs significantly higher number of steps than in the medium but not in the wide sections. No statistically significant differences were found between the mean number of steps made in the wide and in the medium sections for either hindlimb ($p > 0.05$) ($N=32$) (160 test trails).

* Significantly different at the ($p < 0.05$) level

** Significantly different at the ($p < 0.01$) level

The second parameter analyzed was number of errors. Surprisingly, there was a statistically significant effect of SIDE on the number of errors made on the TLBWT ($F_{1,186} = 12.45, p = 0.0005$). The number of errors made with the left hindlimb was significantly higher than those made with the right hindlimb, but only in the narrow section ($p < 0.01$) (Fig. 3.5).

There was also a significant effect by BEAM SECTION on the number of errors made on the TLBWT ($F_{2,186} = 35.17, p < 0.0001$) with both hindlimbs. In the narrow section, the rats made significantly more errors with both hindlimbs than in any other section of the beam ($p < 0.01$) (Fig. 3.5). The interaction effect (SIDE x BEAM SECTION) was not significant ($F_{2,186} = 1.02, p > 0.36$).

To control for the variability of number of steps and errors per beam section, the third parameter analyzed was the percentage of errors/steps. The ratio errors/steps was calculated for each hindlimb and section. To obtain the corresponding percentage, each ratio was multiplied by 100. There was a statistically significant effect of SIDE on the percentage of errors/steps made on the TLBWT ($F_{1,186} = 8.83, p < 0.003$). The percentage of errors/steps of the left hindlimb was significantly higher than that of the right hindlimb, but only in the narrow section ($p < 0.05$) (Fig. 3.6).

There was also a significant effect by BEAM SECTION on the percentage of errors/steps made on the TLBWT ($F_{2,186} = 22.00, p < 0.0001$) with both hindlimbs. In the narrow section of the beam, the rats made significantly more errors/steps with both hindlimbs than in the other two sections ($p < 0.05$) (Fig. 3.6). The interaction effect (SIDE x BEAM SECTION) was not significant ($F_{2,186} = 0.10, p > 0.90$).

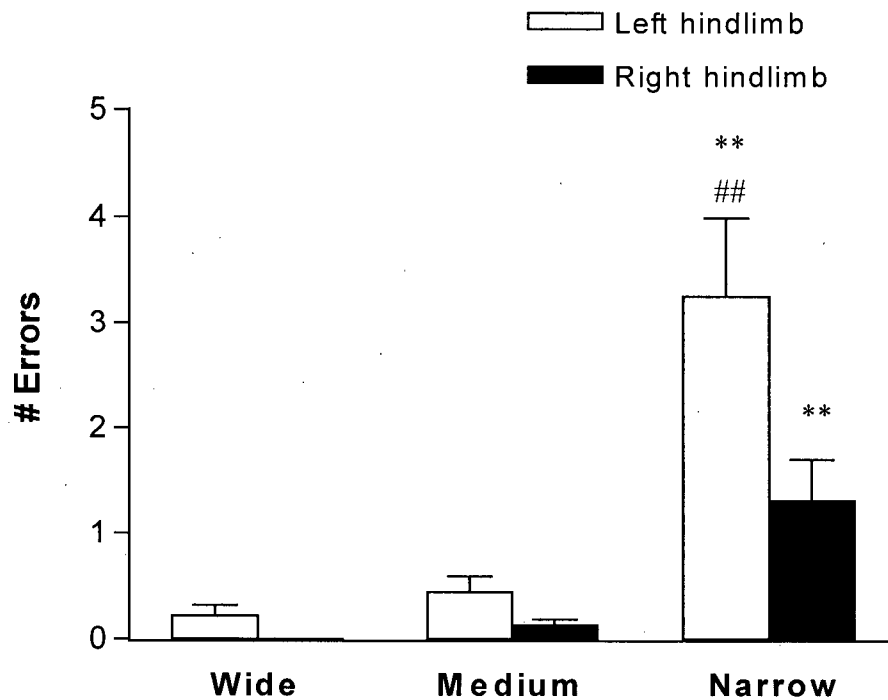


Figure 3.5 Left and right hindlimb mean (\pm SEM) number of errors per beam section in normal rats. In the wide and medium sections of the beam, rats made on average less than one error in 5 trials with the left (wide: \underline{M} =0.22, \underline{SD} =0.55; medium: \underline{M} =0.44, \underline{SD} =0.84) and almost none with the right hindlimb (wide: \underline{M} =0.0, \underline{SD} =0.0; medium: \underline{M} =0.12, \underline{SD} =0.34). However, in the narrow section rats made significantly more errors than in the wide and medium sections with each the left (\underline{M} =3.25, \underline{SD} =4.17) and the right (\underline{M} =1.31, \underline{SD} =2.19) ($p < 0.01$). Rats made significantly more errors with the left than with the right hindlimb in the narrow section, but there were no significant left/right differences in the other two sections ($p < 0.01$) ($N=32$) (160 test trails).

** Significantly different from the other sections of the tapered beam ($p < 0.01$).

Significantly different from the other hindlimb within the same section ($p < 0.01$)

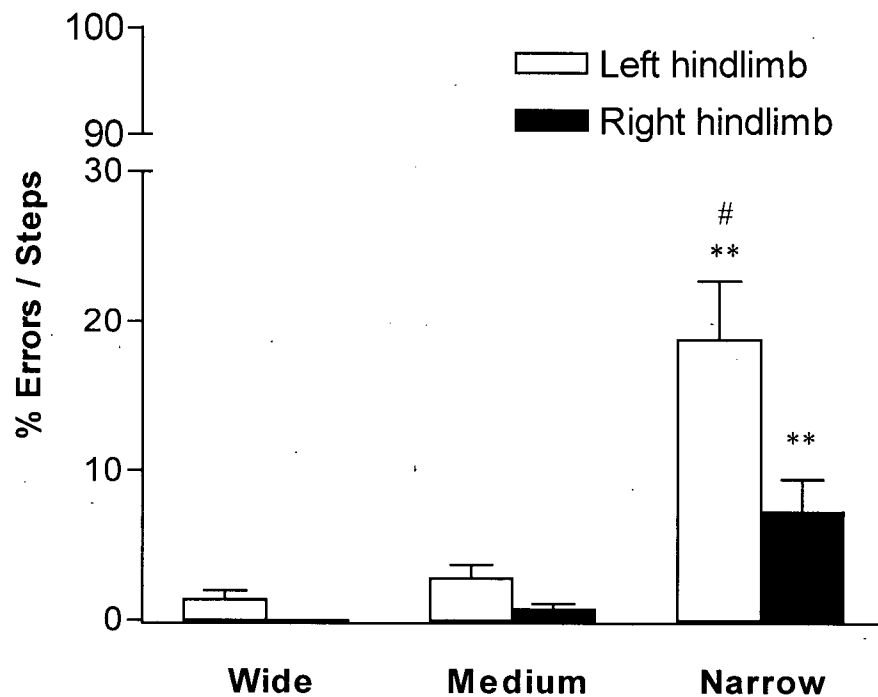


Figure 3.6 Left and right hindlimb mean (\pm SEM) percentage of errors/steps per beam section in normal rats. Rats rarely used the beam ledge for weight support (errors) in the wide (left: \bar{M} =1.33%, \bar{SD} =3.44; right: \bar{M} =0.0%, \bar{SD} =0.0) and medium (left: \bar{M} =2.75%, \bar{SD} =5.35; right: \bar{M} =0.74%, \bar{SD} =1.98) sections of the beam. However, in the narrow and most challenging part of the test, rats had a mean percentage errors/steps significantly higher than in the wide and medium section with the left (\bar{M} =18.75%, \bar{SD} =22.81) and the right (\bar{M} =7.24%, \bar{SD} =12.44) ($p < 0.01$). Rats had a significantly higher percentage of errors/steps with the left than with the right hindlimb in the narrow section, but there were no significant left/right differences in the other two sections ($p < 0.05$) (N=32) (160 trails).

** Significantly different from the other sections of the tapered beam ($p < 0.01$).

Significantly different from the other hindlimb within the same section ($p < 0.05$).

The fourth parameter analyzed was the hindlimb % errors/steps asymmetry score. In the wide section of the beam most of the rats (84.38%) showed no left/right difference in the percentage of errors/steps while in the medium section no left/right asymmetry was found in 75% of the rats. The remaining 15.62% in the wide section, and 25% in the medium, showed a slight tendency to make more errors/step with the left hindlimb compared to the right (Fig. 3.7).

Only the narrow section elicited asymmetries in both directions. Even though this group of rats made on average slightly more errors/step with the left hindlimb than with the right (mean % left errors/steps - % right errors/steps = 11.52, SD=26.63), no left or right consistent bias was found in this group as a population. However, at the individual level, calculating a hindlimb asymmetry score for each subject (% left errors/steps - % right errors/steps), it was found that the mean difference between the non-preferred (limb using the ledge) and the preferred hindlimb (limb kept on the upper surface of the beam) was in fact 19.42%. This mean difference went up to 23.91% when rats with an exact score of 0.0% (no left/right asymmetry) (N=6; 18.75% of the rats) were not included, leaving only lateralized rats (N=26; 81.25% of the rats). In the narrow section of the beam, the percentage of rats with an asymmetry score between 10 and -10 (no strong hindlimb difference in errors/step) was 43.75%, while rats that displayed a preference to keep one hindlimb on the upper surface of the beam (whilst using the ledge with the other), corresponded to 18.75% (preferring the left hindlimb and making more errors/step with the right) and 62.50% (preferring the right hindlimb and making more errors/step with the left) (Fig. 3.7). A histogram and a normality test of the hindlimb errors/step ratio asymmetry scores confirmed normally distributed values (Fig 3.8).

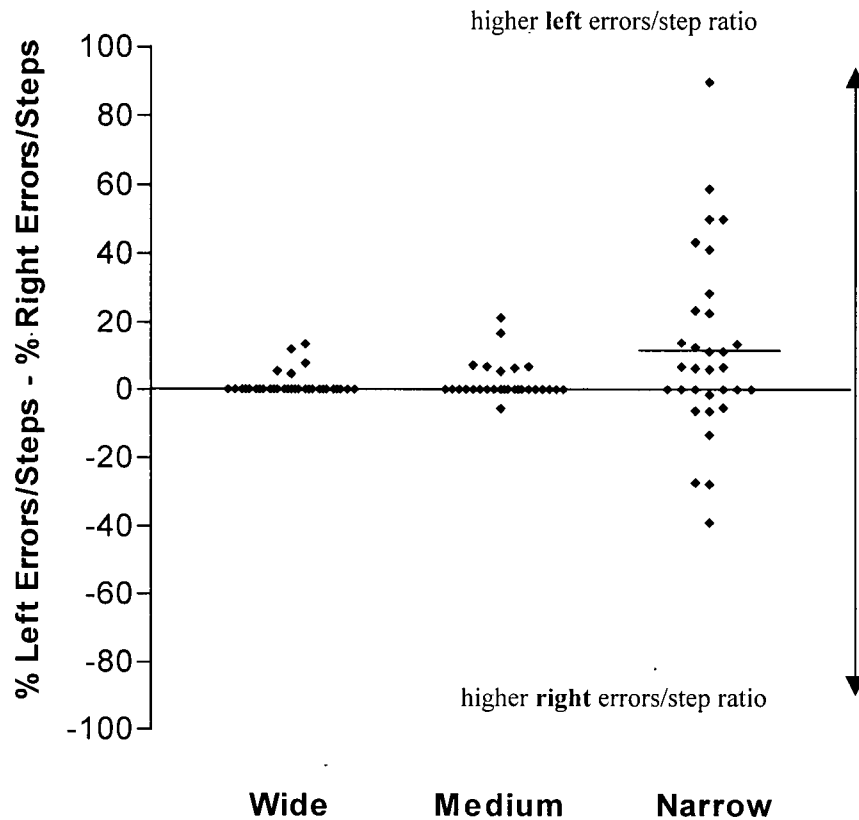


Figure 3.7 Scatter plot of individual left-right % errors/steps asymmetry scores per beam section in normal rats. In the wide (84.38%) and medium (75%) sections of the beam most of the rats showed no left/right difference in errors per step. The remaining smaller proportion, showed a mild tendency to make more errors/step with the left hindlimb. Only in the narrow section were asymmetries in both directions found. In this section, only 18.75% of the rats were symmetrical, 56.25% had a higher left errors/step ratio and 25% had a higher right errors/step ratio. The mean asymmetry score in the narrow section was 11.52%, SD=26.63, range: -38.97% to 89.74% (N=32) (160 test trials).

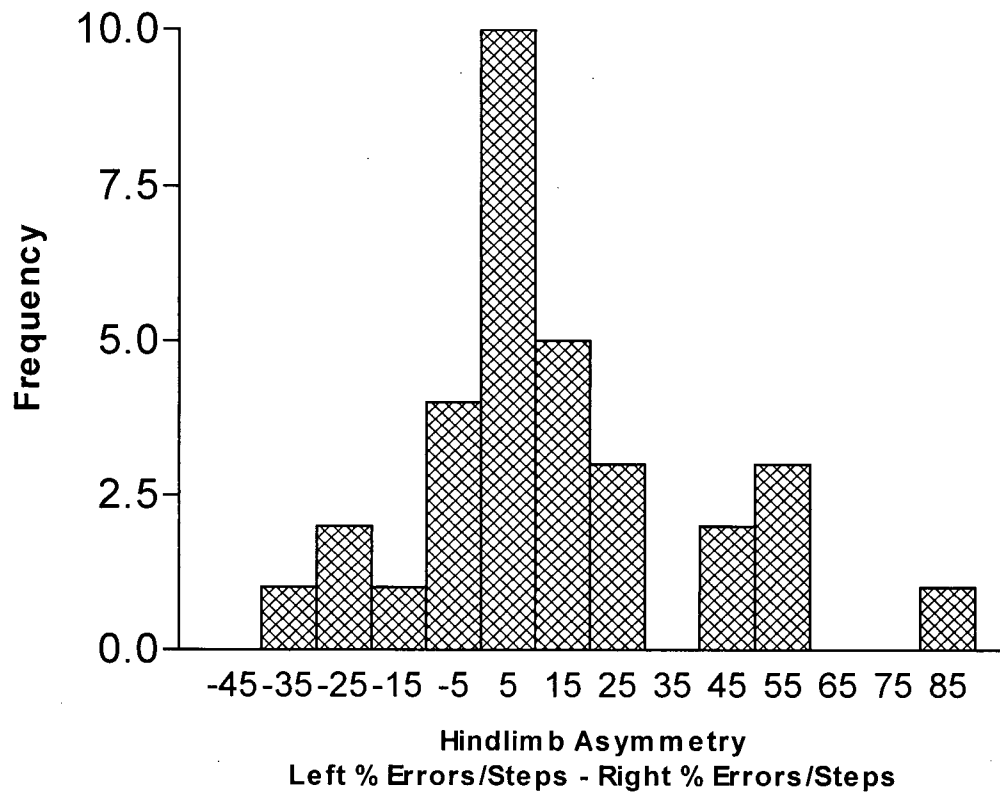


Figure 3.8 Histogram of individual hindlimb % errors/steps asymmetry scores of normal rats.

Limb asymmetry scores followed a normal distribution, demonstrated by the Normality test:

Kolmogorov-Smirnov statistic = 0.1853, $p > 0.10$ (N=32).

3.1.3 Correlation between forelimb function and hindlimb function

To investigate the direction and magnitude of the correlation between forelimb use asymmetry scores obtained with the cylinder test and hindlimb errors/step ratio asymmetry scores obtained with the TLBWT, the Pearson correlation coefficient was calculated. For each rat, forelimb use asymmetry scores were paired with the hindlimb errors/step ratio asymmetry scores obtained for the narrow section of the beam. The wide and medium sections failed to elicit asymmetries in both directions, and therefore were not considered for correlation.

The forelimb and hindlimb asymmetry scores showed a Pearson correlation coefficient r of 0.56, $p < 0.0008$, demonstrating a significant positive correlation between the two scores (Fig. 3.9).

Surprisingly, there was no significant correlation between the percentage of independent use of the left forelimb in the cylinder and the percentage of errors/steps of the ipsilateral (left) hindlimb in the narrow section of the TLBWT ($r = -0.19$, $p < 0.29$), and neither was there between the percentage of independent use of the right forelimb and the percentage of errors/steps of the ipsilateral (right) hindlimb on the same tests ($r = -0.10$, $p < 0.58$). However, strikingly, there was a positive and statistically significant correlation between the percentage of independent use of the left forelimb in the cylinder and the percentage of errors/steps of the contralateral (right) hindlimb in the narrow section of the TLBWT ($r = 0.36$, $p < 0.04$), and an even stronger positive and statistically significant correlation between the percentage of independent use of the right forelimb and the percentage of errors/steps of the contralateral (left) hindlimb on the same tests ($r = 0.59$, $p < 0.0004$).

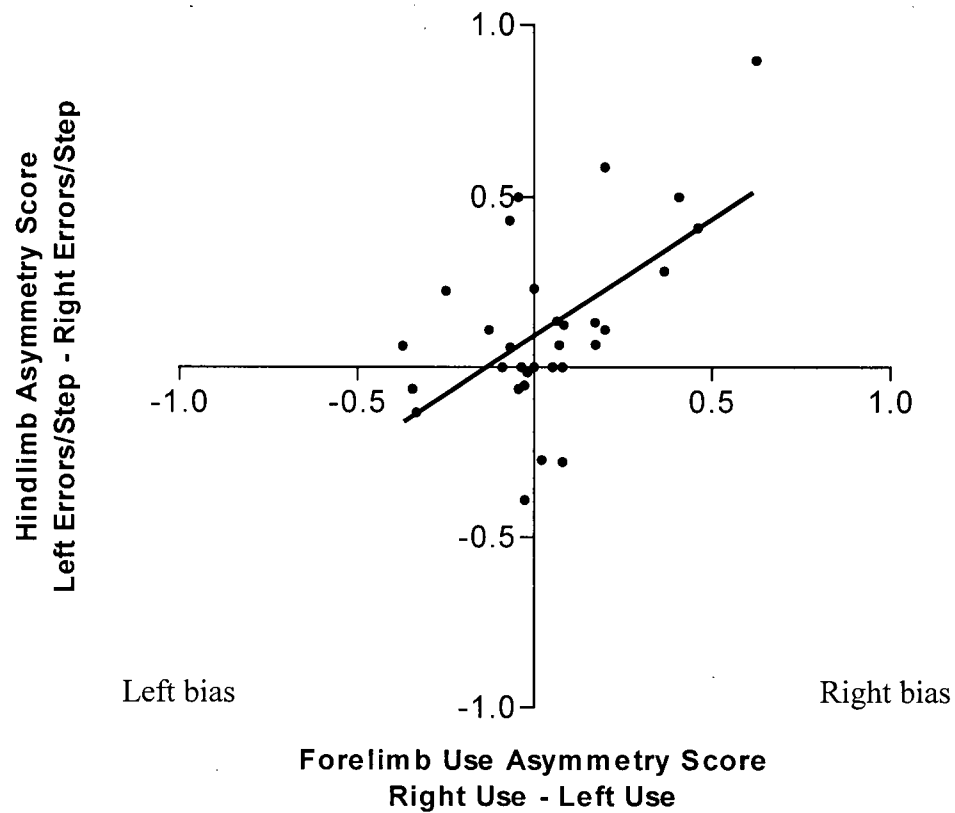


Figure 3.9 Correlation of forelimb and hindlimb asymmetry scores in normal rats. Correlation between forelimb use asymmetry score measured with the cylinder test was positive and significantly correlated with hindlimb errors/steps ratio asymmetry score measured in the narrow section of the tapered ledged beam-walking test (N=32). Coefficient $r = 0.56$, $p < 0.0008$.

3.2 Multiple Testing Effect in Normal Rats

To assess the variability and reproducibility of both tests, as well as the presence of a multiple exposure effect under normal conditions, Group 1 rats (normal, $n=4$) were tested in both tasks at weeks 2, 4, 6, 8, 10, and 20 after arrival to the animal care facility at age 7 weeks. Weight increase during this period is shown as an insert in Figure 3.10.

3.2.1 Forelimb use asymmetry test (cylinder test)

A one-way repeated measures ANOVA was performed to assess the effect of repetitive testing on forelimb use in the cylinder test. Rats were tested on 12 different occasions in a period of 5 months. The results of 2 trials performed during the same week were combined to constitute a complete test increasing reliability of the scores; therefore 6 data points were analyzed.

During the five-month period of multiple testing, there were no significant differences in the mean percentage use for the left forelimb ($F_{5,3} = 0.71, p = 0.63$) or the right forelimb ($F_{5,3} = 0.96, p = 0.48$). However, the percentage of simultaneous use (both) showed a significant time effect ($F_{5,3} = 8.19, p < 0.0001$) (Fig.3.10). A Tukey's post hoc test determined that the mean percentage of "both" use did not differ within the first three tests at weeks 2, 4 and 6, but it was significantly smaller at the fourth test at week 8 ($p < 0.01$), and even smaller at week 20 ($p < 0.001$) compared to the first three tests (Fig.3.10).

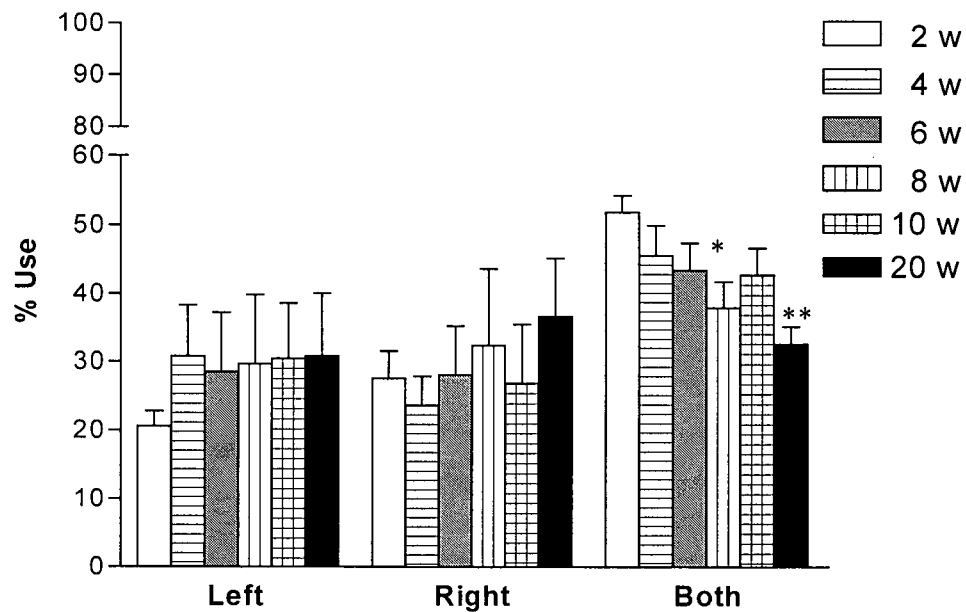
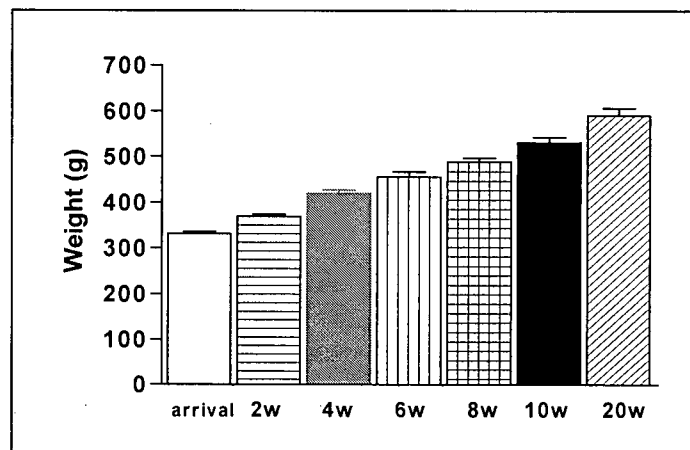


Fig. 3.10 Multiple testing effect on forelimb use in the cylinder test in normal rats. Bars represent mean % use (\pm SEM). The percentage of independent use for both left and right forelimbs did not change significantly over the five-month testing period. The mean percentage of left independent use varied within a 10.16% range, while the mean percentage of right independent use varied within a 13.04% range. However, the mean percentage for simultaneous forelimb placement decreased significantly at week 8 (4th test) ($p < 0.05$) and at week 20 (6th test) ($p < 0.001$) compared to the 1st test at week 2. Corresponding mean weights are:

* Significantly different from week 2, $p < 0.05$.

** Significantly different from week 2, $p < 0.001$.



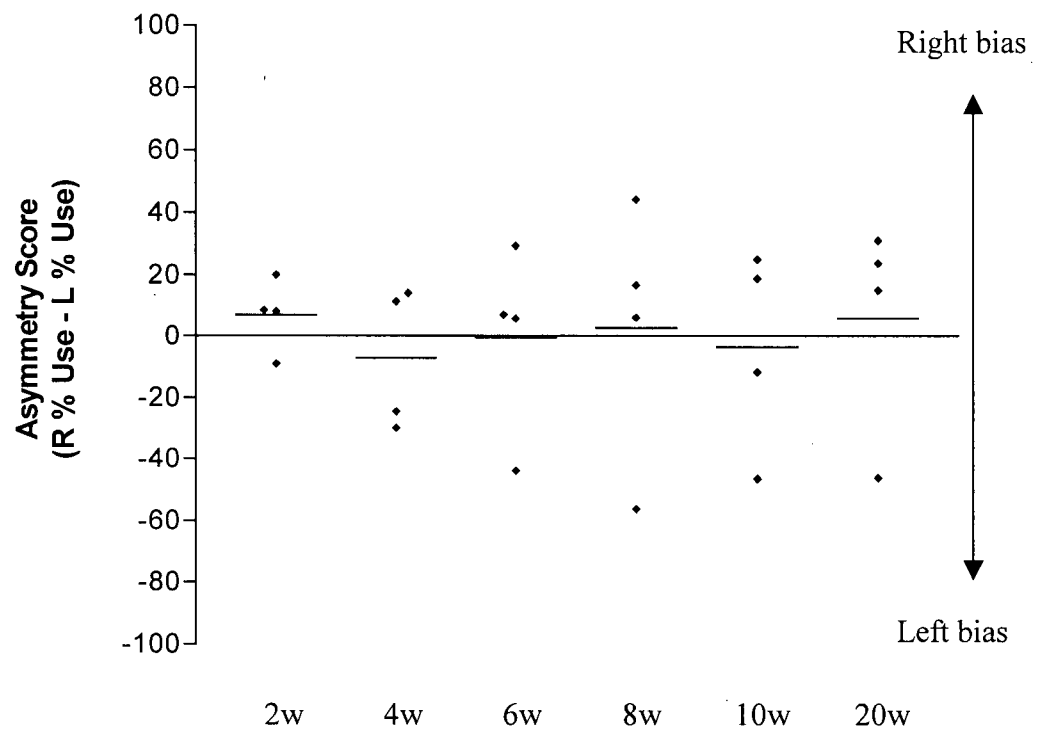


Figure 3.11 Forelimb use asymmetry scores of 6 tests on normal rats. Dots represent individual scores, and the mean score is shown for each test. The mean asymmetry scores varied between 6.92 and -7.12%, variation that did not reach statistical significance. Time period 5 months. (N=4) (48 trials).

There was no statistically significant difference between the mean forelimb use asymmetry scores (% right independent use - % left independent use) for the 6 tests performed during the five-month period ($F_{5,3} = 0.40, p = 0.84$). The mean asymmetry scores varied within 14% (range: 6.92 to -7.12%) (Fig. 3.11).

3.2.2 Tapered ledged beam-walking test

A one-way repeated measures ANOVA was performed to assess the effect of repetitive testing on the percentage of errors/steps in the three different beam sections for each hindlimb. Rats were tested 6 times during a five-month period. There were no statistically significant differences in the mean percentages errors/steps in any of the three sections of the tapered beam between tests for both hindlimbs; left hindlimb: wide section ($F_{5,3} = 1.56, p = 0.23$); medium section ($F_{5,3} = 0.79, p = 0.57$); narrow section ($F_{5,3} = 0.97, p = 0.47$); right hindlimb: wide section ($F_{5,3} = 0.60, p = 0.70$); medium section ($F_{5,3} = 1.31, p = 0.31$); narrow section ($F_{5,3} = 1.85, p = 0.16$) (Fig. 3.12).

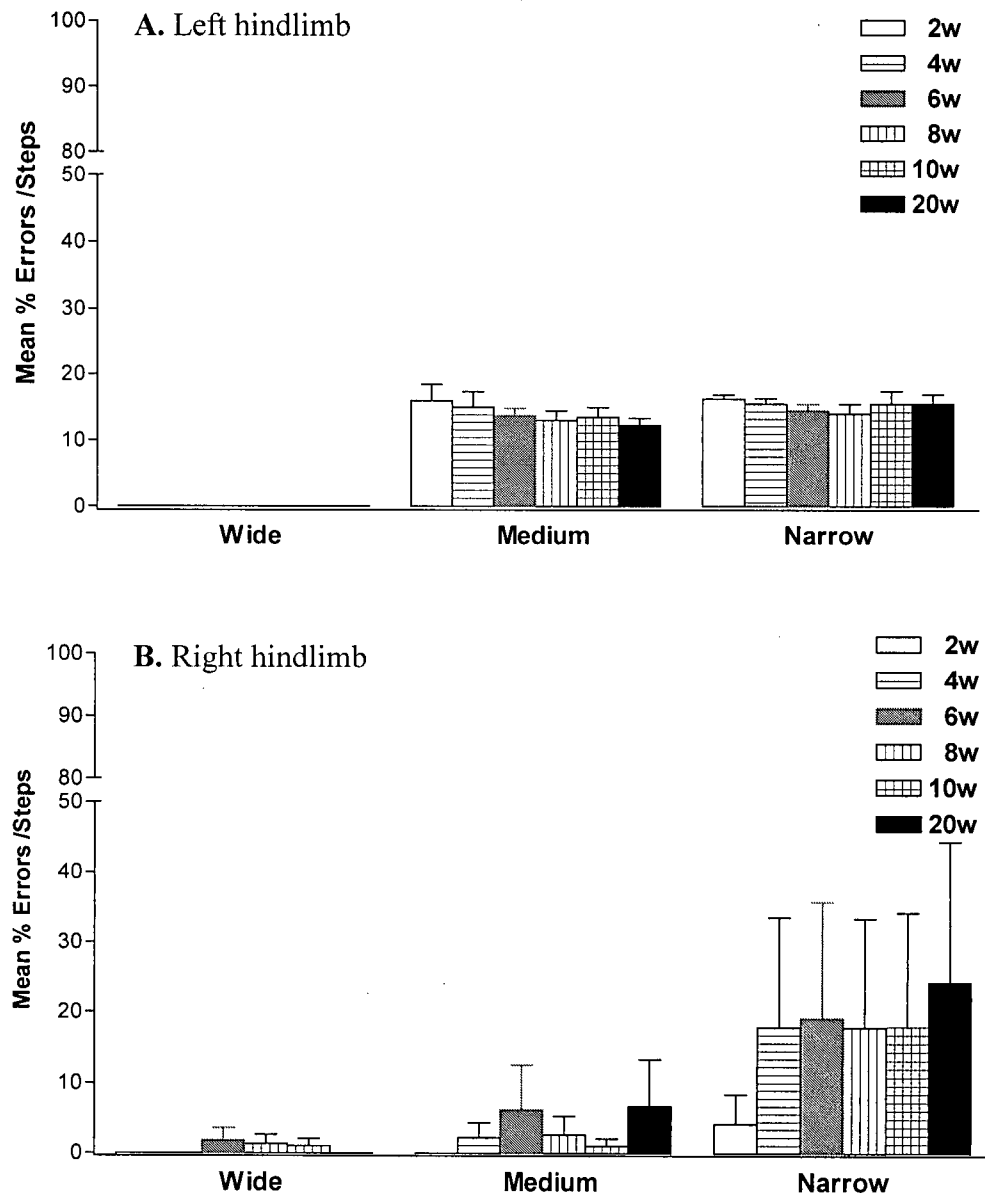


Figure 3.12 Tapered beam-walking test performed 6 times in normal rats. Bars represent % errors/steps (\pm SEM). There was no significant effect of multiple testing in any of the beam sections for both hindlimbs. A. Left hindlimb; B. Right hindlimb. (n=4) (120 trials).

3.3 Multiple Testing Effect in Rats after right MFB 6-OHDA Lesion

To evaluate the development of motor deficits and assess the variability and reproducibility of both tests, as well as the presence of a multiple exposure effect, or spontaneous recovery under lesioned conditions, Group 2 rats (unilateral 6-OHDA lesioned) (n=5) were tested in both tasks prior to lesioning and four times after lesion at weeks 2, 4, 8, and 10.

3.3.1 Forelimb use asymmetry test (cylinder test)

The results of 2 trials performed during the same week were combined to constitute a complete test. A one-way repeated measures ANOVA was performed to assess the effect of unilateral infusion of 6-OHDA on forelimb use in the cylinder. There was a significant time effect on the percentage of left ($F_{4,4}=9.96, p < 0.0001$), and right independent use ($F_{4,4}=9.96, p < 0.0001$), as well as on simultaneous limb use ($F_{4,4}=22.79, p < 0.0001$) (Fig 3.13).

Post-hoc comparisons using the Tukey's multiple comparison test indicated that the percentage of left independent forelimb use decreased significantly after contralateral lesion ($p < 0.001$) compared to the pre-lesion percentage use, but did not differ between post-lesion tests. On the other hand, the percentage of right independent forelimb use increased significantly after ipsilateral lesion ($p < 0.001$) compared to the pre-lesion percentage use, but did not differ between post-lesion tests. Finally, the percentage of simultaneous forelimb use also decreased significantly after lesion compared to pre-lesion "both" use, but did not differ between post-lesion tests (Fig. 3.13).

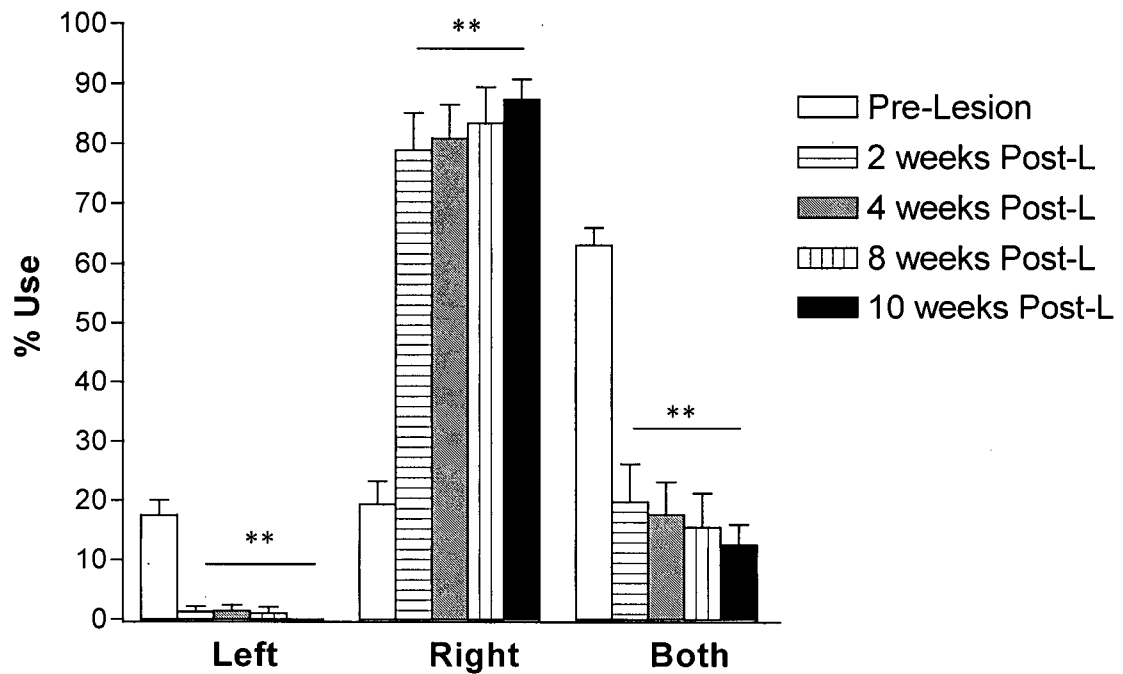


Figure 3.13 Forelimb use before and multiple times after right MFB 6-OHDA lesion.

Bar represent mean % use (\pm SEM). After lesion left forelimb independent use decreased, right forelimb independent use increased and both use decreased significantly. The effect of the lesion was evident from week 2 and slowly progressed over time up to week 10, as shown by a further decreased in both movements, although comparisons between post-lesion tests values did not reach statistical significance.

(n=5) (50 trials)

*** Significantly different from pre-lesion use ($p < 0.0001$).

A one-way repeated measures ANOVA was performed to assess the effect of the lesion on the forelimb use asymmetry score (% contralateral forelimb use - % ipsilateral forelimb use). There was a significant time effect on the mean forelimb use asymmetry scores ($F_{4,4}=75.76$, $p<0.0001$). A Tukey's multiple comparison test indicated a statistically significant decrease of the forelimb use asymmetry score between pre-lesion and all the other post-lesion time points, but not among post-lesion tests (Fig. 3.14).

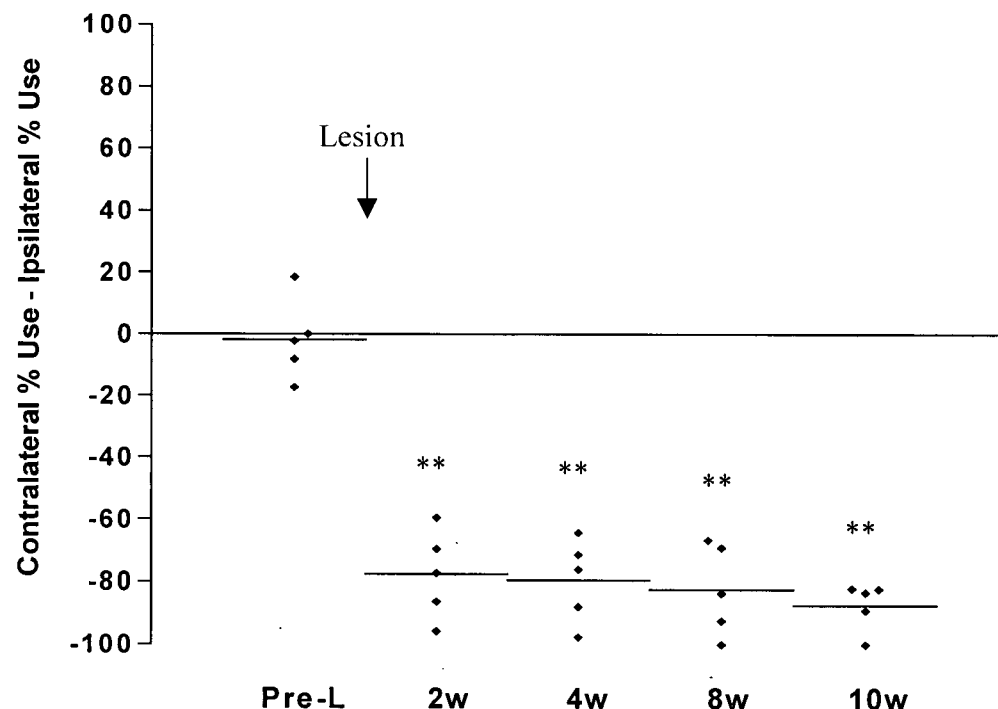


Figure 3.14 Forelimb use asymmetry score before and after right-MFB-6-OHDA lesion.

The mean forelimb use asymmetry score (% contralateral forelimb use - % ipsilateral forelimb use) decreased significantly after 2 weeks post-lesion and continued to decrease up to 10 weeks after lesion. There was no indication of spontaneous recovery at week 10 post-lesion. (N=5) (50 trials)

*** Significantly different from pre-lesion at $p<0.001$ level.

3.3.2 Tapered ledged beam-walking test

A one-way repeated measures ANOVA was performed to assess the effect of right-MFB-6-OHDA lesion on the percentage of errors/steps in the three different beam sections for each hindlimb. Additionally, the effect of repetitive testing, and the possibility of spontaneous recovery was studied by testing the rats four times after lesion at weeks 2, 4, 8, and 10.

The hindlimb contralateral to the lesion (left) (Fig. 3.15A) did not show any statistically significant differences in the mean % errors/steps in the wide section of the tapered beam between the five tests ($F_{4,4} = 1.35$, $p=0.30$). In the medium section, the same hindlimb increased the mean % errors/steps between pre- ($\bar{M} = 3.81\%$, $\underline{SD}=5.63$) and 10 weeks post-lesion ($\bar{M} = 40.57\%$, $\underline{SD}=39.16$), but this difference was borderline significant ($F_{4,4} = 2.92$, $p<0.055$). Surprisingly, at week 4, the mean % errors/steps of the lesioned hindlimb decreased in the medium section for all the rats, but this decrease was only transient, and not statistically significant. In the narrow section there was a significant time effect in the mean % errors/steps for the lesioned hindlimb ($F_{4,4} = 21.15$, $p<0.0001$). Post-hoc comparisons indicated that the % errors/steps increased significantly after contralateral lesion ($p < 0.001$) compared to the pre-lesion levels, but did not differ significantly between post-lesion tests.

The hindlimb ipsilateral to the lesion (right) (Fig. 3.15B) did not show any statistically significant differences in the mean % errors/steps in any section of the tapered beam between the 5 time points tested.

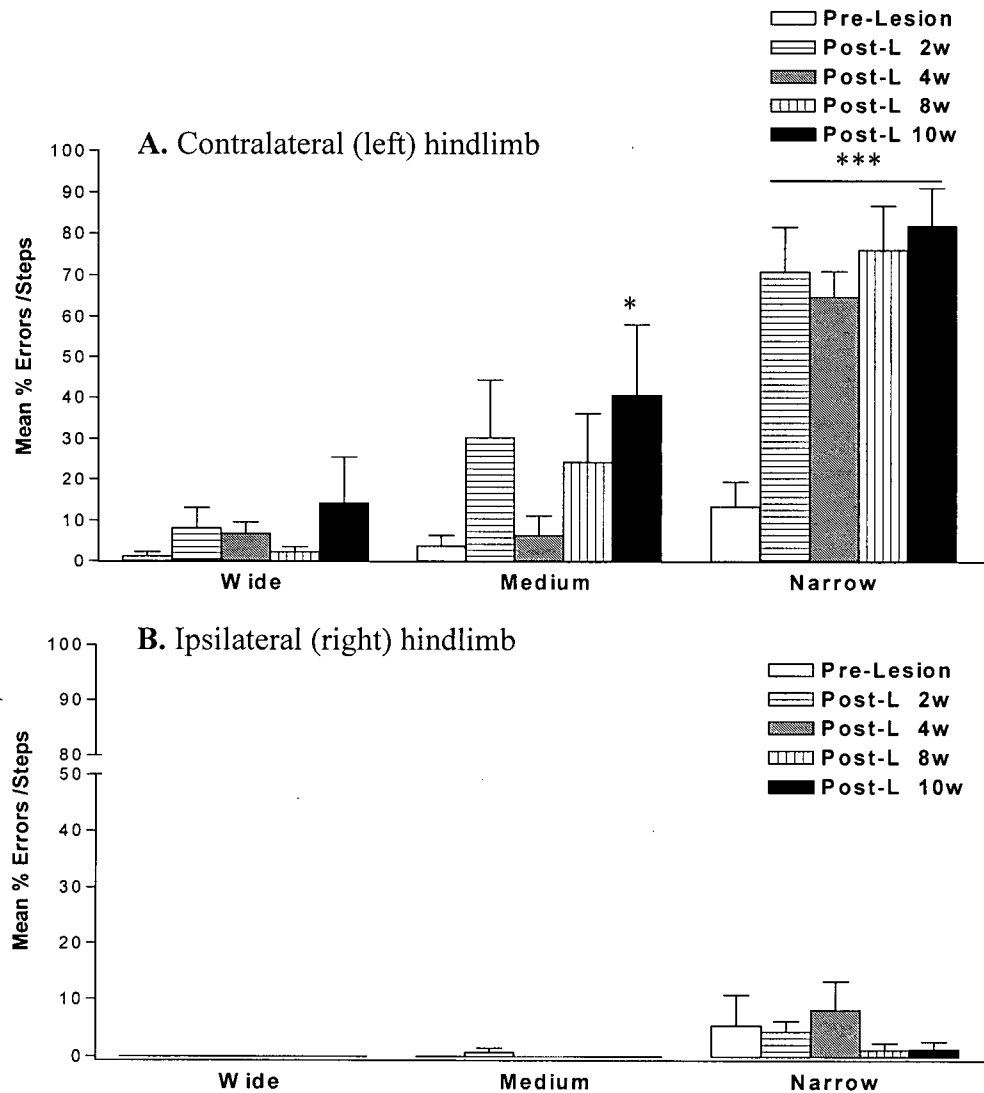


Figure 3.15 Tapered ledged beam-walking test prior to right-MFB-6-OHDA lesion and at 4 time points after lesioning. Bars represent mean %errors/steps (\pm SEM). There was an increase in the %errors /steps after lesion in the contralateral hindlimb in all beam sections A; but not in the ipsilateral hindlimb B (n=5) (125 trials).

* Borderline significantly different from pre-lesion values, $p < 0.055$.

*** Significantly different from pre-lesion values, $p < 0.0001$.

3.4 Behavioural Effects of hRPE-GM Implants in a Unilateral 6-OHDA Rat Model of PD

To assess the behavioural effects of hRPE cell implants in this model, 10 animals were lesioned along the right MFB with 6-OHDA as described before (Groups 2 and 3). Ten weeks after 6-OHDA infusion, animals were implanted with either GM alone (Group 2; n=5) or with RPE-GM (Group 3; n=5). Behavioural testing was done prior to lesion, 10 weeks after lesion (before implant) and two times after implant at weeks 2-4 and 8-10, using the forelimb use asymmetry test (cylinder test) and the TLBWT. One rat that consistently stepped on the ledge along the beam with the left hindlimb prior to lesioning was excluded from the TLBWT analysis, but it was kept in the cylinder test analysis. The 30-week (7 months) experimental timeline is presented in Figure 3.16.

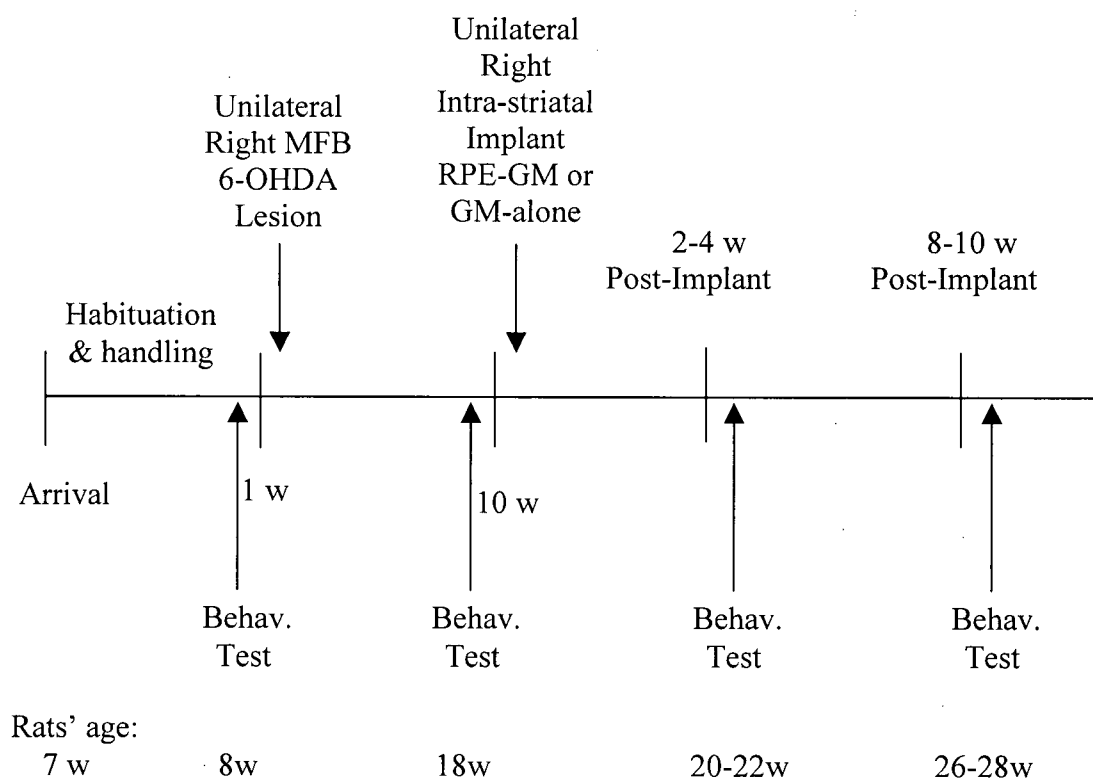


Figure 3.16 Experimental timeline for Groups 2 & 3, unilaterally lesioned, unilaterally implanted with either RPE-GM or GM-alone (n=10).

3.4.1 Forelimb use asymmetry test (cylinder test)

To increase the reliability of the test, scores of 2 trials were combined to constitute a complete test prior to lesioning, while scores of 4 trials were combined to constitute one test in the subsequent testing time points to insure that sufficient number of rearing movements were obtained in the parkinsonian rats. Rats were given a total of 14 trials over a period of 7 months.

A two-way repeated measures ANOVA was performed to assess the effect of TIME (pre-lesion, post-lesion, 2-4 weeks after implant and 8-10 weeks after implant) and TREATMENT (RPE-GM implant or GM-alone implant). There was a statistically significant effect of TIME on the % of independent use of the contralateral forelimb to the lesion/implant ($F_{3,16} = 30.28$, $p < 0.0001$), the ipsilateral ($F_{3,16} = 23.43$, $p < 0.0001$), and the simultaneous use ($F_{3,16} = 15.51$, $p < 0.0001$), in the cylinder test (Fig. 3.17). Tukey's post-hoc comparisons indicated that the % of independent use of the contralateral forelimb decreased significantly after lesion ($p < 0.001$) compared to pre-lesion, but did not differ significantly compared to post-implant time points.

On the other hand, there was a significant increase in ipsilateral forelimb use after lesion compared to pre-lesion ($p < 0.001$), but not compared to post-implant time points. Additionally, the % use of both forelimbs for weight support on the cylinder wall decreased significantly after lesion compared to before lesion ($p < 0.0001$), but not when compared to subsequent testing time points. There was no significant interaction effect

Although the effect of TREATMENT did not reach statistical significance on any of the three parameters calculated, 1). % of contralateral independent forelimb use, 2). % of ipsilateral independent forelimb use and, 3). simultaneous use of the forelimbs,

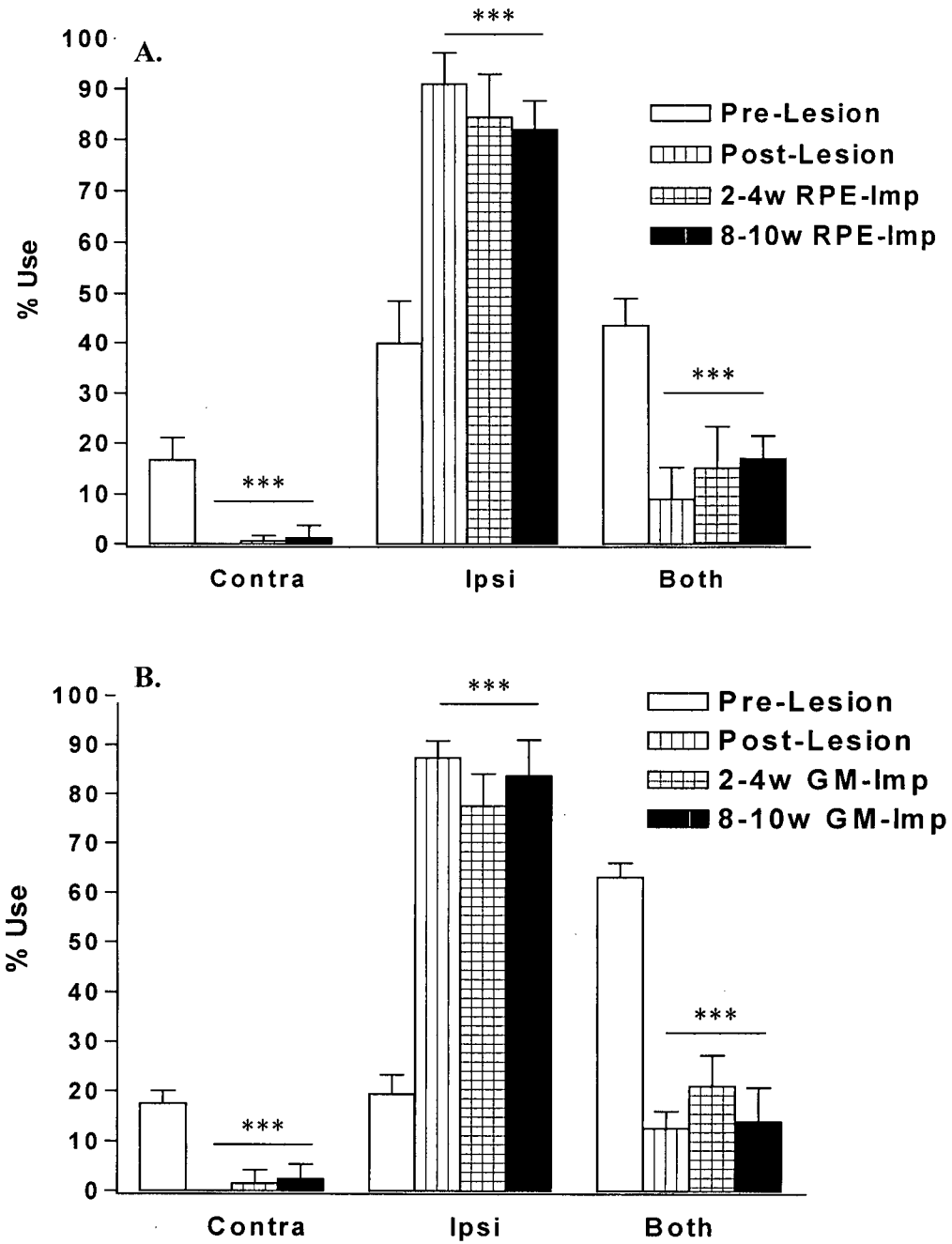


Figure 3.17 The effect of A. microcarrier-attached RPE cell implant or B. microcarrier-alone implant on forelimb use after severe unilateral 6-OHDA lesion. Bars represent % use (\pm SEM) during vertical exploration in the cylinder.

*** Significantly different from pre-lesion (n=5).

all three parameters showed a trend towards improvement over time in the RPE-GM implanted group (Fig. 3.17A), opposite to the trend in the GM-alone implanted rats (Fig. 3.17 B). The percentage of both movements in the RPE-GM implanted rats went from 9% at post-lesion to 15% 2-4 weeks post-implant, and to 17% at weeks 8-10, while in the GM-alone implanted rats, both % use increased initially from 13% to 21% 2-4 weeks after GM-alone implant, but decreased to 14% at weeks 8-10.

The trend of improvement in the RPE-GM implanted group is also suggested by a mild but progressive decrease in the forelimb use asymmetry (induced by the unilateral lesion) in this group, measured by the forelimb use asymmetry score which went from -91% at post-lesion to -80% after 8-10 weeks post-RPE-GM implant, a 9% difference that represents a 15% improvement from base line (Fig. 3.18A). GM-alone implanted rats however, went from -87% at post-lesion to -81% after 8-10 weeks post-GM-implant, a 6% difference that represents only a 3.6% improvement from baseline (Fig. 3.18B).

3.4.2 Tapered ledged beam-walking test

A two-way repeated measures ANOVA was performed to assess the effect of TIME (pre-lesion, post-lesion, 2-4 weeks after implant and 8-10 weeks after implant) and TREATMENT (RPE-GM implant or GM-alone implant) on the % of errors/steps of the contralateral hindlimb to the lesion/implant for each section of the tapered beam. There was a statistically significant main effect for TIME on the % of errors/steps on the wide ($F_{7,21} = 5.29, p < 0.008$), medium ($F_{7,21} = 6.92, p < 0.002$), and the narrow ($F_{7,21} = 19.96, p < 0.0001$) sections of the beam (Fig. 3.19). Post hoc comparisons using Tukey's multiple comparison test determined that in the wide and the narrow beam sections, the mean % errors/steps was significantly higher after lesion than pre-lesion ($p < 0.02$), but

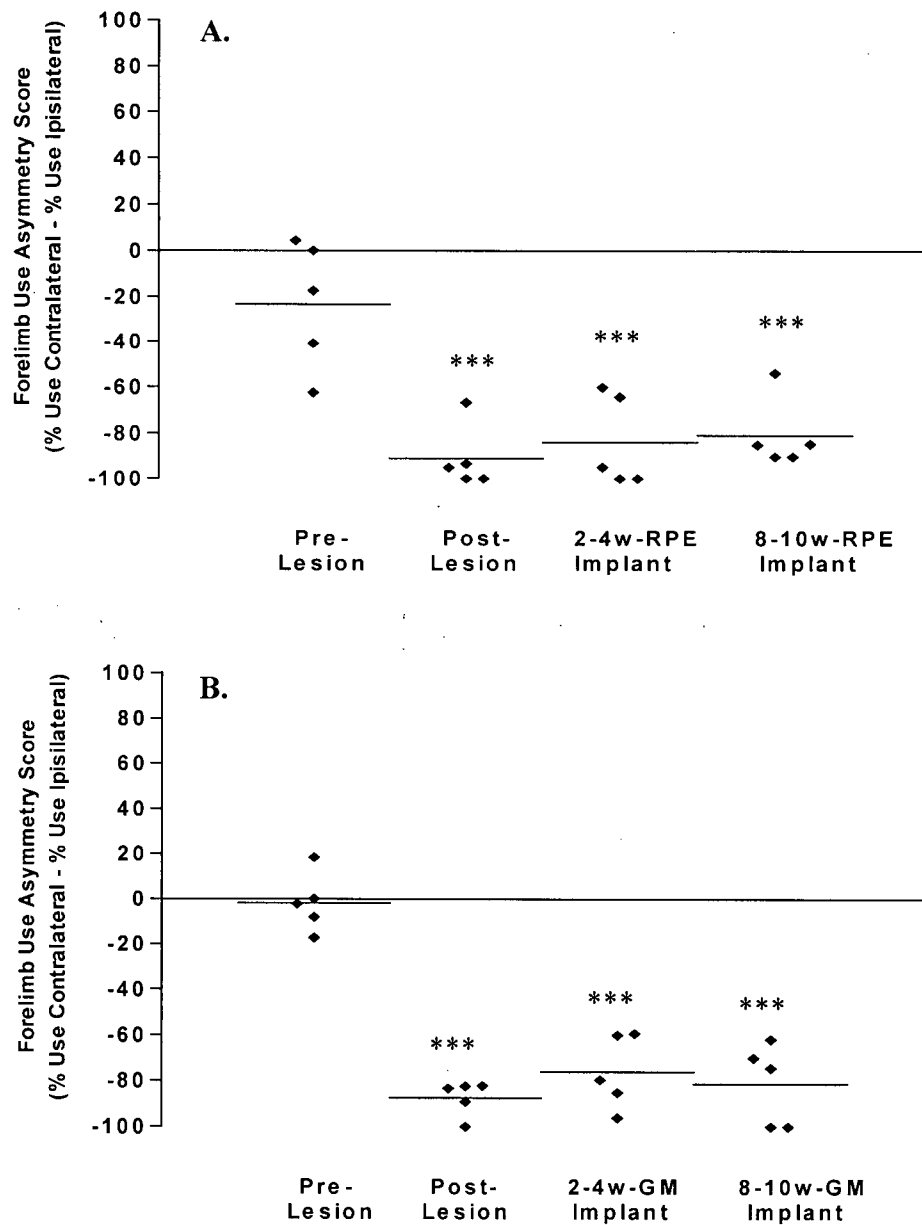


Figure 3.18 Forelimb use asymmetry scores of unilaterally-6-OHDA lesioned rats after A. RPE-GM implant or B. GM-alone implant. Dots represent individual scores and mean scores are shown. (n=5). Treatment effect did not reach statistical significance.

*** Significantly different from pre-lesion, $p < 0.0001$.

did not differ significantly between subsequent testing time points ($p>0.05$). Interestingly, in the medium section, there was no statistically significant difference in the % of errors/steps made with the contralateral hindlimb at pre-lesion compared with 8-10 weeks after implant.

Although the TREATMENT effect did not reach statistical significance for any beam section, the mean % errors/steps of the contralateral hindlimb decreased after RPE-GM implant, RPE-GM implanted rats progressively decreased the % errors/steps in the wide and the medium sections of the beam after implant (Fig. 3.19), while the GM-implanted rats did not show this trend. In both groups, there were no changes in the % errors/steps on the ipsilateral hindlimb (Fig. 3.20).

The percentage of improvement from baseline (pre-lesion) was calculated using the %errors/steps for each beam section and time points in the following formula: $\{[PostLesion - PostImplant] / [PostLesion - PreLesion]\} \times 100$ (Table 1). There was no significant INTERACTION effect.

Table 1: Improvement in the tapered ledged beam walking test in hRPE implanted (RPE-GM) and control (GM-alone) rats

Implant	Beam Section	% Improvement at	
		2-4 w	8-10 w
GM-alone	Wide	-56.17	6.16
	Medium	10.10	13.70
	Narrow	19.58	6.16
RPE-GM	Wide	22.17§	53.92§
	Medium	32.24§	57.39§
	Narrow	13.73	3.52

§ Better than GM-alone implant

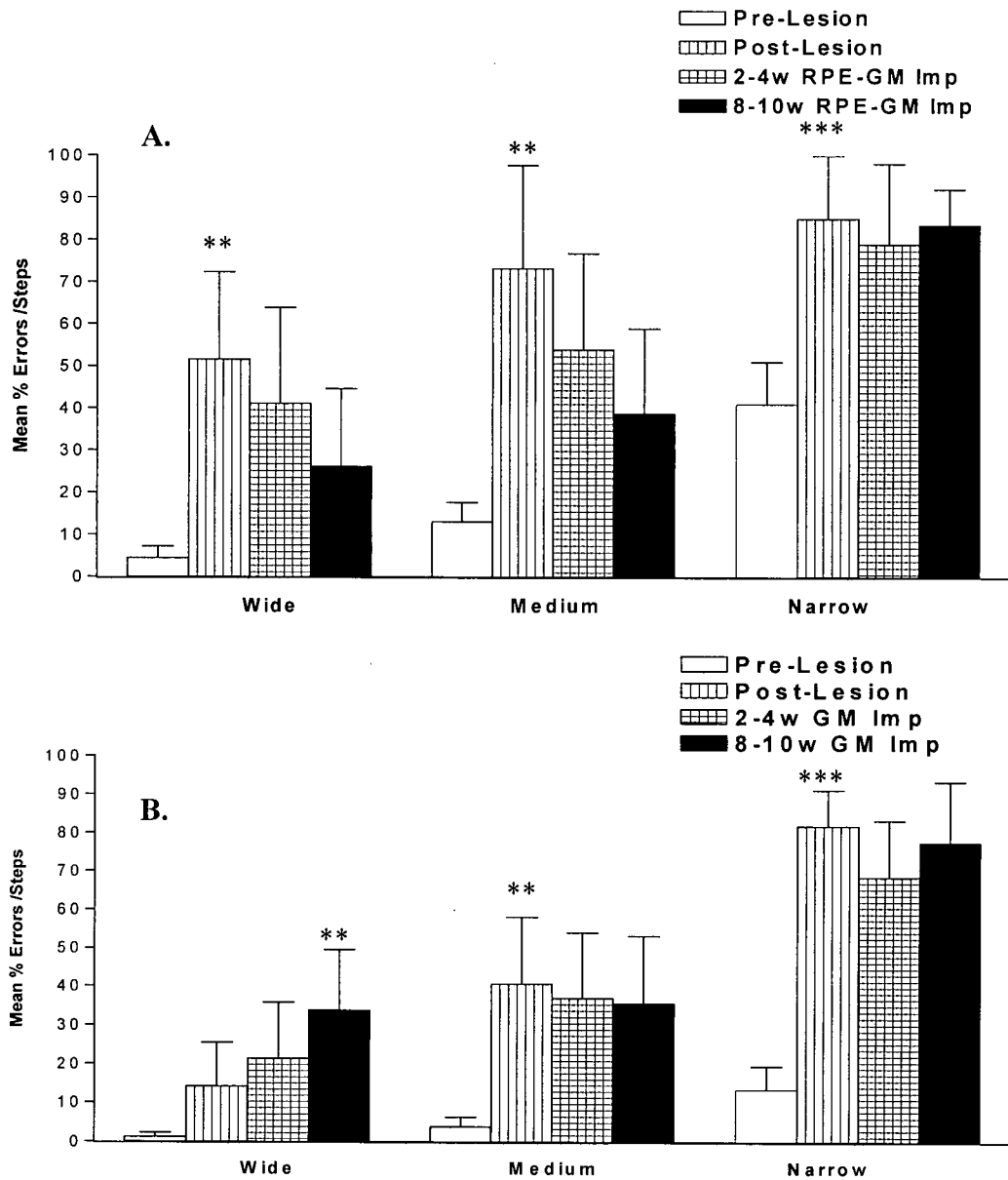


Figure 3. 19 Tapered ledged beam-walking test in the contralateral hindlimb of unilaterally-6-OHDA lesioned rats after A. RPE-GM implant or B. GM-alone implant. Bars represent mean %errors/steps (\pm SEM). After RPE-GM implanted rats decreased the % errors/steps in the wide and the narrow sections of the beam ($n=4$).

** Significantly different from the other sections of the tapered beam ($p < 0.01$).

*** Significantly different from the other sections of the tapered beam ($p < 0.001$).

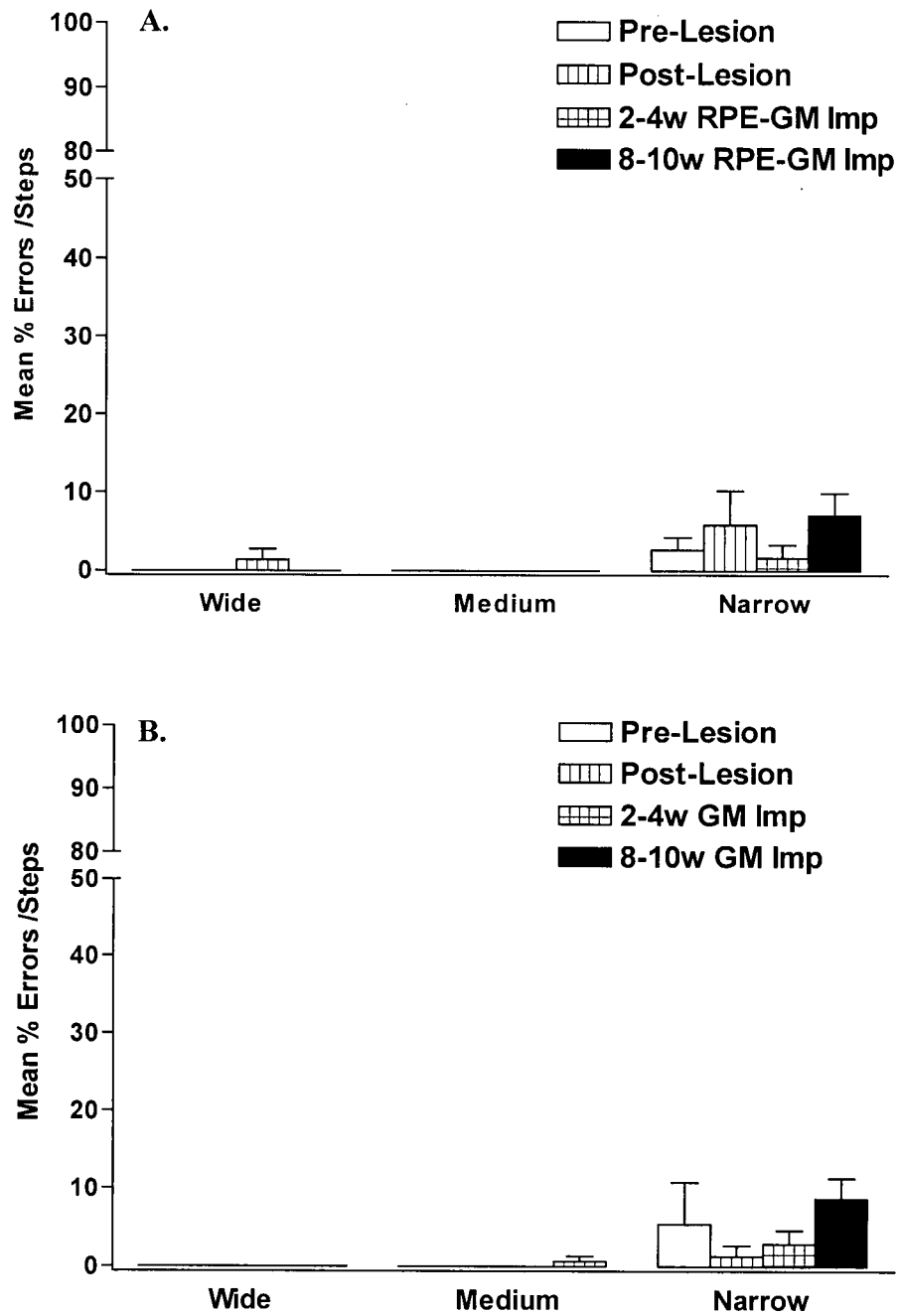


Figure 3.20 Tapered ledged beam-walking test in the ipsilateral hindlimb of unilaterally-6-OHDA lesioned rats after A. RPE-GM implant (n=4) or B. GM-alone implant (n=5). No changes in % errors/steps were seen in the ipsilateral hindlimb. Bars represent mean % errors/steps (\pm SEM).

3.5 Behavioural Effects of RPE-GM Implants in a Bilateral 6-OHDA Rat Model of PD

To assess the behavioural effects of hRPE cell implants in this model, 5 animals were bilaterally infused with 6-OHDA in each cerebral lateral ventricle as described before (Group 4). Ten weeks after lesion, animals were implanted with RPE-GM in the striatum contralateral to the most affected side of the body. Behavioural testing was done prior to lesion, 10 weeks after lesion (before implant) and two times after implant at weeks 2-4 and 8-10, using the forelimb use asymmetry test (cylinder test) and the TLBWT. The 30-week (7 months) experimental timeline is presented in Figure 3.21.

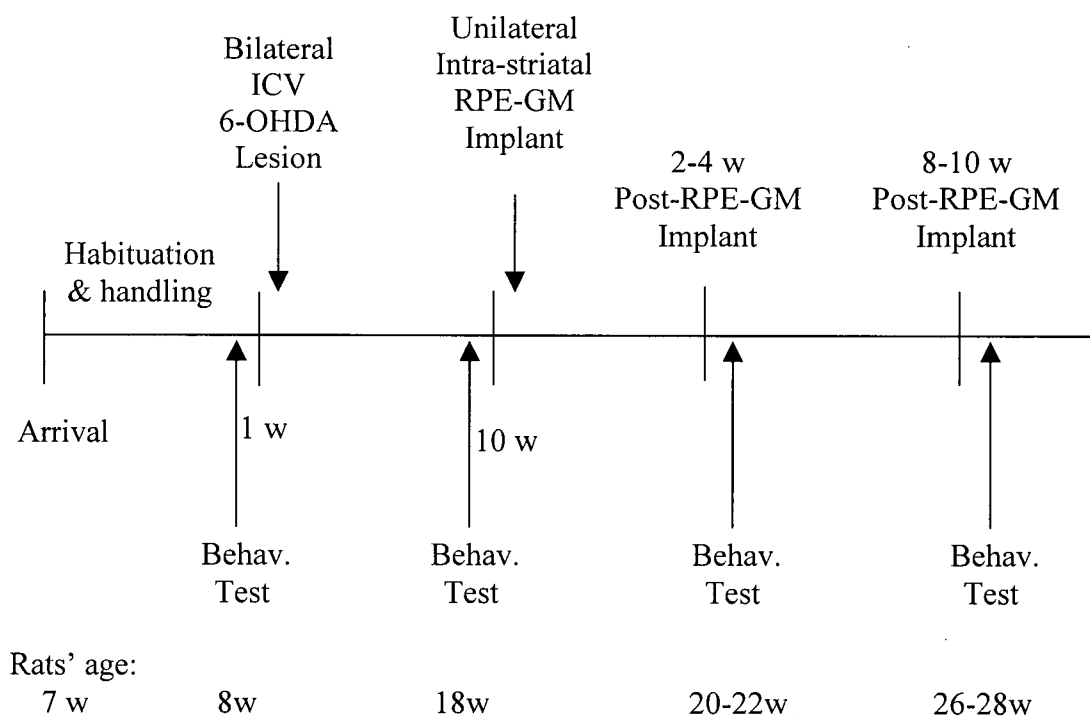


Figure 3.21 Experimental timeline for Group 4, bilaterally lesioned, unilaterally RPE-GM implanted (n = 5).

3.5.1 Forelimb use asymmetry test (cylinder test)

To increase the reliability of the test, scores of 2 trials were combined to constitute a complete test prior to lesioning, while scores of 4 trials were combined to constitute one test in the subsequent testing time points to insure that a sufficient number of rearing movements were obtained in the parkinsonian rats. Rats were given a total of 14 trials over a period of 7 months.

A one-way repeated measures ANOVA revealed that there was a statistically significant effect of TIME on the % of independent use of the contralateral forelimb to the RPE-GM implanted striatum ($F_{2,4} = 6.50, p < 0.03$), as well as on the % of independent use of the ipsilateral forelimb, ($F_{2,4} = 0.93, p < 0.05$), but not on the simultaneous % use ($F_{2,4} = 4.85, p > 0.05$) in the cylinder test (Fig. 3.22). Tukey's post-hoc comparisons indicated that the % of independent use of the contralateral forelimb to the RPE-GM implant increased significantly 8-10 weeks after implant ($p < 0.05$) compared to post-lesion (pre-implant), but not significantly at weeks 2-4 post-implant. On the other hand, there was a significant decrease in ipsilateral forelimb use 8-10 weeks after implant compared to post-lesion ($p < 0.05$), but not at weeks 2-4.

Similarly, a one-way repeated measures ANOVA revealed that there was a statistically significant effect of TIME on the forelimb use asymmetry score ($F_{2,4} = 9.50, p < 0.008$) (Fig. 3.23). Tukey's post-hoc comparisons test indicated that the forelimb use asymmetry score shifted significantly in favour of the forelimb contralateral to the RPE-GM implant for the first time 8-10 weeks after implant ($p < 0.01$) compared to post-lesion (pre-implant).

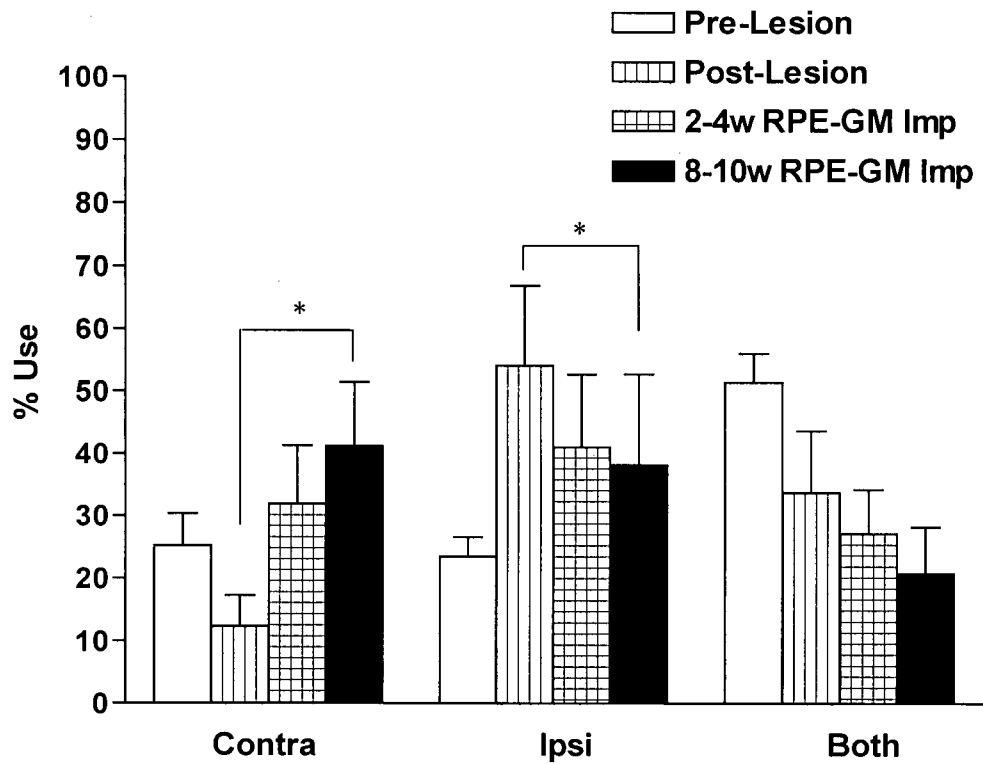


Figure 3.22 The effect of microcarrier-attached RPE cell implant on forelimb use after moderate (60-50% DA striatal depletion) bilateral ICV 6-OHDA lesion. Bars represent % use (\pm SEM) during vertical exploration in the cylinder. After RPE-GM implant, the % use of the forelimb contralateral to the implanted striatum increased, and the % use of the ipsilateral forelimb decreased. What about both? Looks like it decreases to me.

* Significantly different from post-lesion ($n=5$).

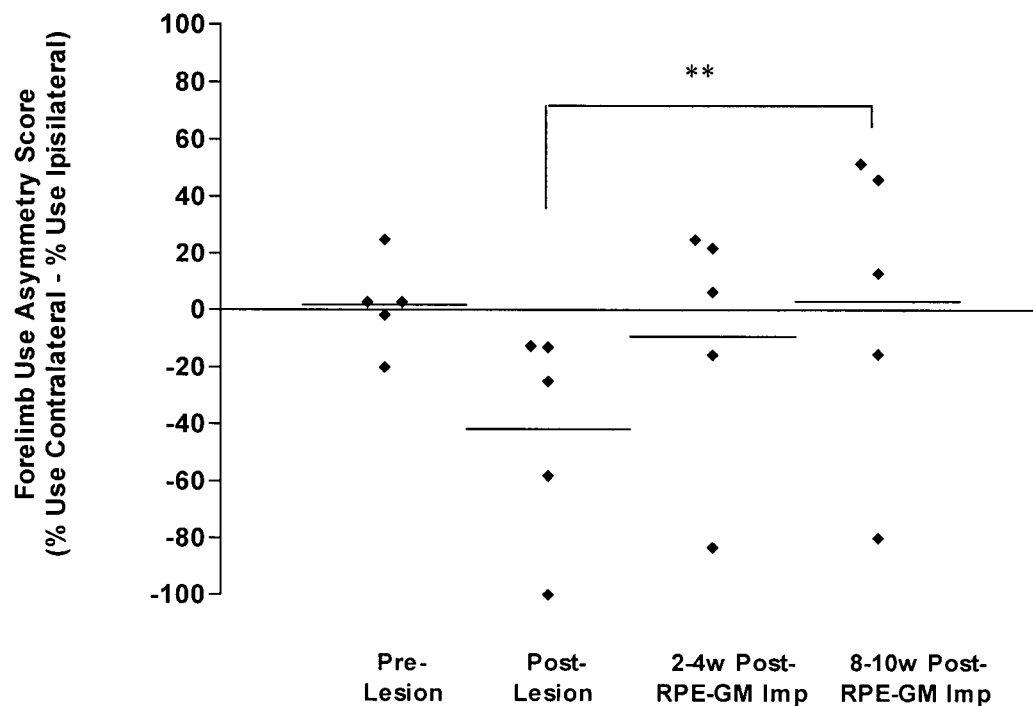


Figure 3.23 The effect of microcarrier-attached RPE cell implant on the forelimb use asymmetry score after moderate (60-50% DA striatal depletion) bilateral ICV 6-OHDA lesion. Dots represent individual forelimb use asymmetry scores. The corresponding mean score for each time point is shown. After RPE-GM implant, the asymmetry score shifted in favour of the forelimb contralateral to the implanted striatum.

** Significantly different from post-lesion at $p < 0.01$ level (n=5).

3.5.2 Tapered ledged beam-walking test

A one-way repeated measures ANOVA was performed to assess the effect of TIME on the % errors/steps of the hindlimb contralateral and the ipsilateral to the RPE-GM implant for each section of the tapered beam. There was no statistically significant main effect for TIME (pre-lesion, post-lesion, 2-4 w and 8-10 w post-implant) on the % of errors/steps on the wide, medium, and the narrow sections of the beam in both the contralateral and the ipsilateral hindlimbs (Fig. 3.24A).

Although after RPE-GM implant there were no statistical significant differences for any beam section, the mean % errors/steps of the contralateral hindlimb to the implanted striatum decreased in the medium and narrow sections, while the % errors/steps of the ipsilateral hindlimb did not change (Fig. 3.24B).

The percentage of improvement from baseline (pre-lesion) was calculated using the % errors/steps for each beam section and time points in the following formula: $\{[PostLesion - PostImplant] / [PostLesion - PreLesion]\} \times 100$ (Table 2).

Table 2: Improvement in the tapered ledged beam-walking test of bilaterally ICV 6-OHDA lesioned rats after unilateral RPE-GM implant

Hindlimb	Beam Section	% Improvement at	
		2-4 w	8-10 w
Contralateral	Wide	67.62	42.22
	Medium	26.42§	70.17§
	Narrow	42.67§	42.33§
Ipsilateral	Wide	100	66.64
	Medium	0.8	25
	Narrow	15.58	6.94

§ Better than Ipsilateral

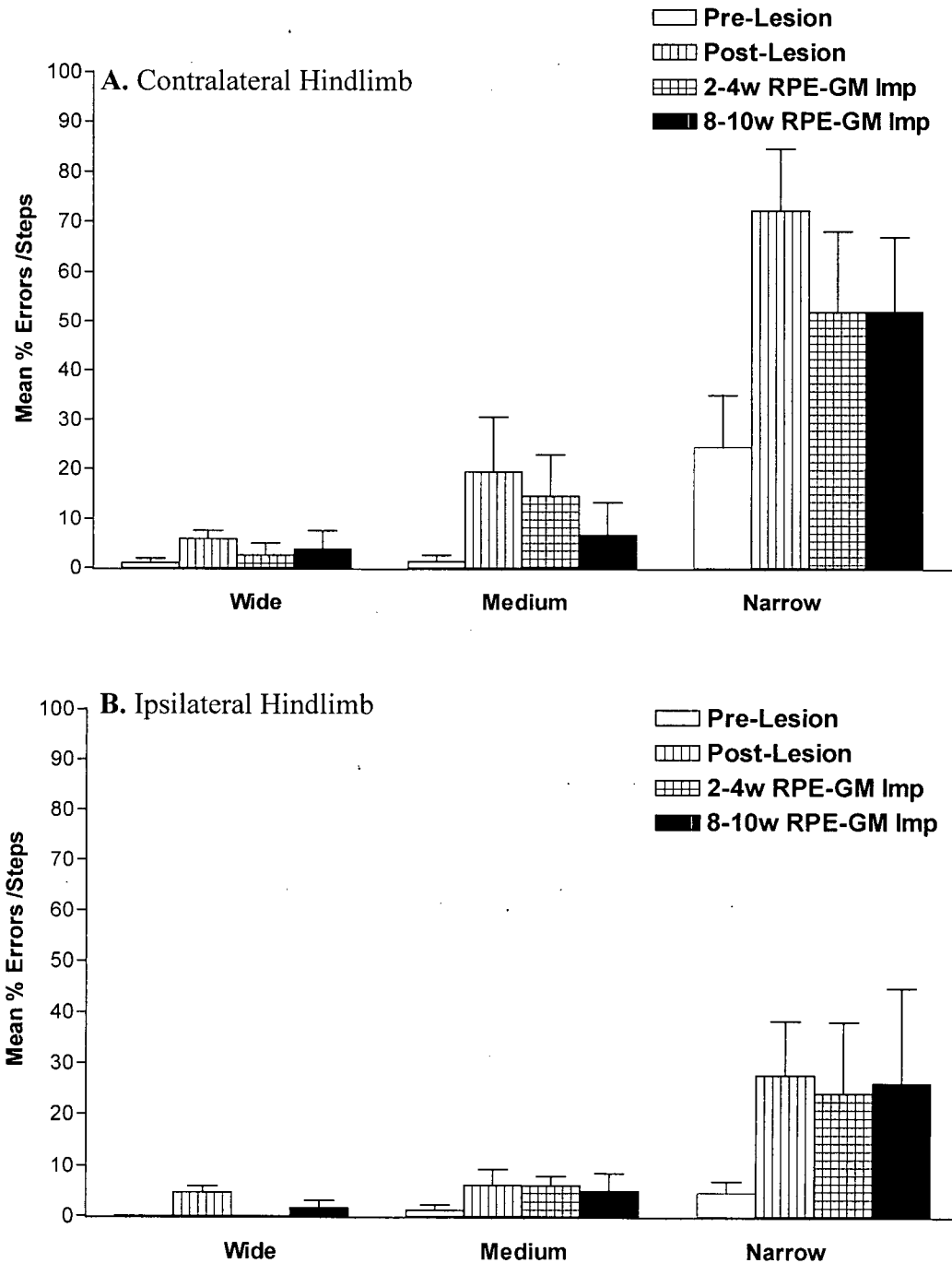


Figure 3.24 Tapered ledged beam-walking test in bilaterally ICV 6-OHDA lesioned rats after unilateral RPE-GM implant. A. Hindlimb contralateral and B. Ipsilateral to the implant. Bars represent mean %errors/steps (\pm SEM). After RPE-GM rats decreased the % errors/steps with the hindlimb contralateral to the implant (n=5).

3.6 Postmortem-Analysis

In unilaterally-lesioned animals, lesion severity was > 97%, whereas in bilaterally-lesioned animals, binding to the dopamine transporter was reduced 60-50% bilaterally in the dorsal striatum, following a medial-lateral gradient (fig. 3.25).

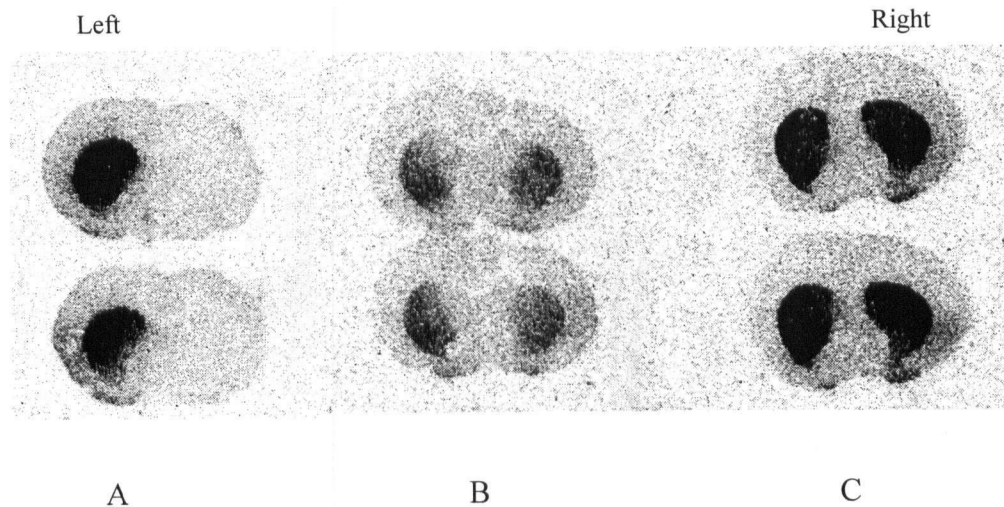


Fig. 3.25 [^3H]WIN 35,428 binding to the dopamine transporter in the striatum of unilateral (A) and bilateral (B) 6-OHDA-lesioned rats, compared to vehicle-infused controls (C).

CHAPTER IV Discussion and Conclusion

RPE cells appear to be a suitable cell therapy option for transplantation in patients with PD for several reasons. RPE cells produce the DA precursor L-dopa, as part of the metabolic pathway leading to melanin synthesis (Pawelek and Korner, 1982b; Boulton, 1998). RPE cells are readily isolated from eyes obtained from eye banks, and can be cultured and expanded in quantities sufficient to allow implant in multiple patients from a single donor. Furthermore, microcarrier-attached RPE cells implanted into the striatum, have shown to cause long-term amelioration of parkinsonian motor deficits in human (Watts et al., 2003; Bakay et al., 2004a) and non-human primates (Subramanian, 2001; Doudet et al., 2004b) without adverse effects. This evidence highlights the advantages of RPE cell implants over other cell options for transplantation as a promising therapy for patients with PD and makes it deserving of further study.

The 6-OHDA rat model of PD has been studied extensively since first developed in the late 1960's (Ungerstedt, 1968b; Ungerstedt, 1971; Schallert et al., 1978a; Schallert et al., 1979a; Lee et al., 1996a; Zigmond and Keefe, 1998), and is currently considered an effective and inexpensive model that complements studies in primates with high clinical validity (Cenci et al., 2002a). Striatal implantation of RPE-GM have shown to reduce apomorphine-induced turning in a unilateral 6-OHDA rat model of PD provoking a minimal host immune response (Subramanian et al., 2002) however, their effects on spontaneous motor function have not been studied in this model.

4. 1 Why Our Choice of Behavioural Tests?

The purpose of this study was to develop and validate behavioural tests to assess the effects of RPE-GM implants on motor deficits in both a unilateral and a bilateral 6-OHDA rat

model of PD. Our aim was to find simple tests which did not require pharmacological agents, and which were relatively insensitive to the effects of learning and motivation. In the future we plan to investigate the mechanism of action of the RPE cells implants in the host striatum.

Pharmacological agents such as the nonselective DA D1/D2 receptor agonist apomorphine or the DA transporter ligand amphetamine, extensively used in the evaluation of the efficacy of potential antiparkinsonian therapies, may affect our capability to study the actual interactions of the RPE cells with the host striatum. Both classes of drugs are well known to induce their own intrinsic responses, such as sensitization, stimulation of trophic factors, and alterations of the DA receptors, even after a single dose (Kashihara et al., 2002; Battaglia et al., 2002). To a lesser extent, but also through adaptative molecular strategies between the striatum and limbic structures, learning and motivation also may influence the performance of behavioural responses (Groenewegen et al., 1996; Mulder et al., 2004) and make longitudinal studies more difficult to interpret and complicate the analysis of the implant effects on motor performance. Thus, we chose two tests of spontaneous motor function that rely on normal rodent behaviours. One test, the cylinder test, allowed us to test the function of the forelimbs and the second test, the TBLWT, allowed us to study the function of the hindlimbs.

4.2. Validation of the Tests in Normal Animals and in Longitudinal Studies

It has been previously found that rats show forelimb preferences at the individual level (Whishaw, 1992a), and it has been proposed that this lateralization is related in part, to asymmetries of the nigrostriatal dopamine system, not only in the rat (Zimmerberg et al., 1974) but also in humans (de la Fuente-Fernandez et al., 2000). Extensive research conducted by Glick et al., (1979; 1980; 1981; 1981a) has established that normal rats have functional and/or biochemical asymmetries in several brain regions. These studies have also shown that

asymmetry in nigrostriatal function; characterized by hemispheric differences in striatal DA content, metabolism and receptor activity, has been related to spontaneous side preferences and nocturnal and drug-induced circling behaviour. Although the nigrostriatal asymmetry was consistent in direction within individual rats, no population bias has been evident (Glick and Ross, 1981b). Other studies, using big samples of animals have also suggested that there is no dominant population left or right bias in rats (Whishaw, 1992b). Similar to the results obtained from the relatively small number of Sprague-Dawley rats in this study, Whishaw et al., (1986) reported almost equal numbers of left, right and ambidextrous rats in a population of the same strain.

Asymmetries in the nigrostriatal system and hemispheric dominance have also been shown to influence susceptibility to DA neurotoxins and recovery after neuronal transplants in a 6-OHDA rat model of PD. Interestingly, left/right nigrostriatal asymmetry in susceptibility to neurotoxic DA depletion with 6-OHDA has been reported, with the right nigrostriatal pathway showing greater vulnerability, probably associated with increased DA turnover in the right striatum (Sullivan and Szechtman, 1994). Additionally, DA-neuronal-grafted rats recovered differently depending on hemispheric dominance prior to lesion, with the rats that did not exhibit a strong lateralization for forelimb use before lesion showing the greatest recovery (Nikkhah et al., 2001). Performing only right MFB 6-OHDA infusions for the unilaterally lesioned animals to obtain consistent lesions and using Sprague-Dawley rats, which as mentioned above have almost equal numbers of left, right and ambidextrous rats in a population, reduced the influence of these two factors in this study.

Surprisingly, no reports have been made on the stability of individual forelimb preferences over time. In this study, we found that normal rats tested 12 times with the forelimb

use asymmetry test over a period of five months showed strikingly stable forelimb preferences. Furthermore, these preferences were found to be highly correlated with hindlimb function measured with the TLBWT. That is, rats that favoured one particular forelimb for weight support on the cylinder wall, showed a consistent tendency to keep the ipsilateral hindlimb on the beam, while making more foot faults (stepping on the ledge) with the contralateral hindlimb. This is the first study to show a correlation between forelimb and hindlimb function lateralization in the rat. Forelimb and hindlimb lateralization in rats resemble the upper and lower limb lateralization in humans. This similarity further validates the use of a rat model to study neurological diseases that are predominantly asymmetrical in their clinical manifestations like PD, and opens a window of possibilities to improve our understanding in the nature and implications of these asymmetries.

Curiously, the percentage of simultaneous forelimb use in the cylinder decreased significantly with time in normal rats. This finding may be explained by considering the growth of the animals relative to the fixed size of the cylinder. When the rat size is no longer small relative to the cylinder, after rearing up, it is easier for the animal to go from one side of the cylinder wall to the other, using only one forelimb at a time for weight support. In contrast, when the cylinder is still big relative to a young smaller rat, opposite sides of the cylinder wall are relatively distant. At the beginning of the experiment, the mean weight for this group of normal rats was 331 g, (\pm SD=4.62), while at week 20 was 590 g (\pm SD=16.68). This minor source of error of the test can be overcome simply by using at least two cylinders of increasing size to keep the cylinder/rat size ratio constant in long-term studies. Despite the relative decrease in simultaneous use of the forelimbs in the cylinder test, the forelimb use asymmetry score remained remarkably stable.

It has been previously shown that a beam-walking task using multiple beams with variable widths in random order, almost completely eliminated practice effects (Drucker-Colin and Garcia-Hernandez, 1991b), however data on repetitive testing in normal rats using the tapered ledged beam to address the possible effects of learning have not yet been reported. For that reason, the effects of repeated testing of normal rats on the TLBWT was addressed in this study. Rats did not significantly change the percentage of errors/steps in any beam section. Normal rats did not “learn” to make fewer errors over time, even though they traversed the beam 30 times in five months. This group of normal rats were not exposed to the beam before the first scored test to assess changes in performance from the very first exposure. Unexpectedly, rats made slightly less errors on the first test (difference that did not reach statistical significance), but after the first beam exposure, the errors/step ratio remained stable. According to these results, giving ten trials before performing the first recorded test may ensure that results will be stable thereafter, unless an experimental intervention is made. These results support the idea that the TLBWT evaluates a spontaneous motor behaviour that is stable and not subject to changes inflicted by repeated testing or weight gain over time. To correct for variability of the test, the scores of 5 trials were not averaged, since the number of steps varied from trial to trial within the same section. Instead, the total ratio of errors/step was calculated for the 5 trials to constitute one complete test.

Not only under normal conditions, but also, after 6-OHDA infusion, scores obtained from both the forelimb use asymmetry test and the TLBWT, were stable even after 10 weeks of multiple testing, and no signs of recovery were seen in either test. Documenting a stable lesion is very important when studying a new treatment, ensuring that any change seen after RPE-GM implant is not an artifact of spontaneous recovery.

These pilot studies were performed to test the sensitivity of the chosen tests to small improvements in motor function. Indeed, many behavioural tests are able to detect large impairments in motor activity as induced by a 6-OHDA lesion. However, few tests are consistently able to demonstrate improvements in spontaneous motor function, especially discrete changes and in a small number of animals.

4.3 Pilot Studies of the Effects of hRPE Implants

Rats implanted with RPE-GM showed improvement in motor function as measured by validated outcome measures such as the forelimb use asymmetry test and the TLBWT. Improvement in forelimb motor function deficits, measured with the forelimb use asymmetry test, was mild to moderate and more importantly, showed a progressively increasing trend. Slow, progressive improvements over the course of 6-12 months have been reported after RPE implants both in human subjects and in non-human primates (Doudet et al., unpublished observations). Average improvement at 8-10 weeks post RPE-GM was 15% while GM-alone implanted rats improved only 3.6%.

Improvement in hindlimb motor function deficits, measured with the TLBWT, also showed a progressively increasing trend. Average improvement at 8-10 weeks post RPE-GM was 53.92% in a task with relatively low level of difficulty (wide beam section), and 57.39% in a task with medium level of difficulty (medium beam section), while for GM-alone implanted rats the improvement was only 6.16% and 13.70% respectively. At 8-10 weeks post-implant, neither of the two groups showed improvement in the part of the task with the highest level of difficulty (narrow beam section), which provides a difficult challenge, even to normal rats. These results are consistent with growing evidence that parkinsonian motor deficits improve in response to human RPE cell implants, but that in subjects with severe degeneration, the improvement

afforded never reaches 100%, and full return to pre-clinical levels of activity without adjunctive therapy has not been reported. Indeed, the RPE implanted patients remain under L-dopa pharmacotherapy (Watts et al., 2003), and the moderate to severely lesioned MPTP-treated monkeys maintain some degree of impairment (Doudet et al., 2004a).

Motor function improvement after severe (>97% striatal DA depletion) unilateral 6-OHDA lesion in the RPE-GM implanted group is unlikely to be explained by the variability of the tests or induced by multiple test exposure. Forelimb use measured with the cylinder test proved to be consistent when testing normal rats multiple times. The group of normal rats tested 12 times over a period of five months, showed an 11% range variability in the forelimb use asymmetry score when 2 trials performed in the same week were combined to constitute one complete test. Variability decreased to half that value when 4 trials, performed within two consecutive weeks (2 trials per week) were pooled to constitute a complete test. To increase reliability and sensitivity of the test, rats implanted with RPE-GM or GM-alone were administered 4 trials of the cylinder test to constitute one complete test at all time points. This number of trials per test was also necessary to ensure that lesioned rats, which have some degree of hypokinesia and are therefore less active than normal animals of the same age and weight range, could have the opportunity to make more movements and thus obtain a relatively constant number of movements per complete test between normal and implanted animals.

Furthermore, a group of rats tested before and 8 times after unilateral 6-OHDA lesion over a period of 10 weeks showed no bi-directional variability. Instead, these rats had a progressive worsening of the contralateral forelimb function, declining 10% from week 2 to week 10 post-lesion. This consistent trend supports the idea that spontaneous recovery did not play a significant role in the recovery displayed by RPE-GM implanted rats.

Similarly, in the TLBWT, multiple exposure effects or score variability do not seem to play a role in the interpretation of the improvement showed by the RPE-GM implants. Bilaterally lesioned rats showed greater improvement on forelimb and hindlimb function contralateral to the RPE-GM implant than unilaterally lesioned animals. This is most likely explained by the difference in the severity of the lesion. While unilateral lesioned rats had a striatal DA depletion of >97%, bilaterally lesioned rats had only 50 to 60% striatal DA terminal loss. Since RPE-GM implant effects rely on the remaining nigrostriatal terminals to have a beneficial effect, it was expected that animals with less severe lesions would show greater benefits. These results support that the mechanism of action proposed recently by Doudet et al., (2004d) is mainly enhanced endogenous DA release, mediated by increased availability of the DA precursor, L-dopa, secreted by hRPE cells and converted to DA by the remaining nigrostriatal DA neurons.

Preliminary data on the behavioural performance of the rats with unilateral lesion and unilateral RPE-GM implant at 5 months post implant in the cylinder test and the TLBWT showed that the trend of improvement continues at week 18-20, with further increase of simultaneous forelimb use, decrease of independent ipsilateral forelimb use (decrease in the asymmetry score) and finally a very discrete increase in independent use of the forelimb contralateral to the implant (Appendix V.A). Similarly, the trend of improvement continues at week 18-20 on the TLBWT, as shown by further decrease of % errors/steps in all beam sections (including the narrow) of the hindlimb contralateral to the implant (Appendix V.B). However, testing and analysis of the GM-alone implanted rats have not been yet completed and as such, this later data point was not incorporated into this report. In all the RPE-GM implanted rats, improvement in the TLBWT progressed from the less challenging part of the test (wide section)

to the most challenging (narrow section) and was evident from the first testing time point at 2-4 weeks suggesting a rapid beneficial implant effect.

Two important questions need to be addressed: (1) how severe was the lesion caused to the unilaterally and bilaterally lesioned animals? and (2) did the hRPE cells implanted survived into the host brain, since no immunosuppression was used?

Post-mortem analysis of two groups of rats (normal, n=4; and rats with unilateral 6-OHDA lesion and implanted with GM alone, n=5) are not included because these animals are still alive and being tested as part of a continuing behavioural study. Post-mortem quantification of the 6-OHDA lesion using DA transporter ($[^3\text{H}]\text{WIN 35,428}$) binding in bilaterally lesioned rats showed 50-60% striatal DA terminal loss, while 2 of the 5 unilaterally lesioned and implanted rats showed 97 and 98% striatal DA terminal loss. The remaining 3 rats in this group, were used in a parallel study to assess the long-term viability of RPE cell implants by developing appropriate biochemical techniques by Flores et al., (2004). Using qualitative immunofluorescence (IF) analysis by confocal microscopy and electron microscopy, striatal tissue from implanted rats 5 months after implants were examined. Two primary antibodies were used for the IF staining analysis: Nuclear Mitotic Apparatus Protein (NuMA), a nuclear matrix protein that is reactive to human, but not to mouse or rat, and EMMPRIM, an epithelial specific antibody reactive to cell membrane of normal epithelium. Intrastratial human RPE cells were successfully identified for the first time with both antibodies and electron microscopy in the host rat brains after 5 months, with no signs of activated microglia or chronic inflammation.

Together, these results provide some of the first evidence for the efficacy of implantation of cultured human RPE cells as a promising therapeutic option for many of the 6.3 million patients suffering from PD all over the world. These data were, however, obtained in a small

group of animals as part of a pilot study. To further validate the findings, a number of studies need to be performed in the near future.

4.4 Limitations and Future Directions

The lack of a significant effect in the unilaterally lesioned and unilaterally implanted rats raises some doubts as to the efficacy of the hRPE implants. However, this finding has to be interpreted in the light of the lesion itself and mode of action of the implant: the unilaterally 6-OHDA lesioned rats had more than 97% loss of striatal DA terminals; the hypothesized mechanism of action of the hRPE cells is through local release of L-dopa, the amino acid precursor of DA which has to be taken into the DA terminals and decarboxylated into DA to produce a beneficial effect on motor function. The loss of >97% of DA terminals in the unilaterally lesioned rats may be responsible for the apparently negligible effect of the hRPE implants in this model. Indeed, PD patients and MPTP-treated monkeys in which the hRPE implants produced significant motor improvement were not in the last stage of the disease and were only moderately to severely impaired (stage III to IV of Hoehn and Yahr).

On the contrary, in support of our assumption, a significant improvement was seen in the two behavioural tests in the mild to moderate bilaterally lesioned rats (50-60% loss of DA terminals). The use of a relatively small sample of animals in this pilot study limited the study's statistical power. However, it is remarkable that even in this small sample, the repeated measure analysis yielded significant results. Unfortunately, in this pilot study, we did not have an adequate control group (i.e. bilaterally lesioned animals with unilateral GM-alone implant) because our initial experiments with bilaterally lesioned rats led to large mortality and we elected to use the survivors mainly for the hRPE-GM implant. Our only bilaterally lesioned animal with a unilateral GM-alone implant (data not shown) did not show any recovery in the cylinder or

LBWT thus suggesting that the effect found in the hRPE-GM animals was due to the RPE cells. However, a matched control group (studies initiated in our laboratory as of now) will allow for comparisons between groups and will further clarify the nature of the effects seen in the bilaterally lesioned and RPE-GM implanted animals. Another potential factor to consider in interpretation of the data, is the to date, unknown sensitivity of the behavioural tests.

Our pilot study shows that the choice of the behavioural tests and the choice of the animal model (severe lesion vs. moderate lesion) are important factors to ponder in the design of our future studies and should be carefully considered and chosen depending on the therapeutic intervention to evaluate.

More experimenters with increased number of animals implanted with either RPE-GM or GM-alone will need to be conducted to enhance the validity of these pilot studies. For further validation of long-term behavioural performance reliability under normal conditions it is necessary to continue our longitudinal studies in normal rats for up to 2 years. Additionally, to test the hypothesis that the main mechanism of the observed amelioration of motor deficits is due to hRPE cell release of L-dopa, which is then taken up by the remaining nigrostriatal DA neurons, behavioural assessment of the improvements in motor function can be carried out after administration of various i.p. doses of L-dopa in sham-implanted (GM-alone) animals. These effects can be characterized and compared to improvements by RPE-GM-implanted animals. Because the specificity of the therapeutic effects of the implant also depends on the implant location, a future experiment should test the hypothesis that implants in non-striatal areas, such as cerebral cortex or ventricles should not produce behavioural recovery.

Additional tests can be developed to complement the motor tasks assessed by the cylinder test and the TLBWT. For example, the evaluation of the effect of RPE –GM implants on

spontaneous motor activity using a computerized activity monitor, in addition to the implementation of motor tests that measure skilled movement, will increase the clinical relevance of the behavioural evaluation of the effects of RPE-GM implants in animal models. Furthermore, tests of cognition, impaired in PD patients later in the disease, and tests of spatial function can also be applied in the future to explore additional dimensions of this new therapy.

Concomitant development of methods to follow *in vivo* the fate and viability of the implanted RPE cells will be necessary to monitor the progression of the implanted cells. It will be also important to adapt quantitative methods to determine viability of RPE cells attached to spherical microcarriers after long periods of time.

Determination of the effects of RPE-GM implants on motor fluctuations induced by prolonged L-dopa therapy will also be of capital importance to assess the possibility that continuous L-dopa release by RPE cells, can reduce or prevent dyskinesias.

4.5 Conclusion

The results of this study have established that forelimb function measured with the forelimb use asymmetry test, as well as hindlimb performance in the TLBWT remains stable over time, under normal conditions and after 6-OHDA infusion; even when rats are exposed to multiple tests, and they increase in body weight, indicating that neither multiple exposures nor weight gain influence test performance. This study provides additional evidence that the tests used are sensitive, reliable, clinically relevant and useful for longitudinal studies of the effects of new therapeutic strategies for PD. One such promising strategy is the intrastriatal implantation of microcarrier-attached hRPE cells.

This study also showed the first evidence that RPE-GM implants ameliorate deficits in spontaneous forelimb and hindlimb motor behaviour, measured with validated tests, in both a bilateral and a unilateral 6-OHDA rat model of PD.

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APPEDIX I Forelimb Use Asymmetry Test Scoring Sheet

FORELIMB USE ASYMMETRY – CYLINDER TEST Scoring sheet

Test Date: _____ Animal Code: _____ Tape: _____.

Rearing up and using forelimbs for weight support on the wall.			Initiating weight shifting laterally		Regaining center of gravity laterally		Using both forelimbs for alternate stepping on the wall
Both	Left forelimb independent wall placement	Right forelimb independent wall placement	Left forelimb independent movement	Right forelimb independent movement	Left forelimb independent movement	Right forelimb independent movement	Both

Totals: Left: _____ **Right:** _____ **Both:** _____.

% Use of LEFT forelimb = $\text{left} / (\text{left} + \text{right} + \text{both}) \times 100$

% Use of RIGHT forelimb = $\text{right} / (\text{left} + \text{right} + \text{both}) \times 100$

% Of simultaneous (BOTH) limb use = $\text{both} / (\text{left} + \text{right} + \text{both}) \times 100$

e.g., 1. % Use of Right (non-lesioned) - % Use of left (lesioned) =

2. % Use of Right (Implanted) - % Use of left (non-Implanted) =

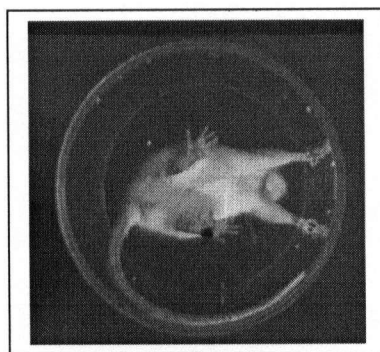
APPENDIX II Tapered Ledged Beam-Walking Test Scoring Sheet

TAPERED LEDGED BEAM-WALKING TEST Scoring sheet

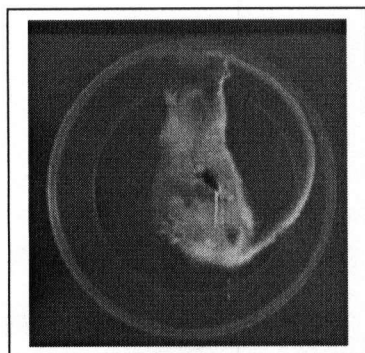
Test Date: _____ **Animal Code:** _____ **Tape:** _____
Scoring Date: _____ (S=step; E= error)

Wide			Medium			Narrow		
Trial #1	0 errors	0 errors						
Left:	S S S	3 steps (0%)	Left:	S E E	3 steps (66.66%)	Left:	E E E	3 errors (100%)
Right:	S S S	0 error 3 steps (0%)	Right:	S S S	0 errors 3 steps (0%)	Right:	S S E	1 error 3 steps (33.33%)
Trial #2			Left:			Left:		
Left:								
Right:			Right:			Right:		
Trial #3			Left:			Left:		
Left:								
Right:			Right:			Right:		
Trial #4			Left:			Left:		
Left:								
Right:			Right:			Right:		
Trial #5			Left:			Left:		
Left:								
Right:			Right:			Right:		

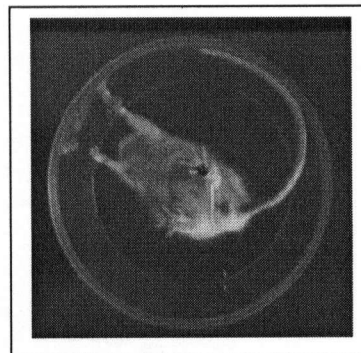
APPENDIX III: Forelimb Use Asymmetry Test Picture Examples



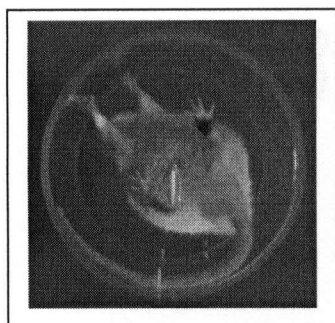
A



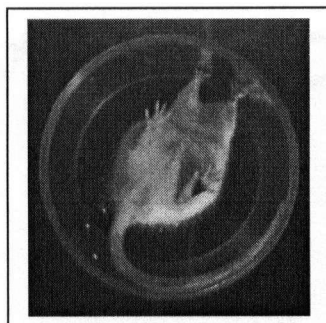
B1



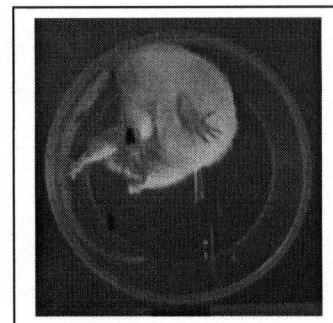
C1



B2



C2

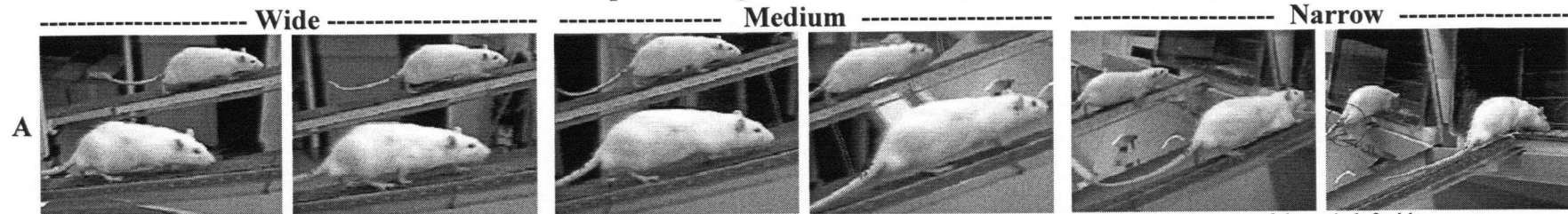


D

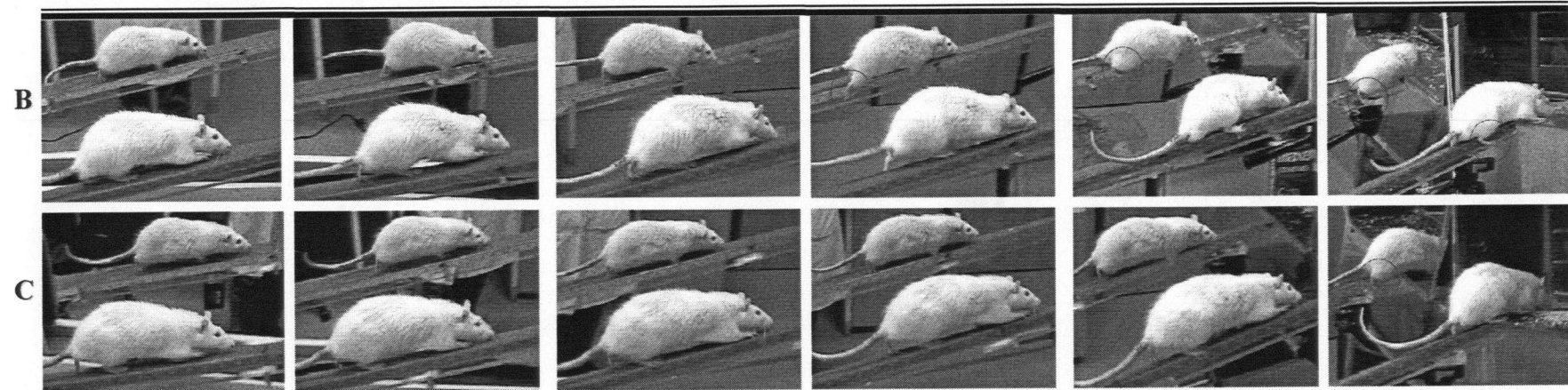
A. A normal rat using both forelimbs for weight support on the cylinder wall; **B1.** After moderate bilateral ICV 6-OHDA lesion, a rat shows a slight forelimb use asymmetry, most frequently affecting more the left forelimb than the right one; **B2.** The same rat from B1, 10 weeks after RPE-GM implant in the right striatum, favours the contralateral forelimb for independent use; **C1.** After 10 weeks of severe unilateral right-MFB-6-OHDA lesion, a rat shows a profound preference to use the non-impaired forelimb almost exclusively; **C2.** The same rat from C1, 10 weeks after RPE-GM implant in the right striatum, still favours the ipsilateral forelimb for independent use, but increases the use of the impaired forelimb for simultaneous use; **D.** A rat with severe unilateral right-MFB-6-OHDA lesion, 10 weeks after implant in the right striatum with GM-alone, shows no recovery of the impaired forelimb.

* The black dot identifies the animals' RIGHT hindlimb.

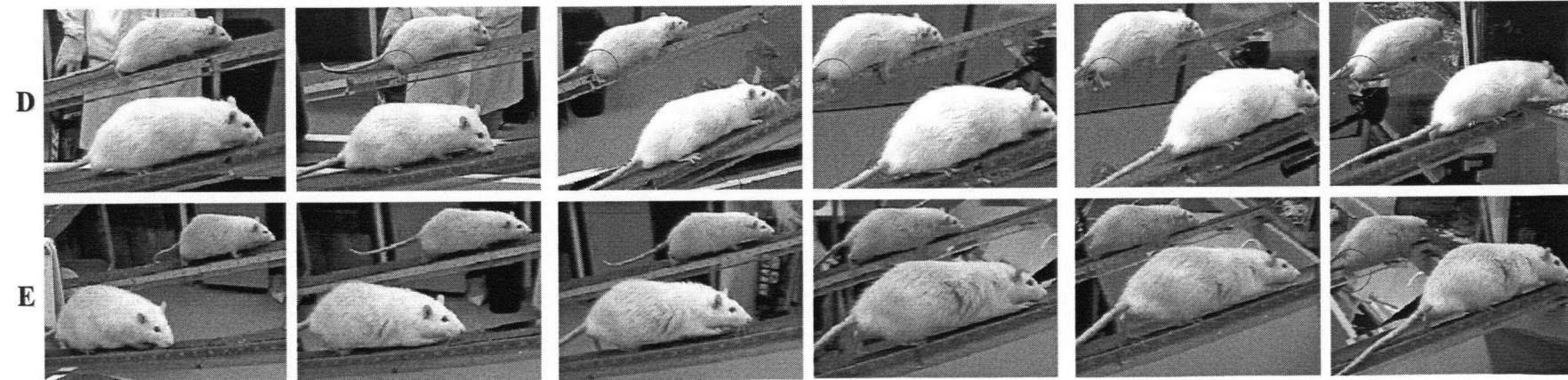
APPENDIX IV: Tapered Ledged Beam-Walking Test Picture Examples



A: A normal rat makes very few left hindlimb footfaults (circled) at the very end of the narrow part of the beam. Top images are mirror images of the rat's left side.

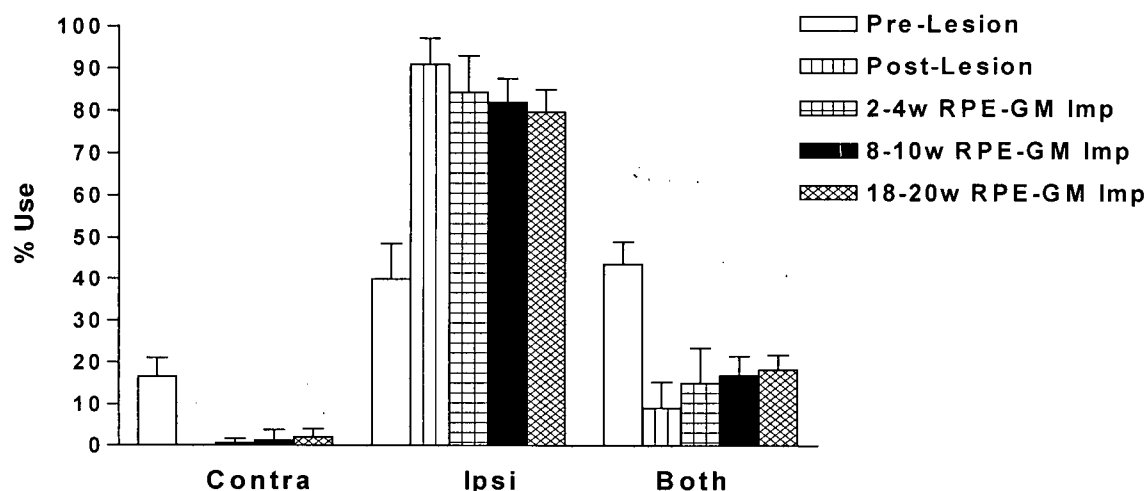


B: 10 weeks after bilateral 6-OHDA lesion, a rat starts to make left hindlimb errors at the end of the medium section of the beam and makes bilateral errors at the end of the narrow section. **C:** 10 weeks after right RPE-GM implant, the same rat makes footfaults only at the end of the narrow section of the beam.

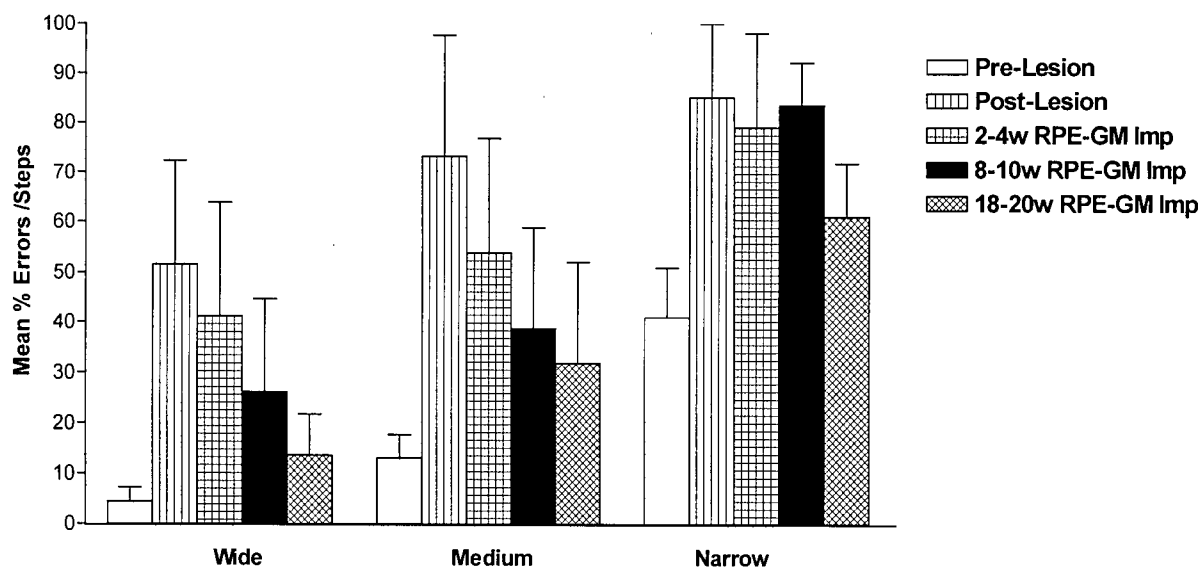


D: 10 weeks after unilateral (right) 6-OHDA MFB lesion, a rat makes very frequent contralateral hindlimb footfaults along the three sections of the beam. **E:** 20 weeks after right RPE-GM implant, the same rat decreased the number of contralateral (left) footfaults along the beam.

APPENDIX V: Effects of RPE-GM implant on unilaterally-lesioned rats at 18-20w



A. Effect of RPE-GM implant on forelimb use in the cylinder of severe unilaterally lesioned rats 18-20 weeks after implant. The trend of improvement continues at week 18-20, as shown by further increase of both use, decrease of independent ipsilateral use and finally a very discrete increase in independent contralateral use.



B. Effects of RPE-GM implant on the contralateral hindlimb on hindlimb on the TLBWT of severe unilaterally lesioned rats 18-20 weeks after implant. The trend of improvement continues at week 18-20, as shown by further decrease of % errors/steps in all beam sections (including the narrow) of the hindlimb contralateral hindlimb to the implant.