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The Potential of Neurotrophin-Secreting Mesenchymal Stem
Cells for the Treatment of Parkinson's Disease

By

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Doctor of Philosophy

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Declaration

I declare that the work presented in this thesis has not been submitted for any degree or diploma at this, or any other university, and that the work described herein is my own with the following exceptions:

Behavioural data acquisition for the studies in sections 4.3.1.1 and 4.3.2.1 were performed by 4th year project students Grace Hogan, Emer Connaughton, Catherine Connaughton and Chloe Thornton, under my guidance.

In vitro cell assays in section 5.3.1 were performed in collaboration with Dr. Ben Newland, Network of Excellence for Functional Biomaterials, National University of Ireland, Galway.

Characterisation and GDNF transduction of MSCs was performed by Dr. Gemma Rooney, Regenerative Medicine Institute, National University of Ireland, Galway.

Signed:..... Date:.....

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Abstract

The most effective experimental neuroprotectant for Parkinson's disease that has been identified from extensive preclinical studies is the neurotrophin, glial cell line-derived neurotrophic factor (GDNF). However, the efficacy of GDNF in clinical trials has been hampered by issues related to its delivery. A possible alternative approach for delivery of this neurotrophin is through *ex vivo* gene therapy, in which suitable cells, such as bone marrow-derived mesenchymal stem cells (MSCs), are genetically engineered to overexpress GDNF prior to transplantation. Thus, the overarching aim of this project was to develop and preclinically validate an *ex vivo* gene therapy approach using MSCs for delivery of GDNF to the Parkinsonian brain.

We first assessed the ability of GDNF-transduced MSCs to enhance the survival of primary dopaminergic neurons (obtained from ventral mesencephalon (VM) dissection) when co-transplanted in the 6-hydroxydopamine-lesioned rat model of Parkinson's disease. Following this, we then assessed the neuroprotective potential of GDNF-MSCs in the inflammation-driven lipopolysaccharide (LPS) model of Parkinson's disease. Based on the results of these studies, we then investigated various strategies to improve the survival of MSCs in the rat brain including encapsulation of the cells in a collagen hydrogel prior to transplantation.

In brief, we found that GDNF-MSCs did not improve the survival of primary dopaminergic neurons when co-transplanted into the 6-hydroxydopamine-lesioned rat striatum. However, when these cells were grafted into the striatum in advance of an intra-nigral LPS lesion, these cells were capable of providing localised protection against inflammation-driven neurodegeneration to the dopaminergic terminals surrounding the graft site. However, due to issues with the poor survival of these bone marrow-derived cells in the ectopic environment of the brain, this neuroprotection did not extend beyond the immediate vicinity of the graft site. We attempted a number of strategies to improve MSC survival in the rat brain. Of these, encapsulation in a type 1 collagen hydrogel proved the most promising approach, as the hydrogel was not

detrimental to cell survival, did not impede the striatal diffusion of GDNF secreted by the transplanted cells, and significantly reduced the host immune response to the graft. Furthermore, the GDNF released from the collagen encapsulated MSCs was also capable of providing local neuroprotection at the site of transplant in the 6-hydroxydopamine-lesioned rat model of Parkinson's disease.

To conclude, GDNF-transduced MSCs are capable of inducing local dopaminergic neuroprotection in both inflammatory and a neurotoxic models of Parkinson's disease when transplanted alone or encapsulated in a collagen hydrogel. Therefore, while we believe the optimisation of this therapeutic approach warrants further study, until the issue of poor survival of these cells in the brain can be addressed, the progression and potential clinical translation of this *ex vivo* gene therapy remains limited.

Publications

Peer Reviewed Published Manuscripts

1. **DB Hoban**, L Howard, E Dowd. (2015) GDNF-secreting mesenchymal stem cells provide localised neuroprotection in an inflammation-driven rat model of Parkinson's disease. Under review of *Neuroscience*.
2. J Samal, **DB Hoban**, C Naughton, R Concannon, E Dowd, A Pandit. (2015) Fibrin-based microsphere reservoirs for delivery of neurotrophic factors to the brain. *Nanomedicine*. 2015 Mar; 10(5), 765-783
3. **DB Hoban**, B Newland, TC Moloney, L Howard, A Pandit, E Dowd. (2013) The reduction in immunogenicity of neurotrophin overexpressing stem cells after intra-striatal transplantation by encapsulation in an *in situ* gelling collagen hydrogel. *Biomaterials*. 2013 Dec;34(37):9420-9
4. **DB Hoban**, E Connaughton, C Connaughton, G Hogan, C Thornton, TC Moloney, P Mulcahy, E Dowd. (2013) Further characterisation of the LPS model of Parkinson's disease: a comparison of intra-nigral and intra-striatal lipopolysaccharide administration on motor function, microgliosis and nigrostriatal neurodegeneration in the rat. *Brain Behav Immun*, 2013 Jan;27(1): 91-100
5. TC Moloney, **DB Hoban**, FP Barry, L Howard, E Dowd. (2012) Kinetics of thermally induced heat shock protein 27 and 70 expression by bone marrow-derived mesenchymal stem cells. *Protein Science*. 2012 Jun; 21(6):904-909.

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2. **DB Hoban**, E Connaughton, C Connaughton, G Hogan, C Thornton, E Dowd. (2012) Modelling Parkinson's disease by direct intranigral administration of lipopolysaccharide. *Movement Disorders* 2012;27 Suppl 1 :1289
3. **DB Hoban**, B Newland, TC Moloney, L Howard, A Pandit, E Dowd. (2012) Assessment of an injectable matrix for ex vivo neurotrophin gene therapy in the rat. *Irish Journal of Medical Science* (182) Supplement 4.

Other Publications

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Other Research Dissemination

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3. **DB Hoban**, E Connaghton, C Connaughton, G Hogan, C Thornton, TC Moloney, P Mulcahy, E Dowd. The Impact of Intra-Nigral LPS on Neuroinflammation, Nigrostriatal Neurodegeneration and Motor Function in Rats. **Poster presentation** at the Movement Disorder Society's 16th Annual Congress of Parkinson's Disease and Movement Disorders, June 2012.
4. **DB Hoban**, B Newland, TC Moloney, L Howard, A Pandit, E Dowd. Assessment of a Collagen Hydrogel for *ex vivo* GDNF Delivery to the Parkinsonian Rat Brain. **Poster Presentation** at Cambridge Centre for Brain Repair Spring School, March 2012.

Irish Conference Presentations:

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List of commonly used abbreviations

ALDH	aldehyde dehydrogenase
ANOVA	analysis of variance
AP	anterioposterior
BDNF	brain-derived neurotrophic factor
CNS	central nervous system
COMT	catechol-O-methyl transferase
DAT	dopamine transporter
DMEM	Dulbecco's Modified Eagle's Medium
DOPAC	3,4-dihydroxyphenyl-acetaldehyde
DV	dorsoventral
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
Fig.	figure
g	gram
GDNF	glial cell line-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
Gpe	globus pallidus externa
Gpi	globus pallidus interna
h	hour
HVA	homovanillic acid
i.p.	intraperitoneal
IHC	immunohistochemistry
IF	immunofluorescence
kg	kilogram
LPS	lipopolysaccharide
MAO	monoamine oxidase
MFB	medial forebrain bundle

µg	microgram
mg	milligram
mg kg ⁻¹	milligram per kilogram
min	minute
ML	mediolateral
mm	millimetre
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSCs	mesenchymal stem cells
ng	nanogram
NGF	nerve growth factor
nm	nanometre
6-OHDA	6-hydroxydopamine
PET	positron emission tomography
s	second
s.e.m.	standard error ± mean
SN	substantia nigra
STR	striatum
STN	subthalamic nucleus
TBS	Tris-buffered saline
TH	tyrosine hydroxylase
TUNEL	TdT-mediated dUTP nick end labelling
UPDRS	Unified Parkinson's Disease Rating Scale
VM	ventral mesencephalon
VTA	ventral tegmental area
3-MT	3-methoxytyramine

Most abbreviations, other than commonly used expressions, are also defined at the first point of occurrence in the text.

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Chapter 1: General introduction

Parkinson's disease is characterised pathologically by the severe and progressive loss of dopaminergic neurons from the nigrostriatal pathway (Fearnley and Lees, 1991, Lang and Lozano, 1998). The subsequent dopamine deficiency clinically manifests with the onset of the classical motor disturbances associated with the disease including resting tremor, stiffness and slowness of movement (Jankovic, 2008), following the development of the pathology to a certain stage (de Lau and Breteler, 2006).

Current clinical therapeutic approaches used to treat Parkinson's disease are based on dopamine replacement strategies, such as the peripheral administration of the dopamine precursor levodopa, or the use of dopamine agonists (Fahn, 2008, Tintner and Jankovic, 2003). Unfortunately these pharmacological approaches merely provide symptomatic relief and do not address the underlying progressive degeneration of dopaminergic neurons. This substantial limitation of the current treatments highlights the clinical need for a novel disease modifying therapy that could effectively halt disease progression and improve prognosis for patients.

One novel therapeutic approach involves the delivery of the neurotrophin, glial cell line-derived neurotrophic factor (GDNF), to the Parkinsonian brain, in order to salvage the 40-50% of dopaminergic neurons still present at disease onset (Kirik et al., 2004). Since its discovery and isolation (Lin et al., 1993), a plethora of studies have demonstrated its potential as a promising neuroprotectant in both preclinical (Kirik et al., 2000a, Tomac et al., 1995) and clinical studies (Gill et al., 2003, Love et al., 2005, Patel et al., 2005). However, issues related to the optimal delivery method have hampered its progression beyond clinical trials and may have masked its true neuroprotective and restorative potential (Sherer et al., 2006).

Therefore, the aim of this thesis was to investigate the potential of an alternative method for the delivery of GDNF to the degenerating brain. *Ex vivo* gene therapy, in which suitable cells, such as bone marrow-derived mesenchymal stem cells (MSCs), are

genetically engineered to overexpress GDNF prior to transplantation, may be more effective for intracerebral delivery of GDNF rather than direct brain infusion of the protein previously investigated in clinical trials (Sherer et al., 2006).

This introductory chapter will provide an evidence-based overview of Parkinson's disease, its current treatments and their associated limitations, and the current standing of neurotrophic therapy and the issues that have hampered its clinical translation. The suitability of MSCs as vehicles for the delivery of GDNF to the brain will also be discussed.

1.1 PARKINSON'S DISEASE

Parkinson's disease is a progressive neurodegenerative disorder with neuropathological features including the chronic degeneration of the dopaminergic nigrostriatal neurons in the brain (Naoi and Maruyama, 1999), accompanied by the presence of Lewy bodies in the surviving dopaminergic neurons (Braak et al., 2003). Parkinson's disease is one of the most common neurological diseases encountered in the clinic (MacDonald et al., 2000) and is the second most common neurodegenerative disease in the world. It has an annual occurrence of 0.1 - 0.5%, less only to Alzheimer's disease in terms of incidence of neurodegenerative diseases (Mayeux, 2003). Parkinson's disease affects about 3% of people over the age of 65 and approximately 4-5% of people over the age of 85 (Whitton, 2007). Sporadic idiopathic Parkinson's disease accounts for approximately 90% of diagnosed patients with just 10% of cases resulting from hereditary factors or genetic links (Tarazi et al., 2014).

Absolute certainty of the diagnosis of Parkinson's disease cannot be confirmed during life as there is no conclusive test to distinguish the diagnosis of Parkinson's disease from a diagnosis of closely related diseases such as other forms of parkinsonism (Foltynie et al., 2004). The pathological examination of *post-mortem* brain tissue from Parkinson's disease patients remains the superior choice to confirm Parkinson's disease diagnosis (Michotte, 2003) and the identification of Lewy bodies in the midbrain is a

definitive feature of the disease (Forno, 1996, Pollanen et al., 1993). However, it is primarily the appearance of clinical symptoms that is used for diagnosis, despite the fact that onset of clinical symptoms only occurs once 80% of dopamine levels have depleted and when 40-50% of dopaminergic neurons have degenerated (Lang and Lozano, 1998).

The diagnosis of the disease is primarily based on the clinical presentation of numerous motor symptoms along with the recording of a detailed patient history. The cardinal symptoms of Parkinson's disease are bradykinesia, tremor at rest, rigidity and postural instability (Jankovic, 2008) along with a myriad of secondary motor symptoms such as loss of spontaneous movements (akinesia), shuffling or festinating gait, changes in speech fluency (dysarthria), freezing, reduced facial expressions (hypomimia) and dystonia (Ben-Shlomo and Sieradzan, 1995, Jankovic, 2008, Tarazi et al., 2014). Non-motor symptoms are also a consequence of Parkinson's disease, however they are often under-recognised and under-reported which leads to delays in treatment and increased treatments costs and hospitalisations for Parkinson's disease patients (Findley et al., 2003, Chaudhuri and Schapira, 2009). The non-motor symptoms of Parkinson's disease include neuropsychiatric symptoms such as depression, anxiety and dementia; sleep disorders such as restless legs and insomnia; autonomic symptoms such as dry eyes and bladder disturbances along with gastrointestinal and sensory disturbances (Chaudhuri et al., 2006). Some of these non-motor symptoms precede the onset of the clinical symptoms of the disease and therefore are said to occur in the sub-clinical phase (Foltynie et al., 2004).

Specific criteria have been set out to aid the diagnostic process for Parkinson's disease. These criteria include 1) presence of two or more of the cardinal symptoms of Parkinson's disease (one of which to be tremor at rest or bradykinesia), 2) asymmetrical onset, 3) response to levodopa, and 4) progression of the disease process over time (Gelb et al., 1999, Jankovic, 2008). Despite the wealth of research that has been undertaken in the last 200 years, the identification of these cardinal symptoms of Parkinson's disease, first described in 1817 by James Parkinson, remains the most identifiable and distinguishable component of the diagnostic process for the disease.

1.1.1 AN HISTORICAL PERSPECTIVE

Almost 200 years ago, a London-based physician, James Parkinson, issued a comprehensive description of six patients (three of which he examined and three he observed on the streets of London) who presented with a movement disorder that eventually would come to bear his name (Parkinson, 1817 (republished 2002)). In this monologue, ‘An Essay on the Shaking Palsy’, Parkinson offered a systematic review of both the medical histories and backgrounds of the patients involved. Although numerous differences were noted between the lives of the patients he described, the observed clinical symptoms of each patient bore striking similarities to each other. These included initial “slight sense of weakness, with a proneness to trembling in some particular part...most commonly in one of the hands or arms”, and difficulty in “preserving an upright posture: this being most observable whilst walking”. Parkinson also referred to the progressive nature of the disease by indicating “As time and the disease proceed, difficulties increase”. Ultimately the combination of these symptoms were to be known as Shaking Palsy (*Paralysis Agitans*), defined by Parkinson as “Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellects being uninjured” (Parkinson, 1817 (republished 2002)). In an era where physical observations or examinations were not yet commonplace as a means of diagnosis, Parkinson offered strikingly detailed descriptions of the disease that were so vivid, the diagnosis of Shaking Palsy in patients who presented with these symptoms was inevitable (Kopin, 1993).

In subsequent years, further symptoms of the disease were catalogued including muscle rigidity and ‘cogwheeling’ as noted by Trousseau in 1867 (Kopin, 1993). In 1877, Jean-Martin Chopard dubbed the disease, thus far known as Shaking Palsy, as Parkinson’s disease, in honour of James Parkinson’s achievement in first describing the motor symptoms of the disease (Lees, 2007). Charcot also further characterised the behavioural characteristics of the disease by delineating the ‘pill-rolling’ tremor that is characteristic of the disease and also noted the loss of facial expression as an unusual

component of the disorder. He classified the disease as a neurological disorder with no known pathological lesion (Goetz, 1986, Charcot, 1880).

The pathological features of Parkinson's disease only began to emerge in the early 20th century, with the discovery of eosinophilic proteinaceous inclusion bodies by Frederic Lewy (Lewy, 1913). These Lewy bodies and Lewy neurites have since become a pathological hallmark of the disease, although they have been discovered in other degenerative disorders (Kopin, 1993). The biochemical nature of the disease was brought to the fore in the 1950's initiated by the detection of dopamine in mammalian brains, including humans (Montagu, 1957, Weil-Malherbe and Bone, 1957). In 1958, Carlsson and others successfully showed that the dopamine concentration in the brain was at its highest in the striatum (Bertler and Rosengren, 1959, Carlsson, 1959) and subsequently that the administration of 3,4-dihydroxyphenylalanine (DOPA) could reverse reserpine-induced dopamine loss and motor deficits in animals (Carlsson et al., 1957, Weil-Malherbe and Bone, 1958). This pioneering work, performed in a relatively short space of time, indicated that dopamine acted as a neurotransmitter in the brain and was involved in the control of motor function. These pathophysiological hallmarks of Parkinson's disease, identified in the mid-20th century, remain the primary pathological features and diagnostic characteristics of the disease today, and have greatly influenced the focus of modern research.

1.1.2 PATHOPHYSIOLOGY

As briefly described earlier, one of the main pathophysiological features of Parkinson's disease is the progressive degeneration of the nigrostriatal pathway in the basal ganglia, namely the dopaminergic neurons and terminals of the substantia nigra and the striatum respectively. Non-dopaminergic systems beyond the nigrostriatal pathway are too thought to be affected and implicated in the pathology of Parkinson's disease. The formation of Lewy bodies is also a pathophysiological feature of the disease. In recent years, there has been substantial research into the involvement of neuroinflammation as a causative factor of the disease.

1.1.2.1 Nigrostriatal Degeneration

Although Parkinson's disease encompasses a spectrum of both motor and non-dopaminergic non-motor manifestations, our understanding of the pathophysiologic origin of these non-motor alterations remains very limited. Dopaminergic neuron loss has been identified as a pathological hallmark of Parkinson's disease and is thought to underpin the motor aspects of the disease, in particular the cardinal features of bradykinesia and akinesia (Wichmann et al., 2011). In order to better understand how loss of dopamine may affect motor control, it is necessary to define the role of the basal ganglia in modulating cortical motor function (Kopin, 1993).

1.1.2.1.1 The basal ganglia

The basal ganglia refers to a collection of subcortical nuclei that are important in the control of movements including: the caudate nucleus and caudate putamen (together deemed the neostriatum); the globus pallidus *pars interna* (GPi) and the globus pallidus *pars externa*; the substantia nigra *pars compacta* (SNc) and the substantia nigra *pars reticulata* (SNr) and the subthalamic nucleus (STN) (Obeso et al., 2000). The inhibitory neurotransmitter gamma-aminobutyric acid (GABA) plays a major role in the functioning basal ganglia where the striatum is composed of approximately 95% GABAergic medium spiny neurons (MSNs) (Kemp and Powell, 1971). There are two major basal ganglia pathways in the motor circuitry: a 'direct' pathway which connects the striatum to the GPi and SNr, and an 'indirect' pathway which also connects the striatum to the output nuclei of the basal ganglia but in this instance the fibres first pass through synaptic connections in the external segment of the GPe and then the STN (Wichmann et al., 2011).

The direct (with high expression of excitatory dopamine D₁ receptors) and indirect (with high expression of inhibitory dopamine D₂ receptors) pathways work in synergy to facilitate normal motor function (**Fig 1.1A**). Activation of D₁ receptors, by release of dopamine in the striatum, activates the direct pathway resulting in the inhibition of GPi

and SNr neurons that reduces inhibition of thalamocortical projection neurons and thus facilitates of movement. Activation of D₂ receptors of the indirect pathway leads to increased inhibition of the GPe, subsequently inhibition of the thalamus (via excitation of the STN), which ultimately reduces the glutamatergic excitation of motor cortical areas that would compete with voluntary movement, a role which is equally essential for normal motor function (Albin et al., 1989, DeLong, 1990).

In the Parkinsonian brain (**Fig 1.1B**), decreased levels of dopamine results in decreased activation of the direct pathway and increased activation of the indirect pathway. Excessive stimulation of the D₂ receptors of the indirect pathway increases the inhibition of the GPe, reduces the inhibition of the STN and therefore, increases excitation of the GPi and SNr. Overall this reduces thalamocortical glutamatergic excitation of the motor cortex and thereby reduces movement (DeLong and Wichmann, 2007). The decreased levels of dopamine which induce this dysregulation in the basal ganglia is due to a severe loss of nigrostriatal dopaminergic neurons.

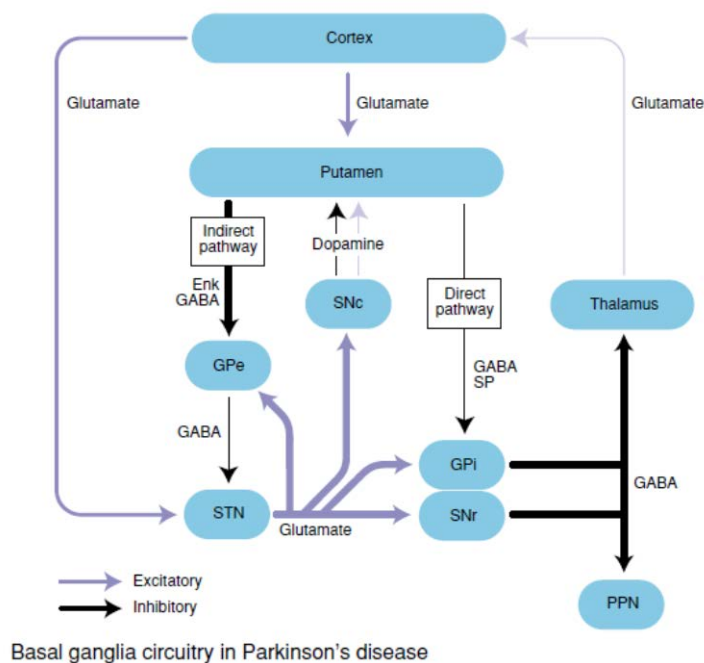


Fig 1.1B In Parkinson's disease, the natural balance of the circuit shown in Figure 1.1A is lost owing to the depletion of dopamine in the striatum. Both the direct and indirect pathways operate through the GPi/SNr output nuclei and their influence is inhibitory on the thalamus. Thus, increased activity in the output nuclei leads to increased inhibition on the glutamatergic excitation of the motor cortex and a subsequent reduction in movement, observed in patients as bradykinesia. These changes in the PD brain are shown here by the differing thickness of arrows, which represents the relative degree of activation in each projection. (Lewis et al., 2003)

1.1.2.1.2 Loss of dopaminergic nigrostriatal neurons

The main pathological hallmark of Parkinson's disease is progressive death of the nigrostriatal pathway in the basal ganglia, as described above, namely the dopaminergic neurons and terminals of the substantia nigra and the striatum respectively (**Fig. 1.2**). The loss of neurons in the disease appears to be heterogeneous and whilst it includes the dopaminergic neurons of the substantia nigra *pars compacta*, other neuronal populations such as selected catecholaminergic and serotonergic nuclei in the brain-stem; neurons in the olfactory bulb; sympathetic ganglia and parasympathetic neurons in the gut are also affected (Lang and Lozano, 1998).

The loss of neurons within the substantia nigra *pars compacta* also appears to be diverse, tending to be greatest in the ventrolateral tier (estimated loss of 60-70% at the onset of symptoms), followed by partial damage in the medial ventral tier and dorsal tier (Damier et al., 1999, Fearnley and Lees, 1991) representing a pattern of cell loss relatively specific to Parkinson's disease. This neuronal loss subsequently results in a reduction of striatal dopamine, most prominently in the dorsal and intermediate subdivisions of the putamen, believed to account for akinesia and rigidity observed clinically in the disease (Kish et al., 1988).

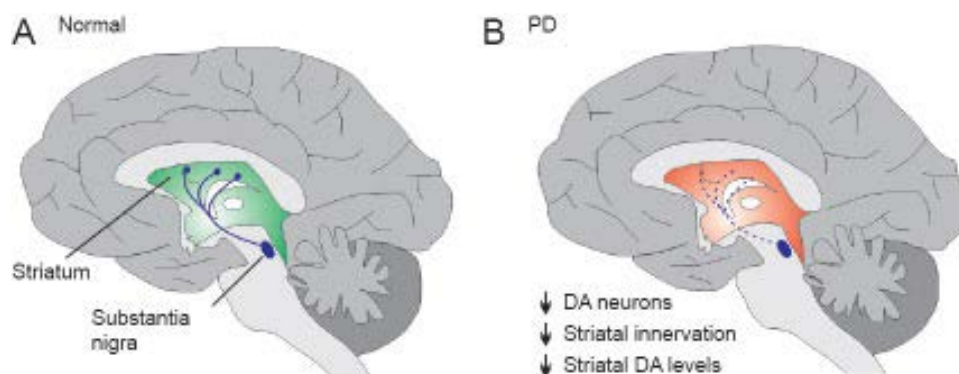


Fig 1.2 (A) Graphical representation of the intact nigrostriatal pathway showing ascending axons (blue) of dopaminergic neurons originating in the substantia nigra innervating the striatum. (B) In Parkinson's disease, the progressive degeneration of these neurons leads to a loss of dopaminergic innervation of the striatum and consequently a reduction in striatal dopamine levels, which is one of the pathological hallmarks of Parkinson's disease. Modified from Hegarty et al., (2014).

The degeneration of the dopaminergic neurons in the basal ganglia can often progress for years prior to the manifestation of motor symptoms. Typically, when patients first present with motor impairment, approximately 40% of dopaminergic neurons in the substantia nigra have already been lost and dopamine levels in the striatum have already been reduced by up to 80% (Bernheimer et al., 1973, Riederer and Wuketich, 1976). The reason for the delay between the initiation of the disease process and the appearance of clinical symptoms is believed to be due to compensatory mechanisms. These compensatory mechanisms in the pre-symptomatic period of the disease effectively mask the underlying degenerative process via hyperactivity of the remaining dopaminergic neurons to maintain dopamine homeostasis (Hornykiewicz and Kish, 1987, Zigmond et al., 1990), as well as increased dopamine receptor number and sensitivity in the striatum (Lee et al., 1978, Thornburg and Moore, 1975). Another pathological process that may be ongoing in the pre-symptomatic phase of Parkinson's disease is the development of Lewy pathology.

1.1.2.2 Lewy Bodies

In addition to the progressive degeneration of the nigrostriatal pathway of Parkinson's disease, the other primary pathological hallmark of the disease is the presence of Lewy bodies and/or Lewy neurites. First identified in 1913 by Frederic Lewy, these eosinophilic proteinaceous inclusion bodies accumulate in the degenerating brain of Parkinson's disease patients (Lewy, 1913) as well as in other neurodegenerative disorders (Kopin, 1993). The accumulation of these proteinaceous deposits, while a common feature of many neurodegenerative diseases, is evident in both genetic and sporadic Parkinson's disease patients with very little exception (Poulopoulos et al., 2012), and thus suggests a role for the involvement of Lewy pathology in the development and progression of the disease process itself (Luk and Lee, 2014).

A typical Lewy body is approximately 8-30 μm in diameter and when stained with eosin, exhibits a densely stained granular core surrounded by a lightly stained halo of radiating filaments (McNaught, 2004). The primary components of Lewy bodies are the

protein α -synuclein (Spillantini et al., 1998, Spillantini et al., 1997), ubiquitin and ubiquitinated proteins (Lennox et al., 1989, McNaught et al., 2002), and neurofilaments (Schmidt et al., 1991).

Seminal research performed by Braak and colleagues has led to the increased appreciation and knowledge of the staging and spread of Lewy body pathology in Parkinson's disease. Braak and colleagues suggested a theory for the progression of the disease from the stage where patients exhibit Lewy pathology but are asymptomatic, to those with late stage clinical symptoms of the disease using 110 α -synuclein positive patients (69 incidental and 41 clinically diagnosed PD patients) (Braak et al., 2006, Braak et al., 2003, Luk and Lee, 2014). The theory proposed that the Lewy pathology advances via cell-to-cell contact, beginning in the periphery and moving into the medulla oblongata and olfactory system. This initiates the onset of autonomic and olfactory defects, followed by the progression of the pathology to the brainstem, causing sleep and motor disturbances. Advancement to the limbic system succeeds until finally the neocortical regions are affected, causing neurobehavioral and cognitive impairment (**Fig. 1.3**) (Braak et al., 2006, Braak et al., 2003, Halliday et al., 2011).

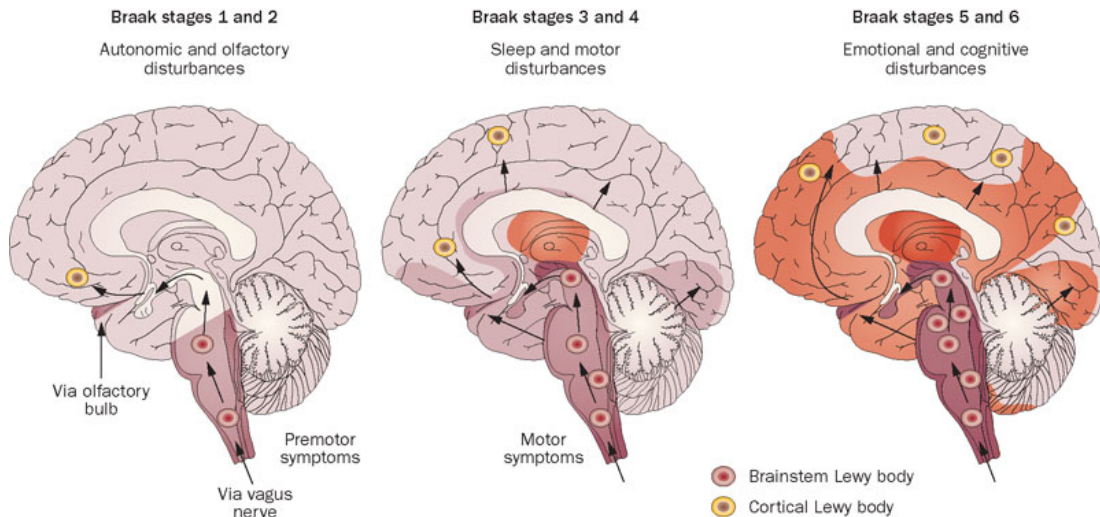


Fig. 1.3 Schematic representation of the Braak staging of Parkinson's disease showing the initiation sites in the medulla oblongata and olfactory bulb through to the later infiltration of Lewy pathology into the cortical regions. Associated symptoms at each stage are also shown (Halliday et al., 2011).

Further reiterating the theory of cell-to-cell transfer of Lewy pathology, is the recent evidence showing the ability of α -synuclein to spread from cell-to-cell, thus introducing the prion hypothesis of Lewy body pathology in Parkinson's disease. This prion hypothesis is supported by the observation of Lewy body pathology in embryonic dopamine neurons transplanted into the putamen of patients with Parkinson's disease (Kordower et al., 2008a, Kordower et al., 2008b, Li et al., 2008) and by compelling evidence that α -synuclein is capable of pathologically spreading from one cell to another in both *in vitro* and *in vivo* studies (Desplats et al., 2009, Hansen et al., 2011). This highlights the role of α -synuclein in the pathogenesis of the disease and has drawn attention to the possibility of targeting this transfer of α -synuclein as a neuroprotective strategy for the disease (Visanji et al., 2013).

1.1.2.3 Neuroinflammation

In recent years, the role of inflammation in the brain as a factor in the pathogenesis of Parkinson's disease has been extensively researched. The contribution of inflammation to the pathogenesis of Parkinson's disease was first suggested by McGeer and co-

workers in 1988 after the discovery of activated microglial cells and increased MHC molecules in the Parkinsonian brain (McGeer et al., 1988). Neuroinflammation is not specific to Parkinson's disease and is thought to contribute to several other neurodegenerative diseases including Alzheimer's disease, Huntington's disease, and amyotrophic lateral sclerosis (Akiyama et al., 2000, McGeer and McGeer, 2003, McGeer and McGeer, 2002, McGeer and McGeer, 2004, Thomas et al., 2003).

It is widely believed that neuroinflammation caused by exposure to infectious agents or toxicants with proinflammatory characteristics may lead to development of Parkinson's disease (de Meira Santos Lima et al., 2006), however, whether neuroinflammation is a cause or consequence of dopamine degeneration is a debatable subject. In theory, it is thought that neurodegeneration may establish a chronic neuroinflammation that may lead to the continuous degeneration of the dopaminergic neurons. Thus a continuous, self-propagating cycle (Tansey and Goldberg, 2010, Tzeng et al., 2005) (**Fig 1.4**) of neuroinflammation and neurodegeneration ensues (Banati et al., 1998, Bronstein et al., 1995, McGeer and McGeer, 1998).

There is an overwhelming amount of evidence for the involvement of neuroinflammatory processes in the pathogenesis of Parkinson's disease. Microglial activation, NF- κ B activation, and astrocytic upregulation have been identified in both patients and animal models of Parkinson's disease. Increases of IL-2, IL-6, TNF- α , and IL-1 β were recorded in many areas including the brain, serum and cerebrospinal fluid (Boka et al., 1994, Mogi et al., 1994a, Mogi et al., 1995, Mogi et al., 1996, Mogi et al., 1994b). Thus, both cellular and molecular evidence have been uncovered in *post-mortem* and *in vivo* analysis of Parkinson's disease patients to indicate that there is a role for neuroinflammatory processes in the pathogenesis of the disease.

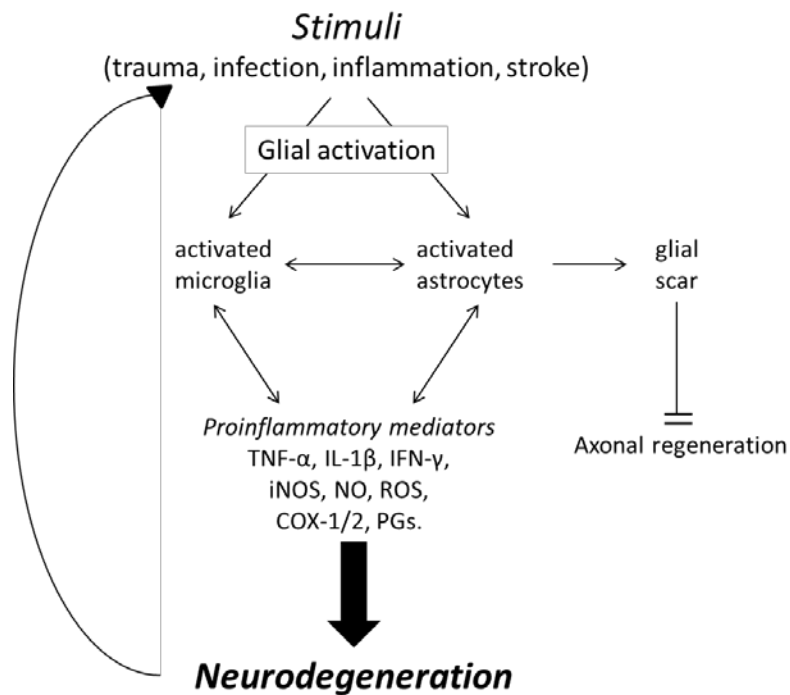


Fig. 1.4 A schematic diagram showing the role of glial activation and glial scar in neurodegeneration and the eventual self-sustaining cycle of neuroinflammation and neurodegeneration. Modified from Tzeng et al., (2005)

Microglial activation is a pathological hallmark for neuroinflammation and has been implicated in the pathogenesis of Parkinson's disease as well as other progressive neurodegenerative disorders (Kim et al., 2000). Microglia are a population of innate immune cells resident in the brain and constitute approximately 5-20% of all glial cells. They are thought to function by monitoring the surrounding environment for potential threat and thus become activated via Toll-like receptor signalling when such a threat is detected (Qin et al., 2007). Activation involves a morphological change from a quiescent resting or ramified state to an activated or amoeboid state (Streit et al., 1988). Microglial activation is necessary for maintenance of CNS integrity but over-activation can cause detrimental and neurotoxic effects to the CNS (Qin et al., 2007). Over-activation of microglia leads to the over-production of pro-inflammatory mediators and this excessive response can induce neuronal damage and death. Recent research suggests that this excessive activation results in dopamine neuron loss in the

progression of Parkinson's disease (Chung et al., 2010). While microglial cells essentially induce pro-inflammatory cytokines to protect the CNS against infection, this action also induces inflammation, and thus results in a self-propagating cycle of inflammation and degeneration, that can continue even when the instigating stimulus has disappeared (Qin et al., 2007).

The substantia nigra contains approximately four to five times the amount of microglia in comparison to other areas of the brain (Kim et al., 2000). Dopaminergic neurons are also more sensitive to microglial activation in comparison to other neurons in the brain because of their low intracellular glutathione (Whitton, 2007). In addition, it has been reported that microglial activation may also be triggered by protein aggregation caused by the disruption of the ubiquitin-proteasome system (Jansen et al., 2014) and mutations in α -synuclein (Zhang et al., 2005b), or by bacterial or viral infections (Castano et al., 1998, Deleidi et al., 2010) of which are thought to be involved in the etiology and pathogenesis of Parkinson's disease. This offers an explanation as to why microglial activation, leading to neurodegeneration, is thought to be particularly involved in the degeneration of the particular population of dopaminergic neurons that are lost in Parkinson's disease.

1.1.3 ETIOLOGY

While the pathophysiology of Parkinson's disease has been relatively well described, the exact etiology of the disease remains elusive. No single causative factor of the disease has been identified, but the etiology of the disease may be multifactorial involving numerous contributing risk factors, including but not limited to, the influence of age, exposure to environmental toxins and genetic predisposition.

1.1.3.1 Age

Aging is the largest single risk factor for the development of Parkinson's disease. The average age of onset is approximately 65 years of age with incidence of the disease increasing with age (Hindle, 2010). The estimated prevalence of Parkinson's disease has

been reported as 600 per 100,000 individuals between the ages of 65 and 69, increasing to 2,600 per 100,000 for individuals aged between 85 to 89 years (Mayeux, 2003). Due to an ever-increasing population and an internationally aging society, it is anticipated that the number of diagnosed Parkinson's disease patients will increase from 4.1 million in 2005 to 8.7 million in 2030 (Tarazi et al., 2014), and as the aging population increases, so too will the financial burden associated with diagnosis, treatment and care (Brown et al., 2005). Studies have shown that the dopaminergic system affected in Parkinson's disease also degenerates in the course of the normal aging process (Reeve et al., 2014, Rodriguez et al., 2014). It has also been shown that the process of normal aging can be associated with extremely mild motor symptoms of Parkinson's disease (Hindle, 2010). However, since Parkinson's disease has a very particular clinical profile and diagnosis of the disease is not ubiquitous in an aged population, it is not regarded as a causative factor and therefore, the extensive dopaminergic cell death associated with the disease and initiation of the disease process itself, must be attributable to a combination of age and exposure to environmental toxins and/or a genetic predisposition.

1.1.3.2 Environment

The origin and development of Parkinson's disease appears to be multi-factorial with the environment playing an important role in susceptibility to the disease. Epidemiological and environmental studies have uncovered both protective and adverse environmental factors associated with the development and/or progression of the disease (Bonnet and Houeto, 1999, Di Monte, 2003).

Evidence for the influence of external environmental factors in the etiology of the disease date back to the beginning of the 20th century, when development of parkinsonism secondary to viral encephalitis occurred subsequent to the influenza epidemic in 1918 (Jang et al., 2009). The search for specific environmental causative factors was revived, when in 1982, intravenous drug users presented to emergency departments in San Jose, California with acute parkinsonism. The signs and symptoms

evident in these patients were reversed upon treatment with levodopa, the gold standard treatment for Parkinson's disease. Langston and colleagues identified the initiating toxic compound as MPTP (1,2,3,6-methyl-phenyl-tetrahydropyridine), a by-product in the synthesis of an illicit meperidine analog (Langston et al., 1983), a compound which was promptly utilised to model the disease in experimental animals (Langston et al., 1984).

Epidemiological studies in humans have identified a link between pesticide exposure and incidence of neurodegeneration and Parkinson's disease (Kanavouras et al., 2011, Le Couteur et al., 1999). Meta-analysis of published studies has also found a correlation between Parkinson disease risk and living in a rural area, drinking well water, and farming as an occupation, all occasions in which there would be a higher probability of pesticide exposure (Priyadarshi et al., 2001). Pesticides are classified as a range of substances that are most frequently used to control insects, fungi and weeds. In general, individuals are exposed to numerous pesticides or combinations of pesticides, either concurrently or consecutively, making it increasingly difficult to attribute the adverse effects of pesticide exposure to one particular agent (Kamel and Hoppin, 2004).

Recently, a study was performed to assess the risk of developing Parkinson's disease with exposure to 31 specific pesticides. Of the screened pesticides, two were found to have approximately a 3-fold increased chance of developing Parkinson's disease, namely, paraquat and rotenone (Tanner et al., 2011, Tanner et al., 2009). The increased risk associated with exposure to these compounds is reflected in the development of animal models of Parkinson's disease using both paraquat and rotenone. Administration of paraquat (Manning-Bog et al., 2002, McCormack et al., 2002), or rotenone (Betarbet et al., 2000, Sherer et al., 2003) results in Parkinsonian motor deficits and associated dopaminergic loss in rodents. Animal models have essentially supported the implication that pesticide exposure is involved in the etiology of Parkinson disease.

Environmental agents may also be beneficial with regards to disease manifestation and progression. Substantial epidemiological evidence has demonstrated the inverse

relationship between smoking and development of the disease. It is thought that some compounds in the cigarette smoke may protect the dopaminergic neurons from degeneration (Morens et al., 1995, Sugita et al., 2001), which is further iterated by the presence of nicotinic receptors on dopaminergic neurons of the substantia nigra (Quik and Kulak, 2002). Moderate consumption of caffeine is also thought to be associated with a decreased incidence of Parkinson's disease (Ascherio et al., 2001, Ross et al., 2000).

Ultimately, these environmental factors are thought to be involved in a contributory manner to the manifestation of the disease, and much focus has been placed on the gene-environment interaction as a hypothesis for the etiology of the disease.

1.1.3.3 Genetic Predisposition

There is overwhelming evidence indicating the involvement of genetics in the development of Parkinson's disease. Twin studies, epidemiological studies, and studies of large families with hereditary cases, all point towards a genetic predisposition to Parkinson's disease. Epidemiological studies investigating the prevalence of familial and sporadic cases of Parkinson's disease have revealed that approximately 90% of diagnosed cases are sporadic in nature and exhibit a late onset (Tanner, 2003). The remaining 5-10% of patients possess some familial form of the disease (Mizuno et al., 2001) and the majority of these experience an early onset of symptoms (Samii et al., 2004). Familial Parkinson's disease can thus be classified as an uncommon entity. However over the last 15 years, essential research has been focused on the identification of potential genetic mutations in order to elucidate possible molecular mechanisms involved in the disease. Familial Parkinson's disease can be inherited in either an autosomal dominant or an autosomal recessive manner (Fan et al., 2013), and genome wide association studies have successfully identified common variants in almost 20 genes that may augment the risk of developing Parkinson's disease (Bonifati, 2014). A selection of specific identified loci are outlined in **Table 1.1** below. It is hoped that an increased understanding of the involvement of genetics in the etiology of the disease

would facilitate the development of novel therapeutic interventions that would halt, or at the very least, slow the progression of the disease, and eventually replace the purely symptomatic treatments currently in use.

Gene Name	Gene product	Inheritance	Pathology	Clinical Phenotype	Progression	Onset	Reference
SNCA	α -synuclein	Dominant	Nigral degeneration with Lewy bodies	Aggressive progressive Parkinsonism, cognitive and autonomic symptoms	Aggressive	Early	(Poulopoulos et al., 2012)
LRRK2	Leucine-rich repeat-containing kinase	Dominant	α -synuclein pathology (Variable)	Parkinsonism and variable additional symptoms	Typical	Late	(Paisan-Ruiz et al., 2004, Zimprich et al., 2004)
GBA	Lysosomal glucocerebrosidase	Dominant	Nigral degeneration with Lewy bodies	Parkinsonism and variable additional symptoms	Typical	Late	(Aharon-Peretz et al., 2004, Neudorfer et al., 1996)
VPS35	Vacuolar protein sorting 35	Dominant	Uncertain pathology	Parkinsonism with possible cognitive and behavioural symptoms	Typical	Late	(Vilarino-Guell et al., 2011, Zimprich et al., 2011)
Parkin	E3 ubiquitin protein ligase	Recessive	Nigral degeneration without Lewy bodies	Young onset classical parkinsonism	Slow	Early	(Kitada et al., 1998)
PINK1	PTEN-induced mitochondrial serine/threonine kinase	Recessive	Nigral degeneration with Lewy bodies	Young onset classical parkinsonism with various cognitive and psychiatric symptoms	Slow	Early	(Valente et al., 2004)
DJ-1	Redox-dependent molecular chaperone in mitochondria	Recessive	No pathology reported	Classical parkinsonism	Slow	Early	(Bonifati et al., 2003)
PLA2G6	Phospholipase A2	Recessive	Lewy body pathology	Classical parkinsonism or parkinsonism-pyramidal syndrome	Unreported	Early	(Paisan-Ruiz et al., 2009)
FBX07	E3 ubiquitin protein ligase	Recessive	Unknown	Classical parkinsonism or pyramidal syndrome	Unreported	Early	(Di Fonzo et al., 2009, Shojaee et al., 2008)
DNAJC6	Neuronal-specific clathrin-uncoating co-chaperone auxilin	Recessive	Unknown	Parkinsonism-pyramidal syndrome	Unreported	Juvenile	(Edvardson et al., 2012, Koroglu et al., 2013)

Table 1.1. Genes involved in the etiology of Parkinson's disease. Modified from Bonifati (2014), Funke et al., (2013), Puschmann (2013), Spatola et al., (2014)

1.2 CURRENT TREATMENTS FOR PARKINSON'S DISEASE

Parkinson's disease, while remaining an incurable disease as of yet, is treated using various agents to try and improve the physical and psychological symptoms as well as the quality of life of the individual patient. Early diagnosis is of utmost importance in the disease as treatment of early Parkinson's disease is far less challenging than treatment of advanced Parkinson's disease. Most treatments available to Parkinson's disease patients are purely to treat the symptoms of the disease as there are not as yet any protective drugs available that may slow or stop the progression of the disease (Salawu et al., 2010). An ideal drug for the disease would be a drug that exerts neuroprotective effects that could be given at early stages of the disease. This could prevent or slow the progression of the disease, improve the prognosis for Parkinson's disease patients, and also reduce the cost of treatment of the disease. Treatment regimens, while purely symptomatic thus far, are ultimately patient-specific and depend on the individual's anticipated risk for side effects and requirement for improvement of symptoms (Hauser, 2010).

1.2.1 LEVODOPA

In 1967, oral levodopa therapy was developed, accounting for one of the most significant developments in pharmacotherapy for Parkinson's disease (Cotzias et al., 1967). Levodopa, also known as L-dopa, is today regarded as the gold standard of Parkinson's disease treatment and its efficacy has not been surpassed since its discovery over 50 years ago. When levodopa was first tested in Parkinsonian patients (intravenous administration), it was found to have few short term side effects and essentially abolished the bradykinesia and rigidity symptoms of the disease (Birkmayer and Hornykiewicz, 1961). The mechanism behind this effect has been explained by the conversion of levodopa to dopamine via decarboxylation by DOPA-decarboxylase enzyme (Fahn, 2008). In its formative days as a therapeutic intervention, peripheral conversion of levodopa to dopamine was found to induce various cardiovascular and gastrointestinal side effects. Subsequently levodopa was administered with a

peripherally-restricted DOPA-decarboxylase inhibitor to ameliorate the side effects of peripheral dopamine (Fahn, 2008). The most common DOPA-decarboxylase inhibitor in clinical use is carbidopa and numerous studies have demonstrated its ability to double the plasma half-life and increase the bioavailability of levodopa, therefore increasing the central therapeutic effect of the drug (Markham et al., 1974, Robertson et al., 1989, Sweet et al., 1975).

1.2.2 DOPAMINE AGONISTS

Dopamine agonists act by directly stimulating post-synaptic dopamine receptors and effectively mimicking the effect of the neurotransmitter (Tintner and Jankovic, 2003), and have been demonstrated to have a moderate positive effect on symptoms of Parkinson's disease (Pahwa and Lyons, 2010). Dopamine agonists are usually most beneficial as a monotherapy for treatment of early Parkinson's disease but are also used as an adjunctive therapy with levodopa to delay the onset of dyskinesia, thereby improving the patient's quality of life (Salawu et al., 2010). When patients received early treatment with dopamine agonists, it was found that they were less likely to develop motor fluctuations and dyskinesia in the advanced disease. Dopamine agonists that have been developed include ergot derivatives bromocriptine, lisuride, pergolide, and cabergoline, as well as other non-ergot agents, such as apomorphine, pramipexole and ropinirole (Tintner and Jankovic, 2003).

1.2.3 MAO/COMT INHIBITORS

Inactivation of dopamine occurs via the action of three enzymes: monoamine oxidase (MAO), aldehyde dehydrogenase (ALDH) and catechol-O-methyl transferase (COMT), producing inactive metabolites including 3,4-dihydroxyphenyl-acetaldehyde (DOPAC), homovanillic acid (HVA) and 3-methoxytyramine (3-MT) (Elsworth and Roth, 1997). Manipulation of dopamine metabolism through the use of enzyme inhibitors in order to prolong the availability of dopamine has been used in the treatment of Parkinson's disease.

Selegiline and rasagline are two clinically approved MAO-B inhibitors that exert their action by preventing the degradation of dopamine. These inhibitors provide mild symptomatic relief in early Parkinson's disease and are of therapeutic use as an adjunctive therapy to reduce motor fluctuations resulting from levodopa treatment (Schapira, 2011). Entacapone is an example of an approved COMT inhibitor for the treatment of Parkinson's disease. While not effective as a monotherapy, there is evidence to show that levodopa/carbidopa/entacapone combinations are effective in reducing 'end of dose' motor fluctuations of levodopa (Ruottinen and Rinne, 1998, Silver, 2004).

1.2.4 NON-DOPAMINERGIC TREATMENTS

The anti-Parkinsonian effect of anti-cholinergic drugs was first discovered in 1867. These remained the only drugs used to address the symptoms of the disease for almost a century (Katzenschlager et al., 2003), and are still used today despite the discovery of other treatments with less risk of adverse events. The most commonly used anticholinergics in the U.S. are the muscarinic antagonists trihexyphenidyl, benzotropine, and procyclidine (Rezak, 2007). Adverse events or side effects associated with cholinergic antagonists include confusion, agitation, hallucinations and dementia (de Smet et al., 1982, Drachman, 1977, Katzenschlager et al., 2003). Despite this, many clinicians still use anti-cholinergic drugs (either as a monotherapy or in combination therapy) as a first-line therapeutic option for treatment of the disease (Schapira, 2007). However, because this class of medication is burdened with side effects, it is usually reserved for tremor that is resistant to dopaminergic treatment (Rezak, 2007).

Amantadine, originally used as an anti-viral agent, has been shown to offer minor symptomatic control of motor features in Parkinson's disease patients via its action as a non-competitive NMDA receptor antagonist, although it is considerably less effective than dopaminergic therapy (Crosby et al., 2003). Treatment with amantadine is most beneficial in the earlier stages of Parkinson's disease and appears to be of particular use

in treating tremor (Rezak, 2007). Emerging data also suggests that chronic amantadine treatment improves levodopa-induced dyskinesia, and that withdrawal of amantadine treatment can exacerbate levodopa-induced dyskinesia (Ory-Magne et al., 2014). However, there are various side effects associated with amantadine treatment including confusion, worsening of hallucinations, nausea and oedema (Crosby et al., 2003, Verhagen Metman et al., 1998).

1.2.5 DEEP BRAIN STIMULATION

DBS involves the implantation of a device which sends electrical pulses to specific regions of the brain (usually the STN or the GPi) via implanted electrodes (Yu and Neimat, 2008, Olanow et al., 2000). DBS candidacy is considered when motor problems produced in Parkinson's disease (slowness, stiffness, shaking, walking problems, wearing off of medication, fluctuations of motor symptoms, dyskinesia) are no longer sufficiently treated by an optimized medication regimen (Chang and Chou, 2006). Similar to available pharmacological treatments, DBS does not slow or halt disease progression, but rather offers symptomatic benefits (Limousin and Martinez-Torres, 2008). In addition, DBS is an invasive therapy and is contraindicated in some patients, such as those with a high surgical risk.

1.2.6 LIMITATIONS OF CURRENT TREATMENTS

Despite the obvious symptomatic relief experienced by patients on levodopa/carbidopa treatment, it is important to note that there are various limitations to the treatment. It has been reported that for some, even high dose carbidopa administered with levodopa may not be sufficient to prevent the peripheral side effects of nausea, vomiting and hypotension (Fahn, 2008). Long term treatment with levodopa has a high propensity to cause side effects such as motor fluctuations (on-off periods) and dyskinesias. Effectiveness of the drug on motor symptoms is also curtailed with prolonged treatment and progression of the disease (Jankovic, 2005). Levodopa therapy is therefore usually

reserved for treatment of advanced Parkinson's disease even though it is by far the most beneficial treatment (Hauser, 2010).

In spite of the benefits of their use, dopamine agonists have been shown to result in non-motor adverse effects such as oedema, sudden onset of sleep, constipation, nausea, dizziness, hallucinations and impulse control disorders (Hauser, 2010, Antonini et al., 2009, Siri et al., 2010). MAO-B inhibitors are limited therapeutically as they are less efficacious than both levodopa and dopamine agonists but conversely they produce far less side effects which makes their use desirable in early treatment of the disease (Pahwa and Lyons, 2010, Fernandez and Chen, 2007). Use of COMT inhibitors, namely entacapone, is hampered by its propensity to increase levodopa-induced dyskinesia, thereby limiting the permissible daily dose of levodopa (Lees, 2008, Rascol et al., 2011, Stocchi et al., 2010).

Despite the many advances that have occurred in PD therapy, it is important to note that there are serious unmet clinical needs. All the pharmacological treatments used in routine clinical practice have failed to provide disease modification and only contribute to symptomatic relief. The ability of a specific therapeutic agent, or indeed a combination of treatments, to adequately address the symptoms of the disease, reduce motor complications of treatment, halt disease progression and provide functional neuroprotection and/or restoration of the dopaminergic neurons in the degenerating brain remains elusive to this day.

The stark insufficiencies in current therapeutic options have prompted a determined effort to identify novel therapeutic agents or strategies to address the degeneration of the nigrostriatal pathway and improve the current inevitably poor prognosis for patients diagnosed with Parkinson's disease.

1.3 NEUROTROPHIC THERAPY FOR PD

In recent years, due to the limitations of therapies currently available to patients with Parkinson's disease, the delivery of trophic factors to regions susceptible to degeneration has gained interest as an experimental strategy for treatment of the disease (Kordower and Bjorklund, 2013). Indeed, neurotrophic factors have the potential to halt the neurodegenerative process and theoretically stimulate the regeneration and repair of dying neurons (Stayte and Vissel, 2014).

1.3.1 RATIONALE FOR NEUROTROPHIC THERAPY

Neurotrophic factors are endogenous proteins that are responsible for the development and maturation of the nervous system, including survival and differentiation of neurons and establishment of various neuronal connections in the brain (Huang and Reichardt, 2001, Snider and Johnson, 1989). While there are many different neurotrophic factors, each serving a specific neuronal population, the levels of these neurotrophins declines rapidly once the nervous system has developed, and they then serve to maintain neuronal function (Hefti et al., 1989, Sofroniew et al., 2001).

In Parkinson's disease, at the stage whereby patients present with clinical characteristics of the disease, the underlying degeneration of dopaminergic neurons has already taken hold, with approximately 40-50% dopaminergic cell loss and 70-80% striatal dopamine depletion (Lang and Lozano, 1998, Bernheimer et al., 1973). Progressive dopaminergic death and worsening of motor symptom ensues in the following 10 years of the disease (Morrish et al., 1996), although this too is highly variable (Hoehn and Yahr, 1967). This prolonged degeneration offers a possible therapeutic window for a disease-modifying therapy, that is, an opportunity to salvage the remaining dopaminergic neurons and slow or arrest disease progression.

Extensive research has consistently shown that when neurodegeneration occurs, neurotrophic factors (at higher than endogenous levels) can decelerate neurodegeneration and even stimulate neuronal repair of degenerating neuronal

populations (Hefti et al., 1989). Thus, neurotrophic factors provide the opportunity to considerably restore degenerating neurons in the brain, at both a morphological and functional level, potentially improving symptoms, prolonging the efficacy of current pharmacological treatments and hypothetically slowing, halting or possibly even reversing disease progression (Bartus, 2012).

1.3.2 GDNF – DISCOVERY AND CLASSIFICATION

GDNF was originally isolated from the supernatant of a rat glial cell-line (Lin et al., 1993), and since its discovery, a plethora of studies have found it to have distinct protective effects on the survival of midbrain dopaminergic neurons both *in vitro* and *in vivo* (Beck et al., 1995, Kearns and Gash, 1995, Lin et al., 1993, Sauer et al., 1995). The effects of GDNF have been shown to be relatively specific for dopaminergic neurons and thus it has substantial promise for the treatment of Parkinson's disease, as the disease is predominantly characterised by progressive degeneration of the dopaminergic cell populations found in the midbrain (Allen et al., 2013, Lin et al., 1993). Interestingly, GDNF has also exhibited protective effects on noradrenergic neurons in the locus coeruleus, as well as peripheral motor neurons, highlighting its therapeutic potential in Huntington's disease and amyotrophic lateral sclerosis (Arenas et al., 1995, Zurn et al., 1994, Zurn et al., 1996).

GDNF is part of the GDNF family of growth factors which also includes neurturin, artemin and persevin (Kotzbauer et al., 1996). The GDNF trophic factors are classified by the structural composition of seven conserved cysteine residues, similarly spaced, that form dimers in order to function (Ibanez, 1998). Due to approximately 20% homology, GDNF and its associated trophic factors are considered to technically be part of the TGF β superfamily (Airaksinen and Saarma, 2002). The GDNF family functions via a two-component receptor complex: GDNF family receptor alpha (GFR α), which is a glucosylphosphoinositol-linked surface receptor, and RET, a receptor tyrosine kinase. Four receptor GFR α subtypes (GFR α types 1–4) have been identified but the specificity

for the receptors is not exclusive; for example, neurturin can act through the GDNF receptor GFR α 1, as well as through GFR α 2 (Bespalov and Saarma, 2007).

Because neurotrophins are large homodimer molecules, they do not readily cross the blood-brain barrier and thus, are delivered directly to the brain using a variety of methods which will be discussed in detail in this section (Kordower et al., 1993).

1.3.3 PRECLINICAL STUDIES

Following the identification of GDNF as a potent dopaminergic neuroprotective agent (Lin et al., 1993), extensive investigative efforts were launched into its neuroprotective effects in animal models of Parkinson's disease. Initial preclinical trials in both rodent and primate models of the disease examined the effects of GDNF injection or infusion into the lateral ventricles or brain parenchyma (Hurelbrink and Barker, 2004). These studies have demonstrated that GDNF is not only protective of dopaminergic neurons when challenged with degenerative toxins, but also may have restorative effects on dying dopaminergic neurons (when the protein is administered before or after a lesion, respectively) (Aoi et al., 2000). Intrastratial administration of the protein resulted in the most consistent improvements in dopamine neuron survival, dopamine fibre outgrowth and behavioural motor impairment (Kirik et al., 2000a, Tomac et al., 1995) however beneficial results of both intra-nigral (Hoffer et al., 1994, Kearns and Gash, 1995, Tomac et al., 1995) and intracerebroventricular administration of GDNF have also been observed (Bowenkamp et al., 1997, Kirik et al., 2001).

As an alternative approach to the direct protein infusion of GDNF, viral vectors have also been assessed for their ability to provide long-term expression of GDNF in animal models of Parkinson's disease. Adenoviral vectors (Choi-Lundberg et al., 1998), adeno-associated viral vectors (Kirik et al., 2000b) and lentiviral vectors (Dowd et al., 2005b, Georgievska et al., 2002) have each been harnessed in order to induce long-term expression of GDNF in the both the substantia nigra and striatum of animal models of Parkinson's disease. These have shown promise by increasing dopaminergic neuronal

survival and attenuating the behavioural motor deficits (Hurelbrink and Barker, 2004). As with the direct brain infusion of the protein, striatal delivery of the viral vectors have shown superior results (Connor et al., 1999) and in fact, injection of the vector into the striatum allows targeting of the dopaminergic neurons in the substantia nigra due to the retrograde transport of the virus from axonal terminals in the striatum to the degenerating nigral cell bodies (Ridoux et al., 1994).

It is however important to note that while the neuroprotective effect of GDNF has been extensively studied in various toxin-induced models of Parkinson's disease such as the 6-hydroxydopamine and MPTP models, viral vector-mediated delivery of GDNF failed to induce neuroprotective effects in the α -synuclein model of Parkinson's disease (Decressac et al., 2011). This is suggested to be due to α -synuclein-induced downregulation of the transcription factor, Nurr1, and its downstream target, the GDNF receptor RET (Decressac et al., 2012). Prior to this discovery however, the benefits of GDNF in preclinical models incited the evaluation of the infused protein in clinical trials for patients with Parkinson's disease.

1.3.4 PROGRESSION OF GDNF TO CLINICAL TRIALS

The neuroprotective and neuroregenerative effects of intracerebral GDNF observed in preclinical studies prompted its investigation in human clinical trials. The first of these trials was a multicentre, randomized, double-blind, placebo-controlled trial which involved the delivery of 0-4,000 μ g of GDNF protein via an implanted intracerebroventricular catheter and access port in patients with advanced Parkinson's disease (Nutt et al., 2003). The study design was largely based on the positive results of monthly intracerebroventricular delivery of GDNF in MPTP-induced hemi-Parkinsonian monkeys (Gash et al., 1996). The trial however proved that this GDNF delivery regime and delivery site lacked therapeutic efficacy and was indeed inappropriate as it was associated with various side effects such as nausea, vomiting, anorexia, weight loss, with some of these adverse events capable of being reversed upon cessation of GDNF. While these results were obviously discouraging, the authors

concluded that the intracerebroventricular-infused GDNF may not have reached the intended target (the putamen and substantia nigra) and therefore this may explain the lack of efficacy observed and various off-target effects (Nutt et al., 2003). Consequently, delivery of the protein directly into the putamen of Parkinson's disease patients was assessed in two open-label clinical trials.

1.3.4.1 The Bristol Trial

In this open-labelled trial performed in Bristol, UK, 5 patients with advanced Parkinson's disease were infused either unilaterally (1 patient) or bilaterally (4 patients) with between 14.4 and 42.4 µg/day of GDNF directly into the posterior putamen via implanted catheters and SynchroMed pumps. An improvement of 39% (on) and 24% (off) in the Unified Parkinson's Disease Rating Scale (UPDRS) was initially reported in the first three months which extended to 17% (on) and 33% (off) at 12 months (Gill et al., 2003). Medication-induced dyskinesias were found to be reduced by 64% and [¹⁸F] Fluorodopa positron emission tomography (PET) scans of dopamine uptake showed a significant 28% increase in putamen dopamine storage after 18 months. These beneficial motor effects subsequent to intraputaminal infusion of GDNF were sustained up to 2 years (Patel et al., 2005). Notably, approximately 3 months after GDNF cessation, following unrelated myocardial infarction, a man who had received 43 months of intraputaminal infusion of GDNF exhibited a marked local increase in tyrosine hydroxylase-immunopositive nerve fibres in the striatum and sprouting of fibres in the substantia nigra at autopsy (Love et al., 2005). Furthermore, the adverse effects that hampered the Nutt trial (Nutt et al., 2003) were not present in this open-label trial which made this dosing regimen and delivery method all the more promising.

1.3.4.2 The Kentucky Trial

In this open-labelled University of Kentucky trial, 10 patients with advanced Parkinson's disease were infused unilaterally with escalating doses of GDNF directly into the anterior putamen via an implanted Medtronic catheter with multiple ports to

attempt increased protein diffusion throughout the target region. The dosing regimen involved a dose-escalation protocol initiating at 3 µg/day for 2 months, rising to 10 µg/day for 2 months and finally peaking at 30 µg/day for 2 months. This was in turn followed by a wash-out period of 1 month. UPDRS motor scores improved by 30% (on and off states) at 24 weeks and continued through the wash-out period. Improvements were reported to have occurred bilaterally, despite unilateral delivery of the protein, although this was measured by balance, gait and increased speed of hand movements rather than reported bilateral UPDRS scores (Slevin et al., 2005). Approximately 30% of patients also reported a reduction in the occurrence of drug-related dyskinesia (Sherer et al., 2006). The trial was halted by the drug sponsor due to cerebellar toxicity in a concurrent GDNF infusion trial in rhesus monkeys (Hovland et al., 2007) and the patients were monitored for an additional 1 year during which the effects of drug withdrawal were evaluated (Slevin et al., 2007). Benefits from GDNF treatment were found to be lost by 9 to 12 months after GDNF infusion was discontinued. UPDRS scores returned to their baseline and the patients subsequently needed higher levels of conventional anti-Parkinsonian drugs than was required prior to GDNF infusion to provide symptomatic relief (Slevin et al., 2007).

1.3.5 DOUBLE-BLIND PLACEBO-CONTROLLED TRIAL (AMGEN-SPONSORED TRIAL)

The observations of the Bristol trial were the basis for the design of a randomized, double-blind, placebo-controlled trial of bilateral continuous GDNF infusion in 34 patients with advanced Parkinson's disease sponsored by Amgen Inc. The patients were randomized to receive 15 µg/putamen/day of GDNF or placebo directly into the motor putamen via a single port catheter implanted bilaterally. Unfortunately, the trial failed to meet the primary outcome measurement of efficacy (pre-defined as an improvement of 25% in UPDRS score between GDNF and placebo), as UPDRS scores improved by just 10% in the GDNF treated group compared to 4.5% improvement in the placebo group. While there was an increase of 23% in [¹⁸F] Fluorodopa uptake, which was comparable to that observed in the Bristol trial, this effect was limited to the immediate vicinity of the implanted catheter tip (Lang et al., 2006). Ultimately, the development of GDNF

antibodies in this trial and the open-label trials (Gill et al., 2003, Slevin et al., 2005), as well as the reported cerebellar toxicity in a concurrent primate study (Hovland et al., 2007), led to the termination of the trial by the drug sponsor.

1.3.6 ISSUES RELATING TO GDNF DELIVERY

Administration of GDNF remains an active area of investigation in Parkinson's disease, despite the various setbacks provided by negative results of the double-blind placebo-controlled trial and the safety concerns highlighted by the development of antibodies to GDNF and side effects observed in this trial. It is of utmost importance that the experts in the Parkinson's disease field of research do not base their judgement regarding the potential of GDNF therapy on such a small number of patients. Promising results in the open label GDNF trials were not replicated in the double-blind placebo-controlled trial, possibly due to the differential designs of these trials. The main differences in trial design are summarized in **Table 1.2** below, including aspects of the trials such as patient selection, dose and delivery of GDNF.

Despite the obvious shortcomings in these clinical trials, these studies were essential as proof-of-principle studies for use of the neurotrophic factor. However the differences in the results of these three trials have led to much controversy, which prompted the Michael J. Fox Foundation for Parkinson's Research and the Kinetics Foundation to convene researchers with expertise in the field to hold a meeting, and discuss the resulting issues and uncertainties involved. This meeting led to a thoughtful comparison of the studies and suggestions of how to move forward the study of trophic factors in Parkinson's disease (Sherer et al., 2006) and ultimately concluded that the uncertainties surrounding the trials of intraputaminial infusion of GDNF should not end the search for therapeutic benefit from neurotrophic factors (Peterson and Nutt, 2008).

The Michael J. Fox Foundation meeting (published by Sherer et al., (2006)) highlighted the importance of a more advanced and uniform delivery and diffusion method. They and others have concluded that the variable catheters, pumps and methods used to

deliver the protein in the clinical trials produced relatively poor distribution throughout the putamen which contributed to the modest results obtained in the open label trials, and indeed the negative results obtained in the double-blind placebo-controlled trial (Lang et al., 2006, Sherer et al., 2006, Salvatore et al., 2006, Morrison et al., 2007). This concern is further supported by the fact that in the seminal paper reporting on the positive results with GDNF in the Bristol open label trial, Gill also reported that a better means of delivering the protein into the brain was required than that provided by the implanted hardware and cannula that they used (Gill et al., 2003). Documented complications associated with the use of implanted hardware and cannula include the formation of neutralising antibodies to GDNF (Hovland et al., 2007, Lang et al., 2006), although this was most likely attributed to leaking of the protein while the subdermally implanted diffusion pump was being routinely filled with GDNF (Hovland et al., 2007).

The scientific evidence for the neuroprotective efficacy of neurotrophic factors is well-established and spans several decades, however, attempts at clinical translation of the promising therapy have been largely disappointing because the technology required to deliver these complex proteins in a safe, controlled and continuous manner to specific, targeted areas of the brain remains grossly inadequate (Bartus, 2012). This has been addressed in part by the development of convection-enhanced delivery (Laske et al., 1997), which provides a more widespread delivery of the agent than simple diffusion (Hadaczek et al., 2006). This method is not without concern as convection-enhanced delivery may cause off-target effects as agents delivered in this manner often do not respect the anatomical boundaries of the nuclei into which they are injected (Peterson and Nutt, 2008), however this may be addressed by intermittent delivery over prolonged periods of time (Barua et al., 2013).

While these technological modifications are promising, it means a lifetime of GDNF injections for the patient which would obviously incur substantial cost. It has therefore been postulated by Sherer and others that 'Future approaches such as gene therapy or cells engineered to produce GDNF, may prove more effective for providing local and sustained delivery of GDNF than simple direct brain infusion' (Sherer et al., 2006). This

would effectively raise the concentration of the therapeutic trophic factor in the target tissue via implantation of cells programmed to make and secrete the trophic factor of interest (Peterson and Nutt, 2008).

	Bristol Trial	Kentucky Trial	Amgen Trial
Trial Design	Open-label	Open-label	Randomized, double-blind, placebo-controlled
Patients	5 PD patients with advanced disease, mostly young onset: 1 unilateral, 4 bilateral	10 PD patients with advanced disease: all unilateral	34 patients with advanced disease (17 receive GDNF, 17 placebo): stable UPDRS before study: all bilateral
Length of study	Initial report at 6 months; patients were treated up to 3.5 years	6 months, followed by washout; patients have continued to be followed	6 months
Dosage	Not standardized; different doses for each patient; range from 14.4 to 42.4 µg/day	3 µg/day for 2 months, 10 µg/day for 2 months, 30 µg/day for 2 months; 1 month washout	15 µg/putamen/day
Delivery	Catheter may have provided convection-enhanced delivery, but was not FDA-approved	Medtronic multi-port catheter; idle phase of delivery followed by pulses every 6 hours	Single-port Medtronic catheter (larger than Bristol catheter)
Outcomes (efficacy)	Improvements in motor UPDRS: <i>on</i> (39%) and <i>off</i> (24%) at 3 months, <i>on</i> (17%) and <i>off</i> (32%) at 6 months; further improvement over time	Improvements in motor UPDRS: <i>on</i> and <i>off</i> (30%) at 6 months, up to 45% at 12 months; diminished effects upon GDNF cessation	No significant improvement observed (25% difference between active and placebo groups sought)
Outcomes (safety)	No major side effects; one patient developed antibodies	No major side effects; one patient developed antibodies	Anti-GDNF antibodies in four patients; cerebellar toxicity in concurrent primate study
[¹⁸F]dopa PET results	28% increase in putamen at 18 months	Unknown	32.5% increase in posterior putamen
Trial completed?	Yes	Trial halted by sponsor	Trial halted by sponsor

Table 1.2 The different clinical designs and study outcomes of the three clinical trials focusing on intraputaminial GDNF delivery in Parkinson's disease. Adapted from Sherer et al., (2006).

1.4 *EX VIVO* GDNF DELIVERY

Ex vivo gene therapy has a number of distinct advantages over direct delivery of a virus to the brain. As the viral transduction occurs ‘in the dish’, all free viral particles that may bear potential risk to the recipient, are eliminated at an early stage prior to transplantation. This avoids the complication of host response to the virus, and with the intelligent selection of the potential candidate cell type, this risk of host immune response can be minimised further. After *ex vivo* transduction, the cells would constitutively express the protein of interest and would effectively become an implanted biological minipump for the release and diffusion of the protein to the region of interest. This section will review the beneficial characteristics of MSCs for use as potential cell candidates for *ex vivo* GDNF gene therapy, ultimately for use in the treatment of Parkinson’s disease.

1.4.1 RATIONALE FOR USE OF MSCs AS A POTENTIAL CELL TYPE FOR NEUROTROPHIN DELIVERY

MSCs have long been viewed as an exciting tool for regenerative cell therapy. MSCs are multipotent adult stem cells that can be isolated from most adult tissues, including bone marrow, adipose, liver, amniotic fluid, lung, skeletal muscle and kidney (Yi and Song, 2012). In terms of the clinical applications of MSCs, they are being tested in four main areas: as tools for tissue regeneration for cartilage, bone, muscle, tendon and neuronal cells; as cell vehicles for gene therapy; to enhance hematopoietic stem cell engraftment; and finally for treatment of conditions such as graft versus host disease (Zhao et al., 2014), stroke (Calio et al., 2014), myocardial infarction (Santos Nascimento et al., 2014), as well as many other conditions (Yi and Song, 2012), including Alzheimer's disease (Lee et al., 2009), amyotrophic lateral sclerosis (Mazzini et al., 2006), Huntington's disease (Lin et al., 2011) and multiple sclerosis (Karussis et al., 2008).

1.4.2 BENEFICIAL CHARACTERISTIC OF MSCs

MSCs were first identified by Friedenstein and colleagues as a small population of nucleated cells that could be isolated from the bone marrow, capable of adhering to tissue culture plastic while remaining distinct from hematopoietic stem cells (Friedenstein et al., 1970). Unfortunately, no definitive marker for the identification of MSCs has been discovered and therefore they are characterised by the positive expression of the surface markers CD73, CD90 and CD 105 and negative expression for CD11b, CD14, CD19, CD79a, CD34, CD45 and HLA-DR (Dominici et al., 2006, Horwitz et al., 2005). MSCs also have the characteristic ability to differentiate into osteoblasts, adipocytes and chondrocytes *in vitro* (Pittenger et al., 1999). While initially MSCs were identified as a cell population in the bone marrow, they have now been isolated from almost all tissues of the body (da Silva Meirelles et al., 2006). MSCs have also been shown to inherently produce neurotrophic factors and growth factors that protect and induce regeneration of damaged tissue (Kurozumi et al., 2005) and their hypoinmunogenic properties have been researched extensively (Bartholomew et al., 2002, Di Nicola et al., 2002, Moloney et al., 2010a).

1.4.2.1 Isolation and Culture of MSCs

MSCs are easily attainable from the bone marrow aspirate of healthy donors (Pittenger et al., 1999) and, if isolated from patient tissue, offer an autologous cell source (Zhang et al., 2008). Using only small volumes of bone marrow aspirate, they are easily cultured and expanded *in vitro* to achieve significant numbers for therapeutic use without imposing major ethical issues, and are also amenable to viral manipulation (Bartmann et al., 2007, Digirolamo et al., 1999). MSCs may also be feasibly stored for extensive periods of time using cryopreservation with dimethyl sulfoxide (DMSO), which prevents the degeneration of the stem cells. While DMSO is toxic, its use is necessary to prevent damage to cells via the formation of ice crystal in the frozen aliquots of cells, however detrimental effects of DMSO are reduced through adequate washing of the cells upon removal from cryo-storage (Veeraputhiran et al., 2010).

1.4.2.2 Inherent Neurotrophin Secretion

MSCs have been reported to exert neurotrophic effects via release of a plethora of molecules such as neurotrophic growth factors, chemokines, cytokines, and extracellular matrix proteins that promote endogenous repair either directly or indirectly. Pro-inflammatory stimuli, hypoxic stimuli and factors associated with tissue damage sensed in the local environment all stimulate MSCs to secrete a myriad of neurotrophic factors dubbed the MSC secretome (Chen et al., 2002, Rosova et al., 2008, Paul and Anisimov, 2013). In all probability, it is this secretion of neurotrophic factors into the neural niche microenvironment that mediates and promotes neuroplasticity, cell protection and cell repair in experimental situations (Zhou and Shine, 2003). This is further evidenced by the fact that MSC can exert their neuroprotective effect without direct cell contact, that is, engraftment of MSC is not necessary to protect neurons from inflammation as treatment with conditioned medium from MSCs is sufficient to elicit both behavioural recovery and neuronal regeneration in a model of multiple sclerosis (Bai et al., 2012) and an *in vitro* model of Parkinson's disease (Shintani et al., 2007). Preclinical animal studies have also reported beneficial effects in behaviour and surviving dopaminergic neurons after implantation of MSCs (Blandini et al., 2010). Significantly higher GDNF levels were observed in these animals implanted with MSCs in comparison to the control animals suggesting a direct relationship between preserved dopaminergic neurons, and the increased expression of GDNF.

1.4.2.3 Immunomodulation

Another mechanism by which MSCs exert their reparative benefits is their immunomodulatory capacity (Yi and Song, 2012, Uccelli et al., 2008). MSCs possess potent anti-inflammatory and immunomodulatory characteristics which is unique to this stem cell type, and the main advantage of using autologous MSCs is the fact that there is no need to suppress the host immune system (de Munter et al., 2014). These immunomodulatory properties include suppression of T cell expansion (Di Nicola et al., 2002), suppression of B cell proliferation and differentiation (Budoni et al., 2013), inhibition of natural killer cell proliferation (Spaggiari et al., 2008), reduction of

dendritic cell maturation and function (Beyth et al., 2005), and release anti-inflammatory molecules such as transforming growth factor- β 1 (TGF β 1), hepatocyte growth factor (HGF), interleukin-10 (IL-10), and interleukin-6 (IL-6) (Uccelli et al., 2008). Since inflammation has been recognised to play a substantial role in the pathogenesis of Parkinson's disease (McGeer et al., 1988), it seems entirely appropriate that a cell type that exhibits immunomodulatory properties be investigated for transplantation, and its potential as a vehicle for neurotrophin delivery be exploited for possible treatment of the disease.

1.4.3 PREVIOUS STUDIES USING MSCs FOR GDNF DELIVERY

A considerable advantage of utilising MSCs for *ex vivo* gene therapy is the ease with which they can be manipulated. Using lentiviral or retroviral transduction of the MSCs, efficient long-term integration and expression of the transgene of interest in the cells can be achieved (Wyse et al., 2014). Cells that have been effectively transduced in the culture dish may then be transplanted into rodent or nonhuman primate models of Parkinson's disease. Previously in our laboratory, rat MSCs were genetically engineered by retroviral transduction to overexpress GDNF, and subsequently transplanted into the 6-hydroxydopamine rat model of Parkinson's disease (Moloney et al., 2010b). While the transplantation of GDNF-transduced MSCs did not ameliorate the behavioural consequences of the 6-hydroxydopamine infusion, enhanced tyrosine hydroxylase labelling and increased dopaminergic neuron sprouting at the site of cell transplantation in the striatum was observed (Moloney et al., 2010b). Glavaski-Joksimovic and colleagues (2010) transplanted human MSCs (lentivirally transduced to express GDNF and transiently pre-differentiated with a Notch plasmid) into the striatum one week following induction of a unilateral 6-hydroxydopamine lesion. From this they observed a decrease in amphetamine-induced rotations accompanied with rejuvenated tyrosine hydroxylase fibres at the graft site (Glavaski-Joksimovic et al., 2010). Another group performed intrastriatal injections of MSCs, transduced with a lentivirus to overexpress GDNF, one week before a lactacystin lesion of the medial forebrain bundle (MFB). The results of this study found that transplantation of cells overexpressing GDNF

significantly rescue dopaminergic neurons from lactacystin-induced neurotoxicity with regard to reducing apomorphine-induced rotations, increased tyrosine hydroxylase levels in nigra and striatum, and significantly increased striatal dopamine levels (Wu et al., 2010). Furthermore, Ren and colleagues (2013) transplanted genetically modified autologous MSCs (transduced using a lentivirus to overexpress GDNF) into the striatum and substantia nigra of MPTP-treated non-human primates. Transplantation of these cells increased levels of dopamine in the striatum and improved contralateral limb function, but did not prevent the loss of nigral dopaminergic neurons (Ren et al., 2013).

While the results of these studies are certainly promising, transplantation of GDNF-transduced MSCs has provided rather modest neuroprotective results evidenced by the confinement of dopaminergic regeneration largely to the site of cell transplantation. Variable outcomes and minimal improvements in motor function have also been reported, which is undoubtedly concerning given that the symptoms of the disease are largely motor related. These issues may be circumvented by addressing the limitations associated with this approach, as outlined in the section below.

1.4.4 LIMITATIONS

While MSCs possess many characteristics which make them seemingly ideal for *ex vivo* gene therapy, there are certain limitations associated with their use. In order to fulfil their function as vehicles for neurotrophin delivery to the brain, transduced MSCs must be capable of surviving in the ectopic transplanted environment for prolonged, if not indefinite, periods of time.

Safe and effective cell delivery remains one of the main challenges in cell-based therapy of neurodegenerative disorders. Unfortunately, low survival rate and poor differentiation of MSCs after transplantation *in vivo* has been reported in many different instances (Moloney et al., 2010b, Rossignol et al., 2009, Swanger et al., 2005). This issue is a substantial one, affecting not just MSCs but also the transplantation of fetal dopaminergic cells where approximately 5-20% of cells survive after grafting (Hagell

and Brundin, 2001). Indeed this issue is not unique to the treatment of Parkinson's disease either as poor cell survival rates post-transplantation is a concern for a wide range of cell types being exploited for the treatment of a myriad of diseases and conditions.

Substantial death of transplanted cells has been observed when MSCs are injected directly into the lesioned tissues (Liu et al., 2011, Bakshi et al., 2005), and indeed the delivery method employed for the transplantation of MSCs can greatly affect their survival *in vivo*. MSCs have demonstrated chemotactic properties and an ability to home to the site of injury, which shows promise for non-invasive systemic delivery of cells. However, after systemic administration of MSCs there is often a wide dispersal of cells throughout many other organs beyond the area of injury. In general, peripheral intravenous administration results in an initial concentration of MSCs in the lungs, followed by a gradual increase in cell number at the site of injury (Kang et al., 2012). The most significant challenge to a non-invasive strategy of MSC delivery is the ability of MSCs to cross the blood-brain barrier following systemic delivery (Liu et al., 2013, Steiner et al., 2012, Gutierrez-Fernandez et al., 2013). While there is growing evidence that MSCs may be able to cross the blood-brain barrier (Kean et al., 2013), it is doubtful whether they would be able to do so in sufficient numbers to elicit a therapeutic benefit, thereby necessitating a once-off intracerebral delivery of MSCs.

After transplantation, MSCs are unavoidably exposed to hostile conditions such as hypoxia (Zhu et al., 2006), oxidative stress (Ko et al., 2012), and lack of structural support and reduced access to nutritional enrichment (Emgard et al., 2003, Sortwell et al., 2000), factors which drastically reduce their survival and their consequent therapeutic efficacy. Survival and differentiation of MSCs is strongly dependent upon a permissive microenvironment and as a result, numerous studies have been performed in order to enhance the survival of MSCs against unfavourable milieu of host tissues (Chen et al., 2012, Li et al., 2012, Uemura et al., 2010, Rosova et al., 2008). Despite the wealth of research invested thus far, the unknown factors in this potential therapeutic intervention remain numerous, and an optimal transplantation strategy, delivery method

and treatment regime to successfully increase MSC survival after transplantation has yet to be fully determined before it can be considered a viable therapeutic option.

1.5 POTENTIAL OF BIOMATERIALS FOR IMPROVING *EX VIVO* GENE THERAPY

The significant hurdles that face the field of stem cell delivery are common across the field, regardless of the specific cell type and particular disease to be addressed. These include, but are not limited to, low cell viability after delivery, lack of extracellular physical support, host immune response and lack of trophic support. Innovative delivery strategies could be the key to achieving greater success in cell delivery to the central nervous system (CNS), and the use of biomaterials for delivery of cells may provide the permissive microenvironment that is essential for the daunting task of cell-induced CNS regeneration. The term biomaterial refers to a varied collection of both natural and synthetic biocompatible materials possessing a diverse range of physical and chemical properties (Williams, 2009). Biomaterial-based delivery of stem cells involves their encapsulation within a material in an attempt to provide a niche that enhances the viability of transplanted cells, isolates the transplanted cells from immune attack and ultimately prolongs their function after transplantation (Kim et al., 2012, Pakulska et al., 2012). Employment of biomaterial-based cell delivery platforms may also provide a possible multi-factorial approach to repairing the injured CNS tissue by the combination of delivering cells and therapeutic molecules into the injured CNS (Elliott Donaghue et al., 2014). Injectable hydrogels, in particular, represent a minimally invasive solution to enhance the effectiveness of potential therapeutic strategies for the treatment of neurodegenerative disorders like Parkinson's disease (Albani et al., 2013, Giordano et al., 2009). While the design and optimisation of an injectable hydrogel requires a certain degree of fine-tuning, utilising this strategy may circumvent some of the issues related to stem cell delivery, and indeed transform their therapeutic potential into a valid clinical approach (Albani et al., 2013).

1.5.1 RATIONALE FOR USE OF BIOMATERIALS

While stem cell transplantation may appear promising, cells that are transplanted into a lesioned or degenerating region are subjected to a hostile host environment and subsequently undergo cell death via various mechanisms (Bakshi et al., 2005, Liu et al., 2011, Moloney et al., 2010a). In addition to this, numerous groups have reported that increased cell survival after transplantation is associated with increased functional recovery (Itosaka et al., 2009, Parr et al., 2008, Patel et al., 2010). Therefore it is imperative that stem cell survival is maintained after transplantation in order for the therapy to achieve clinical success.

Prior to transplantation, there are two main mechanisms by which stem cells may undergo cell death in cell culture. These are detachment of cells from their adherent surface and alteration of optimal growth conditions specific to the cell type (Cooke et al., 2010). Cell death due to lack of attachment or inhibition of cell adhesion was first reported in 1994 by Frisch and Francis, and the condition was termed anoikis (a Greek term, meaning the state of being without a home) (Frisch and Francis, 1994). Anoikis can be rescued, and survival of cells increased, by culturing cells on surfaces that have been coated with natural extracellular matrix proteins such as collagen (Hu et al., 2009). However, once cells in sufficient cell numbers for transplantation have been attained in culture, the cells are usually prepared as a single cell suspension for transplantation. This process initiates apoptosis due to loss of connections between integrins native to the cell and the supplemented extracellular matrix proteins (Cooke et al., 2010). Therefore, the presence of a suitable matrix to which cells are able to adhere to both during and after transplantation, may confer increased cell survival when compared to delivering cells in standard transplantation vehicle (Pakulska et al., 2012) (see **Fig 1.5**).

Stem cell survival post-transplantation is further limited by cell death induced by the phagocytic response launched by the host environment following cell delivery (Bacigaluppi et al., 2009). Indeed exaggerated inflammatory host response and the formation of glial and astrocytic scarring may induce apoptosis and necrosis of

transplanted cells, not to mention exacerbate endogenous neuronal death and worsen the disease process itself (Chung et al., 2010).

Both natural and synthetic biomaterial delivery platforms, with the ultimate goal of transplanting cells in a scaffold in order to maintain cell viability and function via the optimisation of a permissive microenvironment are currently in development (Shoichet, 2009). Hydrogels, which have physical and chemical properties similar to the extracellular matrix, hold great potential as scaffolds for cell delivery to the central nervous system (Cooke et al., 2010). Since it is preferable that the biomaterials elicit minimal inflammatory response, natural materials which form hydrogels, such as collagen and agarose, have received significant attention for this purpose (O'Connor et al., 2001, Zhong and Bellamkonda, 2008).

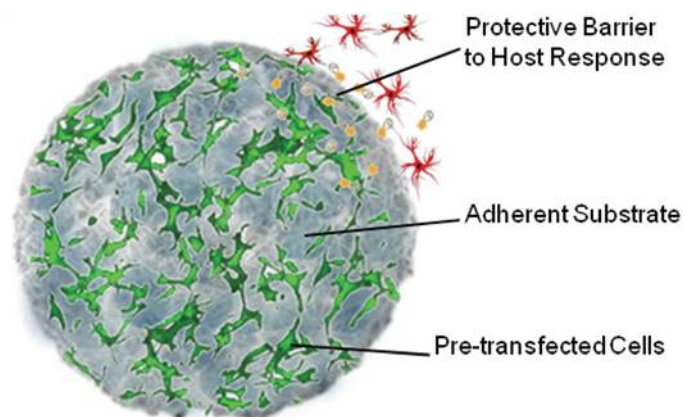


Fig 1.5 Schematic representation of a supportive biomaterial matrix to aid delivery of transduced cells for gene therapy applications in the treatment of neurodegenerative disorders. Modified from Newland et al., (2013b)

1.5.2 WHAT ARE HYDROGELS?

Biological sources are the origin from which natural biomaterials are isolated and purified and these can include chitosan, alginate, methylcellulose, hyaluronan

(polysaccharides) or proteins such as collagen and fibrin. The use of natural polymers bears many advantages resulting from their inherent roles in biological systems. Many natural polymers contain endogenous binding sites for mammalian cells and also possess the ability to elicit minimal inflammatory responses (Heino and Kapyla, 2009, Pradhan and Farach-Carson, 2010). Chemically manufactured synthetic biomaterials also show promise as they are more consistent, and their properties may be manipulated more readily than their natural counterparts. When designing an ideal synthetic biomaterial, or indeed deciding which natural biomaterial to harness for a specific application, properties such as the cell adhesion potential, degradability, shape, pore size, hydrophilicity, mechanical strength, target tissue structure and eventual delivery method must all be considered (Kim et al., 2012). Because of their distinctive properties and potential for biocompatibility, injectable hydrogels are perhaps the most widely investigated materials for local cell or drug delivery to the CNS for regenerative applications (Elliott Donaghue et al., 2014).

Hydrogels are polymer networks which contain high quantities of water dispersed amongst the polymer chains (Hoffman, 2002). Chemical crosslinking of these polymer chains alters the physical properties of the gels allowing their customisation for various tissue engineering applications (Drury and Mooney, 2003). Injectable hydrogels are advantageous for improving tissue regeneration and CNS repair because they have similar mechanical properties to the soft CNS tissue and provide a minimally invasive strategy for drug delivery (Elliott Donaghue et al., 2014). They possess an amenable porous structure which can be altered (by varying the amount of chemical cross-linking (Weber et al., 2009)) to allow for release of growth factors or drugs while also minimising immune cell infiltration. They may also play a role in promoting cell attachment via the potential to attach adhesion factors (Bellamkonda et al., 1995), and even enhance transplanted cell survival by the incorporation of specific growth factors in the hydrogel itself (Wang et al., 2009). The ability of a hydrogel to form a scaffold *in situ* is a very important property, so that intrusive surgical implantation may be avoided, and minimal damage evoked on the delicate tissue of the CNS (Pakulska et al., 2012, Zhong and Bellamkonda, 2008). Additionally, by choosing hydrogel materials that are

biodegradable, the delivery system will eventually be eliminated from the body. Degradable biomaterial systems such as those derived from the natural polymer collagen offer significant translatable potential due to their existing use in the clinic (albeit for alternative applications (Johl and Burgett, 2006)), and thus warrant extensive pre-clinical evaluation as biomaterial matrices in cell transplantation studies.

1.5.3 COLLAGEN HYDROGELS

One of the most common natural hydrogel components investigated as scaffold for biomedical applications is collagen (Chevallay and Herbage, 2000). Collagen accounts for 25-30% of the proteins found in the body, of which approximately 90% is composed of Type 1 collagen (Hosseini et al., 2014). Type 1 collagen can be easily extracted from animal tissues, such as tendons of rat tails and calf skins, and is a fibrous protein composed of three α subunits wound in a triple helical structure forming a rigid superhelix (Cooke et al., 2010). Collagen can be prepared in an injectable form and hydrogels are formed in physiological conditions (through warmth, neutral pH and the presence of salt) by the change in structure of the elongated triple helices to compact coils (Johnson and Christman, 2013, Newland et al., 2013b). This liquid-to-solid transition (gelation) of the collagen hydrogel is advantageous for CNS regeneration as the matrix can be formed *in situ* effectively encapsulating transplanted cells or soluble growth factors (Gonen-Wadmany et al., 2007). MSCs delivered in a collagen hydrogel have previously been shown to have increased cell survival after transplantation in a model of traumatic brain injury, which subsequently led to enhanced functional recovery, in comparison to MSCs delivered in a saline control (Lu et al., 2007).

While natural polymers such as collagen are an attractive option for encapsulation of cells for delivery to the CNS due to their biocompatibility, these protein hydrogels allow limited control of their range of physical properties. These properties are commonly modulated by changing the concentration of the collagen used, however this can simultaneously alter the degradation, cell adhesion properties and the very architecture of the hydrogel itself (Ghajar et al., 2008). Conversely, synthetic polymers

can theoretically provide a range of physical properties that may be easily manipulated. Thus the formation of biosynthetic hydrogels with the biological properties defined by the protein and crosslinking chemistry and architecture of the hydrogel executed by the synthetic polymer, show great promise for the field (Singh et al., 2013).

Once such synthetic polymer that has previously been coupled with both collagen and fibrin is polyethylene glycol (PEG) (Gonen-Wadmany et al., 2007). PEG is a hydrophilic polymer that non-toxic, non-immunogenic, highly water soluble, and which is already approved by the FDA for a number of different clinical indications (Nisbet et al., 2008, Veronese and Pasut, 2005). Covalent attachment of PEG to the collagen hydrogel may protect the protein backbone by slowing the enzymatic degradation of the hydrogel after transplantation to the CNS (Dikovsky et al., 2006, Doillon et al., 1994, Zhang et al., 2006). Attachment of PEG chains to therapeutic peptides or protein drugs has successfully increased their plasma half-life (Veronese, 2001), largely due to the shielding effect PEG has on the protein while simultaneously permitting its natural biological function (Veronese and Pasut, 2005). Crosslinking a collagen hydrogel with PEG may prolong its integrity after transplantation, without affecting the ability of the collagen to act as an extracellular matrix for cell delivery and thus, holds great potential as a strategy to improve *ex vivo* gene therapies for neurological disorders. The employment of a biomaterial matrix may hold benefit for protecting the cells from an inflammatory host response and thus improve this *ex vivo* gene therapy approach.

1.6 AIMS OF THIS THESIS

The overarching aim of this thesis was to develop and validate an *ex vivo* gene therapy approach for the delivery of GDNF, a potential neuroprotective and disease modifying therapy for Parkinson's disease.

Initially we wished to exploit the potent neurotrophic effects that GDNF has been reported to have on dopaminergic neurons (Lin et al., 1993). Specifically, we wanted to assess the ability of GDNF-MSCs to increase the survival of co-transplanted primary

dopaminergic neurons isolated from the developing ventral mesencephalon. Next, we sought to further characterise an inflammation-driven model of Parkinson's disease (which better recapitulates the human disorder), and to determine the neuroprotective capability of transplanted GDNF-MSCs in this model. Finally, we attempted to address the poor survival of transplanted MSCs through a variety of methods including investigating the potential of an alternative delivery method for transplantation (in a Type 1 collagen hydrogel).

Chapter 2: Materials & Methods

2.1 ETHICAL STATEMENT

All procedures were carried out in accordance with the European Union Directive 2010/73/EU and S.I. No. 543 of 2012, were completed under a CAA licence (B100/3827) issued to Dr. Eilís Dowd by the Irish Department of Health and Children, and were reviewed and approved by The Animal Care and Research Ethics Committee of the National University of Ireland, Galway. Any experiments involving the production or use of viral vectors were conducted in accordance with the regulations outlined and approved by the Irish Environmental Protection Agency.

2.2 ANIMAL HUSBANDRY

A total of 215 Sprague Dawley rats were used in this research. Animals were sourced from Charles River, UK or in-house bred where specified. Unless specified otherwise, animals were housed in groups of four per cage, in plastic bottom cages (50.5 x 13 x 24 cm) with a wire grid lid and wood shavings as standard bedding. Animals had access to food and water *ad libitum* (except when food restriction was required for behavioural experiments). In this instance, animals were provided with water *ad libitum*, and provided with enough food to maintain their weight at 85-90% of their free-feeding body weight. This was assessed by comparison of animal weights to an animal growth curve supplied by Charles River. Rats were maintained on a 12:12 h light:dark cycle (lights on at 08:00h), under regulated temperature of 19–23 °C and with the humidity level of the holding room maintained between 40–70%. All behavioural testing and quantitative immunohistochemistry was completed blind to the treatment of the rats.

2.3 GLOBAL EXPERIMENTAL DESIGN

The overarching aim of this project was to develop and validate an *ex vivo* gene therapy approach for delivery of the dopaminergic growth factor, GDNF, to the Parkinsonian

brain. Essentially this project had 3 main objectives: 1) to determine if co-transplantation of GDNF-transduced MSCs with primary dopaminergic neurons can improve the survival of dopaminergic neuron grafts in the Parkinsonian rat brain 2) to determine if delivery of GDNF-transduced MSCs can provide functional neuroprotection in rat models of Parkinson's disease and 3) to improve the survival of adult MSCs in the rat brain, thus permitting their use for long-term intracerebral delivery of GDNF to Parkinsonian rats.

To aid visualisation of intra-cerebrally implanted cells, this project used MSCs extracted from the bone marrow of a GFP transgenic Sprague Dawley rat line (SD-Tg (CAG-EGFP) CZ-004Osb) which will hereafter be referred to as GFP-MSCs. For the GDNF studies, GFP-MSCs that had been stably transduced with a Moloney leukaemia retrovirus to overexpress GDNF were used, which will hereafter be referred to as GDNF-MSCs. All experiments used syngeneic MSC transplants (i.e. Sprague Dawley donor cells to Sprague Dawley hosts).

In the first results chapter, the ability of the secreted GDNF from the GDNF-transduced MSCs to enhance the survival of primary dopaminergic neurons (obtained from ventral mesencephalon dissection) was assessed. Mesencephalic cells were transplanted alone or in combination with GFP-MSCs/GDNF-MSCs to both intact and 6-hydroxydopamine lesioned animals. Primary endpoints included the survival of transplanted tyrosine hydroxylase-positive cells and the effect of MSCs on amphetamine induced rotations in lesioned animals.

In the second results chapter, we sought to further characterise an emerging inflammation-driven model of Parkinson's disease, with a particular focus on the behavioural effects succeeding inflammation and dopaminergic degeneration induced by intracerebral infusion of LPS. Efforts to establish the appropriate dose and site of infusion of LPS to achieve adequate motor dysfunction and nigrostriatal neurodegeneration are included in this chapter. We then went on to assess the potential neuroprotective and neurotrophic effects of transplantation of either GFP-MSCs or

Chapter 2: Materials & Methods

GDNF-MSCs prior to induction of the LPS model. Primary endpoints in this chapter included the emergence of contralateral motor deficit (as assessed by a range of behavioural tests), the induction of neuroinflammation and its ability to lead to nigrostriatal neurodegeneration (by immunohistochemical analysis) and finally, the ability of transplanted MSCs to protect against these events.

Finally, in the third results chapter, a biomaterial-based strategy was employed in an effort to aid and enhance cell delivery and transplantation to the brain. Specifically, a collagen hydrogel was used to deliver GDNF-MSCs to the brain and its effects on cell survival, release of GDNF to the surrounding tissue and host immune response was examined. Once these parameters had been determined, the impact of the collagen hydrogel on the neurotrophic ability of GDNF-MSCs, when transplanted prior to lesion induction, was assessed in the 6-hydroxydopamine model of Parkinson's disease.

This chapter will provide an overview of the different methodologies used throughout the project, while subsequent results chapters will provide more specific methodological detail where relevant.

2.4 CELL CULTURE

2.4.1 ISOLATION, EXPANSION AND CULTURE OF GFP-MSCs AND GDNF-GFP-MSCs

GFP transgenic MSCs (kindly donated by Prof. Anthony Windebank, Mayo Clinic, USA) were extracted from the green transgenic rat SD-Tg (CAG-EGFP) CZ-004Osb as previously described (Okabe et al., 1997). In brief, animals were sacrificed by CO₂ inhalation, after which the femora and tibia were both cleaned of musculature. Bone ends were removed (under sterile conditions) and the bone marrow was flushed from the femora and tibia using Alpha MEM and F-12 and an 18G needle. The flushed marrow was pooled and triturated followed by centrifugation at 500 x g for 5 minutes. Cells were counted and plated at a cell density of 9×10^5 cells cm⁻² in complete rat MSC medium (44.5% Alpha MEM; 44.5% F12; 10% FBS; 1% penicillin/streptomycin). Flasks were cultured under standard conditions (37°C in 5% CO₂ at 90% humidity). After 3 days, any cells which were not adhered to the tissue culture plastic were washed away and the remaining adherent cells were fed with fresh complete medium. The medium was subsequently changed every 3-4 days. After approximately 16-17 days, when colonies began to exhibit a compact appearance, multi-layered growth and/or began to merge into a monolayer, cells were deemed ready for subculture. Cells were enzymatically lifted from the culture plastic by incubation in 0.25% trypsin/1 mM EDTA solution, the enzymatic process was neutralised by addition of complete MSC medium, and cells were re-plated at a cell density of 5.7×10^3 cells cm⁻². Further subculture passages were performed every 4-6 days.

Characterisation of GFP-MSCs was performed by Dr. Gemma Rooney as previously described (Rooney et al., 2008). In brief, MSCs were characterised by differentiation along adipogenic, chondrogenic, and osteogenic lineages. MSCs were also processed for flow cytometric analysis to confirm their MSC phenotype by the expression of markers CD73, CD71 and CD172, and the absence of the markers CD45, CD34, CD14, and CD11b (Rooney et al., 2008).

GFP-MSCs were stably transduced using a Moloney leukaemia virus to overexpress human GDNF driven by the 5' LTR intrinsic promoter (Rooney et al., 2009) and were kindly donated by Dr. Gemma Rooney (NUI Galway's Regenerative Medicine Institute). Expression and secretion of GDNF from GFP-MSCs and GDNF-MSCs was assessed *in vitro* by GDNF ELISA and *in vivo* by GDNF immunohistochemistry.

2.4.2 ENZYME-LINKED IMMUNOSORBENT ASSAY

An enzyme-linked immunosorbent assay (ELISA) was performed in order to assess and quantitatively measure the secretion of GDNF by GFP-MSCs and GDNF-MSCs *in vitro* (human GDNF ELISA; R&D Systems, Minneapolis, MN USA). The assay was performed in triplicate according to the manufacturer's protocol. In brief, wells of a 96 well Nunclon surface microplate (Nunc, Roskilde, Denmark) were incubated overnight with 100 µl of capture antibody in PBS at room temperature. Wells were aspirated and then washed 3 times with wash buffer (0.05% Tween 20 in PBS) using an automated plate washing system (ELx50, Bio-Tek) and blocked for 1 h at room temperature with 100 µl reagent diluent (1% BSA in PBS). Aspiration/wash step was repeated before 100 µl of standards/samples in reagent diluent were added to appropriate wells and incubated at room temperature for 2 h. Aspiration/wash step was repeated once again before 100 µl of detection antibody was added to each well. The plate was incubated for 2 h at room temperature after which the aspiration/wash step was repeated. 100 µl of streptavidin-HRP was added to each well and incubated at room temperature for 20 minutes. Aspiration/wash step was repeated and 100 µl of substrate solution (1:1 ratio H₂O₂ and Tetramethylbenzidine) was then added to each well for 20 minutes. To stop the reaction, 50 µl of stop solution (2N H₂SO₄) was added to each well and the plate gently tapped to ensure thorough mixing. Finally, the plate was read at 450 nm and 570 nm, with the 570 nm reading subtracted from the 450 nm reading to correct for optical imperfections in the plate. Controls included complete MSC medium and MSC medium without FBS.

2.4.3 VENTRAL MESENCEPHALON DISSECTION, PREPARATION AND CULTURE

Mesencephalic tissue was obtained as previously described elsewhere (Dunnett and Bjorklund, 1997). In brief, time-mated Sprague-Dawley rats (either in-house bred or sourced from Charles River, UK) were anaesthetised by inhalational isoflurane (5% in O₂). Anaesthetised pregnant rats were quickly decapitated using a guillotine. Embryonic Day 14 (E14) embryos in the uterine horn were removed from the pregnant female via laparotomy, using a blunt forceps and large scissors. The uterine horn containing the embryos was subsequently immersed in Hank's Balanced Salt Solution (HBSS) (with sodium bicarbonate and without phenol red, Ca²⁺ or Mg²⁺; Sigma H6645) in a 90 mm Petri dish and kept on ice. Further dissections were performed using a dissection microscope. Embryos were removed from the uterine horn and subsequently taken out of their sacs, using a scissors, curved forceps and a tweezers (World Precision Instruments), and placed in a new HBSS containing 90 mm Petri dish on ice. Embryos undergoing dissection were then transferred to the lid of a 90 mm Petri dish containing HBSS. The mesencephalon was dissected out by making an incision at the midbrain-hindbrain boundary, and at the forebrain-midbrain boundary using a dissecting scissors and a curved forceps (World Precision Instruments). The dorsal mesencephalon was then cut lateral to the midline, and the mesencephalon was opened/ flattened out to show the ventral mesencephalon in the centre. A cut at a point between the mid-lateral to medial mesencephalon was then made on one side. The meninges were removed, and a similar incision was subsequently made on the other side of the midline to leave the medial mesencephalon. Cranial and caudal cuts were then made to the medial mesencephalon to ensure no forebrain/ hindbrain tissue was used. Dissected mesencephalic tissue was stored in HBSS in a 15 ml tube on ice until all the embryos were dissected, when tissue culture could be performed immediately.

Dissected mesencephalic tissue was centrifuged at 500 x g for 5 min at 4°C. The tissue pellet was incubated in a 0.1% trypsin-Hank's Balanced Salt solution for 5 min, at 37°C with 5% CO₂. Fetal calf serum (FCS) was then added to the tissue followed by centrifugation at 500 x g for 5 min at 4°C. The resulting cell pellet was resuspended in 1 ml of media (Dulbecco's modified Eagle's medium/F12, 33 mM D-glucose, 1% L-

glutamine, 1% FCS, supplemented with 2% B27) using a P1000 Gilson pipette and carefully triturated using a sterile plugged flame-polished Pasteur pipette, followed by a 25-gauge needle and syringe, ensuring not to add air bubbles into the cell suspension. Once a single cell suspension was obtained, a 20 μl sample of the suspension was taken and mixed with 20 μl of trypan blue. Cells were counted using a haemocytometer. The cell suspension was again centrifuged at 500 x g for 5 minutes and the pellet was re-suspended in the appropriate volume of transplantation medium to give 100,000 cells μl^{-1} (for co-transplant studies, GFP-MSCs/GDNF-MSCs at a density of 33,333 cells μl^{-1} were added to the mesencephalic cells at this point). The cell suspension was maintained on ice for the duration of the surgical procedures and gently triturated prior to infusion to ensure a homogenous solution. 3 μl of the cell suspension was delivered via cannula at a rate of 1 $\mu\text{l min}^{-1}$ with the cannula remaining in site for an additional 2 minutes to allow diffusion of the cells.

2.5 SURGERY

All surgery was performed under isoflurane gaseous anaesthesia (5% in O_2 for induction and 2% in O_2 for maintenance) in a stereotaxic frame with the nose bar set at -2.3 mm (unless otherwise specified). The site of surgery was shaved and rats were secured in the stereotaxic frame using the ear bars. An incision was made through the skin on the head using a scalpel and the skull was exposed. The coordinates for Bregma were established and used to calculate the site for insertion of the cannula. An electric drill was used to expose dura, from which the cannula was lowered to the correct dorso-ventral coordinates. The required suspension of cells or toxin was slowly infused into the desired site by monitoring the movement of a deliberately introduced air bubble in the plastic tubing which connects the steel cannula to a 50 μl Hamilton syringe. The plunger on the Hamilton syringe was depressed at a steady rate of 1 $\mu\text{l min}^{-1}$ using an automated pump (Harvard Apparatus).

2.5.1 MSC CELL TRANSPLANTATION SURGERY

MSCs growing in culture and adhered to culture plastic flasks were washed with Hanks Balanced Salt Solution (Gibco) before being enzymatically lifted from the culture plastic by incubation in 0.25% trypsin/1 mM EDTA solution. The reaction was quenched by adding complete rat MSC medium before transferring the cell suspension to 15 ml tube and triturating the solution a number of times using a sterile Pasteur pipette. The cell suspension was then centrifuged at 500 x g for 5 minutes. The supernatant was discarded and the pellet of cells was re-suspended in 10 ml of complete rat MSC medium. A 20 μ l sample was taken and mixed with 20 μ l of trypan blue. Cells were counted using a haemocytometer. The cell suspension was again centrifuged at 500 x g for 5 minutes and the pellet was re-suspended in the appropriate volume of transplantation medium to give 100,000 cells μ l⁻¹ (unless, where specified, another cell quantity/volume was required). The cell suspension was maintained on ice for the duration of the surgical procedures and gently triturated prior to infusion to ensure a homogenous solution. Cells were delivered via cannula at a rate of 1 μ l min⁻¹ with the cannula remaining in site for an additional 2 minutes to allow diffusion of the cells.

2.5.2 LPS LESION SURGERY

For induction of the LPS lesion, LPS was weighed out into a number of eppendorfs and kept on ice until it was dissolved in 0.09% sterile saline. For the unilateral striatal lesion, rats received 10 μ g of LPS in 2 μ l at the coordinates (from Bregma): AP 0.0 mm, ML \pm 3.7 mm and DV -5.0 mm below dura. For the unilateral nigral lesion, rats received 10 μ g of LPS in 2 μ l at the coordinates (from Bregma): AP -5.3 mm, ML \pm 2.0 mm and DV -7.0 mm below dura. The LPS was delivered via cannula at a rate of 1 μ l min⁻¹ with the cannula remaining in site for an additional 2 minutes to allow diffusion of the inflammagen.

2.5.3 6-HYDROXYDOPAMINE LESION SURGERY

For induction of the 6-hydroxydopamine lesion, 6-hydroxydopamine was weighed out into a number of eppendorfs and kept on ice in the dark until dissolving in 0.01% sterile ascorbate saline, immediately prior to infusion into the brain. For the unilateral striatal lesion, rats received 7 μg of 6-hydroxydopamine (free base) in 2 μl at the coordinates (from Bregma): AP +0.5 mm, ML ± 3.2 mm and DV -5.5 mm below dura. For the unilateral MFB lesion, rats received 12 μg of 6-hydroxydopamine (free base) in 3 μl at the coordinates (from Bregma): AP -4.0 mm, ML ± 1.3 mm and DV -7.0 mm below dura, with the nose bar set to -4.5 mm. The 6-hydroxydopamine was delivered via cannula at a rate of 1 $\mu\text{l min}^{-1}$ with the cannula remaining in site for an additional 2 minutes to allow diffusion of the toxin.

2.5.4 HYDROGEL SURGERY

For the preparation of the collagen hydrogel, all components were placed on ice to prevent premature gelation. For a final hydrogel volume of 100 μl , 50 μl of collagen, neutralised with 1M NaOH, was added to 10 μl of 4x phosphate buffered saline. If cells were transplanted in the collagen hydrogel, 40 μl of the cell suspension was added to an eppendorf containing 40 mg of poly(ethylene glycol) ether tetrasuccinimidyl glutarate (4S-StarPEG) to dissolve this cross-linker. The cell/cross linker solution was then added to the collagen/PBS solution and mixed thoroughly. The cell seeded collagen hydrogel was held on ice prior to transplantation to prevent gelation. For the striatal infusion, rats received 3 μl of collagen/cell-seeded collagen at the coordinates (from Bregma): AP 0.0 mm, ML ± 3.7 mm and DV -5.0 mm below dura. Collagen/cell-seeded collagen was delivered via cannula at a rate of 1 $\mu\text{l min}^{-1}$ with the cannula remaining in site for an additional 2 minutes to allow diffusion.

Section of Thesis	Site	Toxin/Cell Type	Dose/Cell No.	Volume	Coordinates
3.2.1.1	STR	Co-transplant of ventral mesencephalon and GFP/GDNF-MSCs	300,000 mesencephalic cells alone or combined with 100,000 GFP/GDNF-MSCs	3 µl	AP 0.0 mm, ML ±3.7 mm, DV -5.0 mm
3.2.1.2	MFB	6-hydroxydopamine	12 µg of 6-hydroxydopamine (free base)	3 µl	AP -4.0 mm, ML ±1.3 mm, DV -7.0 mm
	STR	Co-transplant of ventral mesencephalon and GFP/GDNF-MSCs	300,000 mesencephalic cells alone or combined with 100,000 GFP/GDNF-MSCs	3 µl	AP 0.0 mm, ML ±3.7 mm, DV -5.0 mm
4.2.1.1	STR	Lipopolysaccharide	10 µg	2 µl	AP 0.0 mm, ML ±3.7 mm, DV -5.0 mm
4.2.1.2	SN	Lipopolysaccharide	10 µg	2 µl	AP -5.3 mm, ML ±2.0 mm, DV -7.0 mm
4.2.1.3	STR	GFP/GDNF-MSCs	200,000 GFP/GDNF-MSCs (100,000 per site)	6 µl	AP +1.5 and -0.5 mm, ML ±2.4 and ±4.0 mm, DV -4.5, -5.5 and -6.5 mm
	SN	Lipopolysaccharide	10 µg	2 µl	AP -5.3 mm, ML ±2.0 mm, DV -7.0 mm
5.2.1.2 A	STR	Hydrogel or Transplantation Media	NA	3 µl	AP 0.0 mm, ML ±3.7 mm, DV -5.0 mm
5.2.1.2 B	STR	GDNF-MSCs in hydrogel or transplantation media	30,000 GDNF-MSCs	3 µl	AP 0.0 mm, ML ±3.7 mm, DV -5.0 mm
5.2.1.3	STR	GDNF-MSCs in hydrogel or transplantation media	60,000 GDNF-MSCs (30,000 per site)	6 µl	AP +1.5 and -0.5 mm, ML ±2.4 and ±4.0 mm, DV -4.5, -5.5 and -6.5 mm
	STR	6-hydroxydopamine	7 µg of 6-hydroxydopamine (free base)	2 µl	AP 0.0 mm, ML ±3.7 mm, DV -5.0 mm
A2.2.1.1	STR	GFP-MSCs	30,000 GFP-MSCs	3 µl	AP 0.0 mm, ML ±3.7 mm, DV -5.0 mm
A2.2.1.2	STR	GFP-MSCs	100,000 GFP-MSCs	3 µl	AP 0.0 mm, ML ±3.7 mm, DV -5.0 mm
A2.2.1.3	STR	GFP-MSCs	100,000 GFP-MSCs	3 µl	AP 0.0 mm, ML ±3.7 mm, DV -5.0 mm

Table 2.1 Summary of the surgical parameters used in this thesis. Section of the thesis corresponds to the experimental design section of each chapter/appendix. All surgeries were performed isoflurane gaseous anaesthesia (5% in O₂ for induction and 2% in O₂ for maintenance) in a stereotaxic frame with the nose bar set at -2.3 mm (except MFB surgeries where the nose bar was set at -4.5 mm). Animal numbers for each study can be found in the relevant experimental design sections of each chapter/appendix.

2.6 BEHAVIOURAL TESTS OF MOTOR IMPAIRMENT

2.6.1 CORRIDOR TEST

The Corridor Test of contralateral sensorimotor neglect was completed as previously described (Dowd et al., 2005b, Fitzsimmons et al., 2006). Rats were food restricted to 85-90% of their free feeding body weight for the duration of testing. The apparatus consisted of two parallel corridors (Dimensions: length=150 cm, height=24.5 cm and width=7 cm). Rats were habituated to the test by allowing the rats to freely explore the empty corridor with CocoPops® scattered along the floor. On days of testing, 10 adjacent pots containing 2-3 CocoPops® were placed at equal intervals along the corridor parallel to the empty corridor, and animals were allowed to freely retrieve CocoPops® from pots to their left or right side (**Fig 2.1 (A)**). Trials were deemed complete once the animal had made a total of 20 retrievals or after the trial time of 5 min was complete. The number of retrievals made from both the ipsilateral and contralateral sides of the rat's body was counted. The number of retrievals made by each rat from the ipsilateral and/or contralateral sides was expressed as a percentage of the total retrievals made. Rats with a unilateral dopaminergic lesion tend to ignore food on their contralateral side and retrieve the CocoPops® almost exclusively from their ipsilateral side.

2.6.2 STEPPING TEST

The Stepping Test of forelimb akinesia was completed as described previously (Olsson et al., 1995). Rats were habituated to the test by restraining them so that both forelimbs were resting on the edge of a table with rear legs resting in the hands of the experimenter. When the rat was habituated to this restraint, one forelimb was restrained and the rat was guided horizontally across a table top at a steady pace (90 cm in 5 s) (**Fig 2.1 (B)**). Habituation of the animals to this test was essential in order to perform the test accurately and prevent false positive results, such as the inclusion of the additional steps that may be taken should a rat attempt to escape from the restraint or

experimenter. On days of testing, the number of adjusting steps made by the free forelimb in the forehand and backhand directions was counted. This was completed on both the ipsilateral and contralateral sides of the rat's body. Data were expressed as the number of ipsilateral or contralateral steps in either the forehand or backhand direction. Unilateral dopaminergic lesions in rats results in impairment in forelimb motor function on their contralateral side.

2.6.3 WHISKER TEST

The Whisker Test of sensorimotor integration was completed as described previously (Schallert et al., 2000). This test is also known as the vibrissae-elicited forelimb placement test and it harnesses the sensorimotor response of the rat - when its whiskers are brushed against a surface (corner of table) a reflex is initiated where the rat reaches out to place the ipsilateral forelimb on the table. This response is impaired on the contralateral side following unilateral dopaminergic lesions. Rats were habituated to this test by restraining the hind limbs and one forelimb of the rat. When the rat was comfortable in this position, the whiskers on the side of the unrestrained forelimb were gently brushed against the side of a table (**Fig 2.1 (C)**). As with the Stepping Test, habituation of animals to this test is of utmost importance to prevent false positive results. On days of testing the number of vibrissae-elicited forelimb placings made by the unrestrained forelimb was counted when the rat's whiskers were brushed against the side of a table top 10 times. This was completed on both the ipsilateral and contralateral sides of the rat's body. Data were expressed as the number of ipsilateral or contralateral forelimb placings.

2.6.4 AMPHETAMINE-INDUCED ROTATIONS

Amphetamine rotational behaviour was assessed as previously described (Ungerstedt and Arbuthnott, 1970). Rats received a single injection of D-amphetamine hydrochloride (2.5 mg kg⁻¹ i.p.) before they are placed in a cylinder containing standard

bedding material (**Fig 2.1 (D)**). Full body rotations were counted in ten 1-minute time bins over a period of 60 minutes. Data was expressed as net ipsilateral turns min^{-1} .

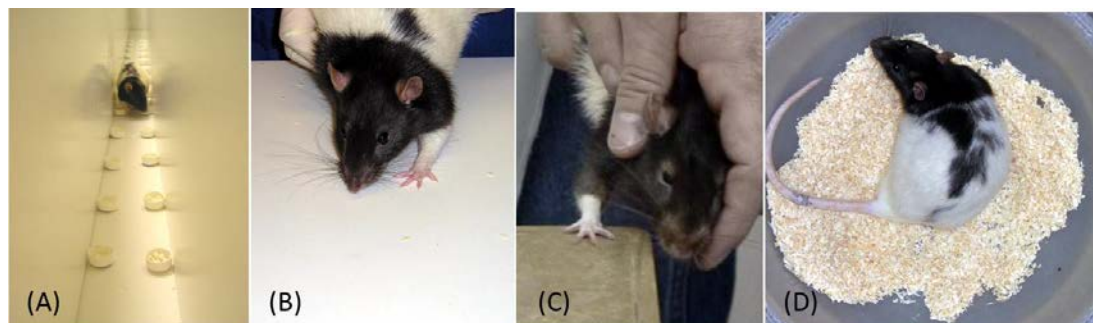


Fig 2.1 Behavioural Tests of motor impairment. (A) The Corridor Test – Rats were free to choose CocoPops® from 10 pairs of adjacent pots on either side of their bodies. (B) The Stepping Test – One forelimb was restrained and the number of adjusting steps made by the unrestrained limb, as the rat was moved along a table top, was counted. (C) The Whisker Test – One forelimb was restrained and the number of forelimb placements made by the unrestrained limb, when the rat's whiskers were brushed against the corner of a table, was counted. (D) Amphetamine-induced rotations – The number of ipsilateral and contralateral turns per minute were counted for 60 minutes in 10 1-minute time bins after the rat has been administered with D-amphetamine hydrochloride at 2.5 mg kg^{-1} i.p.

2.7 IMMUNOHISTOCHEMISTRY

2.7.1 TISSUE PROCESSING

Rats were anaesthetised by injection of sodium pentobarbital (50 mg kg^{-1} i.p., Dolethal, Vétquinol, Dublin, Ireland) and perfused transcardially with heparinised saline (5000 units litre^{-1}) followed by 150 ml of ice cold paraformaldehyde. Brains were rapidly removed, post-fixed in 4% paraformaldehyde for 24 hours and cryoprotected in 25% sucrose plus 0.1% sodium azide solution. Serial coronal sections ($30 \mu\text{m}$) were cut using a freezing stage sledge microtome (Bright, Cambridgeshire, England), and collected in a series of 12. 1:12 or 1:6 (where indicated) series of sections was used for all quantitative immunohistochemistry.

2.7.2 IMMUNOHISTOCHEMISTRY

Free floating immunohistochemical staining was performed using the streptavidin-biotin-peroxidase method (**Fig 2.2**) (see Appendix 3 for more details). In brief, endogenous peroxidase activity was quenched using a solution of 3% hydrogen peroxide/10% methanol in distilled water. Non-specific antibody binding was blocked using a solution of 3% normal serum (dependent on 2° antibody host) in tris-buffered saline (TBS) with 0.2% Triton X-100 at room temperature for 1 hour. Appropriate primary antibody (see **Table 2.2**) diluted in TBS with 0.2% Triton X-100 was added to sections and allowed to incubate at room temperature overnight. Corresponding biotinylated secondary antibody (see **Table 2.3**) was then added to the sections and incubated for three hours. A streptavidin–biotin–horseradish peroxidase solution (Vector, UK (PK6100)) was then added to the sections and allowed to incubate for 2 hours. Staining was developed using a 0.5% solution of diaminobenzidine tetrahydrochloride (DAB) (Sigma, Ireland (D5637)) in TBS containing 0.3 $\mu\text{l ml}^{-1}$ of hydrogen peroxide. Sections were mounted onto gelatin-coated slides, dehydrated in an ascending series of alcohols, cleared in xylene and coverslipped using DPX mountant (BDH chemicals, UK).

For staining using immunofluorescence, the protocol is performed as above until incubation of the sections in secondary antibody. At this stage, an appropriate fluorophore-labelled secondary antibody (see **Table 2.3**) was added to sections and incubated at room temperature for 3 hours. Sections were mounted onto gelatin-coated slides and coverslipped using the fluorescence mounting medium Fluoromount (Sigma).

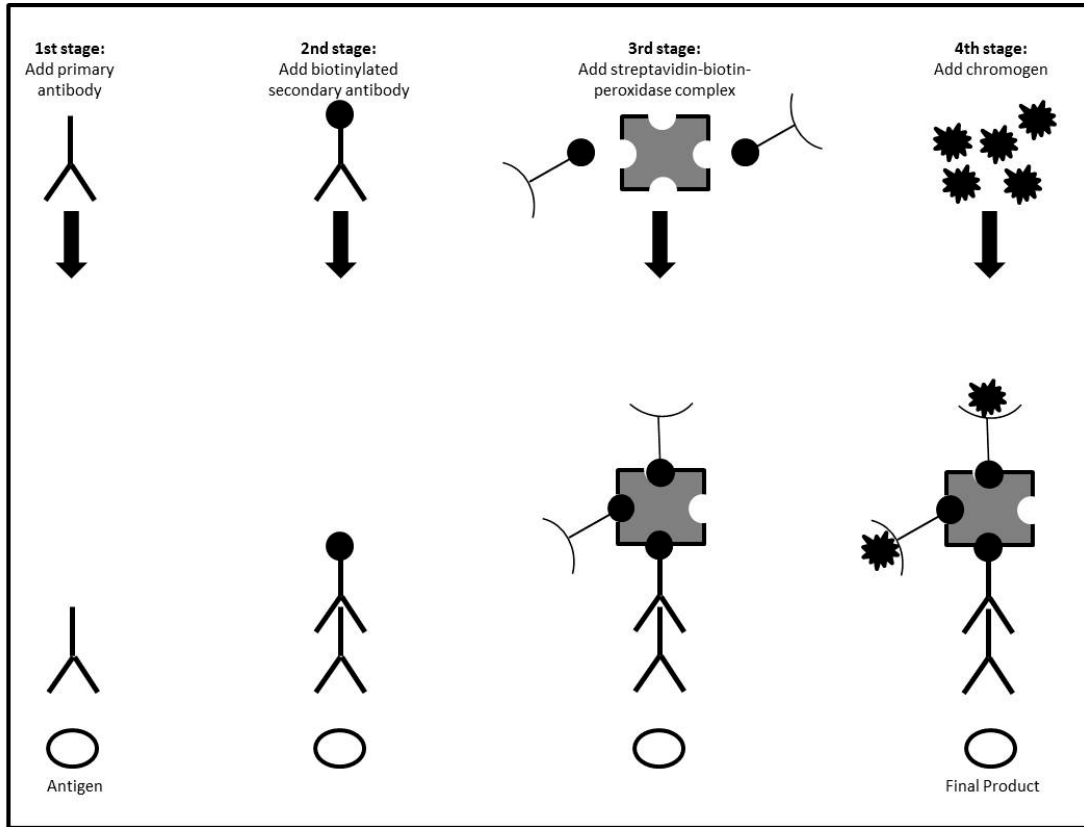


Fig 2.2 Schematic illustrating the stages involved in immunohistochemistry

Primary Antibody	Target	Source	Host	Dilution	Application
Glial cell line-derived neurotrophic factor (GDNF)	GDNF	R&D Systems	Goat	1:200	IHC
Glial fibrillary acidic protein (GFAP)	Astrocytes	DAKO	Rabbit	1:2000	IHC, IF
OX-42/CD11b	Microglia	Chemicon	Mouse	1:400	IHC, IF
Tyrosine Hydroxylase (TH)	Catacholaminergic neurons	Millipore	Mouse	1:1000	IHC
Collagen	Collagen hydrogel	Abcam	Rabbit	1:1000	IHC

Table 2.2 A list of the primary antibodies used in this thesis. IHC: Immunohistochemistry; IF: Immunofluorescence

Secondary Antibody	Source	Host	Reactivity	Dilution	Application
Biotinylated	Vector	Horse	Mouse	1:200	IHC
Biotinylated	Vector	Horse	Goat	1:200	IHC
Biotinylated	Jackson	Goat	Rabbit	1:200	IHC
A.F. 546	Invitrogen	Goat	Mouse	1:200	IF
A.F. 546	Invitrogen	Goat	Rabbit	1:200	IF

Table 2.3 A list of the secondary antibodies used in this thesis. IHC: Immunohistochemistry; IF: Immunofluorescence

2.8 HISTOLOGICAL QUANTIFICATION

All image analysis was performed using ImageJ software (U.S. National Institutes of Health, Bethesda, Maryland, USA). In all instances, graft volume was used as a measure of cell survival and this was quantified using an equation based on Cavalieri's Principle (which is described in detail below). Transplanted fetal dopaminergic cells (dissected from the ventral mesencephalon) were quantified by counting individual tyrosine hydroxylase-positive cell bodies in the transplanted region. Nigral dopaminergic quantification was assessed by counting individual tyrosine hydroxylase-positive cell bodies. Striatal dopaminergic quantification was assessed by measuring the optical density of tyrosine hydroxylase -positive striatal fibres and/or the volume of tyrosine hydroxylase -positive striatal terminals also by application of Cavalieri's Principle. Similarly, microglial reactivity, astrocytic response, GDNF release and collagen staining were quantified using both optical density and volumetric analysis.

2.8.1 CAVALIERI'S PRINCIPLE

Cavalieri's principle was used to perform all volumetric analysis in this thesis. It states that 'the volume (V) of an arbitrary shaped object can be estimated in an unbiased manner from the product of the distance between planes (T_T) and the sum of the areas on systematic random parallel sections through the object' (Garcia et al., 2007). The equation used to determine volume (of the graft and other parameters listed above) was:

$$V = T_T \times \sum_{i=1}^i A_i,$$

$$T_T = NS \times D$$

- NS is the number of section in the series (i.e. 6 series)
- D is the tissue sectioned at known distances (i.e. 30 μm)
- (Area) A_i was determined using ImageJ software as detailed below.

2.8.2 IMAGEJ TO MEASURE AREA/VOLUME

The following method to assess area was used throughout this thesis, including as a step towards determining volume. To determine graft volume, fluorescent photomicrographs of striatal sections containing the transplanted GFP-MSCs/GDNF-MSCs were taken using an Olympus IX81 fluorescent microscope (Olympus UK, London, United Kingdom). An image of a graticule was also captured at the magnification and saved at the same pixel quality as the fluorescent photomicrographs to be analysed. The software programme ImageJ was calibrated using this graticule image by associating the number of pixels with a known distance. For each animal the area of the MSC graft in each striatal section was determined in a 1 in 6 series (unless otherwise indicated in the text), using ImageJ freehand drawing tool to outline and measure the area (see **Fig 2.3**). Using these measurements for area, volume was assessed (using the above equation) according to Cavalieri's Principle. This method was also used to determine the area and volume of staining for microglia, astrocytes, GDNF, collagen and tyrosine hydroxylase staining where indicated in the results chapters of this thesis.

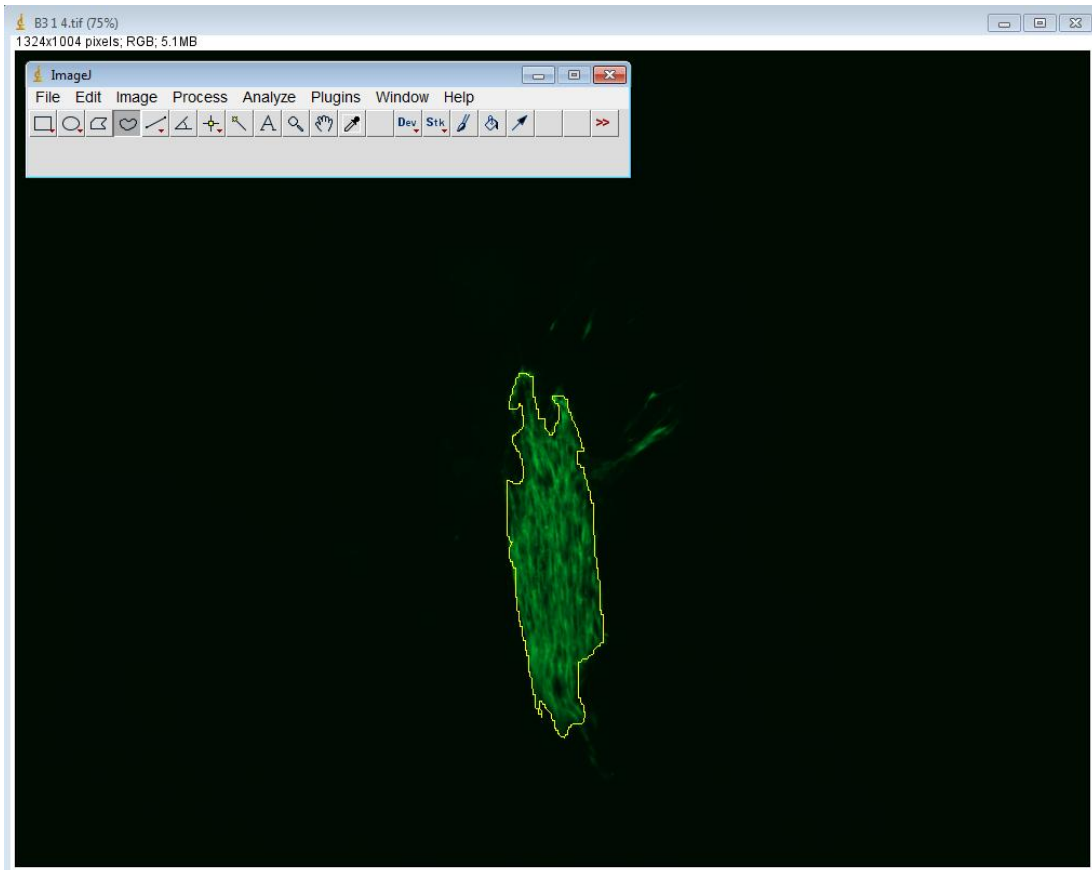


Fig 2.3 Screen grab of the ImageJ software used to determine area. The GFP-MSC graft was outlined using the freehand drawing tool in ImageJ (yellow outline on photomicrograph). As the pixel number of the photomicrograph corresponded to a known distance (calibrated using an image of a graticule taken under the same magnification and pixel quality conditions), the area of the graft in each section can be determined.

2.8.3 QUANTIFICATION OF TYROSINE HYDROXYLASE-POSITIVE CELL BODIES

The number of tyrosine hydroxylase-positive cell bodies in the substantia nigra was counted using the cell counter tool in ImageJ (see **Fig 2.4**). For each animal, photomicrographs of the region were obtained using an Olympus microscope BX40 and Olympus C5060 digital camera. The numbers of tyrosine hydroxylase-positive cell bodies in the substantia nigra were counted both ipsilateral and contralateral to the lesion according to the distinct boundaries as previously defined (Kirik et al., 1998). Briefly, the area was defined to include the tyrosine hydroxylase-positive cell bodies of

the substantia nigra *pars reticulata*, *pars lateralis* and *pars compacta* but to exclude the tyrosine hydroxylase-positive cell bodies of the ventral tegmental area (VTA). Data were expressed as percentage of the intact side. This method of quantification (cell counting) was also employed to determine the number of surviving tyrosine hydroxylase-positive cells after ventral mesencephalon transplantation to the striatum (as indicated in the results chapters of this thesis).

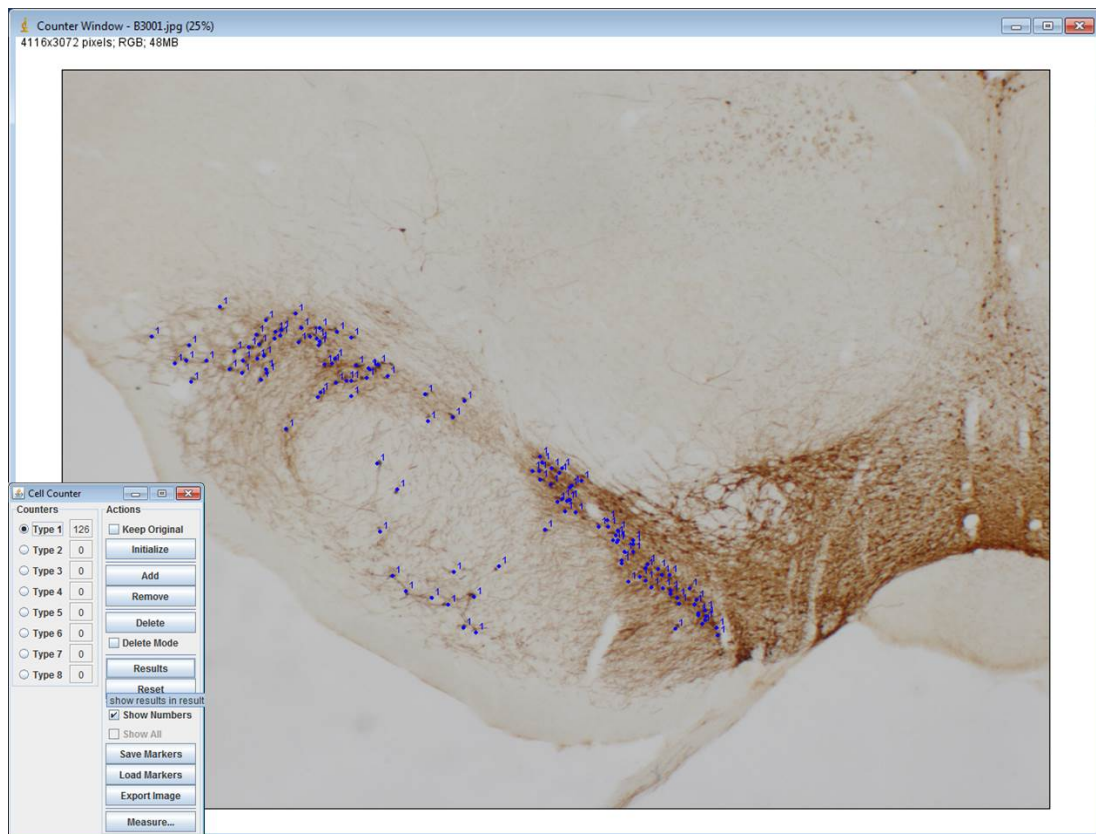


Fig 2.4 Screen grab of the ImageJ software used to determine tyrosine hydroxylase -positive cell bodies in the substantia nigra. Blue counter dots on the image indicate the location of cell bodies. For each section, counts were performed on both the left and right substantia nigra.

2.8.4 STRIATAL FIBRE DENSITY MEASUREMENTS

Tyrosine hydroxylase-positive fibres in the striatum were quantified using optical density measurement in ImageJ. For each animal, photomicrographs were obtained using a Nikon SMZ800 microscope with a DXM1200C digital camera under the same

conditions of exposure magnification and pixel quality. For analysis, images were converted to 8-bit black and white within ImageJ. The entire dorsal striatum was delineated using the freehand drawing tool and the mean grey value was measured. To estimate the specific tyrosine hydroxylase staining density, the optical density readings were corrected for non-specific background density, as measured from unstained tissue in the sections. Measurements were taken on both the ipsilateral and contralateral sides of the brain and data were expressed as a percentage of the intact side.

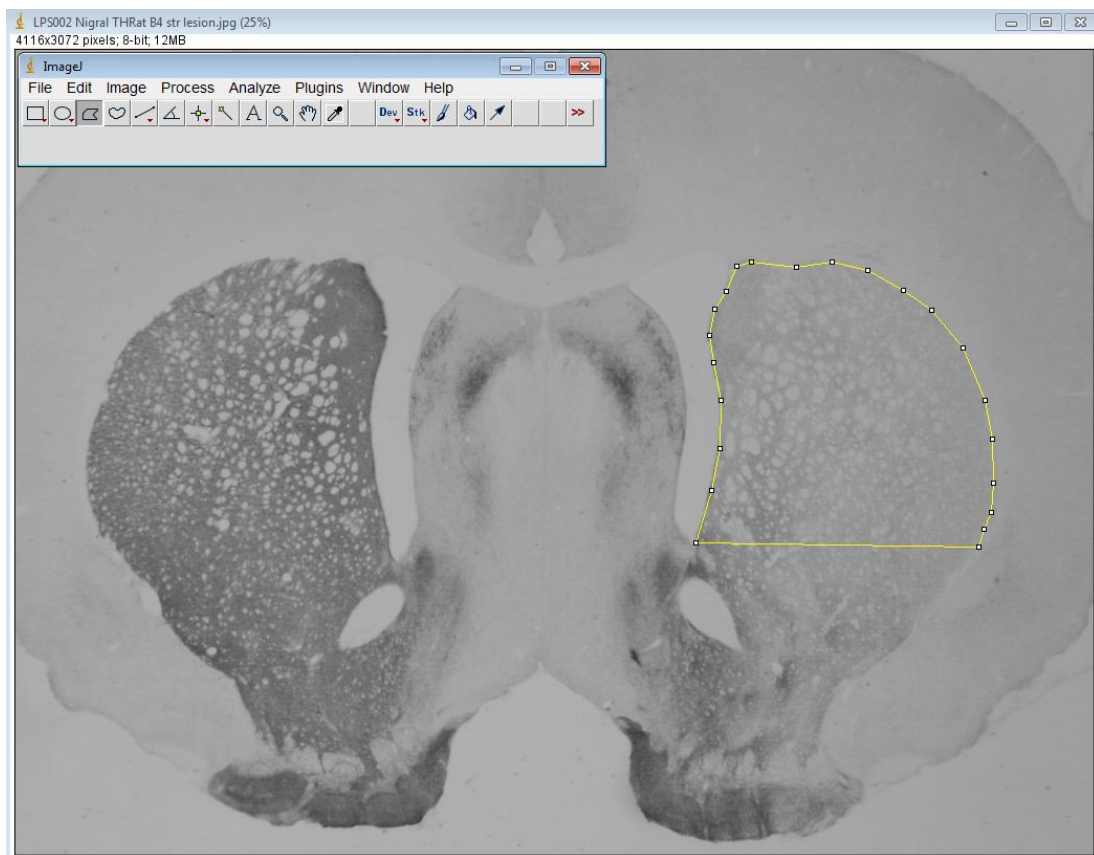


Fig 2.5 Screen grab of the ImageJ software used to quantify optical density of tyrosine hydroxylase-positive staining in the striatum. Images were converted to greyscale prior to analysis. The yellow border depicts the area to be measured. Note that the nucleus accumbens was excluded from each image using a horizontal line originating at the ventral tip of the ventricle. For each section, measurements were taken of the left and right striatum as well as a non-stained background measurement.

2.8.5 HOST IMMUNE CELL MEASUREMENTS

The response of the brain's primary inflammatory cells, namely microglia and astrocytes to an intracerebral injection was quantified by measuring the optical density and/or volume of immunohistochemical staining in representative striatal and/or nigral sections as previously described (Moloney et al., 2010a). In all instances, photomicrographs were obtained using a Nikon SMZ800 microscope with a DXM1200C digital camera and were taken under the same conditions of exposure, magnification and pixel quality. In the case of analysis of volume of staining, an image of a graticule was also captured under the same conditions as the photomicrographs to be analysed. For analysis, images were converted to 8-bit black and white within ImageJ.

2.8.5.1 Microgliosis

In order to measure optical density of host microgliosis (using OX-42 immunostaining), a representative coronal section through the transplant was chosen for each animal. Where no transplant was present, a section was chosen based on either visible needle tract sites or from representative regions in an intact control. An oval shape was centred over the site of the transplant and the density of the staining was measured (see **Fig 2.6**). To estimate the specific staining density, the optical density readings were corrected for non-specific background density. The same oval size was used for all sections/animals. The volume of host microgliosis (OX-42-positive immunostaining) was measured using the method described in section 2.8.2.

2.8.5.2 Astrocytosis

Optical density of host astrocytosis (using GFAP immunostaining) was measured using a representative coronal section through the transplant for each animal. Where no transplant was present, a section was chosen based on either visible needle tract sites or from representative regions in an intact control. An oval shape was placed over the staining at the site of the transplant and the density of staining was measured. To estimate the specific staining density, the optical density readings were corrected for

non-specific background density, as measured from unstained regions in the sections. The same circle size was used for all sections/animals. The volume of host astrocytosis (GFAP-positive immunostaining) was measured using the method described in section 2.8.2.

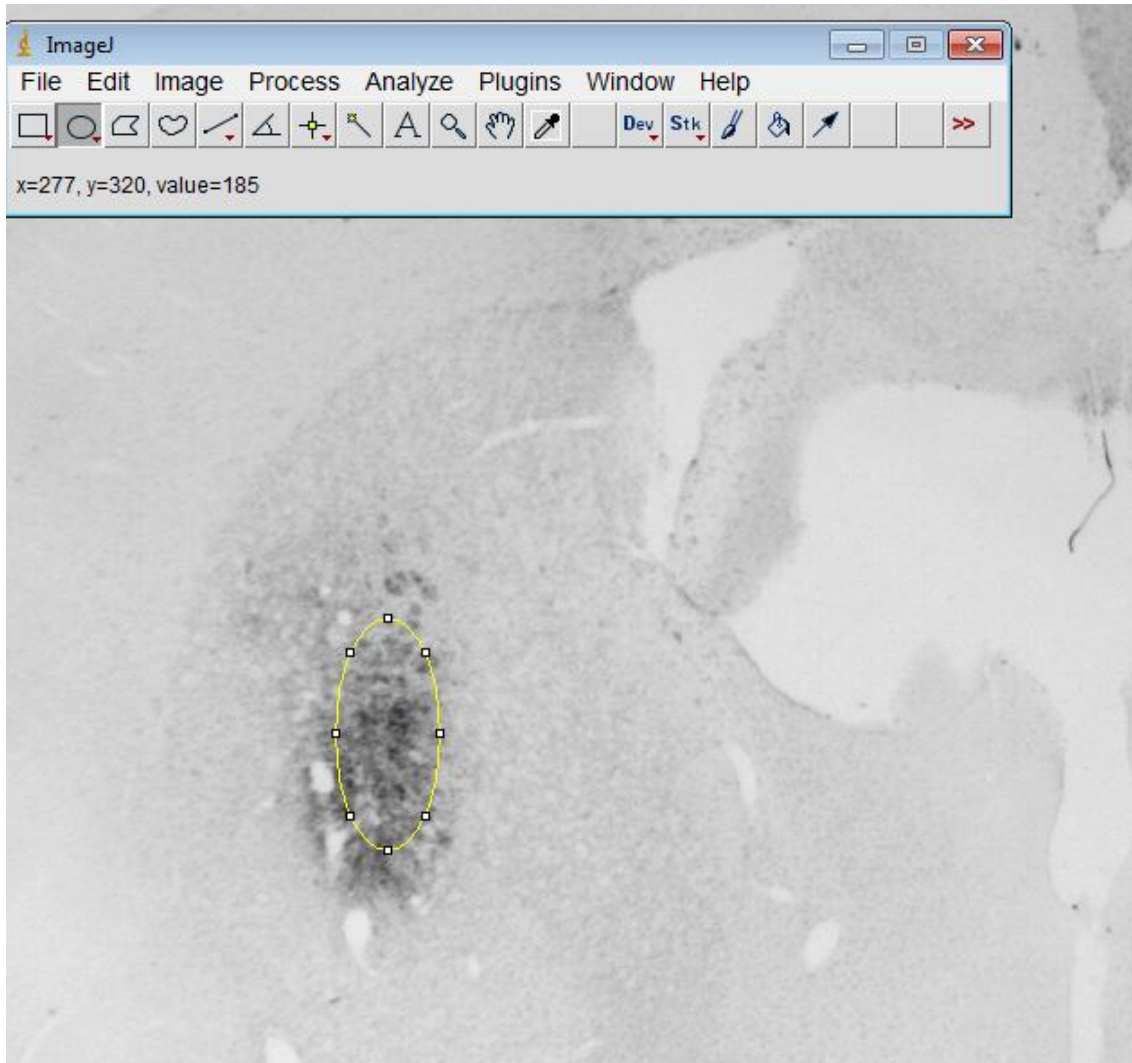


Fig 2.6 Screen grab of ImageJ software used to measure density of microglial/astrocytic staining. The density of microglial/astrocytic staining was measured for each animal around the graft site.

2.9 STATISTICAL ANALYSIS

All data was expressed as mean \pm standard error of the mean. An unpaired *t*-test was used to compare two distinct population means. One-way ANOVA was used to

Chapter 2: Materials & Methods

compare the mean of more than two groups on one factor whereas two-way ANOVA was used to compare the mean of more than two groups on two factors simultaneously. Behavioural data was measured using one-way ANOVA or two-way repeated measures ANOVA (with within-subject factor of time and between subject factor of group). *Post-hoc* testing was conducted using the Student Newman Keuls or Bonferroni analysis as indicated in the text. Analysis were deemed to be significant at $P < 0.05$ in all cases.

Chapter 3: Survival of fetal dopaminergic neurons after co-transplantation with GDNF-MSCs

3.1 INTRODUCTION

Parkinson's disease is a severe and disabling progressive motor degenerative disorder that is characterised pathologically by the progressive loss of dopaminergic neurons from the nigrostriatal pathway (Naoi and Maruyama, 1999). This loss of dopaminergic neurons results in the emergence of a behavioural syndrome with symptoms including resting tremor, bradykinesia, rigidity and postural instability (Jankovic, 2008). Although there are various treatments available for the treatment of the disease, particularly for the motor symptoms, these therapies are limited in that they solely provide symptomatic relief and ultimately do not address the underlying degenerative process associated with the disease (Salawu et al., 2010). Due to the relatively selective loss of nigrostriatal dopaminergic neurons in Parkinson's disease, cell replacement therapy, and in particular the transplantation of fetal dopaminergic cells, has long been seen as a promising disease-modifying approach for the treatment of the disease.

The rationale behind fetal dopaminergic transplantation is that restoration of striatal dopaminergic transmission by grafted dopaminergic neurons would induce long-lasting clinical improvement (Lindvall and Bjorklund, 2004). To this day, a number of patients have received fetal dopaminergic cell transplantation yielding varying degrees of success in terms of improvement in functional deficits (Peschanski et al., 1994, Sawle et al., 1992, Widner et al., 1992). However, the clinical efficacy of neural transplantation has not yet reached a level to justify its use as a routine therapeutic procedure for Parkinson's disease, due in large part to the major limitations of poor graft survival and ethical issues linked to the number of human fetuses required for this cell replacement strategy (Brundin et al., 2000, Ishii and Eto, 2014, Liu and Huang, 2007). In order for cell replacement using fetal dopaminergic cells to be realised as a viable therapeutic option, efforts must be made to increase the quantity of surviving cells after transplantation.

Chapter 3: Co-transplantation of GDNF-MSCs with fetal dopaminergic neurons

Since GDNF was first isolated by Lin and colleagues in (1993), a plethora of studies have found it to have distinct protective effects on the survival of midbrain dopaminergic neurons both *in vitro* and *in vivo* (Beck et al., 1995, Kearns and Gash, 1995, Lin et al., 1993, Sauer et al., 1995). Previously in our laboratory, mesenchymal stem cells transduced with a retrovirus containing the transgene for GDNF were intracerebrally transplanted in a rat model of Parkinson's disease, and were found to be capable of inducing local neuroprotection of nigrostriatal dopaminergic terminals. While this *ex vivo* gene therapy approach was promising, its efficacy was also hindered by the poor survival of the cells after *in vivo* transplantation (Moloney et al., 2010a, Moloney et al., 2010b). However, the characteristic poor survival of MSCs in the brain may actually be advantageous in certain clinical situations. For example, in fetal dopaminergic cell therapy for PD, injection of GDNF protein adjacent to a primary dopaminergic graft has been shown to provide a beneficial, albeit transient, trophic signal to the transplanted cells in the critical period of 1-2 weeks after transplantation when approximately 95% of dopaminergic neurons have been shown to die (Brundin et al., 2000, Rosenblad et al., 1996).

With this in mind, the aim of this chapter was to determine if co-transplantation of MSCs genetically engineered to overexpress GDNF could provide a neurotrophic signal to the transplanted fetal dopaminergic neurons in this critical period, thereby increasing their survival, and ultimately improving the efficacy of this cell replacement approach.

3.2 METHODS

All methods have been described in more detail in Chapter 2. Methods specific to this chapter are also detailed below.

3.2.1 EXPERIMENTAL DESIGN

The studies presented in this chapter assessed the ability of GDNF-MSCs to improve the survival of primary dopamine neurons when co-transplanted into the 6-hydroxydopamine-induced hemi-Parkinsonian rat brain. Single cell suspension transplants were prepared from the developing ventral mesencephalon of embryonic donors (E14) via the method outlined below and as published previously (Dowd and Dunnett, 2004, Torres et al., 2008). Initial studies were designed to determine if co-transplantation of the cells could improve survival of primary dopaminergic cells *in vivo*, when delivered to an intact rat striatum. Following this, we assessed if this co-transplantation approach was capable of conferring long-lasting, improved survival and function of the primary dopaminergic neurons in the 6-hydroxydopamine-lesioned striatum. *Post-mortem* analyses included quantitative tyrosine hydroxylase immunohistochemistry for the survival of primary dopaminergic neurons at the transplant site, and fluorescence microscopy for MSC survival. All *in vivo* experiments included 3 groups (VM transplants, VM + unmodified GFP-MSC co-transplants and VM + GDNF-MSC co-transplants).

3.2.1.1 Effects of co-transplantation with GDNF-MSCs on primary dopaminergic cell survival in an intact rat striatum

In the first pilot *in vivo* study in this chapter, GDNF-MSCs or GFP-MSCs were co-transplanted with primary dopaminergic neurons in an intact rat striatum. To do this, a single cell suspension of ventral mesencephalic cells was obtained as described in the Methods chapter of this thesis. Animals were divided into 3 groups (n=4 per group) and received a striatal transplant of either 300,000 VM cells, 300,000 VM cells + 100,000

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GFP-MSCs or 300,000 VM cells + 100,000 GDNF-MSCs. Animals were sacrificed 4 weeks post-transplantation and their brains processed for tyrosine hydroxylase immunohistochemistry (to determine dopaminergic cell survival).

3.2.1.2 Effects of co-transplantation with GDNF-MSCs on primary dopaminergic cell survival in a 6-hydroxydopamine MFB model of Parkinson's disease

Following the initial *in vivo* study, we next wanted to test the co-transplantation protocol in a rat model of Parkinson's disease, and ultimately assess if co-transplantation of GDNF-MSCs could improve the survival of dopaminergic neurons *in vivo*, and restore motor deficits induced by the lesion. To do this, a 6-hydroxydopamine MFB lesion was performed on all animals prior to cell transplantation. Induction of the lesion was confirmed by amphetamine rotations 2 weeks post infusion of the neurotoxin. Following this, we performed the ventral mesencephalon dissection, as described above, in order to obtain a single cell suspension of ventral mesencephalic cells. Animals were divided into 3 groups (n=4 per group) and received a striatal transplant of either 300,000 VM cells, 300,000 VM cells + 100,000 GFP-MSCs or 300,000 VM cells + 100,000 GDNF-MSCs. Amphetamine rotations were performed at both 4 and 8 weeks post-transplantation before animals were sacrificed and their brains processed for tyrosine hydroxylase immunohistochemistry (to determine dopaminergic cell survival).

3.3 RESULTS

3.3.1 EFFECTS OF CO-TRANSPLANTATION OF GDNF-MSCs ON PRIMARY DOPAMINERGIC CELL SURVIVAL IN AN INTACT RAT STRIATUM

3.3.1.1 GDNF-transduced MSCs secrete GDNF *in vitro*

The expression and secretion of GDNF from normal and GDNF-transduced MSCs was first confirmed *in vitro* using an ELISA of the conditioned culture medium, as previously described in the methods section of this thesis. This ELISA revealed that GFP-MSCs secreted GDNF at a level of approximately 0.6 ± 0.1 ng of GDNF per ml of culture medium while retroviral transduction of the MSCs with a vector carrying the transgene for the neurotrophic factor resulted in the cells secreting significantly more GDNF into the culture medium at a level of 14.7 ± 1.9 ng of GDNF per ml of culture medium ($t_{(4)} = 7.70$, $P < 0.01$, using an unpaired *t*-test) (Fig 3.1).

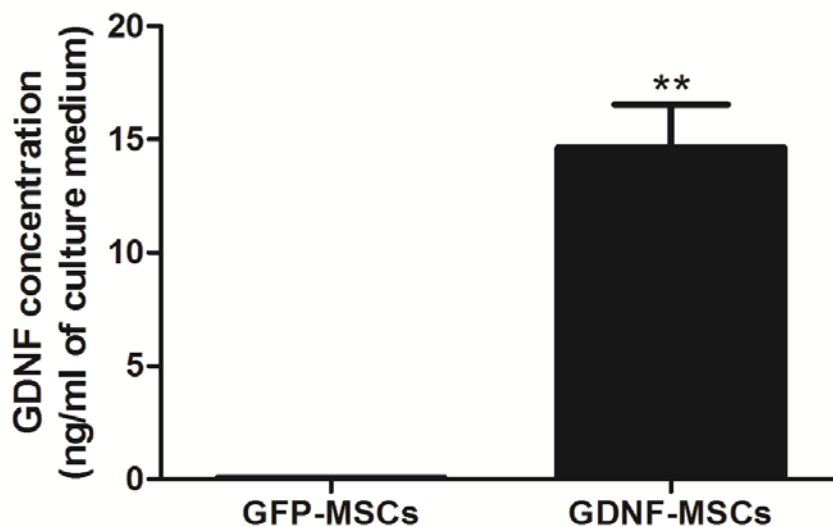


Fig 3.1 GDNF profile of GFP-MSCs and GDNF-MSCs *in vitro*. GDNF ELISA reveals that MSCs are inherently capable of secreting GDNF into the culture medium. Transduction of the cells with a retrovirus to overexpress GDNF resulted in the cells secreting significantly more GDNF into the culture medium (** $P < 0.01$ vs. GFP-MSCs, using an unpaired *t*-test). Data are shown as mean \pm s.e.m.

Chapter 3: Co-transplantation of GDNF-MSCs with fetal dopaminergic neurons

3.3.1.2 GDNF-transduced MSCs secrete GDNF *in vivo*

In a study that will be described in more detail in Chapter 5 of this thesis, we used immunohistochemical analysis to determine the ability of GDNF-MSCs to secrete and release GDNF *in vivo*. In brief, GDNF-MSCs were transplanted to the rat striatum and animals were sacrificed at Days 1, 4, 7 and 14 post-transplantation. The results are also shown in this chapter to demonstrate the ability of cells to release GDNF *in vivo*, in a time frame that would allow transplanted mesencephalic cells to have access to the neurotrophin in the 2 week time period shown to be critical for fetal dopaminergic cell survival (Brundin et al., 2000, Rosenblad et al., 1996). This shows that transplanted GDNF-MSCs release GDNF *in vivo* (**Fig 3.2 A**), with the expression of GDNF reducing over time (**Fig 3.2 B**), due to the progressive death of the transplanted cells as shown previously (Moloney et al., 2010b).

Chapter 3: Co-transplantation of GDNF-MSCs with fetal dopaminergic neurons

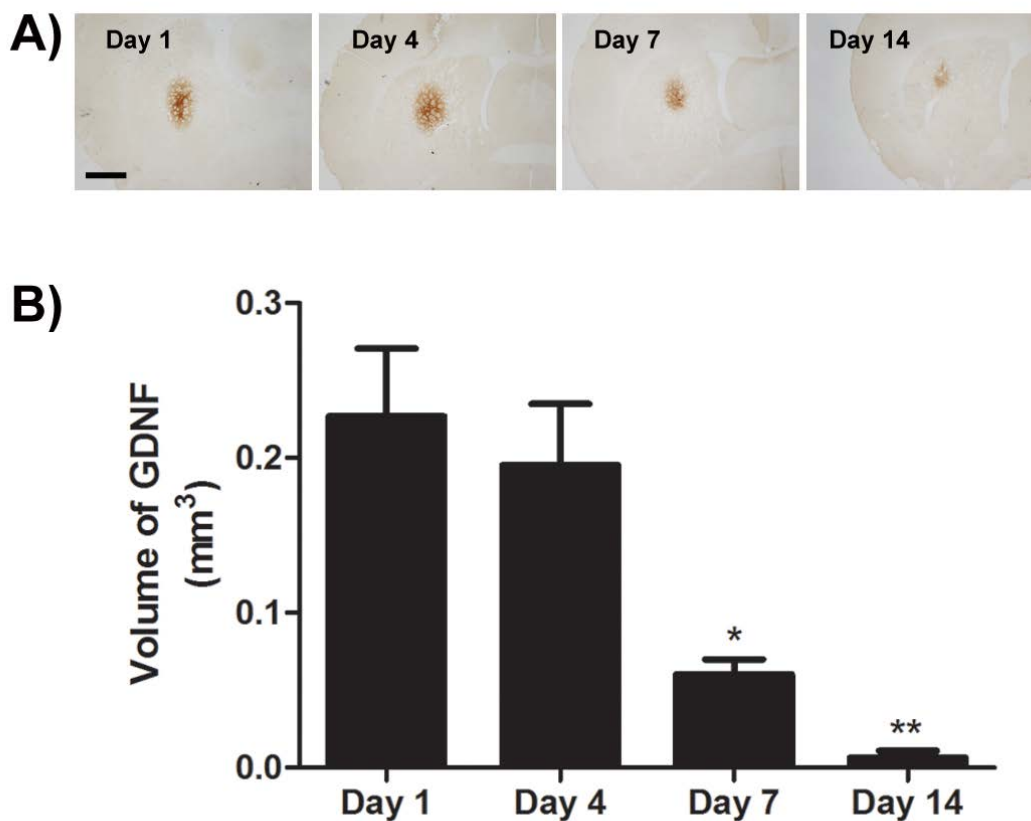


Fig 3.2 GDNF-MSCs release GDNF *in vivo*. GDNF-MSCs were injected into the striatum and GDNF release into the striatal tissue was analysed at Days 1, 4, 7 and 14 after transplantation. A) The GDNF protein could be visualised using immunohistochemistry in the host striatum at each time-point examined. B) Quantification of the volume of striatal tissue occupied by GDNF immunostaining showed that the GDNF released reduces gradually over time, most likely due to progressive cell death. Data are presented as mean \pm s.e.m. * $P < 0.05$, ** $P < 0.01$ vs. Day 1 by 1 way ANOVA with *post hoc* Bonferroni. Scale bar represents 750 μ m.

3.3.1.3 Survival of MSCs after co-transplantation to the rat striatum with primary dopaminergic neurons

Survival of transplanted MSCs was assessed by quantification of the volume of the GFP-positive cells remaining at 28 days post-transplantation. Transplanted cells were easily identifiable due to their expression of the GFP reporter gene (**Fig 3.3 A**). Surviving MSC cell transplants were observed in only half of the transplanted animals at the time point examined in this study (Day 28 after transplantation). However, this level of cell survival is to be expected at this later time-point, and is consistent with a previous report from our laboratory in which we demonstrated the progressive death of these cells *in vivo*, with the majority of cells dying within one week post-transplantation (Moloney et al., 2010a). There was no difference in grafted cell survival between the two transplanted cell types ($t_{(2)} = 0.20$, $P = 0.86$, n.s.) (**Fig 3.3 B**).

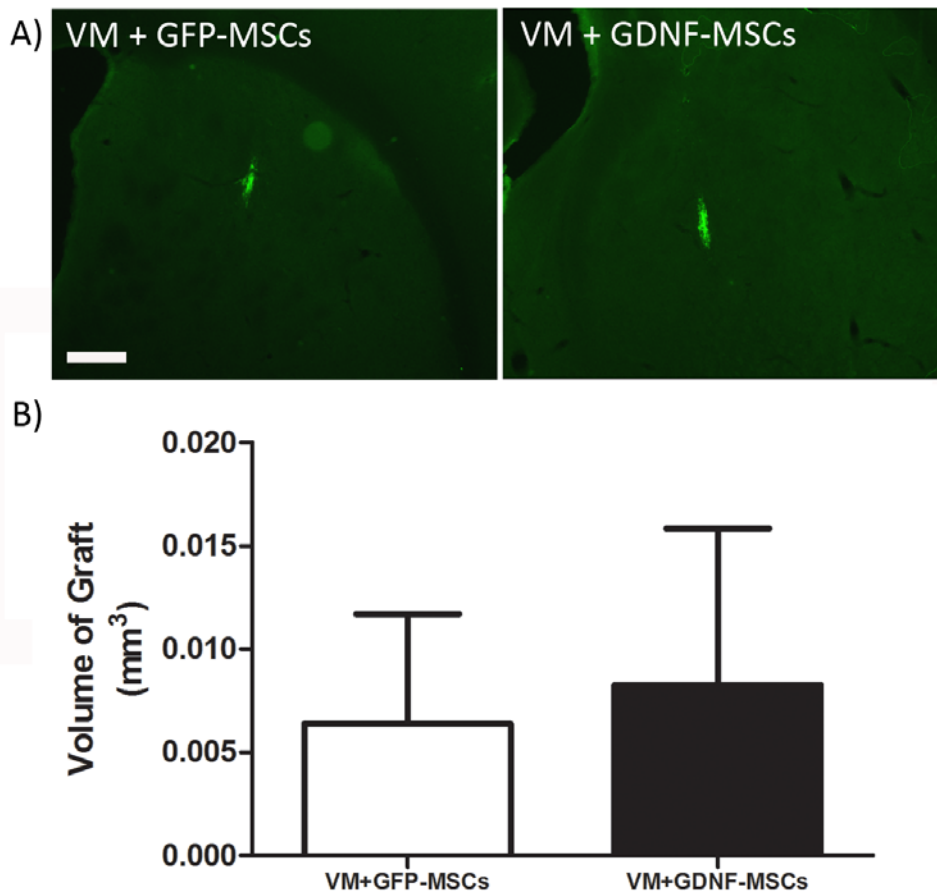


Fig 3.3 Survival of co-transplanted GFP-MSCs and GDNF-MSCs in an intact rat striatum. A) Representative photomicrographs showing that transplanted GFP-MSCs and GDNF-MSCs were identifiable *in vivo*, despite the relatively small graft volume, due to their strong GFP expression at 28 days post-transplantation. B) Quantitative analysis of the volume of the graft showed that there was no significant difference in cell survival between either cell type. Data are shown as mean \pm s.e.m. Scale bar represents 200 μ m.

3.3.1.4 Survival of primary dopaminergic cells after co-transplantation with MSCs

Four weeks post-transplantation surgeries, animals were sacrificed and tyrosine hydroxylase immunohistochemistry was performed to determine the survival of transplanted ventral mesencephalon-derived dopaminergic cells. The cells were counted individually and the effect of co-transplantation of GDNF-MSCs on dopaminergic cell survival was determined. No difference in the number of surviving dopaminergic cells was observed between groups, regardless of whether they were transplanted alone or in combination with GFP-MSCs or GDNF-MSCs (**Fig 3.4 B**; Group, $F_{(2,9)} = 0.61$, $P = 0.57$, n.s.). When examining the photomicrographs for quantification, it was noted that there appeared to be a dense area of tyrosine hydroxylase-positive staining around the transplant site in the VM + GDNF-MSC transplant group, an observation which was not seen in the VM or the VM + GFP-MSC transplant groups (see **Fig 3.4 A**). When the optical density of the tyrosine hydroxylase staining was measured at the site of transplantation (see **Fig 3.4 C**), the density of staining was significantly greater in the VM + GDNF-MSC group, indicating a trophic effect of GDNF from the GDNF-MSCs on the host dopaminergic terminals and/or the transplanted dopamine cells in the striatum (**Fig 3.4 C**; Group, $F_{(2,9)} = 31.79$, $P < 0.001$). While a neurotrophic response from the transplant is desirable, this dense staining may have masked the tyrosine hydroxylase-positive cell bodies from view and therefore, from quantification in this study. Thus moving forward we felt it was important to assess the effects of this transplantation approach in the denervated striatum of a Parkinson's disease rat model, especially since transplanted primary dopaminergic neurons have previously been shown to exhibit enhanced function in terms of fibre outgrowth in a lesioned striatum (Doucet et al., 1990).

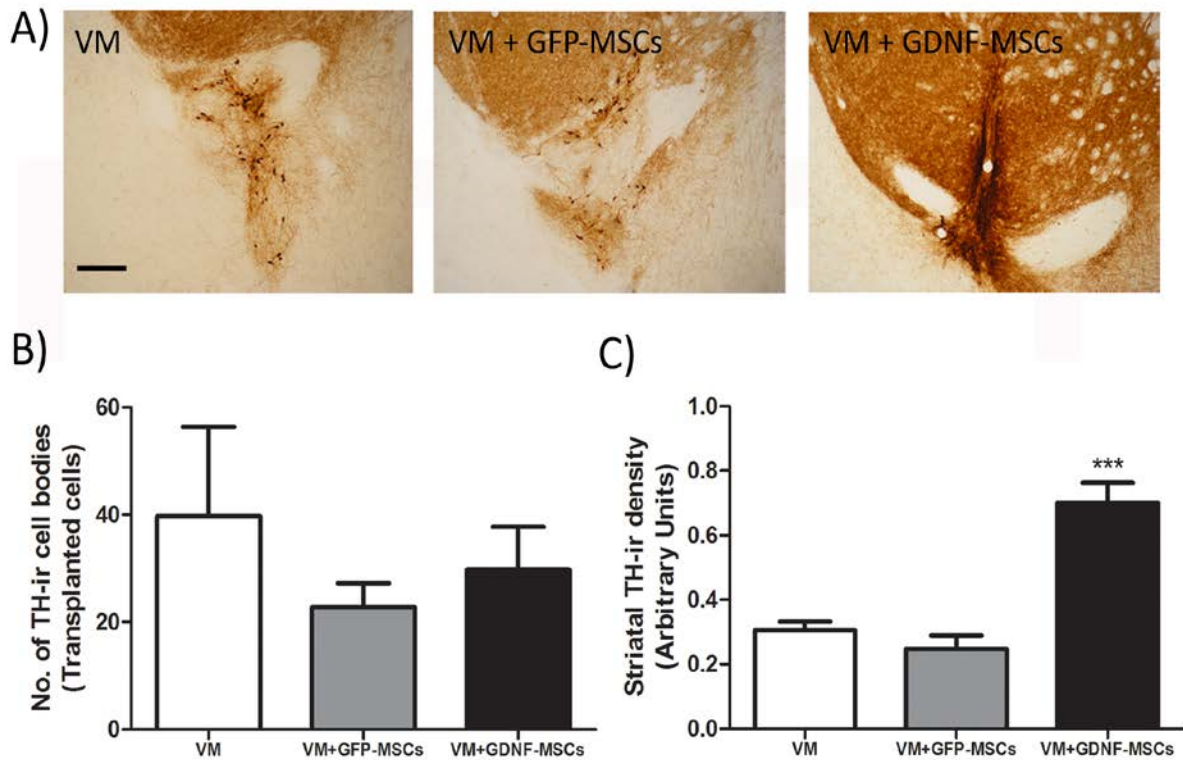


Fig 3.4 Survival of primary dopaminergic cells in an intact rat striatum after co-transplantation with GDNF-MSCs. A) Representative photomicrographs showing that transplanted dopaminergic cells were identifiable using tyrosine hydroxylase immunohistochemistry at 28 days post-transplantation. Dense tyrosine hydroxylase staining at the transplant site can be observed in the VM + GDNF-MSCs transplant group which may have obscured some tyrosine hydroxylase-positive cell bodies from quantification. B) Quantitative analysis of the number of tyrosine hydroxylase-positive grafted cells showed that there was no significant difference in cell survival between any of the transplant groups whether they were transplanted alone, or combined with GFP-MSCs or GDNF-MSCs. C) Quantitative analysis of the density of tyrosine hydroxylase staining at the transplant site revealed a significant local trophic effect in the VM + GDNF-MSC group (** $P < 0.001$ by 1 way ANOVA with *post hoc* Bonferroni test). Data are shown as mean \pm s.e.m. Scale bar represents 500 μ m. TH-ir: Tyrosine hydroxylase immunoreactivity.

Chapter 3: Co-transplantation of GDNF-MSCs with fetal dopaminergic neurons

3.3.2 EFFECTS OF CO-TRANSPLANTATION OF GDNF-MSCs ON PRIMARY DOPAMINERGIC CELL SURVIVAL IN A 6-HYDROXYDOPAMINE MFB MODEL OF PARKINSON'S DISEASE

3.3.2.1 Effect of co-transplantation of GDNF-MSCs with primary dopaminergic neurons on 6-hydroxydopamine induced motor deficits

In order to confirm a successful lesion of the MFB by unilateral infusion of 6-hydroxydopamine, amphetamine rotations were performed 2 weeks after lesion surgery. All animals displayed significant ipsilateral rotational bias, performing an average of 7.4 net ipsilateral rotations per minute over a 60 minute testing period after amphetamine injection (**Fig 3.5 A**). Amphetamine rotations were performed again at 4 and 8 weeks after transplantation surgeries to assess the ability of transplanted dopaminergic cells to reduce the rotational asymmetry induced by 6-hydroxydopamine. When compared to 2 weeks post-lesion surgeries, the lesion-induced motor deficit was reduced at both 4 and 8 weeks post-transplantation (**Fig 3.5 B**; Time, $F_{(2,18)} = 8.22$, $P < 0.01$), however there was no significant difference between animals transplanted alone or in combination with either GFP-MSCs or GDNF-MSCs (**Fig 3.5 B**; Group, $F_{(2,18)} = 0.10$, $P = 0.82$, n.s.).

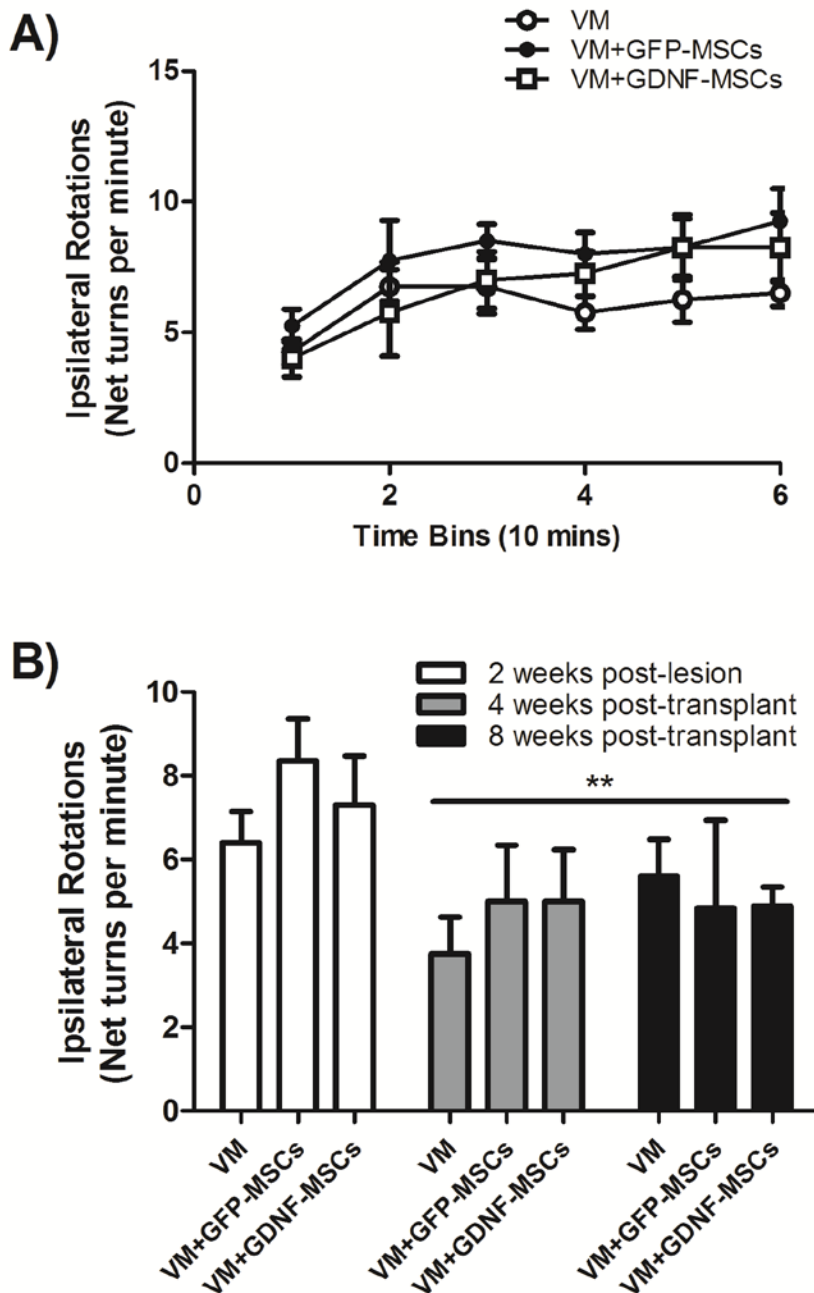


Fig 3.5 Transplantation of primary dopaminergic cells reduced rotational asymmetry induced by 6-hydroxydopamine lesion. A) Unilateral infusion of 6-hydroxydopamine to the MFB induced rotational asymmetry 2 weeks after lesion surgery. B) Transplantation of primary dopaminergic cells (both alone and in combination with GFP-MSCs and GDNF-MSCs) reduced rotational asymmetry at 4 and 8 weeks after transplantation surgeries, however no significant difference in net ipsilateral rotations was observed between transplantation groups (** $P < 0.01$ vs. 2 weeks post-lesion, by 2 way ANOVA with *post hoc* Bonferroni test). Data are shown as mean \pm s.e.m.

3.3.2.2 6-hydroxydopamine administration into the MFB induces pronounced nigrostriatal degeneration

While the success of the MFB lesion by unilateral infusion of 6-hydroxydopamine was assessed 2 weeks after lesion surgery by amphetamine rotation, the loss of dopaminergic cell bodies and terminals as a result of the neurotoxin was confirmed *post mortem* by qualitative tyrosine hydroxylase immunohistochemistry at 8 weeks after cell transplantation. The loss of tyrosine hydroxylase-positive staining on the injected (right) side of brain can be seen clearly in the images in **Fig 3.6** below.

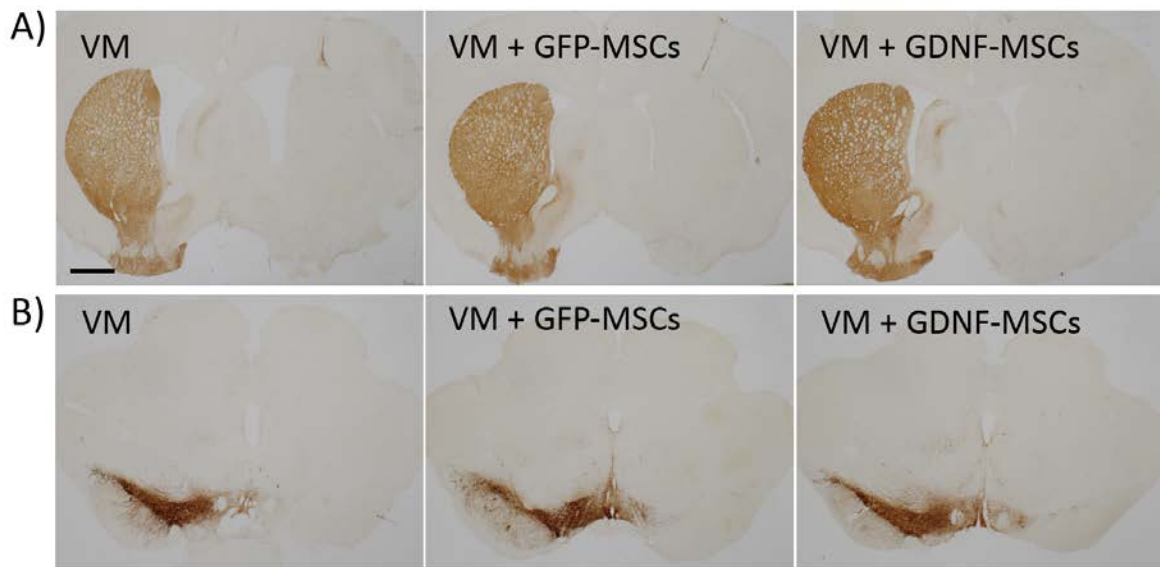


Fig 3.6 Unilateral administration of 6-hydroxydopamine to the MFB induces nigrostriatal degeneration. Qualitative immunohistochemical staining for tyrosine hydroxylase revealed pronounced loss of dopaminergic striatal terminals (A) and cell bodies (B) on the injected (right) side of the brain. Scale bar represents 1.5 mm.

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3.3.2.3 Survival of MSCs after co-transplantation to the rat striatum with primary dopaminergic neurons in the hemi-Parkinsonian rat brain

Survival of transplanted MSCs was assessed by quantification of the volume of the GFP-positive cells remaining at 8 weeks post-transplantation. There were no surviving transplants, of either GFP-MSCs or GDNF-MSCs, detectable in any of the co-transplanted animals in this study. This result is to be expected given the later time-point of sacrifice in this study, a finding which correlates with the results of a previous report from our laboratory in which we demonstrated the progressive death of these cells *in vivo* (Moloney et al., 2010a).

3.3.2.4 Survival of primary dopaminergic cells after co-transplantation with MSCs in the hemi-Parkinsonian rat brain

Eight weeks after cell transplantation surgeries, animals were sacrificed by transcardial perfusion-fixation and their brains were processed for tyrosine hydroxylase immunohistochemical analysis in order to determine the survival of transplanted dopaminergic cells. Dopaminergic cells were clearly visible in the largely denervated striatum (**Fig 3.7 A**), and were counted individually in order to determine the effects of co-transplantation of dopaminergic cells with either GFP-MSCs or GDNF-MSCs on their survival *in vivo* (**Fig 3.7 B**; Group, $F_{(2,9)} = 0.59$, $P = 0.58$, n.s.). No difference between cell transplant groups was observed with regards to cell survival.

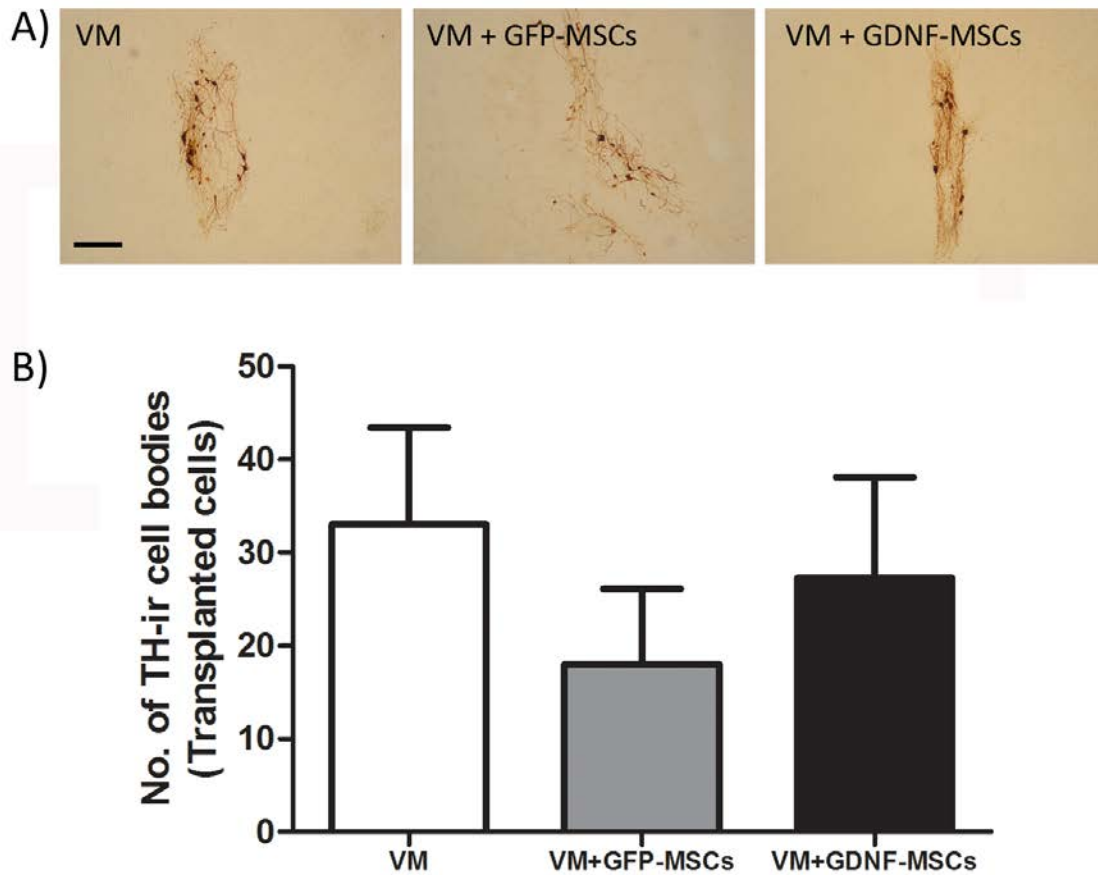


Fig 3.7 Survival of primary dopaminergic cells in a 6-hydroxydopamine lesioned rat striatum after co-transplantation with GDNF-MSCs. A) Representative photomicrographs showing that transplanted dopaminergic cells were easily identifiable in the largely denervated striatum of the 6-hydroxydopamine model, after tyrosine hydroxylase immunohistochemistry at 8 weeks post-transplantation. B) Quantitative analysis of the number of tyrosine hydroxylase-positive grafted cells showed that there was no significant difference in cell survival between any of the transplant groups whether they were transplanted alone, or combined with GFP-MSCs or GDNF-MSCs. Data are shown as mean \pm s.e.m. Scale bar represents 100 μ m. TH-ir: Tyrosine hydroxylase immunoreactivity.

3.4 DISCUSSION

Two promising potential strategies under investigation for the treatment of Parkinson's disease are the delivery of neurotrophic factors to protect degenerating dopaminergic neurons, and the use of fetal tissue to replace the lost dopaminergic neurons. Thus, the aim of this chapter was to examine the effects of using a combination of these two potential therapeutic options by assessing the ability of GDNF-MSCs to increase fetal dopaminergic cell survival when delivered to the brain as a co-transplant of GDNF-MSCs and fetal dopaminergic cells. In this study, we found that while GDNF-MSCs were capable of secreting GDNF to the conditioned medium *in vitro* and to the host striatum *in vivo*, co-transplantation of GDNF-MSCs and fetal ventral mesencephalic cells did not improve the survival of dopaminergic cells *in vivo* in either an intact or 6-hydroxydopamine lesioned striatum.

Transplantation of fetal ventral mesencephalic tissue to patients with Parkinson's disease has been shown to be effective in ameliorating the cardinal symptoms of the disease (Lindvall, 1994, Olanow et al., 1996). However, variable outcomes across patients, accompanied with incomplete striatal innervation and inadequate restoration of motor function, highlight the need for improved and standardised methods of cell transplantation as a potential disease-modifying treatment for Parkinson's disease (Winkler et al., 2005). A major limitation associated with the fetal nigral transplant procedure is the poor survival rate of grafted neurons, which may be as low as 5% in human fetal nigral transplants (Brundin et al., 2000, Rosenblad et al., 1996, Brundin and Bjorklund, 1987). While the reason for this poor survival rate after transplantation is unclear, it has been postulated that it could, at least partly, be due to insufficient neurotrophic support at the graft site, an idea which is supported by the reduced expression of neurotrophic factors such as brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF) and GDNF in the degenerating brain (Weis et al., 2003, Kruttgen et al., 2003, Siegel and Chauhan, 2000).

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The mechanism by which MSCs have been reported to exert regenerative effects is via the release of a complement of molecules such as neurotrophic growth factors, chemokines, cytokines, and extracellular matrix proteins, dubbed the MSC secretome, that promote endogenous repair either directly or indirectly (Chen et al., 2002, Rosova et al., 2008, Paul and Anisimov, 2013). Indeed in this study, we found that GFP-MSCs were capable of secreting GDNF, in line with previous evidence that they are inherently neurotrophic (Kurozumi et al., 2005). This basal level of GDNF release from GFP-MSCs appears somewhat negligible however, when compared to the high level of GDNF secreted from MSCs previously transduced with a retrovirus to overexpress the GDNF transgene (Rooney et al., 2009). The level of secreted protein observed by ELISA *in vitro* in this study is consistent with previous studies in our laboratory assessing the expression profile of these GDNF-transduced MSCs (Moloney et al., 2010b). A number of studies have demonstrated the ability of GDNF to promote dopaminergic neuron survival and neurogenesis both *in vitro* and *in vivo* (Bjorklund et al., 1997, Rosenblad et al., 1996, Redmond et al., 2009). Indeed, we have previously shown that this GDNF expression is easily detectable *in vivo*, and was capable of inducing local neuroprotection of the striatal dopaminergic terminals when transplanted prior to induction of a 6-hydroxydopamine lesion (Moloney et al., 2010b). Thus we aimed to co-transplant the GDNF-MSCs with primary dopaminergic neurons obtained from ventral mesencephalon dissection of E14 rat fetuses in order to harness the inherent neurotrophic properties of the released GDNF from the GDNF-transduced MSCs.

Transplantation experiments were undertaken to test whether the GDNF-MSCs could enhance the survival and/or function of embryonic ventral mesencephalon grafts initially in an intact rat striatum, and subsequently in the hemiparkinsonian rat brain. When ventral mesencephalon cells were transplanted to the intact rat striatum (either alone or as a co-transplant with GFP-MSCs or GDNF-MSCs) in our initial transplant study, we found that inclusion of GFP-MSCs or GDNF-MSCs in the cell suspension to be transplanted had neither a positive nor a detrimental effect on the survival of dopaminergic cells. What we did notice however, is that a dense area of tyrosine

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hydroxylase-positive staining was evident at the site of transplant in the VM + GDNF-MSC co-transplant group. While innervation of tyrosine hydroxylase-positive terminals is a desirable outcome in a transplant study of this nature, we believe that the dense staining may have occluded a number of the transplanted dopaminergic cells from quantification and thus have masked a therapeutic effect of the co-transplantation approach. Thus, we wished to assess the impact of the co-transplantation approach on the survival and function of embryonic ventral mesencephalon grafts in the Parkinsonian rat brain.

In rats, the coordinates for unilateral administration of 6-hydroxydopamine into the MFB are long established, and a single injection of the neurotoxin is normally sufficient to produce 95% ipsilateral depletion of dopamine neurons (Torres et al., 2011). As previously described, the success of the lesion was assessed by quantification of net ipsilateral rotation induced by injection of amphetamine, the extent of which is dependent on the extent of unilateral dopaminergic cell loss (Bjorklund et al., 1980). Animals administered 2.5 mg/kg of methamphetamine, that exhibited a net ipsilateral rotation of at least 6 turns per minute, were deemed to have a successful lesion and thus were included in cell transplantation surgeries. This threshold has previously been acknowledged as an important threshold in order to obtain animals with stable lesions that do not spontaneously recover (Dowd and Dunnett, 2005). The amphetamine induced rotation test was repeated at 4 and 8 weeks post-transplantation surgeries. As expected, all animals that received grafts of ventral mesencephalic cells alone elicited a partial recovery from amphetamine-induced rotational asymmetry at 4 weeks. While a similar level of recovery was seen in recipients of ventral mesencephalic cells co-transplanted with the either GFP-MSCs or GDNF-MSCs at four weeks, the level of recovery exhibited by all groups was minimal given that recovery was not further enhanced at 8 weeks post-transplantation. This minimal functional recovery echoes that observed in a previous study where ventral mesencephalic cells were co-transplanted with neural precursor cells genetically modified to release GDNF (Ostenfeld et al., 2002). Also, in this previous study by Ostenfeld *et al.*, (2002) there were no surviving transplanted GDNF-transduced neural precursor cells observed at the time of sacrifice.

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We too could not detect a surviving MSCs transplant at 8 weeks post-transplantation, unsurprising given that we have previously shown the progressive death of these cell post-transplantation (Moloney et al., 2010a, Moloney et al., 2010b). Despite this result, Ostenfeld *et al.*, demonstrated a highly significant increase in overall tyrosine hydroxylase-positive cell survival in animals that were recipients of both ventral mesencephalic cells and GDNF-transduced neural precursor cells. In contrast to this finding, survival of dopaminergic cells was not enhanced by co-transplantation with either GFP-MSCs or GDNF-transduced MSCs in this study. Possible reasons for the variance from published data may be the difference in experimental paradigm, in that Ostenfeld *et al.*, transplanted the GDNF secreting neural precursor cells 3 days prior to VM cell transplantation, in order to pre-establish local GDNF production (Ostenfeld et al., 2002). However, it has been previously shown that delivery of GDNF via viral transfer at the time of ventral mesencephalon transplantation enhanced the survival and function of ventral mesencephalic cells (Kauhausen et al., 2013, Thompson et al., 2009). Therefore, in our study, GDNF-MSCs and ventral mesencephalic cells were mixed prior to transplantation and infused simultaneously. While the reason for the lack of efficacy of the co-transplantation approach is largely unknown, there are a number of confounding factors. These include the possibility that the ventral mesencephalic cells may have been in competition for survival with the co-transplanted MSCs (e.g. for nutrients, interaction, oxygen), the ratio of GDNF-MSCs to ventral mesencephalic cells may not have been optimal, or indeed the transplant itself may not have been rich with dopaminergic cells to begin with (due to inaccurate dissection technique and/or relative inexperience of the experimenter performing the dissection). In addition, it has been shown that ventral mesencephalic cells dissected from younger E12 embryos, rather than the E14 embryos utilised in this study, yield higher tyrosine hydroxylase-positive cell numbers, and therefore are more widely used for pre-clinical transplantation studies (Torres et al., 2008, Torres et al., 2007).

Improved survival, fibre outgrowth and function of fetal dopaminergic transplants are believed to be crucial in order for translation to the clinical setting to become a feasible option (Rosenblad et al., 1996). Since the survival rate of the grafted fetal dopaminergic

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neurons is quite low, usually between 5% and 20% in different studies, (Sauer and Brundin, 1991, Brundin and Bjorklund, 1987) there is considerable margin for improvement of nigral graft survival. Despite the extensive evidence supporting the ability of GDNF delivery/overexpression to improve fetal dopaminergic cell transplantation (Ostenfeld et al., 2002, Kauhausen et al., 2013, Thompson et al., 2009, Rosenblad et al., 1996), we found that at this particular ratio of GDNF-secreting MSCs to dopaminergic cells, and using this particular donor age of fetal tissue, the co-transplantation approach did not increase dopaminergic cell survival *in vivo*. Further studies investigating the effects of varying the parameters of the co-transplantation approach may yield different results in terms of cell survival, however, given the lack of efficacy in these pilot studies, the following chapters of this thesis will focus on harnessing the neurotrophic potential of GDNF-transduced MSCs when transplanted in models of Parkinson's disease, and the efforts undertaken in order to streamline this approach.

Chapter 4: GDNF-MSCs in an inflammation-driven model of Parkinson's disease

4.1 INTRODUCTION

GDNF was originally isolated from the supernatant of a rat glial cell line (Lin et al., 1993), and since its discovery a plethora of studies have found it to have distinct protective effects on the survival of midbrain dopaminergic neurons both *in vitro* and *in vivo* (Beck et al., 1995, Kearns and Gash, 1995, Lin et al., 1993, Sauer et al., 1995). Initial preclinical trials in both rodent and primate models of the disease examined the effects of GDNF injection or infusion into the lateral ventricles or brain parenchyma (Hurelbrink and Barker, 2004). These studies have demonstrated that GDNF was not only protective of dopaminergic neurons when challenged with degenerative toxins but also may have a restorative effects on dying dopaminergic neurons (when the protein is administered before or after a lesion, respectively) (Aoi et al., 2000). The neuroprotective and neuroregenerative effects of intracerebral GDNF observed in preclinical studies prompted its investigation in human clinical trials. Initial results from an open-label clinical trial in which GDNF was infused directly into the putamen via implanted catheters and mechanical delivery pumps were both positive and encouraging. This study found that infusion of GDNF resulted in an improvement in motor function as assessed by the UPDRS. A reduction in medication-induced dyskinesias and partial restoration of the nigrostriatal pathway was also observed as assessed by [¹⁸F] Fluorodopa PET scans (Gill et al., 2003, Slevin et al., 2005). The results of these open-labelled trials, whilst providing essential proof-of-principle for use of the neurotrophic factor, were undermined by the subsequent results of a randomised, double-blind, placebo-controlled trial of GDNF infusion which was ceased due to lack of efficacy (Lang et al., 2006). However, given that it has been postulated that the delivery method and infusion pumps used in these trials may have minimised the clinical outcomes, it has been suggested that optimisation of a novel method to deliver the neurotrophic factor may unveil the true potential of this therapeutic intervention (Sherer et al., 2006).

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Ex vivo gene therapy offers a possible alternative approach for delivery of GDNF. Previously in our laboratory we have described the neurotrophic ability of GDNF-transduced MSCs after transplantation to the commonly used 6-hydroxydopamine model of Parkinson's disease (Moloney et al., 2010b). Given the substantial evidence to support the inherent anti-inflammatory and immunomodulatory characteristics of MSCs (Yi and Song, 2012, Moloney et al., 2010a, Di Nicola et al., 2002), and the fact that inflammation has long been implicated in the pathogenesis of Parkinson's disease (McGeer et al., 1988), we felt it was important to assess the neurotrophic ability of GDNF-transduced MSCs in the inflammation-driven LPS model of Parkinson's disease.

Although previous studies had investigated the brain's response to intra-cerebral LPS administration (Andersson et al., 1992, Bourdiol et al., 1991, Montero-Menei et al., 1996, Montero-Menei et al., 1994, Szczepanik et al., 1996), the first report of inflammation-driven preclinical Parkinsonism came from two independent laboratories in 1998 who demonstrated that intra-nigral injection of LPS to rats caused microglial activation and degeneration of nigrostriatal dopaminergic neurons (Castano et al., 1998, Bing et al., 1998). Since then, a number of studies have confirmed that direct intra-cerebral injection of LPS in rodents induces microglial activation, proinflammatory cytokine release and dopaminergic neuron death (reviewed in Liu and Bing, 2011). To date, however, the motor impact of intra-cerebral LPS administration has been relatively poorly explored with most studies tending to focus on general measures of locomotor activity or relatively artificial measures of motor asymmetry such as drug-induced rotation (Choi et al., 2009, Hsieh et al., 2002, Hunter et al., 2009, Zhang et al., 2005a).

Given that PD is a progressive neurodegenerative disorder that manifests primarily as a motor impairment, it is vital that the effect of direct intra-cerebral administration of LPS on motor function is more fully characterised. Therefore, the first aim of this chapter was to further characterise the neuropathological and behavioural impact of intra-cerebral LPS administration, and to compare the effects of unilateral intra-nigral and intra-striatal administration of the endotoxin. We particularly focussed on monitoring

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the emergence and stability of contralateral motor dysfunction in LPS-injected rats using a battery of spontaneous and drug-induced tests of lateralised motor function. Following this, we next sought to assess the ability of GDNF-MSCs to provide neurotrophic support, and to protect against the motor dysfunction and neuropathology induced by a unilateral LPS lesion.

4.2 METHODS

All methods have been described in more detail in Chapter 2.

4.2.1 EXPERIMENTAL DESIGN

This chapter will detail the results of three separate studies. The aim of the first two studies was to further characterise the neuropathological and behavioural impact of intra-cerebral LPS administration, and to compare the effects of unilateral intra-nigral and intra-striatal administration of the endotoxin. We particularly focussed on monitoring the emergence and stability of contralateral motor dysfunction in LPS-injected rats using a battery of spontaneous and drug-induced tests of lateralised motor function. Following this, we embarked on a third study involving the transplantation of either GFP-MSCs or GDNF-MSCs to the unilateral intra-nigral LPS model of Parkinson's disease in order to assess their ability to protect against LPS-induced motor impairment, neuroinflammation and nigrostriatal neurodegeneration. Prior to these studies, we completed pilot studies to establish the appropriate dose of LPS for intra-cerebral injection. These studies followed a similar experimental design and the data generated is shown in Appendix 1 of this thesis.

4.2.1.1 Intra-striatal LPS administration

Male Sprague Dawley rats underwent habituation and baseline testing on the Corridor, Stepping and Whisker tests. They were then performance matched to receive either LPS (10 µg in 2 µl sterile saline) or vehicle (2 µl sterile saline) into the striatum (LPS, n = 10; Vehicle, n = 6). Behavioural testing on the spontaneous tests resumed the day after

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lesion surgery and continued for 18 days after which the amphetamine-induced rotation test was completed (Day 19 post lesion). The animals were then sacrificed by transcardial perfusion-fixation 20 days after infusion, and their brains were used for *post-mortem* assessment of nigrostriatal neurodegeneration and microgliosis using quantitative tyrosine hydroxylase and OX-42 immunohistochemistry respectively.

4.2.1.2 Intra-nigral LPS administration

Male Sprague Dawley rats underwent habituation and baseline testing on the Corridor, Stepping and Whisker tests. They were then performance matched to receive either LPS (10 µg in 2 µl sterile saline) or vehicle (2 µl sterile saline) into the substantia nigra (LPS, n = 6; Vehicle, n = 6). Behavioural testing on the spontaneous tests resumed the day after lesion surgery and continued for 18 days after which the amphetamine-induced rotation test was completed (Day 19 post lesion). The animals were then sacrificed by transcardial perfusion-fixation 20 days after infusion and their brains were used for *post-mortem* assessment of nigrostriatal neurodegeneration and microgliosis using quantitative tyrosine hydroxylase and OX-42 immunohistochemistry respectively.

4.2.1.3 The neuroprotective potential of GDNF-MSCs in the LPS model of Parkinson's disease

Male Sprague Dawley rats underwent habituation and baseline testing on the Stepping and Whisker tests and were performance matched into 3 groups (LPS lesion only, LPS lesion + GFP-MSCs, LPS lesion + GDNF-MSCs; n=10 per group). Transplanted rats received dual unilateral injections of either 100,000 GFP-MSCs or 100,000 GDNF-MSCs into the left striatum. One day post-transplantation, all animals received a unilateral infusion of LPS (10 µg in 2 µl sterile saline) into the left substantia nigra. Behavioural testing on the spontaneous tests resumed the day after cell transplant surgery and continued for 14 days after which the amphetamine-induced rotation test was completed (Day 15 post-transplantation surgeries). The animals were then sacrificed by transcardial perfusion-fixation (Day 21 post-transplantation surgery, day 20 post-LPS) and their brains processed for *post-mortem* assessment of nigrostriatal

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neurodegeneration, microgliosis and grafted cell survival using quantitative immunohistochemistry.

4.3 RESULTS

4.3.1 INTRA-STRIATAL LPS ADMINISTRATION

In this study the effects of infusion of 10 µg of LPS to the rat striatum were assessed. Specifically, the effect of unilateral intra-striatal LPS on motor function neuroinflammation, nigrostriatal integrity was assessed.

4.3.1.1 Intra-striatal LPS administration induced contralateral motor dysfunction

In order to assess the impact of intra-striatal LPS administration on motor function, we subjected the rats to a battery of behavioural tests that assess different aspects of the motor disorder. However, we first wanted to confirm that any behavioural impairments were due to the neuroinflammatory effects of LPS on the injected side and not a general 'sickness behaviour' (Hart, 1988) known to affect locomotor activity. To do this, we first assessed motor function on the side ipsilateral to the side of LPS administration and found that intrastriatal LPS administration did not significantly impair ipsilateral motor performance in the Corridor, Stepping or Whisker tests (**Fig 4.1**). Due to their contralateral impairment (see **Fig 4.2**), LPS-injected animals developed a transient bias towards the ipsilateral side in the Corridor test and they preferentially retrieved CocoPops® from this side for the first week after LPS administration (Group x Time, $F_{(18,252)} = 3.37$, $P < 0.0001$). This ipsilateral assessment confirms that LPS did not induce a general 'sickness behaviour' and that any behavioural changes observed on the contralateral side were due to inflammation-driven events on the injected side of the brain.

In contrast to the ipsilateral side, intra-striatal LPS administration induced significant (but transient) contralateral impairment in motor function in the Corridor test of contralateral neglect (Group x Time, $F_{(18,252)} = 3.47$, $P < 0.001$) and the Stepping test of forelimb akinesia (Group x Time, $F_{(18,252)} = 2.83$, $P < 0.001$), and also induced stable deficits in the Whisker test of sensorimotor integration (Group x Time, $F_{(18,252)} = 2.40$, P

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< 0.01) (**Fig 4.2**). Striatically-injected rats also displayed a rotational asymmetry (towards the ipsilateral side) after amphetamine injection ($t_{(14)} = 2.34$, $P < 0.05$) (**Fig 4.2**).

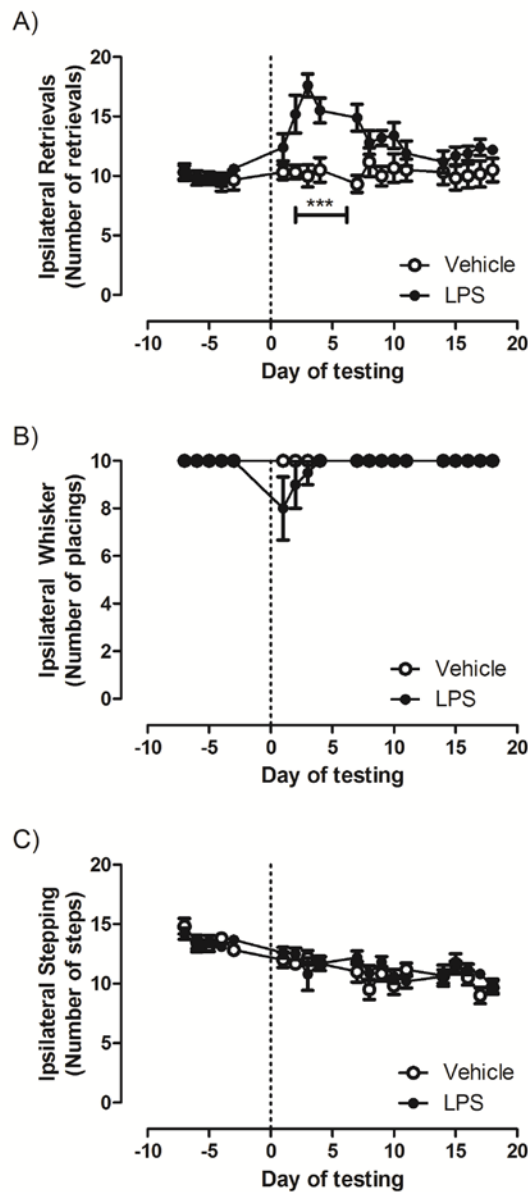


Fig 4.1 Ipsilateral motor function after unilateral intra-striatal LPS administration. Unilateral injection of LPS into the striatum did not cause any ipsilateral dysfunction in the Whisker (B) or Stepping (C) Tests of spontaneous motor function indicating that any impairments observed on the contralateral side were not due to a general sickness behaviour. Because of their contralateral impairment, LPS-injected animals preferentially retrieved CocoPops® from the ipsilateral side in the Corridor Test (A) for the first week after LPS administration. The dotted line at Day 0 indicates the day of surgery. Data are shown as mean ± s.e.m. *** $P < 0.001$ vs. Vehicle by 2-way repeated measures ANOVA with *post-hoc* Bonferroni.

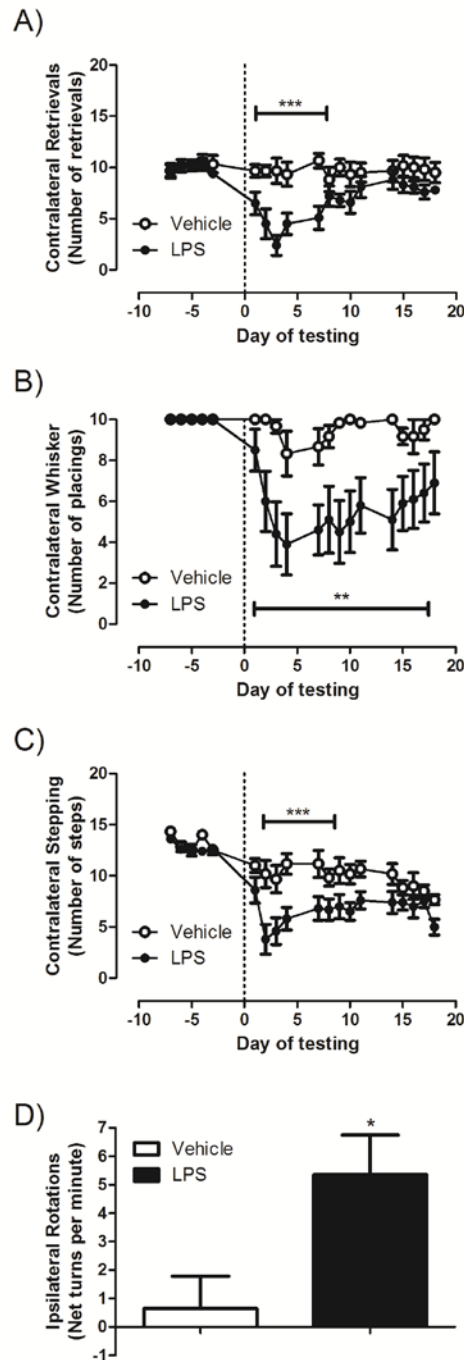


Fig 4.2 Contralateral motor function after unilateral intra-striatal LPS administration. Unilateral injection of LPS into the striatum induced transient impairments in the Corridor (A) and Stepping Tests (C), and stable impairments in the Whisker Test (B). The lesion also caused ipsilateral rotational asymmetry in the Rotation Test at Day 19 post lesion (D). The dotted line at Day 0 indicates the day of surgery. Data are shown as mean \pm s.e.m. $**P < 0.01$, $***P < 0.001$ vs. Vehicle by 2-way repeated measures ANOVA with *post-hoc* Bonferroni. $*P < 0.05$ vs. Vehicle by unpaired *t*-test.

4.3.1.2 Intra-striatal LPS administration induced localised microgliosis at the site of injection

Here we assessed the ability of intra-striatal LPS to activate microglia in the striatum and/or substantia nigra. We found that intra-striatal injection induces a pronounced microgliosis in the striatum ($t_{(14)} = 3.40$, $P < 0.01$), but not in the substantia nigra, as evidenced by the increased density of OX-42 immunopositive cells surrounding the injection site (**Fig. 4.3**).

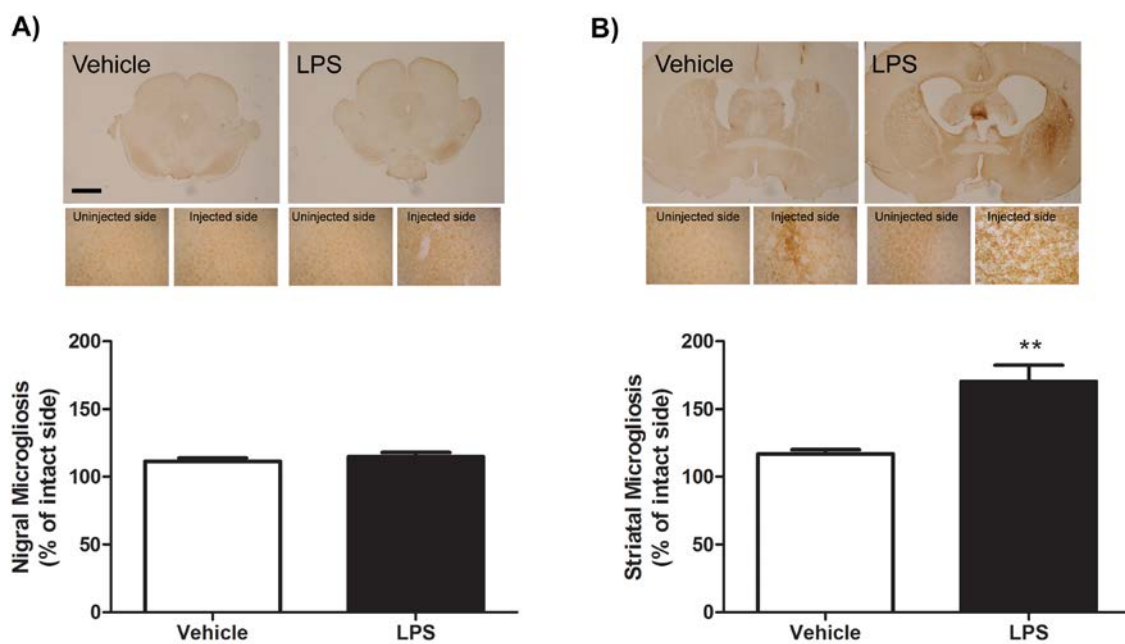


Fig 4.3 Nigrostriatal microgliosis after unilateral intra-striatal LPS administration. OX-42 immunohistochemical staining revealed the extent of microgliosis in the nigrostriatal pathway after injection of LPS into the striatum (substantia nigra shown in A, striatum shown in B). Quantitative analysis of the density of OX-42-positive staining verified that intra-striatal administration of the inflammagen induced a significant microglial reaction at the site of administration. Data are shown as mean \pm s.e.m. ** $P < 0.01$ vs. Vehicle by unpaired t -test. Scale bar represents 1.5 mm (low magnification images) or 400 μ m (high magnification images).

4.3.1.3 Intra-striatal LPS administration did not induce nigrostriatal neurodegeneration

Here we assessed the ability of intra-striatal LPS to cause retrograde neurodegeneration of the nigrostriatal pathway when administered into the striatum. We found that intra-striatal injection of the endotoxin did not cause any statistically significant loss of nigrostriatal cell bodies or terminals despite the significant microgliosis observed in the striatum (**Fig 4.4**).

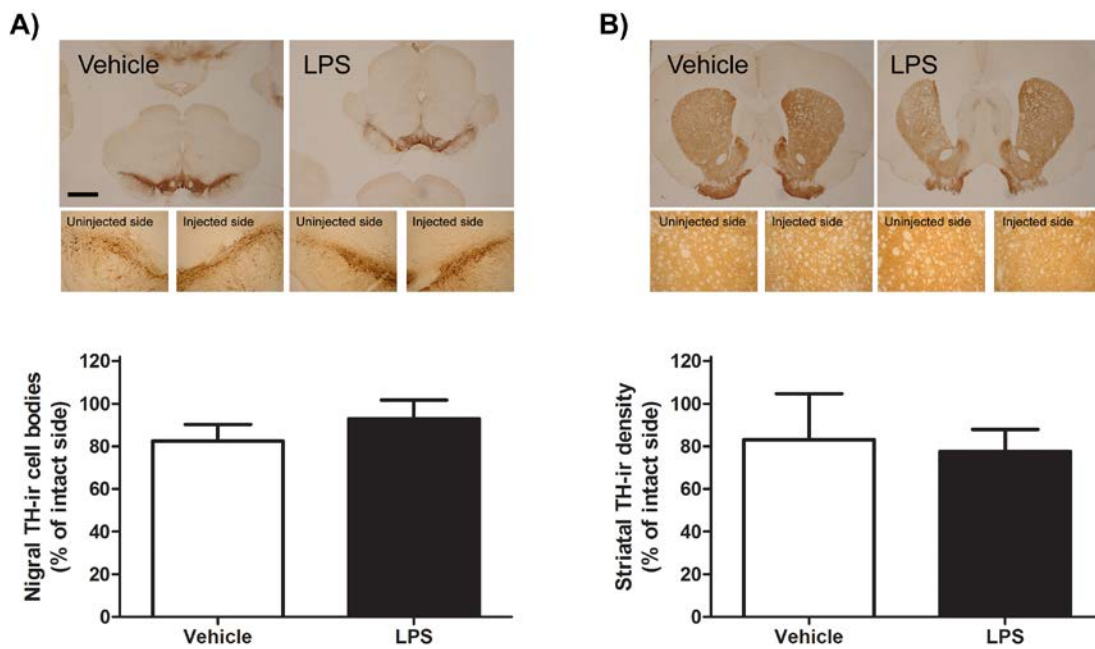


Fig 4.4 Nigrostriatal integrity after unilateral intra-striatal LPS administration. Tyrosine hydroxylase immunohistochemical staining revealed no overt nigrostriatal neurodegeneration after injection of LPS into the striatum (substantia nigra shown in A, striatum shown in B). Quantitative analysis of tyrosine hydroxylase-positive staining confirmed that intra-striatal administration of the inflammagen did not induce degeneration of nigrostriatal cell bodies or terminals. Data are shown as mean \pm s.e.m. TH-ir: Tyrosine hydroxylase immunoreactivity. Scale bar represents 1.5 mm (low magnification images) or 400 μ m (high magnification images).

4.3.2 INTRA-NIGRAL LPS ADMINISTRATION

In this study the effects of infusion of 10 µg of LPS to the rat substantia nigra were assessed. As with the previous study, the effect of unilateral intra-nigral LPS on motor function, neuroinflammation and nigrostriatal integrity was assessed.

4.3.2.1 Unilateral intra-nigral LPS administration induced significant contralateral motor dysfunction

Similarly to the study performed to assess the effects of intra-striatal LPS, we subjected the rats to a battery of behavioural tests that assess different aspects of the motor disorder. We first confirmed that any behavioural impairment was due to the neuroinflammatory effects of LPS on the injected side and not a general 'sickness behaviour' by assessing motor function on the side ipsilateral to the side of intra-nigral LPS administration. Intra-nigral LPS administration did not significantly impair ipsilateral motor performance in the Corridor, Stepping or Whisker tests (**Fig 4.5**). In contrast to the ipsilateral side, on the contralateral side, intra-nigral LPS administration induced pronounced and stable deficits in the Stepping test of forelimb akinesia (Group x Time, $F_{(18,180)} = 14.36$, $P < 0.001$) and the Whisker test of sensorimotor integration (Group x Time, $F_{(18,180)} = 39.08$, $P < 0.001$) (**Fig 4.6**). Intriguingly, despite intra-nigral LPS administration inducing a pronounced nigral microgliosis and nigrostriatal neurodegeneration (see **Figs 4.7** and **4.8**), these pathogenic features only transiently affected Corridor test performance (Group x Time, $F_{(18,180)} = 2.59$, $P < 0.001$) and did not induce any rotational asymmetry after amphetamine injection ($t_{(10)} = 2.06$, $P = 0.07$, ns) (**Fig 4.6**).

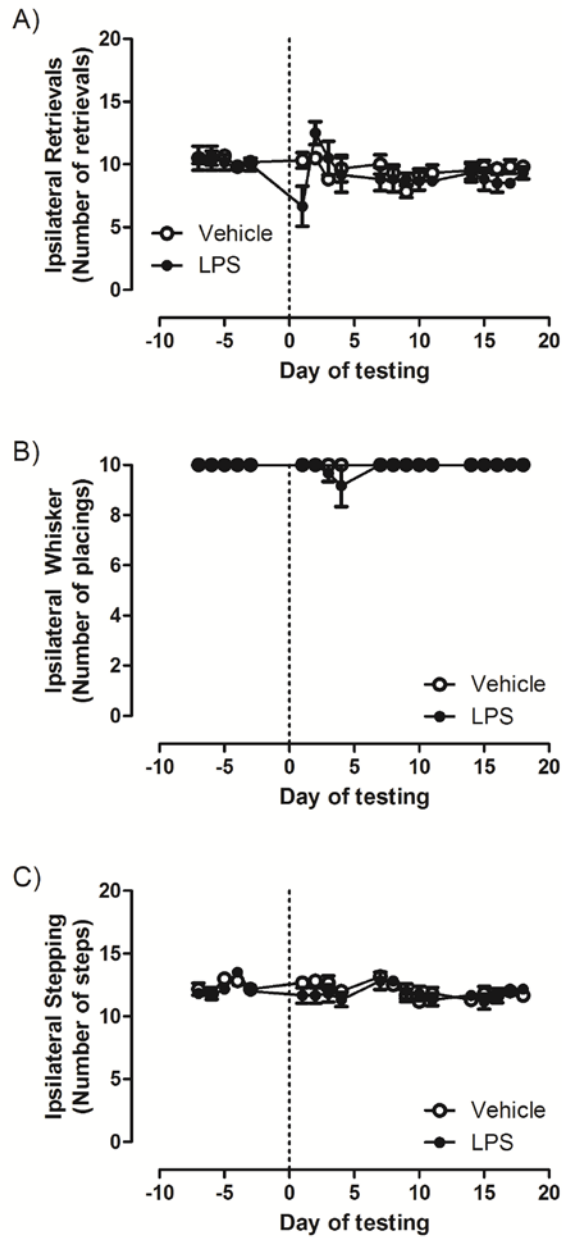


Fig 4.5 Ipsilateral motor function after unilateral intra-nigral LPS administration. Unilateral injection of LPS into the substantia nigra did not cause any ipsilateral dysfunction in the Corridor (A), Whisker (B) or Stepping (C) tests of spontaneous motor function indicating that any impairments observed on the contralateral side were not due to a general sickness behaviour. The dotted line at Day 0 indicates the day of surgery. Data are shown as mean \pm s.e.m.

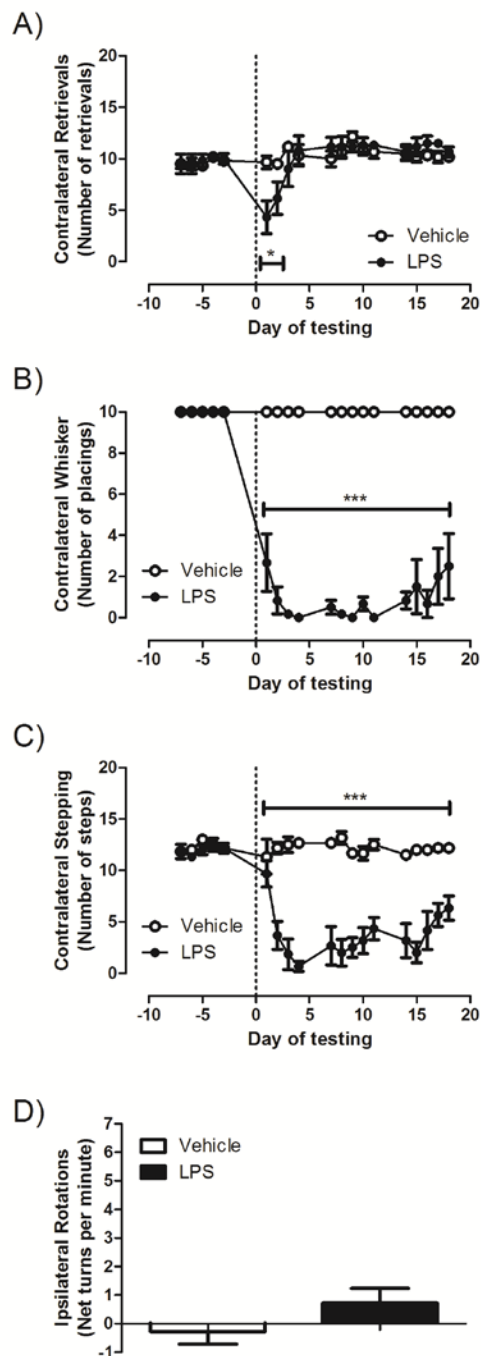


Fig 4.6 Contralateral motor function after unilateral intra-nigral LPS administration. Unilateral injection of LPS into the substantia nigra induced stable deficits in the Stepping (B) and Whisker (C) Tests but not in the Corridor (A) or Rotation (D) Tests. The dotted line at Day 0 indicates the day of surgery. Data are shown as mean \pm s.e.m. * $P < 0.05$, *** $P < 0.001$ vs. Vehicle by 2-way repeated measures ANOVA with *post-hoc* Bonferroni.

4.3.2.2 Intra-nigral LPS administration induced localised microgliosis at the site of injection

In agreement with the results of the previous study where we assessed the impact of intra-striatal infusion of LPS on host microgliosis, here we show that intra-nigral injection of the inflammagen induces a pronounced microgliosis in the substantia nigra ($t_{(10)} = 6.01$, $P < 0.001$) (Fig 4.7). Interestingly, intra-nigral administration of LPS did not induce any microgliosis in the striatum despite the nigrostriatal lesion extending to this site (Fig 4.8).

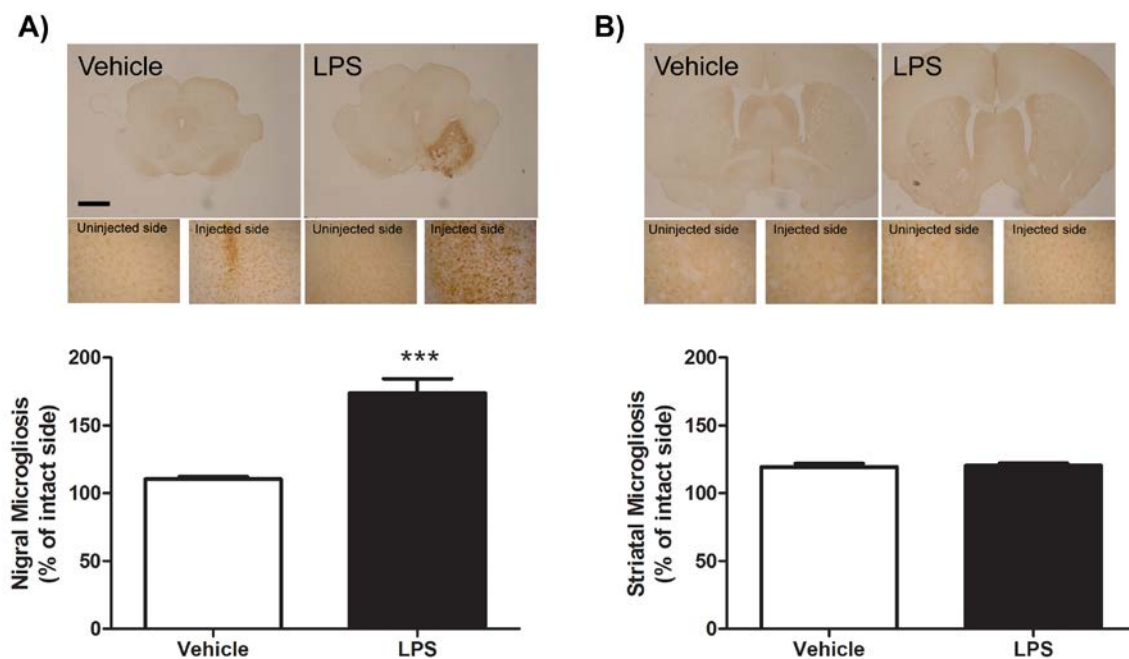


Fig 4.7 Nigrostriatal microgliosis after unilateral intra-nigral LPS administration. OX-42 immunohistochemical staining revealed the extent of microgliosis in the nigrostriatal pathway after injection of LPS into the substantia nigra (substantia nigra shown in A, striatum shown in B). Quantitative analysis of the density of OX-42-positive staining confirmed that intra-nigral administration of the inflammagen induced a significant microglial reaction at the site of administration. Data are shown as mean \pm s.e.m. *** $P < 0.001$ vs. Vehicle by unpaired t -test. Scale bar represents 1.5 mm (low magnification images) or 400 μ m (high magnification images).

4.3.2.3 Intra-nigral LPS administration induced significant nigrostriatal neurodegeneration

In contrast to the previous study where we reported that intra-striatal infusion of LPS failed to induce nigrostriatal degeneration, here we show that intra-nigral infusion of the endotoxin causes a significant loss of tyrosine hydroxylase immunopositive cell bodies from the substantia nigra ($t_{(10)} = 5.42, P < 0.001$) as well as terminals from the striatum ($t_{(10)} = 8.72, P < 0.001$) (**Fig 4.8**).

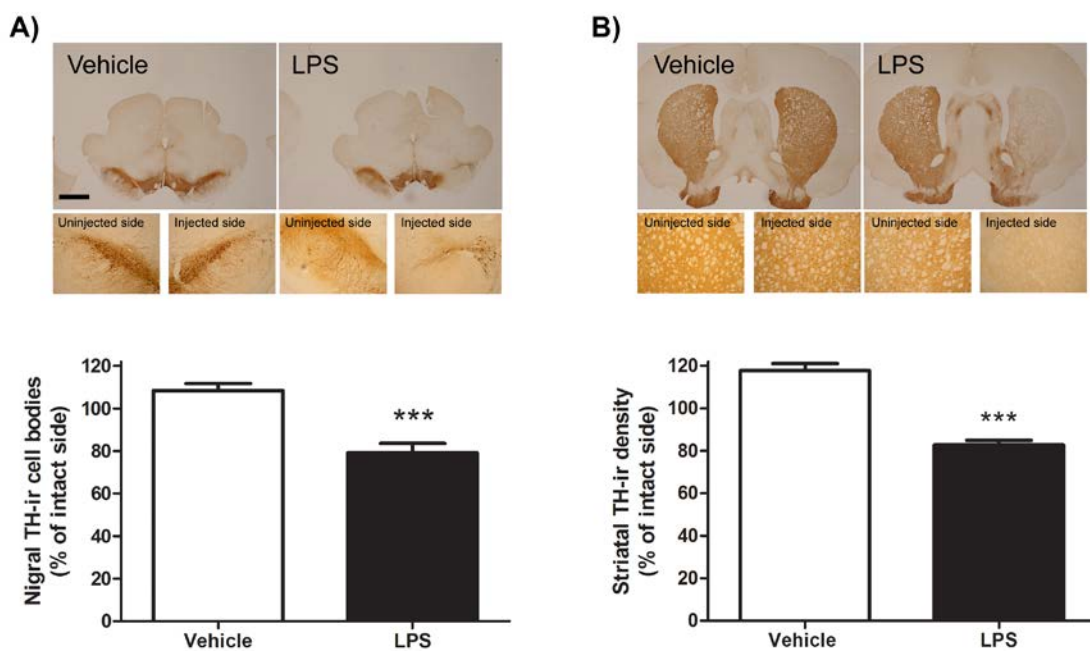


Fig 4.8 Nigrostriatal integrity after unilateral intra-nigral LPS administration. Tyrosine hydroxylase immunohistochemical staining revealed the extent of nigrostriatal neurodegeneration after injection of LPS into the substantia nigra (substantia nigra shown in A, striatum shown in B). Quantitative analysis verified that intra-nigral administration of the inflammagen induced degeneration of the nigrostriatal cell bodies and terminals. Data are shown as mean \pm s.e.m. *** $P < 0.001$ vs. Vehicle by unpaired t -test. TH-ir: Tyrosine hydroxylase immunoreactivity. Scale bar represents 1.5 mm (low magnification images) or 400 μ m (high magnification images).

4.3.3 THE NEUROPROTECTIVE POTENTIAL OF GDNF-MSCs IN THE LPS MODEL OF PARKINSON'S DISEASE

Having determined that unilateral intra-nigral administration of LPS produced nigral microgliosis, nigrostriatal neurodegeneration and stable contralateral motor impairments (Hoban et al., 2013a), we then sought to determine if GDNF-transduced MSCs could protect the nigrostriatal pathway against this inflammation-induced Parkinsonism. To do so, the ability of intra-striatally transplanted GDNF-MSCs or control GFP-MSCs to protect against the motor dysfunction and neuropathology induced by unilateral intra-nigral infusion of 10 µg of LPS was assessed. Since the previous study determined that the motor impairment induced by LPS was not evident in the Corridor test, we excluded this test from this experimental paradigm.

4.3.3.1 GDNF-MSCs did not prevent LPS induced contralateral motor impairment

Akin to the results of the previous study, intra-nigral LPS administration did not impair ipsilateral motor performance in the Stepping test of forelimb akinesia (Group x Time, $F_{(18,297)} = 1.90$, $P = \text{n.s.}$) and the Whisker test of sensorimotor integration (Group x Time, $F_{(18,297)} = 1.06$ $P = \text{n.s.}$) (**Fig 4.9**). However, injection of the inflammagen resulted in a (transient) impairment in the rats' ability to make contralateral adjusting steps in the Stepping Test (Time, $F_{(9,297)} = 30.86$, $P < 0.001$) and reduced contralateral vibrissae-elicited forelimb placings in the Whisker test (Time, $F_{(9,297)} = 14.43$, $P < 0.001$). No functional protection was afforded by the intra-striatal transplantation of either GFP-MSCs or GDNF-MSCs prior to induction of the lesion (**Fig 4.10**). We again confirmed that intra-nigral LPS did not induce any rotational asymmetry after amphetamine injection, and cell transplants had no effect on this result (**Fig 4.10**).

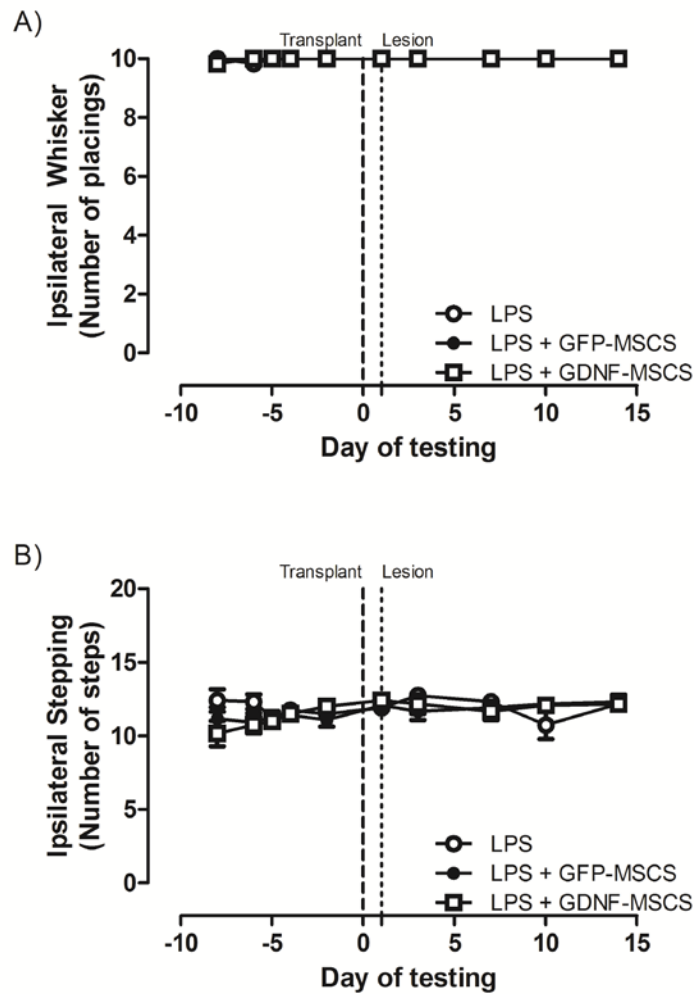


Fig 4.9 Impact of GDNF-MSCs on ipsilateral motor function after unilateral intra-nigral LPS administration. Unilateral injection of LPS into the substantia nigra did not cause any ipsilateral dysfunction in the (A) Whisker or (B) Stepping tests of spontaneous motor function. This was not affected by intra-striatal transplantation of MSCs. The dashed line at Day 0 indicates the day of cell transplantation surgery, the dotted line at Day 1 indicates the day of lesion surgery. Data are shown as mean \pm s.e.m.

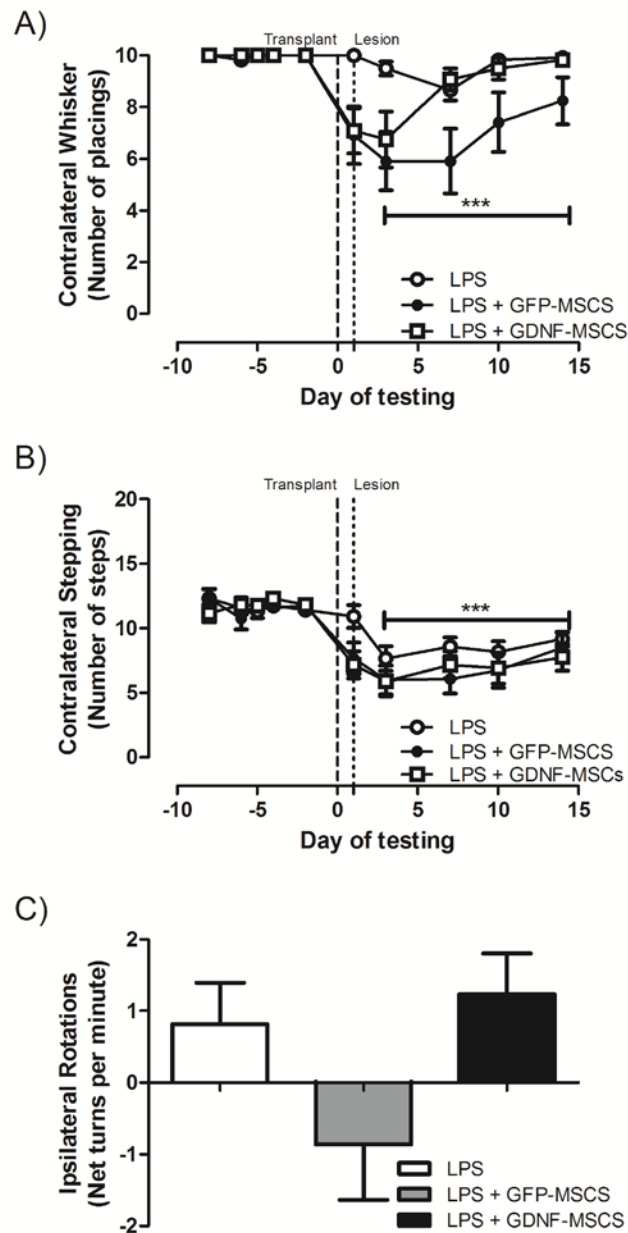


Fig 4.10 Impact of GDNF-MSCs on contralateral motor function after unilateral intra-nigral LPS administration. Unilateral injection of LPS into the substantia nigra induced contralateral deficits in the (A) Whisker and (B) Stepping Tests but not in the (C) Rotation Test. This was not affected by intra-striatal transplantation of MSCs. The dashed line at Day 0 indicates the day of cell transplantation surgery, the dotted line at Day 1 indicates the day of lesion surgery. Data are shown as mean \pm s.e.m. *** $P < 0.001$ vs. Baseline by 2 way ANOVA with *post hoc* Bonferroni.

4.3.3.2 GDNF-MSCs did not prevent LPS induced localised microgliosis at the site of injection

Consistent with both previous studies, intracerebral infusion of LPS resulted in a localised microgliosis at the lesion site, in this case the substantia nigra (Side, $F_{(1,54)} = 16.96$, $P < 0.001$) (**Fig 4.11**). Not surprisingly (given the spatial separation between the site of the lesion (substantia nigra) and the site of cell implant (striatum), intra-striatal transplantation of neither GFP-MSCs nor GDNF-MSCs had any effect on this lesion induced microgliosis (Group, $F_{(2,54)} = 0.43$, $P = \text{n.s.}$).

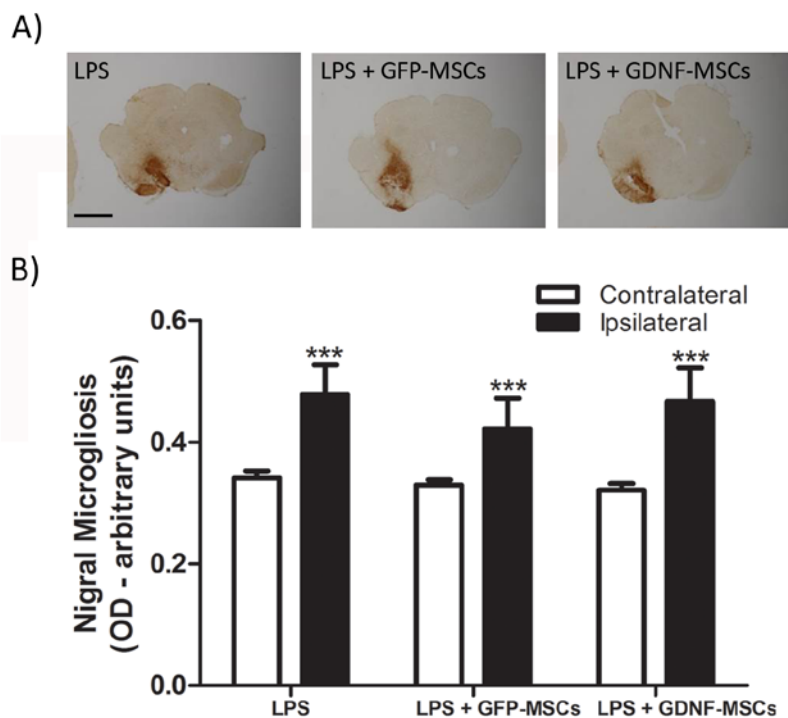


Fig 4.11 Impact of GDNF-MSCs on nigrostriatal microgliosis after unilateral intra-nigral LPS administration. A) Photomicrographs of OX-42 immunohistochemical staining confirmed that injection of LPS into the substantia nigra resulted in pronounced microgliosis at this site. B) Quantitative analysis of density of OX-42 staining confirmed that intra-nigral administration of the inflammagen induced a significant microgliotic reaction at the site of administration (when compared to the intact side), and that this was not affected by intra-striatal transplantation of MSCs. Data are shown as mean \pm s.e.m. *** $P < 0.001$ vs. Contralateral by 2 way ANOVA with *post hoc* Bonferroni. Scale bar represents 1.5 mm.

4.3.3.3 Survival of transplanted MSCs

Survival of transplanted MSCs was assessed by quantification of the volume of the GFP-positive cells remaining at 21 days post-transplantation (**Fig 4.12**). Transplanted cells were easily identifiable due to their expression of the GFP reporter gene. Despite this, surviving cells were observed in only some of the transplanted animals at the time point examined in this study (Day 21 after transplantation). However, this level of cell survival is consistent with a previous report from our laboratory in which we demonstrated the progressive death of these cells *in vivo*, with the majority of cells dying within one week post-transplantation (Moloney et al., 2010a). There was no difference in grafted cell survival between the two transplanted cell types ($t_{(6)} = 0.60$, $P = \text{n.s.}$

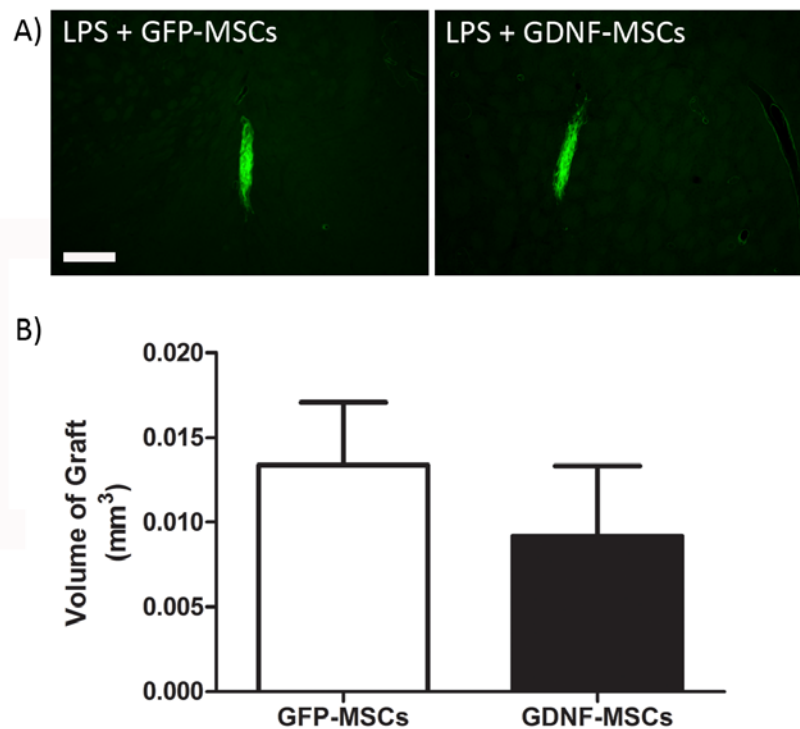


Fig 4.12 Survival of transplanted GFP-MSCs and GDNF-MSCs in the LPS-lesioned striatum. A) Representative photomicrographs showing that transplanted GFP-MSCs and GDNF-MSCs were identifiable *in vivo* due to their strong GFP expression at 21 days post-transplantation. B) Quantitative analysis of the volume of the graft showed that there was no significant difference in cell survival between either cell type. Data are shown as mean \pm s.e.m. Scale bar represents 500 μm .

4.3.3.4 GDNF-MSCs did not protect against LPS-induced nigrostriatal neurodegeneration

As in the previous study, unilateral intra-nigral injection of LPS resulted in significant nigrostriatal neurodegeneration at the level of both the cell bodies and the terminals (**Fig 4.13**). LPS caused the death of approximately 20% of dopaminergic cell bodies in the substantia nigra (Side, $F_{(1,54)} = 11.87$, $P < 0.01$) and loss of 20% of dopaminergic terminals in the striatum (Side, $F_{(1,54)} = 19.43$, $P < 0.001$). This was observed in all groups, regardless of cell transplantation. That is, neither the GFP-MSCs nor the GDNF-GFP-MSCs transplanted into the striatum were capable of protecting the nigrostriatal pathway as a whole (Group, $F_{(2,48)} = 1.04$, $P = \text{n.s.}$).

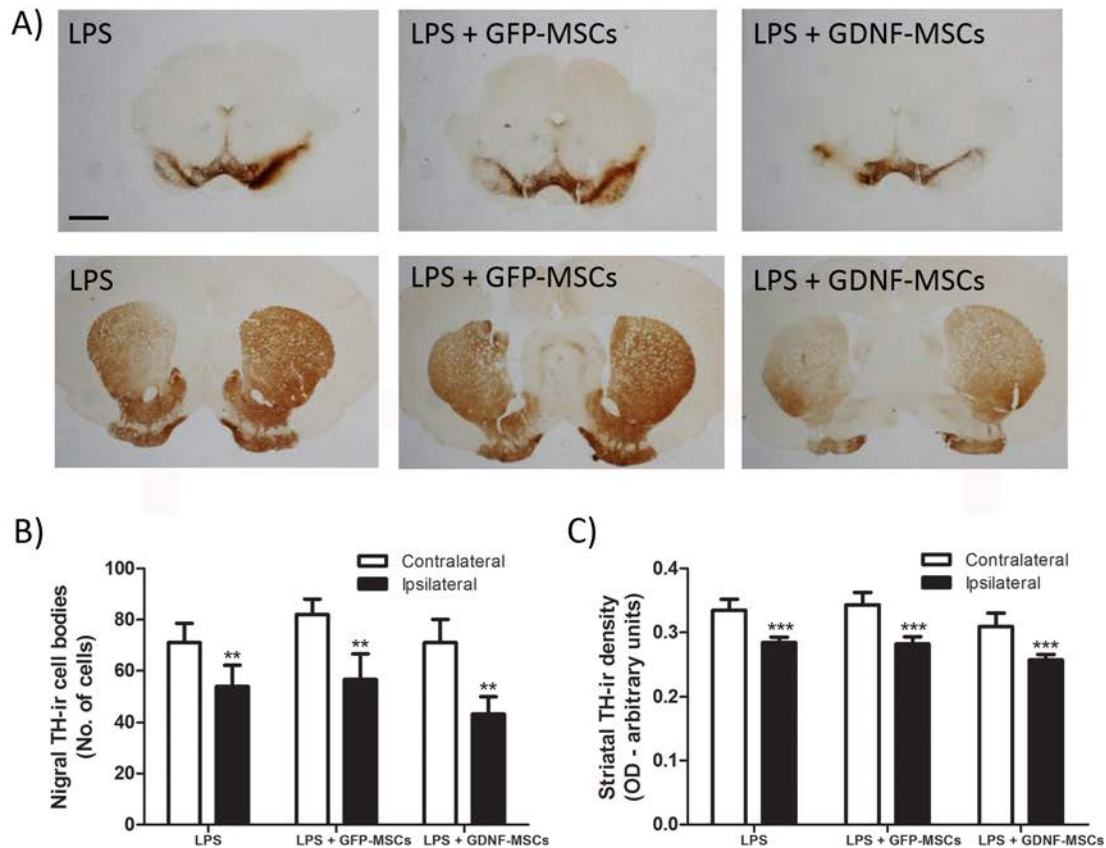


Fig 4.13 Impact of GDNF-MSCs on nigrostriatal integrity after unilateral intra-nigral LPS administration of MSCs. A) Tyrosine hydroxylase immunohistochemical staining revealed the extent of nigrostriatal neurodegeneration after injection of LPS into the substantia nigra (the lesion is on the left side). Photomicrographs show the tyrosine hydroxylase-positive immunostaining of the cell bodies in the substantia nigra and the terminals of the striatum. B) Quantitative analysis revealed that neither GFP-MSCs nor GDNF-MSCs were able to protect the dopaminergic cell bodies from degeneration with the number of cell bodies significantly reduced compared to the contralateral intact (right) side. C) Additionally, neither cell type was capable of protecting the striatal terminals from degeneration with the density of striatal fibres reduced when compared to the contralateral intact (right) side. Data are shown as mean \pm s.e.m. ** $P < 0.01$, *** $P < 0.001$ vs. Contralateral by 2 way ANOVA with *post hoc* Bonferroni). TH-ir: Tyrosine hydroxylase immunoreactivity. Scale bar represents 1.5 mm.

4.3.3.5 Transplanted GDNF-MSCs provided local neuroprotection of dopaminergic terminal in the striatum

Despite the fact that neither GFP-MSCs nor GDNF-MSCs were capable of protecting the nigrostriatal terminals when assessed throughout the entire rostro-caudal axis of the striatum, when the tyrosine hydroxylase-positive staining in the striatum was assessed directly in the vicinity of the transplant site, discrete regions of dense tyrosine hydroxylase-positive staining were observed (**Fig 4.14**). Quantitative analysis of the density of staining proximal to the transplant in comparison with the depleted tyrosine hydroxylase-positive regions distal to the transplant verified this observation. Importantly, this effect was observed only in the GDNF-MSC transplanted group and not the GFP-MSC group (Group x Region, $F_{(1,34)} = 10.16$ *** $P < 0.001$).

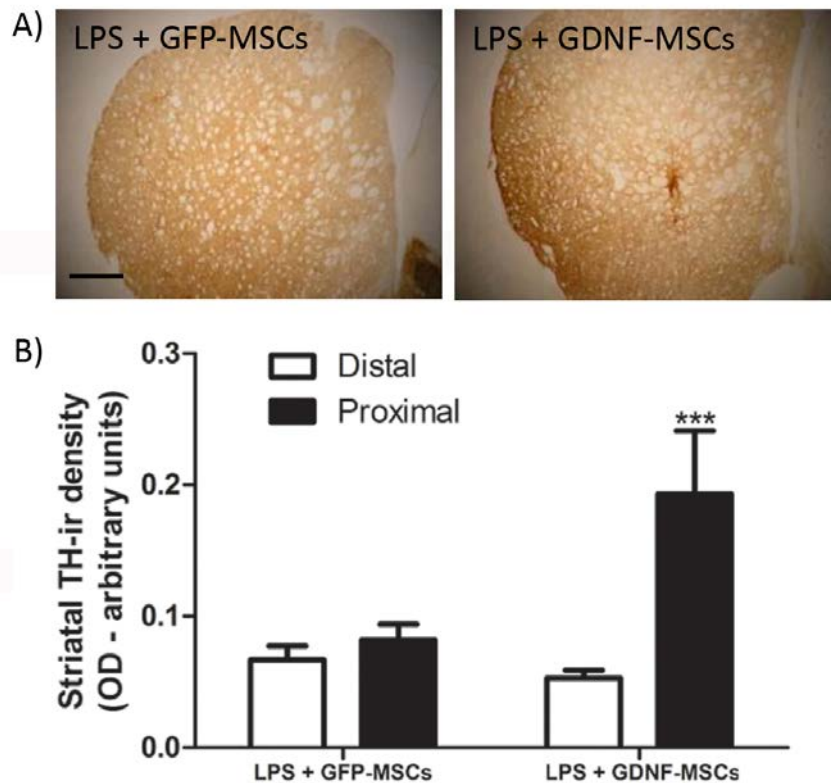


Fig 4.14 GDNF-MSCs induced local neuroprotection of dopaminergic terminals in the striatum. A) Tyrosine hydroxylase-positive staining in the striatum was assessed directly in the vicinity of the transplant site. In animals that received GDNF-MSCs, discrete regions of dense tyrosine hydroxylase-immunoreactive fibres were present in the vicinity of the transplant. This effect was not observed in the animals that received GFP-MSCs. B) Quantitative analysis of the density of tyrosine hydroxylase-positive staining in the denervated striatum (distal) compared with the density of tyrosine hydroxylase-positive staining surrounding the graft (proximal) confirmed that this effect was exclusive to the animals transplanted with GDNF-MSCs and not GFP-MSCS. Data are shown as mean \pm s.e.m. *** $P < 0.001$ vs. distal by 2 way ANOVA with *post hoc* Bonferroni. TH-ir: Tyrosine hydroxylase immunoreactivity. Scale bar represents 750 μ m.

4.4 DISCUSSION

This first section of this chapter sought to further characterise the LPS model of PD, primarily focussing on the behavioural aspect of the model which has been sparsely reported in the literature to date. We compared the impact of unilateral intra-nigral and intra-striatal LPS administration on nigrostriatal microgliosis and neurodegeneration, and particularly the consequences of these neuropathological features on lateralised motor function. We found that injection of LPS into the substantia nigra caused pronounced and stable deficits in contralateral forelimb kinesis (Stepping test) and sensorimotor integration (Whisker test) but did not induce stable contralateral neglect (Corridor test) or rotational asymmetry after amphetamine administration. This behavioural pattern was underpinned by nigral microgliosis and loss of both nigrostriatal cell bodies and terminals. In contrast, injection of LPS into the striatum induced only mild and transient impairments in spontaneous contralateral motor function but did induce amphetamine-induced turning bias. These behaviours were associated with striatal microgliosis and an intact nigrostriatal pathway. This study indicates that intracerebral administration of LPS induces a complex behavioural outcome that is highly dependent on the nigrostriatal site of administration as well as on the choice of motor test (Hoban et al., 2013a).

Microglia are the resident innate immune cell of the CNS and are activated by the bacterial endotoxin, LPS, by virtue of their expression of the TLR4 receptor (Hoshino et al., 1999, Lehnardt et al., 2003). LPS-induced microglial activation results in the release of numerous pro-inflammatory substances including TNF α , IL-1 β , IL-6, proteinases, eicosanoids, and reactive nitrogen and oxygen species (reviewed in Dutta et al., (2008)). Microglia-derived TNF α and IL-1 β have been implicated in dopaminergic neuron death because these neurons express the relevant cytokine receptors (McCoy et al., 2006, Koprach et al., 2008, Long-Smith et al., 2010). Microglia-derived reactive nitrogen and oxygen species have also been implicated in dopamine neuron death as they cause DNA damage, lipid peroxidation and protein damage leading to oxidative stress, neuronal cell injury and death (Mosley et al., 2006). Dopaminergic neurons are particularly sensitive

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to oxidative stress largely owing to low intracellular levels of antioxidant glutathione (Block and Hong, 2005, Gonzalez-Hernandez et al., 2010). In this study we show that injection of LPS into the rat substantia nigra causes nigral microgliosis and degeneration of the nigrostriatal pathway at the level of the cell bodies in the substantia nigra and terminals in the striatum which is in line with previous reports (Bing et al., 1998, Castano et al., 1998, Hsieh et al., 2002, Herrera et al., 2000, Lu et al., 2000, Arimoto et al., 2007). In contrast, injection of the inflammagen into the striatum caused striatal microgliosis (as previously reported (Hunter et al., 2007a, Hunter et al., 2007b, Choi et al., 2009, Hunter et al., 2009)) but did not cause degeneration of the nigrostriatal pathway which is in contrast with previous reports.

The reason why striatal LPS administration did not cause nigrostriatal neurodegeneration in our study is possibly because rostrocaudal distribution of the endotoxin may be required to achieve dopaminergic deafferentation of the striatum with retrograde degeneration of the nigrostriatal neurons. Thus, Hunter et al., (2007b) injected 16 μg at two sites (32 μg in total) and Choi et al., (2009) injected 7.5 μg at four sites (32 μg in total) whereas we injected 10 μg at a single site. The reason that we injected 10 μg at a single striatal site was to allow for a direct comparison with the single 10 μg intra-nigral injection. However, although this dose was sufficient to induce nigrostriatal neurodegeneration after nigral injection (probably due to the high density of microglia in the substantia nigra (Kim et al., 2000)), it is probable that higher doses and more rostro-caudal distribution of the inflammagen is required to obtain retrograde nigrostriatal degeneration after striatal injection. Although intra-striatal injection of LPS did not yield motor impairments in this study, it may still be of relevance to the modelling of PD if it were incorporated into a multi-hit model of the condition. Thus, since cell loss in PD arises as a result of multiple pathogenic events caused by genetic and/or environmental factors, incorporation of LPS-induced neuroinflammation with other environmental or genetic risk factors may yield a relevant model of the human condition and/or render subclinical doses of dopaminergic toxins more effective (Ling et al., 2004a, Ling et al., 2004b, Koprach et al., 2008, Byler et al., 2009, Mulcahy et al., 2012).

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The main focus of this study however, was to further characterise the motor features of the LPS models of Parkinson's disease by comparing and contrasting the behavioural syndrome resulting from unilateral intra-nigral and intra-striatal injection of the inflammagen. An 18 day time-course for behavioural analyses was chosen based on previous literature showing that significant dopaminergic neuron loss is evident from 7–21 days after intra-cerebral LPS injection (Castano et al., 2002, Arai et al., 2004, De Pablos et al., 2005, Harkavyi et al., 2008, Koprach et al., 2008). After nigral injection of LPS, the resultant neuroinflammation and neurodegeneration caused a striking pattern of behavioural impairments on the contralateral side of the rats' bodies. Specifically, performance in the Stepping and Whisker tests was profoundly and stably impaired indicating that intra-nigral LPS can induce dysfunction in forelimb kinesis and sensorimotor integration, both of which are motor functions highly relevant to the human condition (Jankovic, 2008). To our knowledge, this is the first time that the impact of LPS administration on these widely-used tests of lateralised motor function has been assessed as most previous studies have focussed on general measures of locomotor activity or artificial measures of motor asymmetry such as drug-induced rotation (Hsieh et al., 2002, Zhang et al., 2005a, Choi et al., 2009, Hunter et al., 2009). Intriguingly, intra-nigral LPS administration did not result in a stable impairment in the Corridor test of contralateral neglect even though pronounced and stable deficits were observed in the Stepping and Whisker tests. We and others have shown on many previous occasions that this test is sensitive to unilateral nigrostriatal degeneration caused by other Parkinsonian insults including 6-hydroxydopamine, rotenone and AAV- α -synuclein (Dowd et al., 2005a, Fitzsimmons et al., 2006, Grealish et al., 2008, Grealish et al., 2010, Mulcahy et al., 2012, Mulcahy et al., 2011). However the extent of nigrostriatal cell loss caused by intra-nigral administration of 10 μ g of LPS in this study was clearly not of a sufficient magnitude to induce deficits in this task.

Another surprising and unexpected finding of this study was that intra-nigral LPS administration did not result in amphetamine-induced ipsilateral rotation even though the rats had a clear, albeit relatively mild, unilateral nigrostriatal lesion. Ordinarily this test confirms the presence of a unilateral nigrostriatal lesion as amphetamine releases

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dopamine from the intact nigrostriatal neurons causing the rat to turn in an ipsilateral direction (Ungerstedt and Arbuthnott, 1970). Our finding that nigraly lesioned rats did not rotate is at odds with some previous literature showing that unilateral intra-nigral injection of 10 µg of LPS can induce lateralised nigrostriatal neurodegeneration of sufficient magnitude to cause ipsilateral turning after amphetamine challenge (Iravani et al., 2005, Iravani et al., 2008). However, there are a number of important differences between the current study and these previous studies which may account for the differences seen. Firstly, Iravani et al., (2008) used the Wistar strain of rat whereas we used the Sprague Dawley strain. Since previous studies have shown differences in sensitivity to LPS between rat strains (Grota et al., 1997, Tonelli et al., 2001, Taylor et al., 2005), and even within the same strain obtained from different vendors (Turnbull and Rivier, 1999), it is possible that strain differences may have accounted for the discrepancies observed between the studies. Moreover, in the studies by (Iravani et al., 2005, Iravani et al., 2008) a dose of 5 mg/kg amphetamine was used to induce turning whereas we used a dose of 2.5 mg/kg. Thus dose differences may also have accounted for the discrepancy between this study and the previous ones. Whatever the reason, what is clear is that in the present study, the magnitude of the LPS-induced lesion was not sufficient to induce ipsilateral turning after amphetamine challenge. Thus, the unlesioned nigrostriatal neurons probably compensated for the effect of the lesions by increasing dopamine synthesis, release or turnover, or there may have been a compensatory up-regulation of postsynaptic dopaminergic receptors or an increase in receptor sensitivity (Hornykiewicz, 1975). Such compensatory mechanisms are common to many other models of Parkinson's disease as well as to the human condition. Although it is interesting that rats with stable deficits in the Stepping and Whisker tests did not rotate after amphetamine administration, it has been shown previously that rotation levels do not necessarily correlate with deficits in tests of spontaneous motor function (Metz and Whishaw, 2002).

In contrast to the intra-nigral model, after striatal injection of LPS, the resultant neuroinflammation (which failed to induce nigrostriatal neurodegeneration) only led to mild and transient impairments in spontaneous motor function in the Corridor and

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Stepping tests. This is not surprising as the LPS administration regime chosen (to compare with intra-nigral administration) was not sufficient to induce degenerative damage to the nigrostriatal neurons. Interestingly, in the absence of nigrostriatal asymmetry at an anatomical level, rats with intra-striatal LPS administration did rotate significantly after amphetamine administration. This could indicate that although the dopaminergic neurons did not degenerate, LPS may have induced functional alterations in the nigrostriatal dopaminergic system such as dysregulation of presynaptic dopamine levels, post-synaptic dopamine receptor sensitisation, or alterations in dopamine turnover (Choi et al., 2009, Hsieh et al., 2002). Alternatively, the LPS-induced striatal inflammation may have damaged or suppressed the function of the striatal GABAergic output neurons, evidence of which can be seen by the reduced striatal volume and increased ventricular volume in these animals, thereby reducing their ability to respond to the dopamine released by amphetamine on the lesioned side, and an activation of dopamine receptors on the intact side, resulting in ipsilateral rotation (Yan et al., 2015).

To summarise, in the first section of this chapter we further characterised the intra-cerebral LPS models of Parkinson's disease by reporting the neuropathological and behavioural features induced by intra-striatal and intra-nigral administration of the endotoxin (**Table 4.1**). Injecting LPS into the substantia nigra led to a microgliotic reaction in the substantia nigra and nigrostriatal neurodegeneration with consequent impairments in spontaneous motor function relevant to the human condition. Injecting the inflammagen into the striatum led to a microgliotic reaction in the striatum which was not of sufficient magnitude or extent to induce nigrostriatal neurodegeneration or spontaneous motor impairments. We conclude that the intra-nigral LPS model may be a highly relevant model for researchers attempting to gain a better understanding of immune-mediated events in Parkinson's disease (Hoban et al., 2013a) and thus, decided that intra-nigral administration of LPS was an appropriate model to use in order to assess the neurotrophic potential of GDNF-MSCs.

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Feature Modelled	Test	Intra-nigral LPS	Intra-striatal LPS
Nigral Microgliosis	Nigral OX-42 IHC	Yes	No
Striatal Microgliosis	Striatal OX-42 IHC	No	Yes
Dopaminergic cell body degeneration	Nigral TH IHC	Yes	No
Dopaminergic terminal degeneration	Striatal TH IHC	Yes	No
Contralateral neglect	Corridor Test	No	Yes, but transient
Forelimb akinesia	Stepping Test	Yes	Yes, but transient
Sensorimotor disconnect	Whisker Test	Yes	Yes
-	Rotation	No	Yes

Table 4.1 Summary of results obtained in the first part of this chapter. The neuropathological and behavioural effects of intra-cerebral LPS administration are summarised in this table. Intra-nigral LPS administration caused nigral microgliosis and nigrostriatal neurodegeneration which was accompanied by stable deficits in the Stepping and Whisker tests but not in the Corridor or Rotation tests. In contrast, intra-striatal LPS administration caused striatal microgliosis without nigrostriatal neurodegeneration or stable deficits in the spontaneous motor tests – this approach was, however, associated with impairments in drug-induced rotation. IHC: immunohistochemistry; TH: tyrosine hydroxylase.

The final study in this chapter sought to determine the neurotrophic potential of GDNF-transduced MSCs in the inflammation-driven LPS model of Parkinson's disease. Specifically, we wished to assess the ability of GDNF-MSCs to protect against the motor impairment, neuroinflammation and neurodegeneration induced by a unilateral intra-nigral LPS lesion when transplanted prior to induction of the lesion. Animals received intra-striatal transplants of GDNF-MSCs or GFP-MSCs (as a control) followed by an intra-nigral LPS lesion the next day. The ability of the transplanted cells to afford functional protection was assessed by behavioural testing, while anatomical protection against the lesion effects was assessed using quantitative immunohistochemistry for microgliosis and tyrosine hydroxylase. This study found that transplanted GDNF-MSCs were capable of exerting anatomical neuroprotection of the dopaminergic terminals local to the transplant site in the LPS model of Parkinson's disease. This study

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highlights the potential of MSCs as a cell type for *ex vivo* gene therapy for the application of neurotrophin delivery to the degenerating brain.

Mesenchymal stem cells have previously been exploited for their regenerative and reparative effects in neurodegenerative diseases such as Alzheimer's disease (Lee et al., 2009), amyotrophic lateral sclerosis (Mazzini et al., 2006), Huntington's disease (Lin et al., 2011) and multiple sclerosis (Karussis et al., 2008). The rationale for the utilisation of MSCs as a cell type for transplantation stems from their numerous beneficial characteristics including the ease at which they are attainable for isolation and expansion. MSCs are easily attainable from the bone marrow aspirate of healthy donors (Pittenger et al., 1999), and, if isolated from patient tissue, offer an autologous cell source (Zhang et al., 2008). Using only small volumes of bone marrow aspirate, they are easily cultured and expanded *in vitro* to achieve significant numbers for therapeutic use, without imposing any of the major ethical issues often encountered with the use of alternate cell sources such as embryonic stem cells or fetal tissue (Bartmann et al., 2007, Digirolamo et al., 1999). The mechanism by which MSCs have been reported to exert regenerative effects is via the release of a complement of molecules such as neurotrophic growth factors, chemokines, cytokines, and extracellular matrix proteins that promote endogenous repair either directly or indirectly. Pro-inflammatory stimuli, hypoxic stimuli and factors associated with tissue damage sensed in the local environment all stimulate MSCs to secrete a myriad of neurotrophic factors dubbed the MSC secretome (Chen et al., 2002, Rosova et al., 2008, Paul and Anisimov, 2013). Indeed in the previous chapter, we found that GFP-MSCs were capable of secreting GDNF, in line with previous evidence that they are inherently neurotrophic (Kurozumi et al., 2005).

The ability of MSCs to be virally manipulated with relative ease is another attractive attribute of this particular cell type with regards to their potential use for the treatment of Parkinson's disease. Their manipulation *ex vivo* with a retrovirus means that the GDNF transgene is stably integrated into the host genome, GDNF expression is continuous over numerous passages and any free viral particles that may bear potential

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risk to the recipient, are eliminated at an early stage prior to transplantation (Moloney et al., 2010b). In light of the ability of these MSCs to release GDNF, both in this study and as shown previously (Hoban et al., 2013b, Moloney et al., 2010b, Rooney et al., 2009) and their reported hypoimmunogenic properties (Bartholomew et al., 2002, Rossignol et al., 2009, Moloney et al., 2010a, Di Nicola et al., 2002, Ohtaki et al., 2008), we sought to determine their neurotrophic potential in the inflammation-driven LPS model of Parkinson's disease.

In this study, injection of LPS gave rise to contralateral impairment of motor function, local microgliosis and nigrostriatal neurodegeneration. We, and others, have previously shown this inflammatory and degenerative response to intracerebral infusion of the LPS, and its ability to affect both lateralised and spontaneous motor function (Hoban et al., 2013a, Bing et al., 1998, Castano et al., 1998, Choi et al., 2009). Transplantation of neither the GDNF-MSCs nor the GFP-MSCs to the striatum reduced significant local microgliosis in the substantia nigra (i.e. the lesion site). This is hardly surprising given the spatial separation between the site of the lesion (substantia nigra) and the site of cell implant (striatum). While previous studies have shown that GDNF delivery to the substantia nigra has shown beneficial results (Hoffer et al., 1994, Kearns and Gash, 1995, Tomac et al., 1995), more consistent improvements in terms of dopamine neuron survival, dopamine fibre outgrowth and behavioural motor impairment have been observed following intra-striatal delivery of GDNF (Kirik et al., 2000b, Tomac et al., 1995), hence the rationale for transplantation of the cells to this site rather than closer to lesion site. Because of this spatial difference, the reported hypoimmunogenic properties of MSCs (Yi and Song, 2012) may not be as effective on the LPS-induced microgliosis than if they were transplanted proximal to the lesion site.

Intra-nigral injection of LPS also resulted in an approximate loss of 20% of nigrostriatal dopaminergic neurons regardless of whether they had received a cell transplant or not. This degree of loss explains the mild contralateral motor impairment observed in this study, given that sustained motor impairments are generally not observed in the human condition until approximately 60-80% of dopaminergic neurons have degenerated

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(Bernheimer et al., 1973, Riederer and Wuketich, 1976). As previously reported (Hoban et al., 2013a), the resulting motor impairment, while transient, does appear to be LPS-induced given that the ipsilateral side is completely unaffected by either surgical intervention. Despite the modest degeneration observed in this study, transplanted MSCs did not protect against the lesion-induced motor impairment and nor did they protect the nigrostriatal pathway as a whole from lesion-induced degeneration.

Interestingly, when the tyrosine hydroxylase staining in the striatum was examined in greater detail, with particular focus on the vicinity of the cell transplantation site, dense pockets of tyrosine hydroxylase-positive staining were observed, despite the apparent loss of tyrosine hydroxylase immunoreactivity in the striatum as a whole. Importantly these innervated areas were observed in animals that received transplants of GDNF-MSCs, and were not evident in animals that received control GFP-MSCs indicating that the protection of the striatal dopaminergic terminals was as a result of the secreted GDNF rather than the inherent trophic properties of MSCs.

While the neuroprotection afforded in this study may be modest in nature, we believe this is as a result of the poor survival of the cells after transplantation, rather than a lack of efficacy of the released GDNF. Given the later time-point of sacrifice in this study (Day 21 post-transplantation surgeries) it is no surprise that the survival of these cells was relatively low. We have previously reported on the poor survival of MSCs following transplantation to the brain (Moloney et al., 2010a, Moloney et al., 2010b), with approximate loss of ~35% of transplanted cells between one and four days post-transplantation. While a microtransplantation technique of GDNF-MSCs may impart superior GDNF distribution around the affected striatum, careful consideration must be taken with regards to the invasiveness of the transplantation strategy employed so as not to cause further disruption or damage to the already degenerating nigrostriatal pathway. The issue of *in vivo* cell survival is a substantial one, affecting not just mesenchymal stem cells but also the transplantation of fetal dopaminergic cells where only ~5-20% of cells survive after grafting (Hagell and Brundin, 2001). Indeed this issue is not unique to the treatment of Parkinson's disease either, as poor cell survival rates post-

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transplantation is a concern for a wide range of cell types being exploited for the treatment of a myriad of diseases and conditions. Thus, issues relating to the death of transplanted cells must be addressed before this approach can be considered for clinical translation as a long-term therapeutic strategy.

While the field of transplantation continues to be troubled by issues related to cell survival it is important to note that this method of GDNF delivery does appear promising based on the results of this study. To the best of our knowledge, this is the first study to demonstrate the neurotrophic effects of GDNF in an inflammatory model of Parkinson's disease whether it was delivered by *ex vivo* gene therapy or otherwise (Hoban et al., (Submitted)). Future studies in this thesis will endeavour to increase the survival of cells *in vivo* after transplantation and thereby increasing the amount of secreted GDNF, thus revealing the true potential of this *ex vivo* gene therapy approach.

Chapter 5: Delivery of GDNF-MSCs in a type 1 collagen hydrogel

5.1 INTRODUCTION

While *ex vivo* gene therapy may circumvent some of the problems associated with direct delivery of neurotrophic factors to the brain, a major limitation associated with this approach is that cells survive poorly after transplantation into the brain (Moloney et al., 2010a, Sabate et al., 1995, Zietlow et al., 2005, Zietlow et al., 2012, Praet et al., 2012) and are associated with activation of the host brain's neuroimmune microglia and astrocytes (Moloney et al., 2010a, Praet et al., 2012). Biomaterial systems such as preformed scaffolds or *in situ* forming hydrogels, show potential to improve the grafting procedure by acting as supportive and protective matrices for the cells following intracerebral transplantation (Loh et al., 2001, Tian et al., 2005, Crompton et al., 2007, Freudenberg et al., 2009, Uemura et al., 2010, Zhong et al., 2010, Zhang et al., 2011, Park et al., 2012) (reviewed elsewhere (Newland et al., 2013b)). Whilst scaffolds for applications in traumatic brain (cortical) injury (Woerly et al., 1999, Cui et al., 2006) or spinal cord injury (Yao et al., 2010, Daly et al., 2012) may be implanted by invasive surgical techniques, intervention for neurodegenerative diseases of the brain must be injectable. Materials with the ability to form hydrogels *in situ* (Saeed et al., 2012) or in response to stimuli such as body temperature (Dong et al., 2010) offer an injectable biomaterial platform to aid cell transplantation. As cell survival post-transplantation is poor (Moloney et al., 2010a, Sabate et al., 1995, Zietlow et al., 2005, Zietlow et al., 2012), a predominant goal of using hydrogels to deliver cells to the brain is to 1) improve the cell engraftment by providing an adherent substrate and 2) to provide a physical barrier to protect the transplanted cells against the host response (Loh et al., 2001, Tian et al., 2005, Crompton et al., 2007, Freudenberg et al., 2009, Uemura et al., 2010, Zhong et al., 2010, Zhang et al., 2011, Park et al., 2012). The commercially available gel formulation, Matrigel™, composed of extracellular matrix proteins derived from mouse sarcomas, has previously been used to enhance stem cell transplantation (Park et al., 2012, Uemura et al., 2010, Laflamme et al., 2007). In another study, injection of embryonic stem (ES) cell-derived neural precursor cells

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(NPCs) with the Matrigel™ into the mouse striatum resulted in greater cell survival, as determined by graft volume, than cells transplanted in medium (Uemura et al., 2010). Additionally, pan-leukocytic CD45-positive cells (microglia and lymphocytes) were reduced at the early time point (24 h), while another study showed less graft infiltration of GFAP positive cells (astrocytes) when the Matrigel™ was used (Park et al., 2012). Whilst these studies provide proof-of-concept for inclusion of a supportive and protective matrix in cell transplantation studies, the murine derivation of Matrigel™ combined with its multitude of growth factors (even when used as a factor poor version), negates its clinical translatability. Degradable biomaterial systems that have been granted approval for clinical use offer greater translatable potential, warrant pre-clinical evaluation as biomaterial matrices in cell transplantation studies.

The natural extracellular matrix, collagen, is a clinically accepted biomaterial which has received comparatively less attention for cell transplantation in the brain (Zhong et al., 2010). Unlike synthetic hydrogels such as those composed of poly N-(2-hydroxypropyl)-methacrylamide (HPMA) (Loh et al., 2001) or poly(ethylene glycol)epoly(propylene sulfide) (PEGePPS) (Zhang et al., 2011) which require laborious addition of RGD peptide sequences to facilitate cellular attachment, collagen has the advantage having an endogenous RGD sequence allowing cell adhesion without requiring further functionalisation chemistries (Holladay et al., 2011). In order for a collagen hydrogel to prove useful in the assistance of *ex vivo* therapies, it is important that its composition does not hinder therapeutic molecule delivery and diffusion at the target parenchymal tissue. Moreover, the ability of the collagen hydrogel to modulate the host response to transplanted cells could play an important role in cell transplantation procedures in the CNS, potentially allowing better graft integration and/or survival.

To this end, the aim of this chapter was to assess the potential of a collagen hydrogel to improve MSC delivery to the rat brain.

5.2 METHODS

All methods have been described in more detail in Chapter 2. Methods specific to this chapter are also detailed below.

5.2.1 EXPERIMENTAL DESIGN

This chapter will detail the results of four separate studies. The aim of the first study was to assess the *in vitro* characteristics of a type 1 collagen hydrogel. We particularly focussed on the potential toxicity of the hydrogel to both a rat neural cell type and rat MSCs in order to determine its suitability for transplantation to the rat brain. Following this, we performed two separate *in vivo* studies to determine the *in vivo* characteristics of the collagen hydrogel. Specifically we performed a pilot *in vivo* study to assess the host response to delivery of the hydrogel alone. Next we assessed the *in vivo* characteristics of the collagen hydrogel when used as a matrix for delivery of GDNF-MSCs to the rat brain. We specifically investigated the effect of the hydrogel on cell survival, host immune response and GDNF release. Finally, we embarked on a fourth study to evaluate the impact of the collagen hydrogel on the neurotrophic ability of transplanted GDNF-MSCs, and to verify the ability of released GDNF to offer neuroprotection in a rat model of Parkinson's disease. The overall design of the approach used is shown in **Fig 5.1**. Several other strategies to improve cell survival were also attempted. These included i) supplementing the MSC transplantation medium with the MSC growth factor, insulin-like growth factor 1 (IGF-1), ii) employing a multi-component pro-survival treatment regime for the MSCs, and iii) pre-conditioning the cells in a hypoxic environment prior to grafting. The results of these studies are shown in Appendix 2.

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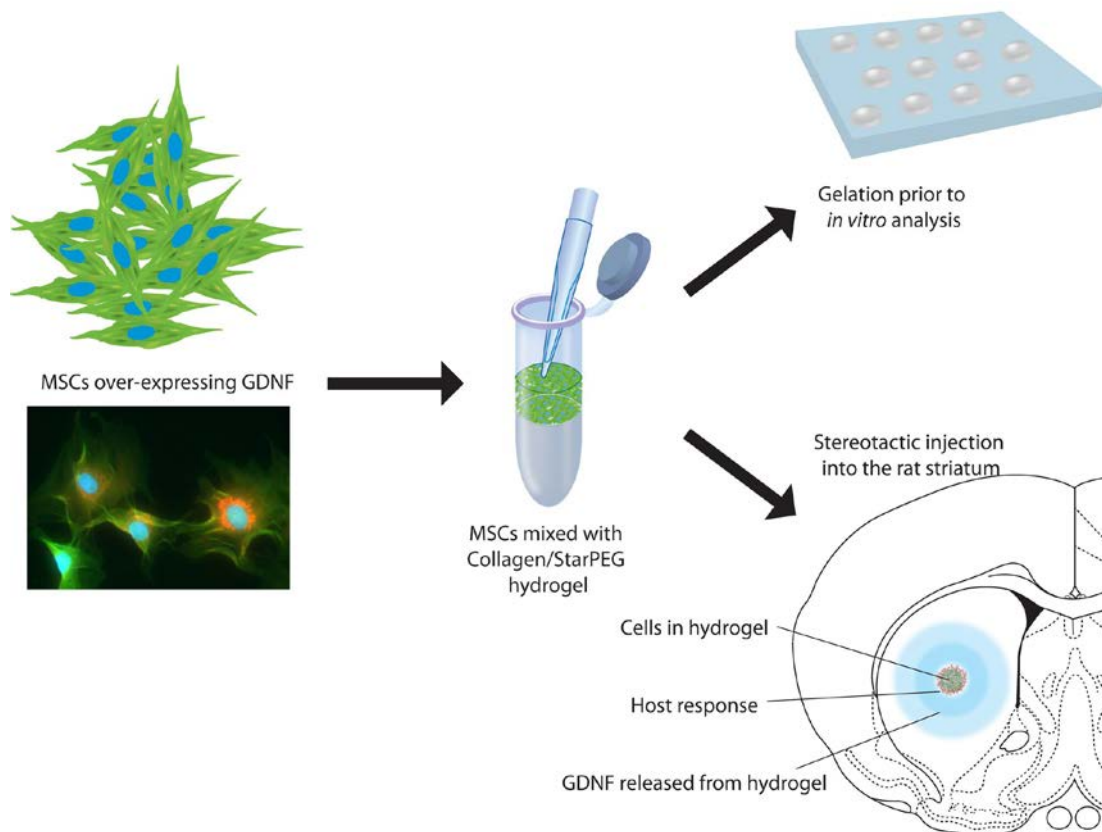


Fig 5.1 Overall approach of this chapter. Bone marrow-derived MSCs were extracted from the femora and tibiae of GFP transgenic Sprague Dawley rats and transduced to over-express human GDNF using a murine leukaemia virus (in the photomicrograph, GDNF and nuclei are stained in red (immunofluorescence) and blue (DAPI) respectively). These were suspended in a Type 1 collagen hydrogel prepared from bovine Achilles tendon which was kept on ice to prevent gelation. The cell-seeded collagen hydrogel was then subjected to a number of *in vitro* validation studies (i.e. impact of the hydrogel on astrocyte viability, MSC viability and GDNF release) which were followed by *in vivo* studies in the adult rat brain to determine the host's response to the hydrogel, and the impact of the hydrogel on the survival of, GDNF release from, and the host response to, the GDNF-MSCs. The neurotrophic ability of the GDNF released from the cells when delivered alone or in the collagen hydrogel in the 6-hydroxydopamine model of Parkinson's disease was also assessed.

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5.2.1.1 *In vitro* assessment of the collagen hydrogel

Initial *in vitro* analysis aimed to assess the potential toxicity of the hydrogel to a neural cell and MSCs. We also sought to determine if encapsulation of GDNF-MSCs in the collagen hydrogel would impede neurotrophin release *in vitro*.

5.2.1.1.1 Fabrication of cell-seeded cross-linked collagen type I hydrogels

Under standard sterile cell culture conditions, MSCs were cultured in a 1:1 F12: Alpha-MEM mix that contained 10% FBS and 1% penicillin/streptomycin in T75 flasks in humid conditions at 37 °C with 5% CO₂. MSCs were enzymatically lifted from the culture surface using trypsin and resuspended in cell transplantation medium, at a density of 25,000 cells/μl so that a final concentration of 10,000 cells/μl could be obtained in the hydrogel. For the preparation of the collagen hydrogel, all components were placed on ice to prevent premature gelation. For a final hydrogel volume of 100 μl, 50 μl of collagen, neutralised with 1M NaOH, was added to 10 μl of 4x phosphate buffered saline. 40 μl of the cell suspension was added to an eppendorf containing 40 mg of poly(ethylene glycol) ether tetrasuccinimidyl glutarate (4S-StarPEG) to dissolve this cross-linker. The cell/cross linker solution was then added to the collagen/PBS solution and mixed thoroughly. For *in vitro* experiments, 4 μl samples were transferred onto a previously sterilised (UV radiation) super hydrophobic surface (Teflon® tape) and placed at 37 °C to gel (~10-15 min). Images of the gels either immediately after formation or after one day in culture were obtained using a fluorescent microscope (Olympus). Image overlays were produced from the intrinsic GFP expression, DAPI nuclear counterstain and bright field images. For *in vivo* experiments, the cell seeded collagen hydrogel was held on ice prior to transplantation to prevent gelation.

5.2.1.1.2 Astrocyte viability assay

Before embarking on *in vivo* studies, confirmation that the collagen hydrogel itself was non-toxic to neural cells was needed. To do so, primary astrocytes were extracted from new-born rat pups, as described previously (Newland et al., 2013c), using a

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combination of two protocols (Allen et al., 2001, Albuquerque et al., 2009). The astrocytes were cultured in a 1:1 F12: Alpha-MEM mix supplemented with 10% FBS and 1% penicillin/streptomycin and seeded at a density of 20,000 cells per well of a 24 well-plate and left overnight to attach. The astrocytes then received either no treatment, or they were incubated with transplantation medium samples (three samples of 4 μ l each) or unseeded collagen hydrogels (three gels of 4 μ l each) for 48 h. As an indicative measure of cell viability, metabolic activity of the cells was measured using the alamarBlue® assay as previously described (Newland et al., 2013a). Briefly, 100 μ l of a 10% solution of alamarBlue® (Invitrogen) solution in HBSS was added to each well and incubated for 3 h. Absorbance at 550 nm and 595 nm was analysed using a Varioskan Flash plate reader (Thermo Scientific) with SkanIt® software. Viability was calculated by normalisation of all results to control wells.

5.2.1.1.3 MSC viability assay

To determine the effect of the collagen hydrogel on the viability of MSCs seeded within it, 120,000 MSCs in either cell transplantation medium or seeded into the collagen hydrogel (three gels of 4 μ l each containing 10,000 cells/ μ l) were added to 24 well-plates containing 1 ml of MSC growth medium. Once again, as a measure of cell viability, metabolic activity of the cells was measured using the alamarBlue® assay as described above (MSCs were incubated with the alamarBlue® solution for 6 h due to 3D hydrogel culture hindering alamarBlue® diffusion time).

5.2.1.1.4 GDNF ELISA

Analysis of the impact of the collagen hydrogel on GDNF release into the surrounding medium was performed by seeding 120,000 GDNF-MSCs per well of a 24 well-plate, either within (three gels of 4 μ l each containing 10,000 cells/ μ l) or without a collagen hydrogel. 50 μ l of the total 1.5 ml cell supernatant was removed one, three and five days post seeding for analysis by the human GDNF enzyme linked immunosorbent assay

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(ELISA, human GDNF DuoSet, R&D Systems) according to the manufacturer's protocol.

5.2.1.2 *In vivo* assessment of the impact of the collagen hydrogel on cell viability, GDNF release, host response

After the initial *in vitro* analyses, three *in vivo* studies were conducted. The first of these was a pilot study designed to assess the host response to the collagen hydrogel in the rat brain. Animals (n=4 per group) received an intra-striatal infusion of either 3 μ l of collagen hydrogel or 3 μ l of transplantation medium. Animals were then sacrificed by transcardial perfusion-fixation 4 days post-infusion before the brains were processed for immune markers for microglia and astrocytes.

For the second *in vivo* study, animals received a bilateral intra-striatal injection of 30,000 GDNF-MSCs in 3 μ l of either collagen hydrogel (n=8 per time point) or transplantation medium (n=4 per time point). The aim of this study was to assess the impact of the collagen hydrogel on the survival of, GDNF release from, and host response to, GDNF-MSCs transplanted into the rat striatum. Animals were sacrificed at days 1, 4, 7 and 14 post-transplantation. Specific parameters assessed in this study included cell survival, released GDNF, immune response to the transplants.

5.2.1.3 *In vivo* assessment of the neuroprotective potential of GDNF-MSCs delivered in the collagen hydrogel

For the final *in vivo* study, animals received a unilateral intrastriatal infusion of 60,000 GDNF-MSCs in 6 μ l of either control transplantation medium or collagen hydrogel. The following day, rats received a unilateral intrastriatal infusion of the catecholamine neurotoxin, 6-hydroxydopamine (7 μ g in 2 μ l), to induce a retrograde degeneration of the nigrostriatal dopaminergic neurons. The ability of cells transplanted alone or in the hydrogel to protect against lesion-induced nigrostriatal degeneration was assessed.

5.3 RESULTS

5.3.1 IN VITRO ASSESSMENT OF THE COLLAGEN HYDROGEL

5.3.1.1 Impact of the hydrogel on cell viability

As an *in vitro* model of intracerebral transplantation, it was first determined if the collagen hydrogel itself had any detrimental impact on the viability of primary astrocytes by plating astrocytes with pre-formed 4 μ l hydrogels. It was seen that the collagen hydrogel had no negative impact on the viability of the plated astrocytes (**Fig 5.2 A**; Group, $F_{(2,9)} = 2.20$, $P = 0.17$, n.s.) indicating that it was non-toxic to neural cells in this *in vitro* model system.

It was then sought to determine if the collagen hydrogel had any impact on the viability of MSCs seeded within it. It was seen that seeding the MSCs in the collagen hydrogel had no impact on their viability (**Fig 5.2 B**; $t_{(4)} = 0.95$, $P = 0.40$, n.s). Because the MSCs were extracted from the bone marrow of GFP transgenic rats, the cell seeded collagen hydrogels could easily be visualised by fluorescent microscopy. This revealed that cell attachment caused the collagen hydrogel to shrink within 1 day of seeding with MSCs (**Fig 5.2 B i & ii**).

5.3.1.2 Impact of the hydrogel on GDNF release

Before embarking on *in vivo* studies, it was essential to confirm that seeding GDNF-MSCs in the collagen hydrogel did not impede GDNF release into the surrounding medium. The quantity of GDNF that was produced by the transduced MSCs, and whether the GDNF could be released from the collagen hydrogel, was analysed using an ELISA. At each time-point examined, the concentration of GDNF in the culture medium was significantly reduced when the GDNF-MSCs were seeded in the 3D collagen hydrogel compared to when they were plated in standard 2D culture (**Fig 5.2 C**; Group, $F_{(1,6)} = 21.22$, $P < 0.01$; *post-hoc* Newman Keuls confirmed differences at

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each time-point). This probably due to differences in the diffusion rates between 2D and 3D cultures. However, the concentration of GDNF in the culture medium increased significantly over days in culture for both groups (Time, $F_{(2,12)} = 511.84$, $P < 0.0001$; *post-hoc* Newman-Keuls confirmed differences between each time-point within each group, see **Fig 5.2 C**). This indicates that, although GDNF release from the hydrogel may have been delayed due to the 3D nature of the culture, the collagen hydrogel ultimately did not prevent diffusion of the protein into the surrounding medium.

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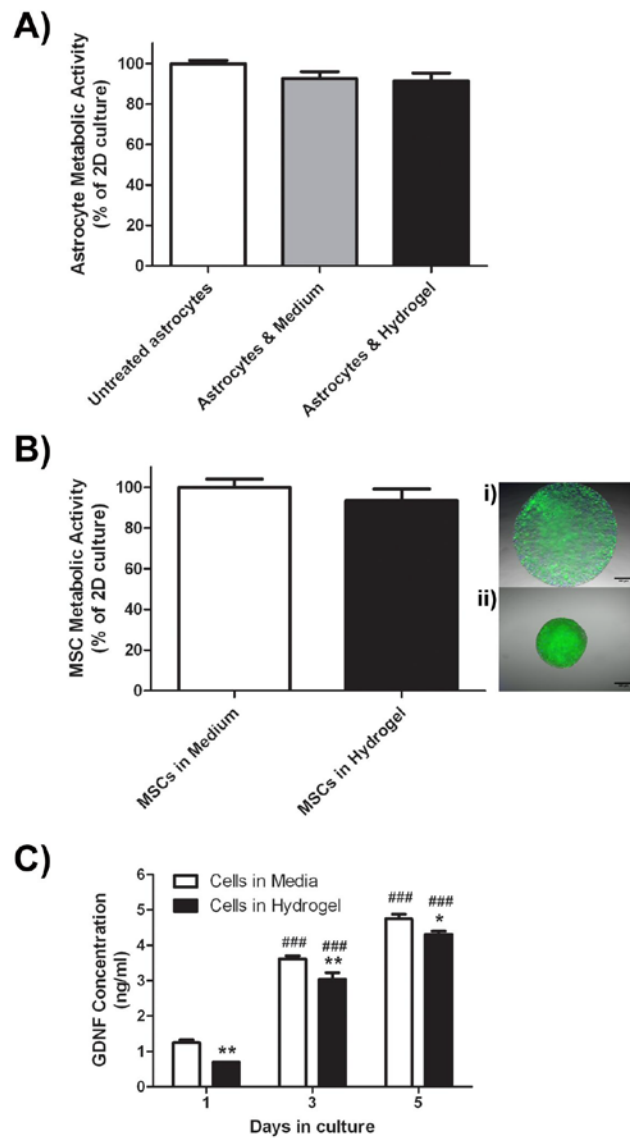


Fig 5.2 The collagen hydrogel did not affect cell viability and GDNF release *in vitro*. *In vitro* validation studies confirmed that the type 1 collagen hydrogel had no negative impact on A) the viability of astrocytes when placed in culture with them or B) the viability of MSCs seeded within them. In B) a typical 4 μl MSC-seeded hydrogel is shown immediately after crosslinking (top photomicrograph Bi) and after 1 day in culture (bottom photomicrograph Bii) highlighting that the seeded gels typically shrink over time. C) Seeding of GDNF-MSCs within the collagen hydrogel significantly reduced the concentration of GDNF in the surrounding medium at each time-point sampled. However, the GDNF concentration in the medium increased over time regardless of whether the cells were plated in standard 2D culture or in the 3D hydrogels. This indicates that the GDNF protein could be secreted from the cells and successfully diffuses through the hydrogel to the surrounding medium. Data are presented as mean ± s.e.m. and were analysed by A) one-way ANOVA, B) unpaired *t*-test, or C) two-way repeated measured ANOVA with *post-hoc* Newman-Keuls. * $P < 0.05$, ** $P < 0.01$ vs. Cells in Media and ### $P < 0.001$ vs. preceding time-point within each group.

5.3.2 *IN VIVO* ASSESSMENT OF THE HOST IMMUNE RESPONSE TO THE COLLAGEN HYDROGEL

Having established that the collagen hydrogel had favourable *in vitro* characteristics (in terms of neural cell toxicity, MSC toxicity and GDNF release), we then sought to determine the host response to it after injection into the rat brain. To do so, the host response to an intra-striatal injection of hydrogel was compared to that caused by an intra-striatal injection of control transplantation medium on Day 4 post-injection. The volume of striatal microgliosis and astrogliosis was similar in rats injected with the collagen hydrogel when compared to rats injected with the control medium (**Fig 5.3**; Microgliosis: $t_{(6)} = 0.31$, $P = 0.76$, n.s.; Astrogliosis: $t_{(6)} = 0.13$, $P = 0.90$, n.s.). Moreover, the collagen hydrogel did not cause any loss of striatal tissue (see photomicrographs in **Fig 5.3**) and nor did it have any effect on the general condition or behaviour of the rats, indicating that it was not overtly toxic after intra-striatal injection. Thus, the collagen hydrogel did not cause any overt adverse reaction in the rat brain.

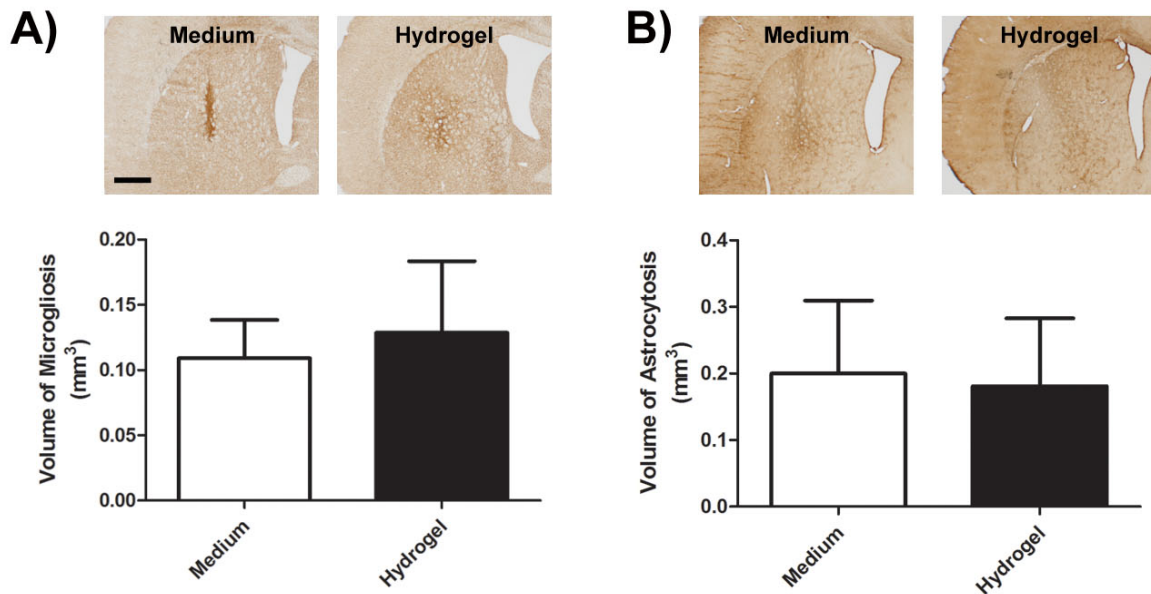


Fig 5.3 The collagen hydrogel did not provoke a marked host response *in vivo*. The host response to the collagen hydrogel in the rat brain was determined by assessing the volume of striatal microgliosis (OX-42 immunoreactivity) and astrocytosis (GFAP immunoreactivity) it induced after striatal injection when compared to that induced by intra-striatal injection of control transplantation medium at the day 4 time-point. The volume of A) microgliosis and B) astrocytosis was similar in both groups indicating that the collagen hydrogel was well tolerated in the rat brain. Data are presented as mean \pm s.e.m. and were analysed by unpaired *t*-test. Scale bar represents 750 μ m.

5.3.3 *IN VIVO* ASSESSMENT OF THE IMPACT OF THE COLLAGEN HYDROGEL ON GDNF-MSC VIABILITY, GDNF RELEASE AND HOST TOLERABILITY

Having established that the collagen hydrogel was permissive for MSC survival and GDNF release *in vitro* and was well tolerated in the rat brain, we then sought to determine its suitability as an intracerebral cell transplantation matrix for GDNF-MSCs.

5.3.3.1 Impact of the hydrogel on GDNF-MSC survival

The GDNF-MSCs were transplanted either within standard cell transplantation medium or the collagen hydrogel. The effect that the collagen hydrogel had on the graft volume was analysed 1, 4, 7 and 14 days post-transplantation. At each of the time-points examined, the collagen hydrogel had no impact on GDNF-MSC graft survival in the rat brain (**Fig 5.4**; Group, $F_{(1,38)} = 0.27$, $P = 0.61$, n.s.). This indicates that although the hydrogel could not prevent the stem cells from dying in their ectopic location in the brain (Time, $F_{(3,38)} = 6.40$, $P < 0.01$), it was not detrimental to graft survival *in vivo* either.

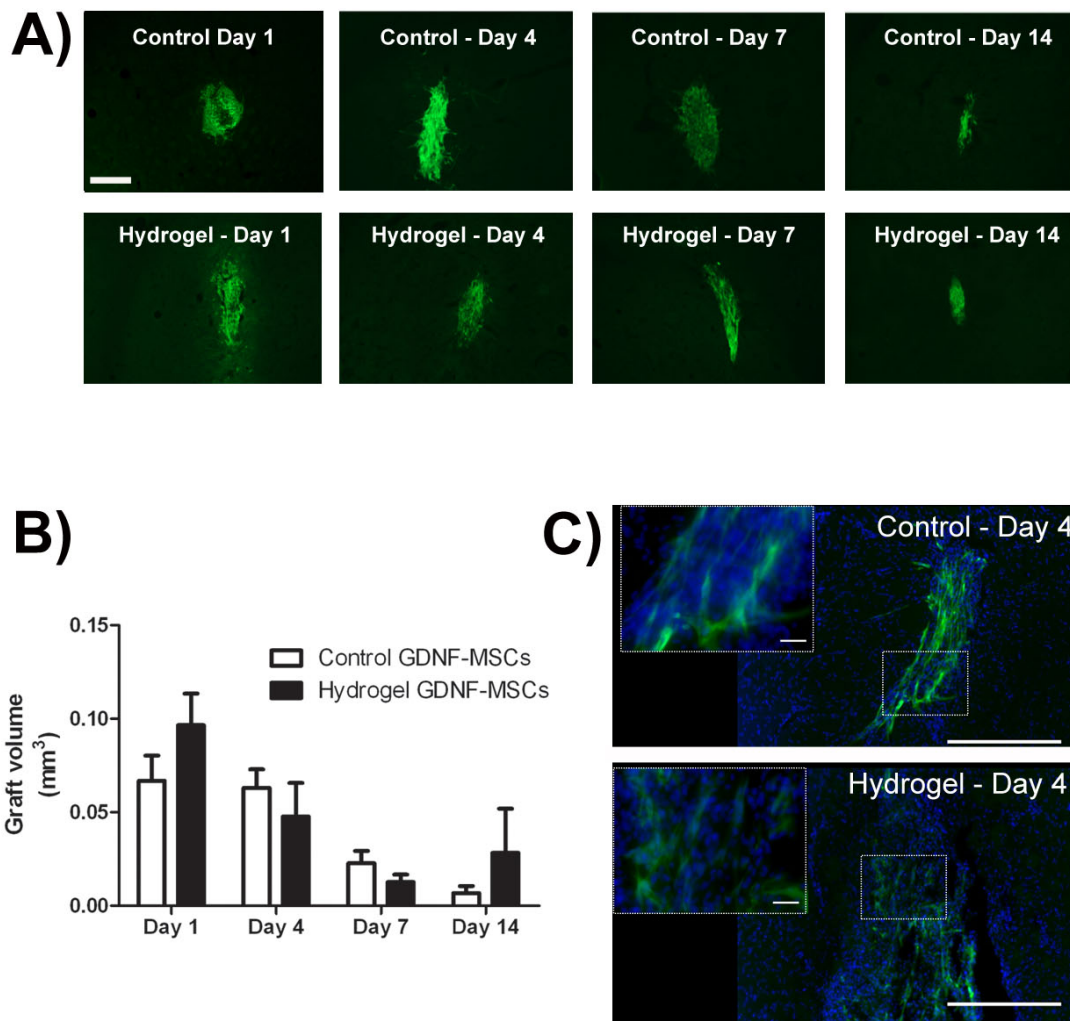


Fig 5.4 The collagen hydrogel was not detrimental to GDNF-MSC graft survival *in vivo*. 30,000 GDNF-MSCs were injected into each striatum either in control transplantation medium or in the collagen hydrogel, and graft volume was analysed at Days 1, 4, 7 and 14 after transplantation. A) Because the MSCs were extracted from the bone marrow of GFP transgenic rats, the grafts could easily be visualised in the striatum using fluorescent microscopy. Scale bar represents 200 μm . B) Quantification of the volume of the transplants showed that although the collagen hydrogel did not significantly improve the graft volume over time, it was not detrimental to the survival of the cells. Data are presented as mean \pm s.e.m. and were analysed by 2-way ANOVA. C) Visualisation of GFP expressing cells at the graft site incorporating a DAPI nuclear counterstain (blue). Scale bars represent 100 μm or 10 μm (insert).

5.3.3.2 Impact of the hydrogel on striatal diffusion of GDNF from GDNF-MSCs

The effect that the collagen hydrogel had on GDNF diffusion into the surrounding striatum was analysed 1, 4, 7 and 14 days post transplantation. At each of the time-points examined, the collagen hydrogel had no significant effect on the diffusion of GDNF into the surrounding striatal tissue (**Fig 5.5**; Group, $F_{(1,38)} = 3.18$, $P = 0.08$, n.s.). The clear decline in the volume of striatum into which GDNF was released (Time, $F_{(3,38)} = 36.67$, $P < 0.001$) is due to the loss of the MSC graft as described above. This data indicates that the collagen hydrogel does not impede striatal neurotrophin penetrability from these engineered cells.

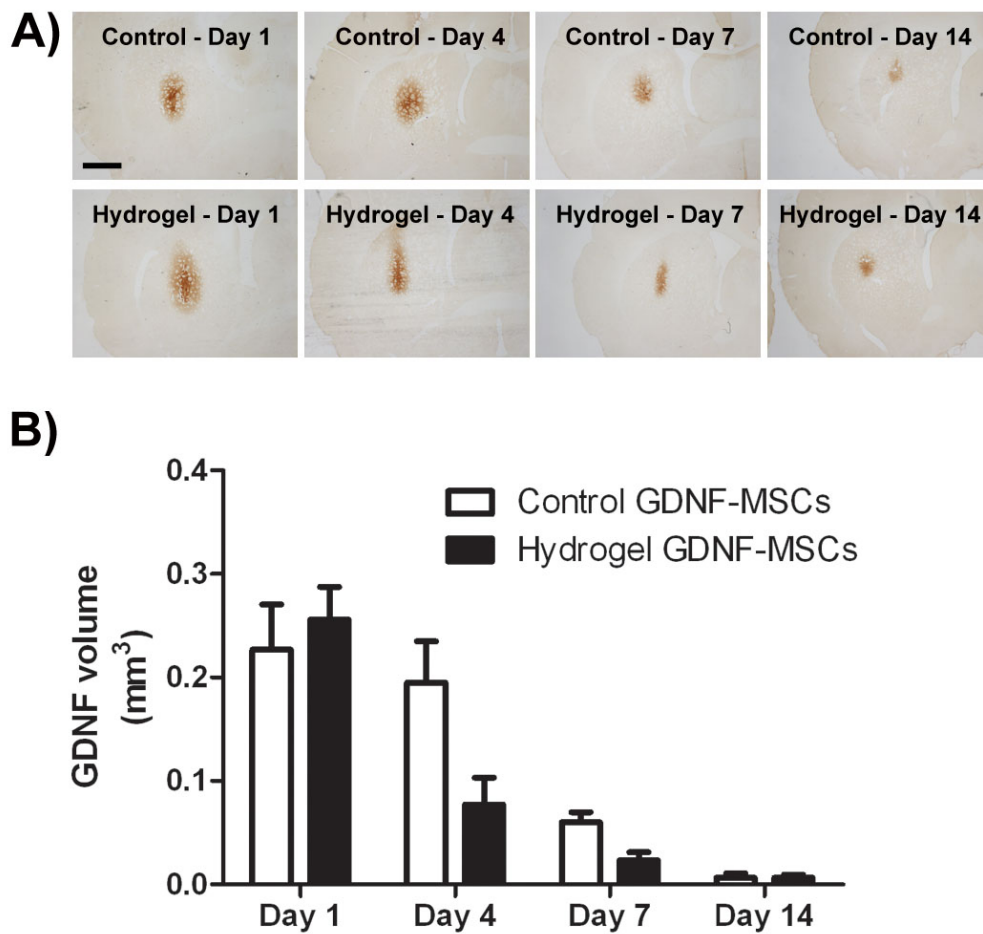


Fig 5.5 The collagen hydrogel permitted the release and diffusion of GDNF *in vivo*. 30,000 GDNF-MSCs were injected into each striatum either in control medium or in the collagen hydrogel, and GDNF release into the striatal tissue was analysed at Days 1, 4, 7 and 14 after transplantation. A) The GDNF protein could be visualised using immunohistochemistry in the host striatum at each time-point examined. B) Quantification of the volume of striatal tissue occupied by GDNF immunostaining showed that the collagen hydrogel did not significantly impede GDNF release from the GDNF-MSCs into the surrounding striatum at any time-point. Data are presented as mean \pm s.e.m. and were analysed by 2-way ANOVA. Scale bar represents 750 μ m.

5.3.3.3 Impact of the hydrogel on host immune response to GDNF-MSCs

We have previously shown that intra-striatal transplantation of MSCs is associated with recruitment and activation of microglia and astrocytes at the graft site (Moloney et al., 2010a). Thus we sought to determine if transplantation of the cells in the collagen hydrogel would provide a physical barrier between the host neuroimmune cells and the transplanted bone marrow-derived MSCs. The effect that the collagen hydrogel had on the volume of microgliosis and astrocytosis was analysed 1, 4, 7 and 14 days post transplantation. Cells transplanted in the control medium invoked a pronounced host microglial response that was maximal at Day 7 after transplantation and subsided thereafter. We found that the collagen hydrogel significantly reduced the volume of striatal tissue occupied by a microgliotic reaction (**Fig 5.6**; Group, $F_{(1,38)} = 16.21$, $P < 0.001$). *Post-hoc* comparison of the group differences revealed a significant reduction in striatal microgliosis at the day 7 time-point with a strong trend towards a reduction at the Day 4 time-point (see **Fig 5.6**). We also found that the collagen hydrogel significantly reduced the volume of striatal tissue occupied by an astrocytic reaction (**Fig 5.7**; Group, $F_{(1,38)} = 44.80$, $P < 0.0001$). *Post-hoc* comparison of the group differences revealed a significant reduction in striatal astrocytosis at the Day 1, Day 4 and Day 7 time-points (see **Fig 5.7**). In line with our previous report (Moloney et al., 2010a), qualitative fluorescent staining for OX-42 and GFAP revealed that the microglia infiltrated into the GFP-MSC grafts (**Fig 5.6 C**) while the astrocytes surrounded, but did not infiltrate, the graft site (**Fig 5.7 C**).

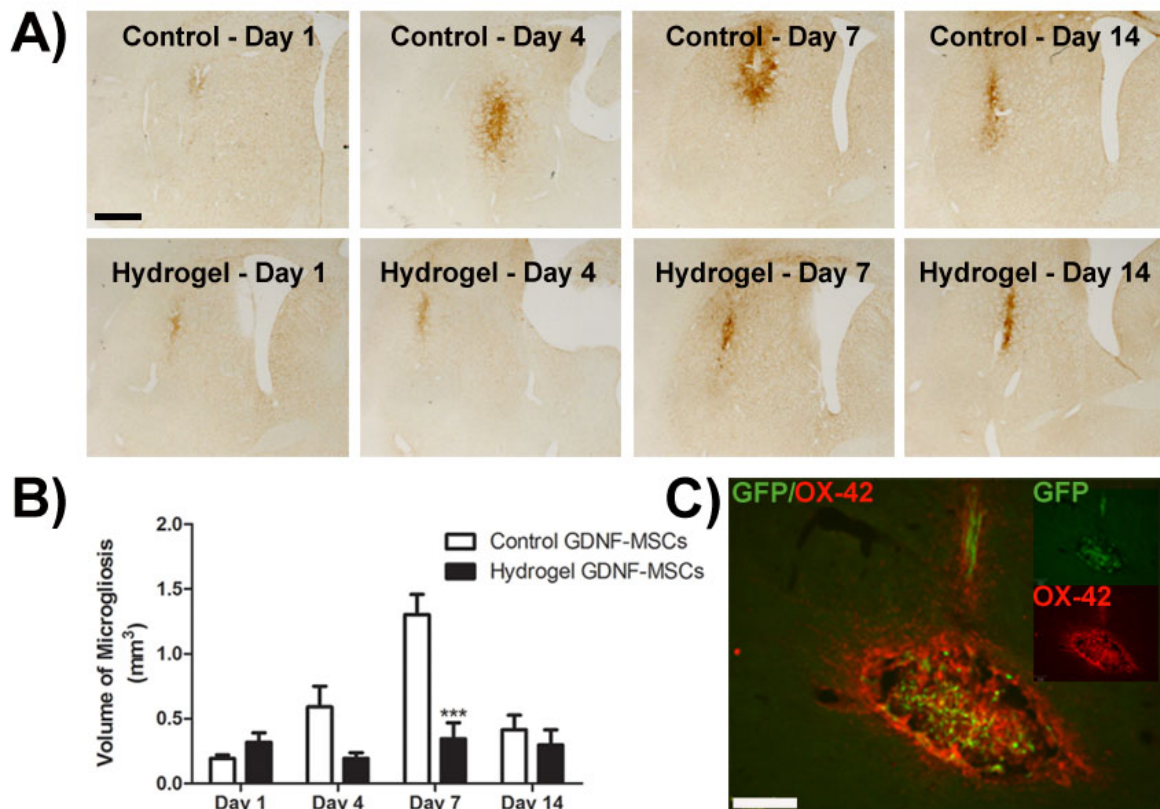


Fig 5.6 The collagen hydrogel reduced the host microglial response to GDNF-MSCs *in vivo*. 30,000 GDNF-MSCs were injected into each striatum either in control medium or in the collagen hydrogel, and the host microglial response to the cells was analysed at Days 1, 4, 7 and 14 after transplantation. A) A striatal microgliotic reaction to the implanted cells could be visualised using OX-42 immunohistochemistry in the host striatum at each time-point examined. B) Quantification of the volume of striatal tissue occupied by microgliosis showed that the collagen hydrogel significantly reduced the host microgliotic reaction to the GDNF-MSCs. C) Representative photomicrographs of OX-42 immunofluorescence show that the microglia infiltrated the GFP-MSC graft site in line with our previous report (Moloney et al., 2010a). Data are presented as mean \pm s.e.m. and were analysed by 2-way ANOVA. *** $P < 0.001$ vs. Control GDNF-MSCs. Black scale bar represents 750 μ m. White scale bar represents 100 μ m.

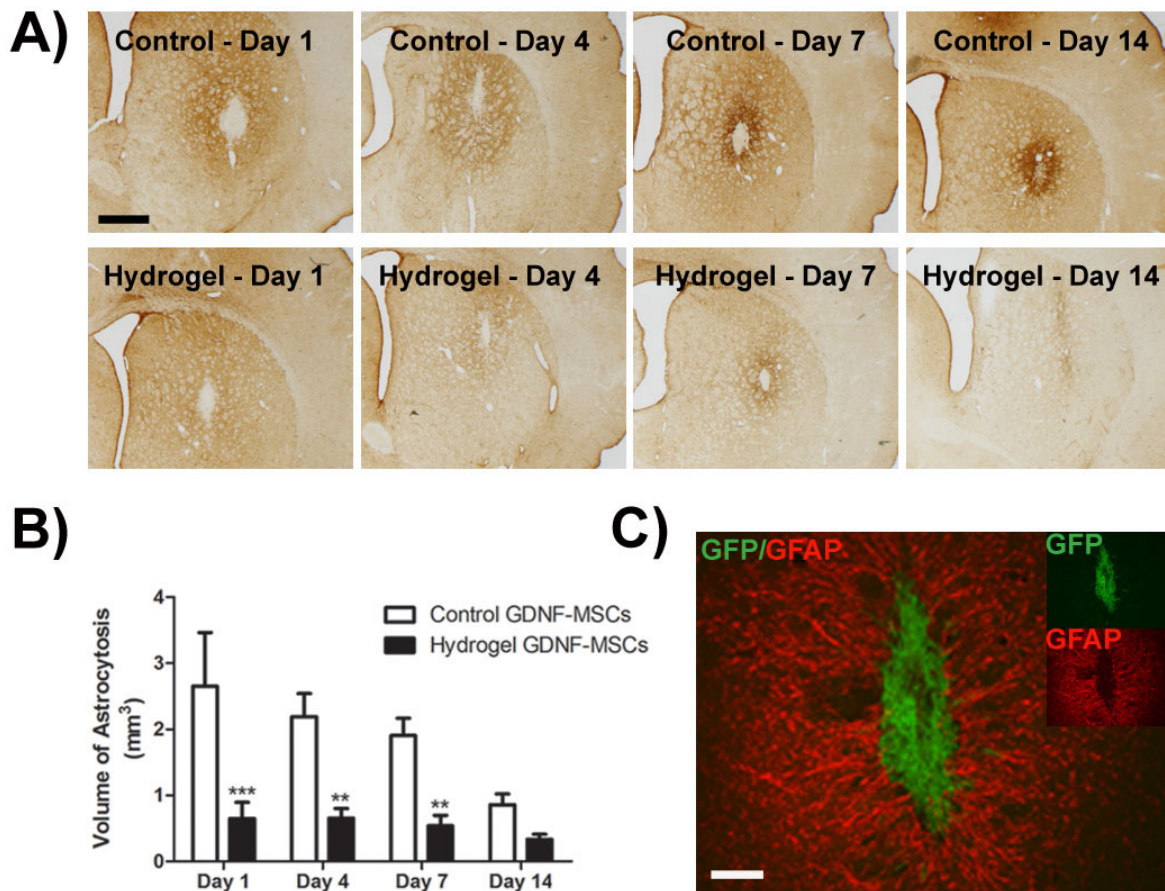


Fig 5.7 The collagen hydrogel reduced the host astrocytic response to GDNF-MSCs *in vivo*. 30,000 GDNF-MSCs were injected into each striatum either in control medium or in the collagen hydrogel, and the host astrocytic response to the cells was analysed at Days 1, 4, 7 and 14 after transplantation. A) A striatal astrocytic reaction to the implanted cells could be visualised using GFAP immunohistochemistry in the host striatum at each time-point examined. B) Quantification of the volume of striatal tissue occupied by astrocytosis showed that the collagen hydrogel significantly reduced the host astrocytic reaction to the GDNF-MSCs. C) Representative photomicrographs of GFAP immunofluorescence show that the astrocytes surrounded the GFP-MSC graft site in line with our previous report (Moloney et al., 2010a). Data are presented as mean \pm s.e.m. and were analysed by 2-way ANOVA. ** $P < 0.01$, *** $P < 0.001$ vs. Control GDNF-MSCs. Black scale bar represents 750 μ m. White scale bar represents 100 μ m.

5.3.4 *IN VIVO* ASSESSMENT OF THE NEUROPROTECTIVE POTENTIAL OF GDNF-MSCs DELIVERED IN THE COLLAGEN HYDROGEL

Having determined that the collagen hydrogel was a suitable matrix to aid cell delivery to the rat brain by reducing the host immune response to the cells, by not detrimentally affecting cell survival and by permitting neurotrophin release and diffusion, we next wished to confirm that the presence of the collagen hydrogel did not affect the neuroprotective ability of released GDNF. To this end, GDNF-MSCs were transplanted into the striatum either in standard transplantation media or in the collagen hydrogel, one day prior to induction of a 6-hydroxydopamine lesion.

5.3.4.1 Impact of the hydrogel on GDNF-MSC survival

In this study, survival of transplanted GDNF-MSCs was assessed by quantification of the volume of the GFP-positive cells remaining at 14 days post-transplantation (**Fig 5.9**). Transplanted cells were easily identifiable due to their expression of the GFP reporter gene. Despite this, surviving cells were observed in only some of the transplanted animals at the time point examined in this study. This level of cell survival is consistent with the previous study in this chapter when cells were transplanted to an intact, unlesioned striatum), and also with a previous report from our laboratory in which the progressive death of these cells *in vivo* (Moloney et al., 2010a). As with the previous study, no difference in grafted cell survival was observed between cells transplanted in standard transplantation medium or the collagen hydrogel ($t_{(8)} = 1.28$, $P = 0.21$, n.s.) (**Fig 5.9**).

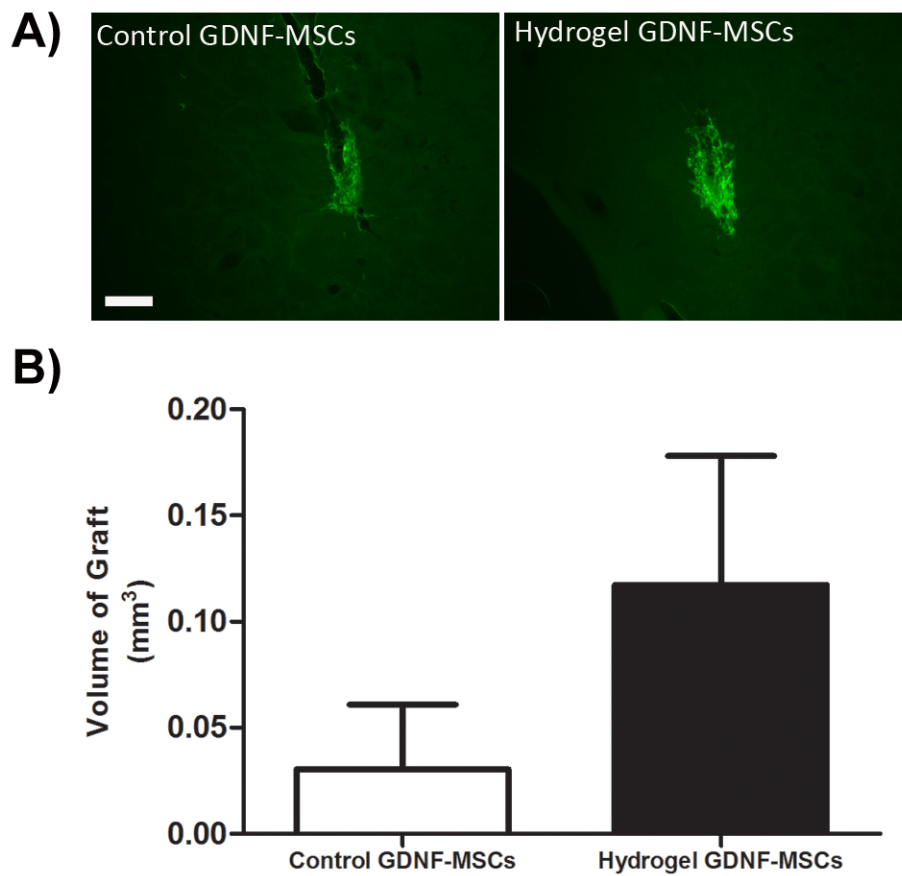


Fig 5.9 GDNF-MSC survival was not affected by encapsulation in a collagen hydrogel. 60,000 GDNF-MSCs were injected into the rat striatum one day prior to induction of a striatal 6-hydroxydopamine lesion. A) Once again, grafts could easily be visualised in the striatum using fluorescent microscopy. B) Quantification of the volume of the transplants showed that the collagen hydrogel was not detrimental to the survival of the cells. Graft volume at this timepoint was similar to the level observed at this timepoint in unlesioned animals in the previous study. Data are presented as mean \pm s.e.m. and were analysed by unpaired *t*-test. Scale bar represents 200 μ m.

5.3.4.2 GDNF-MSCs transplanted in a collagen hydrogel are capable of inducing local neuroprotection of dopaminergic terminals in the striatum

Unilateral, intra-striatal injection of 6-hydroxydopamine induced contralateral nigrostriatal degeneration (degeneration of striatal dopaminergic terminals on the injected side can be seen as seen in **Fig 5.10 A**). When the tyrosine hydroxylase immunohistochemical staining in the striatum was assessed directly in the vicinity of the transplant site, discrete regions of dense tyrosine hydroxylase-positive staining were observed (**Fig 5.10**) in the largely denervated striatum. Quantitative analysis of the density of staining proximal to the transplant in comparison with the depleted tyrosine hydroxylase-positive regions distal to the transplant verified this observation. Importantly, this effect was observed in both transplanted groups, regardless of whether the cells were delivered in the standard transplantation media or in the collagen hydrogel (Group x Region, $F_{(1,16)} = 29.35$ *** $P < 0.001$). This indicates that the collagen hydrogel did not adversely affect the functionality of the released GDNF.

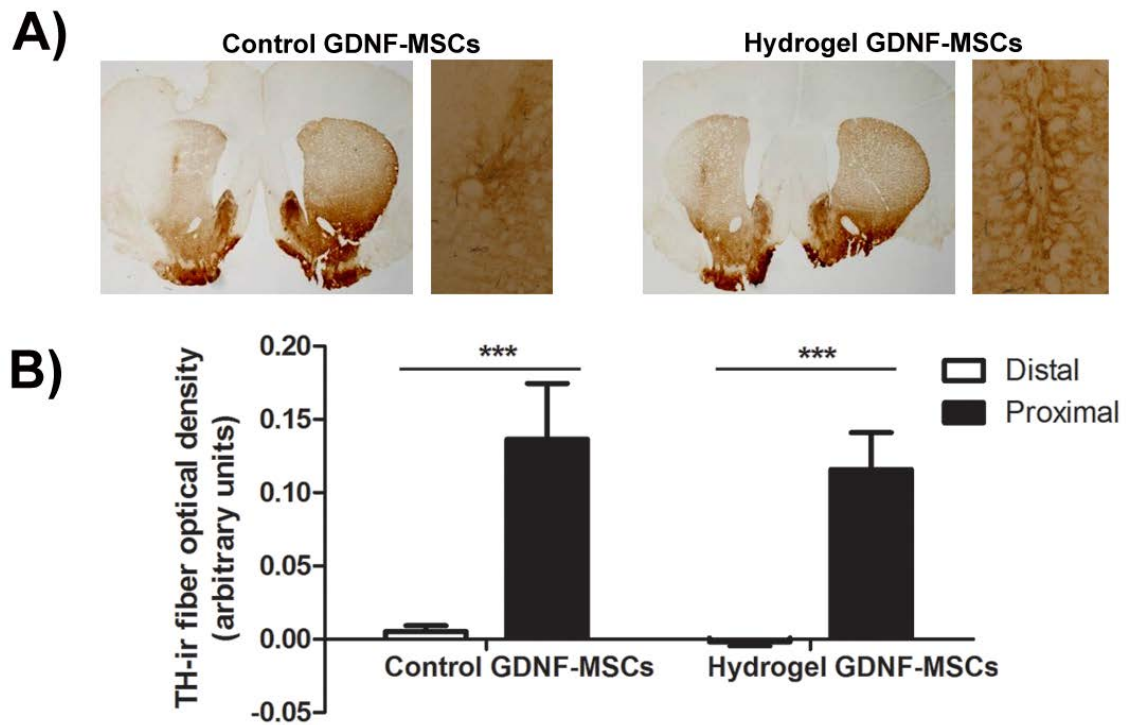


Fig 5.10 Local neuroprotection by GDNF-MSCs delivered in a collagen hydrogel. A) Tyrosine hydroxylase immunohistochemistry (14 days after lesion surgery) revealed a clear loss of nigrostriatal terminals from the injected striatum. However, GDNF-MSCs, whether implanted in control transplantation medium or in the collagen hydrogel, were capable of protecting the nigrostriatal terminals in the immediate vicinity of the transplant from the neurotoxic effects of 6-hydroxydopamine. B) Quantification of the density of tyrosine hydroxylase staining in the lesioned striatum confirmed that the terminals proximal to the transplant site were protected relative to the striatal deafferentation seen more distally from the transplant. Data are shown as mean±s.e.m. *** $P < 0.001$ vs. distal by 2-way ANOVA with *post-hoc* Bonferroni test. TH-ir: tyrosine hydroxylase immunohistochemistry.

5.4 DISCUSSION

The aim of this chapter was to assess the potential of a collagen hydrogel as a cell delivery matrix for neurotrophin overexpressing adult stem cells in the brain. We specifically sought to determine the impact of the hydrogel on the survival of transplanted MSCs, and the brain's tolerance to the collagen hydrogel, that is if it could reduce the host response to striatal grafts of GDNF-MSCs without hindering the diffusion of GDNF into the surrounding brain tissue. In validatory *in vitro* studies, we confirmed that the type 1 collagen hydrogel was non-toxic to neural cells or MSCs seeded within it, and also permitted diffusion of GDNF from GDNF-MSCs into the cell culture medium. We then demonstrated that the collagen hydrogel itself was well tolerated in the rat brain, and went on to evaluate the effect of the collagen hydrogel on the survival of, GDNF release from, and host response to, GDNF-MSCs transplanted into the rat striatum. We found that the collagen hydrogel did not negatively impact on the survival of the cells, it permitted GDNF secretion into the striatal parenchyma, and, importantly, it significantly reduced the host brain's response to the cells by reducing the recruitment of both microglia and astrocytes at the site of delivery. Moreover, when GDNF-MSCs were transplanted to the striatum of a 6-hydroxydopamine model of Parkinson's disease, the cells were capable of inducing local neuroprotection of dopaminergic terminals, despite their encapsulation in the collagen hydrogel (Hoban et al., 2013b). This study suggests that collagen-based hydrogels should warrant further investigation as matrices for intra-cerebral cell transplantation in neurodegenerative diseases.

MSCs provide a potential autologous cell source (Zhang et al., 2008) which can be easily expanded *in vitro* (Bartmann et al., 2007). Uncontrolled, open label clinical analysis of MSCs delivered to the subventricular zone of Parkinson's disease patients showed no adverse effects from the treatment (Venkataramana et al., 2010, Venkataramana et al., 2012). Therefore, as GDNF has been shown to be neuroprotective to dopaminergic neurons (Winkler et al., 1996, Sullivan et al., 1998, Rosenblad et al., 1999, Kirik et al., 2000a), GDNF overexpressing MSCs provide a potential method of

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intracranial neurotrophin delivery (Moloney et al., 2010b). However, previous studies have shown that MSC survival is poor post-transplantation in the rat brain, with a graft volume reduction of ~35% occurring between one and four days after transplantation (Moloney et al., 2010a). Because of this limitation associated with current *ex vivo* intracerebral gene delivery approaches, this chapter sought to examine whether a biomaterial hydrogel based on collagen could potentially be used for assisting *ex vivo* gene therapy to the brain.

The use of any material within the brain necessitates careful analysis of local toxicity/adverse host response to the material. In addition, for the analysis of a hydrogel for applications in *ex vivo* neurotrophin delivery, analysis of whether the material inhibits protein release from the graft site must also be performed. *In vitro* analysis showed that when pre-formed collagen hydrogels were cultured with primary astrocytes, the hydrogel was not detrimental to the viability of these neural cells as revealed by the alamarBlue® assay. Although the alamarBlue® assay is strictly a measure of cell metabolic activity, it may also be used as a gross indication of cell viability, providing higher sensitivity assay than the commonly used MTT assay (Hamid et al., 2004). Thus, using this *in vitro* model of intra-cerebral collagen hydrogel transplantation, we established that the collagen hydrogel was not overtly toxic to a neural cell type. When the GDNF secreting MSCs were seeded into the collagen hydrogel, the hydrogel did not significantly reduce the viability of the cells. The lack of cytotoxic effects of the collagen substrate is in agreement with analysis of other 4S-StarPEG/collagen containing biomaterials (Collin et al., 2011). Reduction in collagen hydrogel volume one day post gelation is likely due to the cell attachment to the surrounding collagen matrix. Previous hydrogel studies have used peptides to improve cell attachment (Loh et al., 2001, Tian et al., 2005, Crompton et al., 2007, Freudenberg et al., 2009, Zhang et al., 2011), however the ability of cells to grow within collagen structures is well reported (Zhong et al., 2010, Daly et al., 2012, Holladay et al., 2011, Collin et al., 2011). One of the most important findings from the *in vitro* studies was that the collagen hydrogel did not prevent diffusion of the secreted GDNF protein into the cell culture medium. Although GDNF release into the culture medium was delayed

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by seeding into the 3D collagen hydrogel, the protein was still able to diffuse through the gel into the surrounding medium, a feature that is imperative if these biomaterials are going to be developed as matrices to support *ex vivo* neurotrophin gene therapies.

Having established that the collagen hydrogel had favourable characteristics *in vitro* (in terms of neural cell toxicity, MSC toxicity and GDNF release), we then injected it directly into the rat brain to assess its local toxicity and any adverse host response to the material. We found that the collagen hydrogel was well tolerated in the brain and did not induce significantly more microgliosis or astrogliosis when compared to control medium injection. This confirmed the tolerability of collagen based biomaterials in the rat brain (Zhong et al., 2010, Newland et al., 2013c). The results from the subsequent *in vivo* study replicated those we had seen in the *in vitro* studies in that the collagen hydrogel had no detrimental effect on the survival of, or GDNF release from, the GDNF transduced bone marrow-derived MSCs in their ectopic location in the brain. Following transplantation of MSCs to the intact striatum of host rats, the transplanted cells were clearly distinguishable from the striatal host tissue by virtue of their expression of the GFP reporter protein. Indeed, GFP expressing MSC grafts were evident at each time point examined and the collagen hydrogel neither improved nor worsened graft survival. While there was no significant difference in graft volume between the control or hydrogel injected cells, the volume of the graft and therefore survival of the cells decreased over time as previously shown (Moloney et al., 2010a, Moloney et al., 2010b). The reasons for this poor survival are not clear but possible contributing factors may include immune rejection (Bergwerf et al., 2011) and/or poor vascularisation at the graft site resulting in oxygen and/or glucose deprivation (Praet et al., 2012). Despite the poor survival of the cells, at each time-point examined, a large volume of GDNF immunopositive staining was detectable surrounding the graft site. One of the main reasons biomaterial-based support matrices are being analysed for transplantation of cells is their ability to improve grafting of cells in the brain (Loh et al., 2001, Tian et al., 2005, Crompton et al., 2007, Freudenberg et al., 2009, Uemura et al., 2010, Zhong et al., 2010, Zhang et al., 2011, Park et al., 2012, Newland et al., 2013b). However, for these materials to be used for *ex vivo* applications it is imperative that they do not

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inhibit neurotrophin release to the surrounding tissue. The results of the *in vivo* analysis were similar to those seen in the *in vitro* study, i.e. the collagen hydrogel did not affect the volume of GDNF staining surrounding the injection site. Thus collagen-based hydrogels do offer the potential for assisting cell-based delivery of neurotrophic factors such as GDNF.

Although MSCs have been shown previously to regulate immune response and have minimal immunogenic properties (Bartholomew et al., 2002, Di Nicola et al., 2002, Ohtaki et al., 2008), we have previously shown that transplantation of MSCs into the striatum resulted in microglial activation and infiltration into the cell graft, accompanied with astrocyte recruitment and scarring around the site of cell transplantation (Moloney et al., 2010a, Moloney et al., 2010b). In this study, this host immune response to the GDNF-MSCs was significantly reduced by delivery of the cells in the collagen hydrogel in comparison to cells delivered in standard transplantation medium. Thus, the hydrogel formed a barrier between the ectopically transplanted cells and the host brain, and did not itself evoke a pronounced host immune response. Previous studies have shown degradable implants to cause a short-term elevated host response to non-degradable counterparts (Bjugstad et al., 2010). This is mainly due to the response generated by breakdown of degradation products (Lewitus et al., 2011). This phenomenon does not hold true with the use of the Star-PEG cross-linked collagen hydrogel. The collagen hydrogel is composed of the extracellular matrix collagen which when degraded results in inert breakdown products. As previously shown, a host response is invoked by the injection process even following intracranial injection of sterile PBS (Newland et al., 2013c, Hoban et al., 2013a), thus the ability of materials to modulate this host response to an injection is of high importance. This is especially the case for applications where cells are transplanted into damaged tissue such as for traumatic brain injury (Tian et al., 2005) or stroke (Zhong et al., 2010) where minimal inflammation is a desirable trait of any proposed therapy.

Finally, in this chapter the MSCs were initially implanted into the intact striatum. However, it should be borne in mind that, if MSCs are to be used for *ex vivo*

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neurotrophin gene therapy for Parkinson's disease, then they will ultimately be transplanted into the striatum of patients with ongoing disease. Thus, it is important to consider the potential impact of the collagen hydrogel on the fate and functionality of the transplanted cells. To determine the impact of the hydrogel on the ability of GDNF-MSCs to provide neuroprotection, we transplanted GDNF-MSCs in the collagen hydrogel one day prior to induction of a 6-hydroxydopamine lesion. We demonstrated that the survival of intrastrially implanted GDNF-MSCs in a collagen hydrogel appeared to be unaffected when implanted into the striatum of 6-hydroxydopamine-lesioned rat. In line with our previous report (Moloney et al., 2010b), we have shown that GDNF released from these GDNF-MSCs encapsulated in the collagen hydrogel is functional *in vivo*, in that it can protect tyrosine hydroxylase immunopositive nigrostriatal terminals from the neurotoxic effects of the catecholamine neurotoxin, 6-hydroxydopamine. Despite this however, the magnitude of the neuroprotection afforded is relatively poor, a result which we believe is in part due to poor MSC survival in the brain (Moloney et al., 2010a, Moloney et al., 2010b). Microtransplantation of these MSCs may also provide an increased diffusion potential of secreted GDNF while increasing graft survival as has been shown previously with fetal nigral suspensions in a PD model (Nikkhah et al., 1994). However, in the first instance, GDNF production would potentially be increased to therapeutically relevant levels by increasing the number of surviving transduced MSCs in the brain. Thus, the death of transplanted cells must be addressed for long term translation of such a therapeutic strategy.

This chapter aimed to examine the feasibility of using an *in situ* gelling hydrogel system to assist with the delivery of mesenchymal stem cells to the brain. Poor cell survival post transplantation represents a substantial problem for *ex vivo* gene therapy thus provoking research into the use of biomaterials-based support matrices for the delivery of cells to the brain. In this study, a collagen hydrogel was employed to assess if delivery of cells in this manner would affect the survival of the GDNF-MSCs, growth factor delivery to the brain tissue and/or reduce the host response to the graft. We have shown that this collagen hydrogel could be delivered to the rat brain along with GDNF-MSCs and form a cell-seeded gel *in situ*. This collagen hydrogel as a cell delivery

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matrix was not detrimental to the survival of the cells themselves both *in vitro* and *in vivo* thereby reiterating its suitability as a substrate for stem cell delivery to the brain. Moreover, this collagen hydrogel did not affect the GDNF release from the graft into the striatum as evidenced by the volume of GDNF immunohistochemical staining, nor did it affect the functionality of this released GDNF in a 6-hydroxydopamine model of Parkinson's disease. Most importantly, this study showed the ability of the collagen hydrogel to attenuate the microglial response to the graft while also significantly reducing the volume of the astrocyte recruitment.

Ultimately, this work shows the ability of a collagen based biomaterial platform to be used for applications in cell delivery to damaged tissue, neurotrophic factor delivery and/or cell replacement transplants (Hoban et al., 2013b). Furthermore, specific tailoring or functionalisation of the collagen hydrogel could be performed to release biological cues, growth factors, glial scar modulating enzymes such as chondroitinase, or pro-survival factors. This, in combination with the structural support afforded by the hydrogel itself, could potentially provide a niche microenvironment for cell transplantation and enhance cell viability, thereby further improving this *ex vivo* gene therapy approach.

Chapter 6: General Discussion

The work presented in this thesis sought to determine the potential of GDNF-secreting MSCs, as an *ex vivo* gene therapy, for the treatment of Parkinson's disease. We particularly focussed on the ability of GDNF-MSCs to enhance two main therapeutic approaches for the treatment of Parkinson's disease, namely the transplantation of fetal dopaminergic neurons (to replace the dopaminergic neurons lost in the disease process), and the delivery of the neurotrophin GDNF to the Parkinsonian brain (to protect the remaining dopaminergic cells from the degenerative process associated with the disease).

The main findings from this body of work are: 1) GDNF-MSCs (that release GDNF both *in vitro* and *in vivo*) were capable of inducing an initial dopaminergic trophic response in the striatum, but did not improve the survival of transplanted fetal dopaminergic cells, 2) transplantation of GDNF-MSCs in the LPS model (an inflammation-driven model of Parkinson's disease evidenced by the motor, neuroinflammatory and neurodegenerative features associated with the disease (Hoban et al., 2013a)) induced a neuroprotective effect on the dopaminergic terminals local to the transplant site, despite poor *in vivo* cell survival (Hoban et al., (Submitted)), and 3) delivery of the GDNF-MSCs in a collagen hydrogel did not affect cell survival nor did it affect the ability of the cells to release GDNF to the surrounding striatum allowing for neurotrophin-induced local neuroprotection of dopaminergic terminals in the 6-hydroxydopamine model of Parkinson's disease. In fact, delivery of GDNF-MSCs in the collagen hydrogel significantly reduced the host immune response to the cell transplants (Hoban et al., 2013b). These findings, while limited by the issue of poor cell survival *in vivo*, highlight the potential of this *ex vivo* gene therapy approach for GDNF delivery for the treatment of Parkinson's disease.

Despite the wealth of ongoing research into novel treatments for Parkinson's disease, and the numerous advances in pharmacotherapy for the disease to date, an effective therapeutic intervention capable of halting or even slowing the progression of the

disease remains an unmet but vital clinical need. Each of the pharmacological treatments routinely used in clinical practice solely provide symptomatic relief and fail to provide a disease modifying effect (Salawu et al., 2010). The stark insufficiencies in current therapeutic options have prompted a determined effort to identify novel therapeutic agents or strategies to address the degeneration of the nigrostriatal pathway and improve the poor prognosis for patients diagnosed with Parkinson's disease.

Due to an ever-increasing population and an internationally aging society, it is anticipated that the number of patients with a diagnosis of Parkinson's disease will increase from 4.1 million in 2005 to 8.7 million in 2030 (Tarazi et al., 2014). Therefore, as the aged population increases, the apparent lack of a viable treatment option for the disease represents a major socio-economic burden associated with the diagnosis and treatment of the disease (Brown et al., 2005). The ability of a specific therapeutic agent, or indeed a combination of treatments, to adequately address the symptoms of the disease, reduce motor complications of treatment, halt disease progression and provide functional neuroprotection and/or restoration of the degenerated dopaminergic neurons in the affected brain remains elusive to this day.

Cell transplantation, in particular the transplantation of fetal dopaminergic cells, has long been regarded as a promising disease-modifying approach for the treatment of the disease. Patients with Parkinson's disease may be particularly responsive to cell transplantation therapy due to the relatively selective loss of nigrostriatal dopaminergic neurons in the disease (Fearnley and Lees, 1991). The main era of cell transplantation for Parkinson's disease started with the pre-clinical evaluation of fetal dopaminergic cells obtained from the developing ventral mesencephalon (Brundin et al., 1986, Dunnett et al., 1981). These pioneering studies provided proof-of-concept for the survival and function of fetal dopaminergic transplants, and indeed prompted the progression of this cell transplantation approach into clinical trials. A series of small, open-label studies initially reported beneficial effects in patients treated with fetal dopaminergic transplants derived from the ventral mesencephalon (Lindvall et al., 1989, Lindvall et al., 1990, Wenning et al., 1997). However, this initial promise was

subsequently diminished by negative results from two larger, double-blind, randomised studies (Freed et al., 2001, Olanow et al., 2003), and the emergence of complications related to the procedure such as graft-induced dyskinesias in a subset of patients (Politis et al., 2010). However, longitudinal follow-up studies of the patients grafted with these cells have revealed the ability of the cells to survive, grow and secrete dopamine for over 15 years (although there is considerable variation between patients (Ma et al., 2010, Hallett et al., 2014, Mendez et al., 2008)). In order to minimise this variation, it has since been identified that multiple aspects of the fetal dopaminergic transplantation trials need to be addressed in order to improve the approach (including patient selection, age, disease severity, existing dyskinesia, and donor cell population preparation and storage) (Barker et al., 2013). While these factors are being addressed in the ongoing TRANSEURO transplantation trial (Evans et al., 2012), the ethical and logistical implications of obtaining the multiple donors required for the transplantation of a single patient may preclude the majority of patients with Parkinson's disease from access to this treatment. In order for cell replacement using fetal dopaminergic cells to be realised as a viable therapeutic option, efforts must be made to increase the quantity of surviving cells after transplantation, thus limiting the number of donors required for each transplanted patient and widening the access to the therapy itself.

GDNF has been found to have distinct protective effects on the survival of midbrain dopaminergic neurons both *in vitro* and *in vivo* (Beck et al., 1995, Kearns and Gash, 1995, Lin et al., 1993, Sauer et al., 1995). Previous studies have shown that injection of GDNF protein adjacent to a graft of fetal dopaminergic cells provided a beneficial, albeit transient, trophic signal to the transplanted cells in the critical period of 1-2 weeks after transplantation when approximately 95% of dopaminergic neurons have been shown to die (Brundin et al., 2000, Rosenblad et al., 1996). In Chapter 3, we sought to establish the effect of GDNF on transplanted fetal dopaminergic neurons when delivered in a co-transplant with GDNF-secreting MSCs. We have shown herein, and previously (Moloney et al., 2010b), the ability of transplanted GDNF-MSCs to release GDNF *in vitro* and *in vivo*, resulting in local neuroprotection of nigrostriatal dopaminergic terminals (Moloney et al., 2010b). As an *ex vivo* gene therapy approach,

transplantation of these cells, while promising, is hindered by the poor survival of the cells after *in vivo* transplantation (Moloney et al., 2010a, Moloney et al., 2010b). However, the characteristic poor survival of MSCs in the brain may actually be advantageous in terms of their ability to provide initial trophic support to the fetal dopaminergic cells and potentially improve their survival. In Chapter 3, we have shown that GDNF-MSCs, when delivered to either an intact or a lesioned rat striatum in combination with fetal dopaminergic cells, did not improve the survival of the latter cell type. However, we found that while animals transplanted with fetal dopaminergic cells experienced an improvement in rotational asymmetry at 4 weeks after transplantation surgery, this effect was not enhanced by the inclusion of GDNF-MSCs. A study by Ostefeld et al., (2002), found a similar modest behavioural improvement after co-transplantation of fetal dopaminergic cells with GDNF-transduced neural precursor cells. The authors concluded that long term GDNF release would be required to enhance behavioural improvement but that a short exposure to GDNF in the initial days after transplantation was sufficient to improve dopaminergic cell survival. However, in contrast to these results, and indeed other studies which have combined GDNF with fetal dopaminergic cell transplantation (Kauhausen et al., 2013, Thompson et al., 2009), we found that inclusion of GDNF-MSCs in a fetal dopaminergic cell transplant did not enhance the survival of the transplanted dopaminergic cells. However, an interesting observation did arise from the first pilot transplant study, in that there was significant neurotrophic response to the GDNF-MSC and dopaminergic cell transplant, a phenomenon which was not observed in either of the control transplantation groups. While this trophic response is most likely host derived, it does however provide further evidence for the neurotrophic potential of transplanted GDNF-MSCs. A number of modifications to the design of the co-transplant studies, including varying the ratio of GDNF-MSCs to dopaminergic cells and assessing an alternative donor age, may improve the limited efficacy of the co-transplantation approach. However given the trophic effect of GDNF we initially observed in this chapter, and indeed previous results from our laboratory demonstrating the neuroprotective ability of GDNF-MSCs (Moloney et al., 2010b), we elected to consolidate our efforts to harness the neurotrophic ability of the GDNF-MSCs in models of Parkinson's disease.

Neurotrophic therapy represents another promising candidate for the treatment of Parkinson's disease, and it is tantalisingly close to adoption into the clinic. GDNF, in particular, holds great promise for the treatment of Parkinson's disease, given that it has potent protective effects on the survival of midbrain dopaminergic neurons pre-clinically. However, the major barrier to neurotrophic factor translation from bench to bedside, is the challenge involved in delivering such a large protein to the appropriate target cells of the nigrostriatal pathway. Pre-clinical and indeed clinical trials have investigated the efficacy of infusion of the GDNF protein to the brain (delivered via an implanted catheter system) as a therapeutic option for Parkinson's disease. Despite initial promising results from open-labelled clinical trials (Gill et al., 2003, Love et al., 2005, Patel et al., 2005), setbacks provided by negative results of the double-blind placebo-controlled trial (Lang et al., 2006) and the safety concerns highlighted by side effects observed in this trial (Slevin et al., 2005) prompted researchers in the field to investigate alternative methods for GDNF delivery. In particular it was suggested that *ex vivo* gene therapy in which a cell is engineered to release GDNF may be a more beneficial for delivery of GDNF rather than direct infusion of the protein itself (Sherer et al., 2006).

MSCs are an attractive candidate for *ex vivo* gene therapy given their ease of isolation and propagation (Pittenger et al., 1999), their immunomodulatory properties (Bartholomew et al., 2002, Di Nicola et al., 2002, Moloney et al., 2010a), their inherent ability to release neurotrophic factors and growth factors that protect and induce regeneration of damaged tissue (Kurozumi et al., 2005, Paul and Anisimov, 2013) and the ease in which they can be virally manipulated in order to exploit their neurotrophic potential (Wyse et al., 2014). Previous studies in our laboratory have demonstrated the ability of GDNF-transduced MSCs to release GDNF at the site of transplantation in the rat brain, and in turn the ability of this released GDNF to provide local neuroprotection against the toxic effects of the catecholaminergic neurotoxin, 6-hydroxydopamine (Moloney et al., 2010b). The delivery of GDNF using this *ex vivo* gene therapy approach removes the potential risks associated with viral delivery of GDNF and

negates the requirement for an implanted pump system for continuous delivery of the GDNF protein. GDNF has been shown repeatedly to be effective in both the 6-hydroxydopamine and MPTP models of Parkinson's disease. However, recent evidence has shown that viral vector-mediated delivery of GDNF failed to induce neuroprotective effects in the α -synuclein model of Parkinson's disease (Decressac et al., 2011), suggested to be due to α -synuclein-induced downregulation of the transcription factor, Nurr1, and its downstream target, the GDNF receptor RET (Decressac et al., 2012). Thus we felt it important to assess the effects of GDNF in the emerging inflammation-driven LPS model of Parkinson's disease in order to fully elucidate its efficacy in an animal model of the disease with a different inductive mechanism.

The ability of intracerebral LPS to induce neuroinflammation and dopaminergic degeneration is relatively well established (Liu and Bing, 2011), however the motor implications of such neuroinflammation and degeneration required some further characterisation before we could assess the impact of transplanted GDNF-MSCs on these features. To this end, in Chapter 4 we have demonstrated the ability of intra-nigral administration of LPS to induce the motor impairment, neuroinflammatory and neurodegenerative features associated with the disease (Hoban et al., 2013a). Following this, we sought to determine the ability of transplanted GDNF-MSCs to protect against these features. While we found that the cells were not capable of preventing LPS-induced motor impairment, or indeed the nigrostriatal pathway as a whole, they did induce a localised neuroprotection of dopaminergic terminals. To our knowledge, this is the first evidence showing the ability of GDNF to protect against inflammation-induced neurodegeneration (Hoban et al., (Submitted)). Although modest in nature, given that the neuroprotective effect is only evident at the site of transplantation, this observation was not completely unexpected given our previous report highlighting local (but not widespread) neuroprotection in the 6-hydroxydopamine model – a finding attributed to the poor cell survival and consequent loss of neurotrophin secretion from GDNF-MSCs in the (Moloney et al., 2010b). Therefore, the poor survival of MSCs after intracerebral transplantation must be addressed in order to sustain neurotrophic support and thus

induce a more widespread protection/restoration of the nigrostriatal pathway as a whole and potential restoration of motor function.

In order for MSCs to prove beneficial as vehicles for neurotrophin delivery to the CNS, they should be capable of surviving for a prolonged period of time once transplanted to the brain. Previous studies in our own, as well as other laboratories, have shown that bone marrow-derived MSCs do not survive for long enough in the ectopic environment of the brain to allow for the sustained delivery of neurotrophins to the chronically degenerating brain (Moloney et al., 2010a, Moloney et al., 2010b, Rossignol et al., 2009, Swanger et al., 2005), which is a serious limiting factor for future progression of this potential cell transplantation therapy. Several factors undoubtedly contribute to the outcome of any cellular transplant into the brain including (but not restricted to) 1) deprivation of nutrients and serum provided by the culture or transplantation medium, 2) insults such as oxidative stress, excitotoxicity and hypoxia once *in situ* in the brain parenchyma, and 3) immunological disparity between the host and donor cells (Potier et al., 2007, Zhu et al., 2006). Our efforts (shown in Appendix 2) to alter the transplantation media through the inclusion of either the MSC growth factor IGF-1 (Huang et al., 2012) or a range of factors (Laflamme et al., 2007) or to prime the cells to the hypoxic environment of the brain did not improve the survival of the cells after transplantation. However, previously in our laboratory we have shown that the transplanted cells are dying by apoptotic means (Moloney et al., 2010a), possibly through anoikis due to a loss of membrane attachment caused by the lack of an extracellular matrix after transplantation. Thus, in Chapter 5, we sought to evaluate the impact of delivering GDNF-MSCs in a collagen hydrogel (to mimic the presence of an extracellular matrix). When GDNF-MSCs were delivered to the rat brain in the collagen hydrogel, we determined that, while the collagen hydrogel did not improve the survival of transplanted GDNF-MSCs, it did not detrimentally affect their survival or inherent ability release GDNF and induce local neuroprotection. Employment of the hydrogel as a cell delivery matrix did significantly reduce the host immune response to the transplanted cells. However, we have previously shown that, while immune suppression of the host (using cyclosporine A) improved MSC survival, this improvement was of

little biological significance since approximately 80% of cells were still lost 14 days after transplantation (Moloney et al., 2010a). Thus, while minimising the host immune response is undoubtedly beneficial in a disease in which neuroinflammation is believed to contribute to its pathogenesis, realistically this *ex vivo* gene therapy approach for GDNF delivery remains limited by the issue of poor cell survival after transplantation.

Future Directions

The use of the collagen hydrogel, although in isolation did not improve cell survival, offers a platform on which to build a regime to potentially increase the survival of the cells after transplantation. Since the hydrogel investigated in this thesis attenuated the cell-induced host immune response, it may therefore be suitable for the delivery of cell types which elicit a greater immune response than MSCs, and those which require immunosuppressive drug treatment after transplantation such as embryonic stem cells and fetal dopaminergic cells (de Munter et al., 2014).

A treatment regime which harnesses the favourable immunosuppressive characteristics and the mechanical support of the hydrogel itself, combined with possible chemical or environmental alteration of the hydrogel, perhaps via the addition of pro-survival factors assessed independently in Appendix 2, may prove advantageous for the survival of cells *in vivo*. In fact, a previous study has shown that a fabricated scaffold functionalised to release IGF-1, significantly improved the survival and growth of MSCs under hypoxic growth conditions *in vitro* when compared with MSCs seeded on non-functionalised scaffolds (Wang et al., 2009). Therefore, future studies could investigate if functionalisation of the collagen hydrogel with IGF-1, thereby providing the encapsulated MSCs with growth factor-rich environment after transplantation, could enhance their survival in the brain.

While the employment of a biomaterial matrix may hold benefit for protecting the cells from an inflammatory host response, the transient treatment regimens that we have investigated (in Appendix 2) may be insufficient to infer long term cell survival *in vivo*. Thus, a combination of cell encapsulation with viral-mediated genetic manipulation of

the cells to address apoptotic cell death may be necessary for long term cell survival *in vivo*. Heat shock proteins are widely known for their protein chaperone role, however members of the heat shock protein 27 (Hsp27) and heat shock protein 70 (Hsp70) family are important regulators of the signal transduction pathway leading to apoptosis (Takayama et al., 2003). Lentiviral overexpression of Hsp27 resulted in increased MSC survival in an *in vitro* model of ischemia and in an *in vivo* model of rat myocardial infarction, and enhanced the therapeutic effect of the MSCs as evidenced by decreased apoptosis in the infarcted tissue and improved cardiac function (McGinley et al., 2013). Meanwhile lentiviral overexpression of Hsp70 enhanced MSC survival, increased resistance to apoptosis and restored their inherent capacity for differentiation in an *in vitro* model of hypoxia and ischaemia (McGinley et al., 2011). These studies offer proof-of-concept for the overexpression of anti-apoptotic proteins in order to increase survival of MSCs after transplantation. MSCs have also been genetically modified to overexpress Bcl-2 for applications in treatment of myocardial infarction (Li et al., 2007). Bcl-2 overexpression in MSCs has been shown to reduce their apoptosis and enhance vascular endothelial growth factor (VEGF) secretion under hypoxic conditions *in vitro*. Additionally, Bcl-2 overexpression increased cellular survival, capillary density in the infarct border zone and exhibited functional recovery in a model of myocardial infarction (Li et al., 2007). These reported beneficial effects of Bcl-2 overexpression on MSCs, show that this method may hold significant potential to increase stem cell viability after transplantation, thereby improving the efficacy of this *ex vivo* gene therapy approach for the treatment of Parkinson's disease.

Concluding remarks

Neurotrophic therapy and fetal dopaminergic transplantation remain exciting and active areas of both pre-clinical and clinical research. The TRANSEURO trial for fetal dopaminergic transplantation (Moore et al., 2014, Evans et al., 2012), and the GDNF convection enhanced delivery trials currently underway by Gill and colleagues in Bristol (Gill et al., 2012), highlight the continued promise these approaches hold. The technological advances and redesigned implantable pump and catheters employed in the new Bristol trial will harness the method of convection enhanced delivery to slowly and

intermittently deliver GDNF directly to the target structures (Barua et al., 2013). However, successful outcomes of this approach will not circumvent the requirement for a lifetime of GDNF injections for the patient. The *ex vivo* gene therapy approach investigated in this thesis would in theory abolish the requirement for long term GDNF infusions via implanted catheter systems, as after a single cell transplantation surgery, these GDNF-MSCs would potentially act as a ‘biological minipump’ for GDNF release and diffusion in the brain. However, until the issues relating to poor cell survival can be overcome, the true potential of this approach remains limited. Nevertheless, if these issues can be overcome, this *ex vivo* gene therapy approach may hold great potential as a disease-modifying treatment for Parkinson’s disease.

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Appendix 1 - Pilot study to assess appropriate LPS dose for Chapter 4

Prior to commencing the studies to assess the effects of intra-nigral LPS on motor function, neuroinflammation and neurodegeneration, we completed pilot studies to establish the appropriate dose of LPS for intra-nigral injection. These studies followed a similar experimental design, and the data generated, and therefore the rationale for choosing the 10 µg dose of LPS, is shown in **Table A1.1**.

Parameter Assessed	Vehicle	3 µg LPS	8 µg LPS	10 µg LPS
Nigral microgliosis (%OD of OX-42-ir)	110.4±1.8	119.8±4.4**	193.9±12.7***	157.6±9.4***
Striatal microgliosis (%OD of OX-42-ir)	119.2±2.7	91.4±4.9	103.0±2.0	100.9±1.6
TH – SN (%OD of TH-ir)	108.4±3.3	81.9±11.8	93.4±9.2	73.0±4.1***
TH – STR (%OD of TH-ir)	117.7±3.5	75.3±8.6	97.9±3.4	70.3±2.0***
Stepping (contralateral steps)	103.9±1.9	86.9±10.9	78.3±4.1***	30.8±6.7***
Whisker (contralateral placings)	100±0.0	78.9±10.5	80.0±10.0	9.0±9.0***
Corridor (contralateral retrievals)	92.2±1.9	102.1±1.3	98.6±2.1	95.2±3.4
Rotation (turns per min)	0.3±0.4	n.d.	1.4 ± 0.9	0.7±0.5

Table A1.1 Data generated in pilot studies to determine the appropriate dose of LPS for intra-nigral injection. LPS (3 µg or 8 µg) was injected unilaterally into the substantia nigra and its effect on microgliosis, nigrostriatal integrity and motor function was assessed. All data is shown as mean±s.e.m. The data generated from the main study (using 10 µg) is also shown for comparison. Neuropathological data is shown as % of intact side whereas behavioural data is shown as % of baseline (except for rotation data which was not completed at baseline). ** $P < 0.001$, *** $P < 0.0001$ vs. appropriate vehicle by 1-way ANOVA with *post-hoc* Newman Keuls. TH-ir: tyrosine hydroxylase immunoreactivity; OX-42-ir: OX-42 immunoreactivity; n.d.: not done.

Appendix 2 - Strategies to increase survival of MSCs

A2.1 Introduction

In order for MSCs to prove beneficial as vehicles for neurotrophin delivery to the CNS, MSCs should be capable of surviving for a prolonged period of time once transplanted to the brain. Thus, the studies detailed in this appendix focus on exploring individual and/or synergistic means of improving the survival of bone marrow-derived MSCs in the rat brain. In the first instance, GFP-MSCs will be delivered to the brain in an enhanced transplantation media containing insulin like growth factor. IGF-1 has been shown to enhance survival of many cell types including mesenchymal stem cells (Huang et al., 2012) and has been shown act as an anti-apoptotic factor on tissue derived stem cells (Gehmert et al., 2008), and thus may be advantageous in this application of improving *in vivo* cell survival.

We also wished to assess the ability of a recently identified (Laflamme et al., 2007) pro-survival “cocktail” to improve the survival of these cells. This treatment regime includes 1) transient heat stress to induce heat shock protein expression, 2) Matrigel® to reduce apoptosis induced by matrix detachment, 3) cell-permeant (TAT) Bcl-XL peptide to block mitochondrial death pathways, 4) cyclosporine A to attenuate cyclophilin D-dependent mitochondrial death pathways, 5) pinacidil to open K⁺ channels thereby mimicking ischemic preconditioning, 6) insulin-like growth factor to activate the pro-survival Akt pathway, and 7) the caspase inhibitor, Z-VAD-FMK. These factors in combination, but not alone, improved the survival of stem cell-derived cardiomyocytes when subsequently transplanted into the infarcted heart (Laflamme et al., 2007) and thus may improve MSC survival following transplantation to the rat brain.

Hypoxic preconditioning has previously been shown to allow cells to survive and adapt to an ischemic environment in applications including cardiac dysfunction and stroke. Cardiac progenitor cells cultured under hypoxic conditions (2%–5% O₂) *in vitro* were found to exhibit enhanced cell survival in a mouse model of myocardial ischemia–

reperfusion injury (Tang et al., 2009). Moreover, preconditioning ESC-derived NPCs under hypoxia enhanced cell survival with 30%–40% reduction in cell death after transplantation into the ischemic brain of rats, compared to the groups cultivated under normoxia (Francis and Wei, 2010, Theus et al., 2008). Similarly, MSCs conditioned under hypoxia promoted angiogenesis and neurogenesis in rat ischemic brain models that mimicked stroke (Wei et al., 2012). MSCs exposed to hypoxia *in vitro* also showed the enhanced survival through the up-regulation of Bcl-2 and Bcl-x_L, leading to the reduced infarct size and the enhanced heart functions (Hu et al., 2008). To this end, we wished to assess the impact of hypoxic preconditioning on the survival of GFP-MSCs after transplantation to the rat striatum.

A2.2 Methods

All methods have been described in more detail in Chapter 2. Methods specific to the studies in this appendix are detailed below.

A2.2.1 Experimental Design

The overall aim of the studies included in this appendix will focus on the different strategies we employed as a means of improving the survival of bone marrow-derived MSCs in the rat brain. In these studies, we applied an array of strategies; including treating MSCs prior to transplantation or altering the transplantation media in which they were delivered to the brain in an effort to enhance their survival *in vivo*. This chapter will detail the results of three separate *in vivo* studies, each utilising a different pro-survival strategy, in attempt to increase the efficacy of this *ex vivo* gene therapy approach.

A2.2.1.1 Transplantation of GFP-MSCs in media supplemented with varying concentrations of IGF-1

IGF-1 has been shown to enhance survival of many cell types including MSCs (Huang et al., 2012) and also been shown act as an anti-apoptotic factor on tissue derived stem cells (Gehmert et al., 2008). Because of these positive attributes, the goal of the first *in vivo* study was determine if supplementation of the transplantation media with the growth factor IGF-1 would lead to an increase in transplanted cell survival. Animals were divided into 4 groups (n=6 per group) and each received intra-striatal transplants of 30,000 GFP-MSCs in 3 μ l of transplantation media supplemented with; 0, 10, 30 or 50 ng/ μ l of recombinant IGF-1. Animals were sacrificed by transcatheter perfusion-fixation 7 days after transplantation, and their brains processed for *post mortem* analysis of graft persistence to determine the effect of IGF-1 in the transplantation media on cell survival.

A2.2.1.2 Treatment of MSCs with a pro-survival cocktail

The aim of this study was to determine if treatment of GFP-MSCs with a previously published pro-survival cocktail (Laflamme et al., 2007) would improve the survival of

the cells *in vivo*. This treatment regime includes 1) transient heat stress, 2) Matrigel®, 3) cell-permeant (TAT) Bcl-XL peptide, 4) cyclosporine A, 5) pinacidil, 6) insulin-like growth factor, and 7) the caspase inhibitor, Z-VAD-FMK and is shown in more detail **Table A2.1** below. Animals were divided into two groups (n=4 per group), and each received intra-striatal transplants of 100,000 GFP-MSCs (in 2 μ l transplantation media). Cell transplants were comprised of GFP-MSCs that had been subjected to either the pro-survival regime (PSC-Treated MSCs) or to the control equivalent as detailed below (Control MSCs). Animals were sacrificed one week after transplantation and their brains were processed for *post mortem* analysis of graft persistence.

	PSC-Treated MSCs	Control MSCs
Step 1: (24 h before transplant)	Culture flask was sealed in a watertight plastic bag and submerged in a water bath set to 42°C for 30 min exposure to induce transient heatshock	Culture flask was sealed in a watertight plastic bag and returned to the incubator for 30 min
Step 2: (directly after heat shock)	Flask was removed from watertight bag and medium was replaced with normal medium supplemented with IGF-1 (100 ng/ml) and cyclosporine A (200 nM)	Flask was removed from watertight bag and medium was replaced with normal medium supplemented with corresponding volumes of BSA and saline (the respective vehicles for IGF-1 and cyclosporine A)
Step 3: (prior to transplant surgeries)	Cells were resuspended, at the appropriate density for transplantation, in standard transplantation medium supplemented with the pro-survival cocktail at a ratio of 1:1. The pro-survival cocktail consisted of 50% (vol/vol) growth factor–reduced Matrigel, supplemented with ZVAD (100 mM), Bcl-XL (50 nM), cyclosporine A (200 nM), IGF-1 (100 ng/ml) and pinacidil (50 mM)	Cells were resuspended, at the appropriate density for transplantation, in standard transplantation medium supplemented with corresponding volumes of vehicles for the pro-survival cocktail (at a ratio of 1:1) (DMSO for ZVAD, Bcl-XL and pinacidil; BSA for IGF-1 and saline for cyclosporine A).

Table A2.1 Treatment regime for PSC-Treated MSCs and Control MSCs

A2.2.1.3 Pre-conditioning of MSCs to a hypoxic environment prior to transplantation

In this study, we sought to determine if exposure of GFP-MSCs to a hypoxic environment for 24 hours prior to transplantation was sufficient to suitably prime the cells and ultimately increase their survival post-transplantation. Animals were divided into two groups (n=8 per group) and each received an intra-striatal transplant of 100,000 GFP-MSCs (in 2 μ l transplantation media). Cell transplanted were comprised of GFP-MSCs that were cultured in hypoxic (5% O₂) (Hypoxic MSCs), or normoxic conditions (21% O₂) (Control MSCs) for 24 hours prior to transplantation. Animals were sacrificed one week after transplantation and their brains were processed for *post mortem* analysis of graft persistence.

A2.3 Results

A2.3.1 Transplantation of GFP-MSCs in media supplemented with varying concentrations of IGF-1

We sought to determine if transplantation of GFP-MSCs in transplantation media containing the growth factor IGF-1 would improve cell survival. Cell transplants were clearly visible in all animals on Day 7 post-transplantation by virtue of their strong GFP expression (see **Fig A2.1 A**). However, when we quantitatively assessed the graft volume of surviving cells, as previously described, there was no significant difference observed in graft volume despite the presence of an escalating concentration of IGF-1 in the transplantation media used (**Fig A2.1 B**; Group, $F_{(3,20)} = 0.6944$, $P = 0.57$, n.s.).

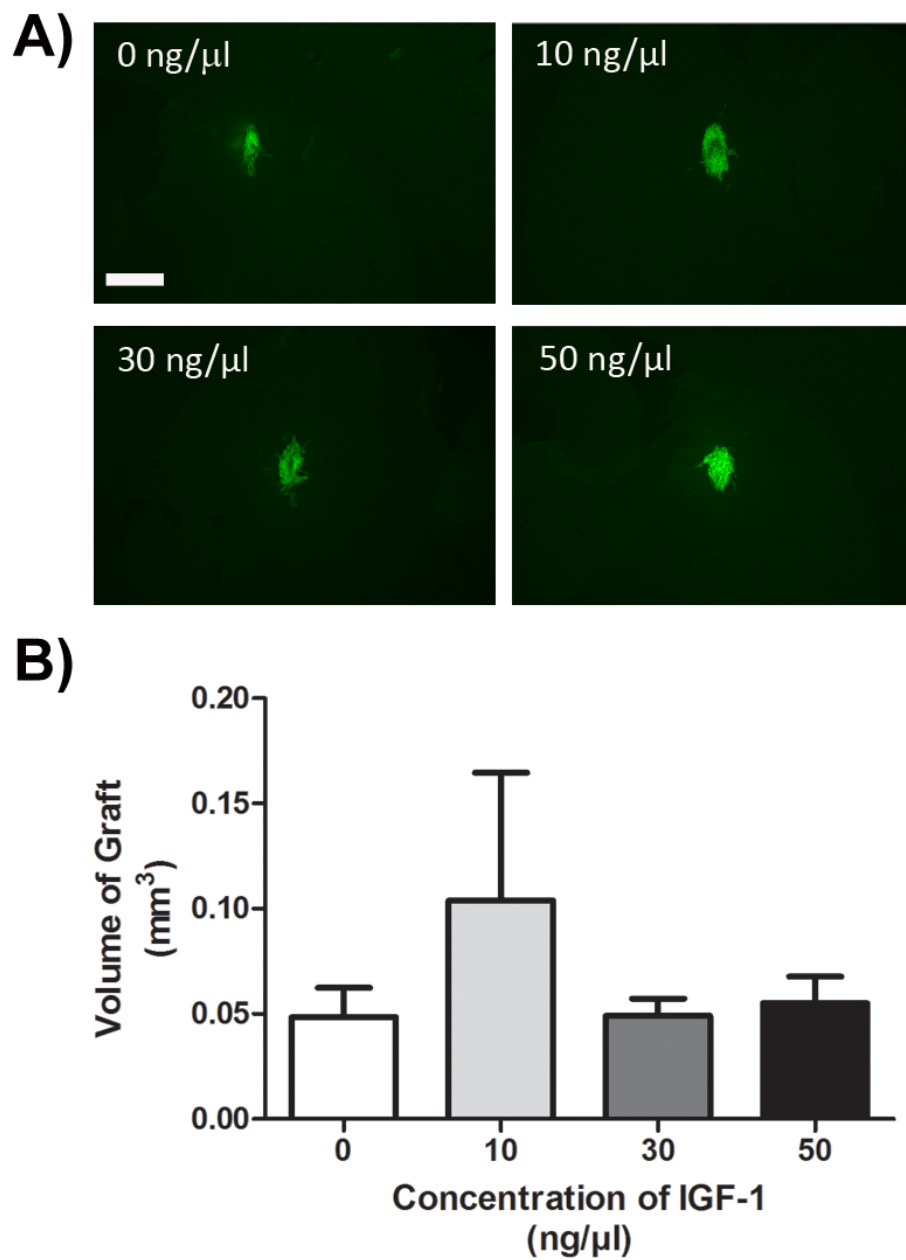


Fig A2.1 Inclusion of IGF-1 in the cell transplantation media does not affect GFP-MSC survival *in vivo*. A) Representative photomicrographs showing representative striatal sections from animals that received intra-striatal GFP-MSC transplants in the presence of 0 ng/μl, 10 ng/μl, 30 ng/μl or 50 ng/μl of recombinant IGF-1. B) Quantification of the volume of the transplants showed that inclusion of IGF-1 did not significantly improve the graft volume regardless of the concentration used. Data are presented as mean \pm s.e.m. and were analysed by 1-way ANOVA. Scale bar represents 500 μ m.

A2.3.2 Treatment of MSCs with a pro-survival cocktail

In this study, we subjected the MSCs to a previously published pro-survival cocktail (Laflamme et al., 2007). The regime of treatment involved in the pro-survival cocktail was initiated 24 hours prior to transplantation of the cells when MSCs were exposed to heat shock preconditioning and the addition of IGF-1 and cyclosporine A was added to the culture medium. The regime resumed immediately prior to cell transplantation surgeries, when the remainder of drug treatments (Bcl-XL peptide, cyclosporine A, pinacidil, IGF-1, Z-VAD-FMK) and the Matrigel™ component were added to the cell suspension to be infused into the brain. After animals were sacrificed on Day 7, and their brains processed for GFP microscopy to determine graft persistence, GFP-MSC grafts were evident in each transplanted animal (see **Fig A2.2 A**). When the graft volume was quantified, we found that treatment of MSCs with the pro-survival did not increase MSC survival *in vivo*, at this time point (**Fig A2.2 B**; $t_{(6)} = 1.912$, $P = 0.1045$, n.s.).

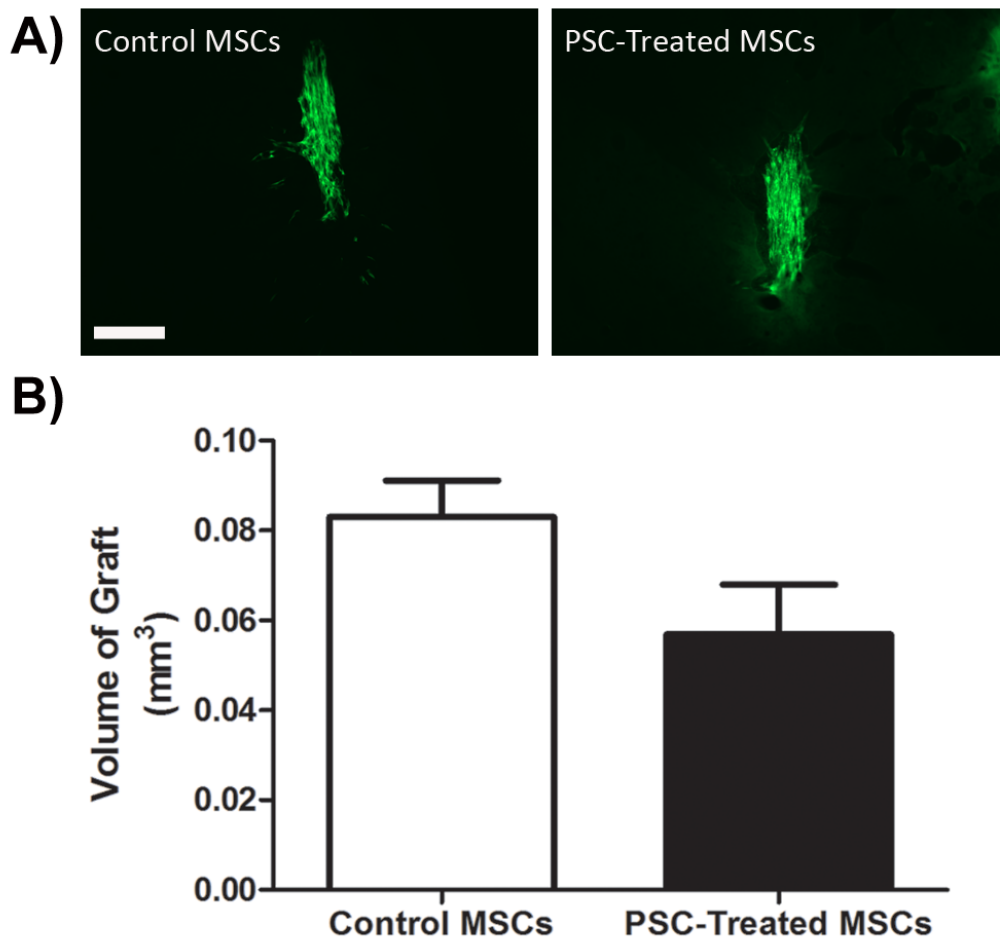


Fig A2.2 Treatment of MSCs with a pro-survival cocktail did not affect MSC survival *in vivo*. A) Representative photomicrographs showing representative striatal sections from animals that received intra-striatal GFP-MSC transplants treated with the pro-survival cocktail (PSC-Treated MSCs) or appropriate vehicles (Control MSCs). B) Quantification of the volume of the transplants showed that treatment with the pro-survival cocktail did not significantly improve the graft volume when compared to control MSCs. Data are presented as mean \pm s.e.m. and were analysed by unpaired *t*-test. Scale bar represents 200 μ m.

A2.3.3 Pre-conditioning of MSCs to a hypoxic environment prior to transplantation

In this study MSCs were placed in a hypoxic environment (an incubator set to 5% O₂) for 24 hours prior to transplantation surgery. Control MSCs were provided with exactly the same growth conditions as hypoxic MSCs (culture flask size, quantity of culture medium) except the incubator in which they were grown was maintained under standard conditions (21% O₂). After this preconditioning stage, cells were transplanted to the rat striatum, ensuring the time of infusion was recorded (i.e. time out of hypoxia). Once again, cell transplants were easily identifiable *post mortem* using GFP microscopy (see **Fig A2.3 A**). Upon quantification of the volume of remaining graft, we found that hypoxic pre-conditioning had no effect on MSC survival when examined 7 days post-transplantation (**Fig A2.3 B**; $t_{(13)} = 1.459$, $P = 0.1684$, n.s.). Time of infusion did not appear to have any effect on cell survival; however no transplantation surgery was performed beyond 2 hours after cells were removed from hypoxic conditions.

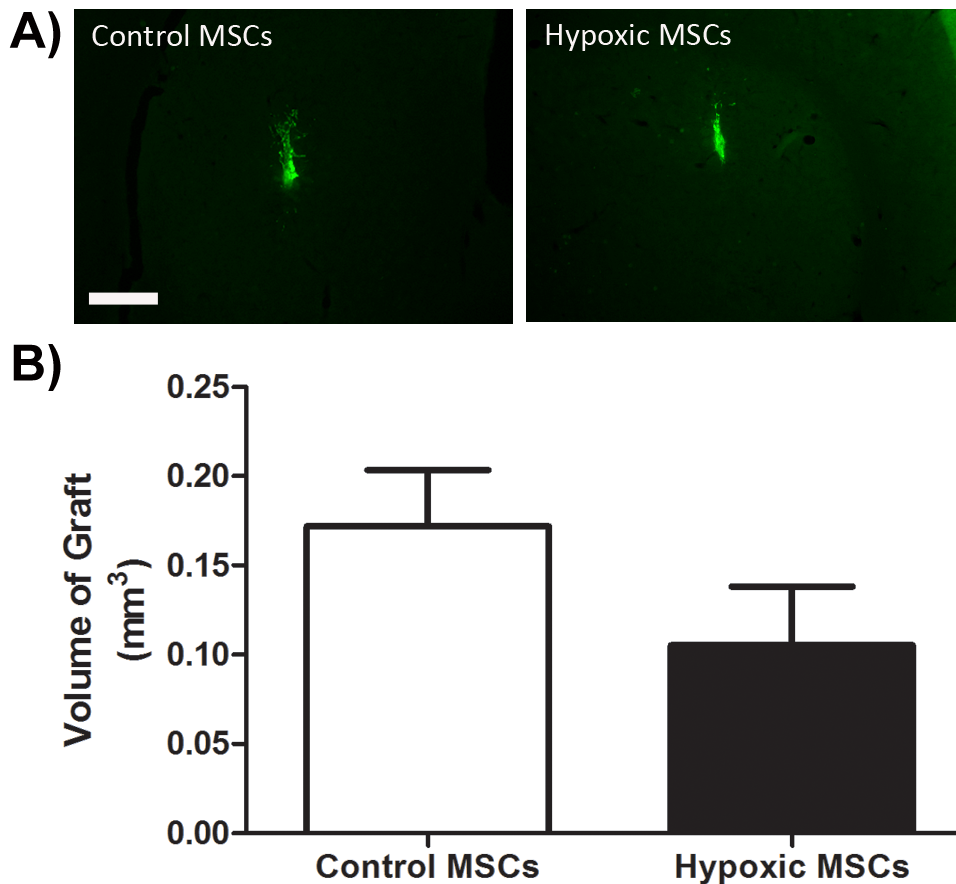


Fig A2.3 Hypoxic pre-conditioning of GFP-MSCs did not increase MSC survival *in vivo*. A) Representative photomicrographs showing representative striatal sections from animals that received intra-striatal GFP-MSC transplants exposed to hypoxic pre-conditioning B) Quantification of the volume of the transplants showed that hypoxic pre-conditioning did not significantly improve the graft volume when compared to control MSCs. Data are presented as mean \pm s.e.m. and were analysed by unpaired *t*-test. Scale bar represents 500 μ m.

A2.4 Conclusion

The studies detailed in this appendix focused on exploring individual strategies of improving the survival of bone marrow-derived MSCs in the rat brain. In contrast to published results, we have found that delivery of GFP-MSCs in transplantation media containing the growth factor IGF-1 (Huang et al., 2012), exposure of GFP-MSCs to a pro-survival cocktail regime (Laflamme et al., 2007), and hypoxic pre-conditioning of GFP-MSCs prior to transplantation (Hu et al., 2008, Wei et al., 2012), did not increase cell survival *in vivo*.

Appendix 3 – Immunohistochemistry Methods**Buffers for Perfusion-Fixation****0.2M Phosphate buffer****Stock A**Sodium dihydrogen phosphate monohydrate (NaH₂PO₄·H₂O MW=137.99)1M=137.99g in 1L dH₂O0.1M=13.799g in 1L dH₂O0.2M=27.598g in 1L dH₂O**Stock B**Disodium hydrogen phosphate dehydrate (Na₂HPO₄·2H₂O MW=177.99)1M=177.99g in 1L dH₂O0.1M=17.799g in 1L dH₂O0.2M=35.598g in 1L dH₂O

→For 100ml PB

9.5ml stock A + 40.5ml stock B + 50ml dH₂O =100ml PB

→For 1000ml PB

95ml stock A + 405ml stock B + 500ml dH₂O =1000ml PB

→For 2000ml PB

190ml stock A + 810ml stock B + 1000ml dH₂O =1000ml PBStock A: 27.598g/L x 0.19L = 5.24362g in 190ml dH₂OStock B: 35.598g/L x 0.81L = 28.83438g in 810ml dH₂OAlternatively add both to 1L of dH₂O and dissolve

Make up to 2L with water

NOTE: Weights must be multiplied by 2 to take into account the 1:1 dilution with PFA**Fixative: 4% Paraformaldehyde (4L) (in fume hood)**

1. Heat 1.5L of dH₂O to 60 °C
2. Add 160g of PFA powder
3. Add a few NaOH pellets in order to dissolve PFA
4. Stir until clear
5. Fill to 2L with dH₂O
6. Stir until clear
7. Add 2L of 0.2M PB to give a final volume of 4L
8. PH to 7.4 and cool to 4 °C

Heparinised Saline

1 ml heparin is added per 1L saline

Each small vial of heparin contains 25,000/5 ml i.e. 1 ml of heparin has 5000 units

25% w/v Sucrose Solution (1L)

1. Dissolve 5 PBS tablets in ~500ml dH₂O (1 tablet per 200ml water)
2. Add 250g of sucrose
3. Stir until dissolved, apply heat if necessary
4. Make up to 1L with dH₂O

0.1% w/v TBS-Azide

1. Weigh out 12g Trizma, 9g NaCl, 1g sodium azide
2. Add to 1L dH₂O and dissolve
3. PH to 7.4

Solutions for Immunohistochemistry

Quench	Methanol (98%)	5 ml
	Hydrogen peroxide (30%)	5 ml
	Distilled Water	40 ml
TBS	Trizma Base	12 g
	Sodium Chloride	9 g
	Distilled water	Make up to 1 l
	Adjust to pH 7.4 with conc HCl	
TXTBS	TBS	250 ml
	Triton X-100	500 µl
ABC	DAKO Streptavidin Kit	
	TBS with 1% serum	1 ml
	Solution A	5 µl
	Solution B	5 µl
TNS	Prepare fresh prior to use	Make up to 1L
	Trizma base 6g	
	Distilled water	
	Adjust to pH 7.4 with conc HCl	
DAB stock	DAB	1 g
	TNS	100 ml
	Aliquot into 2 ml aliquots and store at -20°C	20mg in 2 ml aliquot
DAB working	DAB stock	2ml
	TNS(fresh)	40ml
	Hydrogen peroxide (30%)	12 µl
	This solution may be diluted to 1 in 5 with TNS if the reaction proceeds too quickly.	

General Immunohistochemistry Protocol

Suitable for 30 μm free-floating sections. Cut from tissue which has been perfused with phosphate buffer, fixed in 4% buffered paraformaldehyde, and then equilibrated with 25% buffered sucrose.

Free floating sections are processed in “Greiner pots” on a rotating mixer. The lids of the pots are cut away partly and a gauze square is fitted between the lid and the pot in a way such as to retain the sections but allow the liquid to be tipped away and more added.

1. Wash 1*5 min in TBS.
2. Quench for 5min.
 - Methanol 5 ml
 - 30% H_2O_2 5 ml
 - Dist H_2O 40 ml
3. Wash 3*5 min with TBS.
4. Incubate sections in 3% NHS* for 60 min.
 - 30 $\mu\text{l/ml}$ NHS in 970 $\mu\text{l/ml}$ TXTBS (*freshly made-up*)
 - *serum dependent on 2^o host
5. Draw off excess and incubate in primary in 1% NHS* at room temperature overnight (e.g. 1:1000):
 - 1 $\mu\text{l/ml}$ of 1^o in 10 $\mu\text{l/ml}$ NHS* in 989 $\mu\text{l/ml}$ TXTBS
6. Wash 3*10 min with TBS.
7. Incubate in biotinylated secondary in 1% NHS* for 3 hours (e.g. 1:200):
 - 5 $\mu\text{l/ml}$ of 2^o in 10 $\mu\text{l/ml}$ NHS* in 985 $\mu\text{l/ml}$ TBS
8. Make ABC Complex. Wash 3*10 min with TBS.
 - 5 μl of solution A and 5 μl of solution B per ml in 10 $\mu\text{l/ml}$ NHS in 980 $\mu\text{l/ml}$ TBS.
9. Incubate in ABC Complex for 2 hours.

10. Wash 3*10 min with TBS.
11. Wash with TNS (freshly made-up) overnight @ 4°C.
12. Incubate in H₂O₂/DAB solution until colour develops.
 - TNS 40 ml
 - DAB 20 mg (frozen in 2 ml aliquots).
 - 30% H₂O₂ 12 µl
 - Dilute 1/5 and use.
13. Wash 3*5 min with TNS
14. Mount (in TBS with a little TXTBS) on gelatin-coated slides and air dry overnight.
15. Dehydrate in an ascending series of alcohols
 - 50% EtOH for 5 min
 - 70% EtOH for 5 min
 - 100% EtOH for 5 min
 - 100% EtOH for 5 min
16. Clear in Xylene in the fume hood and coverslip using DPX mountant.
 - 1st Xylene for 5 min
 - 2nd Xylene for 5 min