The Potential of GDF5-Functionalised Collagen Hydrogels to Enhance Dopaminergic Cell Replacement Therapies for Parkinson´s Disease

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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.

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

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Abstract

Parkinson’s disease is a progressive neurodegenerative disorder associated with the loss of dopaminergic neurons from the substantia nigra and striatum. Over time, the gold standard pharmacotherapy, L-dopa, loses its efficacy in alleviating the motor symptoms that characterise this disorder, making it necessary to find alternative treatments that can modify the disease course or repair the damage already done. A promising reparative therapy for Parkinson’s disease is the transplantation of dopaminergic neurons either harvested from foetal tissue or derived from various stem cell sources. However, cell replacement therapies have been limited by poor survival leading to poor striatal reinnervation and limited motor recovery.

Biomaterial-based therapies, like collagen hydrogels, offer significant potential to improve the outcome of cell replacement therapy by mimicking the *in vivo* microenvironment of the transplanted cells via giving them an adhesive matrix for growth and a supply of neurotrophic factors, while acting as a physical protection against inflammatory microglia and astrocytes. One potential neurotrophic factor to use in this context is growth differentiation factor 5 (GDF5) which is a potent dopaminergic neuroprotective agent. Therefore, the aim of this thesis was to determine if a dual approach combining the delivery of GDF5 and cell therapy in a collagen hydrogel could improve the outcome of cell replacement therapy in a rat model of Parkinson’s disease.

Through a series of preliminary *in vitro* and *ex vivo* studies, we first assessed the biocompatibility of collagen hydrogels and the effects of GDF5 alone or GDF5-functionalised collagen hydrogels on a SH-SY5Y cell line and on a ventral mesencephalon (VM) explant system. Subsequently, we did preliminary assessments *in vivo* of the suitability of collagen hydrogels and GDF5-functionalised collagen hydrogels for the delivery of primary dopaminergic neurons (derived from the developing VM of the rat) at early time-points. Based on these results, we sought to investigate the long-term potential of GDF5-functionalised collagen hydrogels to enhance the survival, re-innervation capacity and function after transplantation of embryonic day 14 (E14) rat VM grafts in a rat model of Parkinson's disease. Additionally, we examined the long-term effects of GDF5+IL-10-functionalised collagen hydrogels to enhance the survival, re-innervation capacity and function after transplantation of E14 VM grafts in a rat model of Parkinson's disease.

The *in vitro* and *ex vivo* results demonstrated that exposure to the collagen hydrogels did not have any detrimental effects on a human SH-SY5Y cell line or on explanted E14 rat VM tissue explants. Following this, we found that after a 6-hydroxydopamine (6-OHDA) insult on SH-SY5Y cells and VM explants, GDF5 treatment promoted recovery of cells.

The preliminary *in vivo* studies demonstrated that collagen hydrogels are a suitable matrix for cell and neurotrophic factor delivery, that are well tolerated in the brain, and that support the survival of encapsulated primary cells.

Subsequently, when we sought to determine the long-term survival and efficacy of E14 VM primary dopaminergic grafts in GDF5-loaded collagen hydrogels in a rat model of Parkinson's disease, we found that incorporation of GDF5, but not the collagen hydrogel, into the transplantation process actually reduced the survival, reinnervation and functional capacity of the grafted dopaminergic neurons. When we examined the long-term survival and efficacy of E14 VM primary dopaminergic grafts in GDF5+IL-10-loaded collagen hydrogels, we found that the addition of IL-10 did not have a detrimental effect on the grafts and we confirmed that the incorporation of GDF5 had damaging effects on the survival of the grafted dopaminergic neurons, suggesting that GDF5 was toxic to the transplanted cells.

In conclusion, the results obtained in this thesis suggest that GDF5, at the dose used in this work, was detrimental to primary dopaminergic grafts. Nevertheless, collagen hydrogels functionalised with a different neurotrophic factor have the potential to enhance cell replacement therapies in Parkinson’s disease and should be further studied.

Publications

Peer Reviewed Original Research Manuscripts and Reviews

- Moriarty N, Cabré S, **Alamilla V**, Pandit A, Dowd E. (2019). Encapsulation of young donor age dopaminergic grafts in a GDNF-loaded collagen hydrogel further increases their survival, reinnervation, and functional efficacy after intrastriatal transplantation in hemi-Parkinsonian rats. European Journal of Neuroscience. 49:487-496.

- Olsen L, Cairns A, Ådén J, Moriarty N, Cabré S, **Alamilla V**, Almqvist F, Dowd E and McKernan D. (2019). Viral mimetic priming enhances α-synuclein-induced degeneration: implications for Parkinson's disease. Brain Behaviour & Immunity. pii: S0889-1591(18)30768-2.

Other Research Dissemination

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- **Alamilla V**, Cabré S, Moriarty N, Olsen L, Pandit A, Dowd E. 2018. Utility of ventral mesencephalic tissue explants for assessment of neurotrophin-functionalised biomaterial hydrogels in the context of Parkinson's disease therapeutics. Federation of Neuroscience Societies (FENS), Berlin, Germany.

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- Kelly R, Cabré S, **Alamilla V**, Cairns A, Ådén J, Almqvist F, McKernan D, Dowd E. 2019. Development of a novel rat model of Parkinson's disease induced using AAV-mediated α-synuclein overexpression combined with FN075-mediated α-synuclein aggregation. British Neuroscience Association (BNA). Dublin, Ireland.

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- Santaella A, Wessels H, Gloerich J, Kuiperij B, Bloem B, Van Gool A, Verbeek M, **Alamilla V**, Cabré S, Dowd E. 2018. Proteomic Profiling of Striatal Tissue of a Rat Model of Parkinson's Disease after Implantation of Collagen-Encapsulated Mesenchymal Stem Cells. Federation of Neuroscience Societies (FENS), Berlin, Germany.

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List of commonly used abbreviations

°C	degrees Celsius
4s-StarPEG	poly (ethylene glycol) ether tetrasuccinimidyl glutarate
6-OHDA	6-hydroxydopamine
AAV2	adeno-associated viral type-2
ACREC	Animal Care and Research Ethics Committee
ANOVA	analysis of variance
BBB	blood-brain barrier
BMP	bone morphogenetic protein
BMP-14	bone morphogenetic factor-14
BMPR1b	bone morphogenetic protein receptor type 1B
BMPR2	bone morphogenetic protein receptor type 2
CD11b	integrin alpha M/ CR3
cDNA	complementary DNA
cm	centimetre
CNS	central nervous system
DAB	diaminobenzidine tetra hydrochloride
DAT	dopamine transporter
DBS	deep brain stimulation
DC	dendritic cells
DIV	days *in vitro*
DMEM	Dulbecco's modified eagle's medium
DV	dorso-ventral
E	embryonic day
E14	embryonic day 14
ESC	embryonic stem cell

FACS	fluorescence-activated cell sorting
FBS	foetal bovine serum
FCS	foetal calf serum
g	gram
GDF	growth differentiation factor
GDF5	growth differentiation factor 5
GDNF	glial-derived neurotrophic factor
GFL	GDNF family of ligands
GID	graft-induced dyskinesias
Girk2	G protein-gated inwardly rectifying potassium channel 2
H_2O_2	hydrogen peroxide
HBSS	Hank's balanced salt solution
hr	hour
i.p.	intraperitoneal injection
ICC	immunocytochemistry
ICV	intracerebroventricularly
IF	immunofluorescence
IHC	immunohistochemistry
IL-10	interleukin-10
iPSC	induced pluripotent stem cell
KLF4	kruppel-like-factor-4
LID	levodopa-induced dyskinesia
LPS	lipopolysaccharide
MACS	magnetic-activated cell sorting
MANF	mesencephalic astrocyte-derived factor
MFB	medial forebrain bundle

mg	milligram
mg/kg	milligram per kilogram
min	minute
ML	medial-lateral
mm	millimetre
MPP^+	N-methyl pyridinium
MPTP	N-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine
NIH	National Institute of Health
NK	natural killer cells
NRTN	neurturin
OCT3/4:	octamer-binding transcription factor-3/4
PBS	phosphate buffer saline
PD	Parkinson’s disease
PEG	polyethylene glycol
PET	positron emission tomographic
PFA	paraformaldehyde
PLGA	poly (lactic-co-glycolic acid)
Poly I:C	polyinosinic: polycytidylic acid
ROR2	tyrosine-protein kinase transmembrane receptor
ROS	reactive oxygen species
RRF	retro-rubral field
s	second
SEM	standard error ± mean
SN	substantia nigra
SNpc	substantia nigra *pars compacta*
TBS	tris-buffered saline

TGF-β	transforming growth factor β
TH^+	tyrosine hydroxylase
UPDRS	Unified Parkinson´s Disease Rating Scale
v	volume
VM	ventral mesencephalon
VTA	ventral tegmental area
µg	microgram
µl	microliter

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

Table of Contents

Chapter 1: General Introduction

"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"-this is the original description of the elemental symptoms of a disorder first described by James Parkinson in 1817, and that later would be named after him: Parkinson´s disease (Parkinson, 2002).

Parkinson´s disease is the second most common chronic neurodegenerative disorder and is characterized by the development of movement impairments. It is one of the most common causes of disability and mortality globally with a significant clinical and socioeconomic impact (Findley, 2007). The symptoms of Parkinson´s disease begin subtly but steadily worsen over time due to the severe and progressive loss of dopaminergic neurons from the nigrostriatal pathway (Fearnley and Lees, 1991; Hornykiewicz, 2006; Björklund and Dunnett, 2007). Other neuropathological hallmarks of PD involve the accumulation of the α-synuclein protein in the form of Lewy bodies and/or Lewy neurites, which extend across many areas of the central nervous system (CNS) and also involves the enteric and autonomic nervous system (Cochen *et al*., 2003; Poulopoulos *et al*., 2012; Mittal *et al*., 2017).

The cardinal motor disturbances are resting tremor, rigidity, bradykinesia and postural instability. These symptoms of Parkinson´s disease are first encountered when there is about an 80% reduction of dopamine concentration in the putamen, and the degree of physical disability strongly correlates with the extent of impairment in striatal dopaminergic innervation (Fahn, 2003). People suffering from Parkinson´s disease also exhibit non-motor symptoms such as sleep disturbance, autonomic dysfunction, hyposmia, cognitive decline and depression (Jankovic, 2008). See **Table 1.1** for more details on the symptoms exhibited by Parkinson´s disease patients.

Table 1.1 Symptoms exhibited by Parkinson´s disease patients.

Motor symptoms	Non-motor symptoms
Tremor, bradykinesia, rigidity, postural instability	Cognitive impairment, bradyphrenia, tip-of-the-tongue (word finding) phenomenon
Hypomimia, dysarthria, dysphagia, sialorrhoea	Depression, apathy, anhedonia, fatigue, other behavioural and psychiatric problems
Decreased arm swing, shuffling gait, festination difficulty arising from chair, turning in bed	Sensory symptoms: anosmia, ageusia, pain (shoulder, back), paresthesias
Micrographia, cutting food, feeding, hygiene, slow activities of daily living	Dysautonomia (orthostatic hypotension, constipation, urinary and sexual dysfunction, abnormal sweating, seborrhoea), weight loss
Glabellar reflex, blepharospasm, dystonia, striatal deformity, scoliosis, camptocormia	Sleep disorders (REM behaviour disorder, vivid dreams, daytime drowsiness, sleep fragmentation, restless legs syndrome)

Adapted and modified from Jankovic (2008)

Dopamine replacement therapy has been the major medical approach to treat Parkinson's since 1960. The gold standard dopamine replacement treatment is administration of the dopamine precursor levodopa in conjunction with a peripheral decarboxylase inhibitor to prevent formation of dopamine in the peripheral tissues (Jankovic, 2008). Unfortunately, with the administration of levodopa, there are two significant complications experienced in about 60% of patients, which ultimately limits its effectiveness. First, the development of complications of disabling response fluctuations ("wearing off effect"), and second, the development of levodopa-induced dyskinesias (LIDs). These LIDs are involuntary movements that occur in 50% to 75% of patients on levodopa after 5 to 10 years of treatment or even sooner in patients less than 60 years of age (Marsden and Parkes, 1976; Fahn, 2008).

One surgical approach to treat Parkinson´s disease is stereotaxic deep brain stimulation (DBS). This therapy is only considered when the motor problems of Parkinson's disease patients no longer respond to the medication. DBS involves the implantation of a device that sends electrical pulses via implanted electrodes to stimulate the thalamus, globus pallidus or the subthalamic nucleus aiming to control bradykinesia and/or the tremors that affect patients; by controlling bradykinesia, dosage of levodopa can be reduced, thus reducing the severity of LIDs as well (Limousin and Martinez-Torres, 2008). However, DBS is very expensive, frequent adjustments are needed to optimise the correct functioning of the stimulator, and this treatment does not slow down or halt the

progression of the disease and it only offers symptomatic relief (Fahn, 2003; Graff-Radford *et al*., 2006; Herzog *et al*., 2008; Yu and Neimat, 2008).

So far, available therapies only target the motor symptoms of Parkinson´s disease and neither the pharmacological nor surgical approaches have been shown to slow down the rate of progression, nor provide neuroprotection to the surviving dopaminergic neurons. This substantial limitation in the current treatments emphasises the major unmet clinical need for new disease modifying therapies that could effectively stop the progression of neurodegeneration and/or repair the diseased brain.

Parkinson´s disease is the most promising neurological disorder for brain repair by cell replacement therapy due to its relatively focal neurodegeneration. The aim of cell replacement in this disorder is to restore nigrostriatal dopaminergic transmission by transplantation of dopaminergic neurons in the striatum and substantia nigra. The intraputamenal transplantation of foetal ventral mesencephalon (VM) grafts, containing developing dopamine cells, has been shown to re-innervate the striatum post-transplantation, whilst also restoring motor function (Sauer and Brundin, 1991; Kordower *et al*., 1995, 1998; Piccini *et al*., 1999; Olanow *et al*., 2003; Kuan and Barker, 2005; Petit *et al*., 2014). However, dopaminergic cell survival following transplantation is limited with only 5% to 10% of the foetal nigral dopaminergic neurons surviving the tissue preparation and grafting procedure (Barker *et al*., 1996; Kordower *et al*., 1998). This poor survival results in the need for multiple foetal donors per transplant. Consequently, there is a need to find a way to improve the survival of grafted dopaminergic neurons to substantially decrease the amount of embryonic tissue needed per patient.

Biomaterial systems, such as *in situ* forming hydrogels, have the potential to improve the survival and engraftment of cells during and after the transplantation process. These biomaterials can be functionalised to act as a supportive and protective matrix for the cells, and to deliver therapeutic molecules to enhance their survival and development, while shielding them from the host immune response (Hoban *et al*., 2013; Moriarty *et al*., 2019b).

The work described in this thesis sought to determine the impact on the survival, integration and efficacy of rat embryonic day 14 (E14) VM cells when transplanted in an injectable collagen hydrogel enriched with a growth factor, and to assess the

potential of this biomaterial to improve cell transplantation therapy in Parkinson's disease.

This introductory chapter will provide an evidence-based overview of cell-replacement therapy in Parkinson's disease, the issues that have impeded its clinical translation and how biomaterials in conjunction with therapeutic factors can potentially enhance cell survival in this therapy.

1.1 CELL REPLACEMENT THERAPY

The concept of transplanting cells into the brain is not new, the very first experiments took place as long ago as 1890 (Thompson, 1890), but dopaminergic cell replacement therapy emerged over 40 years ago as one of the most promising treatments for Parkinson´s disease (Perlow *et al*., 1979). This approach targets the unmet clinical needs of the current pharmacological and surgical therapies: to repair the brain and restore function. The transplantation of dopaminergic neurons to the nigrostriatal pathway potentially could restore dopaminergic transmission in the striatum, therefore, ameliorating the motor deficits suffered by the patients and consequently improving their quality of life.

1.1.1 HISTORICAL PERSPECTIVE OF CELL TRANSPLANTATION IN PARKINSON´S DISEASE

1.1.1.1 Pre-clinical studies

Research into cell therapy for Parkinson´s disease began in the 1970s with the transplantation of rat foetal VM tissue, which contain abundant dopaminergic cells, into the denervated lateral cerebral ventricle of an adult rat in which the dopaminergic system had been unilaterally destroyed by 6-hydroxydopamine (6-OHDA). In that study, Perlow and colleges demonstrated that transplantation of rat foetal VM tissue, implanted in proximity to the caudate survives, proliferates and can produce recovery of motor function in the 6-OHDA animal model of Parkinson´s disease, a model that had only been developed a few years previously (Perlow *et al*., 1979).

The 6-OHDA animal model of Parkinson´s disease was developed in Sweden by Ungerstedt and colleagues (Ungerstedt, 1968). This model permits selective degeneration of the dopaminergic cell bodies, axons and terminals in the vicinity of the site of injection. Animals unilaterally lesioned with 6-OHDA show quantifiable motor deficits characterized by turning behaviour both ipsilateral or contralateral to the side of the lesion induced by the systemic administration of dopaminergic drugs, like amphetamine or apomorphine, as well as postural curvature and contralateral sensory neglect (Ungerstedt and Arbuthnott, 1970). Since its development, this model has been extremely valuable for investigating the restoration of dopaminergic tone in the lesioned

nigrostriatal pathway by cell transplantation, although this model does not fully recapitulate Parkinson´s disease.

Then in 1979, Björklund and Stenevi transplanted rat foetal VM tissue into a cavity in the anterior parietal cortex and the corpus callosum of rats unilaterally lesioned with 6-OHDA (Björklund and Stenevi, 1979). This study showed that the transplants were able to establish new dopaminergic input to the denervated neostriatum, and this reinnervation was able to compensate for some of the lesion-induced motor disturbances.

Even though these early studies demonstrated that the cells of the implanted foetal VM tissue were able to survive several months after transplantation, to extend projections into the host striatum and to reduce the number of rotations induced by amphetamine or apomorphine, the results were variable and limited. This method of transplantation did not allow grafting to all sites in the brain and many areas were restricted by the need of axons from the transplants to grow over relatively long distances (Björklund and Stenevi, 1979; Perlow *et al*., 1979; Dunnett *et al*., 1981). These limitations led to the optimization of a different technique for transplantation, which would dissociate the cells from the tissue into suspension (Björklund *et al*., 1980).

Transplantation of cell in suspension has four major advantages over transplants of whole tissue: (1) Dissociated cells can be implanted at defined and selected sites in the brain; (2) cell suspensions can be used to reinnervate large regions of the brain (in this case the striatum) by implanting cells in multiple deposits; (3) transplantation of cell suspensions causes less damage to the host´s brain; (4) the composition of the cell suspension can be altered to mix cells of different types or to incorporate factors to promote the growth and development of the cells (Björklund *et al*., 1980).

Another important discovery was made in 1980, when Björklund and colleagues, and in parallel Freed and colleagues, demonstrated that the behavioural recovery brought about by the transplantation of cells was the result of dopamine released from neurons in the grafts, the fibre ingrowth from these cells and the consequent changes in concentrations of dopamine in the caudate nucleus (Björklund *et al*., 1980; Freed *et al*., 1980).

In 1983, it was determined by Schmidt and colleagues that dopamine tissue-content in the striatum correlated with the reduction of rotating behaviour induced by amphetamine and that restoration of at least 3% of normal dopamine levels in the

striatum was sufficient to reduce the motor asymmetry (Schmidt *et al*., 1983). The same year, Freed and colleagues found that, in conjunction with the decrease in motor asymmetry by the transplantation of foetal VM, grafts caused post-synaptic dopaminergic binding sites to return to normal density. Therefore, the transplanted grafts produce long-term recovery of the lesioned brain responsible for the behavioural deficits (Freed *et al*., 1983).

In 1986, Zetterström *et al*, demonstrated by *in vivo* dialysis assays, that foetal mesencephalic transplants release dopamine spontaneously and the release is enhanced after administration of amphetamine (Zetterström *et al*., 1986). The following year, the same team found that dopamine release was higher in animals with more surviving foetal VM grafted cells and more fibre ingrowth, reaching about 85% of normal dopamine levels under basal conditions (Strecker *et al*., 1987).

In parallel, cell transplantation started to be tested in non-human primate models of Parkinson´s disease. In 1985 and 1986, a Parkinson-like syndrome was induced in nonhuman primates by the chronic administration of N-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP). MPTP is a selective neurotoxin that destroys dopaminergic cells in the nigrostriatal pathway resulting in movement deficits like rigidity and bradykinesia which are characteristic of Parkinson´s disease (Burns *et al*., 1983). Afterwards, these non-human primates received bilateral transplantation of foetal VM cells stereotactically implanted into multiple sites of the caudate. The results in these studies demonstrated a significant improvement of the Parkinson-like motor deficits indicating the successful integration of foetal cells in the caudate (Bakay *et al*., 1985; Redmond *et al*., 1986).

Soon after the findings in the animal models, another crucial step towards clinical application was taken. Cross-species transplants of mesencephalic tissue were done into the striatum of rats previously subjected to a 6-OHDA-lesion (Brundin *et al*., 1986). Brundin and colleagues found that transplantation of human foetal VM xenografts were successful when the human tissue was obtained from 6.5 to 8 week old foetuses and if the host was immunosuppressed with cyclosporine A (Brundin *et al*., 1985b); suggesting that the immunosuppressed rat can provide a good model to explore the utility of human foetal VM as a source for dopaminergic neurons in clinical grafting (Brundin *et al*., 1986). Additionally, the human grafts showed successful survival and maturation of the cells, extensive reinnervation of the denervated caudate-putamen and

improvement of the motor deficits caused by the 6-OHDA-lesion. This indicated that human foetal VM tissue may be an efficient source of dopamine neurons for functional intracerebral grafting in patients with Parkinson´s disease (Brundin *et al*., 1985b, 1986, 1988; Clarke *et al*., 1988; Strömberg *et al*., 1992, 2001).

After all these important findings, the preclinical field of cell replacement therapy in Parkinson´s disease rapidly developed. Several studies have validated the efficacy of foetal VM grafts to restore dopamine transmission and improve motor and sensorimotor performance in the 6-OHDA rat model of Parkinson´s disease (Björklund *et al.*, 1987; Mandel *et al*., 1990; Nikkhah *et al*., 1993; Olsson *et al*., 1995; Dowd and Dunnett, 2004; Dowd *et al*., 2005) and in the non-human primate models of the disease (Sladek *et al*., 1987; Taylor *et al*., 1991; Annett *et al*., 1994, 1997). For a timeline of foetal VM transplantation studies on animal models of Parkinson´s disease see **Figure 1.1**.

The work from the pre-clinical *in vivo* studies shed light on the efficacy of cell replacement to restore dopaminergic transmission in Parkinson´s disease. This efficacy is dependent on the survival and axonal outgrowth of the transplanted cells with appropriate integration and maturation of the dopaminergic cells in the host brain (Brundin and Björklund, 1987; Annett *et al*., 1994; Torres *et al*., 2008). Further evidence has shown that the grafted dopaminergic neurons are capable of the synthesis, release and uptake of dopamine, which maintains a sustained extracellular dopamine concentration improving the behavioural deficits of the host (Rose *et al*., 1985; Zetterström *et al*., 1986; Strecker *et al*., 1987).

As a whole, all the findings in the animal models demonstrated the potential of dopamine cell transplantation as a restorative approach for Parkinson's disease and were fundamental to the evolution of the field towards clinical trials.

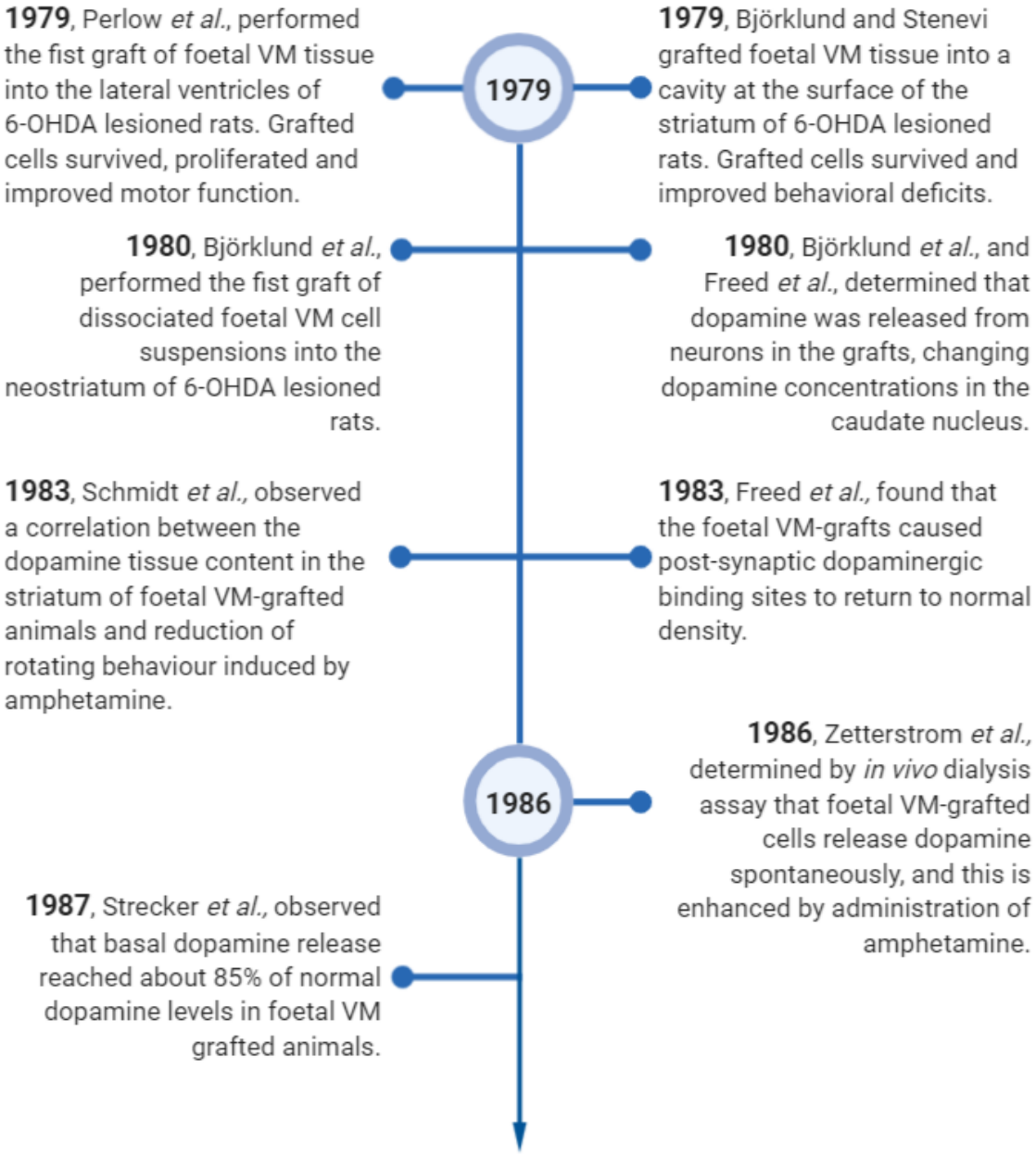


Figure 1.1 Timeline of first studies of foetal VM cell transplantation in animal models of Parkinson´s disease.

This timeline depicts a few of the pioneering studies of foetal VM cell transplantation in animal models of Parkinson´s disease.

1.1.1.2 Early clinical trials

During the 1970s, in the field of cell replacement therapy there were preclinical studies where the cell source was obtained from adrenal medullary tissue (catecholamine producing cells), but the results of those studies showed that this cell source produced grafts with low cell survival, minimal fibre outgrowth, low dopamine release and minimal recovery of functional deficits (Freed *et al*., 1981, 1983; Nishino *et al*., 1988). However, even after the poor results and very limited preclinical data, the first clinical trial using cell replacement for treatment of Parkinson´s disease was performed using adrenal medullary tissue. This clinical trial was done in Lund, Sweden in 1982 where two patients were grafted with adrenal medullary tissue placed in the caudate. This study was followed a few years later by grafting of adrenal medullary tissue in two further patients. Unfortunately the transplants did not have major clinical benefits for the patients and any benefit only lasted a few months (Backlund *et al*., 1985).

Then 1987, Madrazo and colleagues, published the results of their clinical study where solid grafts of adrenal medullary tissue were placed into the head of the caudate of two patients with Parkinson´s. This study showed major recovery of the motor deficits of the patients by this treatment (Madrazo *et al*., 1987). After the publication of this study, a large number of patients were grafted with adrenal medullary tissue, particularly in the United States of America, but the patients did not benefit to a significant extent, there was poor graft survival and some patients suffered from postoperative psychiatric disturbances. Later on, real concerns about its efficacy and safety began to emerge, and eventually this led to the abandonment of this cell replacement approach (Goetz *et al*., 1989, 1990; Jankovic *et al*., 1989; Kelly *et al*., 1989; Cahill and Olanow, 1990).

1.1.1.3 Open-label clinical trials

The positive findings in the preclinical studies using human foetal VM as the cell source for cell transplantation led the way to the first open-label clinical trials to use it as a restorative treatment for Parkinson´s disease.

In 1987, the first open-label clinical trial took place in Lund, in which 2 patients with advanced Parkinson´s disease received a unilateral transplantation of foetal VM cells from human foetuses of 8 to 10 weeks gestational age and were treated with cyclosporine to avoid graft rejection. However, no major therapeutic improvement was found (Lindvall *et al*., 1989). After this trial, the transplantation protocol was modified,

and changes were made to the amount of tissue grafted, the age of foetal tissue harvested, and the method of delivery of the tissue in order to improve the outcome of the transplants. A couple of years later, another patient received a foetal VM transplant from four foetuses of 8 to 9 weeks gestational age in the anterior, middle and posterior part of the putamen. In this case, the patient had a gradual and marked improvement of motor function that was consistent with the slow development of a growing graft, and using 6-L-[^{18}F]fluorodopa ([^{18}F]dopa) positron emission tomographic (PET) scan, it was determined that the graft significantly increased the uptake of the tracer, meaning that there was a recovery in the functional integrity of the dopaminergic system of the patient (Lindvall *et al*., 1990).

After these studies, other open-label human foetal VM clinical transplants were performed over the 1990s. The results were variable, but overall the patients improved following transplantation (Sawle *et al*., 1992; Widner *et al*., 1992; Kordower *et al*., 1995; Brundin *et al*., 2000b). Some patients were able to stop taking their medication for Parkinson´s disease, and the [^{18}F]dopa scanning showed that the grafts restored dopamine signalling to normal in the striatum (Freed *et al*., 1992; Hagell *et al*., 1999; Piccini *et al*., 1999; Mendez *et al*., 2002; Cochen *et al*., 2003). Some of these patients continued to be monitored and the benefits of their grafts were still evident after 20 years (Hauser *et al*., 1999; Kefalopoulou *et al*., 2014). For a direct comparison and more information about some of these open-label trials, refer to **Table 1.2.**

The functional improvements seen in these open-label trials were questionable due to the placebo effect, which can be prominent in patients with Parkinson´s disease (Shetty *et al*., 1999; de la Fuente-Fernández and Stoessl, 2002). These questions led to the next generation of clinical trials for human foetal VM cell transplantation sponsored by the National Institutes of Health (NIH).

Table 1.2 Direct comparison of the functional outcome after intracerebral transplantation of foetal VM in open-label clinical trials of Parkinson's disease.

	Hauser *et al.* 1999	**Hagell *et al.* 1999**	**Brundin *et al.* 2000**	**Mendez *et al.* 2002**	**Cochen *et al.* 2003**
Patient Information					
Study Design	Open-label	Open-label	Open-label	Open-label	Open-label
Number of patients	6	5	5	3	6
Mean age of patients (years)	55.5	48	53	53	55.3
Mean disease duration (years)	18.2	11	12.6	11.7	13.5
Treatment					
Number of VM per putamen	3-4	4-8	2.2-3.6	3-4	1-3
Tissue handling	In hibernation medium (2 days)	Fresh	Fresh	In hibernation medium (6 days)	Fresh
Size of VM	Solid pieces	Suspension	Suspension	Suspension	Suspension
Number of tracts per side	6-8	5	5	4	3
Immunosuppression	C for 6 months	C, A, P continuous	C, A, P for 12-24 months	C for 6 months	C for 7 months, continuous A and P
Outcome	Increased fluorodopa uptake, improved motor function	3 patients improved motor function; 2 patients had side effects	Improved motor function	Increased fluorodopa uptake, improved motor function	Increased fluorodopa uptake, improved motor function

Immunosuppression: C: cyclosporine; A: azathioprine; P: prednisolone. Adapted and modified from Winkler *et al*,. (2005).

1.1.1.4 Double-blind trials

In 2001, Freed and colleagues published the results of the first randomized double-blind, controlled trial that included a sham surgery group to address whether the grafts were really efficacious or simply that a placebo effect was improving the clinical outcome after human foetal VM transplantation (Freed *et al*., 2001). Additionally, patients and investigators were blinded to both treatment group and specific treatment interventions. In this trial, 40 patients were enrolled, and 19 of them received bilateral foetal VM transplants. The results showed that the younger patients in the transplantation group had a significant improvement in their motor performance compared to patients in the sham-surgery group, but older patients did not benefit from the transplantation. Overall, the transplant group did not meet the primary end point since there was no significant improvement in the unified PD rating scale (UPDRS) after 12 months. These results could not be directly compared to the results from the open-label trials, since several untested procedures were done for transplantation in this trial: only 1 foetus per transplant was dissected for foetal VM tissue, this tissue was cultured for up to 4 weeks before transplantation, and no immunosuppressive treatment was given to the patients. All of these factors could account for the failure to meet the primary endpoints in this double-blind trial.

The results of the second NIH sponsored randomized double-blind, sham-surgery-controlled trial were published by Olanow and colleagues in 2003. In this trial, a bilateral transplantation of solid pieces of foetal VM tissue, from 1 (n=11) or 4 donors (n=12), were implanted in the putamen of the patients and compared to the group of sham surgery (n=11). Patients received immunosuppressive treatment for 6 months after surgery and were followed for 2 years. The results of this trial showed that patients with less advanced disease did benefit from the transplantation, but no significant benefit was found when all 3 groups where compared. Additionally, it was found a trend towards improvement in patients who received transplants from 4 donors, but overall, the trial failed to reach significance of the primary endpoint, which was to reach a significant improvement in the UPDRS after 2 years (Olanow *et al*., 2003). For a direct comparison of these double-blind trials, refer to **Table 1.3.**

Several years after the transplantation surgeries of the double-blind trials, it was reported that 15% to 56.5% of the grafted patients started to develop adverse effects in the form of graft-induced dyskinesias (GIDs) (Ma *et al*., 2002; Herzog *et al*., 2008).

These GIDs were so severe that these patients necessitated further neurosurgery, such as DBS, to ameliorate them. Such problems had not previously been reported in the open-label studies, but further analysis revealed their presence in some patients (Hagell *et al*., 2002).

Despite the disappointing outcomes, the results from the two NIH-sponsored double-blind trials were carefully reanalysed in 2006 by all the main investigators involved in these trials, seeking to decide whether foetal VM cell transplantation had a future as a therapeutic approach for Parkinson´s disease. Some important issues were highlighted: 1) Transplantation of dopaminergic neurons remains a viable and highly promising approach; 2) several aspects of the transplantation procedure, tissue handling, and storage, immunosuppressive treatment, and patient selection clearly needed to be improved and standardized; 3) with further improvement and refinement of the grafting procedure, there is every reason to believe that cell transplantation could be developed into a safe and efficacious restorative therapy for advanced, although not too seriously affected, Parkinson´s disease patients (Piccini *et al*., 2005; Barker *et al*., 2013).

As a consequence, the TRANSEURO project was developed. In this new trial, all the previous issues were addressed with the hope of improving the outcome of the human foetal VM transplants in patients of Parkinson’s disease.

Table 1.3 Direct comparison of the functional outcome after intracerebral transplantation of foetal VM in double-blind clinical trials in Parkinson's disease

	Freed *et al.* 2001	**Olanow *et al.* 2003**
Patient Information		
Study Design	Double-blind	Double-blind
Number of patients	20 sham 19 transplants	11 sham 11 transplants from 1 donor 12 transplants from 4 donors
Mean age of patients (years)	56.9	57.5 60
Mean disease duration (years)	13.4	11.6 8.2
Treatment		
Number of VM per putamen	1	1 4
Tissue handling	In culture for 4 weeks	In hibernation medium for 2 days
Size of VM	Tissue strands	Solid pieces
Number of tracts per side	2	8
Immunosuppression	None	Cyclosporine for 6 months
Outcome	Only younger patients had significant motor improvement. Trial failed	Patients with less advanced disease showed improvement. Trial failed 56% of patients developed GIDs

Adapted and modified from Winkler *et al.*, (2005).

1.1.1.5 TRANSEURO

In 2009, the European Union funded a small open-label multicentre trial called TRANSEURO, led by Prof. Roger Barker. The objective of this new study, using human foetal VM transplants, was to adopt a systematic and rigorous approach, with a series of well-defined criteria for patient selection, tissue dissection, preparation, grafting and immunosuppression, and trial design (Petit *et al*., 2014; Barker *et al*., 2019).

The TRANSEURO study had two major arms: first, an observational study, registering the natural history of younger onset and early stage of Parkinson´s disease with an n=150 patients; and second, a transplant arm, that included patients randomly selected from the observational cohort. The observational study is still ongoing, and a significant proportion of the cohort has been followed up to the present day.

The selection criteria of patients, to form part of the transplant arm, was patients with no cognitive impairment, who are younger than 65 years old, have less than 10 years of disease duration and do not show significant LIDs. The transplant arm has now been completed, and 11 patients received a graft over a 3-year period from 2015-2018. Originally 20 patients were going to receive transplants but due to problems with the tissue supply it was reduced to 11 patients (Barker *et al*., 2019).

Patients selected for transplantation had two unilateral stereotactic surgeries, with 5 tracts per hemisphere. The foetal VM cell suspension (tissue was stored up to a maximum of 4 days before transplant) was implanted into 2 tracts in the pre-commissural putamen and 3 tracts in the post-commissural putamen. Immunosuppressive therapy (cyclosporine, azathioprine and prednisolone) was administered for 12 months post-transplantation (Barker *et al*., 2019).

The outcome of these human foetal VM transplants will be evaluated in 2021 using the predefined primary endpoint of the trial, which is change in the UPDRS scores for the patients in the defined ‘OFF’ medication state at 36 months following the second transplant compared to their baseline pretransplant scores (Barker *et al*., 2019).

1.1.2 FACTORS AFFECTING AND CHALLENGES FACING BRAIN REPAIR

The results from the clinical trials described in the previous sections, have demonstrated that ventral midbrain dopaminergic neurons can be safely transplanted into the striatum of Parkinson´s disease patients, and for some of them, the graft enabled them to discontinue their medication. However, variations between clinical studies have highlighted a number of factors and challenges that affect graft efficacy and the generation of GIDs.

1.1.2.1.1 Neuronal subtype

Foetal ventral mesencephalon cells have been shown to give rise to different classes of dopaminergic neurons which integrate and differentiate into the host brain alleviating Parkinsonian symptoms following transplantation.

In 1964, Dahlstöm and Fuxe identified 12 groups of catecholamine containing neurons in the rat brain that were designated A1-A12 (Dahlstom and Fuxe, 1964). This nomenclature has been retained, since it helps to compare cell groups in different animal species. The developing mammalian midbrain contains 3 major dopaminergic neuron subtypes: A8 neurons of the retro-rubral (RRF) field, nigral A9 neurons of the SN pars compacta (SNpc) and the A10 neurons of the ventral tegmental area (VTA) (Dahlstom and Fuxe, 1964; Björklund and Dunnett, 2007).

The A8 neurons innervate limbic and striatal structures and provide local innervation to both A9 and A10 neurons. The A9 neurons are large and angular in shape and form the nigrostriatal pathway in rodents with their ascending axonal projections to the dorsal striatum or to the caudate nucleus and putamen in primates. Most of these neurons co-express the G protein-gated inwardly rectifying potassium channel 2 (Girk2). The A10 neurons are smaller and round in shape and extend their axons primarily to the ventral striatum also known as the nucleus accumbens and cortical areas, but also to the olfactory tubercle, septum and amygdala. Most of these neurons co-express the calcium binding protein Calbindin (Thompson *et al*., 2005; Reyes *et al*., 2012). See **Figure 1.2** for a diagram of the distribution of A8-A10 neurons in the developing and adult rodent brain.

In Parkinson´s disease, the A9 neurons are the most vulnerable to degeneration and contribute to the various motor impairments, while adjacent A8 and A10 dopaminergic

neurons are the last ones to be affected (Damier *et al*., 1999; Mendez *et al*., 2005; Bye *et al*., 2012).

The A9 and A10 subpopulations of dopaminergic cells are present in foetal VM grafts in the dorsal striatum but these grafts mostly contain A9-like neurons. Quantification of Girk2 and Calbindin demonstrated that VM grafts are composed of 60-70% A9 neurons and 30-40% A10 neurons (Bye *et al*., 2012). Therefore, recovery of motor function and correction of dyskinesias by dopamine graft transplantation is mainly due to the presence of A9-like neurons (Bye *et al*., 2012; Somaa *et al*., 2015). Furthermore, several studies have shown that after transplantation, the A9 and A10 neurons retain their distinctive morphological features and markers for Girk2 and Calbindin, which can be used to identify the position of these cell groups within the foetal VM graft (Mendez *et al*., 2005; Thompson *et al*., 2005; Kuan *et al*., 2007; Grealish *et al*., 2010; Fjodorova *et al*., 2017).

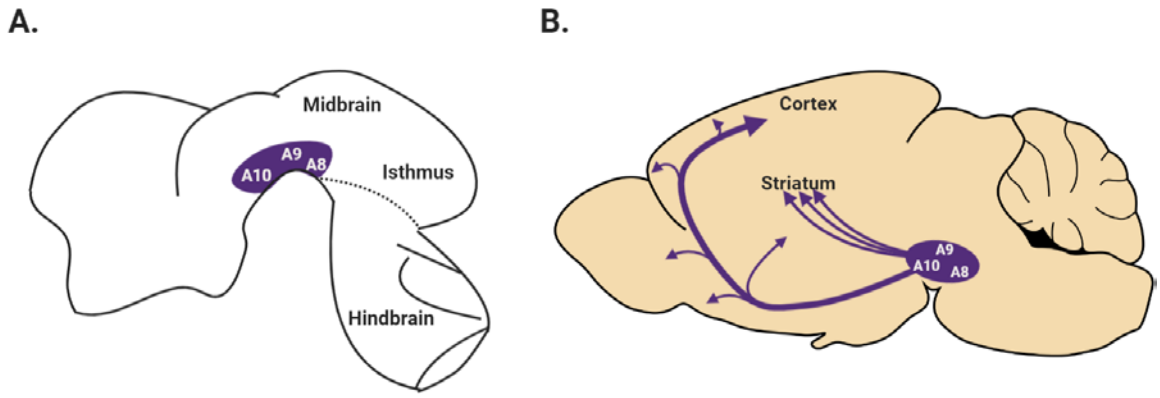


Figure 1.2 Distribution of the A8-A10 dopaminergic cell groups in the developing and adult rat brain.

Diagram of the dopamine neuron groups in a sagittal view from the developing (A) and adult (B) rat brain. The principal projections of the A8-A10 cell groups are depicted by arrows. Adapted and modified from Björklund and Dunnett (2007).

1.1.2.1.2 Embryonic donor age

Another factor that considerably affects the outcome of VM transplantation is the age of the embryonic donor since viability of the dopaminergic cells in suspension declines with age of the donor foetuses. The developing dopaminergic neurons survive the

dissociation process best when they are in the proliferative and migratory stages, and survival declines as the cells start differentiation and grow out axonal processes (Brundin *et al*., 1985a).

Currently, the gold standard for rat transplantation studies is to isolate VM tissue at the peak of neurogenesis from E14 (the equivalent to human embryos is 44 to 47 days (6.5 weeks) post-conception). Recent studies have shown that grafting E12 VMs produce larger grafts with enhanced dopamine neuron yield and greater proportion of A9 neurons, presumably due to an increase in mitotic cells at the time of transplantation than E14 VMs (Torres *et al*., 2008; Bye *et al*., 2012; Fjodorova *et al*., 2017). A study by Torres and colleagues found that the survival rate of dopamine neurons after transplantation was over 35% when the donor tissue was E12 compared to the 12% survival rate of the transplanted E14 (Torres *et al*., 2008).

During development, A9 neurons precede the birth of A10 neurons, thereby the use of younger embryonic donor tissue generates grafts which are composed of more than 75% of A9 neurons (Joksimovic *et al*., 2009; Blaess *et al*., 2011; Bye *et al*., 2012; Somaa *et al*., 2015). These grafts displayed increased plasticity, innervating a greater volume of the striatum and significantly increasing dopamine release. Furthermore, a recent study has demonstrated that A9 neurons from younger embryonic donor tissue are more responsive to environmental cues at the transplantation site when adopting a dopaminergic phenotype during differentiation post-grafting (Fjodorova *et al*., 2017).

Another factor that improves the survival of A9 neurons in grafts is the presence of young meningeal cells overlying the VM (Bye *et al*., 2012; Somaa *et al*., 2015). Somaa and colleagues found that the young meninges secrete a variety of trophins that promote neural survival and development, thus increasing graft reinnervation (Somaa *et al*., 2015).

1.1.2.1.3 Graft placement

The efficacy of foetal VM transplantation is also affected by the placement of the graft in the host brain since the host environment surrounding the transplanted graft has a great influence on the yield and type of surviving cells, degree of reinnervation, axonal outgrowth and restoration of the functional capacity (Thompson *et al*., 2005; Fjodorova *et al*., 2017).

In an ideal scenario, dopamine neuron grafts should be implanted into the SN to allow reconstruction along the entire nigrostriatal dopaminergic pathway. However, experiments where grafts have been placed intranigrally have shown very limited growth of axons along the nigrostriatal pathway towards the striatum and limited behavioural recovery (Schmidt *et al*., 1983; Olsson *et al*., 1995; Mendez *et al*., 2002). The limited results could be due to the restrictive host environment in the adult brain.

Previous studies have investigated this neuron-target interaction and it was shown that when foetal VM grafts were transplanted in the vicinity of the dopamine-depleted dorsal striatum (into the adjacent cortex or ventricle) they exhibited a target specific dopaminergic fibre outgrowth, capable of providing an extensive functional reinnervation (Strömberg *et al*., 1992; Nikkhah *et al*., 1993, 1994; Piccini *et al*., 1999; Mendez *et al*., 2005). Grafts transplanted into the dorsal striatum, which is an A9 input nucleus, are enriched for dopamine neurons that express Girk2, suggesting that the host striatum influences the development and survival of A9-like neurons. These A9-like neurons are more responsive to environmental cues in adopting a dopaminergic phenotype during differentiation post-grafting (Fjodorova *et al*., 2017).

As a whole, all these findings have demonstrated the potential of dopamine cell transplantation as a restorative approach for Parkinson's disease. Despite these encouraging results, the major limitations of this treatment appear to be the tissue supply, high variability of the outcomes and for some patients, the development of GID.

1.1.2.2 Tissue source

Due to the ethical concerns related to the use of aborted foetuses and their limited availability, the use of foetal VM tissue as the source of dopaminergic cells is one of the major problems limiting cell replacement therapy.

As described in previous sections, in order to achieve significant clinical improvement, patients with Parkinson´s disease require VM tissue from four to eight aborted embryos (within a narrow window of gestation time) per hemisphere (Lindvall *et al*., 1990; Hagell and Brundin, 2001). Previous preclinical and clinical studies have shown that the survival rate of grafted cells is around 3 to 20%, of which only 5 to 10% have dopaminergic phenotype (A9-like neurons) (Brundin and Björklund, 1987; Sauer and Brundin, 1991; Kordower *et al*., 1996; Hauser *et al*., 1999; Freed *et al*., 2001).

Therefore, in order to reduce the number of foetuses needed per transplant, the yield of surviving dopaminergic neurons needs to be significantly improved.

1.1.2.2.1 Alternative cell sources for transplantation

Cell replacement therapy in Parkinson´s disease is based on the principle that dopaminergic neurotransmission can be restored by the transplantation of dopamine neurons into the dopamine depleted striatum, providing a functionally efficient substitute for neurons that are lost in this disease. The preclinical and clinical studies have shown that transplantation of foetal VMs are a good substitute of the lost dopaminergic cells in Parkinson´s disease, that they are able to bring clinical benefits, and that, for some patients, it lets them discontinue their medication. Despite these encouraging results, the use of aborted foetuses presents several ethical and logistical issues that impede the effective translation of foetal VM transplantation as a routine therapeutic option. Therefore, alternative cell sources such as stem cells, that are readily available, renewable and that can mimic mesencephalic dopaminergic neurons of the A9 phenotype are needed.

Stem cells are undifferentiated unspecialized cells with an ability to self-renew over long periods and which can give rise to various highly specialized fully functional and mature cell types (Behari and Singhal, 2011). The use of stem cell-derived dopaminergic neurons might be the future of cell replacement therapy in Parkinson´s disease. However, before these cells can be considered for clinical use, it is necessary to verify that their functional efficacy is robust, reproducible, stable, that the cells are able to grow and develop axons to reinnervate the denervated striatum and that they can bring functional recovery like or even better than the foetal VMs. Additionally, no uncontrolled cell proliferation should occur to avoid the formation of tumours. Importantly, these cells must mimic A9-like neurons that express appropriate markers such as tyrosine hydroxylase (TH), dopamine transporter (DAT) and GIRK2 and produce sufficient dopamine (Lindvall *et al*., 2012; Barker *et al*., 2015).

Currently, there are 2 main sources of cells that show the greatest potential for grafting in Parkinson´s disease: dopaminergic neurons derived from human embryonic stem cells (hESCs) or from human induced pluripotent stem cells (iPSCs) (Petit *et al*., 2014).

1.1.2.2.1.1 Human embryonic stem cells

Human ESCs were first isolated in 1998 by Thompson and colleagues. ESCs are pluripotent stem cells with a normal karyotype, derived from the inner mass of early-stage preimplantation embryos that can form derivatives of all 3 embryonic germ layers (endoderm, mesoderm and ectoderm) even after prolonged culture in an undifferentiated state and that theoretically, can provide an unlimited supply of cells of any cell type in the body after differentiation (Thomson *et al*., 1998). Undifferentiated hESCs do not undergo senescence (*in vitro* aging) and can remain nontransformed over multiple passages (Zeng, 2007).

Several protocols have been developed to differentiate these hESCs cells into midbrain dopaminergic neurons *in vitro* by mimicking embryonic development in a dish and activating transcription factor pathways important for VM dopaminergic neuron derivation via dual inhibition of SMAD signalling (Chambers *et al*., 2009; Kriks *et al*., 2011; Kirkeby *et al*., 2012). These cell preparations can also contain non-dopaminergic cell populations or proliferating non-neural cells, which can potentially form tumours after transplantation (Sundberg *et al*., 2013; Barker *et al*., 2015). Therefore, to solve this problem, several sorting methods have been developed for enrichment of dopaminergic cell populations using fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS) (Yuan *et al*., 2011).

As a lot of progress has been made in the generation and purification of dopaminergic neurons from hESCs, such stem cell treatments are expected to reach clinical trials soon (Kirkeby *et al*., 2017).

1.1.2.2.1.2 Human induced pluripotent stem cells

iPSCs are pluripotent stem cells, which are a product of somatic cells (such as skin fibroblasts) reprogramming to an embryonic-like state. This cell source provides an unlimited supply of cells which avoids many of the ethical issues associated with the use of foetal VMs and hESCs (Barker *et al*., 2015). Autologous cell transplantation using iPSCs is especially advantageous because it could avoid possible immune reactions against the graft (since its source is the patient´s own cells) and reduce the risks associated with the use of immunosuppressants (Morizane *et al*., 2013). iPSCs might be an ideal source of cells for cell replacement therapy in countries where the use of embryonic cells is restricted, such as Ireland.

In 2006, Takahashi and Yamanaka turned mouse fibroblasts into pluripotent cells by retroviral-mediated introduction of 4 transcription factors (octamer-binding transcription factor-3/4 (OCT3/4), SRY-related high-mobility-group (HMG)-box protein-2 (SOX2), MYC and Kruppel-like factor-4 (KLF4)) (Takahashi and Yamanaka, 2006). Other studies have shown that by substituting SRY and MYC for other combinations of transcription factors, iPSCs can be produced (Yu *et al*., 2007; Park *et al*., 2008).

The potential of iPSCs to be reprogrammed into dopaminergic neurons of midbrain character was shown by Wernig *et al*., in 2008. These cells were first deprogrammed by retroviral transduction, using OCT4, SOX2, KLF4 and c-Myc transcription factors and then converted into neurons. Subsequently, these cells were tested in a rat model of Parkinson´s disease and the results showed an improvement in behavioural deficits (Wernig *et al*., 2008). As with ESCs, iPSCs also pose the risk of tumorigenesis after transplantation, but this risk can be minimised by separating contaminating pluripotent cells and committed neural cells using FACS, thus leaving only differentiated cells for transplantation (Wernig *et al*., 2008).

In 2017, Kikuchi and colleagues showed that Parkinson´s disease-specific iPSCs (iPSCs from idiopathic PD patients) are not significantly different, or more vulnerable, compared to dopaminergic neurons generated from healthy individuals. Furthermore, upon transplantation into the brain of α-synuclein transgenic mice, these Parkinson´s disease-specific iPSCs did not show an accumulation of pathological α-synuclein. These results suggest that Parkinson´s disease-specific iPSCs are a good source of donor cells for cell transplantation treatment in this disease (Kikuchi *et al*., 2017b). Another crucial finding in the iPSCs field was reported the same year, when Takahashi and colleagues reported the safe and successful transplantation of human-derived iPSC-derived dopaminergic neurons in a primate model of Parkinson´s disease (Kikuchi *et al*., 2017a).

The success of the use of these iPSCs for cell replacement therapy in animal models of Parkinson´s disease led to the launch of the first clinical trial in Japan. The primary endpoints of this ongoing clinical trial, are related to safety (tumorigenicity) and the presence or absence of graft expansion in the brain 24 months after transplantation (Takahashi and Price-Evans, 2019).

Dopaminergic neurons from both ESCs and iPSCs show extraordinary potential as cell sources for cell replacement therapy in Parkinson's disease, but further research needs to be done to ensure that the potential tumorigenesis or the genetic manipulation used on these cells do not hinder their clinical use.

Until the safety and efficacy of the stem cells is confirmed by research, foetal cell replacement is still the main source of dopaminergic cells for cell transplantation therapy. However, a major factor hindering its efficacy is the low survival rate of the grafted dopamine neurons.

1.1.2.3 Low survival rate of grafted dopamine neurons

Preclinical and clinical studies on foetal intrastriatal grafts suggest that most of the dopaminergic neurons die within the first 4 days after transplantation and it is mainly caused by apoptosis (Mahalik *et al*., 1994; Zawada *et al*., 1998; Emgård *et al*., 1999; Sortwell *et al*., 2001). This apoptosis might be triggered by oxidative stress, hypoxia, withdrawal of trophic factors or by detachment from the extracellular matrix (ECM) and neighbouring cells (also known as anoikis) (Marchionini *et al*., 2003).

Four phases have been identified as various points where dopamine neurons may die; two phases during the pre-transplantation process and two post-transplantation.

1.1.2.3.1 Cell death pre-transplantation

Phase one: Cell death begins immediately after the detachment of the donor embryo from the maternal blood supply which causes hypoxic and hypoglycaemic insult that later on produces oxidative damage (Mahalik *et al*., 1994; Marchionini *et al*., 2003).

During dissection, necrosis and apoptosis are caused by the mechanical trauma and axotomy. Also, the dissociation of the VM cells from the ECM and the preparation of cell suspension further damages cells before transplantation (Mahalik *et al*., 1994; Marchionini *et al*., 2003; Reddig and Juliano, 2005). This anoikis is a major trigger of apoptotic cell death since it removes the normal cell-matrix interactions causing damage to the cells (Frisch and Francis, 1994). Furthermore, to avoid additional cell death pre-transplantation, tissue must be stored in the appropriate medium and at the appropriate temperature, and ideally, should be transplanted within a short period of time after dissection (Freed *et al*., 2001).

Phase two: Damage to the cells is caused during transplantation, while they are passed through a cannula for implantation to the host brain. This extra cell loss is caused by sheer stress and mechanical trauma (Barker *et al*., 1996; Steiner *et al*., 2008).

1.1.2.3.2 Cell death post-transplantation

Previous studies have shown that about 80 to 90% of grafted dopaminergic neurons die through apoptosis following transplantation (Brundin *et al*., 1988; Barker *et al*., 1996; Kordower *et al*., 1996; Sortwell *et al*., 2001). This apoptosis is mainly driven by external factors in the cells´ environment rather than a mechanical insult (Mahalik *et al*., 1994; Brundin *et al*., 2000a; Sortwell *et al*., 2001).

Phase three: Right after transplantation, primary cells undergo trophic withdrawal, since they were removed from a neurotrophic-rich environment at the height of neurogenesis and placed into an environment deprived of the growth factors that normally should be present during their development. Therefore, due to the lack of appropriate neurotrophic support most embryonic neurons die, and the surviving ones slowly mature in the new host environment (Sortwell *et al*., 2001; Abeliovich and Hammond, 2007).

Phase four: During the first 3 days after graft transplantation into the new adult host environment, excessive extracellular glutamate and oxidative stress is produced, causing a reduction in the survival of grafted neurons. These detrimental factors are generated by the physical damage to the adult brain and nervous system and by the production of damaging pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6 and tumour necrosis factor (TNF)α, which cause inflammation (Joniec-Maciejak *et al*., 2014). Several studies have shown that the injection of exogenous cells into the brain evokes an elevated host immune response where the activation of microglia and lymphocyte infiltration hinder their survival and consequently have an effect on their clinical efficacy (Duan *et al*., 1995; Shinoda *et al*., 1995, 1996; Barker *et al*., 1996). The immune response may intensify over time and produce a continuous inflammatory response leading to more cell death (Shinoda *et al*., 1995). As seen in the clinical trial by Olanow and colleagues in 2003, deterioration of the motor recovery found a few months after transplantation, begins after the withdrawal of immunosuppression (Olanow *et al*., 2003). See **Figure 1.3** for a diagram of the phases of dopaminergic cell death during the transplantation process.

It is necessary to find new methods to enhance the survival and efficacy of the transplanted cells. The incorporation of biomaterials into the transplantation process, might be the key to improve cell replacement therapy. Biomaterials could be employed during the transplantation process to target and reduce dopaminergic cell death in any of the four phases previously described. By increasing the survival of the transplanted cells, the number of embryos needed per transplant would be reduced and it would considerably improve foetal cell replacement therapy as a restorative approach for Parkinson's disease.

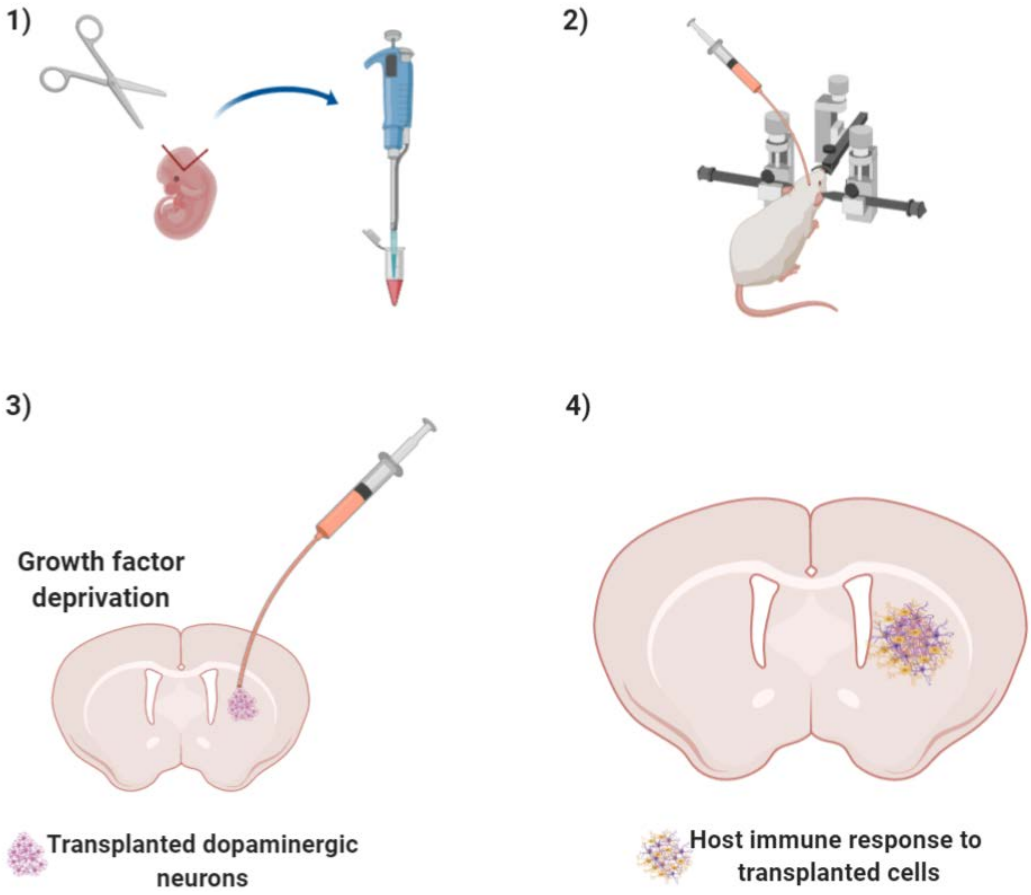


Figure 1.3 Phases of dopaminergic cell death during the transplantation process.

Dopaminergic cell death mainly occurs at four phases throughout the transplantation process: 1) detachment from the extracellular matrix during tissue dissection, followed by mechanical trauma during dissociation, 2) mechanical trauma and sheer stress during transplantation, 3) immediate growth factor deprivation upon transplantation into the adult striatum and 4) the attack of the host neuro-immune cells to the established exogenous graft.

1.1.3 BIOMATERIALS FOR BRAIN REPAIR

The aim of cell replacement therapy in Parkinson´s disease has been to restore dopaminergic transmission in the striatum by replacing the lost dopaminergic neurons and consequently, to ameliorate the motor deficits suffered by the patients. Although this treatment holds an extraordinary potential, its efficacy has been hindered mainly by the low yield of surviving dopaminergic neurons after transplantation. Therefore, it is necessary to find new methods to enhance the survival and efficacy of the transplanted cells. The incorporation of biomaterials into the transplantation process might be the key to improve cell replacement therapy. Biomaterials are materials that are used and adapted for a medical application, intended to interact with a biological system (Orive *et al*., 2009) .

As described in **Section 1.1.2.3,** several studies have shown that about 80 to 90% of grafted dopaminergic neurons die through apoptosis during the transplantation process (Brundin *et al*., 1988; Mahalik *et al*., 1994; Barker *et al*., 1996; Kordower *et al*., 1996; Sortwell *et al*., 2001). This apoptosis might be triggered by different factors such as oxidative stress, hypoxia, lack of trophic factors or by anoikis (Sortwell *et al*., 2001; Marchionini *et al*., 2003). Biomaterials have the potential to help at any of the four phases of dopaminergic cell death during transplantation.

As described earlier, in phase one cell death begins with the detachment of the donor embryo from the maternal blood supply, followed by mechanical damage caused during dissection and dissociation of the VM cells from the ECM to prepare the cell suspension (Mahalik *et al*., 1994; Marchionini *et al*., 2003; Reddig and Juliano, 2005). This anoikis is a major trigger of apoptotic cell death, since it removes the normal cell-matrix interactions, causing damage to the cells (Frisch and Francis, 1994). The addition of a biomaterial scaffold during this phase would provide the cells with a matrix for adhesion and support that should enhance their survival

In phase two, extra cell death is caused by mechanical trauma and sheer stress while they are passed through a cannula for implantation into the host brain (Barker *et al*., 1996; Steiner *et al*., 2008). Thus, a biomaterial could provide physical protection during this process.

In phase three, right after transplantation, cells undergo trophic withdrawal, since they are removed from a neurotrophic-rich environment and placed into a location deprived

of the growth factors needed for development (Collier and Sortwell, 1999; Abeliovich and Hammond, 2007). The incorporation of neurotrophic factors, encapsulated in a biomaterial matrix, holds the potential to further improve the survival and development of the transplanted dopaminergic cells, by proving them with localised and prolonged trophic support during and after transplantation.

In the final phase (first 3 days after transplantation), cells are exposed to excessive extracellular glutamate, oxidative stress and damaging pro-inflammatory cytokines caused by the physical damage to the host brain (Joniec-Maciejak *et al*., 2014). Additionally, the injection of exogenous cells into the brain evokes an elevated host immune response that hinders the survival and efficacy of cells (Duan *et al*., 1995; Shinoda *et al*., 1995, 1996; Barker *et al*., 1996). The encapsulation of cells within a biomaterial matrix can form a physical barrier between the transplanted cells and the hostile host environment, therefore protecting them from damage caused by the immune response.

Thus, the addition of biomaterials into the transplantation process could intervene beneficially at any of the phases of cell death described above, and since it would provide physical and trophic support for the graft, the efficacy of cell replacement therapy, as a restorative approach for Parkinson's disease, could increase.

1.1.4 PROPERTIES OF BIOMATERIALS FOR CELL REPLACEMENT THERAPY

Biomaterials have become more important in the process of developing novel or improved drug delivery systems to protect, repair or regenerate the brain (Zhong and Bellamkonda, 2008). The use of biomaterials shows great potential to improve cell replacement therapy by acting as a supportive and protective matrix for the cells, providing localised and sustained trophic factor delivery and creating a physical barrier between the transplanted cells and the host immune response, following intracerebral transplantation (Orive *et al*., 2009; Hoban *et al*., 2013; Moriarty and Dowd, 2018; Moriarty *et al*., 2019b).

A good biomaterial for intracerebral transplantation must have the following characteristics (Gutowska *et al*., 2001; Jain *et al*., 2006; Zhong and Bellamkonda, 2008; Orive *et al*., 2009):

a) Non-toxic for the host and/or for the transplanted cells. Besides the lack of local toxicity, the material must also be non-toxic systemically and should not generate toxic degradation products.

b) Chemically and mechanically stable. The microstructure and porosity of the material should be controllable while stable long enough to perform its desired biological function.

c) Biocompatible with low immunogenicity. The material must not elicit the host´s inflammatory response that could cause further neuronal death or rejection of the graft.

d) Adaptable. The material should be biomimetic to encourage the cells to survive and functionally integrate into the host tissue.

e) Provide controllable and sustainable release of therapeutic molecules such as neurotrophic factors.

f) Biodegradable or bioresorbable. The material should be structurally stable to allow the delivery of cells while shielding them from the host immune response (first few days after transplantation), but the material should completely degrade over time.

g) Processable. The biomaterial can be shaped *in situ* by direct injection or it can be formed into a variety of shapes such as tubes, sheets, meshes, sponges, etc., as required for different applications.

1.1.5 TYPES OF BIOMATERIALS

In 1999, biomaterials were defined as materials intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body (Williams, 1999). This term can be applied to a diverse set of natural and synthetic materials with a wide range of physical and chemical properties.

Natural biomaterials are obtained and purified from biological sources such as polysaccharides (alginate, methylcellulose) or proteins (collagen, fibrin). The advantages of using natural sources derive from their similarity to biological macromolecules that can be easily metabolized. Additionally, this similarity with the

extracellular matrix helps to avoid the host immune response (Mano *et al*., 2007; Heino and Käpylä, 2009; Kim *et al*., 2012).

Synthetic biomaterials are produced chemically. Compared to natural biomaterials, synthetic sources usually allow for greater product consistency and adjustable properties, such as polyesters poly (glycolide), poly(lactide), and poly(lactic-co-glycolic acid) (PLGA). These synthetic biomaterials have many clinical uses such as absorbable sutures, orthopaedic fixation devices or drug delivery vehicles (Lü *et al*., 2009; Madhavan Nampoothiri *et al*., 2010).

The use of biomaterials for brain repair must take into consideration the characteristics of the particular material to be used such as hydrophilicity, cell-adhesion, degradability, shape, porosity, and mechanical strength in order to ensure its safety and the feasibility of it to serve its purpose (Kim *et al*., 2012). Biomaterial systems, such as preformed scaffolds or *in situ* forming hydrogels, show potential to improve the grafting procedure by retaining the injected cells at the tissue site of interest, providing a microenvironment that supports cell viability and function while acting as a physical barrier between the cells and the inflammatory environment, thus improving the viability of the cells following intracerebral transplantation (Hoban *et al*., 2013; Burdick *et al*., 2016).

Hydrogels are a class of biomaterials that are composed of water-swollen polymeric networks, from natural or synthetic sources (Tsang and Bhatia, 2004). These materials are highly hydrated, with tissue-like mechanical properties that make them good scaffolds for implantation in soft tissue (Burdick *et al*., 2016). The physical properties of the hydrogels can be altered by charge interaction or chemical crosslinking (using poly(ethylene glycol) (PEG)) and can be designed to gelate *in situ* and deliver a range of therapeutic agents such as growth factors or anti-inflammatory cytokines, and/or cells (Lee *et al*., 2016). When designing the appropriate hydrogel for cell replacement therapy, it is essential that the level of gelation (strength of *in situ* formation) and porosity is compatible with cells, that the amount of crosslinker used is not toxic, that it can be injectable through minimally invasive techniques via syringes or catheters, and that it completely degrades over time (Drury and Mooney, 2003; Newland *et al*., 2013).

1.1.6 INJECTABLE COLLAGEN HYDROGELS

Collagen is the most abundant protein in the body, as it is a major component of all mammalian tissues, including skin, bone, cartilage, tendons and ligaments. More than

20 genetically different forms have been identified of which collagen type 1 is the most abundant and extensively studied for biomedical applications (Hayashi, 1994). *In vitro* studies have demonstrated that collagen promotes neural cell attachment and neurite outgrowth (Carbonetto *et al*., 1983; Zhong *et al*., 2001).

Collagen gels seem to be promising matrices for drug delivery and cell replacement therapy due to characteristics such as high mechanical strength, good biocompatibility, low antigenicity and ability of crosslinking that enable the tailoring of the mechanical, degradation and water uptake properties (Wallace and Rosenblatt, 2003). Collagen can form hydrogels *in situ* under physiological conditions (through warmth, neutral pH and the presence of salt) by the change in structure of the elongated triple helices, to that of compact coils (Xu and Kopeček, 2007). Growth factors and other active agents can be combined with collagen hydrogels to prolong their release rate and increase their therapeutic effect (Wallace and Rosenblatt, 2003).

Collagen has been coupled with the synthetic polymer PEG. PEG is a non-toxic, non-immunogenic, water-soluble hydrophilic polymer. This polymer is already approved by the Food and Drug Administration (FDA) for a number of different clinical uses (Drury and Mooney, 2003; Lee *et al*., 2016). When PEG is combined with collagen, the enzymatic degradation of the hydrogel slows down, prolonging the therapeutic effect (Dikovsky *et al*., 2006; Weber *et al*., 2009). Drug delivery in collagen hydrogels crosslinked with PEG have an increased retention time in the body, reduction of immunogenicity and increased stability of the therapeutic peptide or protein drug (Doillon *et al*., 1994; Veronese and Pasut, 2005).

Therefore, the use of PEG-crosslinked collagen hydrogels for cell replacement therapy in Parkinson´s disease has the potential to improve the survival and efficacy of the transplanted dopaminergic cells. In 2013, Hoban and colleagues showed that the delivery of glial-derived neurotrophic factor (GDNF) overexpressing mesenchymal cells (MSCs) encapsulated in a collagen hydrogel into the brain of 6-OHDA-lesioned rats reduced the host immune response to the transplanted cells (Hoban *et al*., 2013). In 2017 and 2019, Moriarty and colleagues showed that the transplantation of VM cells into the denervated striatum of 6-OHDA-lesioned rats, encapsulated in GDNF-loaded collagen hydrogels resulted in a significant increase in cell survival and reinnervation of the striatum. Additionally there was a significant restoration of motor function in the rats of this animal model of Parkinson's disease (Moriarty *et al*., 2017, 2019a).

Materials such as collagen, with the ability to form hydrogels *in situ*, offer an injectable biomaterial platform to aid cell transplantation. As cell survival post-transplantation is limited, the goal of using collagen hydrogels to deliver cells to the brain is to 1) improve the cell engraftment by providing an adherent substrate, 2) to deliver and prolong the release of therapeutic factors and 3) to provide a physical barrier to protect the transplanted cells against the host immune response. See **Figure 1.4**.

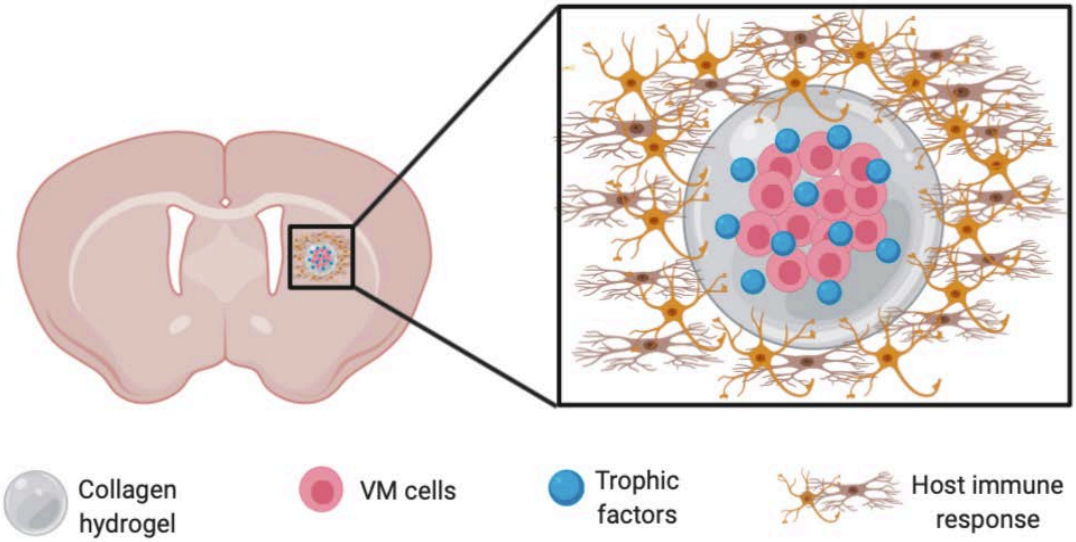


Figure 1.4 Schematic representation of a functionalised collagen hydrogel *in situ* in the brain after transplantation.

Collagen hydrogels have the potential to aid in the delivery of VM cells and trophic factors for the treatment of Parkinson´s disease.

1.2 THERAPEUTIC FACTORS FOR BRAIN REPAIR

Previous preclinical and clinical studies have demonstrated the potential of dopamine cell transplantation as a restorative approach for Parkinson´s disease. We now know that ventral midbrain dopaminergic neurons can be safely transplanted into the striatum of Parkinson´s disease patients with beneficial effects. However, despite the encouraging results, the poor survival and integration of dopaminergic cells after transplantation is one of the major factors hindering the efficacy of this therapy.

As mentioned above, one of the causes of low cell survival is the lack of trophic support after transplantation. Primary cells are removed from a neurotrophic-rich environment at the height of neurogenesis and placed into an environment deprived of the growth factors that normally should be present during their development (Sortwell *et al*., 2001; Abeliovich and Hammond, 2007). This low cell survival can be improved by the supply of neurotrophic factors.

Another factor hindering the survival of grafted neuron is that the injection of exogenous cells into the brain evokes an elevated host immune response followed by the production of pro-inflammatory cytokines such as IL-1β, IL-6, and TNFα, which produce a continuous inflammatory response causing damage to the transplanted cells and deterring their clinical efficacy (Duan *et al*., 1995; Shinoda *et al*., 1995, 1996; Barker *et al*., 1996; Joniec-Maciejak *et al*., 2014). This inflammatory response can be reduced by the addition of an anti-inflammatory factor to the cell transplantation process.

The delivery of these compounds to the brain is complicated and challenging since most of proteins and peptides do not cross the blood-brain barrier (BBB) and are rapidly degraded (Siegel and Chauhan, 2000). As discussed in the previous section, by encapsulating the grafted cells in a collagen hydrogel in combination with therapeutic factors such as GDF5 or IL-10, it creates a trophic and anti-inflammatory microenvironment around the transplanted cells. This strategy has an enormous potential to improve the survival and integration of dopamine cells after transplantation.

1.2.1 NEUROTROPHIC FACTORS AND CELL TRANSPLANTATION

Neurotrophic factors are secreted proteins that act as growth factors and which regulate neuronal phenotypic development, function, survival and plasticity in the developing and adult brain (Huang and Reichardt, 2001). These factors are synthesized in distinct target areas and act on specific neuronal populations (Korsching, 1993). Most of these factors belong to the Transforming Growth Factor β superfamily (TGF-β). This superfamily is composed of several members such as Bone Morphogenetic Proteins (BMP), Growth/Differentiation factors (GDF), and the GDNF family of ligands (GFL), which are widely distributed in the peripheral and central nervous system (Kingsley, 1994; Krieglstein *et al*., 1995).

Neurotrophic factors that have selective effects on dopaminergic neurons are good candidates for protection in Parkinson's disease. These include GDNF, growth/differentiation factor 5 (GDF5), neurturin (NRTN), and mesencephalic astrocyte-derived factor (MANF) among others (Siegel and Chauhan, 2000). Several studies have shown that neurotrophic therapy has beneficial effects on the survival, integration and efficacy of transplanted midbrain dopaminergic neurons (Kuan and Barker, 2005). Although there are many neurotrophic factors, this introduction will concentrate on GDF5 as it is the focus of this research project.

1.2.1.1 Growth/Differentiation factor 5

GDF5 (also known as cartilage-derived morphogenetic protein 1 or bone morphogenetic factor 14 (BMP-14)) was first identified in 1994 by Storm and colleagues as a member of the TGF-β superfamily, and was shown to be a necessary factor for the formation of bones, joints and tendons since it is related to the BMPs (which have osteoinductive and morphogenetic capacities) (Storm *et al*., 1994). In that study, Storm reported that mutations in the mouse GDF5 gene were shown to cause brachypodism phenotype, characterised by pronounced shortening of skeletal elements and/or loss of one or more joints. Additionally, members of the BMP subgroup are involved in the development of the nervous system, where they play roles in early CNS patterning as well as in neural cell fate determination, differentiation and survival (Hall and Miller, 2004).

In its active state, GDF5 forms a dimer that has binding affinity for the cell surface BMP receptors (BMPR) and the tyrosine-protein kinase transmembrane receptor (ROR2). GDF5 has higher affinity for BMPR1b than BMPR1a (Nishitoh *et al*., 1996). Binding of GDF5 to BMPR1a or BMPR1b recruits BMPR2 to form a serine/threonine kinase receptor dimer that activates the SMAD family of nuclear transcription factors, SMAD1, SMAD5, SMAD8 and SMAD4α or SMAD4β (ten Dijke *et al*., 2000). These transcription factors signal functions in many crucial aspects of neural development, and in midbrain dopaminergic neurons they play an endogenous role where they regulate neuron survival and axon growth (O'Keeffe *et al*., 2017).

GDF5 is expressed in many regions of the developing CNS (including the mesencephalon), neonatal and adult rat brain (Storm *et al*., 1994; Krieglstein *et al*., 1995; O'Keeffe *et al*., 2004a). In the rat, GDF5 is expressed in the VM from E12, peaking at E14, the time at which dopaminergic neurons in the developing midbrain are

undergoing terminal differentiation, then decreases with age to reach its lowest levels around the perinatal period (O'Keeffe *et al*., 2004b; Clayton and Sullivan, 2007). In the postnatal period, GDF5 increases to reach maximal levels in the adult rat brain, being expressed in many regions, including the striatum and midbrain (O'Keeffe *et al*., 2004b).

In 1995, shortly after its discovery, Krieglstein and colleagues showed that GDF5 is a potent neurotrophic molecule that acts as a survival promoting molecule for rat dopaminergic midbrain neurons (Krieglstein *et al*., 1995). Since then, it was hypothesised that GDF5 may be a useful protein for the treatment of Parkinson´s disease in two ways: 1) as a neurotrophic factor to protect cells after transplantation in cell replacement therapy or 2) as a neuroprotective factor to support the remaining dopaminergic neurons and protect them against the ongoing disease.

1.2.1.1.1 *In vitro* studies with GDF5

*In vitr*o studies have shown that GDF5 treatment of E14 rat VM cultures increases the survival and morphological differentiation of dopaminergic neurons; there was a significant increase in total neurite length, number of branch points and somal area after 6 days *in vitro* (O'Keeffe *et al*., 2004a; Wood *et al*., 2005). BMPR1b was found to be strongly expressed in freshly dissected E14 VM tissue, but its expression was lost with increasing time in culture. Therefore, the beneficial effects of GDF5 depend on the expression of BMPR1b and decrease with increasing time in culture (O'Keeffe *et al*., 2004a).

Clayton and Sullivan (2007), found that the effects of GDF5 were much greater when cultures were prepared from the lateral part (rich in A9 cells) of rat E14 VM. Furthermore, the BMPR1b receptor was expressed at higher levels in the lateral than in the medial region, confirming that GDF5 acts through this receptor to increase dopaminergic neuronal numbers (Clayton and Sullivan, 2007).

Additionally, *in vitro* treatment with GDF5 attenuates dopaminergic neurotoxicity induced by N-methyl pyridinium ion (MPP^+). In the presence of GDF5, 70% of embryonic rat dopaminergic neurons survived the neurotoxic insult of MPP^+, compared to the 40% survival seen in the groups treated with MPP^+ alone (Krieglstein *et al*., 1995). GDF5 also protects VM neurons in culture from free radical induced damage (using iron or sodium-nitroprusside) (Lingor *et al*., 1999).

In 2012, Toulouse and colleagues, and later Hegarty *et al*. (2013), using the SH-SY5Y human neuronal cell line as a model of human midbrain dopaminergic neurons, found that GDF5 induced neuronal differentiation and neurite extension. Furthermore, treatment of SH-SY5Y cells with GDF5 before a 6-OHDA insult protected the cells from the toxic events, and treatment with GDF5 after the 6-OHDA insult rescued most of the cells from the neurotoxic insult (Toulouse *et al*., 2012; Hegarty *et al*., 2013).

1.2.1.1.2 *In vivo* studies with GDF5

Studies using the 6-OHDA-lesioned rat model of Parkinson´s disease have shown that intracerebral injection of GDF5 can protect and restore the adult rat nigrostriatal dopaminergic neurons (Sullivan *et al*., 1997, 1999; Hurley *et al*., 2004).

The intracerebral injection of recombinant human GDF5, just above the SN and into the lateral ventricles of rats lesioned with 6-OHDA, induced motor recovery, protected nigral dopaminergic neurons, and preserved striatal levels of dopamine (Sullivan *et al*., 1997). A follow-up study by Sullivan and colleagues (1999) found that injection of GDF5 into either the striatum or substantia nigra had neuroprotective effects (Sullivan *et al*., 1999).

Hurley and colleagues (2004) found that administration of recombinant human GDF5, one week after an intrastriatal 6-OHDA-lesion resulted in significant motor recovery and protection of nigral dopaminergic cell bodies (Hurley *et al*., 2004). These results indicate that GDF5 has the potential to rescue the degenerating nigrostriatal pathway in Parkinson´s disease.

In 2012, Costello and colleagues found that a continuous supply of GDF5, produced by transplanted GDF5-overexpressing CHO-cells *in vivo*, has neuroprotective and neurorestorative effects on midbrain dopaminergic neurons after MFB 6-OHDA-lesions of adult rats. Additionally, they found that GDF5 increased the survival and significantly improved motor deficits of the lesioned rats (Costello *et al*., 2012).

1.2.1.1.3 GDF5 for primary dopaminergic cell transplantation

The efficacy of cell replacement therapy has been limited by the poor survival and integration of dopaminergic cells after transplantation due to lack of trophic support (Sortwell *et al*., 2001). Therefore, an exogenous supply of GDF5 has the potential to improve the survival yield of the transplanted dopaminergic cells.

A study by Sullivan and colleagues (1998) found that preincubation of embryonic rat midbrain tissue in recombinant human GDF5 before transplantation, produced significant improvements in cell survival and significant motor recovery in 6-OHDA-lesioned rats (Sullivan *et al*., 1998). Another study reported that transplants of GDF5-over-expressing embryonic rat midbrain grafts survived better in the 6-OHDA-lesioned rat striatum and caused significant improvement of motor deficits (O'Sullivan *et al*., 2010).

The exogenous administration of GDF5 is quickly metabolised in the brain, therefore, the encapsulation of this neurotrophic factor in combination with VM cells in a collagen hydrogel may significantly improve the outcome of cell replacement therapy for Parkinson´s disease.

1.2.2 ANTI-INFLAMMATORY FACTORS AND CELL TRANSPLANTATION

The efficacy of cell replacement therapy has also been hindered by the elevated host immune response reacting to tissue injury caused during transplantation and to the implantation of exogenous cells into the brain (Duan *et al*., 1995; Shinoda *et al*., 1995, 1996; Barker *et al*., 1996; Joniec-Maciejak *et al*., 2014). Inflammation is caused by the accumulation of inflammatory cells such as microglia and astrocytes and other mediators in the brain. Anti-inflammatory factors may be important to minimize the inflammatory reaction of the brain to trauma (Orive *et al*., 2009; Yan *et al*., 2014).

Cytokines are small secreted proteins with growth, differentiation, and activation functions that regulate and determine the nature of immune responses and control immune cell trafficking and the cellular arrangement of immune organs (Borish and Steinke, 2003; Mollazadeh *et al*., 2019). Cytokines such as, IL-1, IL-6, interferon (IFN)-γ, transforming growth factor (TGF)-β and TNF-α are upregulated in the brain as a result of inflammation, but there are other cytokines like IL-10 that inhibit the release of proinflammatory factors, enhances the production of anti-inflammatory mediators and oppose the actions of the major inflammatory cytokines (Yan *et al*., 2014; Kwilasz *et al*., 2015).

1.2.2.1 Interleukin-10

IL-10 is a homodimeric, pleiotropic cytokine that belongs to the IL-10 family which includes IL-19, IL-20, IL-22, IL-24 and IL-26 (Volk *et al*., 2001; Zdanov, 2010). This

cytokine is produced by nearly all leukocytes, dendritic cells (DC), natural killer (NK) cells, neutrophils, eosinophils, B cells, etc. (Hutchins *et al*., 2013). In the brain, IL-10 and its receptor IL-10R are expressed by microglia, astrocytes, oligodendrocytes and neurons in normal conditions and after injury (Ledeboer *et al*., 2002).

IL-10 is one of the most important and best anti-inflammatory cytokines that is essential for the regulation of inflammation and immune response (Johnston *et al*., 2008). Additionally, this cytokine acts as an anti-apoptotic agent by inhibiting the actions of caspase-3 and decreasing microglial activation in response to inflammation (Kwilasz *et al*., 2015; Saxena *et al*., 2015). IL-10 binds to IL-10R, a tetramer composed of two different chains (IL-10R1 and IL-10R2) and activates the IL-10/JAK1/STAT3 anti-inflammatory pathway which subsequently suppresses the pro-inflammatory genes (Saraiva and O'Garra, 2010; Hutchins *et al*., 2013; Kwilasz *et al*., 2015; Zhu *et al*., 2017).

1.2.2.1.1 *In vitro* studies with IL-10

In vitro studies have used the lipopolysaccharide (LPS)-induced model to study inflammatory mechanisms in Parkinson´s disease. LPS is a component of the outer membrane of Gram-negative bacteria that triggers an inflammatory response in animals (Liu and Bing, 2011). Qian and colleagues (2006) found that IL-10 has neuroprotective effects on LPS-induced dopaminergic toxicity through the inhibition of microglial activation (Qian *et al*., 2006a, 2006b). Furthermore, Zhu and colleagues (2017) used this model in E14 VM cells to assess the ability of IL-10 to protect the primary cultured VM neurons from LPS toxicity. In this study they found that pre-treatment with IL-10 reduced neuronal damage after exposure to LPS toxicity by inhibiting neuronal apoptosis mediated by TNF-α (Zhu *et al*., 2017).

1.2.2.1.2 *In vivo* studies with IL-10

The neuroprotective effects of IL-10 have also been tested *in vivo* using the administration of LPS into the striatum of rats. LPS administration causes marked microglial activation and a dose-dependent loss of dopaminergic neurons. In this study, Arimoto and colleagues (2007) found that infusion of IL-10 protected against LPS-induced cell death of dopaminergic neurons with a corresponding decrease in the number of activated microglia (Arimoto *et al*., 2007).

In 2008, Johnston *et al*., investigated the neuroprotective effects of an adeno-associated viral type-2 (AAV2) vector containing the complementary DNA (cDNA) for human IL-10 (hIL-10) in rats unilaterally lesioned with 6-OHDA, and found that AAV2-hIL-10 reduced the loss of TH^+ neurons in the SN, and also reduced loss of striatal dopamine (Johnston *et al*., 2008).

In 2013, Schwenkgrub and colleagues used the MPTP model of Parkinson´s disease to test the effects of IL-10 injected intracerebroventricularly (ICV) before the MPTP injection. In this study, they found that IL-10 administration had neuroprotective effects (Schwenkgrub *et al*., 2013).

There are no previous *in vivo* studies using IL-10, looking at the direct effects of this cytokine on VM grafts. Thus, the co-implantation of a neurotrophic factor with an anti-inflammatory cytokine encapsulated with VM cells in a collagen hydrogel has a greater potential to improve the outcome of cell replacement therapy for Parkinson´s disease and requires further investigation.

1.3 HYPOTHESIS

Taking all of the above together, we hypothesise that a dual approach combining cell therapy and the delivery of a neurotrophic factor in an injectable collagen scaffold will increase the survival, outgrowth and function of the transplanted dopaminergic neurons by providing them with an adhesive substrate, prolonged and site-specific delivery of growth factor support, while preventing the harmful infiltration of inflammatory cells.

1.4 OBJECTIVES

The overall aim of this thesis was to test an injectable GDF5-enriched collagen hydrogel for the delivery of E14 ventral mesencephalic-derived dopaminergic neurons in hemi-Parkinsonian rats. Specifically, we aimed to:

1. Assess *in vitro* and *ex vivo*, the biocompatibility of collagen hydrogels and the neuroprotective effects of GDF5-encapsulated in collagen hydrogels, on a SH-SY5Y neuroblastoma cell line and in VM explants.
2. Assess *in vivo* the suitability of collagen hydrogels for intrastriatal delivery of E14 VM-derived dopaminergic neurons and for neurotrophic factors to the brain.
3. Assess the effects of the GDF5 functionalised collagen hydrogel on the survival and efficacy of E14 VM-derived dopaminergic neurons in a rat model of Parkinson´s disease.
4. Assess the effects of an enriched GDF5 plus IL-10 functionalised collagen hydrogel on the survival and efficacy of E14 VM-derived dopaminergic neurons in a rat model of Parkinson´s disease.

Chapter 2: Materials & Methods

2.1 ETHICAL STATEMENT

All procedures involving the use of animals were:

1. Approved by the Animal Care and Research Ethics Committee (ACREC) of the National University of Ireland, Galway.
2. Completed under project licenses issued to Dr. Eilís Dowd by the Irish Department of Health and Children and the Irish Health Products Regulatory Authority (AE19125/P063), and under an Individual Authorisation issued to Ms. Verónica Rebeca Alamilla Marroquín (A819125/I159).
3. Carried out in compliance with the European Union Directive (2010/63/EU) and the Irish legislation (S.I. No. 543 of 2012).

All procedures were conducted blind to the treatment of rats.

2.2 GLOBAL EXPERIMENTAL DESIGN

The overall aim of this thesis was to determine if a dual approach combining the delivery of GDF5 and cell therapy in a collagen scaffold could improve the outcome of reparative cell therapy in a rat model of Parkinson's disease (**Figure 2.1**). Through a series of preliminary *in vitro* and *ex vivo* studies, we first assessed the efficacy and safety of collagen hydrogels, and GDF5 encapsulated collagen hydrogels, in a SH-SY5Y cell line and in explanted rat E14 VM tissue explants (Chapter 3). Then we proceeded to assess the suitability of collagen hydrogels for intrastriatal delivery of cells and/or neurotrophic factors to the brain (Chapter 4). Following this, we then sought to determine the survival and efficacy of E14 VM primary dopaminergic grafts in GDF5-loaded collagen hydrogel in a rat model of Parkinson's disease (Chapter 5). In parallel, we looked to determine the survival and efficacy of E14 VM grafts in GDF5 and IL-10-loaded collagen hydrogel in a rat model of Parkinson´s disease (Chapter 6).

In Chapter 3, we evaluated the cytocompatibility of the collagen hydrogels and of the GDF5 enriched gels in a SH-SY5Y neuroblastoma cell line and in VM explants. These

studies allowed us to determine the safety, neuroprotective effects of the GDF5 enriched gels and their suitability for *in vivo* assessment.

In Chapter 4, we sought to assess the suitability of collagen hydrogels for delivery of primary dopaminergic neurons and/or GDF5 in the striatum. Additionally, we looked to determine if GDF5 enrichment has early effects on primary dopaminergic neuron grafts. These studies allowed us to determine whether our collagen hydrogels could protect the transplanted cells from the attack of the immune response while enhancing their survival.

In Chapter 5, we sought to assess the effect of our optimised GDF5-loaded collagen hydrogel on the long-term survival, outgrowth and efficacy of encapsulated E14 primary dopaminergic neurons in a rat model of Parkinson's disease. This study allowed us to determine whether our growth factor-loaded collagen hydrogel could provide additional support to dopaminergic neurons and therefore enhance graft survival.

In Chapter 6, we assessed a dual approach: GDF5 and IL-10 encapsulated in collagen hydrogels, on the long-term survival, outgrowth and efficacy of encapsulated E14 VM grafts in a rat model of Parkinson's disease. This study allowed us to determine whether our growth factor/cytokine-loaded collagen hydrogel could provide additional support to dopaminergic neurons and therefore enhance graft survival and behavioural recovery.

Detailed experimental designs of each study are provided in the relevant results chapters, while this chapter will provide details of the different methodologies used throughout this thesis.

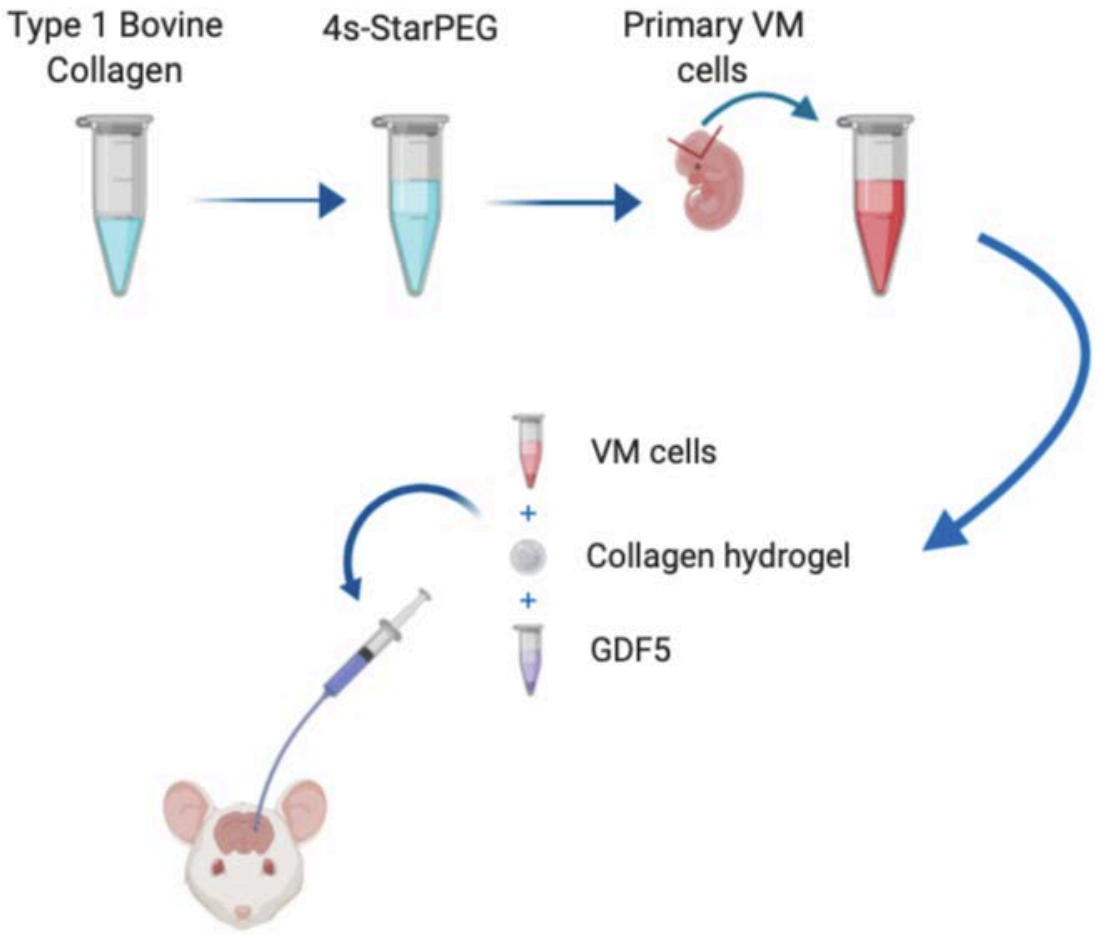


Figure 2.1 Primary thesis objective.

A schematic depicting the overall aim of this thesis: to develop a GDF5-loaded injectable collagen hydrogel for the delivery of dopaminergic neurons to a rat model of Parkinson's disease.

2.3 FABRICATION OF TYPE 1 COLLAGEN HYDROGELS

To prepare the collagen hydrogels used throughout this thesis, collagen type 1 from Vornia Biomaterials was used (except for one study where collagen from Collagen Solutions was also used). During the preparation of the gels, all the components were maintained on ice to prevent premature gelation. For a final volume of 100 µl, 40 µl of 5 mg/ml type 1 collagen was neutralised with 1 M NaOH until pH 7 was reached, then 20 µl of 10x PBS containing 0.1 mg, 0.2 mg or 0.4 mg of poly (ethylene glycol) ether tetrasuccinimidyl glutarate (4s-StarPEG) was added. Then 40 µl of one of the following was added and mixed thoroughly: 1) cell transplantation medium or 2) cell suspension or 3) GDF5 enriched cell suspension or 4) GDNF enriched cell suspension or 5) IL-10 enriched cells suspension. For *in vitro* and *ex vivo* explant experiments, gels of 15 µl, 30 µl or 50 µl samples were formed on a sterilised super hydrophobic surface (Teflon® tape) and placed in an incubator at 37°C to gel. For *in vivo* experiments, the un-seeded and cell-seeded collagen hydrogels were maintained on ice prior to transplantation to prevent premature gelation.

2.4 CELL CULTURE ASSAYS

2.4.1 CULTURE AND MAINTENANCE OF SH-SY5Y NEUROBLASTOMA CELL LINE

The SH-SY5Y cell line is a cloned human catecholaminergic neuroblastoma mixed adherent/suspension subline of the SK-N-SH cell line. This cell line was established in 1970 from a bone marrow biopsy of a metastatic neuroblastoma of a 4-year old female and has undergone three rounds of clonal selection. SH-SY5Y cells have been widely used as a model of dopaminergic neurons since they expresses dopaminergic neuronal markers, such as dopamine and the dopamine transporter (Lopes *et al*., 2010).

SH-SY5Y cells were maintained in Dulbecco's Modified Eagle's Medium with F12 (DMEM/F12, D6421, Sigma, Ireland) supplemented with 10% foetal bovine serum ((FBS) (10270-106, Gibco, USA)) and 1% Penicillin-Streptomycin (P0781, Sigma, Ireland). They were grown in a T-75 flask (83.3911.002, Sarstedt, Germany) in a sterile incubator (Hera Cell 150, Kendro, Germany) at 37°C with 5% CO_2. Cells were sub-cultured approximately every 2-3 days after reaching 80% confluency. For counting,

extraction, and preservation, cells were sub-cultured. In a sterile flow-hood (Hera Safe, Kendro, Germany), old medium was removed and discarded from the T-75 flask. The adherent cells were washed with sterile phosphate buffer saline ((PBS) (D8537, Sigma, Ireland)), followed by 3-minute incubation in warmed trypsin medium (1 ml trypsin (T4174, Sigma, Ireland) + 9 ml Hank's Balanced Salt Solution (H9394, Sigma, Ireland)). After the 3-minute incubation in the trypsin medium in the incubator at 37°C, the flask was tapped to ensure cell suspension before adding warmed medium to neutralise the trypsin. The suspended cells were then centrifuged at 1400 rpm for 5 minutes. Afterwards, the neutralised trypsin was discarded and the pelleted cells were re-suspended in fresh warmed medium before returning the cells back to the T-75 flask for storage in the incubator.

To create a stock of cells for later use, cells were grown in a T-75 flask until ~80% confluency was reached, then they were re-suspended as described above using trypsin. In this case, after the neutralised trypsin was removed, cells from one flask were re-suspended in 1 ml of cryopreservation medium (DMEM/F12 with 5% DMSO (D8418, Sigma, Ireland)). Cells were re-suspended and transferred to a sterile preservation tube (72.380, Sarstedt, Germany) that was placed in a Mr. Frosty Freezing Container (5100-0001, Thermo Fisher, Ireland) to control the rate of freezing once placed in the -80°C freezer. After the cells reached -80°C, cells were transferred to a liquid nitrogen tank for long-term storage.

2.4.2 CELL VIABILITY ASSAYS

First, to determine the effect of the collagen hydrogels on the viability of SH-SY5Y cells, cells were seeded at a density of 125,000 cells per well on a 24 well-plate in 500 µl of plating medium and left overnight to attach at 37°C with 5% CO_2. SH-SY5Y cells were then either incubated with unloaded collagen hydrogels (1 x 30 µl gel per well) or with unloaded collagen of various 4s-StarPEG concentrations (1, 2 or 4 mg/ml) for 24 hr or left untreated. As an indicative measurement of cell viability, metabolic activity of the cells was assessed using the alamarBlue® assay and the 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole (MTT) assay.

For concentration-response experiments, cells were treated 24 hr after plating to determine the effect of 6-OHDA (prepared in sterile medium) on the viability of the SH-SY5Y cells. Cells were seeded at a density of 125,000 cells per well of a 24 well-plate

in 500 µl of plating medium and left overnight to attach at 37°C with 5% CO_2. SH-SY5Y cells were then treated with 0, 20, 50, 100 µM of 6-OHDA. Viability of cells was measured by the alamarBlue® assay and the MTT assay.

For treatment experiments, cells were exposed for 24 hours to 50 µM of 6-OHDA and then treated with 1 or 5 µg of GDF5 or GDNF enriched collagen hydrogels (1 x 30µl gels), to determine if there was recovery of cells after exposure to the neurotoxin. Cells were seeded at a density of 125,000 cells per well on a 24 well-plate in 500 µl of plating medium and left overnight to attach at 37°C with 5% CO_2. Then, SH-SY5Y cells were exposed to 50 µM of 6-OHDA for 24 hr followed by treatment with either GDF5, GDNF, GDF5-gels or GDNF-gels for 24 hr. Viability of cells was measured by the alamarBlue® assay and the MTT assay.

Every experiment had 6 technical replicates per experimental condition, with three biological replicates.

2.4.2.1 The alamarBlue® assay

This assay was used to investigate the metabolic activity of the cells following exposure to the collagen hydrogels or cytotoxic agents, such as 6-OHDA. It may also be used as a gross indicator of cell viability. The alamarBlue® (Invitrogen) assay is a blue sensitive oxidation-reduction non-fluorescent indicator that changes to a pink colour upon reduction of resazurin (7-hydroxy-10-oxidophenoxazin-10-ium-3-one) by living cells. This reduction is mediated by mitochondrial enzymes, therefore the intensity of fluorescence produced is proportional to the number of living cells respiration (Hamid *et al.*, 2004; Rampersad, 2012).

After treatment of the SH-SY5Y cells, all the medium was carefully removed and discarded. Since alamarBlue® is photosensitive all incubations were done in the dark. Then the 10% solution of alamarBlue® (1 ml of alamarBlue® stock + 9 ml of HBSS) was added to each well (i.e. 310 µl of solution per well of a 24-well plate) and placed back in the incubator under standard culture conditions for 1 hour. Following this incubation period, the medium was carefully transferred to a 96-well plate (100 µl per well), after which the plate was placed in a spectrophotometer and the absorbance was read at 570 and 600 nm. Absorbance readings were used according to the formula in **Figure 2.2**. Viability was calculated by normalisation of all results to control wells.

$$\text{\% difference between treated and control cells} = \frac{(O2 \times A1) - (O1 \times A2)}{(O2 \times P1) - (O1 \times P2)} \times 100$$

Figure 2.2 Percentage difference between treated and control cells formula.

The amount of fluorescence or absorbance is proportional to the number of living cells and corresponds to the cells metabolic activity. Damaged and nonviable cells have lower innate metabolic activity and this generate a proportionally lower signal than healthy cells. O1: molar extinction coefficient (E) of oxidized alamarBlue (blue) at 570 nm (E= 80586); O2: E of oxidized alamarBlue at 600 nm (E= 117216); A1: absorbance of test wells at 570 nm; A2: absorbance of test wells at 600 nm; P1: absorbance of positive growth control well (cells plus alamarBlue but no test agent) at 570 nm; P2: absorbance of positive growth control well (cells plus alamarBlue but no test agent) at 600 nm.

2.4.2.2 MTT Assays

This assay was used to investigate mitochondrial activity following exposure to the gels or cytotoxic agents, such as 6-OHDA (Sigma Aldrich, H4381) used over the course of this research. MTT (Sigma Aldrich, M2128) is reduced to purple formazan in the mitochondria of living cells. MTT is only reduced when mitochondrial reductase enzymes are active.

After treatment of SH-SY5Y cells, the MTT solution (5 mg Thiazolyl Blue Tetrazolium Bromide per 1 ml PBS) was added to each well so that the MTT was diluted 1:10 (i.e. 22 µl of MTT was added to wells containing 198 µl of treated cell suspension) and placed back in the incubator under standard culture conditions for 3 hours. Following this incubation period, the medium was carefully removed and discarded. Then the MTT solvent was prepared (for 50 ml: 50 ml NP-40 (Igepal) + 200 µl of 1 M HCL+ 49.75 ml of Isopropyl alcohol). Each well received 150 µl of MTT solvent, after which the plate was covered with tin foil and placed on an orbital shaker for 15 minutes to agitate cells and assist the dissolving of formazan crystals. The plate was placed in a spectrophotometer and the absorbance was read at 570 nm. Absorbance readings were used, according to the formula in **Figure 2.3**, to establish the percentage cell viability following exposure to the agents.

$$\% \text{ Cell Viability} = \left[\frac{(\text{Absorbance} - \text{Blank})}{\text{Average (Absorbance} - \text{Blank) of Untreated Sample}} \right] \times 100$$

Figure 2.3 Percentage cell viability formula.

The blank absorbance was subtracted from each sample absorbance. The average (absorbance-blank) for the untreated sample was divided into each sample. This value was multiplied by 100 to give a percentage cell viability value.

2.4.3 SH-SY5Y CELL IMMUNOCYTOCHEMISTRY

Dopamine and neuronal cell survival was assessed 24 hr after treatment with collagen hydrogels (as described above) using TH^+ and βIII tubulin immunocytochemistry (ICC) respectively. SH-SY5Y cells were fixed with 4% paraformaldehyde for 30 min, followed by 3 washes in Tris-buffered saline (TBS) with 0.2% triton-X-100 for permeabilisation. Cultures were then incubated in blocking serum (5% bovine serum albumin in TBS with 0.2% triton-X-100) for 1 hr at room temperature, before being subsequently incubated with primary antibody (Mouse anti-TH, 1:200, Millipore; Mouse anti-beta III tubulin, 1:333, Millipore) diluted with 1% bovine serum albumin in TBS with 0.2% triton-X-100 at room temperature overnight. Following 3 x 10 min washes with TBS, cultures were incubated in rabbit anti-mouse AF 488 conjugated secondary antibodies (1:800, Biosciences) in 1% bovine serum albumin in TBS, at room temperature for 3 hr in darkness. Cultures were then counterstained with DAPI (1 µg/ml in TBS, Sigma) for 5 min. Following 3 x 10 min washes in TBS, cultures were stored in 0.1% TBS azide at 4°C until imaging. Negative controls where no primary antibody was added were also prepared. Samples were taken for imaging right after finishing the process of this immunostaining.

2.4.4 SH-SY5Y CELLS IMAGE CAPTURE

All images of SH-SY5Y cells were captured using the Olympus Fluoview 300 laser scanning confocal microscope. After loading the samples into the microscope, the region of interest was found using a low-power objective (10x). Once the correct location was found and image was focused, then the objective was switched to a higher power (40x). Using the Olympus Fluoview software, the combination of lasers needed were set up and the filter combination was chosen to acquire the images: FITC for 488 nm laser (βIII tubulin and TH^+) and TRITC for 543 nm laser (DAPI). Three randomly selected sample sites per well per condition were chosen for imaging.

2.5 ANIMAL HUSBANDRY

A total of 170 Sprague Dawley rats were used to complete this research. Most animals were purchased from Charles River, UK, except for 48 rats that were born in-house. All rats were housed in groups of 2 per cage, in plastic bottom cages (50.50 x 13 x 24 cm) with a wire grid lid and standard bedding material of sawdust for pups or 3Rs lab basic bedding for weaned and adult rats. Each cage contained sizzle-nest and hollow plastic tunnels as environmental enrichment. Rats were kept on a 12:12 hr light: dark cycle with lights on at 08:00 AM and off at 8:00 PM, at a temperature of 20-23°C, with relative humidity levels maintained between 40 and 70%. Throughout the duration of the study, rats were allowed access to food and water *ad libitum*. All behavioural testing and *ex vivo* analyses were carried out blind to the treatment of the animals.

2.5.1 EMBRYO HARVESTING

The ventral mesencephalon of rat embryos was obtained following the protocol previously established by Dunnett *et al*., (2001). Time-mated female Sprague-Dawley rats were put under isoflurane anaesthesia (5% in 0.5 L O_2) and decapitated using a guillotine. The uterine horn was removed by laparotomy using rat tooth grasping forceps and a scissors and then submerged in Hank's Balanced Salt Solution (HBSS) (with sodium bicarbonate and without phenol red, Ca^{2+} or Mg^{2+}; Sigma H6645) in a 9 cm petri dish that was kept on ice. E14 embryos found in the uterine horn, were removed from it and from their embryonic sac, using curved forceps, small scissors and fine-point forceps, and subsequently washed 3 times in ice cold HBSS to ensure the

removal of all blood. Embryos were then placed in the lid of a 9 cm petri dish containing ice cold HBSS for dissection under a dissecting microscope.

2.5.2 VENTRAL MESENCEPHALON DISSECTIONS FOR EXPLANT CULTURES

The morning before embryo harvesting, fresh plating medium was prepared (9.7 ml complete medium + 100 µl foetal calf serum (FCS) + 200 µl B27 supplement) and placed into 6-well plates (950 µl/ well). With sterilised tweezers, culture plate inserts (EMD Millipore) were positioned into each well and these plates were stored in the incubator.

Embryos were harvested following the procedure from section **2.5.1**. Each embryo was dissected using curved forceps and dissecting micro-scissors. The mesencephalon was dissected out by making an incision at the midbrain-hindbrain boundary, and at the forebrain-midbrain boundary. The dorsal mesencephalon was then cut lateral to the midline, opening the neural tube and exposing the ventral mesencephalon in its centre. An incision was made at the point where the lateral mesencephalon starts on both sides and the meningeal layer was removed (**Figure 2.4**). The dissected ventral mesencephalon tissue was stored on ice in a 1 ml micro-centrifuge tube (Eppendorf) containing cold HBSS until all embryos were dissected. Immediately after all dissections were completed, explant preparation was performed.

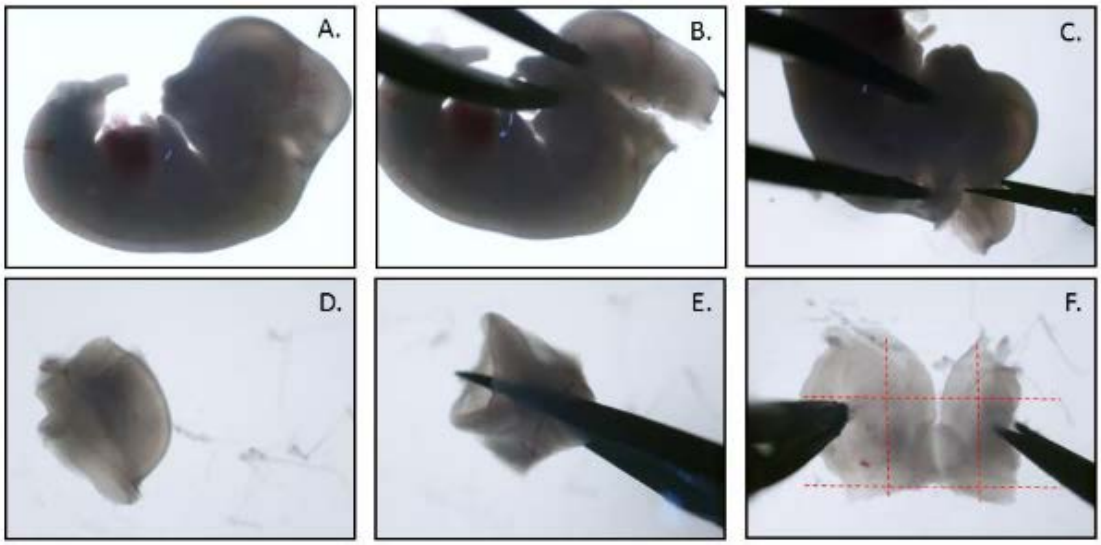


Figure 2.4 Dissection of the ventral mesencephalon for explant cultures.

After removing the embryos from the uterine horn (A). An incision was made at the midbrain-hindbrain boundary (B), and at the forebrain- midbrain boundary (C). This exposed the neural tube (D); then the dorsal mesencephalon was cut laterally to the midline, (E) which opened the neural tube and exposed the ventral mesencephalon. An incision was then made at the points between the mid-lateral to medial mesencephalon on both sides and the meningeal layer was removed (F). The dissected ventral mesencephalon was then maintained in ice cold hibernation medium until all dissections were completed (photos taken from Moriarty, (2018)).

2.5.2.1 Ventral mesencephalon preparation for explant cultures

All of the dissected VMs were poured into a petri dish filled with warmed medium. Then using sterile cut out tips for a P200 (Gilson pipette) set to 50 µl, each one of the dissected VMs were transferred into a warmed culture plate insert (previously prepared 6-well plates). Explants were cultured using the air-medium interface method (Stoppini *et al.*, 1991) in which each explant was placed completely flat against the membrane (0.4 µm pores) of the insert (extra caution was taken to ensure that no air bubbles were introduced between the membrane containing the explant and the medium) and maintained at the interface between the culture medium and the CO_2-enriched atmosphere in the 6-well plate. The medium should not cover the membrane so that the explant remains well exposed to the air. In this way, explants remain covered by a film of medium and obtain the nutrients and/or treatment in the medium by diffusion through the membrane. Explants were incubated at 37°C with 5% CO_2. Medium was changed

every two days by taking out the inserts with sterile tweezers, removing 900 µl of medium and adding fresh DMEM/F12. Explants were incubated for 4 days *in vitro* (DIV) so they flattened against the membrane of the insert before any assay was done.

2.5.2.2 Assays with VM explanted tissue

First, to determine the effect of the collagen hydrogels on the viability of the VM explants, these were incubated up to 4 DIV at 37°C with 5% CO_2. Explants were then incubated with unloaded collagen of various 4s-StarPEG concentrations (1, 2 or 4 mg/ml) for 48 hours (1 x 30 µl gel per well in the medium) and fixed with 4% paraformaldehyde (PFA) after this time (collagen hydrogels degrade over time in the medium exposing the explants to their components by diffusion, passing through the pores of the membrane). In order to assess development of the explants, free floating immunohistochemistry (IHC) for TH^+ was done using the streptavidin-biotin-peroxidase method as previously described by Hoban, (2015) and Moriarty, (2018) (for further details see **Section 2.8**). Measurements of TH^+ optical density were performed using ImageJ software (U.S. National Institutes of Health, Bethesda, Maryland, USA). All VM explant studies had 3 technical replicates per experimental condition, with 3 biological replicates.

For dose-response experiments, explants were incubated up to 4 DIV at 37°C with 5% CO_2. Explants were then exposed to 2 mM of 6-OHDA, 50 µg/ml of LPS or 100 µg/ml of polyinosinic: polycytidylic acid (Poly I:C (Invivogen, USA, tlrl-pic)) mixed in the medium for 48 or 72 hours and fixed with 4% PFA. In order to assess damage to the development of the explants, free floating IHC for TH was performed. Measurements of TH^+ optical density were done using ImageJ software.

For the purpose to determine if after exposure to 6-OHDA there was recovery of the cells in the explants, they were treated with GDF5 functionalised gels. Explants were incubated up to 4 DIV at 37°C with 5% CO_2 and then exposed to 2 mM of 6-OHDA in the medium for 72 hours. After this time, explants were treated with 5 µg or 20 µg of GDF5 enriched gels (1 x 30 µl gels in the medium) and fixed with 4% PFA after 12 DIV (collagen hydrogels degrade over time in the medium exposing the explants to their components by diffusion, passing through the pores of the membrane). In order to assess recovery of the TH^+ cells, free floating IHC was performed. Measurements of TH^+ optical density were done using ImageJ software.

2.5.2.3 Quantification of optical density of TH^+ staining

Optical density measurements were taken to quantify the expression of TH^+ staining. These density measurements are an indirect indicator of dopaminergic cell viability on the explants. Optical density measurements may suggest that there is an increase or decrease in dopaminergic cell numbers/growth or both. In the work presented in this thesis, photomicrographs were obtained using the Olympus slide scanner. All images were taken at the same conditions of magnification and pixel quality. Measurements of optical density were done using the ImageJ program. To do this, images of explants in every group were taken. The site containing staining for TH^+ was located and a circular shape was centred over the site (**Figure 2.5**). Then, the density of staining was measured. To determine the specific staining density, the optical density readings were corrected for non-specific background density. To do this, the same circular shape was placed over an unstained region in the section, the optical density was measured and the corresponding calculation was performed.

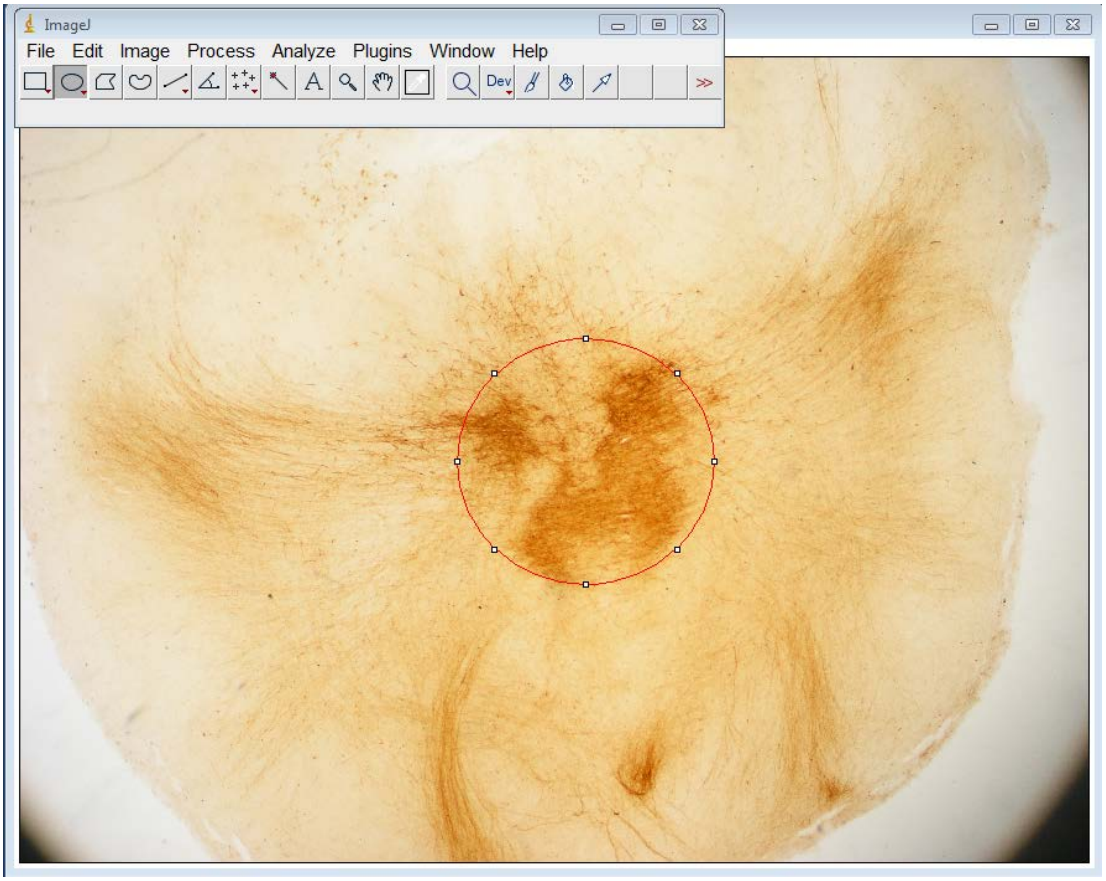


Figure 2.5 Screenshot of ImageJ software used to measure optical density of VM explants.

The density of the immunostaining was measured for each explant around the TH^+ staining (red outline).

2.5.3 VENTRAL MESENCEPHALON DISSECTIONS FOR CELL SUSPENSION

Embryo harvesting was done as per section 2.5.1. Each embryo was dissected using curved forceps and dissecting micro-scissors, the mesencephalon was dissected out by making an incision at the midbrain-hindbrain boundary, and at the forebrain-midbrain boundary. The dorsal mesencephalon was then cut lateral to the midline, opening the neural tube and exposing the ventral mesencephalon in its centre. An incision was made at the point between the mid-lateral to medial mesencephalon on both sides and the meningeal layer was removed. Cranial and caudal cuts were made to the medial mesencephalon to ensure that no forebrain or hindbrain tissue were included (**Figure 2.6**). Dissected ventral mesencephalon tissue was stored on ice in a 15 ml tube containing cold hibernation medium (Hibernate-E; Gibco) or cold HBSS until all

embryos were dissected. Once all dissections were complete, cell suspension preparation was performed immediately.

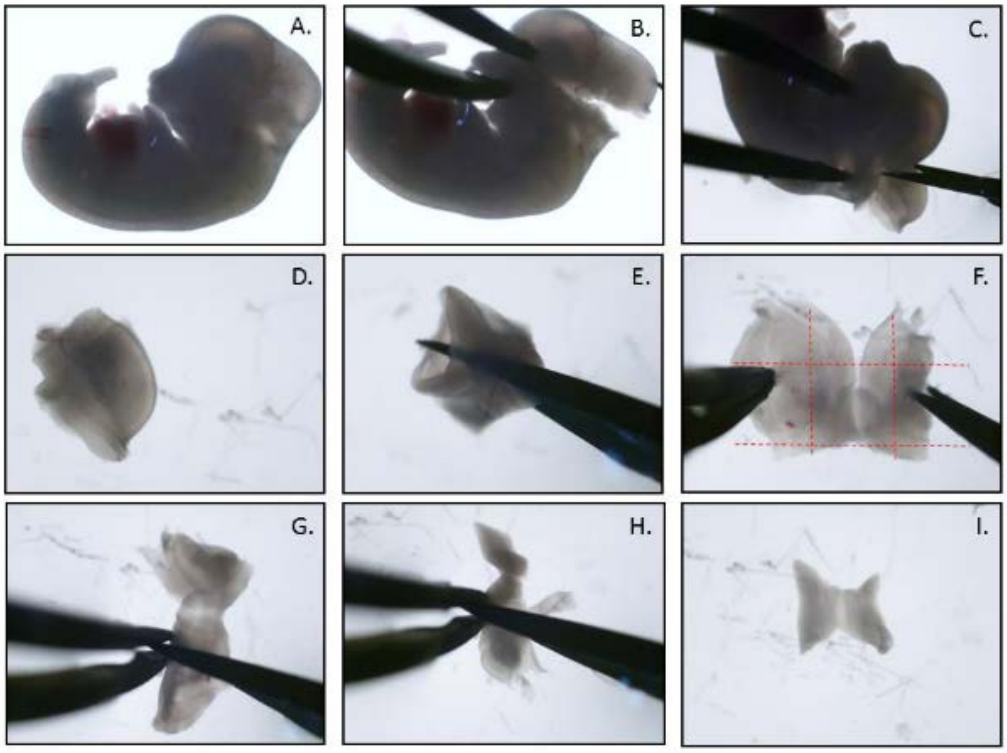


Figure 2.6 Dissection of the ventral mesencephalon for cell suspension.

After removing the embryos from the uterine horn (A). An incision was made at the midbrain-hindbrain boundary (B), and at the forebrain- midbrain boundary (C). This exposed the neural tube (D); then the dorsal mesencephalon was cut laterally to the midline, (E) which opened the neural tube and exposed the ventral mesencephalon. An incision was then made at the points between the mid-lateral to medial mesencephalon on both sides and the meningeal layer was removed (F). Cranial and caudal cuts were made to the medial mesencephalon to ensure that no forebrain or hindbrain tissue was included (G-H). Dissected ventral mesencephalon (I) was then maintained in ice cold hibernation medium until all dissections were completed (Photos taken from Moriarty, (2018)).

2.5.3.1 VM preparation for cell suspension

HBSS was removed from VM tissue by centrifugation at 1400 rpm for 5 minutes, followed by incubation of the tissue pellet in warmed medium (2 ml trypsin + 3 ml HBSS) for 4 minutes at 37°C. FCS was then added to the tissue to neutralize the trypsin and centrifuged at 1400 rpm for 5 min. Dissociation medium and FCS were then removed and the cell pellet was carefully dissociated in 1 ml of plating medium

(Dulbecco's modified Eagle's medium/F12, 0.6% D-glucose, 1% L-glutamine, 1% FCS and 2% B27) by pipetting with a P1000, followed by a 25 gauge needle with a 1 ml syringe. Extra caution was taken to ensure that no air bubbles were introduced to the cell suspension. Once a single cell suspension was obtained, a 10 µl sample of the cell suspension was taken and mixed with 90 µl of trypan blue. Cells were then counted using a haemocytometer (Blaubrand, Neubauer Improved, Germany). Once counted, cell suspension was centrifuged at 1400 rpm for 5 min and the cell pellet was resuspended in the appropriate volume of transplantation medium for surgery. For *in vivo* studies cells were resuspended at a range between 83,333 cells/µl and 166,666 cells/µl.

2.6 SURGERY

2.6.1 STEREOTAXIC SURGERY

Surgeries were performed under sterile conditions and were performed under isoflurane anaesthesia (5% in O_2 for induction and 2% in O_2 for maintenance) in a stereotaxic frame. The nose bar of the frame was set to -4.5 mm for intra-medial forebrain bundle (MFB) lesion with 6-OHDA or -2.3 mm for intra-striatal transplantation. To begin the surgery, the head of the rat was shaved and the animal was secured to the stereotaxic frame using the ear bars. Afterwards, the site of surgery was disinfected with iodine and using a scalpel, an incision on the head was made to expose the skull. Rats´ breathing rate and temperature were monitored every five minutes throughout the duration of the surgery. The coordinates for bregma were measured and used to calculate the site for insertion of the cannula. A 30 gauge injection cannula was connected to a 50 µl Hamilton syringe using polyethene tubing of 0.28 mm of inner diameter filled with saline. An electric drill was used to expose dura and then the cannula was lowered to the correct dorso-ventral coordinates. The required solution or suspension was slowly and carefully delivered to the desired region. To monitor that the cell suspension or neurotoxin was successfully delivered, an air bubble was made inside the tubing between the saline and the suspension and its movement was monitored. The plunger on the Hamilton was slowly pressed by an automated pump (Harvard apparatus), at a constant rate of 1 µl/min, followed by 2 mins of diffusion. Once the delivery finished, the incision was sutured closed and a topical anaesthetic was applied. Then the animal

was taken to a recovery cage where it was monitored every 5 minutes until they regained full consciousness, before being placed back in their home cage.

2.6.2 INTRA-MFB 6-HYDROXYDOPAMINE LESION SURGERIES

For the MFB lesion, 6-OHDA was weighed out, dissolved in 0.01% sterile ascorbate saline and was kept on ice and in the dark until infusion. All 6-OHDA lesion surgeries were performed as described in the previous section. The MFB was infused unilaterally with 6-OHDA (12 µg) at the following coordinates from bregma: AP -4.0, ML ±1.3 and DV -7.0 below dura. Infusions were completed at a total volume of 3 µl at a rate of 1 µl/min with a further 2 min allowed for diffusion.

2.6.3 INTRA-STRIATAL TRANSPLANTATION SURGERIES

For the transplantation of VM cells, neurotrophic factor (GDF5), anti-inflammatory cytokine (IL-10) and collagen hydrogels (with or without cells and/or GDF5 and/or IL-10), all surgeries were performed as described in section 2.6.1. The striatum was infused unilaterally or bilaterally at coordinates from bregma: AP 0.0, ML ±3.7 and DV -5.0 below dura. Infusions were completed at a total volume of 6 µl at a rate of 1 µl/min with a further 2 min allowed for diffusion.

2.7 BEHAVIOURAL TESTS OF MOTOR IMPAIRMENT

2.7.1 METHAMPHETAMINE-INDUCED ROTATIONAL BEHAVIOUR

In this thesis, methamphetamine-induced rotations were used to assess the dopaminergic asymmetry caused by the MFB 6-OHDA lesion (Ungerstedt and Arbuthnott, 1970). Animals were taken from their home cage and placed into plastic bowls containing standard bedding and were left there for 10 minutes to habituate to this new environment. After this time, rats received an intraperitoneal (i.p) injection of 2.5 mg/kg methamphetamine (**Figure 2.7**). Ipsilateral and contralateral rotations were manually counted for 60 mins in time bins of 10 x 1 minute. Data was expressed as net ipsilateral turns/min.

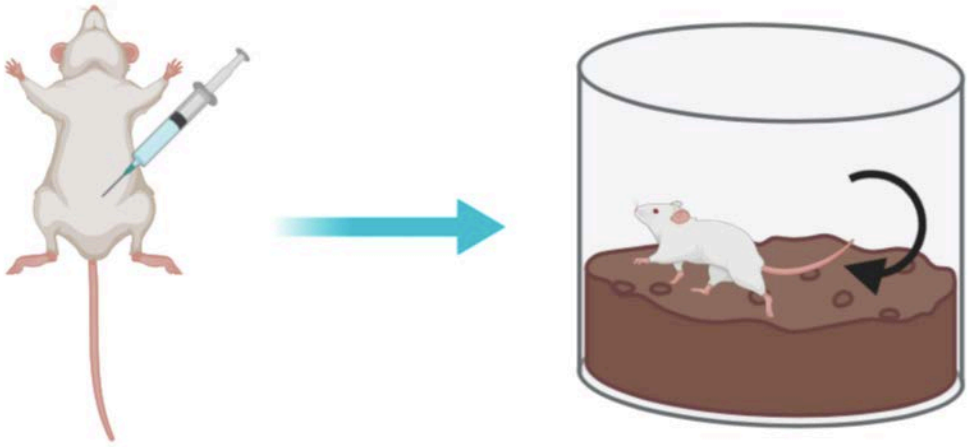


Figure 2.7 Methamphetamine-induced rotational behaviour.

The number of ipsilateral and contralateral rotations per minute were counted for 60 minutes in 10 x 1 min time bins after i.p. administration of methamphetamine (2.5 mg/kg).

2.7.2 CORRIDOR TEST

The corridor test measures sensorimotor neglect of contralateral stimuli (Dowd *et al.*, 2005). 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, one by one, rats are placed for 5 minutes inside the empty corridor (**Figure 2.8a**) and then transferred into the adjacent corridor where containers for CocoPops® (5 to 10 pellets) are arranged as adjacent pairs along the floor. Rats are then free to explore and eat CocoPops® at will, while the side of retrieval and number of retrievals were recorded (**Figure 2.8b**). Each trial lasted 5 minutes or 20 retrievals. Rats with a unilateral dopaminergic lesion tend to ignore food on their contralateral side and retrieve the CocoPops® almost exclusively from their ipsilateral side.

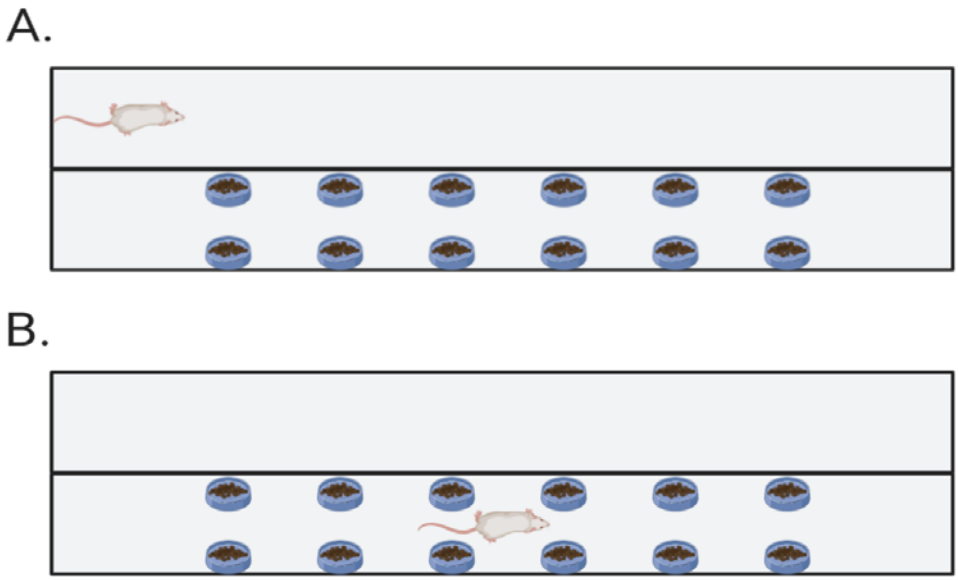


Figure 2.8 Corridor test of sensorimotor neglect.

After a 5-minute habituation in an empty corridor (A), rats were transferred to the adjacent corridor with the containers for CocoPops® (B). The number of retrievals and the side of retrieval were recorded. Trials lasted 5 minutes or 20 retrievals.

2.7.3 WHISKER TEST

The whisker test is also known as the vibrissae-elicited forelimb placement test and it assesses contralateral sensorimotor neglect (Schallert and Tillerson, 2000). In this behavioural test, both hind paws and one forepaw were restrained while the whiskers of the rats were brushed against the side of a table top (**Figure 2.9**). The number of whisker-elicited forepaw placements steps of the unrestrained forepaw out of 10 tests was recorded for both the ipsilateral and contralateral sides of the body.

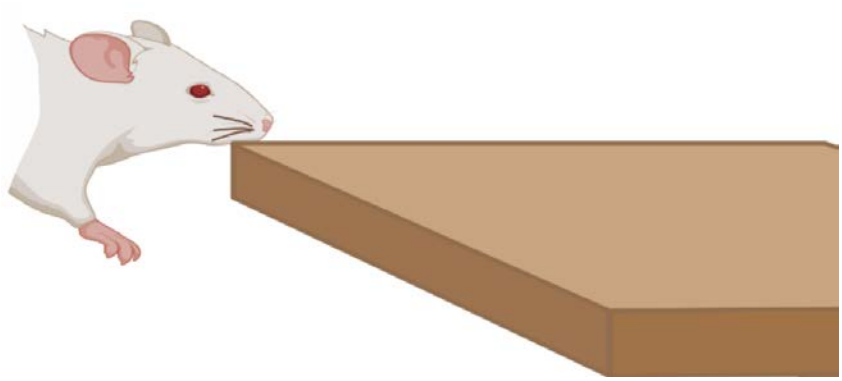


Figure 2.9 Whisker test for sensorimotor integration.

The hind paws and one forepaw were restrained while the rat's whiskers (of the free forepaw) were brushed against the edge of a table consecutively for 10 tests. The number of forepaw placements on the edge of the table was recorded out of 10 tests.

2.7.4 ADJUSTMENT STEP TEST

The adjustment step test evaluates forelimb akinesia (Olsson *et al*., 1995). In this behavioural test, both hind limbs and one forelimb were restrained while the other forelimb touched the table top. Then, the rat was guided across the table top (90 cm in 5 seconds) in a horizontal position and the unrestrained forelimb was assessed for the number of paw adjustments steps while going across in both forepaw and back paw directions (**Figure 2.10**). This task was conducted for both the ipsilateral and contralateral sides of the body. Unilateral dopaminergic lesions in rats result in impairment in forelimb motor function on their contralateral side.

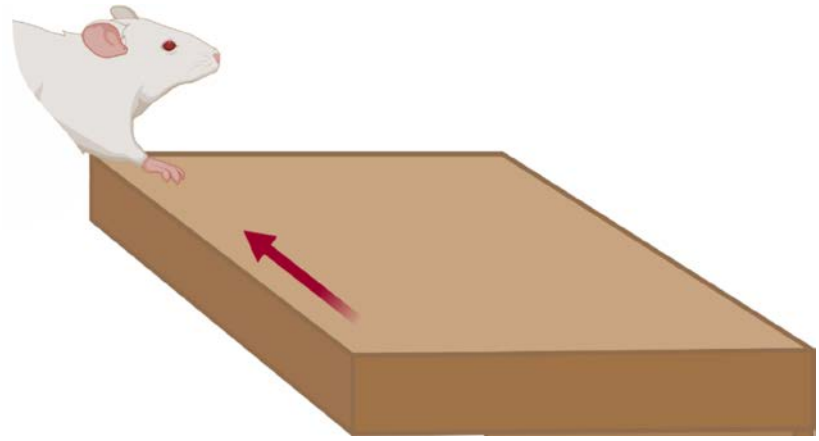


Figure 2.10 Adjustment step test of forelimb akinesia.

The hind limbs and one forelimb were restrained while the free forelimb was guided across a 90 cm space for 5 seconds. The number of forelimb repositions during the 5 seconds were recorded.

2.8 IMMUNOHISTOCHEMISTRY

2.8.1 TISSUE PROCESSING

Animals were sacrificed by terminal anaesthesia with an i.p. injection of 50 mg/kg of pentobarbital and were transcardially perfused with 100 ml heparinised saline (5000 units/litre of heparin) followed by 150 ml of cold 4% PFA. After perfusion of the rats, their brains were rapidly removed and placed in 4% PFA overnight before being cryoprotected in a solution of 25% sucrose with 0.1% sodium azide. Using a freezing stage sledge microtome (Bright, Cambridgeshire, UK), serial coronal sections of 30 µm were cut in series of 12.

2.8.2 IMMUNOHISTOCHEMISTRY

Free floating IHC was performed using the streptavidin-biotin- peroxidase method as previously described (Hoban, 2015; Moriarty, 2018). In brief, endogenous peroxidise activity was quenched using a solution of 3% hydrogen peroxidase and 10% methanol in distilled water. Non-specific binding was blocked using 3% normal serum (goat or horse serum origin was used depending on 2º antibody host) in TBS with 0.2% Triton-X-100 at room temperature for 1 hour. Primary antibody (see **Table 2.1**) was diluted in TBS with 0.2% triton-X-100 and 1:100 normal serum, added to sections and incubated at room temperature overnight. Sections were then incubated in the corresponding

secondary antibody (diluted in TBS) and 1:100 normal serum (see **Table 2.2**) for 3 hours at room temperature. A streptavidin-biotin-horseradish peroxidise solution (Vector, UK) was subsequently added to sections and allowed to incubate for 2 hours. To development the staining, a 0.5% solution of diaminobenzidine tetra hydrochloride (DAB) (Sigma, Ireland) in TNS containing 0.3 µl/ml of hydrogen peroxide was used. Sections were mounted onto gelatin-coated slides, dehydrated in a series of ascending alcohols, cleared in xylene and finally coverslipped using DPX mountant for DAB stained sections (Sigma).

Table 2.1 List of primary antibodies used in this thesis.

Target	Primary antibody	Source	Host	Dilution	Application
Catecholaminergic neurons	TH	Millipore	Mouse	1:1000	IHC/ICC
Astrocytes	GFAP	DAKO	Rabbit	1:2000	IHC
Microglia	CD11b	Chemicon	Mouse	1:400	IHC
Bovine collagen	Collagen	Abcam	Rabbit	1:1000	IHC
Neural outgrowth	βIII tubulin	Millipore	Mouse	1:333	ICC
Human GDF5	GDF5	Abcam and Sigma	Rabbit	Range: 1:50 to 1:500	IHC

IHC: Immunohistochemistry; ICC: Immunocytochemistry

Table 2.2 List of secondary antibodies used in this thesis.

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 488	Millipore	Goat	Mouse	1:800	ICC

IHC: Immunohistochemistry; ICC: Immunocytochemistry

2.9 HISTOLOGICAL QUANTIFICATION

Expression of TH^+, collagen, GDF5, CD11b and GFAP were identified in DAB stained sections. All histological image analysis was performed using ImageJ software. Measurements of graft volume, re-innervation, collagen volume, GDF5 volume and the volume of microgliosis and astrocytosis were quantified using an equation based on Cavalieri's Principle (**Section 2.9.1**). Microglial reactivity (CD11b) and astrocytic reactivity (GFAP) were also quantified using optical density measurements. Finally, the total number of transplanted dopaminergic cells was determined by counting individual TH^+ cell bodies in the transplanted region and correcting using Abercrombie's principle (**Section 2.9.2**).

2.9.1 CAVALIERI'S PRINCIPLE

In this thesis, all volumetric analyses were performed using Cavalieri's Principle. This principle states that: "the volume of an arbitrary shaped object can be estimated in an unbiased manner from the product of the distance between planes and the sum of the areas on systematic random parallel sections through the object" (Garcia *et al*., 2007).

In order to determine graft volume, re-innervation volume, collagen volume and the volume of microgliosis/astrocytosis throughout this thesis, the equation below was used (**Figure 2.11**).

$$V = T_{\mathrm{T}} \times \sum_{i=1}^{i} A_i,$$

$$T_{\mathrm{T}} = \mathrm{NS} \times D,$$

Figure 2.11 Equation of Cavalieri's Principle

V: volume; T_T: product of the distance between the planes; A_i: area determined using ImageJ software; NS: number of sections in the series; D is the known distance between tissue sections.

2.9.2 ABERCROMBIE'S PRINCIPLE

Abercrombie's principle was used to correct the total number of surviving transplanted cells in all relevant studies throughout this thesis (see **Figure 2.12**). This correction is based on the knowledge that "The number of nuclei visible in a microtome section can easily be counted. But not all of the objects thus counted are whole nuclei. Some must be fragments of nuclei, because some nuclei lie partly within the section examined, partly within an adjacent section" (Abercrombie, 1946). Therefore, Abercrombie's principle takes the frequency and thickness of sections, alongside the average cell diameter into account, in order to determine the total number of cells in any given series.

$$T = F \times A \times M / (D/M)$$

Figure 2.12 Abercrombie's principle equation.

T: total number of cells; F: frequency of sections; A: total cell counts; M: thickness of sections; D: average cell diameter.

2.9.3 QUANTIFICATION OF AREA/VOLUME

ImageJ software was used to measure area and subsequently calculate volume. In order to determine graft volume, collagen volume, microglia and astrocyte volume, photomicrographs of striatal sections containing the transplant were taken using an Olympus slide scanner. The ImageJ program was calibrated with a scale by associating the number of pixels with a known distance. The same scale was used for the analysis of all pictures from one immunostaining. Using the ImageJ freehand drawing tool, the area of the graft or the area where the immune response was located, and was measured in each striatal section of a 1 in 6 series (**Figure 2.13**). Using the area measurements obtained, volume was assessed according to Cavalieri's Principle. In these incidences all striatal sections containing DAB staining were measured in a 1 in 6 series.

Figure 2.13 Screenshot of the ImageJ software used to determine area.

The area of interest (i.e. graft area) was outlined using the freehand drawing tool (yellow outline).

2.9.4 QUANTIFICATION OF TH^+ CELL BODIES

In order to quantify the number of surviving transplanted TH^+ cells, the cell counter tool in ImageJ was used to count the number of TH^+ bodies in the grafts (**Figure 2.14**). The complete unilateral denervation of the striatum by the MFB 6-OHDA lesion allowed for the easy identification of transplanted TH^+ cell bodies. Photomicrographs of the transplanted region were captured using an Olympus slide scanner. For each animal, the number of TH^+ cell bodies were counted in each striatal section of a 1 in 6 series containing a dopaminergic cell transplant. The average cell diameter was determined using ImageJ and the total number of transplanted cells was then corrected for using Abercrombie's Principle.

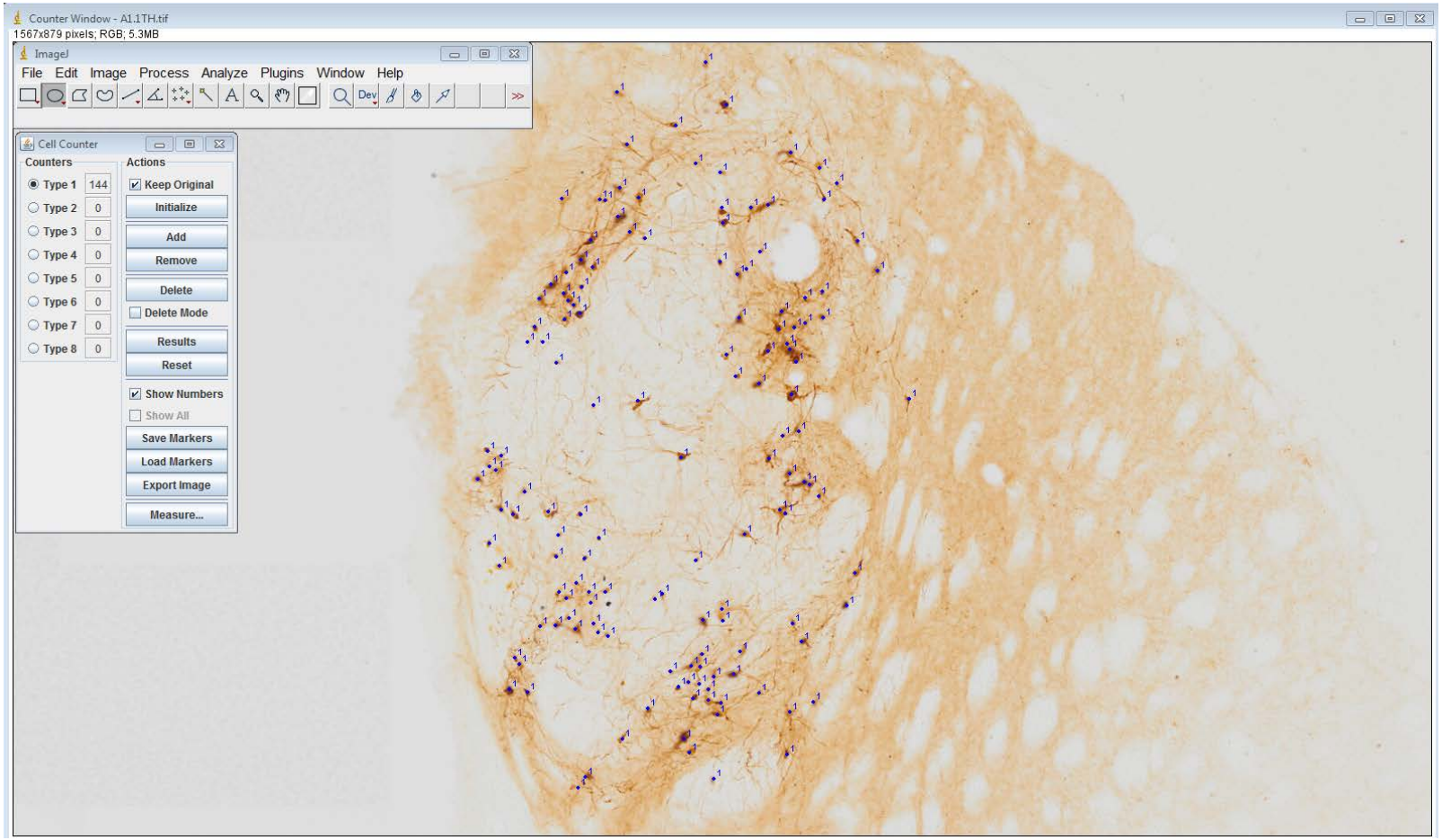


Figure 2.14 Screenshot of the ImageJ software used to quantify the number of TH^+ cell bodies.

Blue dots on the image represent the location of a TH^+ cell body in the transplanted striatum.

2.9.5 QUANTIFICATION OF OPTICAL DENSITY

Optical density measurements were taken to determine the response of the brain's inflammatory cells (microglia and astrocytes) present at the implantation site. In the work presented in this thesis, photomicrographs were obtained using the Olympus slide scanner. All images were taken at the same conditions of magnification and pixel quality. Measurement of optical density was done using the ImageJ program. For this, 3 representative coronal images through the transplant site were chosen for each animal. The site of transplantation was located based on either having the visible transplant (TH^+ or collagen immunostaining) or from visible needle tract sites. A circular shape was centred over the site of transplantation and the density of staining was measured (**Figure 2.15**). To determine the specific staining density, the optical density readings were corrected for non-specific background density. To do this, the same circular shape was placed over an unstained region in the section, the optical density was measured and the corresponding calculation was performed.

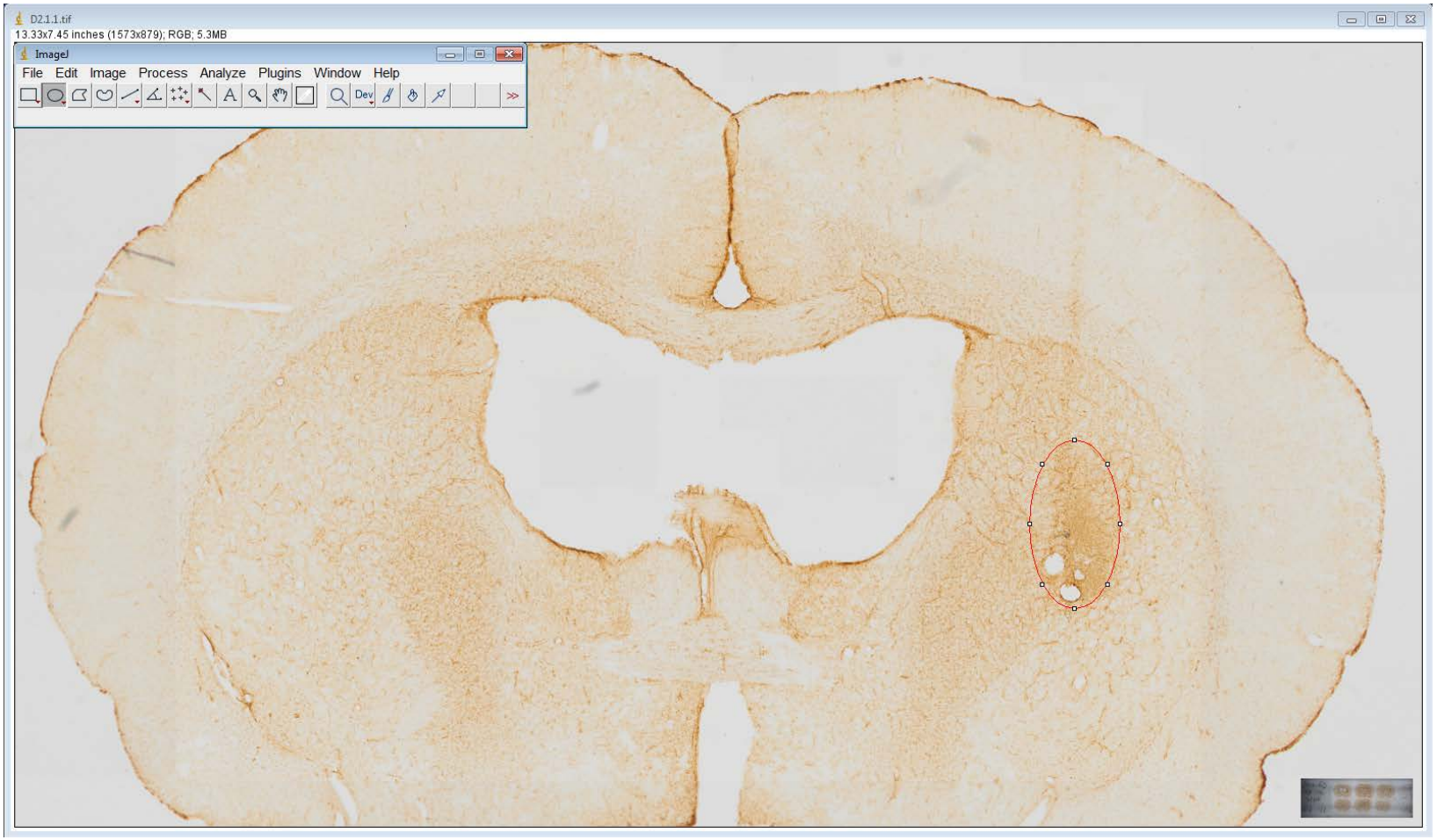


Figure 2.15 Screenshot of ImageJ software used to measure optical density.

The density of the immunostaining was measured for each animal around the graft site (yellow outline).

2.10 STATISTICAL ANALYSIS

Statistics were conducted using SPSS and GraphPad Prism. All data was tested for normality by Shapiro-Wilk's test and homogeneity of variance by Levene's test to determine if the data was parametric or non-parametric, and to analyse it with the appropriate statistical test. Results of parametric data were expressed as mean ± standard error of the mean (SEM) and were analysed using a one-way analysis of variance (ANOVA), two-way ANOVA or a two-way repeated measures ANOVA as appropriate. One-way ANOVA was used to compare more than 2 groups on one factor, whereas a two-way ANOVA was used to compare 2 or more groups on 2 factors. Behavioural data of methamphetamine induced rotations in Chapter 5 were analysed using a two-way repeated measures ANOVA with within subject factor of time and between subject factors of group. When applicable, Bonferroni correction or Tukey's multiple comparison *post-hoc* analyses were carried out as indicated in the text. If the results were non-parametric, data was expressed as median and interquartile range and were analysed using Kruskal-Wallis followed by Dunn's *post-hoc* analyses. In all cases, analysis were deemed significant at $P<0.05$. Throughout the results section, the main effects from the initial statistical test are cited in the body of the results, while the results of the *post-hoc* analyses are shown on the corresponding figure and explained in the figure legend.

Chapter 3: Assessment of the effects of collagen hydrogels and GDF5 on a SH-SY5Y cell line and VM explants.

3.1 INTRODUCTION

After emerging over 40 years ago, dopaminergic cell replacement therapy is now one of the most promising treatments for Parkinson´s disease with one main goal: repair dopamine input to the striatum and therefore motor function (Perlow *et al*., 1979). Transplantation of dopaminergic neurons into the nigrostriatal pathway has the potential to restore dopaminergic transmission in the striatum, whilst also restoring motor function. Unfortunately, most of the dopaminergic cells die within the first 4 days post-transplantation (Brundin *et al*., 1988; Mahalik *et al*., 1994; Zawada *et al*., 1998; Emgård *et al*., 1999; Sortwell *et al*., 2001) hindering the outcome of cell replacement therapy. Therefore, it is necessary to find new methods to enhance the survival and efficacy of the transplanted cells and the key might be the incorporation of biomaterials into the transplantation process.

Biomaterials are materials that are used and adapted for a medical application, intended to interact with a biological system (Orive *et al*., 2009). The addition of biomaterials such as collagen hydrogels has shown potential to improve cell replacement therapy by acting as a supportive and protective matrix for the cells, that can also provide localised and sustained trophic factor delivery, while creating a physical barrier between the transplanted cells and the host immune response following intracerebral transplantation (Orive *et al*., 2009; Hoban *et al*., 2013; Moriarty and Dowd, 2018).

Collagen is the most abundant protein in the body, as it is a major component of skin, bone, cartilage, tendons, and ligaments. More than 20 genetically different forms have been identified of which collagen type 1 is the most abundant and extensively studied for biomedical applications (Hayashi, 1994). Collagen gels seem to be promising matrices for drug delivery and for cell replacement therapy due to characteristics such as high mechanical strength, good biocompatibility, low antigenicity and their ability to be crosslinked which allows the tailoring of their mechanical, degradation and water uptake properties (Wallace and Rosenblatt, 2003). Collagen can form hydrogels *in situ*

under physiological conditions by the change in structure of the elongated triple helices, to that of compact coils (Xu and Kopeček, 2007).

Neurotrophic factors that have beneficial effects on the survival, integration and efficacy of transplanted midbrain dopaminergic neurons, such as GDF5 (Sullivan *et al*., 1998) or GDNF (Andereggen *et al*., 2009), can be encapsulated in collagen hydrogels to prolong their release rate, increase their therapeutic effect and improve the effectiveness of cell replacement therapy (Wallace and Rosenblatt, 2003; Moriarty *et al*., 2019a). GDF5 is a potent neurotrophic molecule that is expressed in many regions of the developing CNS, neonatal and adult rat brain (Storm *et al*., 1994; Krieglstein *et al*., 1995; O'Keeffe *et al*., 2004a).

In this chapter, through a series of *in vitro* and *ex vivo* studies on an immortalised SH-SY5Y cell line and on a VM explant culture system, the assessment of collagen hydrogels and GDF5 for biocompatibility and neurotrophic effects, was completed. The neuroblastoma SH-SY5Y cell line is an *in vitro* model widely used in Parkinson´s disease research, even though these cells do not reproduce all the dopaminergic features of primary VM cultures, this cell line is a good model to study the cytocompatibility of the collagen hydrogels and the neuroprotective effects of GDF5. Nevertheless, cell cultures are two-dimensional and do not accurately reflect the *in vivo*-like situation in the organism. Hence, a more complex model, a VM explant culture system was also used to test our collagen hydrogels and GDF5. Explant cultures are dissected sections of brain (in this case the VM of E14 rats) that are maintained "alive" *ex vivo* as a whole, preserving the three-dimensional structure and synaptic organization of the original tissue.

Therefore, the aims of this chapter are to 1) determine the biocompatibility of collagen hydrogels on an immortalized SH-SY5Y cell line and on a VM explant system, 2) assess the effects of GDF5 on the growth and development of the VM explants and 3) determine if after a 6-OHDA insult, GDF5 enrichment of collagen hydrogels have neurorestorative effects on SH-SY5Y cells and on VM explants.

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

This chapter will detail the results of 5 *in vitro* studies using SH-SY5Y cells and 5 *ex vivo* studies on a VM explant system. These studies were designed to assess the biocompatibility of collagen hydrogels and test the neurotrophic effects of GDF5 on explants and SH-SY5Y cells. The overall experimental design used in this chapter is shown below in **Figure 3.1**.

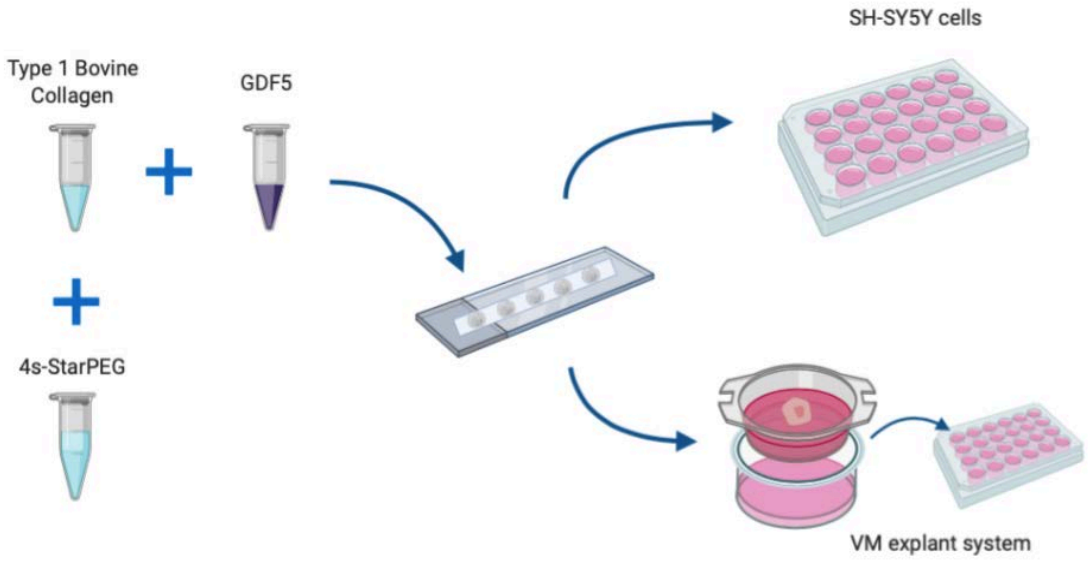


Figure 3.1 *In vitro* and *ex vivo* assessment of collagen hydrogels and GDF5.

Schematic representation of the overall experimental design of this chapter. Through a series of *in vitro* studies on an immortalised SH-SY5Y cell line and *ex vivo* studies on a VM explant system, the assessment of the effects on biocompatibility of type 1 bovine collagen hydrogels and tests of the neurotrophic effects of GDF5 were completed. Collagen hydrogels were prepared with various concentrations of 4s-StarPEG crosslinker and formed on a sterilised super hydrophobic surface (Teflon® tape) and placed in an incubator at 37°C to gel. Collagen hydrogels were then tested in SH-SY5Y cells and on VM explants. Following these experiments, GDF5 was encapsulated in collagen hydrogels and its effects were tested on the same cell line and explants.

3.2.1.1 Experimental designs of *in vitro* studies with SH-SY5Y cells.

To address the aims of this chapter, the following *in vitro* studies were completed:

1) ***In vitro* study 1:** Comparison of gelation times between gels made with 3 concentrations of 4s-StarPEG crosslinker from two sources.

In this study we focused on the effects of crosslinking on gelation time of type 1 collagen hydrogels from 2 different sources. Collagen from Vornia or from Collagen Solutions were used to make unloaded hydrogels with 3 different concentrations of 4s-StarPEG crosslinker (see **Table 3.1** for experimental groups; see **Section 2.3** for details). These hydrogels were formulated on the bench on a super hydrophobic surface (Teflon® tape), placed at 37°C and the time it took gels to gelate was measured. Collagen hydrogels were considered gelled once it was not possible to pipette them up.

Table 3.1 Experimental groups for *in vitro* study 1.

Collagen solutions collagen (1 mg/ml of 4s-StarPEG crosslinker) n=3	Vornia collagen (1 mg/ml of 4s-StarPEG crosslinker) n=3
Collagen solutions collagen (2 mg/ml of 4s-StarPEG crosslinker) n=3	Vornia collagen (2 mg/ml of 4s-StarPEG crosslinker) n=3
Collagen solutions collagen (4 mg/ml of 4s-StarPEG crosslinker) n=3	Vornia collagen (4 mg/ml of 4s-StarPEG crosslinker) n=3

2) ***In vitro* study 2:** Assessment of the cytocompatibility of collagen hydrogels, made with 3 concentrations of 4s-StarPEG crosslinker, using SH-SY5Y cells after 24 hr exposure.

In this study we assessed the cytocompatibility of collagen hydrogels made with increasing crosslinker concentrations (1, 2 or 4 mg/ml). Under standard sterile cell culture conditions, SH-SY5Y cells were cultured in T-75 flasks at 37°C with 5% CO_2. SH-SY5Y cells were enzymatically lifted from the culture surface using trypsin and

resuspended in fresh warmed medium at a density of 250,000 cell per ml. Cells were then seeded in a 24-well plate (500 µl per well) and incubated for 24 hr before exposure to the pre-formed hydrogels.

Collagen hydrogels of 50 µl were made with one of three different concentrations of 4s-StarPEG crosslinker, formed on a previously sterilised (by UV radiation) super hydrophobic surface and placed at 37°C to gel (see **section 2.3** for details). Once all the hydrogels gelated, they were incubated with the cells (2 x 50 µl per well) for 24 hr (see **Table 3.2** for experimental groups). To determine the cytocompatibility of the collagen hydrogels, alamarBlue®, MTT assay and ICC for βIII tubulin and TH^+ were completed (see **Section 2.4.2** and **2.4.3** for details).

Table 3.2 Experimental groups for *in vitro* study 2.

Untreated control n=3	Hydrogels with 1 mg/ml of 4s-StarPEG crosslinker n=3
Hydrogels with 2 mg/ml of 4s-StarPEG crosslinker n=3	Hydrogels with 4 mg/ml of 4s-StarPEG crosslinker n=3

3) ***In vitro* study 3**: Assessment of the effects of different concentrations of 6-OHDA on SH-SY5Y cells after 24 hr exposure.

In this concentration-response experiment, we assessed the toxic effects of 24 hr exposure to 6-OHDA on the cellular metabolic activity of SH-SY5Y cells. SH-SY5Y cells were cultured and resuspended in fresh warmed medium at a density of 250,000 cell per ml. Cells were then seeded in a 24-well plate (500 µl per well) and incubated for 24 hr, following by an incubation with 20, 50 or 100 µM per well of 6-OHDA for 24 hr (see **Table 3.3** for experimental groups). To determine cell viability, alamarBlue®, MTT assay and ICC for βIII tubulin and TH^+ were completed (see **Section 2.4.2** and **Section 2.4.3** for details).

Table 3.3 Experimental groups for *in vitro* study 3.

Untreated control n=3	20 μM of 6-OHDA n=3
50 μM of 6-OHDA n=3	100 μM of 6-OHDA n=3

4) ***In vitro* study 4**: Assessment of the effects of 24 hr treatment with GDNF or GDNF-encapsulated in collagen hydrogels, on SH-SY5Y cells after 6-OHDA exposure.

In this study we focused on assessing the effects of GDNF and GDNF-encapsulated in collagen hydrogels, on SH-SY5Y cells after 24 hr exposure to 50 μM of 6-OHDA. SH-SY5Y cells were cultured and resuspended in fresh warmed medium at a density of 250,000 cell per ml. Cells were then seeded in a 24-well plate (500 μl per well) and incubated for 24 hr before exposure to 6-OHDA, followed by the addition of 50 μl of the 6-OHDA stock for a final concentration of 50 μM per well for 24 hr.

Collagen hydrogels of 30 μl were pre-formed (as specified in **Section 2.3**) containing either 1 or 5 μg per μl of GDNF and placed at 37°C to gel. Once all the hydrogels gelated, they were incubated with the cells (1 x 30 μl per well) for 24 hr (see **Table 3.4** for experimental groups). To assess cell viability, alamarBlue®, MTT assay and ICC for βIII tubulin and TH^+ were completed (see **Section 2.4.2** and **Section 2.4.3** for details).

Table 3.4 Experimental groups for *in vitro* study 4.

Untreated control n=3	
6-OHDA control n=3	Unloaded hydrogel (4 mg/ml of 4s-StarPEG crosslinker) n=3
6-OHDA + GDNF (1 μg/μl) n=3	6-OHDA + GDNF in hydrogel (1 μg/μl) n=3
6-OHDA + GDNF (5 μg/μl) n=3	6-OHDA + GDNF in hydrogel (5 μg/μl) n=3

5) ***In vitro* study 5**: Assessment of the effects of 24 hr treatment with GDF5 or GDF5-encapsulated in collagen hydrogels, on SH-SY5Y cells after 6-OHDA exposure.

In this study we focused on assessing the effects of GDF5 and GDF5-encapsulated in collagen hydrogels, on SH-SY5Y cells after 24 hr exposure to 50 μM of 6-OHDA. SH-SY5Y cells were cultured and resuspended in fresh warmed medium at a density of 250,000 cell per ml. Cells were then seeded in a 24-well plate (500 μl per well) and incubated for 24 hr before exposure to 6-OHDA, followed by the addition of 50 μl of the 6-OHDA stock for a final concentration of 50 μM per well for 24 hr.

Collagen hydrogels of 30 μl were pre-formed (as specified in **Section 2.3**) containing either 1 or 5 μg per μl of GDF5 and placed at 37°C to gel. Once all the hydrogels gelated, they were incubated with the cells (1 x 30 μl per well) for 24 hr (see **Table 3.5** for experimental groups). To assess cell viability, alamarBlue®, MTT assay and ICC for βIII tubulin and TH^+ were completed (see **Section 2.4.2** and **Section 2.4.3** for details).

Table 3.5 Experimental groups for *in vitro* study 5.

Untreated control n=3	
6-OHDA control n=3	Unloaded hydrogel (4 mg/ml of 4s-StarPEG crosslinker) n=3
6-OHDA + GDF5 (1 μg/μl) n=3	6-OHDA + GDF5 in hydrogel (1 μg/μl) n=3
6-OHDA + GDF5 (5 μg/μl) n=3	6-OHDA + GDF5 in hydrogel (5 μg/μl) n=3

3.2.1.2 Experimental designs of *ex vivo* studies with VM explants.

1) **Explant study 1:** Comparison between narrow and wide explanted tissue after 8 days *in vitro*.

In this study we focused on: 1) setting up a VM explant system in our laboratory and 2) assessing the difference between explants obtained by narrow dissections and explants obtained by wide dissections. For this, E14 rat embryos were dissected to obtain the mesencephalic-diencephalic tube (see **Section 2.5.2** for details). Whole tissue explants were dissected from two regions: 1) narrow dissections (comprising only the medial mesencephalon) or 2) wide dissections (comprising the mid-lateral to medial mesencephalon on both sides). The explants were grown using the air medium interface method (Stoppini *et al*., 1991), and were 4% PFA fixed (see **Table 3.6** for experimental groups). Free floating TH^+ immunohistochemistry was completed to identify dopaminergic neurons.

To allow further assessment of the narrow explanted nigral tissue system, explants were cultured for longer time-points (see **Table 3.7** for experimental groups) and TH^+ immunohistochemistry was completed to identify survival of dopaminergic neurons.

Table 3.6 Experimental groups for explant study 1.

Narrow dissection	Wide dissection
2 DIV n=3	2 DIV n=3
4 DIV n=3	4 DIV n=3
6 DIV n=3	6 DIV n=3
8 DIV n=3	8 DIV n=3

DIV: days *in vitro*

Table 3.7 Experimental groups for assessment of explant study 1 at longer time-points.

Narrow dissection
12 DIV n=3
15 DIV n=3
18 DIV n=3
21 DIV n=3
24 DIV n=3

2) **Explant study 2:** Assessment of the effects of collagen hydrogels, made with different concentrations of 4s-StarPEG crosslinker, on the viability of explants, after 48 hr exposure.

In this study we assessed the effects of collagen hydrogels made with increasing crosslinker concentrations (1, 2 or 4 mg/ml) on viability of dopaminergic cells of the explants. Explants were narrowly dissected, and cultured to 4 DIV. On day 4, collagen hydrogels of 30 µl were made with one of three different concentrations of 4s-StarPEG crosslinker, formed on a super hydrophobic surface and placed at 37°C to gel (see **section 2.3** for details; see **Table 3.8** for experimental groups). Once all the hydrogels gelated, they were incubated in the medium of the explants (1 x 30 µl per well) for 48 hr (see **section 2.5.2.2** for details). In order to assess the viability of the explants, TH^+ immunohistochemistry and measurements of TH^+ optical density were completed.

Table 3.8 Experimental groups for explant study 2.

Untreated control n=3	Hydrogels with 1 mg/ml of 4s-StarPEG crosslinker n=3
Hydrogels with 2 mg/ml of 4s-StarPEG crosslinker n=3	Hydrogels with 4 mg/ml of 4s-StarPEG crosslinker n=3

3) **Explant study 3:** Assessment of the effects of GDF5 and GDNF (alone or encapsulated in a collagen hydrogel) on the viability of dopaminergic cells in explants.

In this study we assessed the effects of early exposure to GDF5 or GDNF (alone or encapsulated in a collagen hydrogel) on explant dopaminergic development. For this, explants were dissected and cultured with 5 µg/µl of GDNF or 5 µg/µl of GDF5 (alone or encapsulated in a collagen hydrogel) until 15 DIV (medium and treatment were changed every 3 days; see **Table 3.9** for experimental groups). In order to assess dopaminergic survival in the explants, TH^+ immunohistochemistry and measurements of TH^+ optical density were completed.

Table 3.9 Experimental groups for explant study 3.

Untreated control n=3	Unloaded hydrogel (4 mg/ml of 4s-StarPEG crosslinker) n=3
GDNF (5 μg/μl) n=3	GDNF in hydrogel (5 μg/μl) n=3
GDF5 (5 μg/μl) n=3	GDF5 in hydrogel (5 μg/μl) n=3

4) **Explant study 4:** Assessment of the effects of LPS, Poly I:C or 6-OHDA on dopaminergic cells of explants after 48 or 72 hr exposure.

In this study we assessed the effects of LPS, Poly I:C and 6-OHDA on dopaminergic cells in the explants after 48 or 72 hr exposure to try to establish an explant model of dopaminergic cell loss. For this, explants were dissected and cultured to 4 DIV. Explants were then exposed to either 2mM 6-OHDA, 100 μg/ml Poly I:C or 50 μg/ml LPS for either 48 or 72 hr (see **Section 2.5.2.2** for details; see **Table 3.10** for experimental groups). Doses were adapted for VM explant work based on published literature of *in vitro* assays (Storch *et al.*, 2000; Qian *et al.*, 2006a, 2006b; Lopes *et al.*, 2010; Toulouse *et al.*, 2012). In order to assess the survival of dopaminergic cells in the explants, TH^+ immunohistochemistry and measurements of TH^+ optical density were completed.

Table 3.10 Experimental groups for explant study 4.

48 hr exposure	72 hr exposure
Untreated control n=3	Untreated control n=3
2 mM 6-OHDA n=3	2 mM 6-OHDA n=3
100 μg/ml Poly I:C n=3	100 μg/ml Poly I:C n=3
50 μg/ml LPS n=3	50 μg/ml LPS n=3

5) **Explant study 5:** Assessment of the effects of treatment with GDF5 or GDF5 encapsulated in a hydrogel, on dopaminergic cells of explants after 72 hr exposure to 6-OHDA.

In this study we assessed the effects of GDF5 and GDF5-functionalised collagen hydrogels on dopaminergic cells of explants after 72 hr exposure to 6-OHDA. Explants were dissected and cultured to 4 DIV before exposing them to 2 mM 6-OHDA for 72 hr After this time, explants received treatment of GDF5 (alone or in hydrogel) at a concentration of 5 or 20 μg/μl until 12 DIV (see **Section 2.5.2.2** for details; see **Table 3.11** for experimental groups). In order to assess dopaminergic survival in the explants, TH^+ immunohistochemistry and measurements of TH^+ optical density were completed.

Table 3.11 Experimental groups for explant study 5.

Untreated control n=3	
6-OHDA control n=3	6-OHDA + unloaded hydrogel (4 mg/ml of 4s-StarPEG crosslinker) n=3
6-OHDA + GDF5 (5 µg/µl) n=3	6-OHDA + GDF5 in hydrogel (5 µg/µl) n=3
6-OHDA + GDF5 (20 µg/µl) n=3	6-OHDA + GDF5 in hydrogel (20 µg/µl) n=3

3.3 RESULTS

3.3.1 *IN VITRO* STUDIES

3.3.1.1 *In vitro* study 1: Comparison of gelation times between gels made with three concentrations of 4s-StarPEG crosslinker from two sources.

In this study, we assessed the effects of three increasing crosslinker concentrations on gelation time of collagen hydrogels made from two different sources of type 1 collagen. Overall, hydrogels made with collagen from Vornia took significantly longer to gel in comparison to hydrogels made with Collagen Solutions collagen (**Figure 3.2**; Source, $F_{(1,12)}$=289, P<0.001). Additionally, the results indicate that the higher the concentration of 4s-StarPEG used, the shorter the time taken for gelation (**Figure 3.2**; Crosslinker, $F_{(2,12)}$=2748, P<0.001). Collagen from Vornia was chosen to be used in the work presented ahead in this thesis.

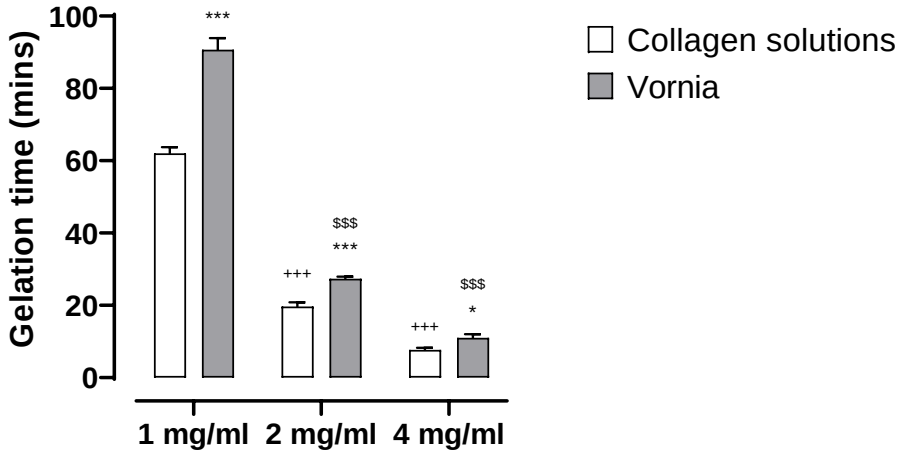


Figure 3.2 Comparison of gelation time between Vornia collagen and Collagen Solutions collagen with different concentrations of 4s-StarPEG crosslinker.

Gels made out of Vornia collagen had greater gelation time than gels made with Collagen Solutions collagen. The crosslinking of hydrogels with rising levels of 4s-StarPEG significantly decreased the time required for gelation. Data are represented as mean ± SEM and were analysed by two-way ANOVA with *post-hoc* Bonferroni. *P<0.05 Vornia vs. Collagen Solutions, ***P<0.001 Vornia vs. Collagen Solutions. $^{\$\$\$}$P<0.001 vs. Vornia's preceding crosslinker concentration. $^{+++}$P<0.001 vs. Collagen Solutions' preceding crosslinker concentration. n= 3 per group.

3.3.1.2 *In vitro* study 2: Assessment of the cytocompatibility of collagen hydrogels, made with 3 concentrations of 4s-StarPEG crosslinker, using SH-SY5Y cells after 24 hr exposure

In order to determine if collagen hydrogels of increasing crosslinker concentrations (1, 2 or 4 mg/ml 4s-StarPEG) were cytocompatible, SH-SY5Y cells were incubated with pre-formed hydrogels. The immunofluorescent staining for βIII tubulin showed that the presence of the hydrogels did not hinder the survival and growth of the SH-SY5Y cells (**Figure 3.3a**). TH^+ immunocytochemistry was also not affected by the collagen hydrogels (**Figure 3.3b**). The results from the alamarBlue® assay (**Figure 3.4a**; Treatment, $F_{(3,8)}$=0.786, P>0.05) and the MTT assay (**Figure 3.4b**; Treatment, $F_{(3,8)}$=0.607, P>0.05) showed that none of the collagen hydrogels had a negative impact on the viability of SH-SY5Y cells when compared to the untreated control.

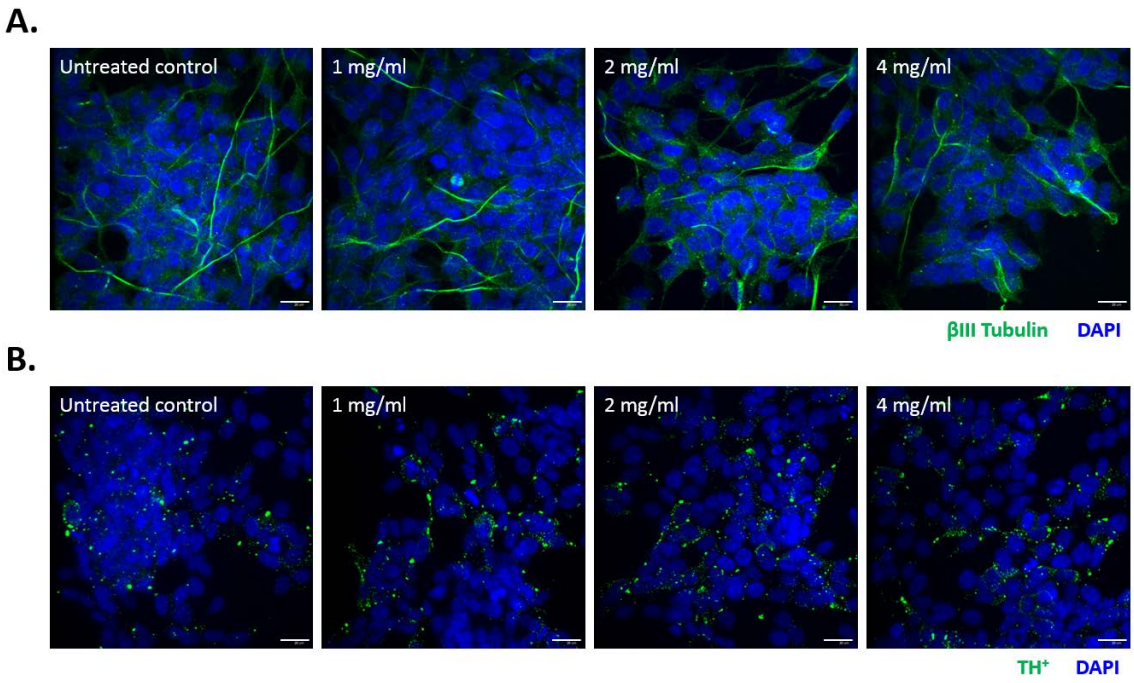


Figure 3.3 Cytocompatibility assessment of collagen hydrogels made with various concentrations of 4s-StarPEG crosslinker.

Photomicrographs represent the immunofluorescent staining for βIII tubulin (A) and TH^+ (B) counterstained with DAPI. The βIII tubulin staining showed that incubation of collagen hydrogels with various 4s-StarPEG concentrations had no negative effect on the survival and outgrowth of SH-SY5Y cells. The TH^+ immunostaining showed fluorescence around some SH-SY5Y cells and was not affected by the collagen hydrogels. Scale bar represents 200 μm.

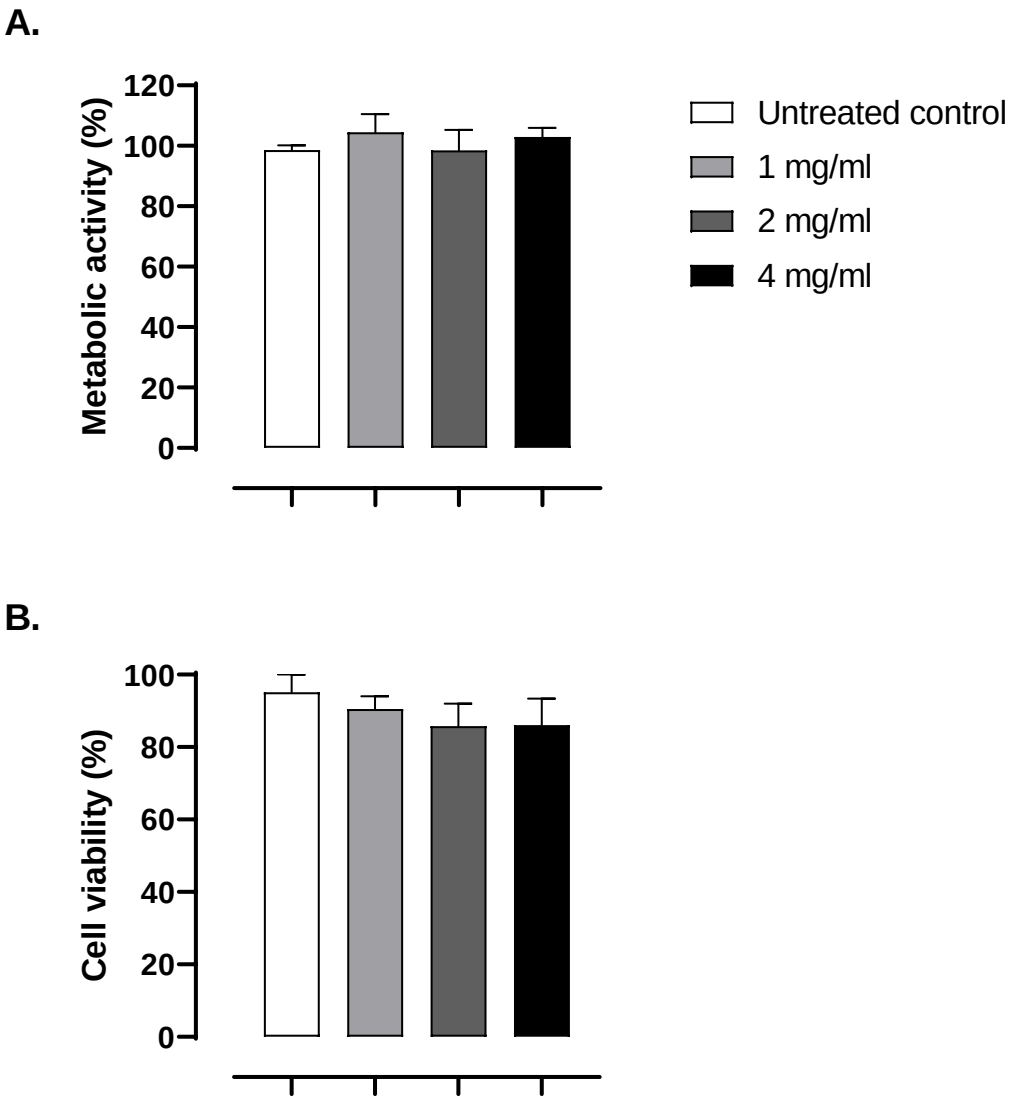


Figure 3.4 Metabolic activity of SH-SY5Y cells after 24 hr exposure to collagen hydrogels made of rising concentrations of 4s-StarPEG crosslinker.

Metabolic activity was measured by alamarBlue® assay (A) and MTT assay (B). The results indicate that the collagen hydrogels are cytocompatible with SH-SY5Y cells. Data are represented as mean ± SEM and analysed using One-way ANOVA. n=3 per group.

3.3.1.3 *In vitro* study 3: Assessment of the effects of different concentrations of 6-OHDA on SH-SY5Y cells after 24 hr exposure

In this study, we assessed the damaging effects of various concentrations of 6-OHDA (20, 50 or 100 μM) on the metabolic activity of SH-SY5Y cells. The

immunofluorescent staining for βIII tubulin showed an observable reduction of axonal growth that intensifies as the concentrations of 6-OHDA increases (**Figure 3.5a**). The TH^+ immunocytochemistry showed some staining around some cells, and this staining decreases with the increasing concentrations of 6-OHDA (**Figure 3.5b**). Additionally, there is an observable loss of DAPI staining, indicating toxicity of 6-OHDA. This loss increases as the concentrations of 6-OHDA increase (**Figure 3.5**). The results from the alamarBlue® indicate that as the concentration of 6-OHDA increases, there is a significant reduction in the metabolic activity (**Figure 3.6a**; Treatment, $F_{(3,8)}$=9.12, P<0.05) of SH-SY5Y cells when compared to the untreated control. This reduction in metabolic activity can be attributed to cell death. These results were confirmed by the MTT assay (**Figure 3.6b**; Treatment, $F_{(3,8)}$=19.14, P<0.001).

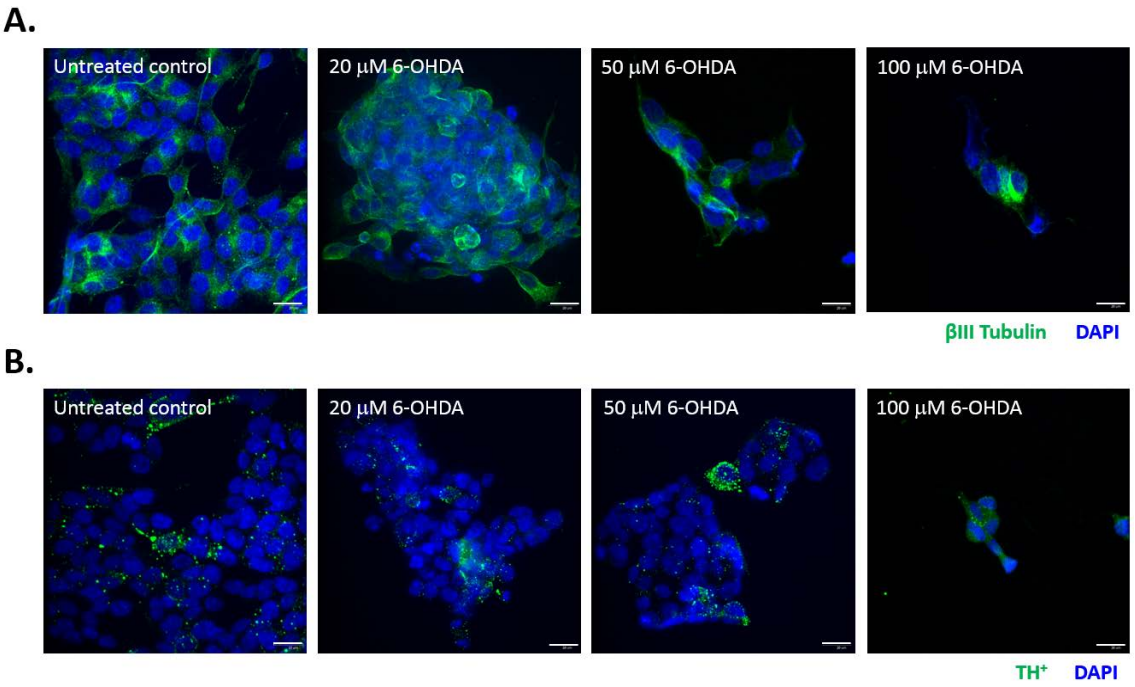


Figure 3.5 Effects on survival and growth of SH-SY5Y cells after 24 hr exposure to different concentrations of 6-OHDA.

Photomicrographs represent the immunofluorescent staining for βIII tubulin (A) and TH^+ (B) counterstained with DAPI. The βIII tubulin staining showed that increasing concentrations of 6-OHDA had detrimental effects on the survival and outgrowth of SH-SY5Y cells. The TH^+ immunostaining showed some fluorescence around some SH-SY5Y cells that decrease with the increasing concentrations of the neurotoxin. DAPI staining decreased with increasing concentrations of 6-OHDA, indicating toxicity of the neurotoxin. Scale bar represents 200 μm.

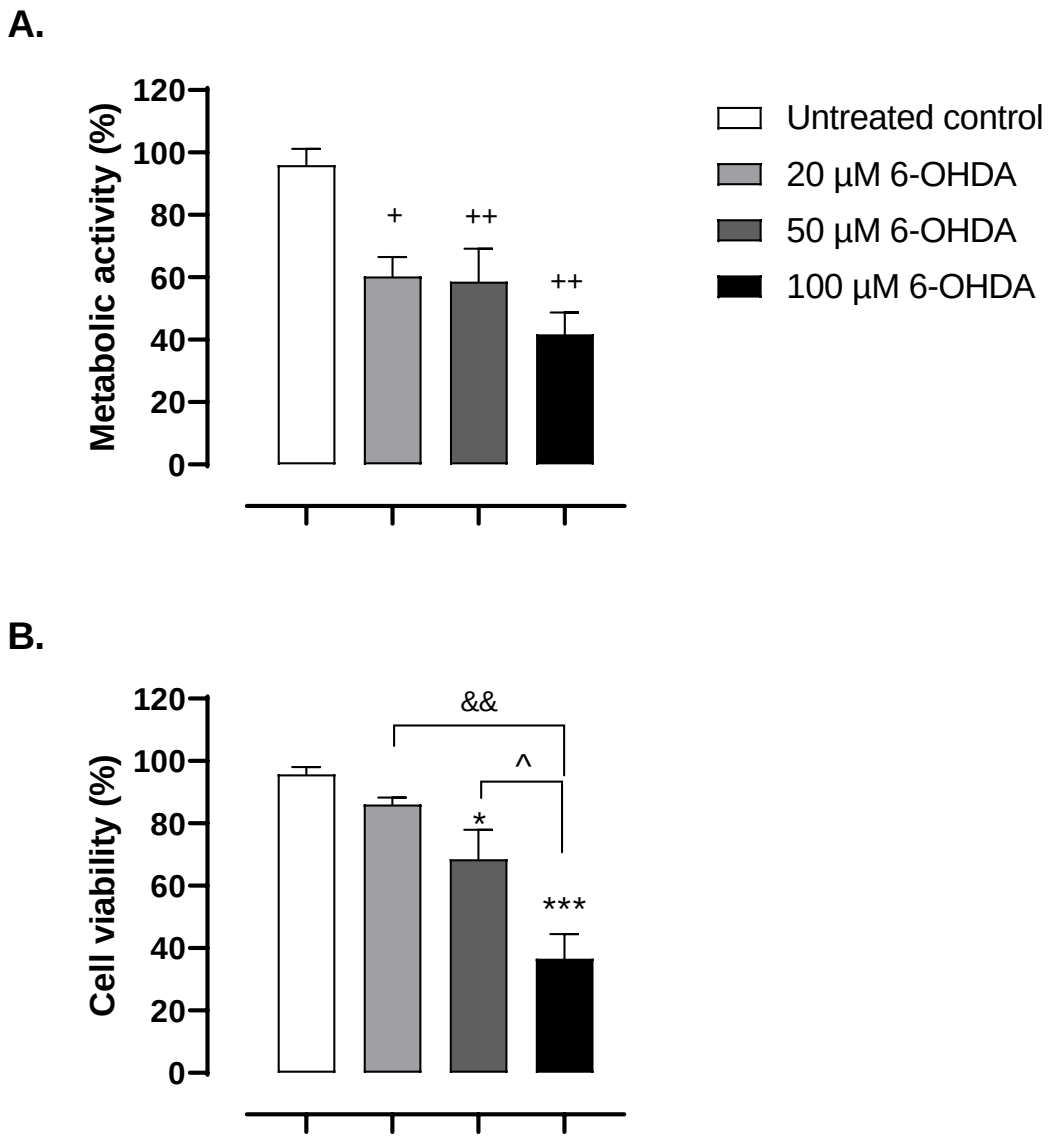


Figure 3.6 Metabolic activity of SH-SY5Y cells after 24 hr exposure to 6-OHDA.

Metabolic activity of SH-SY5Y cells was measured by alamarBlue® assay (A), and MTT assay (B). As the concentration of 6-OHDA increases, there is a significant reduction in the metabolic activity of SH-SY5Y cells compared to the untreated group. This reduction was particularly high in the group exposed to 100 µM 6-OHDA. Data are represented as mean ± SEM and were analysed using One-way ANOVA, followed by Bonferroni *post-hoc* test. n= 3 per group. $^{+}p<0.05$ vs untreated control, $^{++}p<0.01$ vs Control, $^{*}p<0.05$ vs untreated control, $^{***}p<0.001$ vs untreated control, $^{\wedge}p<0.05$ vs 100 µM 6-OHDA, $^{\&\&}$ $p<0.01$ vs 100 µM 6-OHDA.

3.3.1.4 *In vitro* study 4: Assessment of the effects of 24 hr treatment with GDNF or GDNF-encapsulated in collagen hydrogels, on growth and metabolic activity of SH-SY5Y cells after 6-OHDA exposure

After finding a concentration of 6-OHDA (50 μM) that resulted in an intermediate reduction of viable SH-SY5Y cells, we then assessed the effects of GDNF (1 or 5 μg/μl alone or encapsulated in a hydrogel) on this cell line after exposure to 6-OHDA for 24 hr. The immunofluorescent staining for βIII tubulin showed an observable recovery in SH-SY5Y growth induced by both concentrations of GDNF but this effect was greater when GDNF was encapsulated in a hydrogel (**Figure 3.7**). The TH^+ immunocytochemistry was minimal and very few appear to have TH^+ staining (**Figure 3.8**). The alamarBlue® results showed that SH-SY5Y cells exposed to both concentrations of GDNF encapsulated in a hydrogel had higher metabolic activity after lesion (**Figure 3.9a**; Treatment, $F_{(3,14)}$=35.29, P<0.001). Additionally, the treatment with 5 μg/μl GDNF alone promoted recovery in metabolic activity but was not as effective as when it was encapsulated in a hydrogel. The results also showed more activity in cells of the group that received unloaded collagen hydrogels after lesion. The results from the MTT assay, showed that the only treatment that significantly increased cell viability was 5 μg/μl of GDNF alone or encapsulated in a collagen hydrogel (**Figure 3.9b**; Treatment, $F_{(3,14)}$=39.15, P<0.001).

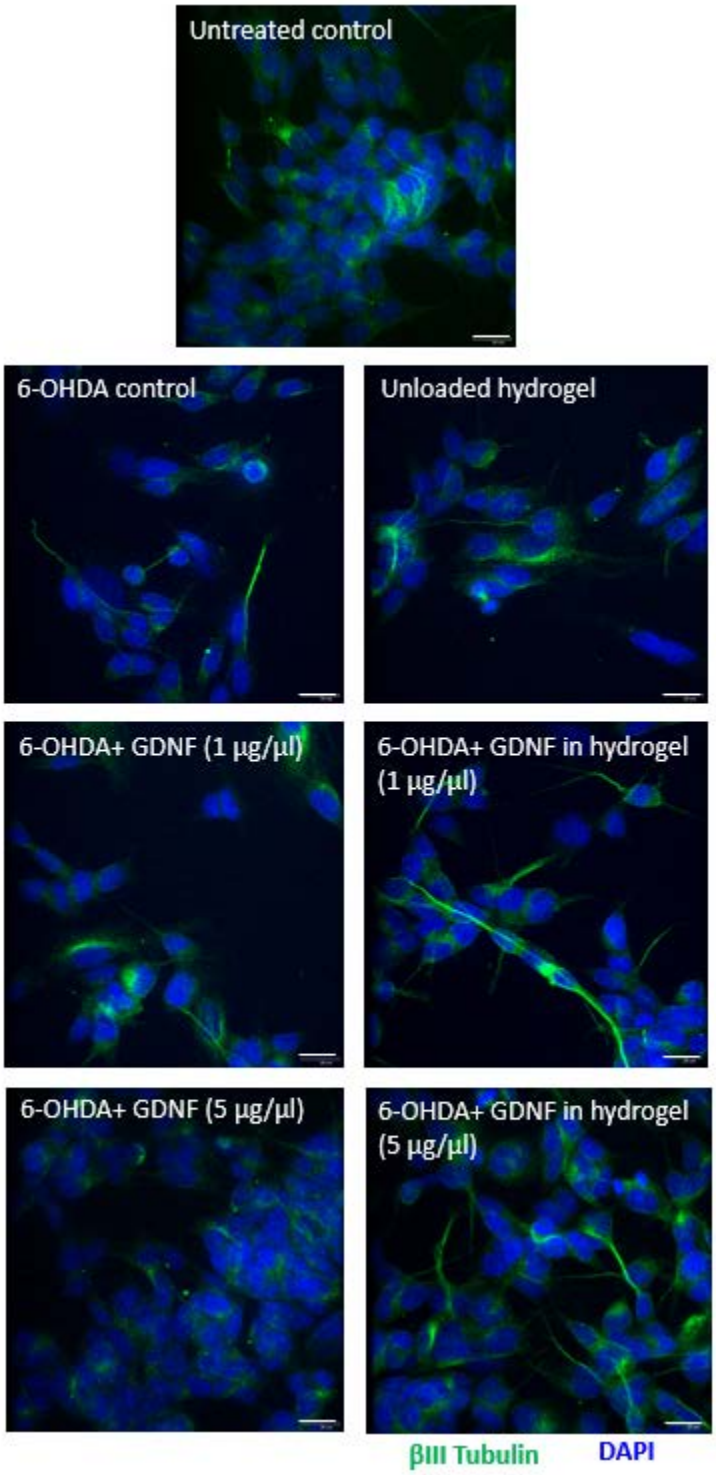


Figure 3.7 βIII tubulin assessment of the effects on growth of SH-SY5Y cells after 24 hr treatment with GDNF after 6-OHDA exposure.

Photomicrographs represent the immunofluorescent staining of βIII tubulin counterstained with DAPI. The immunostaining showed an observable recovery in SH-SY5Y cells induced by both concentrations of GDNF encapsulated in a hydrogel. Scale bar represents 200 µm.

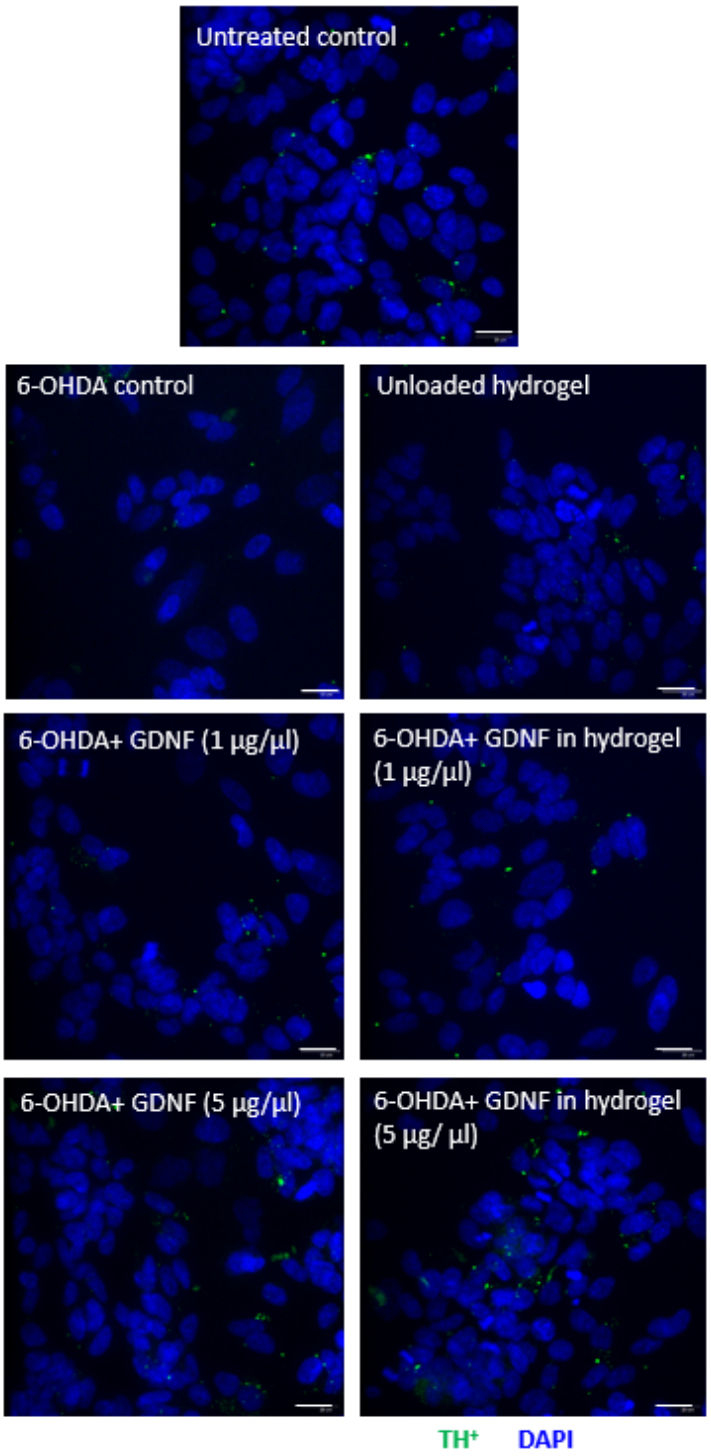


Figure 3.8 TH^+ assessment of the effects on survival and growth of SH-SY5Y cells after 24 hr treatment with GDNF after 6-OHDA exposure.

Photomicrographs represent the immunofluorescent staining for TH^+ counterstained with DAPI. DAPI staining shows the presence of viable cells in the various culture conditions, but very few appear to show TH^+ staining. Scale bar represents 200 µm.

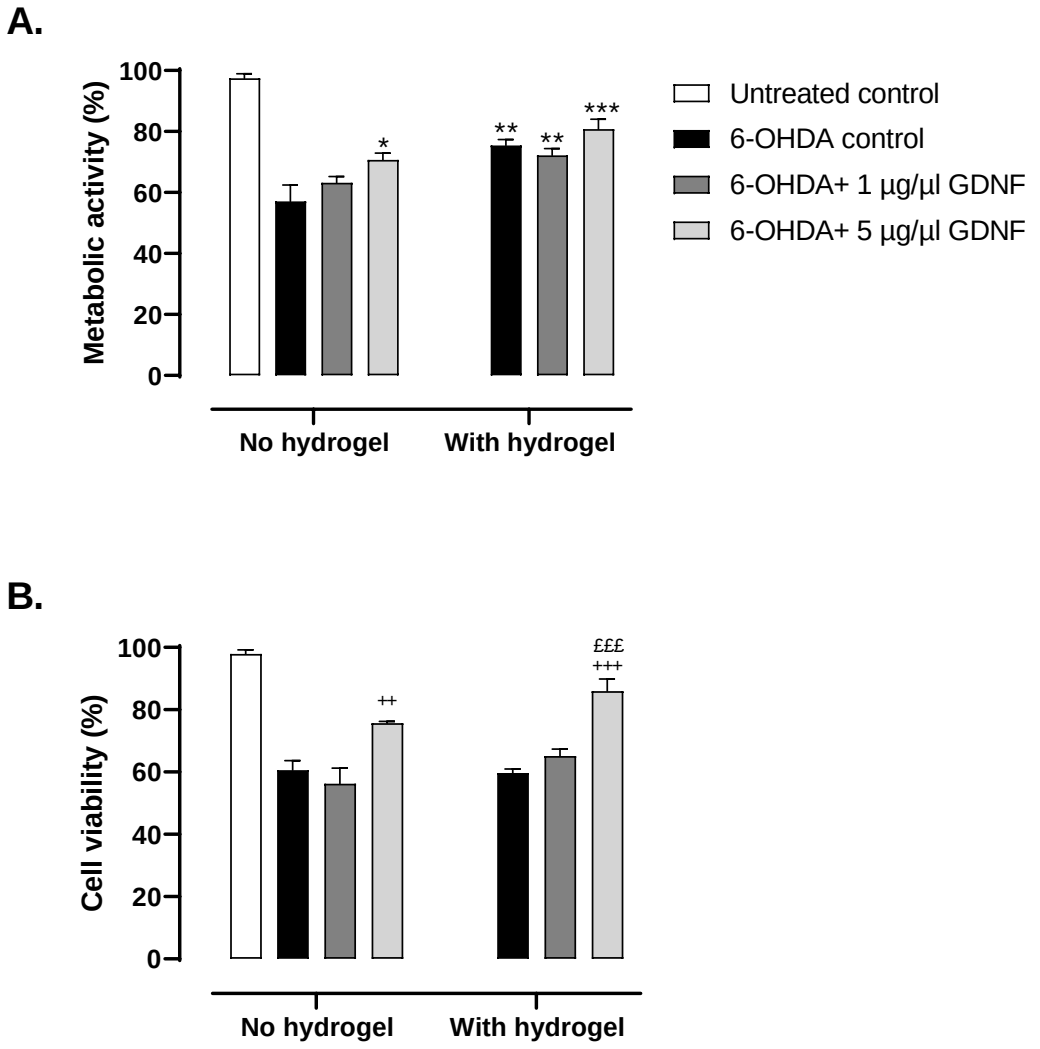


Figure 3.9 Metabolic activity of SH-SY5Y cells after 24 hr treatment with GDNF after exposure to 6-OHDA.

Both concentrations of GDNF encapsulated in a hydrogel significantly increased metabolic activity of SH-SY5Y cells after lesion (A), this effect was greater in the group that received 5 µg/µl GDNF encapsulated in hydrogel. The MTT assay showed that the treatment that significantly increased cell viability was 5 µg/µl of GDNF alone or encapsulated in hydrogel (B). Data are represented as mean ± SEM and were analysed using a two-way ANOVA, followed by Tukey *post-hoc* test. n= 3 per group. $^{*}p<0.05$ vs lesioned control, $^{**}p<0.01$ vs lesioned control $^{***}p<0.001$ vs lesioned control, $^{++}p<0.01$ vs lesioned control, $^{+++}p<0.001$ vs lesioned control $^{£££}p<0.001$ vs 1 µg/µl GDNF.

3.3.1.5 *In vitro* study 5: Assessment of the effects of 24 hr treatment with GDF5 or GDF5-encapsulated in collagen hydrogels, on growth and metabolic activity of SH-SY5Y cells after 6-OHDA exposure

In this study, we assessed the effects of GDF5 (1 or 5 μg/μl alone or encapsulated in a hydrogel) on SH-SY5Y cells after exposure to 50 μM 6-OHDA for 24 hr. The immunofluorescent staining for βIII tubulin showed more axonal growth of SH-SY5Y cells induced by treatment by both concentrations of GDF5 either encapsulated in a hydrogel or alone (**Figure 3.10**). The TH^+ immunocytochemistry was only present in a few cells (**Figure 3.11**). The alamarBlue® results showed that both concentrations of GDF5 either alone or encapsulated in collagen hydrogels significantly promoted recovery of metabolic activity of SH-SY5Y cells after lesion (**Figure 3.12a**; Treatment, $F_{(3,14)}$=121.6, P<0.001). In this study we also found recovery of activity in the group that received unloaded collagen hydrogels after lesion. The results from the MTT assay corroborated the results of the alamarBlue®, that both concentrations of GDF5 alone or encapsulated in a collagen hydrogel, significantly promoted the recovery of activity of the SH-SY5Y cells (**Figure 3.12b**; Treatment, $F_{(3,14)}$=70.19, P<0.001).

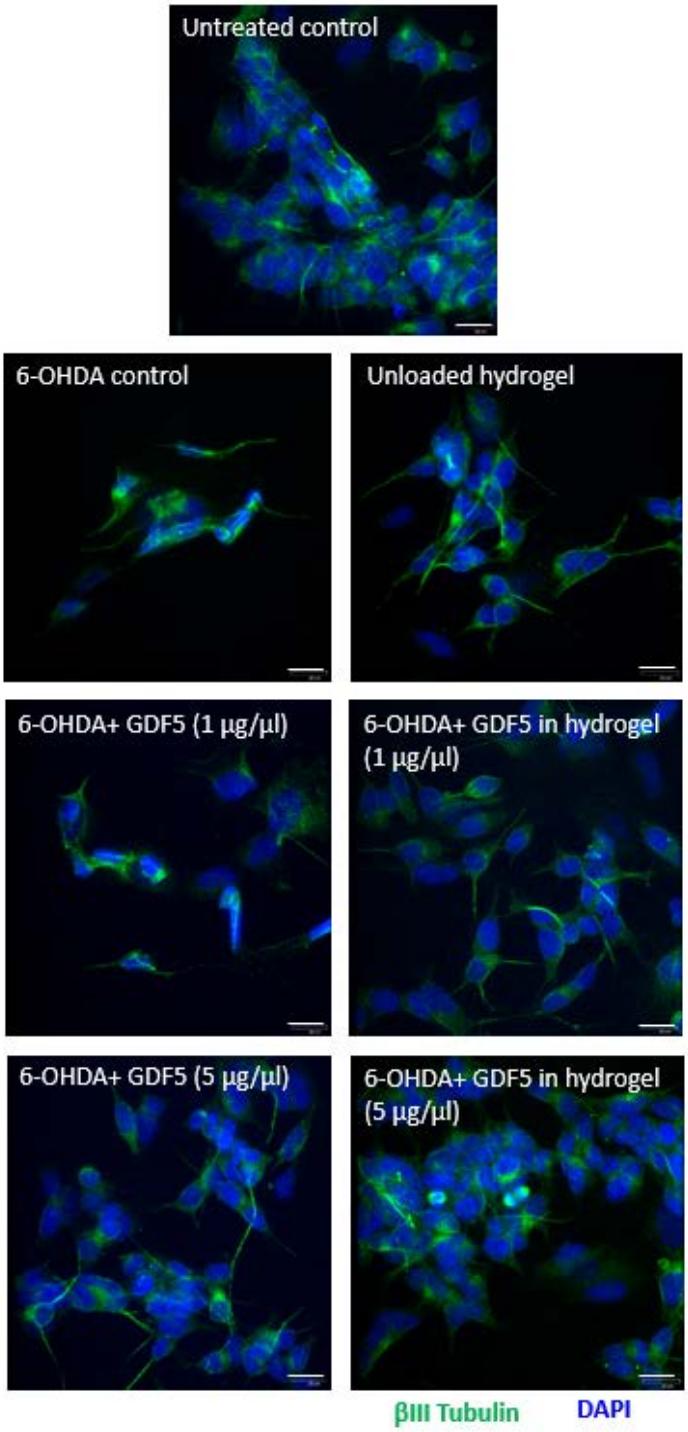


Figure 3.10 βIII tubulin assessment of the effects on growth of SH-SY5Y cells after 24 hr treatment with GDF5 after 6-OHDA exposure.

Photomicrographs represent the immunofluorescent staining for βIII tubulin counterstained with DAPI. The immunostaining showed an observable axonal growth in SH-SY5Y cells promoted by both concentrations of GDF5 either encapsulated in a hydrogel or alone. Scale bar represents 200 µm.

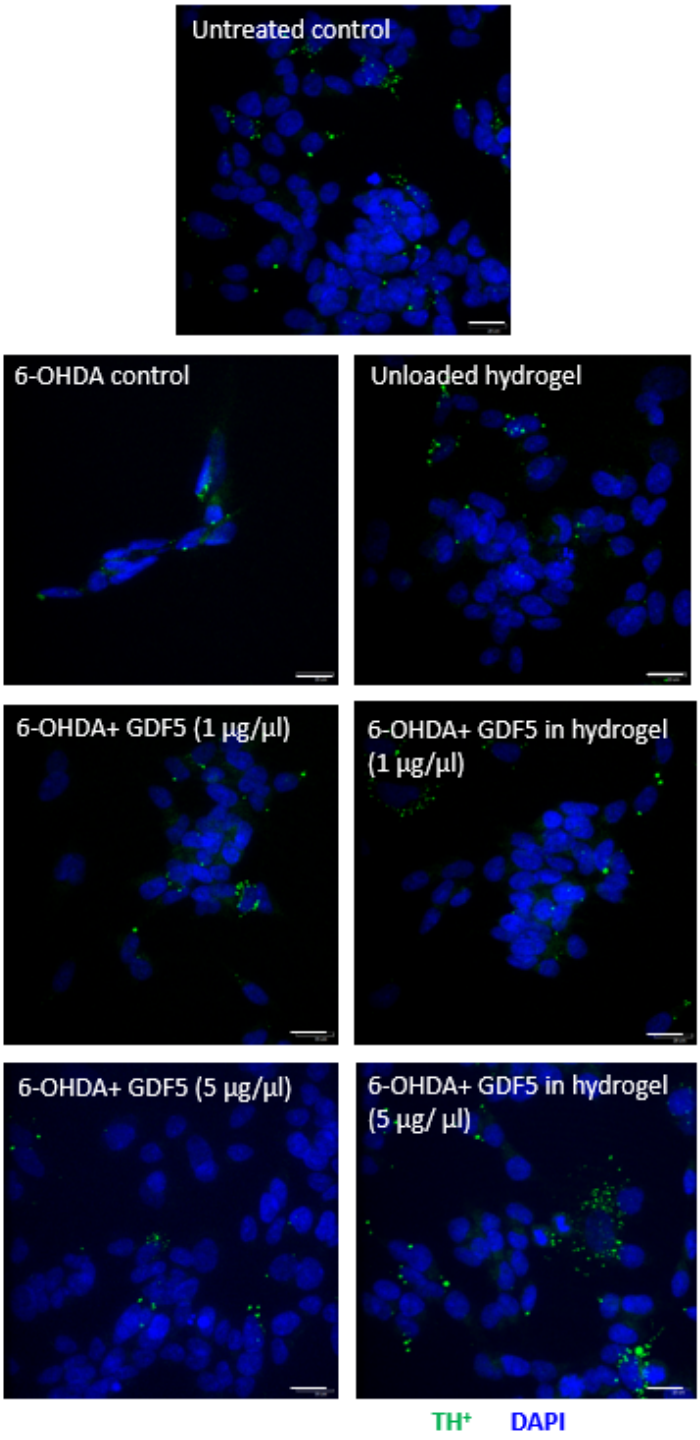
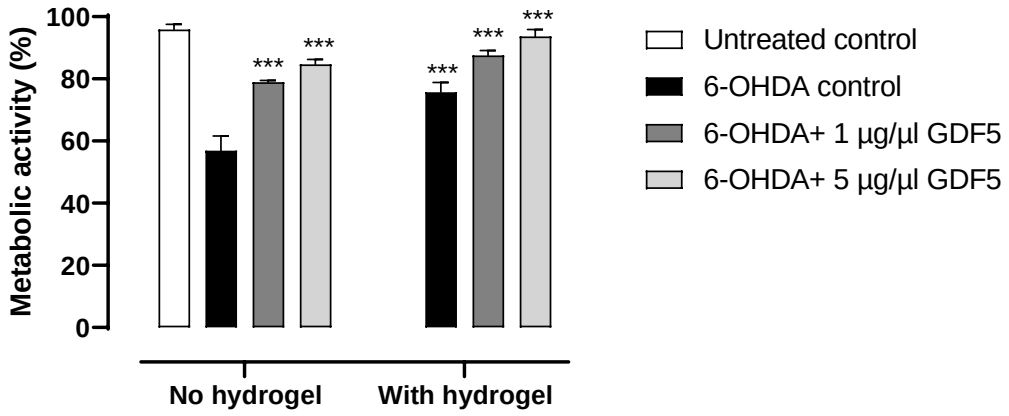


Figure 3.11 TH^+ assessment of the effects on survival and growth of SH-SY5Y cells after 24 hr treatment with GDF5 after 6-OHDA exposure.

Photomicrographs represent the immunofluorescent staining for TH^+ counterstained with DAPI. DAPI staining shows the presence of viable cells in the various culture conditions, but very few appear to show TH^+ staining. Scale bar represents 200 µm.

A.



B.

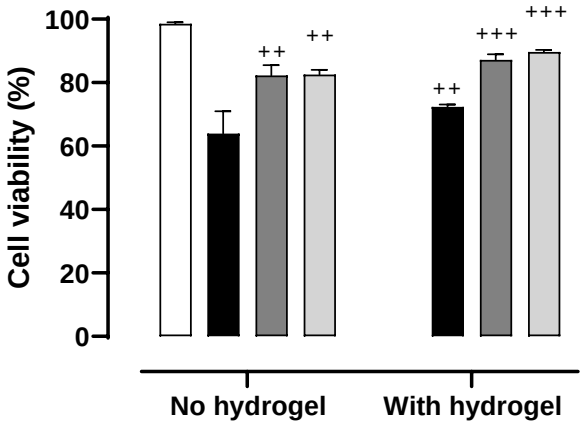


Figure 3.12 Metabolic activity of SH-SY5Y cells after 24 hr treatment with GDF5 after exposure to 6-OHDA.

Metabolic activity was measured by alamarBlue® assay (A) and cell viability by the MTT assay (B). The results showed that both concentrations of GDF5 either alone or encapsulated in collagen hydrogels promoted the recovery of SH-SY5Y cells after lesion. Data are represented as mean ± SEM and were analysed using a two-way ANOVA, followed by Tukey *post-hoc* test. n= 3 per group. $^{***}p<0.001$ vs lesioned control, $^{++}p<0.01$ and $^{+++}p<0.001$ vs lesioned control.

3.3.2 VM EXPLANT SYSTEM STUDIES

Cell cultures are two-dimensional and do not accurately reflect the *in vivo*-like situation in the organism. Therefore, in this chapter, we characterised a more complex model, a VM explant culture system and used it to test our collagen hydrogels and GDF5.

3.3.2.1 Explant study 1: Comparison between narrow and wide explanted tissue after 8 DIV.

The aims of this study were to establish a VM explant system in our laboratory and to determine if there was a difference between explants obtained by narrow dissections or by wide dissections. The TH^+ immunostaining showed that both narrow (**Figure 3.13a**) and wide explanted tissue (**Figure 3.13b**) survived well in culture for up to 8 DIV and that at all time-points, dopaminergic cells were detectable. In addition to the cell bodies in the developing substantia nigra, staining was also observed through the mesencephalic tissue suggesting development of axons from these cells. No significant differences were observed between the two types of explants, therefore in all the following experiments only narrow dissections were used.

In addition, to further assess this explant system, narrowly dissected explants were kept in culture for up to 24 DIV. The TH^+ immunostaining showed that the explanted tissue survived well in culture for up to 24 days (**Figure 3.14**). Dopaminergic cells were detectable at all time points and axonal growth was also observed.

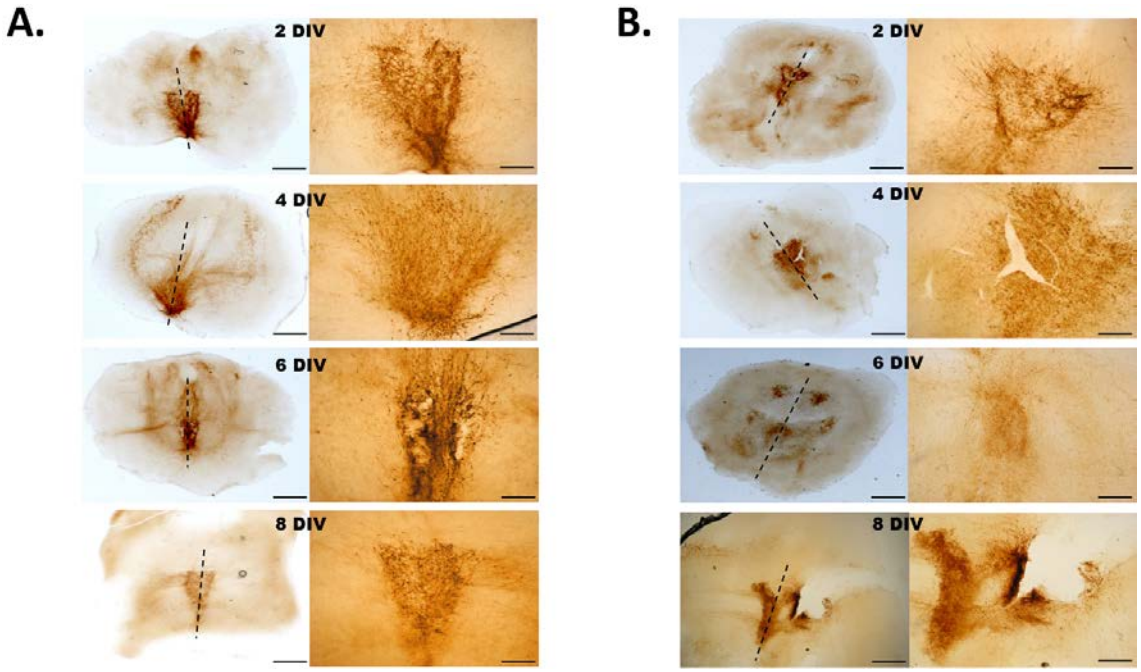


Figure 3.13 Representative photomicrographs of dopaminergic cells in a VM explant system after 2, 4, 6 and 8 DIV.

The TH^+ immunostaining showed numerous dopaminergic cells (dark brown) in both narrow and wide explanted tissue and at all time-points. Narrow explants shown at 2, 4, 6 and 8 DIV (A). Wide explants at 2, 4, 6 and 8 DIV (B). TH^+ cells can be seen extending from the central portion on either side of the explant midline (marked by dashed line). Scale bar represents 500 µm and 200 µm.

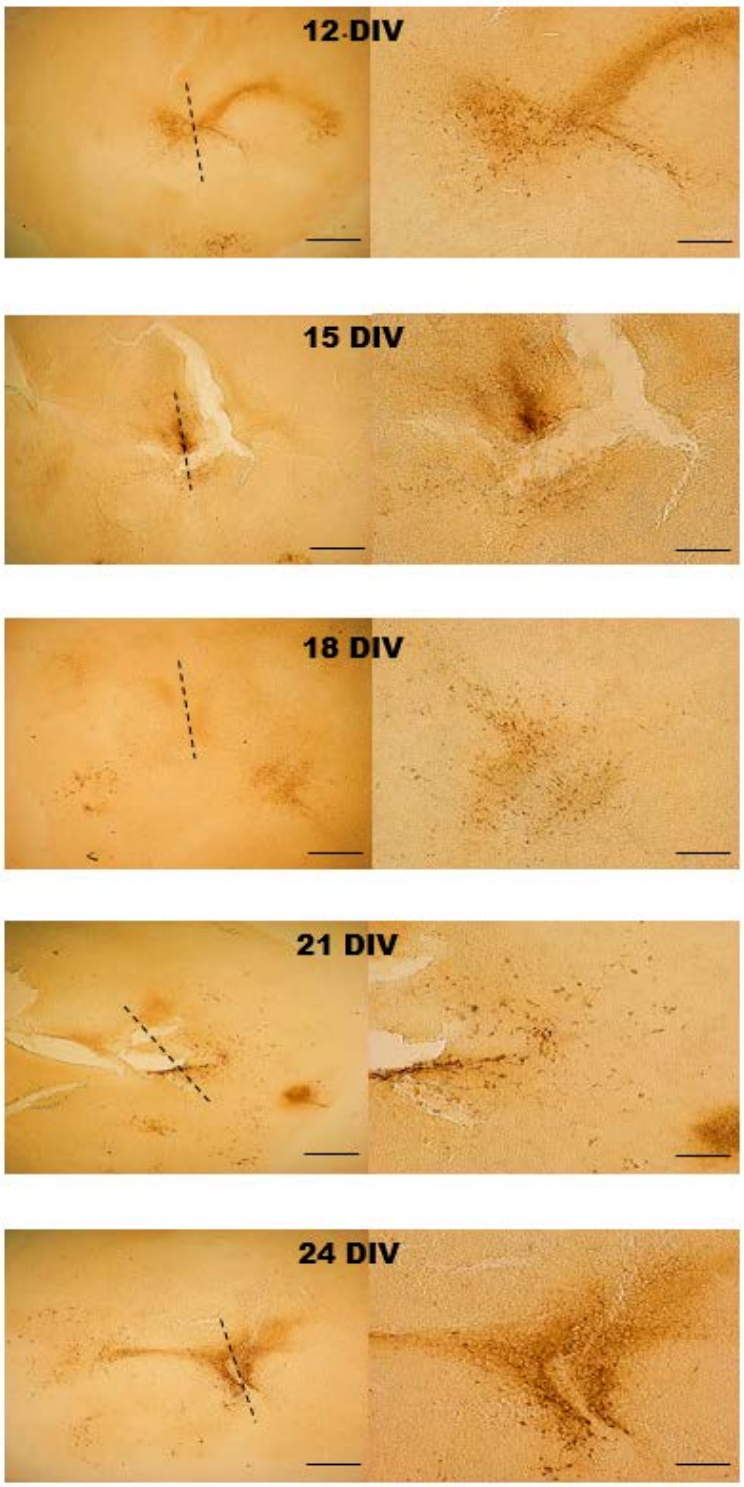


Figure 3.14 Representative photomicrographs of dopaminergic cells in a VM explant system after 12, 15, 18, 21 and 24 DIV.

The TH^+ immunostaining showed numerous TH^+ cells (dark brown) at all time-points. TH^+ cells can be seen extending from the central portion on either side of the explant midline (marked by dashed line). Scale bar represents 500 µm and 200 µm.

3.3.2.2 Explant study 2: Assessment of the effects of collagen hydrogels, made with different concentrations of 4s-StarPEG crosslinker, on the viability of explants, after 48 hr exposure

In order to determine if collagen hydrogels of increasing crosslinker concentrations (1, 2 or 4 mg/ml 4s-StarPEG) were biocompatible with the explants, these were incubated with the pre-formed hydrogels. The TH^+ immunostaining showed that none of the collagen hydrogels had a negative impact on the viability of the dopaminergic cells of the explants when compared to the untreated control (**Figure 3.15**). Dopaminergic cells were detectable in all the groups and axonal growth was also observed. Furthermore, no difference was found in the optical density of TH^+ staining in any of the groups exposed to hydrogels when compared to the optical density measured in the untreated controls (**Figure 3.16**; Group, $F_{(3,8)}$=1.207, P>0.05).

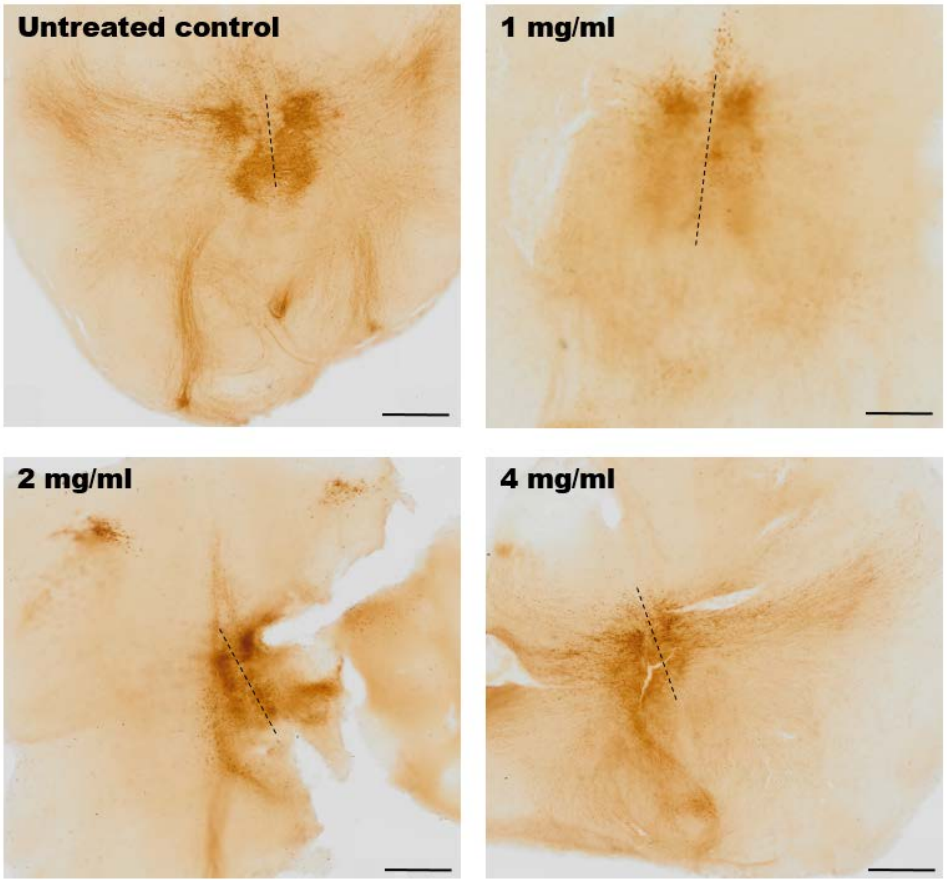


Figure 3.15 Representative photomicrographs of dopaminergic cells in an explanted system after 48 hr exposure to collagen hydrogels of 1, 2, or 4 mg/ml of crosslinker.

After exposure to collagen hydrogels, no detrimental effects were observed on the viability of the dopaminergic cells of the explants. Dopaminergic cells were detectable in all of the groups and axonal growth was also observed. Scale bar represents 500 µm, midline marked by dashed line.

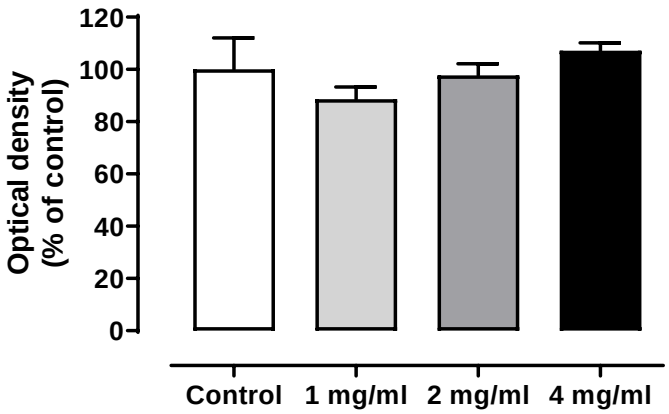


Figure 3.16 Density of TH^+ staining in explants after 48 hr exposure to collagen hydrogels of 1, 2 or 4 mg/ml of crosslinker.

The optical density results showed that the collagen hydrogels were biocompatible. No difference was found in TH^+ density in any of the groups exposed to the collagen hydrogels. Data are represented as mean ± SEM and were analysed using one-way ANOVA. n= 3 per group.

3.3.2.3 Explant study 3: Assessment of the effects of GDF5 and GDNF (alone or encapsulated in a collagen hydrogel) on the viability of dopaminergic cells in explants.

In this study, we compared the effects on the viability of dopaminergic cells in explants by early exposure to 5 µg/µl of GDF5 or GDNF (alone or in a collagen hydrogel). The TH^+ immunostaining showed an observable increase of TH^+ staining in the groups that received treatment with either GDF5 or GDNF (**Figure 3.17**). Furthermore, the optical density measurements showed a trend where explants treated with GDF5 and GDNF had increased TH^+ optical density when compared to the untreated control (**Figure 3.18**; Treatment, $F_{(2,12)}$=1.823, P>0.05). These findings could represent an increase in either the number of TH^+ cells in the tissue or an increase of the amount of growth by TH^+ cells.

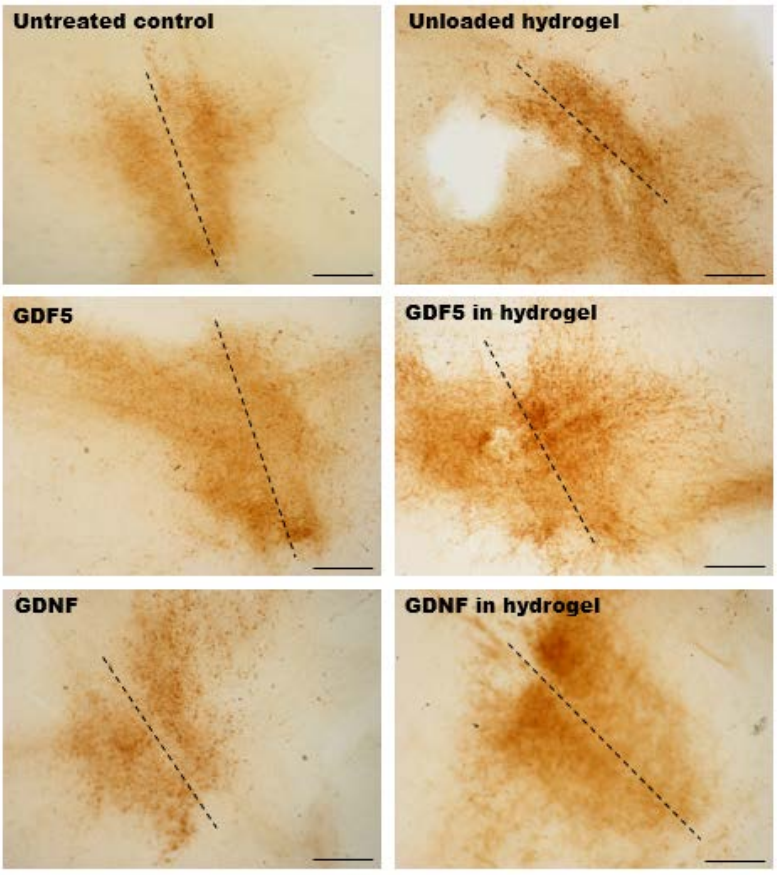


Figure 3.17 Representative photomicrographs of TH^+ cells in an explanted system after early treatment with 5 µg/µl GDF5 or GDNF.

The TH^+ immunostaining showed an observable increase in TH^+ staining in the groups that received treatment with either GDF5 or GDNF (alone or in hydrogel). Scale bar represents 200 µm, midline marked by dashed line.

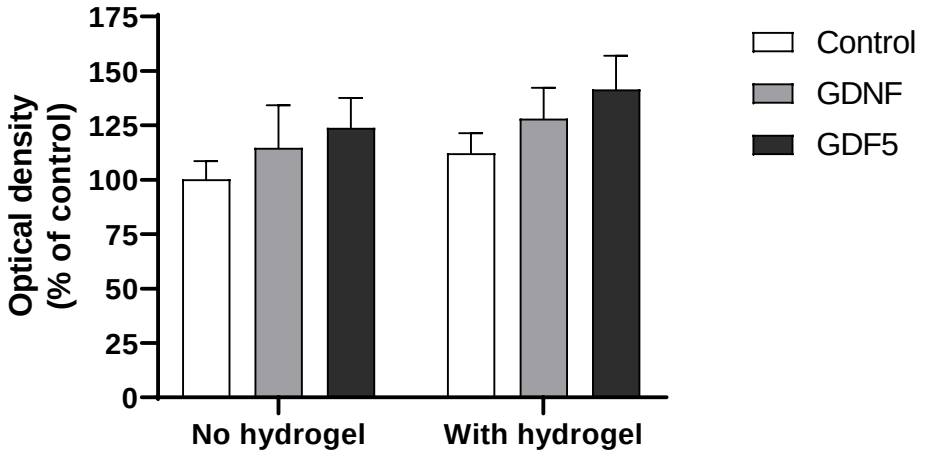


Figure 3.18 Density of TH$^+$ staining in an explanted system after early treatment with GDF5 or GDNF.

Optical density measurements showed a trend where explants treated with either GDF5 or GDNF (alone or in hydrogel) increased the density of TH$^+$ staining. Data are represented as mean ± SEM and were analysed using a two-way ANOVA, followed by Tukey *post-hoc* test. n= 3 per group.

3.3.2.4 Explant study 4: Assessment of the effects of LPS, Poly I:C or 6-OHDA on dopaminergic cells in explants after 48 or 72 hr exposure.

In this study, we assessed the damaging effects of 50 μg/ml LPS, 100 μg/ml Poly I:C and 2 mM 6-OHDA on dopaminergic cells in explants after either 48 or 72 hr exposure to establish a model of dopaminergic cell loss. The immunostaining showed that explants exposed to 6-OHDA and Poly I:C for 48 hr had a visible reduction of TH$^+$ cells (**Figure 3.19a**). Additionally, optical density measurements showed that explants exposed to 6-OHDA and Poly I:C had significantly less density of TH$^+$ staining when compared to the untreated control (**Figure 3.20a**; Treatment, $F_{(3,8)}$=8.841, P<0.01).

Explants exposed to Poly I:C and 6-OHDA for 72 hr showed further reduction of TH$^+$ cells and clear cell body and axonal damage (**Figure 3.19b**). Furthermore, optical density measurements showed that explants exposed to 6-OHDA and Poly I:C had greater loss in density of TH$^+$ staining when compared to the untreated control (**Figure 3.20b**; Treatment, $F_{(3,8)}$=12.02, P<0.01).

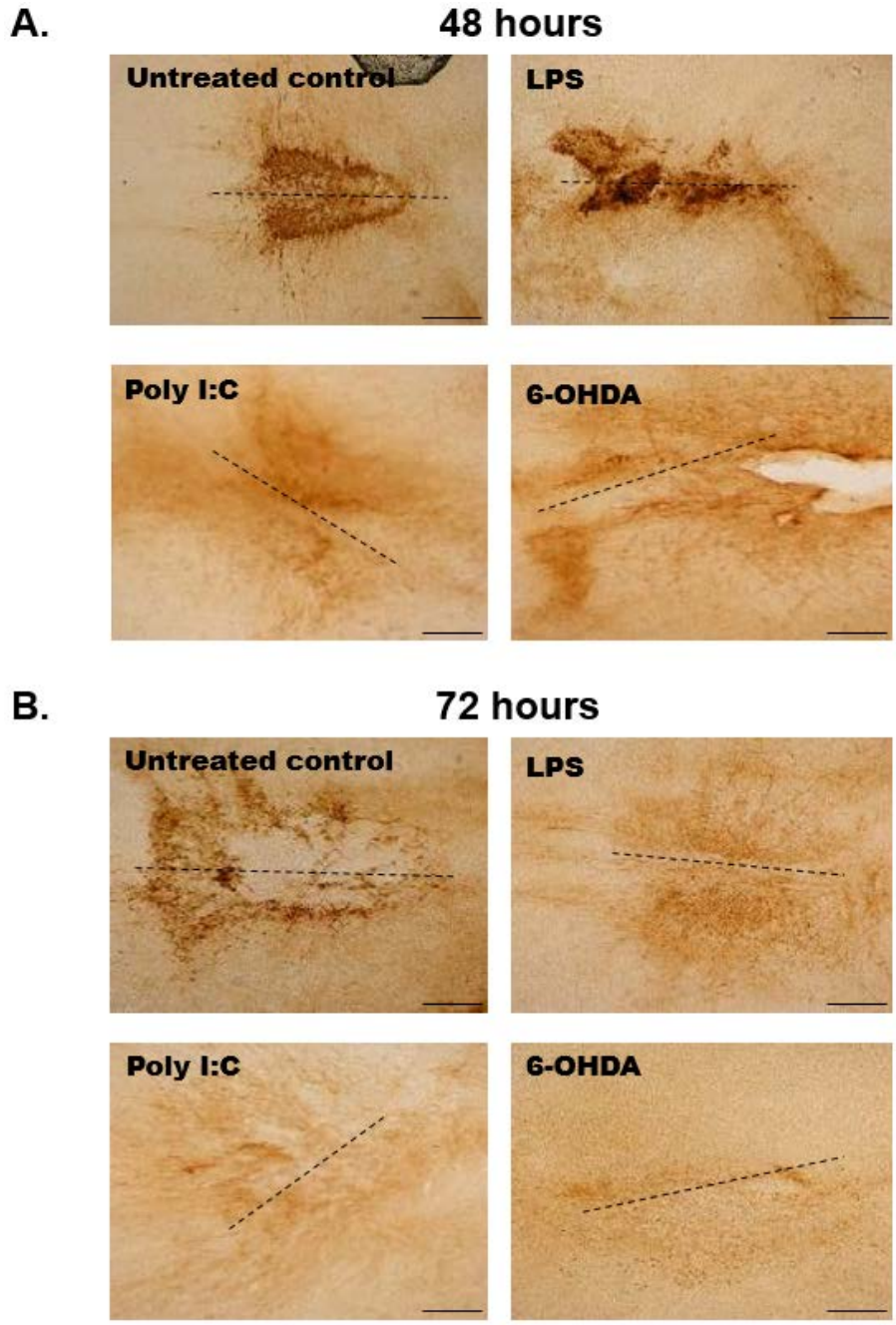


Figure 3.19 Representative photomicrographs of dopaminergic cells in an explanted system after 48 or 72 hr exposure to LPS, Poly I:C or 6-OHDA.

The immunostaining showed that explants exposed to Poly I:C and 6-OHDA but not LPS for 48 hr had a visible reduction of TH^+ cells (A). This damage appeared greater in explants exposed for 72 hr (B). Scale bar represents 200 µm, midline marked by dashed line.

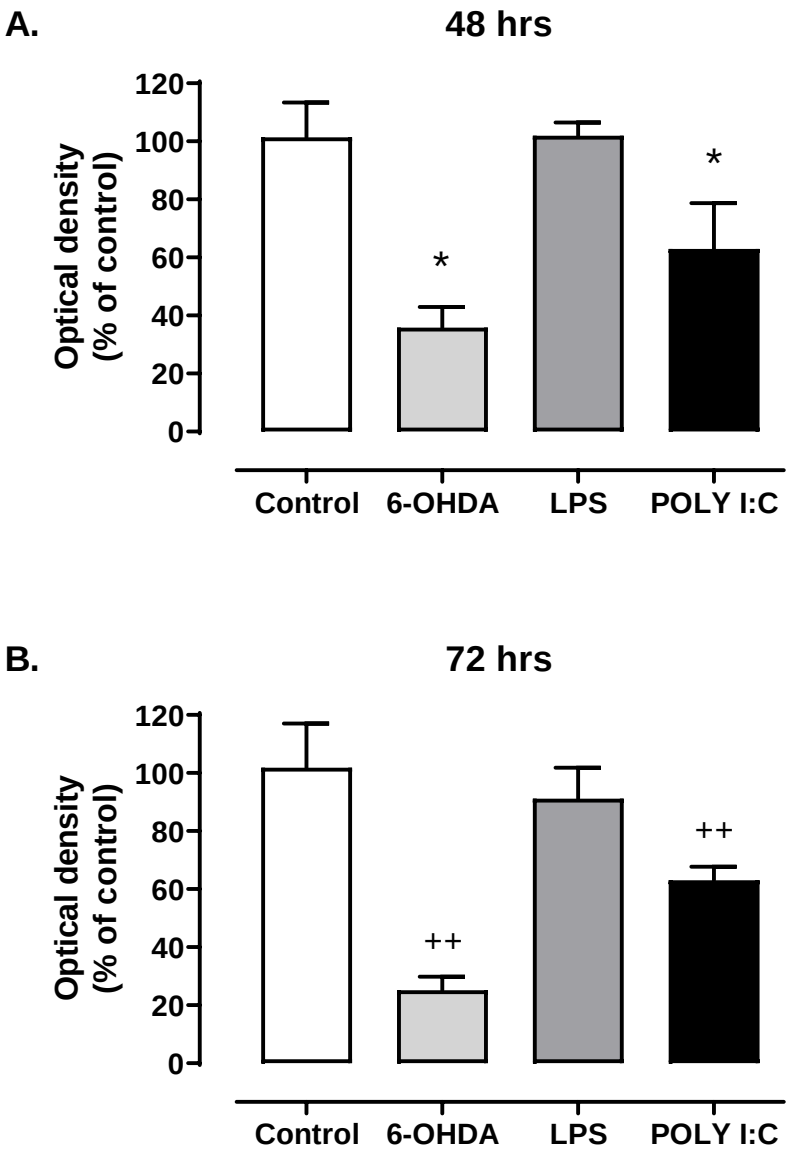


Figure 3.20 Density of TH^+ staining in explants after 48 or 72 hr exposure to LPS, Poly I:C or 6-OHDA.

Optical density measurements show that explants exposed for 48 hr (A) or 72 hr (B) to 6-OHDA and Poly I:C had diminished density of TH^+ staining when compared to the untreated control. Data are represented as mean ± SEM and were analysed using One-way ANOVA, followed by Bonferroni *post-hoc* test. $^{*}p<0.05$ vs 48 hr untreated control, $^{++}p<0.01$ vs 72 hr untreated control. n=3 per group.

3.3.2.5 Explant study 5: Assessment of the effects of treatment with GDF5 or GDF5 encapsulated in a hydrogel, on dopaminergic cells of explants after 72 hr exposure to 6-OHDA.

In this study, we assessed the effects of GDF5 (5 or 20 µg/µl alone or encapsulated in a collagen hydrogel) on dopaminergic cells of explants after 72 hr exposure to 6-OHDA. The TH^+ immunostaining showed an observable recovery of TH^+ cells in explants that received treatment with both concentrations of GDF5 either alone or encapsulated in collagen hydrogels when compared to the untreated lesioned group (**Figure 3.21**). Groups that received GDF5 treatment in collagen hydrogels showed greater recovery of dopaminergic cells and more fibrous staining than the groups that received treatment of GDF5 alone. Additionally, greater recovery and growth was observed in the groups that received treatment of 5 µg/µl of GDF5 than the groups that received 20 µg/µl of GDF5. The optical density measurements showed the same trend (**Figure 3.22**; Treatment, $F_{(2,14)}$=5.410, P<0.05) and that treatment of 5 µg/µl of GDF5 encapsulated in a collagen hydrogel significantly promoted a recovery of TH^+ staining density above 90% when compared to density of the lesioned control.

Furthermore, in this study recovery of TH^+ cells were observed in the group that received unloaded collagen hydrogels after lesion.

Figure 3.21 Representative photomicrographs of dopaminergic cells in an explanted system treated with GDF5 after 72 hr exposure to 6-OHDA.

Both concentrations of GDF5 (alone or encapsulated in hydrogels) seem to increase the expression of TH^+ staining from the midline on explants (marked by dashed line). Higher TH^+ staining was observed in the groups that received treatment of 5 µg/µl of GDF5 encapsulated in collagen hydrogels. Scale bar represents 200 µm.

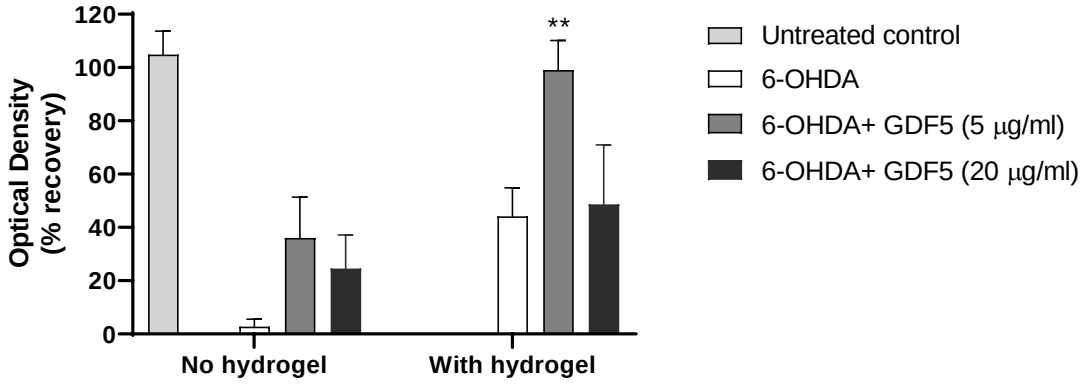


Figure 3.22 Density of TH^+ staining in an explanted system treated with GDF5 after 72 hr exposure to 6-OHDA.

Optical density measurements showed that groups that received GDF5 treatment in collagen hydrogels had greater recovery of TH^+ expression. This recovery was significantly enhanced in the group that received treatment of 5 µg/µl of GDF5 encapsulated in a collagen hydrogel, since it had a recovery of density above 90%. Data are represented as mean ± SEM and were analysed using a two-way ANOVA, followed by Tukey *post-hoc* test. $^{**}p<0.05$ vs lesioned control. n= 3 per group.

3.4 DISCUSSION

The use of biomaterials in cell replacement therapy has the potential to enhance the survival and efficacy of transplanted cells for the treatment of Parkinson´s disease. Biomaterials, like collagen hydrogels, can be adapted for medical applications and be used to create a supportive and protective matrix for cells, provide localised and sustained trophic factor delivery and to protect transplanted cells from the attack of the host immune response following intracerebral transplantation (Orive *et al*., 2009; Hoban *et al*., 2013; Moriarty and Dowd, 2018). In this chapter, we assessed collagen hydrogels and the neurotrophic factor GDF5, through a series of *in vitro* studies on an immortalised SH-SY5Y cell line and on a VM explant culture system to: 1) determine the cytocompatibility of collagen hydrogels on the SH-SY5Y cell line and on VM explants, 2) assess the effects of GDF5 on the growth and development of the VM explants and 3) determine if GDF5 enrichment of collagen hydrogels has neurorestorative effects on SH-SY5Y cells and on VM explants after a 6-OHDA insult.

3.4.1 *IN VITRO* STUDIES ON SH-SY5Y CELLS

In the first *in vitro* study described in this chapter, we compared gelation times of collagen from 2 different sources with various concentrations of 4s-StarPEG crosslinker and we found that the higher the concentration of crosslinker used, the shorter the time taken for the hydrogels to gelate. Additionally, we established that Vornia was the best source of collagen to keep using forward throughout the studies in this thesis, and its gelation times were greater than the ones from Collagen Solutions. Since our ultimate goal is to use the functionalised collagen hydrogels for intrastriatal transplantation of VM cells to enhance the results of cell replacement therapy in Parkinson´s disease, these gels should have a slow enough gelation time that allows its intrastriatal transplantation but that gels fast enough once transplanted into the host. Hence, we decided that Vornia was the best option. In previous studies, Moriarty *et al*., (2017) established that the best crosslinker concentration for intrastriatal transplantation was 4 mg/ml of 4s-StarPEG crosslinker (Moriarty *et al*., 2017). Therefore, we decided to continue using that crosslinker concentration for all of the hydrogel work in this thesis.

Before any biomaterial can be used in the brain it must undergo essential toxicity and cytocompatibility assessment and for this we used the immortalized SH-SY5Y cell line. This cell line has been used since the 1970´s as an *in vitro* model for Parkinson´s disease research (Biedler *et al*., 1978). To assess the effects on metabolic activity of these cells, we used alamarBlue® and MTT assays. In the second study, the results from these assays did not show a difference in metabolic activity or in cell viability indicating that the incubation of pre-formed collagen hydrogels with SH-SY5Y cells did not have any negative effects on the cells. The increasing concentrations of 4s-StarPEG in the collagen hydrogels had no detrimental effects, confirming that neither the collagen nor the crosslinker component were toxic to the cells. Furthermore, these results are in line with previous literature reporting the *in vitro* cytocompatibility of 4s-StarPEG crosslinked collagen hydrogels (Hoban *et al*., 2013; Moriarty *et al*., 2017).

Additionally, the immunofluorescent staining for βIII tubulin and DAPI showed that incubation with the crosslinked hydrogels did not hinder the survival and outgrowth of the SH-SY5Y cells, but the immunofluorescent staining for TH^+ did not show conclusive results since very few cells appear to be TH^+. According to the literature, the SH-SY5Y cell line has the machinery to synthesize both dopamine and noradrenaline so it is considered to display a dopaminergic phenotype. However, a systematic review by Xicoy *et al*., (2017) published that 392 out of 962 papers state that SH-SY5Y cells have a dopaminergic phenotype without actually showing supporting evidence (Xicoy *et al*., 2017). Experiments by Gomez-Santos *et al*., (2002) and McMillan *et al*., (2007) found TH^+ expression by SH-SY5Y cells, while experiments by Mastroeni *et al*., (2009) and Toulouse *et al*., (2012) failed to demonstrate its expression (Gómez-Santos *et al*., 2002; McMillan *et al*., 2007; Mastroeni *et al*., 2009; Toulouse *et al*., 2012). In the *in vitro* work with SH-SY5Y cells completed in this chapter, we found in the TH^+ immunofluorescence some staining in the cells, but this was not defined nor was it consistent to all of the cells.

In the third study, we assessed the damaging effects of various concentrations of 6-OHDA on the growth and metabolic activity of SH-SY5Y cells. 6-OHDA is the most used toxin in experimental models of Parkinson´s disease. It is a catecholaminergic neurotoxin that enters cells via dopamine or noradrenaline transporters (since it has a similar structure to dopamine), then accumulates inside the cell and triggers the formation of reactive oxygen species (ROS), leading to oxidative stress and cell death

(Lehmensiek *et al*., 2006; Simola *et al*., 2007). In this study, we found that as the concentration of 6-OHDA increases, there is an observable decrease of DAPI and βIII tubulin staining indicating a reduction of viable cells and reduced growth. The results from the alamarBlue® and MTT assays confirmed the reduction in metabolic activity and showed that while the highest concentration of 100 μM of 6-OHDA reduced the metabolic activity to only 40%, the lowest concentration of 20 μM only reduced it to 80%. Therefore, due to the results of this study we decided to use the concentration of 50 μM for the rest of the 6-OHDA experiments, as it caused an intermediate reduction of metabolic activity.

In the fourth *in vitro* study with SH-SY5Y cells, we assessed the effects of GDNF on this cell line after exposure to 6-OHDA. GDNF is a potent neurotrophic factor *in vitro* and *in vivo* (Lin *et al*., 1993; Allen *et al*., 2013). It has a relatively high specificity for dopaminergic neurons and thus has significant potential for the treatment of PD. The results from the alamarBlue® showed that both concentrations of GDNF encapsulated in a collagen hydrogel increased the metabolic activity of SH-SY5Y cells treated, particularly with the dose of 5 μg/μl. This dose increased the metabolic activity to about 80%. We also observed and increase in the βIII tubulin immunostaining, while the DAPI staining showed more viable cells. Although the main focus of this thesis is GDF5, we wanted to investigate the effects of another well-known neurotrophic factor on SH-SY5Y cells. Therefore, in the fifth *in vitro* study, we assessed the neuroprotective effects of GDF5 after exposure to 6-OHDA. In the βIII tubulin immunostaining we observed recovery of axonal growth in all of the groups threated with GDF5 and the DAPI staining showed more viable cells in these groups. In the results from the alamarBlue® and MTT assays we found that both concentrations of GDF5 either alone or encapsulated in a collagen hydrogel increased the level of metabolic activity to about 80%. These results are in line with previous literature reporting that GDF5 promoted survival of SH-SY5Y cells following a 6-OHDA insult (Toulouse *et al*., 2012).

Additionally, in the fourth and fifth study, we found that incubation of SH-SY5Y cells with unloaded collagen hydrogels also increased the level of metabolic activity and βIII tubulin immunostaining after a 6-OHDA insult. There are no previous studies reporting beneficial effects of unloaded collagen hydrogels on cells after exposure to 6-OHDA.

3.4.2 VM EXPLANT SYSTEM STUDIES

Since cell cultures are two-dimensional and do not accurately reflect the *in vivo*-like situation in the organism, we characterised a more complex model, a VM explant culture system and used it to test our collagen hydrogels and GDF5.

In the first explant study, we looked to establish a VM explant system in our laboratory. Two types of E14 dissections were done: 1) narrow dissections (comprising only the medial mesencephalon) or 2) wide dissections (comprising the mid-lateral to medial mesencephalon on both sides) and then, these explants were incubated for up to 8 DIV. Since no observable differences were found in the TH^+ immunostaining between the 2 types of explants and dopaminergic cells and axonal growth can be found at all-time points, we decided to only use narrow dissections for the rest of the explant studies.

Once the explant system was established, we used this 3D system for essential toxicity and biocompatibility assessment of our collagen hydrogels. Therefore, in the second explant study, explants were exposed to pre-formed hydrogels composed of various crosslinker concentrations. The results from the optical density measurement showed that the incubation of the collagen hydrogels with the explants did not have any negative effects on the density of TH^+ expression. The increasing concentrations of 4s-StarPEG in the collagen hydrogels had no detrimental effects, confirming that neither the collagen nor the crosslinker component were toxic to the explants. These results are similar to our findings when the hydrogels were tested on SH-SY5Y cells.

For the third explant study, we assessed the effects of early exposure to GDF5 and GDNF (alone or encapsulated in a collagen hydrogel) on dopaminergic cells in explants, while also comparing the neurotrophic properties of both of these proteins. The TH^+ immunostaining showed that, compared to the untreated control, there was an observable increase in axonal growth in the groups that received treatment with either GDF5 or GDNF. Additionally, the optical density measurements showed that compared to the untreated control, explants treated with GDF5 and GDNF tend to show greater density of TH^+ expression. This could represent an increase in the number of TH^+ cells present in the tissue.

In the fourth explant study, we assessed the effects of LPS, Poly I:C and 6-OHDA on the explants to determine which compound would be the best option to induce dopaminergic cell death. LPS, Poly I:C and 6-OHDA are compounds used to model

Parkinson´s disease *in vitro* and *in vivo*. LPS is an endotoxin derived from cell wall gram (-) bacteria, used to induce inflammation (Ramsey and Tansey, 2014). Poly I:C is a Toll-like receptor 3 (TLR-3) agonist that produces a viral-like neuroinflammatory reaction (Deleidi *et al*., 2010). The results from this study showed that prolonged exposure to 6-OHDA (72 hr) produced greatest loss of TH^+ optical density indicating a decrease in the number of dopaminergic cells. Hence, we decided to use this neurotoxin for the rest of the explant experiments.

Once we found that 6-OHDA exposure caused a reduction in TH^+ density greater than 60%, we then assessed the neuroprotective effects of GDF5 after a 6-OHDA insult. The TH^+ immunostaining showed a trend where groups that received GDF5 treatment in collagen hydrogels had an observable recovery of TH^+ staining (indicating the presence of more dopaminergic cells and more axonal growth) greater than in the groups that received treatment of GDF5 alone. Most importantly, greater recovery and growth was observed in the groups that received treatment of 5 µg/µl of GDF5 encapsulated in a collagen hydrogel than in the groups that received 20 µg/µl of GDF5 in collagen; this effect was confirmed by the optical density measurements. These results are similar to our findings when SH-SY5Y cells were treated with GDF5 after a 6-OHDA insult.

Overall, the results obtained in this chapter showed that the collagen hydrogels are biocompatible to SH-SY5Y cells and to the VM explants. Furthermore, GDF5 encapsulated in collagen hydrogels is neuroprotective against the neurotoxic effects of 6-OHDA. These results suggest that our collagen hydrogels and GDF5 are good candidates to be studied to improve the results of cell transplantation. Thus, in an effort to enhance the functionality of cell replacement therapy in Parkinson´s disease, the following chapters in this thesis will assess the potential of GDF5-loaded collagen hydrogels in this context.

Chapter 4: Assessment of the suitability of collagen hydrogels for intrastriatal delivery of cells and/or neurotrophic factors to the brain

4.1 INTRODUCTION

The potential of cell replacement therapy to restore dopaminergic transmission in the striatum has been hindered by the low yield of surviving dopaminergic neurons after transplantation. Several studies have shown that 80 to 90% of the grafted dopaminergic neurons die during the transplantation process due to different causes such as anoikis, oxidative stress, hypoxia or lack of trophic factors (Brundin *et al*., 1988; Mahalik *et al*., 1994; Barker *et al*., 1996; Kordower *et al*., 1996; Sortwell *et al*., 2001; Marchionini *et al*., 2003).

The addition of biomaterials to the transplantation process has shown great potential to improve the survival of cells. Collagen gels seem to be promising matrices for delivery of cells and drugs, due to characteristics such as high mechanical strength, good biocompatibility and low antigenicity (Wallace and Rosenblatt, 2003). Collagen can form hydrogels *in situ* under physiological conditions, offering an injectable biomaterial platform to aid cell transplantation (Xu and Kopeček, 2007).

The goal of using collagen hydrogels to deliver cells to the brain is to 1) improve cell engraftment by providing an adherent substrate, 2) to provide a physical barrier to protect the cells against the host immune response caused by the mechanical trauma during intracranial delivery and 3) to deliver and prolong the release of therapeutic factors (Orive *et al*., 2009; Hoban *et al*., 2013; Moriarty and Dowd, 2018).

One of the main causes of low cell survival is the lack of trophic support after transplantation. Primary cells are removed from a neurotrophic-rich environment at the height of neurogenesis and placed into an environment deprived of the growth factors that normally should be present during their development (Sortwell *et al*., 2001; Abeliovich and Hammond, 2007). This low cell survival can be improved by the supply of neurotrophic factors. Growth factors and other active agents can be combined with

collagen hydrogels to prolong their release rate and increase their therapeutic effect (Wallace and Rosenblatt, 2003).

GDF5 is a potent neurotrophic molecule that acts as a survival promoting molecule for rat dopaminergic midbrain neurons (Krieglstein *et al*., 1995). Since its discovery in 1994 (Storm *et al*., 1994), it has been hypothesised that GDF5 may be a useful protein for the treatment of Parkinson´s disease in two ways: 1) as a neurotrophic factor in cell transplantation therapy or 2) as a neuroprotective factor to support the remaining dopaminergic neurons and protect them against the ongoing disease.

The restorative properties of GDF5 have been well documented in *in vitro* and *in vivo* models of Parkinson's disease (Hurley *et al*., 2004; Sullivan and O'Keeffe, 2005; Toulouse and Sullivan, 2008; Sullivan and Toulouse, 2011; Costello *et al*., 2012; Toulouse *et al*., 2012; Gavin *et al*., 2014); however, the neurotrophic effects of this growth factor on cell transplantation have not been fully studied.

In light of these, the aims of this chapter were:

1. To determine the suitability of the collagen hydrogels for delivery of primary dopaminergic neurons to the striatum.
2. To determine the ability of the collagen hydrogels to deliver and retain the neurotrophic factor, GDF5, in the striatum.
3. To determine if GDF5 enrichment of collagen hydrogels has any early effects on primary dopaminergic neuron grafts.

4.2 METHODS

4.2.1 EXPERIMENTAL DESIGN

In order to address the aims of this chapter, the following studies were completed:

4.2.1.1 Study 1: *In vivo* assessment of the suitability of collagen hydrogels for delivery of primary dopaminergic neurons to the striatum

Twenty-four male Sprague Dawley rats were given a unilateral intra-MFB 6-OHDA lesion (12 µg per 3 µl). Two weeks later, the lesion was assessed by methamphetamine-induced rotations (2.5 mg per kg) and based on their performance rats were matched into 6 groups for the transplantation surgery (for details on groups see **Table 4.1**). Rats were transcardially perfused with 4% paraformaldehyde for the post-mortem assessment, at either 1- or 4-days after transplantation. Free floating immunohistochemistry for TH^+, collagen, GFAP and CD11b were completed to identify graft survival, collagen biodegradability and host immune response. A schematic representation of the experimental design is shown in **Figure 4.1**. Refer to Chapter 2 for more detailed methodology.

Table 4.1 Groups for the assessment of the suitability of collagen hydrogels for delivery of cells to the striatum.

Perfusion at day 1 post-transplantation	Perfusion at day 4 post-transplantation
E14 VM cells alone (400,000 per 6 µl) n=4	E14 VM cells alone (400,000 per 6 µl) n=4
Collagen alone (crosslinked with 4mg/ml 4s-StarPEG) n=4	Collagen alone (crosslinked with 4mg/ml 4s-StarPEG) n=4
E14 VM cells in collagen (400,000 per 6 µl, crosslinked with 4mg/ml 4s-StarPEG) n=4	E14 VM cells in collagen (400,000 per 6 µl, crosslinked with 4mg/ml 4s-StarPEG) n=4

Based on the rat´s performance in the methamphetamine induced rotations, rats were distributed into 6 groups for unilateral intrastriatal transplant.

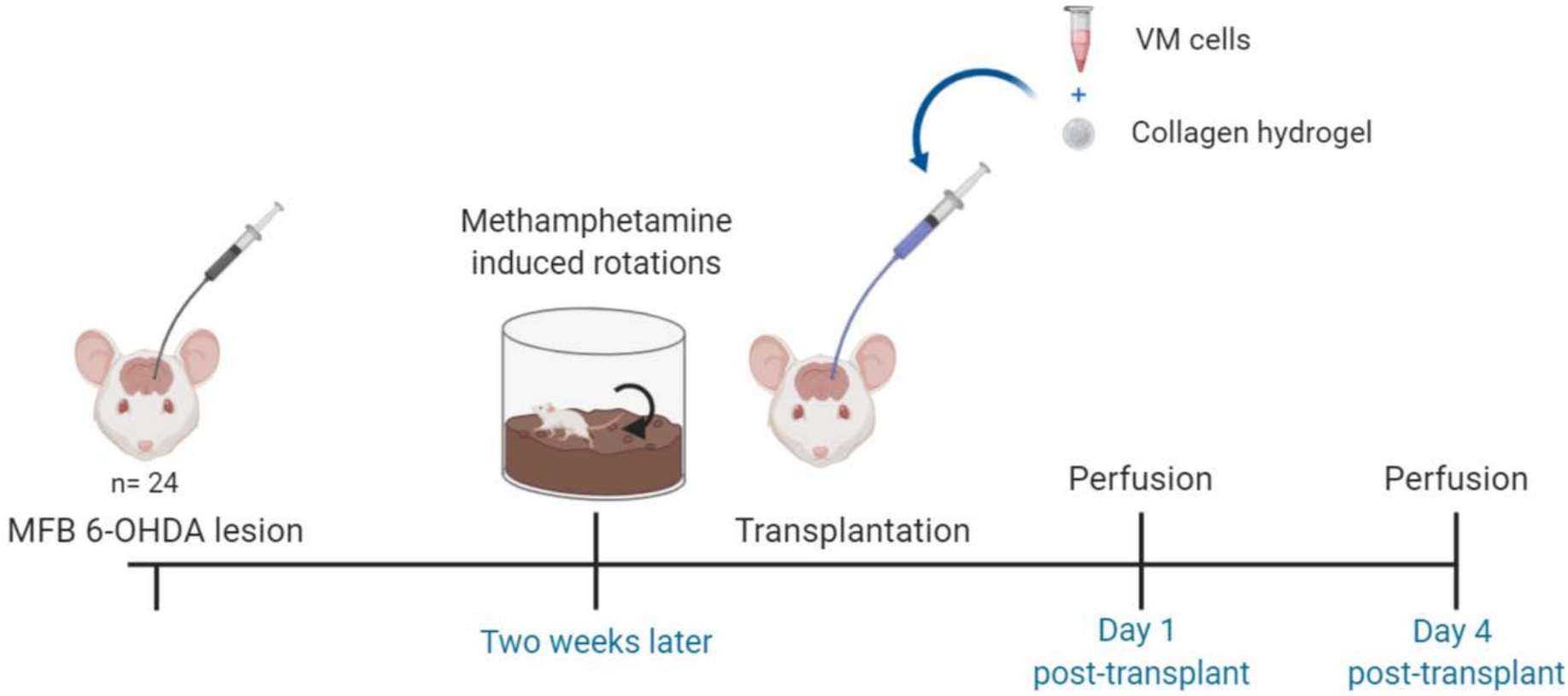


Figure 4.1 Schematic representation of the experimental design of the *in vivo* assessment of the suitability of collagen hydrogels for delivery of primary dopaminergic neurons to the striatum.

Rats received a MFB lesion with 6-OHDA. Two weeks later, the lesion was assessed by methamphetamine-induced rotations. Subsequently, rats received a unilateral intrastriatal transplant (for details on groups and doses see **Table 4.1**) and were transcardially perfused at either 1 or 4 days post-transplantation. n= 4 per group.

4.2.1.2 Study 2: *In vivo* assessment of the ability of the collagen hydrogels to deliver and retain GDF5 in the striatum

Nine male rats were given a bilateral intrastriatal injection of GDF5 (1 µg per 6 µl) in the left striatum and GDF5 in a collagen hydrogel (1 µg per 6µl, crosslinked with 4 mg/ml 4s-StarPEG) in the right striatum (for details on groups see **Table 4.2**). Subsequently, rats were transcardially perfused with 4% paraformaldehyde for the post-mortem assessment at either 1-, 4- or 7-days post intrastriatal delivery. Free floating immunohistochemistry for GDF5, TH^+, collagen, GFAP and CD11b were completed to evaluate polymerisation and biodegradability of collagen, host immune response and retention of GDF5. A schematic representation of the experimental design is shown in **Figure 4.2**. Refer to Chapter 2 for more detailed methodology.

Table 4.2 Groups for the assessment of the ability of the collagen hydrogels to deliver and retain GDF5 in the striatum.

Perfusion day	Left striatum	Right striatum
Day 1	GDF5 (1 µg per 6 µl) n=3	GDF5 in collagen (1 µg per 6 µl, crosslinked with 4 mg/ml 4s-StarPEG) n=3
Day 4	GDF5 (1 µg per 6 µl) n=3	GDF5 in collagen (1 µg per 6 µl, crosslinked with 4 mg/ml 4s-StarPEG) n=3
Day 7	GDF5 (1 µg per 6 µl) n=3	GDF5 in collagen (1 µg per 6 µl, crosslinked with 4 mg/ml 4s-StarPEG) n=3

Rats received a bilateral intrastriatal implantation of: GDF5 (1 µg per 6 µl) in left striatum or GDF5 in collagen (1 µg per 6 µl, crosslinked with 4 mg/ml 4s-StarPEG) in right striatum and were perfused at either day 1-, 4- or 7-days post intrastriatal delivery. n=3 per group.

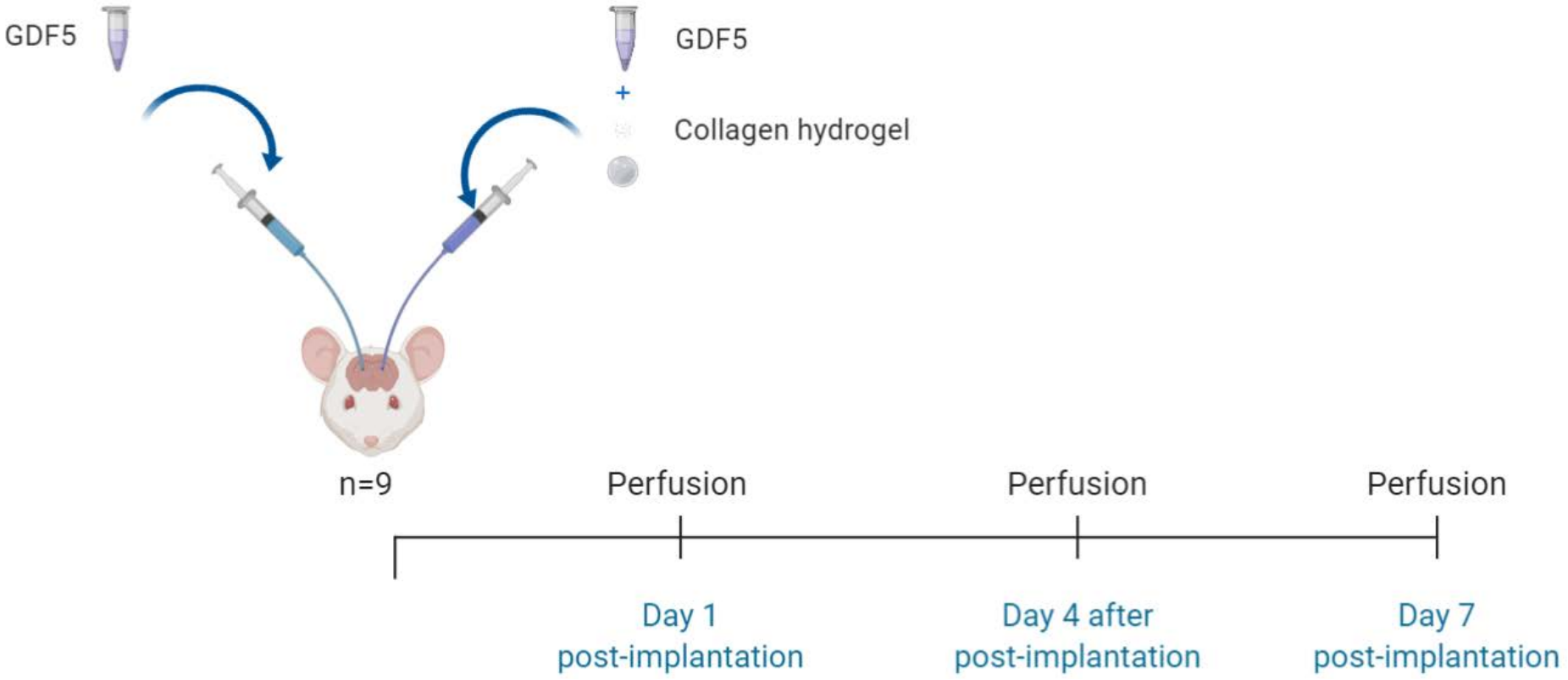


Figure 4.2 Schematic representation of the experimental design of *in vivo* assessment of the ability of the collagen hydrogels to deliver and retain GDF5 in the striatum.

Rats received a bilateral intrastriatal injection of GDF5 in the left striatum and GDF5 in a collagen hydrogel in the right striatum (for details on groups and doses see **Table 4.2**). Subsequently, rats were transcardially perfused at either 1-, 4- or 7-days post-intrastriatal delivery. n=3 per group.

4.2.1.3 Study 3: *In vivo* assessment of the effects of GDF5 enrichment of collagen hydrogels on primary dopaminergic neuron grafts at an early time-point

Sixteen male rats were given a unilateral intra-MFB 6-OHDA lesion (12 µg per 3 µl). Two weeks later, rats were distributed into 4 groups for transplantation surgery of cells in collagen with increasing concentration of GDF5 (for details on groups and doses see **Table 4.3**). Fourteen days later, rats were transcardially perfused with 4% paraformaldehyde for the post-mortem assessment. Free floating immunohistochemistry for TH^+, GFAP and CD11b were completed to identify graft survival and host immune response. A schematic representation of the experimental design is shown in **Figure 4.3**. Refer to Chapter 2 for more detailed methodology.

Table 4.3 Groups and doses for the assessment of the effects of GDF5 enrichment of collagen hydrogels on primary dopaminergic neuron grafts at an early time-point.

E14 VM cells in collagen (300,000 cells per 6 µl, crosslinked with 4 mg/ml 4s-StarPEG) n=4	E14 VM cells + GDF5 in collagen (300,000 cells/1 µg per 6 µl, crosslinked with 4 mg/ml 4s-StarPEG) n=4
E14 VM cells + GDF5 in collagen (300,000 cells/5 µg per 6 µl, crosslinked with 4 mg/ml 4s-StarPEG) n=4	E14 VM cells + GDF5 in collagen (300,000 cells/10 µg per 6 µl, crosslinked with 4 mg/ml 4s-StarPEG) n=4

Rats received a MFB lesion with 6-OHDA. Two weeks later, rats were distributed into four groups for unilateral intrastriatal transplant. Rats were transcardially perfused 14 days later. n= 4 per group.

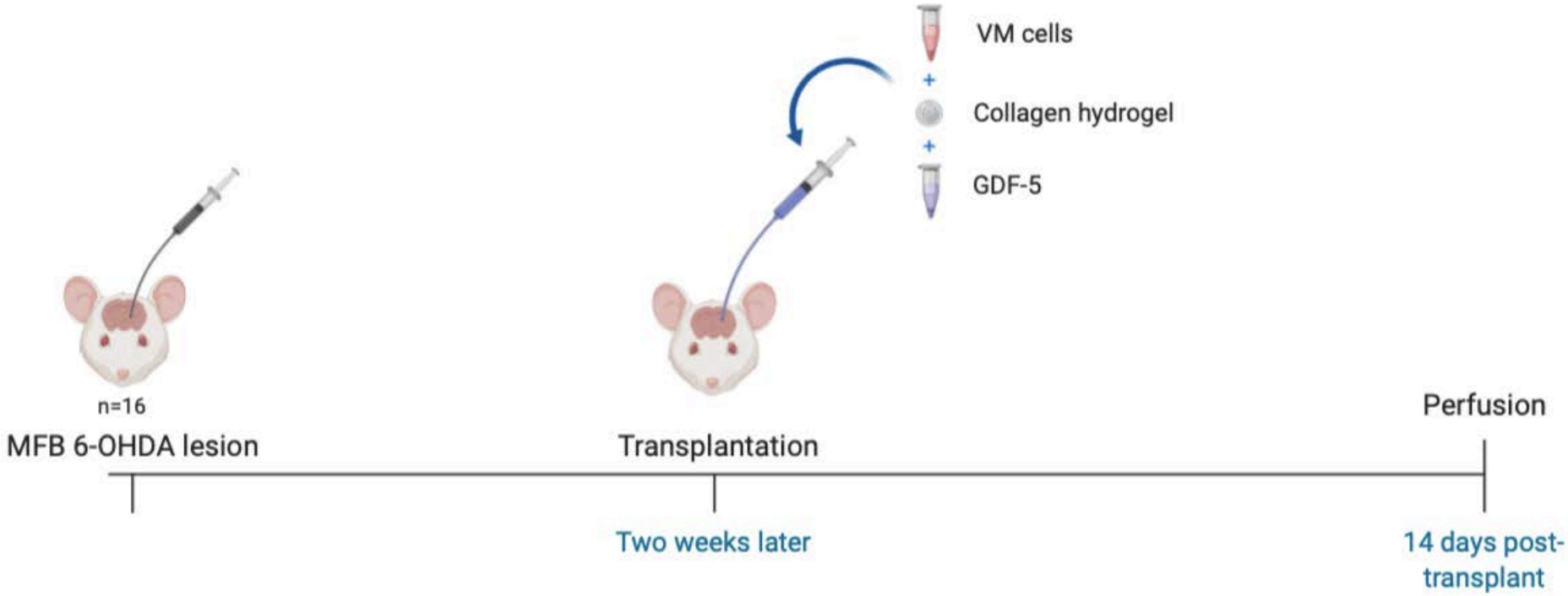


Figure 4.3 Schematic representation of the experimental design of the *in vivo* assessment of the effects of GDF5 enrichment of collagen hydrogels on primary dopaminergic neuron grafts at an early time-point.

Rats received a MFB lesion with 6-OHDA. Subsequently, rats received a unilateral intrastriatal transplant (for details on groups and doses see **Table 4.3**) and were transcardially perfused 14 days later. n=4 per group

4.3 RESULTS

4.3.1 STUDY 1: *IN VIVO* ASSESSMENT OF THE SUITABILITY OF COLLAGEN HYDROGELS FOR DELIVERY OF PRIMARY DOPAMINERGIC NEURONS TO THE STRIATUM

In this preliminary study we investigated the suitability of collagen hydrogels for the intrastriatal delivery of E14 VM cells at early time points (rats were perfused 1- or 4-days post-transplantation). For this assessment, we looked at *in situ* polymerisation, collagen biodegradability, host immune response and graft survival.

4.3.1.1 *In situ* polymerisation and biodegradation of the collagen hydrogel

In this preliminary study, free floating bovine collagen immunohistochemistry was completed to assess collagen polymerisation and biodegradability. The photomicrographs of the immunostaining confirmed the presence of collagen hydrogels at the transplantation site and showed that our collagen hydrogels polymerise *in situ* (**Figure 4.4)**. Importantly, the collagen hydrogels did not undergo rapid degradation and were still present 4 days post-transplantation (**Figure 4.5**; Interaction $F_{(3,12)}$= 0.737, P>0.05).

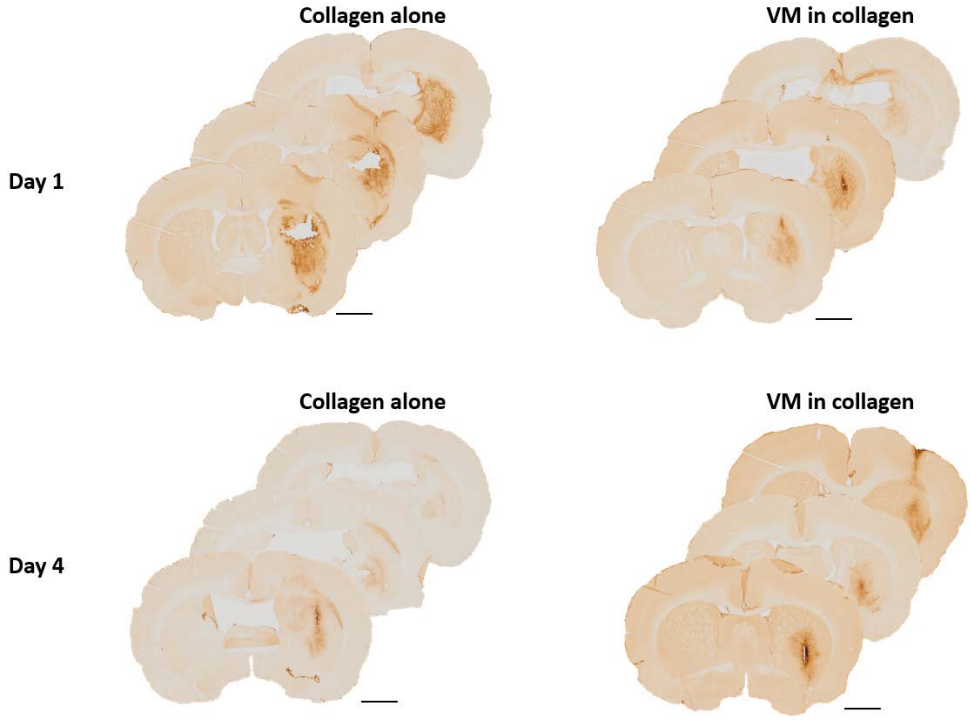


Figure 4.4 Representative photomicrographs of *in situ* gelation of collagen hydrogels.

Representative photomicrographs show striatal collagen staining at day 1 and 4 post-transplantation. Scale bar represents 2 mm. n=4 per group.

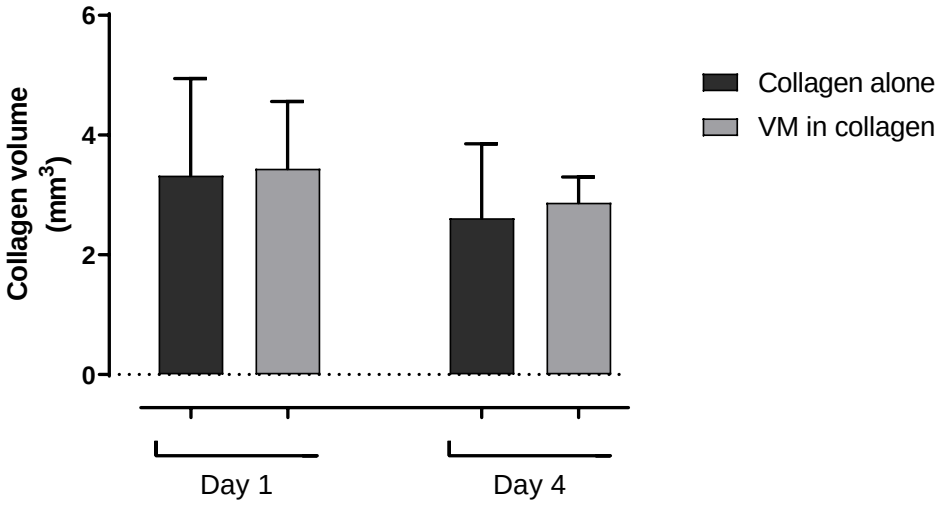


Figure 4.5 *In situ* gelation of collagen hydrogels.

Collagen hydrogels crosslinked with 4 mg/ml of 4s-StarPEG formed *in situ* and were still present at 4 days post-transplantation. Data are represented as mean ± SEM and were analysed by two-way ANOVA. n=4 per group.

4.3.1.2 Effect of the collagen hydrogel on the host immune response to primary dopaminergic neurons

In order to establish whether the collagen hydrogels crosslinked with 4mg/ml of 4s-StarPEG were cytocompatible and did not evoke an exacerbated host immune response to the transplanted E14 VM cells, we quantified the volume and optical density of astrocytes (**Figure 4.6**) and microglia (**Figure 4.8**) via immunostaining for GFAP and CD11b. In order to determine astrocytic and microglial volume we measured the area where the immune response was located, and using these area measurements the volume was assessed according to Cavalieri's Principle (see **section 2.9.3** for more details). Optical density measurements were taken to determine the response of the brain's inflammatory cells (microglia and astrocytes) to the transplanted grafts. The site of transplantation was located based on either having the visible immunostaining or from visible needle tract sites (see **section 2.9.5** for more details).

The immunostaining showed that cell transplantation did elicit an astrocytic and microglial response in the host brain. However the GFAP immunostaining showed that the delivery of cells in a collagen hydrogel did not exacerbate the immune response since no significant increase was found in the volume (**Figure 4.7a**; Group $F_{(5,18)}$= 1.144, P>0.05) or density (**Figure 4.7b**; Group, $F_{(5,18)}$= 4.536, P>0.05) of astrocytes. However, there was a tendency to find less astrocytes present around the graft site, in the groups that received VM cells in collagen after day 1 post-transplantation compared to the group that received only VM cells. Furthermore, the CD11b immunostaining did not show a significant increase in the volume (**Figure 4.9a**; Group, $F_{(5,18)}$= 0.2650, P>0.05) or in the density (**Figure 4.9b**; Group, $F_{(5,18)}$=2.258, P>0.05) of microglia, but there was tendency where the groups that received collagen alone or VM cells in collagen showed less volume and density of microglia compared to the groups that only received VM cells at both time points. These results suggest that the collagen hydrogels are cytocompatible.

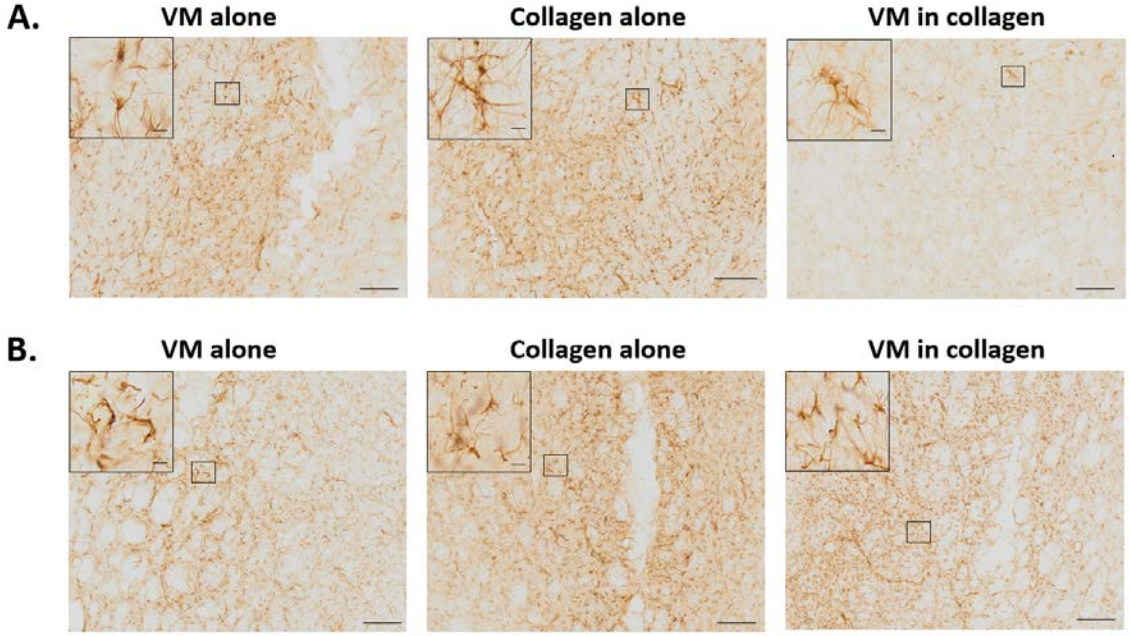


Figure 4.6 Representative photomicrographs of the effects of collagen hydrogel on astrocytic response to E14 VM cells.

Representative photomicrographs show GFAP immunostaining at the transplantation site at day 1 (A) or day 4 post transplantation (B). The GFAP immunostaining confirmed that delivery of cells in a collagen hydrogel did not exacerbate the astrocytic response. Scale bars represent 200 µm and 20 µm. n=4 per group.

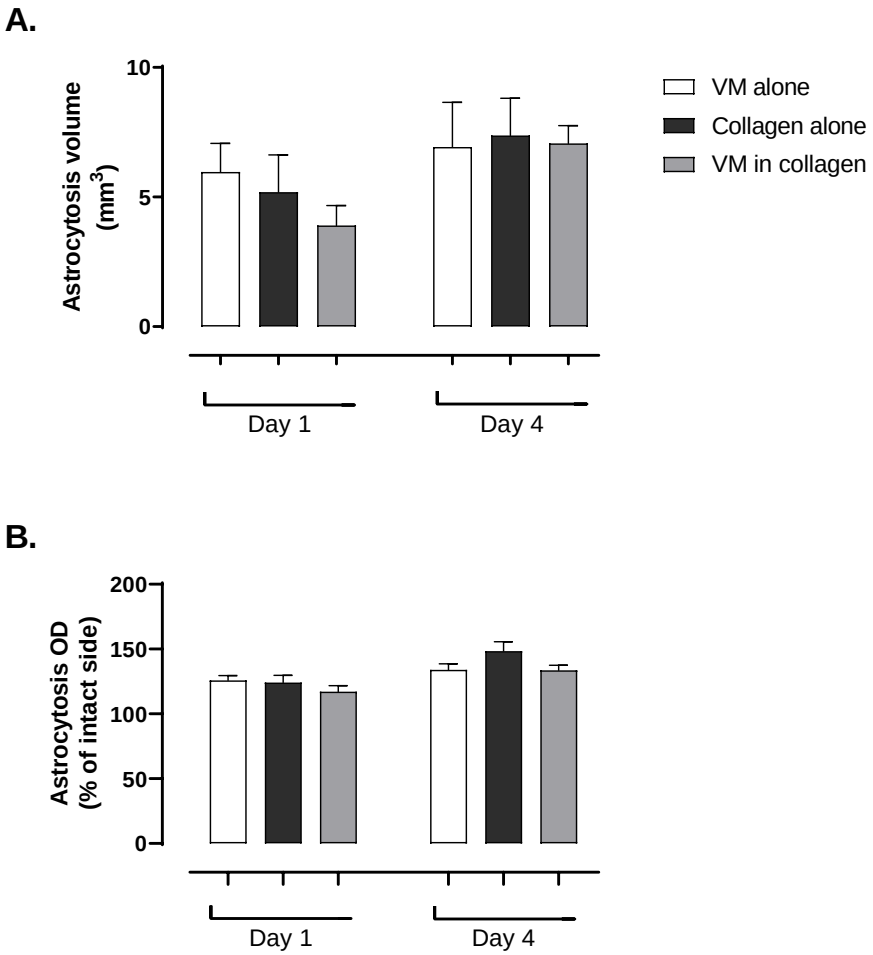


Figure 4.7 Effects of collagen hydrogel on astrocytic response to E14 VM cells.

The GFAP immunostaining confirmed the biocompatibility of the collagen hydrogels. Delivery of cells in collagen hydrogels did not exacerbate the immune response since no significant increase was found in the volume (A) or density of astrocytes (B) present in the transplantation site at day 1 or 4 post-transplantation. There was a trend of less astrocytes around the graft site in the groups that received VM cells in collagen after day 1 post-transplantation. Data are represented as mean ± SEM and were analysed by two-way ANOVA. n= 4 per group.

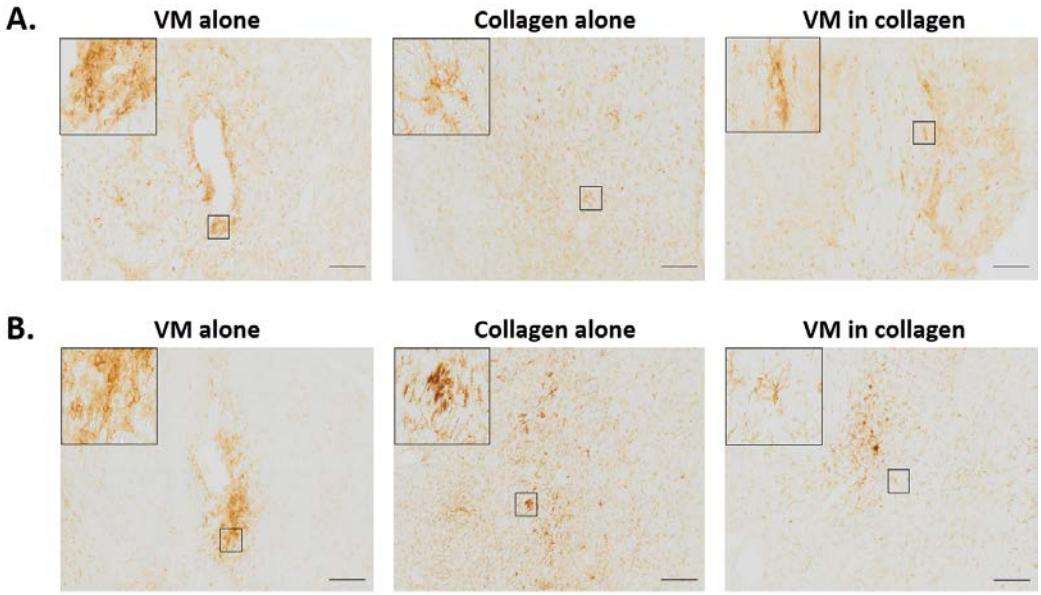


Figure 4.8 Representative photomicrographs of the effects of collagen hydrogel on microglial response to E14 VM cells.

Representative photomicrographs show CD11b immunostaining at the transplantation site at 1-day (A) or 4-days post-transplantation (B). The immunostaining showed that the delivery of cells in a collagen hydrogel did not exacerbate the microglial response. Scale bar represents 200 µm and 20 µm (insert). n= 4 per group.

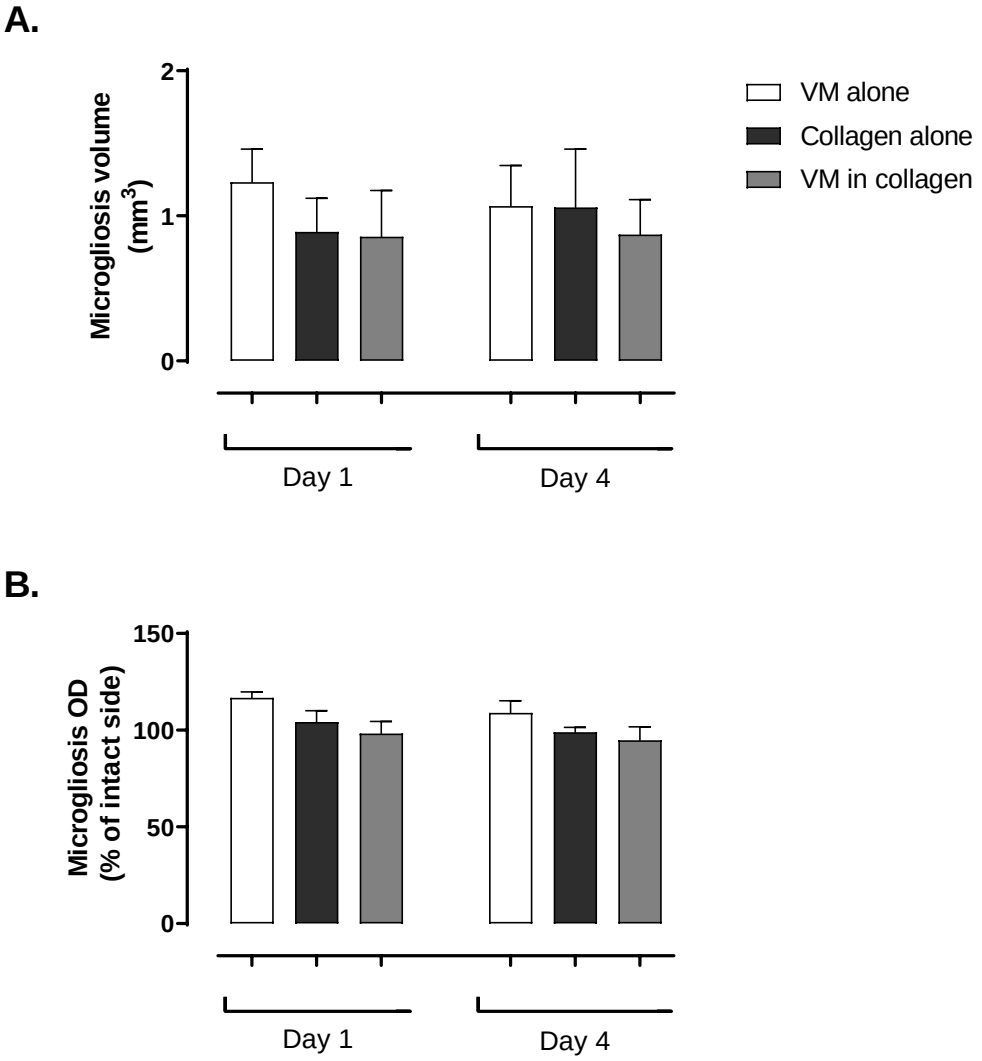


Figure 4.9 Effects of collagen hydrogel on microglial response to E14 VM cells.

The CD11b immunostaining confirmed the biocompatibility of the collagen hydrogels. Delivery of cells in a collagen hydrogel did not elicit an exaggerated response in the volume (A) or density of microglia (B) at the transplantation site day 1 or 4 post-transplantation. In both time-points, there was a trend of less microglia around the graft site in the groups that received VM cells in collagen. Data are represented as mean ± SEM and were analysed by two-way ANOVA. n= 4 per group.

4.3.1.3 Effects of the collagen hydrogel on the survival of primary dopaminergic neurons in the striatum

In order to assess if the collagen hydrogels are suitable for the delivery of VM cells, free floating TH^+ immunohistochemistry was completed (**Figure 4.10**). The total number of transplanted dopaminergic cells was determined by counting individual TH^+ cell bodies in the transplanted region and correcting using Abercrombie's principle (see **Section 2.9.2** for more details). In order to determine graft volume, the area of the graft was measured and graft volume was assessed according to Cavalieri's Principle (see **Section 2.9.3** for more details). The immunostaining showed that the delivery of VM cells in the collagen hydrogel did not have any significant impact on the survival of dopaminergic cells (**Figure 4.11a**; K=9.643, $P>0.05$) or in graft volume (**Figure 4.11b**; K=9.643, $P>0.05$) when compared to cells transplanted alone. These results indicate that the collagen hydrogels are permissive for primary dopaminergic cell survival in the striatum.

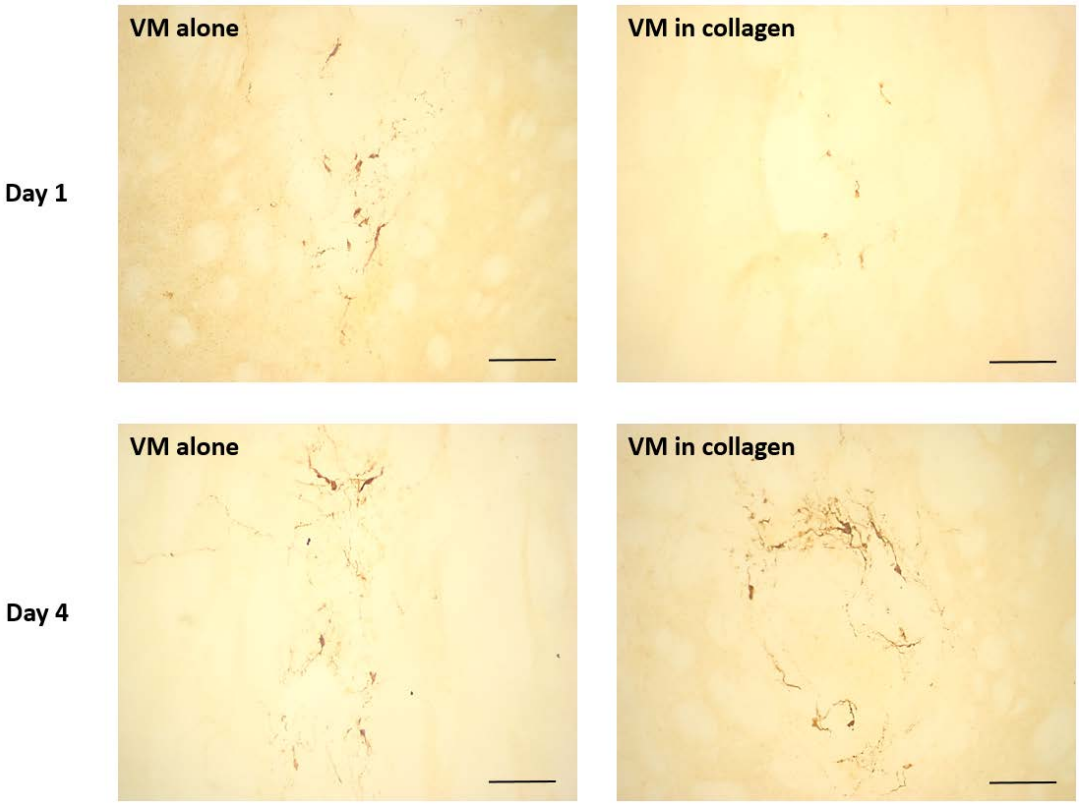


Figure 4.10 Representative photomicrographs of the effects of collagen hydrogel on dopaminergic neurons in the striatum.

Representative photomicrographs show low TH^+ immunostaining at the transplantation site at day 1 (A) or day 4 post-transplantation (B). Scale bar represents 200 µm.

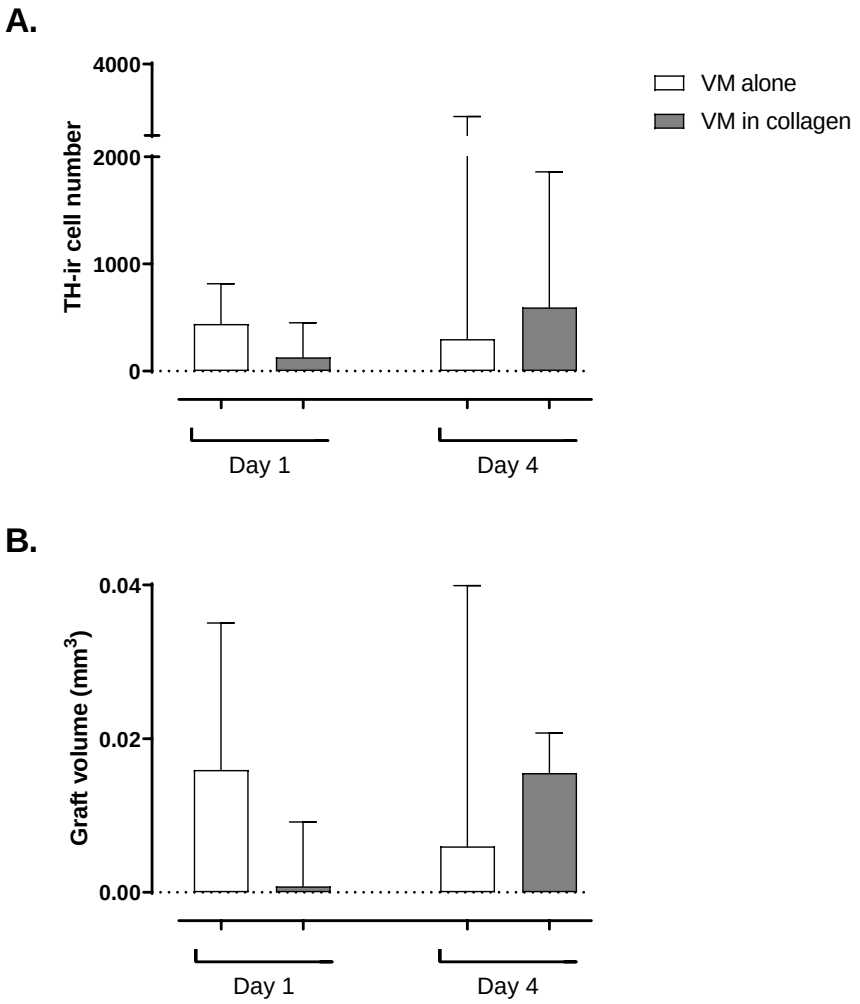


Figure 4.11 *In vivo* assessment of the impact of collagen hydrogels on dopaminergic graft survival at 1 or 4 days post-transplantation.

The TH^+ immunostaining showed that the delivery of VM cells in the collagen hydrogel did not have a negative impact on the survival of dopaminergic cells (A) or in graft volume (B). Therefore, the collagen hydrogels are cytocompatible and suitable for transplantation. Data are represented as median and interquartile range and were analysed by Kruskal-Wallis. n=4 per group.

4.3.2 STUDY 2: *IN VIVO* ASSESSMENT OF THE ABILITY OF THE COLLAGEN HYDROGELS TO DELIVER AND RETAIN GDF5 IN THE STRIATUM

Preclinical and clinical studies on intrastriatal grafts suggest that most of the dopaminergic neurons die within the first 4 days after transplantation (Mahalik *et al*., 1994; Zawada *et al*., 1998; Emgård *et al*., 1999; Sortwell *et al*., 2001). Therefore, it is important that the hydrogel used is able to retain a neurotrophic factor for this short period of time.

In this preliminary study we investigated the ability of collagen hydrogels to deliver and retain GDF5, in the striatum at early time points. Additionally, in order to build up a profile of the *in vivo* behaviour of the collagen hydrogels, we also assessed polymerisation, degradation and host immune response to neurotrophic factor with an additional time-point (perfusion at day 1, day 4 and day 7).

4.3.2.1 *In situ* polymerisation and biodegradation of the collagen hydrogel

The photomicrographs of the bovine collagen immunostaining confirmed the presence of the collagen hydrogels at the transplantation site and showed that they polymerise *in situ* (**Figure 4.12)**. Importantly, there was a trend showing that collagen hydrogels are biodegraded by day 4 and day 7 (**Figure 4.13**; Time $F_{(2,6)}$=0.9006, P>0.05)

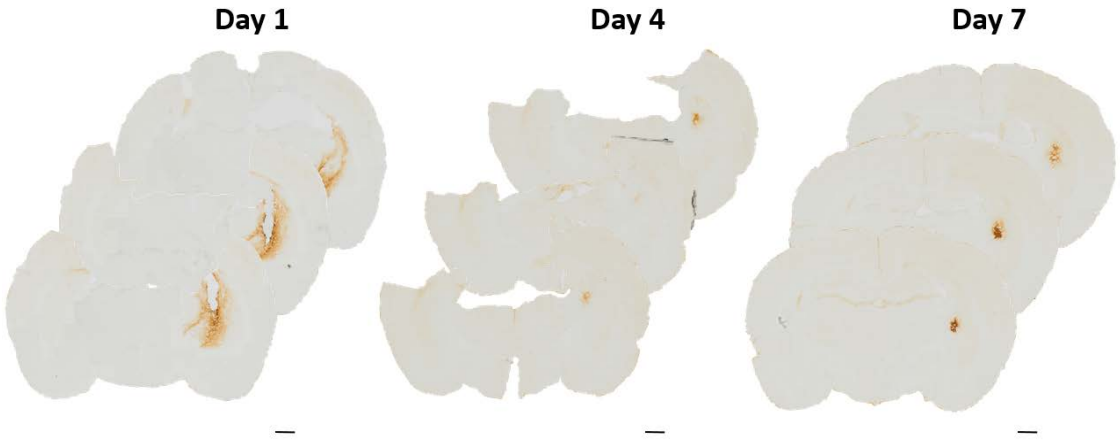


Figure 4.12 Representative photomicrographs of *in situ* polymerisation and degradation of collagen hydrogels.

Representative photomicrographs show striatal collagen immunostaining at 1, 4- and 7-days post-injection. Collagen hydrogels polymerise *in situ* and are biodegraded over time. Scale bar represents 2 mm.

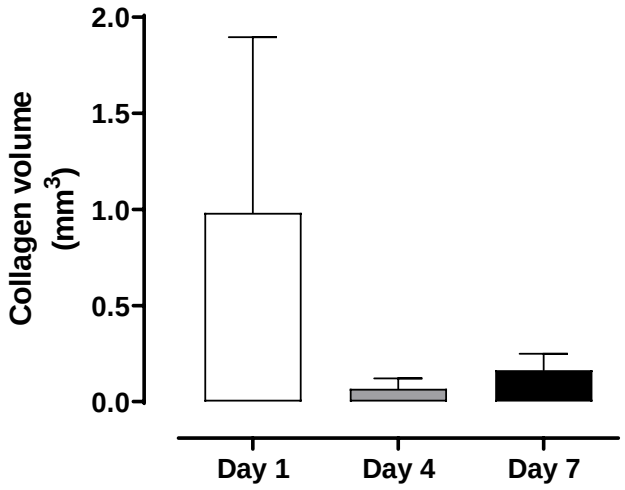


Figure 4.13 *In situ* polymerisation and biodegradation of collagen hydrogels.

Collagen hydrogels crosslinked with 4 mg/ml of 4s-StarPEG formed *in situ* and biodegraded by day 4. Data are represented as mean ± SEM and were analysed by one-way ANOVA. n=3 per group.

4.3.2.2 Effects of the collagen hydrogel on the host immune response to neurotrophic factor administration

In the previous study, we established that our collagen hydrogels are cytocompatible and do not evoke an exacerbated host immune response to the transplanted E14 VM cells. Therefore, in this study we sought to determine whether the collagen hydrogels were biocompatible and did not evoke an exacerbated host immune response to the administration of GDF5 in the striatum. For this, we quantified the volume and optical density of astrocytes (**Figure 4.14**) and microglia (**Figure 4.16**) via immunostaining for GFAP and CD11b.

The immunostainings showed that injection of GDF5 did elicit an astrocytic and microglial response in the host brain. However, the GFAP immunostaining showed that the delivery of GDF5 in a collagen hydrogel decreased the volume (**Figure 4.15a**; Interaction $F_{(2,12)}$= 7.761, P<0.05) and density (**Figure 4.15b**; Group, $F_{(1,12)}$=86.28, P<0.05) of astrocytes found around the injection site particularly at 7 days post-injection. Furthermore, the CD11b immunostaining showed that the delivery of GDF5 in the collagen hydrogel did not exacerbate the volume of microglia present at the

injection site (**Figure 4.17a**; Interaction, $F_{(2,12)}$=1.099, P>0.05) and significantly decreased the density of the CD11b staining at 1 and 7 days post-injection on the site (**Figure 4.17b**; Treatment, $F_{(1,12)}$=37.72, P<0.05). These results suggest that the collagen hydrogels can reduce the volume and density of astrocytes and microglia to neurotrophic factor administration to the brain.

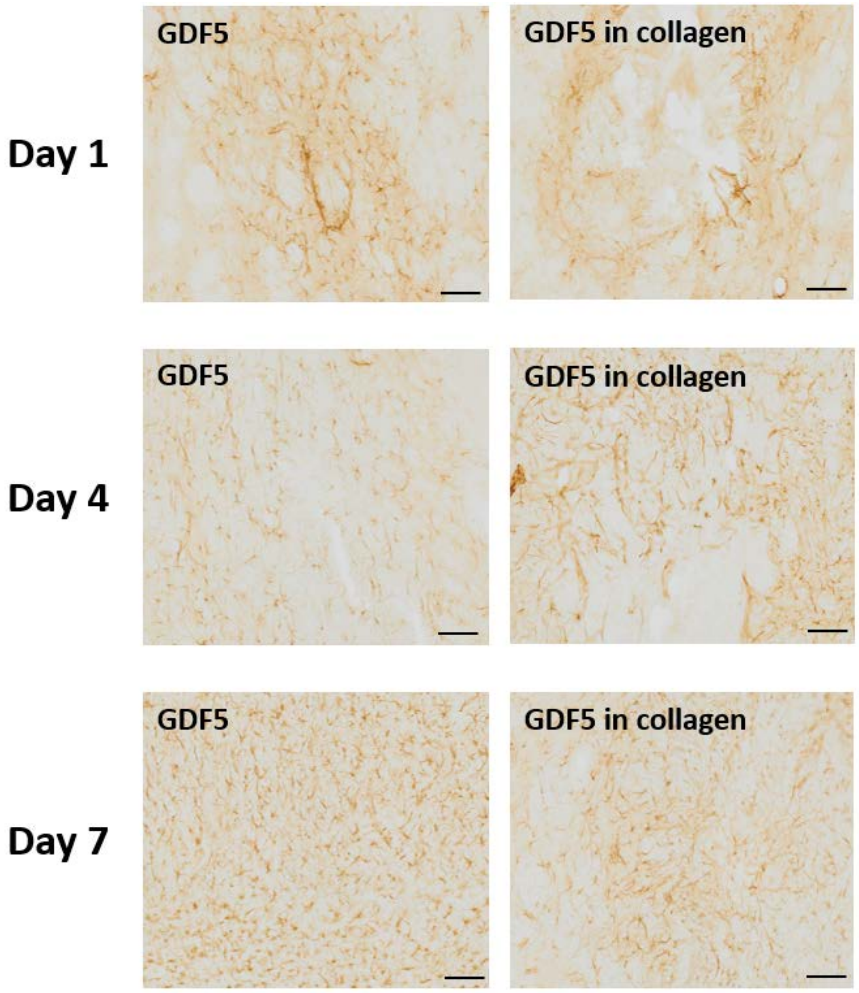


Figure 4.14 Representative photomicrographs of the astrocytic response to injection of GDF5 alone vs. GDF5 in collagen hydrogel.

Representative photomicrographs show the GFAP immunostaining at day 1, 4, and 7 post-injection. The immunostaining showed that the injection of GDF5 alone elicited a substantial astrocytic response. However, the delivery of GDF5 in the collagen hydrogels did not exacerbate the astrocytic response around the injection site. Scale bar represents 100 µm.

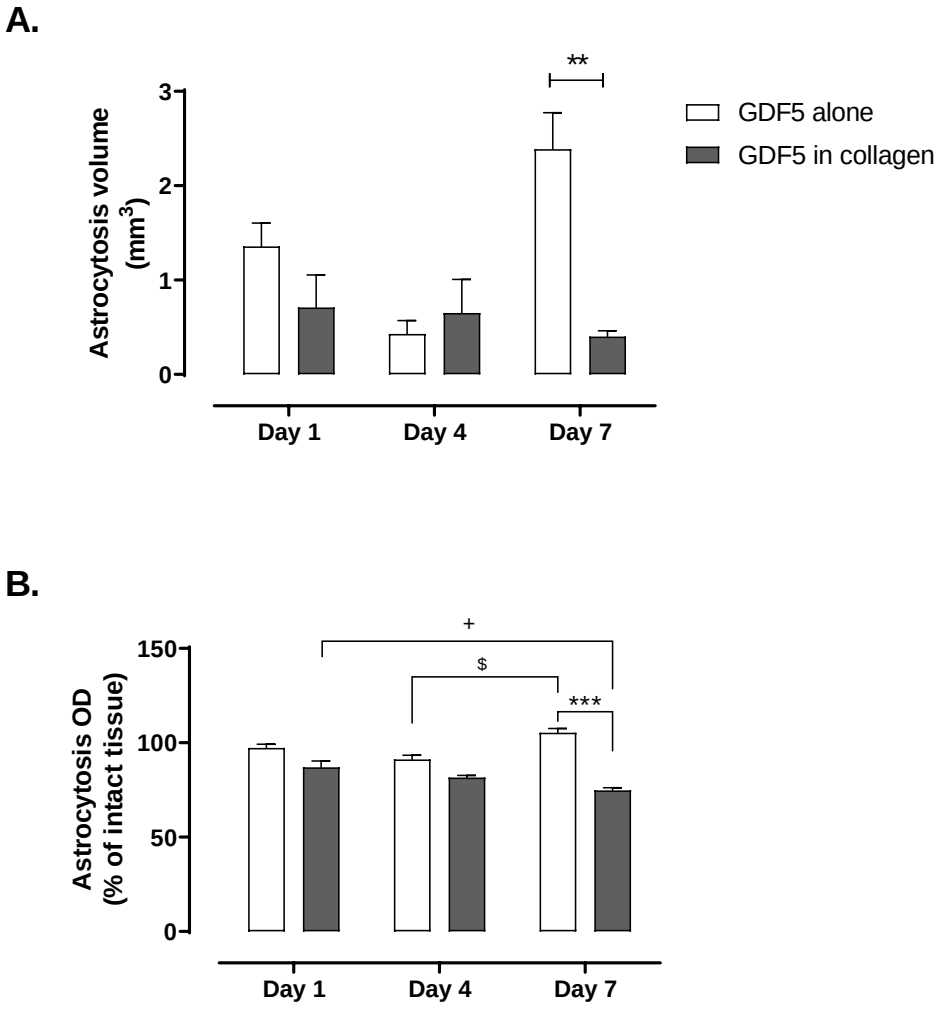


Figure 4.15 Astrocytic response to injection of GDF5 alone vs GDF5 in collagen hydrogel.

The GFAP immunostaining showed that collagen hydrogels encapsulating GDF5 did not elicit an exaggerated immune response. The delivery of GDF5 in the collagen hydrogel significantly decreased the volume of astrocytes (A) around the graft site at day 7 post-injection vs the injection of GDF5 alone. The optical density (B) showed a significant reduction in the density of astrocytes post-injection between the groups that received GDF5 in collagen vs the injection of GDF5. Data are expressed as mean ± SEM and were analysed by two-way ANOVA with *post-hoc* Tukey's multiple comparison test. n=3 per group. (A)***P<0.01* vs. injection of GDF5 alone at 7 days post-injection. (B) $^{+}$*P<0.05* vs. GDF5 in collagen at day 1 post-injection. $^{\$}$*P<0.05* vs. GDF5 alone at 7 days post-injection. ****P<0.001* vs. injection of GDF5 alone at 7 days post-injection.

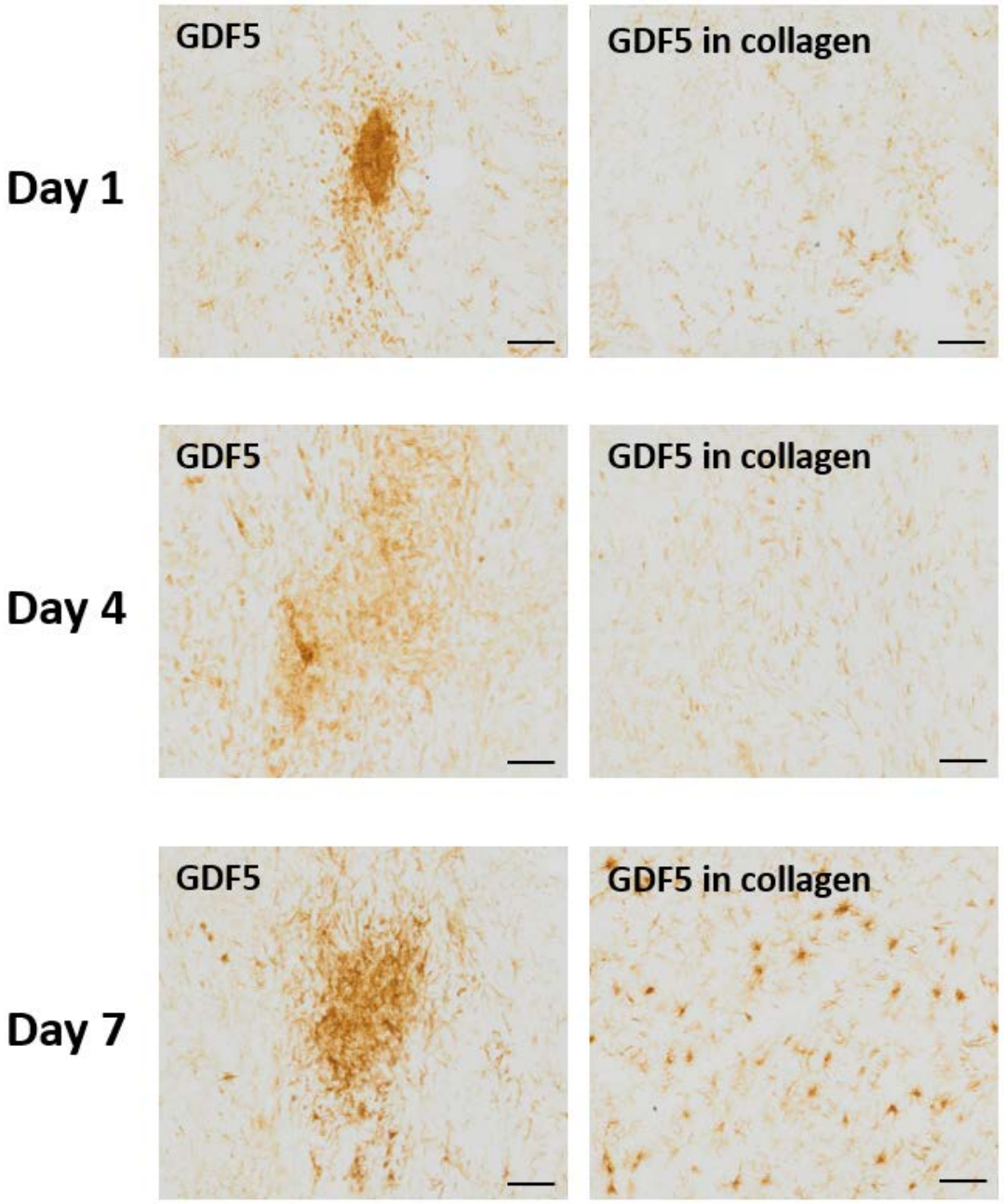


Figure 4.16 Representative photomicrographs of the microglial response to injection of GDF5 alone vs GDF5 in collagen hydrogel.

Representative photomicrographs show the CD11b immunostaining at day 1, 4, and 7 post-injection. The immunostaining showed that the injection of GDF5 alone elicited a substantial microglial response. However, the delivery of GDF5 in the collagen hydrogels did not exacerbate the microglial response around the injection site. Scale bar represents 100 µm. n=3 per group.

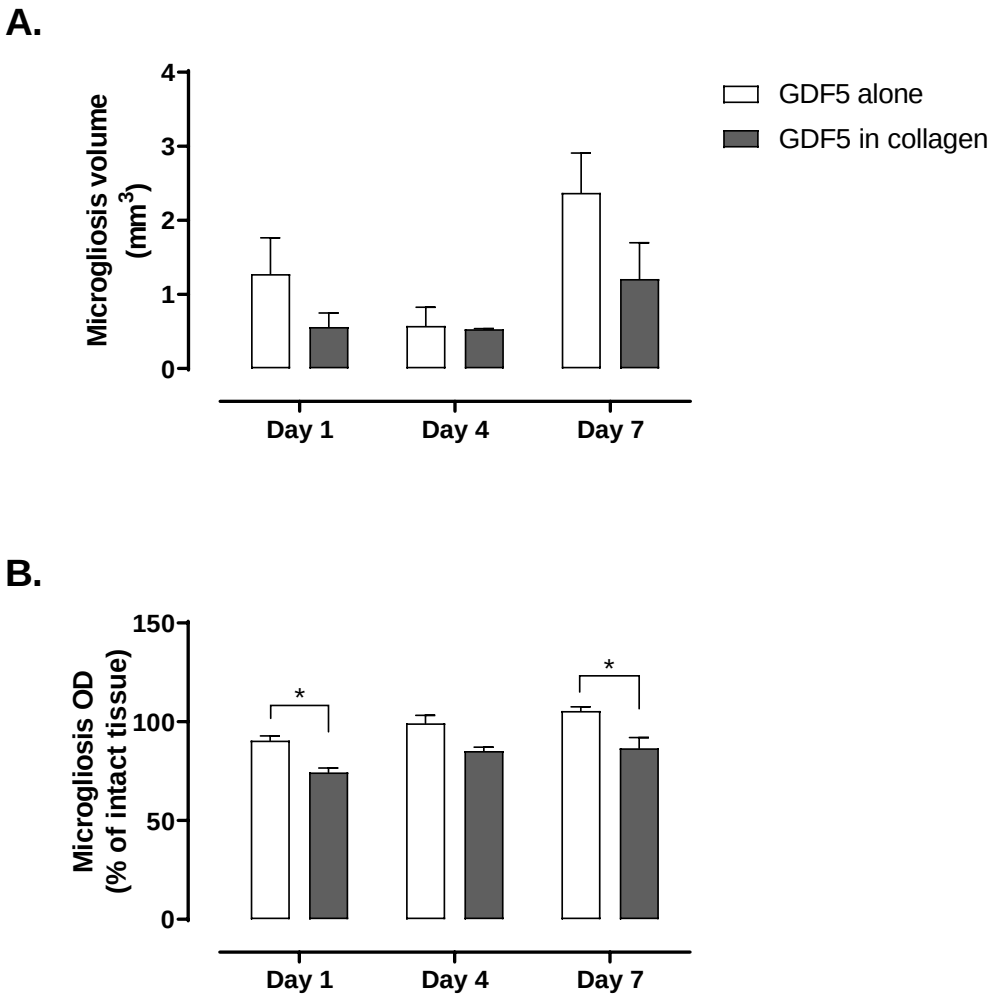


Figure 4.17 Microglial response to injection of GDF5 alone vs. GDF5 in collagen hydrogel.

The CD11b immunostaining showed that GDF5 in collagen hydrogel did not elicit an exaggerated immune response (A). Additionally, GDF5 in collagen hydrogel showed a significant decrease in the density of microglia present at the injection site (B) at 1 and 7 days compared to GDF5 alone at those time points. Data are expressed as mean ± SEM and were analysed by two-way ANOVA with *post-hoc* Tukey's multiple comparison test. n=3 per group. (B)*$P<0.05$ vs. injection of GDF5 alone at day 1 and 7 post-injection.

4.3.2.3 Effects of the collagen hydrogel on the delivery and retention of GDF5 in the striatum

Having established that the collagen hydrogel is biocompatible for the delivery of GDF5, we then sought to determine its ability to deliver and retain the neurotrophic factor in the striatum. Free floating immunostaining for GDF5 was attempted to visualize its retention and delivery, however, we were unable to detect it. Two different primary antibodies for GDF5 were tested using several concentrations (1:50, 1:100, 1:200, 1:500), looking to optimize this immunostaining but no GDF5 staining could be detected (**Figure 4.18**).

Despite being unable to determine if the collagen hydrogel affected the delivery or retention of GDF5 in the striatum, we did establish that collagen hydrogels were biocompatible for its delivery and that the host immune response was reduced. Thus, in the next study, we assessed the effects of GDF5 enrichment of collagen hydrogels on primary dopaminergic neuron grafts at an early time-point.

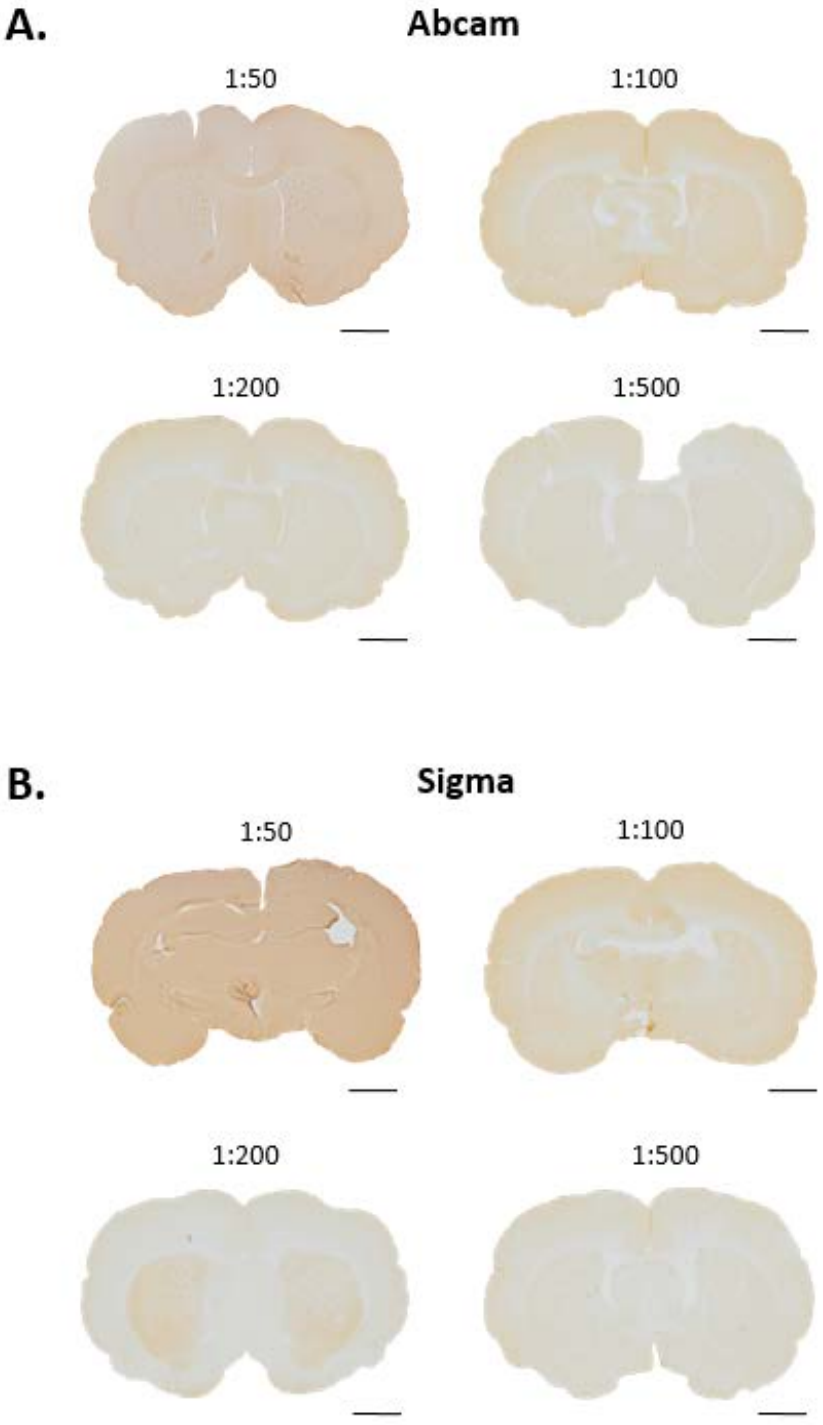


Figure 4.18 Representative photomicrographs of attempts to assess *in vivo* assessment of GDF5 retention within the collagen hydrogel.

Primary antibodies from 2 different providers were used for detection of the GDF5 immunostaining. Abcam (A) and Sigma (B) antibodies were tested at the following concentration 1:50, 1:100, 1:200, and 1:500, however GDF5 was not detected.

4.3.3 STUDY 3: *IN VIVO* ASSESSMENT OF THE EFFECTS OF GDF5 ENRICHMENT OF COLLAGEN HYDROGELS ON PRIMARY DOPAMINERGIC NEURON GRAFTS AT AN EARLY TIME-POINT

In this preliminary study we investigated the effects of GDF5 enrichment of collagen hydrogels on E14 VM cells at an early time-point (rats were perfused 14 days post-transplantation). To do so, we evaluated host immune response and graft survival.

4.3.3.1 Effects of the GDF5-enriched collagen hydrogel on the host immune response to primary dopaminergic neurons

In order to establish whether the encapsulation of VM cells in combination with one of the three different doses of GDF5-enriched in collagen hydrogels had an effect on the host immune response, we quantified the volume and optical density of astrocytes (**Figure 4.19**) and microglia (**Figure 4.21**) via immunostaining for GFAP and CD11b.

The GFAP immunostaining showed that none of the 3 doses of GDF5 provoked a more pronounced immune response since no significant increase was found in the volume (**Figure 4.20a**; Group $F_{(3,12)}$=0.1677, P>0.05) or density (**Figure 4.20b**; Group, $F_{(3,12)}$=0.7161, P>0.05) of the astrocytes at the transplantation site. However, the CD11b immunostaining showed a significant increase in the volume of microglia (**Figure 4.22a**; Group, $F_{(3,12)}$=5.528, P<0.05) in the group that received transplantation of VM cells with 5 µg of GDF5, although this group did not show a significant increase in the density of microglia in the transplantation site (**Figure 4.22b**; Group, $F_{(3,12)}$=0.9398, P>0.05).

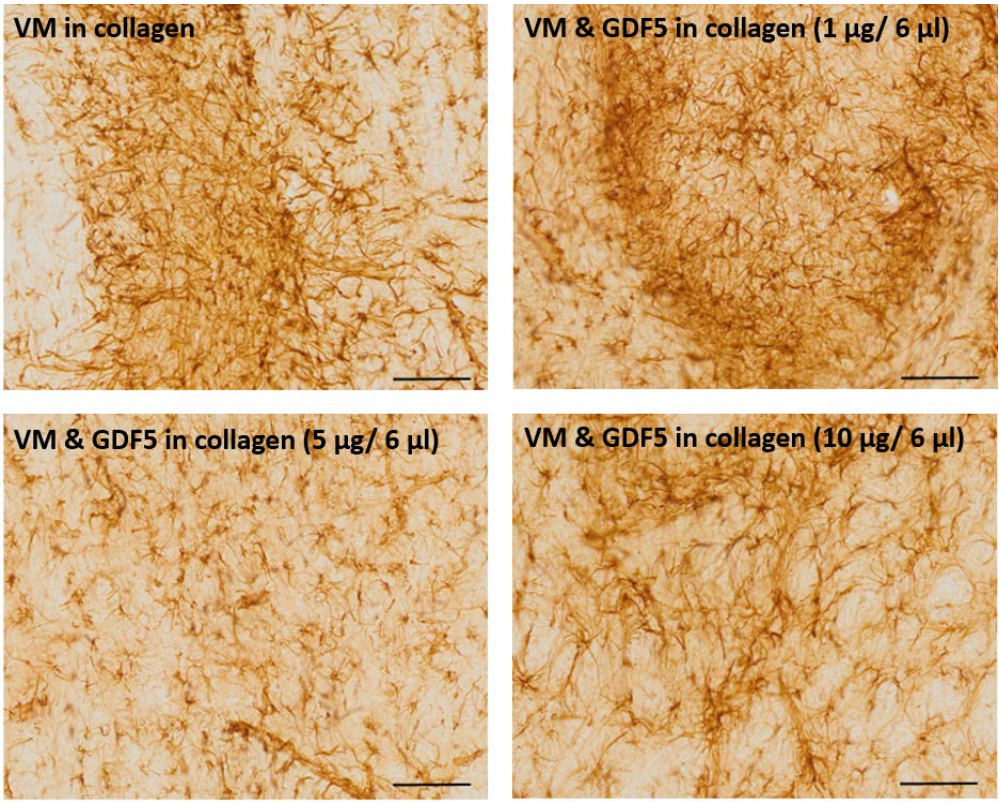


Figure 4.19 Representative photomicrographs of the effects of GDF5-enriched collagen hydrogels on the astrocytic response to primary dopaminergic neurons.

Representative photomicrographs show the GFAP immunostaining at 14 days post-transplantation. The immunostaining showed that the delivery of VM cells in collagen hydrogels functionalised with different doses of GDF5 did not exacerbate the astrocytic response. Scale bar represents 100 µm. n=4 per group.

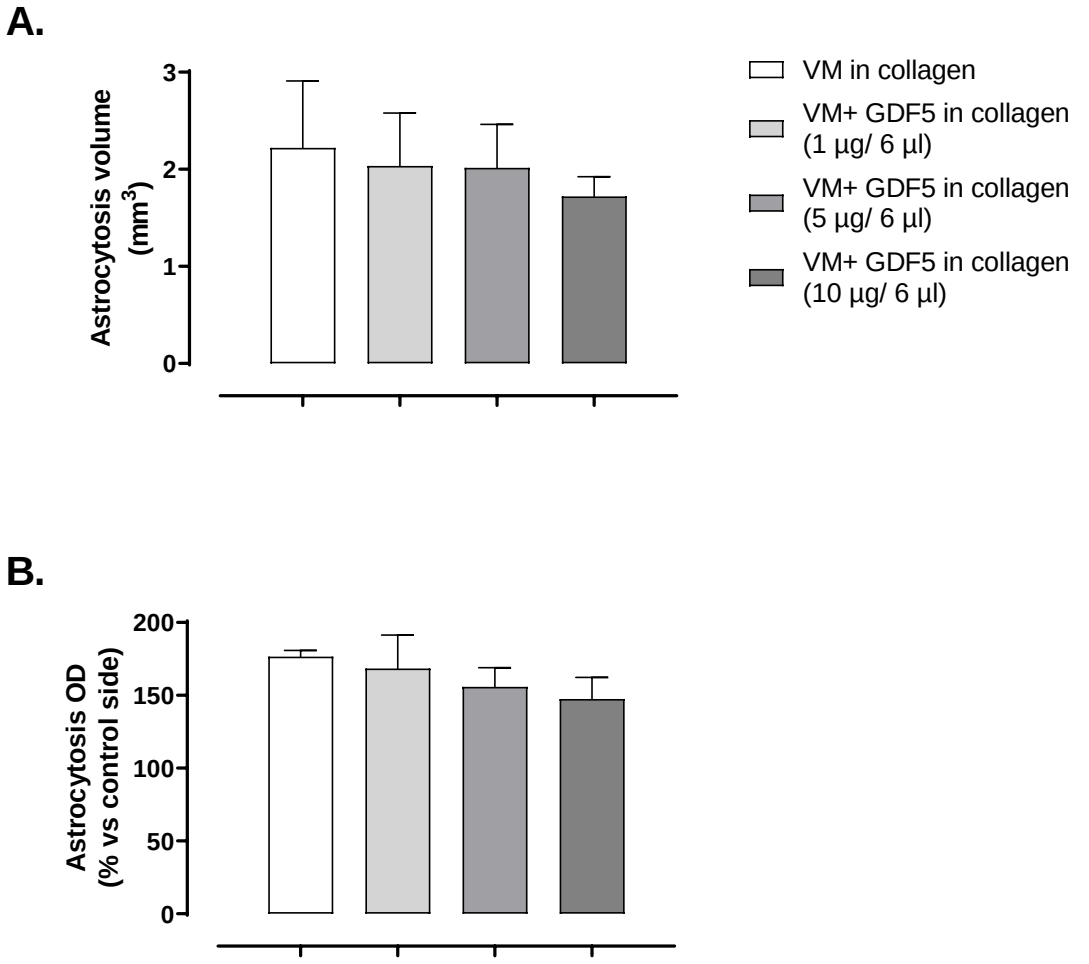


Figure 4.20 Effects of GDF5-enriched collagen hydrogels on the astrocytic response to primary dopaminergic neurons.

The GFAP immunostaining showed that the delivery of VM cells in collagen hydrogels functionalised with one of the 3 different doses of GDF5 did not significantly exacerbate the volume (A) or the density (B) of astrocytes around the graft site. Data are expressed as mean ± SEM and were analysed by a one-way ANOVA with *post-hoc* Newman-Keuls multiple comparisons test. n= 4 per group.

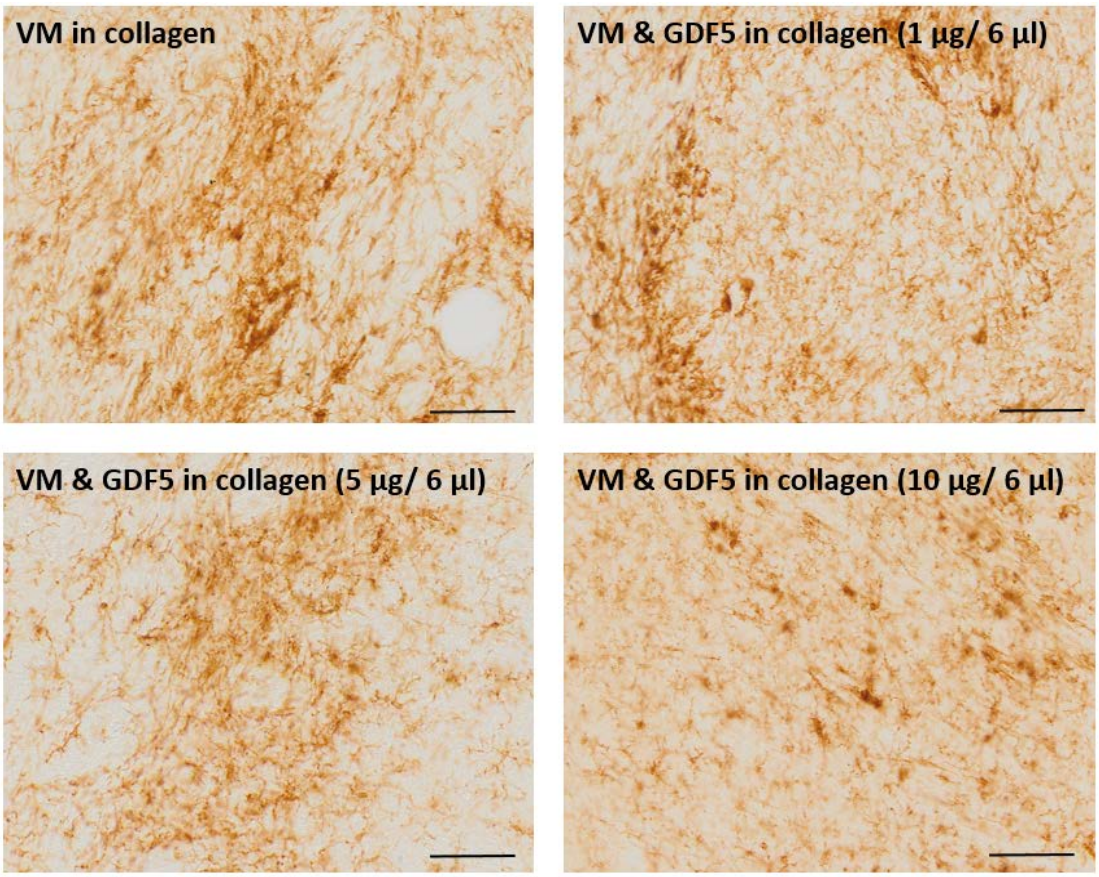


Figure 4.21 Representative photomicrographs of the effects of GDF5-enriched collagen hydrogels on the microglial response to primary dopaminergic neurons.

Representative photomicrographs show the CD11b immunostaining at 14 days post-transplantation. More microglia are observed in the group that received transplantation of VM cells with 5 µg of GDF5-enriched collagen hydrogel. Scale bar represents 100 µm. n=4 per group.

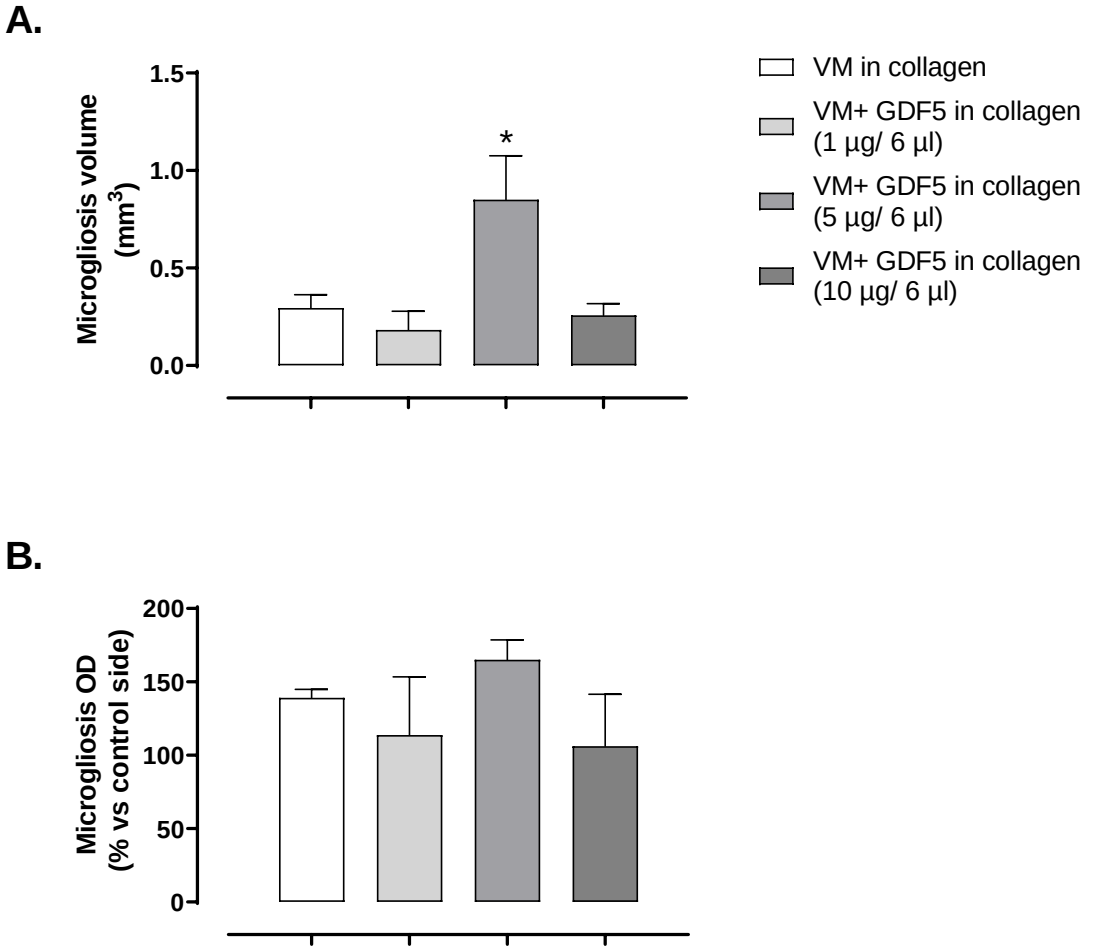


Figure 4.22 Effects of GDF5-enriched collagen hydrogels on the microglial response to primary dopaminergic neurons.

The CD11b immunostaining showed that the delivery of VM cells in collagen hydrogels functionalised with either 1 or 10 µg of GDF5 did not provoked a more pronounced microglial response but the dose of 5 µg increased the volume of microglia present at the transplantation site (A). Furthermore, no significant increase in the density of microglia in the transplantation site was found (B). Data are expressed as mean ± SEM and were analysed by a one-way ANOVA with *post-hoc* Newman-Keuls multiple comparisons test. n=4 per group. **P*<0.05 vs. VM cells delivered in a collagen hydrogel.

4.3.3.2 Effect of the GDF5-enriched collagen hydrogels on the survival of dopaminergic neurons in the striatum

The results from the first study in this chapter indicated that our collagen hydrogels are permissive for primary dopaminergic cell survival in the striatum. Therefore, in this preliminary study we assessed the effects of GDF5-enriched collagen hydrogels on the survival of E14 VM cells in the striatum.

In order to quantify the number of surviving transplanted dopaminergic cells in the de-enervated striatum of the rats, free floating TH^+ immunohistochemistry was completed. At this early timepoint, the immunostaining showed that the GDF5-enriched collagen hydrogel did not have any significant impact on the survival of dopaminergic cells (**Figure 4.23**). Similar numbers of surviving dopaminergic cells where found when the cells were transplanted in GDF5-enriched collagen hydrogels (**Figure 4.24a;** Group $F_{(3,12)}$=0.7556, P>0.05) rather than in an unenriched gel. Additionally, no differences were seen in graft volume (**Figure 4.24b**; Groups $F_{(3,12)}$=0.6058, P>0.05).

Although at this early time-point, the hydrogel was not successful at improving the number of surviving TH^+ dopaminergic cells, it was not detrimental to graft survival. This confirms that GDF5-enrichment of collagen hydrogels is permissive for primary dopaminergic cell survival in the striatum. Further studies are required to see if the neurotrophic enrichment of cells can have positive effects on cells.

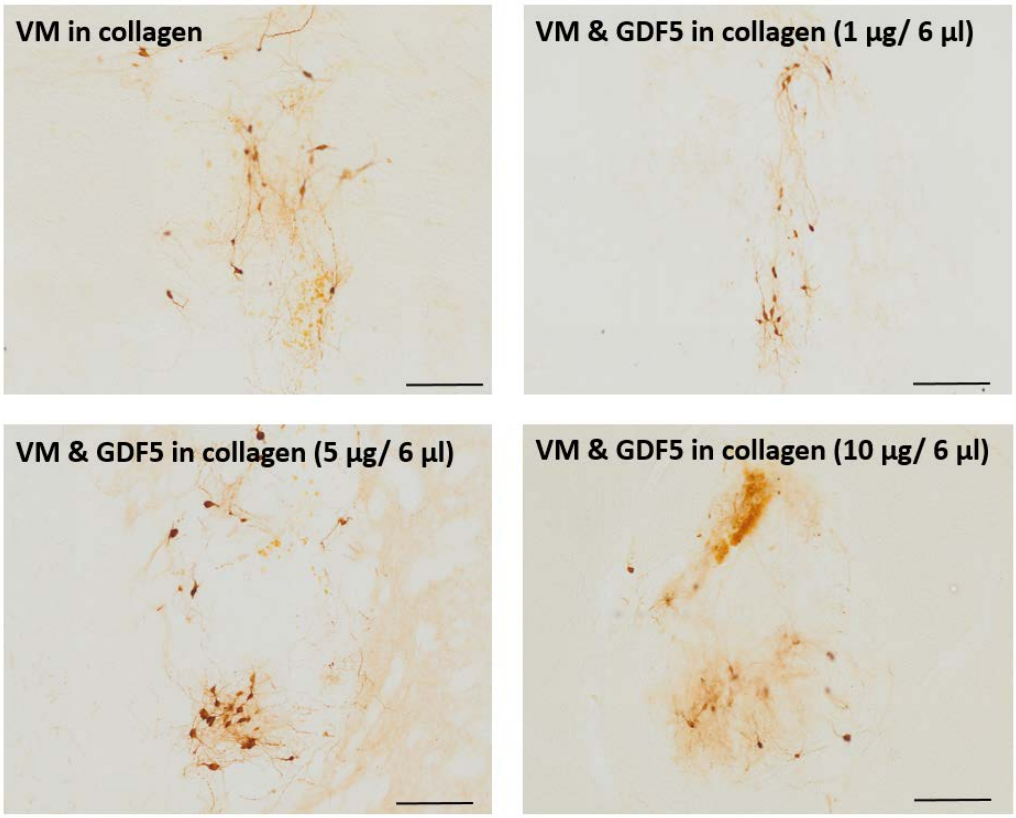


Figure 4.23 Representative photomicrographs of the effects of GDF5-enriched collagen hydrogels on the survival of dopaminergic neurons in the striatum.

Photomicrographs show TH^+ graft survival at the transplantation site 14 days post-transplantation. Scale bar represents 200 µm. n=4 per group.

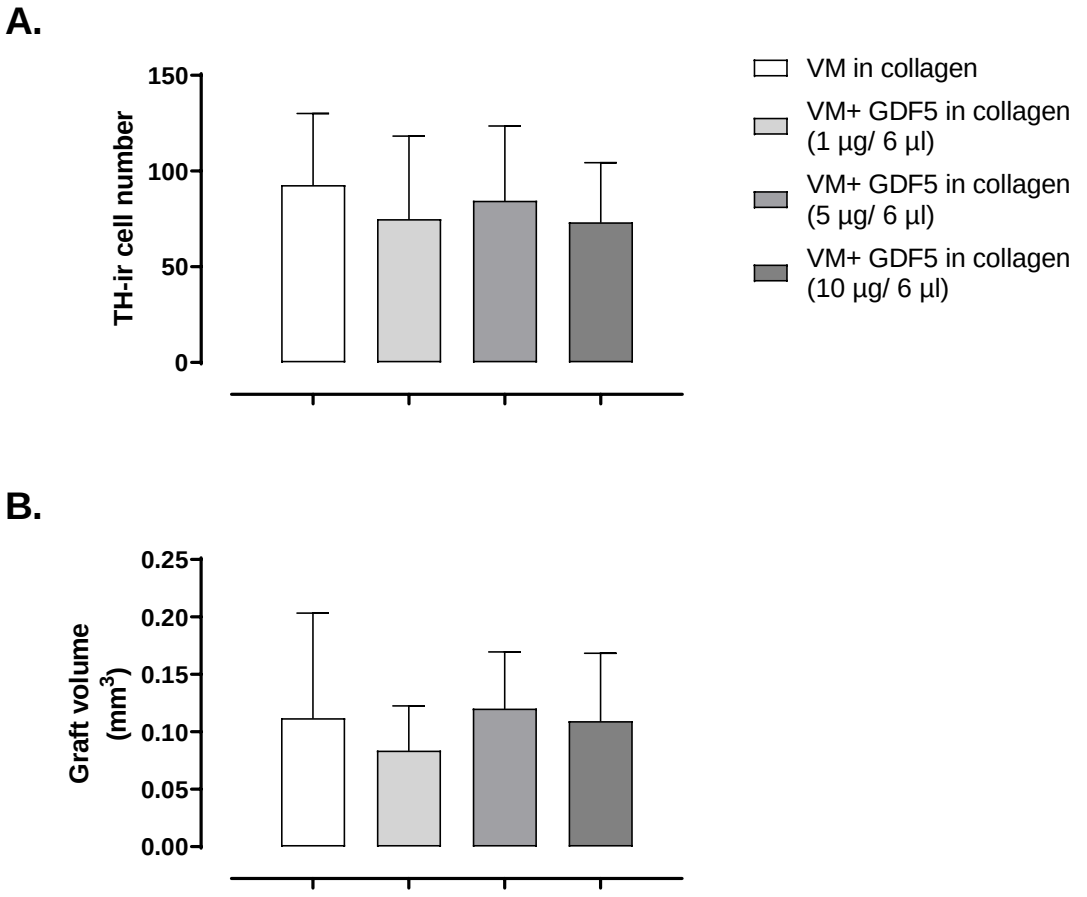


Figure 4.24 Effects of GDF5-enriched collagen hydrogels on the survival of dopaminergic neurons in the striatum.

The TH^+ immunostaining showed that the encapsulation of E14 VM cells in the GDF5 enriched collagen hydrogels of various doses (1, 5 or 10 µg of GDF5) had no negative effects on the number of surviving primary dopaminergic cells (A) or on the volume of the grafts (B). Data are represented as mean ± SEM and were analysed by one-way ANOVA. n=4 per group.

4.4 DISCUSSION

Dopaminergic cell replacement therapy emerged over 40 years ago as a promising treatment for Parkinson´s disease (Perlow *et al*., 1979). This treatment aims to halt the progression of the disease and repair function in the brain, thereby targeting the unmet clinical needs of the current pharmacological and surgical therapies. However, several factors have limited the efficacy of foetal cell transplantation as a restorative approach for Parkinson's disease.

Preclinical and clinical studies on intrastriatal grafts suggest that most of the dopaminergic neurons die within the first 4 days after transplantation caused by apoptosis (Mahalik *et al*., 1994; Zawada *et al*., 1998; Emgård *et al*., 1999; Sortwell *et al*., 2001). Biomaterial systems such as *in situ* forming hydrogels, have the potential to improve the survival and engraftment of cells during and after the transplantation process. These biomaterials can be functionalised to act as a supportive and protective matrix for the cells that can protect them from the host immune response caused by the intracranial delivery. Additionally, these biomaterials can encapsulate therapeutic molecules to enhance the survival and development of the transplanted cells (Hoban *et al*., 2013; Moriarty *et al*., 2019b).

In this chapter, the aims of the preliminary *in vivo* studies were to 1) determine the suitability of the collagen hydrogels for intrastriatal delivery of primary dopaminergic neurons, 2) assess the ability of the collagen hydrogels to deliver and retain the neurotrophic factor GDF5, and 3) determine if GDF5 enrichment of collagen hydrogels has any early effects on primary dopaminergic neuron grafts.

Collagen hydrogels possess many favourable characteristics such as high mechanical strength, good biocompatibility and low antigenicity (Wallace and Rosenblatt, 2003), which make them promising matrices for delivery of cells and drugs. Since collagen can form hydrogels *in situ* under physiological conditions (Xu and Kopeček, 2007), this makes collagen hydrogels an ideal injectable carrier for the intrastriatal delivery of cellular and neurotrophic therapies. The results described in the first pilot study of this chapter, confirmed that collagen hydrogels polymerise *in situ*, do not undergo rapid degradation and are still present up to day 4 post-transplantation.

Additionally, one major obstacle that hinders the survival of cells after transplantation is the substantial host immune response triggered at the graft site (Barker *et al*., 1996). In this study, at these early timepoints, we found that transplantation of VM cells provokes a host immune response at the graft site, but the delivery of these cells in collagen hydrogels did not exacerbate the host astrocytic or microglial response to the implanted cells. These results are in line with the findings of Moriarty and colleagues (2017) who transplanted VM cells encapsulated in collagen hydrogels and reported that the collagen hydrogels did not exacerbate the immune response to the transplanted cells (Moriarty *et al*., 2017).

Furthermore, we found that collagen hydrogels are permissive for primary dopaminergic cell survival in the striatum. The delivery of VM cells encapsulated in collagen hydrogels did not have a negative effect on graft survival when compared to the control group. Therefore, the results found in this first preliminary study confirmed the biocompatible and biodegradable characteristics of collagen hydrogels that make them suitable to use in cell replacement therapy. Once we established the suitability of the hydrogel for intrastriatal delivery of cells, we went on to assess its ability to deliver and retain the neurotrophic factor GDF5 in the striatum.

In the second preliminary study described in this chapter, we continued to build up an *in vivo* profile of the collagen hydrogel´s behaviour, so we looked at the early effects of encapsulation of GDF5 on the host immune response and on the *in situ* polymerisation and biodegradation with an additional early time point (Day 7). In this study, we found that collagen hydrogel encapsulation of GDF5 can reduce the volume and density of astrocytes and microglia present after GDF5 administration to the brain. Moreover, we found that the collagen hydrogel polymerises *in situ* and is biodegraded by day 4. These findings demonstrate the presence of collagen in the window of time where the transplanted VM cells need to be protected the most.

The efficacy of cell replacement therapy is hindered by the lack of appropriate neurotrophic support to the primary dopaminergic neurons in the new host environment (Sortwell *et al*., 2001; Abeliovich and Hammond, 2007). Cell survival is diminished by trophic withdrawal upon transplantation, hence the incorporation of a neurotrophic factor like GDF5, encapsulated in a collagen hydrogel, holds the potential to further improve the survival and development of the transplanted dopaminergic cells, by

providing them with localised and prolonged trophic support during and after transplantation. So, we sought to determine the ability of collagen hydrogels to retain GDF5 in the surrounding striatum; however, it was not possible to detect GDF5 in the brain. Previous *in vivo* studies that have implanted GDF5 in the brain, did not show immunohistochemistry staining for this neurotrophic factor (Sullivan *et al*., 1997, 1999; O'Keeffe *et al*., 2004a) and only mentioned doing TH^+ immunohistochemistry to measure dopaminergic cell survival. Since we were unable to detect GDF5 by immunohistochemistry, we were also unable to determine if the collagen hydrogel affected the delivery or retention of GDF5 in the striatum relative to a bolus injection. Despite being unable to detect GDF5 in the brain, we did establish that collagen hydrogel was favourable for its delivery in that the host immune response to administration of the neurotrophin was reduced. Thus, in the next study, we went on to assess the effect of GDF5 enrichment of collagen hydrogels on primary dopaminergic neuron grafts at an early time-point.

It has been well reported that intracerebral injection of GDF5 can protect and restore the adult nigrostriatal dopaminergic neurons. However, the neurotrophic effects of this growth factor on cell transplantation have not been fully studied (Sullivan *et al*., 1997, 1999; Hurley *et al*., 2004). Therefore, in the third preliminary study of this chapter, we assessed the early effects of GDF5 enrichment of collagen hydrogels on E14 VM cells transplanted to the striatum of 6-OHDA lesioned rats while also looking at the host immune response 14 days post-transplantation. In this study we found that, for the most part, GDF5 enrichment of collagen hydrogels does not provoke a more pronounced host astrocytic or microglial response to the encapsulated VM cells when compared to the control, at this very early time point. Furthermore, we found that GDF5 enrichment of collagen hydrogels is permissive for primary dopaminergic cell survival in the striatum since the delivery of VM cells encapsulated in GDF5-enriched collagen hydrogels did not have a negative effect on graft survival when compared to the delivery of cells in an unenriched collagen hydrogel.

All the findings described in this chapter have shown that our collagen hydrogels are a suitable matrix for cell and/or neurotrophic factor delivery. So, in an effort to enhance the functionality of cell replacement therapy in Parkinson´s disease, the following chapters in this thesis will look into the effects of GDF5 functionalised collagen

hydrogels on primary dopaminergic neuron grafts at long-term time points, in order to allow for assessment of long-term graft survival, striatal dopaminergic reinnervation and functional recovery.

Chapter 5: Assessment of the effect of a GDF5 functionalised collagen hydrogel on E14 primary dopaminergic grafts in a rat model of Parkinson's disease.

5.1 INTRODUCTION

The motor symptoms characteristic of Parkinson's disease are a consequence of the degeneration of striatal and nigral dopaminergic neurons. Therefore, replacement of the lost dopaminergic neurons is a promising brain repair treatment for this disorder (Kordower *et al*., 1995; Hagell and Brundin, 2001; Winkler *et al*., 2005; Mendez *et al*., 2008; Barker *et al*., 2013).

Primary cell transplantation is one of the most promising therapeutic approaches under investigation. However, the success of this treatment is limited by several factors:

- Logistical and ethical concerns: It is difficult to obtain a suitable number of foetal donors for one patient since one transplant requires 4 to 8 donors within a narrow window of gestation time (Kordower *et al*., 1996; Hauser *et al*., 1999; Freed *et al*., 2001). Additionally, only 5 to 10% of donor cells have a dopaminergic phenotype (Sauer and Brundin, 1991).
- Poor survival of transplanted cells: only 3-20% of donor cells survive the transplantation process (Brundin and Björklund, 1987; Kordower *et al*., 1996; Sortwell *et al*., 2001). This poor survival is caused by mechanical damage to cell bodies and axonal projections during dissection, apoptotic cell death caused by the disruption in the cell-cell interactions, neurotrophic factor deprivation and cell damage caused by inflammatory immune response (Lynch and Dawson, 1994; Ghirnikar *et al*., 1998; Brundin *et al*., 2000a).

The results of cell replacement therapy can be improved by the addition of biomaterials in the transplantation process. Cell viability and functionality can be enhanced by the use of naturally-derived materials like collagen hydrogels that can mimic the *in vivo* microenvironment of the transplanted cells by giving them an adhesive matrix for growth while acting as a physical protection against inflammatory microglia and astrocytes (Nakaji-Hirabayashi *et al*., 2012; Lin *et al*., 2016; Wang *et al*., 2016).

Chapter 5: *In vivo* assessment of GDF5 functionalised collagen hydrogel on E14 primary dopaminergic grafts

Previous studies in rat models of Parkinson's disease have provided proof-of-principle of the potential of bioengineered scaffolds such as collagen hydrogels in improving the outcomes of cell replacement therapy by increasing survival, reinnervation and functional efficacy of the transplanted cells (primary dopaminergic cells or mesenchymal stem cells) (Hoban *et al*., 2013; Wang *et al*., 2016; Moriarty *et al*., 2019a).

The outcome of cell replacement therapy can be further improved by the addition of neurotrophic factors to the transplantation process. Growth factors and other active agents can be combined with collagen hydrogels to prolong their release rate and increase their therapeutic effect (Wallace and Rosenblatt, 2003).

GDF5 is a potent neurotrophic factor member of the TGF-β superfamily, that was first identified in 1994 (Storm *et al*., 1994). GDF5 is a great candidate for use in cell transplantation since it is expressed in the rat VM from E12, peaking at E14, the time at which dopaminergic neurones in the developing midbrain are undergoing terminal differentiation and time in which they are used for cell transplantation (O'Keeffe *et al*., 2004b; Clayton and Sullivan, 2007). Previous *in vitro* studies have shown that GDF5 treatment of E14 rat VM cultures increases the survival and morphological differentiation of dopaminergic neurons (O'Keeffe *et al*., 2004a; Wood *et al*., 2005). The restorative properties of GDF5 *in vivo* have been studied using the 6-OHDA-lesioned rat model of Parkinson´s disease and have shown that intracerebral injection of GDF5 can protect and restore the adult rat nigrostriatal dopaminergic neurons (Sullivan *et al*., 1997, 1999; Hurley *et al*., 2004). However, the neurotrophic effects of this growth factor on cell transplantation have not been studied.

Hence, the main aim of the study described in this chapter was to determine whether the encapsulation and delivery of E14 VM cells in a GDF5-loaded collagen hydrogel could enhance the long-term graft survival, striatal dopaminergic reinnervation and functional recovery in a rat model of Parkinson´s disease.

5.2 METHODS

In Chapter 4, we established that collagen hydrogels are a suitable carrier for the delivery of cells and neurotrophic factors, therefore in this chapter we focused on assessing the effects of GDF5 functionalised collagen hydrogels on primary dopaminergic neuron grafts at a long-term time point.

A second study (described in Chapter 6) to investigate the effects of GDF5 plus IL-10 functionalised collagen hydrogels on primary dopaminergic neuron grafts at a long-term time point was run in parallel to the study presented in this chapter.

5.2.1 EXPERIMENTAL DESIGN

The study presented in this chapter was designed to assess the effect of a GDF5 functionalised collagen hydrogel on the survival and efficacy of E14 primary dopaminergic neurons in a rat model of Parkinson's disease.

To do this, 40 male rats were given a unilateral intra-MFB 6-OHDA lesion (12 µg per 3 µl). Two weeks later, the lesion was assessed by methamphetamine-induced rotations (2.5 mg per kg) and based on their performance the rats were matched into 4 groups for the transplantation surgery (for details on groups and doses see **Table 5.1**).

Functional recovery was measured by methamphetamine-induced rotations at 6- and 12-weeks post-transplantation. At 12-weeks post-transplantation, the rats were transcardially perfused with 4% paraformaldehyde for the post-mortem assessment. Free floating immunohistochemistry for TH^+, GFAP and CD11b was completed to identify graft survival, reinnervation and host immune response. A schematic representation of the experimental design is shown in **Figure 5.1** (below). Refer to Chapter 2 for more detailed methodology.

Table 5.1 Groups and doses for the assessment of the effects of GDF5 functionalised collagen hydrogels on E14 primary dopaminergic grafts.

E14 VM cells alone (300,000 cells per 6 µl) n=10	E14 VM cells in collagen (300,000 cells per 6 µl, crosslinked with 4 mg/ml 4s-StarPEG) n=10
E14 VM cells + GDF5 (300,000 cells/20 µg per 6µl) n=10	E14 VM cells + GDF5 in collagen (300,000 cells/20 µg per 6 µl, crosslinked with 4 mg/ml 4s-StarPEG) n=10

Rats received a MFB lesion with 6-OHDA. Two weeks later, the lesion was assessed by methamphetamine induced rotations and based on their performance they were matched into four groups for unilateral intrastriatal transplantation. Rats were transcardially perfused 12-weeks post-transplantation. n= 10 per group.

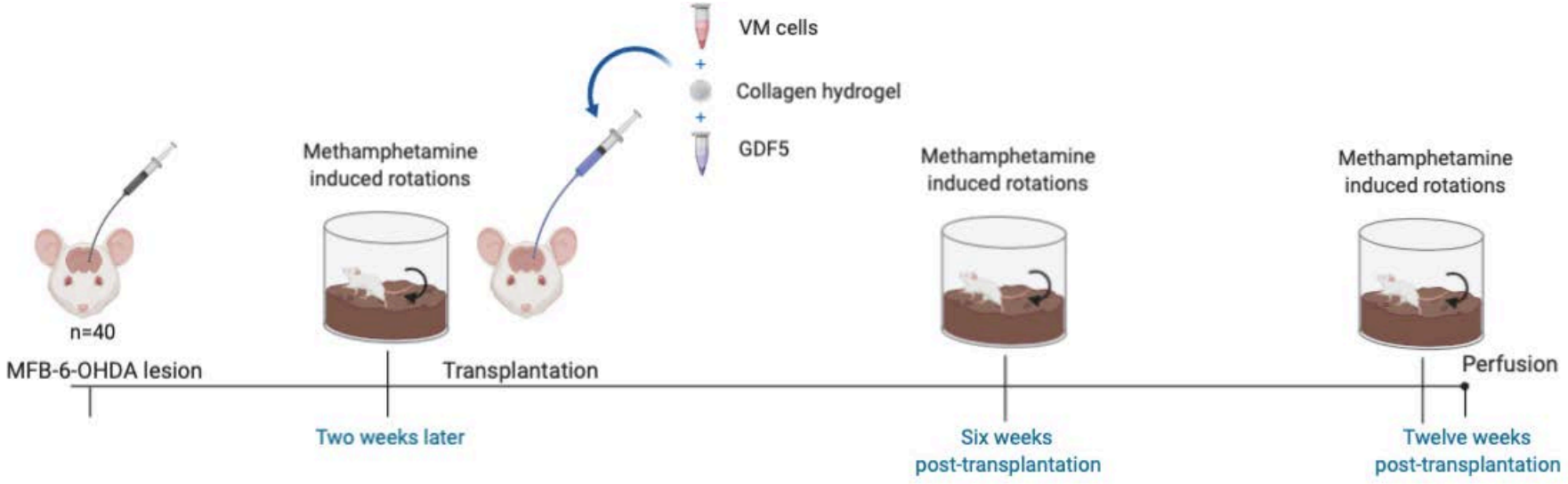


Figure 5.1 Schematic representation of the experimental design of the *in vivo* assessment of the effect of a GDF5 functionalised collagen hydrogel on E14 primary dopaminergic neurons.

Rats received a MFB lesion with 6-OHDA. Two weeks later, the lesion was assessed by methamphetamine induced rotations. Subsequently, rats received a unilateral intrastriatal transplant (for details on groups and doses see **Table 5.1**). Methamphetamine induced rotations were done to assess recovery of motor function at 6- and 12-weeks post-transplantation. Rats were transcardially perfused 12-weeks post-transplantation. n= 10 per group.

5.3 RESULTS

5.3.1 IMPACT OF FUNCTIONALIZED GDF5-LOADED COLLAGEN HYDROGELS ON E14 VM GRAFT FUNCTIONAL RECOVERY

In order to measure functional recovery, methamphetamine-induced rotations were done 6- and 12-weeks post-transplantation in order to assess the ability of the E14 VM grafts to restore motor function to the unilaterally lesioned rats. The delivery of VM cells reduced the number of ipsilateral rotations in all groups (**Figure 5.2**; Time, $F_{(2,70)}$=13.82, P<0.0001). Further *post-hoc* analysis did not show a significant difference between relevant groups, however there was a trend of reduced level of behavioural recovery in the groups transplanted with GDF5.

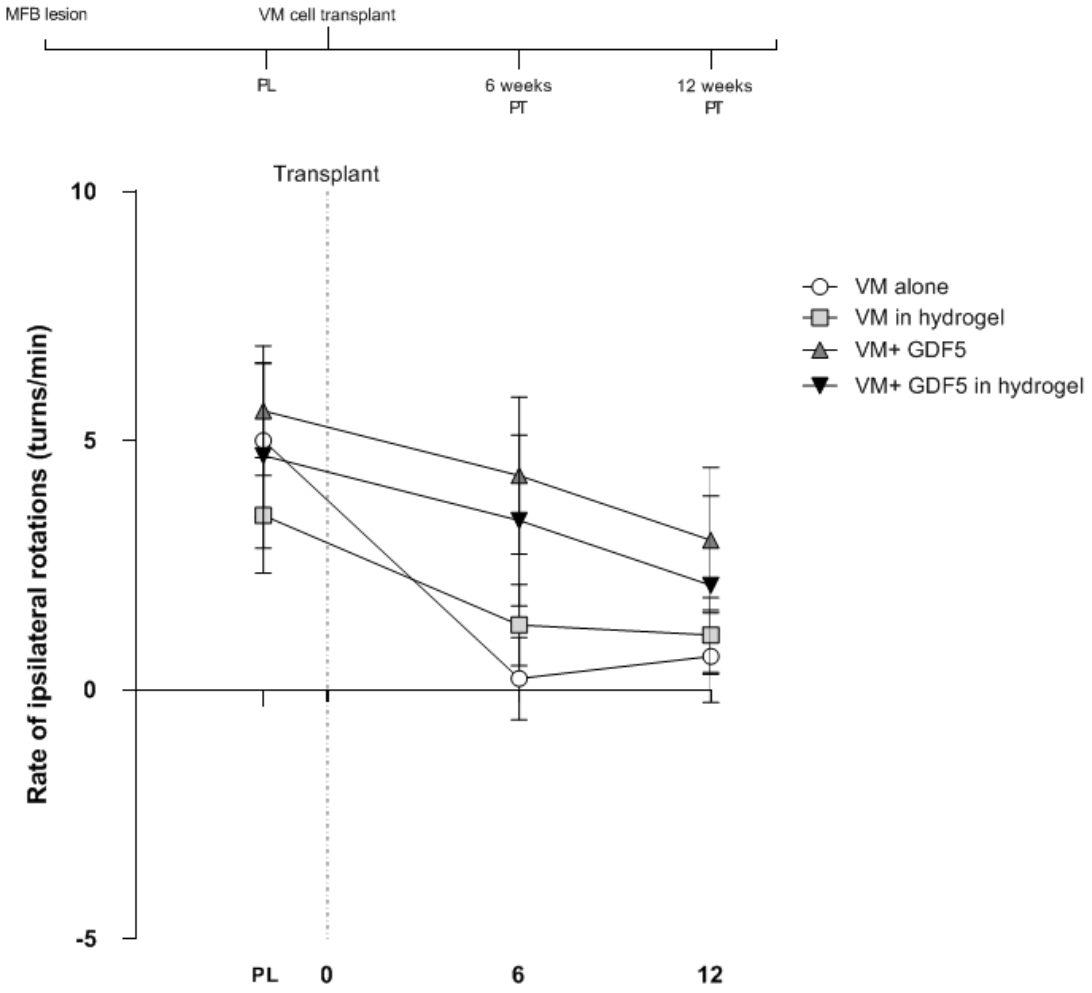


Figure 5.2 Impact of the GDF5-loaded collagen hydrogel on dopaminergic cell graft functionality.

Transplantation of VM cells decreased the number of ipsilateral turns made in each group. However, there was a trend for less recovery when cells were delivered in GDF5 enriched gels. PL; post-lesion, PT; post-transplant. Data are represented as mean ± SEM and were analysed by two-way repeated measures ANOVA followed by Bonferroni test. The *post-hoc* analysis did not show a significant difference between relevant groups. n= 10 per group.

5.3.2 IMPACT OF FUNCTIONALIZED GDF5-LOADED COLLAGEN HYDROGELS ON E14 VM NEURON SURVIVAL

Free floating immunohistochemistry for tyrosine hydroxylase was done in order to quantify the number of surviving dopaminergic cells transplanted in the lesioned striatum. The TH^+ immunostaining identified the successful transplantation of dopaminergic cells in each group (**Figure 5.3** & **Figure 5.4**), however, the incorporation of GDF5 into the transplantation process did not increase the number of surviving cells (**Figure 5.5a**; Treatment, K=5.126, $P>0.05$) nor did it increase the size of the grafts (**Figure 5.5b**; K=4.298, $P>0.05$). Indeed, when cells were delivered with GDF5, either alone or in combination with the collagen hydrogel, there was a trend towards a decrease in the number of surviving cells and smaller grafts when compared to the delivery of VM cells alone.

5.3.3 IMPACT OF FUNCTIONALIZED GDF5-LOADED COLLAGEN HYDROGELS ON E14 VM NEURON STRIATAL RE-INNERVATION

We then measured the ability of the surviving dopaminergic cells to form neural outgrowths and re-innervate the lesioned striatum. The TH^+ immunostaining showed that in all groups the E14 VM grafts successfully re-innervated a portion of the striatum (**Figure 5.4**), however, the incorporation of GDF5 into the transplantation process did not increase the magnitude of re-innervation (**Figure 5.5c**; K=5.042, $P>0.05$). Indeed, in groups where the cells were delivered in combination with GDF5, either alone or in combination with the collagen hydrogel, the re-innervation tended to be of smaller magnitude.

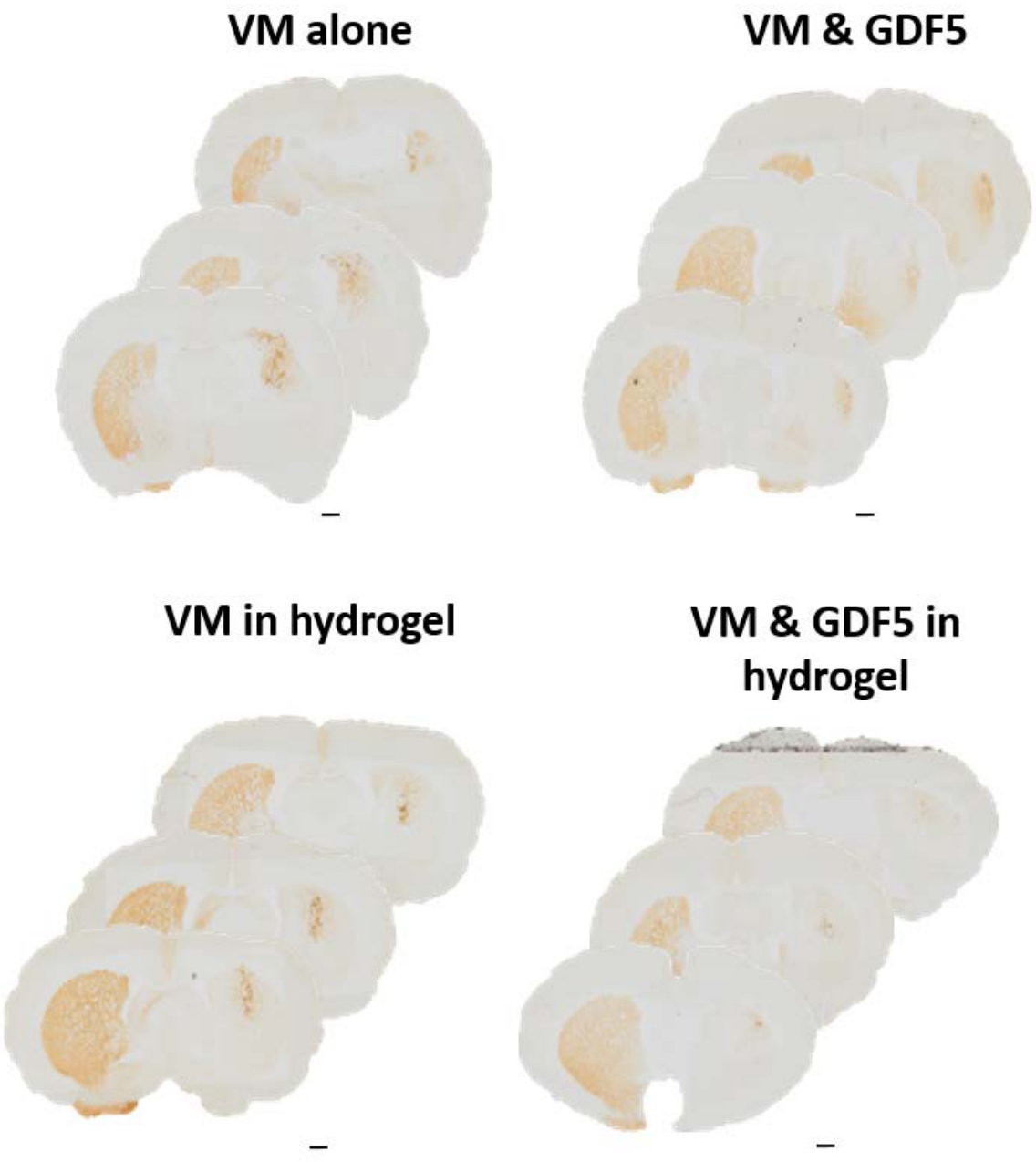


Figure 5.3 Representative photographs of E14 primary dopaminergic cell grafts.

Representative photomicrographs show TH^+ immunostaining at the transplantation site. Re-innervation in a portion of the denervated striatum was found in all the groups. However, when VM cells were transplanted in combination with GDF5 (alone or in collagen hydrogel) grafts seem to be smaller. Scale bar represents 1 mm.

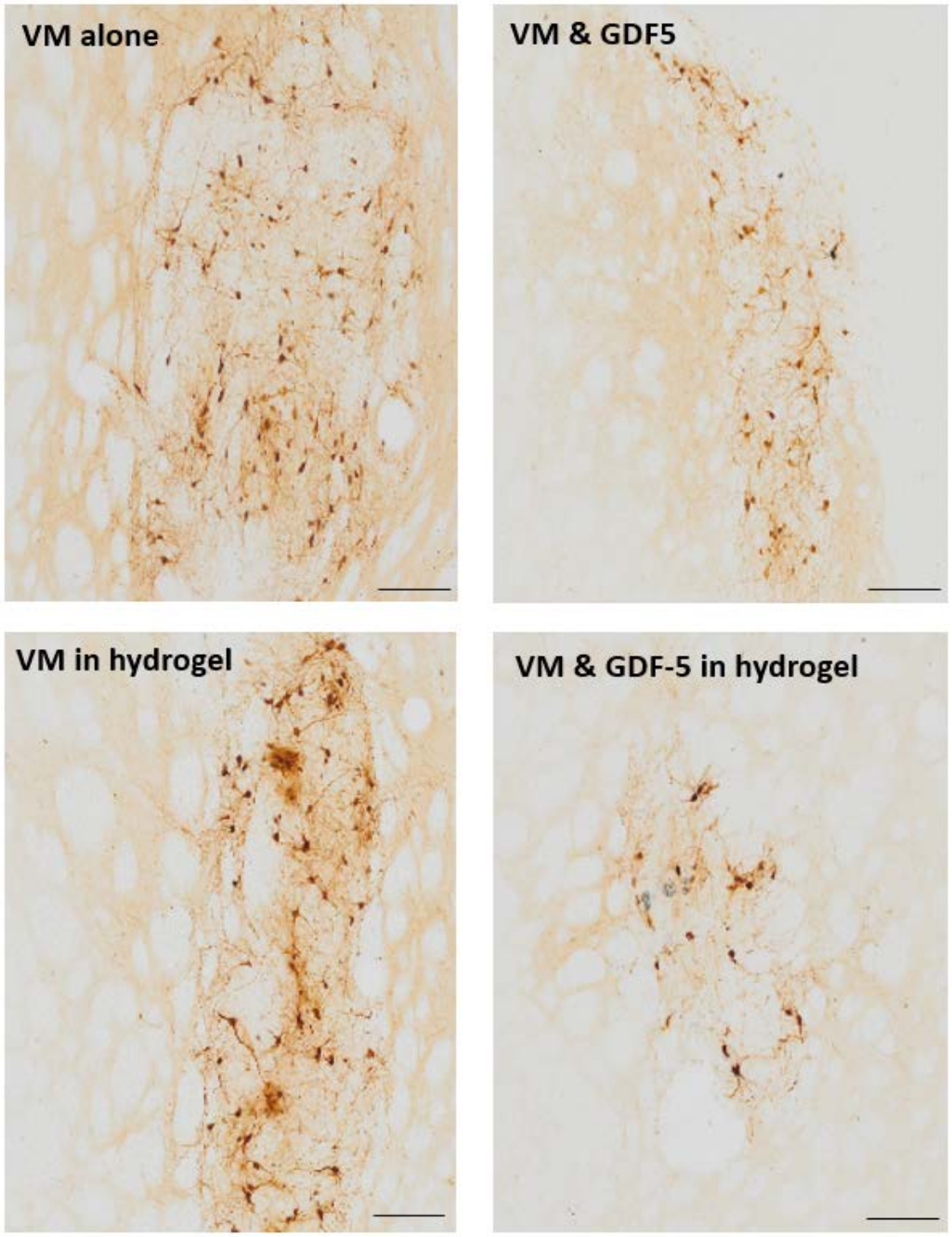


Figure 5.4 Representative photographs of E14 primary dopaminergic neuron survival.

Representative photomicrographs show TH^+ immunostaining. Surviving dopaminergic cells were found in all the groups. However, when VM cells were transplanted in combination with GDF5 (alone or in collagen hydrogel) there seems to be a visual decrease in the number of surviving cells. Scale represents 200 µm.

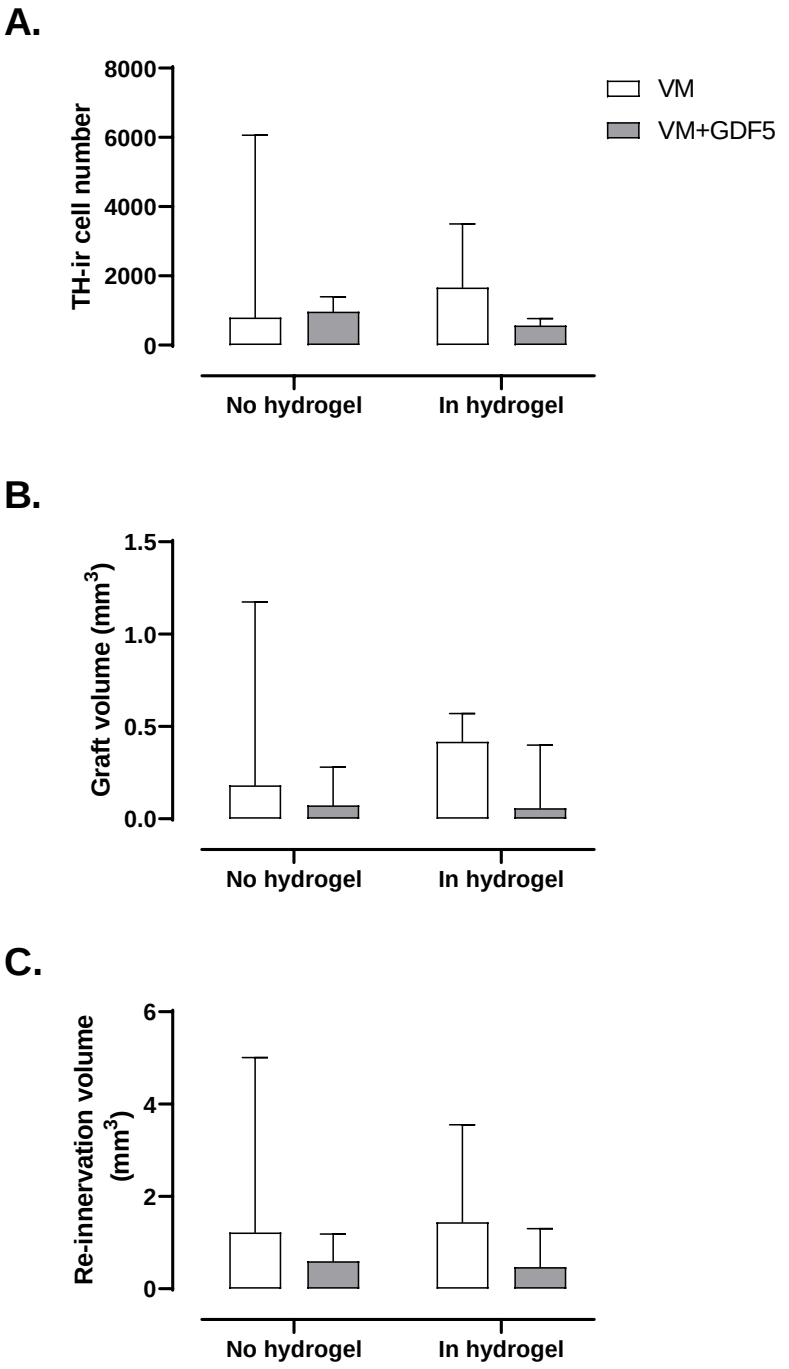


Figure 5.5 *In vivo* assessment of the impact of the GDF5-loaded collagen hydrogel on dopaminergic cell graft survival and striatal re-innervation.

When the VM cells were delivered in a GDF5 loaded collagen hydrogel there was a trend towards a reduced level of TH^+ cell survival (A), smaller grafts (B) and reduced re-innervation (C). Data are represented as median with interquartile range and were analysed by Kruskal Wallis. n=10 per group.

5.3.4 CORRELATION BETWEEN FUNCTIONAL RECOVERY AND E14 VM GRAFTS AT 12-WEEKS POST-TRANSPLANTATION

To determine whether the number of surviving dopaminergic neurons is associated with the observed striatal reinnervation we analysed at the correlation between them. We found a strong correlation (**Figure 5.6a**; r=0.836, $P<0.0001$) that suggests that the grafts containing more surviving dopaminergic cells had a bigger volume of reinnervation in the striatum. However, the behavioural recovery observed was not significantly correlated to the number of surviving TH^+ cells (**Figure 5.6b**; r=0.321, $P>0.05$) or to the volume of striatal re-innervation (**Figure 5.6c**; r=0.322, $P>0.05$).

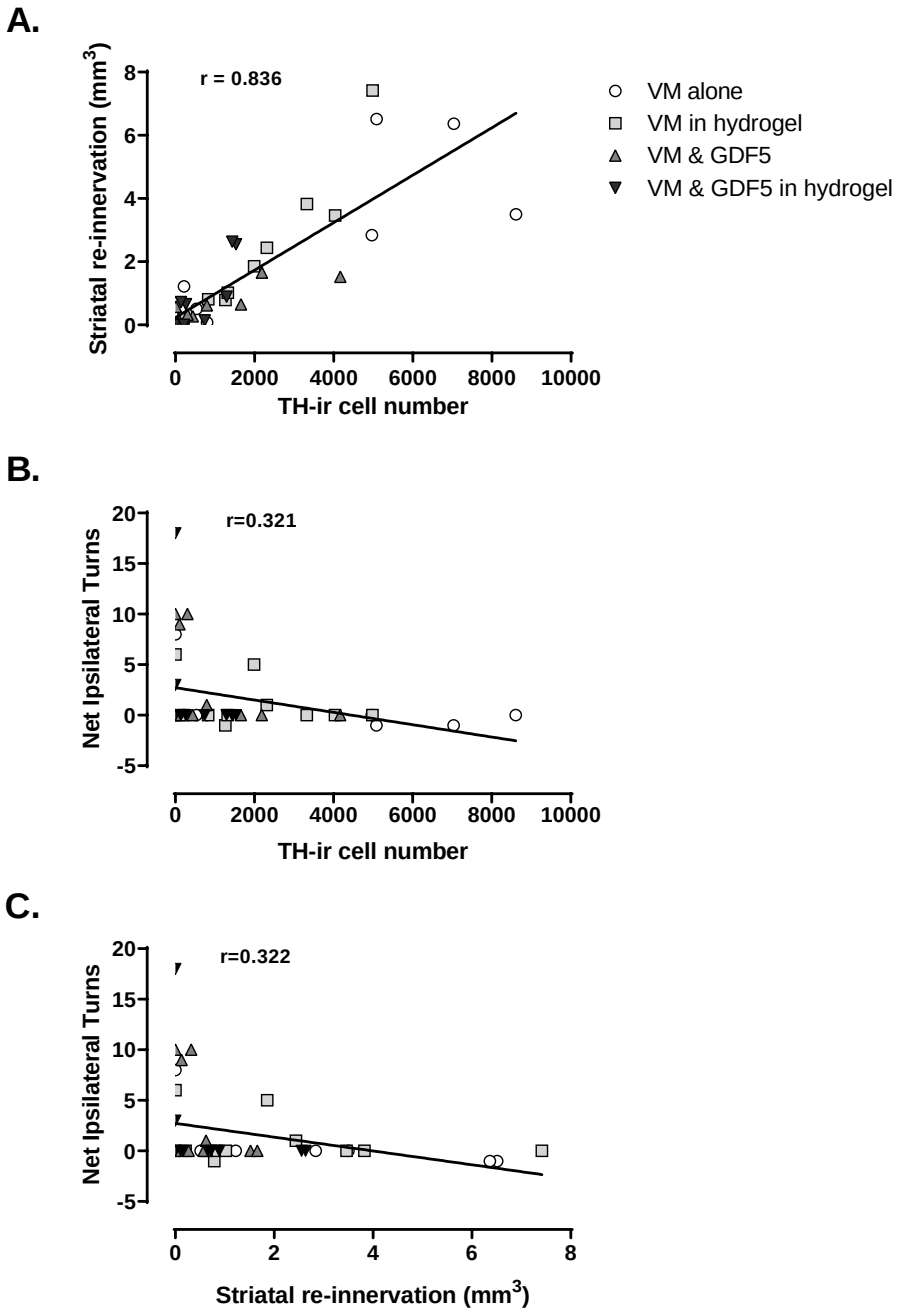


Figure 5.6 Lack of correlation between graft survival and functionality at 12-weeks post-transplantation.

A significant positive correlation was observed between the number of surviving TH^+ neurons and the volume of striatal re-innervation (**A**; r=0.836). No correlation was found between the number of surviving TH^+ cells (**B**; r=0.321) or striatal reinnervation (**C**; r=0.322) and the number of net ipsilateral turns taken (12-weeks post-transplantation).

5.3.5 IMPACT ON THE HOST IMMUNE RESPONSE OF THE GDF5-LOADED COLLAGEN HYDROGELS

In order to evaluate the activation of the host immune response surrounding the graft site, we quantified volume and optical density (see **section 2.9.3 and 2.9.5** for more details) of cells through immunostaining for GFAP and CD11b. The transplantation process elicited proliferation of astrocytes (**Figure 5.7**) and microglia in the striatum (**Figure 5.9**). The GFAP immunostaining did not indicate any beneficial effect of the collagen hydrogels on reducing the volume (**Figure 5.8a**; Treatment $F_{(1,35)}$=0.3286, P>0.05) or density (**Figure 5.8b**; Treatment, $F_{(1,35)}$=2.031, P>0.05) of the astrocytic response. Furthermore, the CD11b immunostaining did not show any beneficial effect of the collagen hydrogel on reducing the volume (**Figure 5.10a**; Treatment, $F_{(1,35)}$=0.0297, P>0.05) or density (**Figure 5.10b**; Treatment, $F_{(1,35)}$=1.094, P>0.05) of the microglia response 12-weeks post-transplant.

5.3.6 EXPRESSION OF HUMAN-GDF5 *IN SITU*

The optimization of the immunostaining for GDF5 was not accomplished but it is likely that after 12-weeks post-transplantation GDF5 cleared out from the brain.

5.3.7 *IN VIVO* BIODEGRADABILITY OF THE COLLAGEN HYDROGEL

The collagen immunostaining showed that our collagen hydrogels completely biodegraded 12-weeks post-transplantation (not shown).

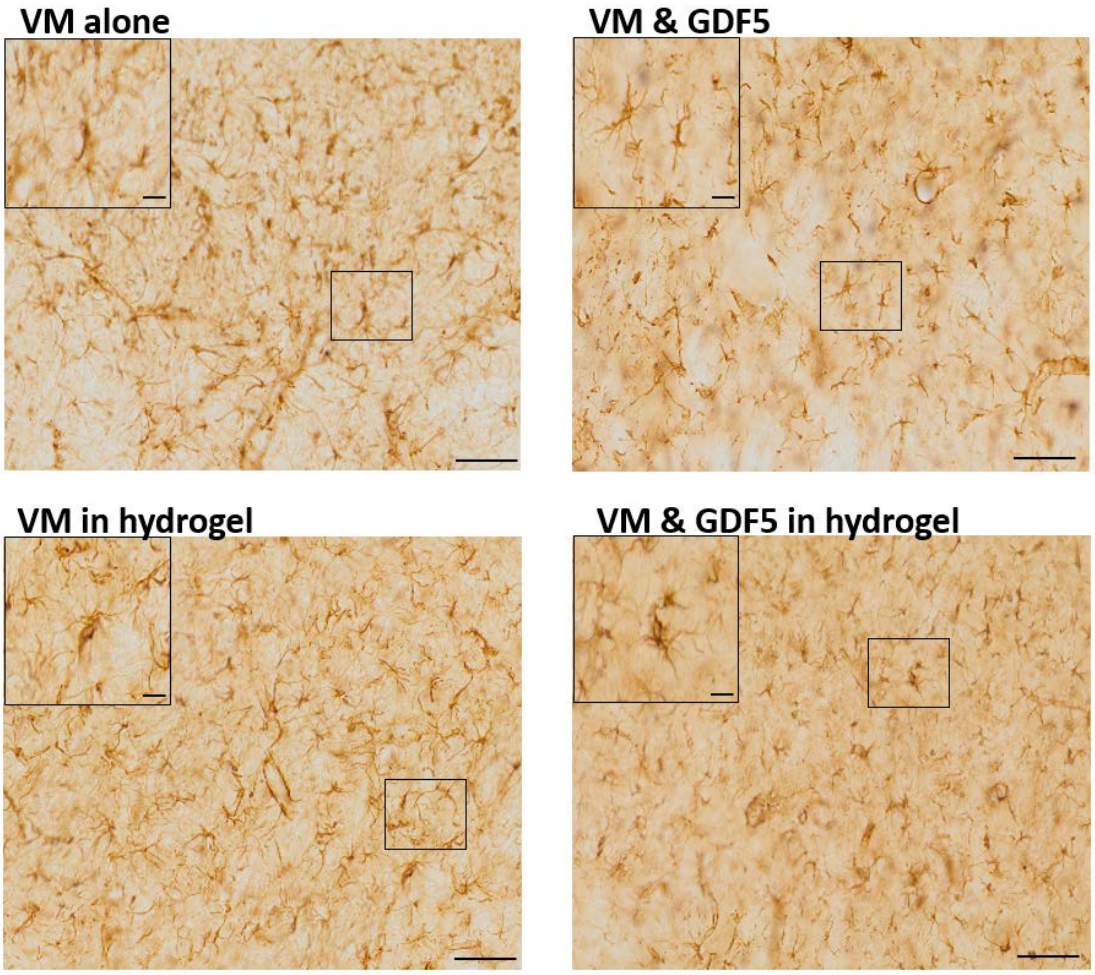


Figure 5.7 Representative photomicrographs of the impact of the GDF5-loaded collagen hydrogel on host astrocytic response.

Representative photomicrographs show GFAP immunostaining at the transplantation site 12-weeks post-transplantation. No beneficial effect of the GDF5-loaded collagen hydrogels on astrocytic response was observed. Scale bar represents 100 µm and 20 µm. n=10 per group.

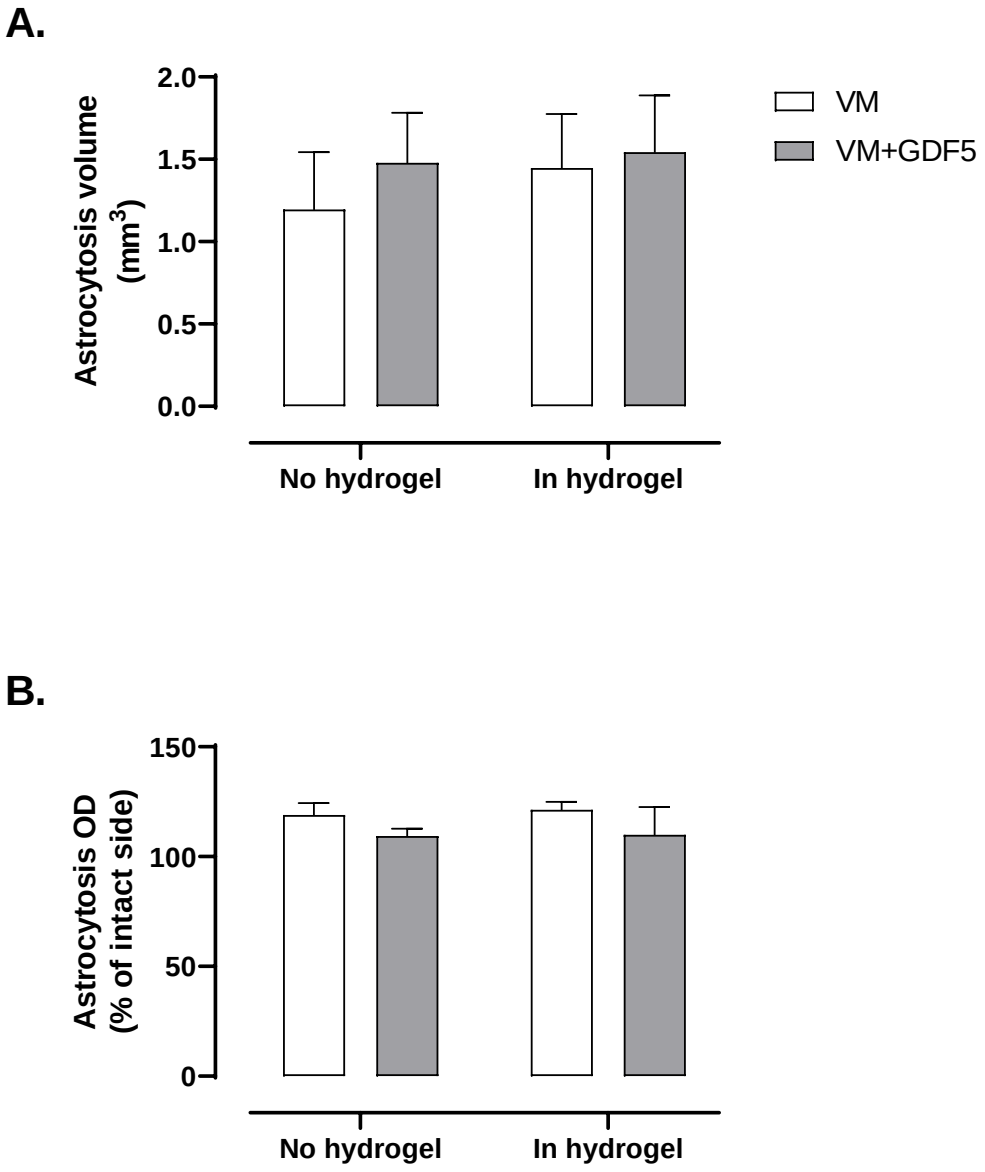


Figure 5.8 Impact of the GDF5-loaded collagen hydrogel on host astrocytic response.

The astrocytic response was measured by GFAP immunostaining. The immunostaining did not show any beneficial effect on the volume (A) or density (B) of astrocytes surrounding the transplantation site 12-weeks post-transplant. Data are represented as mean ± SEM and were analysed by a Two-way ANOVA. n= 10 per group.

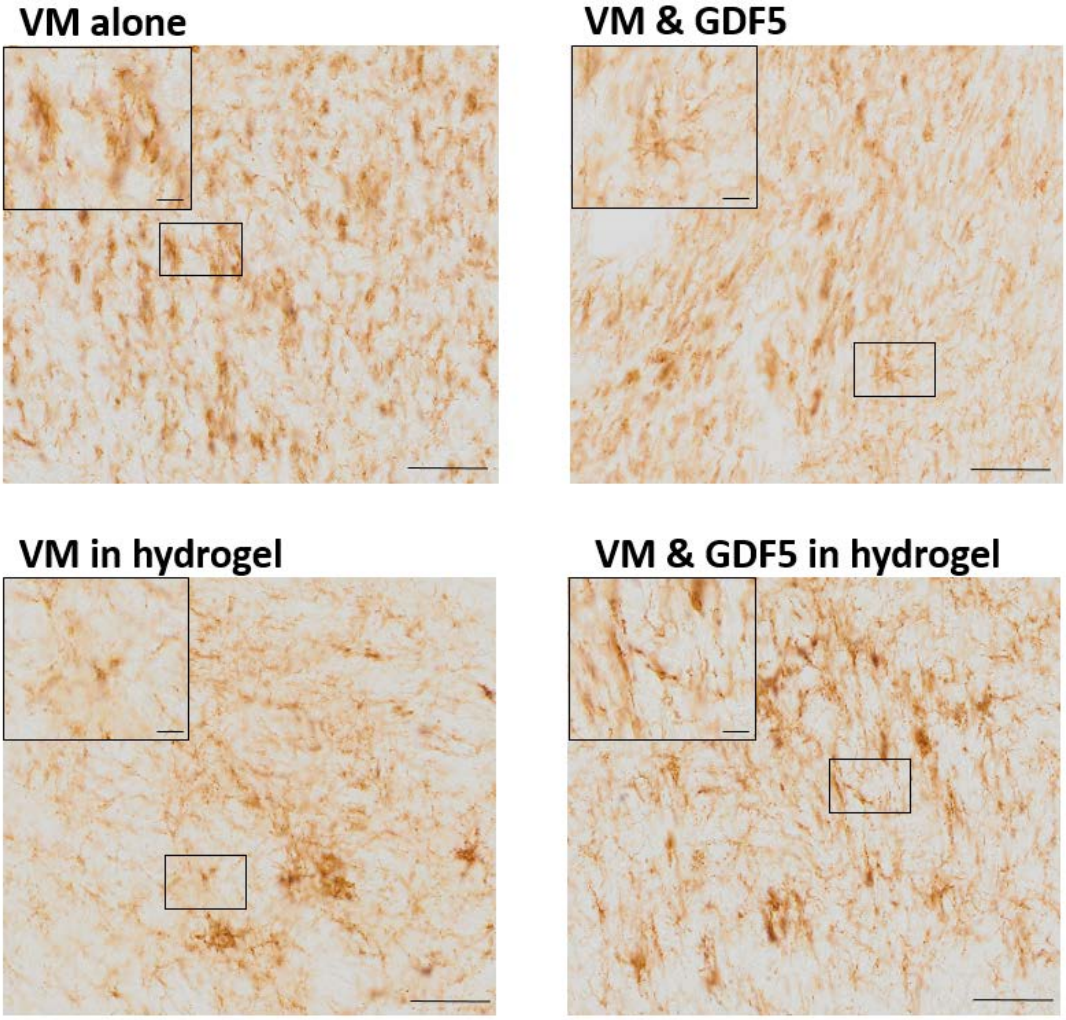


Figure 5.9 Representative photomicrographs of the impact of the GDF5-loaded collagen hydrogel on host microglial response.

Representative photomicrographs show CD11b immunostaining at the transplantation site at 12-weeks post-transplantation. No beneficial effect of the GDF5-loaded collagen hydrogels on microglial response was observed. Scale bar represents 100 µm and 20 µm. n=10 per group.

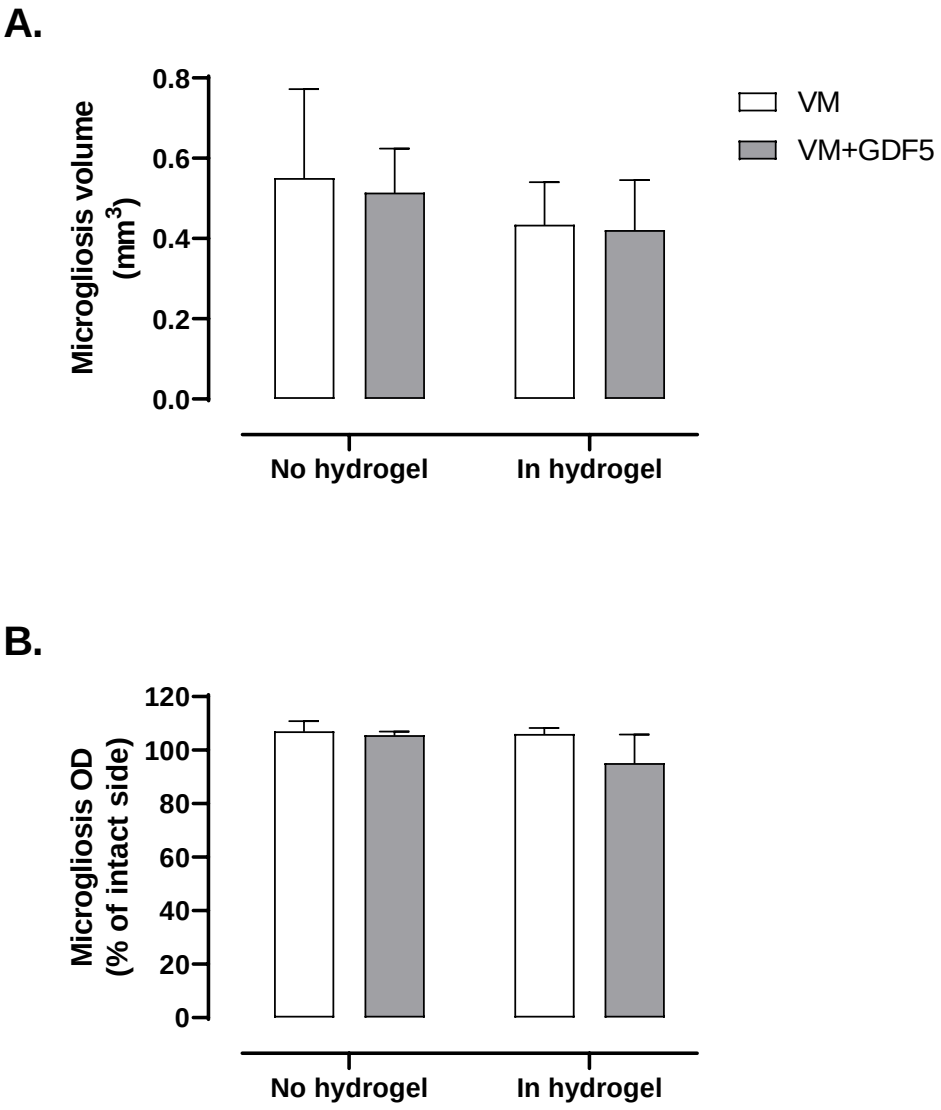


Figure 5.10 Impact of the GDF5-loaded collagen hydrogel on host microglial response.

The microglial response was measured by CD11b immunostaining. The immunostaining did not show a beneficial effect on the volume (A) or density (B) of microglia in the transplantation site 12-weeks post-transplant. Data are represented as mean ± SEM and were analysed by a two-way ANOVA. n= 10 per group.

5.4 DISCUSSION

Transplantation of cells into the brain is not a new concept, the very first experiment took place as long as 1890 (Thompson, 1890), but dopaminergic cell replacement therapy emerged over 40 years ago as a promising treatment for Parkinson´s disease (Perlow *et al*., 1979). Although the outcomes have been limited and inconsistent due to poor tissue availability and low survival of transplanted cells (Freed *et al*., 1992; Kordower *et al*., 1996; Olanow *et al*., 2003), several studies in animal models of Parkinson´s disease have shown the potential of primary dopaminergic neurons to re-innervate the striatum and induce motor function recovery (Perlow *et al*., 1979; Hebb *et al*., 2003; Dowd and Dunnett, 2004; Fricker *et al*., 2012; Perez-Bouza *et al*., 2017; Yasuhara *et al*., 2017; Moriarty *et al*., 2019a).

In cell replacement therapy, one transplant requires multiple foetal donors with similar gestation time, but due to ethical and logistical constrains, it is difficult to obtain enough dopaminergic cells that will go into the transplantation process. Therefore, this chapter aimed to assess the effect of GDF5-loaded in a collagen hydrogel with VM cells, on the enhancement of their long-term survival and efficacy.

Methamphetamine-induced rotations are the gold standard assessment of dopamine depletion in the 6-OHDA MFB lesion model. In rats, a lesion >80% of the nigrostriatal dopaminergic neurons causes an ipsilateral (same side of lesion) spontaneous turning that is exacerbated by the administration of an indirect dopamine agonist like methamphetamine (Schwarting and Huston, 1996; Johnston and Becker, 1997). A lesion is considered successful if the animal rotates an average of 6 ipsilateral turns per minute after administration of methamphetamine. In the work described in this chapter, we evaluated the ability of the VM grafts to synthesize/release dopamine and reduce the ipsilateral turns in response to the administration of methamphetamine to the unilaterally lesioned rats. Before transplantation, rats were rotating 6 turns or more per minute. The results indicate that in all groups, the delivery of VM cells reduced the number of ipsilateral turns. However, we observed a trend where less functional recovery was shown in the groups that received transplants in combination with GDF5. Since GDF5 has not been transplanted before in combination with VM cells, it is difficult to do a direct comparison, but Sullivan and colleagues (1998) examined the

effects of GDF5 pre-treatment on embryonic rat VM grafts in 6-OHDA lesioned rats and found that VM cell exposure to GDF5 enhanced the synthesis and release of dopamine by the grafts and in consequence reduced the number of ipsilateral turns per minute after administration of methamphetamine (Sullivan *et al*., 1998).

When we examined the brains post-mortem, the histology supported the behavioural findings. When VM cells were delivered in combination with GDF5 we observed a trend for fewer surviving cells compared to the group that only received VM cells. When we measured the ability of the surviving dopaminergic cells to form neural outgrowths and re-innervate the lesioned striatum we found that, in all groups, the E14 VM grafts successfully re-innervated a portion of the striatum; however, the incorporation of GDF5 into the transplantation process tended to decrease the volume of the grafts and the magnitude of re-innervation. Since this is the first time that GDF5 was used encapsulated in collagen hydrogels for cell transplantation, direct comparisons with previous studies are difficult to make, but in the study by Sullivan and colleagues (1998), mesencephalic cells were suspended in a solution of GDF5 (500 µg in buffer) before transplantation. This pre-treatment increased the survival of the transplanted cells from 11% to 30% and the grafts successfully re-innervated a portion of the striatum while effectively synthesized and released dopamine (Sullivan *et al*., 1998). However our methodology and dose were different.

The dose implemented in this work was chosen based on previous *in vivo* studies where GDF5 was used as neuroprotection against the 6-OHDA lesion (Sullivan *et al*., 1997, 1999; Hurley *et al*., 2004). Doses of 20 µg, 25 µg, 50 µg or 100 µg of GDF5 were infused into either the striatum or substantia nigra of rats to reduce or avoid dopaminergic neuronal death induced by the neurotoxin 6-OHDA. The study by Sullivan *et al*., (1999) found that 50 µg of GDF5 was the minimal dose to achieve optimal neuroprotection against dopaminergic loss by 6-OHDA. Since no detrimental effects were found with such high doses and because in the work of this thesis GDF5 is used for its neurotrophic qualities, the dose of 20 µg per injection was chosen.

Several studies have demonstrated that the actual injection procedure and the transplantation of exogenous cells to the Parkinsonian brain evokes an elevated host immune response, activating a cascade of destructive events that further diminish the number of surviving cells (Hagell and Cenci, 2005; Kuan and Barker, 2005; Fricker *et*

al., 2012). Within the first three days after graft injection into the new adult host environment, cells are exposed to oxidative stress and inflammatory cytokines activated by tissue damage (Brundin *et al*., 2000a). Previous studies by Hoban *et al*., (2013) and Moriarty *et al*., (2019) showed that the collagen hydrogels used by our group are immune-neutral upon transplantation, while also attenuating the host immune response to the transplanted graft (Hoban *et al*., 2013; Moriarty *et al*., 2019a). The collagen hydrogel acts like a physical barrier between the transplanted cells and the environment, protecting them from the cascade of destructive insults. Therefore, in the work described in this chapter we assessed the volume and density of microglia and astrocytes surrounding the graft site, but no beneficial effect was found from the addition of GDF5 nor collagen into the transplantation process. Since GDF5 is a neurotrophic factor and not an anti-inflammatory protein, these results are not surprising. However, in this study we found that the incorporation of GDF5 to the transplantation process, at the dose used, appears to be detrimental to the VM cells.

The results from cell transplantation are hindered by four stages where cell death occurs. On the fourth stage, cell are attacked by the host immune response. This response is initiated by the mechanical damage of the intracranial delivery, then immediately at the post-grafting period, lasting for at least 10 days (Hudson *et al*., 1994; Shinoda *et al*., 1995). Therefore, the following chapter aims to determine whether a dual approach: a GDF5 loaded collagen hydrogel in combination with an anti-inflammatory cytokine like IL-10 can provide additional support to dopaminergic neurons resulting in greater graft survival and efficacy, while targeting neuroinflammation.

Chapter 6: Assessment of the effect of a GDF5/IL-10 functionalised collagen hydrogel on E14 primary dopaminergic grafts in a rat model of Parkinson's disease.

6.1 INTRODUCTION

Progressive degeneration of the nigrostriatal dopaminergic system is the main pathology underlying the motor symptoms in Parkinson's disease. Over time, the use of the gold standard pharmacotherapy L-dopa, loses its efficacy in alleviating the symptoms that characterise this disorder, making it necessary to find alternative treatments that can slow down the progression of the disease or modify it (Björklund, 1993; Winkler *et al*., 2005; Lindvall, 2015).

Transplantation of foetal VM cells is a promising therapeutic approach to treat Parkinson´s disease patients, however it has not yet been developed into a clinically useful treatment due to poor tissue availability, logistical constraints, poor cell survival and ethical restrictions (Henderson *et al*., 1991; Freed *et al*., 1992, 2001; Kordower *et al*., 1996; Olanow *et al*., 2003).

One of the most critical problems is the poor survival of the transplanted cells. Previous clinical studies have shown that the majority of the cells die at an early stage after transplantation (Freed *et al*., 2001; Olanow *et al*., 2003). Activation of neuroinflammatory mechanisms, caused by tissue damage or bleeding at the graft site, are an important contributor to poor graft survival and functional deterioration (Hudson *et al*., 1994; Shinoda *et al*., 1995; Olanow *et al*., 2003; Piccini *et al*., 2005). Upon tissue injury, inflammatory responses such as microglia migrate to the lesioned site to repair the tissue. These inflammatory factors contribute to mitochondrial dysfunction, oxidative stress and increased synthesis of pro-inflammatory cytokines leading to apoptosis of the transplanted dopaminergic cells (Nagatsu *et al*., 2000; Joniec-Maciejak *et al*., 2014; Zhu *et al*., 2017).

Anti-inflammatory cytokines could be the key to reduce cell death caused by inflammation. For instance, IL-10 is a homodimeric anti-inflammatory cytokine important for the regulation of inflammation and immune response. It is mainly

expressed by immune cells such as monocytes, astrocytes, microglia and oligodendrocytes in the CNS where it modulates their activity, and also in neurons where the IL-10 receptor (IL-10R) is present (Zhu *et al*., 2017). Previous studies have shown that IL-10 reduces neuronal apoptosis by inhibiting the release of pro-inflammatory cytokines, like IL-1β, IL-6, TNFα, which cause inflammation (Joniec-Maciejak *et al*., 2014). These inflammatory factors contribute to mitochondrial dysfunction, oxidative stress, leading to apoptosis of dopaminergic neurons (Hirsch and Hunot, 2009).

The addition of an exogenous growth factor like GDF5, could increase the survival of the transplanted dopaminergic neurons (Brundin *et al*., 2000a; O'Keeffe *et al*., 2004b; Toulouse *et al*., 2012). However, the neurotrophic effects of this growth factor on cell transplantation have not been fully studied. In theory, a dual step approach (neurotrophic factor and anti-inflammatory cytokine in a collagen hydrogel) would provide an adhesive substrate for the transplanted cells, prevent the harmful infiltration of the inflammatory response, limit the development of inflammation in the transplantation site, and improve survival of the cells. Therefore, in the work presented in this chapter, we sought to limit the development of the inflammatory response through the addition of the anti-inflammatory cytokine IL-10 to the GDF5 functionalised collagen hydrogels in order to maximise the yield and efficacy of the surviving E14 VM cells in a rat model of Parkinson´s disease.

6.2 METHODS

The study presented in this chapter was run in in parallel to the study described in Chapter 5.

6.2.1 EXPERIMENTAL DESIGN

This study was designed to assess the effect of GDF5/IL-10 functionalised hydrogels on the survival and efficacy of transplanted E14 primary dopaminergic neurons in a rat model of Parkinson's disease.

To do this, 48 male rats were given a unilateral intra-MFB 6-OHDA lesion (12 µg per 3 µl). Two weeks later, the lesion was assessed by methamphetamine-induced rotations (2.5 mg per kg) and based on their performance rats were matched into 8 groups for the transplantation surgery (for details on groups and doses see **Table 6.1**).

Table 6.1 Groups and doses for the assessment of the effects of GDF5/IL-10 functionalised collagen hydrogels on E14 primary dopaminergic grafts.

Not encapsulated in collagen hydrogel	Encapsulated in collagen hydrogel
E14 VM cells (400,000 cells per 6 µl) n=6	E14 VM cells (400,000 cells per 6 µl, crosslinked with 4 mg/ml 4s-StarPEG) n=6
E14 VM cells+ GDF5 (400,000 cells/20 µg per 6 µl) n=6	E14 VM cells+ GDF5 (400,000 cells/20 µg per 6 µl, crosslinked with 4 mg/ml 4s-StarPEG) n=6
E14 VM cells+ IL-10 (400,000 cells/1 µg per 6 µl) n=6	E14 VM cells+ IL-10 (400,000 cells/1 µg per 6µl, crosslinked with 4 mg/ml 4s-StarPEG) n=6
E14 VM cells+ GDF5+ IL-10 (400,000 cells/20 µg/1 µg per 6µl) n=6	E14 VM cells+ GDF5 + IL-10 (400,000 cells/20 µg/1 µg per 6µl, crosslinked with 4 mg/ml 4s-StarPEG) n=6

Rats received a MFB lesion with 6-OHDA. Two weeks later, the lesion was assessed by methamphetamine-induced rotations and based on their performance they were matched into eight groups for unilateral intrastriatal transplantation. Rats were transcardially perfused 12-weeks post-transplantation. n= 6 per group.

Functional recovery was measured by methamphetamine-induced rotations at 4- and 8-weeks post-transplant. Additionally, sensorimotor recovery of the rats was tested in the adjustment step, corridor and whisker test, which were done at 8-weeks post-transplantation. Twelve weeks post-transplantation rats were transcardially perfused with 4% paraformaldehyde for post-mortem assessment. Free floating immunohistochemistry for TH^+, GFAP and CD11b was completed to identify graft survival, reinnervation and host immune response. A schematic representation of the experimental design is shown in **Figure 6.1**. Refer to Chapter 2 for more detailed methodology.

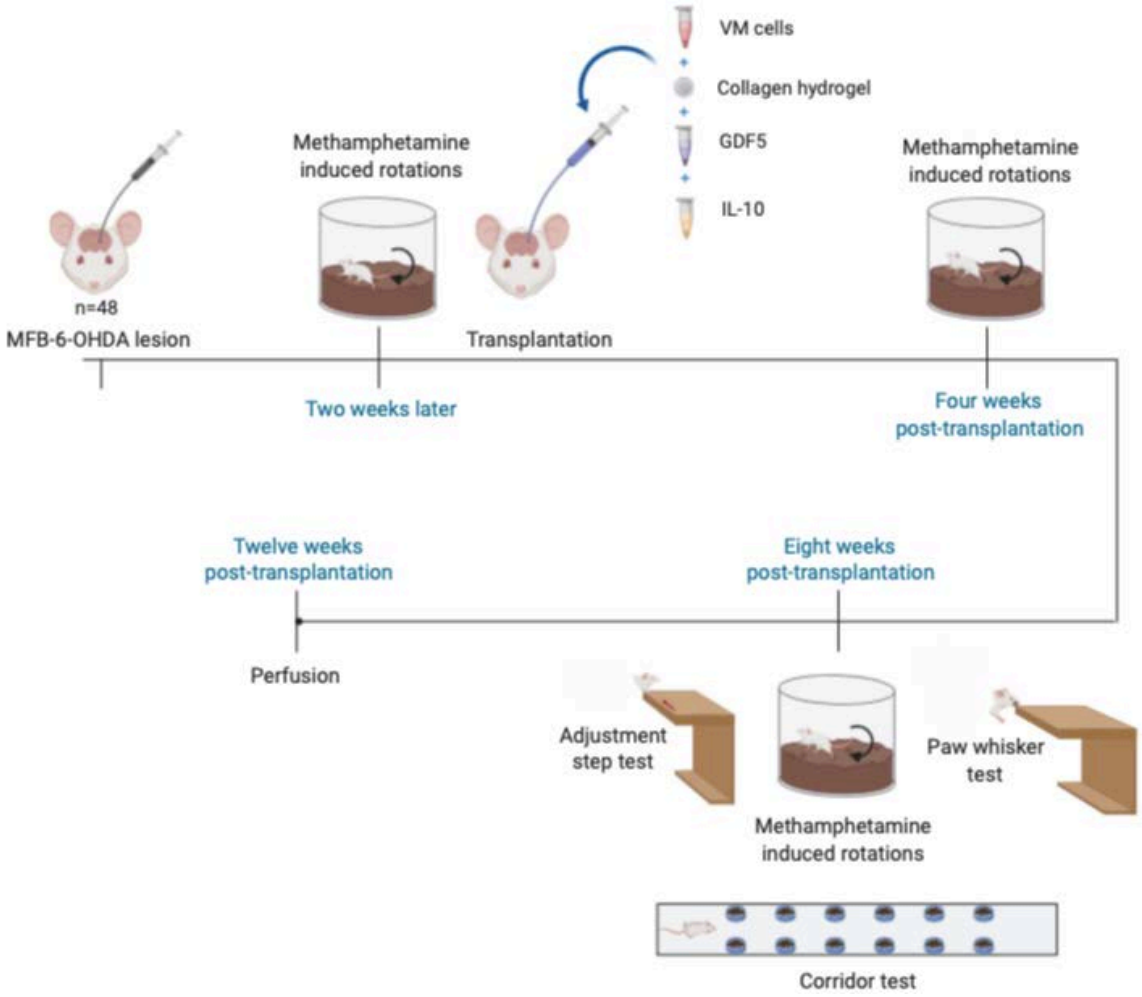


Figure 6.1 Schematic representation of the experimental design of the *in vivo* assessment of the effect of a GDF5 functionalised hydrogel on E14 primary dopaminergic neurons.

Forty-eight rats received a MFB lesion with 6-OHDA. Two weeks later, the lesion was assessed by methamphetamine-induced rotations and based on their performance they were matched into eight groups for unilateral intrastriatal transplantation (for details on groups and doses see **Table 6.1**). Methamphetamine-induced rotations were done at 4- and 8-weeks post-transplantation. Additionally, rats were tested in the corridor, adjustment step and paw whisker test at 8-weeks post-transplantation. Rats were transcardially perfused 12-weeks post-transplantation. n= 6 per group.

6.3 RESULTS

6.3.1 IMPACT OF THE FUNCTIONALIZED GDF5/ IL-10-LOADED COLLAGEN HYDROGELS ON E14 VM GRAFT FUNCTIONAL RECOVERY

In order to assess the ability of the E14 VM grafts to restore motor function to the unilaterally lesioned rats, methamphetamine-induced rotations were done 2-weeks after the MFB 6-OHDA lesion to establish a baseline. All animals displayed ipsilateral rotational bias after methamphetamine injection (**Figure 6.2a**). Methamphetamine rotations were performed again at 4- and 8-weeks after transplantation surgeries to assess the ability of the transplanted dopaminergic cells to reduce the rotational asymmetry induced by 6-OHDA. In the previous chapter we found that rats that received transplantation of VM alone tended to show functional recovery, but in this study when we compared the rotations of the different groups at 2-weeks post-lesion surgeries, the lesion-induced motor deficit was not reduced in any of the groups at both 4- (**Figure 6.2**; Interaction, $F_{(3,40)}$= 1.901, P>0.05) and 8- (**Figure 6.2c**; Interaction, $F_{(3,40)}$= 1.320, P>0.05) weeks post-transplantation.

Additionally, rats were subjected to a battery of behavioural tests (whisker, stepping and corridor tests) to assess the ability of transplanted dopaminergic cells to improve motor deficits at 8-weeks post-transplantation. First, we assessed motor function on the side ipsilateral to the side of 6-OHDA administration to confirm that any behavioural impairment found was due to the lesion and not general sickness behaviour (Hart, 1988) that affects locomotor activity. The results indicated that the 6-OHDA lesion did not significantly impair ipsilateral motor performance in the Whisker (**Figure 6.3a**), Stepping (**Figure 6.4a**) and Corridor test (**Figure 6.5a**).

In contrast to the ipsilateral side, rats displayed a noticeable lesion effect in motor function in all the groups in the different behavioural tests. Therefore, it seems that transplantation of E14 VM cells in combination with GDF5 with or without IL-10 had no beneficial effect in functional recovery of the rats in the Whisker test of sensorimotor integration (**Figure 6.3b**; Interaction, $F_{(3,40)}$= 0.611, P>0.05), in the Stepping test of forelimb akinesia (**Figure 6.4b**; Interaction, $F_{(3,40)}$= 0.990, P>0.05) and also in the Corridor test of contralateral neglect (**Figure 6.5b**; Interaction, $F_{(3,40)}$= 0.155, P>0.05).

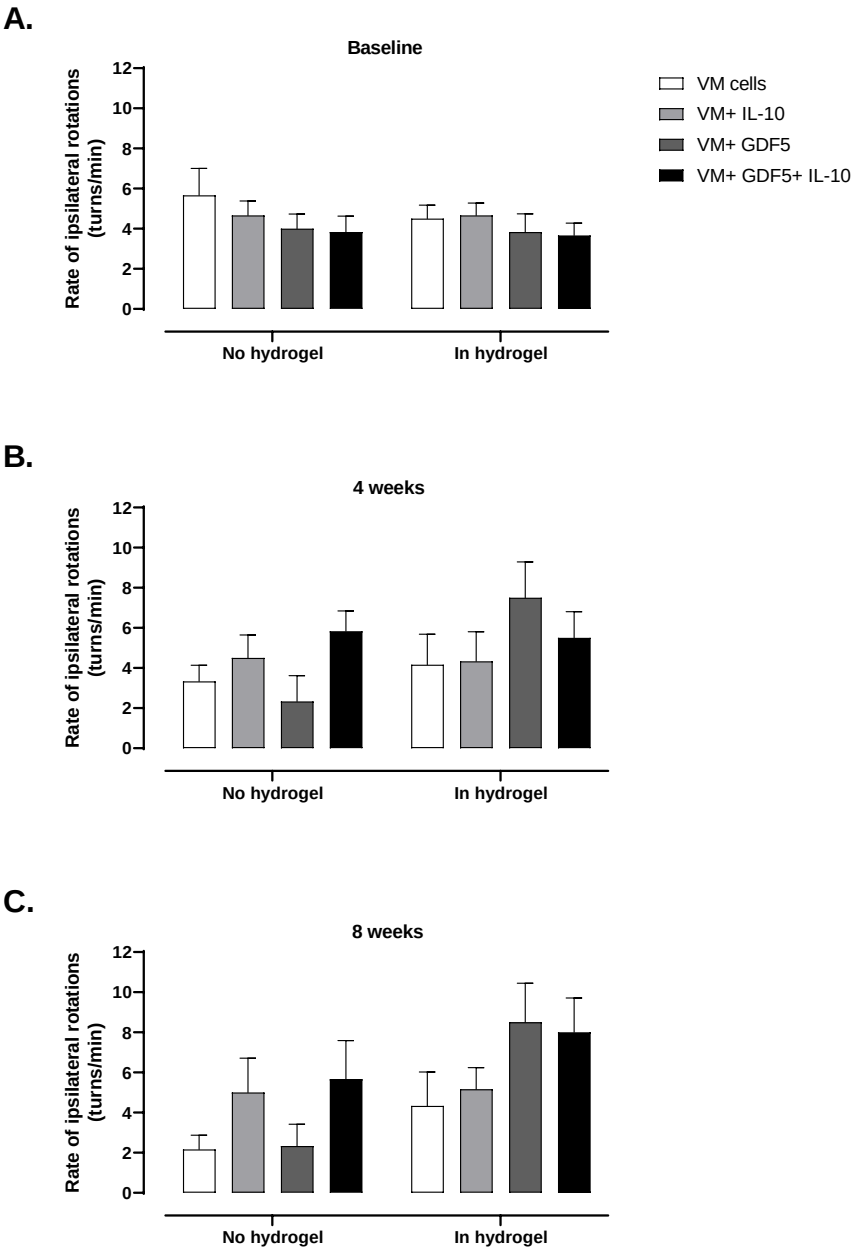


Figure 6.2 Impact of the GDF5/IL-10-loaded collagen hydrogel on dopaminergic graft functionality tested by methamphetamine-induced rotations at 4- and 8-weeks post-transplantation.

Methamphetamine-induced rotations were measured 2-weeks after lesion and at 4- and 8-weeks post-transplantation. Results did not show a significant decrease of ipsilateral rotations in any of the groups at either time point. Data are represented as mean ± SEM and were analysed by two-way ANOVA. n=6 per group.

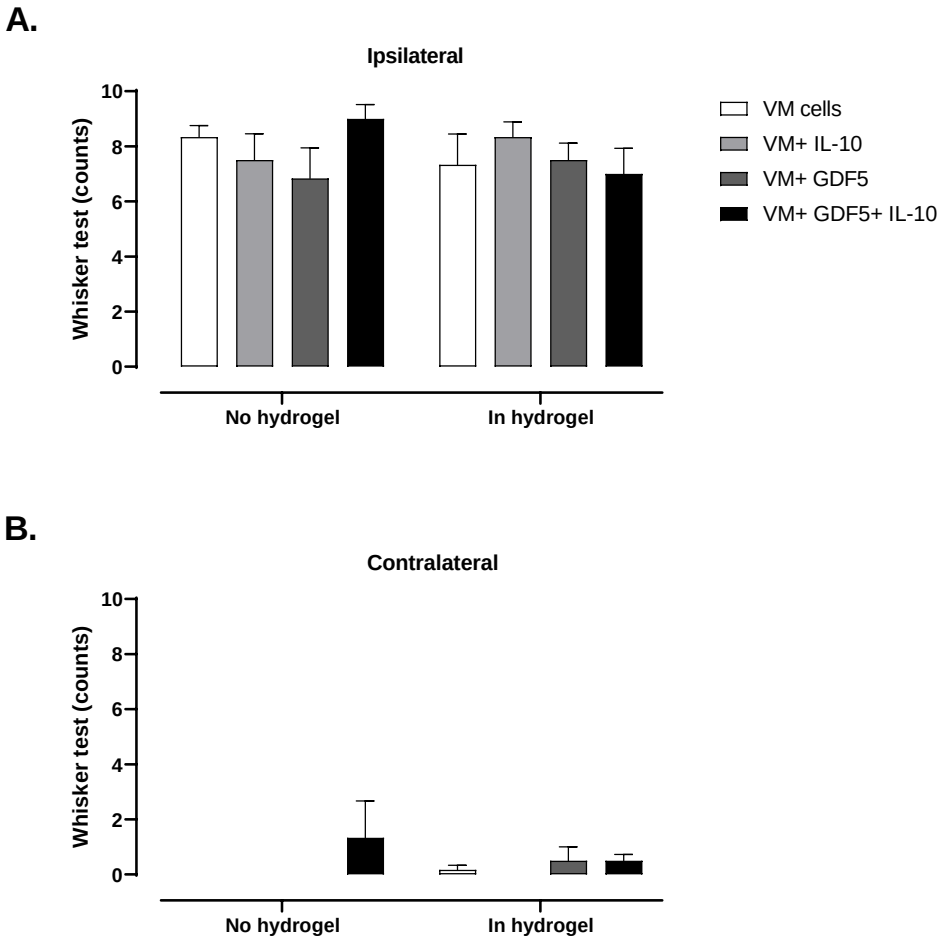


Figure 6.3 Impact of the GDF5/IL-10-loaded collagen hydrogel on dopaminergic cell graft functionality tested by the whisker test at 8-weeks post-transplantation.

Rats were habituated then tested for three consecutive days in the whisker test at 8-weeks post-transplantation. Ipsilateral (A) and contralateral (B) whiskers were stimulated for 10 trials each to evoke paw placement response. Contralateral whiskers showed impaired function caused by the 6-OHDA lesion. Transplantation of VM cells in combination with GDF5 with or without IL-10 had no effect on sensorimotor recovery. Data are represented as mean ± SEM and were analysed by two-way ANOVA. n=6 per group.

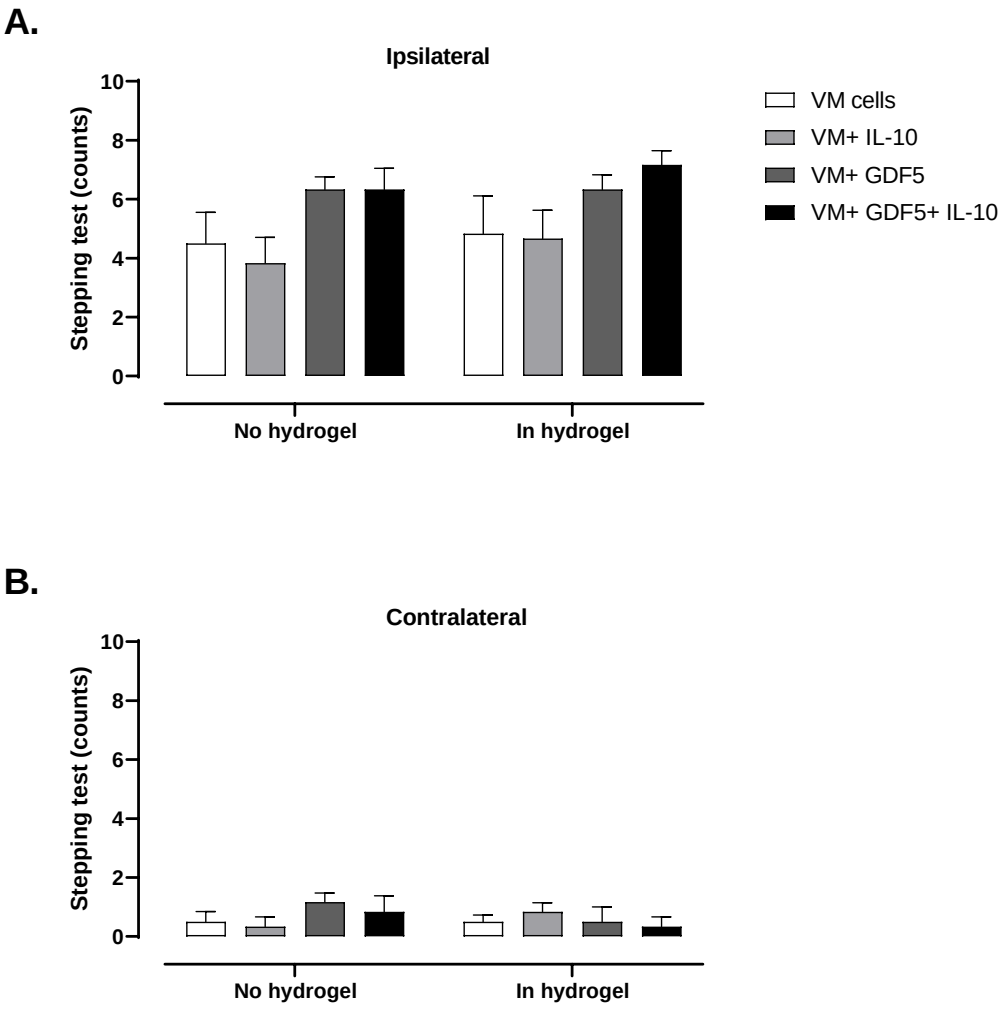


Figure 6.4 Impact of the GDF5/IL-10-loaded collagen hydrogel on dopaminergic graft functionality tested by the forepaw adjustment step test at 8-weeks post transplantation.

Rats were habituated then tested for 3 consecutive days in the forepaw adjustment step test at 8-weeks post-transplantation. The hind paws and one forepaw were restrained such that the free forepaw supported the body weight on a table. Rats were then moved laterally across the table (90 cm) and adjustment steps were counted for 5 seconds for the ipsilateral (A) and contralateral forepaw (B). Contralateral forepaw showed impaired function caused by the 6-OHDA lesion. Transplantation of VM cells in combination with GDF5 with or without IL-10 did not show a beneficial effect in motor recovery. Data are represented as mean ± SEM and were analysed by two-way ANOVA. n=6 per group.

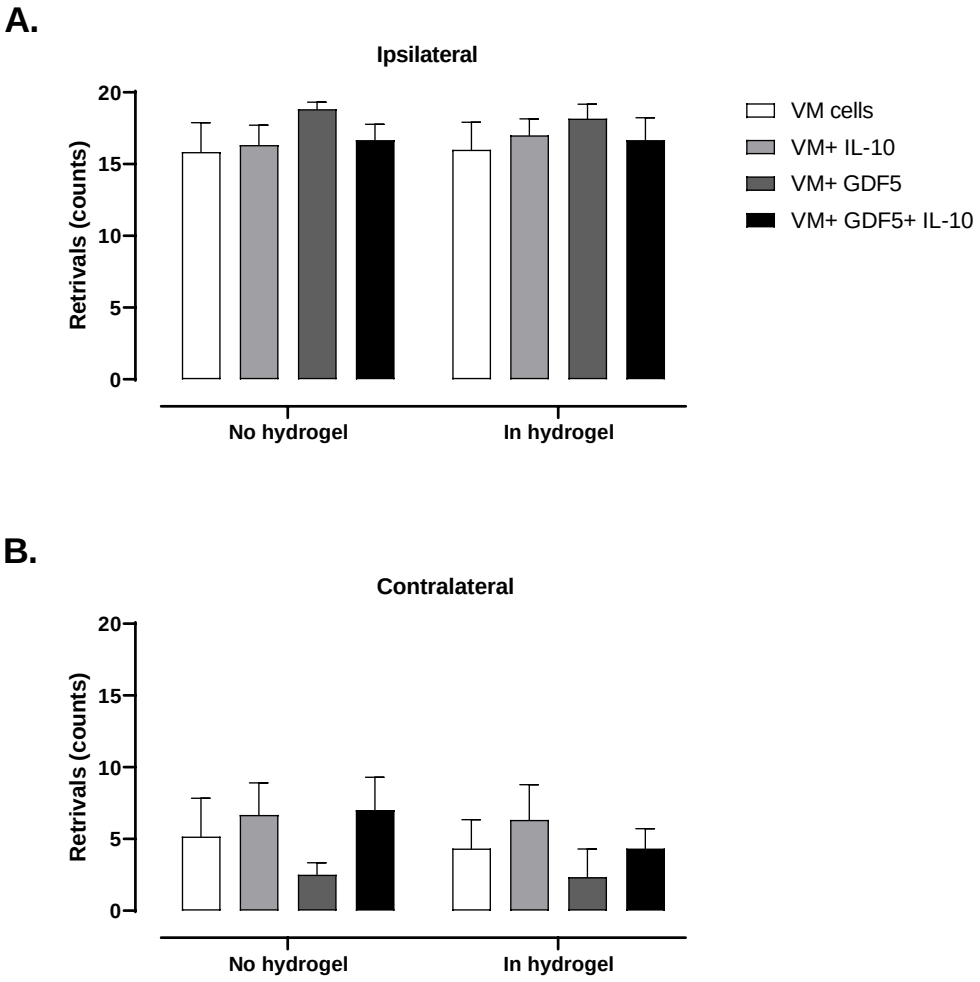


Figure 6.5 Impact of the GDF5/ IL-10-loaded collagen hydrogel on dopaminergic cell graft functionality tested by the corridor test at 8-weeks post-transplantation.

Rats were habituated to the corridor and then tested for 3 consecutive days at 8-weeks post-transplantation. One by one, rats were placed at one end of the corridor and they were free to explore, turn around and eat the CocoPops® at will (CocoPops® were placed in parallel containers at both sides of the corridor). Trials lasted 5 minutes or terminated after 20 retrievals. Ipsilateral (A) and contralateral (B) retrievals were counted. Contralateral side showed impaired function caused by the 6-OHDA lesion. Transplantation of VM cells in combination with GDF5 with or without IL-10 had no effect in sensorimotor recovery. Data are represented as mean ± SEM and were analysed by two-way ANOVA. n=6 per group.

6.3.2 IMPACT OF FUNCTIONALIZED GDF5/IL-10 LOADED COLLAGEN HYDROGELS ON E14 VM NEURON SURVIVAL

In order to quantify the number of surviving transplanted dopaminergic cells in the de-enervated striatum of the rats, free floating TH^+ immunohistochemistry was completed. The immunostaining showed the survival of dopaminergic neurons in each group (**Figure 6.6** & **Figure 6.7**), however, not all groups had the same number of surviving neurons. A trend for reduced survival of dopaminergic cells was observed in all the groups that received transplantation of VM cells in combination with a factor (**Figure 6.8a**; K=17.48, $P>0.05$). Additionally, a trend for smaller grafts was observed in all the groups that received VM cells in combination with GDF5 (**Figure 6.8b**; K=13.79, $P>0.05$). Furthermore, the addition of IL-10 into the transplantation process did not show a beneficial effect to the survival of the cells.

In Chapter 5, we also observed a trend of reduced cell survival and smaller grafts in the groups that received transplantation of E14 VM cells in combination with GDF5.

6.3.3 IMPACT ON E14 VM NEURON STRIATAL RE-INNERVATION BY THE FUNCTIONALIZED GDF5/ IL-10 LOADED COLLAGEN HYDROGELS

Using the TH^+ immunostaining we assessed the ability of the surviving dopaminergic cells to form neural outgrowths *in situ* and re-innervate the lesioned striatum by measuring the volume of striatal tissue occupied by innervation from the transplanted cells. The immunostaining showed that in all of the groups, the E14 VM grafts re-innervated a portion of the striatum (**Figure 6.7**), however, not all groups showed the same magnitude of reinnervation. Additionally, a trend for smaller magnitude of reinnervation was observed in all the groups that received VM cells in combination with GDF5. Moreover, the addition of IL-10 into the transplantation process did not show a beneficial effect on the magnitude of re-innervation (**Figure 6.8c**; K= 15.18, $P>0.05$).

In Chapter 5, we also observed a trend of reduced reinnervation in the groups that were transplanted in combination with GDF5.

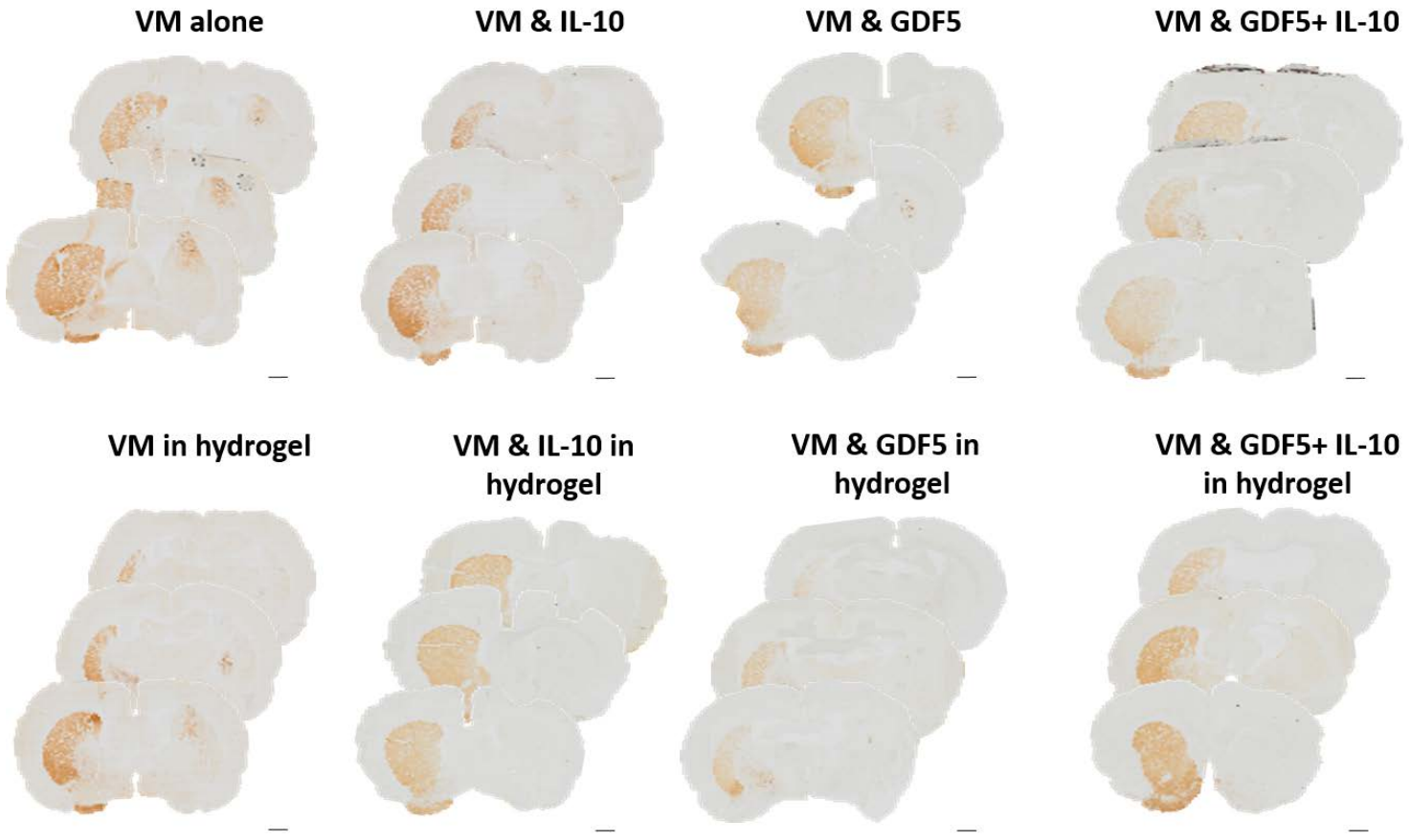


Figure 6.6 Representative photographs of the impact of the GDF5/IL-10-loaded collagen hydrogel on E14 primary dopaminergic cell grafts.

Representative photomicrographs show TH^+ immunostaining at the transplantation site. Small grafts are observed in all the groups that received VM cells in combination with a factor. Scale bars represent 1 mm.

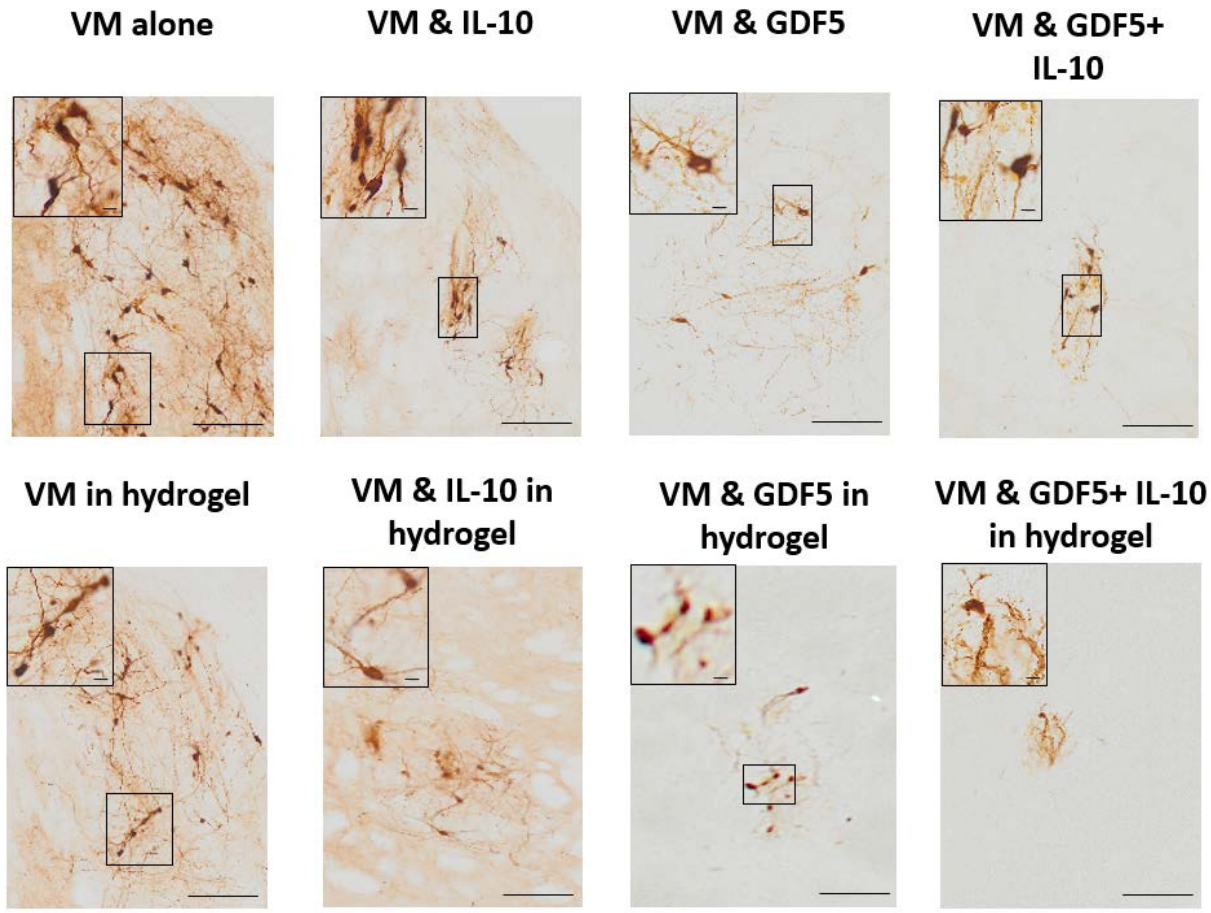


Figure 6.7 Representative photographs of E14 primary dopaminergic neuron survival.

Representative photomicrographs show TH^+ immunostaining. Surviving dopaminergic cells were found in all the groups Fewer cells can be observed in all the groups that received VM cells in combination with a factor. Scale represents 200 µm and 20 µm (insert).

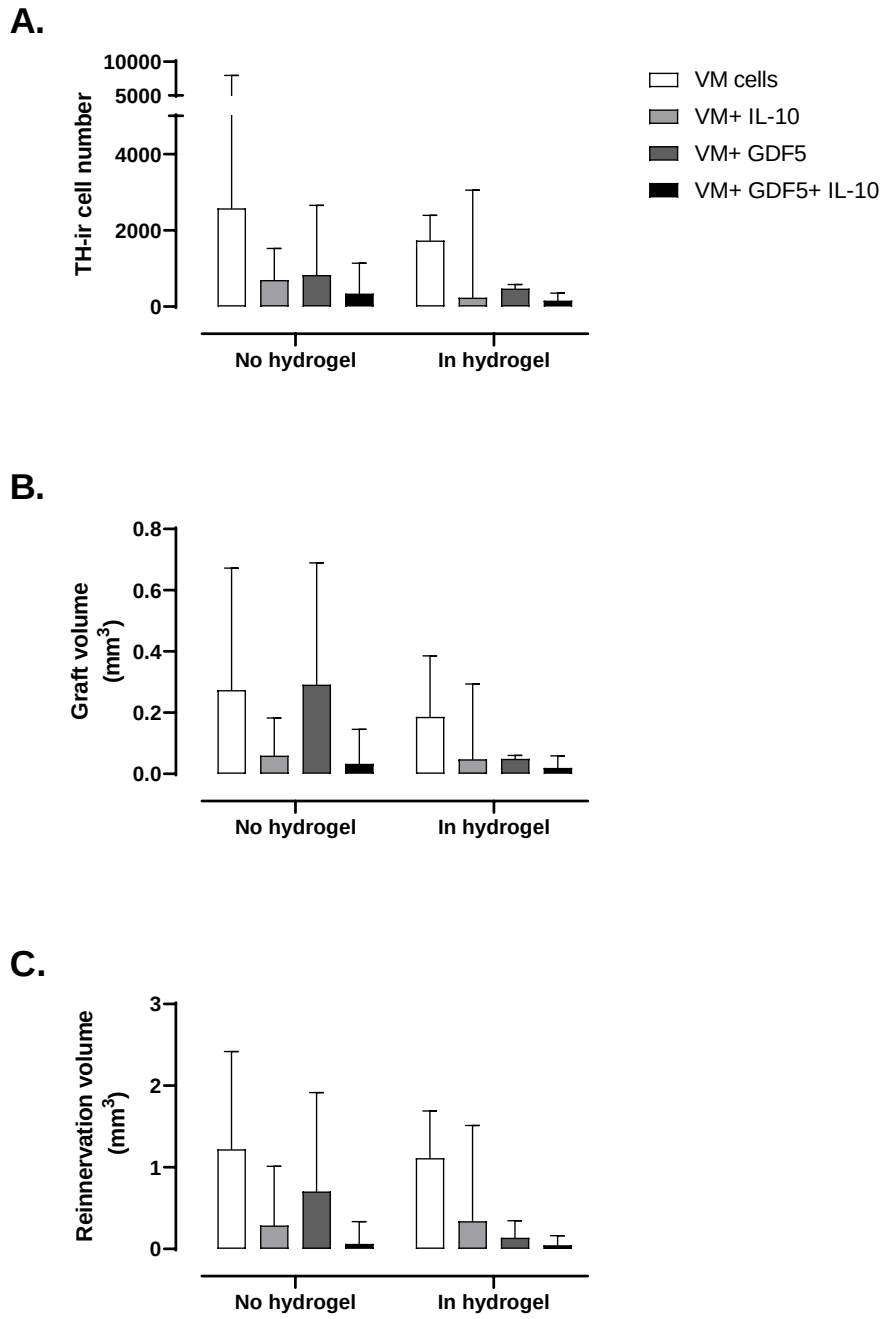


Figure 6.8 *In vivo* assessment of the impact of the GDF5/IL-10-loaded collagen hydrogel on dopaminergic cell grafts survival and striatal re-innervation.

The TH^+ immunostaining showed a trend of reduction in the survival of dopaminergic cells (A), smaller grafts (B) and reduced reinnervation (C) in all the groups that received VM cells in combination with a factor (GDF5). Data are represented as median with interquartile range and were analysed by Kruskal-Wallis. n= 6 per group.

6.3.4 IMPACT ON THE HOST IMMUNE RESPONSE OF THE GDF5/IL-10 LOADED COLLAGEN HYDROGELS

In line with the results from the studies of Chapter 4 and Chapter 5, the transplantation process causes mechanical trauma to the brain. This damage prompts a reaction from the host immune response in the tissue surrounding the graft site. Therefore, to evaluate the immune response, we quantified volume and density of astrocytes (**Figure 6.9**) and microglia (**Figure 6.11**) via immunostaining for GFAP and CD11b.

The GFAP immunostaining did not show any beneficial effect by the addition of the anti-inflammatory cytokine IL-10 or by the addition of GDF5 in the transplantation process. The volume (**Figure 6.10a**; Group $F_{(7,40)}$= 1.698, P>0.05) and density (**Figure 6.10b**; Group, $F_{(7,40)}$= 0.6234, P>0.05) of the astrocytic response did not show a difference between the different groups. Furthermore, the CD11b immunostaining did not show any beneficial effect by IL-10 or GDF5 in volume (**Figure 6.12a**; Group, $F_{(7,40)}$= 0.9013, P>0.05) and density (**Figure 6.12b**; Group $F_{(7,40)}$= 1.912, P>0.05) of the microglia response 12-weeks post-transplant.

6.3.5 EXPRESSION OF HUMAN-GDF5 *IN SITU*

The optimization of the immunostaining for GDF5 was not accomplished but it is likely that after 12-weeks post-transplantation GDF5 cleared out from the brain.

6.3.6 *IN VIVO* BIODEGRADABILITY OF THE COLLAGEN HYDROGEL

The collagen immunostaining showed that our collagen hydrogels completely biodegraded 12-weeks post-transplantation (not shown).

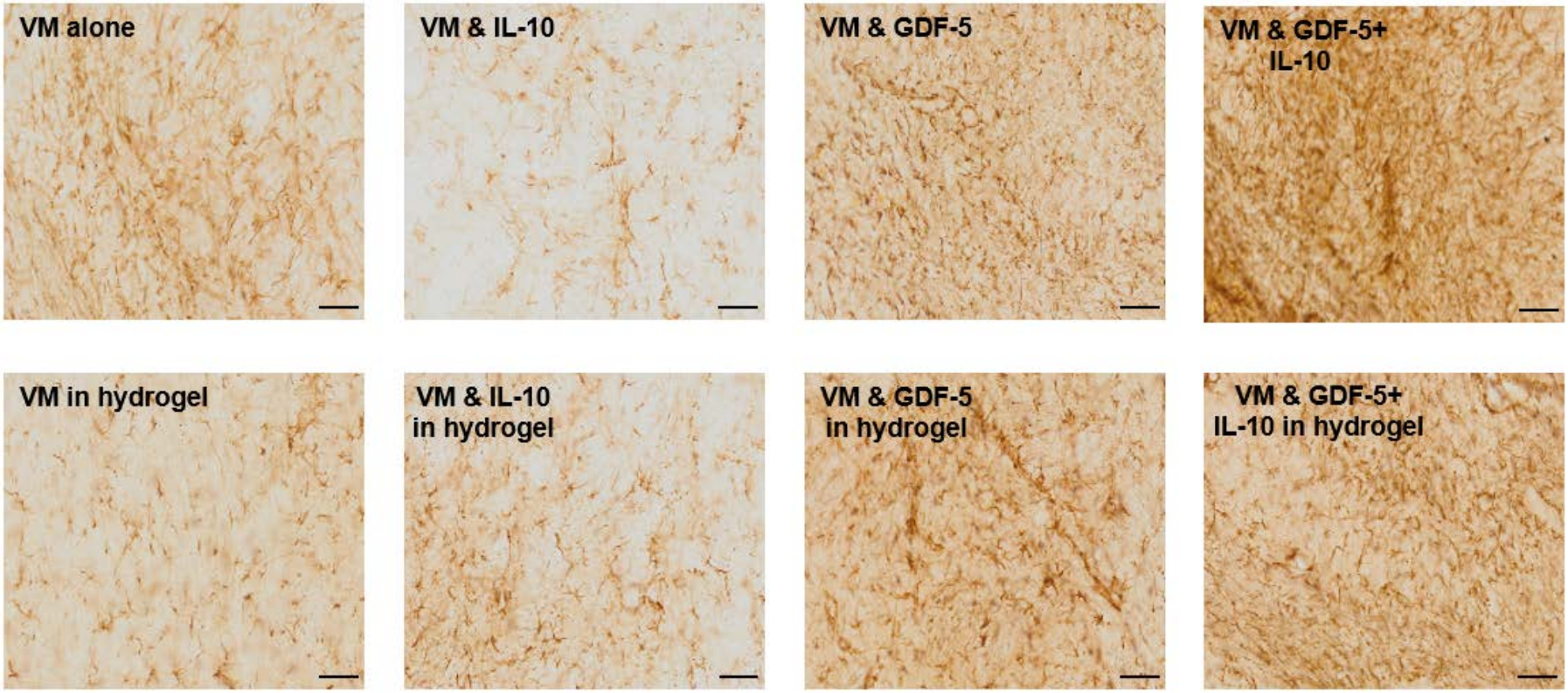


Figure 6.9 Representative photomicrographs of the impact of GDF5/IL-10-loaded collagen hydrogel on host astrocytic response.

Representative photomicrographs show GFAP immunostaining at the transplantation site 12 weeks post-transplantation. No beneficial effect was observed by the addition of the anti-inflammatory cytokine IL-10 or by the addition of GDF5 in the transplantation process. Scale bar represents 100 µm.

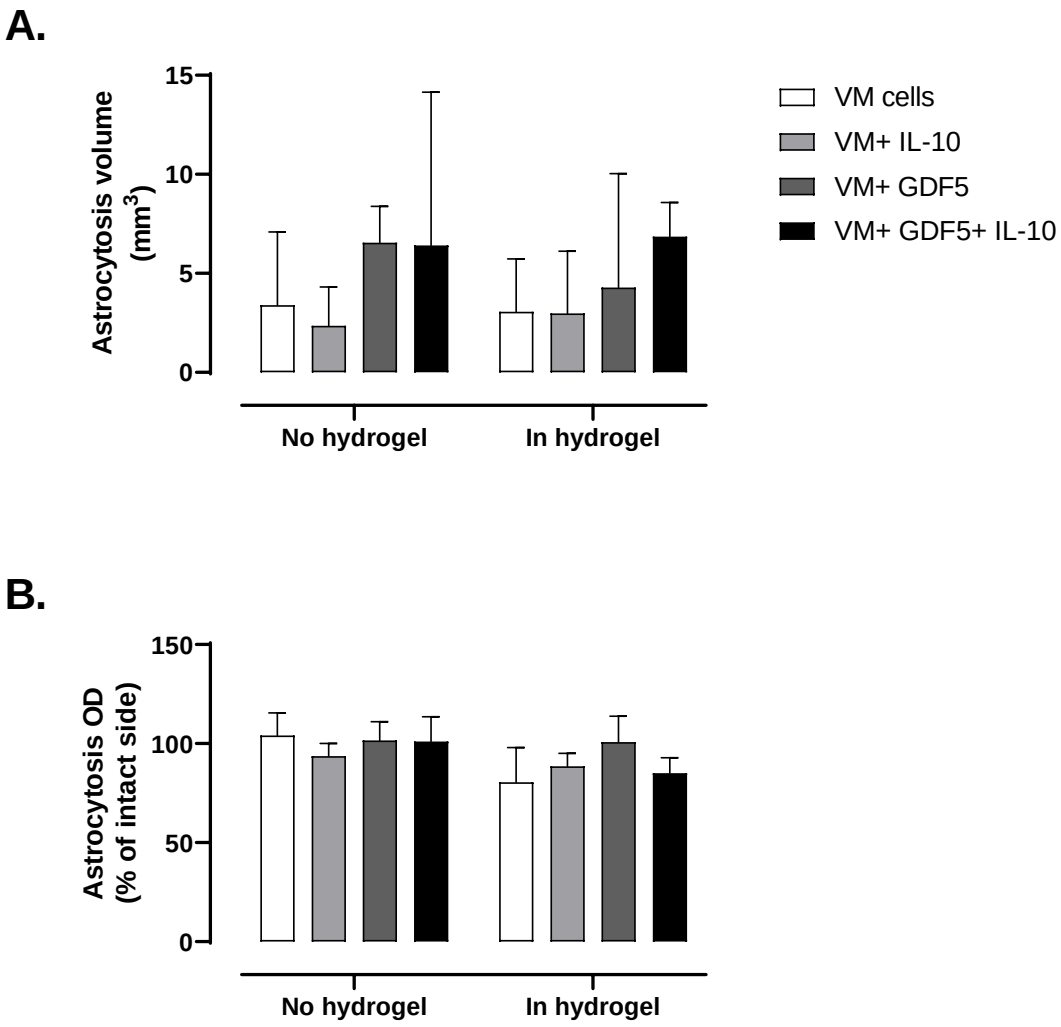


Figure 6.10 Impact of the GDF5/IL-10-loaded collagen hydrogel on host astrocytic response.

The astrocytic response was measured by GFAP immunostaining. The immunostaining did not show any beneficial effect on the volume (A) or density of astrocytes (B) surrounding the transplantation site 12-weeks post-transplant in groups that received VM cells in combination with a factor. Data are represented as mean ± SEM and were analysed by two-way ANOVA. n= 6 per group.

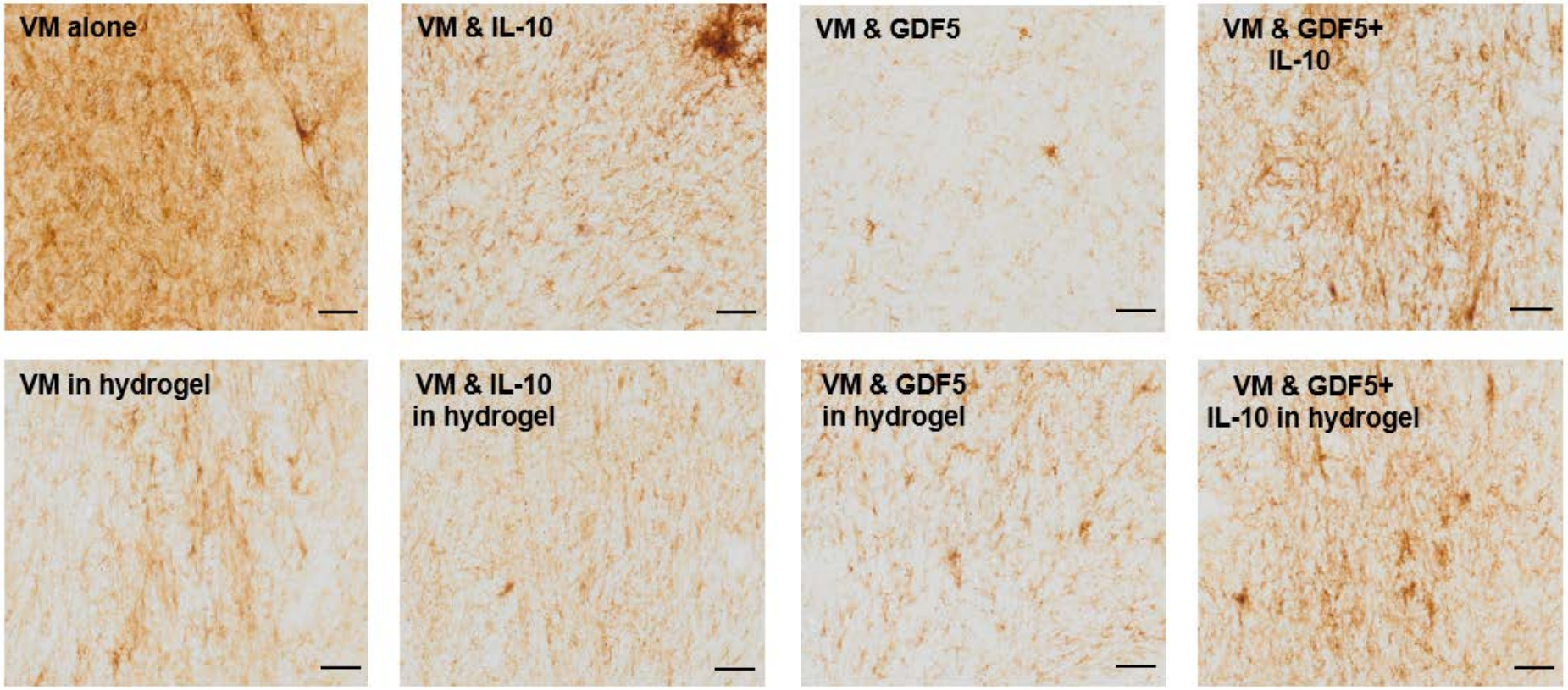


Figure 6.11 Representative photomicrographs of the impact of the GDF5/IL-10-loaded collagen hydrogel on host microglial response.

Representative photomicrographs show CD11b immunostaining at the transplantation site at 12-weeks post-transplantation. No beneficial effect was observed by the addition of the anti-inflammatory cytokine IL-10 or by the addition of GDF5 in the transplantation process. Scale bar represents 100 µm.

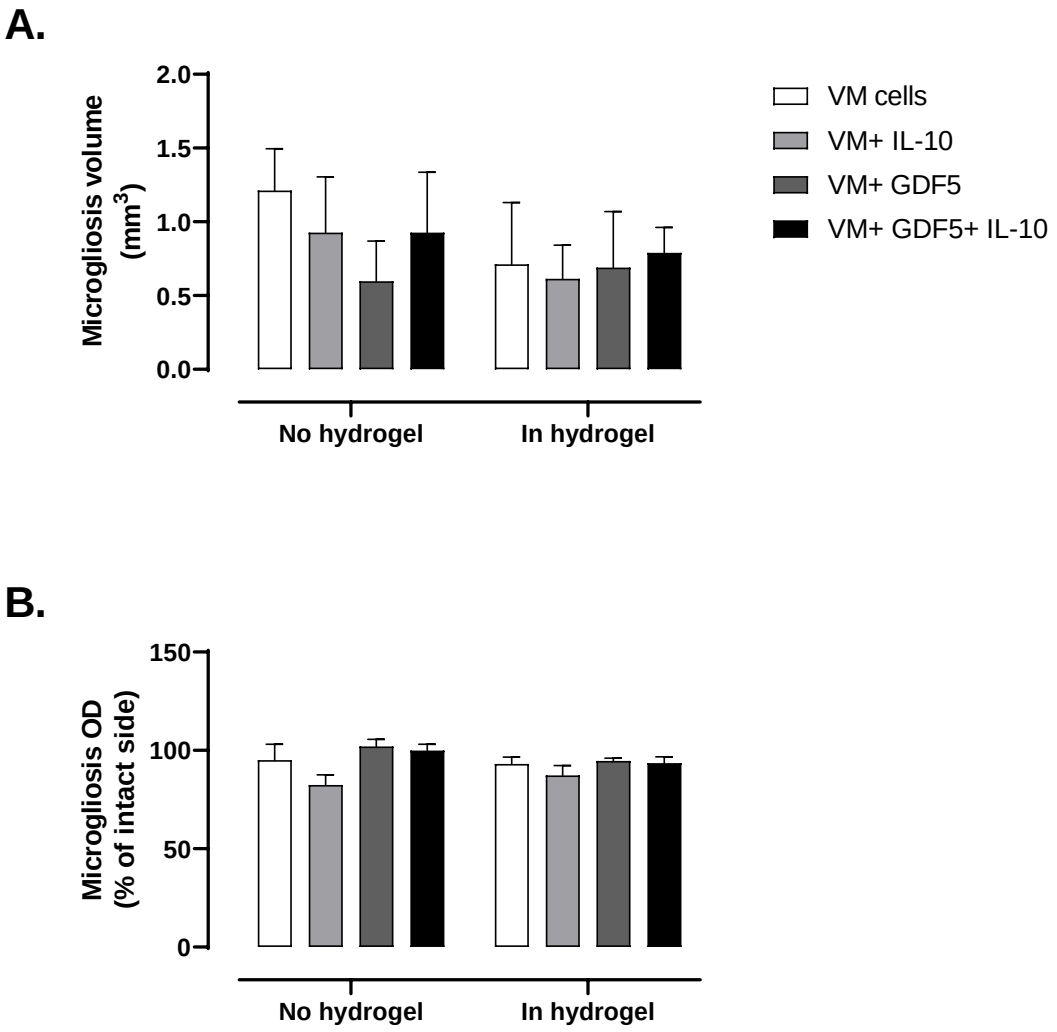


Figure 6.12 Impact of the GDF5/ IL-10-loaded collagen hydrogel on host microglial response.

The microglial response was measured by CD11b immunostaining. The immunostaining did not show a beneficial effect on the volume (A) or density (B) of microglia in the transplantation site 12-weeks post-transplant. Data are represented as mean ± SEM and were analysed by two-way ANOVA. n= 6 per group.

6.4 DISCUSSION

Dopamine cell replacement therapy is a promising alternative treatment for Parkinson's disease, however, its use as a routine clinical procedure is primarily held back by the need of many foetal donors for one transplant caused by poor graft survival (Henderson *et al*., 1991; Freed *et al*., 1992, 2001; Björklund, 1993; Kordower *et al*., 1996; Olanow *et al*., 2003). If we find a way to reduce cell death and maximise the survival of the grafted cells, we could decrease the number of donors needed per transplant and increase the likelihood of a successful outcome. Therefore, the aim of this chapter was to increase the yield and viability of the transplanted E14 VM cells by employing a dual step approach to provide them with a protecting microenvironment enriched with GDF5 and IL-10 during and after transplantation, in a rat model of Parkinson's disease.

In general, as a consequence of the 6-OHDA lesion, animals present behavioural abnormalities such as contralateral sensorimotor neglect, spontaneous and drug induced rotations, contralateral akinesia and impaired contralateral forelimb use (Schwarting and Huston, 1996). In this chapter, restoration of motor function of the rats was measured through a series of behavioural tests (methamphetamine-induced rotations, paw whisker test, adjustment step test and corridor test) in order to assess the ability of the transplanted VM grafts to ameliorate the behavioural deficits caused by the MFB 6-OHDA lesion.

Reduction of drug-induced rotations triggered by injection of methamphetamine is one of the main indicators of successful graft derived restoration of dopamine to the lesioned striatum. In the work described in this chapter, we showed that before transplantation, rats were rotating indicating a successful lesion by 6-OHDA. Then at 4- and 8-weeks post-transplantation rats were tested again but unfortunately the only group that showed reduction of rotations was the one that received transplantation of VM alone. Groups that received a transplant in combination with our functionalised collagen hydrogels did not showed reduction in the number of rotations, suggesting that the factors had a detrimental effect on the grafts and the graft derived dopamine replacement in the de-enervated striatum was not sufficient to induce recovery.

In the study described in this chapter, contralateral sensorimotor neglect was assessed by the paw whisker test (also known as the vibrissae-evoked forelimb placing test) at 8-weeks post-transplantation. This test assesses the effect of the lesion in the ability to translate positional information from the whiskers into movement of the contralateral forepaw and depends on the presence of high levels of extracellular dopamine and striatal activity (Schallert and Tillerson, 2000). Rats that are lesioned by 6-OHDA fail to quickly reach out with the contralateral forepaw after stimulation of the whiskers of one side of the head. Our results did not show functional recovery following stimulation of the whiskers in any of the transplanted groups. This is in line with the results obtained for the adjustment step test and corridor test (below).

Forelimb akinesia caused by the 6-OHDA lesion was tested with the adjustment step test at 8-weeks post-transplantation. This test looks for deficits in motor initiation of the forepaws, comparable to limb akinesia and gait problems in Parkinson´s disease patients. In this behavioural test, rats are held with only one forepaw touching a table while the rat is moved sideways along the table surface in the forehand direction. The number of adjusting steps are counted for both forepaws. Rats that are lesioned by 6-OHDA will passively drag the forepaw contralateral to the lesioned hemisphere while non-lesioned rats will perform frequent adjusting step movements with both forepaws (Olsson *et al*., 1995; Kirik *et al*., 1998). The results of this test showed that in all the groups rats passively dragged their left paw (contralateral to the lesion) when they were moved in the forehand direction, while the right paw performed frequent stepping movements, indicating that the transplantation of cells in the GDF5/IL-10 gels did not show a beneficial effect on the initiation of stepping movements.

The corridor test was the last task done to measure spontaneous recovery of sensorimotor neglect of contralateral stimuli in the rats at 8-weeks post-transplantation. Rats that are lesioned by 6-OHDA neglect food located contralaterally to the lesioned hemisphere while non-lesioned rats will retrieve CocoPops® from containers at both sides of the corridor (Dowd *et al*., 2005). After this test, we found that rats in all of the transplanted groups showed an ipsilateral bias for retrieval of pellets, suggesting that the transplantation of E14 VM cells in combination with factors had no beneficial effect in behavioural recovery.

In the previous chapter and in the work presented in this one, we enriched collagen hydrogels with GDF5 (20 μg per 6 μl) in an attempt to provide neurotrophic support to the VM grafts, during and after transplantation, for enhancement not only of graft survival, but also of fibre outgrowth and of the functional effects. The immunohistochemistry work described in this chapter demonstrated the somewhat successful transplantation of E14 VM cells in the lesioned striatum of rats, however, the results showed reduced survival of cells, smaller grafts and poor re-innervation in all the groups that received a transplant in combination with a factor compared to the group that only received VM cells, therefore, no benefit was found by the addition of either GDF5 or IL-10 into the transplantation process. These results explain the lack of functional recovery found in all of the behavioural tests done in this chapter. In order to get recovery of motor function, higher levels of extracellular dopamine and striatal activity are necessary (Schallert and Tillerson, 2000).

The findings about GDF5 in this chapter are similar to the results obtained in the previous chapter, where the groups transplanted in combination with GDF5 showed limited survival of VM cells and smaller grafts, suggesting that the dose of GDF5 (20 μg per 6 μl) used in both studies might be toxic for the mesencephalic cells. It is difficult to compare these results to previous *in vivo* studies since this is the first time GDF5 has been transplanted simultaneously with VM grafts, but the safety of high doses of GDF5 (suspending the mesencephalic cells in solutions of GDF5 prior transplantation) was seen in a study by Sullivan and colleagues (1998). In that study, exposure of cells to GDF5 (500 μg in buffer) before transplantation increased the survival of cells by up to 30% (Sullivan *et al*., 1998).

Tissue damage is caused during the surgical procedure for transplantation, which triggers a cascade of events where microglia and astrocytes accumulate at the graft site (Tambur, 2004). These inflammatory reactions lead to reduced graft survival and functional deterioration of the dopaminergic grafts (Hudson *et al*., 1994; Shinoda *et al*., 1995). In the work described in this chapter, we attempted to use the cytokine IL-10 to limit the development of the inflammatory response. We quantified the volume and density of microglia and astrocytes on the transplantation site, but no difference in the host immune response was found between any of the transplanted groups. Furthermore, no beneficial effect was found by the addition of IL-10 or by the addition of GDF5 to

the transplantation process. We chose to use IL-10 in this study, because previous *in vitro* studies have shown that IL-10 can protect against inflammation-mediated degeneration of rat mesencephalic neuron glia cultures by decreasing the volume of microglia and inhibition of proinflammatory cytokines (Qian *et al*., 2006b). Additionally, other *in vitro* studies have shown that IL-10 acts on the VM neurons directly and attenuates VM neuronal apoptosis and neuronal loss, by decreasing the expression of pro-inflammatory cytokines (Zhu *et al*., 2017). However, there are no previous *in vivo* studies of IL-10 transplanted in combination with VM grafts, and the *in vitro* protection found in previous studies was not observed in the work of this chapter.

The results obtained in Chapter 5 and in this chapter suggest that GDF5, at the dose used in this work, was detrimental to primary dopaminergic grafts. Given the studies of Moriarty and colleagues (2017, 2019) which showed that the transplantation of VM cells (either E12 or E14) encapsulated in combination with the neurotrophic factor GDNF, dramatically increased cell survival, striatal re-innervation and behavioural recovery (Moriarty *et al*., 2017, 2019a). Therefore, collagen hydrogels enriched with other neurotrophic factors have the potential to enhance cell replacement therapies in Parkinson´s disease.

Chapter 7: General Discussion

The work presented in this thesis sought to determine the potential of a dual approach combining cell therapy and the delivery of a neurotrophic factor in an injectable collagen scaffold to improve dopaminergic cell replacement therapy in Parkinson´s disease. We particularly focused on the ability of GDF5-functionalised collagen hydrogels to improve the survival and efficacy of the transplanted primary dopaminergic neurons by providing them with a supportive and protective matrix, while also supplying localised support from the neurotrophic factor GDF5 to the Parkinsonian brain (see **Figure 7.1**).

The main findings from this body of work are: 1) Collagen hydrogels are biocompatible to SH-SY5Y cells and to VM explants. Exposure to the collagen hydrogels did not hinder the survival and growth of the SH-SY5Y cells or of the dopaminergic cells in the VM explants; 2) GDF5 encapsulated in collagen hydrogels is neuroprotective against the neurotoxic effects of 6-OHDA. Treatment of GDF5 alone or encapsulated in a collagen hydrogel promoted the recovery of SH-SY5Y cells and dopaminergic cells in VM explants after exposure to the neurotoxin 6-OHDA; 3) collagen hydrogels are suitable carriers for the delivery of primary cells and neurotrophic factors. Collagen hydrogels successfully form *in situ*, are well tolerated in the brain given that their injection do not provoke a more pronounced host astrocytic or microglial response to the implanted primary cells and are permissive for primary dopaminergic graft survival in the striatum; 4) the transplantation of E14 VM cells encapsulated in a GDF5-functionalised collagen hydrogel did not increase cell survival, striatal re-innervation or promoted behavioural recovery in a 6-OHDA rat model of Parkinson´s disease; 5) the transplantation of E14 VM cells encapsulated in a GDF5/IL-10-functionalised collagen hydrogel did not increase cell survival, striatal re-innervation, promoted behavioural recovery and did not show a beneficial effect on the host immune response in a 6-OHDA rat model of Parkinson´s disease. Moreover, the incorporation of GDF5 into the transplantation process, at the dose used in this work, was detrimental to primary dopaminergic grafts.

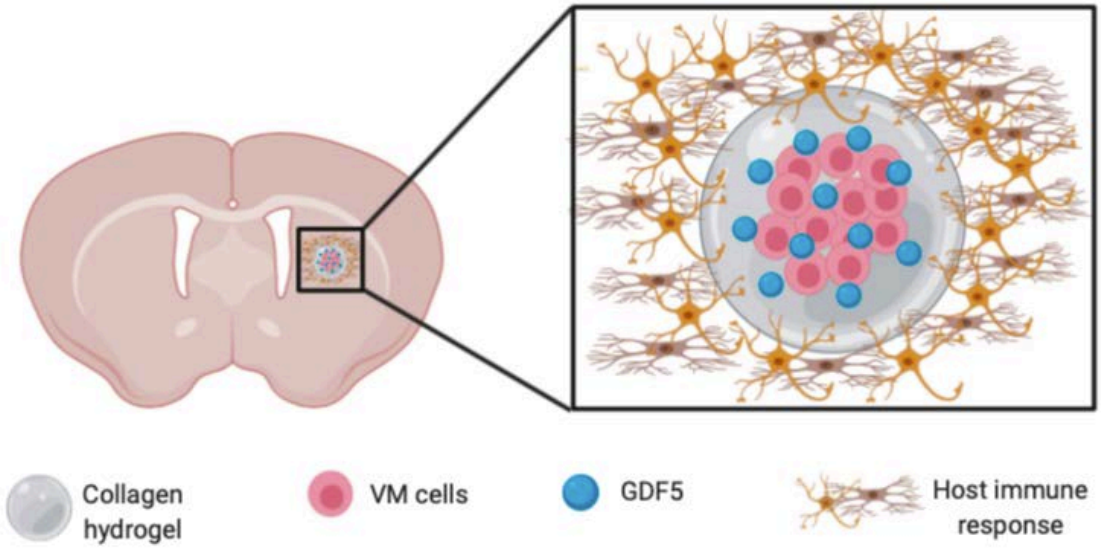


Figure 7.1 Schematic representation of a GDF5-functionalised collagen hydrogel.

GDF5-functionalised collagen hydrogels have the potential to aid in the delivery of primary dopaminergic cells for the treatment of Parkinson´s disease.

Parkinson´s disease is the second most common neurodegenerative disorder characterized by the development of movement impairments. The onset of the disease is usually at an age of 65 to 70 years with higher prevalence in men than women (de Lau and Breteler, 2006). The prevalence of the disease ranges from 100 to 200 per 100,000 people and the annual incidence is thought to be 15 per 100,000 (Tysnes and Storstein, 2017). Parkinson´s disease is one of the most common causes of disability and mortality globally with a significant clinical and socioeconomic impact (Findley, 2007).

The motor disturbances characteristics of this disease are resting tremor, rigidity, bradykinesia and postural instability. These symptoms are first encountered when there is an 80% reduction of dopamine in the substantia nigra pars compacta leading to reduced facilitation of voluntary movements; the degree of physical disability strongly correlates with the extent of impairment in striatal dopaminergic innervation (Fahn, 2003). People with Parkinson´s disease also exhibit non-motor symptoms such as sleep disturbance, autonomic dysfunction, hyposmia, cognitive decline and depression (Jankovic, 2008; Garcia-Ruiz *et al*., 2014). Available therapies for Parkinson´s disease only target the motor symptoms and neither the pharmacological nor surgical approaches have shown to slow down the rate of progression nor provide

neuroprotection to the surviving dopaminergic neurons. This emphasises the major unmet clinical need for new disease modifying therapies that could effectively stop the progression of neurodegeneration and/or repair the diseased brain.

Parkinson´s disease is the most promising neurological disorder for brain repair by cell transplantation due to its relatively focal neurodegeneration. The aim of cell replacement in this disorder is to restore nigrostriatal dopaminergic transmission by transplantation of dopaminergic neurons in the striatum and substantia nigra. To do so, the intraputamenal transplantation of foetal VM grafts, containing developing dopamine cells, have shown to partially re-innervate the striatum post-transplantation, whilst also restoring motor function (Sauer and Brundin, 1991; Kordower *et al*., 1995, 1998; Piccini *et al*., 1999; Olanow *et al*., 2003; Kuan and Barker, 2005; Petit *et al*., 2014). However, preclinical and clinical studies on intrastriatal grafts suggest that most of the dopaminergic neurons die within the first four days after transplantation by apoptosis (Mahalik *et al*., 1994; Zawada *et al*., 1998; Emgård *et al*., 1999; Sortwell *et al*., 2001). This apoptosis might be triggered by oxidative stress, hypoxia, withdrawal of trophic factors or by detachment from the extracellular matrix and neighbouring cells (Marchionini *et al*., 2003).

Additionally, dopaminergic cell survival following transplantation is limited with only 5% to 10% of the foetal nigral dopaminergic neurons surviving the tissue preparation and grafting procedure (Barker *et al*., 1996; Kordower *et al*., 1998). The incorporation of biomaterials into the transplantation process, might be the key to improve cell replacement therapy. Biomaterials such as collagen hydrogels could be employed during the transplantation process to target and reduce dopaminergic cell death. Thus, by increasing the survival of the transplanted cells, the number of embryos needed per transplant would be reduced and it would considerably widening the access to the therapy itself.

Biomaterials show great potential to improve cell replacement therapy. Their characteristics can be tailored to act as a supportive and protective matrix for the cells, provide localised and sustained trophic factor delivery and create a physical barrier between the transplanted cells and the host immune response, following intracerebral transplantation (Orive *et al*., 2009; Hoban *et al*., 2013; Moriarty and Dowd, 2018; Moriarty *et al*., 2019b). Collagen hydrogels seem to be promising matrices to use in cell transplantation due to characteristics such as high mechanical strength, good

biocompatibility, low antigenicity and ability of crosslinking that enable the tailoring of gelation and degradation properties (Wallace and Rosenblatt, 2003). Collagen can be used to form hydrogels *in situ* under physiological conditions and growth factors can be combined with collagen hydrogels to prolong their release rate and increase their therapeutic effect (Wallace and Rosenblatt, 2003).

Thus, before any biomaterial can be used in the brain it must undergo essential toxicity and cytocompatibility assessments and for this, we used an immortalized SH-SY5Y cell line and VM explants. In Chapter 3, we assessed collagen hydrogels and the neurotrophic factor GDF5, through a series of *in vitro* studies and *ex vivo* studies to determine the cytocompatibility of collagen hydrogels and determine if GDF5 enrichment of collagen hydrogels had neurorestorative effects on SH-SY5Y cells and on dopaminergic cells of explants after a 6-OHDA insult. In that chapter we found that our collagen hydrogels are biocompatible, they did not affect the survival and outgrowth of the SH-SY5Y cells or of the dopaminergic cells in the VM explants. These results were in line with the findings of Hoban and colleagues (2013) and Moriarty and colleagues (2017) reporting the *in vitro* cytocompatibility of 4s-StarPEG crosslinked collagen hydrogels (Hoban *et al*., 2013; Moriarty *et al*., 2017).

GDF5 had been found to have protective effects on the survival of E14 rat VM cells both *in vitro* and *in vivo* (Sullivan *et al*., 1998; O'Keeffe *et al*., 2004a; Wood *et al*., 2005; Clayton and Sullivan, 2007) however, the neurotrophic effects of this growth factor on cell transplantation have not been fully studied. A study by Sullivan and colleagues (1998) found that preincubation of embryonic rat midbrain tissue in recombinant human GDF5 before transplantation, produced significant improvements in cell survival and significant motor recovery in 6-OHDA-lesioned rats (Sullivan *et al*., 1998). Therefore, in Chapter 3, we first assessed the neuroprotective effects of GDF5 encapsulated in a collagen hydrogel on SH-SY5Y cells and VM explants after a 6-OHDA insult. Here, we found that GDF5 either alone or encapsulated in a collagen hydrogel promoted neuronal survival by 80%. These results were in line with previous reports from Toulouse and colleagues (2012) that found that treatment with GDF5 after a 6-OHDA insult rescued most of the cells from the neurotoxic insult (Toulouse *et al*., 2012).

Furthermore, in Chapter 3, we also found that exposure to the unloaded collagen hydrogels, had beneficial effects on SH-SY5Y cells and on the dopaminergic cells in the

VM explants after exposure to 6-OHDA. The unloaded collagen hydrogels promoted recovery of cells and axonal growth after a 6-OHDA insult, but there are no previous studies reporting beneficial effects of unloaded collagen hydrogels on cells after exposure to 6-OHDA.

Once we established that our collagen hydrogels were biocompatible and that GDF5 had neuroprotective qualities, we decided to assess in three preliminary *in vivo* studies: 1) the suitability of the collagen hydrogels for delivery of primary dopaminergic neurons to the striatum at early timepoints; 2) its ability to retain GDF5 in the striatum and 3) if encapsulation of GDF5 in the collagen hydrogels had early effects on primary dopaminergic grafts.

In the preliminary *in vivo* studies from Chapter 4, we found that collagen hydrogels exhibit a number of characteristics that make them good candidates for the intrastriatal delivery of cells. First, we determined that it polymerised *in situ,* allowing for relatively non-invasive delivery, and is biodegraded. We found that the normal immune response caused by the intracranial transplantation of VM cells was not exacerbated by the delivery of VM cells encapsulated in collagen hydrogels. This low immunogenicity could be attributed to collagen being one of the most abundant proteins in the human body (Hayashi, 1994). Moreover, we found the collagen hydrogels are permissive for cell survival in the striatum.

Having established that the collagen hydrogels were suitable for primary cell transplantation, we then assessed their ability to retain GDF5 in the striatum. In this preliminary study we found that the injection of GDF5 alone caused an astrocytic and microglial response at the injection site but when this neurotrophic factor was delivered encapsulated in a collagen hydrogel the host immune response to it was reduced. Unfortunately, it was not possible to detect this factor by immunohistochemistry and we were unable to determine if the collagen hydrogel affected the delivery or retention of GDF5 in the striatum relative to the bolus injections. Thus, we then assessed the effects of GDF5 enrichment of collagen hydrogels on E14 VM cells at an early time-point. Here, we found that the enrichment of collagen hydrogels with GDF5 was permissive for dopaminergic cell survival in the striatum and that for the most part this treatment did not provoke a more pronounced host astrocytic or microglial response to the encapsulated dopaminergic cells.

The preliminary studies, showed that our collagen hydrogels are a suitable matrix for cell and neurotrophic delivery. Hence, these results led to the most important question addressed in this thesis, could the application of GDF5-functionalised collagen hydrogels improve the long-term survival and efficacy of dopaminergic neurons after transplantation?

The dose of GDF5 implemented in the work in Chapter 5 and 6, was chosen based on previous *in vivo* studies where GDF5 was assessed as a neuroprotectant against the 6-OHDA lesion (Sullivan *et al*., 1997, 1999; Hurley *et al*., 2004). In previous studies, doses of 20 µg, 25 µg, 50 µg or 100 µg of GDF5 were infused into either the striatum or substantia nigra of rats to reduce or avoid dopaminergic neuronal death induced by the neurotoxin 6-OHDA. The study by Sullivan *et al*., (1999) found that 50 µg of GDF5 was the minimal dose to achieve optimal neuroprotection against dopaminergic loss by 6-OHDA. Since no detrimental effects were found with such high doses and because in the work of this thesis GDF5 is used for its neurotrophic qualities, the dose of 20 µg per injection was chosen to be used for transplantation.

In the work of Chapter 5, we found that delivery of E14 VM cells produced a behavioural recovery (in the form of reducing the number of methamphetamine induced ipsilateral rotations) in all of the transplanted groups, however, we saw a trend where the groups that received GDF5 alone or in collagen hydrogel showed less functional recovery. The *post-mortem* analysis showed that we successfully transplanted E14 VM cells but when these were delivered in combination with GDF5 we observed a trend for fewer surviving cells compared to the group that only received VM cells. When we measured the ability of the surviving dopaminergic cells to form neural outgrowths and re-innervate the lesioned striatum we found that, in all groups, the E14 VM grafts successfully re-innervated a portion of the striatum; however, the incorporation of GDF5 into the transplantation process tended to decrease the volume of the grafts and the magnitude of re-innervation. Due to these results, we concluded that the incorporation of GDF5 into the transplantation was detrimental for the survival of dopaminergic cells, graft volume and magnitude of re-innervation. Then, we looked at the microglial and astrocytic response and no beneficial effect was found.

In parallel to the study from Chapter 5 we ran a second study with GDF5 and IL-10. In this study, we aimed to determine whether a dual approach: a GDF5-functionalised collagen hydrogel in combination with an anti-inflammatory cytokine such as IL-10 can

provide additional support to dopaminergic neurons and result in greater graft survival and efficacy, by targeting neuroinflammation. In the work of that chapter, after a battery of behavioural tests, we did not find functional recovery in any of the transplanted groups. The *post-mortem* analysis showed the somewhat successful transplantation of E14 VM cells in the lesioned striatum of rats, however, the results showed reduced survival of cells, smaller grafts and poor re-innervation in all the groups that received a transplant in combination with a factor compared to the group that only received VM cells, therefore, no benefit was found by the addition of either GDF5 or IL-10 into the transplantation process. Then we looked at the host immune response, to see if IL-10 had protected against inflammation, but no beneficial effect was found in reducing the volume and optical density of microglia and astrocytes at the transplantation site and no difference in the host immune response was found between any of the transplanted groups. Furthermore, no beneficial effect was found by the addition of IL-10 or by the addition of GDF5 to the transplantation process.

The results from Chapter 5 and 6 led us to conclude that the incorporation of GDF5 into the transplantation process, at the dose used in these studies (20 μg per 6 μl) was detrimental to primary dopaminergic grafts since there was limited survival of cells, smaller grafts, small magnitude of re-innervation and no significant functional recovery in the groups that received this factor.

Future Directions

Cell replacement therapy in Parkinson´s disease is based on the principle that neurotransmission could be restored by transplanting dopamine neurons into the dopamine depleted striatum. Preclinical and clinical studies have shown that transplantation of foetal VMs are a good substitute of the lost dopaminergic cells in Parkinson´s disease, however, the use of aborted foetuses presents several ethical and logistical issues that impede the effective translation of foetal VM transplantation as a routine therapeutic option. Alternative cell sources that are readily available, renewable and that can mimic mesencephalic dopaminergic neurons of the A9 phenotype are needed. Therefore, the use of stem cell derived dopaminergic neurons is likely to be the future of cell replacement therapy in Parkinson´s disease.

Currently, the use of iPSCs for brain repair is limited by low *in vivo* maturation rates (6-54%) seen in both rodent and primate models of Parkinson´s disease (Kriks et al., 2011; Kikuchi et al., 2017a) due to the need of transplantation of these cells in a progenitor state. iPSCs cannot be transplanted in an advanced state of differentiation since they die if lifted from cell culture. Therefore, when these cells, in a progenitor state, are lifted for transplantation, they are transplanted into a growth factor deprived and diseased Parkinsonian adult brain, hindering their differentiation and maturation to dopaminergic neurons.

The incorporation of biomaterials such as our collagen hydrogels into the transplantation process might be the key to improve the results of iPSCs transplantation. The incorporation of neurotrophic factors such as BDNF and/or GDNF, encapsulated in a biomaterial matrix, holds the potential to further improve the maturation and survival of the transplanted iPSCs by providing them with localised and prolonged neurotrophic support during and after transplantation.

Dopaminergic neurons from both ESCs and iPSCs show extraordinary potential as cell sources for cell replacement therapy. The somewhat successful results by the use of iPSCs for cell transplantation in animal models of Parkinson´s disease (Wernig *et al*., 2008; Kikuchi *et al*., 2017a, 2017b) have led to the development of the first clinical trial in Japan (ongoing) (Takahashi and Price-Evans, 2019). The primary endpoints are related to safety (tumorigenicity) and the presence or absence of graft expansion in the brain 24 month after transplantation (Takahashi and Price-Evans, 2019).

Many questions still need to be addressed; will they present similar survival problems as foetal VM cells after transplantation? Will they be able to successfully reinnervate and improve motor impairments? As iPSC-based cell replacement therapy continues to advance toward clinical use, it is necessary to continue with preclinical research to find methods to improve the outcome of the transplantation of these cells and ensure that the potential tumorigenesis or the genetic manipulation used on these cells do not hinder their use.

Concluding remarks

Disappointingly in this thesis, the transplantation of dopaminergic cells in GDF5-functionalised collagen hydrogels did not improve cell survival, reinnervation or functional recovery. However, other neurotrophic factors might be better candidates for their encapsulation in the collagen hydrogels for cell replacement therapy. For example, the work of Moriarty and colleagues (2017, 2019) showed that the transplantation of VM cells (either E12 or E14) encapsulated in combination with the neurotrophic factor GDNF, dramatically increased cell survival, striatal re-innervation and behavioural recovery (Moriarty *et al.*, 2017, 2019a). These findings highlight the potential of biomaterial scaffolds to improve cell transplantation in Parkinson´s disease but further work remains to be carried out to identify a better neurotrophic or anti-inflammatory factor.

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Appendices

Appendix I: Solutions for Hydrogels

10X PBS

To make 1 L of 10X PBS:

1. - In 800 ml distilled H_2O dissolve:

    80 g of NaCl

    2.0 g of KCl

    14.4 g of Na_2HPO_4

    2.4 g of KH_2PO_4

2.-Adjust pH to 7.4

3.- Add additional distilled H_2O to adjust volume to 1 L

4.- Autoclave

1X PBS

To make 1 L of 1X PBS:

1.- From the 10X PBS stock solution take 100 ml and add 900 ml of distilled H_2O

2.-Adjust pH to 7.4

3. - Autoclave

1M NaOH

To make 1 L of 1M NaOH:

1.- In 1000 ml of distilled H_2O dissolve 40 g of NaOH

2.- Autoclave

0.1M NaOH

To make 1 L of 0.1M NaOH:

1.- From the 1M NaOH stock solution take 100 ml and add 900 ml of distilled H_2O

2.-Autoclave

Appendix II: Buffers for Perfusion- Fixation

1. 0.2M Phosphate buffer solution

A: Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$ MW=137.99)
1M=137.99g in 1L dH_2O
0.1M=13.799g in 1L dH_2O
0.2M=27.598g in 1L dH_2O

B: Disodium hydrogen phosphate dehydrate ($Na_2HPO_4.2H_2O$ MW=177.99)
1M=177.99g in 1L dH_2O
0.1M=17.799g in 1L dH_2O
0.2M=35.598g in 1L dH_2O

→For 500ml PBS
A: 27.598g/L x 0.095L = 2. 621 g
B: 35.598g/L x 0.405L = 14.417 g
Dissolve both in 500ml of dH_2O

→For 1000ml PBS
A: 27.598g/L x 0.19L =5.2436 g
B: 35.598g/L x 0.81L = 28.834 g
Dissolve both in 1000ml of dH_2O

→For 2000ml PBS
A: 27.598g/L x 0.38L =10.4872 g
B: 35.598g/L x 1.62L = 57.668 g
Dissolve both in 2000ml of dH_2O

Note: Must be used in a 1:1 dilution with PFA

2. Fixative: 4% paraformaldehyde (4L) (<u>in fume hood</u>)

1. Heat 1.5L of d H_2O to 60 C
2. Add 160g of PFA powder (40 g/L)
3. Add a few NaOH pellets in order to dissolve PFA
4. Stir until clear
5. Fill to 2L with dH_2O
6. Stir until clear
7. Add 2L of 0.2M PBS to give a final volume of 4L
8. pH to 7.4 and cool to 4 °C

3. Heparinised saline

1 ml of heparin is added per 1L saline

Each small vial of heparin contains 25000/ 5 ml i.e. 1 ml of heparin has 5000 units.

4. 30% w/v sucrose solution (1L)

1. Dissolve 5 PBS tablets in ~500ml dH_2O (1 PBS tablet per 200ml water)
2. Add 300g of sucrose
3. Stir until dissolved, apply heat if necessary
4. Make up to 1L with d H_2O

5. 30% w/v sucrose solution (1L) with 0.1% sodium azide

1. Dissolve 5 PBS tablets in ~500ml d H_2O (1 PBS tablet per 200ml water)
2. Add 300g of sucrose
3. Add 1g sodium azide
4. Stir until dissolved, apply heat if necessary
5. Make up to 1L with d H_2O

Appendix III: Immunohistochemistry Protocol

Solutions for immunohistochemistry

9% Saline	Sodium Chloride	9 g
	Distilled Water	1 L
0.01% Ascorbate Saline	Ascorbic Acid	0.01g
	Saline	1 L
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	
	Adjust to pH 7.4 with conc HCl	Make up to 1 L
TBS azide	Trizma Base	12g
	Sodium Chloride	9g
	Sodium azide	1g
	Distilled water	
	Adjust to pH 7.4 with conc HCl	Make up to 1 L
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	
	Trizma base 6g	
	Distilled water	
	Adjust to pH 7.4 with conc HCl	Make up to 1 L
DAB stock	DAB	1 g
	TNS	100 ml
	Aliquot into 2 ml aliquots and store at -20^{o}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 30μm thick 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.

Step by step process

Day 1

1. *Wash 1*5min in TBS.*

2. Quench for 5min.
   - Methanol 5ml
   - 30% H_2O_2 5ml
   - Distilled H_2O 40ml

3. *Wash 3*5min with TBS.*

4. Incubate sections in 3% serum (dependant on secondary host) for 60 mins.
   - 30μl/ml serum in TXTBS (freshly made-up)

5. Draw off excess and incubate in primary antibody in 1% serum at room temperature overnight (e.g. 1:1000)
   - 1μl/ml of primary in 10μl/ml serum in TXTBS

==========

Day 2

6. *Wash 3*10min with TBS.*

7. Incubate in biotinylated secondary in 1% serum for 3 hours (e.g. 1:200)
   - 5μl/ml of secondary in 10μl/ml serum in TBS

8. Make ABC Complex (it is light sensitive) and wash 3*10min with TBS.
   - 5μl of solution A and 5μl of solution B per ml in 10μl/ml serum in TBS

9. Incubate in ABC Complex for 2 hours.

*10. Wash 3*10min with TBS.*

11. Wash with TNS (freshly made-up) leave overnight @ 4°C.

DAB INCUBATION can be done on the same day after step 11.

==========

Day 3

12. Incubate in H_2O_2/DAB solution until colour develops
    - TNS 40ml
    - DAB 20mg (frozen in 2ml aliquots).
    - 30% H_2O_2 12µl

13. Wash 3*5min with TNS

14. Mount (in TBS with a little TXTBS) on gelatin-coated slides, air dry overnight.

15. Dehydrate in 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

Slide Subbing

Materials

- Gelatin (10 g/L)

- Chromic potassium sulphate (500 mg/L)

- Distilled H_20

- Slides

Step by step process

1. Heat dH_20 to greater than 40°C and add gelatin slowly allowing it to dissolve before adding more
2. Add chromic potassium sulphate
3. Subbing Medium is then cooled to less than 35°C
4. Slides are placed in holders and dipped into subbing medium for about 1 min
5. Remove slides and allow to dry on aluminium foil for about 1 week

Appendix IV: General Immunocytochemistry Protocol

Suitable for 2D cell cultures, in wells of a 24-well plate, that have been fixed in 4% buffered paraformaldehyde for 30 mins

Solutions

10% Sodium Azide (stock solution)
-1 g sodium azide in 10ml TBS

TBS containing 1% Bovine Serum Albumin
-1 g BSA in 100ml TBS

Step by step process

Day 1

1. Wash wells 1 x 5 min in TBS.
2. Incubate cells in 200 µl blocking solution for 1 hour at RT.

Blocking Solution (40ml)
-2 ml serum (dependant on secondary host)
-120 µl Triton X-100
-40 µl Sodium azide (from 10% Stock Solution (0.01% is final conc))
-37.84 ml TBS with 1% BSA

3. Remove blocking solution and incubate wells in primary antibody at room temperature overnight

Primary – mouse anti-TH (1:800)

5 µl TH antibody

5 µl Sodium Azide (from 10% Stock Solution)

4990 µl TBS containing 1% BSA (can use stock 0.1g BSA in 10ml)

Primary – mouse anti-βIII tubulin (1:333)

18 µl βIII tubulin antibody

6 µl Sodium Azide (from 10% Stock Solution)

5976 µl TBS containing 1% BSA (can use stock 0.1g BSA in 10ml)

Day 2

4. Remove primary antibody
5. Wash wells 3 x 10 min with TBS
6. Incubate wells in fluorophore labelled secondary antibody for 3 hours at room temperature

<u>Secondary – Rabbit anti-mouse A.F. 488</u>
75 µl secondary antibody
150 µl NRS
14775 µl TBS

7. Wash wells 3*5 min with TBS.
8. Incubate sections in 1 µg/ml DAPI in TBS for ~5 min.
9. Wash wells 3*10 min TBS.
10. Store wells in 0.1% TBS azide in the fridge.
11. Image cells